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Source: The Arabidopsis Book, 2002(1)

Published By: The American Society of Plant Biologists

URL: https://doi.org/10.1199/tab.0063

First published on March 27, 2002: e0063. doi: 10.1199/tab.0063

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INTRODUCTION

In the 1940s and 1950s, a wide variety of substances ranging from yeast extract to tomato juice were identified that that could 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(s) that could stimulate cell division. In the 1950s, Skoog and 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 (but not in its absence) 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 abundant cytokinin in coconut milk.

Since their discovery, cytokinins have been implicated to play a role in almost all aspects of plant growth and development, including cell division, shoot initiation and growth, leaf senescence, and photomorphogenic development (Mok and Mok, 1994). Naturally occurring cytokinins are adenine derivatives with distinct substitutions attached to the N6position 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. In higher plants, zeatin occurs in both the cis and the trans configuration, and these forms can be interconverted by an enzyme known as zeatin isomerase. While most of the effects of zeatin have been attributed to the trans form, recent results suggest that the cis form may also be biologically active (Martin et al., 2001).

In addition to the free base forms, cytokinins can also be present in the plant as a riboside (in which a ribose sugar is attached to the 9 nitrogen of the purine ring) or a ribotide (in which the ribose moiety contains a phosphate group). Free cytokinins are readily converted to their respective nucleoside and nucleotide forms, and these conversions may involve enzymes common to purine metabolism (Brzobohaty et al., 1994; Mok and Mok, 2001). The differences in structure are likely to affect the function of the cytokinin, although the precise roles of the various forms are unclear.

Biosynthesis

Mature tRNAs from most organisms, including plants, contain cis-zeatin as a modified base. The breakdown of tRNA was originally suggested as a possible mechanism for cytokinin synthesis (Vremarr et al., 1972) as the released cis-zeatin could subsequently be converted to active trans-zeatin by zeatin isomerase (Mok and Mok, 2001). However, the slow turnover rate of tRNA is not sufficient to account for the amount of cytokinins present in plants.

An enzymatic activity that converts AMP and dimethylallyl pyrophosphate (DMAPP) to the active cytokinin iPMP (isopentenyladenosine-5'-monophosphate) was firstly identified in Dictyostelium discoideum (Taya et al., 1978). Subsequently, the ipt gene 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 and the corresponding genes were not cloned.

In silico searches of the completed Arabidopsis genome sequence revealed the presence of nine ipt-homologues, designated as AtIPT1 to 9 (Table 1). Phylogenetic analysis suggested that AtIPT2 and AtIPT9 encode a putative tRNA-ipt while the other seven AtIPTs formed a distinct

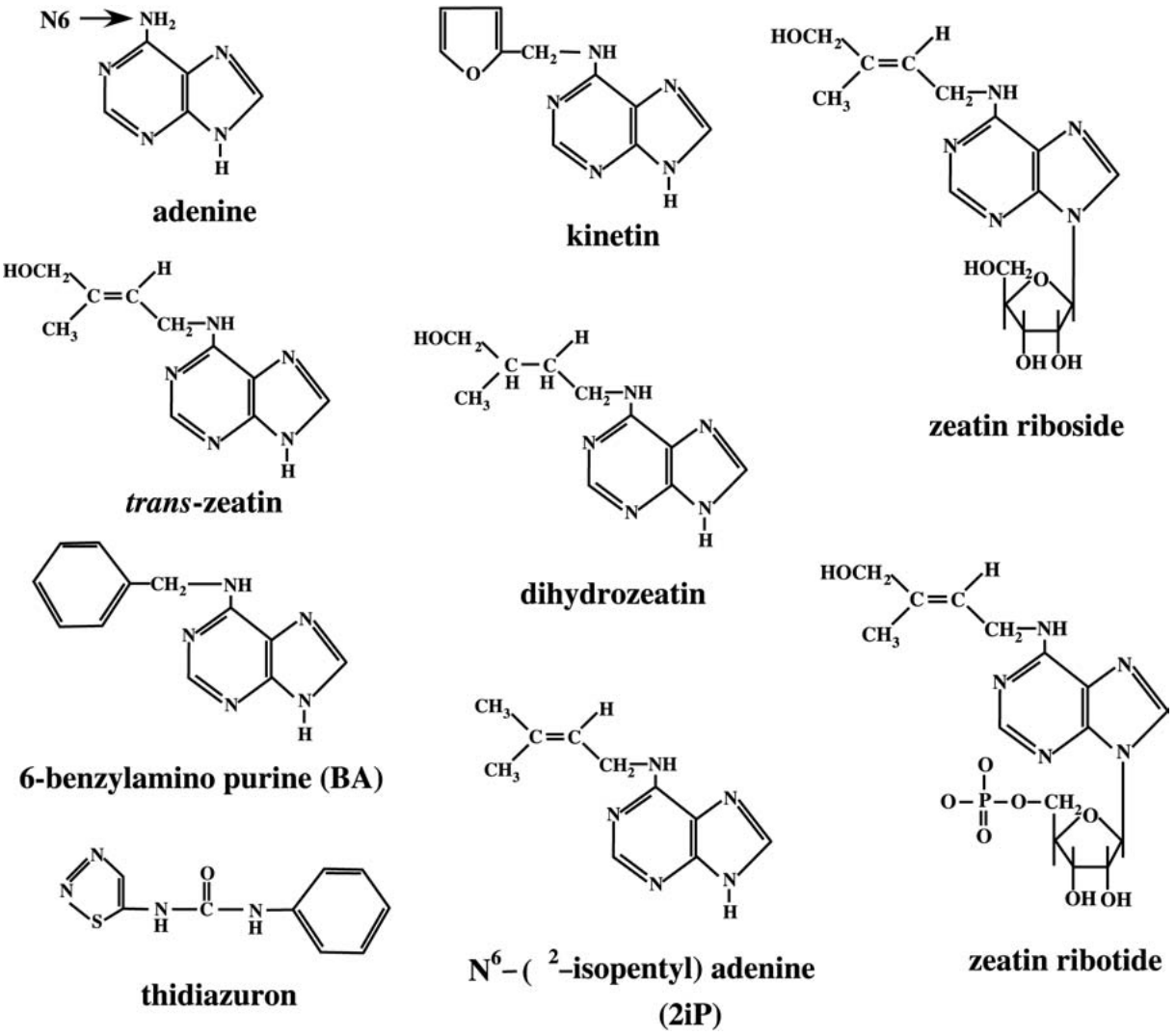

Figure 1. Structures of cytokinins.

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.

clade more closely related to the bacterial ipt gene. 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 enzymes (Takei et al., 2001a). Additionally, calli overexpressing AtIPT4 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). The product of the plant enzyme is likely to be iPTP (isopentenyladenosine-5'-triphosphate) and iPDP (isopentenyladenosine-5'-diphosphate), which can be subsequently converted to zeatin (Figure 2). Several of the AtIPT genes display distinct, tissue-specific patterns of expression, suggesting likely sites of cytokinin production.

Another study in Arabidopsis indicates that an alternative cytokinin biosynthetic pathway exists in plants (Åstot et al., 2000). The authors compared the biosynthetic rate of zeatinriboside-5'-monophosphate (ZRMP; zeatine ribotide) and iPMP in wild-type and transgenic plants

Table 1. AtIPT Genes

Gene Name	Chrom. Locus[a]	Accession No.	Features[b]	References
AtIPT1	At1g68460	AAG52395; AB061400		Kakimoto, 2001; Takei et al., 2001
AtIPT2	At2g27760	AAF00582		Kakimoto, 2001; Takei et al., 2001
AtIPT3	At3g63110	CAB87756; AB061401		Kakimoto, 2001; Takei et al., 2001
AtIPT4	A t4g24650	CAA22998; AB061402		Kakimoto, 2001; Takei et al., 2001
AtIPT5	At5g19040	ACO68809; AB061403		Kakimoto, 2001; Takei et al., 2001
AtIPT6	At1g25410	AAG50809; AB061404	Pseudogene in WS	Kakimoto, 2001; Takei et al., 2001
AtIPT7	At3g23630	BAB02782; AB061405		Kakimoto, 2001; Takei et al., 2001
AtIPT8	A t3g19160	BAB02956; AB061406		Kakimoto, 2001; Takei et al., 2001
AtIPT9	At5g20040	AAK25918	tRNA-IPT	Kakimoto, 2001

[a]Chromosome loci are given by the MIPS designation.
[b]It is noted if the gene is predicted to not encode a cytokinin biosynthetic gene.

designed to inducibly overexpress the Agrobacterial ipt gene. iPMP is the direct product of the transfer of DMAPP to AMP and it can be converted to ZRMP by an endogenous hydroxylase activity (see Figure 2). In vivo deuterium labeling revealed a 66-fold higher biosynthetic rate of ZRMP as compared to that of iPMP, the proposed direct product of IPT. Consistent with this, a feeding experiment using two tracers that allowed the simultaneous determination of iPMP-hydroxylase activity and the de novo synthesis of ZRMP demonstrated that the major precursor for ZRMP was not cytoplasmic iPMP. The authors suggested the presence of an iPMP-independent pathway, in which ZRMP is directly synthesized by IPT from AMP and an as yet unidentified side chain precursor (Figure 2). Inhibition of this pathway by mevastatin suggests that the hypothetical precursor is a terpenoid derivative.

Metabolism

Cytokinins can be conjugated to form a glycoside in which a sugar molecule, generally glucose, is attached to the 3, 7, or 9 nitrogen of the purine ring, or to the oxygen of the zeatin or dihydrozeatin side chain. Glucosyl conjugates at the N7 and N9 but not at the N3 position are usually inactive in bioassays. It is assumed that the N7 and N9 modifications irreversibly inactivate cytokinins, but the precise in vivo role of these N-glucosyl-conjugates is unknown (Mok and Mok, 2001).

O-glucosyl-conjugates of the N6 sidechain are a common modification in all plants. These forms can easily be converted into active cytokinins by b-glucosidases (Brzobohaty et al., 1993). Thus, it is believed that O-glycosylated cytokinins are inactive, stable storage forms that play an important role in the regulation of cytokinin homeostasis. Very little work has been reported on cytokinin conjugation in Arabidopsis.

Many plant tissues contain cytokinin oxidases, enzymes that cleave the N6-side chains from a subset of cytokinins (Figure 3; Jones and Schreiber, 1997). trans-zeatin and iP have unsaturated N6-side chains and are cleaved, while dihydrozeatin and BA are resistant to cytokinin oxidase cleavage. Substitution of other functional groups on the purine ring, as well as O-glycosylation, also prevents cytokinin oxidase cleavage (reviewed in Mok and Mok, 2001). Thidiazuron and other synthetic urea-based cytokinins can non-competitively inhibit cytokinin oxidase activity, while exogenously-applied auxins can increase cytokinin oxidase activity. Cytokinin oxidase irreversibly inactivates cytokinins, and could be important in regulating or limiting cytokinin effects. The activity of the enzyme is induced by high cytokinin concentrations, which is due at least in part to an elevation of the RNA levels for a subset of the genes (J. Kieber, unpublished data).

Recently two groups independently reported the cloning of the first plant cytokinin oxidase gene, ckx1, from Zea mays kernels (Houba-Hérin et al., 1999; Morris et al.,

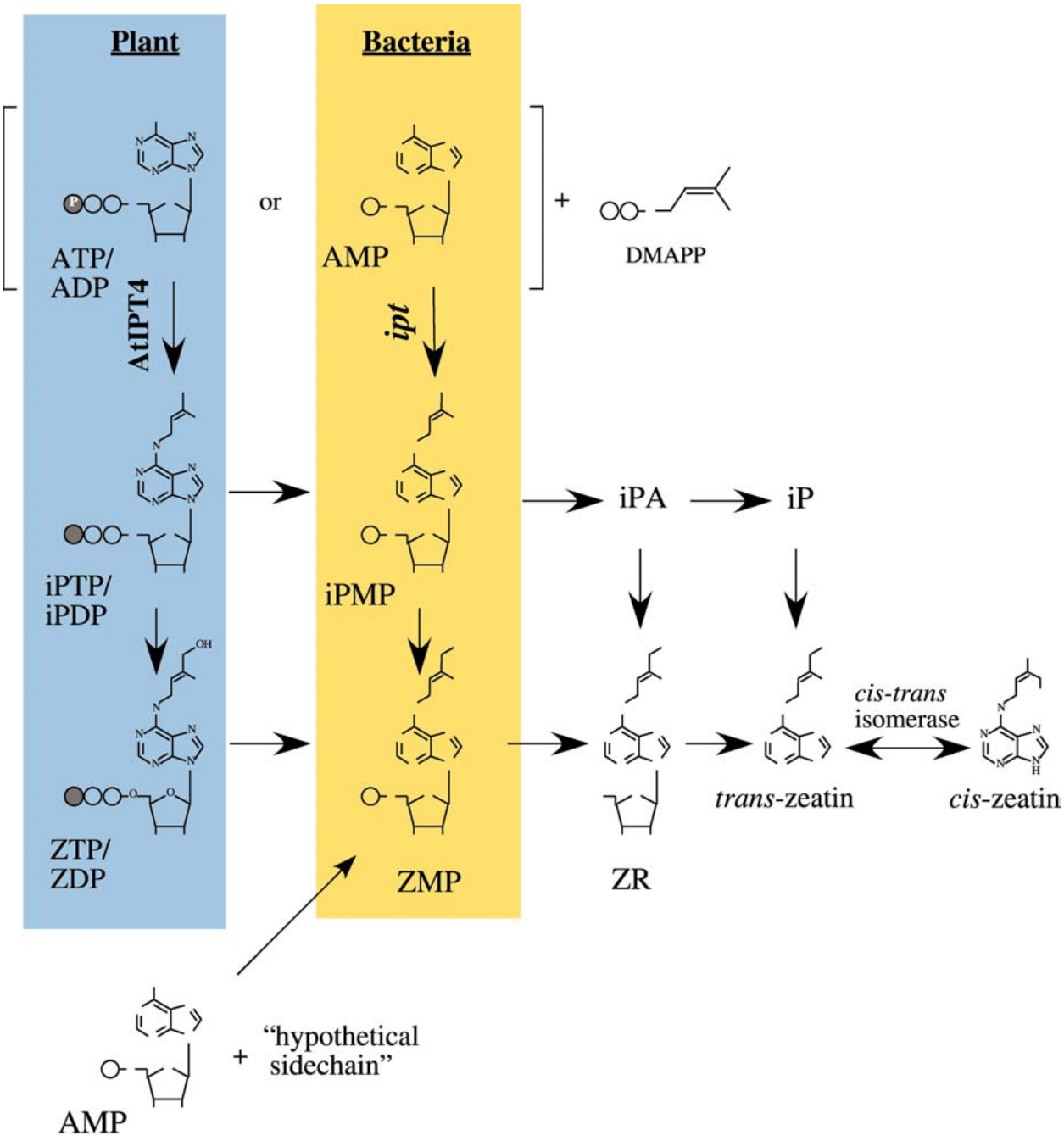

Figure 2. Proposed biosynthetic and metabolic pathway for cytokinins.
The proposed biosynthesis of zeatin tri-/diphosphate in *Arabidopsis* is shown on the left side of the figure. Both ADP and ATP are likely substrates for the plant IPT enzyme, and these and their di- and tri-phosphate derivatives are indicted together (e. g. ATP/ADP). The biosynthesis of cytokinins in bacteria (e.g. *Agrobacterium*) is compared next to it. Several possible modifications and the degradation of zeatin are shown on the right side. The diagram only depicts reactions that are described in the text; cytokinin metabolism is more complex than the pathways shown (see Mok and Mok, 2001). See text for more details.

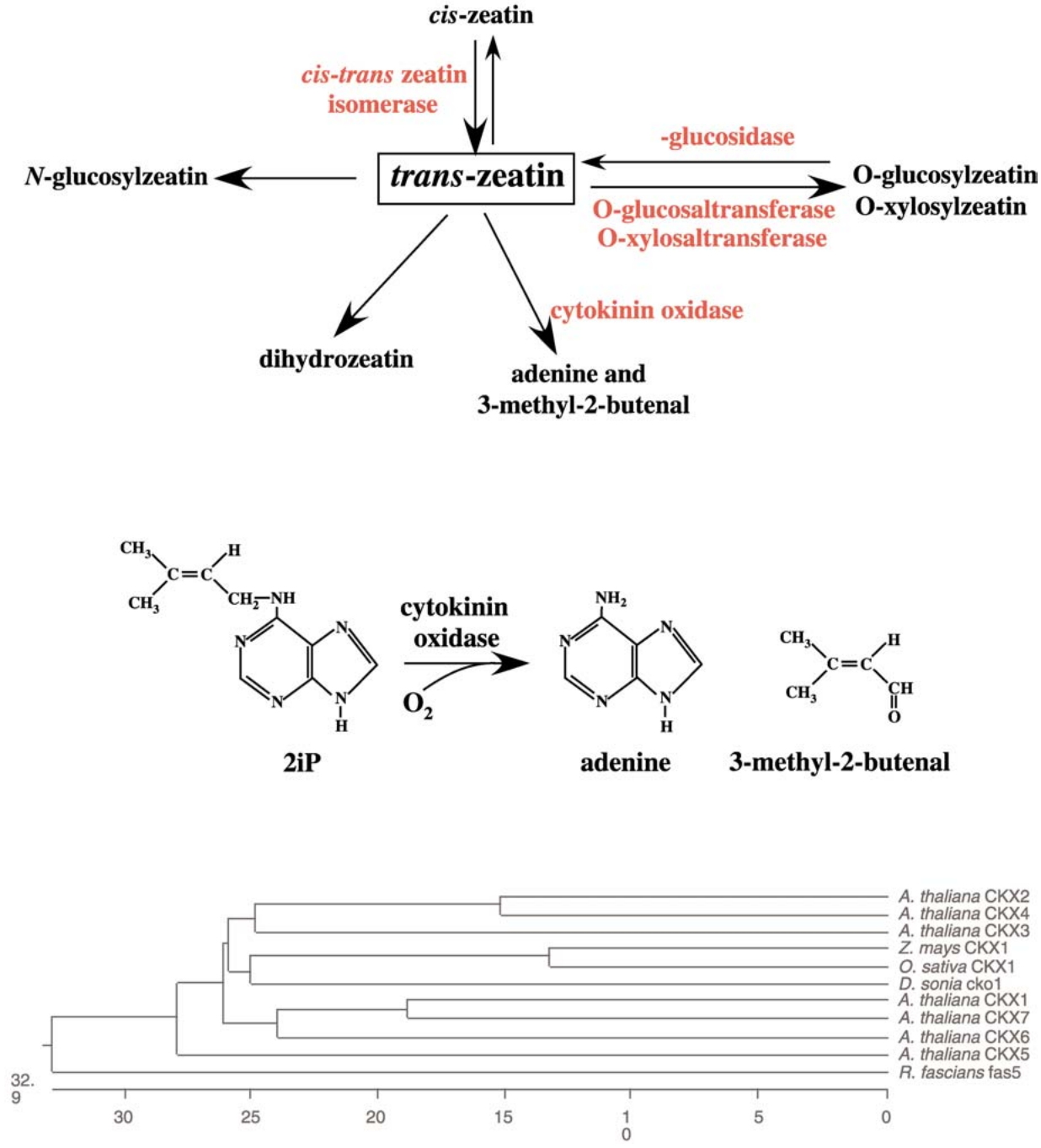

Figure 3. Metabolism of cytokinins.
(A) Metabolic fates of *trans*-zeatin. The enzymes catalyzing each reaction are shown in red. (B) Reaction catalyzed by cytokinin oxidase enzymes. (C) Phylogenic tree of cytokinin oxidase predicted proteins, including all the Arabidopsis enzymes.

1999). In each case, the enzyme was 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 (Table 2; Figure 3; Bilyeu et al., 2001), whose members show distinct patterns of expression. Like their maize counterparts, the predicted Arabidopsis proteins contain a signal sequence for the secretory pathway, and they may be therefore extracellular proteins, but no in vivo localization studies have yet been reported.

Transport of cytokinins

A major location of cytokinin biosynthesis is the root tip, and cytokinins have been found in the xylem sap, suggesting that this class of hormones is transported from the root to the aerial parts of plants. A component possibly involved in this transport, a purine transporter called AtPUP1, has been isolated from Arabidopsis by the functional complementation of a yeast mutant deficient in adenine uptake (Gillissen et al., 2000). The ability of AtPUP1 to transport adenine was competitively inhibited by free cytokinin bases, suggesting that AtPUP1 may be a cytokinin/purine transporter.

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. This suggests that altered nitrate levels in roots leads 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.

Contrasting results with respect to the biological significance of transported cytokinin were obtained from reciprocal grafting experiments with wild-type and ipt transgenic tobacco plants (Faiss et al., 1997). The phenotypic effects of elevated cytokinin were restricted to the part of the plant that was derived from the ipt overexpressing mutant. As seen in other systems, elevated cytokinin levels affect the morphology of the shoot. However, 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 as paracrine signal, at least with respect to apical dominance and leaf senescence.

Table 2. Cytokinin Oxidase Genes

Gene Name	Chrom. Locus[a]	Accession No.	Features	References
CKX1	At2g41510		No oxidase activity detected *in vitro*	Bilyeu et al., 2001
CKX 2	At2g19500	AAG30905	oxidase activity detected *in vitro*	Bilyeu et al., 2001
CKX 3	At5g56970	AAG30906	oxidase activity detected *in vitro*	Bilyeu et al., 2001
CKX 4	At4g29740	AAG30907	oxidase activity detected *in vitro*	Bilyeu et al., 2001
CKX 5	At1g75450	AAG30908		Bilyeu et al., 2001
CKX 6	At1g75450	AAG30909		Bilyeu et al., 2001
CKX 7	At3g63440			Bilyeu et al., 2001

[a]Chromosome loci are given by the MIPS designation.

Roles of cytokinins in plants

Cytokinins have been implicated in many aspects of plant growth and development. The ratio of cytokinin to auxin determines the type of organs regenerated from undifferentiated callus tissue in vitro: callus placed on media with a high cytokinin to auxin ratio usually produces many shoots and few roots while callus placed on media with a low cytokinin to auxin ratio usually produces few shoots and many roots; equal concentrations result in the proliferation of undifferentiated callus (Skoog and Miller, 1957). Treating lateral buds with cytokinin causes them to break dormancy and grow (Phillips, 1975). Application of cytokinin to whole plants or detached leaves tends to delay senescence (Gan and Amasino, 1996). Cytokinins can induce the expression of many light-regulated genes (Chen et al., 1993; Crowell and Amasino, 1994), and etiolated seedlings grown in the presence of cytokinin adopt a morphology similar to light-grown seedlings (Chory et al., 1994). Cytokinins have been shown to influence sink/source relationships, germination, the formation of vascular tissue, and cotyledon expansion in many different plant species (Mok and Mok, 1994).

Most of the research done to determine the role that cytokinins play in growth and development has focused on analyzing the effects of exogenously applied cytokinin. These studies have used many different plant species and experimental designs, sometimes making comparisons difficult. In addition, it is not always clear that the effects of exogenously applied hormones are indicative of the actual physiological role of the hormone. To avoid these problems, endogenous levels of cytokinin have been altered by creating transgenic plants expressing the Agrobacterium ipt (Akiyoshi et al., 1984; Barry et al., 1984), under the control of various promoters (Smigocki and Owens, 1988; Medford et al., 1989; Schmülling et al., 1989; Estruch et al., 1991; Smart et al., 1991; Smigocki, 1991; Li et al., 1992; Ainley and Key, 1993; Klee, 1994). These studies have largely supported the roles established by exogenous application of cytokinins, though the precise role of cytokinins in plant development is still unclear. In one elegant experiment, the ipt gene was expressed from a senescence-specific promoter (Gan and Amasino, 1995). These transgenic tobacco plants developed normally except that senescence was greatly delayed, resulting in dry mass and seed yields 50% greater than wild-type plants.

Induction of expression of ipt under the control of a heat shock promoter in Arabidopsis leads to a large, transient rise in the level of zeatin, mostly in the riboside and ribotide forms (Rupp et al., 1999). Zeatin returned to basal levels within 72 hours after the heat shock. In contrast, the 9-glucoside conjugate of zeatin was elevated after 8 hrs and remained elevated at 72 hrs after a heat shock in these transgenics. Growth for two weeks with a daily heat shock regime resulted in plants with an altered phenotype from the controls. Overall, the biomass of the heat-shocked transgenics increased. The stem was thicker, due to increase in the pith parenchyma, and the leaf thickness increased, partially as a result of an increased number of mesophyll cell layers. The leaves of the heat- shocked transgenics were serrated at the margins, which is similar to the phenotype of Arabidopsis plants that overexpress the KNAT1 and STM genes. Indeed, the ipt transgenics had a higher steady-state level of KNAT and STM mRNA in response to heat shock as compared to the controls (see below).

Cytokinin and the Shoot Apical Meristem

The shoot apical meristem 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 suggest a role for this class of hormones in SAM development.

One mechanism by which cytokinin may influence shoot apical meristem development is by regulating gene expression. Subsets of the knotted1 (kn1) homeobox family of genes are expressed exclusively in the SAM and are involved in its development and maintenance (Jackson et al., 1994; Kerstetter et al., 1994; Kerstetter and Hake, 1997). Transgenic plants that have an elevated level of cytokinin as a result of over-expression of the ipt gene have some phenotypes reminiscent of transgenic plants over-expressing kn1, such as a delay in senescence, reduced apical dominance, and ectopic shoot formation (Kerstetter and Hake, 1997). This suggests that elevated cytokinin levels may induce kn1 expression, which is indeed the case in Arabidopsis (Rupp et al., 1999). These results suggest that cytokinins may act upstream of KNAT1 and STM in regulating shoot apical meristem development.

A similar relationship between cytokinin levels and expression of the maize kn1 gene was observed when kn1 was over-expressed in tobacco. Expressing kn1 under the control of a senescence specific promoter (SAG12) resulted in a delay of senescence, similar to the phenotype seen in plants expressing ipt under control of the SAG12 promoter (Ori et al., 1999). Intact and detached leaves stayed

greener longer and displayed higher chlorophyll content than control plants. Remarkably, older SAG:kn1 leaves had cytokinin levels 15 times higher than wild-type plants, suggesting that kn1 may inhibit senescence by increasing cytokinin levels. These results suggest that the levels of cytokinin and kn1 may positively regulate each other in an interdependent fashion. Alternatively, the elevation of cytokinin in connection with ectopic expression of kn1 may not accurately reflect the endogenous relationship between cytokinin and kn1 homologs.

Further evidence linking cytokinins to apical meristems came from an analysis of transgenic tobacco lines engineered to express four Arabidopsis homologs of the maize cytokinin oxidase from the CaMV 35S promoter, which resulted in reduced endogenous cytokinin levels (Werner et al., 2001). Transgenic lines had elevated cytokinin oxidase activity and significantly reduced amounts of the cytokinins iP and zeatin, including their glycosides, and displayed severely retarded shoot development. In contrast, the growth of the root system was enhanced, indicating that cytokinins may have opposing roles in shoot and root development. These changes were the result of alterations in the rate of cell proliferation in the apical meristems: shoot apical meristems consisted of fewer cells with sizes comparable to wild-type; in contrast, there was an increased number and size of cells in the root apical meristem. Leaves were formed from a significantly decreased number of cells, which was partly compensated for by an increased cell size. Nevertheless, the transgenic leaves were about 15% of the size of their wild-type counterparts. The reduced cell proliferation in the shoot apical meristems as a result of lowered cytokinin levels is consistent with an in vivo role for this hormone in the regulation of cell division.

Cytokinin perception and signal transduction

The proposed cytokinin signal transduction pathway is a phosphorelay pathway similar to bacterial two-component response systems. A brief outline of the relevant details of two-component signaling in prokaryotic and fungal systems is presented in the following section. The involvement of phosphorelay signal transduction in cytokinin signaling will then be considered.

Two-component signal transduction.

Two-component regulators are the major routes by which bacteria sense and respond to various environmental cues (Hoch and Silhavy, 1995; Perraud et al., 1999; West and Stock, 2001). The E. coli genome encodes 62 different two-component proteins that respond to a diverse array of environmental stimuli (Mizuno, 1997). The two components generally consist of a sensor kinase that perceives environmental stimuli and a response regulator

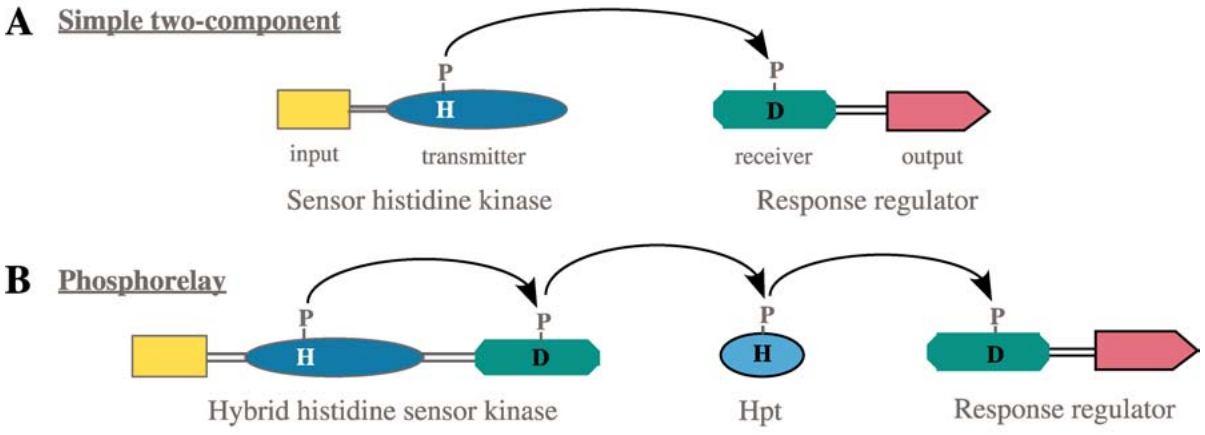

Figure 4. Cartoon representations of two-component phosphotransfer schemes.
(A) A basic prokaryotic two-component system with a sensor histidine kinase and a response regulator. H and D represent the conserved phospho-accepting histidine and aspartate residues involved in phosphorelay signaling. (B) A multistep phosphorelay system involving a hybrid sensor kinase, with input, transmitter and receiver domains, a histidine-containing phosphotransfer protein and a response regulator.

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 sensor kinases are dimers that 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. Most response regulators also contain output domains that typically act as transcription factors (Stock et al., 2000). However, there are also instances in which the receiver domain regulates the activity of a separate protein, as is the case with CheY, which is an E. coli response regulator involved in the chemotaxis response. CheY is comprised 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 (Perraud et al., 1999; Figure 4). The common thread linking multi-step phosphorelays is that they involve four sequential phosphorylation events that alternate between histidine and aspartate residues, although the number of proteins harboring these phosphorylation sites varies. An example of a multistep phosphorelays is the osmosensing pathway in budding yeast. This pathway consists of three components: a hybrid histidine kinase called SLN1 that contains a fused receiver domain, a response regulator (SSK1) that mediates the output of the pathway, and a third component, YPD1, a histidine phosphotransfer protein that mediates phosphotransfer between the receiver domain of the sensor histidine kinase and the receiver domain of the response regulator. The output of this phosphorelay pathway is regulation of SSK2 (a MAPKKK) activity and downstream MAPK signaling (Posas and Saito, 1998). The non-phosphorylated form of the SSK1 response regulator activates SSK2; phosphorylation of SSK1 by SLN1 via YPD renders it inactive.

Genes encoding proteins similar to the various bacterial two-component elements are found in the Arabidopsis genome. These are all found as gene families, and include histidine kinases, histidine phosphotransfer proteins (AHPs), and two classes of response regulators (ARRs). These families will each be discussed separately in the following sections.

The histidine kinase gene family

The Arabidopsis genome encodes a number of genes that share significant sequence similarity to bacterial histidine kinases, including the ethylene receptors, the phytochromes, and cytokinin receptors (Table 3; Figure 5). Sensor histidine kinases typically contain a variable input domain and a conserved transmitter domain, which includes characteristic sequence motifs and a conserved histidine residue that is the site of autophosphorylation (Stock et al., 2000). A subset of the ethylene receptors lacks some of the characteristic transmitter sequence motifs and are thus likely to lack histidine kinase activity (Schaller, 2001). Similarly, the histidine kinase-like domains present in phytochromes are highly divergent and are unlikely to possess histidine kinase activity. Indeed, phytochromes have been shown have serine/threonine protein kinase activity.

Several of the Arabidopsis sensor histidine kinases contain a C-terminal receiver domain, a feature shared by other eukaryotic histidine kinase homologs. In addition, AHK2, AHK3, and AHK4 contain a second receiver domain sandwiched between the transmitter and the C-terminal receiver domains (Figure 5). This second 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.

Most of the Arabidopsis sensor histidine kinases contain multiple predicted transmembrane regions in the N-terminal part of the protein (Ueguchi et al., 2001). The five ethylene receptors contain either 3 or 4 transmembrane domains, and these comprise the site of ethylene binding. AHK2, AHK3 and AHK4 share a region of sequence similarity between the predicted transmembrane regions (Ueguchi et al., 2001; Yamada et al., 2001), which has been shown to contain the cytokinin binding domain (see below). This has recently been recognized as a conserved domain present in receptor-like proteins from bacteria and eukaryotes (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001). It has been named the CHASE (cyclases/histidine kinases associated sensing extracellular) domain and is present in the extracellular or periplasmic regions of receptors containing an intracellular histidine kinase or nucleotide cyclase domains. The CHASE domain senses a diverse set of low molecular weight ligands that affect the development.

Cytokinin receptors are similar to histidine kinases

The first evidence linking cytokinin perception to a histidine kinase came from a screen for Arabidopsis genes whose overexpression resulted in callus proliferation in the absence of exogenously supplied cytokinin (Kakimoto, 1996). The screen was performed on a population of hypocotyl segments transformed with a T-DNA carrying a CaMV35S promoter, and thus the mutants identified were potentially the result of overexpression of the gene into which the T-DNA inserted. This analysis identified a gene called CKI1, which encodes a protein with sequence similarity to histidine kinases. It was proposed that CKI1 may be a cytokinin receptor, though the hypermorphic nature of the mutation precluded a definitive conclusion. Recently, a loss-of-function allele of CKI1 has been identified and shown to result in a female gametophytic lethal phenotype, consistent with the expression pattern of the gene (T. Kakimoto, personal communication). While this indicates an important function in female gametophyte development, it does not address the role of CKI1 in cytokinin responsiveness. Expression of CKI1 in E. coli resulted in a constitutive rescue of a histidine kinase mutation (Yamada et al., 2001), in contrast to CRE1 and AHK3, which displayed a cytokinin-dependent suppression of this same E. coli mutant (see below). Furthermore, when expressed at high levels in isolated Arabidopsis mesophyll protoplasts, CKI1 was able to activate a cytokinin primary response gene, but in a cytokinin-independent manner (see below). Thus, the role of CKI1 in cytokinin signaling remains unclear.

Several recent reports provide compelling evidence that the AHK2, AHK3 and AHK4 genes encode cytokinin receptors (Inoue et al., 2001; Suzuki et al., 2001b). 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.

Further evidence that CRE1 is a cytokinin receptor came from experiments based on the ability of CRE1 to complement histidine kinase deficient yeast and E. coli mutants in a cytokinin-dependent manner. In one report, CRE1 was found to rescue the growth defect of a yeast strain lacking the SLN1 histidine kinase only when cytokinins were present in the media (Inoue et al., 2001). This indicates that CRE1 function was activated in response to cytokinins. Thus, cytokinins alter the function of CRE1, which is one of the key features of receptor/ligand interactions. The complementation of sln1D by CRE1 required YPD1, indicating that CRE1 was acting through the yeast phosphotransfer pathway.

Similar experiments with CRE1, as well as with AHK2 and AHK3, using both a fission yeast and an E. coli multistep phosphorelay system (Suzuki et al., 2001b; Ueguchi et al., 2001; Yamada et al., 2001; T Mizuno, personal communication) gave similar results. As with the complementation of sln1D by CRE1, the complementation in these systems occurred in a cytokinin-dependent manner.

Binding assays using membrane preparations from S. pombe cells designed to express CRE1 has confirmed that CRE1 binds the radiolabeled cytokinin isopentyl adenine with high affinity (Yamada et al., 2001). Competition assays with non-labeled compounds showed that other N6 substituted aminopurines, such as trans-zeatin and aromatic cytokinins such as benzyl adenine could also bind CRE1, but various adenine derivatives that were inactive in cytokinin response assays did not. The structurally distinct diphenylurea-type cytokinin thidiazuron could also compete for binding to CRE1, indicating that this class of synthetic cytokinins are likely to act by directly activating the cytokinin response pathway. Isopentenyl-adenosine (a

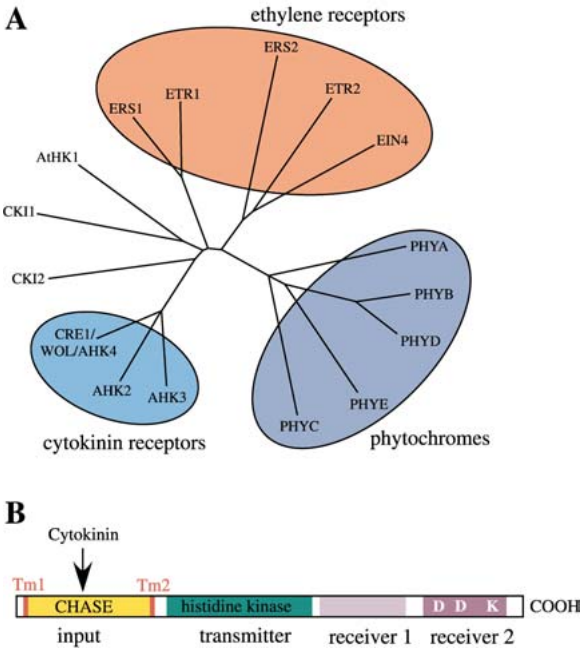

Figure 5. Histidine kinases in Arabidopsis.
An unrooted phylogenetic tree of histidine kinase-related proteins derived using the amino acid sequences of the histidine kinase-like domains of these proteins (adapted from Schaller 2001). Phytochrome, ethylene receptor and cytokinin receptor families are indicated.

Table 3. Histidine Kinase-like Proteins of Arabidopsis

Gene Names	Chrom. Locus[a]	Accession No.[b]	Features[c]	Function	References
ETR1	At1g66340	L24119	HK, Rec	Ethylene Receptor Family	Chang et al. 1993
ETR2	At3g23150	AF047975	Diverged-HK, Rec	Ethylene Receptor Family	Sakai et al. 1998b
ERS1	At2g40940	U21952	HK	Ethylene Receptor Family	Hua et al. 1995
ERS2	At1g04310	AF047976	Diverged-HK	Ethylene Receptor Family	Hua et al. 1998
EIN4	At3g04580	AF048982	Diverged-HK, Rec	Ethylene Receptor Family	Hua et al. 1998
WOL1/CRE1/AHK4	At2g01830	AB049934	HK, Rec	Cytokinin Receptor Family	Mähönen et al. 2000, Inoue et al. 2001, Suzuki et al. 2001
AHK2	At5g35750	AB046869	HK, Rec	Cytokinin Receptor Family	Ueguchi et al. 2001
AHK3	At1g27320	AB046870	HK, Rec	Cytokinin Receptor Family	Ueguchi et al. 2001
CKI1	At2g47430	D87545	HK, Rec		Kakimoto, 1996
AtHK1	At2g17820	AB010914	HK, Rec	Putative Osmosensor	Urao et al. 1999
CKI2	At5g10720		HK, Rec		Kakimoto, 1996

[a]Chromosome loci are given by the MIPS designation.
[b]Accession numbers are for full-length cDNA sequences reported in the literature.
[c]Features noted are conserved histidine-kinase domain (HK), diverged histidine-kinase domain (Diverged-HK), and receiver domain (Rec).

cytokinin riboside) failed to compete for binding to CRE1, consistent with the notion that only the free base forms of cytokinins are active.

cre1 is allelic to the woodenleg (wol) mutation, which was originally identified by a recessive allele that resulted in a 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 predicted extracellular domain (i.e. the CHASE domain) that is assumed to act as a cytokinin-binding domain. Indeed, the wol mutation disrupts cytokinin binding in vitro. These results suggest that the CHASE domain of CRE1 is the site of cytokinin binding and that the wol phenotype results from a loss of cytokinin binding to the CRE1 receptor, and thus a lack of cytokinin action in the affected tissue (Yamada et al., 2001).

Northern analysis revealed that WOL is expressed primarily in the root (Mähönen et al., 2000; Inoue et al., 2001; Ueguchi et al., 2001). 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 hypocotyls, cotyledon shoulders, and in the procambium of the embryonic root (Mähönen et al., 2000). Consistent with the expression pattern of WOL in the embryo, analysis of the mutant phenotype indicates that WOL/CRE1 activity is required for asymmetric cell divisions that occur early in the development of root and hypocotyl and are required to establish phloem and procambium cell lineages in these parts of the plant (Mähönen et al., 2000). 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 to the wol single mutant and the detection of phloem-specific markers in the vascular cylinder. This suppression of wol by fass indicates that WOL/CRE1 is not required to specify phloem development directly, but more likely is required for the production of a sufficient number of vasculature precursor cells.

No shoot phenotype has been described for cre1/wol mutants (Mähönen et al., 2000; Inoue et al., 2001; Ueguchi et al., 2001), which indicates that CRE1 may play little or no role in these tissues, or that its function is redundant with other cytokinin-responsive histidine kinases (e. g. AHK2 and/or AHK3) in the aerial parts of the plant. 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 implicated in

cytokinin signaling

The downstream targets of bacterial and fungal two-component hybrid sensor kinases are histidine phosphotransfer proteins and response regulators. Similarly, the Arabidopsis response regulators (ARRs) and histidine phosphotransfer proteins (AHPs) appear to act downstream of the CRE1/AHK receptors in cytokinin signaling.

Response regulators in Arabidopsis

Response regulators homologs were identified in screens for genes that are rapidly upregulated by cytokinin in Arabidopsis and maize (Brandstatter and Kieber, 1998; Sakakibara et al., 1998). The Arabidopsis response regulators form a large gene family comprised of 22 genes that fall into two main classes (type-A and type-B) based on a phylogenetic analysis of their amino acid sequences and their domain structures (reviewed in: D'Agostino and Kieber, 1999a; Figure 6; Table 4). The type-A ARRs contain a receiver domain, but lack a classic output domain. In contrast, the type-B ARRs are comprised of an N-terminal receiver domain and a fused C-terminal output domain (see below). In addition, most of the type-A ARRs are induced by exogenous cytokinins, while the steady-state level of the type-B ARRs 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). Both the type-A and the type-B ARRs have been implicated in cytokinin signaling.

Type-A ARRs are cytokinin primary response genes

The Arabidopsis genome encodes ten type-A ARRs that fall into five pairs with highly similar amino acid sequences (Figure 6), which may reflect an evolutionarily recent duplication of the Arabidopsis genome (Vision et al., 2001). The amino acid sequences of the receiver domains of the type-A ARRs are very similar, but the sequences of the small C-terminal extensions (< 100 amino acids) are more divergent (Imamura et al., 1999; D'Agostino et al., 2000).

The levels of ARR4 and ARR5 mRNA are rapidly and specifically upregulated by cytokinin and the induction does not require de novo protein synthesis. Thus, these are cytokinin primary response genes. The increase in steady-state transcript levels of the type-A ARRs in response to cytokinin is due, at least in part, to increased transcription (D'Agostino et al., 2000), which implies that a transcription factor(s) is activated in response to cytokinin (see below). Most other type-A ARRs are also induced by cytokinin and with generally similar induction kinetics (Taniguchi et al., 1998; Kiba et al., 1999; D'Agostino et al., 2000). Transcript levels of some type-A ARRs have also 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.

The expression pattern of ARR5 was examined using fusions of the 5' regulatory sequences to a GUS reporter as well as by RNA in situ hybridization (D'Agostino et al., 2000). The promoter-GUS fusion revealed that ARR5 is expressed at high levels in the shoot and root apical meristems, at the junction of the pedicel and immature siliques, and in the central region of mature roots. The expression of ARR5 mRNA in shoot and root apical meristems was confirmed by whole mount in situ analysis of seedlings and is consistent with likely sites of cytokinin action. The steady-state level of ARR4 protein levels has been examined using western blot analysis (Sweere et al., 2001). ARR4 protein was found in stems, leaves and flowers, but, in contrast to the mRNA, the protein was not detected in roots. Furthermore, the level of ARR4 protein was highly elevated in response to light, but the steady-state level of ARR4 mRNA is not altered by light (Brandstatter and Kieber, 1998). Taken together, these data suggest that ARR4 is regulated by a post-transcriptional control mechanism.

Type-B ARRs act as transcription factors

There are eleven type-B ARRs in the Arabidopsis genome that are characterized by the presence of a receiver domain and a large C-terminal extension (Schaller et al., 2001). Unlike type-A ARRs, the steady-state level of type-B ARRs is not affected by application of cytokinins (Imamura et al., 1999; Kiba et al., 1999; Lohrmann et al., 1999). 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 GFP fusion proteins expressed in onion epidermal, parsley, or Arabidopsis cells (Lohrmann et al., 1999; Sakai et al., 2000; Hwang and Sheen, 2001; Lohrmann, 2001). The ARR2 and ARR11 C-terminal domains can activate transcription from a GAL4-driven reporter when fused to the GAL4 DNA-binding domain in

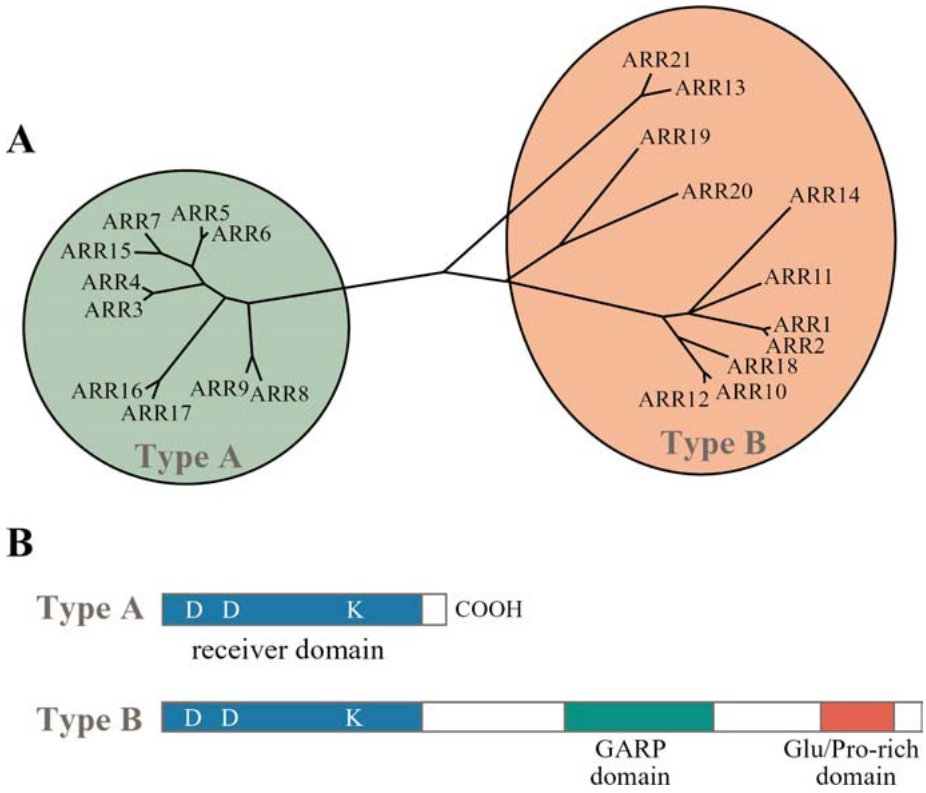

Figure 6. Type-A and type-B response regulators in Arabidopsis.
(A) An unrooted phylogenetic tree made using receiver domain sequences of type-A and type-B ARRs by a heuristic method. Phylogenetic analysis was performed using the PAUP 4.0 program, with 10,000 bootstrap replicates to assess the reliability 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.

both yeast and tobacco cells (Lohrmann et al., 1999; Lohrmann, 2001). The highest activation was observed using a truncated ARR2 protein in which the N-terminal region was removed, indicating that the receiver domain may negatively regulate the activity of the C-terminal transcription activator domain (Sakai et al., 2000).

The C-terminal half of the type-B ARRs contain a conserved GARP domain, so called 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 preferentially to the sequence AGATT and 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). The DNA binding activity of these type-B ARRs is stronger in the absence of the N-terminal part of the protein, again consistent with the idea that the receiver domain regulates the activity of the GARP domain in the type-B ARRs (Lohrmann, 2001).

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 are capable of activating transcription. The amino-terminal receiver domain appears to play a role in negatively regulating the ability of the type-B ARRs to activate transcription.

Histidine phosphotransfer proteins in Arabidopsis

Histidine phosphotransfer proteins mediate transfer of a phosphoryl group from the receiver domain of an activated hybrid sensor histidine kinase to the receiver domain of a

Table 4. Response Regulators of Arabidopsis

Gene Name	Chrom. Locus[a]	Accession No.[b]	Features[c]	References
ARR3	At1g59940	AB008486	Type A	Imamura et al. 1998
ARR4/IBC7/ATRR1	At1g10470	AB008487	Type A	Imamura et al. 1998, Brandstatter and Kieber 1998, Urao et al. 1998)
ARR5/IBC6/ATRR2	At3g48100	AB008488	Type A	Imamura et al. 1998, Brandstatter and Kieber 1998, Urao et al. 1998)
ARR6	At5g62920	AB008489	Type A	Imamura et al. 1998
ARR7	At1g19050	AB008490	Type A	Imamura et al. 1998
ARR8/ATRR3	At2g41310	AB010917	Type A	Imamura et al. 1999, Urao et al. 1998
ARR9/ATRR4	At3g57040	AB010918	Type A	Imamura et al. 1999, Urao et al. 1998
ARR15	At1g74890	AF305720	Type A	D'Agostino et al. 2000
ARR16	At2g40670	AF305721	Type A	D'Agostino et al. 2000
ARR17	At3g56380	AF305722	Type A	D'Agostino et al. 2000
ARR1	At3g16855 (5' end), At3g16857 (3' end)	AB016471	Type B	Sakai et al. 1998a
ARR2	At4g16110	AB016472	Type B	Sakai et al. 1998a
ARR10/ARLP2	At4g31920	AJ005195	Type B	Imamura et al. 1999, Lohrmann et al. 1999
ARR11/ARLP1	At1g67710	AJ005194	Type B	Imamura et al. 1999, Lohrmann et al. 1999
ARR12	At2g25180		Type B	Imamura et al. 1999
ARR13	At2g27070		Type B	Imamura et al. 1999
ARR14	At2g01760		Type B	Imamura et al. 1999
ARR18	At5g58080		Type B	
ARR19	At1g49190		Type B	
ARR20	At3g62670		Type B	
ARR21	At5g07210		Type B	
ARR22	At3g04280			
ARR23	At5g62120		missing 5' end	

[a]Chromosome loci are given by the MIPS designation.
[b]Accession numbers are for full-length cDNA sequences reported in the literature.
[c]Whether response regulators are members of the Type-A cytokinin-induced family (Type A) or contain the B-motif (Type B) is indicated. For pseudo-response regulators (Pseudo), the presence of a CONSTANS-motif (C-motif) or B-motif is indicated.

response regulator in a phosphorelay signal transduction pathway (Figure 4). Five histidine phosphotransfer proteins (AHPs) are encoded by the Arabidopsis genome (Table 5; Suzuki et al., 2000). Each of the predicted proteins is comprised solely of a highly similar phosphotransmitter domain that includes a conserved histidine residue that is predicted to act as the phosphorylation site. There is also a single pseudo-histidine phosphotransmitter in Arabidopsis in which the conserved histidine is replaced with an asparagine residue. Unlike the type-A ARRs, the steady-state level of AHP transcript is not altered by exogenous cytokinin (Miyata et al., 1998; Suzuki et al., 1998; Suzuki et al., 2000).

Several lines of evidence indicate that the AHPs function as phosphotransmitters in a phosphorelay. A number of the Arabidopsis phosphotransfer proteins were able to complement a deletion of YPD1, the histidine phosphotransfer protein component of the budding yeast osmosensing phosphorelay pathway described above (Miyata et al., 1998; Suzuki et al., 1998). Co-expression of AHPs with CRE1 in same E. coli assay system (YojN-RcsB) employed to examine CRE1 function (Suzuki et al., 2000; Suzuki et al., 2001b; see above) suppressed the cytokinin-responsive phosphotransfer, indicating that AHPs were competing with the endogenous E. coli HPt proteins for phosphorylation by CRE1 (Suzuki et al., 2001b). E. coli membrane preparations can phosphorylate the AHP1 and AHP2 at the conserved histidine residue (Suzuki et al., 1998) and phosphotransfer from AHP1 and AHP2 to both type-A and type-B ARRs has also been demonstrated in vitro (Suzuki et al., 1998; Imamura et al., 1999; Suzuki et al., 2001b). Finally, yeast two-hybrid interactions between various AHPs and Arabidopsis histidine kinase homologs and between AHPs and both type-A and type-B ARRs have also been demonstrated (Miyata et al., 1998; Suzuki et al., 2000; Urao et al., 2000; I. D'Agostino and J. Kieber, unpublished). Taken together, these data strongly support the notion that the Arabidopsis AHPs function as histidine phosphotransfer proteins, acting in a phosphorelay mechanisms to mediate the transfer of phosphate from the receiver domains fused to a set of the Arabidopsis histidine kinases to the receiver domains of a set of the type-A and type-B ARRs. How specificity is achieved in this system is question that has yet to be addressed.

Interactions of phosphorelay elements in cytokinin signaling

The work described in the discussions above has shown that several histidine kinases 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 mediate phosphotransfer reactions to the receiver domains of type-A and type-B ARRs. Two recent reports confirm and extend the interactions of these elements and their role in cytokinin signaling (Hwang and Sheen, 2001; Sakai et al., 2001).

Several lines of evidence from these reports indicate that type-B ARRs are involved in cytokinin signaling, including the induction of type-A ARR transcription. A loss-of-function ARR1 mutation results in reduced sensitivity to cytokinin in both root elongation assays in intact

Table 5. HPt Proteins of Arabidopsis

Gene Names	Chrom. Locus[a]	Accession No. (cDNA)[b]	Features	References
AHP1/ATHP3	AT3g21510	AB015141	HPt	Suzuki et al. 1998, Miyata et al. 1998
AHP2/ATHP1	AT3g29350	AB015142	HPt	Suzuki et al. 1998, Miyata et al. 1998
AHP3/ATHP2	AT5g39340	AB015143	HPt	Suzuki et al. 1998, Miyata et al. 1998
AHP4	AT3g16360	AB041766	HPt	Suzuki et al. 2000
AHP5	At1g03430	AB041767	HPt	Suzuki et al. 2000
APHP1	At1g80100		pseudo-HPt	Suzuki et al. 2000

[a]Chromosome loci are given by the MIPS designation.
[b]Accession numbers are for full-length cDNA sequences reported in the literature.

plants and shoot regeneration assays in tissue culture, while overexpression of ARR1 increases the sensitivity to cytokinin in these assays. Analysis of transcript levels of ARR6 (a type-A gene) in these plants correlated with the level of ARR1: ARR1 overexpression resulted in an elevated level of ARR6 while loss-of-function arr1 mutants showed decreased levels, suggesting that ARR1 regulates ARR6 transcription. Transgenic plants engineered to overexpression ARR2 displayed phenotypes consistent with an activation of the cytokinin response pathway, including excess cell proliferation and shoot and leaf formation in cultured cells as well as delayed leaf senescence in excised leaves (Hwang and Sheen, 2001). Although overexpression of full-length ARR1 resulted in only very subtle morphological alteration in intact Arabidopsis, expression of a truncated form of ARR1 lacking the receiver domain resulted in striking changes in the growth of the transgenics that were indicative of activation of cytokinin signaling (). This 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; Lohrmann, 2001).

Further evidence linking the type-B ARRs to the cytokinin signaling came from studies employing a transient expression system in Arabidopsis protoplasts (Hwang and Sheen, 2001). This system utilized 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 protoplast transfected with this construct displayed a strong increase in luciferase activity specifically in response to cytokinin, similar to the induction of the endogenous gene. Using this system, it was found that overexpression of type-B ARRs, especially ARR2, resulted in an elevated basal level and a hyper-response to cytokinin of the ARR6-LUC reporter, 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 aspartate 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 aspartate residue in ARR2 may not be required for transcriptional activation of ARR6 in response to cytokinin, at least as assayed by overexpression in this protoplast system.

Taken 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 indicates that the effect of the type-B ARRs on type-A ARR transcription may be direct. How type-B function is regulated, and in particular the role of phosphorylation in its activation remains unresolved.

In experiments that confirm the heterologous complementation experiments performed in yeast and E. coli (Inoue et al., 2001; Suzuki et al., 2001b; Ueguchi et al., 2001), overexpression of CRE1 in the Arabidopsis mesophyll protoplast system was shown to result in an increased induction of ARR6 transcription in response to exogenous cytokinin (Hwang and Sheen, 2001). CKI1 was localized to the plasma membrane in this system. Surprisingly, overexpression of AHK2 and AHK3 had little or no effect on the induction of ARR6-LUC. Expression of mutant forms of CRE1 in which the conserved histidine and aspartate residues in the transmitter and receiver domains were mutated failed to elicit an elevated ARR6-LUC response to cytokinin, supporting the model that phosphotransfer within CRE1 is required for its signaling function. As with previous results in other systems, expression of CKI1 results in an activation of ARR6-LUC that was not further increased by cytokinin treatment, suggesting that CKI1 is constitutively active or is saturated by the endogenous cytokinin in the protoplasts.

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

Several lines of evidence also 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, were found to transiently accumulate in the nucleus within 30 minutes of application of exogenous cytokinin, as measured by GFP fusions in Arabidopsis protoplasts (Hwang and Sheen, 2001). Secondly, overexpression of AHP2 from a CaMv 35S promoter in transgenic Arabidopsis resulted in modest hypersensitivity to exogenous cytokinin in root and hypocotyl elongation assays (Suzuki et al., 2001a). These results, coupled with the two-hybrid and in vitro phosphorylation experiments described above, indicate that these histidine phosphotransfer proteins are likely to mediate signaling between CRE1 and the type-B ARRs. Definitive demonstration of a role of these genes awaits analysis of loss-of-function alleles.

The data described above are generally consistent with the simple model for phosphorelay signal transduction in cytokinin signaling presented in Figure 7. Cytokinins bind to the CRE1 histidine kinase, and most likely also AHK2 and AHK3, in the conserved extracellular domain. This induces autophosphorylation on a histidine residue within the transmitter domain and subsequent transfer of the

phosphate to an aspartate residue within the fused receiver domain. The phosphate is then probably transferred to a histidine residue on the AHPs, which then translocate to the nucleus where they activate type-B ARRs. The activated type-B ARRs bind to elements within the promoter of the type-A ARRs to increase their rate of transcription. The type-A ARRs feedback inhibit their own expression, and possibly cytokinin signaling in general. Analogy to bacterial systems, in vitro studies and conservation of the sites of phosphorylation all suggest that phosphorylation of the AHPs and ARRs play 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. At least in the cases of ARR2 and ARR6, phosphorylation at the conserved aspartate residue predicted to be the phosphoaccepting residue may not be required for regulation of ARR6 transcription (Hwang and Sheen, 2001). Other targets of type-B ARR transcriptional activation and proteins that interact with type-A ARRs may give clues as to downstream effectors of the cytokinin signal and the role of phosphorylation of these proteins.

Cytokinin signaling beyond the phosphorelay

While it is becoming clear that the signal transduction chain mediating the perception of cytokinin and the induction of the type-A ARRs is mediated by a classic phosphorelay system, how this translates into alteration in cellular function is unknown. It is possible that additional mechanisms of cytokinin perception and or signaling exist. These targets and potential novel elements may be represented in some of the cytokinin action mutants, the cytokinin responsive genes, and/or the yeast two-hybrid interactors that have been identified. These various elements are discussed in the following section.

Proteins that interact with two-component elements

ARR4 was found to interact with the red light photoreceptor PHYB in a yeast two-hybrid screen (Sweere et al., 2001). Overlaps between the action of light and cytokinin on plant development and induction of gene expression have long been observed. For example, the addition of cytokinin can lead to deetiolation of seedlings grown in the dark and induction of several light-regulated genes (Chory et al., 1994). The report of light-dependent ARR4 protein accumulation and an interaction between ARR4 and the N-terminus of the red light photoreceptor phytochrome B (Sweere et al., 2001) provides an interesting and direct link between light and cytokinin signaling. The direct interaction of ARR4 with PhyB was demonstrated by co-immunoprecipitation experiments from both yeast and Arabidopsis. Yeast two-hybrid analysis showed that the interaction of ARR4 with PhyB was mediated by the extreme N-terminus of PhyB. Photoconversion experiments revealed that the interaction with ARR4 stabilizes the active, Pfr-form of PhyB. Consistent with this, overexpression of ARR4 resulted in hypersensitivity to red light as assayed by measuring the lengths of hypocotyls in seedlings grown continuously in varying doses of red light and by comparison of the root length and the number of leaves at flowering between transgenic and wild-type plants. 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.

Additional elements that interact with AHPs and the type-A ARRs have been identified in yeast two-hybrid screens. An Arabidopsis auxin-inducible DNA binding protein, AtDBP, was found in a screen for ARR4 interacting clones, and this gene may providing an link between cytokinin and auxin signaling (Alliotte et al., 1989; Yamada et al., 1998). A novel nuclear localized protein, TCP10, was found to interact with several AHPs. TCP10 contains a bipartite nuclear localization signal and a TCP domain, which is comprised of approximately 50 amino acids that are predicted to adopt a basic helix-loop-helix structure and which is found in a number of other plant proteins (Cubas et al., 1999; Suzuki et al., 2001c).

Cytokinin action mutants

A number of Arabidopsis mutants have been reported that may affect cytokinin action, in addition to the mutations in two-component elements described above. The cyr1 mutant is hypothesized to be defective in cytokinin perception. cyr1 mutants have pale green leaves, abbreviated shoot development and incomplete cotyledon and leaf expansion (Deikman and Ulrich, 1995). cyr1 plants are less responsive to exogenous cytokinin in root inhibition assays, anthocyanin accumulation assays, and in vitro shoot initiation assays. However, cytokinin-primary response genes are induced normally in cyr1 and cytokinin induces ethylene biosynthesis to wild-type levels in cyr1 mutants (J. Kieber, unpublished observations) which, cou-

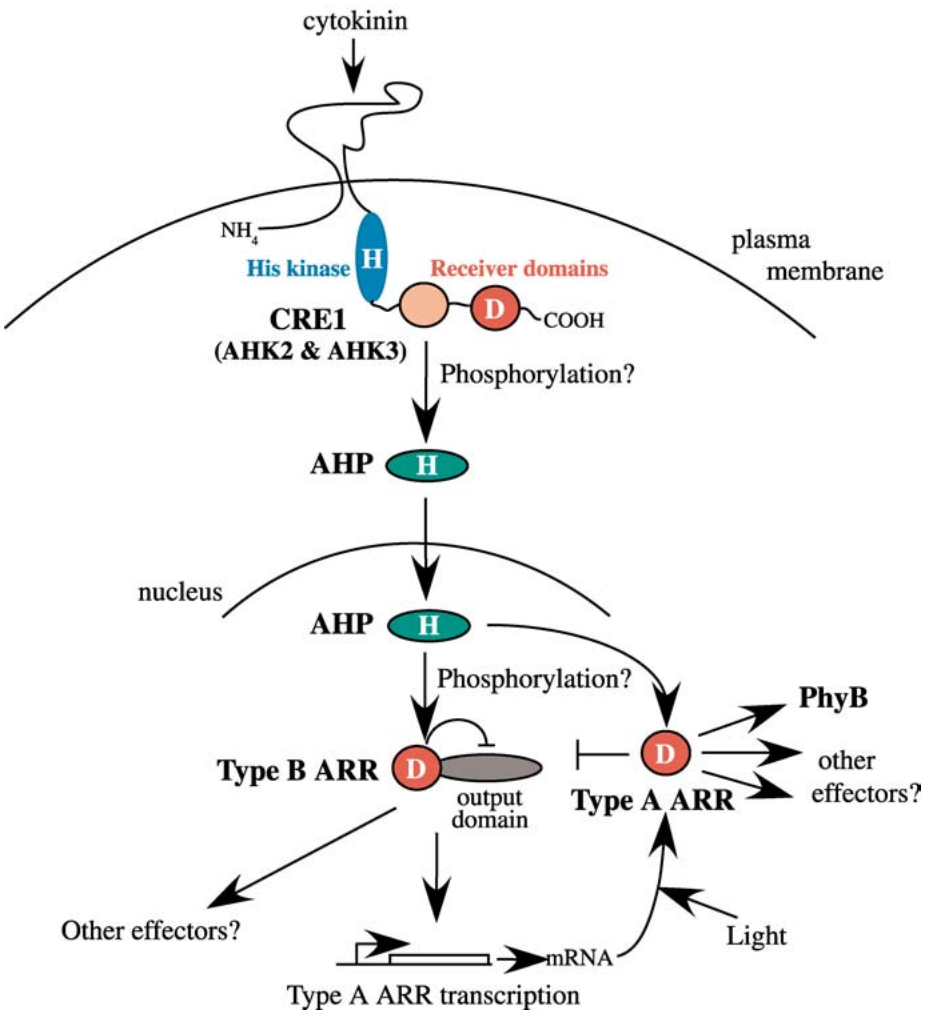

Figure 7. Proposed model for phosphorelay signal transduction in cytokinin signaling.
Cytokinin binds to CRE1, and possibly other histidine kinase-like proteins such as AHK2 and AHK3, within the conserved domain flanked by predicted transmembrane domains. CRE1, by analogy to CKI1 (Hwang and Sheen, 2001), is likely to be located in the plasma membrane. Binding of cytokinin activates the transmitter domain (blue), 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 (red). A second, degenerate receiver domain (pink) is also present. The phosphate is then 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 (indicated by ⊥). Light appears to elevate the level of ARR4 protein levels, as indicated by the arrow. The receiver domain of type-B ARRs inhibits the activity of the output domain (gray). The role of phosphorylation of the type-A and type-B ARRs is unclear. The output of this signaling pathway is mostly unknown, although one likely target is PhyB. See text for additional details

pled with its altered sensitivity to abscisic acid (Deikman and Ulrich, 1995), indicates that cyr1 is not an early or general cytokinin signaling mutant.

Two CYTOKININ-HYPERSENSITIVE mutants, ckh1 and ckh2, were identified as showing increased sensitivity to cytokinin in tissue culture (Kubo and Kakimoto, 2000). Since cytokinin levels are not elevated in these mutants, ckh1 and ckh2 are proposed to identify gene products that negatively regulate the cytokinin signaling pathway that promotes cell proliferation and chloroplast development. Adult ckh1 and ckh2 plants are short, but do not have any other striking morphological alterations.

PASTICCINO1 (PAS1), which encodes an immunophilin-like protein, has been implicated in the negative regulation of cytokinin levels or signaling (Vittorioso et al., 1998). Expression of PAS1 is increased in the presence of cytokinin and pas1 mutations result in an array of phenotypes consistent with increased cytokinin action, 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 reminiscent of wol mutants (Scheres et al., 1995; Mähönen et al., 2000; Inoue et al., 2001). Finally, the rosette of pas1-1 mutant is small and abnormal and pas1 plants rarely produce flowers (Vittorioso et al., 1998).

Mutations in STUNTED PLANT 1(STP1) affect cell division rate and growth rate, but not overall plant morphology. Cytokinin-dependent growth and differentiation in tissue culture is also unaffected in this mutant. However, root growth in the stp1 mutant does show reduced sensitivity to cytokinins. The stp1 root growth phenotype can be copied by treatment of wild-type plants with cytokinin suggesting that STP1 is a target for negative regulation by cytokinin in the root (Beemster and Baskin, 2000).

Several Arabidopsis tissue culture lines were identified that were able to grow in vitro on media lacking added auxin and cytokinin (Frank et al., 2000). These lines grew as shoot-like callus, root-like callus or as undifferentiated tissue. One of the callus-like lines had a 95% reduction in cytokinin oxidase activity, and higher steady-state levels of cytokinins. The steady-state level of transcript of the CKI1 and STM genes were elevated in all six shooty lines, and the level of STM transcript was elevated in three, which, coupled with the observation that they have WT levels of cytokinins, suggests that they may be affected in cytokinin signaling.

A number of mutants that disrupt the induction of ethylene in response to exogenous cytokinin have also been identified (Vogel et al., 1998a; Vogel et al., 1998b). This analysis revealed that cytokinin elevated ethylene production primarily via a post-translational activation of ACS5, which encodes one member of the Arabidopsis ACC synthase gene family. A second mutation, cin1, affected multiple cytokinin responses and may affect the cytokinin response pathway. This mutation does not affect the morphology of the adult plant.

The altered meristem program 1 (amp1) is an Arabidopsis mutant that over-produces cytokinin and several amp1 phenotypes, such as the lack of apical dominance, delayed senescence and increased shoot regeneration in tissue culture, are consistent with what a cytokinin over-producing mutant might look like (Chaudhury et al., 1993). However, other amp1 phenotypes, such as polycotyledons, precocious flowering and abnormal phyllotaxy, have not been observed in plants treated with exogenous cytokinin or in transgenic plants overexpressing ipt. The AMP1 gene has recently been cloned and found to encode a putative glutamate carboxypeptidase (Helliwell et al., 2001). This homology makes it unlikely that this gene directly affects cytokinin biosynthesis. The authors propose that AMP1 may regulate the level of an extracellular peptide signal that controls meristem function.

Cytokinins and gene expression

Many changes in gene expression, in addition to the induction of type-A ARRs, have been detected in response to application of exogenous cytokinins (reviewed in 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. Several genes involved in regulating meristem function and/or cell proliferation are upregulaed by cytokinin in Arabidopsis, including members of the knotted gene family (see above) and CycD3 (see below).

Additional genes responsive to cytokinin have been identified by cytokinin treatment of soybean or tobacco tissue culture cells that were starved for cytokinin. In an extensive study, 20 genes were identified whose transcripts accumulated within four hours of cytokinin application (Crowell et al., 1990). These 20 genes were also responsive to auxin. Sequence analysis revealed that two of these genes were homologous to ribosomal proteins and one was homologous to a pollen allergen protein (Crowell et al., 1990; Crowell, 1994). Several studies have also examined cytokinin-regulated gene expression during tissue differentiation, including nodulation, senescence, floral development, lateral bud induction, and various aspects of light development. Genes identified in these studies include photosynthetic genes, ribosomal protein genes, nitrate reductase as well as many novel genes. However, most of these genes are also induced by other

stimuli, most notably light and auxin, and none of these genes are induced with kinetics suggestive of an immediate early response gene. Cis-acting DNA elements that are responsive to cytokinin have not yet been delineated. Additional cytokinin responsive genes have been identified using microarrays and these may reveal additional elements involved in cytokinin action (T. Schmülling, Arabidopsis Microarray Facility, and A. Roshette and J. Kieber, unpublished observations).

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 are found 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. Cytokinins have been linked to virtually all stages of the cell cycle (reviewed in D'Agostino and Kieber, 1999b; Frank and Schmulling, 1999; den Boer and Murray, 2000).

Several observations suggest that cytokinins may play a role in the G2/M transition (reviewed in Hare and Staden, 1997), though a decisive link is lacking. 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).

Compelling evidence that cytokinin regulates the G1/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 G1 cyclins, and found that one, CycD3, was induced in cultured cells by exogenous cytokinin application (Soni et al., 1995). In Arabidopsis, cytokinin rapidly upregulates 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 the regulating passage through the restriction point of the cell cycle in G1. 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 that were over-expressing CycD3 from a CaMV 35S promoter, healthy green calli were formed independently of cytokinin, whereas wild-type controls only formed calli when cytokinin was present. 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 parallel 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 G1/S transition. These results suggest that cytokinin regulates Arabidopsis cell cycle progression at the G1/S transition, at least partially, by inducing CycD3 transcription.

Summary and Perspectives

Arabidopsis genes encoding key enzymes of 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 revealed a potential cytokinin signal transduction chain from the cell surface to the nucleus. This pathway is, at least in outline, very similar to the bacterial two-component phosphorelay paradigm. We are beginning to understand the roles of cytokinins in Arabidopsis growth and development, including the regulation of meristem function.

It is interesting that the proposed cytokinin signal transduction pathway is remarkably similar in its overall design to the auxin response pathway (reviewed by (Leyser, 2001). In both hormone response pathways, components include a family of sequence specific DNA-binding transcription

factors (type-B ARRs for cytokinin, ARFs for auxin) 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, which negatively regulate the response pathway (type-A ARR or IAA genes). As the genes involved in these pathways share no similarity in their sequence, this overall design of the response pathways and the circuitry involved suggests convergent evolution. The ARF/IAAs have been shown to form homo- and heterodimers, but it remains an open question whether this is also the case of the type-A and type-B ARRs.

Despite this remarkable progress, a multitude of questions remain. How does cytokinin act to regulate cell proliferation, and what others roles does it play in Arabidopsis growth and development? How is the biosynthesis of cytokinin regulated? What is the role of histidine-to-aspartate phosphorylation in the cytokinin signal transduction chain? What are the outputs of the two-component signaling chain? How is specificity of interaction among the various members of the gene families encoding the two-component elements achieved? Is there crosstalk between ethylene and cytokinin signaling at the level of AHP phosphorylation? The cloning of genes corresponding to the receptors, signaling elements, biosynthetic and metabolic enzymes, as well as isolation of gain- and loss-of-function mutants in these genes provides a powerful suite of tools to address these questions.

Acknowledgements

This work was supported by the National Science Foundation grant # IBN-0114965 and National Institute of Health grant # GM64425.

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