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Cytokinin Signaling in Arabidopsis

Claire E. Hutchison and Joseph J. Kieber[1]

Biology Department, University of North Carolina, Chapel Hill, North Carolina 27599-3280

INTRODUCTION

Cytokinins have been implicated in many developmental processes and environmental responses of plants, including leaf senescence, apical dominance, chloroplast development, anthocyanin production, and the regulation of cell division and sink/source relationships. They were identified in the pioneering work of Skoog and Miller (1957) by their ability to promote cell division in cultured cells in combination with another phytohormone, auxin.

Naturally occurring cytokinins are N^6-substituted adenine derivatives (reviewed by Mok and Mok, 2001). There are multiple forms of cytokinin that differ in the structure of their side chain at the N^6 position, and each of these can occur in the free base, riboside, or ribotide form. Cytokinins can be modified by the conjugation of Glc to nitrogen at various positions of the adenine ring. They also can be modified by the conjugation of Glc and, to a lesser extent, Xyl to the hydroxyl group of the N^6 side chain. These modifications generally inactivate the cytokinin and in some cases are reversible. A subset of cytokinins can be degraded irreversibly by cleavage of the N^6 side chain by the enzyme cytokinin oxidase. The existence of pathways for the degradation and conjugation of cytokinins suggests that the level of this signaling compound is tightly regulated. Several of the enzymes encoding the proteins that catalyze these metabolic reactions have been cloned (Houba-Hérin et al., 1999; Martin et al., 1999a, 1999b, 2001; Morris et al., 1999), as have the genes encoding a key enzyme in cytokinin biosynthesis, isopentyl transferase (Kakimoto, 2001; Takei et al., 2001). These advances in the study of cytokinin biosynthesis and metabolism are important in understanding the role of cytokinins in plant growth and development and have been reviewed elsewhere (Haberer and Kieber, 2001; Mok and Mok, 2001). This review will focus on early events in cytokinin signaling and highlight some likely future directions in this field.

A number of recent findings have begun to shed light on the molecular basis of cytokinin signaling. These breakthroughs have come mainly from molecular, genetic, and biochemical studies in *Arabidopsis thaliana* and include the identification of cytokinin primary response genes, genes encoding cytokinin receptors, and a skeletal pathway of elements that mediate signaling between these components. The proposed cytokinin signal transduction pathway is a phosphorelay pathway similar to bacterial two-component response systems, so we will begin with a brief outline of the relevant details of two-component signaling in prokaryotic and fungal systems.

[1] To whom correspondence should be addressed. E-mail jkieber@unc.edu; fax 919-962-1625.

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TWO-COMPONENT SIGNAL TRANSDUCTION

Two-component signal transduction pathways have been studied extensively in bacteria, which use these systems to sense and respond to a diverse array of environmental stimuli (reviewed by Stock et al., 1989; West and Stock, 2001). As the name implies, two basic components are involved (Figure 1). The first is a sensor His kinase that consists of two domains, an input domain and a transmitter domain. The signal is perceived by the input domain, and this modifies the His kinase activity of the transmitter domain, which autophosphorylates on a conserved His residue. The phosphate then is transferred to a conserved Asp residue in the receiver domain of the second component, a response regulator. Many response regulators also contain a second domain, called an output domain. The activity of the output domain is regulated by the phosphorylation state of the receiver domain. Typically, the output domain acts as a transcription factor (West and Stock, 2001). However, there are instances in which the receiver domain regulates the activity of a separate protein, as is the case with the CheY response regulator of *Escherichia coli*. CheY, which is involved in the chemotaxis response, is composed solely of a receiver domain and regulates the activity of the flagellar motor via an interaction with the FliM protein (Welch et al., 1993).

Extended versions of the basic two-component system of histidyl-aspartyl phosphorelay signaling have been discovered in both prokaryotes and eukaryotes. An example of these multistep phosphorelays is the osmosensing pathway in budding yeast (*Saccharomyces cerevisiae*). This pathway consists of three components: a hybrid His kinase (SLN1) that includes a fused receiver domain as part of the His kinase molecule; response regulators, including SSK1, which

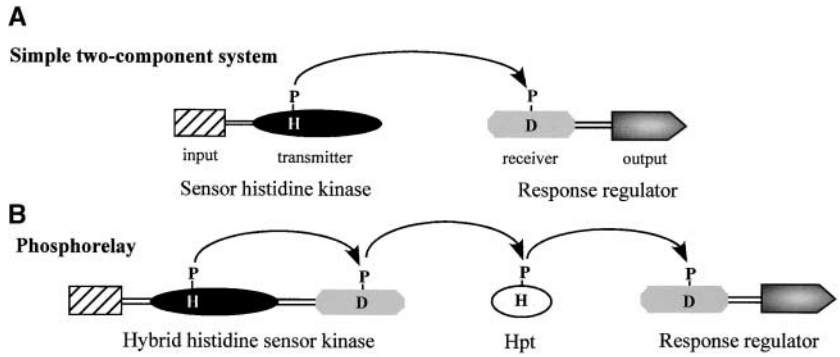

Figure 1. Two-Component Phosphotransfer Schemes.

(A) A basic prokaryotic two-component system with a sensor His kinase and a response regulator. H and D represent the conserved phospho-accepting His and Asp residues involved in phosphorelay signaling.
(B) A multistep phosphorelay system involving a hybrid sensor kinase, with input, transmitter, and receiver domains, a His-containing phosphotransfer protein (Hpt), and a response regulator.

mediate the output of the pathway; and a third component, YPD1, a His-containing phosphotransfer protein that mediates phosphotransfer between the receiver domain of the sensor His kinase and the receiver domain of the response regulators. One output of this phosphorelay pathway is the regulation of SSK2 (a mitogen-activated protein [MAP] kinase kinase kinase) activity and downstream MAP kinase signaling (Posas and Saito, 1998). All of the extended phosphorelay pathways share the common mechanism of phosphotransfer that alternates between His and Asp residues (Figure 1). However, the number of proteins carrying the phosphotransfer domains can differ between pathways (Perraud et al., 1999).

Genes encoding proteins similar to the various bacterial two-component elements are found in the Arabidopsis genome (Schaller et al., 2002). All of these are present as gene families and include His kinases, His-containing phosphotransfer proteins (AHPs), and two classes of response regulator (ARRs). We will consider first the Arabidopsis His kinases and then turn our attention to the likely downstream elements of phosphorelay signaling in Arabidopsis, the ARRs and the AHPs.

THE ARABIDOPSIS HIS KINASE GENE FAMILY

The Arabidopsis genome encodes a family of genes that share significant sequence similarity with bacterial His kinases. This family includes the phytochromes, the ethylene receptors, and the cytokinin receptors (reviewed by Schaller, 2000; Schaller et al., 2002) (Figure 2). Sensor His kinases typically contain a variable input domain and a conserved transmitter domain. The transmitter domain includes characteristic sequence motifs and a conserved His residue that is the site of autophosphorylation (reviewed by West and Stock, 2001). Several of the Arabidopsis sensor His kinases also contain a C-terminal receiver domain, a feature shared by several fungal and some bacterial sensor kinase homologs. In addition, AHK2, AHK3, and AHK4 contain a second receiver domain between the transmitter and the C-terminal receiver domain. This second receiver domain lacks some of the highly conserved residues found in other receiver domains, and in AHK3 and AHK4 the putative phospho-accepting Asp is replaced by a Glu residue (Ueguchi et al., 2001a).

The His kinase–like domains of the phytochromes are highly diverged, and phytochromes have been shown to possess Ser/Thr, rather than His, kinase activity (Yeh and Lagarias, 1998). Similarly, three of the ethylene receptors (ETR2, ERS2, and EIN4) lack some of the characteristic transmitter sequence motifs and thus are likely to lack His kinase activity (Schaller, 2000; Schaller et al., 2002). The remaining eight His kinases contain all of the residues that are likely to be required for His kinase activity.

Most of the Arabidopsis sensor His kinases contain multiple predicted transmembrane regions in the N-terminal part of the protein (reviewed by Schaller, 2000; Ueguchi et al., 2001a). The five ethylene receptors contain either three or four transmembrane domains, and these constitute the site of ethylene binding. AHK2 and AHK3 contain three predicted transmembrane domains, and AHK4 contains two. All three proteins share a region of sequence similarity in the predicted extracytoplasmic region, which is likely to be the cytokinin binding domain (Ueguchi et al., 2001a; Yamada et al., 2001). This domain has been identified in diverse receptor-like proteins from both prokaryotes and eukaryotes and named the CHASE (cyclases/histidine kinases–associated sensory extracellular) domain (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001).

CYTOKININ RECEPTORS ARE SIMILAR TO HIS KINASES

Evidence linking cytokinin signaling to a His kinase came from a screen for Arabidopsis genes whose overexpression resulted in callus proliferation, greening, and shoot formation in the absence of exogenously supplied cytokinin (Kakimoto, 1996). This analysis identified the *CKI1* gene, which encodes a protein with sequence similarity to His kinases. It was proposed that CKI1 might be a cytokinin receptor, although the hypermorphic nature of the mutation precluded a definitive conclusion. Recently, a loss-of-function allele of CKI1 was identified and was shown not to be transmitted through the female gamete (T. Kakimoto, personal communication). Although this indicates an important role of CKI1 in female gametophyte development or function, it does not address the role of CKI1 in cytokinin responsiveness. Other studies suggest that the activity of CKI1 is not responsive to cytokinin, although its overexpression can activate cytokinin-responsive genes (Hwang and Sheen, 2001; Yamada et al., 2001) (see below). Thus, the role of CKI1 in cytokinin signaling remains unclear.

Several reports provide compelling evidence that the *AHK2*, *AHK3*, and *AHK4* genes encode cytokinin receptors

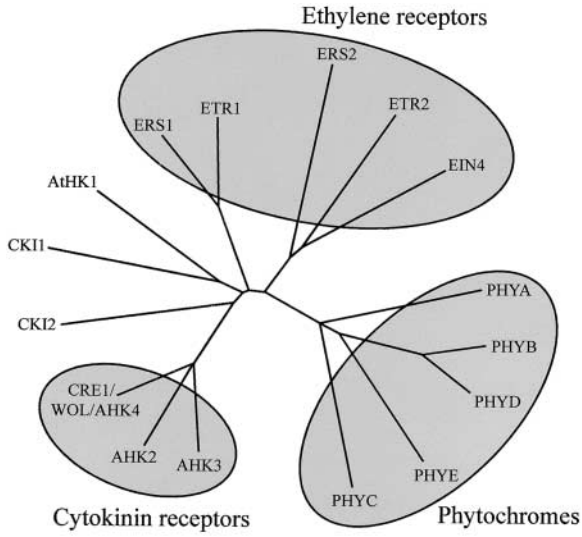

Figure 2. His Kinases in Arabidopsis.

An unrooted phylogenetic tree of His kinase–related proteins derived using the amino acid sequences of the His kinase–like domains of these proteins (adapted from Schaller et al., 2002). Phytochrome, ethylene receptor, and cytokinin receptor families are indicated. For additional information about Arabidopsis His kinases and other Arabidopsis phosphorelay elements, including accession numbers, see http://www.bio.unc.edu/research/two-component/default.htm.

(Inoue et al., 2001; Suzuki et al., 2001a; Ueguchi et al., 2001b; Yamada et al., 2001). The *cytokinin response 1* (*cre1*) mutant was identified in a screen for mutants impaired in the cell division, greening, and shoot formation responses of callus tissue to cytokinin (Inoue et al., 2001). The intact *cre1* mutant seedlings also were less sensitive to cytokinin inhibition of root growth (Inoue et al., 2001). The *cre1* mutation mapped close to *AHK4*, and complementation analysis and sequencing of *cre1* alleles showed that *CRE1* corresponds to *AHK4* (Inoue et al., 2001).

A series of experiments based on the ability of CRE1 to complement His kinase–deficient yeast and *E. coli* mutants in a cytokinin-dependent manner provided further evidence that CRE1 is a cytokinin receptor (reviewed by Schmülling, 2001). Inoue et al. (2001) found that CRE1 complemented a mutation in the SLN1 His kinase of the budding yeast osmosensing pathway described above. Deletion of SLN1 (sln1Δ) is lethal because of the overactivation of the downstream MAP kinase pathway. Expression of *CRE1* in a sln1Δ yeast strain restored viability only when cytokinins were present in the medium (Inoue et al., 2001). This finding indicates that CRE1 function is activated in response to cytokinin, a hallmark feature of a ligand–receptor interaction. Conceptually similar experiments with CRE1 as well as with AHK2 and AHK3, using both a fission yeast and an *E. coli* multistep phosphorelay system, gave similar results, indicating that AHK2 and AHK3 probably are cytokinin receptors as well (Suzuki et al., 2001a; Ueguchi et al., 2001b; Yamada et al., 2001; T. Mizuno, personal communication). As with the complementation of sln1Δ by CRE1, the complementation in these systems occurred in a cytokinin-dependent manner.

Confirmation that CRE1/AHK4 can bind cytokinin was provided by in vitro binding assays using membrane preparations of *Schizosaccharomyces pombe* expressing CRE1 (Yamada et al., 2001). CRE1 binds the radiolabeled cytokinin isopentyl adenine with high affinity. Competition assays with nonlabeled compounds demonstrated that other N^6-substituted aminopurines that have cytokinin activity, such as *trans*-zeatin, and aromatic cytokinins, such as benzyladenine, also bind CRE1, but that various adenine derivatives that are inactive in cytokinin response assays do not. Isopentenyl adenosine (a cytokinin riboside) failed to compete for binding to CRE1, consistent with the idea that only the free-base forms of cytokinins are active. Interestingly, the structurally distinct diphenylurea-type cytokinin thidiazuron also competes for binding to CRE1, indicating that this class of synthetic cytokinins probably acts by activating the cytokinin response pathway directly (Yamada et al., 2001).

The *cre1* mutation is allelic to *wooden leg* (*wol*), which was identified originally by a recessive allele that results in reduced cell number and lack of phloem in the root vasculature (Scheres et al., 1995; Mähönen et al., 2000; Inoue et al., 2001). The mutation in *wol* is a single amino acid substitution in the CHASE domain that is likely to act as a cytokinin binding domain: a *wol* mutant version of CRE1 fails to complement the disruption of the *E. coli* His kinase RcsC and is

unable to bind cytokinins in vitro (Yamada et al., 2001). These results suggest that the presumed extracellular domain of CRE1 is the cytokinin binding site and that the *wol* phenotype results from a loss of cytokinin binding to the CRE1 receptor, and thus a lack of cytokinin signaling in the affected tissue.

RNA gel blot analysis revealed that *WOL* is expressed primarily in the root, but the transcript is detected in aerial tissues as well (Mähönen et al., 2000). RNA in situ analysis showed that *WOL* mRNA is expressed in precursor cells for the vascular tissue in globular-stage embryos and that in later stages of embryo development it can be detected in the procambium of the hypocotyl, cotyledon shoulders, and embryonic root (Mähönen et al., 2000). Consistent with the expression pattern of *WOL* in the embryo, analysis of the mutant phenotype indicated that *WOL*/*CRE1* activity is required for some of the embryonic cell divisions that increase the number of vascular initial cells in the root and also for periclinal cell divisions that increase the number of cell files in the vasculature of the root and hypocotyl (Mähönen et al., 2000). The combination of *wol* with *fass*, a mutation that increases the number of cell layers (Scheres et al., 1995), resulted in an increased number of cell layers in the vasculature compared with the *wol* single mutant, and phloem markers were detected in the vascular cylinder of the double mutant. This suppression of the *wol* phenotype by *fass* indicates that WOL/CRE1 is not required to specify phloem development directly; more likely, it is required for the production of a sufficient number of vasculature precursor cells to allow the correct specification of xylem and phloem to occur (Scheres et al., 1995; Mähönen et al., 2000).

No shoot phenotype has been described for *wol*/*cre1* mutants (Mähönen et al., 2000; Inoue et al., 2001; Ueguchi et al., 2001b), which may indicate that CRE1 plays little or no role in these tissues or that its function is redundant with that of other cytokinin-responsive His kinases (such as AHK2 and/or AHK3) in the aerial parts of the plant. Transcripts of AHK2 and AHK3 have been detected by reverse transcriptase–mediated polymerase chain reaction in RNA prepared from leaves and flowers as well as roots (Ueguchi et al., 2001a). Loss-of-function mutations of these closely related AHK genes may reveal a role for these genes in the growth and development of the shoot.

DOWNSTREAM PHOSPHORELAY COMPONENTS ALSO ARE IMPLICATED IN CYTOKININ SIGNALING

The downstream signal transduction elements of bacterial and fungal two-component hybrid sensor kinases are His-containing phosphotransfer proteins and response regulators. The emerging notion is that, in an analogous manner, the Arabidopsis response regulators and His-containing phosphotransfer proteins act downstream of the CRE1/AHK receptors in cytokinin signaling.

Response Regulators in Arabidopsis

Response regulators were implicated in cytokinin signaling when genes containing a conserved receiver domain were identified in screens for early cytokinin response genes in Arabidopsis and maize (Brandstatter and Kieber, 1998; Sakakibara et al., 1998). The Arabidopsis response regulators form a large gene family composed of 22 genes that includes two major classes (type A and type B) based on a phylogenetic analysis of their amino acid sequences and their domain structures (Imamura et al., 1998, 1999; D'Agostino and Kieber, 1999; D'Agostino et al., 2000; Schaller et al., 2002) (Figure 3). The type-A ARRs consist of a receiver domain with short N- and C-terminal extensions, whereas type-B ARRs have long C-terminal extensions that mediate sequence-specific DNA binding and transcriptional activation (see below). In addition, transcript levels of most of the type-A ARRs are increased in response to exogenous cytokinins, whereas the steady state level of the type-B ARR mRNAs is unaffected by cytokinin (Brandstatter and Kieber, 1998; Taniguchi et al., 1998; Imamura et al., 1999; Kiba et al., 1999; D'Agostino et al., 2000). Members of both classes of ARRs have been implicated in cytokinin signaling.

Type-A ARRs Are Cytokinin Primary Response Genes

Ten genes encoding type-A ARRs are present in the Arabidopsis genome. The type-A ARRs fall into five pairs with highly similar amino acid sequences (Figure 3), reflecting large segmental duplications within the Arabidopsis genome (Arabidopsis Genome Initiative, 2000). Throughout the entire type-A ARR family, the amino acid sequences of the receiver domains are very similar to each other, although there is a variable region of 11 to 21 amino acids in the central portion. The sequences of the C-terminal extensions, which are all $<$100 amino acids long, also are more divergent (Imamura et al., 1999; D'Agostino et al., 2000).

Type-A ARR transcripts have been detected by RNA gel blot analysis in all adult organs of the plant. The highest levels are found in the root, and there are some differences in the expression profiles among the different genes (Brandstatter and Kieber, 1998; Urao et al., 1998; D'Agostino et al., 2000). A more detailed analysis of the pattern of expression of the *ARR5* gene using promoter–β-glucuronidase (GUS) fusions in transgenic Arabidopsis showed that *ARR5* is expressed at high levels in the shoot and root apical meristems. Staining also was seen at the junction of the pedicel and the immature siliques and in the central region of mature roots (D'Agostino et al., 2000). The expression of *ARR5* mRNA in shoot and root apical meristems was confirmed by whole mount in situ analysis of seedlings (D'Agostino et al., 2000). This pattern of expression in regions of proliferating cells is consistent with likely sites of cytokinin action.

ARR4 protein levels have been determined in various adult plant organs using protein gel blot analysis (Sweere et

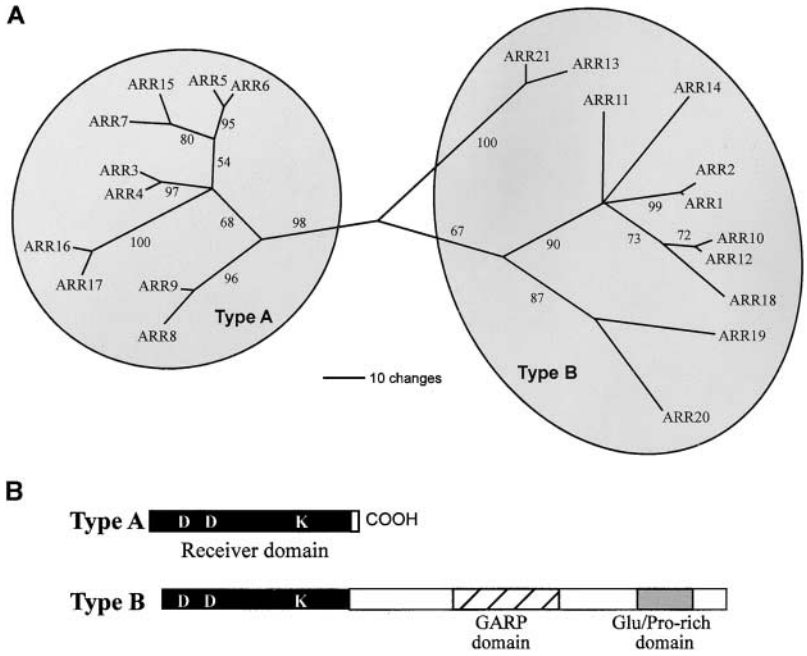

Figure 3. Type-A and Type-B Response Regulators in Arabidopsis.

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

(B) 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 Glu- and Pro-rich region.

al., 2001). ARR4 protein was found in stems, leaves, and flowers, but in contrast to the mRNA, the protein was not detected in roots (Sweere et al., 2001). Furthermore, the steady state level of ARR4 protein was increased significantly in response to light, whereas the steady state level of *ARR4* mRNA was not altered by light (Brandstatter and Kieber, 1998). These results suggest that although ARR4 transcript levels are increased in response to cytokinin, ARR4 expression is regulated post-transcriptionally in response to light as well.

ARR4 and *ARR5* act as cytokinin primary response genes: they are induced rapidly and specifically by cytokinin (within 10 min after cytokinin treatment), and the induction does not require de novo protein synthesis, because it is insensitive to the protein synthesis inhibitor cycloheximide (Brandstatter and Kieber, 1998). Most type-A ARRs are induced by cytokinin with generally similar induction kinetics (Taniguchi et al., 1998; Kiba et al., 1999; D'Agostino et al., 2000). Nuclear run-on transcription studies revealed that the increase in steady state transcript levels of the type-A ARRs in response to cytokinin was, at least in part, the result of increased transcription, which implies that a transcription factor(s) is activated in response to cytokinin (D'Agostino et al., 2000) (see below). Transcript levels of some type-A ARRs also have been reported to be responsive to various environmental stresses and to nitrogen levels (Taniguchi 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.

Evidence that type-A ARRs can function as response regulators in a two-component phosphorelay has been amassed by a number of groups. The interaction of various type-A ARRs with the AHPs, which by analogy to yeast and bacterial multistep phosphorelay systems were proposed to mediate phosphotransfer between the receiver domains of His kinases and those of response regulators, has been demonstrated by yeast two-hybrid analysis. Phosphotransfer from AHP1 and AHP2 to ARR3 and ARR4 has been shown in vitro (Imamura et al., 1999; Suzuki et al., 1998), and ARR6 can acquire a phosphoryl group from an *E. coli* His phosphotransmitter domain labeled in vitro (Imamura et al., 1998). In vitro phosphotransfer from the maize His-containing phosphotransfer protein ZmHP2 to the maize type-A response regulators ZmRR1 and ZmRR2 also has been reported (Sakakibara et al., 1999).

Type-B ARRs Act as Transcription Factors

There are 11 type-B ARRs in the Arabidopsis genome that are characterized by the presence of a receiver domain and a large C-terminal extension that contains features of transcription factors. (Sakai et al., 1998; Imamura et al., 1999; Lohrmann et al., 1999; Schaller et al., 2002). Unlike type-A ARR expression, the steady state level of type-B ARR transcripts is not affected by the application of cytokinins or nitrate and is unresponsive to the application of other plant hormones (Imamura et al., 1999; Kiba et al., 1999; Lohrmann et al., 1999). RNA gel blot analysis has revealed that *ARR1* is transcribed in all tissues of adult plants, with the highest transcript levels occurring in roots. *ARR2*, *ARR10*, and *ARR11* have been detected in all adult tissues tested, but reports differ regarding whether *ARR2* and *ARR10* transcripts are present in the roots (Sakai et al., 1998; Imamura et al., 1999; Lohrmann et al., 1999, 2001). Although high levels of *ARR2* and *ARR10* expression have been reported in roots (Sakai et al., 1998; Imamura et al., 1999), both genes have been reported not to be expressed in these tissues (Lohrmann et al., 1999, 2001). Analysis using a promoter-GUS fusion construct showed ARR2 expression primarily in pollen grains (Lohrmann et al., 2001). The variable reports of expression may reflect a strong effect of environmental factors on the expression of these genes.

The C-terminal domains of the type-B ARRs contain potential nuclear localization signals (Sakai et al., 1998; Lohrmann et al., 1999), and several type-B ARRs have been demonstrated to localize to the nucleus based on observations of GUS or green fluorescent protein fusion proteins expressed in onion epidermal, parsley, and Arabidopsis cells (Lohrmann et al., 1999, 2001; Sakai et al., 2000; Hwang and Sheen, 2001; Imamura et al., 2001). The removal of the receiver domain did not affect the nuclear localization of ARR1 and ARR2 (Sakai et al., 2000).

As their amino acid sequences and nuclear localization suggest, the type-B ARRs can act as transcriptional activators. The C-terminal domains of ARR1, ARR2, and ARR11 can activate transcription from a GAL4-driven reporter when fused to the GAL4 DNA binding domain in both yeast and tobacco cells (Lohrmann et al., 1999, 2001; Sakai et al., 2000). The highest activation was observed using a truncated ARR2 protein in which the N-terminal region was removed, indicating that the N-terminal domains may negatively regulate the activity of the C-terminal transcription activator domain (Sakai et al., 2000).

The C-terminal portion of the type-B ARRs contain a conserved domain called the GARP domain, because it is found in GOLDEN2 in maize, the ARRs, and the Psr1 protein from *Chlamydomonas* (Reichmann et al., 2001). The GARP domains of ARR1 and ARR2 bind DNA in a sequence-specific manner, binding preferentially to the core sequence (G/A)GAT(T/C) in gel-shift assays and requiring the middle GAT sequence for binding to occur. Furthermore, ARR1 and ARR2 are able to activate reporter gene expression from a construct in which copies of this sequence are placed upstream of a minimal 35S promoter (Sakai et al., 2000).

GARP domain binding sites are present in the promoters of type-A ARRs, suggesting that type-B ARRs can bind to these promoters and activate transcription. Sakai et al. (2001) showed that activation of a chimeric protein, consisting of the C-terminal part of ARR1 fused to the glucocorticoid receptor, by treatment with a glucocorticoid in the presence of an inhibitor of protein synthesis induced the expression of type-A ARRs, which is consistent with a direct activation of type-A ARR transcription by the type-B ARRs.

Studies in yeast and in vitro have shown that the ARR2 protein can bind to promoters of nuclear genes for several components of the Arabidopsis mitochondrial respiratory chain complex (Lohrmann et al., 2001). In these studies, the DNA binding activity was stronger in the absence of the N-terminal part of the protein, suggesting that the receiver domain negatively regulates the activity of the C-terminal domains of the type-B ARRs (Lohrmann et al., 2001).

The function of type-B ARRs in phosphorelay signaling is supported by their ability to interact via their receiver domain with His-containing phosphotransfer proteins in the yeast two-hybrid assay (Imamura et al., 1999; Lohrmann et al., 2001; Suzuki et al., 2001b). Phosphotransfer from AHP2 to ARR1 and ARR10 also has been demonstrated (Imamura et al., 2001; Suzuki et al., 2001b). In summary, the C-terminal domains of the type-B ARRs act as classic two-component output domains: they bind DNA in a sequence-specific manner, and they are capable of activating transcription. The N-terminal receiver domain appears to play a role in negatively regulating the ability of the type-B ARRs to activate transcription.

His-Containing Phosphotransfer Proteins in Arabidopsis

His-containing phosphotransfer proteins are predicted to mediate the transfer of the phosphoryl group from the receiver domain of an activated hybrid sensor His kinase to the receiver domain of a response regulator in a multistep phosphorelay signal transduction pathway (Figure 1). Data from yeast and in vitro studies suggest that the His-containing phosphotransfer proteins of Arabidopsis do in fact perform this role.

Five genes encoding His-containing phosphotransfer proteins have been identified in the Arabidopsis genome (Suzuki et al., 2000). Each of these AHPs is composed solely of a phosphotransmitter domain. The predicted amino acid sequences of all five proteins are similar (Suzuki et al., 2000), and each includes a conserved His residue that is predicted to act as the phosphorylation site in phosphorelay signaling.

RNA gel blot expression analysis shows that the steady state level of the *AHP* transcript is not altered by exogenous

cytokinin (I.B. D'Agostino and J.J. Kieber, unpublished data). *AHP1* transcript is expressed at high levels in roots but at low or undetectable levels in other tissues tested. *AHP2* and *AHP3* transcripts have been detected in RNA from all adult tissues (Miyata et al., 1998; Suzuki et al., 1998). *AHP5* message was detected by reverse transcriptase–mediated polymerase chain reaction in RNA from both roots and leaves (Suzuki et al., 2000), but no signal was detected for *AHP4*.

The ability of AHPs to function as phosphotransmitters has been explored in a number of different ways. In a heterologous complementation experiment, several of the Arabidopsis phosphotransfer proteins were able to complement a deletion of YPD1, the His-containing phosphotransfer protein component of the budding yeast osmosensing phosphorelay pathway described above (Miyata et al., 1998; Suzuki et al., 1998). The ability of the AHPs to function in a phosphorelay pathway also has been studied using competition experiments with the same *E. coli* multistep phosphorelay assay system used to examine CRE1 function (Suzuki et al., 2001a). Coexpression of various AHPs with CRE1 in this system suppressed the cytokinin-responsive phosphotransfer to different degrees, suggesting that AHPs were competing with the endogenous *E. coli* His-containing phosphotransfer proteins for phosphorylation by CRE1 (Suzuki et al., 2001a). Finally, in vitro studies have confirmed the ability of the AHP proteins to act in a phosphorelay. Phosphorylation of AHPs at the conserved phosphoaccepting His residue has been shown in vitro by incubation of AHP1 and AHP2 with *E. coli* membrane preparations (Suzuki et al., 1998). Phosphotransfer from AHP1 and AHP2 to type-A ARRs and to type-B ARRs also has been demonstrated in vitro (Suzuki et al., 1998, 2001b; Imamura et al., 1999, 2001). Together, these data strongly support the idea that the AHPs can function as His-containing phosphotransfer proteins.

The interactions between proposed Arabidopsis phosphorelay components in yeast two-hybrid assays have been reported by different groups. In a few cases, the results have differed, but differences in the interactions detected may reflect the inherent variability between the yeast systems that were used. Interactions have been detected between AHPs and His kinases (Imamura et al., 1999; Urao et al., 2000; I.B. D'Agostino and J.J. Kieber, unpublished data) and between multiple AHPs and several type-A and type-B ARRs (Urao et al., 2000; Suzuki et al., 2001b; I.B. D'Agostino and J.J. Kieber, unpublished data). The interaction between AHP2 and ARR1 was supported by in vitro coimmunoprecipitation assays using AHP2 and the receiver domain of ARR1 (Suzuki et al., 2001b).

To summarize, these results are consistent with AHP proteins acting in phosphorelay pathways to mediate the transfer of phosphate from the receiver domains fused to a set of the Arabidopsis His kinases to the receiver domains of a set of type-A and type-B ARRs. How specificity is achieved in this system has yet to be addressed.

IN VIVO ACTION OF PHOSPHORELAY ELEMENTS IN CYTOKININ SIGNALING

The work described in the previous sections has shown that several His kinases are likely to act as cytokinin receptors and that cytokinins act to increase the rate of transcription of the type-A ARRs. Furthermore, yeast two-hybrid and in vitro analyses indicate that the AHPs can mediate phosphotransfer reactions to the receiver domains of type-A and type-B ARRs. Two recent reports confirm and extend our understanding of the interactions of these elements and their role in cytokinin signaling (Hwang and Sheen, 2001; Sakai et al., 2001).

Several lines of evidence indicate that type-B ARRs are involved in cytokinin signaling, including the transcriptional activation of type-A ARR genes. A loss-of-function mutation in *ARR1* resulted in reduced sensitivity to cytokinin in shoot regeneration assays in tissue culture, whereas overexpression of *ARR1* increased the sensitivity to cytokinin in these assays (Sakai et al., 2001). Analysis of the transcript levels of *ARR6* (a type-A gene) in these plants correlated with the level of ARR1 expression: *ARR1* overexpression resulted in an increased level of *ARR6*, whereas a loss-of-function *arr1* mutant showed decreased levels, suggesting that ARR1 mediates the cytokinin induction of *ARR6* transcription. The expression of a truncated form of *ARR1* lacking the receiver domain caused abnormal phenotypes, including disordered cell division at the shoot apex, callus formation on cotyledons and hypocotyls, and the production of ectopic leaves (Sakai et al., 2001). These phenotypes suggest the constitutive activation of cytokinin responses. The difference in phenotype between plants overexpressing full-length and truncated versions of ARR1 is consistent with previous results that suggested that the receiver domain of type-B ARRs inhibits the activity of the C-terminal output domain (Sakai et al., 2000, 2001). Overexpression of *ARR2* in transgenic Arabidopsis also promoted cytokinin responses, including cell proliferation and shoot and leaf formation in cultured cells, and delayed leaf senescence in adult plants (Hwang and Sheen, 2001).

Further evidence linking the type-B ARRs to the cytokinin induction of type-A ARR transcription came from studies using a transient expression system in Arabidopsis protoplasts (Hwang and Sheen, 2001). This system used a reporter gene construct in which the promoter of the cytokinin-inducible type-A ARR gene *ARR6* was used to drive luciferase gene expression (ARR6-LUC). Arabidopsis mesophyll protoplasts transfected with this construct displayed a strong increase in luciferase activity, specifically in response to cytokinin, indicating that in this system *ARR6* is induced in a manner similar to the endogenous gene in planta. Using this system, it was found that the overexpression of type-B ARRs, especially *ARR2*, resulted in an increased basal level and a hyperinduction of the ARR6-LUC reporter in response to cytokinin, which is consistent with the results described

above using stably transformed, intact plants. This indicates that type-B function is likely to be a rate-limiting factor in the activation of the *ARR6* gene. Mutation of the Asp that is the presumed target of phosphorylation did not diminish the effect of ARR2 on ARR6-LUC expression (Hwang and Sheen, 2001), which leads to the surprising conclusion that phosphotransfer to the conserved Asp residue in ARR2 is not required for the transcriptional activation of *ARR6* in response to cytokinin, at least in this system.

Together, these results suggest that the type-B ARRs act as positive regulators of cytokinin responsiveness, including the induction of type-A ARR gene expression. The presence of multiple ARR1 and ARR2 binding sites within the promoter of *ARR6* and other type-A ARRs, and the demonstration that ARR1 can activate *ARR6* transcription in the absence of protein synthesis (Sakai et al., 2000, 2001), indicate that the effect of the type-B ARRs on type-A ARR transcription is direct. The N-terminal part of the type-B ARRs is likely to regulate the activity of the C-terminal output domain, but how type-B function is regulated, and in particular the role of phosphorylation in its activation, remains unresolved.

Using the Arabidopsis mesophyll protoplast system, Hwang and Sheen (2001) further showed that the overexpression of CRE1 results in an increased induction of *ARR6* transcription in response to exogenous cytokinin. Surprisingly, overexpression of *AHK2* and *AHK3* had little effect on the induction of ARR6-LUC. The requirement for His kinase activity and most likely phosphotransfer within CRE1 for the downstream transcriptional activation also was shown in this system: the expression of mutant forms of CRE1, in which the conserved His and Asp residues in the His kinase and receiver domains, respectively, are altered, failed to induce ARR6-LUC expression in response to cytokinin (Hwang and Sheen, 2001). The expression of CKI1 results in an activation of ARR6-LUC that is not further increased by cytokinin treatment, suggesting that CKI1 is active constitutively or is saturated by the endogenous cytokinin in the protoplasts (Hwang and Sheen, 2001). This constitutive, cytokinin-insensitive activity of CKI1 is also seen in *E. coli* complementation assays using CKI1 (Yamada et al., 2001).

The overexpression of several type-A ARRs in the Arabidopsis protoplast system suppressed the cytokinin induction of ARR6-LUC, indicating that type-A ARRs negatively regulate their own expression (Hwang and Sheen, 2001). As with the type-B ARRs in this system, a mutation of the conserved phosphorylation site of type-A ARRs did not affect the repression of ARR6-LUC, indicating that phosphorylation of the type-A ARRs is not required for this repression.

Two lines of evidence implicate the AHPs as mediators of cytokinin signaling, linking the activation of CRE1 by cytokinin binding to the activation of the type-B ARRs. First, AHP1 and AHP2, but not AHP5, accumulate transiently in the nucleus within 30 min of the application of exogenous cytokinin, as measured by green fluorescent protein fusions in Arabidopsis protoplasts (Hwang and Sheen, 2001). Second, the overexpression of AHP2 in transgenic Arabidopsis results in modest hypersensitivity to exogenous cytokinin in root and hypocotyl elongation assays (Suzuki et al., 2002). These results, coupled with the two-hybrid and in vitro phosphorylation experiments described above, indicate that the His-containing phosphotransfer proteins are likely to mediate signaling between CRE1 and the type-B ARRs.

A MODEL FOR PHOSPHORELAY SIGNAL TRANSDUCTION IN RESPONSE TO CYTOKININ IN ARABIDOPSIS

Most of the data described above are consistent with the simple model for phosphorelay signal transduction in cytokinin signaling presented in Figure 4. Cytokinins bind to the CRE1 His kinase, and most likely also to AHK2 and AHK3, in the conserved extracellular CHASE domain. This induces autophosphorylation on a His residue within the transmitter domain and subsequent transfer of the phosphate to an Asp residue within the fused receiver domain. Then, this phosphate probably is transferred to a His residue on an AHP. The AHP translocates to the nucleus, where it activates a type-B ARR. The activated type-B ARR binds to elements within the promoter of the type-A ARRs to increase their rate of transcription. The type-A ARRs feed back to inhibit their own expression and, possibly, cytokinin signaling in general. The analogy to bacterial systems, the results of in vitro studies, and the conservation of the sites of phosphorylation in the proteins all suggest that phosphorylation of the AHPs and ARRs plays a role in regulating their function. The role of phosphorylation in the activation of the type-B ARRs is uncertain, but it seems clear that the receiver domain negatively regulates the function of the output domain. Other targets of type-B ARR transcriptional activation and proteins that interact with type-A ARRs may provide clues to the downstream effectors of the cytokinin signal and the role of phosphorylation of these proteins.

CYTOKININ SIGNALING BEYOND THE PHOSPHORELAY

Many changes in gene expression have been detected in response to the application of exogenous cytokinins (reviewed by Schmülling et al., 1997). Genes showing cytokinin-responsive expression are good candidates for components of cytokinin signaling and effector pathways to various cellular and developmental responses. An interesting example is the induction of cyclin D3 (*CycD3*) transcripts in response to cytokinin (Riou-Khamlichi et al., 1999). CycD3 likely plays a role in the G1-S transition of the cell cycle. Consistent with this, *CycD3* transcripts are expressed in proliferating tissues: in the shoot meristem, young leaf primordia, axillary buds, the procambium, and vascular tissues

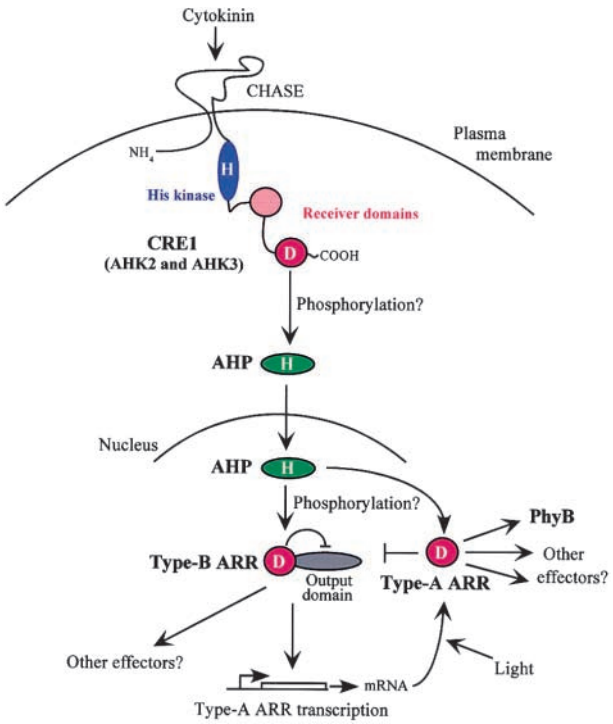

Figure 4. Model for Phosphorelay Signal Transduction in Cytokinin Signaling.

Cytokinin binds to CRE1, and possibly other His kinase–like proteins such as AHK2 and AHK3, within the CHASE domain, which is flanked by predicted transmembrane domains. CRE1 is likely to be located in the plasma membrane and, by analogy to other histidine kinases, to act as a dimer (not shown in model). The binding of cytokinin activates the transmitter domain (blue), which autophosphorylates on a His (H). The phosphate then is transferred to an Asp residue (D) within the fused receiver domain (red). A second, degenerate receiver domain (pink) also is present. The phosphate then is likely to be transferred to an AHP protein, which translocates to the nucleus, where it activates type-B ARRs. The activated type-B ARRs increase the transcription of the type-A ARRs, which feed back to inhibit their own transcription (⊥). Light appears to increase ARR4 protein levels, as indicated by the arrow. The receiver domain of type-B ARRs inhibits the activity of the output domain (gray). The output of this signaling pathway is mostly unknown, although one likely target is PhyB. See text for additional details.

of maturing leaves. The application of exogenous cytokinin increases transcript levels without changing the expression pattern of *CycD3*, indicating a tissue-specific response of this gene to cytokinin (Riou-Khamlichi et al., 1999). The overexpression of CycD3 confers cytokinin-independent cell proliferation and greening in tissue culture but prevents shoot regeneration. These results may indicate a mechanism for the link between cytokinin and the regulation of cell proliferation.

Transgenic Arabidopsis plants that express the *IPT* gene from *Agrobacterium* have increased levels of cytokinin, and this has been shown to result in the increased expression of the *KNAT1* and *STM* genes, which are involved in meristem function (Rupp et al., 1999). Interestingly, cytokinin levels appear to increase in response to the overexpression of *KNAT1* in tobacco (Ori et al., 1999), suggesting a reciprocal relationship between cytokinin levels and *KNAT1* transcript levels.

Many overlaps between the action of light and cytokinin on plant development and the induction of gene expression have been observed (reviewed by Thomas et al., 1997). The report of light-dependent ARR4 protein accumulation and an interaction between ARR4 and the red light photoreceptor phytochrome B (PhyB) provides an interesting and direct link between light and cytokinin signaling (Sweere et al., 2001). The direct interaction of ARR4 with PhyB was demonstrated by coimmunoprecipitation experiments from both yeast and Arabidopsis. Yeast two-hybrid analysis showed that the interaction of ARR4 with PhyB is mediated by the extreme N terminus of PhyB. This interaction appeared to be specific for ARR4, because PhyB did not interact with either ARR5 or ARR2 (Sweere et al., 2001). Photoconversion experiments revealed that the interaction with ARR4 stabilizes the active, Pfr form of PhyB. Consistent with this finding, the overexpression of ARR4 resulted in hypersensitivity to red light, as assayed by measuring the lengths of hypocotyls in seedlings grown continuously in varying amounts of red light. In contrast, there was no difference observed in hypocotyl length between wild-type and ARR4-overexpressing seedlings grown in darkness, far-red light, or blue light. Comparison of root length and the number of leaves at flowering between transgenic and wild-type plants also supports this hypersensitivity to red light (Sweere et al., 2001).

Additional elements that interact with AHPs and the type-A ARRs have been identified in yeast two-hybrid screens and may identify other components of cytokinin signaling or downstream targets of activated AHPs and ARRs. An Arabidopsis auxin-inducible DNA binding protein, AtDBP, was found in a screen for ARR4-interacting clones, perhaps providing a link between cytokinin and auxin signaling (Alliotte et al., 1989; Yamada et al., 1998). A novel nucleus-localized protein, TCP10, was found to interact with AHP1, AHP2, and AHP3 (Suzuki et al., 2001b). TCP10 contains a TCP domain, which is composed of ~50 amino acids that are predicted to adopt a basic helix-loop-helix structure, includes a bipartite nuclear localization signal, and is found in a number of other plant proteins (Cubas et al., 1999; Suzuki et al., 2001b).

Other potential cytokinin signaling elements have been identified in various genetic screens, although it is unclear if any of these affect cytokinin signaling specifically. Two *CYTOKININ-HYPERSENSITIVE* mutants, *ckh1* and *ckh2*, were identified as showing increased sensitivity to cytokinin in tissue culture (Kubo and Kakimoto, 2000). Because cytokinin levels are not increased in these mutants, *CKH1* and *CKH2* were proposed to repress the cytokinin-signaling pathway that promotes cell proliferation and chloroplast development.

PASTICCINO1 (*PAS1*), which encodes an immunophilin-like protein, has been implicated in determining the sensitivity of cell division to cytokinin (Faure et al., 1998; Vittorioso et al., 1998). The expression of *PAS1* is increased in the presence of cytokinin, and *pas1* mutations result in an array of phenotypes consistent with increased cytokinin signaling, including ectopic cell proliferation, alterations in the shoot apical meristem, and short, fat hypocotyls with extra cell layers. The *pas1* mutants also have short primary roots and lack secondary roots, a phenotype similar to that of *wol* mutants (Scheres et al., 1995; Mähönen et al., 2000; Inoue et al., 2001). These mutants do not produce increased levels of cytokinin but show a deregulation of cell proliferation that is enhanced specifically by cytokinin (Faure et al., 1998; Vittorioso et al., 1998). The *stunted plant 1* and the *cytokinin response 1* mutants have been implicated in cytokinin responsiveness as well (Deikman and Ulrich, 1995; Beemster and Baskin, 2000). However, the roles of these genes in cytokinin responses are not yet clear.

A number of mutants that disrupt the induction of ethylene in response to exogenous cytokinin also have been identified (Vogel et al., 1998). This analysis revealed that cytokinin increased ethylene production via a post-translational activation of ACS5, which is encoded by one member of the Arabidopsis 1-aminocyclopropane-1-carboxylic acid synthase gene family.

PERSPECTIVES

The last few years have seen remarkable progress in our understanding of the molecular basis of cytokinin signaling. Signaling events from cytokinin perception to changes in gene expression have been revealed, and this pathway, at least in the basic elements involved, bears striking similarity to the prokaryotic two-component signaling paradigm. Cytokinin has vaulted from arguably one of the least understood hormone signal transduction pathways in plants to perhaps one of the best. However, a multitude of questions remain.

What is the role of His-to-Asp phosphorylation in this signal transduction chain? What are the outputs of the two-component signaling chain? And, given the fact that each Arabidopsis phosphorelay component exists as a gene family, how is the specificity of interaction among the various members of these gene families achieved? The hybrid sensor kinase family contains additional potentially functional receptors, including members of the ethylene receptor subfamily. Indeed, His kinase activity has been established for the ethylene receptor ETR1 (Gamble et al., 1998). Is there communication between ethylene and cytokinin signaling through a phosphorelay signaling pathway? Bacterial two-component signaling elements are known to be promiscuous in their interactions (Stock et al., 1989). Therefore, it is difficult to make definitive conclusions regarding their biological role by interpretation of the various in vitro interactions and overexpression studies alone. Loss-of-function alleles of these elements and analysis of the expression patterns of these genes in plants should elucidate these issues.

One extremely interesting aspect of the proposed cytokinin signal transduction pathway is the remarkable similarity of its overall design to that of the auxin response pathway (reviewed by Leyser, 2001; Kepinsky and Leyser, 2002). In each case, components of the pathway include a family of sequence-specific DNA binding transcription factors (type-B ARRs or auxin response factors) whose expression is not induced by the hormone and that act as positive regulators of the response. These are counteracted by a related family of hormone primary response genes that encode proteins lacking the DNA binding domain that negatively regulate the response pathway (type-A ARR or indoleacetic acid genes). The identification of a role for the 26S proteosome in cytokinin responsiveness (Smalle et al., 2002) reveals a further parallel between the mechanisms of response to these hormones, because protein degradation also has been shown to play a key role in auxin responsiveness (reviewed by Leyser, 2001; Kepinsky and Leyser, 2002). Because the genes involved in these pathways share no similarity in their sequences, the overall design of the response pathways and the circuitry involved provides an interesting suggestion of convergent evolution. One potential difference is that auxin response factors and indoleacetic acid proteins have been shown to dimerize with each other, although it remains an open question whether the type-A and type-B ARRs form either homodimers or heterodimers.

Beyond the phosphorelay signaling elements, the cytokinin signaling pathway remains obscure. The responses of plants to changes in cytokinin levels implicate cytokinin in many aspects of plant development, but in most cases the exact role of cytokinins in these processes is unclear. The cloning of genes corresponding to the receptors, signaling elements, and biosynthetic and metabolic enzymes, as well as the isolation of gain- and loss-of-function mutants in these genes, provides a powerful suite of tools to address these questions. The increasing use of microarray analyses of changes in gene expression in response to different hormones and in mutants and transgenic plants affected in cytokinin signaling also should provide useful indications of the gene products involved in cytokinin responses and help to reveal the role of cytokinin signaling in plant development. The fast pace of discovery in this exciting field is likely to continue in coming years.

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