Cytokinins. New Insights into a Classic Phytohormone

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Cytokinins were discovered in the search for factors that promoted division of plant cells in culture. Naturally occurring cytokinins are N^6-substituted adenine derivatives that generally contain an isoprenoid derivative side chain. These hormones influence numerous aspects of plant development and physiology, including seed germination, de-etiolation, chloroplast differentiation, apical dominance, plant-pathogen interactions, flower and fruit development, and leaf senescence. These processes are also influenced by various other stimuli (e.g. light and other phytohormones), and the physiological and developmental outcomes reflect a highly integrated response to these multiple stimuli. For example, the classical reports of Skoog and Miller (1957) revealed that undifferentiated callus cultures would form into roots or shoots depending on the relative amount of cytokinins and auxin in the medium; the ratio rather than the absolute amount of these two hormones is critical. A balanced ratio keeps the cells in an undifferentiated state, while high cytokinin to auxin ratios promote shoot and low ratios promote root development.

In the last decade, genetic and molecular analysis of mutants has provided valuable insights into the molecular mechanisms underlying the action of other phytohormones. Cytokinin has lagged behind in such studies, but several exciting recent reports show that this is now changing. This review focuses on the dramatic recent progress in understanding cytokinin metabolism, perception, and signaling. The analysis of all three aspects is required to understand the different effects of cytokinin on plant physiology and its role as a developmental signal.

BIOSYNTHESIS AND METABOLISM

Cytokinins occur as a bound form in the tRNA of most organisms, including plants, but plants also possess significant amounts of free cytokinins. Isoprenoid-type cytokinins are the most abundant, but several plant species contain adenine derivatives with aromatic substituents. In addition, there are synthetic cytokinins derived from diphenylurea (DPU) that are structurally unrelated to the adenine-type cytokinins.

The structure and conformation of the side chain are critical to the activity of the respective cytokinin. For example, one of the most abundant cytokinins in higher plants, trans-zeatin, displays a high cytokinin activity in bioassays, but the cis-isomer possesses a significantly lower activity.

In general, a given plant tissue contains several types of cytokinins and their modified forms. The distribution of the various cytokinins differs significantly between plant species. The phenotypic analysis of biosynthetic mutants could reveal the roles of specific cytokinin forms, but unfortunately neither general nor specific mutants with reduced cytokinin content have been isolated, and the overexpression of biosynthetic genes generally affects the whole pool of endogenous cytokinins. Furthermore, it is difficult to quantify cytokinins in vivo, especially with respect to their distribution within different plant tissues or organs. Our knowledge about the in vivo role of individual cytokinins and their modified forms is therefore very limited.

BIOSYNTHESIS

The breakdown of tRNA was originally suggested as a possible mechanism for cytokinin synthesis. The released cis-zeatin could subsequently be converted to active trans-zeatin by the enzyme cis-trans-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.

Enzymatic activity that converts AMP and dimethylallyl-diphosphate (DMAPP) to the active cytokinin isopentenyladenosine-5′-monophosphate (iPMP) was firstly identified in *Dictyostelium discoideum* (Taya et al., 1978). Subsequently, the *tmr* gene (later designated as *ipt*) from *Agrobacterium tumefaciens*, which results in root-like tumors when mutated, 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.

The completion of the Arabidopsis genomic sequence enabled Takei et al. (2001a) and Kakimoto (2001a) in independent studies to identify a total of nine *ipt*-homologs, designated as *AtIPT1* to *AtIPT9*, by an in silico analysis. A phylogenetic analysis suggested that *AtIPT2* and *AtIPT9* encode a putative tRNA-ipt while the other seven *AtIPTs* formed a

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distinct clade more closely related to the bacterial *ipt/tmr* gene. The expression of these seven genes in *Escherichia coli* resulted in the secretion of the cytokinins iP and zeatin, confirming that they encode cytokinin biosynthetic genes (Takei et al., 2001a). Additionally, calli overexpressing *AtIPT4* under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter showed shoot regeneration even in the absence of cytokinin, while CaMV 35S::*AtIPT2* calli were still dependent on cytokinin (Kakimoto, 2001a). 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 isopentenyladenosine-5′-triphosphate (iPTP) and isopentenyladenosine-5′-diphosphate (iPDP), which can be subsequently interconverted to zeatin (Fig. 1). Several of the *AtIPT* genes display distinct, tissue-specific patterns of expression, perhaps providing new insights into the sites of cytokinin production (Kakimoto, 2001b).

Another study in Arabidopsis indicates that an alternative cytokinin biosynthesis pathway exists in plants (Åstot et al., 2000). The authors compared the biosynthetic rate of zeatin riboside-5′-monophosphate (ZMP) and iPMP in wild type and transgenic plants designed to inducibly overexpress the bacterial *ipt* gene. iPMP is the direct product of the transfer of DMAPP to AMP, and it can be converted to ZMP by an endogenous hydroxylase activity. In vivo deuterium labeling revealed a 66-fold higher biosynthetic rate of ZMP than that of iPMP, the product of IPT. By a feeding experiment with two tracers, which allowed the simultaneous determination of iPMP-hydroxylase activity and the de novo synthesis of ZMP, it was shown that the major precursor for ZMP was not cytoplasmic iPMP. The authors suggested the presence of an iPMP-independent pathway, in which ZMP

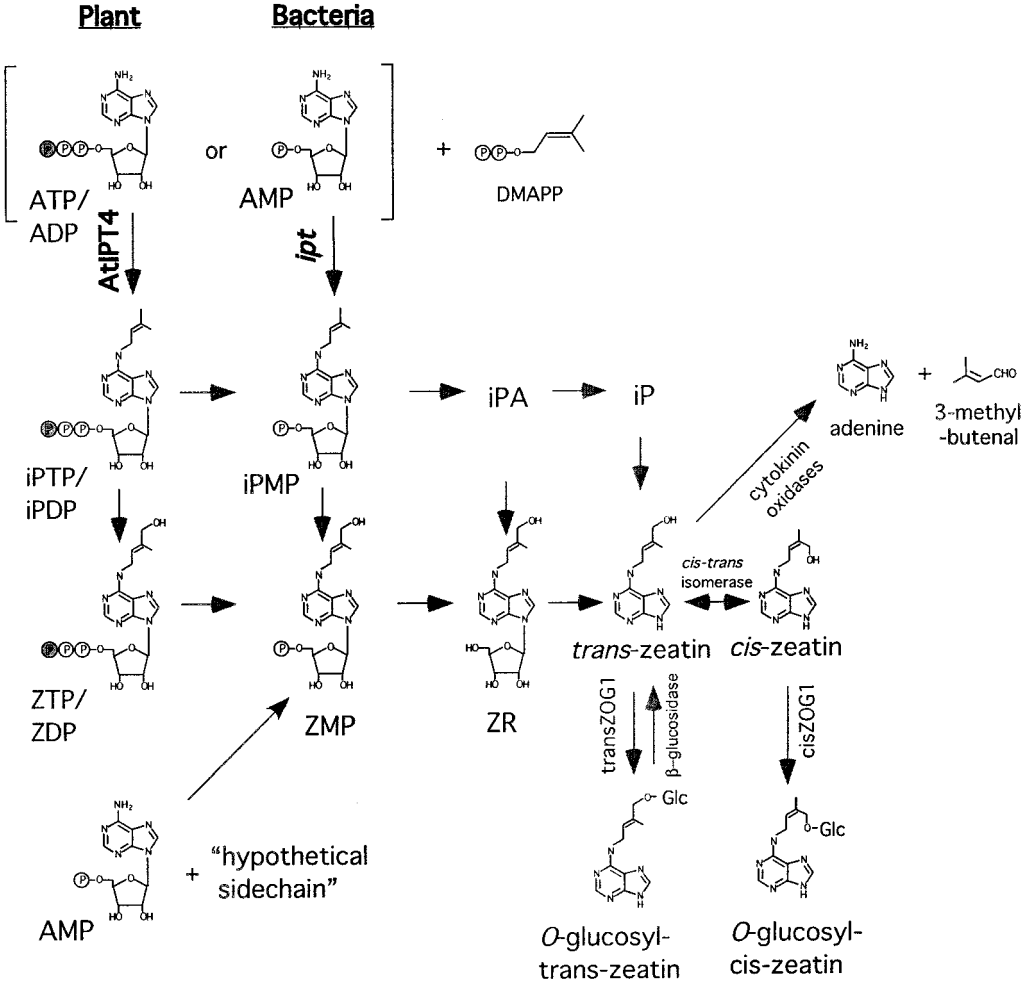

Figure 1. Proposed biosynthetic and metabolic pathway for cytokinins. Left, The proposed biosynthesis of zeatin tri-/diphosphate in Arabidopsis. Both ADP and ATP are likely substrates for the plant IPT enzyme, and these and their di- and triphosphate derivatives are indicted together (e.g. ATP/ADP). The biosynthesis of cytokinins in bacteria (e.g. *A. tumefaciens*) is compared next to it. Right, Several possible modifications and the degradation of zeatin. 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.

is directly synthesized by IPT from AMP and a yet unidentified side chain precursor. This pathway could be strongly inhibited by mevastatin, indicating a terpenoid origin of the hypothetical precursor.

METABOLISM

Almost all cytokinins are present in plants as both the free base and the corresponding nucleosides and nucleotides. The interconversions among these forms are likely to be performed by enzymes involved in the general purine metabolism, but their biological significance is unclear (Mok and Mok, 2001). The adenine ring system can be glucosylated at the N^3-, N^7-, and N^9-position. Glucosyl-conjugates at the N^7- and N^9- but not at the N^3-position are usually inactive in bioassays. It is assumed that the N^7- and N^9- modifications irreversibly inactivate cytokinins, but the precise in vivo function of these *N*-glucosyl-conjugates is unknown (Mok and Mok, 2001).

O-Glucosyl-conjugates of the N^6-side chain are a common modification in all plants. In addition, *O*-xylosyl-conjugates have been reported in *Phaseolus* spp. (Mok and Mok, 2001). The purified corresponding glycosyltransferases of *Phaseolus* spp. exhibited a high substrate specificity both to the cytokinins zeatin and dihydrozeatin and to the glycosyl donor (Dixon et al., 1989). The zeatin-*O*-glucosyltransferase (ZOG1) strongly preferred UDP-Glc while the zeatin-*O*-xylosyltransferase (ZOX1) exclusively utilized UDP-Xyl. The two genes, *ZOG1* and *ZOX1*, have been isolated from *Phaseolus* spp. and the binding site of the UDP-Glc has been mapped by domain swap experiments (Martin et al., 1999a, 1999b; Mok and Mok, 2001). Recently the isolation of a cis-zeatin-*O*-glucosyltransferase (cis-ZOG1) has been reported that specifically glucosylates cis-zeatin but not trans-zeatin or dihydrozeatin (Martin et al., 2001). The authors suggested that the high specificity of cis-ZOG1 to the cis-isomer may indicate an important role for cis-zeatin in plants.

O-Glycosylated cytokinins are resistant to the cleavage of the N^6-side chain by cytokinin oxidases (see below). Additionally, these forms can easily be converted into active cytokinins by β-glucosidases (Brzobohaty et al., 1993). Thus, it is believed that *O*-glycosylated cytokinins are inactive, stable storage forms that play an important role in balancing cytokinin levels.

DEGRADATION

Free cytokinin bases and nucleosides with an unsaturated N^6-side chain are irreversibly degraded by cleavage of the side chain by cytokinin oxidases. Cytokinin oxidases are found in many plant species, and they represent a class of highly diverse proteins (Jones and Schreiber, 1997). The genes of two highly similar cytokinin oxidases have been isolated from maize (*Zea mays*; Houba-Hérin et al., 1999; Morris et al., 1999). Heterologous expression in *Pichia pastoris* and *Physcomitrella patens* confirmed the predicted cytokinin oxidase activity. Maize cytokinin oxidase and its Arabidopsis homologs 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.

Werner et al. (2001) have recently constructed transgenic tobacco (*Nicotiana tabacum*) expressing four Arabidopsis homologs of the maize cytokinin oxidase from the CaMV 35S promoter. For the first time, this allowed the phenotypic consequences of reduced endogenous cytokinin levels to be determined. Transgenic lines exhibited a higher cytokinin oxidase activity and significantly reduced amounts of iP and zeatin metabolites, including their glycosides as compared with wild type. The transgenic lines showed a dwarfed growth as a consequence of a severely retarded shoot development. In contrast, the growth of the root system was enhanced, indicating that cytokinin may have opposing roles in shoot and root development. These defects could be traced back to alterations in the cell number and the rate of cell division in the apical meristems. Shoot apical meristems consisted of fewer cells with sizes comparable to wild type. 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 endogenous cytokinin levels resulted in alterations in cell proliferation in the apical meristems, consistent with an in vivo role for cytokinins in the regulation of cell division. In contrast, there was an increased number and size of cells in the root apical meristem, which contributed to a general enlargement of the root.

TRANSPORT

Based on the occurrence of cytokinins in the xylem sap and the identification of the root tip as a major site of cytokinin biosynthesis, it is generally assumed that cytokinins are transported in the xylem to exert their effects on the aerial parts of plants. A purine transporter, AtPUP1, has been isolated in Arabidopsis by the functional complementation of a yeast mutant deficient in adenine uptake (Gillissen et al., 2000). Adenine uptake was competitively inhibited by free cytokinin bases. Although no direct evidence for cytokinin transport was presented, this competitive inhibition suggests that AtPUP1 represents a possible cytokinin/purine transporter involved in the translocation of root-derived cytokinins to the aerial plant parts.

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 accumulated first in the roots, subsequently in the xylem sap, and finally in leaves. This suggests that in response to nitrate, these plants made cytokinins in the root, which was subsequently transported into the aerial portion of the plant through the xylem. Thus, cytokinin may represent a long-distance signal for nitrogen availability from the root to the shoot, presumably to coordinate shoot and root development.

Somewhat conflicting results with respect to the biological significance of transported cytokinin were obtained from reciprocal grafting experiments with wild-type and cytokinin-overproducing 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. Elevated level 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 cytokinin may act as paracrine signal, at least with respect to apical dominance and leaf senescence.

PERCEPTION AND SIGNAL TRANSDUCTION

The concept of cytokinin as a phytohormone implies that the site of synthesis and the site of action can spatially be separated. Thus, the search for receptors for cytokinin is almost as old as its discovery. Biochemical approaches led to the isolation of cytokinin binding proteins, but none has been unequivocally shown to be a receptor. The report of a G-protein-coupled receptor, GCR1, as a candidate for a cytokinin receptor has recently been retracted (Kanyuka et al., 2001). In an activation-tagging mutagenesis, a His kinase (CKI1) was identified by its ability to confer cytokinin-independent callus growth when overexpressed in Arabidopsis (Kakimoto, 1996). It was proposed that the elevated basal level of CKI1 overcomes the cytokinin requirement for callus growth and greening, and that CKI1 represents a putative cytokinin receptor. Transient expression of CKI1 in Arabidopsis protoplasts indeed showed activation of the promoter of cytokinin primary response gene (Hwang and Sheen, 2001). However, this expression of *ARR6* was constitutive, not responsive to exogenous cytokinin. The constitutive induction of *ARR6* by CKI1 in this system might reflect cross talk between CKI1 and the cytokinin signaling pathway due to the overexpression of CKI1, and may not reflect the situation in vivo. Although the phenotype of the *CKI1* overexpressing mutant implicates an important role for CKI1 or a similar His kinase in the regulation of cell proliferation and/or division, the nature of the mutation (i.e. gain-of-function) does not allow a definitive conclusion regarding the wild-type function of the protein.

The involvement of a His kinase in cytokinin signaling has recently been confirmed by two groups who have reported the identification of a cytokinin receptor in Arabidopsis. This gene, *CRE1*, encodes a His kinase that shows weak similarity to the amino acid sequence of CKI1 (Inoue et al., 2001; Suzuki et al., 2001a). A screen of 19,000 ethyl methanesulfonate-mutagenized Arabidopsis plants identified a single mutant line that displayed reduced sensitivity to cytokinin in tissue culture, including shoot formation and proliferation of green callus (Inoue et al., 2001). Additionally, *cre1* mutant seedlings displayed resistance to cytokinin in a root elongation assay. An independently isolated T-DNA-tagged allele of *cre1* showed a similar phenotype. Both mutations appear to be partial loss-of-function alleles by molecular analysis (see also below), but are semidominant in the root elongation assay. This semidominance of a hypomorphic allele implies haploinsufficiency, indicating a dose-dependent action of the receptor in this response. *CRE1* encodes an unusual type of hybrid kinase with two C-terminal response regulator domains. In addition, CRE1 contains a His kinase domain and two transmembrane regions flanking a novel presumed extracellular domain. All identified mutations affect the N-terminal part of the receptor, and the functional relevance of the two response regulator domains is unknown.

The phenotype of *cre1* mutants and the homology to His receptor kinases are consistent with a role in cytokinin signaling, but compelling evidence that cytokinin can indeed act as a ligand for CRE1 was provided by a series of elegant experiments using yeast and *E. coli* (Inoue et al., 2001; Suzuki et al., 2001a). The experiments were based on the idea that endogenous responses can be influenced by expression of CRE1 in a suitable genetic background of a heterologous system, and that these responses can be altered in a cytokinin-dependent manner. In yeast, the sensor His kinase SLN1, which is involved in osmosensing, inhibits the activity of SSK2 (a mitogen-activated protein kinase kinase kinase) by a phosphorelay consisting of SLN1 itself, the His transfer protein YPD1, and the response regulator SSK1 (Fig. 2A; Posas et al., 1998). In wild-type yeast, unphosphorylated SSK1 enables activation of SSK2 by autophosphorylation. Under normal osmotic conditions, this activation is inhibited by phosphorylation of SSK1 by SLN1 and YPD. Deletion of SLN1 is lethal due to the overactivation of SSK2 by unphosphorylated SSK1. Viability can be restored by the expression of the phosphatase PTP2 that inhibits SSK2. Thus, a yeast strain deleted in SLN1 and carrying the PTP2- gene under the control of a Gal-inducible promoter is conditionally lethal, allowing growth only in the presence of Gal. In the absence of Gal, the expression of CRE1 suppressed the conditional lethality, but only when cytokinins were present in the medium. The cytokinin-dependent rescue by CRE1 required YPD1, indicating that CRE1 acts through the endogenous yeast phosphotransfer mechanism. Fur-

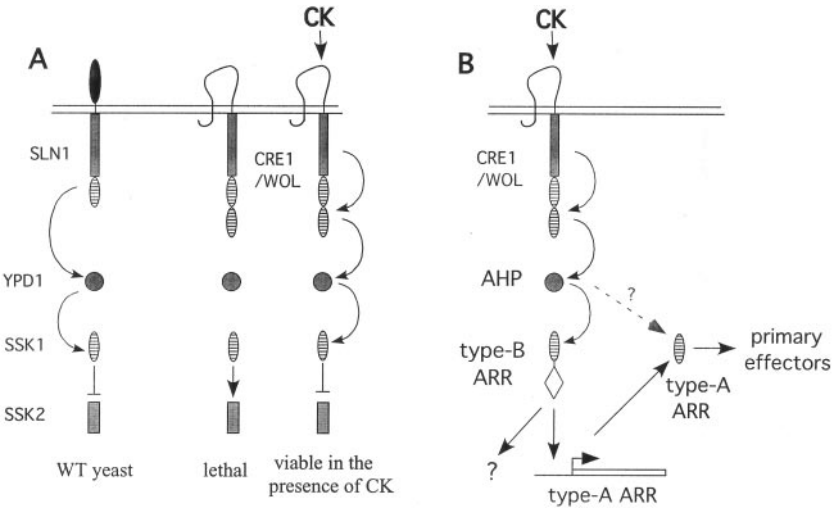

Figure 2. A, Evidence that CRE1 is a cytokinin receptor. The left-most pathway depicts the osmosensing pathway in wild-type yeast: the His kinase SLN1, which suppresses the activity of SSK2 (a mitogen-activated protein kinase kinase kinase) activity via a phosphorelay consisting of YPD1 (an Hpt) and SSK1 (a response regulator). A deletion mutant of SLN1 is lethal due to overactivation of SSK2. CRE1 can suppress the growth defect in an SLN1 deletion only in the presence of cytokinins (two right pathways). B, Model of cytokinin signaling in Arabidopsis. Cytokinin binds to the N-terminal domain of CRE1 (and likely other similar sensor kinase) and activates its His kinase activity. CRE1 phosphorylate the AHPs, which in turn transfer the phosphate to the receiver domain of ARR1 (and presumably to other type-B ARRs), thus activating their output (transcriptional activator) domain. Type-A ARRs (and possibly other primary target genes) are transcriptionally induced by the activated type-B ARRs. The type-A ARRs also interact with the AHPs, and are also likely phosphorylated. The activated type-A ARRs, perhaps in parallel and/or in combination with the activated type-B proteins, interact with various effectors to alter cellular function, including the a feedback inhibition of their own expression. The curved arrows indicate phosphotransfer. CK, Cytokinins. See text for additional details.

thermore, only cytokinins that exhibit high activity in bioassays, such as trans-zeatin, or benzyladenine, could suppress lethality on Glc-containing media; the weakly active cis-zeatin was ineffective (Inoue et al., 2001). Interestingly, thidiazuron, a *N*,*N*′-diphenylurea (DPU)-type cytokinin, also activates CRE1 in this assay. DPU-type cytokinins strongly inhibit cytokinin oxidase activity in vitro, and this has been postulated as their mode of action in vivo (Jones and Schreiber, 1997). However, the activity of thidiazuron in the yeast assay indicates that DPU-type cytokinins likely also exert their in vivo effects by direct stimulation of the receptor. This is quite remarkable given the profound structural differences between DPU- and zeatin-type cytokinins. Recent results from heterologous expression of CRE1 in yeast have confirmed that cytokinin does bind directly to CRE1 with high affinity (Yamada et al., 2001).

What is the biological role of CRE1? Surprisingly, *cre1* has been shown to be allelic to the *wooden leg* (*wol*) mutation, which causes defects in the development of vascular tissue (Mähönen et al., 2000). *wol* seedlings have a reduced number of cells in the vascular cylinder of the root and the lower part of the hypocotyl, and these consist almost entirely of xylem cells (Scheres et al., 1995). Thus, phloem and metaxylem are absent in these parts of *wol*. The defects in *wol* can be traced back to the late torpedo stage of the embryo, which has a reduced number of vascular initials due to the lack of specific asymmetric cell divisions (Scheres et al., 1995; Mähönen et al., 2000). The absence of phloem and metaxylem cells is presumably caused by reduced cell division rather than aberrant cell specification because the *cre1* mutant phenotype can be suppressed by the *fass* mutation, which has supernumerary cell layers (Scheres et al., 1995). It was proposed that the determination of xylem cells precedes the specification of phloem cells and that the reduced number of initials are completely used up as xylem precursors in *wol*, leaving no initials left to specify phloem (Scheres et al., 1995). This model of the *wol* phenotype nicely brings together the role of CRE1/WOL as a cytokinin receptor and the stimulatory effects of cytokinin on cell division. However, CRE1/WOL may also have additional roles in the differentiation of vascular cells. *wol* has a substantially stronger phenotype than the other *cre1* alleles (Mähönen et al., 2000; Inoue et al., 2001), although it is unknown if it is a null allele. Interestingly, the *wol* mutation is a missense mutation affecting the extracellular domain of the protein, which is the putative ligand-binding site (Mähönen et al., 2000). In vitro binding assays have recently shown that the *wol* mutation disrupts binding of cytokinin to CRE1 (Yamada et al., 2001).

CRE1/WOL is expressed primarily in the root, and in the embryo it is restricted to the provascular tissue from the root to the shoulder region of the cotyledons (Mähönen et al., 2000). This expression pattern may explain the rather unexpected absence of a pheno-

type in the aerial portions of *wol* mutant seedlings and adults. In contrast to the lack of a shoot phenotype in intact plants, *cre1* mutants show a strong reduction in the regeneration of shoots from tissue culture cells in response to cytokinin. It may be that the formation of a shoot in tissue culture requires the elaboration of a proper vasculature. In this context, it is interesting that regenerated roots from *wol* callus exhibit identical phenotypic aberrations like roots of *wol* seedlings (Scheres et al., 1995). Alternatively, *CRE1* may be redundant with other genes in the shoots of intact plants, but not with respect to shoot formation in vitro. Indeed, there are two closely related His kinases (AHK2 and AHK3) in Arabidopsis that are mainly expressed in the aerial parts of wild-type plants (Ueguchi et al., 2001). Both His kinases are highly homologous to the entire CRE1 sequence, including the putative ligand binding domain, and also share with CRE1 the presence of two response regulator domains in their C terminus. Furthermore, in a mesophyll protoplast transient expression system, both His kinases may regulate the promoter of a primary cytokinin response gene in a cytokinin-dependent manner (Hwang and Sheen, 2001; see also below). These results strongly suggest that AHK2 and AHK3 indeed act as cytokinin receptors in Arabidopsis.

The involvement of a hybrid His kinase in cytokinin signaling suggests that additional homologs of bacterial phosphorelay elements may be involved in the cytokinin signal transduction pathway. Genes encoding homologs of these other components, phospho-His transfer proteins and response regulators, have been identified in Arabidopsis. The possible role of several of these genes in cytokinin signaling has been addressed by numerous groups. Recently, for example, the induction of the promoter of a cytokinin primary response gene, the type-A *ARR6* (see below), was monitored by a reporter in Arabidopsis mesophyll protoplasts that transiently expressed wild-type and mutated forms of these genes (Hwang and Sheen, 2001). The results, along with numerous findings of other groups, provide important and valuable insights into the putative hardwiring of cytokinin signaling components. The data for each of the three types of components acting downstream of the receptors will first be discussed separately, then we will integrate the information about these components into a working hypothesis of cytokinin signal transduction.

There are five Arabidopsis genes encoding putative His phosphotransfer proteins (AHPs), which function as a bridge in the primary phosphotransfer between the Asp residues present in the receiver domain of the hybrid sensor kinase and the receiver domain of the response regulator (Suzuki et al., 2000). Suzuki et al. (2001a) established a heterologous system in *E. coli* analogous to the above-described yeast system to verify that CRE1 could act as a cytokinin receptor, and to implicate the AHPs as downstream targets of CRE1. The His phosphotransfer protein in *E. coli* used in this analysis was YojN, and the cognate response regulator was RcsB. The activation of RcsB by CRE1 could be quantified by a RcsB-responsive promoter fused to the reporter LacZ. Coexpression of four different AHPs differentially inhibited the reporter expression. It was proposed that the coexpressed AHPs competed with the endogenous YojN for phosphotransfer by CRE1, consistent with an interaction between that CRE1 and these AHPs. The different effectiveness of specific AHPs might reflect specificity of the proposed CRE1-AHP interactions. However, the individual expression levels of the four AHPs in this system were not determined, and thus quantitative measurements should be interpreted carefully. Nuclear localization studies also support the hypothesis that at least some of the AHPs are involved in cytokinin signaling (Hwang and Sheen, 2001). It was shown that AHP1 and AHP2, but not AHP5, transiently translocate from the cytoplasm to the nucleus of Arabidopsis protoplasts in response to exogenously applied transzeatin. Finally, several groups have reported yeast two-hybrid interactions between His kinases and AHPs. Taken together, these reports indicate that at least a subset of AHPs may be involved in cytokinin signaling. These AHPs likely transmit the signal from its site of perception, probably the plasma membrane, to the effectors in the nucleus. Numerous results indicate that these effectors consist, at least in part, of the third class of components of phosphorelays, the response regulators.

The response regulators in Arabidopsis (ARRs) form a gene family that includes the type-A and the type-B ARRs (D'Agostino and Kieber, 1999; Imamura et al., 1999). The type-A ARRs are composed solely of a receiver domain; the type-B ARRs have an output domain fused to the receiver. This C-terminal output domain has been demonstrated to activate transcription in yeast and in planta and to bind to DNA in a sequence-specific manner. The type-B ARRs have been shown to be nuclear-localized proteins (Lohrmann et al., 1999; Sakai et al., 2000; Hwang and Sheen, 2001). The steady-state levels of mRNA of most of the type-A, but not the type-B ARRs are rapidly and specifically elevated in response to cytokinin (Brandstatter and Kieber, 1998; Taniguchi et al., 1998; Kiba et al., 1999; D'Agostino et al., 2000). Homologous response regulators in maize exhibit very similar induction characteristics indicating that the function of type-A ARRs is highly conserved in plants (Sakakibara et al., 1998). The induction by cytokinin of several of the type-A ARRs is due, at least in part, to the transcriptional activation of these genes and is not blocked by cycloheximide, which indicates that these are primary response genes (Brandstatter and Kieber, 1998; D'Agostino et al., 2000). The similarity to bacterial signaling elements

predicted to act downstream of sensor His kinases, combined with the rapid induction by cytokinin, suggest that these type-A ARRs may play a role in cytokinin signaling. Consistent with this, the *ARR5* gene has been found to be expressed primarily in the shoot and root apical meristems, likely sites of cytokinin action (D'Agostino et al., 2000). Recently, studies in protoplasts have shown that the overexpression of type-A ARRs represses their own expression, suggesting that type-A ARRs may provide a negative feedback regulation of cytokinin signaling (Hwang and Sheen, 2001). Interestingly, mutant forms of these genes lacking a conserved Asp, the predicted phosphoaccepting site, were equally effective in their feedback inhibition. Thus, wild-type and mutant proteins may interact with cytokinin signaling elements, such as the AHPs, making these components inaccessible to the pathway. Type-A ARRs may also interact with additional partners and could have dual functions. Their activation by cytokinin may negatively feedback into cytokinin signaling, but would also trigger yet unknown, secondary responses to cytokinin. This model may also explain why there are 10 type-A ARRs in Arabidopsis.

In two recent publications, the transcriptional control of the type-A *ARR*s was shown to be mediated, at least partly, by type-B ARRs (Sakai et al., 2001; Hwang and Sheen, 2001). An *arr1* (a type-B ARR) knockout mutant was found to be partially resistant to cytokinin in shoot regeneration and root elongation assays, and an ARR1 overexpressing transgenic line displayed increased sensitivity to cytokinin in these assays. These results suggest that this type-B gene plays a role in cytokinin responsiveness. Examination of the expression of the type-A genes in the *arr1* mutants revealed that the loss-of-function allele exhibited reduced expression of several type-A *ARR* transcripts and that overexpression of ARR1 resulted in an enhanced induction of the type-A genes, even in the presence of cycloheximide. These results, coupled with the presence of multiple ARR1 binding sites in the promoter of several type-A ARRs suggest that ARR1 directly regulates the transcription of these primary targets of cytokinin signaling (Sakai et al., 2001). The activity of the ARR1 output domain is inhibited by the N-terminal response regulator domain (Sakai et al., 2000). Thus, it was proposed that the inhibition of the transcriptional activator domain by the response regulator domain was modulated by cytokinin. Similar results were independently obtained by transient expression studies in Arabidopsis protoplasts (Hwang and Sheen, 2001). Overexpression of three type-B ARRs, ARR1, ARR2, and ARR10, highly increased both the basal level and the induction of the promoter of a type-A *ARR*, *ARR6*. ARR2 exhibited the strongest induction and was consequently analyzed in greater detail. Expression of the DNA binding domain of ARR2 without the receiver and transactivator domain abolished the cytokinin responsiveness of the *ARR6* promoter, presumably acting as a dominant negative form. The overexpression of ARR2 in planta promoted rapid cell proliferation and shoot and leaf formation in tissue cultures even in the absence of exogenous cytokinin. The transgenic plants also showed delayed leaf senescence as expected for cytokinin overaction.

These results implicate at least several of the type-B ARRs as positive regulators of cytokinin responses. Surprisingly, mutated proteins in the transient assay in which the Asp, the probable site of phosphorylation, was altered to Asn, were equally effective in the induction of the *ARR6*::reporter by cytokinin (Hwang and Sheen, 2001). This raises the question of whether cytokinin signaling acts as a classical His-to-Asp phosphorelay, at least with regard to the induction of the type-A *ARR* genes. The authors propose that an endogenous repressor may be liberated by the overexpression of the type-B ARRs. Several indirect arguments support the phosphorelay hypothesis. The conservation of the presumed phosphorylation sites within all 10 type-A and about 12 type-B ARRs in Arabidopsis as well as the conservation of these sites between maize, rice (*Oryza sativa*), and Arabidopsis homologs strongly suggests the functional relevance of these sites. At least at the level of the receptor, the phosphorelay hypothesis is also experimentally verified. The cytokinin-dependent complementation in yeast and *E. coli* requires an interaction of CRE1 with endogenous His transfer proteins similar to their interaction with their cognate His kinases. In addition, mutant forms of CRE1 in which these sites have been altered are unable to induce the *ARR6*::reporter in protoplasts by application of cytokinin. Thus, it is highly likely that CRE1 acts both in the transient expression system and in the cytokinin-dependent complementation in yeast and *E. coli* as a His kinase. Biochemical studies and loss-of-function mutants of the AHPs and ARRs should enable us to clarify the significance of their presumed phosphorylation sites.

These results are consistent with the following model of cytokinin action. Binding of cytokinin to CRE1 (and possibly other related His kinases) initiates a phosphorelay that culminates, via an AHP intermediate, in the phosphorylation and activation of a set of the type-B ARR(s). These then activate the transcription of the type-A genes, which in turn negatively feedback into the pathway. The type-A ARRs, perhaps together and/or in parallel with the activated type-B ARRs, interact with various effectors to mediate the changes in cell function appropriate to cytokinin (Fig. 2B). The near future should see significant refinement of this model as the tools are now in hand to analyze the interactions among these elements.

CONCLUSION

The field of cytokinin research has seen dramatic advancements during the last 2 years. Several of the

key elements of cytokinin action and metabolism have been identified, but many questions need to be addressed. The in vivo role of most of the members of the phosphorelay in Arabidopsis remains unknown, including their putative function in cytokinin signaling. The function and specificity of the interactions among the various components in vivo represents one of the key questions. The analysis of expression patterns and the identification of mutants in these genes will enable us to understand the numerous effects of cytokinin on plant physiology and development at the molecular level. Furthermore, these genes can now be used to identify additional regulators and downstream targets, which should help unveil the complex network of cytokinin metabolism and signaling.

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