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Cytokinin signaling

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Cytokinins influence many aspects of plant growth and development. The current model for cytokinin signaling is a multi-step phosphorelay similar to the prokaryotic two-component systems that are used in responses to environmental stimuli. Recently, progress has been made in improving our understanding of the molecular mechanism that underlies cytokinin signaling. Molecular and genetic analyses of loss-of-function mutants indicate that the two-component elements that are involved in cytokinin signaling have redundant and overlapping functions. These elements regulate both the shoot and root meristems, are required for the development of fertile flowers, and modulate the response to varying nutrient levels.

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Introduction

Cytokinins were first identified as factors that promote cell proliferation and sustained growth in cultured plant cells [1,2]. They influence many aspects of growth and development, including seed germination, vascular development, cell proliferation, apical dominance and leaf senescence. Lowering endogenous levels of cytokinin causes pleiotropic developmental changes, including delayed leaf initiation and expansion, delayed onset of flowering, increased sterility and enhanced root growth [3,4]. Conversely, increasing endogenous cytokinin levels by ectopic expression of the cytokinin biosynthesis gene *ISOPENTYL TRANSFERASE (IPT)* reduces apical dominance and root development, alters leaf shape and enhances shoot regeneration in culture [5,6].

Recently, our understanding of the molecular elements that underlie cytokinin signal transduction has progressed. This review focuses on these early cytokinin signaling events, which have been studied primarily in

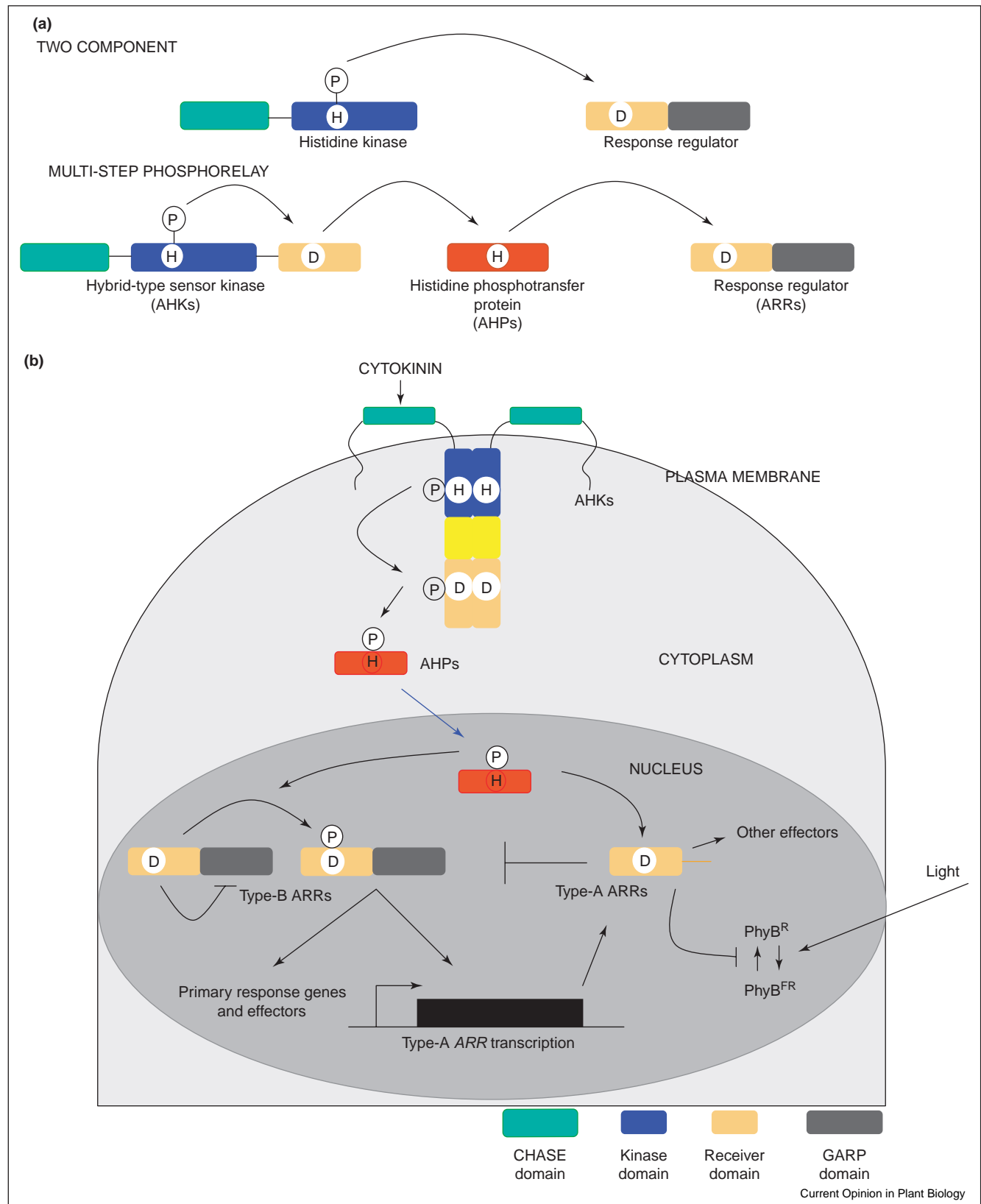
Arabidopsis thaliana. The cytokinin signaling pathway is similar to bacterial and yeast two-component signal transduction pathways; specifically to His-Asp multi-step phosphorelays, which are comprised of sensor kinases, histidine phosphotransfer proteins and response regulators (Figure 1). The *Arabidopsis* cytokinin receptor kinases (*Arabidopsis* *HISTIDINE KINASE2* [*AHK2*], *AHK3*, *AHK4/CYTOKININ RESPONSE 1* [*CRE1*]/*WOODENLEG* [*WOL*]) contain a conserved extra-cellular cytokinin-binding domain called the CHASE (cyclases/histidine kinases associated sensory extracellular) domain, a histidine kinase domain and a receiver domain. The five *Arabidopsis* histidine-phosphotransfer proteins (AHPs) encode small proteins (of about 150 amino acids) that mediate the phosphotransfer from the receptor kinases to the response regulators. There are 23 genes in the *Arabidopsis* genome that are similar in sequence and domain structure to bacterial response regulators, and these encode both positive and negative elements in cytokinin signaling. Molecular and genetic analyses have revealed that there is extensive genetic redundancy in all of these families, and each is discussed in turn in this review.

The cytokinin receptors

An allele of *CRE1/WOL/AHK4* (referred to hereafter as *AHK4*) was isolated in a screen for mutants whose hypocotyl explants fail to form large green calli on shoot-initiating media [7]. *ahk4* mutants are also insensitive to cytokinin in other assays [8], and transient overexpression of *AHK4* in *Arabidopsis* protoplasts enhances the expression of a cytokinin primary response gene [9]. *AHK4* was identified as a cytokinin receptor because of its ability to complement both yeast and *Escherichia coli* histidine kinase mutants in a cytokinin-dependent manner [7,10]. Additionally, *AHK4* binds to the cytokinin 2-iP with high affinity when expressed in *Schizosaccharomyces pombe* membranes [11]. Various active cytokinins can compete for this binding to *AHK4*, but inactive forms cannot [11]. Three-dimensional modeling of the *AHK4* CHASE domain reveals that bound cytokinin is completely buried within the binding pocket [12]. Thr₂₇₈, which is essential for cytokinin binding [13] and for *AHK4* function [14], is positioned at the entry of the binding pocket, within contact distance of the ligand. A subset of the cytokinin receptors from both maize and *Arabidopsis* are activated by *cis*-zeatin and by cytokinin ribosides [15,16] but others are not, suggesting that different receptors might bind distinct cytokinins.

Analysis of a triple receptor knockout mutant indicates that *AHK2*, *AHK3* and *AHK4* are redundant, positive

Figure 1



elements in cytokinin signaling [17^{••},18^{••}]. Inhibition of root elongation by cytokinin is strongly reduced in *ahk4* mutants, but unaffected in *ahk2* or *ahk3* single mutants and in the *ahk2 ahk3* double mutant, which is consistent with the expression pattern of these genes (Figure 2). However, the triple receptor knockout displays a more severe phenotype in the roots and shoots than any double mutant combination, indicating that all three genes have distinct yet overlapping functions in both shoots and roots.

The Histidine-phosphotransfer proteins

The five *Arabidopsis* AHP genes are expressed ubiquitously, and their transcription is not affected by cytokinin treatment ([19]; Figure 2). AHPs have been demonstrated to be genuine histidine phosphotransfer proteins by their abilities to: first, complement a yeast histidine phosphotransfer protein (Hpt) mutant [20]; second, accept phosphoryl groups from *E. coli* membrane preparations and subsequently transfer this phosphate to *Arabidopsis* response regulators *in vitro* [20–22]; and third, compete for phosphotransfer with an *E. coli* Hpt protein *in vivo* [19,23]. In yeast two-hybrid analyses, the AHPs interact with various *Arabidopsis* histidine sensor kinases and *Arabidopsis* response regulators (ARRs) [20,24,25], consistent with their role in mediating phosphotransfer among these elements.

Various additional lines of evidence link the AHPs to cytokinin signaling. Several of the AHPs have been shown to accumulate in the nucleus of cytokinin-treated cells [9,13]. Overexpression of AHP2 in *Arabidopsis* results in a slight hypersensitivity to exogenous cytokinin in both root and hypocotyl elongation assays [23]. In an *Arabidopsis* protoplast system, however, overexpression of AHP1, AHP2 and AHP5 had no effect on the expression of a cytokinin primary response gene [11]. In *Catharanthus roseus* suspension cell lines, the reduction of *CrHpt1* by RNA interference reduces cell proliferation and the expression of a cytokinin-inducible response regulator, *CrRR1* [26].

Compelling evidence that the AHPs mediate cytokinin signaling has come from a reverse genetic approach (CE

Hutchison *et al.*, unpublished). T-DNA insertional alleles of all five AHP genes were identified, and single and multiple mutant combinations analyzed. The single and various double mutants exhibit no differences in cytokinin responsiveness, but the triple *ahp1 ahp2 ahp3* mutant displays reduced sensitivity to exogenous cytokinin in root elongation assays. The quintuple *ahp1 ahp2 ahp3 ahp4 ahp5* mutant is severely impaired in the induction of cytokinin primary response genes and shows developmental defects. Together, these findings indicate that the AHPs are positive, redundant elements in the cytokinin primary signal transduction pathway. Interestingly, a morphology that is consistent with reduced cytokinin function is observed only when there is near-complete loss of AHP function.

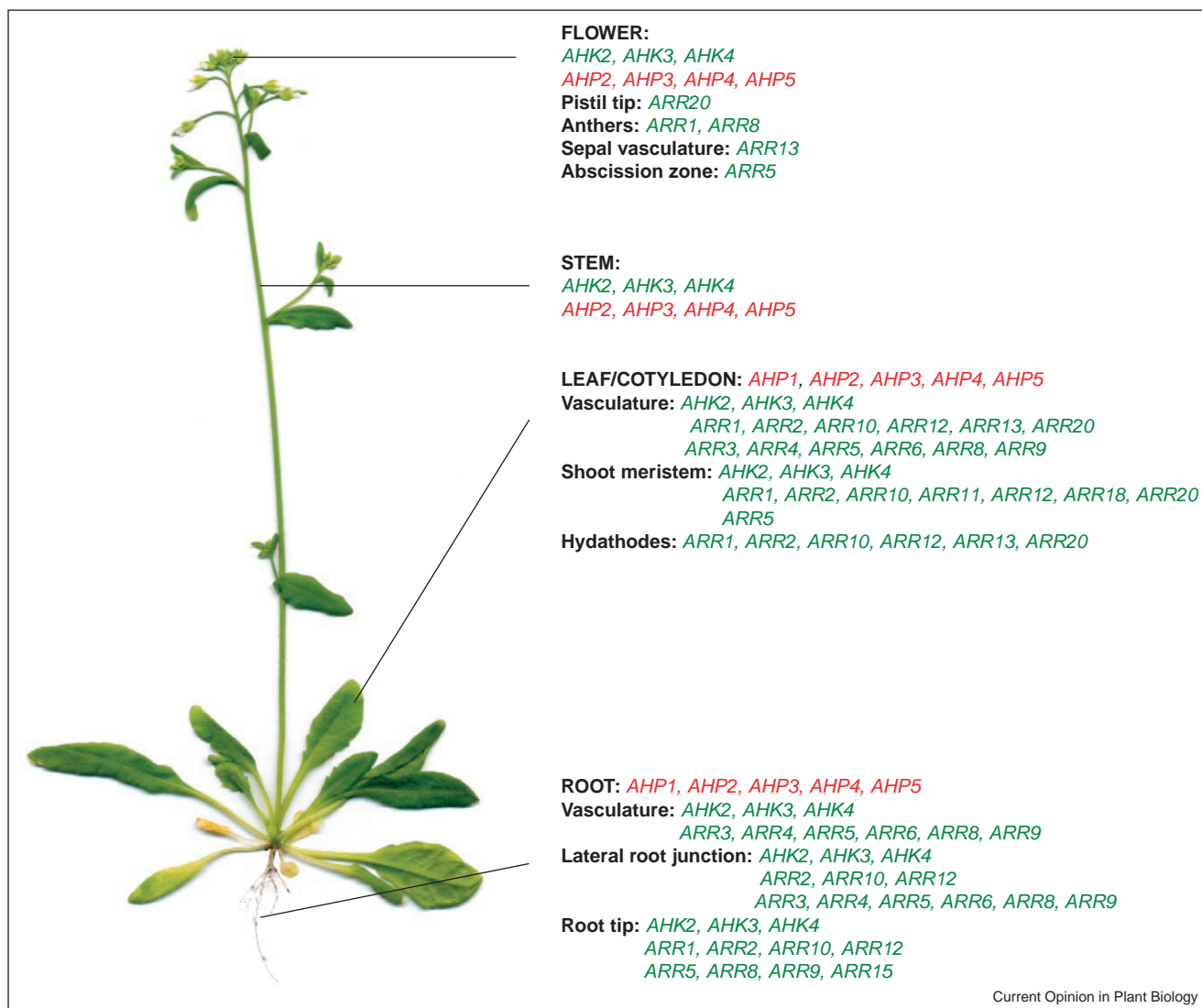
The response regulators

The 23 ARR genes fall into two main groups on the basis of their sequence similarities, domain structure and transcriptional response to cytokinin [27,28]. The type-A ARRs are comprised of a receiver domain and a short carboxyl terminus and their transcription is rapidly elevated in response to exogenous cytokinin; these are considered to be primary response genes [27,29,30]. The type-B ARRs, in addition to the receiver domain, have a carboxy-terminal output domain that has a DNA-binding GARP domain and a transcriptional activation domain. Transcription of the type-B ARRs is not altered by cytokinin. Analysis of promoter:: β -glucuronidase (GUS) fusions for a subset of these ARR genes has revealed distinct, but often overlapping, patterns of expression (Figure 2), which are consistent with the high level of functional redundancy observed in each of these families [28,31,32].

Overexpression of a subset of type-A ARRs in *Arabidopsis* protoplasts causes the repression of a pARR6::LUC luciferase reporter, leading to the hypothesis that these elements might be negative regulators of cytokinin responses [9]. Consistent with this, overexpression of ARR15 decreases sensitivity to cytokinin [33]. This has been further supported by the analysis of loss-of-function type-A ARRs mutants. To *et al.* [34^{••}] analyzed single and

(Figure 1 Legend) Cytokinin signaling. **(a)** Two-component signaling. Simple two-component signaling systems are comprised of a receptor kinase and a response regulator. The kinase auto-phosphorylates in response to an environmental stimulus. The phosphoryl group is subsequently transferred to a conserved Asp residue within the receiver domain of the response regulator. Multi-step phosphorelay systems are comprised of sensor kinase receptors that have both a kinase and a receiver domain, histidine phosphotransfer proteins and response regulators. Ligand binding alters the auto-phosphorylation activity of the sensor kinase. The phosphoryl group is transferred from the auto-phosphorylated His to an Asp residue within the fused receiver domain, then to a His residue in a His-phosphotransfer protein, and ultimately to an Asp residue in a response regulator. The phosphotransfers always occur between histidine and aspartate residues. The *Arabidopsis* cytokinin-signaling proteins are in parentheses. **(b)** General model of cytokinin signaling in *Arabidopsis*. Cytokinin is perceived at the plasma membrane where the hybrid sensor auto-phosphorylates at a conserved His residue that is contained in the kinase domain. The phosphoryl group is then transferred to an Asp residue in the receiver domain of the sensor. There is then an inter-molecular transfer of the phosphoryl group to a His residue in an AHP protein. Activated AHP then translocates to the nucleus and transfers the phosphoryl group to an Asp residue in a response regulator. The type-B ARR receiver domain negatively regulates its own transcriptional activation domain. Upon phosphorylation, this repression is relieved, allowing type-B ARRs to induce the expression type-A ARRs and other primary response genes. Type-A ARRs are negative regulators of cytokinin responses and restrict their own transcription. ARR4 accumulates in the light where it negatively regulates the dark reversion of activated PhyB.

Figure 2



General expression patterns of *Arabidopsis* cytokinin two-component signaling elements. Localization patterns were compiled from Northern analysis data (in red) and from transcriptional- and/or translational GUS fusions (in green) [17[•], 18[•], 19, 28, 29, 32, 33, 34^{••}].

multiple loss-of-function type-A *arr* mutants, including a sextuple *arr3 arr4 arr8 arr6 arr8 arr9* mutant [34^{••}]. Single loss-of-function mutants have no detectable change in cytokinin responsiveness. However, multiple type-A *arr* mutants are hypersensitive to cytokinin in various assays, including inhibition of root elongation and lateral root initiation, leaf senescence, and callus formation. The severity of the hypersensitive phenotype generally correlates with the number of type-A ARR proteins that are disrupted. The shift in the cytokinin sensitivity of the *arr* mutants is the result of an altered primary response; analysis of the cytokinin primary response genes (*ARR7* and *STEROID SULFOTRANSFERASE [SST]*) showed an increase in the amplitude and duration of induction following cytokinin treatment in the quadruple *arr3*

arr4 arr5 arr6 and the sextuple *arr3 arr4 arr5 arr6 arr8 arr9* mutants [34^{••}]. None of the type-A ARR mutants examined had a strong morphological phenotype [34^{••}]. These results reveal highly overlapping function among members of this large gene family. The level of genetic redundancy might be different in monocots, as a single type-A mutation (*ZmRR3*) in maize strikingly alters leaf phyllotaxy, which is probably the result of an increase in the size of the apical meristem [35].

Type-B ARRs are transcription factors that localize to the nucleus [9, 28, 36]. A consensus DNA-binding sequence for type-B ARRs has been delineated (G/A)GGAT(T/C), and this sequence signature is found in the promoters of many of the cytokinin primary response genes [30].

Several type-B ARR have been shown to be positive elements in cytokinin signaling [9,36]. Overexpression of ARR1, ARR2 or ARR10 is sufficient to activate cytokinin primary responses in the absence of exogenous cytokinin in *Arabidopsis* protoplasts [9]. The expression of truncated forms of ARR1 and ARR2 that lack the receiver domain resulted in a greater induction of the cytokinin response than that obtained by expression of the respective full-length proteins [9,37,38], suggesting that the receiver domain might negatively regulate the output function of these proteins [9,37,38]. Plants that overexpress truncated forms of other type-B ARRs (ARR1, ARR11, ARR14 and ARR21) had unusual and unordered cell proliferation around the shoot apex. These plants show reduced apical dominance, presumably the result of increased secondary meristem activity [22,32,36]. Despite elevated cytokinin sensitivity in these plants, the transcripts of only a few type-A ARRs are elevated [22,32], suggesting that there might be specific interactions among the type-B and type-A ARRs.

Role of cytokinin signaling in plant growth and development

Since their discovery in the 1950s, cytokinins have been shown to be involved in many plant growth and developmental processes. However, much of the work on cytokinin has been based on experiments using exogenously applied hormone. The disruption of cytokinin perception and signaling by mutations in the genes that encode various two-component elements provides novel tools that can be used to elucidate the role of cytokinins in plants.

Disruption of the cytokinin AHK receptors blocks the ability of plants to perceive the hormone, and thus the phenotype of this triple mutant is particularly revealing as to the function of cytokinin. Single *ahk* mutants and the *ahk2 ahk4* and *ahk3 ahk4* double mutants are morphologically indistinguishable from wildtype plants, partly because of the functional redundancy among these three genes. By contrast, the *ahk2 ahk3* double mutant has short petioles and smaller leaf blades, and the triple receptor *ahk2 ahk3 ahk4* mutant is severely impaired in growth, with a reduced leaf size and number [17^{••},18^{••}]. The decreased rosette size of the *ahk2 ahk3 ahk4* mutant is the result of decreased initiation of leaf primordia and reduced leaf cell numbers; cell size is comparable to that of the wildtype [17^{••},18^{••}]. Transgenic plants that overexpress cytokinin oxidase also have decreased cell division [4]. Like the triple receptor knockout, these plants have a smaller shoot apical meristem and reduced vasculature. These results are consistent with a role for AHKs and cytokinin as positive regulators of cell division [17^{••}].

Perturbation of cytokinin function also affects floral development. The triple receptor mutant only rarely

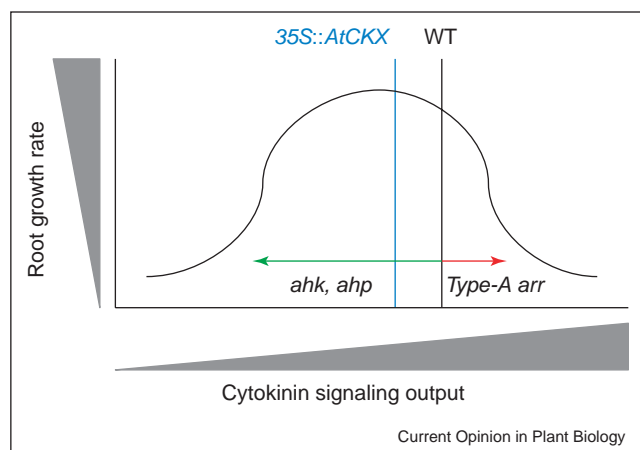
flowers, and when it does, it produces only a few flowers, which are all sterile [17^{••},18^{••}]. These results suggest that the *ahk2 ahk3 ahk4* floral meristem is depleted of cells rapidly. Although not as severe as the receptor mutants, strong reduction of cytokinin function either by loss-of-function mutations in the *AHP* genes or by overexpression of cytokinin oxidase, also resulted in decreased fertility (CE Hutchison *et al.*, unpublished; [4]). Interestingly, cytokinin levels increase in the apical meristem following the transition to flowering in *Arabidopsis* [39]. These findings indicate that cytokinin plays an important role in floral development.

Like shoot growth, root growth in the *ahk2 ahk3 ahk4* mutant is very much reduced; the root elongation rate of this mutant is only 20% of wildtype rates and the number of cell files is reduced [17^{••}]. Interestingly, root development in the triple receptor mutant resembles that of plants that have the anti-morphic *wol* allele of *AHK4* (Thr₂₇₈ → Ile) [14]. *wol* mutants have abnormal cell division during embryogenesis [14]. As a result, too few vascular precursor cells are available for proper phloem development. The *ahp1 ahp2 ahp3 ahp4 ahp5* mutant has a very stunted primary root that lacks phloem and metaxylem, similar to that of the triple receptor mutant [17^{••},18^{••}]. In both *ahk2 ahk3 ahk4* triple mutants and *ahp1 ahp2 ahp3 ahp4 ahp5* quintuple mutants, the short root phenotype appears to be the result of a premature exhaustion of the root meristem [17^{••},18^{••}]. By inference, AHK receptors might function via the AHPs to maintain meristematic competence at the root tip.

These results differ from those of the cytokinin oxidase studies [4]. Overexpression of a subset of cytokinin oxidase genes resulted in a larger root system and a more active root meristem. This led to the proposal that cytokinins play opposing roles in the root and shoot, a proposal that is distinct from the conclusions drawn from studies of signaling mutants. One model to reconcile this difference is that endogenous cytokinin levels in roots might be supraoptimal; that is, the response curve is bell-shaped. If this were the case, then a small reduction in cytokinin levels or signaling would increase root growth, but reduction beyond a threshold would result in decreased growth (Figure 3).

A second *wol* allele of *AHK4* has been isolated [40]. The *wol* mutants display intragenic complementation, indicating that signal transduction involves a homodimer. This complementation, however, is specific for the embryonic root defect: trans-heterozygous adult plants are still insensitive to exogenous cytokinin. How do these recessive mutations cause a dominant-negative effect on procambial cell proliferation, and display intragenic complementation specifically in vascular differentiation? One model is that *AHK4* represses procambial proliferation as a monomer [40]. *AHK4* loses this repression capability

Figure 3



Model correlating cytokinin signaling output and root growth. The horizontal axis represents the cytokinin signaling output in seedlings. The vertical axis represents root growth rate. The hypothetical response is bell-shaped. Endogenous cytokinin levels are supraoptimal and are represented by the intersect. Lowering endogenous cytokinin levels by overexpressing cytokinin oxidase genes lowers the cytokinin signaling output, resulting in increased root growth [34**]. Reduction beyond a threshold results in decreased growth. Reducing type-A ARR function increases cytokinin signaling output, inhibiting root growth [4]. A reduction in AHK and AHP function results in insensitivity to cytokinin. Slight reduction in AHK and AHP function can have a stimulatory effect on root growth (FJ Ferreira, JJ Kieber, unpublished). However, complete loss of AHK and AHP function results in decreased in root growth [17**,18**].

by binding to its ligand and dimerizing. The *wol* alleles of AHK4, when homozygous, are unable to dimerize and thus repress procambial development. Alternatively, the unphosphorylated AHK4 receiver domain might negatively regulate procambial proliferation in the embryo, perhaps by strongly interacting with its downstream effectors, such as the AHPs. Phosphorylation of the receiver domain relieves this repression. In *wol* mutants, mutant proteins cannot phosphorylate the receiver domain either because they cannot bind to cytokinin or because they have a defective kinase domain and thus block cytokinin signaling by binding to the AHPs in an inactive state. These plants are unable to de-repress procambial development and thus display the *wol* phenotype. Intragenic complementation of *wol* mutants results in a receptor of diminished function, which is sufficient for procambial development but not for the high signaling flux that is required for responses to high levels of exogenous cytokinin. Thus, the roots of these intragenic seedlings display cytokinin insensitivity.

The lack of detectable cytokinin responses in the triple *ahk* mutant, including the absence of primary response gene induction and the inability to respond appropriately to cytokinin in calli and shoot initiation assays [17**,18**], raises the question of whether cytokinin function is

required *in vivo* for plant development. For example, it is possible that cytokinin is required solely for the maintenance of shoot apical meristem (SAM) activity, and not for its establishment. An alternative model is that there is another, as yet unidentified divergent cytokinin receptor that compensates for the reduced AHK function. A second alternative is that, in the absence of AHKs, non-cognate histidine kinases may initiate flux through the phosphorelay. Cross-talk occurs in prokaryotic systems when non-cognate proteins are in excess and/or when the cognate sensor is absent [41]. For example, in low phosphate conditions, the non-cognate sensor CreC can phosphorylate PhoB in *E. coli* strains that lack the cognate sensor PhoR (reviewed in [41]).

It is astonishing that the disruption of six out of ten type-A ARRs does not result in a dramatic morphological phenotype, despite a ten-fold increase in cytokinin sensitivity. The sextuple mutant might compensate for the increased sensitivity to cytokinin by lowering the endogenous level of active hormones. Consistent with this model, expression analyses of cytokinin-treated *Arabidopsis* reveals rapid upregulation of genes that function to decrease endogenous cytokinin levels or responsiveness [30]. Alternatively, cytokinin regulation of development might overlap with other regulatory mechanisms. Finally, it could be that changes in cytokinin sensitivity play a minor role in regulating the function of this hormone, and that the primary regulation is at the level of active hormone.

There is increasing evidence that cytokinin might relay the nutritional status of the plant. Supplying nitrogen to nitrogen-starved plants leads to increases in cytokinin transport from the roots. Cytokinin is translocated via the xylem to the leaves, where it leads to the accumulation of type-A response regulators [42,43]. Cytokinins block the induction of several genes that are upregulated in response to phosphate starvation. This repression is mediated through AHK4 [44]. Interestingly, the expression of AHK4 itself is downregulated by phosphate starvation, suggesting a positive feedback loop. More recently, AHK4 was shown to mediate sulfate uptake in *Arabidopsis* roots [45]. Cytokinin reduces the expression of two sulfate transporter genes. This inhibition is dependent on a functional AHK4 protein, suggesting that cytokinin is a negative regulator of sulfate uptake in sulfate-replete conditions. ARR4, a type-A ARR, modulates red-light responses by inhibiting the dark reversion of activated Phytochrome B, and overexpression of ARR4 results in red light hypersensitivity [46]. Consistent with this, the *arr3 arr4 arr5 arr6 arr8 arr9* mutant displays reduced sensitivity to red light [34**]. Contrary to these results, however, single or double mutants in *ARR3*, *ARR4*, *ARR5*, and *ARR6* show increased sensitivity to red light, suggesting that the role of the type-As in light signaling might be complex. Together, these results

suggest that changing cytokinin levels and/or changing sensitivities might play a key role in modulating the balance between shoot and root growth in response to altering carbon/nutrient ratios.

Conclusions

Each step of the cytokinin signal transduction pathway is encoded by a gene family, whose members function redundantly. Despite the significant recent advances in our understanding of cytokinin signaling, there are still many questions to be answered. For instance, with such a large number of players in the pathway, how is the specificity of cytokinin responses determined? Are there auxiliary proteins that mediate specific responses? What is the output from the cytokinin-response pathway? How do the type-A ARRs negatively regulate cytokinin signaling? With many loss-of-function mutants now available, it will be possible to decipher branch points in the pathway, to discover new inputs and to determine the outputs.

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- of special interest
- of outstanding interest

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