The Role of Cytokinin in Female Gametophyte Development in Arabidopsis

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ABSTRACT

CHIA-YI CHENG: The Role of Cytokinin in Female Gametophyte Development in Arabidopsis
(Under the direction of Joseph J. Kieber)

Plants, unlike animals, have distinct haploid and diploid phases of their life cycle. The haploid female gametophyte develops within the diploid maternal sporophytic tissue. The signaling events regulating the development of the female gametophyte are just beginning to be understood. The plant hormone auxin plays a role in the development of the female gametophyte, but the contribution of other phytohormones has not been examined in depth.

Here we explore and demonstrate a role for the plant hormone cytokinin in female gametophyte development. We show that cytokinin function is enriched at one end of the sporophytic tissue that supports the development of the female gametophyte. This localized cytokinin signal in the maternal tissue is required for the initial specification of functional megaspore that will ultimately give rise to the female gametophyte.

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LIST OF ABBREVIATIONS

AHK	Arabidopsis histidine kinase
ARR	Arabidopsis response regulator
BA	N6-Benzyladenine
CHASE	Cyclase/histidine kinase-associated sensing extracellular
Che	Chemotaxis
CKI	Cytokinin independent
CKX	Cytokinin oxidase
CRF	Cytokinin response factor
DM	Degenerating megaspore
FG	Female gametophyte
FM	Functional megaspore
GARP	DNA binding domain found in GOLDEN2 in maize, the ARRs, and the Psr1protein from *Chlamydomonas*
GUS	ß-glucuronidase
HK	Histidine kinase
HP	Histidine phosphotransfer protein
Hpt	Histidine-containing phosphotransfer proteins
IPT	Isopentenyl transferase
MMC	Megaspore mother cell
RR	Response regulator
T-DNA	Transferred-DNA from agrobacterium
X-gluc	5-bromo-4-chloro-3-indolyl-glucuronic acid

CHAPTER 1: Cytokinin Signaling

Introduction

In animals and plants, hormones generally act pleiotropically to regulate growth and development. The phytohormone cytokinin has been linked to a wide array of developmental processes since its identification in the 1950s as a factor which, in concert with auxin, induced cell division in tobacco tissue[1, 2]. These processes include organ initiation, meristem maintenance, chloroplast development, vascular differentiation, and leaf senescence. Further, cytokinin also plays an important role in the responses to biotic factors, such as pathogen defense and rhizobial symbiosis, and abiotic factors such as cold, drought, and salt stress[3, 4]. How a signal mediates such diverse biological outputs and how these responses are intertwined with other signaling pathways remain fundamental questions in plant biology.

Molecular genetic studies in Arabidopsis have revealed that cytokinin perception in this dicot model system is similar to bacterial two-component phosphotransfer signal transduction systems. Two-component elements are also present in monocots[5, 6], as well as in lower plants such as the moss *Physcomitrella patens*[7]. The prototypical two-component system consists of two conserved proteins: a sensor kinase (histidine kinase, HK) and a response regulator (RR) (Figure 1.1). In response to the environmental stimuli, the HK autophosphorylates on a conserved His residue using the γ-phosphate from ATP and then transfers the phosphoryl group to a conserved Asp residue within the receiver

domain of the RR[8, 9]. In addition to the conserved N-terminal receiver domain, RRs also have a variable C-terminal output domain that confers diversity in regulatory strategies. The phosphorylation state of the RRs changes the function of the output domain, which can participate in DNA binding and transcriptional control, perform enzymatic activities, or mediate protein-protein interactions. Thus, RRs function as phospho-mediated switches that couple environmental cues to cellular responses in a simple, direct manner[10, 11].

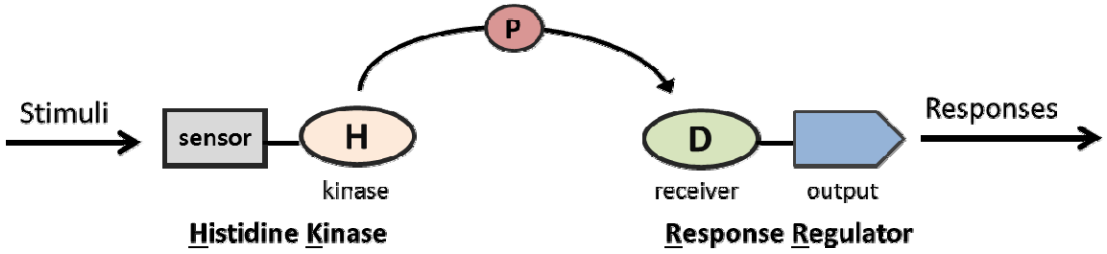

Figure 1.1. Schematic diagram of two-component (TCS) system. The prototypical TCS features a phosphoryl transfer (P) between the conserved His residue (H) of the kinase to the conserved Asp residue (D) in the receiver domain of the response regulator.

Cytokinin signaling, as well as all other known eukaryotic two-component-like signaling, involves a more elaborate version of the two-component system known as a multi-component phosphorelay (Figure 1.2). This involves hybrid kinases containing both histidine kinase (HK) and receiver domains in a single protein, a His-containing phosphotransfer proteins (HP), and response regulators (RR)[12]. The phosphotransfer scheme occurs via a His-Asp-His-Asp phosphorelay that provides more targets for modulation. The multiple-step phosphorelay systems are present in both prokaryotes and eukaryotes.

The elucidation of the mechanism of cytokinin signaling has been hampered by the genetic redundancy of the two-component genes in plants. Nevertheless, molecular

genetic studies, primarily in the model species *Arabidopsis thaliana*, have revealed the various two-component elements involved in cytoknin signaling, and the analysis of high order loss-of-function mutants has shed light on the overlapping and distinct biological roles of these two-component proteins[13-18]. However, the understanding of the underlying mechanisms by which cytokinin achieves signaling specificity in its myriad roles throughout plant growth and development, and how it integrates with other signaling pathways is only beginning to be understood.

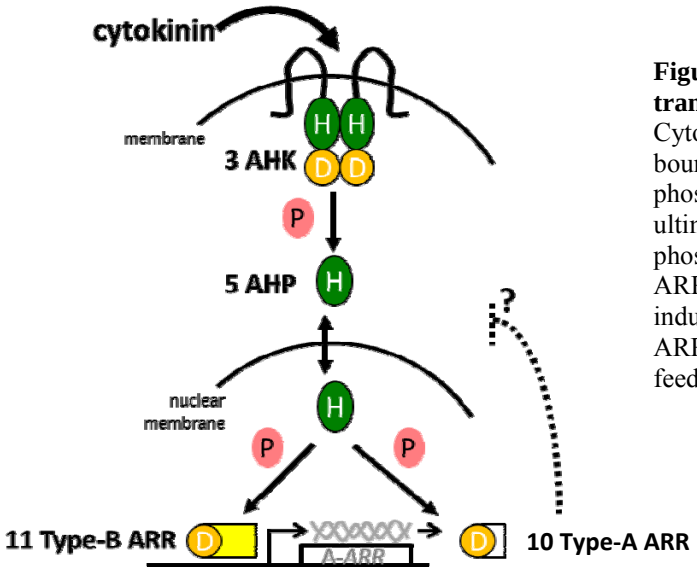

Figure 1.2. Cytokinin signal transduction in *Arabidopsis*. Cytokinin binds to the membrane-bound AHK receptors, which initiates a phosphorelay through the AHPs and ultimately results in the phosphorylation of type-B and type-A ARRs. The activated type-B ARRs induce the transcription of the type-A ARRs, which in turn act to negatively feedback the pathway.

1. Two-component elements are involved in cytokinin signaling

1.1 Phosphotransfer chemistry

Two-component signaling acts as a ubiquitous mechanism by which bacteria sense and respond to environmental cues. Most bacteria possess numerous two-component systems to respond to a variety of environmental changes, such as temperature (thermotaxis), light (phototaxis), salinity (osmotaxis), oxygen (aerotaxis), and chemicals (chemotaxis). To date, two-component signal transduction has been found in all domains: eubacteria, archaea, and eukarya; However, while these systems are common in eubacteria and archaea, they are relatively rare in eukarya, and have not as yet been found in animals, which instead rely on Ser/Thr/Tyr phosphorylation for much of their signaling needs[9]. It is important to note that the chemistry of the phosphorelay differs substantially from the phosphoesters involved in Ser/Thr/Try phosphorylation[9].

The chemistry of the basic two-component system involves three phosphotransfer reactions[8, 9]:

1. Autophophorylation: HK-His + ATP ↔ HK-His~P + ADP
2. Phosphotransfer: HK-His~P + RR-Asp ↔ HK-His + RR-Asp~P
3. Dephosphorylation: RR-Asp~P + H_2O ↔ RR-Asp + P_i

The phosphorylation at His and Asp residues is thermodynamically distinct from the phosphorylation at Ser, Thr, and Tyr residues, which are highly exothermic and thus essentially irreversible. In contrast, the free energy associated with the phosphotransfer between His and Asp residues is close to zero, which allows bidirectional flow of the phosphoryl group. That is, upon cessation of the stimulus, the phosphoryl group on the

RRs is able to flow back to the HKs, effectively terminating the response and allowing rapid, adaptive responses to environmental conditions.

1.2 His-Asp phosphorylation pathway in Arabidopsis thaliana

In 1993, the identification of a candidate ethylene receptor ETR1 with putative His kinase and receiver domains in *Arabidopsis thaliana* was the first indication that eukaryotes in general, and specifically plants harbored two-component signaling systems[19]. In 1996, the second His-kinase, CKI1, was discovered as a potential mediator of cytokinin signaling[20], and shortly thereafter response regulators (RRs) were identified as cytokinin primary response genes. Similarly, two-component elements were identified in a number of fungal species. When the entire genome sequence of *Arabidopsis* was elucidated in 2000, the complete two-component element repertoire was revealed: the *Arabidopsis* genome encodes 11 genes predicted to be functional HKs, 5 HPs, and 23 RRs. Among the eleven HKs in Arabidopsis, five belong to the ethylene receptor family members, though three of these are missing residues essential for histidine kinase activity[21]. Further, the output of the ethylene receptor HKs occurs largely through a Raf-like Ser/Thr kinases CTR1, which does not involve a His-Asp phosphorelay[22]. Among the six other non-ethylene receptor HKs, AHK2, AHK3, and AHK4 are cytokinin receptors; CKI1 functions upstream of the AHPs, both in vegetative and reproductive stages[23]; AHK1/AtHK1 is a positive regulator of drought and salt stress responses[24]; and CKI2/AHK5 is a positive regulator in biotic and abiotic stress responses[25].

1.3 Cytokinin two-component elements

In Arabidopsis, multiple two-component elements have been shown to act in cytokinin signaling. The three cytokinin receptors (AHK2, AHK3, and AHK4/CRE1/WOL) contain a conserved CHASE (Cyclase/Histidine kinase-associated sensing extracellular) domain, which confers the ability to bind cytokinin with high affinity. They also contain a histidine kinase domain, and both an authentic and a pseudo receiver domain[13, 18, 26]. AHKs act partially redundantly as positive elements in cytokinin signaling. The direct target of the AHKs are the five His-containing phosphotransfer proteins (AHPs), which also act redundantly as positive elements in the primary cytokinin signaling pathway[15, 27, 28]. There are 23 response regulators (ARRs) in Arabidopsis that fall into two major classes based on phylogenetic analysis and domain structure: type-A ARRs and type-B ARRs. The eleven type-B ARRs have a conserved myb-like GARP DNA-binding domain following the N-terminal receiver domain and act as partially redundant, positive elements in cytokinin signaling[29-32]. In contrast, the ten type-A ARRs are comprised of essentially only a receiver domain and they act as negative elements in cytokinin signaling[17, 33].

Cytokinin signal transduction initiates when cytokinin binds to the CHASE domain of the receptors AHKs to initiate autophosphorylation on a conserved His residue within the histidine kinase domain (Figure 1.2). This phosphoryl group is then transferred to an Asp residue within the C-terminal receiver domain of the AHKs. The AHPs then shuttle the phosphoryl group from the AHKs to the type-B and type-A ARRs[15, 27]. The phosphorylation activates the type-B ARRs, which regulate the expression of the primary cytokinin-responsive genes, including type-A ARRs[16, 31, 34]. In turn, the type-A ARRs act

as negative feedback regulators of the primary signaling pathway[35]. Overall, the phosphorelay in cytokinin signal transduction involves four sequential phosphorylation events in the order His-Asp-His-Asp. This more elaborate architecture of the phosphorelay provides additional opportunities for crosstalk with other signaling pathways and provides a robust mechanism for shuttling the cytokinin signal to multiple compartments within a eukaryotic cell.

2. Cytokinin receptors are histidine kinases

2.1 Discovery

A histidine kinase (HK), CKI1 (CYTOKININ INSENSITIVE 1), was isolated from an activation T-DNA tagging screening as a gene that when overexpressed conferred shoot initiation in the absence of exogenous cytokinin. The CKI1 protein consists of a histidine kinase domain, a single transmembrane domain, and a receiver domain. Overexpression of *CKI1* induces typical cytokinin responses to cultured cells, including rapid proliferation, greening, shoot formation and inhibition of root formation. The activation of cytokinin responses by *CKI1* implicates it as a cytokinin signaling element, but the gain-of-function nature of the allele complicates this conclusion. Subsequent studies identified *cre1* (*cytokinin response 1*) as a mutant that showed reduced cytokinin sensitivity. *CRE1* also encodes a histidine kinase. However, in contrast to CKI1, CRE1 was demonstrated to bind cytokinin with high affinity and specificity, and could complement yeast and bacterial HK mutants in a cytokinin-dependent manner. This Arabidopsis Histidine Kinase (AHK4/CRE1/WOL) and its paralogues (AHK2 and AHK3) contain a CHASE domain that binds cytokinin. How binding of cytokinin to the CHASE domain transduces the signal across the membrane is not known. CKI1 and other hybrid HKs that do not include the CHASE domain do not bind cytokinin; nevertheless, they could feed into cytokinin signaling by phosphorylation of the AHPs in response to the sensing of other signals beside cytokinin, and/or by forming heterodimers with the cytokinin receptor AHKs.

2.2 Cytokinin receptors differ in biochemical nature

Naturally occurring cytokinins are adenine derivatives with a N^6-side chain and are classified as isoprenoid or aromatic depending on the nature of the side chain [36]. The binding preferences for the different AHKs have been studied in *Escherichia coli.* Strains of *E. coli* expressing AHK3 or AHK4 have different sensitivity in recognizing various cytokinin compounds [37]. Both are most sensitive to the isoprenoid-type *trans*-zeatin (*tZ*) and isopentenyladenine (iP) but differ significantly in the recognition of other cytokinin compounds. Interestingly, all maize HKs recognize *cis*-zeatin (*cZ*) with high affinity [38]. Results from *in planta* experiments confirm the *in vitro* data, and further reveal different sensitivity of each AHK towards *t*Z and iP; AHK2 and AHK4 show a comparable activity by *t*Z and iP, while AHK3 shows the higher sensitivity to *t*Z compared with iP. A higher functional similarity between AHK2 and AHK4 is supported by the promoter-swap experiment, in which AHK4 expressed under the control of the *AHK2* promoter (but not the *AHK3* promoter) is sufficient to complement the *ahk2 ahk3* loss-of-function phenotype [39]. A chimeric protein that includes the CHASE-TM (transmembrane) of AHK3 and the cytoplasmic domain of AHK4 could partially complement *ahk2 ahk3* phenotype [39]. The partial complementation suggests the property of the CHASE-TM domain is critical but not the sole feature required for proper AHK3 function.

Disrupting *AHK4* via T-DNA insertion or nonsense mutation does not cause a substantial effect on plant growth and development. However, certain point mutations within the AHK4 coding region produce the *wooden leg (wol)* alleles of *CRE1,* which have fewer numbers of vascular initials during embryogenesis, and as a result, cause defects in vascular morphogenesis post-embryonically [40]. One of these point mutations

T278I in the CHASE domain of AHK4 blocks its cytokinin binding activity *in vitro*. The recently resolved crystal structure of the CHASE domain of AHK4 has rationalized that the T278I mutation likely restricts the overall size of the binding pocket and thus affects the binding capacity [41]. The dominant-negative nature of *wol*^{T278I} may result from the phosphatase activity of unbound AHK4 [42], which is observed in some prokaryotic histidine kinases that possess both kinase and phosphatase activity[9]. Biochemical analysis show that CRE1 can dephosphorylate multiple AHPs and this phosphatase activity requires the conserved Asp residue in its receiver domain[42]. These results suggest that AHK4 acts as both a kinase and a phosphatase in a bidirectional phosphorelay. Thus, in the absence of cytokinin, phosphate from the ARRs could be removed via flow to AHK4, through the AHPs, reflecting the reversibility of the various phosphorylation events in this pathway.

A distinct *wol*-like allele of *AHK4* has been isolated [43]. This mutation results in a *wol*-like root phenotype. Interestingly, the *wol-2*^{L506F} displays intragenic complementation of *wol-1*^{T278I}, implying that the signal transduction involves dimerization or higher order oligomerization. In the *trans*-hetergozygous plants (*wol-1/wol-2*), the root vascular phenotype is wild-type, though they remain insensitive to exogenous cytokinin. It is puzzling how these recessive mutations cause dominant-negative effects on procambial cell proliferation, and display intragenic complementation exclusively in vascular development. One explanation is that AHK4 represses, perhaps via its phosphatase activity, cambium morphogenesis as a monomer [44]. Binding of cytokinin triggers the dimerization (or higher order oligomerization) and subsequently de-represses the negative effect on vascular initiation. Homozygous *wol* plants lack the

ability either to bind cytokinin (*wol-1*) or to dimerize (*wol-2*) and thus repress procambial development. In this model, the *trans*-heterozygotes *wol-1/wol-2* would only have compromised receptors including one copy of WOL-2 that can bind cytokinin but not dimerize and one copy of WOL-1 that can dimerize but not bind cytokinin, which is sufficient to release the repressing effect on vascular initiation but not for the response to elevated levels of exogenous cytokinin.

2.3 Functions

Since their discovery, cytokinins have been shown to positively regulate shoot growth and negatively regulate root elongation. Much of the work, however, has been based on experiments using overexpressors and exogenous cytokinin treatment. Mutations in the genes encoding various cytokinin two-component elements provide novel ways to explore the functions of cytokinin in plant growth and development.

Primary root elongation and lateral root formation are inhibited in the *ahk2 ahk3 ahk4* mutant, which is associated with cell cycle arrest as the transition from $G_2 \rightarrow M$ phase is delayed[18]. Moreover, the *wol* mutant displays a lack of the periclinal cell divisions that occurs during vascular morphogenesis [40]. However, single mutation in *AHK3* and multiple mutations in isopentenyltransferases (*IPTs*), which are essential to cytokinin biosynthesis, results in longer primary root and a larger root meristem [45]. These results led to a model in which the cytokinin response curve, at least in the root, is bell-shaped rather than linear [44]. If this were the case, a minor reduction of cytokinin signaling would induce root growth, while reduction beyond the threshold would abolish growth. This is similar to the bell-shape response curve observed for cytokinin in shoot initiation assays in cultured cells.

Disruption of the cytokinin receptors also perturbs shoot and floral development. The shoot meristem size and leaf cell number are smaller in *ahk2 ahk3 ahk4* mutants, consistent with a role for AHKs as positive regulator of cell division [13, 14, 18]. The *ahk2 ahk3 ahk4* mutants only occasionally form an inflorescence stem and produce few sterile flowers. These results suggest the transition from vegetative to inflorescence meristem is defective, and that the floral meristem activity is depleted in *ahk2 ahk3 ahk4* mutants. The cytokinin receptors AHKs are also required in gametophyte development as the strong triple mutant combinations result in complete male and female sterility [13, 46]. Interestingly, the weak *ahk* triple mutants produce a few flowers with reduced fertility, but are capable of producing a few seeds. These findings indicate that cytokinin is essential in floral development, however, the dosage of cytokinin signaling required differs for different developmental stages[47].

2.4 Cytokinin receptors have overlapping and specific expression pattern

The expression of the *AHK* genes overlap but display different levels in specific tissues. In roots, *AHK4* is expressed at a higher level as compared to *AHK2* and *AHK3;* in rosette leaves, *AHK2* and *AHK3* display the predominant expression and *AHK4* is barely detectable. Consistent with the expression patterns, *ahk2 ahk3* mutants have smaller rosettes while *ahk3 ahk4* and *ahk2 ahk4* are similar in size to the wild type; single *ahk4* mutants display reduced cytokinin sensitivity in the root while the *ahk2* and *ahk3* mutants exhibit normal sensitivity. The cytokinin-dependent induction of *ARR15* and *ARR16* are compromised in the roots of the *ahk4* mutants, but not in leaves. This suggests the transcriptional induction of a subset of type-A *ARRs* in root by exogenous cytokinin treatment is dependent on AHK4. Although *AHK2* and *AHK3* both have high expression

level in leaves, a specific role in regulating senescence is exclusively mediated by *AHK3*. A gain-of-function mutation in *AHK3* causes delayed leaf senescence whereas a loss of function *ahk3* mutant (but not *ahk2* nor *ahk4* single mutant) confers reduced cytokinin sensitivity in leaf senescence. Chlorophyll retention is impaired in the *ahk3* mutant, and addition of a *ahk2* mutation further magnifies this effect. The involvement of *AHK4* only becomes significant in the triple mutant, consistent with its weak expression level in rosette leaves.

2.5 The majority of cytokinin receptors localize in ER

In addition to the redundant roles of the receptors in some contexts, genetic studies have also reported specific roles for individual AHKs[14, 46, 48]. These unique receptor functions could be the result of differences in expression patterns[39, 46, 48], ligand binding[37, 39], protein structures[41, 49], interacting targets[50], or subcellular localizations[51, 52]. Hydrophobicity analysis suggests putative transmembrane segments in the N-terminus of AHK2, AHK3, and AHK4. Bioinformatic analysis using the PSORT (Prediction of Protein Sorting Signals and Localization Sites) program suggested that they are localized in the plasma membrane (PM). However, more recent data from biochemical and cell biological assays show that, in Arabidopsis and maize, the majority of cytokinin receptors are localized in the endoplasmic reticulum (ER) and expose the cytokinin-binding CHASE domain to the ER lumen[51-53]. Consistent with this, endomembranes have higher cytokinin binding affinity than the PM. Transiently expressed fluorescent fusion proteins support the predominant localization to the ER for all three AHKs. Nevertheless, there is a minor but functional relevant fraction of the receptors at the PM[52]. The canonical model for cytokinin signaling has assumed these receptors sense extracellular cytokinins. The

finding that their location is predominantly in the ER suggests that active cytokinins must cross the plasma membrane and ER membrane in order to bind to the lumen localized CHASE domain. Purine permeases (PUP) have been suggested, in Arabidopsis cell culture and in yeast, to transport cytokinin and adenine into the cytosol[54, 55]. Genetic evidence for the role of PUPs in cytokinin *in planta* is still lacking due to the large number of *PUP* genes present in the Arabidopsis genome. Therefore, more studies are needed to understand the mechanisms by which cytokinin enters the cell and is transported into the ER lumen.

2.6 Non-receptor kinases also feed into two-component signal transduction

2.6.1 CKI1

As noted above, overexpression of CKI1 induced cytokinin-independent callus formation in cultured Arabidopsis cells[20]. However, the lack of a CHASE domain in CKI strongly suggests that this HK is not a cytokinin receptor per se. Recent studies have shown that CKI1 can feed into downstream two-component signal transduction via the phosphoproteins AHPs and the type-B ARRs[23]. The receiver domains of CKI1 can interact with AHP2, AHP3, and AHP5 in yeast and plant protoplasts[56]. The phenotype induced by overexpression of CKI1 is eliminated in the *ahp1,2,3,4,5* mutant, suggesting that *AHPs* act epistatically to *CKI1*[23]. Further, phenotypic analysis has shown that in high order *ahp* and type-B *arr* mutants, a subset of female gametophytes have similar phenotypes as those observed in *cki1* loss-of-function alleles, which supports a role for AHPs and type-B ARRs acting downstream of *CKI1*[47]. Expression of ARR1, one of the

type-B ARRs, under the control of *CKI1* promoter is able to partially rescue the *cki1* phenotype, further suggesting *ARR1* is epistatic to *CKI1*.

Despite the lack of cytokinin-binding CHASE domain, overexpression of CKI1 was found to partially rescue multiple phenotypes in a *wol* mutant, including the shortened primary root, defects in xylem development, and cytokinin insensitivity in shoot regeneration assay[23]. In addition, ectopic expression of cytokinin biosynthetic isopentenyltransferase *IPT8* under the control of the *CKI1* promoter is able to partially rescue *cki1* phenotype. The mechanisms by which elevated cytokinin levels complement the *cki1* phenotype is not clear, though likely involve increased activity of the downstream phosphorelay.

2.6.2 Other non-receptor histidine kinases

The role of AHK1 in plant growth and development is only obvious in a triple *ahk1 ahk2 ahk3* mutant background. Addition of *ahk1* to an *ahk2 ahk3* mutant significantly reduces the plant size and retards growth[24]. In addition, the *ahk1* mutant is more sensitive to drought stress, while *ahk2* and *ahk3* are more tolerant[24].This suggests that cytokinin receptors act in stress responses in a manner opposite to that of *AHK1.* Comprehensive coexpression analysis reveals that *AHK1* is coexpressed with a set of type-A ARRs (*ARR4, ARR5, ARR6, ARR8, ARR9*) under abiotic stress conditions and cytokinin treatment[57]. These results suggest a potential interaction between AHK1 and cytokinin signaling in abiotic responses, though further studies are needed to confirm this.

CKI2/AHK5 is a histidine kinase that lacks a transmembrane domain. The genetic role of *AHK5* in the cytokinin two-component phosphorelay is not clear. AHK5 interacts with multiple phosphotransfer proteins, except AHP4,[58] though the biological

significance of these interactions remains an open question. AHK5 is an active histidine kinase, but this activity is not dependent on cytokinin. Disruption of *AHK5* results in a normal shoot, and the roots displayed wild-type sensitivity to cytokinin, but were hypersensitive to abscisic acid and ethylene, suggesting a potential role as a negative regulator of these signaling pathways[59].

2.7 Are AHKs the only cytokinin receptors?

Cytokinins were long thought to be essential for plant growth and development as they regulated essential processes such as cell division and organogenesis. Surprisingly, three independent *ahk* triple mutants harboring non-overlapping T-DNA alleles are seedling viable, albeit quite stunted. One allelic combination has marginally reduced fertility while the other two mutants are completely male and female sterile[47]. Recent studies have revealed that even in the strongest *ahk* triple mutant, there is residual full-length *AHK3* transcript (~0.02% compared to wild type), indicating that none of these three triple mutants represent null alleles[47]. This raises the question as to whether or not cytokinin is essential for plant growth and development. One possibility is that it is not, notwithstanding the residual AHK3 transcript. A second possibility to explain the viability of the *ahk2 ahk3 ahk4* mutants is that the residual *AHK3* transcript, although insufficient for male and female gametophyte development, is sufficient to support some vegetative development. A final possibility is that there are additional cytokinin receptors beyond AHK2, AHK3 and AHK4. Several cytokinin-binding proteins have been isolated from various plant species including barley, maize, oat, and tobacco. However, the evidence linking these to a physiological function in cytokinin signaling is lacking. Another candidate for a novel cytokinin receptor is *CHARK*, a gene found in rice that

encodes a protein containing a CHASE domain at the N-terminus followed by a serine/threonine kinase domain[60]. The CHASE domain of CHARK is 49% to 67% identical to the cytokinin HK receptors in rice, maize, and *Arabidopsis*[60]. *CHARK* may be a cytokinin-binding element unique to rice or monocots, although the cytokinin-binding activity of the encoded product CHARK has not been verified. Further genetic analysis is needed to resolve this important question.

3. The phosphotransfer proteins in Arabidopsis

3.1 AHPs shuttle phosphate between AHKs and ARRs

Examination of the Arabidopsis genome reveals five genes encoding predicted proteins with high sequence similarity to histidine-containing phosphotransfer (Hpts) proteins from *E. coli* and yeast[28]. These Arabidopsis Hpts (AHPs) were shown to complements a loss-of-function mutation in YPD1 in yeast, which is a Hpt protein involved in osmosensing[28]. The AHPs were shown to rapidly transfer phosphoryl groups from their own conserved His residue to the Asp residue of two response regulators[61]. In addition to these functional AHPs, there is a sixth gene that lacks the conserved His residue that is the site of phosphorylation called AHP6 (see below)[15, 49]. The AHPs directly interact with both the upstream cytokinin receptors AHKs and the downstream type-A and type-B ARRs. The AHPs act to shuttle a phosphoryl group from the Asp residue of the receiver domains present in the cytokinin AHK receptors to the Asp residue present in the receiver domains of the ARRs, thus transducing the signal from the site of perception (the ER membrane) to the nucleus to regulate gene expression, and to the type-A ARRs, some of which are in the nucleus and some of which are cytoplasmic.

3.2 AHPs act downstream of AHKs and CKI1

Reverse genetic experiments provided direct evidence for the role of five *AHPs* as positive regulators in cytokinin signaling. Various combinations of T-DNA insertion alleles in the five *AHP* loci were analyzed. AHP1, AHP2, AHP3 and AHP5 were found to have overlapping roles as positive elements in cytokinin signaling using multiple different cytokinin response assays, including the induction of primary response genes[15].

The quintuple *ahp1ahp2-1 ahp3 ahp4 ahp5* mutant displayed phenotypes similar to *ahk2 ahk3 ahk4* triple receptor mutants, including inhibition of primary root growth and loss of metaxylem development. Interestingly, shoot development in the *ahp1ahp2-1 ahp3 ahp4 ahp5* mutant was not as severely affected compared to the *ahk2 ahk3 ahk4* mutant, which is likely due to the residual full-length *AHP2* transcript from the *ahp2-1* allele used. A quintuple *ahp1ahp2-2 ahp3 ahp4 ahp5* mutant that incorporates a null *ahp2-2* allele is severely delayed in true leaf formation and dies at the seedling stage, which has not been reported even in the strongest *ahk2-7 ahk3-3 cre1-12* triple receptor mutant. As noted above, the discrepancy in the phenotypic strength of these lines as compared to the null AHP mutant is likely due to the fact that none of the *ahk* triple mutant combinations represent complete receptor nulls (see 2.8). Alternatively, as the AHKs are likely not the sole upstream regulators of the AHPs (see 2.6.1), the stronger phenotype of the *ahp1ahp2-1 ahp3 ahp4 ahp5* mutant may reflect disruption of the *AHK*- and/or *CKI1*-dependent signaling pathways.

3.3 The subcellular localization of AHPs suggests a function in a bidirectional phosphorelay

Phospho-His and phospho-Asp residues are high energy molecules. As noted above (see 1.1), the free energy associated with the various phosphorylation reactions that occur in the phosphorelay is close to zero, which allows HP domains to act both as phosphodonors and phosphoreceivers, and so to shuttle a phosphoryl group between two or more receiver domains. That is, the high energy cytosolic phospho-AHP is capable of donating the phosphoryl group to both the type-A and type-B response regulators, or to the upstream AHKs[50, 62]. Early studies suggested that the AHP proteins moved into the

nucleus in response to cytokinin treatment, but a more quantitative analysis demonstrated that the AHPs are persistently nucleo-cytosolic and non-responsive to cytokinin or phosphorylation[62]. Further, the size of the AHP-GFP fusion proteins exceeds the exclusion limit of the nuclear pore, suggesting they are actively transported into and out of the nucleus[62]. Together, this suggests that the AHPs can mediate the phosphorelay from the membrane bound receptors to the mainly-nuclear localized response regulators. In addition, the flow of phosphate can proceed from the receptors to the ARRs in response to cytokinin activation of the AHK receptors, or from the ARRs back to the AHKs upon cessation of the cytokinin signal.

3.4 AHP6 is a negative element in cytokinin two-component signaling

AHP6 was isolated in a genetic screen as a suppressor of the determinate root phenotype of *wol*[49]. AHP6 lacks the conserved His residue that is the site of phosphorylation, and thus is not a functional Hpt protein and does not participate in phosphotransfer. AHP6 was shown to inhibit the phosphorelay *in vitro* from the His kinase domain of SLN1 to its fused receiver domain. SLN1 is a hybrid HK involved in the *Saccharomyces cerevisiae* osmosensing pathway. AHP6 also inhibits the phosphotransfer from phosphorylated AHP1 to ARR1 in vitro, which suggests that AHP6 acts as an inhibitor of phosphotransfer, likely through a dominant negative mechanism[49]. The role of AHP6 as a negative regulator of cytokinin signaling is also supported by functional analysis in vivo. The *ahp6-1* loss-of-function mutant has elevated basal expression of the cytokinin primary response gene *ARR15* and is hypersensitive to the effects of exogenous cytokinin on adventitious root formation and protoxylem

differentiation. Interestingly, cytokinin also negatively regulates *AHP6* expression, forming a mutual-regulatory circuit in regulating root development[49].

3.5 Nitric oxide regulates phosphotransfer proteins through S-nitrosylation

In cells, nitric oxide (NO) can directly modify the cysteine thiol of a protein as a redox-based posttranslational modification mechanism, which is known as *S*-nitrosylation. It has been suggested that NO can regulate cytokinin signaling[63]. The expression of the cytokinin reporter TCS-GFP as well as multiple cytokinin primary response genes are reduced in the *nox1* and *gsnor1*-3 mutants, which have elevated levels of endogenous NO[64]. These NO-overexpressors are less sensitive to cytokinin in root and hypocotyl elongation, root apical meristem size, and shoot regeneration assays, as well as in the induction of cytokinin primary response genes, suggesting that NO negatively regulates cytokinin signaling. This is likely the result of the *S*-nitrosylation of the AHP proteins on the conserved cysteine residue, which reduces their phosphorylation level and hence functionality. AHP1^{C115S}, a non-nitrosylatable mutant protein, was resistant to the inhibitory effect of NO donors and was able to complement the cytokinin-insensitivity of high order *ahp* mutants. In contrast, an AHP1^{C115W} mutant protein that mimics the *S*-nitrosylation modification displayed reduced phosphorylation even in the absence of an NO donor and did not complement *ahp* mutants. *In vitro S*-nitrosylation of AHP1 repressed its phosphotransfer activity to ARR1, a type-B ARR, demonstrating that *S*-nitrosylation reduced the activity of the AHP. This represents a novel mechanism by which environmental stimuli intertwine with endogenous signal transduction pathways. Furthermore, *S*-nitrosylation by NO may not be exclusive to AHPs as putative *S*-

nitrosylated cysteine residues are also present in AHKs and ARRs, although experimental data are needed to verify the hypothesis.

4. Response regulators in Arabidopsis

Response regulators were first implicated in cytokinin signaling when they were identified as early cytokinin response genes in Arabidopsis and maize[65, 66]. Response regulators contain a conserved receiver domain with a conserved Asp residue that is the site of phosphorylation, which generally regulates their output activities. The Arabidopsis response regulators (ARRs) fall into four major classes based on their domain structure and the similarity of the amino acid sequences of the receiver domains[67]: type-A, type-B, type-C ARRs, and the *Arabidopsis* pseudoresponse regulators (APRRs) (Figure 1.3). The ten type-A ARRs are primary transcriptional targets of cytokinin signaling and contain short C-terminal extensions following the conserved receiver domain. The eleven type-B ARRs contain a receiver domain followed by an output domain that has DNA-binding activity. The two type-C ARRs are structurally similar to type-A ARRs as they contain only the receiver domains, however, they are not transcriptionally induced by cytokinin. The role of type-C ARRs in cytokinin signaling remains unclear, although overexpression of one of them confers reduced cytokinin sensitivity[68]. The APRRs contain complete receiver domains but lack the conserved Asp residue for phosphorylation, although many appear to be phosphorylated. A subset of the APRRs play a role in modulating circadian rhythms and their phosphorylation status oscillates throughout the day.

Biochemical and genetic analyses have demonstrated that bacterial response regulators (RRs) function as phosphorylation-mediated switches[10, 11]. Phosphorylation of the highly conserved Asp residue in the receiver domain inactivates the protein in some RRs and activates it in others. In agreement with a regulatory role of phosphorylation, phosphorylation of the conserved Asp residue of type-A ARRs is critical for proper

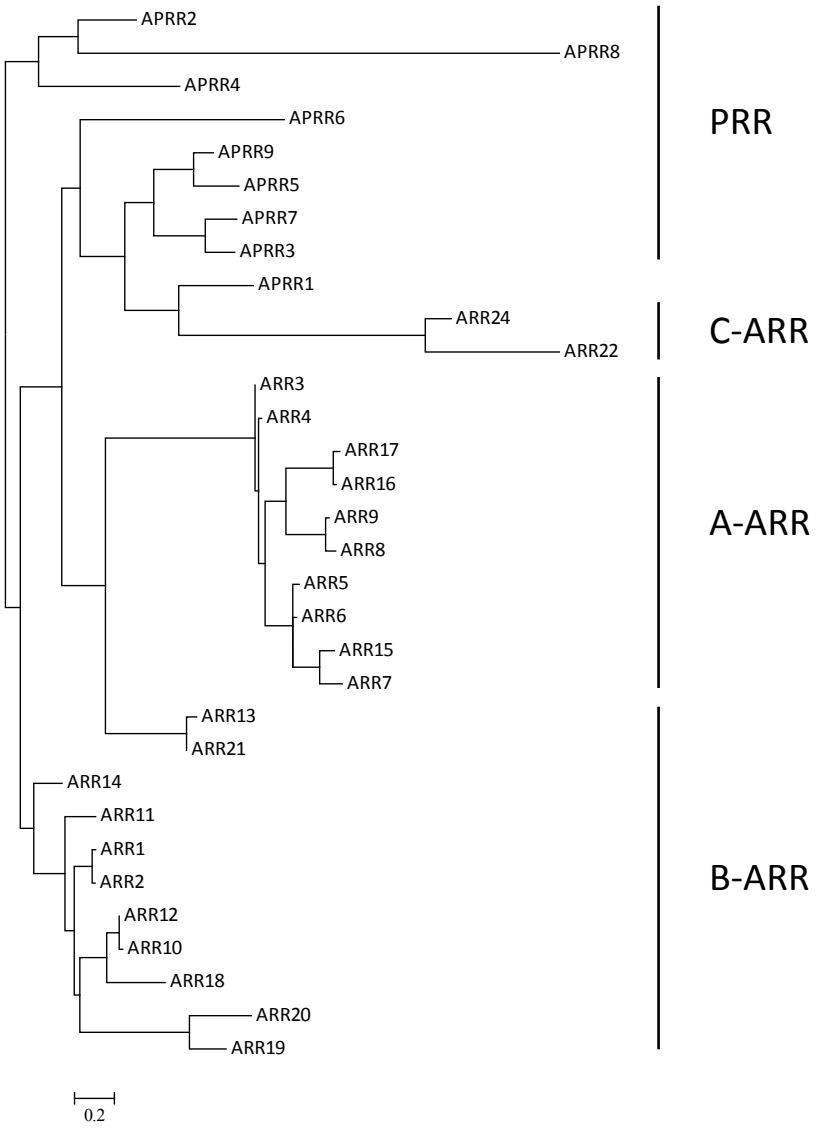

Figure 1.3. Phylogenetic relationship of the Arabidopsis response regulators (ARR). The amino acid sequences of the receiver domains were aligned using MUSCLE. Gaped regions that were poorly conserved were first removed from each alignment. The phylogenetic trees were generated with MEGA5.1. PRR, pseudo-response regulator. Scale bar indicates substitutions per site.

function. A subset of type-A ARR proteins is stabilized by cytokinin via phosphorylation of the Asp residue[33] (see 5.2). Similarly, mutation of the phospho-receiving Asp to phospho-insensitive Asn in type-B ARRs abolished its activity to transactivate type-A *ARR6*[69] (see 6.2). Together, these data suggest that phosphorylation is a common strategy utilized by the ARRs to modulate their output response.

5. Type-A response regulators

5.1 Type-A ARRs are primary response genes in cytokinin signaling

The Arabidopsis type-A ARRs are a family of ten genes that fall into five distinct pairs which, based on the analysis of the locations of the genes within the genome, likely arose from the most recent genome duplication event in the evolution of Arabidopsis[70]. The amino acid sequences of type-A ARRs are somewhat similar to that of the bacterial single-domain response regulator CheY, which is comprised of only a receiver domain[71]. The transcripts of type-A ARRs are rapidly and specifically induced by cytokinin, and this induction is insensitive to inhibition of protein synthesis, and they are thus primary response genes[65]. The cytokinin receptor AHKs, the AHPs, and type-B ARRs are required for the rapid induction of type-A *ARRs*, indicating that their induction requires an intact phosphorelay.

5.2 Type-A response regulators negatively regulates cytokinin signaling

Overexpression of type-A ARRs results in repression of *ARR6-LUC* activity, leading to the hypothesis that they are negative regulators of cytokinin response[72]. Consistent with this hypothesis, analysis of loss-of-function type-A *arr* mutants shows that at least eight of the ten type-A ARRs are negative regulators of cytokinin signaling in multiple cytokinin response assays[35, 73]. Single loss-of-function type-A *arr* mutants show no significant difference from the wild type in the response to exogenous cytokinin treatment in root elongation assays, while double and higher order multiple mutations in type-A *ARRs* show increasing hypersensitivity to cytokinin[35]. In monocots and dicots, disruption of type-A response regulators has been reported to cause enlarged shoot apical

meristem, presumably through increased cytokinin signaling output [74, 75]. Consistently, overexpression of the rice type-A response regulator *OsRR6* leads to repression of shoot regeneration in tissue culture and an aberrant dwarf phenotype in transgenic plants[76].

Despite the well-described role of type-A ARRs as negative regulators in cytokinin signaling, mutation of eight out of ten type-A *ARR*s does not cause dramatic morphological phenotypes. It is possible that additional negative feedback loops might compensate for the cytokinin hypersensitivity in this octuple mutant by decreasing cytokinin levels, as several cytokinin degrading oxidases (*CKX*) are induced upon cytokinin treatment[77]. The alternative, not mutually exclusive possibility is that type-A ARRs might functionally overlap with other signaling elements to regulate plant growth and development. Thus, the octuple mutations in type-A *ARR*s attenuate but not abolish the output responses.

5.3 Phosphorylation of receiver domain is essential for type-A ARR function

The type-A ARR proteins exhibit *in vitro* activity typical of bacterial receivers as they can be phosphorylated on the conserved Asp residue using a phospho-HP domain as a phosphodonor[71]. Further, a subset of type-A ARR proteins is stabilized by cytokinin via phosphorylation of the Asp residue[33]. Type-A ARR5^{D87A}, a non-phosphorylatable mutant protein, fails to complement the cytokinin hypersensitivity of a *arr3 arr4 arr5 arr6* mutant, indicating that phosphorylation of the conserved Asp residue is required for its proper function. In contrast, ARR5^{D87E}, a phosphomimic version of the protein, can partially rescue the cytokinin hypersensitivity of *arr3 arr4 arr5 arr6* mutant. As this ARR5^{D87E} protein cannot be phosphorylated in the phosphorelay, it suggests that the type-A ARRs act via phospho-dependent interactions with other proteins to negatively

feedback cytokinin signaling. Such phospho-dependent interactions are a common strategy used by bacterial single domain response regulators that lack a distinguishable output domain. At least two independent yeast two-hybrid screenings have revealed that type-A ARRs interact with candidate proteins in addition to the two-component elements[50, 78], which potentially may include such target proteins.

5.4 Other signals cross-talk with cytokinin signaling via regulating type-A ARRs

In addition to cytokinin, type-A *ARRs* are regulated by other inputs, presumably as a means to modulate cytokinin signaling. For example, WUSCHEL (WUS), a homeodomain transcription factor, specifically represses the expression of *ARR5, ARR6, ARR7,* and *ARR15* in shoot apical meristem to reduce their negative attenuation on cytokinin signaling, thus maintaining stem cell fate[79]. Overexpression of the phosphomimic form of ARR7 (35S::ARR7^{D85E}) results in arrested shoot apical meristem that is similar to the phenotype observed in a *wus* mutant[79]. Similarly, auxin, another plant hormone, also cross talks with cytokinin signaling by regulating two type-A *ARRs*, *ARR7* and *ARR15*. Whereas cytokinin induces *ARR7* and *ARR15* in shoot apical meristem, auxin represses the expression of these two type-A *ARRs* via AUXIN RESPONSE FACTOR 5 (ARF5)[80]. Mutations of the ARF-binding site in the promoter region of *ARR15* result in ectopic expression of *ARR15*. Further, mutations of *ARR7* and *ARR15* in *arf5* background could partially rescue *arf5* phenotype. These experiments provide examples in which endogenous signals cross talk with cytokinin signaling via manipulation of the expression of type-A *ARRs*.

6. Type-B response regulators

The type-B *ARR* family is comprised of eleven transcription factors that belong to three sub-families based on the amino acid sequences of their receiver domains[81, 82]. The subfamily I type-B *ARRs* have been the most thoroughly studied as they seem to take the predominant role in cytokinin-mediated outputs. The type-B ARRs contain a receiver domain at the N-terminus followed by a conserved plant-specific myb-related GARP DNA-binding domain.

6.1 Type-B ARRs are positive regulators in cytokinin signaling

Type-B *ARRs* play overlapping roles in cytokinin signaling and plant development[16, 83]. Analysis of loss-of-function subfamily I type-B *arr* mutants has enable the elucidation of the endogenous functions of these genes. Single type-B *arr* mutants show only slight cytokinin insensitivity in seedlings, while multiple mutations in subfamily I *ARR1, ARR10, ARR12* confer additive cytokinin insensitivity in primary root growth, hypocotyl elongation, and shoot formation in tissue culture. These results suggest that subfamily I type-B ARRs acts as redundant positive elements in cytokinin signaling. Mutations in type-B *ARRs* also compromise the induction of cytokinin primary response genes[16, 32], indicating that they mediate the immediate transcriptional response to cytokinin. The role of subfamily II and III type-B *ARRs* is not clear as our understanding has been based on overexpression experiments[81], which may not reflect their endogenous functions. Subsequent analysis of loss-of-function mutations in subfamily II and III *ARR* showed that there is no obvious phenotype in flower development, silique development, and seed size, even though they are primarily detected in reproductive organs[84].

Subfamily I type-B *ARR*s are broadly detectable by RT-PCR in vegetative and reproductive tissues, with particular high expression in regions where phenotypes have been reported in loss-of-function mutants, including young developing leaves and meristems[82]. On the contrary, subfamily II and III type-B *ARR*s are barely detected by RT-PCR, but their expression can be seen using promoter fusion to GUS reporter protein[82]. This may reflect the rapid RNA turnover of type II and III type-B *ARRs* or very specific expression domains. The restricted expression profile suggests that subfamily II and III type-B ARRs may function in specific developmental processes. Consistent with this, a recent report shows that *ARR20*, which belongs to subfamily III type-B *ARR*, is a positive regulator of cytokinin signaling in regulating pavement cell morphogenesis. Similar to the cytokinin double receptor mutant *ahk3-3 cre1-12*, the single *arr20* mutant displays a modest enhancement pavement cell interdigitation. This phenotype is not observed even in *arr1-3 arr10-5 arr12-1* mutant, which shows almost complete insensitivity to exogenous cytokinin in multiple other assays[16].

6.2 The receiver domains of type-B ARRs have inhibitory effect on DNA-binding domains

Cytokinin signaling influx activates type-B *ARRs* via phosphorylation at the conserved Asp residue in the receiver domain. The N-terminal receiver domains of type-B ARRs interact with the AHP phosphotransfer proteins as shown by *in vitro* and *in vivo* experiments[50, 61]. Mutation of phospho-receiving Asp in ARR2 to phospho-insensitive Asn abolishes the ability of this protein to be phosphorylated via phosphotransfer activity[69]. This $ARR2^{D80N}$ mutant protein lacks the ability to transactivate type-A *ARR6*

promoter in response to cytokinin[69], indicating that phosphorylation of the receiver domain is required for type-B ARR function.

Transgenic plants overexpressing full-length type-B ARRs display wild-type morphology; however, deletion of the receiver domain leads to constitutive activation of this transcription factor[31]. Overexpressing several N-terminal truncated type-B ARRs (ARR2, ARR11, ARR18, ARR19, ARR20, and ARR21), which contain only the C-terminal DNA-binding domain, results in their constitutive activation and pleiotropic phenotypes[31, 81, 85, 86]. These results indicate potential novel roles of type-B ARRs in plant growth and development, although the ectopic and overexpression of these activated type-B ARRs may not faithfully reflect their endogenous functions.

One simple model for the autoinhibitory effect of the type B N-terminal domains is that the receiver domain blocks, through steric hindrance or direct interaction, the activity of the DNA-binding domain. This is similar to the mechanism in many bacterial RRs. Phosphorylation of the receiver domain triggers conformational change that de-represses the inhibitory effect on the DNA-binding domain. However, the precise mechanism underlying the inhibitory regulation has not been experimentally validated.

6.3 Type-B ARRs function as transcription factors

In vitro assays have shown that the GARP motifs of at least four type-B ARRs bind to DNA in a sequence-specific manner; the sequences that ARR1/ARR2, ARR10, and ARR11 preferentially bind are GAT(T/C), AGATT, and GGATT respectively[30, 87, 88]. The core sequences are too short to specify direct targets because they appear too frequently in DNA genomes. A study analyzing the *cis*-elements of the target genes induced by ARR1 extended the ARR1-binding sequences to AAGAT(C/T),

GAT(C/T)TT, and AAGAT(C/T)TT, which were found to be tandemly enriched in the target promoters[89]. A meta-analysis of cytokinin regulated genes further showed that the ARR1 consensus binding site AAGAT(C/T)TT was substantially overrepresented in the regulatory regions of robustly cytokinin-responsive genes[90]. Single mutations of the ARR1 binding sites AGATT to ACATT in the promoter region of type-A *ARR15* was sufficient to eliminate its cytokinin responsiveness *in planta*[91], demonstrating that this regulatory element is required for cytokinin-responsiveness.

Despite sharing a core binding sequence (GAT), ARR11, unlike other type-B ARRs, does not bind to the cytokinin-responsive element AGATT[88], suggesting non-overlapping targets and hence function of type-B ARRs. Indeed, seven of the type-B ARRs (ARR11, ARR14, and ARR18 of subfamily I; ARR13 of subfamily II; ARR19 and ARR20 of subfamily III) under the control of the *ARR1* native promoter cannot complement the root growth phenotype of *arr1 arr12*, suggesting these proteins may have distinct functions.

6.4 The direct targets of type-B ARRs

Type-B ARRs-regulated genes have been identified by microarray analyses of wild type and multiple type-B *arr* mutants in response to cytokinin treatment[16, 77, 92]. These studies indicate that type-B ARRs are essential for nearly all cytokinin-regulated gene expression. Further, the endogenous levels of many genes not identified as cytokinin-responsive are differentially expressed in the type-B *arr* mutants compared with wild-type seedlings[16]. The different experimental conditions and combinations of high order mutants used in these experiments make it difficult to decipher the target specificity for individual type-B ARRs. Transgenic plants expressing ARR1ΔDDK–GR,

a glucocorticoid (DEX) inducible chimeric transcription factor fused to ARR1 lacking its receiver domain, have been used to identify the genes rapidly activated by ARR1ΔDDK upon DEX treatment. These genes potentially represent direct targets of ARR1 and included cytokinin oxidase, cytokinin hydroxylase, putative disease resistance response proteins, and IAA3/SHY2[89]. Chromatin immunoprecipitation and gel mobility shift analyses confirmed that ARR1 directly associates with the promoter region of *IAA3/SHY2*.[93]

7. Cytokinin response factors

Microarray results from different labs have shown that *CYTOKININ RESPONSE FACTORS (CRFs)*, a subset of AP2/ERF superfamily of transcription factors, are transcriptionally up-regulated by cytokinin[77, 89, 94]. CRF includes a group of six core members that contain a AP2/ERF domain and a CRF motif and represents a large clade of AP2/ERF genes in land plant species[95]. In Arabidopsis seedlings, *CRF2* and *CRF5* show rapid (<30 minutes) induction upon cytokinin treatment while *CRF6* induction does not peak until later (>8 hours)[96], indicating different kinetics in their response to cytokinin. Despite the name, not every *CRF* has been found to be cytokinin-responsive. Similarly, cytokinin up-regulates only a subset of the tomato *CRFs*, which also display distinct kinetics in response to cytokinin [97].

The rapid induction of *CRFs* by cytokinin is compromised in type-B *arr* mutants, leading to the hypothesis that CRFs regulate part of the transcription network downstream of type-B ARRs[96]. Indeed, multiple cytokinin-regulated genes exhibit reduced responsiveness in the loss-of-function *crf* mutants, suggesting CRFs are responsible for a subset of cytokinin responses[96]. The induction of type-A *ARRs,* however, is not dependent on CRFs as they retain the wild-type level of induction in *crf* mutants. Overall, *CRFs* are hypothesized to form a side branch of the cytokinin response downstream of type-B *ARRs*.

Future directions

Twelve years after the initial discovery of a cytokinin receptor, remarkable progress has been made in our understanding of cytokinin signaling. The two-component elements modulate, via sequential phosphorelay events, cytokinin signal transduction. Loss-of-function and gain-of-function mutants have, at least in part, overcome the gene redundancy in this system and revealed the roles of cytokinin signaling in plant growth and development. Meanwhile, the elucidation of the pathway has raised many outstanding questions from perception of cytokinins to determine the output responses. For example, the subcellular localization of cytokinin receptors in ER raises the question as to how cytokinins are transported across plasma membrane and ER membrane to reach the receptors. How is specificity in outputs achieved in the pathway, and how is this pathway integrated with other signals to achieve appropriate growth and development? How does the non-receptor histidine kinase CKI1 sense stimuli and feed into cytokinin signaling? How does elevated cytokinin level partially complement *cki1* phenotype?

The phosphotransfer proteins that shuttle the phosphoryl group between the receptors and the response regulators provide another layer of regulation in this system. At the cellular level, studies are needed understand the mechanism underlying the transport of AHPs in and out of the nucleus, which allows the phospho-AHPs to maintain the appropriate phosphorylation status of the system elements. As for the ARRs, the mechanism by which type-A ARRs negatively feedback on cytokinin signaling is not yet clear. Likewise, how phosphorylation de-represses the inhibitory effect of the receiver domain on the DNA-binding domain of type-B ARR proteins has not been explored.

Disruption of cytokinin signal transduction, at multiple branch points, implicates the requirement of cytokinin in many aspects of plant growth and development. The next step is to illuminate the mechanisms by which cytokinin signaling elicits the biological outputs and intertwines with endogenous and environmental stimuli.

CHAPTER 2: Cytokinin-Dependent Specification of the Functional Megaspore in the *Arabidopsis* Female Gametophyte

Preface

The following chapter was published in The Plant Journal volume 73 and my contribution to the publication was that I designed the experiments and wrote the manuscript. This work was the first example that specified the role of cytokinin in female gametophyte development.

Summary

The life cycle of higher plants alternates between the diploid sporophytic and the haploid gametophytic phases. In angiosperms, male and female gametophytes develop within the sporophyte. During the female gametophyte (FG) development, a single archesporial cell enlarges and differentiates into a megaspore mother cell, which then undergoes meiosis to give rise to four megaspores. In most species of higher plants, including *Arabidopsis thaliana*, the megaspore closest to the chalaza develops into the functional megaspore (FM) and the remaining three megaspores degenerate. Here, we examined the role of cytokinin signaling in FG development. We characterized the FG phenotype in three triple mutants harboring non-overlapping T-DNA insertions in cytokinin AHK receptors. We demonstrate that even the strongest line is not a complete null for the cytokinin receptors. Only the strongest line displayed a near fully penetrant disruption of FG development, and the weakest triple *ahk* mutant had only a modest FG

phenotype. This suggests that cytokinin signaling is essential for FG development, but that only a low threshold of signaling activity is required for this function. Further, we demonstrate that there is elevated cytokinin signaling localized in the chalaza of the ovule, which is contributed by the asymmetric localization of cytokinin biosynthetic machinery and receptors. We show that FM-specific marker is absent in the multiple *ahk* ovules, suggesting that disruption of cytokinin signaling elements in Arabidopsis blocks the FM specification. Together, this study reveals a chalazal-localized, sporophytic cytokinin signal that plays an important role in FM specification in the FG development.

Introduction

In angiosperms, both male and female gametophytes are embedded in the sporophytic tissues and the haploid and diploid generations coexist in a single organ. The surrounding sporophyte supports meiosis and subsequent gametophyte development in anthers and ovules, the male and female organs. The female gametophyte (embryo sac), unlike pollen, remains physically connected to the maternal tissues before and after fertilization. Ovules arise from the placenta within the carpels in flowering plants and are the site of gametogenesis, which can be divided into megasporogenesis and megagametogenesis[98-101]. Ovules have three structural domains along the proximal-distal axis: the funiculus, the chalaza, and the nucellus (Figure 2.1a). During megasporogenesis, a hypodermal archesporial cell enlarges and differentiates into megaspore mother cell (MMC) that undergoes meiosis and gives rise to a tetrad of four megaspores (Figure 2.1b). In higher plants with Polygonum-type embryo sac development, including Arabidopsis, rice, and maize, the megaspore closest to the chalaza develops into the functional megaspore (FM), while the other three degenerate (Figure 2.1c). During the subsequent megagametogenesis, the FM undergoes three rounds of mitosis, resulting in an eight nuclei syncytium that partitions into four cell types after cellularization: two synergid cells that are important for pollen tube guidance; the egg and central cells that receive sperm cells for double fertilization; and three antipodal cells which degenerate by the last developmental stage of female gametophyte (FG7) (Figure 2.1d)[99, 102].

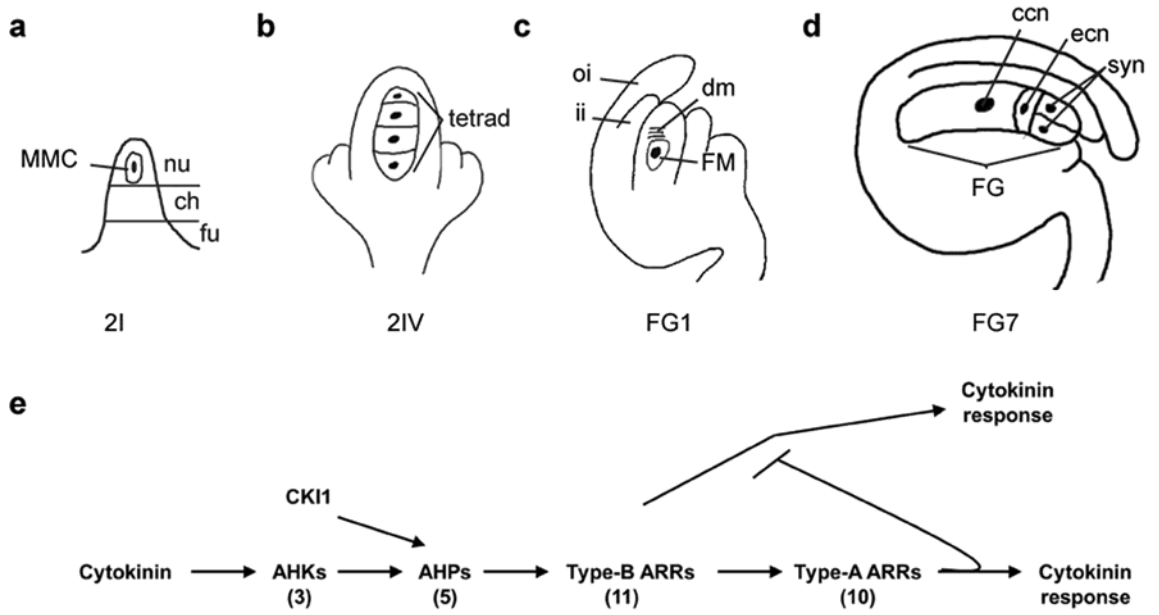

Figure 2.1. Two-component elements and female gametophyte development. (a-d) Schematic depiction of wild-type female gametophyte development in Arabidopsis. Abbreviations: MMC, megaspore mother cell; nu, nucellus; ch, chalaza; fu, funiculus; oi, outer integuments, ii, inner integuments; FM, functional megaspore; dm, degenerating megaspores; ccn, central cell nucleus; ecn, egg cell nucleus; syn, synergid cell nuclei. (e) Model of cytokinin response pathway in Arabidopsis. Cytokinin binds to the AHK receptors, which initiate a phosphorelay through AHPs and ultimately result in the phosphorylation of type-B ARRs. The activated type-B ARRs elevate the transcription of the type-A ARRs, which in turn act to negatively regulate the pathway. CKI1, which acts gametophytically to regulate FG development after FG4, also acts through the AHPs. The number of genes in each family in Arabidopsis is shown in parentheses below each element.

Given the proximal connection between the sporophytic tissues and gametophytic cells, it is anticipated that cellular communication is important during the developmental processes[103, 104]. A sporophytic siRNA pathway involving ARGONAUTE9 (AGO9) is crucial to specify cell fate in the *Arabidopsis* ovule[105]. In *ago9* plants, more than one sub-epidermal cell enlarges and contains a conspicuous nucleus in the ovule[105]. Recent studies have begun to reveal the interactive role of hormone signaling between two generations[103, 106-108]. The cell fate specification during syncytial development depends on a micropylar auxin gradient correlated with local auxin biosynthesis[107]. Mutations in the

cytokinin receptors disrupt female gametogenesis through a sporophytic effect[13, 106], yet the underlying mechanisms remain to be elucidated.

Cytokinin is important in a wide array of developmental processes. Cytokinin perception in plants is similar to bacterial two-component phosphorelay signal transduction systems (TCS). In Arabidopsis, there are three cytokinin receptors (Arabidopsis Histidine Kinases 2, [AHK2], AHK3, and AHK4/CRE1/WOL) that autophosphorylate in response to the binding of cytokinin[18, 26]. AHKs then relay this phosphoryl group to the Arabidopsis histidine phosphotransfer proteins (AHPs), which in turn transfer the phosphoryl group to the Arabidopsis response regulators (ARRs) (Figure 2.1e)[15, 28]. CKI1, which encodes a histidine kinase that lacks a cytokinin-binding domain, can also feed into downstream TCS signaling[20, 72] and acts in the female gametophyte to regulates its development[23, 109, 110]. The ARRs fall into four classes based on phylogenetic analysis and domain structure: type-A ARRs, type-B ARRs, type-C ARRs, and the Arabidopsis pseudoresponse regulators (APRRs). The eleven type-B ARRs are positive elements in the primary cytokinin signal transduction network. The ten type-A ARRs are rapidly transcriptionally up-regulated in response to cytokinin via direct activation by the type-B ARRs and act to negatively regulate cytokinin signaling[31, 34, 35, 65, 71, 72].

In *Arabidopsis* and rice, mutants defective in biosynthesis and perception of cytokinin displayed reduced female fertility, suggesting that cytokinin has a conserved role in regulating ovule development[13, 18, 106, 111, 112]. However, a triple receptor mutant that showed cytokinin deficiency in multiple bioassays was able to form seeds[14], raising the possibility that the female-sterile phenotype was conditional. Here, we examined the role of cytokinin in female gametogenesis using three different *ahk* triple mutants, high

order *ahp* and type-B *arr* mutants. We demonstrate that sporophytic cytokinin signaling is essential for the female gametophyte development. In addition, we demonstrate that FM specification depends on cytokinin signaling in the surrounding sporophyte, which is contributed at least in part by the chalaza-enriched expression of the cytokinin receptors and of *IPT1*, which encodes an enzyme involved in cytokinin biosynthesis. These results provide evidence for a cytokinin-dependent pathway involved in the communication between the sporophytic and gametophytic tissues during female gametophyte development.

Results

Loss of cytokinin receptors results in female gametophytic lethality

Previous studies suggest that disruption of the three cytokinin histidine kinase receptors (AHKs) affected viability of the female gametophyte, though the effect appeared to vary depending on which *ahk* alleles were examined[13, 14, 18]. The *ahk2-5 ahk3-7 cre1-2* line was reported to form some, albeit few seeds under favorable conditions[14]. In contrast, three other allele combinations (*ahk2-1 ahk3-1 ahk4-1*, *ahk2-2tk ahk3-3 cre1-12*, *ahk2-7 ahk3-3 cre1-12*) failed to produce any seeds[13, 18, 106]. To determine if the differences in female sterility reported for these mutants results from different growth conditions or if it reflects the specific allele combinations used in each study, we examined the three triple mutants that have the least overlap in *ahk* alleles (Figure 2.2a). Grown simultaneously under identical conditions, the *ahk2-5 ahk3-7 cre1-2* line was capable of producing a few seeds, while the other two allele combinations failed to generate any seeds. We examined the morphology of the female gametophyte at the last developmental stage (FG7; Figure 2.1d) in each of these allele combinations. There were substantially fewer ovules per gynoecium in the *ahk2-5 ahk3-7 cre1-2* line (13.2±1.1, $n = 76$) as compared to the wild type (58.3±5.1, $n = 260$). Similarly, the numbers of ovules in each gynoecium were also severely reduced in *ahk2-1 ahk3-1 ahk4-1* (12.5±3.7, $n = 50$) and *ahk2-7 ahk3-3 cre1-12* (14.2±0.4, $n = 108$), consistent with previous studies linking cytokinin to the placenta activity and thus the number of ovules per gynoecium[113].

While there were fewer ovules in the *ahk2-5 ahk3-7 cre1-2* mutant, most (89%, $n = 76$) were morphologically indistinguishable from wild-type ovules (Figure 2.2b). In contrast,

half of the ovules in *ahk2-1 ahk3-1 ahk4-1* (51%, *n* = 50) and most in the *ahk2-7 ahk3-3 cre1-12* (97.1%, *n* = 108) mutant lacked a differentiated embryo sac (Figure 2.2b), consistent with previous observations of *ahk2-2tk ahk3-3 cre1-12* ovules[106]. Significantly, we observed female gametophytic lethality in two independent triple *ahk* mutants, each composed of different *ahk* alleles, demonstrating that the mutant phenotype is the result of loss of the cytokinin receptors.

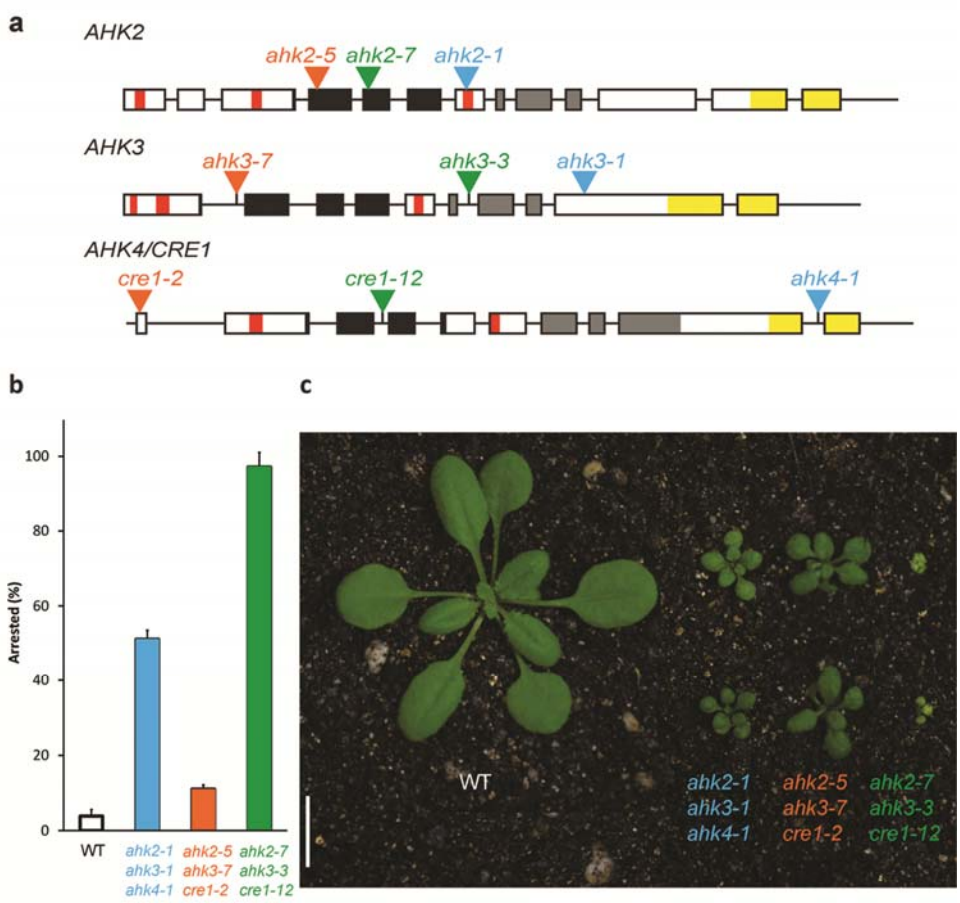

Figure 2.2. Phenotypic analysis of different *ahk* triple mutants. (a) Cartoon representation of the three *AHK* genes with the positions of the various T-DNA insertions present in three multiple mutants used in this study. Boxes represent exons and triangles represent the sites of integration of the T-DNA in each line. The three *ahk* alleles that compose each triple mutant are coded with the same color. Black boxes, CHASE domains; gray boxes, histidine kinase domains; yellow boxes, receiver domains; red bars, transmembrane domains. (b) Percentage of arrested female gametophytes in wild type and various *ahk* triple mutants (*n*>50 from at least three plants for each mutant line). (c) Aerial phenotype of four-week-old wild type and various *ahk* triple mutants. Scale bar = 1 cm.

We examined the phenotypes of these three *ahk* triple mutants at the vegetative stage to determine if the strengths of the phenotypes at this stage matched the relative strengths of the ovule phenotypes. Four-week-old *ahk2-5 ahk3-7 cre1-2* mutant rosettes were substantially smaller than wild type, yet relatively larger than the two other *ahk* triple mutants examined (Figure 2.2c), consistent with the relative strengths of the ovule phenotypes. This data suggest that the threshold of AHK function necessary to support gametogenesis is extremely low as *ahk2-5 ahk3-7 cre1*-2 is strongly insensitive to cytokinin in a number of response assays[14], yet has relatively normal female gametes. For further analysis of the role of cytokinin in female gametophyte development, we used the *ahk2-7 ahk3-3 cre1-12* line, which consistently has the highest percentage of arrested gametophytes.

The *ahk2-7 ahk3-3 cre1-12* line displayed the strongest effect both on the morphology of the rosette and on FG development, and thus likely represents the line closest to a cytokinin receptor null. However, even for this line, it has been suggested that there could be residual full-length transcript from the *ahk3-3* allele[18]. To explore this, we performed qRT-PCR using gene-specific primers spanning the T-DNA insertion sites for all three *AHK* genes in *ahk2-7 ahk3-3 cre1-12*. *AHK4/CRE1* was not detected even with saturating PCR cycles, while *AHK2* and *AHK3* were detected at severely reduced levels (~0.02% and ~0.8% respectively compared to the wild type (Figure 2.3b). Sequence analysis revealed that the splice junction spanning the intron containing the T-DNA insertion in *ahk3-3* was correctly spliced in this residual transcript. Thus, none of the published AHK triple mutants represent a complete disruption of cytokinin receptors,

leaving open the possibility that cytokinin may be essential for sporophytic plant development.

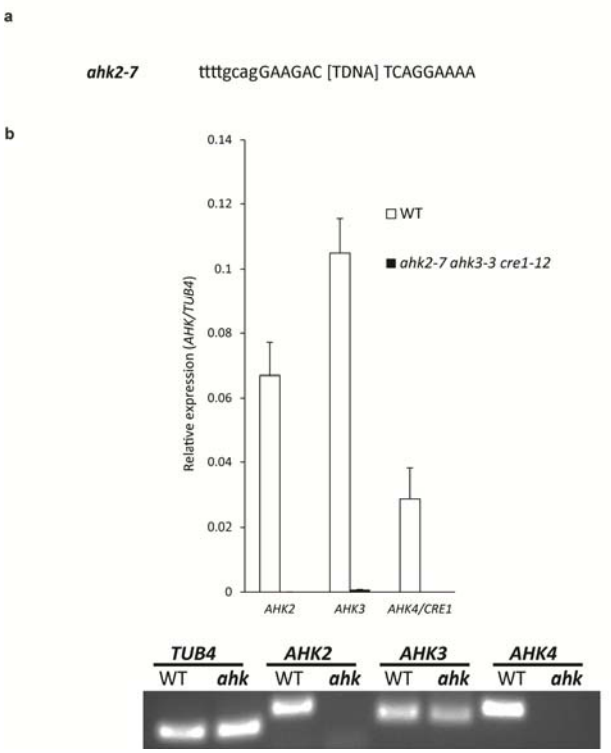

Figure 2.3. Expression of *AHK* genes in *ahk2-7 ahk3-3 cre1-12*. (a) Sequence of the T-DNA insertion junction in *AHK2* locus. The position of the T-DNA insertion in the sequence is indicated. (b) (top) qRT-PCR analysis of *AHK* gene expression in the *ahk2-7 ahk3-3 cre1-12* mutant. (bottom) Gel electrophoresis analysis of the qRT-PCR products. The level of *AHK2* was severely reduced relative to the WT (<0.02% compared to wild type) and was not visible on the gel. *TUB4* was used as in internal control.

Impaired cytokinin signaling results in incomplete megasporogenesis

We analyzed the female gametophyte lethality of the double and triple *ahk* mutants after allowing megagametogenesis to progress to FG7. The double mutants (*ahk2-7 ahk3-3, ahk3-3 cre1-12,* and *ahk2-7 cre1-12* were indistinguishable from the wild type (n >207 ovules observed for each mutant) (Figure 2.4a). Female gametophyte development did not differ from wild type in the *ahk2-7 ahk3-3/AHK3 cre1-12* line (one copy of *AHK3* remaining) (n = 304), yet there was a slight increase in the frequency of arrested ovules in *ahk2-7/AHK2 ahk3-3 cre1-12* (13.3%, n = 352) and *ahk2-7 ahk3-3 cre1-12/CRE1* (7.2%, n = 270) (Figure 2.4b). In contrast, in the *ahk2-7 ahk3-3 cre1-12*

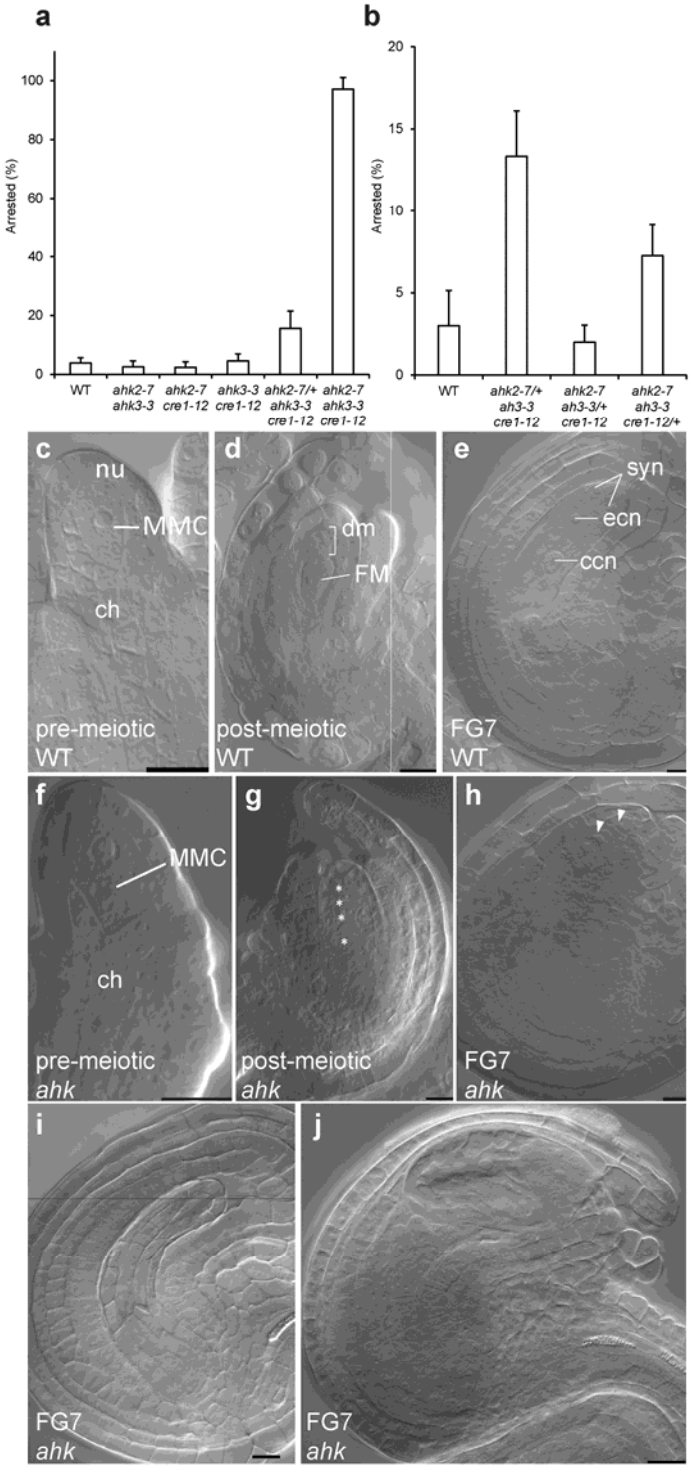

Figure 2.4. Characterization of the ovule phenotypes in the *ahk2-7 ahk3-3 cre1-12* mutant. (a) Analysis of female gametophyte development at FG7 in multiple *ahk* mutants (*n* >250 per line). (b) Analysis of female gametophyte development at FG7 in double homozygous single heterozygous *ahk* mutant**.** The frequency of arrested female gametophyte in the *ahk2-7 ahk3-3/AHK3 cre1-12* (*n* =304) line did not differ from wild type. However, there is an increase in the *ahk2-7/AHK2 ahk3-3 cre1-12* (*n* =352) and *ahk2-7 ahk3-3 cre1-12/CRE1-12* (*n* =270) mutants. (c-j) Wild-type ovule development. (c) The ovule, shortly after initiation, consists of nucellus (nu), chalaza (ch), and a funiculus (fu). A sub-epidermal cell enlarges and becomes megaspore mother cell (MMC). (d) The MMC undergoes meiosis and gives rise to four megaspores. The cell closest to the chalaza becomes functional megaspore (FM). The three nonfunctional megaspores (dm) degenerate shortly after meiosis. (e) The last stage of megagametogenesis, FG7. The FM undergoes three rounds of mitoses, resulting in an eight-nucleate syncytium that is later partitioned into seven cells consisting of four cell types. Antipodal cells degenerate by FG7 and are not shown. ccn, central cell nucleus; ecn, egg cell nucleus; syn, synergid cell nuclei. (f-j) Ovule development in the *ahk2-7 ahk3-3 cre1-12* mutant. (f) The mutant ovules prior to meiosis with a single enlarged MMC. Note that there are fewer cells in the chalaza (ch) compared to wild type as in Figure 3a. (g) The mutant ovule after meiosis. Note that none of the tetrad of megaspores enlarges as functional megaspore as in wild type (Figure 2.4d) (h-j) Distinct phenotypes of the *ahk2-7 ahk3-3 cre1-12* female gametophytes at FG7 stage. (h) A cavity containing two nuclei, indicated by the arrowheads. (i) Absence of embryo sac. (j) A developed embryo sac with a central cell-like nucleus. Note the altered morphology of this embryo sac compared to wild type as in Figure 3c. Scale bars in c-*j* = 10 µm.

triple mutant line, an embryo sac was absent in nearly all of the ovules (97.1%, *n* = 108 from seven different plants) (Figure 2.4a). Together, these results suggest that the three cytokinin receptors have redundant functions in female gametophyte development.

We next examined the development in wild type and *ahk2-7 ahk3-3 cre1-12* ovules to determine the stage at which the morphology of the mutants first departs from wild type. During megasporogenesis, one sub-epidermal archesporial cell differentiates into a megaspore mother cell (MMC) (Figure 2.4c). In *ahk2-7 ahk3-3 cre1-12* ovules, the specification of MMC, meiotic division, and tetrad formation did not differ from wild type (Figure 2.4c, f). The earliest stage at which morphological defects were observed in the *ahk* triple mutant was at the specification of the functional megaspore (FM) after meiosis (Figure 2.4d, g). In wild type, the megaspore closest to the chalaza enlarges and gives rise to the FM, and ultimately the embryo sac, while the other three megaspores degenerate shortly after meiosis (Figure 2.4d). In the *ahk2-7 ahk3-3 cre1-12* mutant, ovule development was generally asynchronous as compared to the wild type. The gametogenesis proceeded normally through the tetrad formation. However, the subsequent specification of the FM often did not occur in the mutant ovules (37.1%, *n* = 108) (Figure 2.4g, i). In some cases (59.9%, *n* = 108), an embryo sac-like cavity formed, but contained only one or two nuclei (Figure 2.4h). A few ovules (~3%, *n* = 108) contained a developing embryo sac but the morphology differed from wild type (Figure 2.4j). We observed similar phenotypes in another triple cytokinin receptor mutant, *ahk2-1 ahk3-1 ahk4-1* (Figure S2.1).

CKI1, an Arabidopsis histidine protein kinase, is similar to the AHKs the cytokinin receptors but it lacks the cytokinin-binding CHASE domain. CKI1 is essential

for megagametogenesis: the earliest stage at which phenotypic abnormalities occurred in *cki1* female gametophytes is at the four-nucleate stage (FG4), after the FM undergoes two rounds of mitosis. The *cki1* phenotype becomes completely penetrant by the eight-nucleate stage (FG5/6)[109, 110]. Previous studies have shown that the AHPs act downstream of CKI1 in megagametogenesis[23]. Here, we explored if the AHPs also act downstream of cytokinin-receptor AHKs to regulate megasporogenesis.

The quintuple *ahp1 ahp2-1 ahp3 ahp4 ahp5* mutant is disrupted for all five *AHP* genes in the Arabidopsis genome, although the *ahp2-1* allele retains some AHP2 function[15]. This *ahp* quintuple mutant displays strongly reduced sensitivity to cytokinin and reduced fertility[15]. Most (81.7%, $n = 278$) of the ovules in the *ahp1 ahp2-1 ahp3 ahp4 ahp5* mutant arrested at FG7 (Figure 2.5a). The majority of these (65.9%, $n = 278$) displayed a *cki1*-like phenotype (increased number of nuclei in embryo sac or degenerating embryo sac) (Figure 2.5c and S2.2), but a substantial number (15.8%, $n = 278$) showed an *ahk*-like phenotype (lack of embryo sac) (Figure 2.5b).This suggests that the AHPs act downstream of the AHKs in megaspore-genesis, in addition to their function downstream of CKI1 in megagametogenesis.

To further decipher the downstream two-component elements in regulating megasporogenesis, we analyzed ovules from a quadruple type-B *arr* mutant (*arr1-3 arr2-2 arr10-2 arr12-1*), which, like the multiple *ahp* and *ahk* mutants, displays insensitivity to high levels of exogenous cytokinin[96]. We observed a substantial number of ovules arrested at FG7 in this quadruple type-B mutant line (21.9%, $n = 276$) (Figure 2.5a), with 10.0% ($n = 276$) showing an *ahk*-like phenotype (Figure 2.5e) and 11.9% ($n = 276$) a *cki1*-like phenotype (Figure 2.5f).

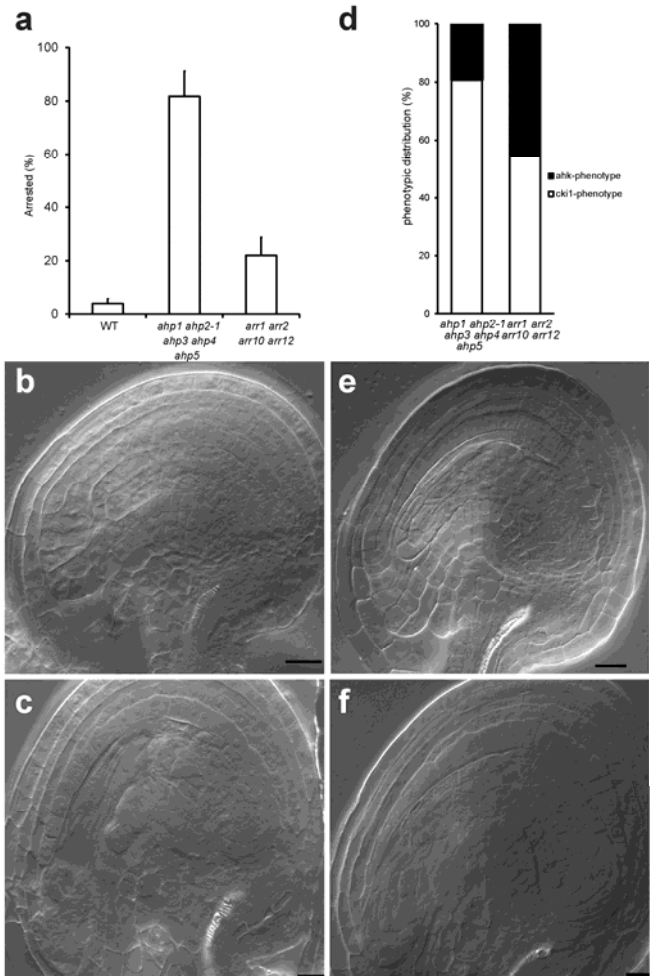

Figure 2.5. Both *ahk*- and *cki1*-like phenotype are present in high order *ahp* and type-B *arr* mutants. (a) Percentage of arrested female gametophytes in wild type and various multiple *ahp* and *arr* mutants (n>270 for each line). (b-c) The morphology of the arrested ovules in *ahp1 ahp2-1 ahp3 ahp4 ahp5*. (b) Lack of embryo sac formation in the *ahp* quintuple mutant, which is similar to the ovule phenotype of the *ahk* triple mutants (Figure 3g). (c) Degenerating embryo sac in the *ahp* quintuple ovule, which is similar to the ovule phenotype of *cki1-5* (Figure S4a). (d) Percentage of *ahk*- and *cki1*-like ovule phenotypes present in multiple *ahp* and *arr* mutants. (e-f) The morphology of the arrested ovules in the *arr1-3 arr2-2 arr10-2 arr12-1* mutant. (e) *ahk*-like phenotype (lack of embryo sac) (f) *cki1*-like phenotype (degenerating embryo sac). Scale bars = 10 μm.

Mutations in *ARR7* and *ARR15*, two of the type-A ARRs, were previously reported to affect female gametophyte development[79]. More recent studies indicate that the lethality is the result of chromosome rearrangements present in the first alleles analyzed[73]. Most (98.9%, *n* = 188) of the ovules in octuple *arr3,4,5,6,7,8,9,15* mutants were morphologically indistinguishable from wild-type, suggesting that these type-A ARRs do not play an essential role in female gametophyte development. Taken together, the dual ovule phenotype observed in the high-order *ahp* and type-B *arr* mutants (Figure

2.5d) suggest that these elements are essential in both megasporogenesis and megagametogenesis, acting downstream of both *AHK* and *CKI1* respectively.

Cytokinin receptors and activity are enriched in the chalaza of the ovule

Mutations affecting embryo sac development may act through either the diploid sporophyte or the haploid gametophyte. The phenotypes of gametophytic mutations are solely determined by the haploid genotype of the embryo sac. In contrast, sporophytic mutations affect all gametophytes within the ovaries of a mutant plant; thus the progeny of a selfed heterozygous recessive mutation will segregate wild-type and mutant genotypes in a Mendelian ratio. Previous studies have suggested that female gametophyte (FG) development depends on the cytokinin receptors (AHKs) in the sporophyte[13, 106]. Single heterozygous *ahk2* showed a Mendelian segregation in double homozygous receptor mutant (*ahk3 ahk4*) background[13, 106]. Consistently, we observed that 25% of progeny seedlings from selfed *ahk2-7/AHK2 ahk3-3/ahk3-3 cre1-12/cre1-12* or *ahk2-7/ ahk2-7 ahk3-3/AHK3 cre1-12/cre1-12* mutants displayed the triple mutant phenotype (Table 2.1).

Table 2.1. Segregation of single *AHK* in double *ahk* background

Parent	Normal seedling: small seedling*		χ^2
	Observed	Expected	
ahk2-7/AHK2 ahk3-3 cre1-12	419:147	424.5:141.5 (3:1)	0.593, $P > 0.5$
ahk2-7 ahk3-3/AHK3 cre1-12	458:143	450.75:150.25 (3:1)	0.494, $P > 0.45$

* Small seedling results from triple mutant.

To better understand the functions of cytokinin during female gametophyte development, we analyzed transgenic plants carrying a GUS reporter driven by the native promoter of *AHK2, AHK3*, or *CRE1*. The intensity of the AHK2, AHK3 and *CRE1* signal was high throughout megasporogenesis. At ovule stage 2III[101] when the outer integument initiates and meiosis has not occurred, AHK2, AHK3 and *AHK4* were expressed preferentially at the chalazal end of the ovule (Figure 2.6a-f). AHK2 and *AHK4* were also expressed in the inner integument primordia (Figure 2.6a, b, e, f). When stained for extended times, AHK2 and AHK3 expression was observed diffusely spread throughout the nucellus, but still with more intense expression at the chalaza (Figure 2.6b, d). *AHK4* was expressed almost exclusively in the chalaza, with little or no expression in the nucellus during megasporogenesis (Figure 2.6e, f). Expression of these *AHK* genes in the chalaza continued through at least stage FG2 of FG development. This pattern of expression is similar to that presented previously, though in that study, the authors did not note the enrichment of AHK expression in the chalaza[111].

To analyze the cytokinin responses in these tissues, we examined the expression of *ARR4*, one of the type-A ARRs that is a cytokinin primary response gene[65]. The expression of *ARR4* was localized in the chalaza in stage 2III (Figure 2.6g), overlapping with the expression pattern of the cytokinin receptors (Figure 2.6a-f). This is consistent with the pattern of expression of the TCS-GFP transgene[65], which reports cytokinin signaling[80]. To further explore the origin of the cytokinin gradient, the expression of different isopentenyl transferases (IPTs) that catalyze the rate-limiting step of cytokinin biosynthesis in Arabidopsis were studied in the ovules. Plants carrying a fusion of the promoter and coding region of *IPT1* to GUS were used to study cytokinin biosynthesis in

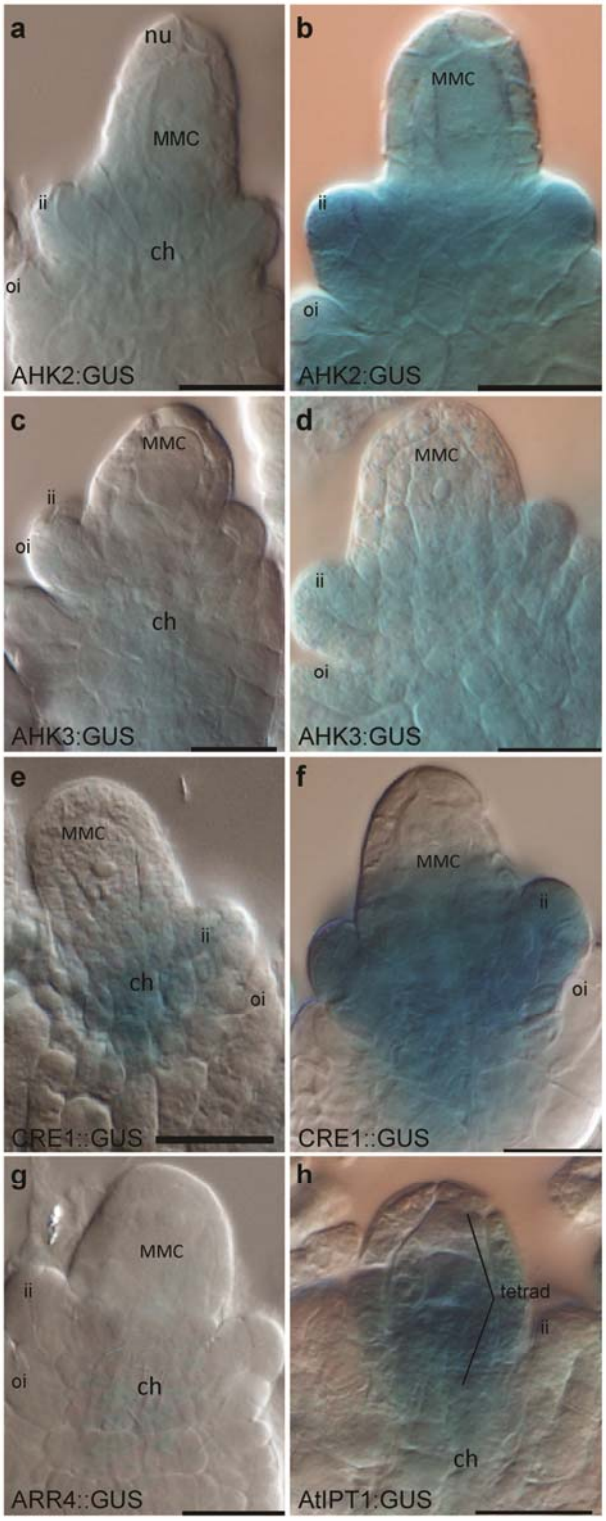

Figure 2.6. Expression of cytokinin signaling elements in ovules. (a-f) Expression of cytokinin receptors as revealed by fusions of the respective regulatory element to a GUS reporter. Lines harboring a AHK2 promoter-protein:GUS fusion (a-b), AHK3 promoter-protein:GUS fusion (c-d), or CRE1 promoter::GUS fusion (e-f) were stained for GUS activity before meiosis when the phenotype in *ahk2-7 ahk3-3 cre1-12* about to occur . Unsaturated (a, c, e) and saturated (b, d, f) staining are shown to reveal the preferential expression in each domain. (a-d) AHK2 and AHK3 are diffusedly detected in the ovule, but are enriched in the chalaza and the integument primordial. (e-f) *AHK4* is specifically localized at the chalaza and inner integument primordia. (g) Visualization of expression of cytokinin-responsive *ARR4::GUS* transgene in pre-meiotic ovules. (H) Expression of IPT1 as revealed by the fusion of promoter and coding region to a GUS reporter. Abbreviations: MMC, megaspore mother cell; nu, nucellus; ch, chalaza; ii, inner integument; oi, outer integument. Scale bars = 10 μm.

the developing ovules. IPT1 expression was observed during megasporogenesis and megagametogenesis (Figure 2.6h and S2.3). In stage 2III when the ovule phenotype in

ahk2-7 ahk3-3 cre1-12 has not occurred, IPT1 was observed in the MMC, companion cells, and the surrounding sporophytic tissues (Figure S2.3). During tetrad formation, IPT1 expression was observed in the haploid megaspore and the diploid sporophytic cells with higher intensity in the chalaza end of the ovule (Figure 2.6h and S2.3). Together, these data indicate that cytokinin signaling and biosynthesis are asymmetrically distributed in the ovule, predominantly at the chalaza during megasporogenesis.

Chalazal cytokinin signaling is required for the selection of functional megaspore

Megasporogenesis and megagametogenesis occur along the chalazal-micropylar axis of the ovule. In most species of flowering plants, including Arabidopsis, the megaspore located closest to the chalaza survives after meiosis and differentiates into the functional megaspore (FM). The lack of development of the FM in the triple cytokinin receptor mutant suggests that the elevated cytokinin signaling in the surrounding chalazal maternal tissues conveys positional information involved in the specification of the FM. To examine this hypothesis, we introduced an FM-specific marker (*pFM2*::GUS) into the *ahk2-7 ahk3-3 cre1-12* background. The *pFM2* promoter first drives expression specifically in the functional megaspore, but not in the degenerating megaspores, and its expression is sustained through FG7[105]. In wild-type plants, *pFM2* expression was detected in the chalazal-most megaspore (Figure 2.7a). In contrast, in an F_3 population homozygous for *pFM2*::GUS in *ahk2-7 ahk3-3 cre1-12/CRE1* background, *pFM2* was absent in the ovules that failed to form a morphologically recognizable FM (Figure 2.7b), while the sibling ovules in the same gynecium with a morphologically recognizable FM displayed GUS expression (Figure 2.7c). Because female gametogenesis is asynchronous

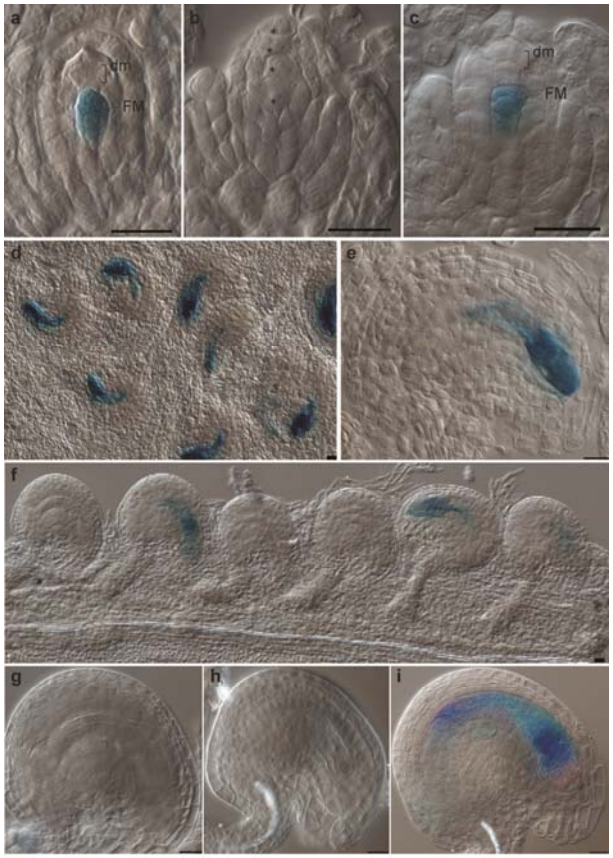

Figure 2.7. Cytokinin signaling is required for the functional megaspore selection. (a) pFM2 expression in wild-type ovules after meiosis. Note the marker specifically stains the functional megaspore (FM) but not the degenerating megaspores (dm). (b-c) Expression of *pFM2::GUS* in ovules from the *ahk2-7 ahk3-3 cre1-12/CRE1* mutants. (b) After meiosis, pFM2 was absent in the ovules that failed to form a morphologically identifiable FM. (c) pFM2 expression was detected in the sibling ovules from the same gynecium as in (b). (d-e) pFM2 expression in wild-type ovules persist through FG6/7. (f-i) FM specification is compromised in ovules from *ahk2-7 ahk3-3 cre1-12/CRE1*. (f) At FG6/7, pFM2 was absent in 30.1% of the ovules (n = 302) that had a cavity (g) or did not form an FM (h). (i) pFM2 was present in a subset of the sibling ovules of (g) that had a specified FM.

in the *ahk* mutants and *pFM2*::GUS expression persists through FG7, we allowed megagametogenesis to progress to FG6/7 to avoid false negative results. At FG6/7, *pFM2* was present in 96.2% of the wild-type ovules (n = 373) (Figure 2.7d, e). In *pFM2*::GUS; *ahk2-7 ahk3-3 cre1-12/CRE1* plants, however, *pFM2* was present in only 69.9% of the ovules (n = 302) (Figure 2.7f-i). An embryo sac-like cavity (Figure 2.7g) was observed in some of the pFM2-negative ovules, while in others, the embryo sac was complete absent (Figure 2.7h). This frequency of FM2-negative ovules in the *pFM2*::GUS; *ahk2-7 ahk3-3 cre1-12/CRE1* line (30.1%), is higher than the frequency of morphologically defective FG (8.9%, n>200) in this line examined at the same time, suggesting that the AHKs are important in both the specification and maintenance of FM identity, or at least maintenance of FM2 expression.

Discussion

Two-component signaling in female gametophyte development

In this study, we demonstrate that all three elements of two-component signaling, the AHKs, AHPs and type-B ARRs, are necessary in the sporophyte for proper female gametophyte development (Figure 2.8). We grew three different *ahk* triple mutants simultaneously and concluded that the differences in their fertility were not due to growth condition, but rather to the strength of the various alleles in these mutants. The cytokinin threshold required for female gametophyte development appears to be very low, as the partially fertile *ahk2-5 ahk3-7 cre1-2* line is strongly insensitive to cytokinin in multiple response assays. We characterized the FG phenotype in the strongest *ahk2-7 ahk3-3 cre1-12* and found that the first deviation from the wild type occurred during functional megaspore (FM) specification, which resulted in the subsequent absence of embryo sac formation (*ahk*-phenotype). This is distinct from the phenotype described recently by Bencivenga *et al.*, which focused on the lack of integument development in the *ahk2-2tk ahk3-3 cre1-12* line[111].

Figure 2.8. Two-component signaling in female gametophyte development. In megasporogenesis, SPL/NZZ is required for the megaspore mother cell specification (Schiefthaler, *et al.* 1999, Yang, *et al.* 1999). The data presented here suggests that an AHK-AHP-type-B ARR signaling pathway acting in the sporophyte is responsible for FM specification. CKI1 is essential in the gametophyte during megagametogenesis (Hejatko *et al.* 2003, Pischke *et al.* 2002). The AHPs (Deng *et al.* 2010) and type-B ARRs also act in the CKI1-dependent pathway in the megagametogenesis.

A previous study has demonstrated an essential role for the AHPs in the megagametogenesis[23]. Here, we demonstrate that disruption of the AHPs results in two distinct ovule phenotypes, suggesting that these elements act downstream of both the AHKs and CKI1, in the sporophyte and gametophyte respectively. A sporophytic role for the AHPs is supported by the transmission efficiency of T-DNA insertions at *AHP3* or *AHP5* locus in *ahp1,2-1,3/+,4,5* or *ahp1,2-1,3,4,5/+*, respectively, which were transmitted at the expected 1:1 ratio in reciprocal crosses[23]. Together these results indicate that the AHPs act in the sporophyte as well in the gametophyte.

Reproductive defects have not been reported in previous analyses of type-B *arr* mutants[16, 32, 82, 92]. The *arr1-3 arr2-2 arr10-2 arr12-1*quadruple mutant examined in this study had two distinct (*ahk*- and *cki1*- like) phenotypes with moderate penetrance. This quadruple mutant has mutations in four out of seven members of the subfamily of type-B ARRs linked to cytokinin signaling; therefore, other members may account for the remaining fertility as these genes have been shown to be functionally redundant in other contexts[16, 32, 83, 92]. Our data reveal a role for type-B ARRs in the FG development, acting in the sporophyte and gametophyte through AHK- and CKI1-dependent pathway respectively.

Cytokinin is essential for functional megaspore specification

Several lines of evidence support the notion that sporophytic cytokinin plays a role in the specification of the functional megaspore (FM). Disruption of cytokinin signaling in the sporophytic tissue results in a lack of embryo sac formation. The earliest defects were observed in FM specification, as shown both morphologically and by using an FM-specific marker. Further, cytokinin signaling is enriched in the chalazal

sporophytic tissue, as revealed by localized expression of the cytokinin primary response gene *ARR4* (Figure 5g) and by expression of the cytokinin reporter, TCS-GFP[111]. Together, these results suggest that chalazal-enriched cytokinin signaling confers positional information involved in specifying and maintaining the FM development.

While cytokinin signaling is clearly involved in the FM specification, the FM-absent phenotype is not completely penetrant, even in the strongest cytokinin receptor mutant, *ahk2-7 ahk3-3 cre1-12*. In this line, however, residual full-length *AHK3* transcript was detected from the *ahk3-3* allele (Figure S1b), indicating that it is not a complete null for cytokinin receptors. Our analysis of three different *ahk* mutantssuggests that the threshold of cytokinin function required for FG development is extremely low; thus the cytokinin signaling derived from the residual *AHK3* in *ahk2-7 ahk3-3 cre1-12* may be sufficient to drive some FM formation.

An alternative, not mutually exclusive model for the residual FM formation in the triple cytokinin receptor mutants is that there are overlapping pathways mediating FM specification. One possibility for such redundant factor is SPOROCYTELESS/NOZZLE (SPL/NZZ), the master transcription factor that links pattern formation and growth control during ovule development[114, 115]. Mutation in *SPL/NZZ* results in complete male and female sterility through a sporophytic effect[114, 115]. Recent transcriptome analysis of developing ovules revealed that multiple cytokinin-responsive type-A ARRs are elevated in the *spl* ovules[116]. In addition, *AHK4* expression, but not *AHK2* or *AHK3*, is abolished in *spl/nzz* ovules compared to wild type[117], and *SPL/NZZ* is induced in ovules upon cytokinin treatment[111]. Overall, these results suggest a intriguing, though complicated interaction between *SPL/NZZ* and cytokinin signaling.

A recent study proposes that *SPL/NZZ* is required for the cytokinin-induced expression of auxin efflux protein PIN1 in the FG, and this regulation accounts for integument development in the ovules[111]. The lack of integuments (leading to finger-like structures), however, represents only ~10% of the phenotypes observed in the *ahk2-2tk ahk3-3 cre1-12* line examined in this study and is not observed in *spl/nzz* ovules[111]. In *spl/nzz* ovules, the sub-epidermal archesporial cell in the nucellus fails to differentiate into the megaspore mother cell[114, 115]. The phenotype of *spl/nzz* ovules occurs in a developmental window earlier than that which requires AHK function (FM specification post-meiotically; Figures 2.8 and 2.9).

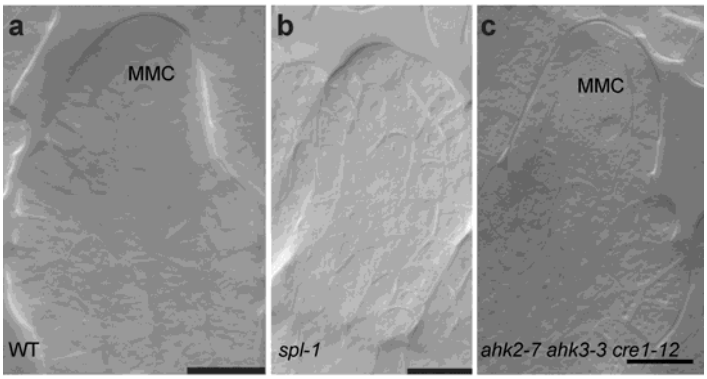

Figure 2.9. Phenotypic difference between *spl-1* and *ahk2-7 ahk3-3 cre1-12* ovules. (a) Wild-type ovule at stage 2I when a single archesporial cell enlarged as megaspore mother cell (MMC). (b) In *spl-1* ovules, the archesporial cells fail to differentiate into a single MMC. (c) MMC formation in *ahk2-7 ahk3-3 cre1-12* ovules.

Signaling between the sporophyte and the gametophyte involves cytokinin

While cytokinin is important in FM specification, it is unlikely that cytokinin itself acts as the signal to determine FM fate in the gametophyte as cytokinin receptor function is not required in the gametophytic cells (Table 2.1). Thus, the chalazal-enriched cytokinin signaling likely results in the generation of a distinct signal to direct FM fate, though further studies are needed to elucidate the nature of this signal. While our studies

suggest a cytokinin-dependent signal from the sporophyte to the gametophyte, the effect of the *spl* mutation on the expression of multiple cytokinin-responsive type-A ARRs[116] suggests that gametophyte also regulates cytokinin signaling in the surrounding sporophytic tissues. Thus, it appears that there is bi-directional communication between the sporophyte and the gametophyte that involves cytokinin.

The question arises as to when during female gametogenesis the putative sporophytic, cytokinin-dependent signal acts. It has been hypothesized that the developmental cues mediating the selection of the FM may be present in the sporophytic tissues before meiosis[100, 118, 119] as prior to meiosis, cellular organelles are asymmetrically distributed in the megaspore mother cell: more plastids are located in the chalazal region, while rough endoplasmic reticulum and vacuoles are more abundant at the micropylar region[120]. This asymmetry in the MMC may lead to an asymmetry in resulting meiotic products, and may be set up by the localized cytokinin in the sporophytic tissue. Alternatively, the cytokinin may signal the single megaspore closest to the chalaza to adopt an FM fate after meiosis has occurred.

Origin of chalaza-localized cytokinin

Cytokinin signaling is enhanced in the chalaza of the sporophytic tissue of the ovule as revealed by the localized expression of ARR4 (Figure 5g) and the TCS-GFP cytokinin reporter[111]. This likely results in part from localized expression of cytokinin signaling elements, but also from localized cytokinin biosynthesis as revealed by localized *IPT1* expression. However, in the *atipt1,3,5,7* quadruple mutant, in which four of the seven *IPT* genes are disrupted, no ovule nor seed set phenotypes were reported[121]. This suggests that residual local cytokinin biosynthesis coming from other lowly expressed IPTs in this

tissue may be sufficient for the ovule development, consistent with the notion that the threshold of cytokinin function required for FG development is very low. Alternatively, the mobile nature of cytokinin may allow it to serve systematically as a long distance signal. Other elements of cytokinin biosynthesis, such as the *LOG* genes[122], and cytokinin metabolism, such as the cytokinin oxidase genes[113], may also contribute to the generation of a localized cytokinin signal.

Conclusion

Plant hormones are involved in all aspects of plant growth and development. Perturbation the signaling pathways of a number of phytohormones, including auxin, brassinosteroid (BR), cytokinin, and ethylene results in defects in the development of ovules[107, 108, 123]. Our data demonstrate that a chalazal-enriched cytokinin signal conveys positional information to specify FM. Future studies are needed to elucidate the nature of this cytokinin-dependent signal emanating from the diploid sporophytic tissues and to determine how it regulates FM specification.

Experimental Procedures

Plant materials and growth conditions

The ahk2-1 ahk3-1 ahk4-1 (Nishimura, et al. 2004), ahk2-5 ahk3-7 cre1-2 (Riefler, et al. 2006), ahk2-7 ahk3-3 cre1-12 (Figure S1) (Argyros, et al. 2008), ahp1 ahp2-1 ahp3 ahp4 ahp5 (Hutchison and Kieber 2007), arr1-3 arr2-2 arr10-2 arr12-1 (Rashotte, et al. 2006), AHK2:GUS (Nishimura, et al. 2004), AHK3:GUS, CRE1::GUS (Higuchi, et al. 2004a), ARR4::GUS (To, et al. 2004), and pFM2::GUS (Olmedo-Monfil, et al. 2010) are in the Columbia (Col-0) ecotype. spl-1 (Yang et al. 1999) is in Landsberg erecta (Ler) background. cki1-5 (Pischke, et al. 2002) is in Ws background.

Arabidopsis seeds were surface-sterilized, cold-treated at 4°C for three days, and germinated on MS/1% sucrose vertical plates under constant light as previously described (To*, et al.* 2004). Seedlings were transferred to soil and kept in a growth chamber under long days (16 h light/8 h dark) at 22° C.

Histology and microscopy

For phenotypic analysis, we used the 6th flower or older, except for the *ahk* triple mutants, which had very few flowers. Ovules were dissected on a slide and fixed in Carnoy's fixative (3:1 ethanol:acetic acid) for 15 min, incubated in 70% ethanol for 15 min, transferred to water for 20 min, mounted in chlorohydrate:water (8:3), and finally photographed with a Nikon E800 photomicroscope equipped with a Nikon Plan Apo 100/1.40 oil immersion objective (http://www.nikon.com/) using differential interference contrast (DIC) optics.

For β-glucuronidase staining, dissected ovules were fixed in cold 90% acetone for 20 min, washed for 10 min in cold rinsing buffer (100 mM sodium phosphate, pH 7.0,

0.5% Triton X-100, 0.5 mM potassium ferro/ferricyanide) and vacuum infiltrated for 5 min in 1 mg/ml X-gluc (5-bromo-4-chloro-3-indolyl-β-D glucuronic acid). The samples were incubated at 37°C for 6 to 48 h depending on lines. Ovules were mounted in chlorohydrate:water (8:3) and photographed under DIC optics as described above.

Expression of *AHK* in *ahk2-7 ahk3-3 cre1-12*

RNA was isolated from the shoots of 4-week-old plants using an RNeasy kit (Qiagen, http://www.qiagen.com/). cDNA was prepared from the total RNA using SuperScript III reverse transcriptase (Invitrogen, http://www.invitrogen.com/) following the manufacturer's instructions. Real-time PCR was performed sing SYBR Premix Ex Taq polymerase (TaKaRa, http://www.takara-bio.com/) in a ViiA^{TM} 7 Real-Time PCR system (ABI, http://www.appliedbiosystems.coml). The following primers were used for gene expression, *AHK2*, 5'- ACGCGCCAGTTATATTTGCT - 3' and 5'- GCGGTAGGCTCGTGTCATAG- 3'; *AHK3*, 5'- CAGCTCAAGAAAAAGGCTGAA- 3' and 5'- TGCGGTCCTAACATAATCCTG - 3'; *AHK4/CRE1*, 5'- ACAATGGATAG AGGAGAGCCTTC- 3' and 5'- ATGGTGAGTTTCCAACAACCTAA- 3'; *TUB4*, 5'- AGAGGTTGACGAGCAGATGA - 3' and 5'- ACCAATGAAAGTAGACGCCA - 3'.

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Supporting Information

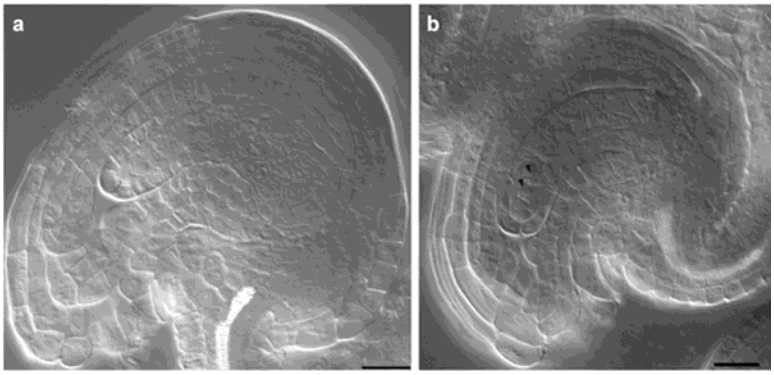

Figure S2.1. Phenotypic analysis of female gametophyte in *ahk2-1 ahk3-1 ahk4-1*. (a) Phenotype of *ahk2-1 ahk3-1 ahk4-1* at the last female gametophyte development stage, FG7; note the absence of embryo sac in the ovule. (b) At FG7, a cavity with two nuclei (arrowheads). Scale bars = 10 μm.

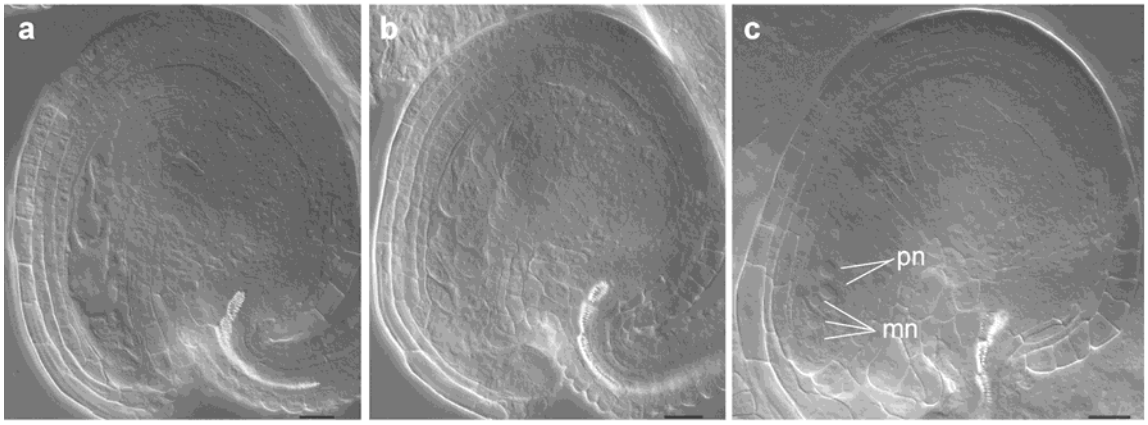

Figure S2.2. Phenotypic analysis of *cki1-5* female gametophytes at FG7. (a) A degenerating *cki1-5* embryo sac. (b) A *cki1-5* embryo sac with an abnormal number (>8) of nuclei. (c) A *cki1-5* embryo sac with defects in cell morphology and identity. The nuclei at the micropylar end are labeled as polar nuclei (pn) and micropylar nuclei because of the abnormal morphology. The presumed polar nuclei (pn) are did not fuse to form central cell. Scale bars = 10 μm.

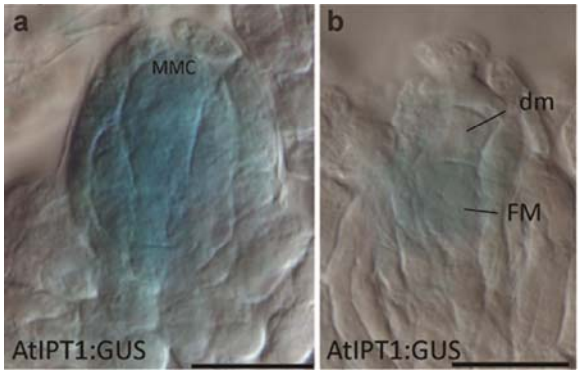

Figure S2.3. Expression of IPT1 during megasporogenesis. (a) AtIPT1:GUS expression in the megaspore mother cell (MMC) and the surrounding sporophytic cells before meiosis occurs. (b) Enrichment of IPT1 expression in the functional megaspore (FM) during tetrad formation. Scale bars = 10 μm.

CHAPTER 3: The Role of Cytokinin in the Ovule Development in Arabidopsis

Preface

The following chapter was published in Plant Signaling and Behavior volume 8 as an addendum to the previous chapter. This work revealed the enhanced mitotic activity in the integuments of a cytokinin insensitive mutant and further discussed the role of cytokinin in ovule development.

Summary

The life cycle of higher plants alternates between the haploid gametophyte and diploid sporophyte. The female gametophyte (FG), surrounded by the sporophyte, develops within the ovule and orients along the chalazal/micropylar axis. This polarity is important in cell specification and development for both the ovule and FG. Previously, cytokinin was shown to act in the sporophytic tissue to regulate FG development[13, 106]. In the highlighted study[47], we further showed that enriched cytokinin signaling in chalaza, the central domain of the ovule, is required for the specification of the functional megaspore, which usually occurs in the chalazal-most megaspore after meiosis. The restricted cytokinin signaling in the chalaza is achieved by localized cytokinin biosynthesis and perception. Here, we discuss the implications of this and other studies for the understanding of the role of two-component signaling in FG development and the genetic and cellular interactions between gametophytic and sporophytic cells. Further, we show that cytokinin-deficient mutants display distorted cell morphology in the inner

integument and elevated mitotic activity in the maternal sporophyte. These results suggest that cytokinin negatively regulates cell proliferation in the sporophytic tissues surrounding the developing FG, consistent with previous results indicating that cytokinin deficiency causes an increase in the number of cells in the embryos and consequently an enlarged seed size.

In most flowering plants, including Arabidopsis, female gametogenesis begins when one of the somatic hypodermal cells in the protruding ovules adopts germ cell fate and differentiates into megaspore mother cell (MMC). Before entering meiosis, the MMC displays polarity, with an enrichment of plastids and plasmodesmata connections at the proximal chalaza and an abundance of small vacuoles and dictyosomes in the distal micropyle[120, 124]. Following meiosis, the four haploid meiotic megaspores line up along the chalazal/micropylar axis and the megaspore closest to the chalazal end is selected to become the functional megaspore (FM), which develops into the mature female gametophyte (FG). The transition between diploid and haploid phases occurs within the sporophytic tissues, and the intimate connection between the diploid sporophyte and haploid gametophyte allows cellular interactions between these generations.

Cell Fate Determination in Ovule

While it has long been suggested that positional information and/or local signaling plays a crucial role in cell fate determination in ovule growth and development[125], only recently has experimental evidence for this been obtained. In Arabidopsis, sporophytic small RNAs (sRNA) controls germline formation by restricting single MMC differentiation from the somatic hypodermal cells[105]. In *argonaute9* mutants, a portion of the ovules exhibited more than one enlarged archesporial cells similar to the MMC in wild type[105]. This suggests that all somatic hypodermal cells are competent to become MMCs and that the AGO9-dependent pathway is involved in restricting differentiation to a single MMC.

The understanding of FM determination has been limited in part due to the lack of mutants that block FG development at this stage. Multiple reports have suggested that the

position rather than the genetic lineage of the haploid megaspore is crucial for adoption of the FM fate[120, 124, 125]. An enrichment of organelles at the chalazal end of the MMC has been implicated to affect FM selection as the chalazal-most megaspore will receive most of the organelles after meiosis[124 120]. Additionally, mutation of *DYAD/SWITCH1* leads to apomeiosis with two unreduced female gametes; however, the FM-specific marker FM1 was only detected in the chalazal-most cell of the dyad[126]. Together, these data imply the FM determination occurs prior to meiosis.

Cytokinin two-component signaling is important in many stages of reproductive growth, including ovule number[113], FG development[47, 106, 111], and seed size[14-16]. Although studies from different labs have shown that disruption of the three cytokinin receptors (Arabidopsis Histidine Kinases, AHKs) blocks FG development[13, 106], one combination of the three *ahk* alleles (*ahk2-5 ahk3-7 cre1-2*) has been reported to be fertile[14]. We recently showed that the discrepancy in the phenotypic strength of the FG defects in these mutants is due to differences in the penetrance of the various *ahk* alleles used[47]. The strongest combination of alleles (*ahk2-7 ahk3-3 cre1-12*) displays nearly complete FG arrest, while FG development in the weakest line (*ahk2-5 ahk3-7 cre1-2*) is comparable to the wild type. Furthermore, even in the *ahk2-7 ahk3-3 cre1-12* triple mutant, some full-length, wild-type *AHK3* transcript is present, indicating that some residual cytokinin signaling remains. These data unequivocally demonstrate a requirement for cytokinin signaling in FG development, but also suggest the threshold is extremely low. Further, cytokinin signaling is enriched in the chalaza as a result of localized cytokinin biosynthesis and receptor expression, and this plays a role in specifying FM, as evidenced by the lack of expression of an FM-specific marker

(pFM2::GUS) in the triple receptor background. The low threshold of cytokinin signaling required for FG development may explain the lack of mutants that specifically block FM determination; alternatively, a parallel signaling pathway may contribute to specify the FM.

Sporophytically Acting Genes in Regulating Integument Growth and Development

A number of genes that act in the sporophyte to regulate FG development also affect the morphogenesis of integument[127-130], which initiates from the chalaza before meiosis[101]. A recent study reported that the strong *ahk2-2 ahk3-3 cre1-12* triple mutant displayed impaired integument initiation and finger-like ovule structure in a fraction of the ovules (~10%)[111]. Most of the ovules we observed in *ahk2-7 ahk3-3 cre1-12* mutants also had normal integument initiation[47]; however, we noticed aberrant integument morphology in many of the ovules of this triple mutant (Figure 3.1a-d). In wild-type ovules, there are 2-3 cell layers in the inner integument when the FG is mature (Figure 3.1a). In *ahk2-7 ahk3-3 cre1-12* ovules, the cell layers in the inner integuments are disorganized (Figure 3.1b). Further, in the *ahk2-7/AHK2 ahk3-3 cre1-12* mutants, the inner layer of the inner integument that contacts the FG often displays an aberrant cell width/length ratio (Figure 3.1c). Interestingly, the ovules with distorted cell shapes in this layer also contained aborted FGs, while the ovules in the same silique with well-developed FGs displayed less severe integument phenotypes (Figure 3 .1c and d). This suggests that either the sporophytic integument affects FG development, or vice versa. Consistent with the later note, transcriptome analysis has revealed that multiple cytokinin signaling elements are differentially expressed in mutant ovules that lack FG formation[116, 117].

The distorted integument morphology in cytokinin signaling mutants could be due to defects in cell division, cell expansion, or a combination of both. To explore this, we examined the expression of a mitotic marker, *cyclinB:GUS*, in the ovules of a cytokinin-deficient mutant *ahp1 ahp2-1 ahp3 ahp5*[15]. Surprisingly, we found substantially enhanced expression of *cyclinB:GUS* in the sporophytes of *ahp1 ahp2-1 ahp3 ahp5* ovules (Figure 3.1i-l) as compared to the wild type (Figure 3.1e-h), which is counter-intuitive to the described role of cytokinin in promoting cell division in the shoot and in cultured cells. The increased level of *cyclinB:GUS* in the *ahp1 ahp2-1 ahp3 ahp5* sporophytic tissue is observed as early as pre-meiotic MMCs and specifically in the chalaza (Figure 3.1e, f, i, and j). The enhanced mitotic activity in *ahp1 ahp2-1 ahp3 ahp5* ovules continues and expands to inner and outer integuments through megagametogenesis (Figure 3.1g, h, k and l).

The accumulation of *cyclinB:GUS* in the *ahp1 ahp2-1 ahp3 ahp5* background could reflect either an enhanced mitotic activity or a blockage of mitotic progression, as cyclin B is a substrate of anaphase-promoting complex before the cell exists cell-cycle[131, 132]. If the former is true, one would expect larger seeds in *ahp1 ahp2-1 ahp3 ahp5* mutants. Consistent with this, plants with decreased cytokinin levels or signaling, including the *ahp1 ahp2-1 ahp3 ahp5* mutants, have enlarged seeds[14-16]. Microscopic examination showed that *ahk* triple embryos had increased cell size and cell numbers as compared to the wild type[14]. Genetic analysis indicated that the maternal genotype with

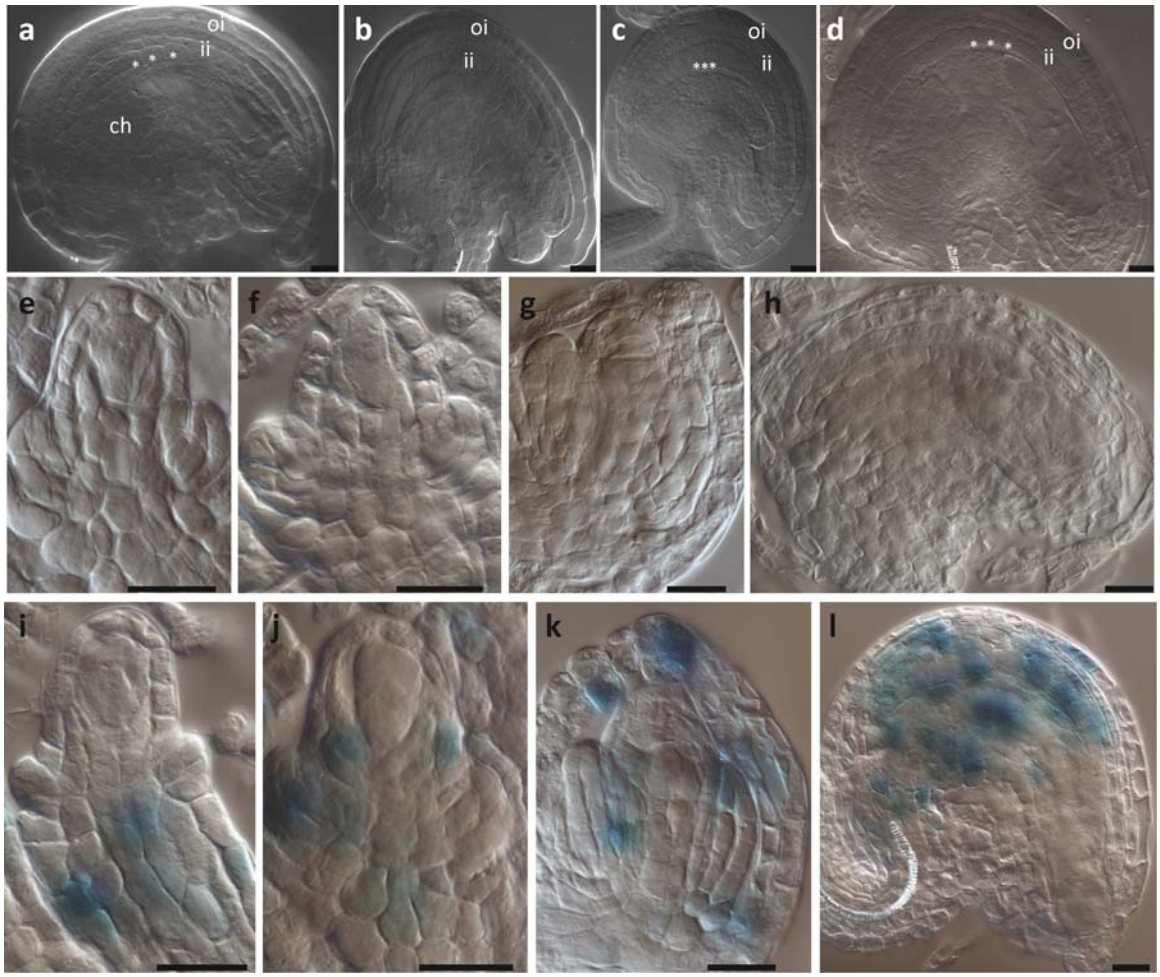

Figure 3.1. Cytokinin signaling regulates mitotic activity in the sporophytic tissues of ovule. (a-d) Integument phenotypes of the wild type (a), *ahk2-7 ahk3-3 cre1-12* (b), or *ahk2-7/AHK2 ahk3-3 cre1-12* (c-d). The cells of the inner integument in *ahk2-7 ahk3-3 cre1-12* (b) ovules are disorganized and altered in morphology as compared to wild type (a). The *ahk2-7/AHK2 ahk3-3 cre1-12* ovules displayed moderate integument phenotype which allowed comparison of the same cell layer with that in the wild type. The asterisks label the cell layer that contacts the embryo sac. Note that the labeled cells in *ahk2-7/AHK2 ahk3-3 cre1-12* display a distinct cell shape relative to comparable cells in the wild type. (e-l) Expression of *cyclinB:GUS* in wild-type (e-h) or *ahp1 ahp2-1 ahp3 ahp5* (i-l) background . The increased GUS signal was observed in megasporogenesis (e, f, i, j) and continued through megagametogenesis (g, h, k, l). Staining was performed as previously described[3] with a two hour incubation at 37˚C for optimal comparison. ch, chalaza, ii, inner integument, oi, outer integument, Scale bars = 10 μm.

regard to cytokinin signaling is the most influential factor in determining seed size[14]. Only seeds from *ahk* triple mutants pollinated with wild-type plants were at least as large as self-pollinated *ahk* triple mutant seeds[14]. The data suggests a model in which cytokinin negatively regulates cell division in the sporophyte, and a key factor determining seed size is programmed prior to fertilization.

Perspectives

Our knowledge of the mechanisms underlying the influence of cytokinin signaling on FM selection remains incomplete. The progress achieved so far raises some important questions. What is the gametophytic factor regulated in response to sporophytic cytokinin signaling that acts to specify FM? Is the integument phenotype in cytokinin receptor mutants a primary or secondary effect? Does the mitotic activity in the maternal integuments contribute to FG development? The FG molecular markers that have been identified allow researchers to diagnose the stage at which mutants arrest in FG development. Molecular and genetic studies have led to models for the regulatory network involved in ovule development, which sets the stage for further studies to define the complex role of cytokinin and its interaction with other signals in regulating FG development.

CHAPTER 4: Characterization of Phospho-Specific Type-A ARR Interacting Targets

Preface

The following chapter will be published in the next year. A pilot yeast-2-hybrid screening was performed in the lab using a random-primed cDNA library with phospho-mimic forms of ARR4, ARR5, and ARR9. Jayson A. Punwani and I confirmed a subset of interactions in Arabidopsis protoplast system. Jayson generated the construct, 35S::ARID:GFP, Tracy Raines performed the transformation, and I isolated the T2 lines with single insertion and studied the morphology and phenotype in T3 homozygous plants.

Introduction

Most bacteria possess numerous two-component systems to respond to a variety of environmental changes, such as temperature (thermotaxis), light (phototaxis), salinity (osmotaxis), oxygen (aerotaxis), and chemicals (chemotaxis). The two-component architecture consists of a conserved receiver domain and a variable effector domain enables response regulator (RR) proteins to function as phosphorylation-mediated switches. Recent completion of approximately 400 sequenced bacterial and archaeal genomes reveal approximately 9000 RRs. Among them, 65% of RRs consist of DNA-binding domains that can be divided into different subfamilies based on predicted functions or structures. Other domains include enzymatic (~11%), protein-binding (2%),

RNA-binding (~1%), and uncharacterized (~7%)[10, 133]. Notably, ~17% of RRs consist of an isolated receiver domain and are known as stand-alone RRs. How a conserved receiver domain mediates, in a phosphorylation-dependent manner, a myriad of distinct ouputs is one of the outstanding questions in the field of two-component signaling.

Chemotaxis response regulator CheY is a well-studied stand-alone RR that elicits its biological function via phosphorylation-dependent interactions. Chemoreceptors sense chemical attractants or repellents in the environment and control the phosphotransfer from the histidine kinase CheA to the response regulator CheY. CheY interacts with flagellar motors and promotes clockwise rotation and tumbling in a phosphorylation-dependent manner[134, 135]. In this system, the phosphorylation status of CheY regulates its ability to interact with its effector, the flagellar motors.

In Arabidopsis, the 23 response regulators (ARRs) fall into two major classes based on phylogenetic analysis and domain structure: type-A ARRs and type-B ARRs (Figure 1.4). The ten type-A ARRs are comprised of a receiver domain and a short C-terminal extension. The eleven type-B ARRs, in addition to the receiver domain, have a longer C-terminus containing a Myb-like DNA-binding and an activation domain[29, 35, 71, 82]. Biochemical studies and functional analyses have shown that type-B ARRs serve as transcription factors that induce the expression of many genes, including type-A ARRs[16, 31, 34]. Type-B ARRs are partially redundant positive elements in the transcriptional network in response to cytokinin. Type-A ARRs mediate downstream responses to cytokinin and negatively feedback the primary signal pathway[35]. Genetic studies have shown that loss of type-A ARRs confers cytokinin hypersensitivity, though our understanding of the underlying mechanisms of action yet remains limited.

In agreement with the regulatory role of phosphorylation, removal of the receiver domain of ARR1 and ARR11, two of the type-B ARRs in Arabidopsis, increase their transactivation activity[31, 136]. In addition, previous study also revealed that phosphorylation of the conserved Asp residue of type-A ARR is critical for proper function [33]. Mutation of the highly conserved phosphorylation site Asp87 in the receiver domain of ARR5 to Ala ($ARR5^{D87A}$) or Glu ($ARR5^{D87E}$), which act as phosphoinsensitive or phosphomimic forms respectively, changes the protein stability, suggesting that ARR5 is stabilized by cytokinin via phosphorylation of the Asp residue[33]. The cytokinin effect on protein stability is observed in a subset of type-A ARRs and type-B ARRs[33, 137]. In addition, introducing genomic $ARR5^{WT}$is able to complement the hypersensitivity of *arr3,4,5,6* to exogenous cytokinin in seedling root elongation assays. Furthermore, $ARR5^{D87A}$ was not functional in this assay while $ARR5^{D87E}$ transgene partially restored the cytokinin sensitivity to the *arr3,4,5,6* parental line[33]. This data shows that ARR5 function requires aspartyl phosphorylation, and predicts that the phosphorylated form is functional in the regard of cytokinin signaling. Taken together, phosphorylation is a common molecular strategy utilized by the ARRs to elicit their output responses.

Mechanistically, there are two general models by which type-A ARRs can act to negatively regulate cytokinin signaling. The type-A ARRs may act like a sponge (or 'sink') by absorbing the phosphoryl groups from the upstream AHPs, and thus compete in an antagonistic fashion with the type-B ARRs. A second model is that type-A ARRs may function as a phosphorylation-mediated switch through direct and/or indirect interactions with target proteins. The functionality of the phosphomimic form of ARR5 indicates that type-A ARR cannot act solely as phosphate sink, though these two models

are not mutually exclusive. The phosphorylation level of the receiver domain may promote activating and/or inhibitory protein-protein interactions, which determine the physiological responses. This predicts that the type-A ARRs interact with targets in a phosphorylation-dependent manner. To further explore this hypothesis, we have identified several candidate proteins that interact with type-A ARRs in a phospho-dependent manner through a yeast two hybrid (Y2H) screen. We have confirmed some of these interactions in an *Arabidopsis* protoplast system and have demonstrated that one of these interacting proteins acts as a positive element in cytokinin response.

Results and Discussion

Identification of phospho-specific type-A ARR interacting proteins

Studies in bacterial and eukaryotic two-component system have shown examples of target proteins that play important roles in integrating the signal outputs by interacting with specific response regulators [138-140]. We performed a pilot yeast two-hybrid screen with phospho-mimic forms of ARR4, ARR5, and ARR9 as baits. Novel proteins that interact with type-A ARRs were identified in addition to the known elements of the cytokinin signal transduction, primarily AHPs. The screening was done using a random-primed cDNA library with three type-A ARRs, and has not as yet been done exhaustively. Nevertheless, we have already identified several strong candidates, including a protein kinase, a transcription factor, and an exocyst subunit (Table 4.1), which will suffice to test and extend the hypothesis that the type-A ARRs interact with some targets in a phospho-dependent manner. These have been independently validated via Y2H re-screening in yeast and tested for specificity by direct yeast two-hybrid assays with the wild-type, phospho-mimic and phospho-insensitive versions of the bait ARR. Several of these positive clones interact preferentially with the phospho-mimic versions

Table 4.1 Type-A ARR interacting proteins

Name	AGI	Description	Interactor
ARID1	AT1G20910	ARID/BRIGHT-domain containing protein	ARR4
TPR8	AT4G08320	Tetratricopeptide repeat (TPR)-containg protein	ARR4
BPC6	AT5G42520	Transcription factor	ARR4
BSK1	AT4G35230	BR-signaling kinase	ARR5
EXO70D3	AT3G14090	Exocyst subunit	ARR5

of ARR4 and ARR5. These data suggest a model in which the interaction between target protein and specific response regulators is dependent upon the phosphorylation level of type-A ARRs.

Establishment of the protoplast system to validate the phospho-specific interactions *in vivo*

Transient transfection of *Arabidopsis* mesophyll protoplast is a rapid platform utilized to study hormone response, subcellular localization, and protein-protein interactions [141-144]. We established and optimized this approach to examine the subcellular localization of a variety of GFP fusion proteins. In addition, we generated Gateway®-compatible bimolecular fluorescence complementation (BiFC) vectors to validate the protein-protein interactions in this *in vivo* system. We fused the wild-type, phospho-mimic, and phospho-insensitive versions of ARR4 (ARR4, $ARR4^{DE}$, $ARR4^{DA}$) to both the N- and C-terminal of the YFP coding region (YFPn and YFPc, respectively). One of the ARR4-interacting candidate proteins (ARID1) was fused to both YFPn and YFPc. If two proteins interact in this assay, the physical interaction will bring the complementary halves of YFP (YFPn and YFPc) together and fluorescence will occur. We tested several combinations of interactions between type-A ARRs and candidate target proteins, and found that ARR4-YFPn interacts with ARID1-YFPc in the nucleus, preferentially in the presence of exogenous cytokinin (Fig. 4.1). Additionally, $ARR4^{DE}$-YFPn interacts with ARID1-YFPc constitutively above the level of their wild-type counterparts. On the contrary, $ARR4^{DA}$-YFPn interacted with ARID1-YFPc at a significantly reduced level, even in the presence of cytokinin. Together, these results support a model in which type-A ARRs function through phosphorylation-dependent

binding of target proteins. We will further quantify the results by co-transforming an organelle marker, and count the percentage of transformed cells that show a positive interaction. In addition, we will also examine the specificity of the interactions by testing the interaction between ARID1 with ARR3, the closest homologue of ARR4, and several other type-A ARRs. Further, there are at least two other ARIDs that contain the same domains as ARID1 (Figure 4.2) and we will examine whether they also interact with

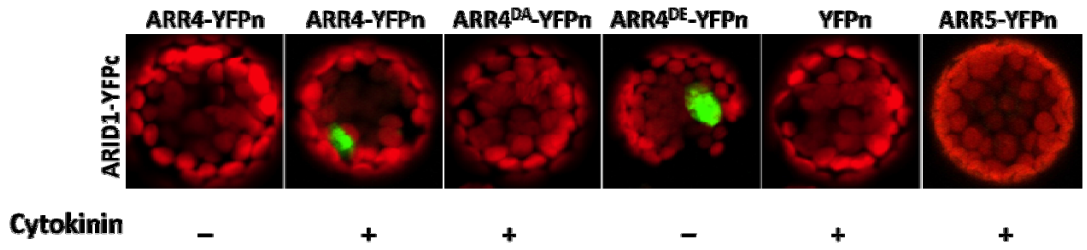

Figure 4.1. Bimolecular fluorescence complementation validates phospho-specific interaction between ARID1 and ARR4. All protoplasts were transfected with *ARID1-YFPc*. Co-transfected constructs are shown above each image while the addition of exogenous cytokinin (+) or a vehicle control (-) is indicated below each image. Interactions are revealed by YFP fluorescence in green, chlorophyll autofluorescence is shown in red. YFPc, C-terminal half of YFP; YFPn N-terminal half of YFP.

ARR4. Together, these data will define the specificity of the type-A ARR-ARID interaction.

Phylogenetic analysis of Arabidopsis ARID-containing proteins

The AT-rich interaction domain (ARID) has been found in many eukaryotic organisms including yeast, *Caenorhabditis elegans*, plants, *Drosophila*, and mammals [145, 146]. ARID-containing proteins are known to be involved in development by functioning as transcription activators and repressors. The extensive sequence data now available shows that the ARID-containing proteins have diverse sequences, structure, size, and predicted functions outside the ARID region. ARID1, the interacting target of ARR4, is

composed of one ARID/BRIGHT DNA-binding domain and one alpha-crystallin domain (ACD). In Arabidopsis, there are ten ARID-containing proteins, yet only three include ARID and ACD domains: *ARID1* (AT1G20910), *ARID2* (AT1G76510), and *ARID3* (AT2G17410) (Figure 4.2). In addition, ARID3 has a longer N-terminal sequence compared with ARID1 and ARID2 and is thus phylogenetically more distant from ARID1 and ARID2. Furthermore, the phylogenetic analysis based on the amino acid sequences of the ARID domains is consistent with that of the whole protein structure (Figure 4.2) Therefore, the following functional analyses have been focused on the three closest homologous of ARID family members with similar domain structures.

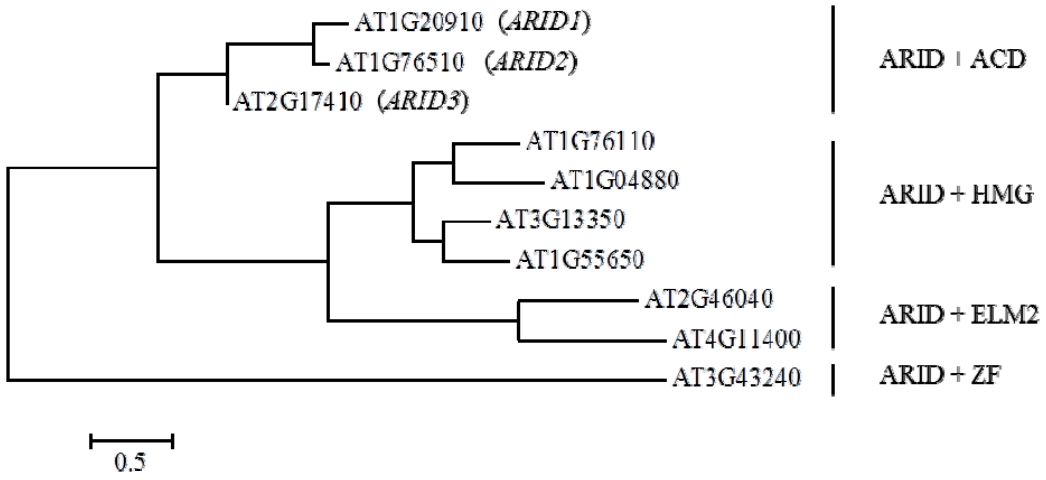

Figure 4.2. . Phylogenetic relationship of ARID-containing proteins in Arabidopsis. The amino acid sequences of the ARID domains were aligned using ClustalW. Gapped regions that were poorly conserved were first removed from each alignment. The phylogenetic trees were generated with MEGA5.1. ACD, alpha-crystallin domain; HMG, high-mobility group; ELM2, Egl-27 and MTA1 homology 2 domain; ZF, PHD-type zinc finger. Scale bar indicates substitutions per site.

Loss of *ARID1* and *ARID2* may cause zygotic lethality

To investigate the function of *ARIDs* in Arabidopsis, we obtained T-DNA insertion lines *arid1-1* (SALK_141443), *arid2-1* (SALK_007400), *arid2-2* (SALK_035980), *arid3-1* (SALK_026385), and *arid3-2* (SALK_104394) from the

ABRC stock center. The single mutants were indistinguishable at all stages when compared with wild-type counterparts (data not shown). We have been able to generate *arid2-2 arid3-1* and *arid1-1 arid3-2* double mutants, but have not been able to isolate *arid1 arid2* double mutant. The *arid 1-1 arid 2-1/ARID2* line displays a dwarf phenotype with retarded shoot growth; however, the progeny from selfed *arid 1-1 arid 2-1/ARID2* are all *arid1-1 arid2-1/ARID2* (*n*=62). The distorted segregation may imply the chromosomal rearrangement resulting from the insertion events and the dwarf phenotype might not be the result of loss-of-function of *ARID* genes, but rather to a second mutation in one the alleles, or to a chromosome rearrangements (s). To test this, I have attempted to generate an *arid1-1 aird2-2* double mutant line that harbors a different *arid2* allele. From the selfed *arid1-1/ARID1 arid2-2* plant, we found 15/22 of the F_1 progeny are *arid1-1/ARID1 arid2-2* and *7/22* are *arid2-2*. The segregation follows Mendelian ratio and the lack of *arid1-1 arid2-2* may due to zygotic lethality. I will perform complementation experiments to confirm the lethality is caused by the loss of *ARID* function, and will characterize the developmental defect in the double mutant. Introducing second allele *arid1-2* into *arid2-2* background will also address the same question.

Overexpressing ARID-GFP transgene results in retarded shoot development

We have generated gain-of-function mutants by transforming Arabidopsis with the *ARID* gene fused to a GFP reporter under the control of the constitutive *35S* promoter of Cauliflower mosaic virus. Five independent transgenic lines were obtained with a single insertion and examined in T3 homozygous plants. After five weeks of growth, two transgenic lines showed retarded shoot growth and early senescence while three lines

appeared slightly smaller than wild-type counterparts (Figure 4.3A). The different phenotypic strength of these lines correlates with the expression level of the transgene as determined by immunoblotting assay (Figure 4.3B).

We also performed seedling root elongation assay to further explore cytokinin responses in these transgenic lines. We found that the strong overexpressors are hypersensitive in primary root elongation (Figure 4.3C), suggesting that ARID1 acts as a positive regulator in cytokinin response. To confirm this, we will examine the expression of cytokinin primary response genes in ARID overexpressing lines upon cytokinin

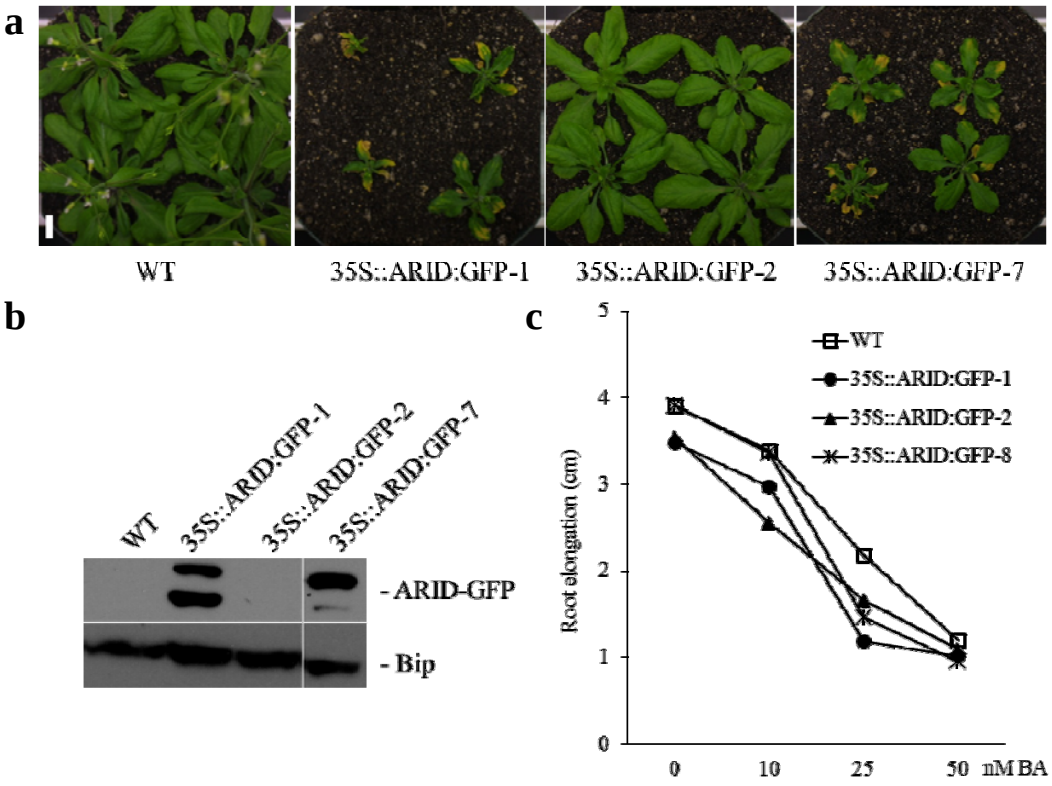

Figure 4.3. Adult phenotype of transgenic plants overexpressing ARID-GFP transgene. (a) Five-week-old wild type and three independent 35S::ARID:GFP lines. The phenotypic strength is corresponding to the expression level of the transgene as detected by immunoblotting in (b). Note the weakest line 35S::ARID:GFP-2 is slightly smaller than wild-type counterparts. Scale bar, 1 cm. (b) Different expression level of the ARID-GFP transgene in three overexpressor lines. The lower band may be the degraded product but needs further confirmation. (c) Primary root elongation in response to exogenous cytokinin (BA) treatment. The strongest line 35S::ARID-1 displays the strongest hypersensitivity to 25nM BA.

treatment. The next challenge is to elucidate the mechanisms by which the interaction between ARR4 and ARID1 affects the cytokinin output responses. One possibility is that ARID1 and type-B ARRs form a transcription complex whose function is hindered by the interaction between ARID1 and ARR4. Preliminary data show that ARID1 interacts with two of the type-B ARRs in protoplast system, and it would be of interest to examine whether the addition of ARR4 has a inhibitory effect on this interaction.

BPC6 belongs to a GAGA-binding transcription factor family

Another ARR4 interacting protein, BASIC PENTACYSTEIN (BPC) 6, is a member of a land plant-specific transcription factor family that preferentially binds to GAGA-motifs in regulatory region of genes [147]. There are seven *BPC* genes in Arabidopsis that can be subdivided into three classes: class I, *BPC1*, *BPC2*, and *BPC3*; class II, *BPC4, BPC5,* and *BPC6*; class III, *BPC7* [147]. BPC6 can heterodimerize with BPC4, which also belongs to class II, but not with BPC1 that belongs to class I [148]. Phenotypically, mutation in multiple *BPC* genes results in pleiotropic effects, suggesting they might be defective in hormone signaling [149]. It has been reported that *bpc1-1 bpc2 bpc4 bpc6* exhibits wild-type responses in multiple hormone responses including auxin, gibberellin, and abscissic acid [149]. This quadruple *bpc* mutant is slightly insensitive to ethylene [149], but cytokinin sensitivity was not examined in this report. Mechanistically, a recent study has shown the regulatory model by which BPC1 mediates downstream gene expression. The binding of BPC1 to the *cis*-element of direct target gene *SEEDSTICK (STK)* is essential for recruiting the transcription repressor complex [150]. Therefore, mutations of the GA-consensus sequences in the promoter region of *STK* disable the regulation from the repressor complex and result in ectopic expression of *STK* [150].

To further explore whether BPC6 is involved in cytokinin signaling as well as the biological meaning of its interaction with ARR4, I first examined whether cytokinin response is affected in *bpc4 bpc6* plants which have mutations on all class II *BPC* loci. I examined the cytokinin response in flowers as *BPC* genes have been reported to regulate ovule identity gene *STK* and integument morphology gene *INNER NO OUTER* [148, 150]. Interestingly, several cytokinin-induced genes, including primary response type-A *ARR7* and *ARR15* as well as cytokinin oxidase *CKX3*, are less-induced in *bpc4 bpc6* plants (Figure 4.4). The reduced induction is similar to the cytokinin receptor double mutant *ahk2 ahk3*, suggesting BPC4 and BPC6 may play a positive role in the cytokinin primary response network in flowers. We will extend the examination to other developmental stages, i.e. seedling shoot and root, rosette leaves, to further explore whether the effect is stage- and/or tissue- specific. It is tempting to hypothesize that BPC4 and BPC6, acting similarly to BPC1, may facilitate the activity of type-B ARRs in inducing cytokinin regulated genes. The interaction with ARR4 may have repressing effect on the role of BPC6 as a transcription facilitator, although follow-up experiments are needed to verify this hypothesis.

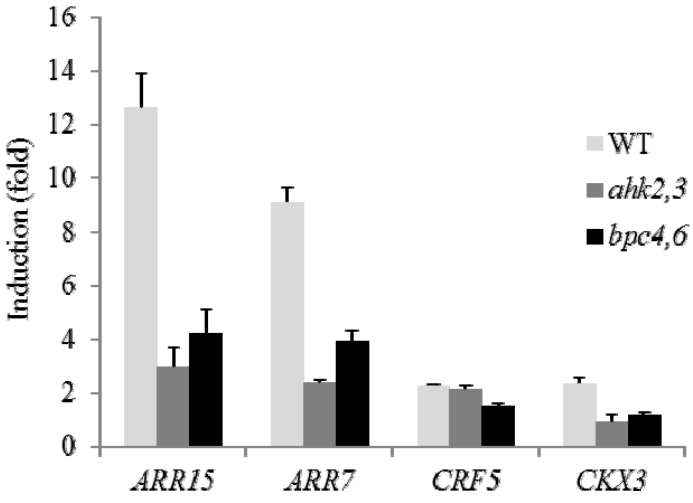

Figure 4.4. Reduced cytokinin response in *bpc4 bpc6* flowers. Six-week-old plants were sprayed with 50 μM BA for one hour and flower buds were collected for RNA isolation. qRT-PCR was performed using gene-specific primers for several known cytokinin-responsive genes. The reduced induction of cytokinin-responsive genes in *bpc4 bpc6* is comparable to cytokinin receptor double mutant *ahk2 ahk3*. Induction is normalized to TUB4. Error bars, SD.

Experimental procedures

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) is the wild type. *arid1-1* (SALK_141443), *arid1-2* (SALK_022359), *arid2-1* (SALK_007400), *arid2-2* (SALK_035980), *arid3-1* (SALK_026385), and *arid3-2* (SALK_104394) were obtained from the Arabidopsis Biological Resource Center (ABRC, http://abrc.osu.edu). For protoplast experiments, the plants were grown under the conditions as previously described [62].

Plant transformation

The coding sequence of *ARID1*(AT1G20910) was cloned into pENTR-D entry clone vector followed by LR reaction to Gateway destination vector pK7WGF2 [151] to generate 35S::ARID:GFP T-DNA construct. Transformed progeny were selected by germinating T_1 seeds on MS medium containing selective antibiotics (50 μg/mL kanamycin). Total proteins were extracted from T_3 seedlings with 2× SDS sample buffer and subjected to immunoblotting assay to detect fusion proteins. Anti-GFP antibody (Roche) and Pierce West Pico Chemiluminescence substrate (Thermo Scientific) were used for immunoblotting assay.

Protoplast isolation and transformation

Protoplast were isolated and transformed as described previously [152] except that 5μg of plasmid DNA were used for transformation. To construct the plasmids for transformation, coding sequences of genes of interest were cloned into Gateway entry clones and transferred into Gateway-compatible destination clones pUC-gw-SPYNE and pUC-gw-SPYCE [62].

Primary root elongation assay

Arabidopsis seeds were germinated on plates containing 1 × Murashige and Skoog (MS) salts supplemented with 0.05% 2-(N-Morpholino)-ethanesulfonic acid (MES) (Research Products International Corporation, http://www.rpicorp.com), 1.0% sucrose, and 0.6% phytoagar. Seedlings were transferred to vehicle (0.01% DMSO) or BA plates at 4 day after germination (dpg) and root lengths were marked on the plates. Root elongation between 4 and 9 dpg was traced and measured using the ImageJ program (http://rsb.info.nih.gov/ij).

Real-time PCR analysis of cytokinin response

Six-week-old plants were sprayed with vehicle (0.01% DMSO supplemented with 0.05% Silwet) or 50 μM BA. Unfertilized flower buds were collected one hour after the spray and total RNA was isolated using Qiagen RNeasy kit following the manufacturer's protocol (http://www.qiagen.com/). 1 μg of RNA were reverse transcribed using the Invitrogen SuperScript III kit (Invitrogen) following the manufacturer's protocol, and 1 μl of the reaction mixture was used for real-time PCR using SYBR Premix Ex Taq (http://www.takara-bio.com/) in a volume of 10 μl. The PCR reaction mixture consisted of 1 μl cDNA, 0.5 μM primers, and 1 × Master Mix and was run on a ViiATM 7 Real-Time PCR system (ABI, http://www.appliedbiosystems.com).). The primers for real-time PCR are as follows: ARR7 (5'- TCTCTTCTTGTAAAGTGACGACTG -3' and 5'- TCA AATTCACCTTCAAATCCTT -3'); ARR15 (5'- GAGATTGCTTAAGATCTCTG GTTG -3' and 5'- CAAATCCTTAAGACCAGAAGATCC -3'), CKX3 (5'- TCTCAAT ACACA GTCAACGAGGA -3' and 5'- TCGTACATAAACCCTCTTACATGG -3'),

CRF5 (5’- GATGACGAACCTAAACCGGCG -3’ and 5’- CCAGAGTCTAGTACGAC TCG -3’).

Chapter 5: Discussion

Sporophytic signals in specifying gametophytic cell fate

One of the features of sexual reproduction in plants is the alternation between haploid gametophytic and diploid sporophytic generations. In higher plants, the sporophytes dominate while the gametophytes only represent a transient phase of the life cycle. With only seven cells, the female gametophyte in higher plants is a highly reduced organism that has become a unique platform for the study of fundamental developmental processes, including three sequential cell fate determination events: the designation of germ line, the selection of functional megaspore, and cellularization of the female gametophyte. During ovule development, one of the hypodermal cells in the sporophytic tissue adopts germ cell fate and differentiates into megaspore mother cell. Among the four meiotic products, one survives and becomes the functional megaspore. The female gametophyte contains seven clonally derived cells that ultimately differentiate into four distinct cell types: three antipodal cells, one central cell, one egg cell, and two synergid cells. The important role of the female gametophyte in plant reproduction has drawn research attention on the molecular and genetic mechanisms underlying the tight regulation of cell specification. Although our understanding in this regard remains fragmentary, sporophytic signals appear to play essential roles regarding each cell fate specification process. Firstly, the master transcription factor *SPOROCYTELESS (SPL)* is required in the sporophyte for designating megaspore mother cell formation [115]. Secondly,

ARGONAUTE 9 (AGO9) in the sporophytic cells acts non-cell-autonomously to restrict single gamete lineage through small RNA silencing pathways [105]. Thirdly, the polarized auxin signal generated in the micropylar end of the sporophyte is required for cellularization along the micropylar-chalazal axis in female gametophyte [107]. Further, our work shows the sporophytic cytokinin signaling directs the functional megaspore specification from the chalazal-most megaspore [47].

A polarized auxin signal has been suggested to act as a morphogen that orchestrates apical-basal pattern formation of the ovule [153]. It was recently found that disruption of the asymmetrical auxin gradient perturbs the cell fate specification along the micropylar-chalazal axis in female gametophyte [107]. Moreover, ectopic auxin biosynthesis was sufficient for cells in the positions of antipodal cells to express egg cell or synergid cell marker [107]. This finding indicates that the functional megaspore-derived eight nuclei are capable of adopting any cell type, and positional information is critical for the specification. Although this auxin gradient was reported to affect cellularizaion, the auxin maximum was observed in the micropylar end as early as the pre-meiotic megaspore mother cell[107]. Interestingly, a two-component signal sensor (TCS), an artificial reporter that reflects the transcriptional activity of type-B ARRs and presumably cytokinin signaling, shows polarized expression pattern complementary to the auxin maximum [154]. The expression pattern of the TCS::eGFP is similar to what we have observed for cytokinin receptors and the type-A *ARR, ARR4*. One model consistent with this is that auxin and cytokinin act in concert to specify cell fate specification in the developing ovule: auxin signals the micropylar megaspores to degenerate while cytokinin signals the chalazal one to adopt functional megaspore fate (Figure 5.1). This is

reminiscent of auxin-cytokinin interactions, synergistically or antagonistically, in regulating many developmental processes. Our preliminary experiments have found that ubiquitous cytokinin signaling in wild-type background is insufficient to induce extra functional megaspore formation (data not shown). Therefore, ectopic cytokinin signaling in the micropylar end, driven by a micropylar-specific promoter in an auxin deficient mutant background is required to verify this hypothesis.

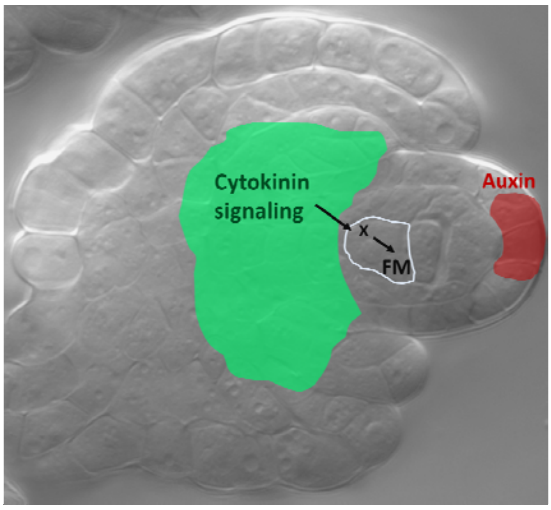

Figure 5.1 The hypothetic model of cytokinin and auxin signalings regulate functional megaspore pecification. Cytokinin signaling enriched in the chalaza is required for functional megaspore specification. In this model, we hypothesize that the opposing auxin signaling elicits the degeneration of the micropylar megaspores. The concerted auxin and cytokinin signalings are responsible for the cell fate determination in the developing ovule.

The role of cytokinin two-component elements in ovule development

The role of cytokinin in female gametophyte development was unknown when I started working on this project in 2009. Three papers had been published describing cytokinin receptor triple mutants, all reported as being null alleles, yet with obviously different results in regards to female gametophyte: two alleles were completely infertile while one maintained marginal fertility[13, 14, 18]. In addition, mutations in *ARR7* and

ARR15, two of the type-A *ARRs*, were reported in two Nature papers to cause female gametophytic and embryonic lethality[79, 80]. However, a former graduate student (Jennifer To) was able to isolate a quadruple type-A *arr* mutant *arr5 arr6 arr7 arr15*, provoking us to explore the antagonistic effect of *ARR5/6* on *ARR7/15*. In the following year, several pieces of data from segregation studies and genotyping analyses that I performed suggested that the gametophytic lethality in *arr7 arr15*, male and female, observed was due to chromosomal translocation resulting from the T-DNA insertional events into the *ARR7* and *ARR15*. Firstly, the gametes aborted after meiosis in approximately 50% of the gametes, which is the diagnostic feature of chromosomal translocation. Secondly, this phenotype was present in the single heterozygous mutants (*arr7/ARR7* and *arr15/ARR15*) but not in single homozygous mutants, suggesting it was caused by the nature of the alleles rather than loss of gene function. Thirdly, two different double mutants harboring independent alleles (*arr7-1 arr15-1* and *arr7-1 arr15-2*) were obtained and found to be fully viable [73]. Overall, we concluded that *ARR7* and *ARR15* are not essential for female gametophyte development, nor in fact for seedling viability.

Our work here shows that the various strengths of phenotypes observed in different combinations of receptor mutants was due to the nature of each mutant allele rather than the growth condition and handling [47]. We further demonstrated that cytokinin signaling acting through the AHKs-AHPs-type-B ARRs in the sporophyte is required for functional megaspore specification [47]. The non-receptor histidine kinase CKI1 has been reported to play an essential role in megagametogenesis after the functional megaspore has completed two rounds of mitosis [109, 110]. CKI1-dependent two-component signaling acts as a shortcut in the gametophyte to substitute for cytokinin signaling as cytokinin per

se is not required in the gametophyte. The advantage of adopting CKI1-AHP-type-B ARRs pathway over AHK-AHP-type-B ARRs pathway in the gametophyte is not clear.

Our work reveals that sporophytic cytokinin signaling provides the positional information to direct functional megaspore specification. An unknown factor emanating from the chalazal cytokinin signaling acts non-cell autonomously in the gametophyte to elicit this function. Our data have excluded two groups of cytokinin response genes, type-A *ARRs* and *CRFs*, as the gametophyte determining factor based on segregation analyses and phenotypic studies. The defect may first occur in the pre-meiotic mother cell and/or surrounding sporophytic cells which, although morphologically indistinguishable from wild type, are insufficient to support the functional megaspore specification. In line with this hypothesis, increased cell division as revealed by cyclinB:GUS expression in the sporophytic cells of *ahp2,3,5* was observed as early as megasporogenesis when megaspore mother cell was specified (Figure 3.1). Whether the increased cell division in the chalazal sporophytic cells is causative to the gametophytic defect is unknown. One approach for future direction is to compare the transcriptome profile of the sporophytic cells surrounding the megaspore mother cell between wild type and cytokinin receptor mutant using Laser capture microdissection. The genes down-regulated in the mutant may reveal the nature of the cell autonomous factor that directs functional megaspore specification.

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