Arabidopsis Response Regulators in Cytokinin Signaling and Development

Jennifer PokChun To

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology (Molecular, Cell and Developmental Biology Program).

Chapel Hill
2007

Approved by

Joseph J. Kieber, Ph.D. (Advisor)

Robert B. Bourret, Ph.D.

Jeffrey L. Dangl, Ph.D.

Sarah R. Grant, Ph.D.

Jason W. Reed, Ph.D.

ABSTRACT

Jennifer PokChun To

Arabidopsis Response Regulators in Cytokinin Signaling and Development

(Under the direction of Dr. Joseph J. Kieber)

The plant hormone cytokinin is involved in many processes in the plant, including cell division, seed germination, photomorphogenesis, shoot and root development, leaf senescence and seed set. The model for cytokinin signaling is similar to a two-component phosphorelay with which bacteria sense and respond to environmental stimuli. The cytokinin receptors are Hybrid Histidine Kinases that autophosphorylate on a conserved histidine residue in response to cytokinin binding. The phosphoryl group is transferred via an intermediate Histidine Phosphotransfer Protein to a conserved aspartate residue on the receiver domain of a Response Regulator.

Members of the Response Regulator protein family in *Arabidopsis thaliana* (ARRs) contain the conserved N-terminal receiver domain required for phosphorylation by two-component elements and can be classified into three groups based on sequence similarity and protein structure: type-A, type-B and type-C ARRs. The ten type-A *ARR*s are rapidly up-regulated by cytokinin treatment but their sequences do not predict known outputs. The eleven type-B ARRs have DNA binding and transactivating activity and are positive activators of cytokinin-regulated transcription. The pair of type-C ARRs are less similar in sequence to the two other groups of ARRs, are not transcriptionally regulated by cytokinin and do not have transcriptional activity.

In order to study the role of type-A ARR in cytokinin signaling and development, I have isolated multiple type-A *arr* loss-of function mutants up to a septuple *arr3,4,5,6,7,8,9* mutant. Type-A *arr* mutants exhibit additive hypersensitivity to cytokinin, indicating that type-A ARRs play overlapping roles in negatively regulating cytokinin response. Subsets of type-A *arr* mutants show specific responses consistent with their patterns of expression. In particular, a subset of type-A *ARR*s interact with the meristem maintenance gene *WUSCHEL* to modulate shoot meristem activity. To further investigate the role of phosphorelay on type-A ARR function, I constructed site-directed mutants targeting the conserved aspartate phosphorylation site and tested their functions *in planta*. My results indicate that type-A ARR proteins are activated by phosphorylation and are likely to function by phospho-dependent interactions, with implications for functional specification.

To my family:

my parents, K.P. To and Mei Ling Chan,
my brother, Ernest To and
my husband, Aaron Wiig.

ACKNOWLEDGEMENTS

The completion of this doctoral dissertation would not have been possible without many individuals who have helped me along the way.

I am grateful to my advisor, Joe Kieber, for his mentoring over the years. His enthusiasm for science and constant flow of new ideas has been inspiring. Joe has always been approachable and receptive to my questions. I thank Joe for directing my research and also for encouraging me to think and work on my own in order to develop as an independent scientist.

I have had the opportunity to work with and learn from many talented postdoctoral fellows in the Kieber lab. I am deeply indebted to Georg Haberer and Jean Deruère, who have initiated parts of this work and have taught me the practical laboratory and critical thinking skills needed to move forward. I also thank Claire Hutchison, Hyun-Sook Chae, Susan Carson, Aaron Rashotte, Jennifer Umphress, Bridey Maxwell, Fernando Ferreira, Cristiana Argueso and Koji Tanase, who have all been very generous with sharing their expertise, protocols and reagents. I thank all the postdoctoral fellows and my fellow graduate students in the lab Veronica Morris, Maureen Hansen and Shouling Xu for making the lab a productive and fun environment. I thank Bridey and Cristiana for their friendship. I also thank all the lab technicians, undergraduates and graduate rotation students who have assisted me in various projects along the way, in particular Monica Gonzalez, Alicia Brandt and Celine Ma.

I would like to thank members of the Reed, Grangl, Jones, Copenhaver and Liljegren labs for many insightful scientific discussions and for sharing reagents.

I thank my collaborators Michael Mason, G. Eric Schaller, Andrea Liebfried, Jan Lohmann, Patrice Salomè and Rob McClung for sharing materials and data and also for teaching me about areas of research outside the expertise of our lab. I also thank Victoria Madden and Michael Vernon at UNC core facilities for technical assistance.

I would like to thank members of my graduate committee, Bob Bourret, Jeff Dangl, Sarah Grant and Jason Reed, for their patient guidance through this process. I thank Bob for carefully reading and editing this dissertation. I thank Sarah and Jeff for their guidance since my first day in graduate school. I also thank Jason for perceptive comments and helpful suggestions at group meetings.

Funding for this work has been provided by National Institutes of Health and National Science Foundation grants to the Kieber lab. I have also been financially supported by a W.C. Coker Dissertation Fellowship, Alma Holland Beers Summer Scholarships and travel grants from the North Carolina Biotechnology Center, the International Plant Growth Substance Association and the Graduate and Professional Student Federation at UNC.

On a personal level, I have been extremely fortunate to be surrounded by a supportive network of family and friends during my time in graduate school and to all of them I am very thankful. I am most grateful to my parents, K.P. To and MeiLing Chan, for instilling in me, from an early age, a desire to learn and also for their wholehearted support through my years of education. I thank my brother, Ernest, who has always challenged me to think critically and from whom I have first learned how to learn. I also

thank my extended family with my grandmothers, my aunts, uncles and cousins for their words of encouragement. It is from support of my family that I have acquired the confidence to take on this challenge. I thank my family-in-law, Randy and Rose Wiig, Ericka, Amanda and Amber Spencer, for providing me a “home-away-from-home” whenever I needed time away from graduate school. I also thank my cats Max and Mia for keeping my feet warm during these last few weeks of intensive writing and for occasionally reminding me that the sun still shines outside. Last but not at all least, I must thank my husband and best friend, Aaron Wiig, for being by my side through this entire process, constantly cheering me on and also making sure that my life is otherwise well-balanced.

TABLE OF CONTENTS

CHAPTER2

CHAPTER 3

CHAPTER 4

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABPH1/ ABPHYL1	Abnormal Phyllotaxy 1
ADP	Adenosine diphosphate
AG	Agamous
AHK	Arabidopsis Histidine Kinase
AHP	Arabidopsis Histidine Phosphotransfer Protein
AP2	Apetala2
APHP	Arabidopsis Pseudo Histidine Phosphotransfer Protein
APRR	Arabidopsis Pseudo Response Regulator
Arc	Anoxic Redox Control
ARF	Auxin Response Factor
ARR	Arabidopsis Response Regulator
Asp	Aspartic Acid, also abbreviated as D
ATP	Adenosine triphosphate
AUX/IAA	Genes rapidly induced by Auxin and Indoleacetic Acid
BA	Benzyladenine
CaMV	Cauliflower Mosaic Virus
cDNA	Complementary DNA
Che	Chemotaxis
CHX	Cycloheximide
CLV	Clavata
CKI	Cytokinin Independent
CKX	Cytokinin Oxidase

COP9/CIN4/FUS10	Constitutive Photomorphogenesis9/Cytokinin Insensitive4/Fusca10
CRF	Cytokinin Response Factor
CTR	Constitutive Triple Response
DEX	Dexamethasone
DMSO	Dimethyl Sulfoxide
DMA5	Dexamethasone-inducible myc-tagged ARR5
DNA	Deoxyribonucleic Acid
EBF	EIN3 Binding F-box protein
EIN	Ethylene Insensitive
ETR	Ethylene Response
ERF	Ethylene Response Factor
ERS	Ethylene Response Sensor
F-box	Sequence domain first found in Cyclin-F
GARP	DNA binding and domain also found in GOLDEN2 in maize, the ARRs, and the Psr1protein from *Chlamydomonas*
Glu	Glutamic Acid, also abbreviated as E
GR	Glucocorticoid Receptor
GUS	ß-glucuronidase
His	Histidine, also abbreviated as H
HK	Histidine Kinsase
HP	Histidine Phosphotransfer Protein
Hpt	Histidine-containing Phosphotransfer proteins
IPT	Isopentenyl Transferase
KNOX	Knotted1-like Homeobox

LFY	Leafy
LUC	Luciferase
NAA	1-Naphthaleneacetic Acid
Myc-tag or Myc	Peptide tag derived from cellular myelocytomatosis oncogene
OsHK	*Oryza sativa* (rice) Histidine Kinase
OsHP	*Oryza sativa* (rice) Histidine Phosphotransfer Protein
OsRR	*Oryza sativa* (rice) Response Regulator
OX	Overexpressor
PCR	Polymerase Chain Reaction
PHYB	Phytochrome B
PRR	Pseudo Response Regulator
qRT-PCR	Quantitative real-time RT PCR
RNA	Ribonucleic Acid
RPN12	Proteasome Regulatory Particle, Non-ATPase-like 12
RR	Response Regulator
RT PCR	Reverse Transcriptase PCR
SLN1	Yeast HK in Osmosensing pathway
SSK1	Yeast RR in Osmosensing pathway
SST1	Steroid Sulfotransferase 1
sSTM	Shootmeristemless
T-DNA	Transferred-DNA from Agrobacterium
TUB	Tubulin
WT	Wild type or Wild-type

WUS	Wuschel
YPD1	Yeast Hpt in Osmosensing pathway
ZmHK	*Zea mays* (maize) Histidine Kinase
ZmHP	*Zea mays* (maize) Histidine Phosphotransfer Protein
ZmRR	*Zea mays* (maize) Response Regulator

CHAPTER 1

An Introduction to Cytokinin Signaling: Components, Mechanisms and Outputs

Jennifer P.C. To and Joseph J. Kieber

ABSTRACT

The plant hormone cytokinin has been linked to a variety of processes including cell division, shoot and root development and leaf senescence. In our current understanding of cytokinin signaling, the cytokinin signal is perceived and transduced via a phosphorelay similar to a two-component system with which bacteria sense and respond to environmental stimuli. Recent progress on characterizing two-component elements in Arabidopsis, maize and rice show that cytokinin responses are mediated via partially redundant two-component protein families: Histidine Kinases, Histidine Phosphotransfer Proteins and Response Regulators. Novel players in cytokinin signaling have recently been identified, such as the Cytokinin Response Factors. Cytokinin regulates these signaling components through a variety of mechanisms, including modulating transcription, controlling phosphate flux through the pathway and regulating protein localization and stability. Genetic analysis of cytokinin signaling components have helped to clarify the roles of cytokinin signaling in development and have revealed novel functions.

REVIEW OF RECENT LITERATURE

Cytokinins were originally discovered by their property of promoting cell division (Miller et al., 1955). These N^6-substituted adenine-based molecules have been associated with various plant developmental roles including germination, shoot and root development and leaf senescence (reviewed in (Mok and Mok, 1994)). In plants, cytokinins are mainly synthesized by ATP/ADP isopentenylation, and the regulation of cytokinin biosynthesis and metabolism has been described in other reviews (Mok and Mok, 2001; Miyawaki et al., 2006; Sakakibara, 2006; Sakakibara et al., 2006). Since the recent discovery of the cytokinin receptor in Arabidopsis, which is a hybrid histidine kinase similar to bacterial two-component sensor kinases (Inoue et al., 2001; Ueguchi et al., 2001a; Yamada et al., 2001), a model for cytokinin signal transduction has emerged that is similar to bacterial two-component systems and has been reviewed extensively (Heyl and Schmulling, 2003; Kakimoto, 2003; Ferreira and Kieber, 2005; Maxwell and Kieber, 2005). Two-component elements in Arabidopsis are encoded by multi-gene families with high levels of functional overlap, and homologous gene families have also recently been identified in monocots maize and rice (Asakura et al., 2003; Ito and Kurata, 2006; Jain et al., 2006; Pareek et al., 2006; Schaller et al., 2007). Until recently, our understanding of the role of two-component elements in the plant has been limited by genetic redundancy of these genes. Over the past few years, higher order loss-of-function mutants have been analyzed and their phenotypic analyses have been reported, which provide evidence for the role of two component elements in cytokinin signaling and begin to reveal functional specificities among two-component proteins (Higuchi et al., 2004; Nishimura et al., 2004; To et al., 2004; Mason et al., 2005; Hutchison et al., 2006;

Riefler et al., 2006; Yokoyama et al., 2007).

In this review, we aim to highlight recent progress on genetic characterization of two-component elements and novel players in cytokinin signaling. We present evidence for a variety of mechanisms with which cytokinin regulates these signaling components, beyond a simple linear phosphorelay mechanism. Finally, we integrate data from studies of cytokinin signaling components to identify subsets of genes that regulate cytokinin dependent processes such as shoot meristem function, root development, leaf senescence, seed set and circadian phase, and cytokinin independent functions such as circadian period. A model is proposed for how these cytokinin signaling components may specify various biological outputs.

Two component elements are involved in cytokinin signaling

Two-component signaling systems are used by prokaryotic and eukaryotic organisms to sense and respond to changes in the environment (reviewed in (Stock et al., 2000; West and Stock, 2001)). In a canonical two-component system, a stimulus is perceived by a sensor kinase, which autophosphorylates on a conserved His in the kinase domain. The signal is transmitted by transfer of the phosphoryl group to a conserved aspartate residue on the receiver domain of a response regulator. Receiver domain phosphorylation induces conformational changes that release repression of the output domain to allow activation of downstream processes, often by transcriptional regulation or direct protein interactions. Variations of the simple two-component system involve intermediate histidine phosphotransfer proteins (HPs) in the phosphotransfer from the sensor histidine kinase (HKs) to the response regulator (RRs), via a His→Asp→ His→Asp phosphorelay.

The cytokinin receptors *ARABIDOPSIS HISTIDINE KINASE 4* (*AHK4*) (also known as *CYTOKININ RESPONSE 1* (*CRE1*) and *WOODENLEG 1* (*WOL1*)), and its homologs *AHK2* and *AHK3* were isolated in Arabidopsis and found to be hybrid kinases similar to histidine kinases in bacterial two-component systems (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001b; Ueguchi et al., 2001a; Yamada et al., 2001). These three hybrid kinases are transmembrane proteins with an extracellular cytokinin-binding CHASE (Cyclase/Histidine kinase-Associated Sensing Extracellular) domain, and a cytoplasmic tail containing a histidine transmitter domain and a receiver domain

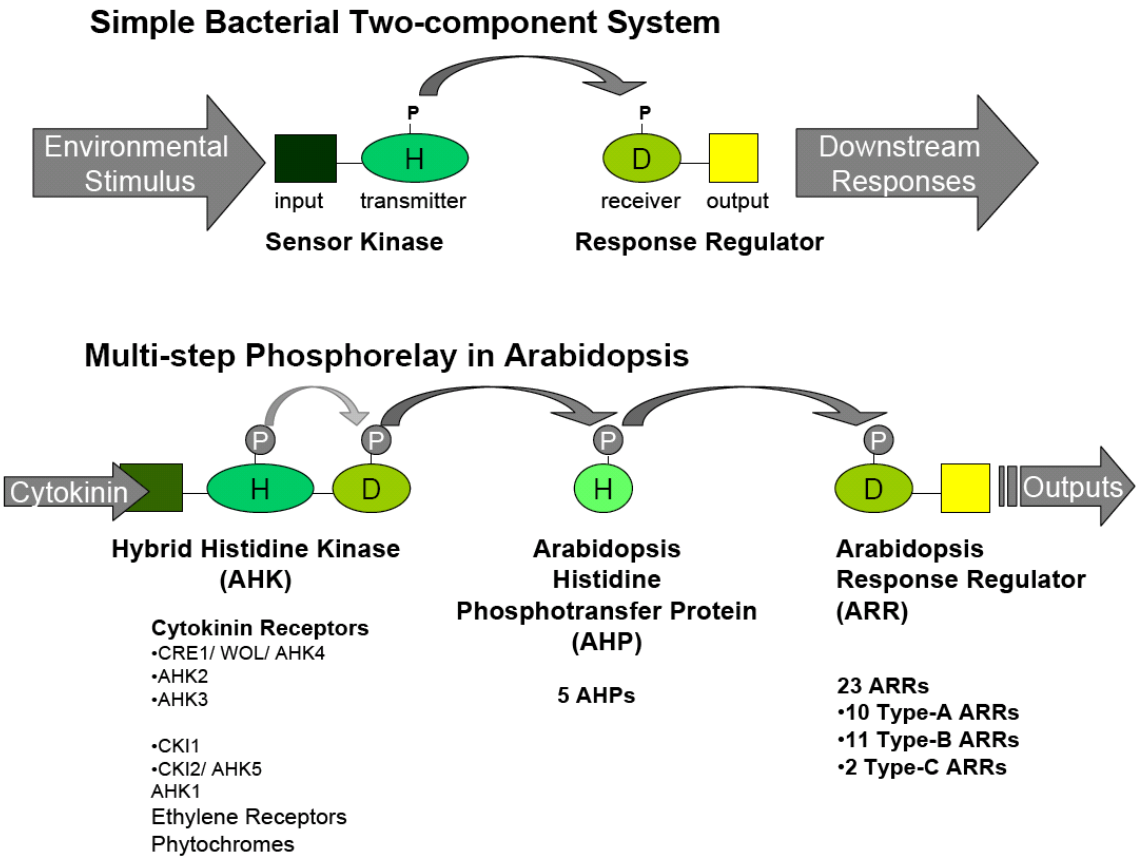

Figure 1.1 Cartoon of two-component phosphorelay

The cytokinin signaling pathway is similar to a simple two-component signaling pathway. Conserved His (H) and Asp (D) residues required for phosphorelay are depicted.

(Kakimoto, 2003). They are the only members containing a CHASE domain in the histidine kinase family in Arabidopsis (Kakimoto, 2003). The closest histidine kinase relatives in Arabidopsis include *AHK1*, *CYTOKININ INDEPENDENT 1* (*CKI1*) and *CKI2/AHK5* (Schaller et al., 2002). *AHK1* has been implicated in osmosensing (Urao et al., 1999). *CKI1* was originally isolated in an overexpressor that had the ability to initiate shoots without exogenous cytokinin application in tissue culture (Kakimoto, 1996). Analysis of transcript expression and multiple loss-of-function *cki1* alleles indicate that this gene regulates female gametophytic development (Pischke et al., 2002; Hejatko et al., 2003). *CKI2/AHK5* has been shown to play a role in ethylene and abscisic acid responses in the root (Iwama et al., 2007). Other distantly related members of the Arabidopsis Histidine Kinase family include the five ethylene receptors, the five phytochrome red-light receptors and a pyruvate dehydrogenase kinase, which have all diverged and lost conserved sequences required for histidine kinase activity except for two of the ethylene receptors *ETR1* and *ERS1* (Schaller et al., 2002). In this review, we refer to the *AHK*s as the cytokinin receptors *AHK2, AHK3* and *AHK4.*

Homologs of two-component phosphorelay elements downstream of the Arabidopsis cytokinin receptors are encoded by multi-gene families that include five authentic *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEINS* (*AHP*s) and 23 *ARABIDOPSIS RESPONSE REGULATORS* (*ARR*s) (Kakimoto, 2003; Ferreira and Kieber, 2005; Maxwell and Kieber, 2005). The five AHPs carry conserved amino acids required for phosphotransfer via a conserved histidine residue (Suzuki et al., 1998; Hutchison et al., 2006). An additional *PSEUDO HPT* (*APHP1*, also referred to as *AHP6*) carries a substitution in the conserved histidine and does not encode a functional

phosphotransfer protein (Mähönen et al., 2006b). The *ARR* gene family falls into four groups by similarity of core receiver domain sequence and C-terminal domain structure: ten type-A *ARR*s, eleven type-B *ARR*s, two type-C *ARR*s and nine *ARABIDOPSIS PSEUDO RESPONSE REGULATORS (APRRs)* (Kiba et al., 2004; Schaller et al., 2007). The type-A *ARR*s contain short C-terminal sequences that do not encode known outputs. Type-A *ARR* transcripts are rapidly upregulated in response to cytokinin and are cytokinin primary response genes (D'Agostino et al., 2000). The C-termini of type-B ARRs contain a MYB-like GARP DNA binding (also found in GOLDEN2 in maize, the ARRs, and the Psr1protein from *Chlamydomonas*) and transactivating domains. Type-B ARRs regulate transcription of cytokinin-activated targets, including the type-A *ARR*s (Hwang and Sheen, 2001; Sakai et al., 2001; Mason et al., 2005; Rashotte et al., 2006; Yokoyama et al., 2007). Type-C *ARR*s are less similar to type-A and type-B *ARR* receiver domain sequences. They do not contain the output domain of type-B ARRs and are not transcriptionally regulated by cytokinin (Kiba et al., 2004). The *APRR*s carry a substitution in the conserved phosphorylation target of the receiver domain and do not encode targets of two-component phosphorelay. Some APRRs play a role in the regulation of circadian rhythms (McClung, 2006).

Homologous two-component gene families have also been identified in monocots maize and rice (Asakura et al., 2003; Jain et al., 2006; Pareek et al., 2006; Du et al., 2007; Schaller et al., 2007). The rice (Os) genome sequence encodes four CHASE domain containing *OsHK*s, two *OsAHPs (Authentic HP*s), three *OsPHPs (Pseudo HP*s), *13* type-A *OsRRs*, seven type-B *OsRRs*, two type-C *OsRRs* and seven *OsPRRs* (Murakami et al., 2005; Ito and Kurata, 2006; Jain et al., 2006; Du et al., 2007; Schaller et al., 2007). In

addition, a novel protein carrying a CHASE domain and a serine/threonine kinase domain, but no histidine kinase domains, was identified and may be a novel cytokinin-signaling protein (Ito and Kurata, 2006). In maize (Zm), four *ZmHK*s, three *ZmHPs*, seven type-A *ZmRRs* and three type-B *ZmRRs* have been identified from leaf and ear cDNA libraries (Asakura et al., 2003). Phylogenetic analysis of two-component genes from the three different plant species reveals different dynamics in the evolution of *HKs*, *HPs* and *RR*s. The *HKs* are highly conserved between the three species (51%-92% identity within CHASE, histidine transmitter and receiver domains) and fall into three main groups, each group containing members from each species, with the monocot *ZmHKs* and *OsHKs* being more similar to each other than to the dicot *AHKs* (Ito and Kurata, 2006; Du et al., 2007). This suggests that subfunctionalization of the *HKs* may have occurred prior to divergence of monocots and dicots and further expansion of monocot *HK* families may have occurred after the monocot/dicot split. The *HPs* are less similar across species (42-88% identical) and mostly claded in a species-specific manner (Hutchison et al., 2006; Ito and Kurata, 2006; Du et al., 2007). This indicates that the common ancestral *HPs* may have expanded to form gene families mostly after divergence of monocots and dicots. Phylogenetic analysis of the *RRs* show that type-A *RR*s clade separately from the type-B and pseudo *RR*s (To et al., 2004; Du et al., 2007), suggesting that the ancestral type-A *RRs* separated from the common *RR* ancestors. The type-A *RR* and type-B *RR* genes further fall into subgroups, with pseudo *RRs* interdigitating between type-B *RR* subgroups, indicating that the pseudo *RRs* separated from the type-B *RRs* via multiple events (Du et al., 2007). Most of the type-A *ARR*s clade separately from the monocot type-A *ZmRRs* and *OsRRs* (To et al., 2004; Ito and Kurata, 2006; Du et al.,

2007), suggesting that the ancestral type-A *RRs* were comprised of a small family that expanded after separation of monocots and dicots, similar to the *HP*s. Type-B *RR*s from different species appear to clade into subgroups together (Ito and Kurata, 2006), suggesting that some expansion of the family may have occurred before the monocot-dicot split.

AHK2, AHK3 and *AHK4* are the Arabidopsis cytokinin receptors

CRE1/AHK4 was the first cytokinin receptor identified: it is required for cytokinin induction of shoots in tissue culture, binds cytokinin with a K_d of ~4.6 nM and can complement yeast and bacteria HK mutants in a cytokinin-dependent manner (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001b; Ueguchi et al., 2001a; Yamada et al., 2001). *AHK4* overexpression in protoplasts can enhance cytokinin induction of *ARR6:LUC,* a type-A *ARR* Luciferase reporter construct, in a manner dependent on conserved His and Asp residues required for kinase activity (Hwang and Sheen, 2001). All three AHKs and a subset of ZmHKs and OsHKs have been shown to directly bind or be stimulated by active cytokinins in heterologous yeast or bacterial *HK* complementation assays. There are some differences in individual HK binding affinities for specific cytokinin moieties (Inoue et al., 2001; Yamada et al., 2001; Asakura et al., 2003; Yonekura-Sakakibara et al., 2004; Romanov et al., 2006; Du et al., 2007).

Analysis of T-DNA insertional mutants reveal *AHK* functions specified by patterns of expression with partially overlapping roles. *AHK4* is expressed most abundantly in root tissues and a single *ahk4* mutation results in a significant decrease in sensitivity to inhibition of root elongation by cytokinin in 8- or 10-day-old plants, but single *ahk2* and *ahk3* mutations exert no effect (Higuchi et al., 2004; Nishimura et al.,

2004). *ahk3* shows the strongest reduction of cytokinin delay of leaf senescence (Kim et al., 2006; Riefler et al., 2006), consistent with *AHK3* having the highest expression in leaves relative to the other two *AHK*s (Higuchi et al., 2004; Nishimura et al., 2004). The *ahk2,3* and *ahk3,4* double mutants but not any of the single mutants were inhibited in cytokinin-dependent de-etiolation, suggesting functional redundancy in this shoot response (Riefler et al., 2006). In all cytokinin response assays tested, increasing the number of *ahk* mutations increased the severity of the phenotype, indicating overlapping roles among *AHK*s. The triple *ahk2,3,4* mutant exhibits near complete insensitivity to the effects of cytokinin on induction of calli, inhibition of root elongation, delay of senescence and transcriptional induction of type-A *ARRs*, indicating that these three *AHK*s are required for cytokinin perception (Inoue et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004). In addition, the cytokinin content of *ahk3* shoots is higher than WT (Riefler et al., 2006). This increase in cytokinin levels is further elevated in *ahk3* double mutant combinations and in the *ahk2,3,4* triple mutant, trans-zeatin levels are increased 16-fold as compared to WT, suggesting that there is feedback control regulating cytokinin homeostasis.

AHPs mediate cytokinin signaling

The five AHPs have been linked to cytokinin signaling through various reports. In a yeast two-hybrid system, all five AHPs can interact with upstream cytokinin receptors AHKs and downstream phosphorelay components ARRs (Dortay et al., 2006). Phosphotransfer from the AHK4 to AHP1, from AHP1 and AHP2 to a subset of members of type-A, type-B and type-C ARRs has also been demonstrated (Suzuki et al., 1998; Imamura et al., 2001; Imamura et al., 2003; Kiba et al., 2004; Mähönen et al., 2006a). In

maize, yeast two-hybrid and *in vitro* phosphotransfer experiments have shown that HPs can interact and transfer phosphoryl groups to type-A and type-B ZmRRs (Sakakibara et al., 1999; Asakura et al., 2003). *AHP*1 can complement a yeast Hpt mutant, indicating that they can also function as phosphotransfer proteins in a heterologous yeast system (Suzuki et al., 1998). In Arabidopsis protoplasts, cytokinin treatment can alter cellular localization of AHP1 and AHP2 to result in protein accumulation in the nucleus (Hwang and Sheen, 2001). In periwinkle tissue culture, RNAi silencing of a *HP* resulted in reduced induction of a *RR*, suggesting that *HP*s are involved in cytokinin signaling (Papon et al., 2004).

Compelling evidence for the role of the five *AHP*s in cytokinin signaling is provided by analysis of loss-of-function mutants (Hutchison et al., 2006). T-DNA insertion alleles in all five *AHP*s were isolated; all were transcript nulls except for *ahp2*. Single and double *ahp* mutants generally show little differences in cytokinin responses as compared to the WT, suggesting a high level of genetic redundancy among *AHP*s, which is consistent with their overlapping expression patterns (Suzuki et al., 2000; Tanaka et al., 2004). The triple *ahp1,2,*3 and *ahp2,3,5* mutants show reduced sensitivity to cytokinin in hypocotyl elongation assays and *ahp1,2,3* also exhibits decreased sensitivity to cytokinin in inhibition of primary root elongation, lateral root formation and shoot chlorophyll content (Hutchison et al., 2006). These data indicate that *AHP*s are positive regulators of the cytokinin response, consistent with observations from *AHP2* overexpression (Suzuki et al., 2002). The quadruple mutant *ahp1,2,3,5* and quintuple mutant *ahp1,2,3,4,*5 show increased resistance to cytokinin in these assays, indicating additive and overlapping function (Hutchison et al., 2006). Interestingly, *AHP4* may act as a weak negative

regulator of cytokinin response in lateral root formation, because the quadruple *ahp1,2,3,4* is less resistant to cytokinin than *ahp1,2,3* in this assay. The quintuple *ahp1,2,3,4,*5 is greatly reduced in cytokinin induction of cytokinin primary response genes, *ARR5, ARR8* and *ARR9*, indicating that AHPs are required for the primary cytokinin signal transduction pathway. However, there still remains a significant residual response to cytokinin in *ahp1,2,3,4,5*, which may be explained by the 10% remaining *AHP2* activity. Alternatively, other pathways may compensate for loss of the *AHP*s in the quintuple mutant.

APHP1/AHP6, the pseudo *AHP* that cannot participate in phosphotransfer, has recently been identified as novel negative regulator of cytokinin signaling (Mähönen et al., 2006b). *ahp6* loss-of-function mutations can partially suppress the *wol* mutation of *AHK4*. *ahp6* mutants are hypersensitive to the effects of cytokinin on root vascular differentiation, indicating that *AHP6* is a negative regulator of cytokinin signaling (see below). *AHP6* is expressed in protoxylem and adjacent pericycle cells and its expression is further repressed by cytokinin, thus providing a positive feedback loop on cytokinin signaling. *In vitro,* AHP6 itself cannot be phosphorylated by the yeast HK, but addition of AHP6 can decrease phosphotransfer between the histidine transmitter and receiver domains of the yeast HK, and can also decrease phosphotransfer between AHP1 and ARR1, suggesting that AHP6 acts as a negative regulator of cytokinin signaling by interfering with phosphorelay.

Type-A ARRs are negative regulators of cytokinin signaling

Type-A ARRs are a family of ten genes originally identified as cytokinin primary response genes (Brandstatter and Kieber, 1998; Imamura et al., 1998; D'Agostino et al.,

2000). The type-A RRs of Arabidopsis, maize and rice have all been reported to be rapidly upregulated in response to cytokinin treatment in a variety of tissues (Brandstatter and Kieber, 1998; Sakakibara et al., 1999; D'Agostino et al., 2000; Asakura et al., 2003; Rashotte et al., 2003; Jain et al., 2006). Cytokinin induction of type-A *ARR*s is dependent on AHKs, AHPs and type-B ARRs (Sakai et al., 2001; Kiba et al., 2003; Higuchi et al., 2004; Nishimura et al., 2004; Hutchison et al., 2006; Riefler et al., 2006; Taniguchi et al., 2007; Yokoyama et al., 2007). Analysis of loss-of-function mutants and overexpressors of type-A *ARR*s indicate that at least eight of the ten type-A ARRs are negative regulators of cytokinin signaling. Single T-DNA loss-of-function type-A *arr* mutants show no significant difference from WT in their responses to cytokinin, while double and higher order mutants up to an *arr3,4,5,6,8,9* sextuple show increasing hypersensitivity to cytokinin inhibition of root elongation and lateral root formation, cytokinin delay of leaf senescence and cytokinin induction of callus and shoot formation in tissue culture (To et al., 2004). This indicates that the type-A ARRs additively contribute to repression of these cytokinin responses. Consistent with these results, overexpression of *ARR4, ARR5, ARR6, ARR7, ARR9* and *ARR15* result in a decrease in cytokinin responsiveness in inhibition of root elongation (Kiba et al., 2003; Lee et al., 2007; To et al., 2007), and overexpression of a rice type-A *ARR, OsRR6,* results in cytokinin hypersensitivity in callus formation (Hirose et al., 2007). In *arr3,4,5,6,8,9,* the induction of *ARR7* (a type-A ARR not included in the sextuple mutant combination) is also enhanced, indicating that these six type-A *ARRs* are negative regulators of the primary cytokinin response (To et al., 2004). Consistent with this result, overexpression of *ARR4, ARR5, ARR6* and *ARR7* in protoplasts can repress *ARR6:LUC* (Hwang and Sheen, 2001), and ARR7 overexpression

in plants also represses induction of cytokinin-regulated genes, including type-A *ARR*s (Lee et al., 2007). The high degree of redundancy among type-A ARRs observed in cytokinin responses is consistent with upregulated and generally overlapping expression patterns under cytokinin assay conditions. However, a subset of the type-A *arr* mutants show tissue-specific phenotypes consistent with their patterns of expression, including reduced rosette size, altered shoot patterning, elongated petioles and lengthened circadian clock (described in following sections).

A recent study explores the mechanism by which type-A ARRs negatively regulate cytokinin signaling (To et al., 2007). An unphosphorylatable *ARR5* ^{D85A} mutant cannot complement *arr3,4,5,6* hypersensitivity to cytokinin inhibition of root elongation, indicating that phosphorylation of ARR5 is required for function. An ARR5^{D87E} partial phosphomimic can partially complement *arr3,4,5,6* cytokinin hypersensitivity, which suggests that the phosphorylated type-A ARR protein is the functional form. Consistent with activation of type-A ARR activity by phosphorylation, overexpression of an ARR7^{D85E} phosphomimic, but not WT ARR7, can alter shoot development (Leibfried et al., 2005) (see section below). A subset of type-A ARR proteins, including ARR5, ARR6 and ARR7 are stabilized in the presence of cytokinin and stabilization of these type-A ARR proteins requires upstream phosphorelay components, AHKs and AHPs (To et al., 2007). Furthermore, unphosphorylatable ARR5^{D87A} and ARR7^{D85A} proteins are less stable than their respective WT proteins, while phosphomimic ARR5^{D87E} and ARR7^{D85E} are more stable, suggesting that phosphorylation regulates type-A ARR protein turnover. However, protein turnover of the mutant type-A ARRs is still partially responsive to cytokinin, and cytokinin-mediated stabilization of ARR5 is reduced in the quadruple

type-B *arr1,2,10,12* mutant background, indicating that other factors, possibly transcriptional targets of type-B ARRs, are also required for cytokinin stabilization of type-A ARRs. These results indicate that type-A ARRs may be activated by phosphorylation in part through protein stabilization, thus reinforcing the negative feedback loop on cytokinin signaling. Phosphorylation can further activate type-A ARR functions in other plant processes.

Type-B ARRs are positive activators of cytokinin signaling

The eleven type-B ARRs are transcriptional activators that regulate transcription of cytokinin activated targets, including the type-A ARRs (Sakai et al., 2000; Hwang and Sheen, 2001; Sakai et al., 2001; Mason et al., 2005; Taniguchi et al., 2007). The 11 type-B ARRs are further subdivided into groups I, II and III (Mason et al., 2004). Analysis of loss-of-function and overexpressors indicates that at least six of the eight group I type-B *ARR*s are positive regulators of cytokinin response (Imamura et al., 2003; Tajima et al., 2004; Mason et al., 2005; Yokoyama et al., 2007). A single loss-of-function T-DNA insertion in *ARR1* resulted in reduced cytokinin response in inhibition of root elongation, in initiation of shoot formation in tissue culture*; ARR1* overexpression resulted in the opposite phenotypes (Sakai et al., 2001). Loss-of-function T-DNA insertion mutations *arr1, arr2, arr10, arr11, arr12* and *arr18* additively increased seedling resistance to cytokinin inhibition of root elongation (Mason et al., 2005; Yokoyama et al., 2007), indicating that these genes have overlapping functions as positive regulators of cytokinin signaling. The contribution of the *arr18* mutation to the root response was much weaker than the other type-B *ARRs* (Mason et al., 2005), consistent with low levels of *ARR18* expression in the root relative to *ARR1, ARR2, ARR10, ARR11* and *ARR12* (Sakai et al.,

2001; Imamura et al., 2003; Mason et al., 2004; Tajima et al., 2004; Mason et al., 2005; Yokoyama et al., 2007). The *arr1,10,12* triple mutant is almost completely insensitive to cytokinin inhibition of root elongation, lateral root formation, induction of callus formation and also shows a strong reduction in cytokinin upregulation of multiple type-A *ARR* transcripts, indicating that these type-B ARRs are required for type-A ARR transcription in cytokinin primary response (Mason et al., 2005). Consistent with this, *arr1* exhibits reduced induction of a type-A *ARR, ARR6* (Sakai et al., 2001), and overexpression of *ARR2* in Arabidopsis protoplasts has been reported to activate an ARR6:GFP reporter (Hwang and Sheen, 2001; Kim et al., 2006).

Similar to bacterial response regulators, type-B ARR transcriptional activity in the C-terminal domains is predicted to be repressed by the N-terminal receiver domain; activation of type-B ARR transcription can be achieved by eliminating the receiver domain or mutating it to a phosphomimic form (Sakai et al., 2001; Tajima et al., 2004; Kim et al., 2006). Overexpression of truncated versions of type-B *ARRs*, which contained the C-terminal DNA binding and transactivating domains but lacked the repressive N-terminal receiver domain, resulted in hypersensitivity to cytokinin in a callus greening assay for group I members *ARR1, ARR11* and group II member *ARR21*, suggesting that both these two groups of type-B *ARRs* may act as positive regulators of cytokinin signaling (Sakai et al., 2001; Tajima et al., 2004; Kim et al., 2006). However, while the *ARR21* C-terminus overexpressor resulted in upregulation of type-A *ARR*s, the *ARR11* C-terminus overexpressor did not significantly change cytokinin induction of type-A *ARRs*, indicating that individual type-B *ARR*s may have distinct transcriptional targets (Imamura et al., 2003; Tajima et al., 2004). Microarray experiments for *arr1,12* seedlings, *arr10,12*

roots and an *ARR21* C-terminus overexpressor and a high coverage expression profiling experiment for an overexpressor of the *ARR1* C-terminus have been pursued to identify genes regulated by type-B *ARRs* (Hass et al., 2004; Tajima et al., 2004; Kiba et al., 2005; Rashotte et al., 2006; Yokoyama et al., 2007). Due to the differences in experimental conditions, it is difficult to make conclusions about target specificities. Collectively, besides the type-A ARRs, these studies have recovered a subset of the previously identified cytokinin targets (Rashotte et al., 2003) including the cytochrome P450 genes involved with cytokinin metabolism, a cytokinin oxidase that degrades cytokinins, several expansins, putative glutaredoxins, putative transferase-familiy genes, putative disease resistance-responsive genes and many transcription factors including the Cytokinin Response Factors (CRFs) (Hass et al., 2004; Tajima et al., 2004; Kiba et al., 2005; Rashotte et al., 2006; Yokoyama et al., 2007). Interestingly, a subset of these genes, including the CRFs, were not induced by cytokinin in the presence of the protein synthesis inhibitor CHX, indicating that the effect of type-B ARRs on their transcription is indirect and may be mediated via other transcription factors downstream of the type-B ARRs (Yokoyama et al., 2007).

Cytokinin Response Factors are novel regulators of cytokinin response

The *Cytokinin Response Factors* (*CRF*s) were originially identified in microarray experiments as a target of cytokinin regulated transcription in Arabidopsis (Rashotte et al., 2003). The *CRF*s are a family of six genes belonging to the greater *APETALA2*-like class of plant specific transcription factors and are distantly related to the *Ethylene Response Factors* (Rashotte et al., 2006). Three of the six *CRF* transcripts are rapidly induced in seedlings by cytokinin and all six CRF:GFP fusion proteins rapidly

accumulate in the nucleus in response to cytokinin treatment. Accumulation of CRF2:GFP in the nucleus is dependent on *AHK*s and *AHPs* but not type-A or type-B *ARR*s, showing a novel mechanism for cytokinin regulation. CRF proteins may be imported into the nucleus in response to cytokinin to allow DNA binding and activation of target gene transcription. Indeed, microarray experiments show that cytokinin regulation of gene expression is altered in *crf1,2,5* and *crf2,3,6*: 55% and 48% of cytokinin regulated transcripts show reduced responsiveness to cytokinin *crf1,2,5* and *crf2,3,*6 respectively, and the genes misregulated in each of the *crf* mutants overlap with approximately two thirds the genes misregulated in the type-B *ARR* double mutant *arr1,12*, indicating that *CRF*s and type-B *ARRs* transcriptional targets overlap and together *CRF*s and type-B *ARRs* may regulate gene expression in response to cytokinin (Rashotte et al., 2006). Interestingly, cytokinin induction of type-A *ARR*s is not greatly affected in *crf1,2,5* or *crf2,3,*6. In addition, T-DNA insertion mutants of the *CRF*s do not exhibit significant changes in sensitivity to cytokinin in seedling root elongation or shoot initiation in tissue culture. However, *crf* mutants display low penetrant defects in cotyledon and leaf expansion mostly due to reduced cell expansion. The penetrance and severity of the cotyledon phenotype increases with increasing *crf* mutations, resulting in a 96% reduction in cotyledon size in the triple *crf1,2,5*. The *ahk2,3,4* mutant also exhibits a 93% reduction in cotyledon size, suggesting that cotyledon expansion may be associated with the cytokinin signaling pathway. However, the *crf1,2,5* are also display loss of pigmentation and the *crf5,6* double mutant exhibits embryonic lethality, both of which are phenotypes not observed in mutants of two-component system genes. The differences between two-component mutant phenotypes and *crf* mutant phenotypes may reflect the

differences in cytokinin-regulated target transcription, including the type-A *ARR*s, further suggest that a subset of processes downstream of CRFs are distinct from those the cytokinin primary signaling pathway. The response of CRF protein localization to cytokinin represents a novel branch point off of the known cytokinin-activated two-component phosphorelay. How CRF localization is regulated by cytokinin, and how this rapid cytokinin response is mediated from the AHKs and AHPs to these CRF proteins, which bear no resemblance to evolutionarily conserved phosphorelay components, are intriguing questions.

Cytokinin modulates shoot meristem function

The shoot meristem consists of a central group of undifferentiated cells at the shoot apex, which divide and feed into the peripheral regions to produce organs (Shani et al., 2006). Reducing cytokinin levels in Arabidopsis, by overexpressing cytokinin degrading CYTOKININ OXIDASES (CKX) and in cytokinin biosynthetic *isopentenyl transferase (ipt)* multiple loss-of-function mutants, results in reduced rosette and shoot meristem size (Werner et al., 2003; Miyawaki et al., 2006), indicating that cytokinins are required for vegetative meristem function. In rice, loss of a shoot meristem specific cytokinin activating enzyme further results in meristem termination, indicating that active cytokinin levels directly regulate meristem activity (Kurakawa et al., 2007). *CKX* overexpressors and *ipt* also develop a smaller inflorescence and a rice cytokinin oxidase was found to regulate inflorescence patterning to alter grain yield (Werner et al., 2003; Ashikari et al., 2005; Miyawaki et al., 2006), indicating that cytokinins also play a role in inflorescence meristems. In addition, a rice loss-of-function type-B OsRR mutant and an *ARR7* overexpressor both flower early (Lee et al., 2007), suggesting that cytokinin

signaling components may also play a role in controlling the transition from vegetative to reproductive phase.

Cytokinin upregulates members of a family of Class 1 *KNOTTED1-like homeobox* (*KNOX*) transcription factors that specify shoot meristem identity (Hamant et al., 2002). Overexpression of *KNOX* genes have been reported to increase cytokinin content and result in phenotypes associated with increased cytokinin responsiveness and meristematic activity (Frugis et al., 1999; Hamant et al., 2002; Sakamoto et al., 2006). In fact, a member of the KNOX gene family, SHOOTMERISTEMLESS (STM), can directly upregulate *IPTs* and increase cytokinin content in seedlings (Jasinski et al., 2005b; Yanai et al., 2005). These results suggest that cytokinin and shoot meristem identity genes interact in a positive feedback loop. In addition, exogenous application of cytokinin and expression of *IPT* driven by *STM* promoter can partially complement an *stm* loss-of-function phenotype, indicating that *STM* acts in part through activating cytokinin biosynthesis (Jasinski et al., 2005b; Yanai et al., 2005). Furthermore, the Arabidopsis *ahk2,3* double mutant exhibits reduced rosette size and stunted growth, suggesting cytokinin signaling positively regulates meristem function (Higuchi et al., 2004; Nishimura et al., 2004). The shoot phenotype of *ahk2,3* is not observed in any of the single *ahk* mutants or either of the double mutant carrying the *ahk4* mutation, suggesting that AHK2 and AHK3 play redundant roles and more prominent roles than *AHK4* in the shoot meristem, which is consistent with their relative levels of expression in the shoot (Higuchi et al., 2004; Nishimura et al., 2004). In *ahk2,3,4*, the overall size and cell number of the shoot apical meristem is reduced and the inflorescence meristem is further impaired, generating a short and thin inflorescence that terminates after producing

only a few flowers (Higuchi et al., 2004; Nishimura et al., 2004). This indicates that the three cytokinin receptors additively contribute to shoot and inflorescence meristem function. Consistent with the role of cytokinin signaling pathway in the meristem, *STM* activation upregulates *ARR5*, a cytokinin primary response gene, and a weak *stm* mutant phenotype is enhanced by the a *AHK4* allele *wol* (Jasinski et al., 2005b; Yanai et al., 2005), suggesting that *KNOX* function also involves the primary cytokinin signaling pathway.

One function of the cytokinin signaling pathway in the meristem is to antagonize gibberellins. Cytokinin application can partially rescue an *stm* mutant, and this effect is repressed by application of another hormone gibberellin (Yanai et al., 2005). Cytokinin and KNOX both downregulate gibberellin levels in the meristem by transcriptionally regulating gibberellin metabolism: KNOX represses gibberellin biosynthetic GA20 oxidase whereas cytokinin upregulates GA2 oxidases to deactivate gibberellins (Hay et al., 2002; Rashotte et al., 2003; Jasinski et al., 2005a). Expression of GA2 oxidase is reduced in the *AHK4* loss-of-function mutant *wol*, indicating that cytokinin signaling via AHK4 is required for GA2 oxidase expression (Jasinski et al., 2005a). Thus, KNOX may also downregulate gibberellin in part through upregulating cytokinin biosynthesis and signaling to activate GA2 expression. In addition, increasing gibberellin signaling by exogenous gibberellin application or in a constitutive gibberellin signaling mutant *spindly*, decreases cytokinin responses, indicating that there is also feedback regulation between gibberellin and cytokinin signaling in the meristem (Greenboim-Wainberg et al., 2005).

Type-A RRs, which are negative regulators of cytokinin signaling, may also act as negative regulators in the meristem. In maize, a type-A *ZmRR3* loss-of-function mutant *abphyl1* develops an enlarged shoot apical meristem with increased *KNOTTED1* expression and develops an abnormal, parallel phyllotactic pattern (Jackson and Hake, 1999; Giulini et al., 2004). These results suggest that this type-A *RR* may repress meristem function by interfering with the positive feedback loop between *KNOX* genes and cytokinin signaling, thus loss of this type-A *RR* results in increased meristem activity and abnormal phyllotaxy. Consistent with this observation, overexpression of a closely-related type-A *RR* in rice, *OsRR6,* results in meristem arrest (Hirose et al., 2007).

In Arabidopsis, a septuple type-A *arr3,4,5,6,7,8,9* mutant also displays abnormal phyllotaxy in the inflorescence, and overexpression of an $ARR7^{D85E}$ phosphomimic results in meristem arrest, suggesting that these type-A *ARRs* negatively regulate meristem function by phosphodependent interactions (Leibfried et al., 2005; To et al., 2007). Furthermore, a subset of type-A *ARR* transcripts*: ARR5*, *ARR6*, *ARR7* and *ARR15* are directly repressed by the homeodomain protein WUSCHEL (WUS*)*, which is required for shoot meristem maintenance (Leibfried et al., 2005). A model can be proposed for a feedback regulatory mechanism in which *WUS* expression can repress type-A *ARRs* to upregulate cytokinin signaling, and cytokinin signaling can in turn upregulate type-A *ARR*s to repress *WUS* expression and meristem maintenance.

Overexpression of truncated C-terminal versions of type-B *ARRs, ARR1*, *ARR11* and *ARR14,* which have constitutive transcriptional activity, results in phenotypes which are associated with a hyperactive shoot meristem (Sakai et al., 2001; Imamura et al., 2003; Tajima et al., 2004). These results point to a model that cytokinin may signal

through the receptor *AHK*s and the signal activates, probably via phosphorylation of a subset of type-B ARRs, transcription of target genes to positively regulate meristem function. The primary cytokinin signaling pathway may interact with local transcription factors in the shoot meristem, such as KNOX and WUS to regulate meristem maintenance. Cytokinin signaling may also interact with other hormones, such as gibberellin, to regulate meristem function, and potentially auxin, which regulates organ initiation (Kuhlemeier, 2007). The cytokinin signaling pathway may serve to coordinate signals among positive and negative feedback loops to balance meristem maintenance and proper organ initiation.

Cytokinin signaling determines root meristem size

A recent study reported that inhibition of root elongation by exogenous cytokinin application is due to an overall decrease in root meristem size, defined as the region between the quiescent center and the differentiation zone (Dello Ioio et al., 2007). Reduced cytokinin levels in *CKX* overexpressing plants or in loss-of-function *ipt* mutants result in increased primary elongation and lateral root formation (Werner et al., 2003; Miyawaki et al., 2006). An *ipt3,5,7* mutant also develops an enlarged root meristem and this effect can be recapitulated by specifically overexpressing *CKX1* in the root vasculature but not in other root tissues (Dello Ioio et al., 2007). Cytokinin signaling mutants *ahk3* and *arr1,12* exhibit enlarged root meristems and accelerated root growth in very young seedlings, indicating that these cytokinin signaling elements are involved in inhibition of root meristem function (Dello Ioio et al., 2007). Consistent with these results, basal root elongation in a cytokinin hypersensitive type-A *arr3,4,5,6,8,*9 sextuple mutant is reduced (To et al., 2004). Interestingly, the *ahk2,3,4* triple receptor mutant

displays reduced root growth, with a decrease in size and activity of the root meristem (Higuchi et al., 2004; Nishimura et al., 2004), similarly, the *ahp1,2,3,4,*5 quintuple mutant shows a dramatic reduction in cell division activity in the meristem region (Hutchison et al., 2006), suggesting that although high levels of cytokinin in the root inhibit meristem activity, some cytokinin signaling in the root is still required for meristem function. Exogenous auxin increases meristem size and it is possible that a threshold level of cytokinin signaling is necessary to antagonize the effects of auxin in the root (Beemster and Baskin, 2000; Dello Ioio et al., 2007).

Cytokinin regulates root vascular differentiation via two-component phosphorelay

Cytokinin signaling was intially linked to root vascular differentiation by isolation of the *wol* mutant, which carries a recessive missense mutation in *AHK4* (Mähönen et al., 2000). Unlike *cre1* and other loss-of-function mutations in *AHK4* that confer reduced cytokinin responsiveness and no obvious morphological changes (Inoue et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006), the *wol* mutant develops a short primary root and no lateral roots (Mähönen et al., 2000). The *wol* mutant has a reduced number of embryonic root vascular initials, which give rise to a seedling root with reduced vascular cell files that form only protoxylem and lack metaxylem, phloem and procambial cells (Mähönen et al., 2000).

Two recent papers (Mähönen et al., 2006b; Mähönen et al., 2006a) provide an explanation for the long standing puzzle of the *wol* allele (de Leon et al., 2004) and demonstrate a divergence from the simple and linear AHK→ AHP→ ARR phosphorelay. The authors show that *wol* can be phenocopied by depleting cytokinins specifically in *AHK4* expressing cells in the seedling root, suggesting that the root defects in *wol* are

caused by reduced cytokinin signaling in these specific cell types (Mähönen et al., 2006b). In addition, exogenous cytokinin application reduces protoxylem cell files and increases cell files of other vascular cell identities, indicating that cytokinin is sufficient to inhibit protoxylem formation and promote vascular differentiation (Mähönen et al., 2006b). In a heterologous yeast system and *in vitro*, AHK4/CRE1 displays distinct biochemical properties in the presence and absence of cytokinin: in the absence of cytokinin, it functions as a phosphatase; on cytokinin binding, AHK4/CRE1 is activated as a kinase to phosphorylate AHPs to activate downstream response regulators (Mähönen et al., 2006a). The WOL/CRE^{T278I} protein is insensitive to cytokinin activation of kinase activity and has constitutive phosphatase activity (Mähönen et al., 2006a). Hence the two separable activities of AHK4 can account for both the recessive *wol1* phenotype and the other recessive cytokinin insensitive *cre1/ahk4* mutant alleles. WOL may act by constitutively dephosphorylating AHPs and reducing the pool of phosphorylated AHPs required for vascular differentiation in the primary root. In fact, a *cre1ahk3* double mutant also exhibits a weak defect in vascular differentiation, indicating that reduced kinase activity can result in similar though less pronounced vascular defects (Mähönen et al., 2006b). Consistent with this model, primary root length and xylem development are reduced in a *ahp2,3,5* triple mutant and differentiation of metaxylem is further abolished in a *ahp1,2,3,4,5* quintuple mutant primary root (Hutchison et al., 2006). A model for bi-directional phosphorelay was proposed for AHK4/CRE1 to control phosphate flux through the pathway (Mähönen et al., 2006a). A similar bi-directional phosphorelay has been described in the bacterial Arc system to facilitate signal decay (Georgellis et al., 1998; Pena-Sandoval et al., 2005). Interesting to note, neither AHK2 nor AHK3

demonstrated phosphatase activity *in vitro* nor in yeast (Mähönen et al., 2006a), which may suggest that the phosphatase activity may be a specific mechanism for root vascular development. It would be interesting to determine if the monocot HKs display similar specificities in expression patterns and biochemical properties.

A further level of control on phosphorelay and vascular differentiation is imposed by the pseudo *AHP*, *APHP1/AHP6*. The *ahp6-1* allele was identified as a suppressor of *wol* (Mähönen et al., 2006b). The *wol1ahp6-1* double mutant can generate some procambial and phloem cell files and can also form a few lateral roots. *ahp6* mutants develop reduced protoxylem cell files and are hypersensitive to cytokinin inhibition of protoxylem formation, indicating that *AHP6* is a negative regulator of cytokinin signaling in vascular differentiation. *In vitro* experiments suggest that AHP6 may interfere with phosphotransfer between two-component elements *in vitro* to reduce flux of phosphoryl groups through the pathway (Mähönen et al., 2006b). A type-A ARR, *ARR15*, is expressed in the procambial cells and the expression pattern is expanded to protoxylem cell files in the *ahp6-1* background (Mähönen et al., 2006b), indicating that AHP6 downregulates the cytokinin primary signaling pathway. It remains to be determined if ARR15 plays a role in vascular differentiation.

Overexpression of a type-C ARR, ARR22, has also been reported to result in a phenotype similar to *wol* (Kiba et al., 2004). While it remains unclear whether ARR22 acts as a negative regulator of cytokinin signaling at endogenous levels, overexpressed ARR22 proteins probably act by dephosphorylating AHPs, and thus reducing phosphate flow in the cytokinin signaling pathway, resulting in increased protoxylem formation and reduced differentiation of other vascular cell types.

The output of the system has been proposed to be determined in part by type-B ARRs *ARR1, ARR10* and *ARR12* (Yokoyama et al., 2007). A strong type-B *arr1,10,12* loss-of-function mutant produces a short, *wol*-like primary root with only protoxylem cell files in the root vasculature. Cytokinin-activated transcription is also reduced in *arr1,10,12*, suggesting that *ARR1, ARR10* and *ARR12* may activate transcription to inhibit protoxylem differentiation and allow specification of other vascular cell types .

A model may be proposed for cytokinin regulation of vascular differentiation: cytokinins are perceived by the three AHKs and the signal is transmitted via phosphorelay from the AHKs to the five AHPs to the type-B ARRs ARR1, ARR10 and the phosphorylated type-B ARRs may activate transcription of genes necessary for proper vascular differentiation. Negative feedback regulation of this phosphorelay may be introduced by phosphatase activity of AHK4, which has also been shown to be transcriptionally upregulated by cytokinin (Rashotte et al., 2003; Yokoyama et al., 2007), while positive feedback regulation may be imposed by inhibition of phosphotransfer by APHP1/AHP6, which is transcriptionally repressed by cytokinin (Mähönen et al., 2006b). Type-C ARRs may also add another level of feedback control.

Cytokinin controls leaf senescence via AHK3 and ARR2

Exogenously-applied cytokinin can delay leaf senescence (Mok and Mok, 1994), as can expression of the cytokinin biosynthetic gene *IPT* under a senescence-specific promoter (Gan and Amasino, 1995). A multiple loss-of-function type-A *arr3,4,5,6,8,9* mutant is hypersensitive to cytokinin inhibition of leaf senescence, suggesting that this process may be regulated by the cytokinin signaling pathway (To et al., 2004). Recently *ore1*-12, a missense gain-of-function mutation in *AHK3* was identified in a screen for

mutants that exhibited delayed senescence in the plant and in dark induced leaf senescence and suppressed senescence associated gene expression (Kim et al., 2006). Overexpression of *AHK3* recapitulated the phenotype of *ore1-12* and a loss-of-function *ahk3* allele exhibited early senescence, indicating that *AHK3* is a positive regulator for delay of senescence. Overexpression of *ARR2* also results in delay of dark-induced leaf senescence and upregulation of *ARR6:LUC* reporter in protoplast cells in a manner dependent on the conserved Asp phosphorylation target in the receiver domain, suggesting that ARR2 can modulate cytokinin signal in delaying leaf senescence and that phosphorylation of ARR2 is required for protein function. ARR2 phosphorylation in response to cytokinin in protoplasts is dependent on *AHK3*, but not *AHK2* or *AHK4*. In addition, *ahk3* is the only single *ahk* mutant showing a reduction in sensitivity to cytokinin delay of dark-induced leaf senescence, which is further reduced in the *ahk2,3* mutant, while the *ahk4* mutation did not contribute to the phenotype (Kim et al., 2006; Riefler et al., 2006). These results support a model in which AHK3 is the main cytokinin receptor regulating leaf senescence. Activation of AHK3 results in phosphorylation of type-B ARRs, including ARR2, and induction of target gene transcription. Interestingly, ARR2 has also been associated with ethylene signaling (Hass et al., 2004), and the hormone ethylene also regulates the timing of senescence (Grbic and Bleecker, 1995). It remains to be determined if the role of ARR2 in regulating leaf senescence is linked to ethylene signaling.

Cytokinin affects seed set and germination

Seed maturation is varied in cytokinin oxidase overexpressors, with frequent seed abortions resulting in fewer, larger seeds per sillique and the larger seeds contain larger

embryos (Werner et al., 2003). Multiple loss-of-function *ipt* mutants may also exhibit similar phenotypes (Miyawaki et al., 2006), suggesting that cytokinin is involved in embryo development. The *ahk2,3,4* triple mutant and *ahp1,2,3,4,5* quintuple mutant also form larger seeds with larger embryos (Hutchison et al., 2006; Riefler et al., 2006), implicating a role for cytokinin signaling. Cross-pollination experiments have determined that the effect of the triple *ahk3,4* mutations is maternal (Riefler et al., 2006). Furthermore, *ahk* mutants additively germinate earlier than WT, and triple *ahk2,3,4* mutants are resistant to far-red light inhibition of germination (Riefler et al., 2006). It is currently unclear whether the embryo size and germination traits are related. The signaling components downstream of the *AHP*s remain to be determined. Furthermore, regulation of seed germination is coordinately regulated by sugars and other phytohormones, such as gibberellin, abscisic acid and ethylene (Gibson, 2004; Chiwocha et al., 2005; Yuan and Wysocka-Diller, 2006). It is not known if cytokinin interacts with these hormones during seed development and germination and if the interactions use mechanisms similar to that in other developmental pathways.

A subset of type-A ARRs modulate circadian rhythms via cytokinin dependent and independent mechanisms

Circadian rhythms have periods of about 24 hours and by definition, can persist under constant environmental conditions after entrainment. They function to provide the plant with a measure of time (reviewed in (McClung, 2006)). The *ARR*s are ancestrally related to the *APRRs*, a subset of which regulate circadian rhythms (reviewed in (McClung, 2006)). The light receptor *PHYTOCHROME B* (*PHYB*) entrains the circadian clock and a *phyB* loss-of-function mutant displays lengthened period in red light and a leading phase in white light (Salomé et al., 2002). The type-A ARR protein, ARR4, has

been reported to interact with PHYB and stabilize it in the active far-red light absorbing form (Sweere et al., 2001). The *arr3,4* and *arr3,4,5,6* mutants display longer periods and a leading phase similar to *phyB*, suggesting that these type-A *ARRs* modulate PHYB signaling to the circadian clock (Salomé et al., 2005). *arr3,4* and *arr3,4,5,6* mutants exhibit longer periods, but not their component single mutants or the *arr5,6* double mutant, indicating that *ARR3* and *ARR4* function redundantly in this response (Salomé et al., 2005). *arr8* and *arr9* suppress the effect of the *arr3,4* mutations and an *arr3,4,8,9* quadruple mutant exhibits WT period length (Salomé et al., 2005), consistent with previous observations that the two gene pairs act antagonistically in longer petioles, another phenotype observed in *phyB* mutants (To et al., 2004). However, the *phyB* mutation lengthens the period under red but not blue light, while a *arr3,4* mutant shows a lengthened period under red, blue and even no light when PHYB is inactive, indicating that in addition to PHYB, *ARR3* and *ARR4* may interact with other targets to modulate circadian period.

Interestingly, the lengthened circadian period of the *arr3,4,5,6* mutant appears to be a cytokinin independent effect (Salomé et al., 2005). Exogenous application of cytokinin on WT, *arr3,4* and *arr3,4,5,6* seedlings did not increase period length and the relative cytokinin sensitivity of other cytokinin signaling mutants, such as *ahk3,4, arr3,4,8,*9 and *arr3,4,5,6,8,9,* and type-A *ARR* overexpressors also did not correlate with their period length. In addition, complementation of *arr3,4,5,6* cytokinin hypersensitivity by *ARR5* failed to rescue the lengthened period, indicating that type-A ARR function in cytokinin response and circadian period are separable. A cytokinin hypersensitive mutant *ckh* has been reported to show a shortened period in the dark (Hanano et al., 2006). *CKH*

is not a known component of the cytokinin primary signaling pathway and it is currently unclear how this mutant affects cytokinin response.

Although the cytokinin application has little effect on circadian periodicity, the phase of circadian rhythm can be altered by cytokinin (Salomé et al., 2005; Hanano et al., 2006). Cytokinin application results in a lagging phase and the effect is enhanced in an *ARR4* overexpressing line and reduced in a *phyb* mutant (Hanano et al., 2006). Furthermore, the effect of *ARR4* overexpression is epistatic to *phyb*, indicating that cytokinin may delay circadian phase through *ARR4* in another *PHYB* dependent mechanism.

Hence, two different paramenters of circadian rhythms can be modulated by type-A *ARRs*. It will be interesting to determine the mechanism for antagonistic interactions between ARR3/ARR4 and ARR8/ARR9 and how their interactions may affect PHYB function in regulating period length, and if these four type-A ARRs interact through similar mechanisms to regulate circadian phase through PHYB. It is intriguing that the same set of type-A ARRs may have cytokinin dependent and independent effects on PHYB, and also PHYB dependent and independent effects on the clock. How these interactions are specified remains to be determined.

CONCLUDING REMARKS

In summary, characterization of loss-of-function mutants in cytokinin signaling component has led us to an understanding of their overlapping roles in cytokinin signal transduction, and have begun to uncover specific roles in shoot and root development, leaf senescence, seed development and circadian rhythms. Detailed examination of gene expression and mutant phenotypes in root vascular tissues have revealed specific interactions between a subset of two-component genes and revealed mechanisms for signal transduction. Further study of gene expression and mutant phenotypes at higher tissue-specific resolution will be necessary overcome genetic redundancy at the gross whole plant level and elucidate the function of cytokinin signaling in other developmental processes.

The role of cytokinin signaling in many of these developmental pathways involves coordinating other hormonal and environmental signals. Mutants in the cytokinin signaling pathway, particularly the receptor mutants, will be important tools for dissecting the interactions with other signaling pathways.

Furthermore, three different classes of effectors of the pathway, the type-A ARRs, type-B ARR and the CRFs, have now been identified. These three classes of effectors are each activated by cytokinin in a different way and are likely to activate different outputs of the cytokinin response. Other effectors may be identified by screens for elements downstream of known signaling components. Studying the mechanisms for activation of these effectors, such as protein stabilization and protein localization, and defining the specific targets of these effectors will be important areas to pursue.

REFERENCES

Asakura, Y., Hagino, T., Ohta, Y., Aoki, K., Yonekura-Sakakibara, K., Deji, A., Yamaya, T., Sugiyama, T., and Sakakibara, H. (2003). Molecular characterization of His-Asp phosphorelay signaling factors in maize leaves: implications of the signal divergence by cytokinin-inducible response regulators in the cytosol and the nuclei. Plant Mol. Biol. **52,** 331-341.

Ashikari, M., Sakakibara, H., Lin, S., Yamamoto, T., Takashi, T., Nishimura, A., Angeles, E.R., Qian, Q., Kitano, H., and Matsuoka, M. (2005). Cytokinin oxidase regulates rice grain production. Science **309,** 741-745.

Beemster, G.T.S., and Baskin, T.I. (2000). STUNTED PLANT 1 mediates effects of cytokinin, but not of auxin, on cell division and expansion in the root of Arabidopsis. Plant Physiol. **124,** 1718-1727.

Brandstatter, I., and Kieber, J.J. (1998). Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in Arabidopsis. Plant Cell **10,** 1009-1020.

Chiwocha, S.D.S., Cutler, A.J., Abrams, S.R., Ambrose, S., Yang, J., Ross, A.R.S., and Kermode, A.R. (2005). The etr1-2 mutation in Arabidopsis thaliana affects the abscisic acid, auxin, cytokinin and gibberellin metabolic pathways during maintenance of seed dormancy, moist-chilling and germination. Plant J. **42,** 35-48.

D'Agostino, I., Deruère, J., and Kieber, J.J. (2000). Characterization of the response of the Arabidopsis *ARR* gene family to cytokinin. Plant Physiol. **124,** 1706-1717.

de Leon, B.G.-P., Zorrilla, J.M.F., Rubio, V., Dahiya, P., Paz-Ares, J., and Leyva, A. (2004). Interallelic complementation at the Arabidopsis CRE1 locus uncovers independent pathways for the proliferation of vascular initials and canonical cytokinin signalling. Plant J. **38,** 70-79.

Dello Ioio, R., Linhares, F.S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., and Sabatini, S. (2007). Cytokinins determine Arabidopsis root-meristem size by controlling cell differentiation. Curr. Biol. **17,** 678-682.

Dortay, H., Mehnert, N., Burkle, L., Schmulling, T., and Heyl, A. (2006). Analysis of protein interactions within the cytokinin-signaling pathway of Arabidopsis thaliana. FEBS J. **273,** 4631-4644.

Du, L., Jiao, F., Chu, J., Chen, M., and Wu, P. (2007). The two-component signal system in rice (Oryza sativa L.): A genome-wide study of cytokinin signal perception and transduction. Genomics **In Press, Corrected Proof**.

Ferreira, F.J., and Kieber, J.J. (2005). Cytokinin signaling. Curr. Opin. Plant Biol. **8,** 518-525.

Frugis, G., Giannino, D., Mele, G., Nicolodi, C., Innocenti, A.M., Chiappetta, A., Bitonti, M.B., Dewitte, W., Van Onckelen, H., and Mariotti, D. (1999). Are homeobox knotted-like genes and cytokinins the leaf architects? Plant Physiol. **119,** 371-374.

Gan, S., and Amasino, R.M. (1995). Inhibition of leaf senescence by autoregulated production of cytokinin. Science **270,** 1986-1988.

Georgellis, D., Kwon, O., De Wulf, P., and Lin, E.C.C. (1998). Signal decay through a reverse phosphorelay in the Arc two-component signal transduction system. J. Biol. Chem. **273,** 32864-32869.

Gibson, S.I. (2004). Sugar and phytohormone response pathways: navigating a signalling network. J. Exp. Bot. **55,** 253-264.

Giulini, A., Wang, J., and Jackson, D. (2004). Control of phyllotaxy by the cytokinin-inducible response regulator homologue ABPHYL1. Nature **430,** 1031-1034.

Grbic, V., and Bleecker, A.B. (1995). Ethylene regulates the timing of leaf senescence in Arabidopsis. Plant J. **8,** 595-602.

Greenboim-Wainberg, Y., Maymon, I., Borochov, R., Alvarez, J., Olszewski, N., Ori, N., Eshed, Y., and Weiss, D. (2005). Cross talk between gibberellin and cytokinin: The Arabidopsis GA response inhibitor SPINDLY plays a positive role in cytokinin signaling. Plant Cell **17,** 92-102.

Hamant, O., Nogue, F., Belles-Boix, E., Jublot, D., Grandjean, O., Traas, J., and Pautot, V. (2002). The KNAT2 homeodomain protein interacts with ethylene and cytokinin signaling. Plant Physiol. **130,** 657-665.

Hanano, S., Domagalska, M.A., Nagy, F., and Davis, S.J. (2006). Multiple phytohormones influence distinct parameters of the plant circadian clock. Genes Cells **11,** 1381-1392.

Hass, C., Lohrmann, J., Albrecht, V., Sweere, U., Hummel, F., Yoo, S.D., Hwang, I., Zhu, T., Schafer, E., Kudla, J., and Harter, K. (2004). The response regulator 2 mediates ethylene signalling and hormone signal integration in *Arabidopsis*. EMBO J. **23,** 3290-3302.

Hay, A., Kaur, H., Phillips, A., Hedden, P., Hake, S., and Tsiantis, M. (2002). The gibberellin pathway mediates KNOTTED1-type homeobox function in plants with different body plans. Curr. Biol. **12,** 1557-1565.

Hejatko, J., Pernisova, M., Eneva, T., Palme, K., and Brzobohaty, B. (2003). The putative sensor histidine kinase CKI1 is involved in female gametophyte development in Arabidopsis. Mol Genet Genomics **269,** 443-453.

Heyl, A., and Schmulling, T. (2003). Cytokinin signal perception and transduction. Curr. Opin. Plant Biol. **6,** 480-488.

Higuchi, M., Pischke, M.S., Mahonen, A.P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S., Helariutta, Y., Sussman, M.R., and Kakimoto, T. (2004). In planta functions of the Arabidopsis cytokinin receptor family. Proc. Natl. Acad. Sci. U S A **101,** 8821-8826.

Hirose, N., Makita, N., Kojima, M., Kamada-Nobusada, T., and Sakakibara, H. (2007). Over-expression of a Type-A Response Regulator alters rice morphology and cytokinin metabolism. Plant Cell Physiol. **48,** 523-539.

Hutchison, C.E., Li, J., Argueso, C., Gonzalez, M., Lee, E., Lewis, M.W., Maxwell, B.B., Perdue, T.D., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2006). The Arabidopsis Histidine Phosphotransfer Proteins are redundant positive regulators of cytokinin signaling. Plant Cell **18,** 3073-3087.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in *Arabidopsis* signal transduction. Nature **413,** 383-389.

Imamura, A., Yoshino, Y., and Mizuno, T. (2001). Cellular localization of the signaling components of Arabidopsis His-to-Asp phosphorelay. Biosci. Biotechnol. Biochem. **65,** 2113-2117.

Imamura, A., Kiba, T., Tajima, Y., Yamashino, T., and Mizuno, T. (2003). In vivo and in vitro Characterization of the ARR11 response regulator implicated in the His-to-Asp phosphorelay signal transduction in Arabidopsis thaliana. Plant Cell Physiol. **44,** 122-131.

Imamura, A., Hanaki, N., Umeda, H., Nakamura, A., Suzuki, T., Ueguchi, C., and Mizuno, T. (1998). Response regulators implicated in His-to-Asp phosphotransfer signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. U S A **95,** 2691-2696.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., and Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from Arabidopsis. Nature **409,** 1060-1063.

Ito, Y., and Kurata, N. (2006). Identification and characterization of cytokinin-signalling gene families in rice. Gene **382,** 57-65.

Iwama, A., Yamashino, T., Tanaka, Y., Sakakibara, H., Kakimoto, T., Sato, S., Kato, T., Tabata, S., Nagatani, A., and Mizuno, T. (2007). AHK5 histidine kinase regulates root elongation through an ETR1-dependent abscisic acid and ethylene signaling pathway in Arabidopsis thaliana. Plant Cell Physiol. **48,** 375-380.

Jackson, D., and Hake, S. (1999). Control of phyllotaxy in maize by the abphyl1 gene. Development **126,** 315-323.

Jain, M., Tyagi, A.K., and Khurana, J.P. (2006). Molecular characterization and differential expression of cytokinin-responsive type-A response regulators in rice (Oryza sativa). BMC Plant Biol. **6,** 1.

Jasinski, S., Piazza, P., Craft, J., Hay, A., Woolley, L., Rieu, I., Phillips, A., Hedden, P., and Tsiantis, M. (2005a). KNOX action in Arabidopsis is mediated by coordinate regulation of cytokinin and gibberellin activities. Curr. Biol. **15,** 1560-1565.

Jasinski, S., Piazza, P., Craft, J., Hay, A., Woolley, L., Rieu, I., Phillips, A., Hedden, P., and Tsiantis, M. (2005b). KNOX action in Arabidopsis is mediated by coordinate regulation of cytokinin and gibberellin activities. Curr Biol **15,** 1560-1565.

Kakimoto, T. (1996). CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. Science (New York, N.Y **274,** 982-985.

Kakimoto, T. (2003). Perception and signal transduction of cytokinins. Annu. Rev. Plant Biol. **54,** 605-627.

Kiba, T., Aoki, K., Sakakibara, H., and Mizuno, T. (2004). Arabidopsis response regulator, ARR22, ectopic expression of which results in phenotypes similar to the *wol* cytokinin-receptor Mutant. Plant Cell Physiol. **45,** 1063-1077.

Kiba, T., Naitou, T., Koizumi, N., Yamashino, T., Sakakibara, H., and Mizuno, T. (2005). Combinatorial microarray analysis revealing arabidopsis genes implicated in cytokinin responses through the His->Asp phosphorelay circuitry. Plant Cell Physiol. **46,** 339-355.

Kiba, T., Yamada, H., Sato, S., Kato, T., Tabata, S., Yamashino, T., and Mizuno, T. (2003). The type-A response regulator, ARR15, acts as a negative regulator in the cytokinin-mediated signal transduction in *Arabidopsis thaliana*. Plant Cell Physiol. **44,** 868-874.

Kim, H.J., Ryu, H., Hong, S.H., Woo, H.R., Lim, P.O., Lee, I.C., Sheen, J., Nam, H.G., and Hwang, I. (2006). Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. Proc. Natl. Acad. Sci. U S A **103,** 814-819.

Kuhlemeier, C. (2007). Phyllotaxis. Trends Plant Sci. **12,** 143-150.

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., Sakakibara, H., and Kyozuka, J. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature **445,** 652-655.

Lee, D.J., Park, J.Y., Ku, S.J., Ha, Y.M., Kim, S., Kim, M.D., Oh, M.H., and Kim, J. (2007). Genome-wide expression profiling of ARABIDOPSIS RESPONSE REGULATOR 7(ARR7) overexpression in cytokinin response. Mol. Genet. Genomics **277,** 115-137.

Leibfried, A., To, J.P.C., Stehling, S.K., A., Busch, W., Demar, M., Kieber, J.J., and Lohmann, J.U. (2005). WUSCHEL controls meristem size by direct transcriptional regulation of cytokinin inducible response regulators. Nature **438,** 1172-1175.

Mähönen, A.P., Bonke, M., Kauppinen, L., Riikonon, M., Benfey, P., and Helariutta, Y. (2000). A novel two-component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root. Genes Dev. **14,** 2938-2943.

Mähönen, A.P., Higuchi, M., Tormakangas, K., Miyawaki, K., Pischke, M.S., Sussman, M.R., Helariutta, Y., and Kakimoto, T. (2006a). Cytokinins regulate a bidirectional phosphorelay network in Arabidopsis. Curr. Biol. **16,** 1116-1122.

Mähönen, A.P., Bishopp, A., Higuchi, M., Nieminen, K.M., Kinoshita, K., Tormakangas, K., Ikeda, Y., Oka, A., Kakimoto, T., and Helariutta, Y. (2006b). Cytokinin signaling and its inhibitor AHP6 regulate cell Fate during vascular development. Science **311,** 94-98.

Mason, M.G., Li, J., Mathews, D.E., Kieber, J.J., and Schaller, G.E. (2004). Type-B response regulators display overlapping but distinct expression patterns in Arabidopsis. Plant Physiol. **135,** 927-937.

Mason, M.G., Mathews, D.E., Argyros, D.A., Maxwell, B.B., Kieber, J.J., Alonso, J.M., and Ecker, J.R.S., G.E. (2005). Multiple type-B response regulators mediate cytokinin signal transduction in Arabidopsis. Plant Cell **17,** 3007-3018.

Maxwell, B.B., and Kieber, J.J. (2005). Cytokinin signal transduction. In Plant Hormones: Biosynthesis, Signal Transduction, Action!, P.J. Davies, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 321-349.

McClung, C.R. (2006). Plant circadian rhythms. Plant Cell **18,** 792-803.

Miller, C.O., Skoog, F., Von Saltza, M.H., and Strong, F. (1955). Kinetin, a cell division factor from deoxyribonucleic acid. J. Am. Chem. Soc. **77,** 1392-1293.

Miyawaki, K., Tarkowski, P., Matsumoto-Kitano, M., Kato, T., Sato, S., Tarkowska, D., Tabata, S., Sandberg, G., and Kakimoto, T. (2006). Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. Proc. Natl. Acad. Sci. U S A **103,** 16598-16603.

Mok, D.W., and Mok, M.C. (2001). Cytokinin metabolism and action. Annu. Rev. Plant Physiol. Plant Mol. Biol. **52,** 89-118.

Mok, D.W.S., and Mok, M.C. (1994). Cytokinins: chemistry, activity and function. (Boca Raton, FL: CRC Press).

Murakami, M., Matsushika, A., Ashikari, M., Yamashino, T., and Mizuno, T. (2005). Circadian-associated rice Pseudo Response Regulators (OsPRRs): Insight into the control of flowering time. Biosci. Biotechnol. Biochem. **69,** 410-414.

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S., and Ueguchi, C. (2004). Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. Plant Cell **16,** 1365-1377.

Papon, N., Vansiri, A., Gantet, P., Chenieux, J.C., Rideau, M., and Creche, J. (2004). Histidine-containing phosphotransfer domain extinction by RNA interference turns off a cytokinin signalling circuitry in Catharanthus roseus suspension cells. FEBS Lett. **558,** 85-88.

Pareek, A., Singh, A., Kumar, M., Kushwaha, H.R., Lynn, A.M., and Singla-Pareek, S.L. (2006). Whole-genome analysis of Oryza sativa reveals similar architecture of two-component signaling machinery with Arabidopsis. Plant Physiol. **142,** 380-397.

Pena-Sandoval, G.R., Kwon, O., and Georgellis, D. (2005). Requirement of the receiver and phosphotransfer domains of ArcB for efficient dephosphorylation of phosphorylated ArcA in vivo. J. Bacteriol. **187,** 3267-3272.

Pischke, M.S., Jones, L.G., Otsuga, D., Fernandez, D.E., Drews, G.N., and Sussman, M.R. (2002). An Arabidopsis histidine kinase is essential for megagametogenesis. Proceedings of the National Academy of Sciences of the United States of America **99,** 15800-15805.

Rashotte, A.M., Carson, S.D., To, J.P., and Kieber, J.J. (2003). Expression profiling of cytokinin action in Arabidopsis. Plant Physiol. **132,** 1998-2011.

Rashotte, A.M., Mason, M.G., Hutchison, C.E., Ferreira, F.J., Schaller, G.E., and Kieber, J.J. (2006). A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Proc. Natl. Acad. Sci. U S A **103,** 11081-11085.

Riefler, M., Novak, O., Strnad, M., and Schmulling, T. (2006). Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell **18,** 40-54.

Romanov, G.A., Lomin, S.N., and Schmulling, T. (2006). Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. J. Exp. Bot. **57,** 4051-4058.

Sakai, H., Aoyama, T., and Oka, A. (2000). Arabidopsis ARR1 and ARR2 response regulators operate as transcriptional activators. Plant J. **24,** 703-711.

Sakai, H., Honma, T., Aoyama, T., Sato, S., Kato, T., Tabata, S., and Oka, A. (2001). ARR1, a transcription factor for genes immediately responsive to cytokinins. Science **294,** 1519-1521.

Sakakibara, H. (2006). CYTOKININS: Activity, Biosynthesis, and Translocation. Annu. Rev. Plant Biol. **57,** 431-449.

Sakakibara, H., Takei, K., and Hirose, N. (2006). Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends in plant science **11,** 440-448.

Sakakibara, H., Hayakawa, A., Deji, A., Gawronski, S.W., and Sugiyama, T. (1999). His-Asp phosphotransfer possibly involved in the nitrogen signal transduction mediated by cytokinin in maize: molecular cloning of cDNAs for two-component regulatory factors and demonstration of phosphotransfer activity in vitro. Plant Mol. Biol. **41,** 563-573.

Sakamoto, T., Sakakibara, H., Kojima, M., Yamamoto, Y., Nagasaki, H., Inukai, Y., Sato, Y., and Matsuoka, M. (2006). Ectopic expression of KNOTTED1-like homeobox protein induces expression of cytokinin biosynthesis genes in rice. Plant Physiol. **142,** 54-62.

Salomé, P.A., To, J.P.C., Kieber, J.J., and McClung, C.R. (2005). Arabidopsis response regulators ARR3 and ARR4 play cytokinin-Independent roles in the control of circadian period. Plant Cell **18,** 55-69.

Salomé, P.A., Michael, T.P., Kearns, E.V., Fett-Neto, A.G., Sharrock, R.A., and McClung, C.R. (2002). The out of phase 1 mutant defines a role for PHYB in circadian phase control in Arabidopsis. Plant Physiol. **129,** 1674-1685.

Schaller, G.E., Mathews, D.E., Gribskov, M., and Walker, J.C. (2002). Two-component signaling elements and histidyl-aspartyl phosphorelays. In The Arabidopsis Book, C.R. Somerville and E.M. Meyerowitz, eds (Rockville, MD: American Society of Plant Biologists), pp. DOI: 10.1199/tab.0086.

Schaller, G.E., Doi, K., Hwang, I., Kieber, J.J., Khurana, J.P., Kurata, N., Mizuno, T., Pareek, A., Shiu, S.-H., Wu, P., and Yip, W.K. (2007). Nomenclature for two-component signaling elements of rice. Plant Physiol. **143,** 555-557.

Shani, E., Yanai, O., and Ori, N. (2006). The role of hormones in shoot apical meristem function. Curr. Opin. Plant Biol. **9,** 484-489.

Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000). Two-component signal transduction. Annu. Rev. Biochem. **69,** 183-215.

Suzuki, T., Ishikawa, K., and Mizuno, T. (2002). An Arabidopsis histidine-containing phosphotransfer (Hpt) factor implicated in phosphorelay signal transduction: Overexpression of AHP2 in plants results in hypersensitivity to cytokinin. Plant Cell Physiol. **43,** 123-129.

Suzuki, T., Imamura, A., Ueguchi, C., and Mizuno, T. (1998). Histidine-containing phosphotransfer (HPt) signal transducers implicated in His-to-Asp phosphorelay in Arabidopsis. Plant Cell Physiol. **39,** 1258-1268.

Suzuki, T., Zakurai, K., Imamura, A., Nakamura, A., Ueguchi, C., and Mizuno, T. (2000). Compilation and characterization of histidine-containing phosphotransmitters implicated in His-to-Asp phosphorelay in plants: AHP signal transducers of *Arabidopsis thaliana*. Biosci. Biotechnol. Biochem. **64,** 2482-2485.

Suzuki, T., Miwa, K., Ishikawa, K., Yamada, H., Aiba, H., and Mizuno, T. (2001). The Arabidopsis sensor His-kinase, AHK4, can respond to cytokinins. Plant Cell Physiol. **42,** 107-113.

Sweere, U., Eichenberg, K., Lohrmann, J., Mira-Rodado, V., Bäurle, I., Kudla, J., Nagy, F., Schäfer, E., and Harter, K. (2001). Interaction of the response regulator ARR4 with the photoreceptor phytochrome B in modulating red light signaling. Science **294,** 1108-1111.

Tajima, Y., Imamura, A., Kiba, T., Amano, Y., Yamashino, T., and Mizuno, T. (2004). Comparative studies on the type-B response regulators revealing their distinctive properties in the His-to-Asp phosphorelay signal transduction of Arabidopsis thaliana. Plant Cell Physiol. **45,** 28-39.

Tanaka, Y., Suzuki, T., Yamashino, T., and Mizuno, T. (2004). Comparative studies of the AHP histidine-containing phosphotransmitters implicated in His-to-Asp phosphorelay in Arabidopsis thaliana. Biosci. Biotechnol. Biochem. **68,** 462-465.

Taniguchi, M., Sasaki, N., Tsuge, T., Aoyama, T., and Oka, A. (2007). ARR1 directly activates cytokinin response genes that encode proteins with diverse regulatory functions. Plant Cell Physiol. **48,** 263-277.

To, J.P., Deruère, J., Maxwell, B.B., Morris, V.F., Hutchison, C.E., Schaller, G.E., and Kieber, J.J. (2007). Cytokinin regulates Type-A Arabidopsis Response Regulator activity and protein stability via two-component phosphorelay. In Arabidopsis Response Regulators in cytokinin signaling and plant development (This Thesis: Chapter 4), pp. 109.

To, J.P.C., Haberer, G., Ferreira, F.J., Deruère, J., Mason, M.G., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2004). Type-A ARRs are partially redundant negative regulators of cytokinin signaling in Arabidopsis. Plant Cell **16,** 658-671.

Ueguchi, C., Sato, S., Kato, T., and Tabata, S. (2001a). The AHK4 gene involved in the cytokinin-signaling pathway as a direct receptor molecule in Arabidopsis thaliana. Plant Cell Physiol. **42,** 751-755.

Ueguchi, C., Koizumi, H., Suzuki, T., and Mizuno, T. (2001b). Novel family of sensor histidine kinase genes in Arabidopsis thaliana. Plant Cell Physiol. **42,** 231-235.

Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T., and Shinozaki, K. (1999). A transmembrane hybrid-type histidine kinase in Arabidopsis functions as an osmosensor. Plant Cell **11,** 1743-1754.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmulling, T. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15,** 2532-2550.

West, A.H., and Stock, A.M. (2001). Histidine kinases and response regulator proteins in two-component signaling systems. Trends Biochem. Sci. **26,** 369-376.

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., Yamashino, T., and Mizuno, T. (2001). The Arabidopsis AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol. **42,** 1017-1023.

Yanai, O., Shani, E., Dolezal, K., Tarkowski, P., Sablowski, R., Sandberg, G., Samach, A., and Ori, N. (2005). Arabidopsis KNOXI proteins activate cytokinin biosynthesis. Curr. Biol. **15,** 1566-1571.

Yokoyama, A., Yamashino, T., Amano, Y., Tajima, Y., Imamura, A., Sakakibara, H., and Mizuno, T. (2007). Type-B ARR transcription factors, ARR10 and ARR12, are implicated in cytokinin-mediated regulation of protoxylem differentiation in roots of Arabidopsis thaliana. Plant Cell Physiol. **48,** 84-96.

Yonekura-Sakakibara, K., Kojima, M., Yamaya, T., and Sakakibara, H. (2004). Molecular characterization of cytokinin-responsive histidine kinases in maize. Differential ligand preferences and response to cis-zeatin. Plant Physiol. **134,** 1654-1661.

Yuan, K., and Wysocka-Diller, J. (2006). Phytohormone signalling pathways interact with sugars during seed germination and seedling development. J. Exp. Bot. **57,** 3359-3367.

CHAPTER 2

Type-A ARRs are Partially Redundant Negative Regulators of Cytokinin Signaling in Arabidopsis

Jennifer P.C. To* Georg Haberer*, Fernando J. Ferreira, Jean Deruère, Michael G. Mason, G. Eric Schaller, Jose M. Alonso, Joseph R. Ecker and Joseph J. Kieber

*These authors contributed equally to this publication

PREFACE

This work was initiated by Dr. Georg Haberer, a postdoc who was in the lab at the beginning of my graduate career. Dr. Haberer isolated T-DNA insertion mutants in the type-A ARRs from the Alonso-Ecker (Salk) T-DNA collection and constructed double and quadruple *arr3,4,5,6* and *arr5,6,8,9* mutants. I constructed the *arr3,4,5,6,8,9* sextuple mutant, *arr3,4,8,9* quadruple mutant and also generated the lines for complementation analysis. Dr. Jean Deruère and Dr. Fernando Ferreira, two other postdocs in the lab, generated and characterized the type-A ARR promoter: GUS reporter lines. Dr. Michael Mason and Dr. G. Eric Schaller are our collaborators who analyzed seedling responses to red light. I characterized general phenotypes and cytokinin responses of all the single, double, quadruple and sextuple mutant lines and wrote the paper.

This paper has been published in: To, J.P.C.*, Haberer, G.*, Ferreira, F.J., Deruère, J., Mason, M.G., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2004). Type-A ARRs are partially redundant negative regulators of cytokinin signaling in Arabidopsis. Plant Cell 16, 658-671. (*These authors contributed equally to this publication.)

ABSTRACT

Type-A Arabidopsis Response Regulators (ARRs) are a family of ten genes that are rapidly induced by cytokinin and are highly similar to bacterial two-component response regulators. We have isolated T-DNA insertions in six of the type-A ARRs and constructed multiple insertional mutants, including the *arr3,4,5,6,8,9* hextuple mutant. Single *arr* mutants were indistinguishable from the wild type in various cytokinin assays; double and higher order *arr* mutants showed progressively increasing sensitivity to cytokinin, indicating functional overlap among type-A ARRs and that these genes act as negative regulators of cytokinin responses. The induction of cytokinin primary response genes was amplified in *arr* mutants, indicating that the primary response to cytokinin is affected. Spatial patterns of ARR gene expression were consistent with partially redundant function of these genes in cytokinin signaling. The *arr* mutants show altered red light sensitivity, suggesting a general involvement of type-A ARRs in light signal transduction. Further, morphological phenotypes of some *arr* mutants suggest complex regulatory interactions and gene-specific functions among family members.

INTRODUCTION

Cytokinins are N^6-substituted adenine derivatives that have been implicated in nearly all aspects of plant growth and development, including cell division, shoot initiation and development, light responses and leaf senescence (Mok and Mok, 2001b). Lowering endogenous levels of cytokinin inhibits shoot development and increases primary root growth and branching, indicating that cytokinin plays opposite roles in the shoot and root meristems (Werner et al., 2001). Ectopic and overexpression of cytokinin biosynthetic genes have also demonstrated that elevated levels of cytokinin can release apical dominance, reduce root development, delay senescence and enhance shoot regeneration in cultured tissues (Medford et al., 1989; Smigocki, 1991; Li et al., 1992; Gan and Amasino, 1995; Sa et al., 2001; Zubko et al., 2002).

The current model for cytokinin signaling in plants is similar to the two-component phosphorelay system with which bacteria sense and respond to environmental changes. A simple two-component system involves a histidine sensor kinase and a response regulator (Stock et al., 2000; West and Stock, 2001). The histidine kinase perceives environmental stimuli via the input domain and autophosphorylates on a conserved histidine residue within the kinase domain. The phosphoryl group is subsequently transferred to a conserved aspartate residue on the receiver domain of a response regulator, which mediates downstream responses via the output domain. Multi-component phosphorelay systems occur in most eukaryotic and some prokaryotic systems which employ histidine kinase signal transduction in a multistep His-Asp-His-Asp phosphotransfer process (Stock et al., 2000; West and Stock, 2001). The Arabidopsis

cytokinin receptors (CRE1, AHK2 and AHK3) are similar to bacterial histidine sensor hybrid kinases in two-component signaling, containing a receiver domain fused to the histidine kinase domain (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001b; Ueguchi et al., 2001a; Yamada et al., 2001). The cytokinin receptors are predicted to signal through histidine phosphotransfer proteins (AHPs) to ultimately alter the phosphorylation state of the Arabidopsis response regulators (ARRs) in a multi-step phosphorelay (Hutchison and Kieber, 2002).

Arabidopsis Response Regulators (ARRs) can be broadly classified into two groups (type-A and type-B) by the similarity of their receiver domain sequences and by their C-terminal characteristics. Like most bacterial response regulators, type-B ARRs have C-terminal domains that contain DNA binding, nuclear localization and transcription activator domains (Sakai et al., 1998; Sakai et al., 2000; Sakai et al., 2001). C-terminal sequences of type-A ARRs are short and have yet to be assigned functions. Type-A and type-B ARR homologs are found in other dicotyledonous and monocotyledonous plants, including maize and rice (Kieber, 2002; Asakura et al., 2003).

There are ten type-A ARRs that fall into five very similar pairs (Fig. 2.1A). The rates of transcription of most of the type-A ARRs, but not the type-B ARRs, are rapidly and specifically induced in response to exogenous cytokinin, and this induction occurs in the absence of *de novo* protein synthesis (Taniguchi et al., 1998; D'Agostino et al., 2000). Gene expression differs among various type-As, with *ARR4, ARR8* and *ARR9* displaying relatively high basal levels, and *ARR5, ARR6, ARR7* and *ARR15* showing the greatest fold-induction in response to cytokinin (D'Agostino et al., 2000; Rashotte et al., 2003). Transcription of type-A ARRs is regulated in part by type-B ARRs (Hwang and Sheen,

2001; Sakai et al., 2001). Overexpression of some type-A ARRs inhibits expression of an *ARR6* promoter-luciferase reporter in cultured Arabidopsis cells, suggesting that type-A ARRs have the ability to negatively regulate their own transcription (Hwang and Sheen, 2001). Consistent with this, ectopic overexpression of ARR15 leads to decreased cytokinin sensitivity (Kiba et al., 2003). ARR4 has been shown to interact with and stabilize the far red active form of phytochrome B (PhyB); overexpression of ARR4 in Arabidopsis also confers hypersensitivity to red light (Sweere et al., 2001), indicating a role in light-regulated development.

Using the model plant Arabidopsis, we took a reverse genetic approach to study the function of type-A ARRs. We isolated T-DNA insertions in six of the ten type-A ARRs (three of the five most similar pairs) and have constructed various combinations of these mutations including the *arr 3,4,5,6,8,9* hextuple mutant. Overall, we show that these genes have overlapping functions and act as negative regulators of cytokinin signaling. We show that the mutants are affected in their reponse to light. In addition, we identify morphological phenotypes in a susbset of *arr* mutants that support some functional specificity within the type-A family of ARRs.

RESULTS

Isolation of insertions in response regulator loci

To study the function of type-A ARRs, we isolated T-DNA insertions in six of the ten genes: *ARR3* (At1g59940), *ARR4* (At1g10470), *ARR5* (At3g48100), *ARR6* (At5g62920), *ARR8* (At2g41310) and *ARR9* (At3g57040). These mutations cover three of the five gene pairs, *ARR3/ ARR4*, *ARR5/ ARR6* and *ARR8/ ARR9*, identified by phylogenetic analysis (Fig. 2.1A; (D'Agostino et al., 2000). We identified individual insertions in each gene by PCR screening, and located the sites of insertions by DNA sequencing (Table 2.S2). In *arr3*, the T-DNA inserted in the C-terminal domain, 26 base pairs downstream of the sequence encoding the receiver domain (Fig. 2.1B). The insertions in *arr4*, *arr5*, *arr6*, *arr8* and *arr9* are predicted to disrupt the receiver domain of the respective genes. Furthermore, the insertions in *arr5*, *arr6*, *arr8* and *arr9* occur in the coding region prior to an invariant Lysine residue in the receiver domain, and thus are unlikely to produce functional proteins (Fig. 1.2B).

We examined RNA expression of the type-A ARRs to determine if the T-DNA insertions affected the level of RNA in each of the mutant lines. Northern analysis showed that *arr4*, *arr6* and *arr9* mutants had substantially reduced levels of the transcripts corresponding to the mutated genes (Fig. 2.1C). The *arr5* mutant displayed a shift in transcript size, as well as a decrease in transcript levels (Fig. 2.1C). RT-PCR analysis showed that the T-DNA insertions in *ARR3* and *ARR8* abolished expression of the respective transcripts (Fig. 2.1C). We conclude that the T-DNA insertions in *arr3* and *arr8* result in null alleles, while the remaining insertions result in hypomorphic alleles.

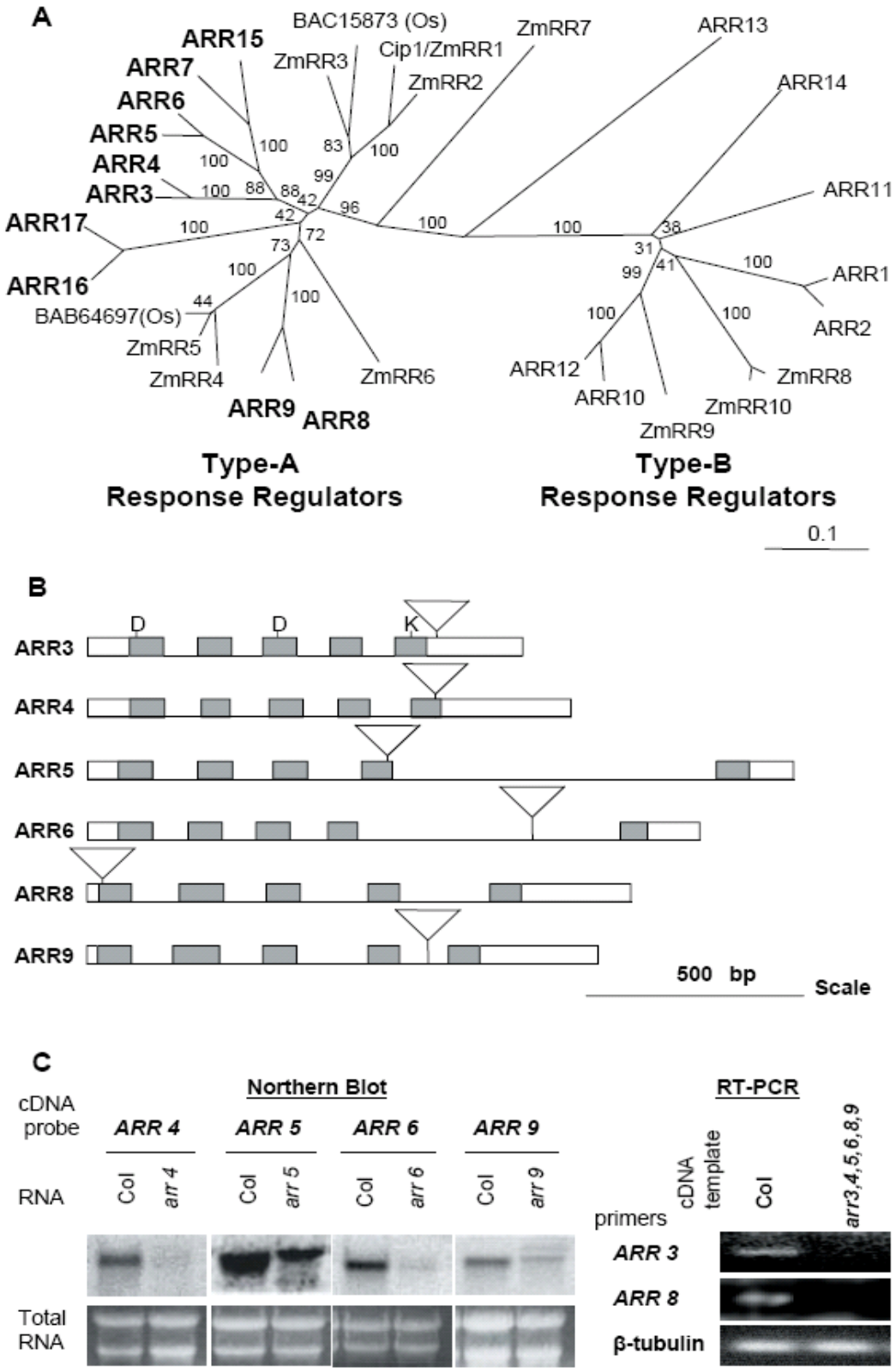

Figure 2.1 Type-A ARR phylogeny and positions of T-DNA insertions

Adult phenotype of *arr* mutants

When grown under long day conditions on soil, the six single *arr* insertion lines were indistinguishable at all stages of growth when compared to their wild-type counterparts (data not shown). Likewise, *arr3*, *arr6*, *arr8* and *arr9* grown under short days were also indistinguishable from the wild type (Fig. 2.2A). However, *arr4* and *arr5* displayed subtle alterations in rosette morphology when grown under short day conditions: *arr4* adult plants developed mildly elongated petioles and the rosette size of the *arr5* mutant was reduced (Figs. 2.2A and 2.S1).

To examine the genetic interactions among the six type-A *arr* mutations, higher order mutants were generated. These include double mutants between each highly similar

Figure 2.1 Type-A ARR phylogeny and positions of T-DNA insertions

(A) An unrooted phylogenetic tree made using receiver domain sequences of type-A and type-B response regulators from Arabidopsis (ARR), maize (ZmRR) and rice (Os with Acession numbers). Full length protein sequences of the response regulators were obtained from Entrez Protein Database (NCBI) and their receiver domain sequences were identified by searching Conserved Domain Database (CDD v1.62, NCBI). Receiver domain sequences were aligned using the CLUSTALW program (v. 1.81, University of Nijmegen, http://www.cmbi.kun.nl/bioinf/tools/clustalw.shtml) and the phylogenetic tree was constructed with 1000 bootstrapping replicates. The unrooted tree is presented in TreeView (version 1.6.6, R. Page, 2001). The bootstrap values are indicated on the tree. Scale bar represents 0.1 amino acid substitution per site.
(B) Positions of T-DNA insertions in the type-A arr mutants. The insertional mutants were identified by PCR screening and the site of insertion determined by DNA sequencing of the border fragment. Boxes represent exons, lines represent introns and T-DNA insertions are indicated by an inverted triangle. Receiver domains are shaded. The DDK residues that are conserved in two-component receiver domains are indicated.
(C) Expression of type-A ARRs in insertional mutants. RNA from 3 day-old seedlings was either blotted to nylon for northern analysis (left panel) or transcribed *in vitro* to cDNA for use in an RT-PCR reaction (right panel) as described in Experimental Procedures. For the northern blot, different cDNA clones were used as hybridization probes as indicated above the figure and the ethidium bromide stained agarose gel is shown below. For RT-PCR, primers were designed to amplify the first three exons of *ARR3*, or the entire β-tubulin gene as a control.

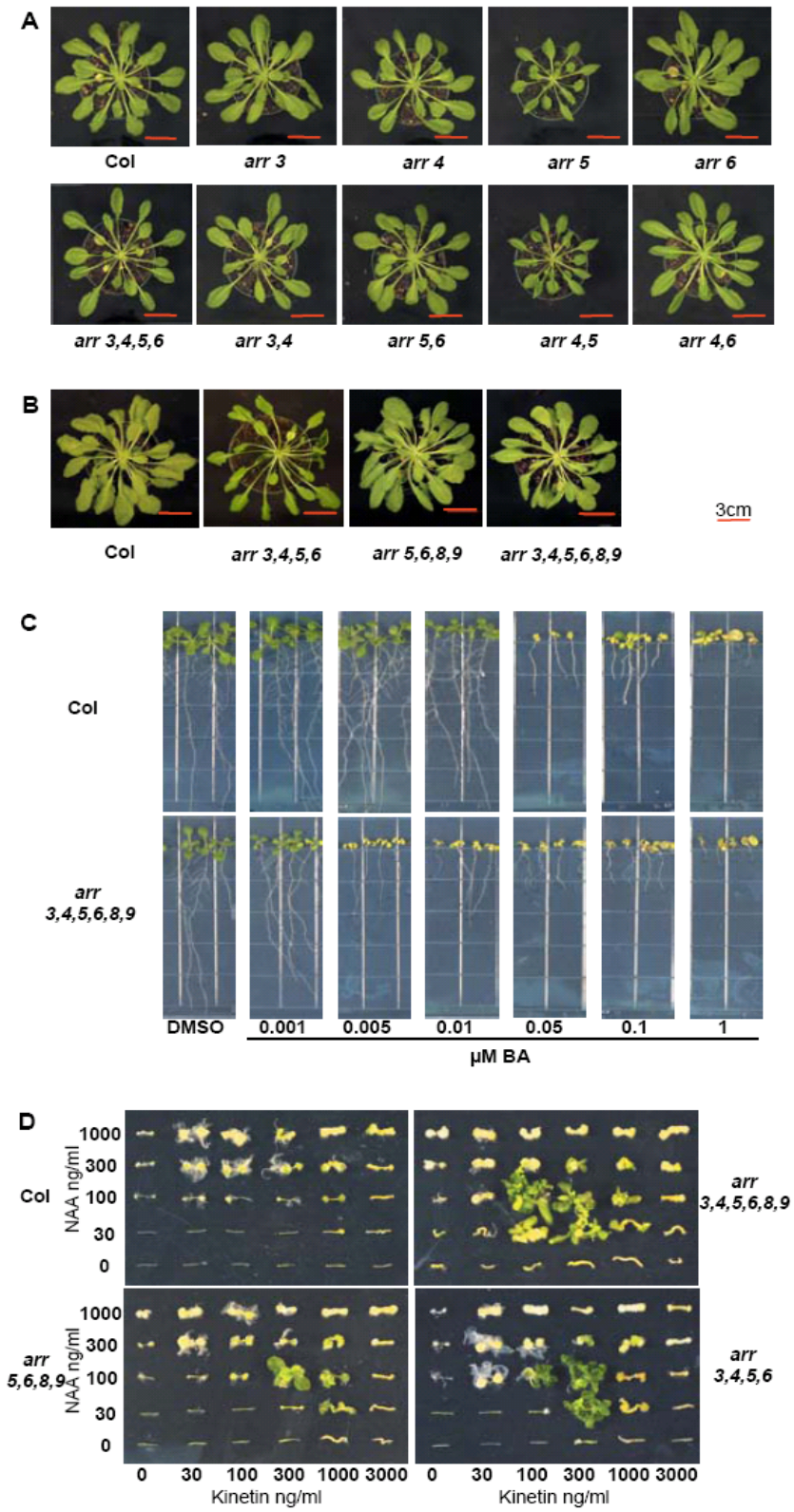

Figure 2.2 *arr* mutant phenotypes

pair (*arr3,4*, *arr5,6* and *arr8,9*), double mutants across pairs (*arr4,5* and *arr4,6*), quadruple mutants (*arr3,4,5,6*, *arr3,4,8,9* and *arr5,6,8,9*) and the *arr3,4,5,6,8,9* hextuple mutant. The elongated petioles of the *arr4* single mutant were enhanced in the *arr3,4* double mutant, indicating functional redundancy between the two members of this gene pair (Figs. 2.2A and 2.S1). Surprisingly, the reduced rosette size of *arr5* was not enhanced, but suppressed by the *arr6* mutation, suggesting antagonistic function. The *arr4,5* double mutant appeared similar to the *arr5* parent, and the *arr4,6* double mutant was similar to the *arr4* parent. The elongated petioles of *arr4* and *arr3,4* were further enhanced in *arr3,4,5,6*, but the overall rosette size was similar to that of the wild-type parent (Figs. 2.2A, 2.2B and 2.S1). The increased petiole elongation in the *arr3,4,5,6* quadruple mutant suggests that although *ARR5* and *ARR6* may act antagonistically to each other in regulating rosette size, as a pair they still function

Figure 2.2 *arr* mutant phenotypes

(A) and (B) *arr* adult plants are affected in short days. Plants of the genotypes noted were grown in short-day conditions (8 hr light, 16 hr dark) for nine weeks. At least eight plants per genotype were examined and photographs of representative plants for each line are shown. The experiment was conducted three times with similar results. The red scale bar in each photograph corresponds to 3 cm. Note: plants in (A) and (B) are from separate experiments
(C) *arr* seedlings are more sensitive to cytokinin. Seedlings were grown vertically on plates supplemented with the specified concentrations of BA or a DMSO vehicle control under constant light conditions at 23°C. Seedlings were photographed at ten days.
(D) *arr* mutants form elaborate shoot structures on low cytokinin concentrations and fewer roots on high auxin concentration in shoot initiation assay. Hypocotyls were excised from seedlings grown for three days in the dark followed by three days in dim light and transferred to media containing various concentrations of auxin (NAA) and cytokinin (kinetin) for four weeks under constant light. Five hypocotyls of each genotype were examined at each concentration. One hypocotyl representative of the response at each concentration was selected and arranged to create a composite photograph for each genotype. Note: (A) and (B) are from two separate experiments.

additively with *ARR3* and *ARR4* in the regulation of petiole elongation.

The *arr5,6,8,9* quadruple mutant was indistinguishable from the wild type, as were the *arr5,6* and *arr8,9* double mutants (Figs. 2.2A and 2.2B). However, the *arr3,4,5,6,8,9* hextuple mutant had intermediate petiole length between *arr3,4,5,6* and the wild type (Fig. 2.2B and 2.S1), suggesting complex interactions between these genes.

arr mutant seedling root elongation is more sensitive to cytokinin inhibition

To assess the role of type-A ARRs in the cytokinin response pathway, we examined root elongation in response to exogenous cytokinin. We compared root elongation of wild-type and *arr3,4,5,6,8,9* mutant seedlings across a range of cytokinin concentrations between 1 nM and 10 μM benzyladenine (BA) (Figs. 2.2C and 2.3). Wild-type root elongation was not affected by BA concentrations below 5 nM. Upon further increase in BA concentration, primary root elongation decreased sharply, with a half maximal inhibition at ~12 nM (Fig. 2.3A). In the absence of exogenous cytokinin, roots of the *arr3,4,5,6,8,9* hextuple mutant were shorter than roots of the wild type (Students' t-test $p < 10^{-4}$). In the presence of low doses (< 50 nM) of BA, the *arr3,4,5,6,8,9* mutant displayed increased sensitivity to BA as shown by a greater inhibition of root elongation than wild-type roots at comparable concentrations. The *arr3,4,5,6,8,9* hextuple mutant reached half maximal inhibition at ~2 nM BA. At higher BA concentrations (≥ 50 nM), the mutant response was similar to that of the wild type (Fig. 2.3A). This resulted in a change in the overall shape of the dose response curve from primarily monophasic in the wild type to biphasic in the hextuple mutant. Interestingly, the central part of the response curve in the hextuple mutant showed little

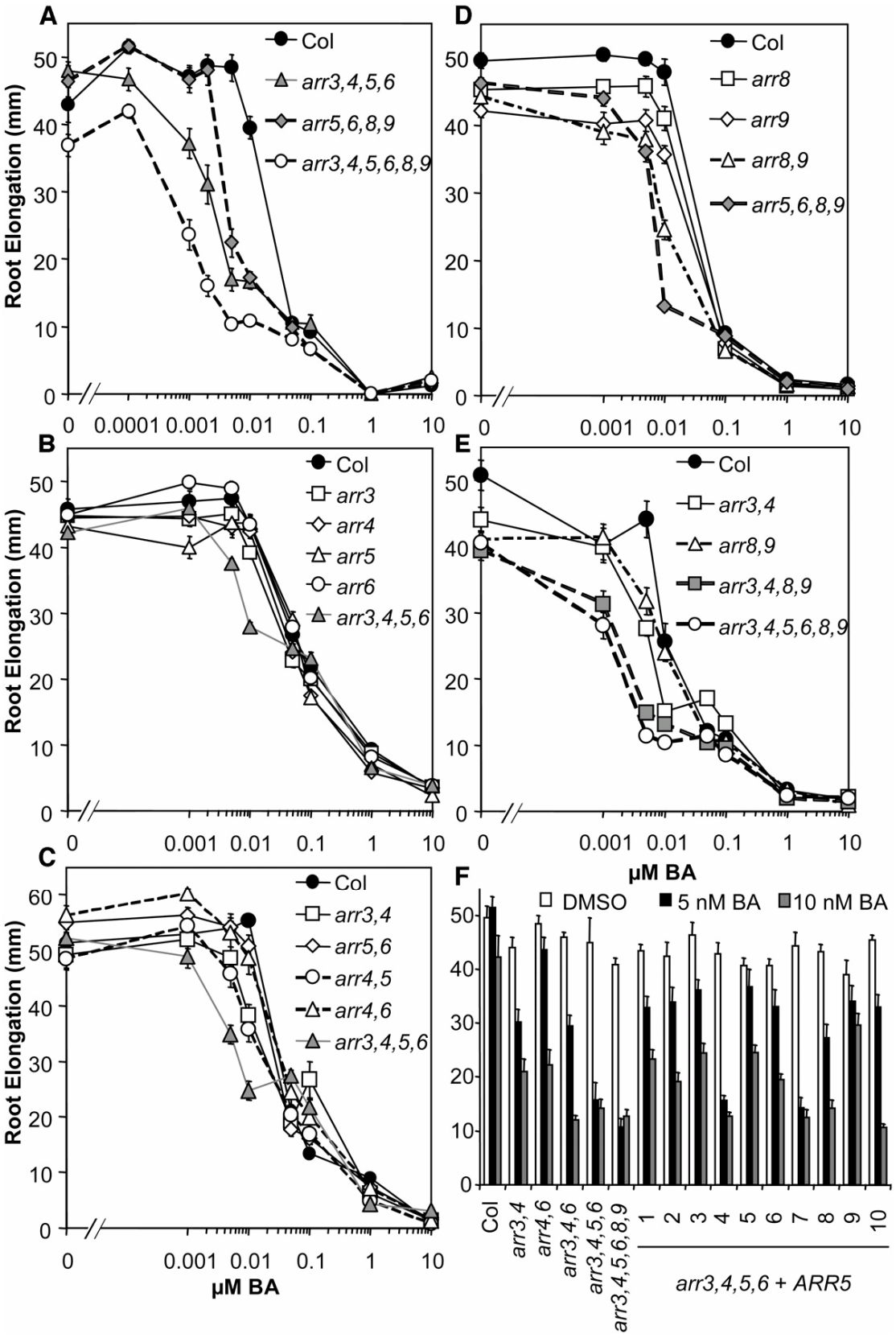

Figure 2.3 *arr* seedlings are more sensitive to cytokinin inhibition of root elongation

or no change in inhibition of root elongation as the concentration of BA was increased from 8 nM to 100 nM BA. This dramatic shape change in the dose response curve was very reproducible, consistently observed among three separate experiments (Fig. 2.3A, E and data not shown). To examine the contributions of individual *ARR* genes to cytokinin responsiveness and their interactions, inhibition of primary root elongation of single, double and quadruple mutants in response to increasing concentrations of exogenous BA were examined. Single *arr* mutants were indistinguishable from the wild type in this cytokinin response (Fig. 2.3B and D), which coupled with the cytokinin-hypersensitve phenotype of the higher order mutants indicates genetic redundancy among these genes. The *arr5,6* and *arr4,*6 double mutants showed subtle differences in cytokinin sensitivity compared to the wild type, while the *arr3,4* and *arr4,5* double mutants exhibited a significant increase in cytokinin inhibition of root elongation intermediate between *arr3,4,5,6* and the wild type (Fig. 2.3C). *arr8,*9 also exhibited a significant increase in cytokinin sensitivity intermediate between the wild type and the *arr5,6,8,9* or *arr3,4,8,9* quadruple mutants (Fig. 2.3D and E), indicating that all these mutations additively

Figure 2.3 *arr* seedlings are more sensitive to cytokinin inhibition of root elongation

(A-E) Seedlings were grown vertically on plates supplemented with the specified concentrations of BA or a DMSO vehicle control under constant light conditions at 23°C. Root elongation between days four and nine was measured as described in the Experimental Procedures. Results shown were pooled from an experimental set of three independent samples of 10-15 individual seedlings. Error bars represent standard error, n>30. Each experiment was repeated at least twice with consistent results.
(F) Complementation of *arr 3,4,5,6* phenotype with *ARR5*. A construct containing a wild-type *ARR5* cDNA driven by the *ARR5* promoter was transformed into *arr 3,4,5,6*. WT seedlings, various *arr* mutant seedlings and ten transformed lines were grown as in (A-E) in the presence of 5 nM BA (black bars), 10nM BA (grey bars) or a DMSO vehicle control (white bars). Ten independent T1 lines are denoted 1-10. Error bars represent standard error, n=15.

contribute to this phenotype of *arr3,4,5,6,8,9*.

The *arr3,4,5,6* and *arr5,6,8,9* quadruple mutants showed root elongation responses intermediate between *arr3,4,5,6,8,9* and the wild type (Fig. 2.3A). The *arr3,4,8,9* mutant exhibited the greatest increase in cytokinin sensitivity among the three quadruple mutants examined, almost approaching the hypersensitivity of the *arr3,4,5,6,8,9* hextuple mutant (Fig. 2.3E), indicating that the component *ARR*s play a key role in this cytokinin response. However, *ARR5* and *ARR6* still contribute to the effect of cytokinin on root elongation as *arr3,4,8,9* is significantly less sensitive than *arr3,4,5,6,8,9* at 5 and 10 nM BA (t-test $p< 0.01$), while *arr3,4,5,6* and *arr5,6,8,9* are also significantly more sensitive than *arr3,4* and *arr8,9* (t-test $p<10^{-5}$ and $p<10^{-10}$ at 10 nM BA) respectively (Fig. 2.3A, C and D).

arr mutant seedling lateral root formation is more sensitive to cytokinin inhibition

Formation of lateral roots is inhibited by cytokinin in plants (Werner et al., 2001). We examined the number of lateral roots on wild-type and all the *arr* mutant 10-day-old seedlings across the same concentration range used in the root elongation assay. In wild-type seedlings, the effect of BA on lateral root formation decreased dramatically between 5 and 50 nM BA, reaching half maximal inhibition at ~12 nM BA, and essentially no lateral roots were detected at BA concentrations greater than 1 μM (Fig. 2.2C and 2.4A). In the *arr3,4,5,6,8,9* hextuple mutant, significantly fewer lateral roots than the wild type were formed in the absence of BA (Students' t-test $p< 10^{-7}$) (Figs. 2.2C and 2.4A). The range of inhibition of lateral roots was also markedly shifted to lower BA concentrations in *arr3,4,5,6,8,9*, with a half-maximal inhibition of ~1 nM BA (Fig. 2.4A).

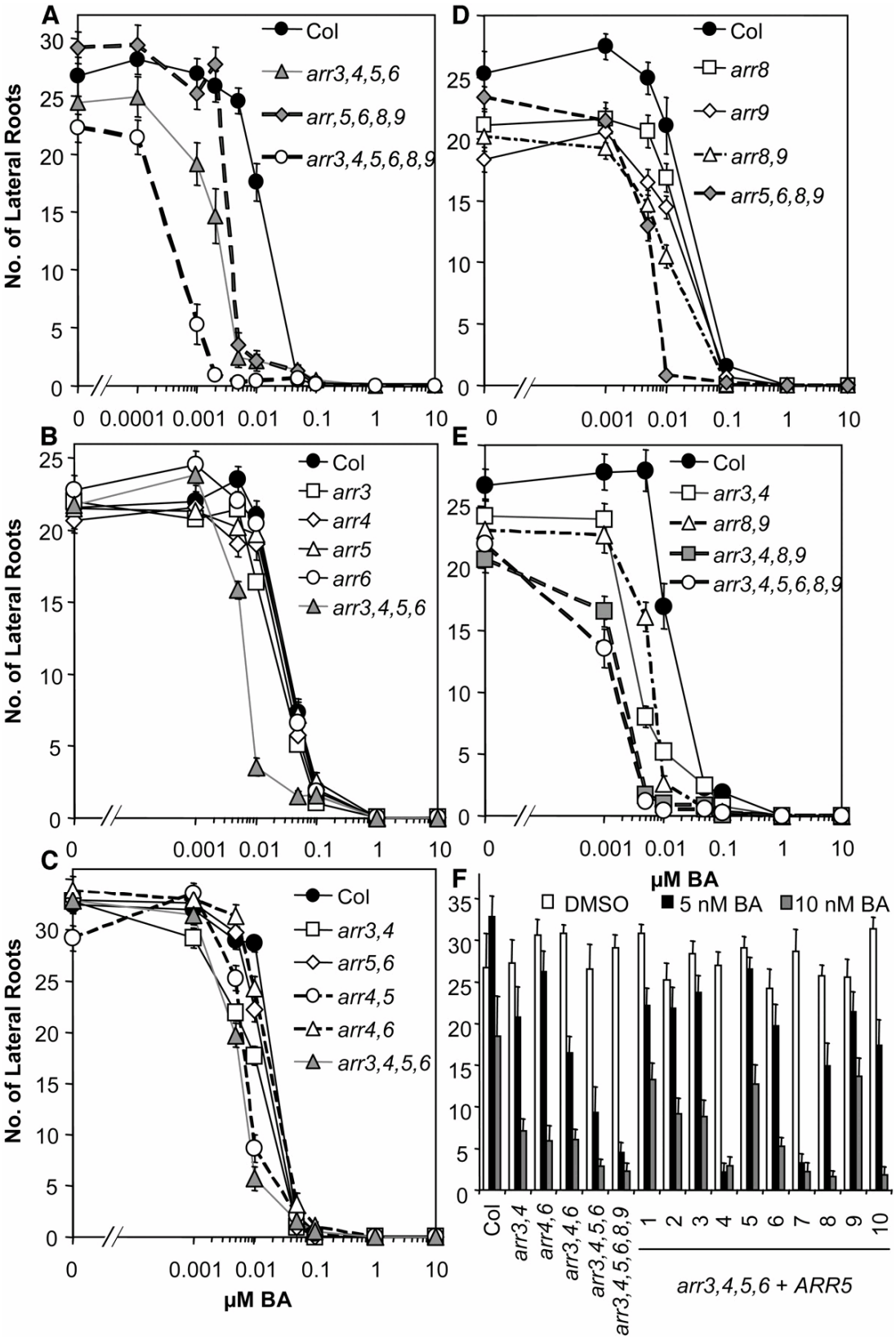

Figure 2.4 *arr* seedlings are more sensitive to cytokinin inhibition of lateral root formation

Overall, the partial genetic redundancy among these type-A ARRs in the lateral root assay was similar to that observed in the root elongation response. In general, the single mutants exhibited near wild-type cytokinin sensitivity (Fig. 2.4B and D), while the double mutants displayed cytokinin sensitivity that was intermediate between the wild type and the quadruple mutants (Fig. 2.4C, D and E). The *arr3,4,5,6*, *arr5,6,8,9* and *arr3,4,8,9* quadruple mutants showed intermediate responses between the wild type and the *arr3,4,5,6,8,9* hextuple mutant (Fig. 2.4A and E), with the sensitivity of *arr3,4,8,9* closest to *arr3,4,5,6,8,9*.

The *arr8* and *arr9* single mutants, and the *arr8,9* double mutant developed slightly fewer lateral roots in the absence of exogenous BA (Students' t-test $p<0.01$). The difference in lateral root number in the absence of exogenous BA was further enhanced in *arr3,4,8,9* and *arr3,4,5,6,8,9*, but not in *arr5,6,8,9* (Fig. 2.4D and E). This indicates that *ARR5* and *ARR6* do not act redundantly with *ARR8* and *ARR9* in the root without exogenous application of cytokinin, and that *ARR8* and *ARR9* may be key elements in cytokinin inhibition of lateral root formation.

Figure 2.4. *arr* seedlings are more sensitive to cytokinin inhibition of lateral root formation

(A-E) Seedlings were grown vertically on plates supplemented with the specified concentrations of BA or a DMSO vehicle control under constant light conditions at 23°C. The total number of lateral roots was quantified at nine days. Results shown were collected from the same experimental sets as in Fig. 2.2. Error bars represent standard error, n>30. (F) Complementation of *arr 3,4,5,6* phenotype with *ARR5*. A construct containing a wild-type *ARR5* cDNA driven by the *ARR5* promoter was transformed into *arr 3,4,5,6*. WT seedlings, various *arr* mutant seedlings and seven transformed lines were grown as in (A-E) in the presence of 5 nM BA (black bars), 10 nM BA (grey bars) or a DMSO vehicle control (white bars). Ten independent T1 lines are denoted 1-10. Error bars represent standard error, n=15.

arr seedlings develop pale rosettes on lower concentrations of cytokinin

When grown in the presence of exogenous BA, rosettes of wild-type seedlings were smaller and the leaves were progressively paler with increasing concentrations of the hormone. The transition from dark to pale green rosettes occurred at similar doses to those that inhibited root formation in wild-type and mutant seedlings, respectively (Fig. 2.2C). Chlorophyll content was quantified for wild-type Columbia and *arr3,4,5,6* seedlings grown in the presence and absence of BA. In the absence of BA, chlorophyll content of the wild type and the *arr3,4,5,6* quadruple mutant were not significantly different (1180 ± 185 and 862 ± 161 nmol/g freshweight, respectively). As observed in the seedling root assays, the most dramatic difference occured at 10 nM BA. Chlorophyll levels in the wild type decreased to 790 ± 220 nmol/g freshweight in the presence of 10 nM BA (~67 % of chlorophyll content in the absence of BA), while chlorophyll levels in *arr3,4,5,6* decreased further to 234 ± 47 nmol/g freshweight (~27 % of chlorophyll content in the absence of BA). This analysis confirmed that wild-type seedlings contained significantly less chlorophyll (Students' t-test p-value= 0.025) when grown in the presence of BA and that the *arr3,4,5,6* mutant was hypersensitive to cytokinin this assay.

Complementation of *arr* seedling response to cytokinin

To confirm that the altered cytokinin responses were due to the disruption of type-A ARRs, a wild-type *ARR5* gene (see Methods) was re-introduced into *arr3,4,5,6* mutants. T1 transformants were selected on hygromycin and homozygous T3 progeny from independent T1 lines were analyzed. The selected T3 progeny were assayed for cytokinin responsiveness in the seedling root assay. Eight of eleven selected lines showed

strong complementation based on analysis of cytokinin-regulated root elongation, lateral root formation and shoot chlorophyll content on 10 nM BA (Figs. 2.3F, 2.4F and data not shown). Three of the 11 lines did not complement these mutant phenotypes (Figs. 2.3F, 2.4F and data not shown). These results indicate that the altered cytokinin sensitivity of the *arr3,4,5,6* mutant is the result of disruption of the type-A *ARR* genes. Re-introducing *ARR5* into the *arr3,4,5,6* quadruple background restored the cytokinin response to the levels of *arr3,4,*6 in two of the eleven lines, while six of the eleven lines resulted in a cytokinin responsiveness intermediate between the wild type and the *arr3,4* mutant, suggesting that re-introduction of an *ARR5* construct lacking introns (see methods), multiple and or tandem T-DNA insertions, or positional effects may have resulted in higher levels of expression.

arr mutations affect the response to auxin: cytokinin ratios in shoot initiation assays

Cytokinins promote cell division and initiate shoots in concert with auxin in cultured plant tissues (Miller et al., 1955; Miller et al., 1956; Mok and Mok, 2001a). We examined the response of excised hypocotyls from wild-type and several type-A *arr* mutant seedlings in response to various concentrations of the cytokinin kinetin and the auxin NAA.

Wild-type Columbia hypocotyl explants formed green foci only at high cytokinin: auxin ratios. However, no recognizable shoots were formed under these conditions, which is consistent with previous reports indicating that the Columbia ecotype does not efficiently form shoots from undifferentiated tissues in culture (Valvekens et al., 1988). At low cytokinin: auxin ratios, initiation of root primordia was observed, with the most prominent root structures observed at 30 ng/ml kinetin 1000 ng/ml NAA; at intermediate

ratios of these hormones, undifferentiated calli predominated (Fig. 2.2D). The *arr* mutants formed larger calli on comparable concentrations of hormones that were able to induce wild-type calli (Fig. 2.2D). *arr3,4,5,6,8,9*, *arr3,4,5,6* and *arr5,6,8,9* mutants also formed recognizable shoot structures; large leafy and flowering structures were found in the *arr3,4,5,6,8,9* hextuple mutant between 100 to 300 ng/ml kinetin and 30 to 100 ng/ml NAA (Figs. 2.2D and 2.S2). The range of calli-inducing media was expanded to lower cytokinin: auxin ratios relative to the wild type, and the ability to form shoots on concentrations where the wild type was only able to form calli indicates an increase in both cytokinin sensitivity and responsiveness. The effect of the *arr* mutations was additive in this assay. *arr3,4*, *arr5,6* and *arr8,9* all formed larger calli than the wild type on comparable concentrations of hormones (data not shown). *arr3,4* and *arr5,6* generated small leaves at 300 ng/ml kinetin 100 ng/ml NAA and 1000 ng/ml kinetin 100 ng/ml NAA, respectively, whereas *arr8,9* did not produce obvious shoot structures (data not shown). *arr3,4,5,6* was more sensitive than *arr5,6,8,9* in this assay, and produced prominent shoot structures at a lower range of cytokinin concentrations than *arr5,6,8,9* (Figs. 2.2D and 2.S2), consistent with the seedling responses of the component double mutants. Further, root formation in the *arr3,4,5,6,8,9* hextuple mutant was inhibited by cytokinin, resulting in elimination of root structures in some concentrations, most prominent at 30 ng/ml kinetin 1000 ng/ml NAA (Fig. 2.2D). Interestingly, in the absence of exogenous hormones, *arr3,4,5,6,8,9* hypocotyl explants appeared swollen from disorganized cell divisions, suggesting a shift in the response to endogenous hormone levels (Fig. 2.2D).

The increase in sensitivity and responsiveness of the *arr3,4,5,6,8,9*, *arr3,4,5,*6 and

arr5,6,8,9 in callus formation and root inhibition, and the ability to form recognizable shoots in this assay, further indicate that these type-A ARRs act as negative regulators of cytokinin signaling with overlapping function.

Leaf senescence is delayed in *arr* mutants

Cytokinins inhibit leaf senescence in a variety of plant species (Gan and Amasino, 1995; Mok and Mok, 2001b). We used chlorophyll loss in a detached leaf assay to determine the effect of *arr* mutations on senescence. After 10 days of dark-induced senescence, wild-type leaf chlorophyll levels were substantially reduced relative to the initial content (Fig. 2.5). This decrease in chlorophyll levels was inhibited in the presence of cytokinin in wild-type leaves, with maximal inhibition at ~100 nM BA (Fig. 2.5). *arr3,4,5,6* exhibited a higher rate of chlorophyll retention in the absence of exogenous

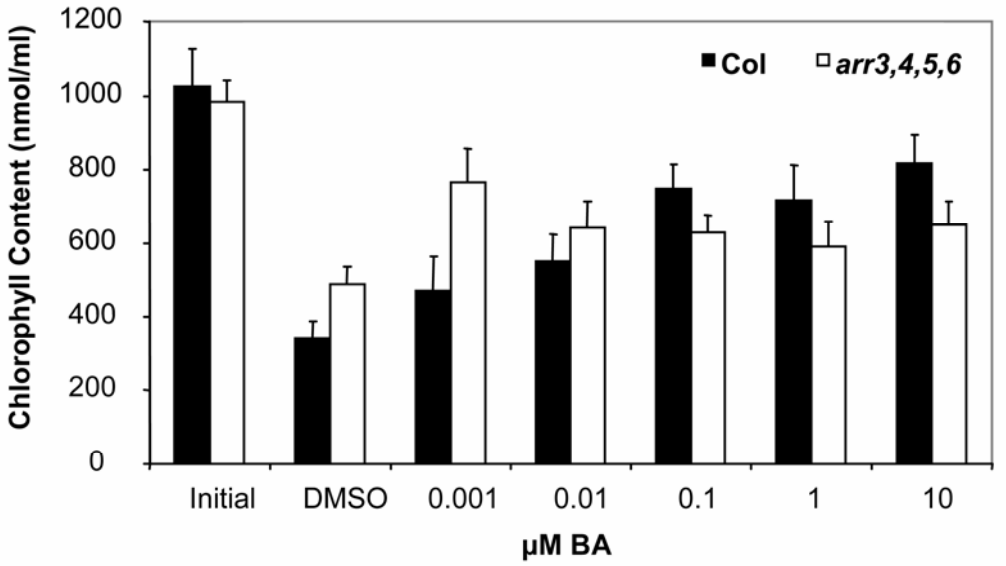

Figure 2.5 *arr3,4,5,6* shows delayed leaf senescence

Fully expanded leaves were excised from 3.5 week-old plants and floated on water supplemented with various concentrations of cytokinin for 10 days in the dark. Chlorophyll content was determined spectrophotometrically as described in Experimental Procedures. Three independent plates with six leaves per plate were examined at each concentration. Two chlorophyll measurements were taken per plate. Results shown are pooled from three independent experiments ± standard error, n=18.

cytokinin (Students' t-test $p < 10^{-4}$), and the maximal response occurred at lower cytokinin concentrations than the wild type (Fig. 2.5). As in the root assays, these results indicate that the *arr* mutant is hypersensitive to cytokinin in adult leaves.

Expression patterns of type-A ARRs

Functional redundancy of the type-A ARRs predicts that the genes would have overlapping patterns of expression. To test this hypothesis, we generated GUS reporter constructs fused to promoters of these six type-A ARRs. We examined the expression of these genes both in the presence and the absence of 10 nM BA, which is the concentration of BA at which the greatest differences in seedling response was observed. Consistent with northern analysis (Taniguchi et al., 1998; D'Agostino et al., 2000), lines harboring the *ARR5* and *ARR6* promoter fusions displayed the highest level of induction by cytokinin, while the *ARR3*, *ARR4, ARR8* and *ARR9* promoter fusions only showed a moderate increase in reporter activity in response to cytokinin (Fig. 2.6). Members of the most similar pairs showed similar patterns of expression (Fig. 2.6).

ARR3:GUS and *ARR4:GUS* were constitutively expressed in the vasculature of both shoots and roots, with stronger expression in the shoot. When grown on 10 nM BA, the region of expression was expanded to tissues surrounding vasculature in the root, but was excluded from the root tip/ meristematic region. *ARR5* expression was as previously reported (D'Agostino et al., 2000), primarily found in the root and shoot meristems in the absence of exogenous cytokinin. In the presence of 10 nM BA, the *ARR5:GUS* expression region was enlarged to include tissues around the shoot meristematic region; strong *ARR5:GUS* expression was induced in all tissues in the root, from the hypocotyl-root junction through the root tip. Basal *ARR6:GUS* expression was detected in the shoot

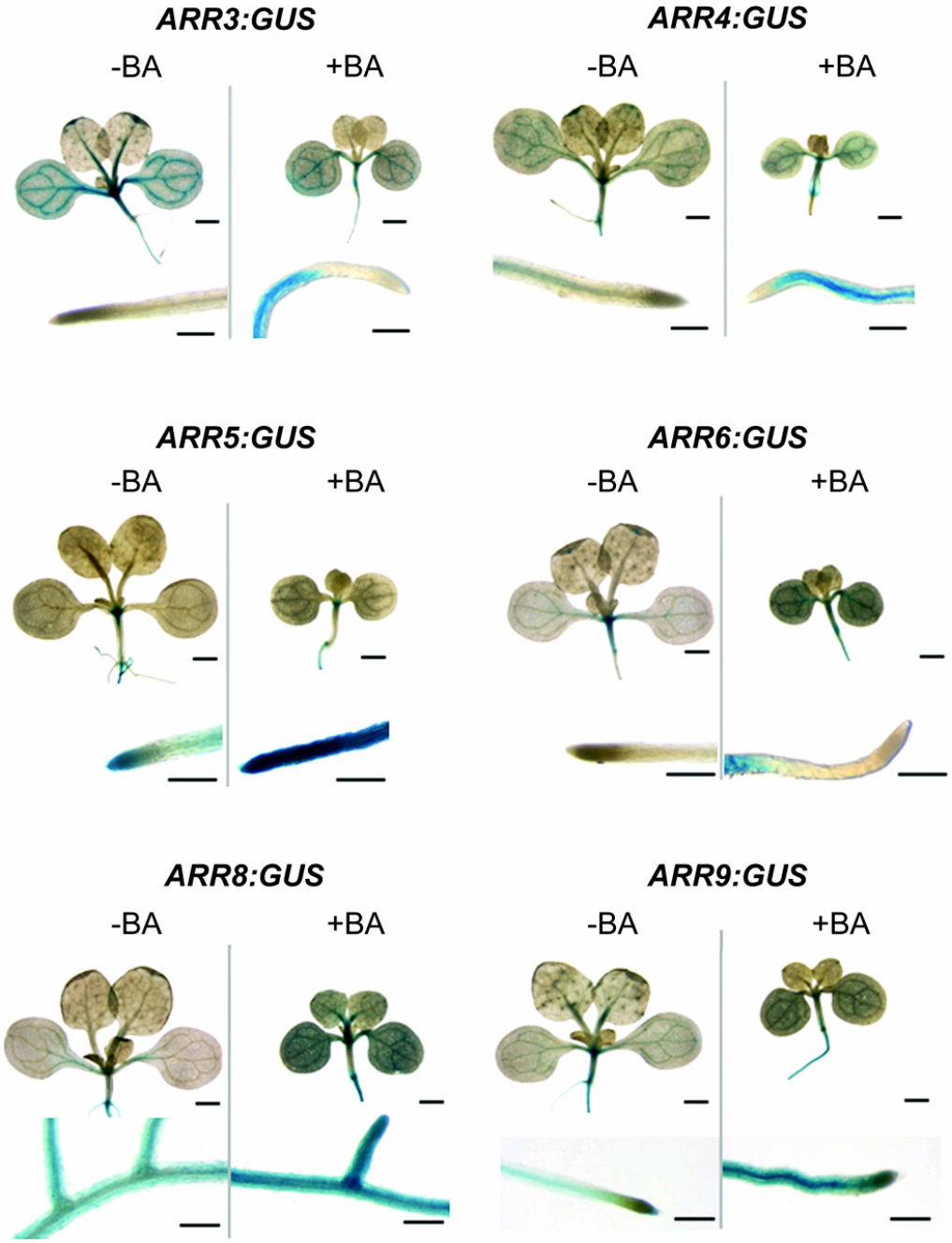

Figure 2.6 Expression analysis of *ARR* gene promoters

ARR-promoter-driven β-glucuronidase (GUS) constructs were generated and introduced into wild-type Col background. Transgenic seedlings were grown on MS media (-BA) or media supplemented with 10 nM BA (+BA) for nine days and assayed for GUS activity. 10 transformed lines were examined and one representative line for each construct was photographed. With the exception of ARR8:GUS, close-up panels show the relative GUS activity at the primary root tip. For ARR8:GUS, the close up panels show lateral root junctions in the expansion zone of the primary root. (Scale bars: For aerial tissues: 1 mm; For roots: 250 μm).

meristematic region and cotyledon vasculature. Cytokinin treatment resulted in overall higher levels of *ARR6:GUS* expression, with GUS staining expanded to the hypocotyl and root tissues, but excluded from the root tip. *ARR8* and *ARR9* were expressed strongly throughout the root and weakly in the seedling vasculature, with an overall increase in GUS activity in the same tissues on exogenous cytokinin.

While basal expression patterns differed among the ARR gene pairs, their expression patterns mostly overlap in the presence of exogenous cytokinin, particularly in the root. This is consistent with the functional redundancy that we observe among type-A ARRs in root assays in the presence of BA.

arr mutations affect cytokinin primary response

To investigate whether the increase in cytokinin sensitivity of the *arr* mutants was due to altered primary response, we examined gene expression in response to cytokinin.

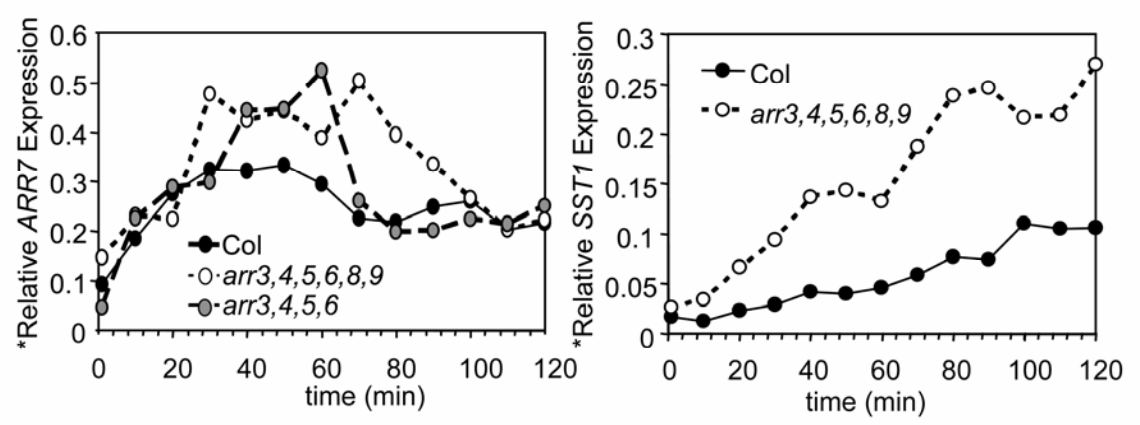

Figure 2.7 *arr* mutants are affected in the cytokinin primary response pathway

RNA was extracted from ten-day-old light-grown seedlings treated with 10 nM BA in liquid MS with 1% sucrose for the indicated time. The RNA was analyzed by northern blotting. The blots were probed with either an *ARR7*, *SST1* or a *β-tubulin* radiolabeled probe. The signal obtained for each was quantified using a PhosphorImager, and the *ARR7* and *SST1* signals were normalized to the *β-tubulin* signal. The experiment was conducted twice with similar results.

Ten-day-old light-grown seedlings were treated with 10 nM BA and the expression levels of two cytokinin primary response genes, *ARR7* and a steroid sulfotransferase (*SST1*) (D'Agostino et al., 2000); J. To and J. Kieber unpublished results), were analyzed by northern blot. Two independent full experiments were conducted and critical time points were further repeated in triplicate, all of which produced consistent results. The results from one of the experiments is presented in Fig. 2.7.

In wild-type seedlings, *ARR7* was induced rapidly by cytokinin treatment and reached two-fold above basal level after 10 min, after which the signal continued to increase to maximal levels of about 3.5-fold at 30 min (Fig. 2.7). The *arr3,4,5,*6 quadruple mutant exhibited a greater amplitude in cytokinin induced *ARR7* expression. The *arr3,4,5,6,8,9* hextuple mutant displayed an induction amplitude similar to that seen in *arr3,4,5,*6, but also showed an extended peak of elevated *ARR7* expression. As with the *ARR7* genes, the rapid induction of *SST1* was magnified in the *arr3,4,5,6,8,9* hextuple mutant (Fig. 2.7). The amplified rapid induction of cytokinin response genes in *arr3,4,5,6* and *arr3,4,5,6,8,9* mutants indicates that type-A ARRs negatively regulate the primary cytokinin signal transduction pathway.

arr mutants exhibit altered responses to red light

ARR4 has previously been implicated in modulating red light responses in Arabidopsis, based on its ability to interact with phytochrome B and the effects of ARR4 over-expression upon the red light sensitivity of seedlings (Sweere et al., 2001). However, no loss-of-function mutants within the type-A ARR family have been characterized for their red light sensitivity. We therefore investigated the response of *arr* seedling hypocotyl elongation to red light.

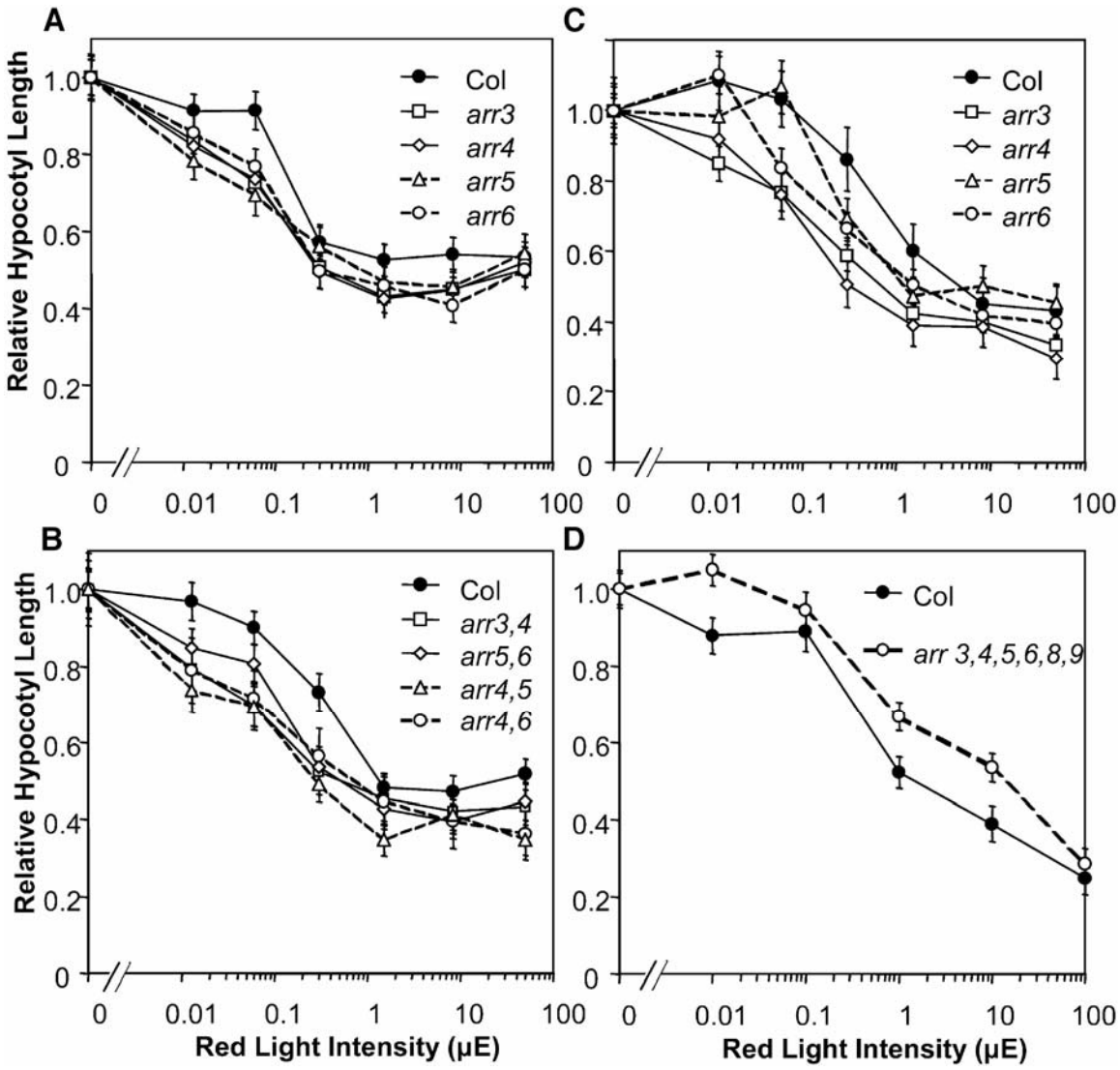

Figure 2.8 *arr* seedlings exhibit altered hypocotyl growth response to red light

Mutant and WT seeds were stratified and pre-treated with fluorescent light before incubation under various red light intensities for 3 days (A, B and D) or directly irradiated with red light after stratification (C). Mean hypocotyl lengths at various light intensities are normalized to the mean value of the etiolated seedlings of the respective genotypes. Mean etiolated hypocotyl heights (mm) are 9.7, 8.5, 8.7, 9.3, and 10 for *arr3*, *arr4*, *arr5*, *arr6*, and WT in (A), 8.6, 8.8, 8.2, 9.7, and 9.6 for *arr3,4*, *arr4,6*, *arr4,5*, *arr5,6*, and WT in (B), 8.6, 7.8, 6.4, 7.0, and 6.7 for *arr3*, *arr4*, *arr5*, *arr6*, and WT in (C) and 9.4 and 9.2 for *arr3,4,5,6,8,9* and WT in (D), respectively. Bars represent standard error, n>13. The experiment was conducted twice with consistent results.

Differences between the single *arr3*, *arr4*, *arr5*, *arr6* mutant and wild-type hypocotyl lengths were observed over the entire red light range from 0.013 to 50 μE (Fig. 2.8A). Among the double mutants, *arr3,4*, *arr4,5* and *arr4,6* demonstrated the greatest increase in sensitivity to red light, while *arr5,6*, although more sensitive to red light than the wild type, did not show as dramatic a shift in response as the three double mutants carrying the *arr4* mutation (Fig. 2.8B). These results suggest that ARR3 and ARR4 play a more substantial role in the red light response than ARR5 and ARR6. Interestingly, the *arr3,4,5,6,8,9* hextuple mutant was less sensitive to red light than the wild type (Fig. 2.8D), suggesting complex interactions among type-A ARRs as previously observed in the rosette phenotypes.

Because the initial ratios of active (P_{fr}) and inactive (P_r) forms of phytochrome in the seeds may affect the red light sensitivity, we also conducted an experiment without the 15-hour light pre-treatment. The results showed a similar trend to the experiment with light pre-treatment, with *arr3* and *arr4* showing the most pronounced increase in red light sensitivity (Fig. 2.8C). Thus, the red light hypersensitivity of the mutants is not an artifact of pre-treatment with fluorescent light. The higher order mutants were delayed in germination relative to the wild type under these growth conditions, hence their sensitivity to red light could not be assessed.

DISCUSSION

We have described the characterization of six type-A response regulator genes in Arabidopsis. A variety of cytokinin response assays indicate that all six of these type-A ARRs act as negative regulators of cytokinin function. This is observed in both root and shoot tissues in seedlings, in fully expanded adult leaves, and in tissue culture. Furthermore, consistent with their highly similar sequences, our analyses indicate that these genes have at least partially overlapping functions. However, we also detect morphological differences among the mutants that are consistent with gene-specific functions and potential antagonistic functions within this gene family.

arr mutations increase cytokinin sensitivity

arr mutants display increased cytokinin sensitivity at low concentrations of cytokinin in various responses, including seedling root elongation and lateral root formation, hypocotyl shoot initiation assays, senescence delay and induction of cytokinin response genes. Intriguingly, in the root elongation assay, mutations in the type-A ARRs only affect the response at lower concentrations of cytokinin (< 0.1 μM), thus changing the shape of the dose response curve from monophasic in the wild type to biphasic in the quadruple and higher order *arr* mutants. This suggests that the monophasic response in the wild-type may be comprised of a more complex response. Alternatively, root inhibition at the higher doses (0.1 – 10 μM BA) could represent a non-physiological, "toxic" effect on root elongation. However, cytokinin receptor mutants are insensitive to such concentrations of cytokinin with no observable toxic effects (Inoue et al., 2001; Ueguchi et al., 2001a), and a similar range of concentrations of BA has been shown to elevate the induction of cytokinin primary response genes (D'Agostino et al., 2000).

Together, these results suggest that these higher doses of BA are not simply toxic but rather constitute part of the cytokinin responsive range.

Hwang and Sheen (2001) have previously shown that overexpression of a subset of type-A ARRs in plant protoplasts inhibits the expression of an *ARR6:GFP* reporter. Here we demonstrate that multiple loss of function type-A *arr* alleles result in an increase in both the amplitude and period of cytokinin induction of cytokinin primary response genes. This effect occurs with kinetics that strongly suggest that type-A ARRs modulate the sensitivity of the cytokinin primary response pathway.

Role of Type-A ARRs in cytokinin signaling

Type-A ARRs are generally rapidly up-regulated by exogenous cytokinin (D'Agostino et al., 2000) which, in conjunction with our results here, suggests that type-A ARRs mediate a feedback mechanism by which the plant decreases its sensitivity to the hormone. Type-B ARRs have been shown to be transcription factors that positively mediate cytokinin responses (Hwang and Sheen, 2001; Sakai et al., 2001). Type-A ARRs may negatively regulate cytokinin responses by interfering with type-B ARR activity. This could occur via direct protein-protein interactions between type-A and type-B ARRs in a manner similar to the IAAs and ARFs in auxin response (Hutchison and Kieber, 2002; Leyser, 2002), though evidence for direct protein-protein interactions between type-A and type-B ARRs is lacking. A more likely model is that type-A ARRs inhibit type-B ARR activation by competing for phosphotransfer from upstream AHPs, as has been demonstrated in a few bacterial two-component systems (Rabin and Stewart, 1993; Li et al., 1995; Sourjik and Schmitt, 1998). An additional possibility is that type-A ARRs may act indirectly, by increasing the function of a negative regulator of type-B ARRs.

arr mutants have weak morphological phenotypes

Cytokinin has been linked to fundamental processes in plant growth and development, including the regulation of cell division, and altering endogenous cytokinin levels can have dramatic consequences on plant development and morphology (Miller et al., 1955; Miller et al., 1956; Medford et al., 1989; Werner et al., 2001). Thus, it is somewhat surprising that a shift in cytokinin sensitivity of greater than ten-fold, as is seen in the *arr3,4,5,6,8,9* mutant, does not result in a strong morphological phenotype. Furthermore, it is remarkable that disruption of six out of ten members of a gene family involved in cytokinin response does not significantly impact basal development. The T-DNA insertions in the type-A ARRs described herein do not all result in transcript nulls, and thus the hextuple mutant may still retain partial function in these genes, which may contribute to the lack of a substantial phenotype. However, this would not explain why a ten-fold shift in cytokinin sensitivity does not affect basal development. The plant may compensate for increased cytokinin sensitivity by decreasing active hormone levels. Attempts to increase cytokinin levels by transient overexpression of bacterial isopentenyl transferases in whole plants resulted in no striking morphological effects, as the plant may compensate for elevated biosynthesis by increasing the conjugation and degradation of the hormone (Medford et al., 1989; Smigocki, 1991; Mok and Mok, 2001a). Consistent with this model, a global analysis of gene expression has revealed that a primary response of Arabidopsis seedlings treated with high levels of exogenous cytokinin is to alter genes whose combined function is to decrease cytokinin levels and responsiveness (Rashotte et al., 2003).

Another explanation for the lack of a phenotype is that while the type-A *arr*

mutants alter cytokinin sensitivity, this change is not beyond a threshold that dramatically affects basal development under laboratory conditions. These genes may play a role in response to some factor not present in laboratory growth conditions, or they may play a role in environmental transitions, which are minimized under controlled growth conditions. A more dynamic environment that requires intact mechanisms for developmental plasticity (and thus fluctuations in hormonal responsiveness) may reveal more pronounced morphological alterations in the *arr* mutants.

Finally, cytokinin regulation of development may be redundant with other control mechanisms. For example, cell division is controlled by multiple regulatory inputs, some subset of which may compensate for the altered cytokinin function of the type-A *arr* mutants.

arr mutants are affected in light responses

We found that mutations in *ARR3*, *ARR4*, *ARR5*, and *ARR6* independently or together result in increased sensitivity to red light, similar to PhyB over-expressors (McCormac et al., 1993; Krall and Reed, 2000), suggesting that these genes function as negative regulators of red light signal transduction. The *arr* double mutants did not show an obvious increase in red light sensitivity over their component single mutants, which may indicate that type-A ARRs modulate only part of the seedling red light response and/or that there is not substantial redundancy in this function of the type-A ARRs. The elongated petiole phenotypes of the *arr3,4,5,6* mutant also suggest an altered shade avoidance response mediated by light and/or ethylene signaling pathways (Finlayson et al., 1999). The long petiole phenotype in *arr3,4,5,*6 is similar to that observed for *phyB* mutants, albeit the *arr3,4,5,6* petiole phenotype is weaker. However, the *arr3,4,5,6,8,9*

hextuple mutant exhibited a decrease in red light sensitivity compared to *arr3,4,5,6*, suggesting that *arr8* and *arr9* may antagonize the effects of the other four *arr* mutations, or that an overall decrease in the abundance of ARRs beyond a certain threshold may have an opposite effect on the light response.

Sweere *et al.* (2001) have shown that ARR4 over-expression resulted in increased red light sensitivity in hypocotyls, and proposed that this was due to a direct interaction between ARR4 and PhyB that inhibited the conversion of PhyB from the active (P_{fr}) to the inactive form (P_r). Our data supports the involvement of ARR4 as well as other type A ARRs in red light signal transduction. However, the over-expression data predicts a decrease in red light sensitivity in a loss-of-function *arr4* mutant, in contrast to what we observe in our mutant analysis. It is possible that over-expression of ARR4 dramatically changes the stoichiometry between ARR4 and PhyB or other interacting proteins. If interactions with phytochrome play a significant role, it may be that the activity of the ARRs is regulated by phytochromes rather than the ARRs regulating phytochrome activity as originally proposed (Sweere et al., 2001). Alternatively, the type-A ARRs could be involved in a cytokinin signaling pathway that impinges upon the phytochrome-mediated pathway (Su and Howell, 1995), and thus indirectly regulate red light sensitivity. Finally, differences in growth conditions may alter the role of the type-A ARRs in red light responses.

Redundancy and specificity among type-A ARRs

Phylogenetic analysis reveals that the ten type-A ARRs fall into five distinct pairs (Fig. 2.2A), and analysis of the positions of these genes within the genome indicates that these pairs arose from a genome duplication event (Vision et al., 2000). Interestingly,

most of the Arabidopsis type-A ARRs generally fall into a clade that is distinct from those formed by the rice and maize type-A ARR genes (Fig. 2.2A), and thus the progenitor of monocots and dicots may have had only a relatively small number of type-A ARRs. If this is the case, then it is likely that the expansion of this family occurred in both monocots and dicots. Alternatively, common ancestral genes may have been deleted in each lineage. Evidence for accelerated gene loss in duplicated regions of the Arabidopsis genome (Ku et al., 2000), suggests that there has been pressure for maintenance of all ten type-A ARRs despite the partial redundancy found in our analysis. Furthermore, the commonality of a large type-A ARR gene family in both monocots and dicots also suggests some selective advantage.

While our studies suggest that there is significant functional overlap among members of the type-A ARR gene family, several lines of evidence also suppport a model for some gene-specific function. Analysis of basal patterns of expression reveal some differences among the type-A ARRs, largely defined by the most similar pairs. *ARR3* and *ARR4* are expressed mainly in the shoot vasculature, *ARR5* and *ARR6* are expressed in the shoot meristematic region, and *ARR8* and *ARR9* are expressed strongly throughout the root. Several of the single and double mutants have subtle but distinct morphological phenotypes, which are in general consistent with their patterns of expression. Disruption of *ARR8* and *ARR9* loci affect lateral root number in seedlings in the absence of cytokinin application, but do not affect shoot development. Under short day conditions, adult plants of *arr5* develop smaller rosettes and *arr4* develop longer petioles, but neither mutant is affected in basal root development. Thus, it is likely that these genes have acquired some specificity that may have contributed to their retention.

Interactions between type-A ARRs

A previous study examined the effect of overexpression of ARR4 and ARR8 on shoot formation from cultured Arabidopsis roots. Interestingly, ARR4 overexpression resulted in a cytokinin hypersensitive phenotype, but overexpression of ARR8 caused cytokinin insensitivity in this assay (Osakabe et al., 2002). The authors concluded that ARR4 and ARR8 have opposing effects on cytokinin responsiveness. Our loss-of-function analysis does not support a positive role for ARR4 in cytokinin signaling, and the discrepancy may reflect complications arising from overexpression in the prior study.

However, phenotypes of adult *arr* mutant plants are consistent with some members of these gene pairs having antagonistic effects. For example, the small rosette phenotype of the *arr5* mutant is suppressed by the *arr6* mutation (its closest homolog), but not by *arr4*. Additionally, the *arr8* and *arr9* mutations appear to partially suppress the elongated petiole phenotype of the *arr3,4,5,6* mutant and antagonize the red light hypersensitivity of single and double mutants containing mutations in *arr3*, *arr4*, *arr5* and *arr6*. These results suggest that there may be interactions among the type-A ARRs involving both additive and antagonistic functions.

Implications in tissue culture

The change in the response of type-A ARR hypomorphic mutants in tissue culture is both quantitative (i.e. shoot formation is shifted to lower concentrations of cytokinin) and qualitative (i.e. well developed shoots form in the mutant, but only green foci form in the wild type). Plant tissue and species vary widely in their regenerative potential, which poses major obstacles for transformation of some species. This conversion of a tissue that is recalcitrant to regeneration (i.e. Columbia hypocotyls) to one that readily forms shoots

in culture (i.e. the mutant hypocotyls) is intriguing and implies that the relative level of functional type-A ARRs may be one of the factors underlying the differences in regenerative capacity.

In conclusion, we have shown that type-A ARRs are negative regulators with overlapping function in cytokinin signaling. These genes also affect light-regulated development. Morphological differences among *arr* mutants predict some specific functions and suggest regulatory interactions among these genes. Additional genetic studies may further dissect the role of type-A ARRs in development and their complex interactions, and biochemical analyses may reveal the mechanism by which these genes inhibit cytokinin signaling.

METHODS

Isolation of *arr* mutants

80,000 Arabidopsis lines from the Salk T-DNA collection in the Columbia ecotype were screened for T-DNA insertions in the type-A ARRs using a PCR-based method as previously described (Alonso et al., 2003). Gene specific primers used and sites of T-DNA insertions are described in supplementary materials.

Single mutants *arr3* and *arr4*, *arr5* and *arr6*, *arr8* and *arr9*, were crossed to generate double mutants *arr3,4*, arr5*,6* and *arr8,9* respectively. Double mutants *arr3,4*, *arr5,6* and *arr8,9* were crossed to generate quadruple mutants *arr3,4,5,6*, *arr5,6,8,9* and *arr3,4,8,9*. Quadruple mutants *arr3,4,5,6* and *arr5,6,8,9* were crossed to generate the hextuple mutant *arr3,4,5,6,8,9*. Double mutants *arr4,*5 and *arr4,*6 were generated by crossing the component single mutants. Insertions were confirmed by genomic PCR with gene specific and T-DNA border primers.

Growth conditions for adult plants and seedlings

Plants were grown at 23°C in ~75 uE light under short day conditions (eight hour light, 16 hour dark), long day (16 hour light, eight hour dark) and constant light as noted.

For seedling assays, seeds were surface sterilized and cold treated at 4°C for 3 days in the dark and then treated with white light for 3 hours. Unless otherwise specified, seedlings were grown on vertical plates containing 1X Murashige and Skoog salts (MS) 1% sucrose, 0.6% phytagel (Sigma) at 23°C in ~100 uE constant light. For growth on horizontal plates, seedlings were grown on 1X MS, 1% sucrose, 0.8% bactoagar at 23°C in ~75 uE constant light.

Seedling cytokinin response assays

Arabidopsis seeds were grown on vertical plates containing the appropriate concentration of the cytokinin benzyladenine (BA) or 0.1% dimethyl sulfoxide (DMSO) vehicle control for ten days. Root lengths at days four and nine were marked on the plates. The plates were photographed at 10 days and root growth between days four and nine were measured using NIH image (v. 1.62, Research Services Branch, NIMH, NIH). At ten days, total lateral roots emerged from the primary root (stage four and beyond) were quantified under a dissecting microscope. For chlorophyll assays, seedlings were grown on horizontal plates supplemented with BA. Shoot systems from two week old seedlings were harvested and chlorophyll was extracted with methanol. Chlorophyll content was determined spectrophotometrically and normalized to freshweight as previously described (Porra et al., 1989).

Analysis of *ARR* expression

For analysis of *ARR* expression in the T-DNA insertion lines, five-day-old etiolated seedlings of single mutant lines were treated with 50 μM cycloheximide and 1 μM BA for 40 min and RNA was extracted and analysed by Northern blotting as previously described, using the appropriate type-A cDNAs as hybridization probes (D'Agostino et al., 2000). For RT-PCR, seedlings were grown on horizontal plates layered with Whatman filter paper for ten days under constant light and harvested for RNA extraction. cDNA was generated using Superscript III Reverse Transcriptase (Invitrogen). ARR cDNA was amplified with a 5' primer at the ATG and a 3' primer in the third exon for 30 cycles. Primer sequences are listed in supplementary materials.

Cytokinin treatment time-course

Seedlings were grown on horizontal plates layered with Whatman filter paper for ten days under constant light. Seedlings were treated in liquid MS supplemented with 10 nM of BA in 0.1% DMSO for the appropriate duration and RNA was extracted and analysed by northern blotting as described above. *ARR7* and *β-tubulin* cDNA probes were described previously (D'Agostino et al., 2000); the *SST1* probe was generated from full length cDNA of *SST1* (At1G13420).

Complementation Analysis

ARR5 wild-type cDNA was amplified and cloned downstream of the 1.6 kb *ARR5* promoter (D'Agostino et al., 2000). The resulting promoter-cDNA construct was inserted into the pCambia1303 binary vector and transformed into *arr3,4,5,6* by the floral dip method (Clough and Bent, 1998). Transformants were selected on MS plates supplemented with 30 μg/ml hygromycin and 50 μg/ml carbenicillin. 11 independent T1 hygromycin resistant lines were selected and homozygous T3 progeny were examined in seedling cytokinin response assays as described above.

Shoot initiation assay

Arabidopsis seedlings were grown on the vertical plates in the dark for three days and then in dim light (~5 μE) for three days to produce elongated and firm hypocotyls. Hypocotyls of about 7 mm were excised from the seedlings. Hypocotyl explants were transferred to MS 1% sucrose 0.4% phytagel plates containing combinations of kinetin and NAA ranging from 0 to 3000 ng/ml for four weeks at 23°C in ~75 μE continuous light. One representative callus at each concentration was selected and arranged to create a composite photograph for each genotype.

Other assays for cytokinin response

For senescence assays, seedlings were grown on horizontal plates for 25 days. Fully expanded leaves (~7th leaf) were excised from the seedlings. To induce senescence, leaves were floated on water in parafilm-sealed petri plates supplemented with various concentrations of BA in 0.1% DMSO at 23°C in the dark for ten days. Chlorophyll was extracted and quantified spectrophotometrically from freshly cut leaves and senesced leaves as in the seedling chlorophyll analysis.

Analysis of *ARR* patterns of expression

Promoter regions to 1.6-2.0 kb upstream of ATG of *ARR3, ARR4, ARR6, ARR8* and *ARR9* were amplified by PCR and cloned upstream of the β-glucuronidase (GUS) gene in the pCambia3301 binary vector. Primers used are listed in supplementary materials. The resulting *ARR:GUS* translational fusion constructs were introduced into wild-type Col plants by the floral dip method (Clough and Bent, 1998). Ten plant lines per construct were selected by kanamycin drug resistance and examined for GUS expression. To detect GUS expression, seedlings were grown on horizontal plates supplemented with 10 nM BA or 0.1% (v/v) DMSO vehicle control. Nine day old seedlings were vacuum infiltrated at 7 x 10^{-2} kg cm^{-2} for 10 mins in X-Gluc buffer (100 mM sodium phosphate pH 7.0, 0.5% Triton X-100, 100 uM X-Gluc). The color reaction was allowed to proceed at 37°C overnight. Chlorophyll was extracted with 3 washes of 100 % ethanol and the seedlings were examined under a dissecting microscope. Representative plant lines from each construct were selected. These seedlings, as well as the previously characterized *ARR5:GUS* line (D'Agostino et al., 2000), were analyzed in parallel.

Analysis of Red light Response

The response of seedlings to red light was performed as described (Krall and Reed, 2000), with minor modifications. Mutant and wild-type seeds were sown on plates containing 1X MS, 0.1 % sucrose, 0.8% Phytagar (Gibco). The seeds were cold-treated and then pre-treated with fluorescent lights for 15 h first or immediately exposed to a red light emitting diode light source (670nm) (Quantum Devices) filtered with bronze-tinted Plexiglass filters to obtain a range of light intensities. After 3 days of red light exposure, the seedlings were scanned and the hypocotyls measured using NIH Image (v1.62).

SUPPLEMENTARY MATERIALS

	Forward	Reverse
ARR3	5'ggaactagtagcaatatctctcttctatcttttc	5'cacagaggtaaactgtcacacattatttg
ARR4	5'tttatgtgcgacacgttgatgactacttt	5'ggaggcgcgagagattaaagggacatctat
ARR5	5'tctctctgtggtacatttcttgaaaaatggg	5'cttggggaaatttctaagaaaagccatgta
ARR6	5'tgtagaagttaaatgcgtgaacttccaca	5'gctatggtgaatcctcttgacaagttactc
ARR8	5'caaatggctgttaaaacccaccaata	5'ccattgttagtgtgctatcacctgagtg
ARR9	5'ggatcccagactctttatttctcttcctc	5'cccacatacaacatcatcatcatattcc

Table 2.S1 Primers used for screening Salk T-DNA collection

	Locus	Site of T-DNA insertion on genomic sequence (**A**TG=1)
arr3	At1g59940	801
arr4	At1g10470	817
arr5	At3g48100	689
arr6	At5g62920	1021
arr8	At2g41310	35
arr9	At3g57040	782

Table 2.S2 Sites of T-DNA insertions

	Forward	Reverse
ARR3	5'tgtcgtcggagaatgtaatga	5'agattccatcgaggatgtgg
ARR8	5'tggaaacagagtcaaagttcca	5'tgtggcgatgtagagagtgc

Table 2.S3 Primers for RT-PCR

	Forward	Reverse
ARR3	5'catgtctagaactccaacacatcctttcaatagc	5'ctttggccatcctgagaaaagagtagg
ARR4	5'aaagtcgacgattttatgtgcgacacgtt	5'aaactcgagagcttatagtaactgtgagg
ARR5	5'tcgggagagagccaagcttctctaaa	5'tgatcaacgaatgttgagggatttggaa
ARR8	5'aagcttgggttaatgtggggcacc	5'tacgtagatattcaatcgaaa
ARR9	5'gaattcgccggtctaaaagtgacgagt	5'tgcgcagaaacttgaagataacaa

Table 2.S4 Primers for cloning ARR promoters

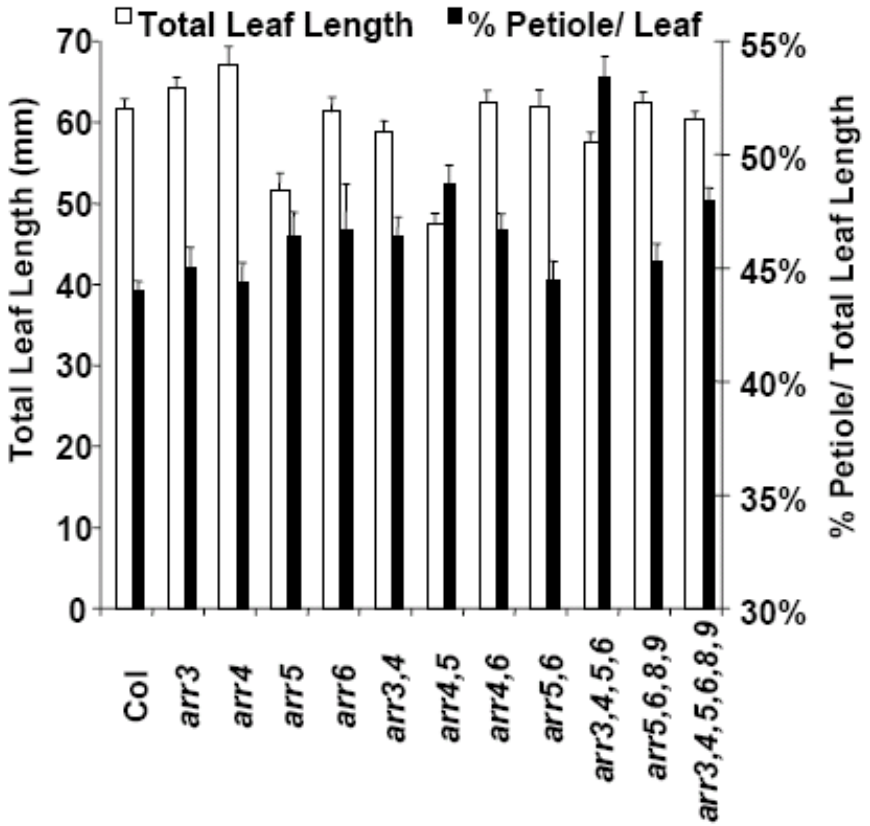

Figure 2.S1 *arr* mutants display subtle morphological differences under short day conditions

Plants were grown in short–day conditions (8hr light, 16hr dark) for nine weeks. Four fully expanded rosette leaves from at least five plants per genotype were measured. Open bars represent total length of rosette leaves, closed bars represent % length of petioles / total leaves and error bars represent standard error, n>20.

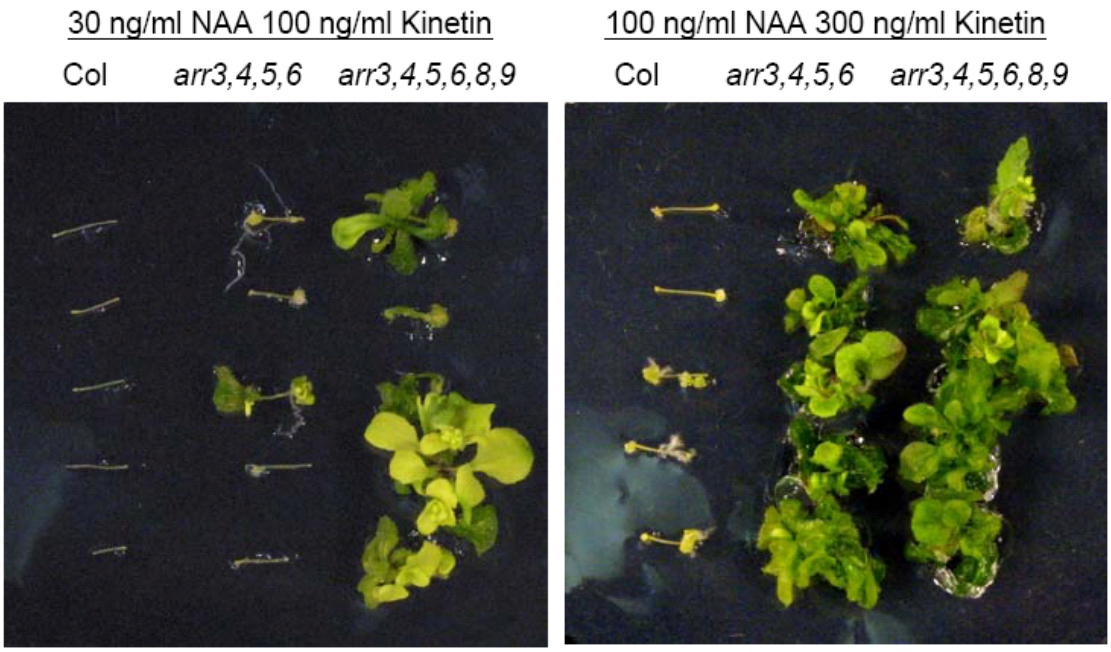

Figure 2.S2 *arr* mutants show increased propensity to generate shoots on low auxin: cytokinin ratios

Shoot initiation assays were conducted as described in methods. Photographs show all five hypocotyls of the indicated genotypes incubated on the same plate at the concentrations noted.

REFERENCES

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., and Ecker, J.R. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301,** 653-657.

Asakura, Y., Hagino, T., Ohta, Y., Aoki, K., Yonekura-Sakakibara, K., Deji, A., Yamaya, T., Sugiyama, T., and Sakakibara, H. (2003). Molecular characterization of His-Asp phosphorelay signaling factors in maize leaves: implications of the signal divergence by cytokinin-inducible response regulators in the cytosol and the nuclei. Plant Mol. Biol. **52,** 331-341.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. **16,** 735-743.

D'Agostino, I.B., Deruere, J., and Kieber, J.J. (2000). Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. Plant Physiol. **124,** 1706-1717.

Finlayson, S.A., Lee, I.J., Mullet, J.E., and Morgan, P.W. (1999). The mechanism of rhythmic ethylene production in sorghum. The role of phytochrome B and simulated shading. Plant Physiol. **119,** 1083-1089.

Gan, S., and Amasino, R.M. (1995). Inhibition of leaf senescence by autoregulated production of cytokinin. Science **270,** 1986-1988.

Hutchison, C.E., and Kieber, J.J. (2002). Cytokinin signaling in Arabidopsis. Plant Cell **14 Suppl,** S47-59.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature **413,** 383-389.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., and Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from Arabidopsis. Nature **409,** 1060-1063.

Kiba, T., Yamada, H., Sato, S., Kato, T., Tabata, S., Yamashino, T., and Mizuno, T. (2003). The type-A response regulator, ARR15, acts as a negative regulator in the cytokinin-mediated signal transduction in Arabidopsis thaliana. Plant Cell Physiol. **44,** 868-874.

Kieber, J.J. (2002). Cytokinins. In The Arabidopsis Book, E.M. Meyerowitz, ed (Rockville, MD: Am. Soc. Plant Biol.), DOI: 10.1199/tab.0063, www.aspb.org/publications/arabidopsis/.

Krall, L., and Reed, J.W. (2000). The histidine kinase-related domain participates in phytochrome B function but is dispensable. Proc. Natl. Acad. Sci. U S A **97,** 8169-8174.

Ku, H.M., Vision, T., Liu, J., and Tanksley, S.D. (2000). Comparing sequenced segments of the tomato and Arabidopsis genomes: large-scale duplication followed by selective gene loss creates a network of synteny. Proc. Natl. Acad. Sci. U S A **97,** 9121-9126.

Leyser, O. (2002). Molecular genetics of auxin signaling. Annu. Rev. Plant Biol. **53,** 377-398.

Li, J., Swanson, R.V., Simon, M.I., and Weis, R.M. (1995). The response regulators CheB and CheY exhibit competitive binding to the kinase CheA. Biochemistry **34,** 14626-14636.

Li, Y., Hagen, G., and Guilfoyle, T.J. (1992). Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. Dev. Biol. **153,** 386-395.

McCormac, A.C., Wagner, D., Boylan, M.T., Quail, P.H., Smith, H., and Whitelam, G.C. (1993). Photoresponses of Transgenic Arabidopsis Seedlings Expressing Introduced Phytochrome-B-Encoding cDNAs - Evidence That Phytochrome-a and Phytochrome-B Have Distinct Photoregulatory Functions. Plant J. **4,** 19-27.

Medford, J.I., Horgan, R., El-Sawi, Z., and Klee, H.J. (1989). Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. Plant Cell **1,** 403-413.

Miller, C.O., Skoog, F., Von Saltza, M.H., and Strong, F. (1955). Kinetin, a cell division factor from deoxyribonucleic acid. J. Am. Chem. Soc. **77,** 1392-1293.

Miller, C.O., Skoog, F., Okomura, F.S., von Saltza, M.H., and Strong, F.M. (1956). Isolation, structure and synthesis of kinetin, a substance promoting cell division. J. Am. Chem. Soc. **78,** 1345-1350.

Mok, D.W., and Mok, M.C. (2001a). Cytokinin Metabolism and Action. Annu. Rev. Plant Physiol. Plant Mol. Biol. **52,** 89-118.

Mok, D.W., and Mok, M.C. (2001b). Cytokinins: Chemistry, Activity and Function. (Boca Raton, FL.: CRC Press).

Osakabe, Y., Miyata, S., Urao, T., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2002). Overexpression of Arabidopsis response regulators,

ARR4/ATRR1/IBC7 and ARR8/ATRR3, alters cytokinin responses differentially in the shoot and in callus formation. Biochem. Biophys. Res. Commun. **293,** 806-815.

Porra, R.J., Thompson, W.A., and Kriedemann, P.E. (1989). Determination of Accurate Extinction Coefficients and Simultaneous-Equations for Assaying Chlorophyll-a and Chlorophyll-B Extracted with 4 Different Solvents - Verification of the Concentration of Chlorophyll Standards by Atomic-Absorption Spectroscopy. Biochim Biophys Acta **975,** 384-394.

Rabin, R.S., and Stewart, V. (1993). Dual response regulators (NarL and NarP) interact with dual sensors (NarX and NarQ) to control nitrate- and nitrite-regulated gene expression in Escherichia coli K-12. J. Bacteriol. **175,** 3259-3268.

Rashotte, A.M., Carson, S.D., To, J.P., and Kieber, J.J. (2003). Expression profiling of cytokinin action in Arabidopsis. Plant Physiol. **132,** 1998-2011.

Sa, G., Mi, M., He-chun, Y., Ben-ye, L., Guo-feng, L., and Kang, C. (2001). Effects of ipt gene expression on the physiological and chemical characteristics of Artemisia annua L. Plant Sci. **160,** 691-698.

Sakai, H., Aoyama, T., and Oka, A. (2000). Arabidopsis ARR1 and ARR2 response regulators operate as transcriptional activators. Plant J. **24,** 703-711.

Sakai, H., Aoyama, T., Bono, H., and Oka, A. (1998). Two-component response regulators from Arabidopsis thaliana contain a putative DNA-binding motif. Plant Cell Physiol. **39,** 1232-1239.

Sakai, H., Honma, T., Aoyama, T., Sato, S., Kato, T., Tabata, S., and Oka, A. (2001). ARR1, a transcription factor for genes immediately responsive to cytokinins. Science **294,** 1519-1521.

Smigocki, A.C. (1991). Cytokinin content and tissue distribution in plants transformed by a reconstructed isopentenyl transferase gene. Plant Mol. Biol. **16,** 105-115.

Sourjik, V., and Schmitt, R. (1998). Phosphotransfer between CheA, CheY1, and CheY2 in the chemotaxis signal transduction chain of Rhizobium meliloti. Biochemistry **37,** 2327-2335.

Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000). Two-component signal transduction. Annu. Rev. Biochem. **69,** 183-215.

Su, W., and Howell, S.H. (1995). The effects of cytokinin and light on hypocotyl elongation in Arabidopsis seedlings are independent and additive. Plant Physiol. **108,** 1423-1430.

CHAPTER 3

WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators

Andrea Leibfried, Jennifer P. C. To, Wolfgang Busch*, Sandra Stehling*, Andreas Kehle, Monika Demar, Joseph J. Kieber & Jan U. Lohmann

*These authors contributed equally to this publication

PREFACE

This work was conducted as a collaborative project between the Kieber and Lohmann labs to study the interaction between type-A ARRs and meristem function. I determined lethality of the loss-of-function *arr7,15* double mutant. I constructed the septuple loss-of-function *arr3,4,5,6,7,8,9* mutant and characterized the irregularities of inflorescence patterning in this mutant. I also participated in discussions, contributed to writing and provided comments on the entire manuscript.

This work has been published in: Leibfried, A., To, J.P.C., Stehling, S.K.*, A., Busch, W.*, Demar, M., Kieber, J.J., and Lohmann, J.U. (2005). WUSCHEL controls meristem size by direct transcriptional regulation of cytokinin inducible response regulators. Nature 438, 1172-1175. (*These authors contributed equally to this publication.)

ABSTRACT

Plants continuously maintain pools of totipotent stem cells in their apical meristems from which elaborate root and shoot systems are produced. In Arabidopsis thaliana, stem cell fate in the shoot apical meristem is controlled by a regulatory network that includes the CLAVATA (CLV) ligand–receptor system and the homeodomain protein WUSCHEL (WUS) (Brand et al., 2000; Schoof et al., 2000). Phytohormones such as auxin and cytokinin are also important for meristem regulation (Leyser, 2003). Here we show a mechanistic link between the CLV/WUS network and hormonal control. WUS, a positive regulator of stem cells, directly represses the transcription of several two-component *ARABIDOPSIS RESPONSE REGULATOR* genes (*ARR5*, *ARR6*, *ARR7* and *ARR15*), which act in the negative feedback loop of cytokinin signalling (Kiba et al., 2003; To et al., 2004). These data indicate that ARR genes might negatively influence meristem size and that their repression by WUS might be necessary for proper meristem function. Consistent with this hypothesis is our observation that a mutant ARR7 allele, which mimics the active, phosphorylated form, causes the formation of aberrant shoot apical meristems. Conversely, a loss-of-function mutation in a maize *ARR* homologue was recently shown to cause enlarged meristems (Giulini et al., 2004).

RESULTS AND DISCUSSION

Genetic analyses have led to the discovery of several essential regulators of stem cell fate in the shoot apical meristem of the reference plant Arabidopsis thaliana. Among them, the homeodomain transcription factors WUSCHEL (WUS) and SHOOTMERISTEM-LESS (STM) have positive functions (Laux et al., 1996; Long et al., 1996), whereas the CLAVATA (CLV) genes negatively influence meristem size (Clark et al., 1993, 1995; Kayes and Clark, 1998). *WUS* is expressed in the organizing centre and induces stem cell fate in the overlaying cells (Mayer et al., 1998) that in turn express CLV3, a small secreted peptide (Fletcher et al., 1999; Rojo et al., 2002) that is thought to act as ligand for the CLV1–CLV2 heteromeric receptor complex (Clark et al., 1997; Jeong et al., 1999). Activation of the CLV1–CLV2 receptor leads to the suppression of *WUS* expression, creating a negative feedback loop that controls the size of the stem cell pool (Brand et al., 2000; Schoof et al., 2000).

Despite the central role of the WUS transcription factor in the initiation and maintenance of stem cell fate, only a single direct target, the floral homoeotic gene *AGAMOUS* (*AG*), which represses the maintenance of stem cells in the flower, has been described (Lohmann et al., 2001). To identify target genes of WUS and other meristem regulators, we performed a comparative microarray screen using plants with ethanol-inducible overexpression alleles (Roslan et al., 2001) of *WUS* as well as *STM* and *LEAFY* (LFY), a floral regulator that interacts with *WUS* (Lenhard et al., 2001; Lohmann et al., 2001). After 12 h of treatment with ethanol we harvested the shoot apex and surrounding tissue (Fig. 3.1A) and subjected it to expression profiling with Affymetrix Ath1 arrays. A combination of per-gene and common variance (Lemon et al., 2003) filtering was used to

identify 148 genes responsive to *WUS* but not to *STM* or *LFY* induction. Of these 148 genes, 44 were repressed, including *ARR5*, *ARR6*, *ARR7* and *ARR15*, which belong to the 10-member type-A *ARABIDOPSIS RESPONSE REGULATOR* gene family (D'Agostino et al., 2000) (Fig. 3.1b). Type-A ARR proteins contain a phosphate-accepting receiver domain similar to bacterial two-component response regulators, but in contrast to type-B ARR proteins they lack a DNA-binding motif in their output domain

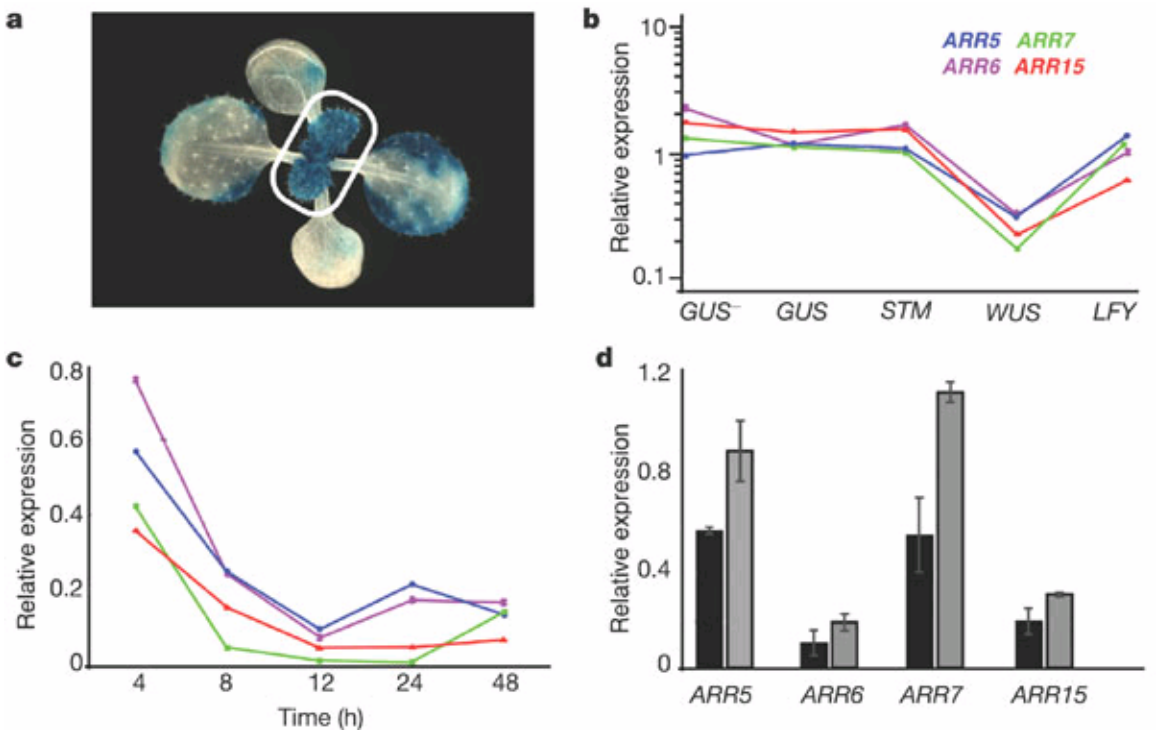

Figure 3.1 Expression profiles of ARR5, ARR6, ARR7 and ARR15

(a) A 12-day-old seedling showing ectopic *AG::GUS* reporter gene activation in response to *WUS* induction. Tissue used for expression profiling is indicated. (b) Expression of *ARR5* (blue), *ARR6* (purple), *ARR7* (green) and *ARR15* (red) is specifically repressed by WUS as detected by microarrays. (c) Real-time qRT–PCR confirms rapid repression of *ARR* genes by WUS. Relative expression is normalized to induced *AlcA::GUS* controls. Line colours are as in (b). (d) *ARR* expression in response to downregulation of *WUS* by induction of *AlcA::CLV3* (grey bars). Black bars, *AlcA::GUS*. Relative expression measured by realtime qRT–PCR is normalized to TUBULIN. Error bars indicate s.e.m.

(D'Agostino and Kieber, 1999). Their expression is rapidly induced by cytokinin (D'Agostino et al., 2000), which has been shown to be a potent inductor of cell proliferation when applied exogenously together with auxin and to induce shoot development when acting alone (Skoog and Miller, 1957). Type-A ARR proteins have been implicated in the negative feedback regulation of cytokinin signalling on the basis of the observation of decreased hormone sensitivity in plants overexpressing type-A *ARR* genes (Hwang and Sheen, 2001; Kiba et al., 2003). Furthermore, in Arabidopsis, type-A *arr* multiple mutants have increased cytokinin sensitivity. However, even in sextuple type-A *arr* mutants (*arr3 arr4 arr5 arr6 arr8 arr9*) morphological changes are minimal, indicating strong redundancy within the gene family (To et al., 2004). *ARR5* and *ARR6*, as well as *ARR7* and *ARR15*, constitute closely related pairs within the gene family (D'Agostino et al., 2000), and inspection of the AtGenExpress expression atlas (Schmid et al., 2005) revealed co-expression of each pair, marked by widespread transcription with highest levels in meristematic tissue for *ARR7* and *ARR15*, and in roots for *ARR5* and *ARR6*.

By using quantitative real-time reverse transcriptase-mediated polymerase chain reaction (qRT–PCR), we found that 4 h after *WUS* induction by ethanol, RNA levels of *ARR5*, *ARR6*, *ARR7* and *ARR15* were already decreased, and after 12 h they reached a minimum at about 10% of control levels. Expression levels remained low for at least 48 h after treatment with ethanol (Fig. 3.1c). To test whether WUS is not only sufficient but also necessary for the repression of *ARR5*, *ARR6*, *ARR7* and *ARR15* in wild-type meristems, we used inducible *CLV3* to transiently repress *WUS*, because the morphology of *wus* mutants deviates strongly from the wild type even at very early stages of

development (Laux et al., 1996). Besides a strong reduction of *WUS* expression, we observed by qRT–PCR a moderate increase in expression of the ARR genes after 24 h of *CLV3* induction (Fig. 3.1d), which is consistent with the idea that ARR expression extended into the small *WUS* domain in these plants.

In situ hybridization on sections of inflorescence meristems demonstrated that *ARR7* RNA accumulates in a subdomain of the meristem consistent with a potential function in this tissue (Figs. 3.2a, and 3.S1). Reporter gene analysis confirmed this pattern and showed in addition that *ARR5*, *ARR6* and *ARR15* promoters are also active in the meristem (Fig. 3.S2).

In plants with an inducible *WUS* transgene (Fig. 3.2h), *ARR7* RNA could no longer be detected 24 h after *WUS* induction (Fig. 3.2b), which is similar to the situation

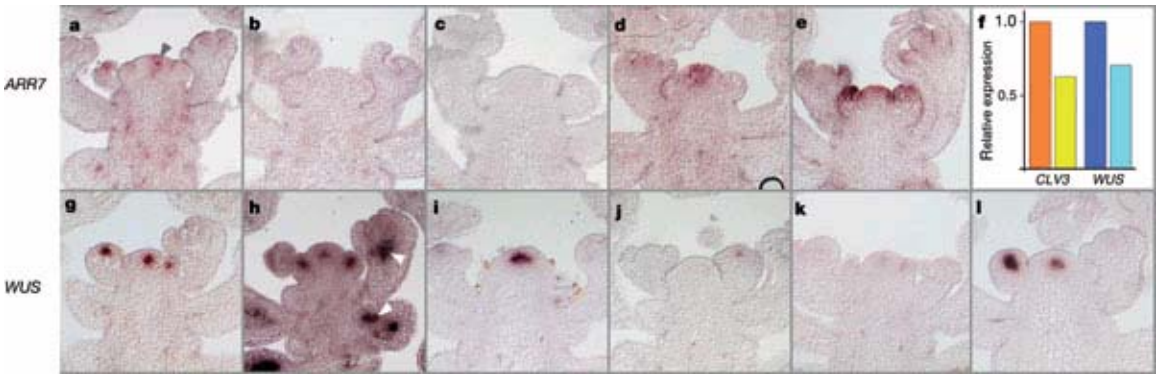

Figure 3.2 Expression patterns of ARR7 and WUS in response to meristematic signals

Upper panels show in situ hybridizations of *ARR7*; lower panels show *WUS*. (a and g) Wild type. (b and h) Induced *35S::AlcR AlcA::WUS*. *WUS* is moderately expressed in all cells with hot spots in more mature tissue (arrowheads). (c and i) *clv3-7* mutant. (d and j) Induced *35S::AlcR AlcA::CLV3*. (e) 6-Benzylaminopurine-treated wild type. (f) *CLV3* and *WUS* expression in inflorescence apices of wild-type and *arr3 arr4 arr5 arr6 arr7 arr8 arr9* septuple mutants as measured by qRT–PCR. Dark colours represent wild-type, light colours indicate mutant. (k) *35S::ARR7*. (l) *arr3 arr4 arr5 arr6 arr7 arr8 arr9* septuple mutant.

in *clv3* mutants (Fig. 3.2c), in which *WUS* expression is expanded (Figs. 3.2g and i). Conversely, after suppression of *WUS* by *CLV3* induction (Fig. 3.2j), we observed an expansion of the *ARR7* expression domain (Fig. 3.2d), confirming the qRT–PCR results. Activation of *ARR7* in cells outside the *WUS* domain might indicate a more direct effect of CLV3 on *ARR7* expression in parallel to its WUS dependent activity. Similarly to what has been observed for the maize homologue *ABPH1* (Giulini et al., 2004), a 30-min treatment with the synthetic cytokinin 6-benzylaminopurine caused an expansion of *ARR7* expression in the wild type (Fig. 3.2e).

An additional level of regulation is provided by negative feedback of ARR7 on *WUS*, because plants that overexpress *ARR7* from the constitutive 35S promoter have lower *WUS* RNA levels (Fig. 3.2k). However, residual *WUS* activity in *35S::ARR7* plants is sufficient for correct function of the meristem, because *35S::ARR7* plants have no obvious defects in the shoot apical meristem, similar to induced *AlcA::CLV3* plants, which show a *wus* mutant phenotype only in flowers (data not shown).

Having established a regulatory interaction between *WUS* and *ARR7*, we next asked whether this interaction is direct. To this end, we first made use of an inducible form of WUS by means of a translational fusion to the ligand-binding domain of the rat glucocorticoid receptor (*WUS:GR*). Application of a steroid such as dexamethasone causes translocation of the fusion protein from the cytoplasm to the nucleus, allowing activation or repression of direct targets in the absence of protein synthesis (Brand et al., 2002; Lenhard et al., 2002). After treatment of *35S::WUS:GR* plants with dexamethasone for 4 h, we observed robust repression of *ARR5*, *ARR6*, *ARR7* and *ARR15*. Repression of the *ARR* genes also occurred in the presence of the protein synthesis inhibitor

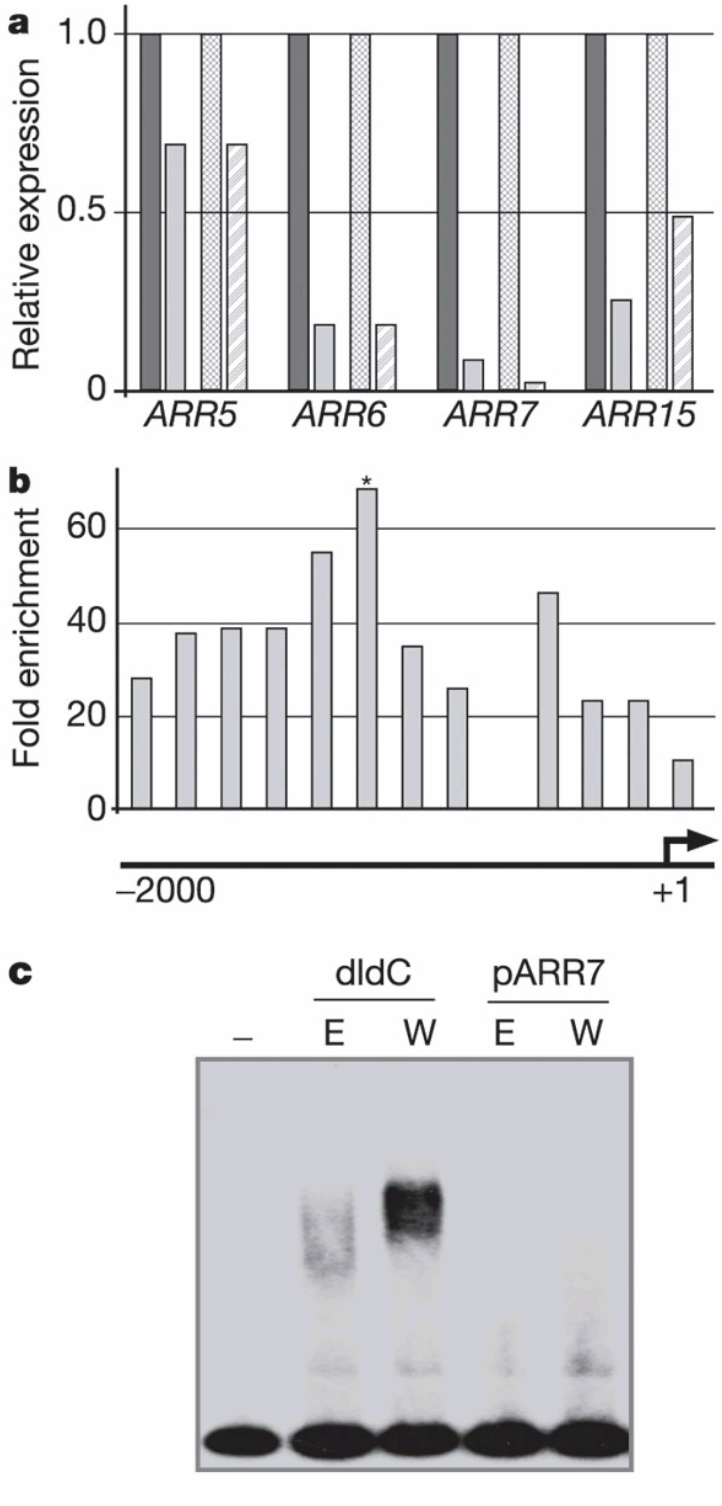

Figure 3.3 Direct interaction of WUS with regulatory sequences of ARR7

(a) Real-time qRT–PCR on *35S::WUS:GR* plants. Dark grey bars represent mock treatment, light grey bars induction with dexamethasone, crosshatched bars mock treatment in the presence of cycloheximide, and hatched bars induction with dexamethasone in the presence of cycloheximide. Expression values are normalized to the respective mock treatment controls (see Fig. 3.S3a for alternative normalization). (b) Detection of *ARR7* regulatory sequences by real-time qRT–PCR after ChIP with anti-WUS antiserum (see Fig. 3.S3b). Enrichment of overlapping genomic fragments upstream of the *ARR7* start codon is shown after normalization to unrelated control sequences (see also Fig. 3.S3c for alternative normalization). ChIP was performed on induced *35S::WUS:GR* tissue. Asterisk, promoter fragment used for gel shifts. (c) EMSA using *ARR7* promoter sequences identified in (b); -, free probe; E, control protein extract from yeast expressing *LEAFY*; W, protein extract from yeast expressing *WUSCHEL*; dIdC, poly(dIdC) used as unspecific competitor; pARR7, unlabelled probe used as specific competitor.

cycloheximide (Figs. 3.3a, and 3.S3a), which is compatible with a direct interaction of WUS with the regulatory elements of the *ARR* genes. We then confirmed in vivo binding of WUS to *ARR7* promoter sequences by chromatin immunoprecipitation (ChIP) with a polyclonal anti-WUS antiserum (Fig. 3.3b, 3.S3b and c). We observed a twofold enrichment of *ARR7* promoter DNA in wild-type inflorescences in comparison with leaves, in which *WUS* is not expressed, whereas in *WUS* overexpressing tissue *ARR7* promoter DNA was enriched 68-fold. The ChIP results indicated binding of WUS to sequences located about 1,000 base pairs upstream of the start codon of *ARR7* in a region harbouring multiple TAATelements, which have been shown to be the core binding sites for WUS (Fig. 3.3b)(Lohmann et al., 2001). Subsequently, we were able to confirm sequence-specific binding of WUS protein to this promoter element by electrophoretic mobility-shift assays (EMSAs) (Fig. 3.3c).

It has recently been shown that maize mutants defective for *ABPH1*, a type-A *ARR* homologue, have defects in phyllotaxis and meristem size regulation (Giulini et al., 2004). In contrast, neither Arabidopsis plants lacking individual type-A *ARR* genes nor plants overexpressing *ARR5*, *ARR6*, *ARR7* or *ARR15* have obvious phenotypes (data not shown, and (Kiba et al., 2003; To et al., 2004)). We therefore constructed *arr7 arr15* double mutants, because they are closely related and both are expressed in meristematic tissue. However, the double mutant combination caused female gametophytic lethality, precluding analysis of the progeny. To reduce redundancy outside the *ARR7/ARR15* pair, we then extended our analysis to *arr3 arr4 arr5 arr6 arr7 arr8 arr9* septuple mutants. These plants were viable, although they had defects in phyllotaxis and organ initiation (Figs. 3.4a and b), indicating that the redundant function of *ARR7* and *ARR15* might be

sufficient for meristem maintenance. *WUS* expression in the inflorescence meristem of septuple mutants was decreased (Figs. 3.2f and l), indicating that, in addition to the negative regulatory activity of *ARR7* on *WUS*, there might be positive effects on *WUS* expression by other type-A ARR genes.

As an alternative to exploring *ARR7* function, we constructed alleles that either mimic the active, phosphorylated state or the inactive non-phosphorylated state of ARR7 by mutating aspartate 85 to glutamate or asparagine, respectively (Hass et al., 2004). Whereas ubiquitous overexpression of the dominant-negative form (Asp 85 → Asn) did not cause any morphological defects, the constitutively active form (Asp 85 → Glu) had

Figure 3.4 Phenotypes of type-A ARR mutant plants

(a) Wild type. (b) *arr3 arr4 arr5 arr6 arr7 arr8 arr9* septuple mutant; note irregular organ positioning indicated by arrowheads. (c–f) Activity of the shoot apical meristem is arrested in *35S::ARR7* (Asp 85 → Glu) plants similar to wus mutants 5 days after sowing. Scale bars, 1mm for seedlings and 100 mm for meristem insets unless otherwise noted. (c) *35S::ARR7* (Asp 85 → Glu) plant with wild-type morphology. Scale bar, 200 mm. (d) *35S::ARR7* (Asp 85 → Glu) plant with intermediate phenotype. (e) *35S::ARR7* (Asp 85 → Glu) plant with strong phenotype. (f) *wus* mutant seedling. (g) *35S::ARR7* (Asp 85 → Glu) seedling shortly after recovery of meristematic activity. Arrowheads indicate duplicated meristems. (h) Phenotype of an adult *35S::ARR7* (Asp 85 → Glu) plant after recovery. Note duplicated rosettes. Arrowheads indicate irregular side-shoot positions.

severe effects on the function of the shoot apical meristem. In some of the transgenic seedlings meristems were arrested for several days after expansion of the cotyledons, resulting in an almost complete block of organ formation, very similar to that observed in wus mutants (Figs. 3.4c–f). Subsequently, shoot apical meristems recovered proliferative activity, but often split into two or three independent meristems (Fig. 3.4g), giving rise to multiple primary shoots. Similarly to the *abph1* mutant of maize (Giulini et al., 2004), these shoots had defects in phyllotaxis (Fig. 3.4h) and flower formation; in addition they did not produce seeds. Our results show that direct interaction between the *CLV/WUS* network and the cytokinin signalling circuitry is required for proper meristem function. Together with the recently uncovered role of the type-A response regulator *ABPH1* in maize (Giulini et al., 2004), our findings are a first step towards understanding how global hormonal signals are integrated with local transcriptional inputs in the regulation of cell behaviour at the shoot apical meristem.

METHODS

Plant material and treatments

Plants were of Columbia background and grown at 23 °C in continuous light. Inductions with ethanol were performed at 20 °C by watering with 1% ethanol. For inductions with dexamethasone, tissue was incubated in 15 mM dexamethasone and 0.015% Silwet L-77. Cycloheximide was used at 10 mM. For 6-benzylaminopurine treatments, tissue was incubated in 1 mM 6-benzylaminopurine and 0.1% DMSO. The Columbia *wus* allele corresponds to *wus-4* (provided by Martin Hobe and Rüdiger Simon); details on the *arr3 arr4 arr5 arr6 arr7 arr8 arr9* septuple mutant are available in Table 3.S1 .

Microarray experiments

Affymetrix Ath1 microarrays were hybridized as described (Schmid et al., 2003) in duplicates using RNA from pools of 20 plants for each replicate. Expression estimates were calculated by gcRMA (Wu et al., 2004) and statistical testing for differential expression was performed with LogitT (Lemon et al., 2003). Quantitative real-time RT–PCR. qRT–PCR was performed as described (Schmid et al., 2003) with the use of either SYBR-green or Taq-Man probes (Fig. 3.1d). Experiments were performed in triplicates from RNA of pooled tissue. Amplification of *TUBULIN* served as control. Oligonucleotides are listed in Table 3.S2.

In situ hybridization.

In situ hybridization was performed in accordance with standard protocols, with the addition of 10% poly(vinyl alcohol) (molecular mass 70–100 kDa) to the staining solution. ChIP. Genomic fragments were analysed by real-time qRT–PCR in triplicates.

Unrelated sequences in the experimental tissue and *ARR7* sequences in leaves, where *WUS* should not be present, served as controls.

EMSA

EMSA was performed as described in (Lohmann et al., 2001).

Transgenes

Complementary DNAs flanked by the *AlcA* promoter and the *OCS* terminator were inserted into a pMLBART-derived binary vector, which harbours a *35S::AlcR* cassette (Roslan et al., 2001). Constitutive overexpression constructs were made in pMLBARTor pART27 binary vectors using a *35S* promoter and an *OCS* terminator.

SUPPLEMENTARY MATERIALS

For construction of the *arr3,4,5,6,7,8,9* septuple mutant the T-DNA insertions mapped to the following positions relative to the ATG:

	Mutant Locus	Insertion site		Mutant Locus	Insertion site
arr3	At1g59940	801	*arr7*	At1g19050	642-660
arr4	At1g10470	817	*arr8*	At2g41310	35
arr5	At3g48100	689	*arr9*	At3g57040	782
arr6	At5g62920	1021			

Table 3.S1 *arr3,4,5,6,7,8,9* mutant loci

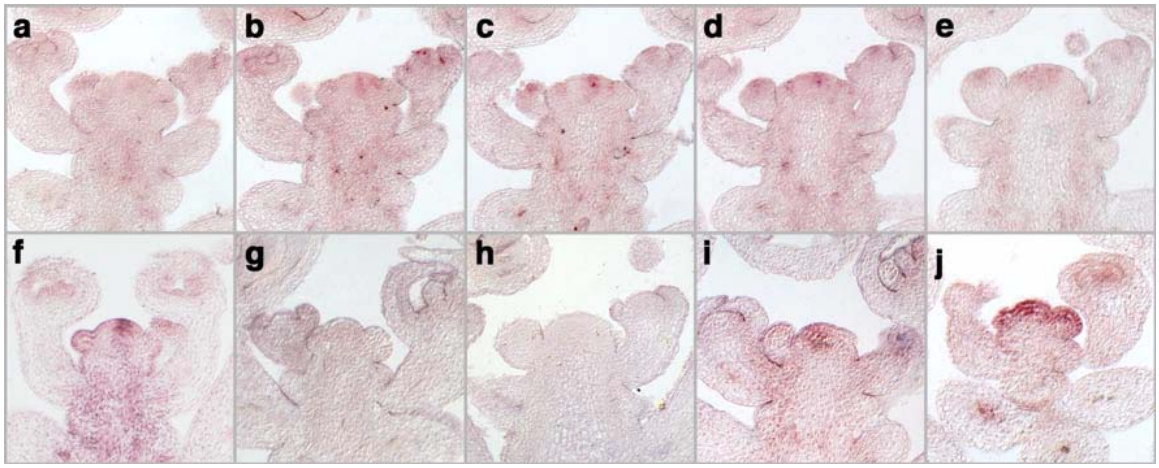

Figure 3.S1. Expression patterns of *ARR7* RNA.
(a–e) Serial sections through a wild-type inflorescence meristem hybridized with *ARR7* probe; (c) section through the center of the meristem shown in Figs. 3.2a and f independent in situ hybridization of wild-type meristem; (g) induced *35S::AlcR AlcA::WUS*; (h) *clv3-7* mutant; (i) induced *35S::AlcR AlcA::CLV3*; (j) BA treated wild type.

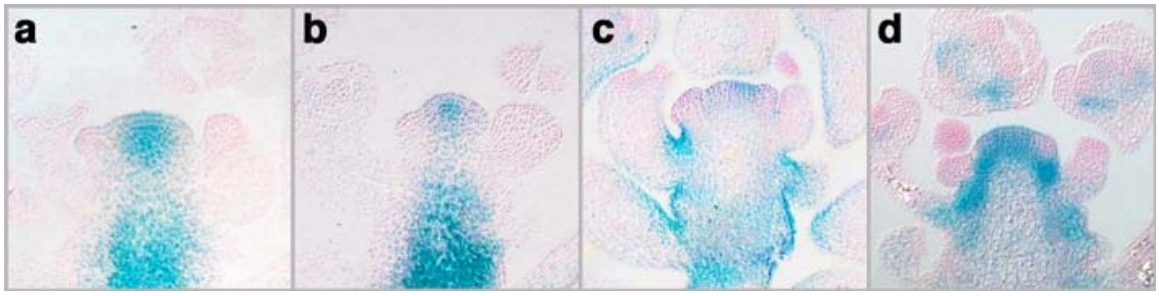

Figure 3.S2. Activity of *ARR5*, *ARR6*, *ARR7* and *ARR15* GUS reporter genes.
(a) *ARR5::GUS* (To et al, Plant Cell. 2004 16:658-71); (b) *ARR6::GUS* (To et al, Plant Cell. 2004 16:658-71); (c) *ARR7::GUS;* (d) *ARR15::GUS.*

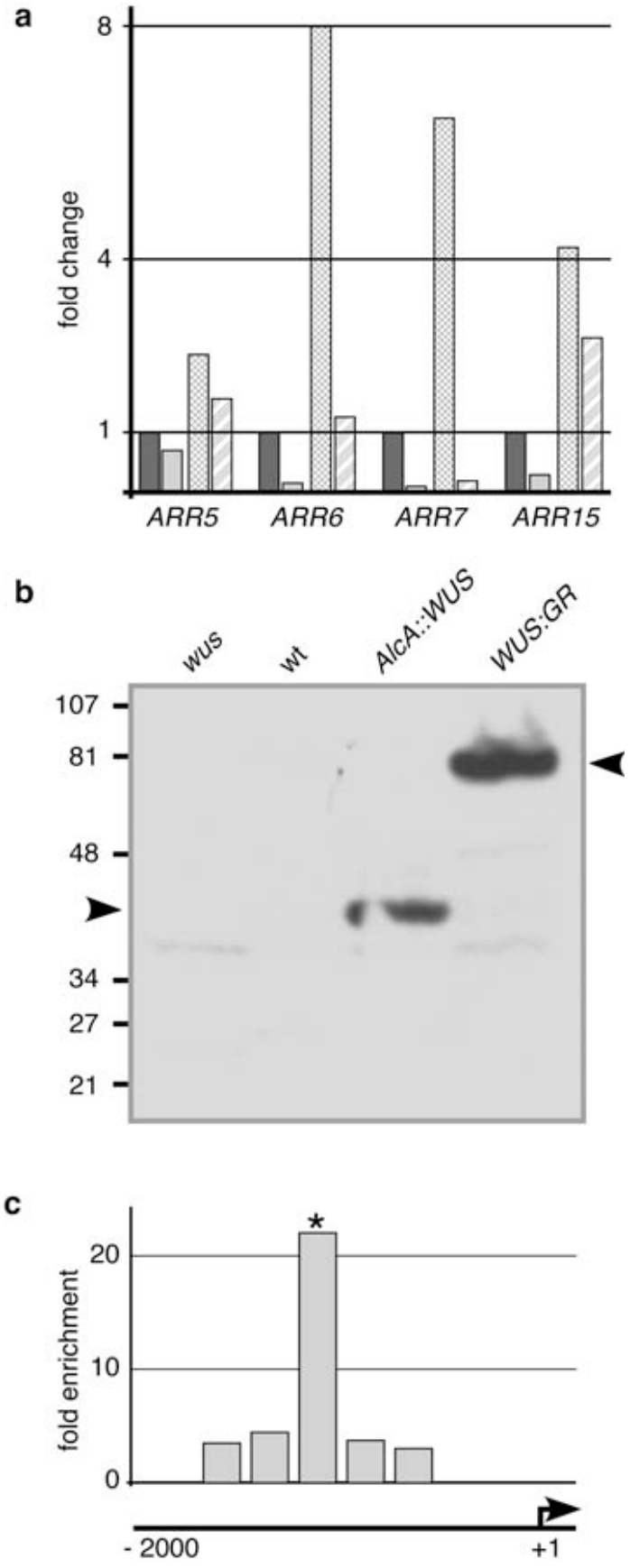

Figure 3.S3. Direct interaction of WUS with regulatory sequences of *ARR7*.
(a) Real-time qRT-PCR following 4 hours DEX induction of *35S::WUS:GR* plants. Dark grey bars represent mock treatment, light grey bars represent DEX induction. Crossed bars indicate mock treatment in the presence of cycloheximide, hatched bars represent DEX induction in the presence of cycloheximide. Expression values are normalized to mock treatment controls. (b) Western-blot of crude protein extract from *wus* mutants, wild-type, induced *35S::AlcR AlcA::WUS* and *35::WUS:GR* plants detected with the polyclonal anti-WUS antiserum used for ChIP. (c) Chromatin-immunuprecipitation of ARR7 promoter sequences using anti-WUS antiserum. ChIP was performed on leaves of *35S::WUS:GR* plants 4h after induction and compared to uninduced leaves without normalization to unrelated sequences. Fold enrichment of overlapping genomic fragments (approx size 200bp) upstream of the *ARR7* start codon is shown. Asterisk indicates promoter fragment used for gel shifts.

Purpose	Gene	Name	Sequence
TaqMan probes	*TUBULIN*	G-4558	CAC TCA TCA GCA TTC TCA ACA AG
	ARR5	G-4560	CTC TTC ACA TCA GCT AAT TTC ACA G
	ARR6	G-4561	CTC TCT CTT CTG AAT ACT CTT TGT G
	ARR7	G-4562	TGT CAT CTG AGA ACA TCT TAC CT
	ARR15	G-4563	AAC TCC TCT GCT CCT TCT ATC AT
GUS reporters	*ARR7*	G-3523	CAG CTG CAG TTG TAG CTA TCG TGA C
		G-3524	GTC CTG CAG GTT CCA AGA AGA GGA AAG
	ARR15	G-3529	ACG CTG CAG AAG TGA CTT CTT GGG TA
		G-3530	GTC CTG CAG CTC TCG GGA AAG TAA
qRT-PCR *WUS:GR* and *AlcA::CLV3*	*TUBULIN*	N-0078	GAG CCT TAC AAC GCT ACT CTG TCT GTC
		N-0079	ACA CCA GAC ATA GTA GCA GAA ATC AAG
	ARR5	G-0935	CAT CTT GCC TCG TAT CGA TAG
		G-0830	GTC TCT AGA TCA GAT CTT TGC GCG T
	ARR6	G-0936	CGG TAT GAC TGG ATA TGA AC
		G-3549	ATT TGC ATC GGA GAG CTC
	ARR7	G-0937	GCA TTC AGA GAA GTA CCA GTA GTG
		G-3490	GCT AAG GTC TTG GCC TCT ATA C
	ARR15	G-0938	CTT CAG CAC TCA GAG AAA TCC
		G-0836	GTC TCT AGA TTA ACC CCT AGA CTC T
mutagenic *ARR7* primers	*ARR7* D85=> E	G-4151	GGT GAA TTT GAT AGT GAC GGA ATA CTC AAT GCC
		G-4152	GGC ATT GAG TAT TCC GTC ACT ATC AAA TTC ACC
	ARR7 D85=> N	G-4149	GGT GAA TTT GAT AGT GAC GAA TTA CTC AAT GCC
		G-4150	GGC ATT GAG TAA TTC GTC ACT ATC AAA TTC ACC
qRT-PCR *AlcA::WUS* time series	*TUBULIN*	N-0078	GAG CCT TAC AAC GCT ACT CTG TCT GTC
		N-0079	ACA CCA GAC ATA GTA GCA GAA ATC AAG
	ARR5	G-0935	CAT CTT GCC TCG TAT CGA TAG
		G-0830	GTC TCT AGA TCA GAT CTT TGC GCG T
	ARR6	G-0936	CGG TAT GAC TGG ATA TGA AC
		G-0832	GTC TCT AGA TCA GAT CTT TGC GCG TT
	ARR7	G-0937	GCA TTC AGA GAA GTA CCA GTA GTG
		G-0834	GTC TCT AGA TCA AAG TAG AGA AAA A
	ARR15	G-0938	CTT CAG CAC TCA GAG AAA TCC
		G-0836	CTT CAG CAC TCA GAG AAA TCC
ChIP	*ARR7* promoter	G-4435	CGT GAC TAT ATC ATC AAT AGT CCG
		G-4436	TCA TCC ATT TGC TCC GTA CCT A
		G-4425	ACG GAG CAA ATG GAT GAC TGT
		G-4426	CGT GCC AAA CAA AAC ATT GG
		G-4423	GTT TGG CAC GAT CAT CAA ATG
		G-4424	TGT TGA GAT CTA CAA GAA TCT AGC A
		G-4421	TGC TAG ATT CTT GTA GAT CTC AAC A
		G-4422	TCT TCA AAA GTT GTG GAG GAG G
		G-4419	CCT CCT CCA CAA CTT TTG AAG ATT
		G-4420	ACC AGG ACA ACA GCC ATA TCA T
		G-4417	GGC TGT TGT CCT GGT ATT ATT TCT C
		G-4418	TAT TGC GGG TGG ATC CTT TT
		G-4415	ATC CAC CCG CAA TAC TTT GT
		G-4416	AGT GGA CCA TTG TGT GTG ATT TG
		G-4413	CAC ACA ATG GTC CAC TAA ACA GA
		G-4414	GTC ATC GTT TTC AGT TGA GTT TCC
		G-4411	GGA AAC TCA ACT GAA AAC GAT GAC
		G-4412	TGT GTA ATG GTA TGA TGG TGA TAC T
		G-4433	AGT ATC ACC ATC ATA CCA TTA CAC A
		G-4434	CCT CTC TCT CTC TTC TTT TTT CTT T
		G-4431	AAA GAA AAA AGA AGA GAG AGA GAG G
		G-4432	GGA AGT GTC AGA AAA CAC AAA TAA
		G-4429	CTG ACA CTT CCC AAA CAC ATA ACA
		G-4430	GAA GAA GAA GTT AGG TTA GGT GAC A
		G-4427	TGC CGT CGA TGA TAG TAT TGT G
		G-4428	GAG GAG AGT TTG TTA ATG AAG AAG A
	MYB97 coding (control)	G-3262	TCT CCG ACC TTT CTT CAC ACC CAT TCC
		G-3263	GTC TCC GCT TAG GAG CAC GAA AGC TAT C
	FLC promoter (control)	G-2622	ATT GAT TCA TAT TTT TCA TAC ACA G
		G-1793	AAT TAG ACC AGT TTA TGT ACA GCA
	ARR6 coding (control)	G-4270	GCC ACC ATG GTT TCA CAT CAT ATC
		G-4271	CCT TTG CAA GAA GAT ACT CTG AGC
	HSF1 promoter (control)	G-4680	GCT ATC CAC AGG TTA GAT AAA GGA G
		G-4681	GAG AAA GAT TGT GTG AGA ATG AAA

Table 3.S2 Primers used

REFERENCES

Brand, U., Grunewald, M., Hobe, M., and Simon, R. (2002). Regulation of CLV3 expression by two homeobox genes in Arabidopsis. Plant Physiol. **129,** 565-575.

Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M., and Simon, R. (2000). Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. Science **289,** 617-619.

Clark, S.E., Running, M.P., and Meyerowitz, E.M. (1993). CLAVATA1, a regulator of meristem and flower development in Arabidopsis. Development **119,** 397-418.

Clark, S.E., Running, M.P., and Meyerowitz, E.M. (1995). CLAVATA3 is a specific regulator of shoot and floral meristem development affecting the same processes a CLAVATA1. Development **121,** 2057-2067.

Clark, S.E., Williams, R.W., and Meyerowitz, E.M. (1997). The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. Cell **89,** 575-585.

D'Agostino, I.B., and Kieber, J.J. (1999). Phosphorelay signal transduction: the emerging family of plant response regulators. Trends Biochem. Sci. **24,** 452-456.

D'Agostino, I.B., Deruere, J., and Kieber, J.J. (2000). Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. Plant Physiol. **124,** 1706-1717.

Fletcher, J.C., Brand, U., Running, M.P., Simon, R., and Meyerowitz, E.M. (1999). Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. Science **283,** 1911-1914.

Giulini, A., Wang, J., and Jackson, D. (2004). Control of phyllotaxy by the cytokinin-inducible response regulator homologue ABPHYL1. Nature **430,** 1031-1034.

Hass, C., Lohrmann, J., Albrecht, V., Sweere, U., Hummel, F., Yoo, S.D., Hwang, I., Zhu, T., Schafer, E., Kudla, J., and Harter, K. (2004). The response regulator 2 mediates ethylene signalling and hormone signal integration in Arabidopsis. EMBO J. **23,** 3290-3302.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature **413,** 383-389.

Jeong, S., Trotochaud, A.E., and Clark, S.E. (1999). The Arabidopsis CLAVATA2 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. Plant Cell **11,** 1925-1934.

Kayes, J.M., and Clark, S.E. (1998). CLAVATA2, a regulator of meristem and organ development in Arabidopsis. Development **125,** 3843-3851.

Kiba, T., Yamada, H., Sato, S., Kato, T., Tabata, S., Yamashino, T., and Mizuno, T. (2003). The type-A response regulator, ARR15, acts as a negative regulator in the cytokinin-mediated signal transduction in Arabidopsis thaliana. Plant Cell Physiol. **44,** 868-874.

Laux, T., Mayer, K.F., Berger, J., and Jurgens, G. (1996). The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. Development **122,** 87-96.

Lemon, W.J., Liyanarachchi, S., and You, M. (2003). A high performance test of differential gene expression for oligonucleotide arrays. Genome Biol. **4,** R67.

Lenhard, M., Jurgens, G., and Laux, T. (2002). The WUSCHEL and SHOOTMERISTEMLESS genes fulfil complementary roles in Arabidopsis shoot meristem regulation. Development **129,** 3195-3206.

Lenhard, M., Bohnert, A., Jurgens, G., and Laux, T. (2001). Termination of stem cell maintenance in Arabidopsis floral meristems by interactions between WUSCHEL and AGAMOUS. Cell **105,** 805-814.

Leyser, O. (2003). Regulation of shoot branching by auxin. Trends Plant Sci. **8,** 541-545.

Lohmann, J.U., Hong, R.L., Hobe, M., Busch, M.A., Parcy, F., Simon, R., and Weigel, D. (2001). A molecular link between stem cell regulation and floral patterning in Arabidopsis. Cell **105,** 793-803.

Long, J.A., Moan, E.I., Medford, J.I., and Barton, M.K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the STM gene Arabidopsis. Nature **379,** 66-69.

Mayer, K.F., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G., and Laux, T. (1998). Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. Cell **95,** 805-815.

Rojo, E., Sharma, V.K., Kovaleva, V., Raikhel, N.V., and Fletcher, J.C. (2002). CLV3 is localized to the extracellular space, where it activates the Arabidopsis CLAVATA stem cell signalling pathway. Plant Cell **14,** 969-977.

Roslan, H.A., Salter, M.G., Wood, C.D., White, M.R., Croft, K.P., Robson, F., Coupland, G., Doonan, J., Laufs, P., Tomsett, A.B., and Caddick, M.X. (2001). Characterization of the ethanol-inducible alc gene-expression system in Arabidopsis thaliana. Plant J. **28,** 225-235.

Schmid, M., Uhlenhaut, N.H., Godard, F., Demar, M., Bressan, R., Weigel, D., and Lohmann, J.U. (2003). Dissection of floral induction pathways using global expression analysis. Development **130,** 6001-6012.

Schmid, M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., Scholkopf, B., Weigel, D., and Lohmann, J.U. (2005). A gene expression map of Arabidopsis thaliana development. Nature Genet. **37,** 501-506.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K., Jurgens, G., and Laux, T. (2000). The stem cell population of Arabidopsis shoot meristems in maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. Cell **100,** 635-644.

Skoog, F., and Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. **54,** 118-130.

To, J.P.C., Haberer, G., Ferreira, F.J., Deruère, J., Mason, M.G., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2004). Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signalling. Plant Cell **16,** 658-671.

Wu, Z., Irizarry, R.A., Gentleman, R., Murillo, F.M., and Spencer, F.A. (2004). A model based background adjustment for oligonucleotide expression arrays. In Dept of Biostatistics Working Papers (Baltimore Maryland: Johns Hopkins Univ).

CHAPTER 4

Cytokinin regulates Type-A Arabidopsis Response Regulator activity and protein stability via two-component phosphorelay

Jennifer P.C. To*, Jean Deruère*, Bridey B. Maxwell, Veronica F. Morris,
Claire E. Hutchison, Fernando J. Ferreira, G. Eric Schaller and Joseph J. Kieber

*These authors contributed equally to this publication

PREFACE

This work is a result of convergence of two studies. I initiated one project to study the mechanism for type-A ARR function and generated mutant constructs targeting the conserved phosphorylation target Asp of ARR5 and ARR7. These constructs were introduced into various WT and mutant background to test how they affected ARR function. At the same time, Dr. Jean Deruère, a postdoc in the lab, initiated a project to study how type-A ARR proteins may be regulated. Dr. Jean Deruère characterized the dex-inducible myc-tagged ARR5 (DMA5) line and introduced it into *ahk3,4* and *ahp1,2,3,4* mutant backgrounds. He found that cytokinin stabilized myc-ARR5 and stabilization of myc-ARR5 was disrupted in *ahk3,4* and *ahp1,2,3,4* backgrounds, suggesting that phosphorylation may be the mechanism for ARR5 stabililzation. I continued to characterize protein turnover of other WT and phosphoryl-Asp targeted mutant type-A ARR proteins and we combined the findings in this story. Other people who contributed to this work include: Dr. Bridey Maxwell, who generated *ARR4OX* and *ARR7OX* lines, Veronica Franco who initially generated the DMA5 line, Dr. Claire Hutchison, Dr. Fernando Ferreira and Dr. G. Eric Schaller who provided the *ahp1,2,3,4*, *ahk3,4* and *arr1,2,10,12* mutants, respectively. I conducted all the other experiments and wrote the paper.

This paper has been submitted for publication as: To, J.P.C.*, Deruère, J.*, Maxwell, B.B., Morris, V.F., Hutchison, C.E., Ferreira, F.J., Schaller, G.E., and Kieber, J.J. Cytokinin regulates Type-A Arabidopsis Response Regulator activity and protein stability via two-component phosphorelay. (*These authors contributed equally to this publication.)

ABSTRACT

The plant hormone cytokinin regulates many aspects of growth and development. Cytokinin signaling involves histidine kinase receptors that perceive cytokinin and transmit the signal via a multi-step phosphorelay similar to bacterial two-component signaling systems (reviewed in (Kakimoto, 2003; Ferreira and Kieber, 2005; Maxwell and Kieber, 2005)). The final targets of this phosphorelay are a set of Arabidopsis Response Regulator (ARR) proteins containing a receiver domain that is phosphorylated on a conserved aspartate. One class of these, the type-A ARRs, are negative regulators of cytokinin signaling that are transcriptionally up-regulated rapidly in response to cytokinin. In this study, we tested the role of phosphorylation in type-A ARR function. Our results indicate that phosphorylation of the receiver domain is required for type-A ARR function, and suggest that negative regulation of cytokinin signaling by the type-A ARRs most likely involves phospho-dependent interactions. Furthermore, we show that a subset of the type-A ARR proteins are stabilized in part via phosphorylation in response to cytokinin. These studies shed light on the mechanism by which type-A ARRs act to negatively regulate cytokinin signaling and reveal a novel mechanism by which cytokinin controls type-A ARR function.

INTRODUCTION

Cytokinins are plant hormones that were discovered by their ability to promote cell division (Miller et al., 1955) and have been implicated in almost every aspect of plant growth and development and in the responses to various biotic and abiotic environmental cues (Mok and Mok, 2001; Sakakibara, 2006). The cytokinin signal transduction pathway involves a phosphorelay between two-component signaling elements that include histidine kinases, histidine phosphotransfer proteins, and response regulators (Fig. 4.1A)(reviewed in (Kakimoto, 2003; Ferreira and Kieber, 2005; Maxwell and Kieber, 2005)). In Arabidopsis, the three cytokinin receptors (Arabidopsis Histidine Kinase 2 (AHK2), AHK3 and AHK4) are hybrid histidine kinases that contain a fused receiver domain in addition to an input (a cytokinin-binding CHASE domain) and a histidine kinase domain. In response to cytokinin binding, these receptors autophosphorylate on a conserved histidine residue and relay this phosphoryl group to the Arabidopsis Response Regulators (ARRs) via an intermediate set of histidine phosphotransfer (Hpt) proteins called the Arabidopsis Hpt proteins (AHPs)(reviewed in (Kakimoto, 2003; Ferreira and Kieber, 2005; Maxwell and Kieber, 2005)). Similar cytokinin signaling components have been characterized in other plant species (Asakura et al., 2003; Ito and Kurata, 2006).

The Arabidopsis response regulator gene family falls into four classes based on phylogenetic analysis and domain structure: type-A *ARR*s, type-B *ARR*s, type-C *ARR*s (Schaller et al., 2007) and the *Arabidopsis Pseudo Response Regulators* (*APRRs*) (Kiba et al., 2004). The ten type-A *ARR*s are primary transcriptional targets of cytokinin and contain short C-terminal regions of unknown function (Brandstatter and Kieber, 1998; Imamura et al., 1998; D'Agostino et al., 2000). The eleven type-B ARRs contain C-

terminal output domains that have DNA binding and transactivating activity (Sakai et al., 1998; Sakai et al., 2000). Type-B ARRs are positive regulators of cytokinin signaling that control transcription of a subset of cytokinin-regulated targets, including the type-A *ARR*s (Hwang and Sheen, 2001; Sakai et al., 2001; Tajima et al., 2004; Mason et al., 2005). The ARRs all contain the conserved Asp required for receiver domain phosphorylation, and phospho-transfer from an AHP to representative members of all three ARR groups has been demonstrated *in vitro* (Suzuki et al., 1998; Imamura et al., 2001; Imamura et al., 2003; Kiba et al., 2004; Mahonen et al., 2006b). The APRRs lack the conserved Asp phosphorylation site and some play a role in modulating circadian rhythms (reviewed in (McClung, 2006)).

At least eight of the ten type-A *ARR*s act as partially redundant negative regulators of cytokinin signaling (Kiba et al., 2003; To et al., 2004; Lee et al., 2007). ARR4 interacts directly with the red-light receptor Phytochrome B and, along with other type-A ARRs, modulates the response to red light (Sweere et al., 2001; To et al., 2004). A subset of type-A ARRs are direct targets of the transcription factor *WUSCHEL* and regulate shoot apical meristem function (Leibfried et al., 2005). *ARR3* and *ARR4* are involved in controlling the circadian clock, and this function is opposed by *ARR8* and *ARR9* (Salomé et al., 2005). While it is clear that type-A *ARR*s play a role in multiple signaling pathways, there is little known with regard to their mechanism of action.

There are two general models by which type-A *ARR*s can act to negatively regulate cytokinin signaling. In the first, the type-A ARRs may compete with positively acting type-B ARRs for phosphoryl transfer from the AHPs, similar to the chemotaxis system in *S. meliloti* (Schmitt, 2002). A second model is that type-A ARRs regulate the pathway

through direct or indirect interactions with pathway components, as observed in *E. coli* chemotaxis (Bourret and Stock, 2002).

Here we explore the mechanism by which the type-A *ARR*s negatively regulate cytokinin signaling and the role of phosphorylation in this process. We show that type-A ARR function requires phosphorylation and that the type-A ARRs probably interact with other components in a phosphorylation-dependent manner to generate negative feedback on the signaling pathway. In addition, we show that a subset of the type-A ARR proteins are stabilized by cytokinin, revealing a novel level of control on these components.

RESULTS

To investigate the role of phosphorylation in type-A ARR function, we generated site-directed mutations in *ARR5* that alter the phosphorylation target in the receiver domain (Fig. 4.1B). The conserved phospho-accepting Asp87 was mutated to Ala ($ARR5^{D87A}$) to test if phosphorylation of the type-A ARRs is necessary for their function. This residue was also mutated to Glu ($ARR5^{D87E}$), which can partially mimic the phosphorylated form of the protein (Klose et al., 1993; Moore et al., 1993; Gupte et al., 1997; Lan and Igo, 1998). We had previously shown that an analogous D→E change in ARR7, another type-A ARR, acts as a gain-of-function mutation (Leibfried et al., 2005). All WT and mutant ARR5 constructs could interact with AHP2 in a yeast-two hybrid assay (Fig. 4.S1), indicating that the Asp87 mutations do not strongly disrupt ARR5 protein folding, and that the interaction between ARR5 and AHP2 is not dependent on ARR5 Asp87 phosphorylation.

ARR5 function requires receiver domain phosphorylation

To test if $ARR5^{WT}$, $ARR5^{D87A}$ and $ARR5^{D87E}$ are functional *in planta*, an *arr3,4,5,6* mutant, which is hypersensitive to cytokinin, was transformed with genomic constructs expressing myc-tagged WT and mutant ARR5 from the endogenous *ARR5* promoter (Fig. 4.1). We identified multiple independent transgenic lines, and four lines that represented a range of expression levels of the different transgenes were tested for cytokinin sensitivity.

Re-introduction of a WT genomic *ARR5* gene was sufficient to restore WT-like cytokinin sensitivity to the *arr3,4,5,6* mutant (Figs. 4.1C and D). If the *ARR5* transgene were expressed identically to the endogenous *ARR5* gene, then the $ARR5^{WT}$ transgenic

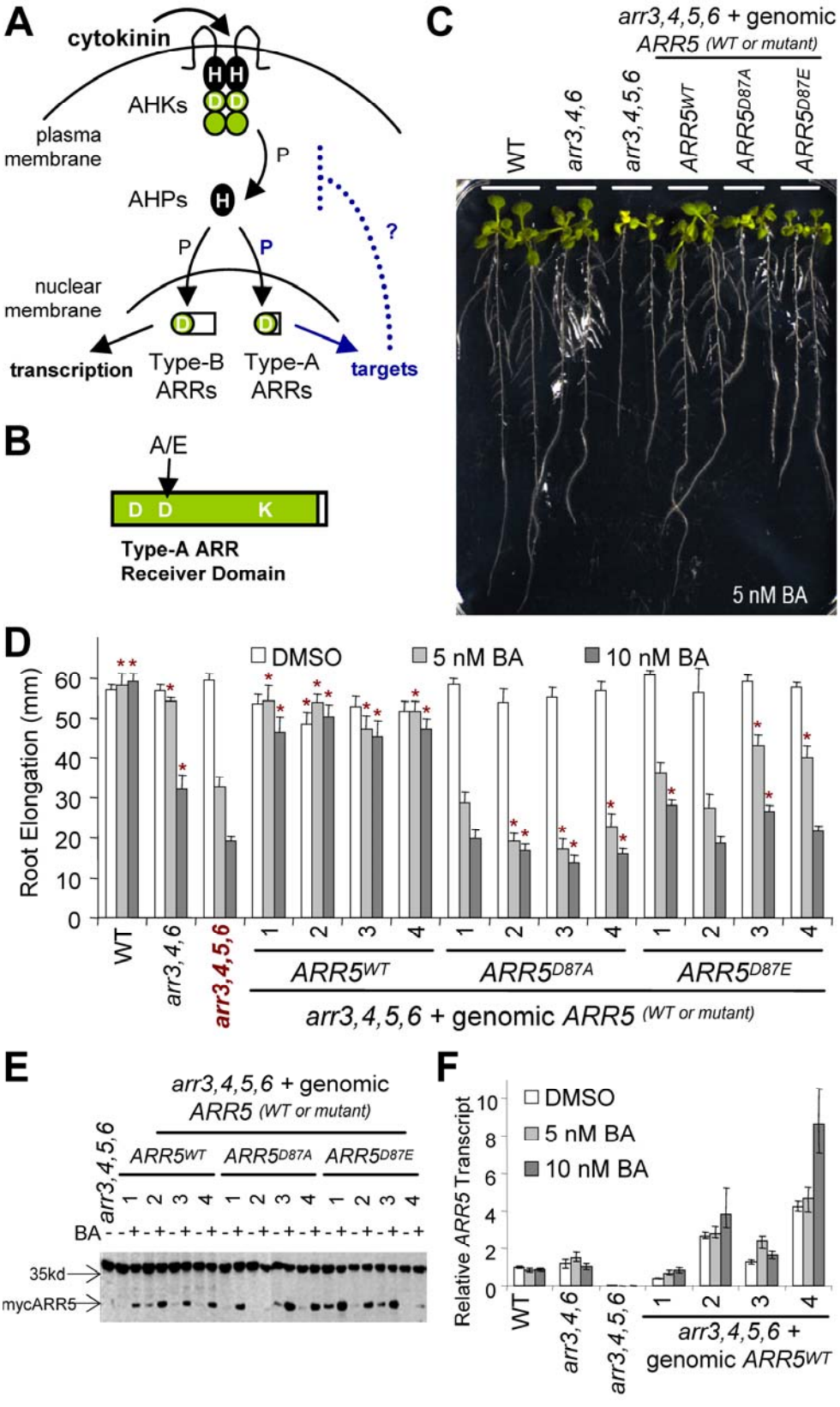

Figure 4.1 ARR5 function is dependent on phosphorylation of its receiver domain

lines should closely resemble the *arr3,4,6* mutant. However, in the four lines examined, cytokinin resistance was restored beyond that of *arr3,4,6*, to nearly WT levels (Figs. 4.1C and D). One explanation for this is that the transgenic copy of *ARR5* in these lines is overexpressed and that the roles of *ARR3, ARR4, ARR5* and *ARR6* are interchangeable in this cytokinin assay. We analyzed of the level of *ARR5* transcripts by real-time PCR. In three of the four *arr3,4,5,6+ genomicARR5*^{WT} lines, the steady-state level of *ARR5* transcripts is significantly higher than in the WT in the assay conditions tested (5 or 10 nM BA) (Fig. 4.1F). Consistent with the model that the level of *ARR5* correlates with

Figure 4.1 ARR5 function is dependent on phosphorylation of its receiver domain

(A) Model of type-A ARR function in cytokinin signaling: cytokinin is perceived by AHKs which autophosphorylate and transmit the signal via AHPs to ARRs in a His (H) to Asp (D) multi-step phosphorelay. Type-A ARRs may compete for phosphotransfer with type-B ARRs or interact with targets to negatively regulate the pathway. **(B)** Type-A ARR protein is shown with conserved Asp (D) and Lys (K) residues characteristic of receiver domains. The conserved phoshorylation target Asp (D) in receiver domain is mutated to Ala (A) or Glu (E). **(C-F)** Complementation of *arr3,4,5,6* hypersensitivity to cytokinin inhibition of root elongation. Homozygous T3 seedlings were grown on vertical plates supplemented with the specified concentrations of BA or 0.1% DMSO control under constant light for 10 days. **(C)** Two representative seedlings grown on 5 nM BA per genotype are pictured. Note: line 3 of *arr3,4,5,6+ genomicARR5*^{WT}, line 4 of *arr3,4,5,6+ genomicARR5*^{D87A} and line1 of *arr3,4,5,6+ genomicARR5*^{D87E} in part D are shown. **(D)** Root elongation of seedlings of four independent transgenic lines were quantified between days 4 and 9 at the indicated cytokinin concentrations. Error bars represent standard error, n>30. Asterisks indicate a statistically significant difference from *arr3,4,5,6* (indicated in red) at the given concentration of BA (Students' t-test $p<0.05$). **(E)** Transgenic seedlings express cytokinin inducible myc-tagged WT and mutant ARR5 proteins. Proteins were extracted from seedlings treated with 1 μM BA or 0.1% DMSO control and separated by SDS PAGE. ARR5-myc proteins were detected by Western blotting with anti-c-myc antibody. **(F)** *arr3,4,5,6+ genomicARR5*^{WT} express *ARR5* transcript. RNA was extracted from seedlings grown under the same conditions as part A and used for real-time RT PCR analysis. *ARR5* relative expression was normalized to *β-tubulin* levels and to WT DMSO control using REST 2005 version 1.9.12. Error bars represent upper and lower limits of 95% confidence interval.

cytokinin resistance, the two lines displaying the highest level of *ARR5* (#2 & 4) also showed the strongest cytokinin resistance at 25-100 nM BA (Fig. 4.S2). Overexpression of *ARR5* in these lines is most likely due to positional effects of the transgene and/ or the insertion of multiple copies of *ARR5*. Surprisingly, one line (#1) displayed close to WT levels of *ARR5,* despite displaying nearly WT cytokinin sensitivity in root assays. This line may overexpress *ARR5* specifically in the root, which may not be detected in our analysis of RNA from whole seedlings.

If phosphorylation is required for ARR5 function, then introducing a $ARR5^{D87A}$ genomic fragment should not rescue the cytokinin hypersensitive phenotype of *arr3,4,5,6*. We analyzed four independent transgenic lines that expressed $ARR5^{D87A}$ protein at levels comparable to the four *arr3,4,5,6+ genomic*$ARR5^{WT}$ lines (Fig. 4.1E). In all four lines, introduction of the $ARR5^{D87A}$ transgene into *arr3,4,5,6* did not decrease the sensitivity to cytokinin, and in three lines $ARR5^{D87A}$ expression further increased cytokinin sensitivity as compared to the parental line (Figs. 4.1C and D). Thus, phosphorylation of the receiver domain is required for *ARR5* function. The increased sensitivity some transgenics may be explained by $ARR5^{D87A}$ acting in a dominant negative manner.

$ARR5^{D87E}$ phosphomimic is partially active

In bacterial systems, altering the Asp phosphorylation target to a Glu can sometimes mimic the phosphorylated form, resulting in a partially activated response regulator (Klose et al., 1993; Moore et al., 1993; Gupte et al., 1997; Lan and Igo, 1998). This change can also block phosphorylation of the activated response regulator, thus preventing further activation (Klose et al., 1993; Moore et al., 1993). If type-A ARRs

negatively regulate cytokinin signaling by acting as phosphate sinks and thus reducing the flow of phosphates to the type-B ARRs, then ARR5^{D87E} should be completely non-functional. In contrast, if type-A ARRs act by interacting with other proteins in a phosphorylation-dependent manner, then a phosphomimic mutant may partially complement the *arr5* loss-of-function mutation in the *arr3,4,5,6* parental line. To test this, we introduced a genomic ARR5^{D87E} transgene into *arr3,4,5,6*. Four independent transgenic lines showed transgenic protein expression comparable to *arr3,4,5,6+ genomicARR5^{WT}* and *arr3,4,5,6+ genomicARR5^{D87A}* (Fig. 4.1E). In three out of the four lines examined, ARR5^{D87E} partially restored cytokinin resistance significantly above the *arr3,4,5,6* parental line (Fig. 4.1D). Importantly, in three *arr3,4,5,6+ genomicARR5^{D87E}* lines, cytokinin resistance was restored significantly above the ARR5 ^{D87A} mutant (Students' t-test $p<0.05$ at 5 nM BA), indicating a phosphorylation-dependent role in ARR5 function. The effect of ARR5^{D87E} is weaker than ARR5^{WT}, which is consistent with a partial activation of the receiver domain and inability of the response regulator to be fully activated by phosphorylation (Fig. 4.1D, (Moore et al., 1993)). This partial complementation by *ARR5^{D87E}*, which is unlikely to receive a phosphoryl group from the AHPs, indicates that ARR5 does not function entirely as a phosphate sink. Further, it suggests that the conformational state of phosphorylated ARR5 is likely to be the active state for interactions with target proteins.

Overexpression of Type-A ARRs confers cytokinin resistance

To test if increasing the levels of type-A ARRs can confer cytokinin resistance, we expressed ARR4, ARR5, ARR6, ARR7 and ARR9 in WT Arabidopsis as myc-epitope tagged fusion proteins from the constitutive CaMV 35S promoter. One

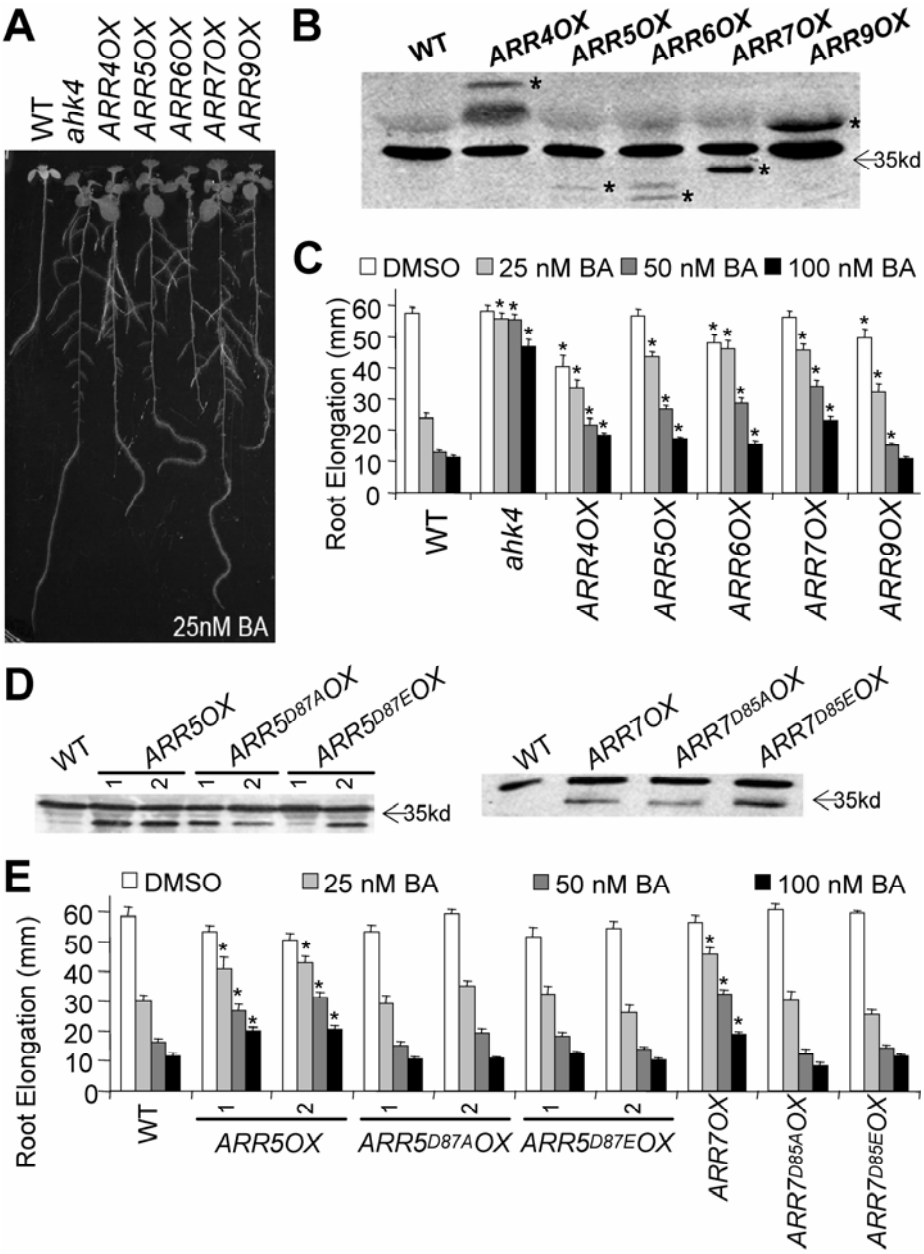

Figure 4.2 WT Type-A *ARR* overexpression confers cytokinin resistance

(A-C) Overexpression of *ARR4, ARR5*, *ARR6*, *ARR7* and *ARR9* confers cytokinin resistance. **(D and E)** Overexpression of ARR5 and ARR7 with mutations targeting the conserved phosphorylation site at Asp87 and Asp85 respectively do not confer cytokinin resistance. **(A)** Seedlings were grown as in Fig. 4.1 with the specified concentrations of BA or 0.1% DMSO control. **(B and D)** Transgenic seedlings express myc-tagged ARR proteins, detected as in Fig. 4.1E. In B, the bands corresponding to protein products of the appropriate sizes are noted with asterisks. **(C and E)** Root elongation was measured as in Fig. 4.1. Error bars represent standard error, n>30. Asterisks indicate a statistically significant difference from WT at the given concentration of BA (Students' t-test $p<0.05$).

representative line that expressed a detectable level of myc-ARR fusion protein was selected and analyzed for cytokinin responsiveness (Figs. 4.2A and B). All transgenic lines tested were significantly more resistant to 25 nM BA than the WT in root elongation assays (Fig. 4.2C, Students' t-test $p<0.05$), but less resistant than the loss-of-function cytokinin receptor mutant *ahk4*.

To test if mutations in the conserved Asp87 alter the function of overexpressed ARRs, we expressed ARR5^{D87A}, ARR5^{D87E}, ARR7^{D87A} and ARR7^{D85E} from this same CaMV 35S promoter and analyzed the effect on cytokinin sensitivity in representative lines (Fig. 4.2D). *ARR5^{D87A}OX* and *ARR7^{D87A}OX* did not show significant differences in cytokinin response compared to the WT (Fig. 4.2E), similar to the results obtained by complementation of the *arr5* loss-of-function allele. Surprisingly, *ARR5^{D87E}OX* and *ARR7^{D85E}OX* also did not show significant differences in cytokinin response (Fig. 4.2E), in contrast to results in complementation studies.

A subset of type-A ARR proteins are stabilized by cytokinin

The regulation of protein turnover plays an important role in controlling several phytohormone signaling and biosynthetic pathways (reviewed in (Dreher and Callis, 2007)). We analyzed ARR5 protein turnover using a Dexamethasone (DEX)-inducible myc-tagged ARR5 line (DMA5). Continuous growth of DMA5 seedlings on 10 nM DEX results in reduced sensitivity to cytokinin, indicating that the ARR5 myc-fusion protein in DMA5 is functional (Fig. 4.S3).

The myc-ARR5 protein is rapidly degraded following inhibition of *de novo* protein synthesis by cycloheximide (CHX). To test if ARR5 protein turnover is regulated by cytokinin, we compared ARR5 protein steady-state levels and degradation rates in the

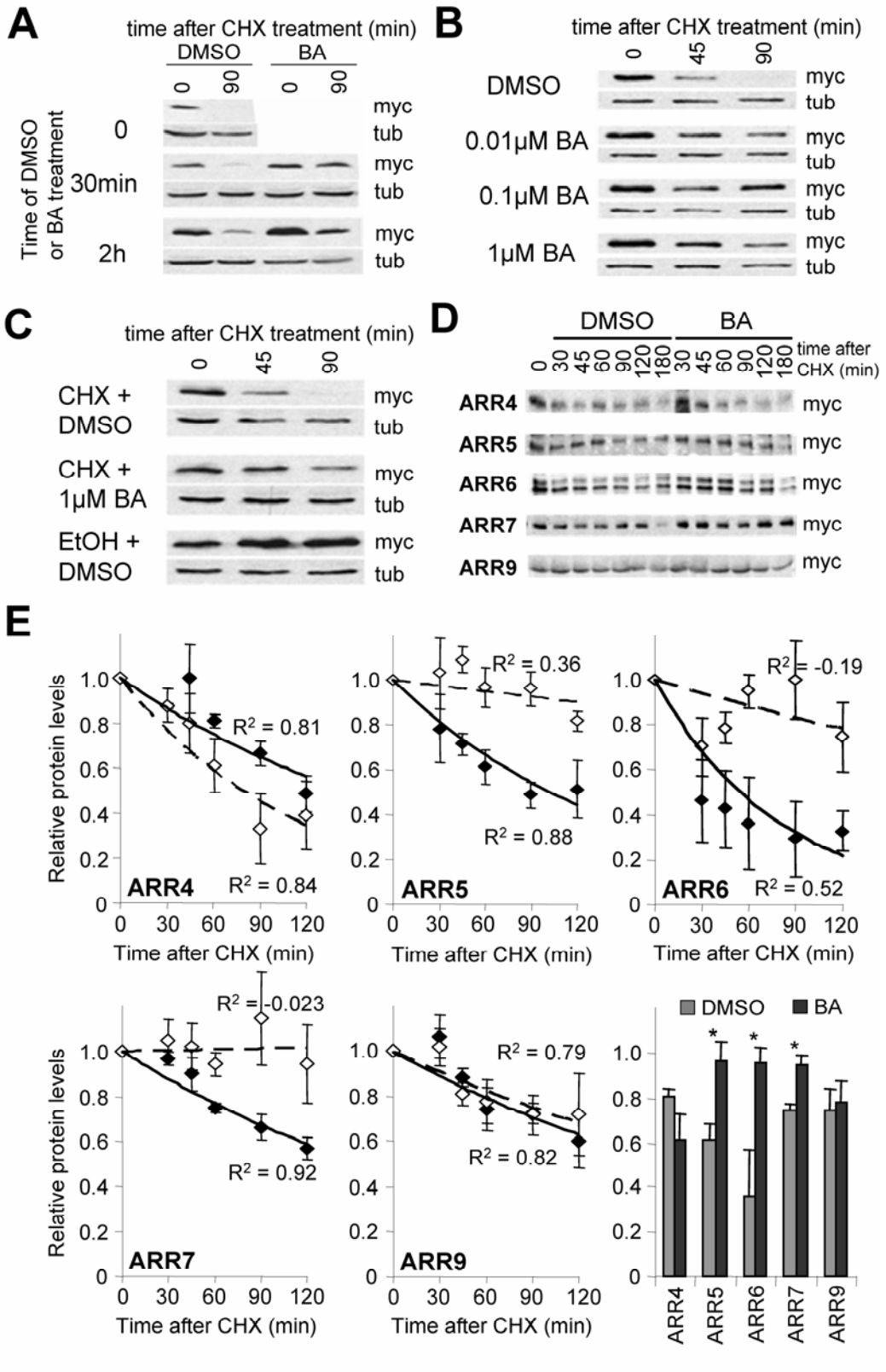

Figure 4.3 A subset of type-A ARR proteins are stabilized by exogenous cytokinin application

presence and absence of cytokinin. ARR5 protein accumulated to higher steady-state levels in the presence of cytokinin, and this is the result of a decreased rate of protein degradation (Fig. 4.3B). Stabilization of ARR5 was effective within 30 min of cytokinin application and was sensitive to concentrations of BA as low as 10 nM (Figs. 4.3B and C). Cytokinin increased ARR5 protein stability when added simultaneously with the CHX treatment, indicating that stabilization of ARR5 protein by cytokinin does not require *de novo* protein synthesis (Fig. 4.3D).

To test if other type-A ARR proteins are stabilized by cytokinin, we analyzed the

Figure 4.3 A subset of type-A ARR proteins are stabilized by exogenous cytokinin application

(A-D) myc-ARR5 protein is stabilized by exogenous cytokinin. myc-ARR5 protein was generated in 7-d-old light-grown seedlings in a DEX-inducible myc-ARR5 (DMA5) line by 2 h 1 μM DEX treatment. **(A)** Cytokinin stabilization of myc-ARR5 protein occurs within 30 minutes and is effective at 2h. After DEX induction of myc-ARR5 protein production, 1 μM BA or 0.1% DMSO control was added for the times indicated on the left before CHX treatment. **(B)** Cytokinin stabilization of ARR5 is sensitive to low concentrations of BA. Seedlings were treated with indicated concentrations of BA or DMSO control during DEX treatment, followed by CHX application. **(C)** Cytokinin stabilization of myc-ARR5 does not require new protein synthesis. After DEX treatment, 200 μM CHX (or ethanol (EtOH) control) and 1 μM BA (or 0.1% DMSO control) were applied simultaneously and myc-ARR5 protein turnover was analyzed as in part A. **(D)** A subset of type-A ARRs are stabilized by exogenous cytokinin application. 7-d-old light-grown *ARR4OX*, *ARR5OX*, *ARR6OX*, *ARR7OX* and *ARR9OX* seedlings were treated simultaneously with 200 μM CHX and 1 μM BA or 0.1% DMSO control. Three independent experiments were conducted with consistent results and one representative blot is shown. **(E)** Relative myc-ARR protein levels were normalized to loading control and to myc-ARR protein levels at time 0. The results from three independent experiments were averaged and shown with standard error bars. Note that the bottom band for ARR6 was quantified. An exponential best-fit curve was fitted through the data points to estimate protein half life. Correlation coefficient (R^2) values are indicated as a measure of curve fit. Closed symbols and solid lines represent DMSO control. Open symbols and broken lines represent BA treatment. The bottom right panel shows relative protein levels at 60 min after CHX treatment. Asterisks indicate a statistical difference between BA treatment and DMSO control (Student's t-test $p<0.05$).

turnover of their respective myc-fusion proteins expressed from the CaMV 35S promoter. The five type-A ARR proteins that we examined exhibited different rates of protein turnover. The half-life of the myc-ARR5 and myc-ARR6 fusion proteins were estimated to be 100 and 60 min respectively. myc-ARR4, myc-ARR7 and myc-ARR9 proteins exhibited longer protein half-lives, approximately 140, 160 and 180 min respectively. In the presence of exogenous cytokinin, the myc-ARR5, myc-ARR6 and myc-ARR7 fusion proteins were stabilized, with protein half-lives estimated to be greater than 300 min. The turnover of the myc-ARR4 and myc-ARR9 fusion proteins was not significantly affected by cytokinin.

Cytokinin-mediated stabilization of ARR5 involves two-component phosphorelay

To test if stabilization of ARR5 by cytokinin is mediated by the two-component signaling pathway, we expressed myc-ARR5 in the background of two-component element mutants. In the *ahk3,4* and *ahp1,2,3,4* mutants, cytokinin treatment fails to stabilize myc-ARR5 (Figs. 4.4A and B). These data indicate that an intact AHK-AHP phosphorelay is required for cytokinin to delay the turnover of type-A ARR proteins. Interestingly, cytokinin-mediated stabilization of myc-ARR5 was also reduced in a multiple type-B ARR loss of function mutant (*arr1,2,10,12)* (Fig. 4.4C). As *de-novo* protein synthesis is not required for the stabilization of ARR5 by cytokinin, this result suggests that type-B ARRs are required for transcription of an element involved in stabilization of ARR5 that is expressed prior to cytokinin application in this assay. However, *arr1,2,10,12* mutants still retain some response to cytokinin stabilization of myc-ARR5, supporting the model that phosphorelay plays a role in regulating myc-ARR5 turnover.

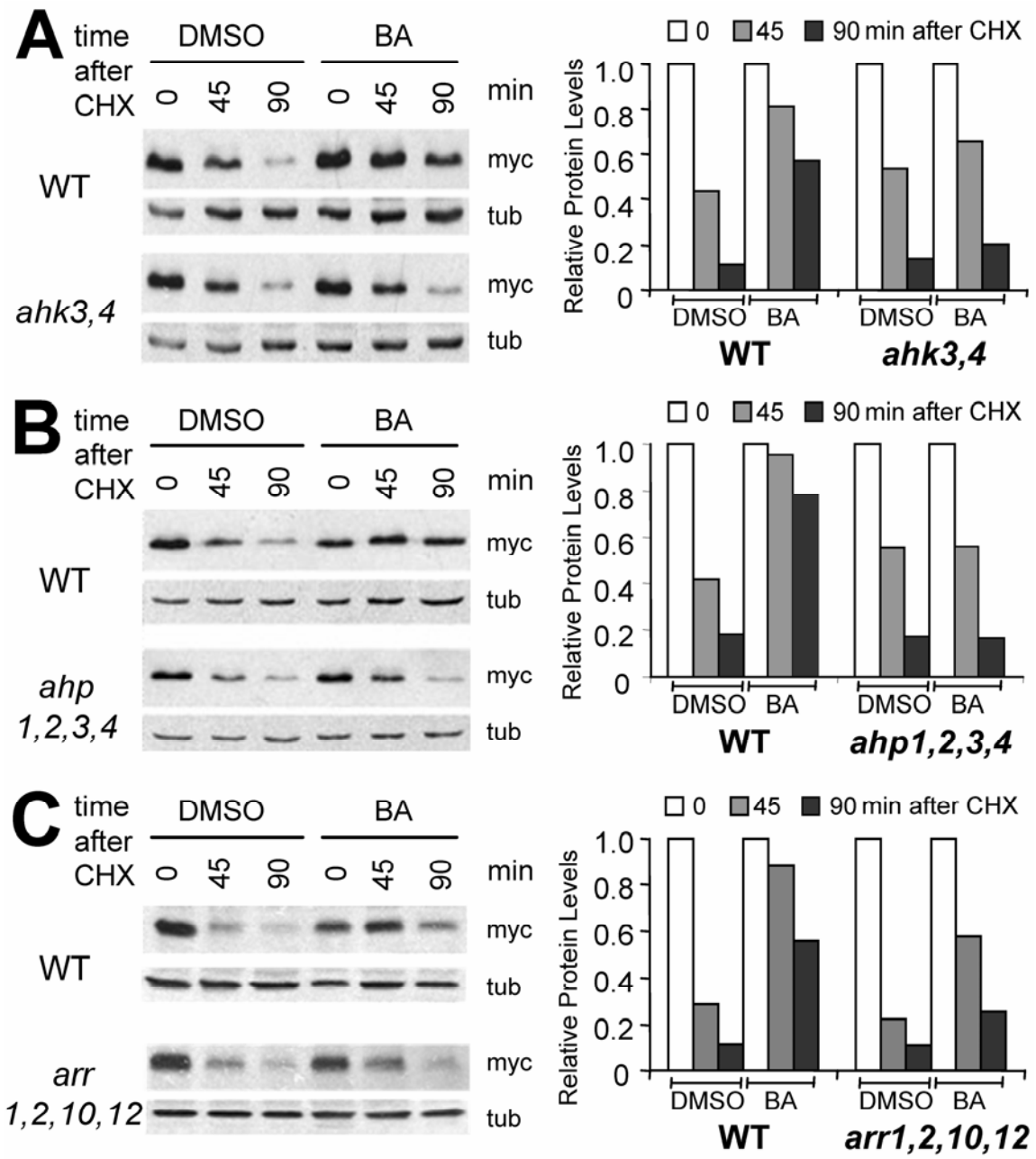

Figure 4.4 Cytokinin stabilization of ARR5 requires upstream cytokinin signaling genes

Protein turnover of DEX-inducible myc-ARR5 was examined in the background of the cytokinin signaling mutants indicated. Seedlings were treated and analyzed as in Fig. 4.3B. Relative protein levels were normalized to tubulin and to myc-ARR5 levels at 0 mins after CHX treatment.

We tested the hypothesis that type-A ARR proteins are stabilized by phosphorylation by analyzing the turnover of ARR proteins mutated in the conserved Asp phosphorylation target. The myc-ARR5^{D87A} protein was degraded more rapidly than myc-ARR5^{WT} in the absence of exogenous cytokinin; 15 minutes after CHX treatment, myc-ARR5^{WT} levels decreased 20% whereas myc-ARR5^{D87A} levels decreased approximately 40% as compared to the initial protein levels (Figs. 4.5A, B and C). Cytokinin treatment resulted in a strong stabilization of myc-ARR5^{WT} protein, but this was not observed with the Myc-ARR5^{D87A} protein (Figs. 4.5A, B and C). Consistent results were observed in WT and mutant ARR5 proteins expressed from genomic constructs used for complementation of *arr3,4,5,6* (Fig. 4.5D). Similarly, in the absence of cytokinin, myc-ARR7^{D85A} protein was turned over more rapidly than myc-ARR7^{WT} (Figs. 4.5E, F and G), and cytokinin treatment resulted in a stabilization of ARR7^{WT}, but not ARR7^{D85A} (Figs. 4.5E, F and G). The rapid turnover of ARR5^{D87A} and ARR7^{D85A} both in the presence and absence of cytokinin suggests that phosphorylation of Asp 85/87 plays a role in regulating the turnover of these ARR proteins.

To further test the role of phosphorylation in type-A ARR protein stability, we analyzed the protein turnover of myc-ARR5^{D87E} and myc-ARR7^{D85E} phosphomimic mutants. When expressed from the CaMV 35S promoter, basal myc-ARR5^{D87E} protein turnover was slower than myc-ARR5^{WT}. At 60 minutes after CHX addition, myc-ARR5^{WT} proteins decreased by more than 40% whereas myc-ARR5^{D87E} proteins only decreased by 10% (Figs. 4.5A, B and C). In the presence of cytokinin, myc-ARR5^{D87E} may be weakly stabilized (Figs. 4.5A, B and C), but the response is greatly muted relative to myc-ARR5^{WT}. Degradation of the myc-ARR7^{D85E} protein was also reduced in the

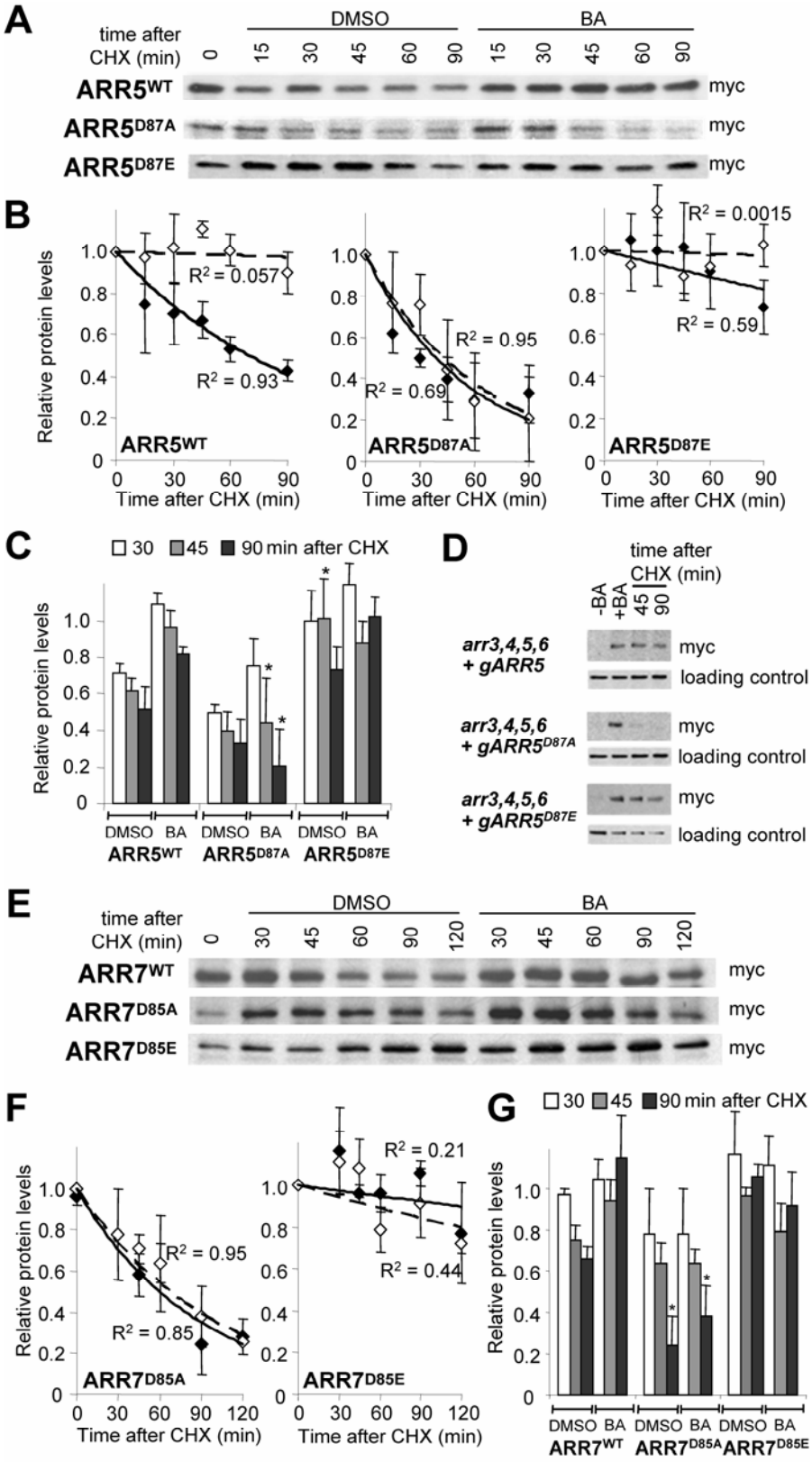

Figure 4.5 ARR5 and ARR7 protein stability is dependent on the conserved phosphorylation target Asp

absence of cytokinin and was not significantly altered on cytokinin application (Figs. 4.5E, F and G). The delayed protein turnover of myc-ARR5^{D87E} and myc-ARR7^{D85E} in the absence of cytokinin suggests that the protein conformation induced by phosphorylation of the conserved Asp contributes to protein stability.

Figure 4.5 ARR5 and ARR7 protein stability is dependent on the conserved phosphorylation target Asp

(A,B,C,E,F and G) Seedlings expressing the proteins indicated were grown, treated and analyzed as in Fig. 4.3E**. (C and G)** Asterisks indicate a significant difference in relative protein levels from ARR5^{WT} or ARR7^{WT} after the same treatment (Students' t-test $p<0.05$). The data for triplicate analysis of ARR7^{WT} protein degradation is presented in Fig. 4.3E. Note that the genomic versions of myc-ARR5 (**D**) show elevated protein levels as a results of the transcriptional induction of the transgenes in response to cytokinin.

DISCUSSION

Type-A ARRs are likely to act by phospho-dependent interactions

We show that type-A ARRs require phosphorylation for function, and that a non-phosphorylatable, partially activated form of type-A ARR can partially complement a loss-of-function mutant. This indicates that phosphorylated type-A ARRs can function to negatively regulate cytokinin signaling independently of their ability to compete for phosphoryl groups with the type-B ARRs, possibly by interacting with other targets.

Although our results suggest that type-A ARRs act in cytokinin signaling through phospho-dependent interactions, they do not rule out a role for type-A ARRs in phospho-competition. Cytokinin-resistance conferred by overexpression of WT ARR5 and ARR7 was disrupted by mutating the conserved phosphorylation target Asp to either an unphosphorylatable Ala or a phosphomimic Glu. Consistent results have also been reported for a rice type-A RR, as well as ARR22, which does not belong to either the type-A or the type-B ARR groups (Kiba et al., 2004; Hirose et al., 2007). One explanation is that the cytokinin-insensitive phenotype conferred by overexpression of WT type-A ARRs may reflect an inappropriate diversion of phosphate flow from the AHP to the abnormally high levels of type-A ARR proteins, which would decrease activation of the type-B ARRs. Whether this proposed phospho-competition is an artifact of overexpression or accurately reflects the role of endogenous type-A ARR proteins is an open question. An alternative for the lack of effect of overexpression of the Asp to Glu mutants is that this mutation only partially mimics the activated form of the response regulator and the expression level is insufficient to increase type-A ARR function above a threshold necessary to alter the response in root elongation studies. In contrast,

disruption of the conserved phosphorylation site did not significantly alter the ability of type-A ARRs to reduce or enhance a cytokinin responsive reporter when overexpressed in protoplasts (Hwang and Sheen, 2001), which may be due to differences in the assay system.

A previous study examined shoot formation from cultured Arabidopsis roots overexpressing ARR4 and ARR8 and reported that overexpression of ARR4 resulted in cytokinin hypersensitivity, whereas overexpression of ARR8 resulted in cytokinin insensitivity (Osakabe et al., 2002). While we have not examined the effect of ARR8 overexpression, our analysis of ARR4 (as well as ARR5, ARR6, ARR7 and ARR9) overexpression in this study and loss-of-function mutants in our previous work (To et al., 2004) are consistent with ARR4, as well as the other type-A ARRs, acting as negative regulators of cytokinin signaling. One explanation for this discrepancy is that ARR4 may act as a positive element in a subset of cytokinin responses, such as shoot initiation. Indeed, we have found antagonistic interactions among type-A ARRs in other physiological roles, such as in controlling rosette size, petiole length and circadian rhythms (To et al., 2004; Salomé et al., 2005).

A recent study indicates that the cytokinin receptor AHK4 determines phosphate flux through the system by regulating a bi-directional phosphorelay to and from the AHPs (Mahonen et al., 2006a). A bi-directional phosphorelay is also used by the bacterial Arc two-component system to mediate signal decay: the phosphoryl group from the ArcB response regulator is transferred back to the receiver domain of the ArcA tripartite hisitidine kinase via its His transmitter domain (Georgellis et al., 1998; Pena-Sandoval et al., 2005). While we cannot rule out that some type-A ARR function may act by a similar

mechanism of reverse phospho-transfer from type-B ARRs to type-A ARRs via AHPs, our data supports a model for direct or indirect type-A ARR interactions with components of the pathway in a phospho-dependent manner.

Cytokinin regulates Type-A ARR function in part by protein stabilization

Control of protein stability through the proteasome degradation machinery is a common mechanism for regulation of plant hormone responses (reviewed in (Dreher and Callis, 2007)). Indeed, mutants of *RPN12* and *COP9/CIN4/ FUS10*, which are subunits of proteasome regulatory structures (reviewed in (Dreher and Callis, 2007)), are cytokinin insensitive (Vogel et al., 1998; Smalle et al., 2002), suggesting that cytokinin signaling may also be regulated by the proteasome. One possible explanation is that these mutants have higher levels of type-A ARR protein due to decreased degradation. However, ARR5 protein stability is not altered in *rpn12a-1* or *cin4/cop9/fus10* (Fig. 4.S4), indicating that cytokinin insensitivity in these mutants is probably due to a distinct mechanism.

In this study, we have shown that cytokinin regulates turnover of a subset of type-A ARR proteins, and that this occurs in the absence of *de novo* protein synthesis. Cytokinin-mediated stabilization of ARR5 is disrupted in mutants of upstream phosphorelay components, suggesting that phosphorylation of type-A ARRs by two-component elements is required for protein stabilization by cytokinin. In addition, the unphosphorylatable $ARR5^{D87A}$ and $ARR7^{D85A}$ mutant proteins are less stable, whereas the partial phospho-mimics, $ARR5^{D87E}$ and $ARR7^{D85E}$, exhibit reduced protein turnover as compared to the WT proteins, consistent with the idea type-A ARR protein turnover is determined by the phosphorylation state of the receiver domain. However, ARR5 and ARR7 mutant proteins still retain some response to cytokinin stabilization. Furthermore,

stabilization by cytokinin is compromised in the *arr1,2,10,12* mutant, which is disrupted in cytokinin activated transcription factors and thus should have no direct effect on the phosphorylation state of the type-A ARRs. Together, these results suggest that there is a Asp85/Asp87 phosphorylation-independent mechanism for the stabilization of ARR5/ ARR7. This mechanism is likely to be dependent on Type-B ARRs basal transcription because *de novo* protein synthesis is not required for type-A ARR stabilization.

A model in which ARR5 and ARR7 turnover is regulated by phosphorylation status of their receiver domains is consistent with the finding that the yeast response regulator, SSK1, is degraded by the 26S proteasome pathway and degradation of SSK1 is inhibited by the upstream phosphotransfer protein YPD1 (Sato et al., 2003). In our yeast two-hybrid analysis, the steady-state protein levels of $ARR5^{D87E}$ prey fusion proteins are higher than ARR5 or $ARR5^{D87A}$ fusion proteins, suggesting that the ARR5 protein may also be subject to phosphorylation-dependent proteasome degradation in yeast.

Why are a subset of type-A ARRs stabilized?

The finding that cytokinin stabilizes type-A ARRs, apparent negative regulators of cytokinin signaling appears distinct from other known phytohormone signaling pathways involving proteasome degradation machinery, such as auxin and ethylene, which generally function to activate or stabilize positively acting transcription factors (reviewed in (Dreher and Callis, 2007)). However, expression of the phosphomimic $ARR5^{D87E}$ can partially complement a multiple type-A ARR loss-of-function mutant, and $ARR7^{D85E}$ overexpression can further induce meristem arrest at a low frequency (Leibfried et al., 2005). These results suggest that phosphorylated and stabilized type-A ARR proteins may interact with other components to regulate outputs. Targets of

phosphorylated and activated type-A ARRs may modulate cytokinin signaling and other processes that remain to be determined.

MATERIALS AND METHODS

Plasmid Constructs

A genomic ARR5 DNA fragment (from 1.6kb upstream of ATG through entire length of cDNA excluding the stop codon (D'Agostino et al., 2000)) was PCR amplified from genomic DNA isolated from WT Col seedlings and inserted into the into pENTR/D-TOPO vector (Invitrogen) to generate Gateway entry clone pAR5g. Full length cDNAs of ARR4, ARR5, ARR6, ARR7, ARR9 and AHP2 were PCR amplified and inserted into the pENTR-D Gateway entry clone vector (Invitrogen) to generate Gateway entry clones pAR4cs, pAR5cs, pAR6cs, pAR7cs, pAR9cs and pAP2cs. In the coding region for ARR5 in pAR5g and pAR5cs, the 87^{th} codon GAT encoding Asp87 of ARR5 cDNA was changed to GCT encoding Ala by site directed mutagenesis to generate pAR5gDA and pAR5DAcs respectively. The same codon for Asp87 was changed to GAG encoding Glu to generate pAR5DEs and pAR5gDE. In pAR7s, Asp85 was changed to Ala and Glu by site directed mutagenesis to generate pAR7DAcs and pAR7DEcs respectively. All entry clones were sequence verified.

For ARR5 complementation constructs, a genomic ARR5 fragment was transferred from each of pAR5g , pAR5gDA and pAR5gDE into Gateway compatible binary vector pGWB16 (a gift from Dr. Tsuyoshi Nakagawa, Shimane University, Japan) to generate pB16-5gw, pB16-5gDA and pB16-5gDE respectively. Each of the resulting constructs carried the endogenous *ARR5* promoter driving expression of WT or mutant ARR5 with a 4X C-terminal myc tag.

For ARR-overexpression constructs, full length ARR cDNAs were transferred from gateway entry vectors pAR4cs, pAR5cs, pAR6cs, pAR7cs, pAR9cs, pAR5DAcs,

pAR5DEcs, pAR7DAcs and pAR7DEcs into the Gateway compatible binary vector pGWB18 (a gift from Dr. Tsuyoshi Nakagawa, Shimane University, Japan) by LR recombination (Invitrogen) to generate pB18-4w, pB18-5w, pB18-6w, pB18-7w, pB18-9w, pB18-5DA, pB18-5DE, pB18-7DA and pB18-7DE. In each of the resulting constructs, expression of an *ARR* cDNA carrying a 4X N-terminal myc tag is driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter.

To generate a dexamethosome (DEX) inducible 6X N-terminal myc tagged ARR5 construct, a full length ARR5 cDNA fragment was introduced into a 6X-myc vector via EcoRI sites and subcloned into pTA7002 (Aoyama and Chua, 1997) to generate pDMA5.

Plant materials and transgenic lines

Arabidopsis thaliana of the Columbia ecotype were used in all experiments as the WT control unless otherwise stated. Mutant lines *arr3,4,5,6* (To et al., 2004), *ahk3,4* (Rashotte et al., 2006), *arr1,2,10,12* (Rashotte et al., 2006), *ahp1,2,3,4* (Hutchison et al., 2006) have been previously described.

All transgenic plant lines described in this paper were generated in the Columbia (Col) ecotype background by introducing binary plasmid constructs via *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). pB16-5gw, pB16-5gDA and pB16-5gDE were introduced into *arr3,4,5,6* to generate *arr3,4,5,6+ genomicARR5*^{WT}, *arr3,4,5,6+ genomicARR5*^{D87A} and *arr3,4,5,6+ genomicARR5* ^{D87E} respectively. At least eight independent lines were analyzed in the T2 generation and taken to T3 homozygositiy. Detailed characterization of four representative T3 lines are presented in this paper.

pB18-4w, pB18-5w, pB18-6w, pB18-7w, pB18-9w, pB18-5DA, pB18-5DE,

pB18-7DA, pB18-7DE and pDMA5 were introduced into WT Col to generate *ARR4OX*, *ARR5OX*, *ARR6OX*, *ARR7OX*, *ARR9OX*, $ARR5^{D87A}OX$, $ARR5^{D87E}OX$, $ARR7^{D85E}OX$, $ARR7^{D85E}OX$ and *DMA5* respectively. Transgenic T1 seedlings were selected on MS agar plates (see plant growth conditions) supplemented with 30μg/ml hygromycin 50 μg/ml carbenicillin. Transgene expression was confirmed in homozygous hygromycin resistant T3 seedlings by protein gel blotting of whole seedling protein extracts and detecting with anti-c-myc POD antibody (Roche Applied Science). For each construct the results from one representative line is presented.

To generate Dex-inducible myc-ARR5 lines in the various genetic backgrounds, *ahk3,4* was crossed to *DMA5.* pDMA5 was introduced into *ahp1,2,3,4* and *arr1,2,10,12* and selected as described above.

Plant growth conditions

Seeds were surface sterilized and cold treated at 4ºC for 3 d in the dark and grown at 23ºC under constant white light (~100 μE). Seedlings were grown on MS media containing 1X Murashige and Skoog salts, 0.05% MES buffer and 1% sucrose pH 5.7. For cytokinin response assays, seedlings were grown on vertical MS plates with 0.6% phytagel (Sigma) supplemented with a dose range of N^6-benzyladenine (BA) or 0.1% (v/v) dimethyl sulfoxide (DMSO) carrier control as previously described (To et al., 2004). For protein assays and transgenic seedling selection, seedlings were grown on horizontal MS plates with 0.8% bactoagar.

Quantitative Real-Time RT PCR

10-d-old light grown seedlings in cytokinin response assays were harvested and total RNA was extracted using an RNeasy kit according to the manufacturer's

instructions (Qiagen). cDNA was generated from the RNA with MMLV reverse transcriptase (Invitrogen). qRT-PCR was performed with *Taq* DNA Polymerase Hot-Start Version, buffer and dNTPs per manufacturer's instruction (Takara Mirus Bio) supplemented with 0.3X Sybr Green (Molecular Probes) and ARR5 primers: ARR5F3 5'TCTGAAGATTAATTTGATAATGACGG and ARR5R2 5'TCACAGGCTTCAATAAGAAATCTTCA, or β-tubulin primers: TUB4s 5'AGAGGTTGACGAGCAAGATGA and TUB4a 5'AACAATGAAAGTAGACGCCA. Real-time PCR reactions were performed in an Opticon2 PCR machine (MJ Research) using the thermocycler program: (*i*) 2 min at 95°C, (*ii*) 15 s at 95°C, (*iii*) 15s at 60°C, (*iv*) 15 s at 72°C, (v) optical read, repeat 34 cycles of steps (ii) through (v) followed by a final analysis of product melting temperature to confirm PCR product. Each biological sample was analyzed at least twice in triplicate. The relative expression for ARR5 (normalized to β-tubulin as reference gene and WT grown on DMSO as control sample) and 95% confidence interval were determined using REST 2005 version 1.9.12 (Pfaffl et al., 2002; Herrmann et al., 2006). Two independent experiments were performed with consistent results. The data from one triplicate analysis are presented.

Analysis of protein stability

For Dex-inducible myc-tagged ARR5, myc-ARR5 protein expression was induced by incubating 7 d old light grown seedlings in liquid MS media with 1 μM Dex supplemented with 1 μM BA or 0.1 % (v/v) DMSO carrier control for 2 hours. Protein synthesis was inhibited by 200 μM cycloheximide (CHX). Seedlings were harvested by flash freezing in liquid nitrogen at the time points indicated.

For lines constitutively overexpressing ARRs, 7 d old light grown seedlings were

incubated in liquid MS media with 200 μM CHX supplemented with 1 μM BA or DMSO carrier control. Seedlings were harvested at the time points indicated.

Protein extracts were prepared in 250 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 1 X Complete protease inhibitors (Roche Applied Science) and 0.5 % ß-mercaptoethanol. Protein extracts were separated by SDS-PAGE and transferred to Nitropure membranes (GE). Myc-tagged proteins were detected with anti-c-myc POD (Roche Applied Science), tubulin was detected by rabbit polyclonal anti-tubulin and secodary goat anti-rabbit POD antibody (Chemicon), and visualized by chemiluminescent detection (Perkin Elmer) on autoradiography. Films were quantified using image quant software (Molecular Dynamics). Myc-ARR protein levels were normalized to signal from β-tubulin or from non-specific anti-c-myc hybridization to a ~35 kD protein. Three independent ARR protein degradation time-course experiments were conducted for each line and the results were averaged. Protein half-life of myc-ARRs was estimated by plotting an exponential best-fit curve to the averaged data from three independent experiments.

SUPPLEMENTARY MATERIALS

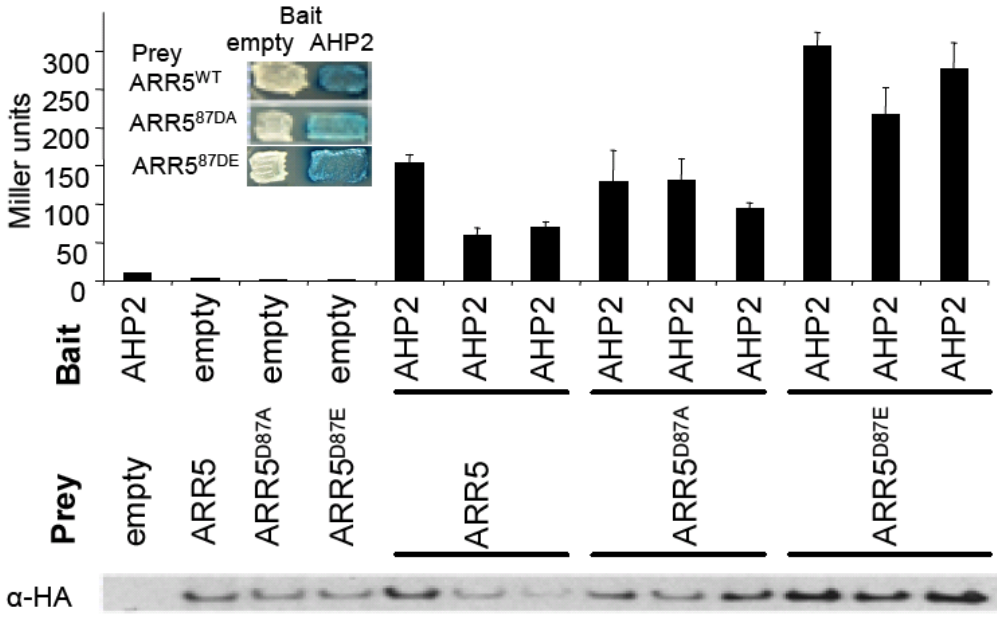

Figure 4.S1. Mutations targeting conserved phosphorylation target Asp do not disrupt ARR5 protein interaction with AHP2 in yeast two-hybrid

Full length cDNAs were transferred from gateway entry vectors pAR5cs, pAR5DAcs, pAR5DEcs and pAP2cs (see methods) into both bait and prey vectors pEG202gw and pjG4-5gw (a gift from Dr. Hironori Kaminaka and Dr. Jeff Dangl, UNC Chapel Hill (Gyuris et al., 1993; Holt et al., 2005)) via LR recombination (Invitrogen) according to manufacturer's instructions to generate bait and prey plasmids. Bait and prey plasmid pairs including all combinations of pEG202-GW, pEG-AHP2, pEG-AR5, pEG-AR5DA and pEG-AR5DE, pjG4-5-GW, pjG-AHP2, pjG-AR5, pjG-AR5DA and pjG-AR5DE were co-transformed into yeast strain EGYpSH18 and selected as previously described (Gyuris et al., 1993). Three independent transformants were analyzed for each bait and prey combination. Yeast cultures with equalized cell density were analyzed for protein expression and two-hybrid interactions. Protein expression was confirmed by protein gel blotting and detecting with anti-HA POD antibody (Roche Applied Science), shown on the bottom panel. Yeast two-hybrid interactions were quantified using a liquid colorimetric o-nitrophenyl-beta-D-galactopyranoside (ONPG) (Invitrogen) assay (adapted from (Clontech Laboratories, 2001)). The same experiment was conducted using ARR7 WT and Asp85 mutant constructs with consistent results (data not shown). Inset shows yeast grown on colorimetric substrate β-galactosidase.

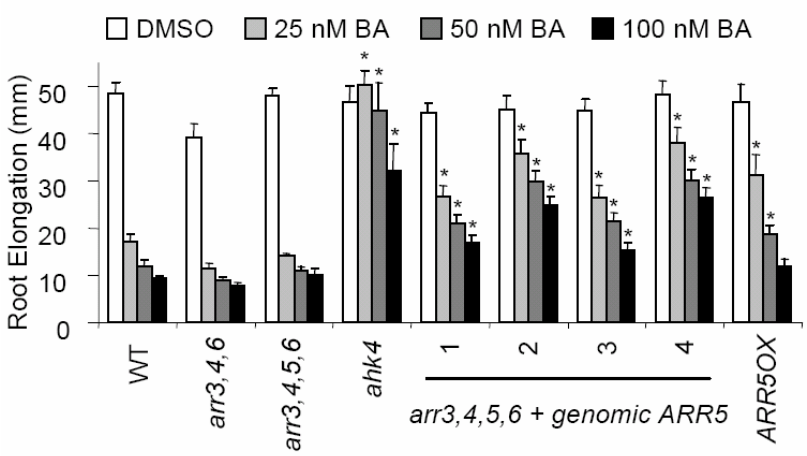

Figure 4.S2. An *ARR5* transgene confers cytokinin resistance to *arr3,4,5,6*

Seedlings were grown on higher doses of cytokinin, where root elongation of WT seedlings is inhibited. Seedlings were grown and measured as described in Fig. 4.1. Asterisks indicate a significant difference from WT root elongation at the given cytokinin concentration (Student's t-test, $p<0.01$). Note that lines #2 and #4 exhibit a stronger resistance to cytokinin than lines #1 and #3, which is consistent with *ARR5* expression levels. Note that Lines 2 and 4 show even stronger resistance to cytokinin than the *ARR5OX* line.

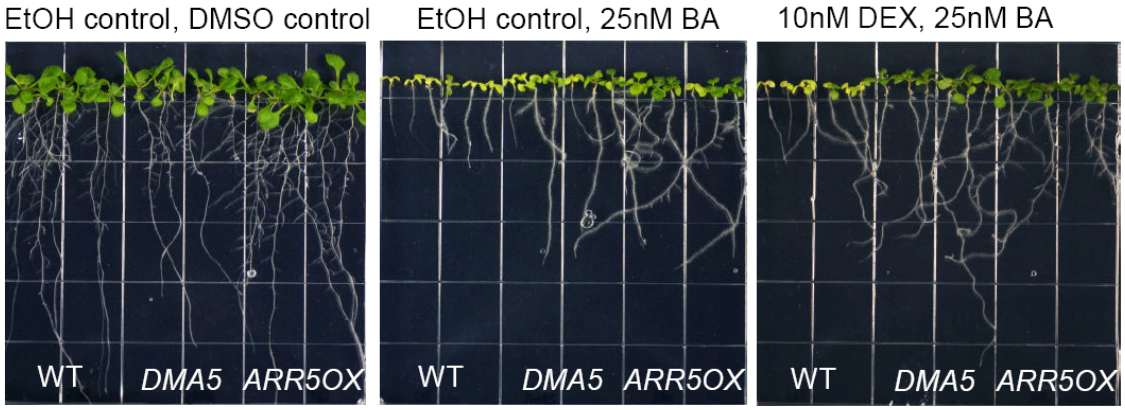

Figure 4.S3. Exogenous Dex application ehances cytokinin resistance in *DMA5* seedlings

Seedlings were grown on various combinations of 10nM dex or ethanol control and 25nM BA or DMSO control. Seedlings were grown as described in Fig. 4.1. At 25nM cytokinin, WT seedling root elongation is inhibited. Note that *DMA5* exhibits weak resistance to cytokinin in the absence of dex, suggesting that the activity of the *DMA5* construct maybe leaky. In the absence of dex, *DMA5* is more resistant to cytokinin than WT, but more sensitive than *ARR5OX*. On 10nM dex, *DMA5* shows enhanced resistance to 25nM cytokinin and may be more resistant than the *ARR5OX* line.

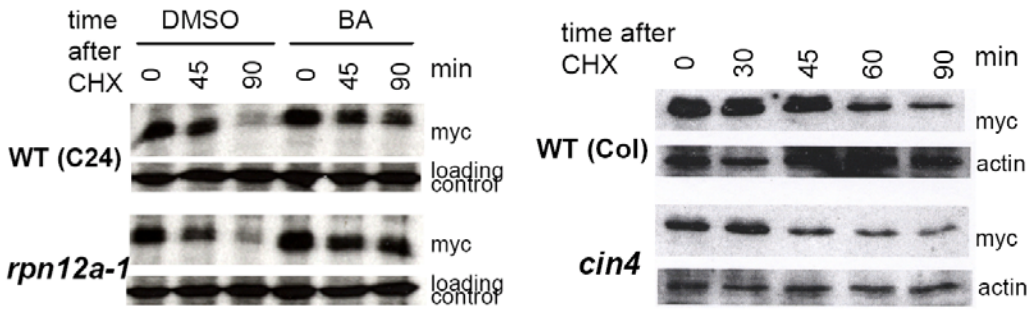

Figure 4.S4. Mutations in *RPN12a* and *COP9/CIN4/FUS10* do not alter mycARR5 protein stability

Seedlings of the indicated genotypes were treated with 1 μM DEX for 2 h to induce myc-ARR5 protein synthesis. New protein synthesis is inhibited by 200 μM CHX addition and protein degradation is monitored as in Fig. 4.3. *rpn12a-1*(Smalle et al., 2002) and *cin4/cop9/fus10* (Vogel et al., 1998) were generated as previously described. *rpn12a-1* (in C24 ecotype), WT C24 and *cop9/cin4/fus10* , were crossed to DMA5 and selected for hygromycin resistance and dex-inducible myc-tagged ARR5 protein expression.

REFERENCES

Aoyama, T., and Chua, N.-H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. Plant J. **11,** 605-612.

Asakura, Y., Hagino, T., Ohta, Y., Aoki, K., Yonekura-Sakakibara, K., Deji, A., Yamaya, T., Sugiyama, T., and Sakakibara, H. (2003). Molecular characterization of His-Asp phosphorelay signaling factors in maize leaves: implications of the signal divergence by cytokinin-inducible response regulators in the cytosol and the nuclei. Plant Mol. Biol. **52,** 331-341.

Bourret, R.B., and Stock, A.M. (2002). Molecular information processing: Lessons from bacterial chemotaxis. J. Biol. Chem. **277,** 9625-9628.

Brandstatter, I., and Kieber, J.J. (1998). Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in Arabidopsis. Plant Cell **10,** 1009-1020.

Clontech Laboratories, I. (2001). Yeast Protocols Handbook, pp. Protocol No. PT3024-3021, Version No. PR3131-3003.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. **16,** 735-743.

D'Agostino, I., Deruère, J., and Kieber, J.J. (2000). Characterization of the response of the Arabidopsis *ARR* gene family to cytokinin. Plant Physiol. **124,** 1706-1717.

Dreher, K., and Callis, J. (2007). Ubiquitin, hormones and biotic stress in plants. Ann. Bot. **99,** 787-822.

Ferreira, F.J., and Kieber, J.J. (2005). Cytokinin signaling. Curr. Opin. Plant Biol. **8,** 518-525.

Georgellis, D., Kwon, O., De Wulf, P., and Lin, E.C.C. (1998). Signal decay through a reverse phosphorelay in the Arc two-component signal transduction system. J. Biol. Chem. **273,** 32864-32869.

Gupte, G., Woodward, C., and Stout, V. (1997). Isolation and characterization of rcsB mutations that affect colanic acid capsule synthesis in Escherichia coli K-12. J. Bacteriol. **179,** 4328-4335.

Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993). Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell **75,** 791-803.

Herrmann, M., Chiew, D., and Pfaffl, M.W. (2006). REST 2005 NEW stand-alone software for gene expression analysis. In REST 2005 (Corbett Research), http://rest-2005.gene-quantification.info.

Hirose, N., Makita, N., Kojima, M., Kamada-Nobusada, T., and Sakakibara, H. (2007). Over-expression of a type-A Response Regulator alters rice morphology and cytokinin metabolism. Plant Cell Physiol. **48,** 523-539.

Holt, B.F., III, Belkhadir, Y., and Dangl, J.L. (2005). Antagonistic control of disease resistance protein stability in the plant immune system. Science **309,** 929-932.

Hutchison, C.E., Li, J., Argueso, C., Gonzalez, M., Lee, E., Lewis, M.W., Maxwell, B.B., Perdue, T.D., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2006). The Arabidopsis Histidine Phosphotransfer Proteins are redundant positive regulators of cytokinin signaling. Plant Cell **18,** 3073-3087.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in *Arabidopsis* signal transduction. Nature **413,** 383-389.

Imamura, A., Yoshino, Y., and Mizuno, T. (2001). Cellular localization of the signaling components of Arabidopsis His-to-Asp phosphorelay. Biosci. Biotechnol. Biochem. **65,** 2113-2117.

Imamura, A., Kiba, T., Tajima, Y., Yamashino, T., and Mizuno, T. (2003). In vivo and in vitro characterization of the ARR11 response regulator implicated in the His-to-Asp phosphorelay signal transduction in *Arabidopsis thaliana*. Plant Cell Physiol. **44,** 122-131.

Imamura, A., Hanaki, N., Umeda, H., Nakamura, A., Suzuki, T., Ueguchi, C., and Mizuno, T. (1998). Response regulators implicated in His-to-Asp phosphotransfer signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. U S A **95,** 2691-2696.

Ito, Y., and Kurata, N. (2006). Identification and characterization of cytokinin-signalling gene families in rice. Gene **382,** 57-65.

Kakimoto, T. (2003). Perception and signal transduction of cytokinins. Annu. Rev. Plant Biol. **54,** 605-627.

Kiba, T., Aoki, K., Sakakibara, H., and Mizuno, T. (2004). Arabidopsis Response Regulator, ARR22, ectopic expression of which results in phenotypes similar to the wol cytokinin-receptor mutant. Plant Cell Physiol. **45,** 1063-1077.

Kiba, T., Yamada, H., Sato, S., Kato, T., Tabata, S., Yamashino, T., and Mizuno, T. (2003). The Type-A Response Regulator, ARR15, acts as a negative regulator in the cytokinin-mediated signal transduction in Arabidopsis thaliana. Plant Cell Physiol. **44,** 868-874.

Klose, K.E., Weiss, D.S., and Kustu, S. (1993). Glutamate at the site of phosphorylation of nitrogen-regulatory protein NTRC mimics aspartyl-phosphate and activates the protein. J. Mol. Biol. **232,** 67-78.

Lan, C.Y., and Igo, M.M. (1998). Differential expression of the OmpF and OmpC porin proteins in Escherichia coli K-12 depends upon the level of active OmpR. J. Bacteriol. **180,** 171-174.

Lee, D.J., Park, J.-Y., Ku, S.-J., Ha, Y.-M., Kim, S., Kim, M.D., Oh, M.-H., and Kim, J. (2007). Genome-wide expression profiling of ARABIDOPSIS RESPONSE REGULATOR 7(ARR7) overexpression in cytokinin response. Mol. Genet. Genom. **V277,** 115-137.

Leibfried, A., To, J.P.C., Stehling, S.K., A., Busch, W., Demar, M., Kieber, J.J., and Lohmann, J.U. (2005). WUSCHEL controls meristem size by direct transcriptional regulation of cytokinin inducible response regulators. Nature **438,** 1172-1175.

Mahonen, A.P., Higuchi, M., Tormakangas, K., Miyawaki, K., Pischke, M.S., Sussman, M.R., Helariutta, Y., and Kakimoto, T. (2006a). Cytokinins regulate a bidirectional phosphorelay network in Arabidopsis. Curr. Biol. **16,** 1116-1122.

Mahonen, A.P., Bishopp, A., Higuchi, M., Nieminen, K.M., Kinoshita, K., Tormakangas, K., Ikeda, Y., Oka, A., Kakimoto, T., and Helariutta, Y. (2006b). Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. Science **311,** 94-98.

Mason, M.G., Mathews, D.E., Argyros, D.A., Maxwell, B.B., Kieber, J.J., Alonso, J.M., and Ecker, J.R.S., G.E. (2005). Multiple type-B response regulators mediate cytokinin signal transduction in Arabidopsis. Plant Cell **17,** 3007-3018.

Maxwell, B.B., and Kieber, J.J. (2005). Cytokinin Signal Transduction. In Plant Hormones: Biosynthesis, Signal Transduction, Action!, P.J. Davies, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 321-349.

McClung, C.R. (2006). Plant circadian rhythms. Plant Cell **18,** 792-803.

Miller, C.O., Skoog, F., Von Saltza, M.H., and Strong, F. (1955). Kinetin, a cell division factor from deoxyribonucleic acid. J. Am. Chem. Soc. **77,** 1392-1293.

Mok, D.W., and Mok, M.C. (2001). Cytokinins: Chemistry, activity and function. (Boca Raton, FL.: CRC Press).

Moore, J.B., Shiau, S.P., and Reitzer, L.J. (1993). Alterations of highly conserved residues in the regulatory domain of nitrogen regulator I (NtrC) of Escherichia coli. J. Bacteriol. **175,** 2692-2701.

Osakabe, Y., Miyata, S., Urao, T., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2002). Overexpression of Arabidopsis response regulators, ARR4/ATRR1/IBC7 and ARR8/ATRR3, alters cytokinin responses differentially in the shoot and in callus formation. Biochem. Biophys. Res. Commun. **293,** 806-815.

Pena-Sandoval, G.R., Kwon, O., and Georgellis, D. (2005). Requirement of the receiver and phosphotransfer domains of ArcB for efficient dephosphorylation of phosphorylated ArcA in vivo. J. Bacteriol. **187,** 3267-3272.

Pfaffl, M.W., Horgan, G.W., and Dempfle, L. (2002). Relative expression software tool (REST(C)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucl. Acids Res. **30,** e36-.

Rashotte, A.M., Mason, M.G., Hutchison, C.E., Ferreira, F.J., Schaller, G.E., and Kieber, J.J. (2006). A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. PNAS **103,** 11081-11085.

Sakai, H., Aoyama, T., and Oka, A. (2000). Arabidopsis ARR1 and ARR2 response regulators operate as transcriptional activators. Plant J. **24,** 703-711.

Sakai, H., Aoyama, T., Bono, H., and Oka, A. (1998). Two-component response regulators from *Arabidopsis thaliana* contain a putative DNA-binding motif. Plant Cell Physiol. **39,** 1232-1239.

Sakai, H., Honma, T., Aoyama, T., Sato, S., Kato, T., Tabata, S., and Oka, A. (2001). *Arabidopsis* ARR1 is a transcription factor for genes immediately responsive to cytokinins. Science **294,** 1519-1521.

Sakakibara, H. (2006). CYTOKININS: Activity, biosynthesis, and translocation. Annu. Rev. Plant Biol. **57,** 431-449.

Salomé, P.A., To, J.P.C., Kieber, J.J., and McClung, C.R. (2005). Arabidopsis Response Regulators ARR3 and ARR4 play cytokinin-independent roles in the control of circadian period. Plant Cell **18,** 55-69.

Sato, N., Kawahara, H., Toh-e, A., and Maeda, T. (2003). Phosphorelay-regulated degradation of the yeast Ssk1p response regulator by the ubiquitin-proteasome system. Mol. Cell Biol. **23,** 6662-6671.

Schaller, G.E., Doi, K., Hwang, I., Kieber, J.J., Khurana, J.P., Kurata, N., Mizuno, T., Pareek, A., Shiu, S.-H., Wu, P., and Yip, W.K. (2007). Nomenclature for two-component signaling elements of rice. Plant Physiol. **143,** 555-557.

Schmitt, R. (2002). Sinorhizobial chemotaxis: a departure from the enterobacterial paradigm. Microbiology **148,** 627-631.

Smalle, J., Kurepa, J., Yang, P., Babiychuk, E., Kushnir, S., Durski, A., and Vierstra, R.D. (2002). Cytokinin growth responses in Arabidopsis involve the 26S proteasome subunit RPN12. Plant Cell **14,** 17-32.

Suzuki, T., Imamura, A., Ueguchi, C., and Mizuno, T. (1998). Histidine-containing phosphotransfer (HPt) signal transducers implicated in His-to-Asp phosphorelay in Arabidopsis. Plant Cell Physiol. **39,** 1258-1268.

Sweere, U., Eichenberg, K., Lohrmann, J., Mira-Rodado, V., Bäurle, I., Kudla, J., Nagy, F., Schäfer, E., and Harter, K. (2001). Interaction of the response regulator ARR4 with the photoreceptor phytochrome B in modulating red light signaling. Science **294,** 1108-1111.

Tajima, Y., Imamura, A., Kiba, T., Amano, Y., Yamashino, T., and Mizuno, T. (2004). Comparative studies on the Type-B Response Regulators revealing their distinctive properties in the His-to-Asp phosphorelay signal transduction of *Arabidopsis thaliana*. Plant Cell Physiol. **45,** 28-39.

To, J.P.C., Haberer, G., Ferreira, F.J., Deruère, J., Mason, M.G., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2004). Type-A ARRs are partially redundant negative regulators of cytokinin signaling in Arabidopsis. Plant Cell **16,** 658-671.

Vogel, J.P., Schuerman, P., Woeste, K.W., Brandstatter, I., and Kieber, J.J. (1998). Isolation and characterization of Arabidopsis mutants defective in induction of ethylene biosynthesis by cytokinin. Genetics **149,** 417-427.

CHAPTER 5

Perspectives

Where have we come from and where are we now?

Research in the area of cytokinin signaling has made a lot of progress over the course of my graduate career. When I first began my work in the Kieber lab, the type-A *ARABIDOPSIS RESPONSE REGULATORS* (*ARR*s) had only recently been identified as cytokinin primary response genes (Brandstatter and Kieber, 1998; Imamura et al., 1998; D'Agostino et al., 2000). Two-component signaling had been implicated in cytokinin signaling by the cytokinin-independent phenotype exhibited by an overexpressor of a histidine kinase homolog, *CKI1* (Kakimoto, 1996), which was later determined to function specifically in female gametophytic development and is not likely to be a cytokinin receptor (Pischke et al., 2002; Hejatko et al., 2003). Some of the two-component elements have been studied in terms of their biochemical properties, but little was known at the time about their involvement in cytokinin signaling. Since then, the cytokinin receptors have been identified to be the ARABIDOPSIS HISTIDINE KINASES (AHKs), and the role of other two-component elements, ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEINS (AHPs) and ARRs in cytokinin signaling has been established (reviewed in Chapter 1).

My graduate work has mainly focused on elucidating the function of members of the type-A Response Regulator gene family in Arabidopsis. Through reverse genetic analysis, we have shown that type-A ARRs are negative regulators of the cytokinin signaling pathway (To et al., 2004). Analysis of loss-of-function mutant phenotypes also indicate that some type-A ARRs also participate in a variety of plant processes, including shoot meristem function, root development and circadian rhythms (To et al., 2004; Leibfried et al., 2005; Salomé et al., 2005), which complement findings in other loss-of-

function cytokinin signaling mutants (Higuchi et al., 2004; Nishimura et al., 2004; Mason et al., 2005; Hutchison et al., 2006; Riefler et al., 2006; Yokoyama et al., 2007). These functions involve specific subsets of type-A ARRs and sometimes involve antagonistic interactions between type-A ARRs . My work has also shown that type-A ARRs function in these processes in part by phospho-dependent interactions and that these interactions may be activated in part by cytokinin-mediated protein stabilization (To et al., 2007). We have made much progress in our understanding of the function of type-A ARRs in cytokinin signaling and other biological processes. However, many questions still remain and point to interesting future areas of research.

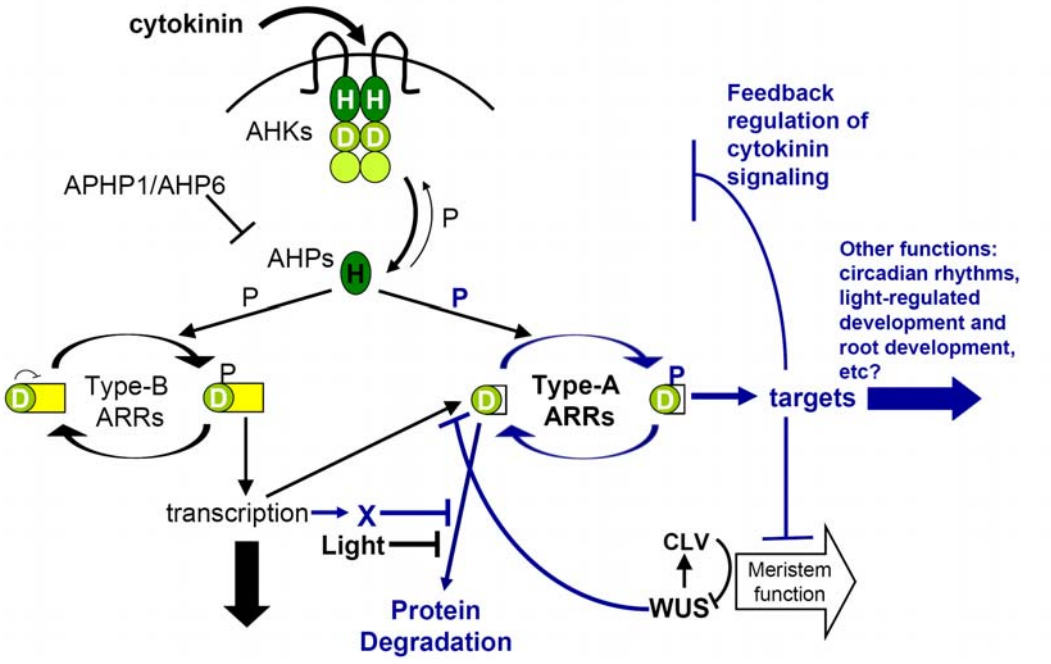

Fig. 5.1 Model of cytokinin signaling

A summary of cytokinin signaling pathway as described in Chapter 1 is presented. The parts of the model relevant to this thesis are highlighted in blue.

Are the type-A ARRs truly functionally redundant in cytokinin signaling?

In various assays for cytokinin sensitivity, we observe similar trends in type-A *arr* mutant behavior: single mutants do not show obvious differences in response, double and higher order mutants show increasing sensitivity, and sometimes responsiveness, to lower doses of cytokinin (To et al., 2004). Our initial assays used to characterize type-A *arr* mutant cytokinin responses have led us to the discovery that type-A ARRs are negative regulators of cytokinin signaling. Similar assays have revealed opposite effects in loss-of-function mutants of the AHKs, AHPs and type-B ARRs (Inoue et al., 2001; Sakai et al., 2001; Imamura et al., 2003; Higuchi et al., 2004; Nishimura et al., 2004; Mason et al., 2005; Hutchison et al., 2006; Riefler et al., 2006), supporting overall antagonistic function of type-A ARRs to these positive signaling components.

These cytokinin response assays were conducted by growing seedlings or culturing calli constantly on media supplemented with cytokinin, or floating excised leaves on buffered solutions containing cytokinin, or immersing whole seedlings in liquid media containing cytokinin. Given that all the type-A ARRs are transcriptionally upregulated by cytokinin, though with somewhat different kinetics and responsiveness (D'Agostino et al., 2000; Rashotte et al., 2003), it is not surprising to find that most of the type-A ARRs characterized appear to function additively in tissues subject to a blanket cytokinin treatment (To et al., 2004). Although our studies have used cytokinin treatments in the nanomolar range, similar to the cytokinin binding capacity of AHK4 (Yamada et al., 2001), the conditions are likely to be very different from endogenous conditions experienced by the plant. In addition, the function of cytokinin signaling differs from tissue to tissue. A classic example is that cytokinin enhances shoot growth

while it inhibits root growth. Recently, studies have shown that within the root, cytokinin inhibits meristem size but promotes formation of vascular tissues (Mähönen et al., 2006b; Mähönen et al., 2006a; Dello Ioio et al., 2007). General assays for cytokinin response, especially at the whole plant level, may risk masking the specific effects within tissues.

Analysis of patterns of expression of cytokinin biosynthetic genes (Miyawaki et al., 2004; Tanaka et al., 2006), cytokinin activating genes (Kurakawa et al., 2007) and cytokinin degrading genes (Werner et al., 2003) further show that cytokinin metabolism is regulated spatially and temporally in the plant by a variety of environmental and developmental cues. Basal expression of subsets of type-A ARR transcripts have been localized to specific tissues and are also regulated by various external and internal signals (Mähönen et al., 2000; To et al., 2004; Zimmermann et al., 2004; Leibfried et al., 2005). It is likely that cytokinin transiently regulates expression of subsets of type-A *ARR*s in specific cell types to regulate negative feedback on the signaling pathway and activate other downstream responses. Indeed, further analysis of type-A *arr* mutant phenotypes have revealed specificities among subsets of type-A *ARR*s that correlate with their patterns of expression (see below). Consistent with this, detailed phenotypic analysis of the cytokinin receptor mutants has also revealed subfunctionalization among different biological processes consistent with the expression patterns of individual AHKs, such as shoot meristem function, root meristem function, root vascular differentiation, leaf senescence and seed development (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). It would be interesting to find out if and which type-A ARRs participate in these cytokinin receptor-regulated processes.

In order to further our understanding of the function of the type-A ARRs and their

interaction with other cytokinin signaling components, it is necessary to first gain better resolution of their expression patterns at the tissue level. The next step would be to evaluate the cytokinin response in specific tissues and develop assays for simple outputs. These steps would enable analysis of type-A ARR function in the context of specific tissues where they are expressed, and determine their genetic interactions with other cytokinin signaling genes co-expressed in those tissues. These studies should determine how type-A ARRs may be involved in specific developmental functions of the cytokinin receptors. Furthermore, narrowing down genes relevant to specific tissues would allow lower-order mutant combinations to be used in analysis of genetic interactions, which may also avoid some of the problems of general redundancy encountered in the initial studies.

How do type-A ARRs antagonize type-B ARR function?

In all of the cytokinin responses and many of the plant developmental processes characterized so far, the type-A ARRs generally play an antagonistic role to the type-B ARRs. There are multiple examples in bacterial two-component systems where two different response regulators function antagonistically within a signaling pathway. The soil bacterium *S. meliloti* chemotaxis system employs two different response regulators: a conventional response regulator, CheY2 that is activated by phosphorylation to interact with the flagella motor to control tumbling, and a modulator, CheY1 with no direct output which negatively regulates flagella motion by diverting phosphate flow away from the positive regulator (Schmitt, 2002). A similar phospho-competition between type-A and type-B ARRs may be the mechanism by which the type-A ARRs negatively regulate cytokinin signaling, with type-A ARRs acting as phosphate sinks. In contrast, the *E. coli*

chemotaxis system employs two different response regulators with distinct targets: CheY interacts with the flagella motor to control tumbling behavior and CheB demethylates and deactivates the chemoreceptor (Baker et al., 2006). Similarly, type-A ARRs may regulate the pathway through direct or indirect interactions with components of the pathway.

Both Arabidopsis and maize type-A and type-B RR proteins are capable of dephosphorylating HPs *in vitro* (Suzuki et al., 1998; Imamura et al., 2001; Asakura et al., 2003; Imamura et al., 2003; Mähönen et al., 2006a), and results from overexpressing WT and phospho-directed mutant type-A RRs in Arabidopsis and rice are consistent with this role (Hirose et al., 2007; To et al., 2007). In order to efficiently dephosphorylate type-B ARRs, unphosphorylated type-A ARRs would need to be in high abundance. This might be achieved either by type-A ARRs having a short phosphorylated half-life, which allows rapid turn-over the pool of phosphorylated proteins to readily absorb more phosphoryl groups, or by increased production of unphosphorylated type-A ARRs. Transcript levels of type-A RRs in Arabidopsis, maize and rice have all been shown to be upregulated by cytokinin treatment and are likely to be more abundant than type-B RRs (Brandstatter and Kieber, 1998; Sakakibara et al., 1999; D'Agostino et al., 2000; Asakura et al., 2003; Rashotte et al., 2003; Jain et al., 2006), though relative protein levels have not been determined. In maize, the turnover of phosphorylated type-A RRs has been shown to be faster than the turnover of phosphorylated type-B RRs characterized *in vitro* (Asakura et al., 2003). However, in Arabidopsis, the stability of phosphorylated type-A ARRs relative to type-B ARRs vary and do not appear to follow the same general tendency (Suzuki et al., 1998; Imamura et al., 2001; Imamura et al., 2003; Mähönen et al., 2006a). In addition, our finding that phosphorylated type-A ARR proteins are stabilized and that a

phosphomimic type-A ARR can complement cytokinin response in a multiple loss-of function type-A *arr* mutant is also at odds with the phospho-competition model (To et al., 2007). It remains to be determined if the maize type-A RR protein turnover is subject to similar regulation as the type-A ARRs and differences may reflect a divergence of monocot and dicot type-A RRs.

Alternatively, type-A ARRs may directly interact with type-B ARRs and inhibit type-B ARR transcriptional function, similar to the mechanism used in auxin signaling. Auxin induces transcription of *AUX/IAA* genes, which encode transcriptional repressors that directly bind to transcriptional activators, AUXIN RESPONSE FACTORS (ARFs), to inhibit their function (Woodward and Bartel, 2005). A large-scale interaction study of Arabidopsis two-component elements in yeast two-hybrid has not identified interactions between type-A and type-B ARRs (Dortay et al., 2006). It may be argued that the response regulators may be subject to phosphorylation and dephosphorylation in yeast, thus hindering the detection of transient phosphorylation-specific interactions. However, further analysis of WT, unphosphorylatable and phosphomimic mutant forms of representative type-A ARRs, ARR5 and ARR7, and a type-B ARR, ARR1, in yeast two-hybrid have also failed to demonstrate interactions between the WT or mutant type-A and type-B ARRs in any combination (Kiba et al., 2004; To et al., 2007). These results suggest that a direct interaction between type-A and type-B ARRs is unlikely.

Another possibility is that type-A ARRs interfere with phosphotransfer from AHPs to type-B ARRs by directly interacting with the AHPs. In yeast two-hybrid, all WT, unphosphorylatable ARR5^{D87A} and ARR5^{D87E} phosphomimic mutant proteins showed strong interaction with AHP2, however, when these constructs were re-

introduced into the plant, only WT and the ARR5^{D87E} phosphomimic, but not the unphosphorylatable ARR5^{D87A}, were able to complement a loss-of-function *arr3,4,5,6* mutant (To et al., 2007), indicating that protein interaction with AHP2 cannot fully explain ARR5 function. Furthermore, the dominant negative activity exhibited by ARR5^{D87A} in the complementation experiments implies further complexities in ARR5 function.

Other speculations may predict that type-A ARRs may interact with the remaining known cytokinin signaling components: the AHKs or the CYTOKININ RESPONSE FACTORS. Preliminary data indicate that WT ARR5 proteins do not interact with AHK3 or CRF2 in yeast two-hybrid (To and Kieber, unpublished data), but interactions with other AHK and CRF family members have not been tested. It will also be necessary to examine tissue-specific expression and subcellular localization of these proteins, in order to determine if physical interaction between type-A ARRs and the AHKs or the CRFs is feasible. The AHKs are currently placed at the plasma membrane, and the CRFs are localized non-specifically in the cell prior to cytokinin application and rapidly accumulate in the nucleus in response to cytokinin treatment (Rashotte et al., 2006). Type-A ARRs have been reported to be localized to the nucleus (ARR6, ARR7 and ARR15) and cytoplasm (ARR16) in transient expression systems (Hwang and Sheen, 2001; Imamura et al., 2001; Yamada et al., 2004). These data have not been confirmed by expression patterns in planta and subcellular localization of ARR5 and other type-A ARRs have not been determined. Alternatively, type-A ARRs may interact with novel proteins to be identified by protein interaction screens such as yeast two-hybrid or co-immunoprecipitation assays. Our data from complementation studies suggest that these

interactions may be specific to the phosphorylated form of type-A ARRs and the phosphomimic mutants may serve as a useful tool for identifying novel type-A ARR interactors.

Overall, neither of the models appear to be able to fully explain the data at hand and the two models may not be mutually exclusive. The function of type-A ARRs in cytokinin signaling may involve additional interacting factors to be identified in future screens.

How is specificity of type-A ARR function defined?

As discussed in the previous section, type-A ARR function can be specified in part by regulation of gene expression. A subset of type-A ARRs, ARR5, ARR6, ARR7 and ARR15 are expressed in the meristem and their transcription is regulated by the meristem maintenance gene WUSCHEL (Leibfried et al., 2005). Consistent with their specific expression pattern in the meristem, *arr5,6,7,15* mutant displays the strongest phenotype in shoot branching among type-A *arr* loss-of-function quadruple mutant combinations analyzed (To and Kieber, unpublished data). ARR8 and ARR9 show stronger basal expression in the roots than ARR3, ARR4, ARR5 and ARR6, and single *arr8* and *arr9* mutants also show subtle but reproducible defects in lateral root formation (To et al., 2004). Further study of tissue specific and subcellular localization of type-A ARRs and identification of colocalized genes will help to place type-A ARRs in specific biological functions.

The type-A ARRs fall into five pairs that are most similar in receiver domain sequence and structure of the C-terminus. To date, the specific functions identified for the type-A ARRs appear to co-segregate among gene pairs with the most similar receiver

domain sequence and C-terminal structure. In many prokaryotic response regulators, C-terminal sequences often contain the sequences that specify outputs. ARR5, ARR6 have the shortest C-termini and regulate shoot meristem function together with ARR7 and ARR15, which have longer C-terminal sequences rich in Ser, Thr and charged residues (D'Agostino et al., 2000; Leibfried et al., 2005).ARR3 and ARR4 modulate circadian period and have the longest C-terminal sequences enriched in acidic residues and Ser, Thr and Pro. ARR8 and ARR9 also have longer C-terminal sequences enriched in charged residues. *ARR8* and *ARR9* antagonize *ARR3* and *ARR4* in regulating circadian rhythms and also play a role in lateral root formation (D'Agostino et al., 2000; Salomé et al., 2005).

The type-A ARR C-termini may confer specificity to interactions with other proteins to activate downstream processes. Our results have also shown that phosphomimic mutant forms of ARR5 and ARR7 are active (Leibfried et al., 2005; To et al., 2007), pointing to a screen for outputs of type-A ARR signaling (both general for function in cytokinin signaling and specific for biological functions) using the phosphomimic mutant proteins in yeast two hybrid or by immunprecipitation. Further specific interactions for biological functions may be identified by verifying interactions for specific subsets of type-A ARR gene pairs with similar C-terminal sequences.

In addition, the C-terminal sequence may also mediate regulation of type-A ARR protein turnover (see below).

Moreover, further analysis of type-A *arr* mutant phenotypes reveal antagonistic interactions within the most similar pairs. For example, *arr5* develops a smaller rosette under short day conditions, and this phenotype is suppressed by *arr6* (To et al., 2004).

ARR7 and *ARR15* also appear to play antagonistic roles in regulating inflorescence branching (To and Kieber, unpublished data). These data suggest further specification of type-A ARR function within most similar gene pairs. Antagonistic interactions have also been observed among AHPs and type-B ARRs (Mason et al., 2005; Hutchison et al., 2006). It is unclear how these antagonistic interactions are established and is an intriguing question.

Why are type-A ARR proteins stabilized?

Regulation at the protein levels is emerging as one of the major mechanisms used by hormone signaling pathways to control outputs in plants, and much progress has been made in understanding the mechanisms used to target proteins for protein degradation (Lechner et al., 2006; Dreher and Callis, 2007). For example, in auxin signaling, auxin binding to the receptor F-box proteins targets transcriptional repressors, AUX/IAAs, for degradation to allow ARF transcription factors to activate auxin targets (Moon et al., 2004). Gibberellin also activates F-box proteins to target transcriptional repressors DELLA and RGA for degradation, thus allowing transcriptional activation of gibberellin ouputs (Fleet and Sun, 2005).

The finding that cytokinin stabilizes type-A ARRs, apparently negative regulators of cytokinin signaling, appears to differ from the auxin and gibberellin signaling pathways that use proteasome machinery to degrade antagonistic transcriptional repressors and activate positive regulators (Huq, 2006). However, our results indicate that type-A ARR proteins are likely to perform functions in cytokinin signaling and other developmental processes through phosphodependent interactions (Leibfried et al., 2005; To et al., 2007), which suggest that modulation of protein stability may be a regulatory

mechanism for type-A ARR-specific outputs.

Interestingly, the effect of cytokinin on type-A ARR protein turnover is not correlated with their intrinsic protein stability, but appears to correlate with their phylogenetic and functional relationships. The type-A ARR proteins that are found to be stabilized by cytokinin, ARR5, ARR6 and ARR7, fall into a subset of ARRs which are more similar in sequence. ARR5, ARR6, ARR7 transcription are also highly induced by cytokinin and are regulated by WUSCHEL to mediate interaction between cytokinin signaling and meristem activity (D'Agostino et al., 2000; Rashotte et al., 2003; Leibfried et al., 2005). The type-A ARR proteins that are found not to be stabilized by cytokinin, ARR4 and ARR9, are less similar to ARR5, ARR6 and ARR7 in sequence. In addition, ARR4 and ARR9 are also less transcriptionally upregulated by cytokinin and play a cytokinin independent role in modulating circadian period (D'Agostino et al., 2000; Rashotte et al., 2003; Salomé et al., 2005). ARR4 protein accumulation has also been shown to be affected by light and is involved PHYTOCHROME B dependent processes (Sweere et al., 2001; Salomé et al., 2005; Hanano et al., 2006).

Cytokinin regulation of protein turnover of a subset of type-A ARRs may be a mechanism for modulating their activity in specific cytokinin-regulated plant processes, such as meristem function. Type-A ARRs proteins may be stabilized by additional signals to regulate other functions, such as ARR4 in light-regulated development. The regulation of type-A ARR proteins by other signals remains to be determined and may be an interesting area to explore the interaction between cytokinin signaling and other signaling pathways.

How is type-A ARR protein stability regulated by cytokinin?

Our data shows that ARR5 protein is stabilized by cytokinin in a manner dependent on cytokinin signaling components (To et al., 2007). It appears that there are at least two separate mechanisms involved in regulation of ARR5 protein turnover. One is mediated by two-component phosphorelay, where phosphorylation of ARR5 protein likely confers protein stability. The second may be mediated by type-B ARRs.

Interestingly, the first mechanism proposed, phosphorylation by two component phosphorelay is a property common to all type-A ARRs yet only a subset of type-A ARRs are stabilized by cytokinin. It remains to be determined if phosphorylation-directed substitutions on ARR4 or ARR9 can affect their protein stability. It is possible that subsets of type-A ARRs are targeted for degradation by different mechanisms, which is consistent with the differences observed in basal protein half-life. This further implies that it is not phosphorylation of the receiver domain itself, but that it is the resulting changes in overall conformation of the ARR protein, or of its C-terminal structure, which determines whether the protein will be targeted for degradation. Interestingly, cytokinin stabilizes the type-A ARRs, ARR5, ARR6 and ARR7, which have shorter C-terminal sequences, while the type-A ARR proteins which are not regulated by cytokinin, ARR4 and ARR9, contain longer C-terminal sequences. One may further speculate that the C-terminal sequence plays a role in regulating type-A ARR protein turnover and thus confer specificity in function, such as meristem activity, circadian rhythms or light-regulated development.

Many of the characterized hormone signaling and developmental pathways employ F-box proteins to target proteins for ubiquitination and subsequent proteasome

degradation (Dreher and Callis, 2007). Over 694 putative F-box proteins have been identified in the Arabidopsis genome by computational methods (Gagne et al., 2002) and to date, targets have been identified for only a limited number of them. The yeast response regulator has been shown to be ubiquitinated and targeted for proteasome degradation, though the E3 ligase has not yet been identified (Sato et al., 2003). Ubiquitination or proteasome-dependent degradation of type-A ARR proteins has not yet been shown, but given the widespread role of F-box proteins in Arabidopsis, it is possible that type-A ARR protein degradation may also be mediated by members of the F-box protein family. Some of these F-box protein-target interactions have been identified by forward genetic screens for mutants with altered response to the signal in question and some have been identified by yeast-two hybrid screens (Lechner et al., 2006; Dreher and Callis, 2007). The F-box proteins regulating turnover of the transcription factor ETHYLENE INSENSITIVE 3 (EIN3), EIN3 BINDING F-BOX 1 (EBF1) and EBF2, were identified by first screening microarray data for genes transcriptionally regulated by ethylene (Guo and Ecker, 2003). A similar initial approach may be used to screen for genes involved in regulation of type-A ARR degradation by first looking for F-box proteins or other proteins that may be involved in protein degradation that are transcriptionally regulated by cytokinin. So far, such efforts have identified an E2 ligase, but no E3 ligases (To and Kieber, unpublished data). E3 ligases are usually specify the targeted substrate, and allows the E2 ligase attach a tag (such as ubiquitin) to the substrate either directly or via the E3 (Dreher and Callis, 2007). This may be because the tissues in which the subset of cytokinin-regulated type-A ARR proteins are expressed are very specific, such as the meristem, and changes in expression levels may be difficult to

detect from whole seedling expression analysis. Alternatively, the protein turnover mechanisms for type-A ARRs may not be subject to the same transcriptional control as for EIN3. In fact the *EBF*s are transcriptionally upregulated by ethylene, and their transcript levels are also repressed by the ethylene signaling pathway (see below).

Because the unphosphorylatable ARR5^{D87A} protein exhibits shorter protein half-life than the ARR5^{D87E} phosphomimic, another strategy may be to look for F-box proteins that show preferential interaction with the unphosphorylatable ARR5^{D87A} mutant over the phosphomimic ARR5^{D87E} mutant protein.

Clues for how type-A ARR proteins may be stabilized by cytokinin could be found in other hormone signaling pathways. A subset of type-A ARRs are rapidly turned over in the absence of cytokinin and stabilized by cytokinin application. Similarly, EIN3 is rapidly turned over in the absence of ethylene and accumulates in the presence of ethylene application. In the absence of ethylene, EIN3 is targeted by the F-box proteins EBF1 and EBF2 for proteasome degradation; in the presence of ethylene, the process for targeting EIN3 to the proteasome is inhibited and EIN3 is stabilized, thus allowing activation of ethylene transcriptional targets (Guo and Ecker, 2003; Gagne et al., 2004). *EBF1* and *EBF2* transcript levels are regulated by a 5' to 3' exoribonuclease *EIN5/XRN4* which functions downstream of the ethylene signaling component CTR1 (Olmedo et al., 2006). Loss-of-function *ein5* mutants accumulate higher levels of *EBF1* and *EBF2* mRNA, accumulate lower levels of EIN3 protein and exhibit reduced response to ethylene-mediated transcriptional control, suggesting that EIN5/XRN4 may play a role in stabilizing EIN3 by inhibiting *EBF1* and *EBF2* expression.

Similarly, the mechanism for type-A ARR protein degradation may be

antagonized by cytokinin to allow stabilization of type-A ARR protein. It is possible that type-B ARRs, ARR1, ARR2, ARR10 and ARR12 are required for transcription of factors necessary to antagonize ARR5 degradation in the presence of cytokinin. Candidates for these factors may be found by among genes with altered transcript levels in *arr1,2,10,12*.

To identify elements involved in cytokinin regulation of type-A ARR protein turnover, a genetic screen can also be conducted using the myc-tagged ARR5, ARR6 and ARR7 overexpressors, with a similar strategy used for identifying genes involved post-transcriptional regulation of ethylene biosynthesis genes (Hansen and Kieber, unpublished data). The myc-tagged ARR5, ARR6 and ARR7 overexpressors exhibit a robust but intermediate resistance to cytokinin inhibition of root elongation, which can be enhanced or repressed to find genes which may affect degradation or stabilization of the type-A ARR proteins in response to cytokinin. A subsequent secondary screen can be conducted to identify mutants with altered myc-ARR5 basal or cytokinin regulated protein turnover.

Are type-A RR functions conserved across plant species?

Recent identification of type-A *RRs* from maize cDNAs and from the rice genome has allowed phylogenetic analysis of this gene family (To et al., 2004; Ito and Kurata, 2006; Jain et al., 2006; Pareek et al., 2006; Du et al., 2007). The type-A *RRs* fall into four major groups. All ten of the type-A ARRs group together, separate from the monocot RRs in the other groups, indicating that expansion of the Arabidopsis type-A ARR gene family occurred after monocots and dicots diverged. In this group, eight of the Arabidopsis type-A ARRs, ARR3, ARR4, ARR5, ARR6, ARR7 and ARR15 further clade into one subgroup, with ARR5, ARR6, ARR7 and ARR15 being the most similar

gene pairs. ARR16 and ARR17 fall into a second subgroup, and ARR8 and ARR9 fall into the remaining group with three maize and three rice type-A RRs.

The type-A RRs of Arabidopsis, maize and rice can all be induced by cytokinin treatment in a variety of tissues (Brandstatter and Kieber, 1998; Sakakibara et al., 1999; D'Agostino et al., 2000; Asakura et al., 2003; Rashotte et al., 2003; Jain et al., 2006), indicating that type-A RRs in all three species are cytokinin response genes. *In vitro* experiments have also shown that both Arabidopsis and maize type-A RRs can be phosphorylated by HPs (Suzuki et al., 1998; Imamura et al., 2001; Asakura et al., 2003; Imamura et al., 2003), indicating maize type-A RRs can also function in phosphorelay. Moreover, similar to Arabidopsis, overexpression of type-A *RR* in rice can confer cytokinin resistance (Kiba et al., 2003; Hirose et al., 2007; Lee et al., 2007; To et al., 2007), indicating that type-A RRs in rice are likely to be negative regulators of cytokinin signaling. These data suggest that the type-A RR functions in cytokinin response are conserved.

Interestingly, the type-A ARR genes that have been found to play a major role in meristem function clade distinctly from the maize type-A *RR*, *ZmRR3/ABPHYL1*, which also functions in the meristem. A single maize *ZmRR3/ABPHYL1* loss-of-function mutant was sufficient to disrupt phyllotaxy whereas in Arabidopsis, a phyllotactic defect was not detectable until at least a quadruple mutant was constructed. Other type-A ARRs, including ARR3, ARR4, ARR8 and ARR9 also appear to function additively in regulating the shoot meristem. Furthermore, the *zmrr3/abphyl* mutant also exhibits increased meristem size and altered pattern of organ intiation. While the *arr3,4,5,6,7,8,9* septuple mutant also shows defects in spatial patterning of organ primordia in the

inflorescence meristem (Leibfried et al., 2005; To and Kieber, unpublished data), it does not show obvious differences in vegetative or inflorescence meristem size (Leibfried et al., 2005; Lewis et al., unpublished data). One possibility is that type-A ARRs may have a higher level of genetic redundancy in the shoot meristem than the maize type-A RRs and loss of seven type-A ARR loci is insufficient to affect meristem size. Alternatively, these differences in phenotypes may reflect a divergence between Arabidopsis and maize type-A RR function. The *zmrr3/abphyl* shows an increased expression in a *KNOX* gene, which is required for meristem maintenance. So far, analysis of microarray data of mRNA collected from *arr3,4,5,6,7,8,9* inflorescences has not detected differences in expression of *KNOX* genes (Leibfried et al., unpublished data) and *in situ* analysis may be required to acquire resolution at the tissue level.

In Arabidopsis, the homeodomain protein *WUSCHEL* interacts with the type-A *ARR*s to regulate meristem function (Leibfried et al., 2005). WUSCHEL also belongs to a larger family of genes with homologs across plant species. A recent study has reported that, unlike the Arabidopsis WUS, which is expressed in the organizing center in the meristem, maize and rice WUS homologs are expressed in new phytomers and reproductive organs (Nardmann and Werr, 2006). It is thus unlikely that the maize WUS homologs interact with *ZmRR3*. These results suggest that mechanisms regulating meristem function, and possibly the role of type-A RRs in meristem function may have diverged between monocots and dicots. It is also possible that different maize type-A RRs, other than *ZmRR3*, are expressed in the monocot WUS expression domain to interact with CLV/WUS function in those tissues.

Thus while Arabidopsis, maize and rice type-A RRs appear to behave similarly in

general cytokinin response, their functions may differ in tissue specific functions, such as regulating shoot meristem function. These ideas are consistent with the finding that monocot and dicot type-A RR families may have expanded largely independently after the two groups diverged. Thus it is important to exercise caution when drawing conclusions about orthologs, as mechanisms for function may not always translate.

Where do we go from here?

My work has shown that type-A ARRs have overlapping function in regulating cytokinin signaling and has begun to uncover specific functions for type-A ARRs. Hence while type-A ARRs retain their ancestral functions in overall cytokinin response and two-component phosphorelay, similar to the monocot type-A ARRs, specification of other functions have also evolved and diverged between monocots and dicots. These findings also indicate type-A ARRs are not only negative regulators of two-component phosphorelay but may also have independent outputs. Further study of type-A ARR functions will require resolution at the tissue and cellular level and identification of co-localized components. Identifying factors involved in regulating type-A ARR protein stability may also shed light on the mechanisms that confer functional specificity to subsets of type-A ARRs and may reveal novel interactions with other signaling pathways. Isolation of type-A ARR-specific downstream outputs is an obvious area to pursue. One method to identify factors involved in specific functions of type-A ARRs is to screen for interactors specific for the phosphorylated or phosphomimic protein form. Interactors may reveal additional roles for type-A ARRs in cytokinin receptor-regulated functions and other cytokinin-independent processes.

REFERENCES

Asakura, Y., Hagino, T., Ohta, Y., Aoki, K., Yonekura-Sakakibara, K., Deji, A., Yamaya, T., Sugiyama, T., and Sakakibara, H. (2003). Molecular characterization of His-Asp phosphorelay signaling factors in maize leaves: implications of the signal divergence by cytokinin-inducible response regulators in the cytosol and the nuclei. Plant Mol. Biol. **52,** 331-341.

Baker, M.D., Wolanin, P.M., and Stock, J.B. (2006). Signal transduction in bacterial chemotaxis. Bioessays **28,** 9-22.

Brandstatter, I., and Kieber, J.J. (1998). Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in Arabidopsis. Plant Cell **10,** 1009-1020.

D'Agostino, I., Deruère, J., and Kieber, J.J. (2000). Characterization of the response of the Arabidopsis *ARR* gene family to cytokinin. Plant Physiol. **124,** 1706-1717.

Dello Ioio, R., Linhares, F.S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., and Sabatini, S. (2007). Cytokinins determine Arabidopsis root-meristem size by controlling cell differentiation. Curr. Biol. **17,** 678-682.

Dortay, H., Mehnert, N., Burkle, L., Schmulling, T., and Heyl, A. (2006). Analysis of protein interactions within the cytokinin-signaling pathway of Arabidopsis thaliana. FEBS J. **273,** 4631-4644.

Dreher, K., and Callis, J. (2007). Ubiquitin, hormones and biotic stress in plants. Ann. Bot. **99,** 787-822.

Du, L., Jiao, F., Chu, J., Chen, M., and Wu, P. (2007). The two-component signal system in rice (Oryza sativa L.): A genome-wide study of cytokinin signal perception and transduction. Genomics **In Press, Corrected Proof**.

Fleet, C.M., and Sun, T.-p. (2005). A DELLAcate balance: the role of gibberellin in plant morphogenesis. Curr. Opin. Plant Biol. **8,** 77-85.

Gagne, J.M., Downes, B.P., Shiu, S.-H., Durski, A.M., and Vierstra, R.D. (2002). The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in Arabidopsis. Proc. Natl. Acad. Sci. U S A **99,** 11519-11524.

Gagne, J.M., Smalle, J., Gingerich, D.J., Walker, J.M., Yoo, S.-D., Yanagisawa, S., and Vierstra, R.D. (2004). Arabidopsis EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. Proc. Natl. Acad. Sci. U S A **101,** 6803-6808.

Guo, H., and Ecker, J.R. (2003). Plant Responses to Ethylene Gas Are Mediated by SCFEBF1/EBF2-Dependent Proteolysis of EIN3 Transcription Factor. Cell **115,** 667-677.

Hanano, S., Domagalska, M.A., Nagy, F., and Davis, S.J. (2006). Multiple phytohormones influence distinct parameters of the plant circadian clock. Genes Cells **11,** 1381-1392.

Hansen, M.E., and Kieber, J.J. (unpublished data).

Hejatko, J., Pernisova, M., Eneva, T., Palme, K., and Brzobohaty, B. (2003). The putative sensor histidine kinase CKI1 is involved in female gametophyte development in Arabidopsis. Mol Genet Genomics **269,** 443-453.

Higuchi, M., Pischke, M.S., Mahonen, A.P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S., Helariutta, Y., Sussman, M.R., and Kakimoto, T. (2004). In planta functions of the Arabidopsis cytokinin receptor family. Proc. Natl. Acad. Sci. U S A **101,** 8821-8826.

Hirose, N., Makita, N., Kojima, M., Kamada-Nobusada, T., and Sakakibara, H. (2007). Over-expression of a Type-A Response Regulator alters rice morphology and cytokinin metabolism. Plant Cell Physiol. **48,** 523-539.

Huq, E. (2006). Degradation of negative regulators: a common theme in hormone and light signaling networks? Trends Plant Sci. **11,** 4-7.

Hutchison, C.E., Li, J., Argueso, C., Gonzalez, M., Lee, E., Lewis, M.W., Maxwell, B.B., Perdue, T.D., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2006). The Arabidopsis Histidine Phosphotransfer Proteins are redundant positive regulators of cytokinin signaling. Plant Cell **18,** 3073-3087.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in *Arabidopsis* signal transduction. Nature **413,** 383-389.

Imamura, A., Yoshino, Y., and Mizuno, T. (2001). Cellular localization of the signaling components of Arabidopsis His-to-Asp phosphorelay. Biosci. Biotechnol. Biochem. **65,** 2113-2117.

Imamura, A., Kiba, T., Tajima, Y., Yamashino, T., and Mizuno, T. (2003). In vivo and in vitro Characterization of the ARR11 response regulator implicated in the His-to-Asp phosphorelay signal transduction in Arabidopsis thaliana. Plant Cell Physiol. **44,** 122-131.

Imamura, A., Hanaki, N., Umeda, H., Nakamura, A., Suzuki, T., Ueguchi, C., and Mizuno, T. (1998). Response regulators implicated in His-to-Asp phosphotransfer signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. U S A **95,** 2691-2696.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., and Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from Arabidopsis. Nature **409,** 1060-1063.

Ito, Y., and Kurata, N. (2006). Identification and characterization of cytokinin-signalling gene families in rice. Gene **382,** 57-65.

Jain, M., Tyagi, A.K., and Khurana, J.P. (2006). Molecular characterization and differential expression of cytokinin-responsive type-A response regulators in rice (Oryza sativa). BMC Plant Biol. **6,** 1.

Kakimoto, T. (1996). CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. Science **274,** 982-985.

Kiba, T., Aoki, K., Sakakibara, H., and Mizuno, T. (2004). Arabidopsis Response Regulator, ARR22, ectopic expression of which results in phenotypes similar to the *wol* cytokinin-receptor mutant. Plant Cell Physiol. **45,** 1063-1077.

Kiba, T., Yamada, H., Sato, S., Kato, T., Tabata, S., Yamashino, T., and Mizuno, T. (2003). The type-A response regulator, ARR15, acts as a negative regulator in the cytokinin-mediated signal transduction in *Arabidopsis thaliana*. Plant Cell Physiol. **44,** 868-874.

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y., Sakakibara, H., and Kyozuka, J. (2007). Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature **445,** 652-655.

Lechner, E., Achard, P., Vansiri, A., Potuschak, T., and Genschik, P. (2006). F-box proteins everywhere. Curr. Opin. Plant Biol. **9,** 631-638.

Lee, D.J., Park, J.Y., Ku, S.J., Ha, Y.M., Kim, S., Kim, M.D., Oh, M.H., and Kim, J. (2007). Genome-wide expression profiling of ARABIDOPSIS RESPONSE REGULATOR 7(ARR7) overexpression in cytokinin response. Mol. Genet. Genomics **277,** 115-137.

Leibfried, A., To, J.P., Lohmann, J.U., and Kieber, J.J. (unpublished data).

Leibfried, A., To, J.P.C., Stehling, S.K., A., Busch, W., Demar, M., Kieber, J.J., and Lohmann, J.U. (2005). WUSCHEL controls meristem size by direct transcriptional regulation of cytokinin inducible response regulators. Nature **438,** 1172-1175.

Lewis, M.W., To, J.P., and Kieber, J.J. (unpublished data).

Mähönen, A.P., Bonke, M., Kauppinen, L., Riikonon, M., Benfey, P., and Helariutta, Y. (2000). A novel two-component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root. Genes Dev. **14,** 2938-2943.

Mähönen, A.P., Higuchi, M., Tormakangas, K., Miyawaki, K., Pischke, M.S., Sussman, M.R., Helariutta, Y., and Kakimoto, T. (2006a). Cytokinins regulate a bidirectional phosphorelay network in Arabidopsis. Curr. Biol. **16,** 1116-1122.

Mähönen, A.P., Bishopp, A., Higuchi, M., Nieminen, K.M., Kinoshita, K., Tormakangas, K., Ikeda, Y., Oka, A., Kakimoto, T., and Helariutta, Y. (2006b). Cytokinin signaling and its inhibitor AHP6 regulate cell Fate during vascular development. Science **311,** 94-98.

Mason, M.G., Mathews, D.E., Argyros, D.A., Maxwell, B.B., Kieber, J.J., Alonso, J.M., and Ecker, J.R.S., G.E. (2005). Multiple type-B response regulators mediate cytokinin signal transduction in Arabidopsis. Plant Cell **17,** 3007-3018.

Miyawaki, K., Matsumoto-Kitano, M., and Kakimoto, T. (2004). Expression of cytokinin biosynthetic isopentenyltransferase genes in Arabidopsis: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant J. **37,** 128-138.

Moon, J., Parry, G., and Estelle, M. (2004). The ubiquitin-proteasome pathway and plant development. Plant Cell **16,** 3181-3195.

Nardmann, J., and Werr, W. (2006). The shoot stem cell niche in angiosperms: expression patterns of WUS orthologues in rice and maize imply major modifications in the course of mono- and dicot evolution. Mol. Biol. Evol. **23,** 2492-2504.

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S., and Ueguchi, C. (2004). Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. Plant Cell **16,** 1365-1377.

Olmedo, G., Guo, H., Gregory, B.D., Nourizadeh, S.D., Aguilar-Henonin, L., Li, H., An, F., Guzman, P., and Ecker, J.R. (2006). ETHYLENE-INSENSITIVE5 encodes a 5'->3' exoribonuclease required for regulation of the EIN3-targeting F-box proteins EBF1/2. Proc. Natl. Acad. Sci. U S A **103,** 13286-13293.

Pareek, A., Singh, A., Kumar, M., Kushwaha, H.R., Lynn, A.M., and Singla-Pareek, S.L. (2006). Whole-genome analysis of Oryza sativa reveals similar architecture of two-component signaling machinery with Arabidopsis. Plant Physiol. **142,** 380-397.

Pischke, M.S., Jones, L.G., Otsuga, D., Fernandez, D.E., Drews, G.N., and Sussman, M.R. (2002). An Arabidopsis histidine kinase is essential for megagametogenesis. Proceedings of the National Academy of Sciences of the United States of America **99,** 15800-15805.

Rashotte, A.M., Carson, S.D., To, J.P., and Kieber, J.J. (2003). Expression profiling of cytokinin action in Arabidopsis. Plant Physiol. **132,** 1998-2011.

Rashotte, A.M., Mason, M.G., Hutchison, C.E., Ferreira, F.J., Schaller, G.E., and Kieber, J.J. (2006). A subset of Arabidopsis AP2 transcription factors mediates cytokinin responses in concert with a two-component pathway. Proc. Natl. Acad. Sci. U S A **103,** 11081-11085.

Riefler, M., Novak, O., Strnad, M., and Schmulling, T. (2006). Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell **18,** 40-54.

Sakai, H., Honma, T., Aoyama, T., Sato, S., Kato, T., Tabata, S., and Oka, A. (2001). ARR1, a transcription factor for genes immediately responsive to cytokinins. Science **294,** 1519-1521.

Sakakibara, H., Hayakawa, A., Deji, A., Gawronski, S.W., and Sugiyama, T. (1999). His-Asp phosphotransfer possibly involved in the nitrogen signal transduction mediated by cytokinin in maize: molecular cloning of cDNAs for two-component regulatory factors and demonstration of phosphotransfer activity in vitro. Plant Mol. Biol. **41,** 563-573.

Salomé, P.A., To, J.P.C., Kieber, J.J., and McClung, C.R. (2005). Arabidopsis response regulators ARR3 and ARR4 play cytokinin-Independent roles in the control of circadian period. Plant Cell **18,** 55-69.

Sato, N., Kawahara, H., Toh-e, A., and Maeda, T. (2003). Phosphorelay-regulated degradation of the yeast Ssk1p response regulator by the ubiquitin-proteasome system. Mol Cell Biol **23,** 6662-6671.

Schmitt, R. (2002). Sinorhizobial chemotaxis: a departure from the enterobacterial paradigm. Microbiology **148,** 627-631.

Suzuki, T., Imamura, A., Ueguchi, C., and Mizuno, T. (1998). Histidine-containing phosphotransfer (HPt) signal transducers implicated in His-to-Asp phosphorelay in Arabidopsis. Plant Cell Physiol. **39,** 1258-1268.

Sweere, U., Eichenberg, K., Lohrmann, J., Mira-Rodado, V., Bäurle, I., Kudla, J., Nagy, F., Schäfer, E., and Harter, K. (2001). Interaction of the response regulator ARR4 with the photoreceptor phytochrome B in modulating red light signaling. Science **294,** 1108-1111.

Tanaka, M., Takei, K., Kojima, M., Sakakibara, H., and Mori, H. (2006). Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. Plant J. **45,** 1028-1036.

To, J.P., and Kieber, J.J. (unpublished data).

To, J.P., Deruère, J., Maxwell, B.B., Morris, V.F., Hutchison, C.E., Schaller, G.E., and Kieber, J.J. (2007). Cytokinin regulates Type-A Arabidopsis Response Regulator activity and protein stability via two-component phosphorelay. In

Arabidopsis Response Regulators in cytokinin signaling and plant development (This Thesis: Chapter 4), pp. 109.

To, J.P.C., Haberer, G., Ferreira, F.J., Deruère, J., Mason, M.G., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2004). Type-A ARRs are partially redundant negative regulators of cytokinin signaling in Arabidopsis. Plant Cell **16,** 658-671.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmulling, T. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15,** 2532-2550.

Woodward, A.W., and Bartel, B. (2005). Auxin: regulation, action, and interaction. Ann. Bot. **95,** 707-735.

Yamada, H., Koizumi, N., Nakamichi, N., Kiba, T., Yamashino, T., and Mizuno, T. (2004). Rapid response of Arabidopsis T87 cultured cells to cytokinin through His-to-Asp phosphorelay signal transduction. Biosci. Biotechnol. Biochem. **68,** 1966-1976.

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., Yamashino, T., and Mizuno, T. (2001). The Arabidopsis AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol. **42,** 1017-1023.

Yokoyama, A., Yamashino, T., Amano, Y., Tajima, Y., Imamura, A., Sakakibara, H., and Mizuno, T. (2007). Type-B ARR transcription factors, ARR10 and ARR12, are implicated in cytokinin-mediated regulation of protoxylem differentiation in roots of Arabidopsis thaliana. Plant Cell Physiol. **48,** 84-96.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004). GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol. **136,** 2621-2632.