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REVIEW

Cytokinin: Perception, Signal Transduction, and Role in Plant Growth and Development

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Cytokinins are essential hormones for the proper growth and development of plants. They exert their actions through the phosphorylation of two-component signaling factors. The two-component elements in cytokinin signaling display not only overlapping, but also specific functions throughout a life cycle. These elements regulate the development of shoots, roots, and inflorescence meristems in *Arabidopsis*; shoot meristems in rice; and nodule formation in the lotus. They are also involved in interactions between plants and pathogens. In this review, we examine the mechanism for signaling events initiated by cytokinins in *Arabidopsis*.

Keywords: cytokinin, development, perception, signal transduction

SUMMARY OF CYTOKININ SIGNALING AND ITS FUNCTION

Cytokinins play a role in many aspects of plant growth and development, including cell division, apical dominance, organ formation and regeneration, vascular development, nutrient mobilization, and senescence (Mok and Mok, 2001; Sa et al., 2001; Kurakawa et al., 2007).

Pathways have been elucidated for cytokinin biosynthesis, metabolism, and signal transduction. The action of cytokinins is mediated through a non-canonical, two-component signaling mechanism (Kakimoto, 1996; Hwang and Sheen, 2001) (Fig. 1). Three histidine protein kinase cytokinin receptors -- AHK2, AHK3, and AHK4 -- bind cytokinins at the plasma membrane. These receptors initiate the Asp-His-Asp phosphorelay via histidine phosphotransfer proteins (AHPs) to response regulator proteins (ARR). The translocation of AHP proteins into the nucleus is thought to cause the transfer of a phosphoryl group to type-B ARRs. AHPs activate type-B ARR proteins, which function as transcriptional activators in cytokinin signaling. Individual activation of type-B ARRs induces the expression of a subset of primary cytokinin-response genes, including type-A *ARRs*. These appear to be negative regulators in the cytokinin signal transduction pathway, and provide a feedback mechanism that is thought to fine-tune cytokinin signaling (Hwang and Sheen, 2001; Kiba et al., 2004; To et al., 2004).

Recently, novel and unexpected roles have been proposed for cytokinins in the de-differentiation process that occurs during nodule formation (Murray et al., 2007; Tirichine et al., 2007). Furthermore, cytokinins may be directly involved in defense responses (Felix and Meins, 1985; Shinshi et al., 1987). Nitrogen assimilation and resistance to disease pathogens are very important issues in agricultural science. The possible involvement of cytokinins in those processes highlights the overall importance of these phytohormones to plant health.

In this review, we focus on the recent progress made in understanding cytokinin signal transduction mechanisms, their multi-phosphorylation systems, and potential applications that exploit their novel roles in plant growth and development.

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THE CYTOKININ RECEPTORS

Six histidine kinases are now known, in addition to a number of ethylene receptors. These are thought to be hybrid histidine kinases. Structurally, the receiver domain containing a conserved Asp is fused to the kinase domain, which has a conserved His phosphorylation site. Among the cytokinin receptors, AHK2, AHK3, and AHK4 display a cyclase/histidine-kinase-associated extracellular (CHASE) domain at the N-terminus of the protein. The CHASE domain is believed to function as the cytokinin-binding region (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001). These receptors also have a histidine kinase region, a receiver-like domain, and a receiver domain at the cytosolic C-terminus. In contrast, AtHK1 and CKI1 have both a kinase and a receiver domain, but lack a CHASE and receiver-like region. Consistent with this absence of a CHASE domain, CKI1 is constitutively active in an *E. coli* mutant, and does not appear to bind cytokinins in yeast *in vitro* (Yamada et al., 2001). Additionally, CKI2/AHK5 is missing the N-terminal transmembrane domain and an extracellular domain.

Based on the expression of *AHK:GUS* fusion genes, Nishimura et al. (2004) have suggested that *AHK* genes are expressed ubiquitously, but at low levels, and that their expression is predominantly in the cells comprising the meristematic and vascular tissues.

Using microarray expression analysis, Schmid et al. (2005) have shown that overall transcript levels of *AHK2*, *AHK3*, and *AHK4* are similar during *Arabidopsis* development (Fig. 2). Interestingly, *AHK4* shows an organ-specific expression pattern when compared with *AHK2* and *AHK3*. Because cytokinins are involved in many growth and development processes, AHK4 may not be able to mediate some of those responses due to its organ-specificity. In contrast, *AHK2* and

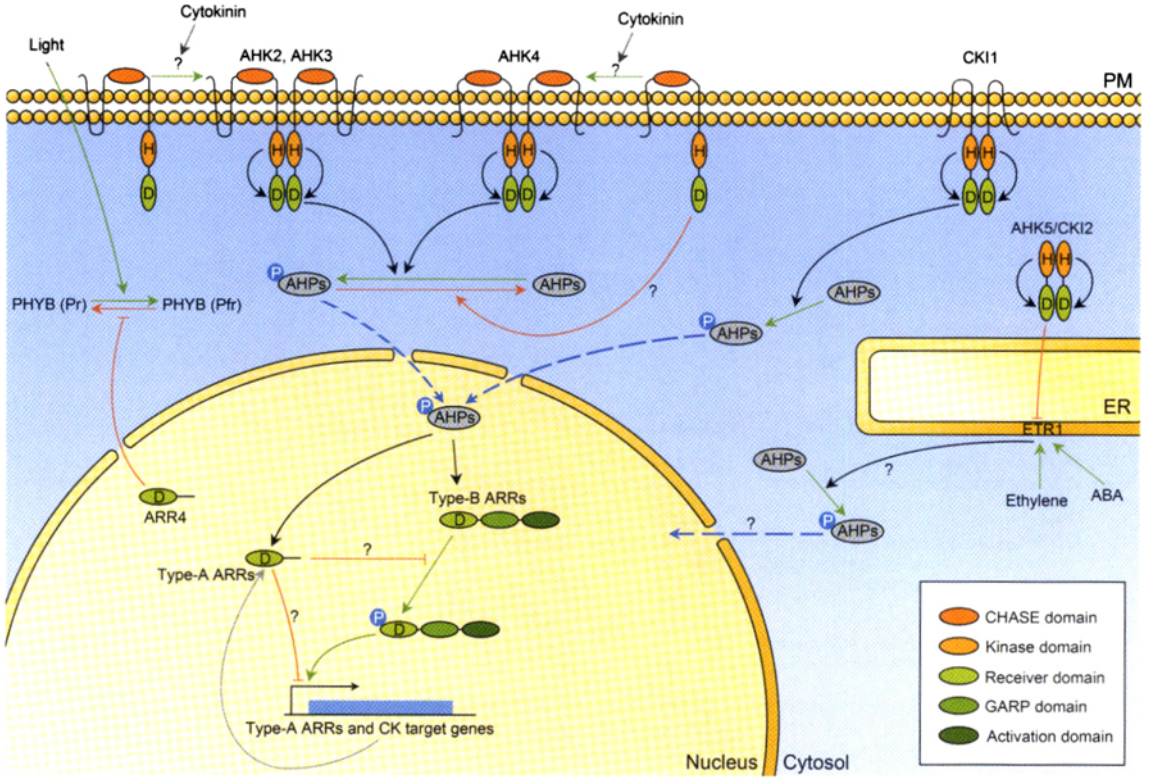

Figure 1. Multiple phosphorelay signal transduction pathways in *Arabidopsis*. Cytokinin receptors, AHK2, AHK3, or AHK4, perceive cytokinins at plasma membrane and are subsequently dimerized to induce autophosphorylation at conserved His residue in kinase domain. Phosphorylated receptors initiate Asp-His-Asp phosphorelay via AHP to ARR proteins. Phosphorylated AHP proteins are translocated to nucleus and transfer phosphoryl group to type-B ARRs. Each activated type-B ARR induces expression of subset of cytokinin target genes, including type-A ARRs. Type-A ARRs appear to provide negative feedback regulation in cytokinin signaling, but underlying regulatory mechanism(s) are virtually unknown. Type-A ARRs may inhibit cytokinin signaling by interfering in phosphorelay by AHPs or competing with type-B ARRs for other not-yet identified interacting proteins or target *cis*-elements. In absence of cytokinins, AHK4 suppresses phosphorelay through AHPs due to phosphatase activity in monomeric and/or dimeric forms (Mähönen et al., 2006b). CKI1 appears to constitutively function as homodimer and modulates cytokinin signaling pathway (Ryu et al., unpublished data). Ethylene signaling pathway could be mediated by two-component signaling cascade, in which AHK5/CKI2 suppresses ETR1-dependent ethylene/ABA signaling specifically in root elongation (Iwama et al., 2007). Light- and cytokinin signaling converge at ARR4, which stabilizes activated PHYB by inhibiting dark-conversion (Sweere et al., 2001). Black arrows, phosphorelay; green arrows, activation; red arrows, inactivation or inhibition; blue dashed arrows, translocation; gray arrows, transcription and translation.

AHK3 demonstrate broad expression patterns throughout many plant tissues. Thus, we might speculate that AHK2 and AHK3 are the general cytokinin receptors found in *Arabidopsis*.

During the development of rosette leaves, AHK2 and AHK3 receptors are predominantly expressed in a similar pattern (Schmid et al., 2005). In contrast, *AHK4* receptor expression in those leaves is much lower. Consistent with this, the *ahk2 ahk3* double mutants display short petioles and smaller leaf blades whereas the double mutants of *ahk2 ahk4* and *ahk3 ahk4* are morphologically indistinguishable from wild-type plants (Higuchi et al., 2004; Nishimura et al., 2004). This may be due to an extensive functional redundancy between AHK2 and AHK3 receptors during rosette-leaf formation.

A loss-of-function mutation in *AHK3* causes a reduced sensitivity to cytokinins (Kim et al., 2006), which is manifested by a decrease in the cytokinin-dependent delay of leaf senescence and the abolishment of cytokinin-dependent phosphorylation of ARR2. Likewise, transgenic overexpression of the wild-type *ARR2* leads to delayed leaf senescence, but not in the mutant form, which cannot be phosphorylated. Unlike with other AHKs, the expression level of *AHK3* is highest during senescence (Schmid et al., 2005). Despite the similar expression patterns for *AHK2* and *AHK3* during rosette development, those receptors have specific functions, thereby validating their partially, but not completely, overlapping roles.

cre1-1, an allele of *AHK4*, has an amino acid conversion of Gly 467 to Asp 467 in the histidine kinase domain. This allele is resistant to cytokinin activation in tissue culture, and fails to form large green calli when exposed to a shoot-initiating medium (Inoue et al., 2001). AHK4 apparently is able to complement both yeast and *E. coli* histidine kinase-mutants in a cytokinin-dependent manner (Inoue et al., 2001; Ueguchi et al., 2001b). The direct evidence for AHK4

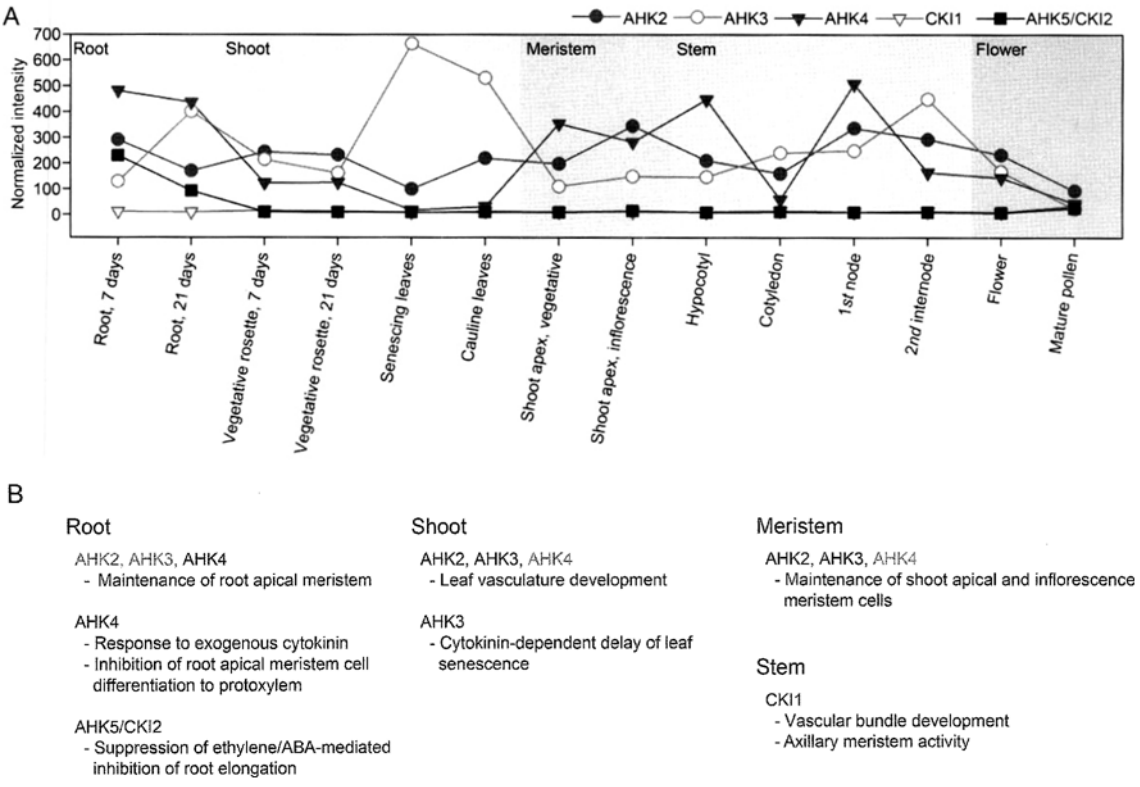

Figure 2. Expression and roles of histidine kinases during *Arabidopsis* development. (**A**) In root, *AHK4* is major cytokinin receptor while *AHK5/CKI2* shows highest expression. *AHK2* and *AHK3* are predominantly expressed in leaves, and expression of *AHK3* is highest at senescence. Although mRNA expression level of CKI1 is low, it is still detectable in *CKI1* promoter-driven *GUS* reporter transgenic plants and by *in situ* hybridization in various tissues, including vascular bundle and axillary meristems. (**B**) AHK4 is required for maintenance of root apical meristems and inhibition of their cellular differentiation to protoxylem. AHK4 is partially involved in maintenance of shoot apical and inflorescence meristems. AHK2 and AHK3 control cell division at shoot apical and inflorescence meristems, and regulate development of leaf vasculature and roots. AHK3 is major receptor in control of leaf longevity. CKI1 is associated with vascular bundle development and regulates axillary meristem activity (Ryu et al., unpublished data). AHK5/CKI2 is involved in suppression of ethylene/ABA-mediated inhibition of root elongation (Iwama et al., 2007).

being a cytokinin receptor is presented by binding assays with membrane vesicles derived from the AHK4 receptor-expressing *Schizosaccharomyces pombe*. AHK4 proteins will directly bind to various active cytokinins, but not to their inactive forms (Yamada et al., 2001). In addition, mutation of Thr 278 in the CHASE domain is responsible for the loss of function from *AHK4* (Mähönen et al., 2000) demonstrating that this region is essential for cytokinin-binding (Yamada et al., 2004). Hence, AHK4 appears to be a cytokinin receptor that is activated in a cytokinin-dependent manner.

The *wol* mutant, allelic to the *cre1* mutation, shows impaired phloem and metaxylem development in the roots. Both the reduced number of cells in the mutants and the suppression of the *wol* mutant via mutation of *fass* (whose function is to increase the number of vascular cells) indicates that AHK4/CRE1 is required for the proliferation and the maintenance of vascular primordial cells (Mähönen et al., 2000). However, only some of the *AHK4* loss-of-function alleles have impaired root development, and other loss-of-function or null *AHK4* alleles exhibit normal growth. AHK4 also shows phosphatase activity in the absence of cytokinin, and *wol* produces a mutant AHK4 that lacks cytokinin-binding activity and remains locked in the phosphorylated form (Mähönen et al., 2006b). Therefore, in the case of *wol*, its deficiency in root viability may be due to its phosphatase activity, which allows for the constant de-phosphorylation of AHPs and suppresses the residual cytokinin signaling mediated by other receptors. Consistent with this, a pseudo-His-phosphotransfer protein AHP6 can suppress cytokinin signaling by competing with the transfer of a phosphorus from AHP to ARRs. In the presence of other AHPs, the loss-of-function *ahp6-1* and *ahp6-2* can suppress the *wol* mutants. Moreover, under the control of the *AHK4* promoter, the expression of cytokinin oxidase results in specific degradation of cytokinins within the *AHK4* expression domain, and can mimic the *wol* phenotype (Mähönen et al., 2006a). These results indicate that cytokinins and AHK4 play a role in the production of procambial cells that then differentiate to protophloem or protoxylem.

Both *wol* and a second *wol* allele, *wol-2* (de León et al., 2004), can be complemented by wild-type *AHK4* or the weak *cre1-4* allele which exhibit a wild-type root phenotype and mild cytokinin insensitivity. However, this does not happen in the presence of strong *cre1-3* or *cre1-7* alleles, which have the wild-type root phenotype but strong cytokinin insensitivity. This may indicate a dose-dependent complementation in the system. Interestingly, *trans*-heterozygotes of *wol* and the *wol-2* allele display interallelic complementation in their vasculature formation but not in canonical cytokinin signaling. This suggests that the cytokinin-independent

signal mediated by AHK4 regulates the proliferation of procambial cells. However, because these findings are not in accord with the results mentioned above, further investigation is needed into the underlying molecular mechanism for *trans*-heterozygote complementation of *wol* and *wol-2*.

Synthetic lethality in *Saccharomyces cerevisiae* has been monitored to measure functional redundancy of paralogous gene pairs (Kafri et al., 2005). These data might suggest that those gene pairs have a partially overlapping expression pattern are functionally redundant and can provide back-up for one another in the case of a mutation. However, this does not appear to be true in the tightly co-regulated paralogous gene pairs. Although a transcriptional re-programming of AHKs to provide an alternative for the knockout of receptors has not been reported yet, this partially overlapping expression pattern and the lack of a visual phenotype in *AHK* single or even double knockout mutants suggests that some type of protection exists amongst the genes. If so, the function of each AHK can be elucidated by the complementation of *ahk2 ahk3 ahk4* knockout mutants by the expression of each *AHK* under tissue-specific promoter control.

The features of AHK4 seem to differ from those of AHK2 and AHK3. First, the abundance of AHK4 transcript is controlled in both a tissue-specific and a cytokinin-dependent manner (Nishimura et al., 2004). Its expression, induced by exogenous cytokinins, is observed predominantly in the roots (Mähönen et al., 2000; Ueguchi et al., 2001a; Che et al., 2002; Rashotte et al., 2003). Second, AHK4 largely controls cellular responses to exogenously supplied cytokinins (Hashimoto et al., 2004). Even the loss-of-function *ahk4-1* single mutant demonstrates defects in both the greening and shoot induction of root explants when exogenous cytokinins are supplied. In addition, the *ahk4-1* mutant shows cytokinin-insensitive root growth whereas the *ahk2* and *ahk3* single mutants and the *ahk2 ahk3* double mutants exhibit a reduction in root growth when exogenous cytokinins are applied, similar to the behavior of the wild type. Third, AHK4 is involved in the phosphate-starvation response (Franco-Zorrilla et al., 2002). An EMS-mutagenized population has been screened to isolate mutants with reduced sensitivity to phosphate-starvation. Four independent lines have manifested a point mutation within *AHK4*, suggesting that AHK4 has an additional role in the adaptive response to external stimuli. Fourth, only AHK4 has phosphatase activity in the absence of cytokinins (Mähönen et al., 2006b). AHK4 can suppress phosphorylation through SLN1, a histidine kinase osmosensor found in yeast. In contrast, neither AHK2 nor AHK3 can cause this suppression. Likewise, wol, the cytokinin-unbound form of AHK4, triggers a decrease in cytokinin signaling by dephosphorylating AHPs. Therefore, AHK4 may play a role in modulating the receptiveness to cytokinins in certain cells in response to various environmental stimuli, resulting in plant adaptations to altered growing conditions (Nishimura et al., 2004).

Overexpression of *AHK5* results in the induction of calli in a cytokinin-independent manner (Kakimoto, 1996). AHK5 can function as a histidine kinase in *E. coli*; this kinase activity is independent of cytokinin concentration (Iwama et al., 2007). In root-elongation assays, two null mutants of *AHK5*, *ahk5-1* and *cki2-1*, are hypersensitive to both ABA and ethylene (Iwama et al., 2007). However, AHK5 is not related to canonical ABA or ethylene signaling during germination or the triple response, respectively. Thus, AHK5 is specifically associated with the inhibition of root elongation that is known to be caused by these two phytohormones. This observation points to a possible involvement of cytokinin signaling components in ethylene and ABA signaling.

THE HISTIDINE-PHOSPHOTRANSFER PROTEINS

Arabidopsis has a pseudo-AHP gene and five AHP genes, the latter group (AHP1 through AHP5) containing a highly conserved His residue that is required for the transfer of phosphate. In AHP6, the His residue is replaced by Asn, making AHP6 unable to transfer phosphoryl groups to the response-regulator genes downstream (Mähönen et al., 2006a). AHPs are His-phosphotransfer proteins and can complement a yeast His-phosphotransfer protein mutant (Imamura et al., 1999). AHPs can also accept phosphate groups from *E. coli* membrane fractions, and subsequently, transfer them to the *Arabidopsis* response regulators *in vitro* (Imamura et al., 1999, 2001, 2003). Furthermore, AHPs compete with an *E. coli* His-phosphotransfer protein *in vivo* for transferring phosphate (Suzuki et al., 2002; Tanaka et al., 2004). In yeast and *E. coli* two-hybrid assays, AHP proteins can interact with hybrid His-protein kinases, such as AtHK1, AHK4, CKI1, and ETR1 (Urao et al., 2000; Suzuki et al., 2001). Dortay et al. (2006) have demonstrated that four AHPs (AHP1, AHP2, AHP3, and AHP5) directly interact with AHK2, AHK3, and AHK4, seven type-A ARRs (ARR3, ARR4, ARR5, ARR7, ARR8, ARR9, and ARR16), and three type-B ARRs (ARR1, ARR2, and ARR14) within the yeast two-hybrid system and by in vitro co-affinity purification. Moreover, AHP1 and AHP2, but not AHP5, translocate from the cytoplasm to the nucleus in a cytokinin-dependent manner (Hwang and Sheen, 2001). These results indicate that AHPs may act as phosphotransfer proteins to transfer phosphate groups from plasma membrane-localized His kinases to the nuclear response regulators.

Because AHPs are able to interact with members of other protein groups within the cytokinin signaling pathway, they may act systemically as signal integrators. To support this hypothesis, Hutchison et al. (2006) have shown that the single and various double *AHP* T-DNA insertional alleles exhibit no differences in their cytokinin responsiveness. However, the quintuple *ahp1 ahp2 ahp3 ahp4 ahp5* mutant is severely impaired in the induction of cytokinin primary-response genes, and it shows such developmental defects as impaired fertility, increased seed size, reduced vascular development, and shorter primary roots.

AHP6, which lacks the conserved His residue required for phosphotransfer to type-B ARRs, can suppress cytokinin signaling by competing with AHPs for the transfer of phosphate to ARRs (Mähönen et al., 2006a). *AHP6* is expressed in the protoxylem and adjacent pericycle cells within the root, where, ordinarily, cytokinins negatively regulate this process. A loss-of-function mutant of *AHP6*, *ahp6-1*, can suppress the *wol* root phenotype by compromising the inhibition of the AHP-mediated phosphorelay that is initi-

ated by mutation on *AHK4*. This *wol* mutant lacks cytokinin-binding activity and remains in the phosphatase form (Mähönen et al., 2006a). All of these observations indicate that the pseudo-His-phosphotransfer protein AHP6 negatively regulates cytokinin signaling during root vascular development.

THE RESPONSE REGULATORS

Arabidopsis has 23 known *ARR* genes, which can be broadly classified into two groups -- type-A and type-B -- based on their sequence similarities, domain structures, and transcriptional responses to cytokinins (D'Agostino et al., 2000; Mason et al., 2004). Type-B ARRs have C-terminal domains that contain the DNA-binding domain GARP as well as nuclear-localization and transcription-activator domains. The C-terminal sequences of type-A ARRs are short, and currently have no known function. Type-A and type-B ARR homologs are found in other dicotyledonous and monocotyledonous plants, including maize and rice (Kieber, 2002; Asakura et al., 2003).

Type-A ARRs

When a subset of type-A *ARR*s is over-expressed in *Arabidopsis* protoplasts, the expression of the *pARR6::LUCIFERASE* reporter gene is repressed (Hwang and Sheen, 2001). This leads one to hypothesize that type-A ARRs function as negative regulators of cytokinin responses. In support of this, overexpression of *ARR15* has been shown to weakly decrease cytokinin-sensitivity (Tajima et al., 2004). Additional evidence has come from studies of transgenic plants that over-express *ARR22* (Kiba et al., 2004). These produce a dwarf phenotype with poorly developed root systems, similar to those found in the *wol* mutant. In addition, the response to cytokinins is attenuated in plants that over-express *ARR22*.

Analysis of single- and multiple-loss-of-function type-A *arr* mutants, including a hextuple *arr3 arr4 arr5 arr6 arr8 arr9* mutant, has demonstrated that only multiple type-A *arr* mutants are hypersensitive to cytokinins (To et al., 2004). These mutants are inhibited in their root elongation, lateral root initiation, leaf senescence, and callus formation. The severity of this hypersensitive phenotype generally correlates with the number of type-A *ARR*s that are disrupted, thereby indicating that the type-A ARRs are functionally redundant, negative regulators of cytokinin responses.

The regulatory mechanism(s) that underlie negative-feedback regulation of type-A ARRs is still not known. Direct protein-protein interactions between type-A and type-B ARRs have not been detected in the yeast two-hybrid system (Dortay et al., 2006). Therefore, it is unlikely that type-A ARRs negatively regulate cytokinin signaling through direct interaction with type-B ARRs. It is possible that such a negative-feedback regulation can be caused by AHPs (Dortay et al., 2006). One proposed mechanism is that the interaction of type-A ARRs with AHPs prevents them from associating with other proteins, e.g., type-B ARRs. Another mechanism could involve the dephosphorylation of phosphorylated AHPs by type-A ARRs. Finally, one might hypothesize that type-A ARRs and type-B ARRs compete for the phosphoryl group of AHPs. In that case, the level of feedback inhibition would be proportional to the number of type-A ARRs present in the cell. Such a mechanism has been demonstrated in the *E. coli* system (Imamura et al., 1998), where expression of type-A ARRs can suppress phosphorylation from the bacterial His-phosphotransfer domain of the ArcB to the response regulator OmpR. Consistent with this, transcription of type-A *ARR*s is activated by the exogenous application of cytokinins, and the overexpression of type-A *ARR*s reveals a varying degree of phenotypes associated with impaired cytokinin signaling (Kiba et al., 2004; Tajima et al., 2004). However, the ectopic overexpression of a dephosphorylated state (Asp85 to Asn) mimics the inactive form of ARR7, and does not cause any morphological defects (Leibfried et al., 2005). In contrast, the overexpression of a phosphorylated state (Asp85 to Glu) resembles the active form of ARR7 but has severe defects in the maintenance of the apical meristem. This may indicate that the phosphorylation of type-A ARRs leads to a conformational change in these proteins, resulting in their ability to tightly bind with AHPs. Another possibility is that the activation of type-A ARRs by phosphorylation at the conserved aspartic acid can launch a signaling pathway that is independent of type-B ARRs, and that this pathway could act as a negative regulator of cytokinin signaling. However, additional experiments are needed to confirm the role of phosphorylation by type-A ARRs in that signaling. For example, the generation of transgenic plants that harbor the active form of ARR7 under the control of the native *ARR7* promoter might then exclude the effect of ectopic expression by *ARR7*. It will be necessary to analyze the expression of cytokinin-responsive genes in transgenic plants that over-express the *ARR7* active form if we are to confirm whether the phenotype of this mutant is directly caused by suppressed cytokinin signaling.

Type-A ARRs may indirectly down-regulate the activity of type-B ARRs. Although a predicted DNA-binding domain is obvious within type-A ARRs, they may bind to type-B ARR-specific *cis*-elements and compete for DNA-binding. In addition, type-A ARRs might bind to an unknown partner of type-B ARRs that could be required for their functioning. This interaction could inhibit the binding of type-B ARRs to these proteins via steric hindrance or through dose-dependent competition.

Type-B ARRs

Type-B ARRs are transcription factors that activate the primary cytokinin-responsive genes (Hwang and Sheen, 2001; Mason et al., 2004). A consensus DNA-binding sequence (G/A)GGAT(T/C) has been defined in type-B ARRs; this sequence has been found via microarray analysis in the promoters of cytokinin primary-response genes (Rashotte et al., 2003).

Mason et al. (2005) have constructed and examined multiple T-DNA insertional knockout and loss-of-function mutants of six type-B *ARR*s (*ARR1, ARR2, ARR10, ARR11, ARR12, and ARR18*), double-, and triple- mutants. Compared with the single mutants, cytokinin sensitivity progressively decreases in the multiple knockout mutants, with the latter showing a gradient of inhibition in their root elongation, induction of callus formation and greening, inhibition of lateral root for-

mation, and repressed induction of the cytokinin primary-response genes. The *arr1 arr10 arr12* mutant is almost completely insensitive to cytokinins. These results indicate a partial functional overlap amongst type-B ARRs, and suggest that they act as positive regulators of cytokinin signal transduction.

Overexpression of *ARR1*, *ARR2*, or *ARR10* is sufficient to activate the cytokinin primary responses in the absence of exogenous cytokinins in *Arabidopsis* protoplasts (Hwang and Sheen, 2001). The expression of truncated forms of *ARR1* and *ARR2*, mutants that lack the receiver domain, results in greater induction of the cytokinin response than what is obtained with full-length proteins (Sakai et al., 2000; Hwang and Sheen, 2001). This suggests that the receiver domain of these ARRs negatively regulates the transduction of cytokinin signaling. Consistent with this, *ARR1, ARR11, ARR14, ARR20,* and *ARR21* comprise a set of transgenic plants over-expressing truncated proteins that lack the N-terminal receiver domain and show an unusual and disordered proliferation of cells around the shoot apex as well as reduced apical dominance (Sakai et al., 2001; Imamura et al., 2003; Tajima et al., 2004).

ROLE OF CYTOKININ SIGNALING IN PLANT GROWTH AND DEVELOPMENT

Cytokinin perception, signaling, and endogenous production can be disrupted by mutation of the genes that encode for various signaling components and for the degradation enzyme cytokinin oxidase. Such interruptions provide novel tools that can be used to elucidate the role of this hormone in plants.

The knockout of single *AHK* genes and the *ahk2 ahk4* and *ahk3 ahk4* double mutants are morphologically indistinguishable from wild-type plants (Nishimura et al., 2004), perhaps because of functional redundancy amongst these genes. By contrast, *ahk2 ahk3* double mutants have short petioles, smaller leaves, and decreased vein densities due to the impairment of higher-order veins. The *ahk2 ahk3 ahk4* triple mutant displays severely impaired growth as a reduction in leaf size and number (Higuchi et al., 2004; Nishimura et al., 2004). The diminished size of rosette leaves in the triple mutant results from reduced initiation of leaf primordial cell division. In contrast, the size and development of stomatal guard cells and trichomes appear to be normal, which indicates that these differentiation processes are not affected by an absence of cytokinin signaling (Nishimura et al., 2004). Transgenic plants that over-express cytokinin oxidase also have less cell division, smaller shoot apical meristems, and reduced vasculature (Werner et al., 2003). These results demonstrate that cytokinins are positive regulators of cell division and stem cell maintenance.

Cytokinins also affect floral development. The triple receptor mutant rarely flowers due to its sterility (Nishimura et al., 2004). Impaired cytokinin signaling, either by loss-of-function mutations in the *AHP* genes or by overexpression of cytokinin oxidase, also lead to decreased fertility (Werner et al., 2003). *CKX1*- and *CKX3*-overexpressing plants form very few flowers per single inflorescence stem. These findings suggest that the apical inflorescence meristem cells are depleted in mutants and their ability to form new flower primordia is reduced. Nevertheless, the structure, morphology, and size of those flowers are similar to the wild types. These observations imply that cytokinins play an important role in the maintenance of the floral meristem, but not in the development of floral organ identity.

The triple receptor mutant also displays reduced root growth, with postembryonic formation being severely impaired, by about 20%, compared with the wild-type controls (Nishimura et al., 2004). Lateral root formation also is greatly compromised. In contrast, adventitious root formation at the junction of the hypocotyls and main root is enhanced. Root development in the triple receptor mutant resembles that of the *wol* mutant, which is allelic to the *cre1* mutation (Mähönen et al., 2000). This *wol* mutation can suppress AHP-mediated cytokinin signaling events because of a rise in phosphatase activity. Thus, it appears that cytokinin signaling is required for maintaining vascular primordial cells.

The root vascular system of the *wol* mutant consists of primary xylem cells, but phloem and parenchyma cells are lacking. This phenotype can be mimicked by depleting cytokinins through the expression of *CYTOKININ OXIDASE 2* gene under the control of a procambium-specific *AHK4/CRE1* promoter (Werner et al., 2001). Although the *wol* mutant has fewer cells, this is not a prerequisite for the exclusive differentiation of the protoxylem (Mähönen et al., 2006a). Inhibiting the production of postembryonic cytokinins through the expression of the cytokinin oxidase1-yellow fluorescent protein (YFP), under the *AHK4/CRE1* promoter and in an estrogen-inducible fashion, can prevent the loss of vascular primordial cells during embryogenesis and result in the differentiation of all cell files within the root vascular cylinder as protoxylem cells. Moreover, in wild-type seedlings grown on media containing cytokinins, the protoxylem is severely impaired or even eliminated. These results indicate that, in addition to maintaining vascular primordial cells, cytokinin signaling is required for suppressing the differentiation of procambial cells into protoxylem cells.

Cytokinins play a role in the formation and activity of the shoot apical meristem. For example, the class-I KNOTTED1-like homeobox (KNOX) transcription factor, including SHOOT MERISTEMLESS (STM), promotes meristem-functioning through the activation of cytokinin biosynthetic gene expression, and by repressing the production of gibberellin (Jasinski et al., 2005; Yanai et al., 2005). When an inducible system is used, activation of the different KNOXI proteins results in a rapid increase in the levels of mRNA for the cytokinin biosynthesis gene *isopentenyl transferase 7* (*AtIPT7*) and a cytokinin-response factor, *ARR5*. A combination of constitutive gibberellin signaling and reduced cytokinin levels is detrimental to the functioning of shoot apical meristems. Moreover, another homeodomain transcription factor, WUSCHEL (WUS), which determines stem cell fate in the overlaying cells, directly suppresses expression of several type-A *ARR*s (Leibfried et al., 2005). This may result in the activation of cytokinin signal transduction. These reports strongly suggest that the regulation of cytokinin signaling in apical meristems plays an important role in their mainte-

nance.

Overexpression of a type-A ARR, *ARR7*, suppresses *WUS* expression (Leibfried et al., 2005). When STM is activated at the apical meristem, the expression of type-A *ARR*s is induced by the activation of cytokinin signaling (Jasinski et al., 2005; Yanai et al., 2005). This may lead to reduced expression of *WUS*. However, that hypothesis is controversial because both STM and WUS have positive functions in the maintenance of the apical meristem. Interestingly, *WUS* expression is decreased in the inflorescence meristem of the septuple mutant *arr3 arr4 arr5 arr6 arr7 arr8 arr9* (Leibfried et al., 2005). This implies that, even though they are in the same family, various type-A ARRs may function as both positive and negative regulators of *WUS* expression. Further investigation is needed to elucidate the roles of different type-A ARRs in the expression of *WUS* and *STM* and in cytokinin signaling to assist in the maintenance of apical meristems.

Legume root nodules originate from differentiated cortical cells that re-enter the cell cycle and form organ primordia. Two independent laboratories have performed genetic screening to isolate the allele responsible for this transition. Szczyglowski's group has isolated the genetic suppressors of the *Lotus japonicus har1-1* hypernodulation phenotype, which is characterized by low nodulation and the excessive formation of infection threads (Murray et al., 2007). They identified the corresponding locus and named it *HYPERINFECTED 1* (*HIT1*). At the same time, Stougaard's group (Tirichine et al., 2007), has isolated lotus mutants that spontaneously develop root nodules, including mutant *snf2*, which produces white rhizobia-free nodules in the absence of *Mesorhizobium loti*. *HIT1* and *snf2*, respectively, are two loss-of-function and gain-of-function alleles of *Lotus histidine kinase 1* (*LHK1*). Both the mutant snf2 and the wild-type LHK1 can activate, in a cytokinin-independent and -dependent manner, respectively, the two-component phosphorylation signaling in *E. coli* that otherwise lacks the two-component receptor (Tirichine et al., 2007). This suggests that LHK1 is a cytokinin receptor protein in lotus. In both reports, mutant analyses have shown that cytokinin signaling is required for the cellular division that initiates nodule development and, furthermore, that in the lotus, cytokinin signaling is necessary and sufficient to induce nodule formation. Because cytokinin signaling is conserved in major crop plants, e.g., maize and rice (Kieber, 2002; Asakura et al., 2003), it may possibly induce nodule formation in these species by integrating the downstream genes required for organogenesis.

ROLE OF CYTOKININ DURING PLANT-PATHOGEN INTERACTIONS

The production of auxins and/or cytokinins is usually regarded as a factor that contributes to plant virulence (Comai and Kosuge, 1980; Silverstone et al., 1993; Lichter et al., 1995; Manulis et al., 1998). In fact, many disease symptoms related to pathogenic microbes appear to be caused by the action of auxins and cytokinins. Examples include the 'green islands' associated with some bacterial, fungal, and viral infections; pathogenic tumor or gall formations; and the fasciation diseases that arise in conjunction with bacterial infections. Crown gall, caused by *Agrobacterium tumefaciens*, can stimulate cytokinin production by integrating *Tmr*, an *Agrobacterium IPT* gene, into the plant genome, then constitutively expressing it under the plant promoter (Sakakibara et al., 2005). The ratio of auxins to cytokinins produced by *Agrobacterium* determines the shape and size of the gall (Akiyoshi et al., 1983). These findings support the possible role of cytokinin during plant-pathogen interactions.

Transcriptome analysis of *Arabidopsis* clubroots has indicated the involvement of cytokinins in club development (Siemens et al., 2006). Three genes associated with cytokinin homeostasis (*IPT3*, *CKX1*, and *CKX6*) are strongly down-regulated early in pathogen colonization. Although it is not clear whether cytokinin production and cytokinin signaling are repressed or activated, transgenic plants over-expressing *CKX1* and *CKX3* appear to be resistant to *Plasmodiophora brassicae*, a pathogen that causes clubroot disease. Interestingly, only the transgenic plants that over-express vacuole-localized *CKX*s (*CKX1* and *CKX3*) have significant resistance to *P. brassicae*, while those that over-express secreted *CKX2* do not. *P. brassicae* induces the growth of root galls presumably from actions inside the pericycle cells (Kobelt et al., 2000). Therefore, the overexpression of intracellularly localized *CKX* may be more effective than the overexpression of extracellular *CKX2*.

During infection, green islands reflect the movement of host nutrients to those sites; exogenous cytokinins also can cause such a redirection of that flow (Mothes and Engelbrecht, 1961). Therefore, cytokinin production has been implicated in green island formation by fungal pathogens. Resource allocation to the site of infection also may be caused by cytokinin accumulation, thereby enhancing pathogenicity. However, there is also evidence that cytokinins act as a direct antagonist of the activity of disease resistance-related proteins, such as chitinase, beta-1,3-glucanase, or glucan endo-1,3-beta-glucosidase (Felix and Meins, 1985; Shinshi et al., 1987). For example, in cultured tobacco tissues, the accumulation of a 33-kDa polypeptide with beta-1,3-glucanase activity is inhibited by kinetin, a cytokinin (Felix and Meins, 1985). In tobacco tissues sub-cultured on a hormone-free medium, the chitinase content increases by about five-fold. This induction can be inhibited by about 90% if auxins and cytokinins are added to the culture media. The patterns reported in that study are similar to those seen with glucan endo-1,3-beta-glucosidase. That is, chitinase and glucan endo-1,3-beta-glucosidase mRNA accumulations also are inhibited by endogenous auxins and cytokinins (Shinshi et al., 1987). All of these results indicate that, in tobacco plants, cytokinins may antagonize their defense responses by repressing the expression or accumulation of defense-related enzymes.

A tobacco cytokinin-binding protein shows identity with endochitinase (Kobayashi et al., 2000). Two such proteins -- CBP1 and CBP2 -- have been isolated via chromatography on a benzyladenine-linked sepharose 4B affinity column. CBP1 shares significant homology (90%) with endochitinase. Moreover, the *PATHOGENESIS-RELATED 10* (*PR10*) protein,

with cytokinin-binding ability, has been detected in both mung bean and moss (Fujimoto et al., 1998). In rice, a PR10 protein, RSOsPR10, is specifically induced in roots by biotic and abiotic stresses (Hashimoto et al., 2004). Those results indicate that cytokinins may regulate the activity or functioning of these possibly disease-related enzymes by directly binding to them. Therefore, if cytokinins suppress their activity, it might be probable to generate cytokinin-insensitive forms of the protein by either a point mutation in, or the deletion of, its binding domain. However, if, instead, cytokinins activate these enzymes, then directed evolution coupled with random mutation following an assay of their enzymatic activity, or manipulation of the cytokinin-binding residue, could lead to the generation of a constitutively active enzyme. This may help researchers in eventually generating a disease-resistant plant with no changes in its cytokinin-mediated growth and development.

Six type-A *ARR*s (*ARR4, ARR5, ARR7, ARR9, ARR15*, and *ARR16*) are repressed by the phytotoxin coronatine following inoculation with *Pseudomonas syringae* pv. tomato DC3000 (Thilmony et al., 2006). *IPT3*, a member of the cytokinin-synthesis isopentenyl transferase gene family, also is known to be repressed by coronatine. Two cytokinin oxidase genes, *CKX4* and *CKX5*, are induced by type three secretion system (TTSS) effectors or the coronatine toxin, respectively. This repression of *IPT3* and cytokinin-responsive type-A *ARR*s, together with the induction of *CKX4* and *CKX5*, suggests that endogenous cytokinin levels decline following inoculation. Moreover, when the pathogen elicitor flg22 or salicylic acid is applied to *Arabidopsis* seedlings, *CKX4* is rapidly induced and two of the type-A *ARR*s (*ARR4* and *ARR6*) are reduced (Choi et al., unpublished data). Because induction of cytokinin production during infection is generally correlated with pathogenicity, it can be assumed that the host plant will repress cytokinin production to achieve pathogen resistance.

CONCLUDING REMARKS

Although some key genes in cytokinin biosynthesis, metabolism, and perception have been identified and functionally characterized, several areas need further elucidation to understand the multiple roles of cytokinins. First, we still do not know how signaling output is regulated by cytokinins in various plant organs and tissues. Specifically, it is important to resolve how the functionally redundant cytokinin receptors and downstream AHPs and ARRs can execute such different activities. Second, we must determine the identity and role of target genes downstream of the cytokinin two-component signaling cascade. Third, the actions of cytokinins during embryogenesis require further investigation.

Three cytokinin receptors -- AHK2, AHK3, and AHK4 -- have extensive functional redundancy. In addition, although they demonstrate only partially overlapping expression patterns, most plant tissues have, at least, low levels of expression (Nishimura et al., 2004; Schmid et al., 2005). Nevertheless, it is also clear that each cytokinin receptor has a specialized function. For example, AHK3 is specifically involved in the cytokinin-dependent delay of leaf senescence while AHK4 plays an important role in maintaining the root stem cells (Kim et al., 2006; Mähönen et al., 2006a). However, it is unclear how the specificity of cytokinin signal transduction is mediated by each receptor. AHK proteins have different binding affinities for the various cytokinins (Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004; Romanov et al., 2005). Therefore, cytokinin receptor tissue-specificity is apparent, and can be better understood by examining the types of cytokinin species that are produced or found in each tissue type. Another potential explanation is that the differentiation of downstream signaling of each cytokinin receptor can arise from the specific components with which the cytosolic domain of each receptor interacts. Future research can confirm either possibility by swapping the extracellular or the cytosolic domain of each cytokinin receptor, and testing whether these chimeric receptors can complement the phenotype of the *ahk2 ahk3 ahk4* triple mutant in a specific tissue or organ. This will help us ascertain which domain determines that phenotype.

It is now apparent that cytokinins play an important role in the formation and maintenance of shoot and root apical meristems (Jasinski et al., 2005; Leibfried et al., 2005; Yanai et al., 2005; Kurakawa et al., 2007). However, it remains unclear how cytokinin signaling regulates meristem activity. Temporal cytokinin deficiency that arises from the overexpression of cytokinin oxidase in an inducible fashion in the meristem cells, coupled with analysis of the differentially expressed genes within the meristematic zone, can help us elucidate the downstream target genes of cytokinin signaling that are involved in this process. By utilizing a similar approach, the molecular mechanism by which cytokinins and AHK4 function together to maintain procambium cells also can be better understood. Root procambium cells can be labeled with GFP by using promoter trap lines (Birnbaum et al., 2005). After exogenous application of cytokinin and its subsequent depletion, either by inducing cytokinin oxidase expression or through RNAi that targets AHK4, we can implement the cell-sorting method FACS to isolate single GFP-labeled procambium cells in the root. Analyzing differential gene expression within the procambium cells after cytokinin signaling is perturbed may make it possible to identify the direct target genes that function in the maintenance of those cells.

Much of the cytokinin signaling pathway has already been elucidated, including the cytokinin receptors for primary-response genes such as type-A ARRs. However, microarray data (Rashotte et al., 2003; Brenner et al., 2005; Lee et al., 2007) suggest that there are many other unidentified cytokinin-responsive genes and novel transcription factors. Because these genes may be directly involved in cytokinin-functioning, they are good candidates for further investigation to define the molecular mechanisms by which cytokinins regulate downstream target genes. For example, Rashotte et al. (2006) have reported on the AP2 gene-family transcription factors, CYTOKININ RESPONSE FACTOR (CRFs); these genes are transcriptionally up-regulated by both cytokinins and the CRF proteins, and rapidly accumulate in the nucleus in response to cytokinins. Furthermore, the *crf1 crf2 crf5* triple mutant exhibits a lack of cell expan-

sion in the cotyledon that results in an abnormal shape. Analysis of the genome-wide transcriptome for wild-type, *arr1 arr12*, *crf1 crf2 crf5*, and *crf2 crf3 crf6* seedlings has revealed that CRFs may regulate the expression of a large number of cytokinin-responsive genes that largely overlap with type-B ARR targets. This implies that CRFs either mediate the cytokinin response through type-B ARRs or else function in tandem with type-B ARRs to mediate the initial response.

Surprisingly, the *ahk2 ahk3 ahk4* triple mutant demonstrates normal embryogenesis despite the fact that the mutants are completely insensitive to cytokinins in the induction of cytokinin-responsive genes, calli formation, and shoot initiation (Higuchi et al., 2004; Nishimura et al., 2004). We might infer from these results that AHK-mediated cytokinin signaling is not required for the establishment of a shoot apical meristem during embryogenesis. However, the *crf5 crf6* double mutant is embryo-lethal (Rashotte et al., 2006), implying that the CRFs mediate cytokinin signaling during embryogenesis independent of AHK2, AHK3, and AHK4, and that another cytokinin receptor is at play. It is possible that His kinases, e.g., CKI1 and AHK5, are involved in embryogenesis. The mutant *cki1* loss-of-function is lethal to the female gametophytes and is expressed in these cells and in the endosperm (Pischke et al., 2002; Hejátko et al., 2003). However, it is not clear whether CKI1 is involved in embryogenesis after the fertilization of male and female gametophytes. Therefore, identification of the upstream signaling components of CRF5 and CRF6 is necessary to clarify the role of cytokinins during embryogenesis. It may be possible to identify these upstream partners by applying the yeast two-hybrid screening system, using CRF5 and CRF6 as bait.

The recently discovered role of cytokinins during root nodule formation is exciting in terms of its agricultural applications (Murray et al., 2007; Tirichine et al., 2007). In lotus, cytokinin signaling is both necessary and sufficient to induce nodule formation. Because many of the known genes involved in cytokinin signaling process are conserved in both maize and rice (Kieber, 2002; Asakura et al., 2003), further understanding of the downstream genes involved in root nodule formation could be used to induce root nodule in crop plants.

It is interesting that cytokinins are involved in plant responses to and defenses against pathogens. For example, endogenous cytokinins affect plant resistance to fungal attacks (Siemens et al., 2006). Further investigation is needed to clarify how endogenous cytokinins are regulated by both plants and pathogens during these interactions, and also how the particular levels of cytokinins alter this response. Plants that have constitutively activated defense mechanism often show growth retardation, and researchers have suggested that this phenomenon is caused indirectly by the allocation of energy to maintain that defense. However, if there is a direct antagonistic relationship between that response and growth, in this case the latter being mediated by cytokinin signaling, then it may be possible to enhance disease resistance in plants without affecting their growth by manipulating the target genes for those interactions between cytokinin signaling and the defense response.

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