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Source: The Arabidopsis Book, 2014(12)

Published By: The American Society of Plant Biologists

URL: https://doi.org/10.1199/tab.0168

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First published on January 13, 2014: e0168. doi: 10.1199/tab.0168
This is an updated version of a chapter originally published on March 27, 2002, e0063. doi:10.1199/tab.0063

# Cytokinins

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**Cytokinins are *N*[6] substituted adenine derivatives that affect many aspects of plant growth and development, including cell division, shoot initiation and growth, leaf senescence, apical dominance, sink/source relationships, nutrient uptake, phyllotaxis, and vascular, gametophyte, and embryonic development, as well as the response to biotic and abiotic factors. Molecular genetic studies in Arabidopsis have helped elucidate the mechanisms underlying the function of this phytohormone in plants. Here, we review our current understanding of cytokinin biosynthesis and signaling in Arabidopsis, the latter of which is similar to bacterial two-component phosphorelays. We discuss the perception of cytokinin by the ER-localized histidine kinase receptors, the role of the AHPs in mediating the transfer of the phosphoryl group from the receptors to the response regulators (ARRs), and finally the role of the large ARR family in cytokinin function. The identification and genetic manipulation of the genes involved in cytokinin metabolism and signaling have helped illuminate the roles of cytokinins in Arabidopsis. We discuss these diverse roles, and how other signaling pathways influence cytokinin levels and sensitivity though modulation of the expression of cytokinin signaling and metabolic genes.**

## INTRODUCTION

In the 1940s and 1950s, a wide variety of substances ranging from yeast extract to tomato juice were identified that could, in combination with auxin, initiate and sustain the proliferation of normal plant stem tissues in culture. Coconut milk, which is liquid endosperm, was found to have the strongest positive effect (Caplin and Steward, 1948), indicating that it contained a substance that could stimulate cell division. In the 1950s, Folke Skoog and Carlos Miller found that autoclaved herring sperm DNA was a potent activator of the proliferation of cultured tobacco pith cells (Miller et al., 1955; Miller et al., 1956). They identified an adenine derivative, *6*-furfurylaminopurine, as the active compound and named it kinetin. In the presence of auxin, kinetin stimulated tobacco pith parenchyma tissue to proliferate in culture. Later, zeatin was identified as the first naturally occurring cytokinin in immature maize endosperm (Letham, 1973), and turns out to be the predominant cytokinin in coconut milk.

Since their discovery, cytokinins have been implicated in almost all aspects of plant growth and development, including cell division, shoot initiation and growth, leaf senescence, apical dominance, sink/source relationships, nutrient uptake, phyllotaxis, and vascular, gametophyte, and photomorphogenic development (Mok and Mok, 1994; Argueso et al., 2012; Hwang et al., 2012). Further, cytokinins play important roles in the interaction with both biotic and abiotic factors (Argueso et al., 2012). Naturally occurring cytokinins are adenine derivatives with distinct substitutions attached to the *N*[6] position of the adenine ring (Figure 1). The most common class of cytokinins have isoprenoid side chains, including the most abundant cytokinin in Arabidopsis, *trans*-zeatin (*t*Z). In higher plants, zeatin occurs in both the *cis* and the *trans* configuration. The *trans* form is an active cytokinin in all plant species; the *cis* form (*c*Z) is present ubiquitously in plants (Gajdošová et al., 2011), but is active only in a subset, and in Arabidopsis, the *cis* form shows at most only limited cytokinin activity (Spichal et al., 2004; Romanov et al., 2006; Heyl et al., 2012). In addition to the free bases, which are the active forms, cytokinins are also present as ribosides (in which a ribose sugar is attached to the *N*[9] nitrogen of the purine ring) and ribotides (in which the ribose moiety contains a phosphate group). Further, cytokinins can also be conjugated to sugar moieties, most commonly glucose.

## BIOSYNTHESIS

Mature tRNAs from most organisms, including plants, contain *c*Z as a modified base. Thus, the breakdown of tRNA was originally suggested as a possible mechanism for cytokinin biosynthesis (Vremarr et al., 1972) as the released *c*Z was thought be converted to active *t*Z by zeatin isomerase (Mok and Mok, 2001). However, more recent results suggest that there is little interconversion between the *cis* and *trans* forms of zeatin (Gajdošová et al., 2011). Further, the slow turnover rate of tRNA is likely insufficient to account for the amount of cytokinins present in plants. Thus,

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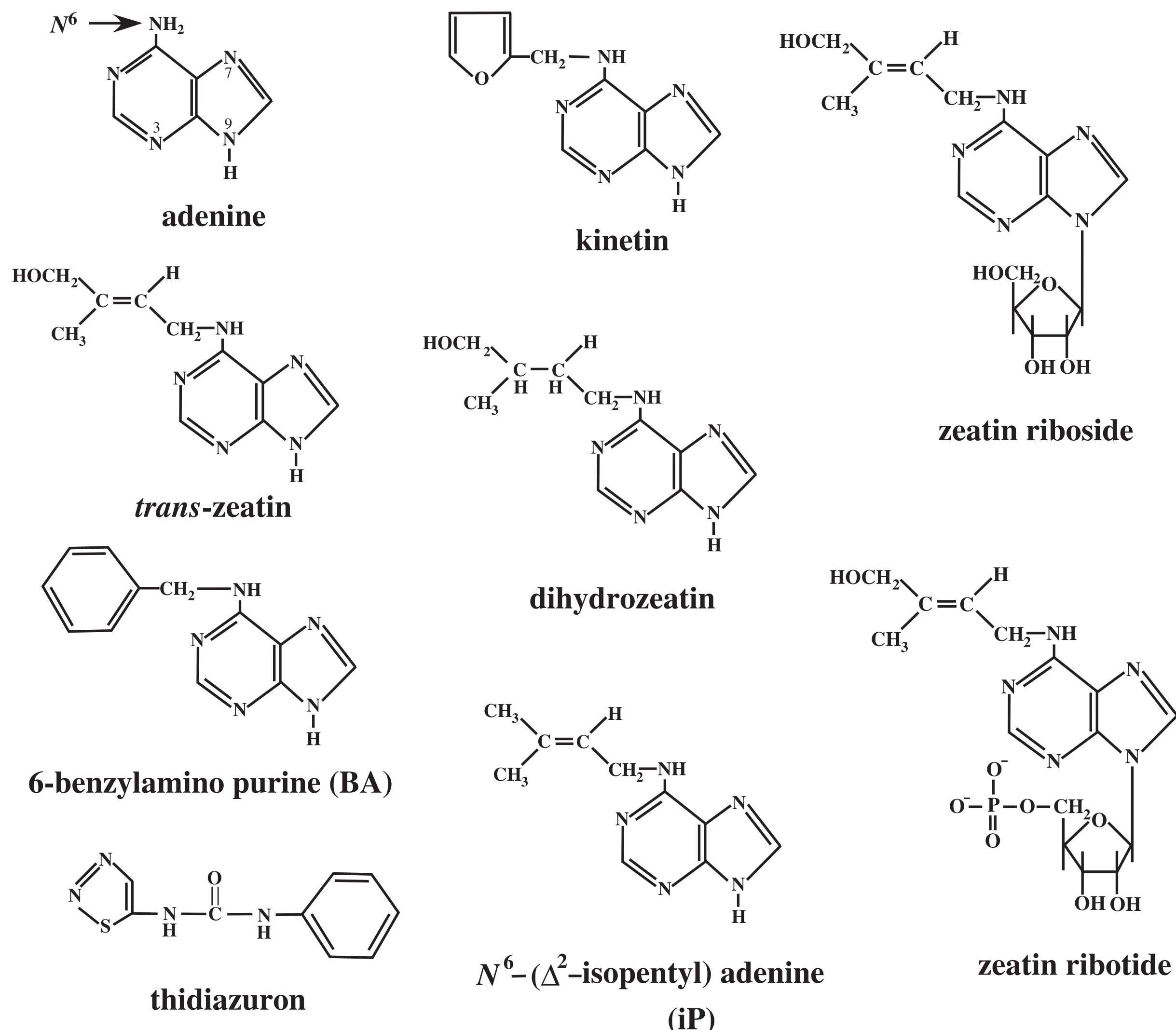


**Figure 1.** Structures of cytokinins.

Adenine is the parent compound of naturally occurring cytokinins, though it does not activate cytokinin responses; the $N^6$ position is indicated with an arrow. *trans*-zeatin is the most abundant cytokinin in Arabidopsis, and the free base as well as the riboside and ribotide forms are shown. Kinetin is an artificial, aromatic cytokinin and benzyladenine is an example of a naturally occurring aromatic cytokinin. Thidiazuron is a diphenylurea-type cytokinin.

with the possible exception of *c*Z, tRNA likely does not contribute to cytokinin biosynthesis.

An enzymatic activity that converts AMP and dimethylallyl pyrophosphate (DMAPP) to the active cytokinin iPMP ($N^6$-($\Delta^2$-isopentenyl)adenosine-5'-monophosphate) was first identified in *Dictyostelium discoideum* (Taya et al., 1978). Subsequently, the *ipt* gene (for isopentenyltransferase) from *Agrobacterium tumefaciens* was shown to encode an enzyme with similar activity (Akiyoshi et al., 1984). *ipt* genes have also been identified in several other bacteria, and IPT activity was detected in crude extracts from a variety of plant tissues, but the plant enzymes were not purified.

*In silico* searches of the sequence of the Arabidopsis genome revealed the presence of nine *ipt*-homologues, designated as *AtIPT1* to *9* (Table 1). Phylogenetic analysis suggested that *AtIPT2* (At2g27760) and *AtIPT9* (At5g20040) encode putative tRNA-IPTs while the other seven *AtIPTs* formed a distinct clade more closely related to the bacterial *ipt* gene. Consistent with this, whereas disruption of *AtIPT2* and *AtIPT9* led to a decrease in the level of *c*Z, but not *t*Z, disruption of the other seven *IPT* genes resulted in decreased levels of *t*Z and its derivatives. The expression of these seven genes (but not *AtIPT2*) in *E. coli* resulted in the secretion of the cytokinins iP (isopentenyladenine) and zeatin, confirming that they encode cytokinin biosynthetic

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enzymes (Takei et al., 2001a). Additionally, calli overexpressing *AtIPT4* (At4g24650) under the control of the CaMV 35S promoter regenerated shoots even in the absence of cytokinin, while CaMV 35S::*AtIPT2* calli were still dependent on exogenous cytokinin (Kakimoto, 2001). Surprisingly, unlike the bacterial *ipt* enzymes, purified AtIPT4 utilized ATP and ADP preferentially over AMP as a substrate (Kakimoto, 2001). Several of the *AtIPT* genes display distinct, tissue-specific patterns of expression, suggesting likely sites of cytokinin production (Miyawaki et al., 2004; Takei et al., 2004b).

There are two possible origins for the isoprenoid side chain on *t*Z. In one pathway, IPT uses DMAPP produced from the methylerythritol phosphate (MEP) pathway, which occurs in the plastid. This would yield iP ribotide, which is then hydroxylated to produce *trans*-zeatin (Figure 2). A second pathway utilizes the mevalonate (MVA) pathway, which is inhibited by lovastatin, to produce the DMAPP precursor. A dual labeling study indicated that most *trans*-zeatin in Arabidopsis is produced through the MEP pathway (Kasahara et al., 2004).

The initial products of the plant enzyme are iPRTP (isopentenyladenosine-5'-triphosphate) and iPRDP (isopentenyladenosine-5'-diphosphate), which can be subsequently converted to *t*Z by hydroxylation of the isoprenoid side chain by a cytochrome P450 enzyme (Figure 2). The genes encoding these cytokinin *trans*-hydroxylase enzymes were identified in Arabidopsis by a screen employing an (*AtIPT4*)/*P450* co-expression system in *Saccharomyces cerevisiae* (Takei et al., 2004a). Various candidate cytochrome P450s were expressed in this strain and two paralogous genes, CYP735A1 (At5g38450) and CYP735A2 (At1g67110) were identified that encode proteins that catalyze the conversion of iP nucleotides, but not the nucleoside or free base forms, to produced *tZ* nucleotides.

The conversion of the cytokinin ribotides to their active, free base forms was thought to occur via a two-step pathway (Chen and Kristopeit, 1981a, b). However, more recent results suggest that cytokinin ribotides are primarily directly converted into the free-base cytokinins by the LONELY GUY (LOG) family of enzymes. These were first identified in rice by a genetic screen for defects in the maintenance of shoot meristems (Kurakawa et al., 2007). In Arabidopsis, there are seven genes that encode active LOG enzymes (Table 1) (Kuroha et al., 2009). Disruption of multiple *LOG* genes leads to severe retardation of shoot and root growth and defects in the maintenance of the apical meristems, with LOG7 (At5g06300) and LOG4 (At3g53450) playing the major role in the shoot apical meristem (SAM) and LOG3 (At2g37210) and LOG4 in the root (Tokunaga et al., 2012). *LOG4* expression is restricted to the L1 layer of the SAM and to the floral meristem in Arabidopsis, and has been hypothesized to be involved in the synthesis of apically derived active cytokinins in the SAM proper (Chickarmane et al., 2012) (see below).

**Table 1.** Genes involved in cytokinin biosynthesis

| IPT genes | AGI Codes | Location | Notes |
|---|---|---|---|
| *IPT1* | At1g68460 | chloroplast | |
| *IPT2* | At2g27760 | cystol | tRNA isopentyl transferase |
| *IPT3* | At3g63110 | chloroplast | upregulated by nitrate; expressed primarily in phloem |
| *IPT4* | At4g24650 | cystol | |
| *IPT5* | At5g19040 | chloroplast | |
| *IPT6* | At1g25410 | not yet determined | Pseudogene in some accessions |
| *IPT7* | At3g23630 | mitochondria | Induced by STM in SAM |
| *IPT8* | At3g19160 | chloroplast | |
| *IPT9* | At5g20040 | not yet determined | tRNA isopentyl transferase |
| Cytokinin trans-hydroxylase genes | | | |
| *CYP735A1* | At5g38450 | not yet determined | |
| *CYP735A2* | At1g67110 | not yet determined | |
| Cytokinin nucleoside 5' monophosphate phosphoribohydrolase genes | | | |
| *LOG1* | At2g28305 | Cystol and nucleus | |
| *LOG2* | At2g35990 | Cystol and nucleus | |
| *LOG3* | At2g37210 | Cystol and nucleus | |
| *LOG4* | At3g53450 | Cystol and nucleus | |
| *LOG5* | At4g3519 | Cystol and nucleus | |
| *LOG7* | At5g06300 | Cystol and nucleus | |
| *LOG8* | At5g11950 | Cystol and nucleus | |

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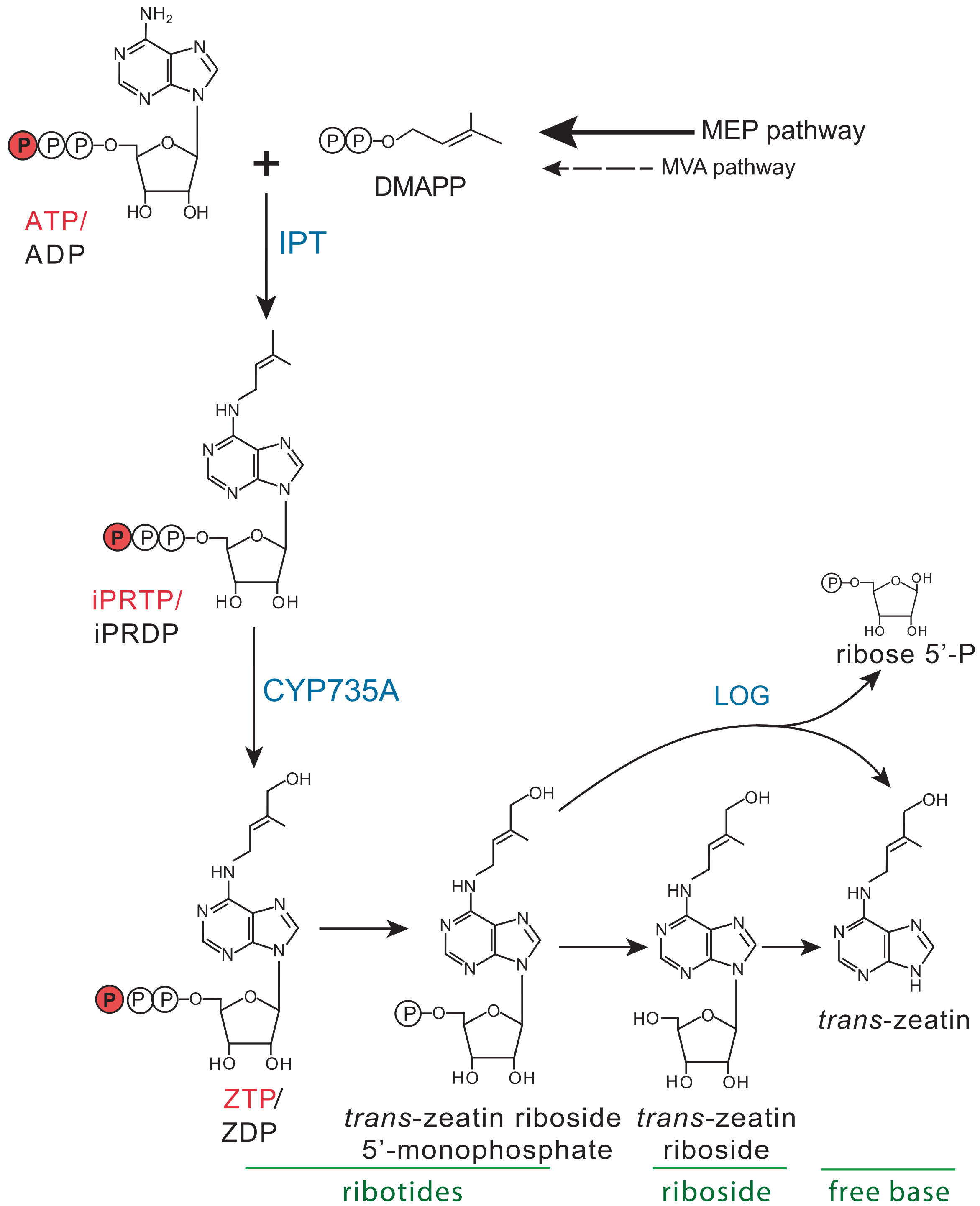


**Figure 2.** Proposed biosynthetic and metabolic pathway for cytokinins.

The proposed biosynthesis of *trans*-zeatin tri-/diphosphate in Arabidopsis is shown. Both ADP and ATP are likely substrates for the plant IPT enzyme, and these and their di- and tri-phosphate derivatives are indicted together (e. g. ATP/ADP). See text for more details.

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## METABOLISM

The levels of active cytokinins in a cell can be decreased through either conjugation to glucose or through irreversible cleavage by cytokinin oxidases. *N*-glycosylation of cytokinin occurs primarily on the nitrogens at positions $N^7$ or $N^9$ of the purine ring, and is thought to be irreversible (Figure 3). *O*-glycosylation occurs at the oxygen on the side chains of zeatin or dihydrozeatin (dhZ) (Figure 3). The *O*-glycosylated forms can be converted into active cytokinins by β-glucosidases (Brzobohaty et al., 1993) and thus the *O*-glycosylated cytokinins are thought to act as stable storage forms of the hormone. Glucosyl conjugates are inactive in bioassays and these conjugated cytokinins also fail to bind to the Arabidopsis AHK cytokinin receptors (Spichal et al., 2004).

In Arabidopsis, five genes encoding cytokinin glucosyl transferases have been identified (Table 2). Two (*UGT76C1* (At5G05870) and *UGT76C2* (At5g05860)) conjugate most cytokinin species on the $N^7$ and $N^9$ position of the adenine moiety; three encode proteins that *O*-glycosylate *t*Z and dhZ (Hou et al., 2004). Disruption of *UGT76C2* leads to a decrease in cytokinin *N*-glycosides and hypersensitivity to exogenous cytokinin, and overexpression has the opposite effects (Wang et al., 2011). Surprising, alteration of *UGT76C1* function did not cause any substantial effects on the growth and development of Arabidopsis, consistent with a lack of an effect on the level of free, active cytokinins (Wang et al., 2013). This is likely the result of compensatory changes in the expression of cytokinin signaling and metabolism genes to maintain the appropriate level of cytokinin function (Wang et al., 2013). Similarly, overexpression of *UGT85A1* (At1g22400) in Arabidopsis resulted in insensitivity to exogenous *t*Z and an increased level of *t*Z *O*-glucosides, with no effect on the level of free, active cytokinin or on growth and development (Jin et al., 2013). This suggests that plants have a fairly high capacity to buffer cytokinin function to an appropriate level in response to perturbation through changes in cytokinin metabolism and sensitivity.

Many plant tissues contain cytokinin oxidases, enzymes that cleave the $N^6$-side chains from a subset of cytokinins (Figure 3). *t*Z and iP have unsaturated $N^6$-side chains and are cleaved, while dihydrozeatin and BA are resistant to cytokinin oxidase cleavage. The free bases and their ribosides are the preferred substrates. Substitution of other functional groups on the purine ring, as well as *O*- and *N*-glycosylation, also prevents cytokinin oxidase cleavage (Schmülling et al., 2003; Werner et al., 2006). Thidiazuron and other synthetic urea-based cytokinins can non-competitively inhibit cytokinin oxidase activity, while exogenously-applied auxins can increase cytokinin oxidase activity (Bilyeu et al., 2001). Cytokinin oxidase irreversibly inactivates cytokinins, and could be important in regulating or limiting cytokinin effects.

Cytokinin oxidase was first cloned from *Zea mays* kernels (Houba-Hérin et al., 1999; Morris et al., 1999). The enzyme was

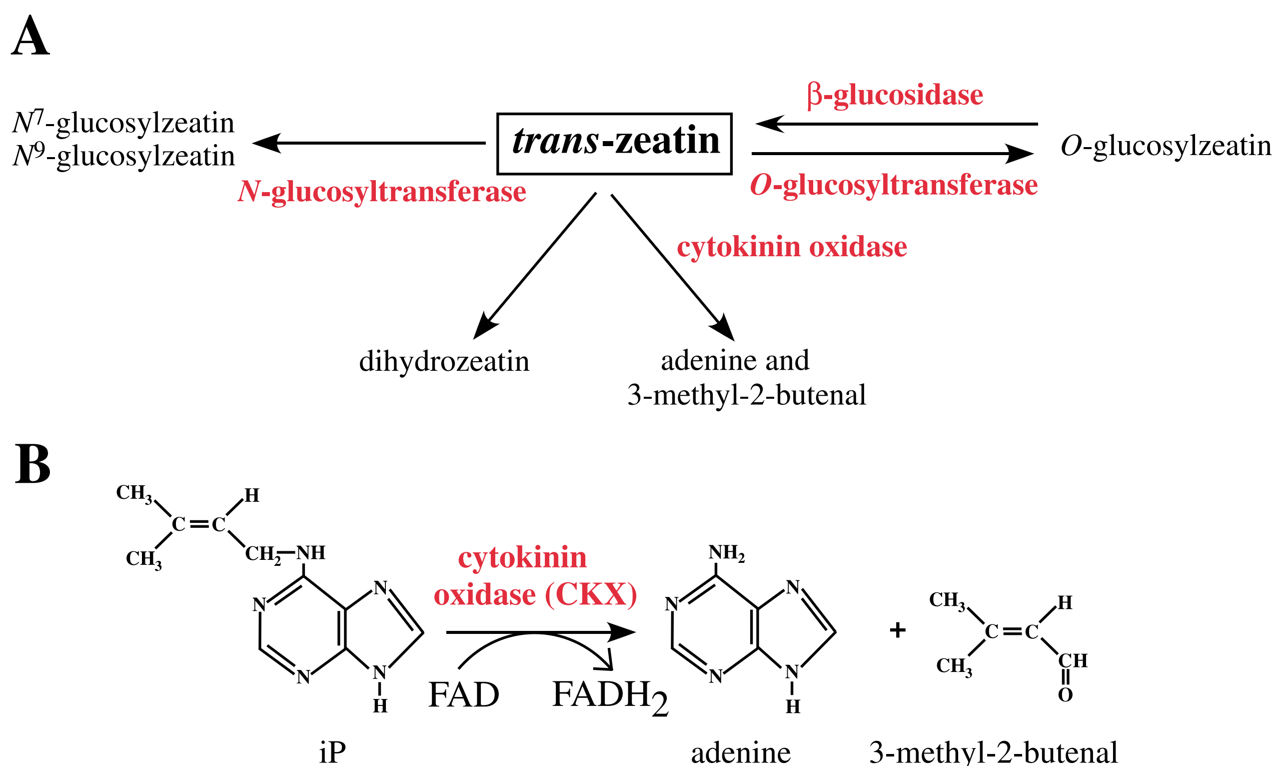


**Figure 3.** Metabolism of cytokinins.

**(A)** Metabolic fates of *trans*-zeatin. The enzymes catalyzing each reaction are shown in red.

**(B)** Reaction catalyzed by cytokinin oxidase enzymes.

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**Table 2.** Genes encoding cytokinin modifying enzymes

| Genes | AGI Codes | Location | Notes |
|---|---|---|---|
| Cytokinin Oxidases | | | |
| *CKX1* | At2g41510 | vacuole | |
| *CKX2* | At2g19500 | extracellular[1] | |
| *CKX3* | At5g56970 | vacuole | |
| *CKX4* | At4g29740 | extracellular (predicted) | |
| *CKX5* | At1g75450 | extracellular (predicted) | |
| *CKX6* | At3g63440 | extracellular (predicted) | |
| *CKX7* | At5g21482 | cystol (predicted) | may not be functional |
| Cytokinin glycosyltransferases | | | |
| *UGT76C1* | At5G05870 | not yet determined | *N*-Glycosylates all cytokinin species at $N^7$ and $N^9$ |
| *UGT76C2* | At5g05860 | not yet determined | *N*-Glycosylates all cytokinin species at $N^7$ and $N^9$ |
| *UGT85A1* | At1g22400 | cytoplasm and nucleus | *O*-Glycosylates tZ and dhZ |
| *UGT73C5* | At2g36800 | Chloroplast (predicted) | *O*-Glycosylates tZ and dhZ |
| UGT73C1 | At2g36750 | Chloroplast (predicted) | *O*-Glycosylates tZ and dhZ |

[1]The GFP fusion was localized to to the ER, but the protein is likely to be secreted

purified sufficiently to obtain peptide sequence and the gene was subsequently cloned using degenerate PCR. Cytokinin oxidases were previously thought to be copper-dependent amine oxidases, but the recombinant cytokinin oxidase from maize was found to be a flavoprotein. This and other data suggest that these enzymes are FAD-dependent amine oxidases. Heterologous expression in *Pichia* and *Physcomitrella* confirmed the predicted cytokinin oxidase activity.

The Arabidopsis genome encodes seven cytokinin oxidase genes (*CKX*) whose members show distinct patterns of expression, intracellular locations, and enzymatic properties (Table 2) (Schmülling et al., 2003; Kowalska et al., 2010). The expression of several *CKX* genes is induced rapidly upon cytokinin treatment (Bhargava et al., 2013). Overexpression of several *CKX* genes in transgenic Arabidopsis lines leads to a reduced level of endogenous cytokinin, resulting in a variety of developmental defects (see below).

## TRANSPORT OF CYTOKININS

Classically, it was thought that cytokinins were synthesized in roots and transported into shoots, but more recent studies indicate that cytokinins are made throughout the plant, including in aerial tissues (Sakakibara, 2006; Hirose et al., 2008; Kamada-Nobusada and Sakakibara, 2009). The *IPT* and *LOG* gene family members, which encode enzymes involved in cytokinin biosynthesis, are expressed in diverse locations in Arabidopsis, including aerial tissues. Long distance transport of cytokinins occurs in the xylem, primarily as *t*Z-riboside (*t*ZR), and in the phloem, primarily as iP type cytokinins (Kudo et al., 2010). Based on this vascular localization, it has been proposed that plants may use *t*ZR as a long-distance messenger for shootward transport and iP for rootward transport (Kudo et al., 2010). This model for cytokinin flow has been supported by grafting experiments between wild-type and *ipt* mutant lines (Matsumoto-Kitano et al., 2008).

Insight into the biological role of cytokinin transport was provided by feeding roots of nitrogen-depleted maize with nitrate (Takei et al., 2001b). In response to the applied nitrate, cytokinin first accumulated in the roots, subsequently in the xylem sap, and finally in leaves. The level of cytokinin observed in the xylem sap was sufficient to elevate the expression of a cytokinin primary response gene in detached leaves. In Arabidopsis, the *IPT3* (At3g63110) transcript accumulates in response to nitrates in roots, followed by accumulation of *t*ZR and *t*Z-ribotides (Miyawaki et al., 2004; Takei et al., 2004b). This suggests that altered nitrate levels in roots lead to changes in gene expression in the leaves as a result of transported cytokinin. Thus, cytokinins may represent a long-distance signal for nitrogen/nutrient-availability from the root to the shoot, presumably to coordinate shoot and root development.

Grafting experiments have been used to explore the biological significance of transported cytokinin. Initial reciprocal grafting experiments, employing wild-type and *ipt* transgenic tobacco plants, suggested that long-distance transport of cytokinins played a minimal role in signaling (Faiss et al., 1997). In the tobacco experiments, the phenotypic effects of elevated cytokinin were restricted to the part of the plant that was derived from the *ipt* overexpressing mutant. Elevation of cytokinin levels in the shoot led to a delay in leaf senescence, and at higher levels to stunted growth. In reciprocal grafting experiments, elevated levels of cytokinins in the root led to only a slight increase in cytokinin levels in the xylem, and had no phenotypic consequences in the scion. Thus, it was concluded that cytokinins may act primarily as paracrine signal, at least with respect to apical dominance and leaf senescence. More recent studies employing Arabidopsis mutants, however, support a model in which long-distance transport of cytokinins coordinates cytokinin responses between the shoot and root (Matsumoto-Kitano et

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al., 2008). For these studies, the *ipt1,3,5,7* quadruple mutant was used, which results in decreased levels of both *t*Z and iP type cytokinins in the shoot and root compared to wild-type plants. A grafted wild-type shoot onto a mutant root recovered wild-type levels of iP but not *t*Z in the root; a grafted wild-type root onto a mutant shoot recovered wild-type levels of *t*Z but not iP in the shoot. These studies thus support a role for long distance transport of shootward directed *t*Z-type cytokinins and rootward directed iP-type cytokinins, although local changes in cytokinin production also likely play a significant role in mediating responses. The AHK3 cytokinin receptor displays a higher affinity for *t*Z than iP (Romanov et al., 2006; Stolz et al., 2011) and thus may potentially mediate the response of the shoot to root-derived *t*Z.

In addition to long distance transport, cytokinins must be also transported across the plasma membrane and into the lumen of the ER, as the site of cytokinin perception is primarily in the ER lumen (see below). A component possibly involved in this transport, a purine transporter called AtPUP1 (At1g28230) and AtPUP2 (At2g33750), has been isolated from Arabidopsis by the functional complementation of a yeast mutant deficient in adenine uptake (Gillissen et al., 2000; Burkle et al., 2003). The ability of AtPUP1 to transport adenine was competitively inhibited by free cytokinin bases, suggesting that AtPUP1 may be a cytokinin/purine transporter. A second candidate for a cytokinin transporter is the equilibrative nucleoside transporter (ENT) family. Competitive uptake studies, again in yeast, demonstrated that the Arabidopsis ENT6 (At4g05110) protein is capable of transporting iP-riboside and *t*Z-riboside (Hirose et al., 2008). Further, a genetic screen identified a T-DNA insertion mutation in the *ENT8* gene as a suppressor of AiIPT8 overexpression (Sun et al., 2005). Despite this progress, there is no definitive demonstration that either the PUP or ENT proteins play a key role in the transport of cytokinin *in planta*.

## CYTOKININ PERCEPTION AND SIGNAL TRANSDUCTION

The cytokinin signal transduction pathway is a phosphorelay similar to bacterial two-component response systems. A brief outline of the relevant details of two-component signaling in prokaryotic, fungal, and plant systems is presented in the following section. The specific involvement of phosphorelay signal transduction in cytokinin signaling will then be considered, with sections on each of the key elements in the signaling pathway. For additional information, readers are also referred to recent reviews focusing on cytokinin signaling (Argueso et al., 2010; Müller, 2011; Heyl et al., 2012; Hwang et al., 2012; Lomin et al., 2012; El-Showk et al., 2013).

### Two-Component Signal Transduction

Two-component signal transduction systems are the major routes by which bacteria sense and respond to various environmental cues (Parkinson, 1993; Hoch and Silhavy, 1995; Perraud et al., 1999; Stock et al., 2000; West and Stock, 2001; Gao and Stock, 2009; Cheung and Hendrickson, 2010). Many bacterial species contain more than a dozen two-component signaling pathways. For example, the *E. coli* genome encodes over 60 different two-component signaling elements that respond to a diverse array of environmental stimuli (Mizuno, 1997; Wuichet et al., 2010). The two components generally consist of a membrane-localized sensor kinase that perceives environmental stimuli and a response regulator that propagates the signal, often by directly regulating transcription of target genes (Figure 4). The input domain of the sensor kinase perceives the signal and controls the autophosphorylation of the histidine kinase domain. Active histidine kinas-

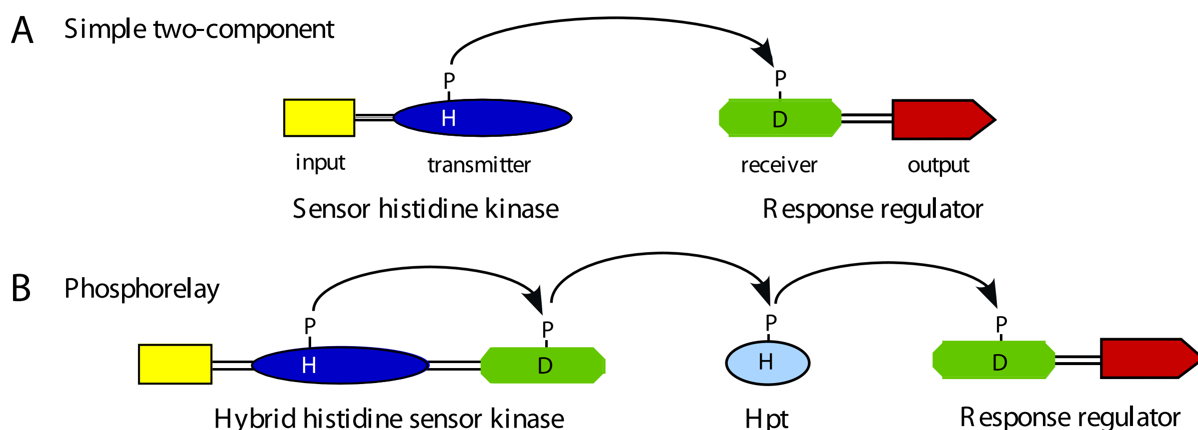


**Figure 4.** Cartoon representations of two-component phosphotransfer schemes.

**(A)** A basic prokaryotic two-component system with a sensor histidine kinase and a response regulator. H and D represent the conserved phospho-accepting histidine and aspartate residues involved in phosphorelay signaling.

**(B)** A multistep phosphorelay system involving a hybrid sensor kinase, with input, transmitter and receiver domains, a histidine-containing phosphotransfer protein and a response regulator.

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es are dimers that generally *trans*-phosphorylate on a conserved histidine residue in the transmitter domain. This phosphate is then transferred to a conserved aspartate residue in the receiver domain of a cognate response regulator. Many response regulators function as transcription factors and contain DNA-binding output domains in addition to their receiver domains (Stock et al., 2000). However, not all response regulators are transcription factors, examples of these 'single domain' response regulators being found in bacteria, archae, fungi, and plants. For example, CheY, which is an *E. coli* response regulator involved in the chemotaxis response, is comprised solely of a receiver domain and regulates the activity of the flagellar motor via a phospho-dependent interaction with the FliM protein (Welch et al., 1993).

Extended versions of the basic two-component system are present in some prokaryotes and predominate in eukaryotic two-component signaling (Swanson et al., 1994; Appleby et al., 1996; Schaller et al., 2011) (Figure 4). Such multi-step phosphorelays typically involve four sequential phosphorylation events that alternate between histidine and aspartate residues, although the number of proteins harboring these phosphorylation sites varies. In eukaryotes the typical multi-step phosphorelay makes use of a "hybrid" kinase that contains both histidine kinase and receiver domains in one protein, a histidine-containing phospho-transfer (HPt) protein, and a separate response regulator (Appleby et al., 1996; Schaller et al., 2011).

Genes encoding proteins similar to the bacterial two-component signaling elements are found in the Arabidopsis genome (Mizuno, 2005; Schaller et al., 2008). These are all found as gene families, and include histidine kinases, histidine-containing phosphotransfer proteins (AHPs), and response regulators (ARRs) (Figures 5 and 6). Biochemical and genetic analyses support their function in a multi-step phosphorelay. Genetic analysis has indicated that a major role for many of these genes is in cytokinin signal transduction (Figure 7); evolutionary analysis has revealed that all the elements involved in cytokinin signaling first appear together in early land plants such as the mosses (Pils and Heyl, 2009). Readers are referred to a separate chapter in The Arabidopsis Book for a discussion of the full complement of two-component signaling elements in Arabidopsis (Schaller et al., 2008). In the following sections, we focus on the two-component signaling elements that act in cytokinin signaling, from the initial perception by the membrane-bound AHK receptors, translocation through the AHPs, and ultimately to the regulatory phosphorylation of the ARRs, with type-B ARRs serving to regulate the transcriptional output from the phosphorelay, and type-A ARRs serving as negative feedback regulators to desensitize plants to cytokinin.

## Cytokinin Receptors Are Sensor Histidine Kinases

### *A family of cytokinin receptors*

The cytokinin receptor family of Arabidopsis is composed of three histidine kinases: AHK2 (At5g35750), AHK3 (At1g27320), and AHK4 (At2g01830)(also called CRE1 or WOL1) (Table 3; Figures 5 and 7). These share a similar structure, having transmembrane domains that yield a predicted topology in which there is an extracytosolic region for signal input and a cytosolic region for signal output. The extracytosolic portion has a CHASE (cyclases/histidine kinases associated sensor extracellular) domain that functions in cytokinin binding (Anantharaman, 2001; Heyl et al., 2007). The cytosolic portion has histidine kinase and C-terminal receiver domains that contain all the highly conserved residues required for enzymatic function. In addition, the receptors contain a diverged second receiver domain sandwiched between the histidine kinase and C-terminal receiver domains. This diverged receiver domain lacks some of the highly conserved residues found in other receivers, and in AHK3 and AHK4 the putative phospho-accepting aspartate is replaced by a glutamate residue. The phylogenetic relationship of the cytokinin receptors to other histidine-kinase related proteins of Arabidopsis is shown in Figure 5.

Genetic, biochemical, and molecular studies all support the role of AHK2, AHK3, and AHK4 as cytokinin receptors (Inoue et al., 2001; Suzuki et al., 2001b; Ueguchi et al., 2001; Yamada et al., 2001). The *cytokinin response 1* (*cre1*) mutant was identified in a screen for mutants impaired in the responses of callus tissue to cytokinin, namely greening and shoot formation (Inoue et al., 2001). The *cre1* mutants are also less sensitive to cytokinin inhibition of root growth in intact seedlings. Complementation analysis and sequencing of a *cre1* allele revealed this mutation corresponded to the *AHK4* gene. *cre1* is also allelic to the *woodenleg* (*wol*) mutation, which was originally identified as a mutant allele that resulted in a reduced cell number and lack of phloem in the root vasculature (Scheres et al., 1995; Mähönen et al., 2000). We refer to AHK4/CRE1/WOL1 as AHK4 in this review for simplicity and consistency with the nomenclature of the other cytokinin receptors.

Convincing initial evidence that AHK4 functioned in cytokinin perception came from experiments in which it was expressed in bacteria and yeast and shown to signal through a phosphorelay in a cytokinin-dependent manner. In one report, AHK4 was found to rescue the growth defect of a yeast strain lacking the SLN1 osmosensing histidine kinase, but only if cytokinins were present in the media (Inoue et al., 2001). This indicates that AHK4 function was activated in response to cytokinins. As ligand-dependent changes in activity are a key feature of receptor/ligand interactions, this strongly supports the notion that AHK4 functions as a cytokinin receptor. Furthermore, the complementation of sln1$\Delta$ yeast by AHK4 required the downstream phosphorelay protein YPD1, indicating that AHK4 was acting through the endogenous yeast phosphotransfer pathway. Similar experiments with AHK4, as well as with AHK2 and AHK3, using both a fission yeast and an *E. coli* multistep phosphorelay system gave similar results (Inoue et al., 2001; Suzuki et al., 2001b; Ueguchi et al., 2001; Yamada et al., 2001). As with the complementation of sln1$\Delta$ by CRE1, the complementation in these systems occurred in a cytokinin-dependent manner.

### *The role of subcellular localization in signal transmission*

The cytokinin receptors contain two predicted transmembrane domains: one near the amino terminus, and a second between the input ligand-binding region and the output region containing the histidine kinase and receiver domains (Figure 5). Topological analysis places the input domain on the extracytosolic side of a membrane, and the output domain on the cytosolic side. This topology conforms to the typical structure of a transmembrane en-

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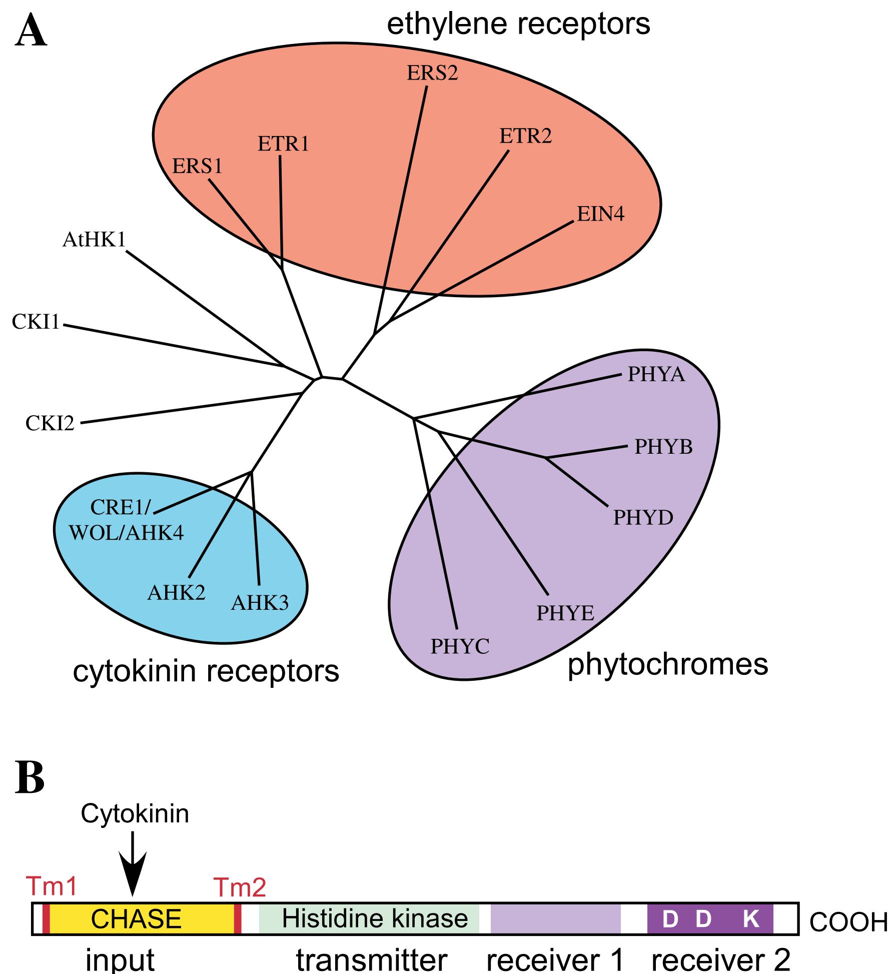


**Figure 5.** Histidine kinases in Arabidopsis.

**(A)** An unrooted phylogenetic tree of histidine kinase-related proteins derived using the amino acid sequences of the histidine kinase-like domains of these proteins (adapted from Schaller 2001). Phytochrome, ethylene receptor and cytokinin receptor families are indicated.

**(B)** Cartoon diagram of the domain structure of the AHK cytokinin receptors. TM1 and TM2 refer to the transmembrane domains.

zyme-linked receptor, in which binding of the ligand to the extracytosolic region results in activation of cytosolic enzymatic activity and propagation of the signal to downstream signaling elements (Wiley, 1992; Schlessinger, 2000; Cheung and Hendrickson, 2010). In this case, cytokinin binding to the extracytosolic portion results in activation of the cytosolic histidine-kinase activity and autophosphorylation on the conserved His residue, followed by transfer of the phosphate to the conserved Asp within the receptor's receiver domain (Inoue et al., 2001; Suzuki et al., 2001b; Ueguchi et al., 2001; Yamada et al., 2001). The phosphate will subsequently be transferred to the downstream AHPs and type-B ARRs. These elements form a positive regulatory circuit by which the cytokinin signal originating from membrane-bound receptors results in a transcriptional change in the nucleus (Hwang and Sheen, 2001). The membrane-associated histidine kinases of prokaryotes are dimers (Parkinson, 1993; Cheung and Hendrickson, 2010) and, similarly, studies of the Arabidopsis cytokinin receptors indicate that they interact with each other, forming potential homo- and hetero-dimers (Dortay et al., 2008; Caesar et al., 2011; Hothorn et al., 2011). By analogy to the prokaryotic histidine kinases, signal transmission across the membrane by the cytokinin receptors is likely to occur via conformational changes brought about by ligand binding. These conformational changes facilitate *trans*-phosphorylation of the cytosolic histidine kinase domains.

Interestingly, subcellular localization studies indicate that the cytokinin receptors are predominantly localized to membranes of the endoplasmic reticulum (ER), both in the dicot Arabidopsis and the monocot *Zea mays* (Caesar et al., 2011; Lomin et al., 2011; Wulfetange et al., 2011). Similar ER localization was found when the Arabidopsis receptors were transiently expressed in tobacco or when expressed from their native or ubiquitin promoters in Ara-

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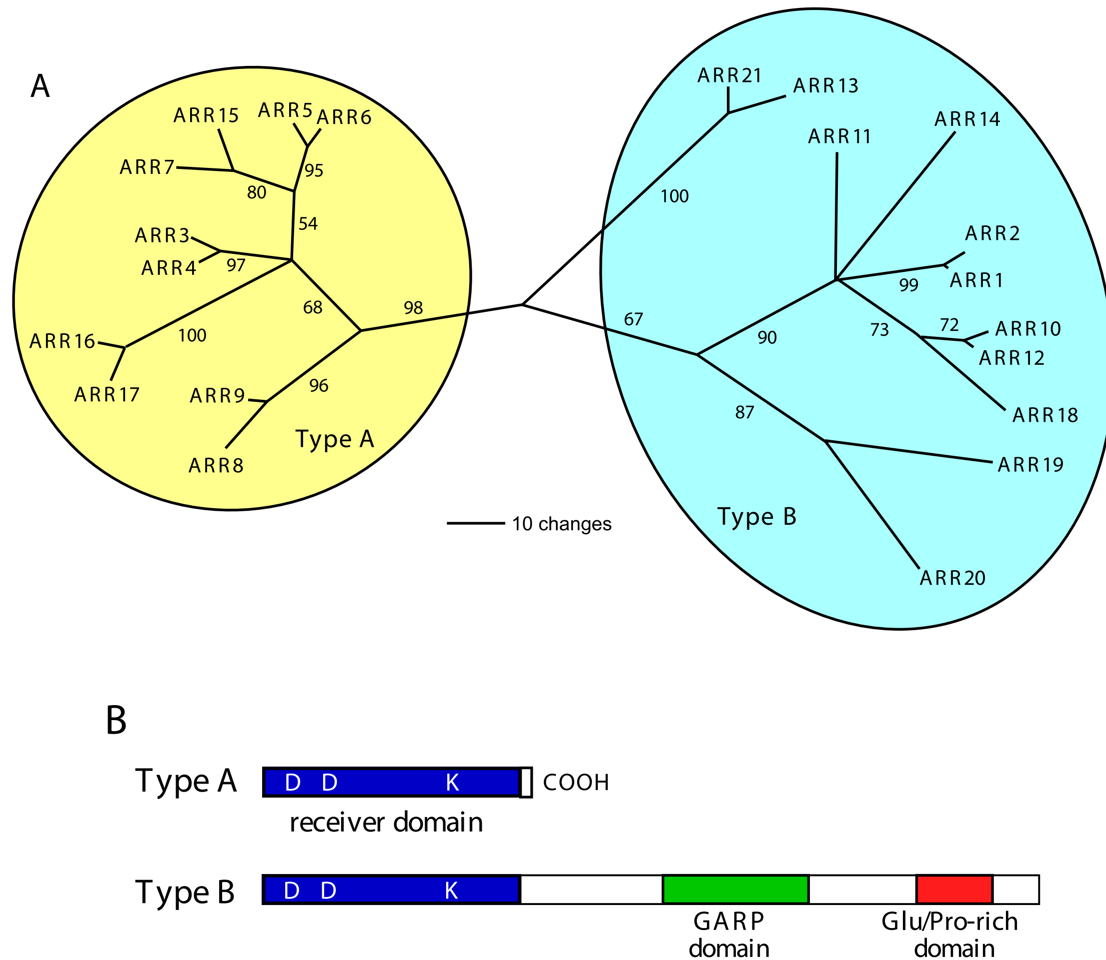


**Figure 6.** Type-A and type-B response regulators in Arabidopsis.

**(A)** An unrooted phylogenetic tree made using receiver domain sequences of type-A and type-B ARRs by a heuristic method. Phylogenetic analysis was performed using the PAUP 4.0 program, with 10,000 bootstrap replicates to assess the reliability of the tree. The bootstrap values are indicated on the tree.

**(B)** Cartoon of the domain structure of type-A and type-B ARRs. Both classes of ARRs contain receiver domains. Type-B ARRs have long C-terminal extensions that include a GARP domain and a glutamine- and proline-rich region.

bidopsis (Caesar et al., 2011; Wulfetange et al., 2011). The biological relevance of this ER localization is supported by the finding that more than 98% of the saturable cellular cytokinin binding sites are located in endomembranes when endomembranes are fractionated from the plasma membrane and analyzed for binding using [$^3$H] *trans*-zeatin (Wulfetange et al., 2011). In addition, binding of cytokinins to the receptors is pH dependent (Romanov et al., 2006), being optimal at neutral pH, such as is found within the cytosol and ER lumen, and contrasting with the acidic pH of the apoplast (Tian et al., 1995). These results indicate that the majority of cytokinin signaling originates from ER-bound receptors, although it does not exclude the possibility that a small receptor population at the PM may also function in signaling.

The ER localization for the receptors raises a number of questions (Wulfetange et al., 2011). First, the membrane topology of the receptors would place the cytokinin-binding domain within the ER lumen, raising the question as to how cytokinins access the binding site. To date no intracellular cytokinin transporters have been identified (Kudo et al., 2010), however the ER membrane is permeable to small molecules, potentially resolving this concern (Le Gall et al., 2004). Second, how are cytokinin concentrations controlled within the ER? This question is more readily resolved as cytokinin degrading activities have been reported in multiple cellular compartments, including the ER, extracellular space, and cytosol (Motyka et al., 1996; Werner et al., 2003; Wulfetange et al., 2011). Third, what advantage(s), if any, does cytokinin binding in the ER confer? Such a localization may facilitate cross-talk with other ER-localized signaling systems, most notably the ethylene receptors which, like the cytokinin receptors, are histidine kinase-linked receptors (Chen et al., 2002; Chen et al., 2007; Grefen et

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al., 2008). In addition, since the ER is contiguous with the nuclear membrane, ER localization may facilitate communication between the receptors and the nucleus.

### *Cytokinin binding by the receptors*

A variety of adenine derivatives exhibit cytokinin-like action in plants and should therefore bind to and stimulate the activity of the receptors (Mok and Mok, 2001; Sakakibara, 2006). These cytokinins include the abundant natural cytokinins *trans*-zeatin (*t*Z) and isopentyladenine (iP), as well as dihydrozeatin, benzyladenine, kinetin, and in some cases *cis*-zeatin. Benzyladenine and kinetin were initially produced synthetically but also occur at low levels naturally in some species. In addition, the synthetic phenylurea derivative thidiazuron stimulates cytokinin responses when applied exogenously to plants. Plants also synthesize inactive cytokinin conjugates such as the cytokinin ribosides, ribotides, and glucose derivatives, which are therefore predicted to lack functionality in cytokinin binding assays (Sakakibara, 2006).

The ability of the AHK family of cytokinin receptors to specifically perceive bioactive cytokinins has been demonstrated through biochemical and genetic approaches. One approach has been to express the receptors in *E. coli* or yeast, systems that allow the binding characteristics for individual receptors to be determined independently from that of other family members (Inoue et al., 2001; Suzuki et al., 2001b; Ueguchi et al., 2001; Yamada et al., 2001; Spichal et al., 2004; Romanov et al., 2005; Romanov

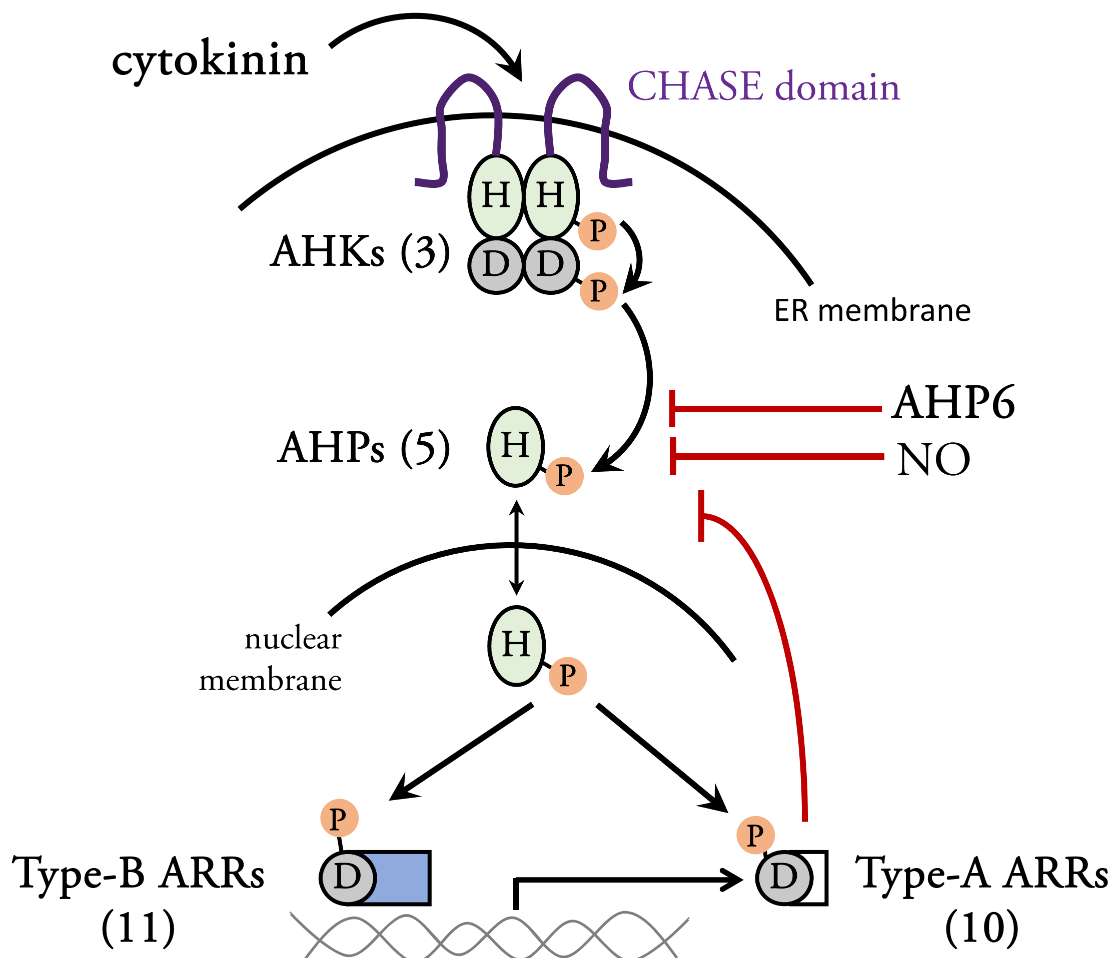


**Figure 7.** Proposed model for phosphorelay signal transduction in cytokinin signaling.

Cytokinin binds to the CHASE domains of the AHK2/AHK3/AHK4 cytokinin receptors within the lumen of the ER. Binding of cytokinin activates the transmitter domain, which autophosphorylates on a His (indicated by an H). The phosphate is then transferred an Asp residue (indicated by a D) within the fused receiver domain. The phosphate is then transferred to an AHP protein, which shuttles back and forth between the cytoplasm and the nucleus. In the nucleus, the AHPs transfer the phosphate to type-B ARRs, which then regulate the expression of many target genes, including the type-A ARRs. The type-A ARRs, which are also phosphorylated by the AHPs, in turn feedback to inhibit cytokinin signaling (indicated by ⊥). The pseudo HPt protein AHP6 and nitric oxide (NO) also negatively regulate cytokinin signaling. See text for additional details.

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et al., 2006; Stolz et al., 2011). A bacterial expression system has been used for a quantitative bioassay in which activation of the cytokinin receptor results in stimulation of a *lacZ* reporter (Inoue et al., 2001; Suzuki et al., 2001b; Spichal et al., 2004). In addition, direct binding assays with radiolabelled cytokinins have been performed using bacterial and yeast expression systems (Romanov et al., 2005; Romanov et al., 2006; Stolz et al., 2011). *In planta* based evidence for the role of individual receptors in cytokinin binding has been obtained from double mutants of Arabidopsis, each of which presumably only has a single remaining receptor. The Arabidopsis double *ahk* mutants have been analyzed for their ability to respond to a variety of exogenously applied cytokinins as well as for the ability of extracted membrane fractions to bind to radiolabelled cytokinins (Stolz et al., 2011; Wulfetange et al., 2011).

**Table 3.** Genes encoding cytokinin signaling elements

| Genes | AGI Codes | Location | Notes |
|---|---|---|---|
| Cytokinin AHK receptors | | | |
| *AHK2* | At5g35750 | ER membrane | |
| *AHK3* | At1g27320 | ER membrane | |
| AHK4 | At2g01830 | ER membrane | also called CRE1 and WOL |
| Related AHK | | | |
| *CKI1* | At2g47430 | Plasma membrane | Lacks a CHASE domain and does not bind cytokinin |
| Histidine Phosphotransfer proteins | | | |
| *AHP1* | At3g21510 | nucleus and cytoplasm | |
| *AHP2* | At3g29350 | nucleus and cytoplasm | |
| *AHP3* | At5g39340 | nucleus and cytoplasm | |
| *AHP4* | At3g16360 | nucleus and cytoplasm | |
| *AHP5* | At1g03430 | nucleus and cytoplasm | |
| *AHP6* | At1g80100 | not yet determined | Pseudo HP; negative reulator of cytokinin signaling |
| Type-A Response Regulators (negative regulators; induced by cytokinin) | | | |
| *ARR3* | At1g59940 | not yet determined | |
| *ARR4* | At1g10470 | not yet determined | |
| *ARR5* | At3g48100 | not yet determined | |
| *ARR6* | At5g62920 | nucleus | |
| *ARR7* | At1g19050 | nucleus | Regulated by auxin in RAM and SAM |
| *ARR8* | At2g41310 | not yet determined | |
| *ARR9* | At3g57040 | not yet determined | Regulated by the circadian clock |
| *ARR15* | At1g74890 | nucleus | Regulated by auxin in RAM and SAM |
| *ARR16* | At2g40670 | cytoplasm | |
| *ARR17* | At3g56380 | not yet determined | |
| Type-B Response Regulators (positive elements; Include a GARP Myb-like DNA binding domain) | | | |
| *ARR1* | At3g16857 | nucleus | |
| *ARR2* | At4g16110 | nucleus | |
| *ARR10* | At4g31920 | nucleus | |
| *ARR11* | At1g67710 | nucleus (predicted) | |
| *ARR12* | At2g25180 | nucleus | |
| *ARR13* | At2g27070 | nucleus (predicted) | |
| *ARR14* | At2g01760 | nucleus (predicted) | |
| *ARR18* | At5g58080 | nucleus | |
| *ARR19* | At1g49190 | nucleus | |
| *ARR20* | At3g62670 | nucleus (predicted) | |
| *ARR21* | At5g07210 | nucleus (predicted) | |

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Several key findings have come from these studies. First, they demonstrated that cytokinins directly bind to these AHK proteins, and that binding occurs through the CHASE domain. Second, these studies demonstrated that the receptors have a high affinity for cytokinins, exhibiting $k_D$ values for optimal binding of 1-10 nM, a binding affinity consistent with the endogenous concentrations of the bioactive cytokinins *t*Z and iP that are reported to range from 0.7-2.5 nM in Arabidopsis (Takei et al., 2004b; Riefler et al., 2006; Werner et al., 2010). Third, these studies demonstrated that the cytokinin receptors exhibit different affinities for various cytokinin species. At a gross level, the receptors all bind bioactive cytokinins such as *t*Z and iP with high affinity, but exhibit low affinity for unsubstituted adenine itself or for the inactive cytokinin conjugates. At a more subtle level, the receptor isoforms exhibit differences in their relative affinities for bioactive cytokinins; AHK3 exhibits a high affinity for *t*Z and a relatively low affinity for iP, contrasting with AHK2 and AHK4, both of which exhibit high affinity for iP.

The physical basis for cytokinin binding was recently determined by crystallization of the CHASE domain from AHK4 in complex with various cytokinins (Hothorn et al., 2011). This study revealed that the cytokinin binding site occurs within a PAS domain and that, although there is low sequence similarity, the structure of the sensor domain is similar to that found in some bacterial histidine kinases. The biologically active cytokinins exhibited a similar structure in complex with the CHASE domain, however *trans*-zeatin formed an additional hydrogen bond compared to *cis*-zeatin, providing a physical basis for their differences in affinity. The binding site itself is small and involves approximately 20 amino acids (Hothorn et al., 2011; Lomin et al., 2012). The small size of the binding site explains the inactivity of the cytokinin conjugates, which are too large to fit into the site. The limited volume of the binding site also provides a physical basis for the *wooden-leg* (*wol*) mutant phenotype, one of the initial mutations identified in *AHK4* (Mähönen et al., 2000). The *wol* mutation arises due to the single amino-acid substitution of Thr with the bulkier Ile within the cytokinin binding pocket, thereby disrupting cytokinin binding. The greater strength of the *wol* mutation, compared to a simple null mutation of the receptor, is likely due to the *wol*-mutant receptor being unable to respond to cytokinins but still interacting with other members of the receptor family as well as downstream signaling components.

The differences in cytokinin specificity of the receptors is likely of physiological consequence and may facilitate long-distance communication between the root and shoot. As described earlier, there are differences in the production and transport of cytokinins: the iP-based cytokinins are transported predominantly from shoot to root via the phloem, whereas the *t*Z-based cytokinins are transported predominantly from root to shoot via the xylem (Kudo et al., 2010). The AHK3 receptor isoform is more abundant in shoots than in roots, and so is primarily responsive to the *t*Z cytokinin produced in the roots and transported shootward. In contrast, the AHK4 receptor isoform is more abundant in roots than in shoots (Mähönen et al., 2000; Inoue et al., 2001; Ueguchi et al., 2001), and would be responsive to iP produced in the shoots and transported rootward. Consistent with cytokinin specificity being of physiological relevance, *AHK4* can substitute for *AHK2* but not for *AHK3* in genetic complementation studies (Stolz et al., 2011).

### *Functional overlap between members of the cytokinin receptor family*

There are three cytokinin receptors and, as with many of the gene families of Arabidopsis, there is functional overlap within the receptor family such that multiple receptors regulate the best-characterized cytokinin responses. Overlap is apparent based on expression patterns and, most significantly, analysis of loss-of-function mutants of the receptors. Expression based on GUS-fusions and tissue-specific mRNA analyses indicates that the receptors are predominantly expressed in meristematic and vascular tissues, although low levels of expression are detected throughout the plant (Birnbaum et al., 2003; Nishimura et al., 2004; Yadav et al., 2009). Among the proliferating tissues exhibiting enhanced AHK expression are the meristematic regions of the root and shoot, lateral root primorida, and the mitotically active regions of young leaves. There are however, as indicated earlier, differences in the expression levels such that some AHK isoforms predominate within certain tissues. This is apparent for *AHK4*, northern analysis having initially revealed its expression being primarily in the root (Mähönen et al., 2000; Inoue et al., 2001; Ueguchi et al., 2001). Subsequent GUS expression indicated that this expression is primarily restricted to vascular tissues in the apical part of the root meristem (Nishimura et al., 2004).

The isolation and characterization of T-DNA insertion mutations has demonstrated roles for the cytokinin receptors in diverse cytokinin-regulated processes, with the analysis of single and higher order mutant combinations clarifying the extent of overlap among the family members (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). Mutants were initially analyzed for effects on such well-characterized cytokinin responses as *in vitro* shoot regeneration, the inhibition of root elongation, the inhibition of hypocotyl growth in the dark, and, at the molecular level, the ability to induce expression of the type-A *ARR*s. In addition, mutants were analyzed for their effects on cell division within the shoot meristem and in the leaves, with cytokinin acting as a positive regulator of cell division in the shoot. The identity of the cytokinin receptors having been confirmed through these genetic approaches, researchers have since employed *ahk* mutants to carefully dissect the role of cytokinin in a host of growth and developmental processes, including unexpected roles not previously linked to cytokinins. We will explore some of these specific roles of cytokinin in growth, development, and responses to biotic and abiotic factors later in this review.

One point became clear from the initial characterization of the receptor mutants: no single receptor acted alone in mediating the cytokinin responses, although genetic analysis demonstrated that subsets of the receptors played predominant roles for certain responses (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). For example, AHK4 is the principal regulator for primary root growth, involving the control of cell proliferation and elongation, as well as for *in vitro* shoot regeneration. The role of AHK4 in root growth is consistent with its high expression in the root. AHK3 is the principal regulator for cytokinin-induced photomorphogenesis and chlorophyll retention during dark-induced senescence, with AHK2 also contributing to a lesser extent. AHK2 and AHK3 share predominant roles in the control of leaf cell formation and root branching. In general, unlike *ahk3* and

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*ahk4* mutants, *ahk2* single mutants did not display mutant phenotypes, but *ahk2* mutations do enhance the phenotypes resulting from *ahk3* and *ahk4* mutations. All three receptors share similar contributions to the regulation of seed size and the regulation of cytokinin metabolism, based on elevated levels of cytokinins being observed in receptor mutants. Nevertheless, even though a single receptor may predominate in its control, none of these responses is controlled solely by one receptor. Similarly, there is also significant functional overlap for downstream elements in the cytokinin signal transduction pathway.

Phenotypic characterization of *ahk* triple mutants point to the extent by which plant growth and development are regulated by cytokinins (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006; Cheng et al., 2013). The triple mutants are extreme dwarfs, the shoots smaller than those found in any of the *ahk* double mutant combinations. Dwarfism correlates with a decrease in cell division, with both the shoot apical meristem and the leaves having reduced cell numbers as compared to those of wild-type plants. The primary root of the triple *ahk* mutant prematurely aborts growth, exhibiting a phenotype similar to that observed in the *wol* mutant; the *ahk* triple mutant plants resume growth upon formation of functional adventitious roots. The *ahk* triple mutant plants are infertile due to defects in female gametophyte development (Cheng et al., 2013). That the triple mutant still produces a growing plant with the normal diversity of cell types may be surprising given the historic demonstration that cytokinins are critical for cell division and shoot formation during *in vitro* plant regeneration. It may be that cytokinins are not required for basal plant functions, but rather primarily serve to modulate plant growth. However, there is also evidence for some functional overlap with signaling by the histidine kinase CKI1 (At2g47430), although the interacting ligand for CKI1 is yet to be uncovered (Kakimoto, 1996; Hejátko et al., 2009). In addition, although loss-of-function mutants have been isolated in all three members of the receptor family, there is no null for all three receptors. Even in the triple mutant exhibiting the strongest mutant phenotype, low levels (< 1% wild-type levels) of full-length, wild-type *AHK3* transcript are present (Cheng et al., 2013). Thus the complete extent of cytokinin receptor contribution to plant growth and differentiation has yet to be determined.

### Histidine-Containing Phosphotransfer Proteins (AHPs)

Arabidopsis Histidine-containing Phosphotransfer proteins (AHPs) act downstream of the AHK receptors in cytokinin signaling (Table 3; Figure 7). The AHPs mediate transfer of a phosphoryl group from the receiver domain of an activated hybrid sensor histidine kinase to the receiver domain of a response regulator in the multistep phosphorelay. The Arabidopsis genome encodes five HPt proteins (AHP1 through 5) that contain the conserved residues required for activity, as well as one pseudo-HPt (APHP1/AHP6; At1g80100) that lacks the histidine phosphorylation site (Miyata et al., 1998; Suzuki et al., 1998; Suzuki et al., 2000; Schaller et al., 2008). Each of the predicted proteins is comprised solely of a highly similar phosphotransmitter domain that includes a conserved histidine residue (except for AHP6) that serves as the phosphorylation site. The AHPs interact with both hybrid histidine kinases and response regulators based on yeast two-hybrid analysis (Imamura et al., 1999; Urao et al., 2000; Tanaka et al., 2004; Dortay et al., 2006), and have been shown capable of participating in a phosphorelay with Arabidopsis response regulators (Suzuki et al., 1998), consistent with an ability to function in a multi-step phosphorelay.

Analysis of loss-of-function mutations has revealed that *AHP1* (At3g21510), *AHP2* (At3g29350), *AHP3* (At5g39340), and *AHP5* (At1g03430) function as redundant positive regulators of cytokinin signaling (Hutchison et al., 2006). *AHP4* (At3g16360) only contributed slightly to the cytokinin responses, and in some cases appeared to act as a negative regulator (Hutchison et al., 2006). Individual *ahp* mutants were indistinguishable from the wildtype in cytokinin response assays. In contrast, various higher order mutants displayed reduced cytokinin sensitivity in such assays as the inhibition of primary root elongation, lateral root formation, and hypocotyl elongation, as well as the ability to induce cytokinin primary response genes, consistent with functional overlap of the AHPs in transduction of the cytokinin signal. A quintuple *ahp1 ahp2 ahp3 ahp4 ahp5* mutant displays phenotypes similar to those found in a cytokinin receptor triple mutant, including reduced shoot development, aborted primary root growth, and enlarged seed size. Quintuple *ahp* mutants also exhibit reduced fertility, evidence indicating that this effect is due to early defects in female gametophyte development such as is found with *ahk* mutants, as well as later defects in megagametogenesis operating downstream of the CKI1 histidine kinase (Deng et al., 2010; Cheng et al., 2013).

The AHP proteins function as mobile elements of the primary cytokinin signaling pathway, transferring the phosphate signal from the ER-localized cytokinin receptors to the nuclear-localized type-B ARRs. Consistent with such a role, GFP fusions of the AHP proteins localize to both the cytosol and nucleus. Initial studies suggested that the AHPs might accumulate in the nucleus in response to cytokinin (Hwang and Sheen, 2001; Yamada et al., 2004). However, subsequent quantitative analysis demonstrated that the subcellular distribution of the AHPs was independent of cytokinin signaling, the AHPs maintaining a constant cytosolic/nuclear distribution in the absence or presence of cytokinin (Punwani et al., 2010; Punwani and Kieber, 2010). This distribution is stable, but dynamic, with the AHPs constantly cycling between the nucleus and the cytosol, but is not dependent on cytokinin or their phosphorylation status. Such cycling between the cytosol and nucleus has also been found with the yeast phosphotransfer protein YPD1, which likewise maintains this distribution independently of activation for its phosphorelay (Lu et al., 2003). This mechanism for actively redistributing AHPs between the cytosol and nucleus may serve several purposes. First, phosphorylated AHPs will still relocate from the cytosol to the nucleus, allowing for transmission of the cytokinin signal from receptors to nuclear-localized ARRs. Second, the presence of a phosphorylated pool of the AHPs in the cytosol allows for phospho-transfer to cytosolic ARRs (a subset of the type-A ARRs). Third, the phosphorylated AHPs may interact in a phospho-dependent manner with other cytosolic or nuclear proteins to regulate their activity, the potential for such regulation arising from the finding that AHPs interact with a protein containing a TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) domain (TCP10; At2g31070); such TCP-domain proteins may function as transcriptional regulators in the control of growth and development (Suzuki et al., 2001a).

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

The *AHP6* gene was isolated in a genetic screen for suppressors of the determinate root phenotype of *wol* (Mähönen et al., 2006b). As noted above, AHP6 is predicted to be a non-functional HPt protein as it lacks the conserved His phosphorylation site. AHP6 inhibits phosphotransfer reactions *in vitro*, both the intramolecular transfer from the His in the transmitter domain of a hybrid kinase to Asp residue in the fused receiver domain, and from AHP1 to ARR1, which indicated that AHP6 likely acts as an inhibitor of phosphotransfer (Bishopp et al., 2011). Disruption of the *AHP6* gene results in an elevated basal expression of the cytokinin primary response gene *ARR15* (At1g74890) and hypersensitity to exogenous cytokinin for the formation of adventitious roots and protoxylem differentiation. These results suggest that AHP6 acts as a negative regulator of cytokinin signaling in Arabidopsis.

***Nitric oxide regulates phosphotransfer proteins through S-nitrosylation***

Nitric oxide (NO) can directly modify the cysteine thiol of proteins as a redox-based posttranslational modification, which is known as S-nitrosylation. The AHP proteins have a conserved cysteine residue that is *S*-nitrosylated by NO *in vitro* and *in vivo* (Feng et al., 2013). The S-nitrosylation of the AHPs reduces their ability to act as phosphotransfer proteins *in vitro*, reducing both their ability to receive a phosphate from a histidine kinase and to transfer a phosphate to ARR1. Consistent with this, the *nox1* and *gsnor1-3* mutants, which have elevated levels of endogenous NO, display decreased expression of the TCS-GFP cytokinin reporter as well as multiple cytokinin primary response genes. Further, these NO-overexpressing lines are less sensitive to exogenous cytokinin in multiple assays, consistent with NO acting as a negative regulator of cytokinin signaling. This represents a novel mechanism by which environmental stimuli can interact with the cytokinin signal transduction pathways. In addition to the nitrosylation of the AHPs, cytokinin can also suppress NO function, possibly by a direct chemical reaction with NO (Liu et al., 2013). The authors suggest that cytokinin may act as an NO scavenger to dampen NO levels in the cell.

**Type-B ARRs Function as Transcription Factors to Mediate Cytokinin-Regulated Gene Expression**

The initial cytokinin transcriptional response is mediated through action of the type-B ARRs (Table 3; Figures 6 and 7). There are eleven type-B ARRs in the Arabidopsis genome, which fall into three subfamilies based on phylogenetic analysis: subfamily 1 contains seven members (ARR1: At3g16857; ARR2: At4g16110; ARR10: At4g31920; ARR11: At1g67710; ARR12: At2g25180; ARR14: At2g01760; and ARR18: At5g58080); subfamily 2 contains two members (ARR13: At2g27070; and ARR21: At5g07210); and subfamily 3 is also comprised of two members (ARR19: At1g49190; and ARR20: At3g62670) (Mason et al., 2004; Schaller et al., 2008). The type-B ARRs are characterized by the presence of a receiver domain and a large C-terminal extension. The key conserved feature of the C-terminal extension is a Myb-like DNA binding domain, referred to the GARP domain because it is found in GOLDEN2 in maize, the ARRs, and the Psr1 protein from Chlamydomonas (Imamura et al., 1999; Hosoda et al., 2002). Multiple lines of evidence support the role of the type-B ARRs as transcription factors (Sakai et al., 2000; Imamura et al., 2001; Lohrmann, 2001; Sakai et al., 2001; Hosoda et al., 2002; Imamura et al., 2003; Mason et al., 2004; Mason et al., 2005; Rashotte et al., 2006). Type-B ARRs are capable of transcriptional activation when expressed in yeast, and directly bind to target DNA sequences through their GARP domain (Lohrmann et al., 1999; Sakai et al., 2000; Lohrmann, 2001; Hosoda et al., 2002). The C-terminal region is quite variable outside of the conserved GARP domain, but does contain activation regions and potential nuclear localization signals, the type-B ARRs being constitutively nuclear localized where examined (Lohrmann et al., 1999; Sakai et al., 2000; Hwang and Sheen, 2001; Lohrmann, 2001; Mason et al., 2004). The C-terminal extensions may also allow for interactions with different regulatory partners.

Genetic analyses indicate that at least five subfamily-1 members mediate cytokinin signaling: ARR1, ARR2, ARR10, ARR11, and ARR12 (Mason et al., 2005; Yokoyama et al., 2007; Ishida et al., 2008b). Of these, ARR1, ARR10, and ARR12 play the predominant roles and regulate the majority of the responses typical of cytokinin (Mason et al., 2005; Yokoyama et al., 2007; Argyros et al., 2008; Ishida et al., 2008b). As with other elements in the cytokinin signaling pathway, there is functional overlap in the gene family, as substantial effects on cytokinin signaling are only observed in multiple mutant combinations. The multiple type-B ARR mutants affect the same cytokinin responses previously described for the *ahk* and *ahp* mutants, such as inhibition of root elongation, lateral root formation, and hypocotyl elongation, and the *in vitro* induction of callus tissue and shoot formation (Mason et al., 2005; Yokoyama et al., 2007; Argyros et al., 2008; Ishida et al., 2008b). An *arr1 arr10 arr12* triple mutant exhibits reduced shoot development, aborted primary root growth, enlarged seed size, and defects in female gametophyte development (Argyros et al., 2008; Ishida et al., 2008b; Cheng et al., 2013).

The type-B ARRs regulate the Arabidopsis transcriptional response to cytokinin. Treatment of plants with exogenous cytokinin results in substantial changes in gene expression. A meta-analysis of multiple microarray experiments has established a ‘golden list’ of genes whose expression level robustly changes following short-term cytokinin treatment (Brenner et al., 2012; Bhargava et al., 2013). The cohort of cytokinin-regulated genes is enriched for those involved in cytokinin signaling and metabolism, auxin function, disease resistance, abiotic stress response, nutrient assimilation (e.g. nitrate transport), secondary metabolism (e.g. anthocyanin biosynthesis), and redox regulation (e.g. glutaredoxins). The cytokinin transcriptional response is substantially reduced in type-B mutant backgrounds, supporting a central role of the type-B ARRs in the cytokinin signaling pathway (Rashotte et al., 2006; Yokoyama et al., 2007; Argyros et al., 2008). Analysis of the type-B ARR-regulated transcriptional response supports the following points. First, none of the genes induced by cytokinin in wild type plants exhibit the same level of induction in an *arr1 arr10 arr12* triple mutant, consistent with the type-B ARRs regulating the global induction of primary cytokinin-response genes (Argyros et al., 2008). Second, the type-B *arr* mutants also have

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broad effects on the repression of cytokinin-regulated genes, even though the type-B ARRs do not contain known repressor motifs, suggesting that the type-B ARRs either induce or interact with transcriptional repressors. Third, one of the largest categories of cytokinin-regulated genes are those encoding additional transcription factors (Brenner et al., 2012; Bhargava et al., 2013), consistent with a model in which the type-B ARRs operate at the head of a transcriptional cascade, with the secondary wave of transcription factors regulating subsets of the cytokinin response.

Transcriptional regulation by the type-B ARRs involves interplay between the N-terminal receiver domain and the GARP DNA-binding domain. Analysis of the *in vitro* DNA-binding specificity for the subfamily-1 members ARR1, ARR2, ARR10, and ARR11 indicates that they bind to a core sequence of: (A/G) GAT(T/C) (Sakai et al., 2000; Hosoda et al., 2002; Imamura et al., 2003). Concatemers of this core binding sequence, driving expression of GFP, serve as a tool to evaluate the activation of type-B ARR transcription *in planta*, this reporter construct being referred to as the TCS-GFP reporter (Müller and Sheen, 2008; Zürcher et al., 2013). Although sufficient for type-B binding, the core sequence is not complex enough to demonstrate clear enrichment in the promoters of cytokinin-regulated genes (Taniguchi et al., 2007; Bhargava et al., 2013). However, the extended sequence AAGAT(T/C)TT, related to the core sequence and identified based on examination of ARR1-regulated genes (Taniguchi et al., 2007), is enriched in the promoters of cytokinin up-regulated genes (Bhargava et al., 2013). The receiver domain of the type-B ARRs apparently serves to negatively regulate the transcriptional activity of the type-B ARRs, based on elimination of the receiver domain resulting in a constitutively active type-B ARR with increased DNA binding activity (Sakai et al., 2001; Liang et al., 2012). Phosphorylation of the receiver domain, in response to cytokinin signaling, thus serves to relieve this inhibitory activity; this model of type-B activation is supported by the increased activity of ARR2 that results when the conserved Asp is replaced by a phosphomimic Glu (Lohrmann, 2001). Both inter- and intra-molecular interactions among the type-B ARRs may control their ability to regulate gene expression, as it is likely that the type-B ARRs homo- and heterodimerize (Veerabagu et al., 2012). Further, analysis of cytokinin cis-acting regulatory regions suggests that two type-B binding sites, located on the same side of the DNA helix, are required to impart strong cytokinin responsiveness to a target gene (Ramireddy et al., 2013; Zürcher et al., 2013), consistent with the binding of a type-B ARR dimer.

Protein degradation of type-B ARRs serves as another mechanism to regulate their function. In this regard, the cytokinin signaling pathway is similar to other plant hormone signaling pathways, such as those for auxin, jasmonate, gibberellin, and ethylene, in which key transcriptional regulators are targeted for degradation by the ubiquitin-proteasome pathway (Santner and Estelle, 2010). In one study, the stability of the type-B response regulator ARR2 decreased in the presence of cytokinin, and one could enhance cytokinin signaling by transgenic expression of a more stable mutant version of ARR2 (Kim et al., 2012). However, cytokinin has little effect on the stability of other type-B ARRs, which appear to undergo continuous degradation in either the absence or presence of cytokinin. The kinetics for turnover vary among the type-B ARR family members (Kim et al., 2012; Kim et al., 2013), and are controlled by an S-PHASE KINASE-ASSOCIATED PROTEIN1 (SKP1)/Cullin/F-box (SCF) E3-ubiquitin ligase complex. Specificity of the SCF complex for the type-B ARRs is determined by the four-member family of KISS ME DEADLY (KMD1: At1g15670; KMD2: At1g80440; KMD3: At2g44130; KMD4: At3g59940) F-box proteins (Kim et al., 2013). The KMDs directly interact with multiple type-B ARRs, the strongest interactions occurring with ARR1 and ARR12 based on yeast two-hybrid analysis (Kim et al., 2013). These are the two type-B ARRs that contribute most substantially to the Arabidopsis cytokinin response (Argyros et al., 2008; Ishida et al., 2008b). An ability of $SCF^{KMD}$ to mediate degradation of both inactive and activated forms of the type-B ARRs could serve two purposes based on well-established models for signal transduction (Muratani and Tansey, 2003). First, $SCF^{KMD}$ could regulate the abundance of type-B ARRs, thereby determining the threshold level for a cytokinin response, a mechanism that may facilitate cross-talk with other signaling pathways. Second, $SCF^{KMD}$ could remove activated type-B ARRs, thereby preventing continued transcriptional activation by cytokinin.

As previously mentioned, genetic analysis based on loss-of-function mutations identifies only five out of the 11 type-B ARRs (ARR1, ARR2, ARR10, ARR11, and ARR12) as clearly functioning in the control of cytokinin signaling. This raises the question as to the role of the other type-B ARRs, in particular those of the more divergent subfamilies 2 and 3. These ARRs are not as broadly expressed as those type-B ARRs that mediate the majority of the cytokinin response (Mason et al., 2004; Tajima et al., 2004), and so may mediate a cytokinin response limited to specific tissues or cell types. Consistent with this possibility, transient and stable overexpression analyses of multiple type-B ARRs (ARR14, ARR18, ARR19, ARR20, and ARR21) indicate they can alter or activate cytokinin signaling (Tajima et al., 2004; Kiba et al., 2005; Müller and Sheen, 2008; Liang et al., 2012; Veerabagu et al., 2012; Zürcher et al., 2013). However, the physiological relevance of such overexpression studies is not clear. The potential for cross-talk is a common feature of two-component signaling pathways, and was actually exploited in the identification of cytokinin receptors (Inoue et al., 2001; Suzuki et al., 2001b; Ueguchi et al., 2001; Yamada et al., 2001). Indeed, we are only just beginning to understand how specificity is controlled in bacterial systems (Mitrophanov and Groisman, 2008; Skerker et al., 2008). In addition, when the type-B ARRs were examined for their ability to complement an *arr1 arr12* mutant, expression being driven by the native *ARR1* promoter, a more limited cohort of type-B *ARR*s were functional (Hill et al., 2013). These included *ARR1*, *ARR2*, *ARR10*, *ARR12*, and *ARR21*, the last being somewhat surprising because it is a member of the more diverged subfamily-2. There are thus functional differences among the type-B ARRs, although the significance of these differences for the cytokinin transcriptional response is yet to be fully elucidated.

### Type-A ARRs Are Primary Response Genes that Function as Negative Regulators of Cytokinin Signaling

The Arabidopsis genome encodes ten type-A ARRs that fall into five pairs with highly similar amino acid sequences (Table 3; Figure 6) (D'Agostino et al., 2000; Schaller et al., 2008), which may reflect an evolutionarily recent duplication of the Arabidopsis genome (Vi-

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sion et al., 2000; Zhang et al., 2001). The type-A ARRs contain a receiver domain but, unlike the type-B ARRs, lack a classic output domain for transcriptional regulation. The amino acid sequences of the receiver domains of the type-A ARRs are very similar, but the sequences of the short N- and C-terminal extensions (< 100 amino acids) are more divergent and may serve to generate specificity in downstream outputs (Brandstatter and Kieber, 1998; Imamura et al., 1998; Urao et al., 1998; D'Agostino et al., 2000).

Most of the type-A ARRs are transcriptionally induced in response to cytokinin (Brandstatter and Kieber, 1998; Taniguchi et al., 1998; D'Agostino et al., 2000; Taniguchi et al., 2007), and were, in fact, initially identified in screens for genes that are rapidly up-regulated by cytokinin in Arabidopsis (Brandstatter and Kieber, 1998; Taniguchi et al., 1998). The cytokinin induction kinetics are similar among members of the type-A ARR gene family, initial induction being detected within 10-15 min of exogenous cytokinin application, increasing over the next hour or two, then decreasing to a steady-state level intermediate between basal and maximal levels (D'Agostino et al., 2000). The type-A ARRs are primary response genes for the cytokinin signaling pathway. Their induction occurs in the absence of *de novo* protein synthesis, and their promoters typically contain multiple type-B ARR binding sites, which accounts for their strong and reproducible upregulation by cytokinin (Taniguchi et al., 2007; Ramireddy et al., 2013). Because of this cytokinin regulation, type-A ARR promoters have been used in the development of reporter systems to evaluate cytokinin responses. Of particular significance has been their use to drive luciferase expression in a transient protoplast reporter system, in which various genes and treatments have been evaluated for their ability to regulate cytokinin signaling (Hwang and Sheen, 2001; Niu and Sheen, 2012). Transcript levels of some type-A ARRs have also been reported to be responsive to various environmental stresses and to nitrogen levels (Sakakibara et al., 1998; Urao et al., 1998; Kiba et al., 1999), which may reflect an alteration of endogenous cytokinin levels in response to these stimuli or cross-talk with these other pathways. Regulation of type-A ARR expression through other signaling pathways may allow modulation of cytokinin signaling, as has been found with regulation of *ARR7* (At1g19050) and *ARR15* (At1g74890) by auxin in the apical meristems (Zhao et al., 2010) and repression of multiple type-A ARRs by WUSCHEL in the shoot apical meristem (Leibfried et al., 2005)(see below).

Genetic analyses indicate that *ARR3* (At1g59940), *ARR4* (At1g10470), *ARR5* (At3g48100), *ARR6* (At5g62920), *ARR7*, *ARR8* (At2g41310), *ARR9* (At3g57040 ), and *ARR15* function as negative regulators of cytokinin signaling, thus participating in a negative feedback loop to reduce the sensitivity to cytokinin (Kiba et al., 2003; To et al., 2004; Leibfried et al., 2005; Lee et al., 2007; To et al., 2007). This contrasts with the primary cytokinin signaling pathway which, as described above, is a positive regulatory circuit chiefly comprised of the hybrid histidine kinases AHK2, AHK3, and AHK4 (Inoue et al., 2001; Suzuki et al., 2001b; Ueguchi et al., 2001; Yamada et al., 2001; Kakimoto, 2003; Kim et al., 2006), the HPt proteins AHP1, AHP2, AHP3, and AHP5 (Hutchison et al., 2006), and the type-B response regulators ARR1, ARR2, ARR10, ARR11, and ARR12 (Sakai et al., 2001; Mason et al., 2005; Yokoyama et al., 2007; Ishida et al., 2008b). The type-A ARRs functionally overlap in the regulation of cytokinin signaling (To et al., 2004). Single mutants exhibit wild-type-like cytokinin responses but double and higher-order mutant combinations exhibit increasing cytokinin sensitivity based on various cytokinin assays, consistent with their role as negative regulators (To et al., 2004). The higher order type-A *ARR* mutants affect the same cytokinin responses previously described for mutants of the primary response pathway elements, but in the opposite direction, resulting in increased cytokinin sensitivity for the inhibition of root elongation, lateral root formation, and the *in vitro* induction of callus tissue and shoot formation. At the molecular level, higher-order type-A ARR mutants exhibited increased sensitivity for the induction of cytokinin-regulated gene expression (To et al., 2004).

Protein stability of the type-A ARRs plays a key role in their ability to negatively regulate cytokinin signaling. Significantly, the presence of cytokinin stabilizes many type-A ARR proteins in a phosphorylation-dependent manner (To et al., 2007). Thus, not only does cytokinin stimulate type-A *ARR* transcription, but it also stabilizes the resultant proteins, thereby accentuating the ability of the type-A ARRs to down-regulate the cytokinin response. Based on the analysis of ARR5, degradation of the type-A ARRs is at least partially dependent on AXR1 , a subunit of the E1 enzyme in the RUB (related to ubiquitin) pathway (Li et al., 2013b). Characterization of the role of AXR1(At1g05180) indicates that the as–yet-unidentified E3 ligase involved in type-A ARR proteolysis requires RUB modification for optimal activity.

Two distinct, but not mutually exclusive mechanisms, can account for the ability of the type-A ARRs to negatively regulate cytokinin signal transduction. First, the type-A ARRs may compete with the type-B ARRs for phosphorylation by the AHPs. Both type-A and type-B ARRs interact with the AHPs (Dortay et al., 2006), and thus the relative concentration of these two classes of phospho-receivers will direct flux through the cytokinin signaling pathway. Based on this model, an increase in the concentration of the type-A ARRs will result in a decrease in phosphorylation of the type-B ARRs, and a corresponding decrease in transcriptional output from the type-B ARRs. The kinetics of type-A *ARR* expression, characterized by an initial increase in levels followed by a decrease, are consistent with this model (D'Agostino et al., 2000). Second, phosphorylated type-A ARRs may participate in phospho-specific interactions with regulatory proteins, such as has been found with bacterial single-domain response regulators (Jenal and Galperin, 2009). The finding that a type-A ARR phosphomimic, in which the conserved Asp is replaced with a Glu, can partially rescue an *arr3 arr4 arr5 arr6* loss-of-function mutant is consistent with this mechanistic model (To et al., 2007). The type-A ARR phosphomimic version of the proteins cannot be phosphorylated, and so should not compete with the type-B ARRs for phosphorylation, yet this protein is still functional and so must negatively regulate cytokinin signaling at least in part though phospho-dependent interactions. The phospho-dependent targets of the type-A ARRs remain to be determined.

### Negative Regulation of the Cytokinin Signaling Pathway

An ability to inactivate a signaling pathway is just as critical to its regulation as an ability to activate it. For this reason, multiple inactivation mechanisms are typically employed to control signaling pathways. These not only serve to inactivate signaling but also

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serve to modulate the sensitivity of cells such that the organism can respond to increasing signal concentrations (Lan et al., 2012). As detailed below, multiple negative regulatory circuits have been identified that control cytokinin function, both through the regulation of cytokinin levels as well as the sensitivity to cytokinin:

(1) AHK4, like some bacterial histidine kinases, has both kinase and phosphatase activities (Mähönen et al., 2006a). In the absence of cytokinin, AHK4 acts as a phosphatase to dephosphorylate AHPs, thereby decreasing signaling through the phosphorelay. Upon cytokinin binding, AHK4 switches to act as a histidine kinase to initiate the multi-step phosphorelay, resulting in phosphorylation of AHPs and downstream response regulators. When cytokinin levels drop, the phosphatase activity of AHK4 likely contributes to shutting off the signaling pathway

(2) As noted above, the AHP6 protein lacks the conserved phosphorylation site present in the other functional phosphotransfer proteins, with the His being replaced with an Asn. *AHP6* expression is induced by cytokinin and functions as part of a negative feedback loop to reduce the sensitivity of a cell to cytokinin (Mähönen et al., 2006b), potentially by interacting with the receiver domains of the cytokinin receptors to prevent phosphotransfer to bona-fide AHPs.

(3) The transcriptional and post-transcriptional induction of type-A ARRs, which negatively regulate cytokinin signaling, in response to cytokinin provides a strong negative feedback loop to dampen the response to cytokinin, thereby limiting the output of the cytokinin activated phosphorelay in a cell (To et al., 2007).

(4) Genes encoding the cytokinin oxidases *CKX4* (At4g29740) and *CKX5* (At1g75450) are reproducibly induced in response to cytokinin (Bhargava et al., 2013), *CKX4* having been identified as a cytokinin primary response gene that is directly regulated by the type-B ARR, ARR1 (Taniguchi et al., 2007). Cytokinin oxidases catalyze the degradation of cytokinins, and so an increase in *CKX* expression is predicted to decrease the concentration of bioactive cytokinins, thereby reducing signaling through the transduction pathway. Transgenic overexpression of *CKX* genes in Arabidopsis, including *CKX4* and *CKX5*, all result in decreased cytokinin levels and a corresponding decrease in cytokinin signaling (Werner et al., 2003), consistent with the cytokinin induction of *CKX* genes being of physiological relevance. Likewise, genes encoding enzymes that conjugate cytokinins to glucose to inactivate them, are also up-regulated in response to cytokinin (Bhargava et al., 2013).

## ROLES OF CYTOKININS IN PLANTS

Cytokinins have been implicated in many aspects of plant growth and development. Until the emergence of cytokinin signaling and metabolism mutants, much of the research done to determine the roles of cytokinin focused on analyzing the effects of exogenously applied cytokinin (Gan and Amasino, 1996). These studies used many different plant species and experimental designs, sometimes making comparisons difficult. Further, it is not always clear that the effects of exogenously applied hormones are indicative of the actual physiological role of the hormone. Many recent studies have taken advantage of mutants disrupted in cytokinin perception or metabolism. These studies generally support and have extended much of the classical work and have revealed novel roles for cytokinin in plant growth and development. The roles of cytokinin, the interaction with other hormonal signals, esp. auxin, and the signaling pathways underlying these effects have been discussed recently in several excellent reviews (Argueso et al., 2009; Dettmer et al., 2009; Moubayidin et al., 2009; Werner and Schmülling, 2009; Müller, 2011; Pernisová et al., 2011; Su et al., 2011; Durbak et al., 2012; Hwang et al., 2012; Naseem and Dandekar, 2012; Vanstraelen and Benková, 2012; El-Showk et al., 2013). Here, we discuss the role of cytokinin in various aspects of Arabidopsis growth and development.

### Cytokinins and the Cell Cycle

Cytokinins are required, in concert with auxin, for cell division in culture in a wide variety of plant cells. There is also evidence that *in vivo* cytokinin may play a role in stimulating cell division. Immunocytochemistry and direct measurements of cytokinin revealed high cytokinin levels in mitotically active areas, such as the root and shoot meristems, and very low levels in tissues where the cell cycle is arrested (Mok and Mok, 1994; Dewitte et al., 1999). Application of exogenous cytokinin to some organs that normally lack this hormone has been shown to induce cell division and cytokinins have been linked to all stages of the cell cycle (reviewed in (D'Agostino and Kieber, 1999; Frank and Schmülling, 1999; den Boer and Murray, 2000).

Compelling evidence that cytokinin regulates the $G_1$/S transition in the cell cycle has been obtained by Murray and co-workers (Riou-Khamlichi et al., 1999). This group previously identified three different Arabidopsis genes encoding D-type cyclins by complementation of a yeast strain deficient in $G_1$ cyclins, and found that one, *CYCD3* (At4g34160) was induced in cultured cells by exogenous cytokinin application (Soni et al., 1995). In Arabidopsis, cytokinin rapidly up-regulates the expression of *CYCD3*, a cyclin that plays a key role in the regulation of plant cell division both *in vitro* and *in vivo* (Soni et al., 1995; Riou-Khamlichi et al., 1999). In animal cells, D-type cyclins are regulated by a wide variety of growth factors and play a key role in regulating the passage through the restriction point of the cell cycle in $G_1$. Riou-Khamlichi and co-workers found, using *in situ* hybridization, that in Arabidopsis *CYCD3* was expressed in the shoot meristem, leaf primordia and axillary meristems, and its induction by cytokinin was also specific to those tissues (Riou-Khamlichi et al., 1999). Thus, this gene is expressed primarily in proliferating tissues, as expected if it is an important element regulating cell division. If CYCD3 acts downstream of cytokinin in promoting cell division or differentiation, then constitutive expression of *CYCD3* should bypass the cytokinin requirement for proliferation in culture. Normally, when explanted into culture, cells require both auxin and cytokinin in the media in order for cell division and callus formation to occur. When leaf explants were obtained from lines over-expressing CYCD3, green calli formed independently of exogenous cytokinin. To demonstrate a role for CYCD3 in cell division, the levels of S-phase associated histone H4 mRNA were examined in the leaf explants. Like the callus tissues, wild-type explants only expressed histone H4 in the presence of cytokinin, whereas lines over-expressing *CYCD3* expressed histone H4 both in the presence and absence of cytokinin. Finally, the expression of *CYCD3* and histone H4 mRNA was observed in par-

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allel with DNA synthesis during synchronous activation of quiescent Arabidopsis cells and S phase was found to occur significantly after the induction of *CYCD3*, which implies that CYCD3 may be involved in the $G_1$/S transition. These results suggest that cytokinin regulates Arabidopsis cell cycle progression at the $G_1$/S transition, at least partly, by inducing *CYCD3* transcription.

Several observations suggest that cytokinins may also play a role in the $G_2$/M transition (reviewed in: Hare and Staden, 1997; Francis, 2011; Lipavská et al., 2011). For example, cytokinins induce the expression of the *cdc2* gene in a number of plant tissues, including intact Arabidopsis roots (Hemerly et al., 1993), and cytokinins were demonstrated to influence the activity, via the phosphorylation state, of a *cdc2*-like kinase in tobacco protoplasts (Zhang et al., 1996). More compellingly, the requirement for cytokinin in cultured tobacco cells can be bypassed by expression of a gene encoding the *S. cerevisiae cdc25* (Orchard et al., 2005; Zhang et al., 2005), a protein phosphatase regulating the G2/M transition. Further, the activity of an endogenous CDK Tyr phosphatase in the cultured tobacco cells peaked at the $G_2$/M transition in cells induced to divide by cytokinin treatment.

Disruption of cytokinin signaling elements has also provided insight into the mechanisms by which cytokinin regulates the cell cycle. The *ahk2 ahk3 ahk4* triple receptor mutant has a smaller shoot and root apical meristem as a result of reduced cell division. Interestingly, fluorescence-activated cell sorting of cells from root tips indicated a reduction in diploid content of DNA in cells and a concomitant increase in cells with a 4N content, suggesting that depletion of cytokinin function *in planta* causes a delay in the $G_2 \rightarrow M$ phase transition (Higuchi et al., 2004). Thus, while overexpression of *CYCD3,* which regulates progression from $G_1$ into the S phase, may bypass the need for cytokinin in cultured cells, the results from the triple receptor mutant suggest that the primary point of control for cytokinin in the cell cycle may be at the $G_2 \rightarrow M$ transition. Alternatively, cytokinin may regulate the cell cycle via distinct mechanisms in different cell types. Consistent with cytokinin acting to promote progression through the cell cycle, exogenous cytokinin, or increased cytokinin sensitivity as a result of type-A ARR mutations, promoted division in the quiescent center cells in the root, which are generally mitotically inactive (Zhang et al., 2011; Zhang et al., 2013) (see below).

### Cytokinin and the Shoot Apical Meristem

The shoot apical meristem (SAM) is a highly specialized group of cells from which the majority of the aerial portion of the plant is derived by reiterative development (Kerstetter and Hake, 1997). The ability of cytokinins to initiate shoots from undifferentiated callus cultures and the initiation of ectopic meristems in transgenic plants engineered to overexpress cytokinins suggested a role for this hormone in SAM development. More recently, a number of studies have shed light on the role of cytokinin in SAM function and its interaction with other hormonal and developmental signaling pathways (Shani et al., 2006; Kyozuka, 2007; Galinha et al., 2009; Veit, 2009; Werner and Schmülling, 2009; Su et al., 2011; Durbak et al., 2012; Hwang et al., 2012).

A decrease in cytokinin levels via overexpression of a *CKX* gene (Werner et al., 2003) or disruption of multiple *IPT* genes (Miyawaki et al., 2006), or disruption of the cytokinin receptors (Higuchi et al., 2004; Nishimura et al., 2004) resulted in a smaller SAM, suggesting that cytokinin is a positive regulator of cell proliferation in the SAM. The KNOTTED-LIKE (KNOX) homeobox transcription factors are required to establish and maintain the SAM (Kerstetter et al., 1994; Kerstetter et al., 1997; Jackson and Hake, 1999). The *KNOX* genes are expressed in the SAM, but excluded from incipient leaf primordia. A major mechanism by which the KNOX transcription factors regulate SAM function is by controlling the relative levels of cytokinin and GA. The KNOX proteins act to decrease GA levels by repressing expression of genes encoding GA20 oxidase, which is involved in GA biosynthesis, and by inducing the expression of GA2 oxidase, which catabolizes GA. In contrast, the KNOX proteins increase cytokinin levels in the SAM at least in part by inducing the expression of *IPT7*(At3g23630). Thus, KNOX expression results in a low GA/high cytokinin environment specifically in the SAM, which is favorable for meristem function. Interestingly, the KNOX genes are up-regulated in Arabidopsis in response to induced elevation of cytokinin levels (Rupp et al., 1999), which suggests that there may be a positive feedback loop between cytokinin levels and KNOX gene expression in the SAM.

The level of cytokinin in the SAM is also the result of localized expression of the *LOG* genes in the apical region of the SAM where the stem cells reside (Kurakawa et al., 2007; Chickarmane et al., 2012). In Arabidopsis, the *LOG4* gene is expressed in the L1 layer of the SAM, providing a localized apical source of cytokinin (Kurakawa et al., 2007; Chickarmane et al., 2012). The *LOG* genes encode enzymes that convert inactive cytokinin ribotides to the active free bases, thus providing a spatially localized source of active cytokinin in the SAM.

Recently, it has been shown that SHOOT-MERISTEMLESS (STM: At1g62360) acts to inhibit cellular differentiation and endoreduplication by acting through cytokinin and through the cytokinin induction of *CYCD3*, which encodes a cyclin that is involved in regulating the progression through the cell cycle (Scofield et al., 2013). In addition, STM was found to organize the SAM and induce the formation of ectopic meristems through a mechanism not strictly dependent on the elevation of cytokinin levels, suggesting that not all of the functions of STM in the SAM require the regulation of cytokinin levels (Scofield et al., 2013).

In addition to the regulation of cytokinin biosynthesis, cytokinin signaling is also modulated in the SAM. The activity of cytokinin signaling in cells of the SAM is determined by the level of active cytokinin and the sensitivity of the SAM cells to cytokinin. The sensitivity relates to the expression level of the various two-component signaling elements, including the negative inputs from the type-A ARRs. The homeodomain transcription factor WUSCHEL (WUS: At2g17950) positively regulates cell proliferation in the SAM (Laux et al., 1996). Several of the type-A ARRs are directly repressed by WUS, which increases the sensitivity to cytokinin in a subdomain of the SAM. A further regulatory input into the level of cytokinin output comes from the repression of a pair of type-A ARRs (*ARR7* and *ARR15*) by auxin in the SAM, which is mediated at least in part by ARF5/MONOPTEROS (At1g19850) (Zhao et al., 2010). Thus, auxin and cytokinin signaling converge on the regulation of type-A ARRs in the SAM, and this provides a mechanism by which auxin can provide input to regulate the sensitivity of a subdomain of the SAM to cytokinin.

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While auxin and WUS increase the sensitivity to cytokinin by repression of type-A ARRs, cytokinin in turn up-regulates *WUS* expression by CVL1/CLV3 (At1g75820/ At2g27250)-dependent and CVL1/CLV3-independent mechanisms, but only at very high concentrations of cytokinin (Lindsay et al., 2006; Gordon et al., 2009). Interestingly, the ERECTA (ER) family of LRR-RLKs play a role in buffering the SAM against changes in cytokinin levels; a mutant disrupted for all ER family members is hyper-responsive for the induction of CLV3 expression and the morphological changes in the SAM in response to exogenous cytokinin (Uchida et al., 2013). Computational modeling of the interactions of cytokinin, WUS, ARR5 and the CLV pathway, coupled with detailed analysis of the expression patterns of these elements in the SAM, led to a model in which multiple feedback loops act among these factors to ultimately regulate the number of cells in the SAM (Gordon et al., 2009). A subsequent study extended this model by incorporating the localized expression of the *LOG4* gene in the apical, L1 layer of the SAM (Chickarmane et al., 2012). The computation model suggested a novel regulatory interaction in the SAM in which WUS represses cytokinin biosynthesis. This was indeed shown to be the case as *LOG4* expression, as well as the level of the TCS::GFP cytokinin reporter (Zürcher et al., 2013) were found to be decreased in the SAM of the *clv3-2* mutant, which has an increased WUS expression domain. This provides an elegant model for the dynamic positioning of the WUS domain within the growing SAM, which maintains a consistent position even in the face of constant turnover of cells within the SAM throughout plant growth and development.

**Cytokinin in the Root Apical Meristem**

Since their first discovery, cytokinins have been known to inhibit root growth and development (Skoog and Miller, 1957). While reduced cytokinin function in Arabidopsis leads to reduced shoot growth, it results in an increase in overall root mass, with longer roots, an increased number of lateral roots and an enlarged root apical meristem (RAM) (Werner et al., 2003; Mason et al., 2005; Miyawaki et al., 2006; Riefler et al., 2006). The inhibitory role of cytokinin on primary root growth arises from effects on cell division in the root meristem and on cell expansion in the root elongation zone. Cytokinin-mediated inhibition of cell expansion occurs in part through stimulation of the ethylene signaling pathway, as inhibition of ethylene biosynthesis or signaling reduces the effects of cytokinin (Cary et al., 1995; Vogel et al., 1998; Ruzicka et al., 2009). Here, we focus on how cytokinin interacts with auxin to control cell division and regulate the size of the RAM.

Auxin and cytokinin act antagonistically in the root to regulate the size of the RAM. Cytokinin regulates RAM size, at least in part, by promoting the rate of cell differentiation in the transition/elongation zone (Dello Ioio et al., 2007; Dello Ioio et al., 2008). In contrast, auxin promotes cell proliferation in the RAM and it is the interplay between auxin and cytokinin that determines the size of the RAM and hence the rate of root growth. One mechanism linking the function these hormones in the RAM is the induction of the auxin signaling repressor *SHY2* (At1g04240) by cytokinin through the AHK3 cytokinin receptor and the type-B ARRs (Dello Ioio et al., 2007; Moubayidin et al., 2010; Hill et al., 2013). SHY2, which is degraded in the presence of high auxin, in turn regulates auxin responsiveness and the expression of several PIN auxin efflux carriers in the RAM. Thus, in the RAM the high cytokinin/low auxin environment in the transition/elongation zone promotes cell differentiation and the high auxin/low cytokinin environment level further down the root inhibits cell differentiation and promotes cell proliferation. A more recent study suggested that the primary mechanism regulating PIN expression by cytokinin occurred postranscriptionally (Marhavý et al., 2011; Zhang et al., 2011). Exogenous cytokinin or disruption of multiple type-A ARRs in general reduced PIN protein expression in the root tip without affecting the level of the cognate transcripts. These findings are not fully consistent with SHY2 primarily meditating the transcriptional control of the PINs in response to cytokinin in the RAM.

A recent study has shed light on how cytokinin regulates cell differentiation in the elongation/differentiation zone (Dello Ioio et al., 2012). These authors found that PHABULOSA (PHB: At2g34710), an HD-ZIPIII transcription factor, binds directly to the *IPT7* DNA regulatory regions to promote its expression. Intriguingly, cytokinin in turn represses both *PHB* and microRNA165, which in turn acts to inhibit PHB. Thus, cytokinin represses both PHB itself, and also represses a repressor of PHB. Further, while cytokinin represses PHB, PHB in turn activates cytokinin expression via induction of *IPT7*. The authors suggest that this incoherent regulatory loop provides a robust cytokinin input into the balance of cell division and differentiation during RAM growth and development.

While previous studies focused on the role of cytokinin in the transition/elongation zone of the root, more recent work suggest that cytokinin also plays a key role in regulating cell division in the quiescent center (QC). The QC is a group of 4-8 mitotically inactive cells in the center of the stem cell niche in the RAM that are essential for the maintenance of the stem cell fate of the surrounding cells (van den Berg et al., 1997). Application of exogenous cytokinin, or disruption of multiple type-A ARR genes, leads to a reactivation of cell division in the QC (Zhang et al., 2011; Zhang et al., 2013). This suggests that low cytokinin signaling is essential to inhibit cell division in the QC. This effect of cytokinin is mediated through a down-regulation of the auxin influx carrier LAX2 (At2g21050). The repression of *LAX2* requires ARR1 and ARR12 and occurs through direct binding of these type-B ARRs to the regulatory region of LAX2 (Swarup and Péret, 2012). Thus, the mitotic inactivity and function of the QC appears to require a high auxin/low cytokinin environment, with cytokinin regulating auxin transport in the RAM to ensure an auxin maximum in the QC cells. Cytokinin also appears to repress the expression of the *SCARECROW* (*SCR)*( At3g54220) gene in the RAM (Zhang et al., 2013), which plays an important role in the specification of the QC cells (Sabatini et al., 2003).

Cytokinin also plays a role in the specification of the root stem cell niche in the developing Arabidopsis embryo (Müller and Sheen, 2008). Using the TCS cytokinin reporter, cytokinin function was determined to be high in the hypophysis, the founding cell of the root system (Müller and Sheen, 2008). Following division of the hypophysis, the apical cell retained high cytokinin signaling as measured by the TCS:GFP reporter, but the basal most cell displayed reduced cytokinin function as a result of the induction of *ARR7* and *ARR15* by the high auxin levels in this cell. Expression of an artificial miRNA designed to silence these two type-A ARRs caused severe defects in the development of the embryonic root, suggesting that these genes act to regu-

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late the relative auxin:cytokinin ratio in the two daughter cells of the hypophysis. However, there are two potential discrepancies that raise concerns about this elegant model. First, disruption of all three cytokinin receptors, which would severely alter the auxin:cytokinin ratio in these cells, does not apparently affect development of the root in the embryo. Second, a double *arr7 arr15* mutant, containing null T-DNA insertion alleles in these genes, is aphenotypic in the embryonic root (Zhang et al., 2011). More studies are needed to resolve these discrepancies.

## Role of Cytokinin in Lateral Root Development

Auxin and cytokinin interact antagonistically to regulate the formation of lateral root primordia. Auxin is a positive regulator of lateral root formation; the appropriate spatial distribution of auxin at the developing lateral root tip, mediated by auxin efflux carriers, is essential for the proper development of the lateral root. In contrast, cytokinin inhibits the formation of lateral roots in Arabidopsis. Exogenous application of cytokinin results in fewer lateral roots (Li et al., 2006). Consistent with this, mutants with decreased cytokinin signaling have an increased number of lateral roots (Riefler et al., 2006) and mutants with increased cytokinin sensitivity have fewer (To et al., 2004). Lateral roots initiate from anticlinal cell divisions of pericycle founder cells. Cytokinin acts directly on pericycle founder cells to disrupt lateral root initiation and patterning. Laplaze et al. (2007) increased cytokinin levels specifically in either xylem-pole pericycle cells or young lateral root primordia using GAL4-GFP enhancer trap lines designed to express Agrobacterial *ipt* in these cells (Laplaze et al., 2007). The authors found that the xylem-pole pericycle cells were sensitive to inhibition by cytokinin, but that the young lateral root primordia were not. Consistent with this, Bielach et al found that young lateral root primordia were more sensitive to mutations that disrupt cytokinin biosynthesis (*ipt3,5,7*) or signaling (*arr1, 11*) than are developmentally more advanced primordia (Bielach et al., 2012). This effect of cytokinin was the result of alteration of PIN levels in lateral root founder cells, which perturbs the distribution of auxin required for lateral root initiation. This is consistent with other results indicating that cytokinin has a major effect on the expression and distribution of the PIN auxin efflux carriers in the root (Dello Ioio et al., 2008; Pernisová et al., 2009; Ruzicka et al., 2009; Zhang et al., 2011). In the developing lateral roots, cytokinin reduces PIN1 protein levels by regulating the endocytic recycling of PIN1 (At1g73590) during lateral root development, redirecting it for lytic degradation in vacuoles (Marhavý et al., 2011). Similar to its role to protoxylem formation, the AHP6 pseudo-HPt inhibits cytokinin signaling during early lateral root development (Moreira et al., 2013). AHP6 is necessary for the correct orientation of cell divisions at the onset of lateral root development and is involved in localizing PIN1. Thus, cytokinin acts to regulate auxin flow required early in the development of lateral roots.

## Cytokinin in Vascular Development

Cytokinin plays key roles in the development of the vasculature system, acting both to promote protoxylem differentiation and the development of the vascular cambium (Dettmer et al., 2009). The first demonstration of a role for cytokinin in vascular development in Arabidopsis came from the analysis of the *woodenleg* (*wol*) mutant, which is the result of an antimorphic allele of the *AHK4* cytokinin receptor (Mähönen et al., 2000)(see above). In *wol* mutants, the root vasculature differentiates exclusively into protoxylem, resulting in roots that lack both phloem and metaxylem; further, there is a reduction in cell proliferation in the procambial cells, resulting in a reduced vascular system. Other mutations that reduce cytokinin signaling (multiple *ahk*, *ahp*, or *type-B arr* mutants) lead to a similar root phenotype in which the vascular system is comprised solely of protoxylem (Higuchi et al., 2004; Nishimura et al., 2004; Hutchison et al., 2006; Yokoyama et al., 2007; Argyros et al., 2008). This suggests that cytokinin is necessary to specify vascular cell identities other than protoxylem. The vascular phenotypes of the *wol* mutant can be suppressed by loss-of-function mutations in *AHP6* (Mähönen et al., 2006b). The expression of *AHP6* is induced by auxin in the root, which acts as a mechanism by which auxin down-regulates cytokinin sensitivity (Bishopp et al., 2011), similar to the induction of type-A ARRs by auxin in the embryonic root (Müller and Sheen, 2008). Further, cytokinin regulates the level of the PIN proteins in the developing vasculature, resulting in an interactive regulatory loop between auxin and cytokinin that plays a key role in the specification of the vascular pattern (Bishopp et al., 2011).

A second role for cytokinin in vascular development is as a positive regulator of the vascular cambium. Disruption of multiple *IPT* genes (*ipt1,3,5,7)* results in the complete loss of vascular cambium in the root and stem (Matsumoto-Kitano et al., 2008). Exogenous cytokinin rescued this cambial defect in a dose-dependent manner. Further, when an *ipt1,3,5,7* mutant shoot was grafted onto a wild-type root, cambial activity (as well as *t*Z levels) was restored in the shoot. Similarly, a wild-type shoot restored cambial activity to an *ipt1,3,5,7* root. These results suggest that cytokinin biosynthesis in either the root or the shoot is sufficient to promote cambium development throughout the plant. Furthermore, they suggest that cytokinin is a mobile signal that can move rootward or shootward in a functionally relevant manner.

The CKI1 histidine kinase, which lacks the cytokinin-binding CHASE domain, also appears to play a role in cambial development. CKI1 was first identified by its ability, when overexpressed, to promote cell proliferation in culture independent of cytokinin (Kakimoto, 1996). Strong loss-of-function *cki1* mutants results in female gametophytic lethality (Pischke et al., 2002; Hejátko et al., 2003). Overexpression of CKI1 partially rescues the decreased procambial proliferation defects of an *ahk2 ahk3* mutant, and expression of a dominant negative CKI1 enhances this defect (Hejátko et al., 2009). This suggest that CKI1 may feed into the cytokinin response pathway to regulate cambial development, consistent with its ability to activate cytokinin responses when overexpressed (Kakimoto, 1996; Hwang and Sheen, 2001).

## Cytokinin and Gametophyte Development

The life cycle of higher plants alternates between haploid gametophytic and diploid sporophytic phases. The female gametophyte is surrounded by the sporophyte and develops within the ovule of the maternal plant. The CKI1 histidine kinase is required for female gametophyte development (Pischke et al., 2002; He-

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játko et al., 2003), as are the downstream AHPs (Deng et al., 2010). Cytokinin has been shown to be required in the sporophytic tissue for female gametophyte development (Nishimura et al., 2004; Kinoshita-Tsujimura and Kakimoto, 2011). While some allelic combinations of the AHK cytokinin receptors are able to form viable seeds, the strongest combination of alleles (*ahk2-7 ahk3-3 cre1-12*) displays nearly complete female gametophyte arrest (Cheng et al., 2013). Further, in a fraction of *ahk2-2tk ahk3-3 cre1-12* triple mutant female gametophytes, integument initiation was impaired and finger-like ovule structures were identified, which resulted in part from a down-regulation of PIN1 expression in the ovules of the triple receptor mutant (Bencivenga et al., 2012). The transcription factor SPL/NZZ (At4g27330), which is a key regulator of ovule development, was found to be required for the cytokinin regulation of PIN1. Cytokinin was also found to down-regulate the expression of the BEL1 (At5g41410) homeodomain transcription factor, another important regulator of ovule development. BEL1 also likely plays a role in the cytokinin-mediated down-regulation of PIN1 expression in the ovule.

Cytokinin appears to provide positional information for the development of the female gametophyte. Cytokinin signaling is enriched in the chalaza in the maternal, sporophytic tissue via localized expression of genes involved in cytokinin biosynthesis (*IPT1;* At1g68460) and perception (*AHKs*) (Cheng et al., 2013). Intriguingly, this gradient of cytokinin mirrors an auxin gradient in the developing female gametophyte (Pagnussat et al., 2009), likely formed through the action of PIN1 auxin efflux carriers (Ceccato et al., 2013), and suggest that, as in other developmental contexts, auxin and cytokinin may play opposing roles during female gametophyte development. This sporophytic localized cytokinin signaling is essential for the production of the functional megaspore following meiosis of the megaspore mother cell (Cheng et al., 2013), suggesting that elevated cytokinin signaling is required for the production of a signal in the sporophytic tissue that promotes the specification of the functional megaspore.

### Role in Leaf Development

The pavement cells of Arabidopsis leaves are puzzle-shaped with highly interdigitated lobes. Previous studies have indicated that auxin acts to coordinate this interdigitation through the activation of a ROP GTPase (Xu et al., 2010). A recent study has also linked cytokinin to this process (Li et al., 2013a). In a genetic screen for mutants that displayed altered pavement cell morphogenesis using an activation-tagged T-DNA insertional library, the *ARR7* and *ARR20* genes were identified (Li et al., 2013a). Increasing cytokinin levels via dexamethasone-induced expression of *IPT7* reduced pavement cell interdigitation in cotyledons. In contrast, dexamethasone-induced expression of *CKX3* (At5g56970) enhanced interdigitation. Furthermore, disruption of other signaling elements (multiple *ahk* and *ahp* mutants) resulted in leaves that displayed enhanced pavement cell interdigitation. Epistasis analysis of an *ARR7* overexpression transgene and the *rop2 rop4* (At1g20090/At1g75840) mutations indicated that cytokinin acts upstream of the ROPs to regulate the degree of interdigitation (Li et al., 2013a). Thus, as in several other contexts, auxin and cytokinin counterbalance each other to regulate this developmental process.

Cytokinin positively regulates cell division in a growing leaf, promotes leaf serrations at the margins and inhibits senescence (see below). In contrast, the CIN-TCP basic-helix-loop helix (bHLH) transcription factors promotes progression of cell cycle arrest in a maturing leaf and induces senescence by elevating jasmonic biosynthesis. A recent study has provided a mechanistic link between these opposing pathways (Efroni et al., 2013). The authors demonstrate that TCP4 (At3g15030) interacts with the SWI/SNF chromatin remodeling complex, and that this complex binds directly to the *ARR16* (At2g40670) promoter (a type-A ARR) to induce its expression. The induction resulted in a decrease in sensitivity of leaves to cytokinin, accounting for the antagonistic relationship between TCPs and cytokinin in leaf development.

### The Interaction of Cytokinin and Light Signaling

Cytokinin and light responses interact in several contexts. The growth and developmental response of seedlings to light, called photomorphogenesis, can be partially mimicked by growth of seedlings in the presence of exogenous cytokinin or by elevation of endogenous cytokinin (Chory et al., 1994; Lochmanova et al., 2008). This activation of photomorphogenesis by cytokinin requires the two-component response pathway (Argyros et al., 2008). A direct coupling of light and cytokinin signaling was inferred from the finding that the red light photoreceptor PhyB (At2g18790) interacts with ARR4, a the type-A ARR (Sweere et al., 2001) and that this interaction stabilizes the active, Pfr-form of PhyB. Consistent with this, overexpression of ARR4 resulted in hypersensitivity to red light. However, subsequent studies demonstrated that the *arr3,4,5,6* quadruple mutant was more sensitive to red light as compared to wild-type seedlings (To et al., 2004), suggesting that these type-A ARRs, which include ARR4, may act as negative regulators of phytochrome function. These conflicting data may be the result of differences in the growth conditions used in these two studies. Indeed, Mira-Rodado et al. found that an *arr4* loss-of-function mutant was hyposensitive to red light in their growth conditions (Mira-Rodado et al., 2007). In any case, these results implicate multiple type-A ARRs in the regulation of phytochrome function.

The bZIP transcription factor HY5 (At5g11260) acts as another point of convergence between light and cytokinin signaling. HY5 is a positive regulator of photomorphogenesis and acts downstream of the phytochrome and cryptochrome photoreceptors (Chang et al., 2008). *hy5* mutants are partially insensitive to cytokinin in root elongation and callus initiation assays (Cluis et al., 2004). Cytokinin increases the abundance of HY5 protein by increasing its stability (Vandenbussche et al., 2007). HY5 is also necessary for the cytokinin induction of anthocyanin production in blue light, but not for the inhibition of hypocotyl elongation by cytokinin in the dark (Vandenbussche et al., 2007). These results suggest that a subset of cytokinin inputs into light responses may be mediated through HY5.

Among the events of photomorphogenesis regulated by cytokinin is chloroplast biogenesis. A long history of research has implicated cytokinin in the control of chloroplast development and maintenance (Chory et al., 1994). Higher-order mutants of the cytokinin receptors (*ahk2 ahk3 ahk4*) and type-B ARRs (*arr1 arr10*

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*arr12)* exhibit reduced levels of chlorophyll, consistent with the cytokinin regulating chloroplast development (Riefler et al., 2006; Argyros et al., 2008). Recent data implicate two families of transcription factors, members of which are induced by cytokinin, in controlling aspects of chloroplast development. Genetic analysis indicates that the GNC/CGA1 family regulates chloroplast development from the proplastid as well as later chloroplast growth and division (Bi et al., 2005; Naito et al., 2007; Hudson et al., 2011; Köllmer et al., 2011; Chiang et al., 2012). The CRF family has also been implicated in controlling chloroplast division, based on overexpression of CRF2 (At4g23750) resulting in increased chloroplast division, the same effect found when plants are treated with exogenous cytokinin (Okazaki et al., 2009).

Cytokinin also plays a role in the response of Arabidopsis to shade. Plants grown in the shade of other plants receive a low ratio of red/far red light (R/FR), which induces a number of developmental changes including increased hypocotyl elongation and an arrest of leaf primordia growth (Carabelli et al., 2007). Exposure to low R/FR was found to result in a rapid increase in auxin signaling in the leaf primordia, which in turn led to increased expression of the *CKX6* (At3g63440) gene (Carabelli et al., 2007), which encodes a cytokinin oxidase enzyme that irreversibly inactivates cytokinin via side chain cleavage. This induction of *CKX6* in response to low R/FR light likely results in a reduction in cytokinin levels in the leaf primordia, and hence a reduction in cell proliferation. Consistent with this, *ckx6* mutants did not arrest leaf primordia growth in low R/FR conditions as is observed in wild-type plants (Carabelli et al., 2007).

### Cytokinin and Circadian Rhythm

Elements of the two-component cytokinin response pathway feed into the regulation of the circadian clock in Arabidopsis. The ARR3 and ARR4 type-A ARRs play a redundant role in regulating the circadian clock in Arabidopsis (Salomé et al., 2005). *arr3,4* and *arr3,4,5,6* mutants have a longer circadian period and in white light, a leading phase similar to *phyB* mutants (Salomé et al., 2005). Introduction of an *ARR5* genomic transgene into the *arr3,4,5,6* mutant restored cytokinin sensitivity to wild-type levels (due to modest overexpression of the *ARR5* transgene) (To et al., 2004; To et al., 2007), but did not restore the altered periodicity of the circadian rhythm (Salomé *et al.*, 2005), indicating that ARR3 and ARR4 regulate the clock through a cytokinin-independent mechanism. Surprisingly, disruption of both *ARR8* and *ARR9* suppressed the altered periodicity of the *arr3,4* mutant, even though no photoperiod phenotype was observed in the *arr8,9* mutant itself. This suggests that these pairs of type-A ARRs act antagonistically in the regulation of the circadian clock.

ARR4 influences the circadian clock by both PhyB-independent and PhyB-dependent mechanisms (Salomé et al., 2005; Hanano et al., 2006), the latter of which could reflect the effect of ARR4 on of PhyB function noted above (Sweere et al., 2001). Intriguingly, the expression of *ARR9*, but not other two-component genes, displays a strong circadian oscillation and the timing of this expression is controlled by the major clock components (Ishida et al., 2008a). Exogenous cytokinin treatment causes a shift in the phase of the circadian clock (Hanano et al., 2006) in an ARR4 and PhyB-dependent manner (Zheng et al., 2006). A mechanistic link for this effect is suggested by the observation that cytokinin induces the expression of *LHY* (At1g01060) and *CCA1* (At2g46830) genes, but represses the expression of *TOC1* (At2g43010) (Zheng et al., 2006). Thus, there appears to be an interdependent regulatory loop between the clock genes and cytokinin response genes (i.e. *ARR9*). Further, the clock-regulated PIF4 (At2g43010) basic helix-loop-helix transcription factor, regulates the expression of *CKX5* (At1g75450), which encodes a cytokinin oxidase that could play role in mediating the diurnal and photoperiodic control of plant growth (Nomoto et al., 2012).

### Cytokinin and Leaf Senescence

Cytokinins have long been known to inhibit leaf senescence (Gan and Amasino, 1996). In Arabidopsis, the *ahk2 ahk3* double mutant did not display the cytokinin inhibition of dark-induced leaf senescence observed in wild-type detached leaves (Riefler et al., 2006). Consistent with this, disruption of multiple type-A ARRs inhibits dark-induced senescence of detached leaves and increases the sensitivity of the leaves to the effect of exogenous cytokinin in delaying leaf senescence (To et al., 2004). The *ore12* mutation displays delayed leaf senescence in intact Arabidopsis plants. *ore12* is the result of a recessive, gain-of-function missense mutation in the predicted extracellular domain of *AHK3* (Kim et al., 2006). The recessive nature of the gain-of-function *ore12* allele is likely the result of a dosage requirement for this mutation to affect leaf senescence. Disruption of *AHK3* displayed earlier leaf senescence; genetic analysis of the three cytokinin AHK receptors indicated that AHK3 plays the major role in regulating leaf senescence. This effect of AHK3 on leaf senescence was due to the phosphorylation and activation of ARR2 (Kim et al., 2006), a type-B ARR. While AHK3 plays the major role of the cytokinin receptors in this response, the specificity of the type-B ARRs has not as yet been examined. Indeed, a loss-of-function *ARR2* mutant does not affect leaf senescence, indicating that other type-B ARRs likely play a role in the regulation of this process.

The Cytokinin Response Factors (CRFs), which are cytokinin-regulated AP2/ERF transcription factors, also play a role in regulating leaf senescence (Zwack et al., 2013). Disruption of *CRF6* (At3g61630) results in a decrease in the sensitivity of leaves to the inhibitory effect of cytokinin on dark-induced leaf senescence, and in intact plants, *crf6* mutants display accelerated leaf senescence (Zwack et al., 2013). Surprisingly overexpression of *CRF6* resulted in an even stronger acceleration of senescence. The authors suggest that CRF6 generally acts as a negative regulator of leaf senescence and may be involved in fine-tuning the timing of leaf senescence.

### Cytokinin and plant defense

A number of plant pathogen interactions involve cytokinin (Argueso et al., 2009; Choi et al., 2011; Robert-Seilaniantz et al., 2011; Naseem and Dandekar, 2012). Some pathogens are capable of synthesizing cytokinins, and/or of directing the plant to elevate cytokinin biosynthesis, which has often been closely tied to the

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success of the pathogen. These include gall-forming pathogenic bacteria such as Agrobacterium and the biotrophic actinomycete *Rhodococcus fascians*, and biotrophic fungal and bacterial pathogens that form green bionissia (formerly known as green islands). In addition to potentially altering the development of the plant, pathogen-derived cytokinins may also act to delay senescence and increase sink activity.

*Rhodococcus fascians* secretes a mix of cytokinins, including *t*Z and 2-methylthio-*cis*-zeatin to induce the production of leafy galls (Depuydt et al., 2008; Pertry et al., 2009). This mixture, which is not fully degraded by cytokinin oxidases in Arabidopsis, is perceived primarily by the AHK2 and AHK3 receptors and results in a stronger activation of cytokinin responses as compared to application of a single cytokinin species, likely due to the activation of multiple AHK cytokinin receptors that have distinct affinities for different cytokinin species.

The characterization of the *uni1-d* mutant in Arabidopsis also links cytokinins to pathogen resistance signaling (Igari et al., 2008). *uni1-d* carries a gain-of-function mutation in a CC-NB-LRR resistance protein. The pathogen responsive genes *PR1* (At2g14610) and *PR5* (At1g75040) are induced in *uni1-d* plants via an SA-dependent mechanism. Intriguingly, *uni1-d* mutant plants display an elevation of type-A *ARR* gene expression and phenotypes consistent with an activation of the cytokinin responses, including loss of apical dominance and ectopic auxiliary meristem formation. These phenotypes, as well as induced *PR1* expression, are suppressed by *CKX1* (At2g41510) overexpression, indicating that the *uni1-d* mutation most likely causes an increase in endogenous cytokinin levels (Igari et al., 2008).

Plant-derived cytokinins promote resistance of Arabidopsis to the gram negative bacterial biotrophic pathogen *Pseudomonas syringae* (Choi et al., 2010). Increasing endogenous cytokinin levels via overexpression of *IPT* led to increased resistance to *Pseudomonas syringae* pv. tomato DC3000. Conversely, decreasing cytokinin levels by overexpression of *CKX* signaling or disruption of *AHK2* and *AHK3* led to reduced resistance to this pathogen. Further, the salicylic acid (SA)-responsive transcription factor TGA3/NPR1 (At1g22070) was found to bind directly to the ARR2 protein, and disruption of TGA3/NPR1 abolished the effect of cytokinin treatment on *Pseudomonas syringae* resistance. This indicates a clear link between cytokinin and SA response pathways.

Cytokinin levels in Arabidopsis are important in determining the amplitude of immune responses, and ultimately influencing the outcome of plant-pathogen interactions (Argueso et al., 2012). While low concentrations of cytokinin were found to increase susceptibility to the virulent oomycete *Hyaloperonospora arabidopsidis*, high concentrations increased defense responses through a process that depends on SA accumulation and activation of defense gene expression (Argueso et al., 2012). Interestingly, the *eds16* (At1g74710) mutant, which is defective in SA biosynthesis, is hypersensitivity to cytokinin, suggesting that SA feedback inhibits cytokinin signaling (Argueso et al., 2012). These functions for cytokinin are mediated in part by type-A response regulators, which negatively regulate basal and pathogen-induced SA-dependent gene expression.

The vascular pathogen *Verticillium longisporum* is a soil-borne fungal pathogen that colonizes the xylem of its host. Late in infection, the pathogen switches from a biotrophic to a necrotrophic lifestyle as it spreads into senescing tissue. There is a decrease in *t*Z levels in infected leaves that coincides with the onset of senescence, possibly due to induction of *CKX* gene expression. Increasing endogenous cytokinin levels inhibited the proliferation of this pathogen and reduced disease symptom development, suggesting that reducing cytokinin levels in the host by this pathogen is important for its optimal growth (Reusche et al., 2013).

**Cytokinin and abiotic stress**

Cytokinin function has been linked to a variety of abiotic stresses (Hare et al., 1997). Cold stress rapidly up-regulates the expression of multiple type-A *ARRs* by an AHK2/AHK3-dependent mechanism that surprisingly does not appear to require cytokinin (Jeon et al., 2010). This suggests other potential inputs into the activation of these AHK hybrid sensor kinases. ARR1, AHP2, AHP3, and AHP5 were also involved in the induction of type-A ARRs by cold (Jeon and Kim, 2013), indicating that the canonical two-component phosphorelay acts downstream of the cold activation of AHK2/AHK3. Overexpression of the cold-induced type-A *ARR* genes enhanced freezing tolerance in Arabidopsis seedlings (Jeon et al., 2010; Shi et al., 2012). Further, the *ahk2 ahk3* and *arr7* mutants were found to be hypersensitive to ABA. This suggests the following pathway for the role of two-component signaling in cold acclimation: (AHK2, AHK3) → (AHP2, AHP3, AHP5) → ARR1 → type-A ARRs → ABA → cold acclimation. Further studies are needed to elucidate the mechanism by which cold activates the AHKs.

Cytokinin also appears to play a role in the response to drought and salt stress. Exposure of plants to drought results in a decrease in the level of cytokinins in the xylem sap (Bano et al., 1994; Shashidhar et al., 1996). Nishiyama *et al*. found that drought and salt stress decreased the level of cytokinins in Arabidopsis, in part through a down-regulation of multiple *IPT* genes (Nishiyama et al., 2011). In contrast, Alvarez et al. found that while isoprene-type cytokinins (*t*Z and *t*Z riboside) are indeed decreased in the xylem in response to drought stress, the level of the aromatic cytokinin 6-benzylaminopurine was actually elevated (Alvarez et al., 2008). Treatment of Arabidopsis with either osmotic or salt stress has a strong effect of on the expression of the *AHK* cytokinin receptors. *AHK2* and *AHK4* were down-regulated, both in the root and the shoot, in response to osmotic or salt stress. Conversely, *AHK3* was up-regulated in response to these conditions. This altered suite of *AHK* receptor expression may have important effects on receptor output under stress conditions.

Consistent with the notion that elevated cytokinin levels may promote survival in drought conditions, a recent study found that expression of *Agrobacterium ipt* from a drought/maturation-induced promoter (*SARK*) resulted in a remarkable tolerance to extreme drought conditions in tobacco (Rivero et al., 2007) and cotton (Kuppu et al., 2013). Transgenic plants showed almost complete recovery following a drought regime that killed wild-type plants. In Arabidopsis, reduction of endogenous cytokinin, via either overexpression of *CKX3* or *CKX4*, or through disruption of multiple *IPT* genes (*ipt1,3,5,7*) resulted in a drought- and salt-tolerant phenotype as a result of ABA hypersensitivity and

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increased membrane integrity (Nishiyama et al., 2011). Similarly, disruption of *AHK2* and/or *AHK3* resulted in increased tolerance to drought and salt stresses (Tran et al., 2007). Interestingly, *AHP4*, but none of the other *AHPs*, is down-regulated in response to salt and osmotic stress (Tran et al., 2007). AHP4 is evolutionarily distinct from the other AHPs, and, in contrast to the other functional HPts in Arabidopsis, may play a negative role in cytokinin signaling in some contexts (Hutchison et al., 2006). The expression of several of the *CRF* genes, which were identified as cytokinin-responsive AP2 transcription factors (Rashotte et al., 2003; Rashotte et al., 2006), are down-regulated in response to salt stress, especially in roots (Argueso et al., 2009; Zwack et al., 2013). These genes may play an important role in mediating the input of cytokinin into the salt-stress response pathway.

These results suggest that endogenous cytokinin, acting through the two-component signaling pathway, negatively regulates drought and salt stress signaling. A transcriptome analysis of wild-type and the *ipt1,3,5,7* mutant in response to salt stress revealed that decreased cytokinin levels affected the expression of many known stress-related genes, both in basal conditions and in response to salt stress (Nishiyama et al., 2012), which the authors hypothesize may contribute to the salt tolerance of the *ipt1,3,5,7* mutant.

An analysis of sodium accumulation revealed that cytokinin treatment increased sodium accumulation in the shoot, but not in roots of wild-type Arabidopsis seedlings (Mason et al., 2010). Consistent with this, disruption of cytokinin signaling elements resulted in changes in sodium accumulation: cytokinin-insensitive mutants (*ahk, ahp,* and type-B *arr* mutants) accumulated less sodium in leaves, and cytokinin hypersensitive mutants (type-A *arr* mutants) accumulated more (Mason et al., 2010). Interestingly, the expression of the high-affinity potassium transporter *AtHKT1.1*, which removes sodium from the root, was repressed by cytokinin treatment and was elevated in the *arr1 arr12* double type-B mutant; thus, this transporter may play a role in regulating sodium accumulation in response to cytokinin (Mason et al., 2010).

**Cytokinin and Nutrient Uptake**

Cytokinin regulates the ability of plants to take up various nutrients from the environment, including nitrogen, phosphorous, sulfur, and iron, and the nutrient status of the plant can, in some cases, regulate cytokinin function and hence the growth of the plant (Argueso et al., 2009). The role of cytokinin in nitrogen assimilation is perhaps the best understood of these (Kiba et al., 2011). Nitrate levels regulate the expression of genes involved in its assimilation and reduction, including nitrate transporters, nitrate reductase, glutamine synthetase and glutamate synthase, as well as the enzyme activities of their encoded proteins (Sakakibara et al., 2006).

The level of nitrate available to the plant regulates the level of cytokinins; plants grown on low levels of nitrogen have reduced levels of cytokinins and the addition of nitrate leads to an increase in various cytokinin species (Salama and Waering, 1979; Samuelson and Larsson, 1993; Kiba et al., 1999; Takei et al., 2001b; Takei et al., 2004b). Added nitrate increases cytokinin in part by inducing expression of *AtIPT3* and *AtIPT5*, predominantly in the roots (Miyawaki et al., 2004; Takei et al., 2004b). Disruption of the *IPT3* gene attenuated the induction of cytokinin observed in response to nitrate, indicating that *IPT3* is the primary target for nitrate-induced cytokinin biosynthesis (Takei et al., 2004b). Interestingly, *IPT3* is also regulated by phosphate, sulfate and iron, suggesting that perhaps *IPT3* acts to integrate multiple nutrient signals (Kiba et al., 2011). However, the addition of ammonium to N-starved Arabidopsis plants leads to an increase the levels of *AtIPT5*, but not *AtIPT3*, indicating that the targets for regulating cytokinin biosynthesis depend on which forms of nitrogen are available to the plant (Takei et al., 2004b). The expression of the *CYP735A2* gene, which encodes another enzyme involved in the synthesis of *t*Z (see above), is also regulated by nitrate levels (Wang et al., 2004).

The expression of the nitrate- and cytokinin-inducible low-affinity nitrate transporter gene *NRT1.3* is compromised in a type-B *arr1, 10, 12* triple mutant (Argyros et al., 2008), suggesting that its expression is dependent on these type-B ARRs. Genome-wide microarray analysis revealed that treatment with either cytokinin or nitrate induces the expression of various genes involved in primary metabolism (Wang et al., 2000; Rashotte et al., 2003; Wang et al., 2003; Wang et al., 2004; Brenner et al., 2005; Kiba et al., 2005; Bhargava et al., 2013), and a significant overlap among the sets of genes induced by each treatment is observed (Sakakibara et al., 2006).

The correlation among nitrate, cytokinin levels and their effects on gene expression has led to the suggestion that cytokinin can act as a root to shoot signal to regulate tissue-specific nitrogen metabolism (Takei et al., 2001b; Takei et al., 2002; Gessler et al., 2004; Sakakibara, 2006). Whereas iP is the most abundant cytokinin species present in leaf exudates and in the phloem (Weiler and Ziegler, 1981; Emery and Atkins, 2002; Corbesier et al., 2003), *trans*-zeatin riboside is actively translocated through the xylem (Beveridge et al., 1997; Takei et al., 2001b). The localized expression of *AtIPT3* in the phloem (Miyawaki et al., 2004) and the high levels of expression of *CYP735A2* in the roots and stems, but low *CYP735A2* levels in the leaves (Takei et al., 2004a), suggest a model in which increased nitrate in the roots leads to the induction of *AtIPT3* and *CYP735A2* and thus the biosynthesis of *t*Z ribosides in the root, which can subsequently be transported to the shoot via the xylem. In the shoot, up-regulation of *AtIPT3* and possibly *AtIPT5* would lead to the accumulation of iP cytokinins, which are translocated via the phloem to other parts of the plant. The balance of different cytokinin species, perceived by two-component elements, would signal the availability of nitrate forms, and would ultimately lead to the expression of cytokinin-responsive and potentially also nitrate-responsive genes, to regulate nitrogen metabolism in the plant (Sakakibara et al., 2006).

A role for cytokinins in the phosphate (Pi)-starvation response has been suggested based on evidence that cytokinin levels are reduced in Pi-starved plants (Salama and Waering, 1979; Horgan and Waering, 1980). Consistent with this, the Arabidopsis *pho1* (At3g23430) and *pho2* (At2g33770) mutants, which fail to accumulate and hyper-accumulate Pi in shoots, respectively, show altered sensitivity to cytokinin (Lan et al., 2006). Pi-starvation leads to complex changes in gene expression (Hammond et al., 2003; Wu et al., 2003). An initial transient change in the

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expression of genes encoding general stress response factors is observed, followed by the induction of genes directly involved in the response to Pi starvation (Hammond et al., 2003). In general, cytokinins down-regulate Pi-starvation responsive genes (Martin et al., 2000; Hou et al., 2005). Microarray analysis of rice plants under Pi starvation confirmed these results, but also identified genes that were up-regulated or unchanged upon cytokinin addition, indicating that the effect of cytokinin in Pi-starvation gene expression is complex (Wang et al., 2006).

The *ahk3,4* double mutant is defective for the cytokinin repression of gene expression in the local response to Pi starvation, but is unaffected in the systemic response (Martin et al., 2000; Franco-Zorrilla et al., 2005). This suggests that while AHK3 and AHK4 are necessary for the local response, AHK2 may play an important role in the systemic response, perhaps redundantly with AHK3. Consistent with this model, AHK4 is primarily expressed in roots, and AHK2 and AHK3 most highly in shoots (Higuchi et al., 2004; Nishimura et al., 2004). The expression of AHK4 was found to be repressed by addition of Pi, indicating a negative feedback regulatory role for cytokinin (Franco-Zorrilla et al., 2002).

Sulfate-responsive genes are up-regulated in response to cytokinin in either sulfur-depleted or non-depleted conditions, and are only marginally up-regulated by other plant hormones (Ohkama et al., 2002). The expression of *APR1* (At4g04610), which encodes an enzyme involved in the key step of sulfate assimilation, is induced by cytokinin (Ohkama et al., 2002). The genes encoding sulfate transporters are also regulated by cytokinin (Maruyama-Nakashita et al., 2004), as well as by sucrose and/or nitrate (Ohkama et al., 2002; Rouached et al., 2008). However, the concentration of cytokinins is not altered after sulfate-starvation (Ohkama et al., 2002), though a different study indicated that *IPT3* was up-regulated in response to sulfate (Hirose et al., 2008). Further, application of cytokinin does not change the concentration of O-acetyl-L-serine (Ohkama et al., 2002), a cysteine biosynthetic precursor that acts as a positive regulator of sulfate starvation-responsive genes (Kim et al., 1999; Hirai et al., 2003), suggesting that the action of cytokinin in the regulation of sulfate uptake and sulfate-responsive genes is most likely indirect.

Cytokinins act to negatively regulate the expression of a subset of iron-responsive genes (Séguéla et al., 2008). This repression requires the AHK3 and AHK4 receptors, but is independent of iron status and of FIT1 (At2g28160), which is a basic helix-loop-helix (bHLH) transcription factor regulating a subset of iron-responsive genes (Briat et al., 2007). A transient rise in *IPT3* and type-A ARR gene expression occurred in response to iron re-supply to iron-starved plants (Séguéla et al., 2008), which is reminiscent of the induction of these same genes in response to nitrogen resupply. It was found that other factors that inhibit root growth, such as mannitol and NaCl, also repress iron-starvation response genes (Séguéla et al., 2008). The authors propose that cytokinin down-regulates iron-responsive gene expression through a growth-dependent pathway ((Séguéla et al., 2008), which may underlie the effect of cytokinin on other nutrient assimilation pathways. Further studies that carefully examine the timing of the effects of cytokinin on root growth and nutrient-regulated genes expression, coupled with a better understanding of the transcription circuits regulating iron-responsive gene expression should help clarify this issue.

## Summary and Perspectives

The previous decade has witnessed remarkable progress in our understanding of cytokinin synthesis, metabolism and signaling. Further, we now have a much clearer understanding of the roles of cytokinin in plant growth and development, the molecular mechanisms underlying those roles, and how cytokinin signaling interacts with other hormonal and developmental signaling pathways. Arabidopsis genes encoding the key enzymes involved in cytokinin biosynthesis and metabolism have been identified, and the near future should reveal much about the mechanism and regulation of cytokinin biosynthesis and metabolism. Molecular, genetic and biochemical studies have elucidated the cytokinin signal transduction chain, from the perception in the lumen of the ER to the alterations of gene expression in the nucleus. This pathway is quite similar to the bacterial two-component phosphorelay paradigm.

A common theme in the role of cytokinin in plant growth and development is the often antagonistic interactions with auxin (Galinha et al., 2009; Moubayidin et al., 2009; Su et al., 2011; Durbak et al., 2012). This was recognized at the very outset, following the discovery of cytokinin, as the earliest studies indicated that the ration of auxin to cytokinin determined tissue differentiation in cultured cells (Skoog and Miller, 1957). These two hormones influence each other through multiple mechanisms. Cytokinin influences the distribution of auxin within the plant, both by regulation of the PIN auxin efflux and the LAX auxin influx transporters and by the regulation of auxin synthesis. Cytokinin also regulates the sensitivity to auxin via regulation of the Aux/IAA protein SHY2. Auxin in turn also regulates cytokinin at multiple levels, through the regulation of the type-A ARRs and *AHP6*, which encode negative regulators of cytokinin signaling, or through the regulation of cytokinin oxidase genes that encode proteins that degrade cytokinin.

In addition to auxin, cytokinin interacts with other hormonal, environmental and developmental pathways. A common theme regarding the mechanisms by which these other signals modulate cytokinin function is, similar to auxin, via up-regulation of type-A ARRs or AHP6, which dampens the response of a cell to cytokinin, or by regulation of cytokinin oxidase genes. Undoubtedly, there will be other regulatory inputs into cytokinin function, targeting processes that are currently poorly understood, such as cytokinin transport and conjugation.

Despite this remarkable progress, a multitude of questions remain. How is *c*Z made in plants? How is the biosynthesis of cytokinin regulated? How is cytokinin transported across cellular membranes? How is specificity of interaction among the various members of the gene families encoding the two-component elements achieved? What is the transcriptional network downstream of the cytokinin-regulated phosphorelay and how does this bring about the myriad of changes prompted by cytokinin. The next decade should see progress on these and other fundamental questions about cytokinin function.

## ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation grants # IOS-1238051 and IOS-1022053 to JJK and GES and USDA grant 2011–67013–30069 to JJK.

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