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The wealth of mechanistic insight into how the Hh pathway functions has revealed both the sophistication of this signal transduction network and the challenges that remain in the treatment of Hh-related diseases. Of urgency is the development of rational therapeutic approaches using knowledge of the pathway to specifically target the events underlying aberrant pathway response. Success in this endeavor will require an understanding of how primary cilia, lipoproteins, and sterol biosynthesis contribute to Hh-related diseases.

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PERSPECTIVE

# Advances in Cytokinin Signaling

**Bruno Müller and Jen Sheen***

**Cytokinins are essential plant hormones that control various processes in plants' development and response to external stimuli. The *Arabidopsis* cytokinin signal transduction pathway involves hybrid histidine protein kinase sensors, phosphotransfer proteins, and regulators as transcription activators and repressors in a phosphorelay system. Each step is executed by components encoded by multigene families. Recent findings have revealed new functions, new feedback loops, and connections to other signaling pathways.**

The plant hormone cytokinin comprises a class of adenine-derived signaling molecules involved in diverse processes throughout a plant's life, such as stem-cell control in root and shoot; vascular differentiation; chloroplast biogenesis; root, shoot, and inflorescence growth and branching; nutrient balance; leaf senescence; stress tolerance; and seed development (*1*–*6*). More than 50 years ago, Skoog, Miller, and collaborators purified the first cytokinin crystal from autoclaved herring sperm DNA extracts and demonstrated its ability to strongly stimulate proliferation in tobacco tissue culture (*7*). It then took some 40 years to identify the first genes involved in cytokinin signaling. Kakimoto and colleagues pioneered in performing large screens based on the effects of cytokinin on cultured *Arabidopsis* tissues and uncovered a role for histidine kinases (HKs) in cytokinin signal transduction (*8*, *9*). HKs are prevailing sensors in prokaryotes that initiate a signaling system in which phosphoryl groups are transferred between histidines and aspartates (phosphorelay signaling system) to activate or inhibit cognate downstream partners called response regulators (RRs). Completion of the *Arabidopsis* genome sequence facilitated the identification of all potential components of phosphorelay signaling: There are eight transmembrane HKs, six histidine phosphotransfer proteins (HPTs), and more than 20 RRs (*1*, *2*, *10*, *11*). Isolated leaf cells were systematically transfected with putative tagged phosphorelay components to test how these components affected the responsiveness of a cytokinin reporter. This analysis resulted in a model (Fig. 1) that distinguishes four major steps of the cytokinin phosphorelay from the plasma membrane to the nucleus: (i) cytokinin sensing and initiation of signaling by receptor HKs; (ii) phosphoryl group transfer to HPTs and their nuclear translocation; (iii) phosphotransfer to nuclear B-type RRs, which activate transcription; and (iv) negative feedback through cytokinin-inducible A-type RRs, which are products of the early cytokinin target genes (*11*). Identification of the orthologs for cytokinin signaling components in other plant species suggests evolutionary conservation of this pathway.

Careful and extensive analyses of plants harboring loss-of-function mutations in signaling components have corroborated the core logic of the cell-based model. Mutant phenotypes became apparent only after multiple family members were knocked out, which suggests extensive functional redundancy at each signaling step (*2*). However, individual components seem to accomplish specific tasks as well, as illustrated by the following findings. Receptors exhibit differential affinities for different cytokinins (*1*, *5*, *12*). One out of three well-characterized cytokinin receptors specifically mediates a delay of senescence in *Arabidopsis* leaves (*2*, *13*). Plants mutated in some RR gene pairs (out of the large RR family) display subtle differences in phenotypes (*2*). In addition, overexpressing different A- or B-type RR family members results in plants with different phenotypes (*1*, *2*, *11*). A comprehensive protein interaction map for all potential components involved in signaling shows distinct patterns of interaction between protein family members (*10*). The molecular basis and biological importance of these observations will need further studies.

To understand the pathway mechanism in more detail, several questions need to be addressed. Not all eight HKs found in the *Arabidopsis* genome encode cytokinin receptors. For example, two HKs encode ethylene receptors, one encodes a putative osmosensor, and another one shows constitutive HK activity when overexpressed (*1*–*4*, *8*, *10*). Their precise roles with respect to cytokinin signaling remain unclear. B-type RRs bind similar cis elements in vitro and induce transcription (*14*). How are they involved in generating tissue- and cell-specific signaling outputs? Do they interact with specific but unknown partners? And what is the molecular mechanism by which A-type RRs attenuate signaling?

Recent findings have added some twists to the pathway. Aside from its kinase function, a cytokinin receptor was found to exhibit phosphatase activity that removes phosphoryl groups from interacting *Arabidopsis* histidine phosphotransfer proteins (AHPs) when no cytokinin is bound. Many prokaryote HKs have such phosphatase activity, and they are associated with phosphorelay systems that need to be shut off quickly. In *Arabidopsis*, the phosphatase activity of this HK may help to ensure that, in the absence of cytokinin, the pathway is quickly and completely inactivated (*15*). One of the six *Arabidopsis* HPTs, AHP6, was identified as a "pseudo-HPT" because of a mutation in the conserved histidine residue required to accept the incoming phosphoryl group from the recep-

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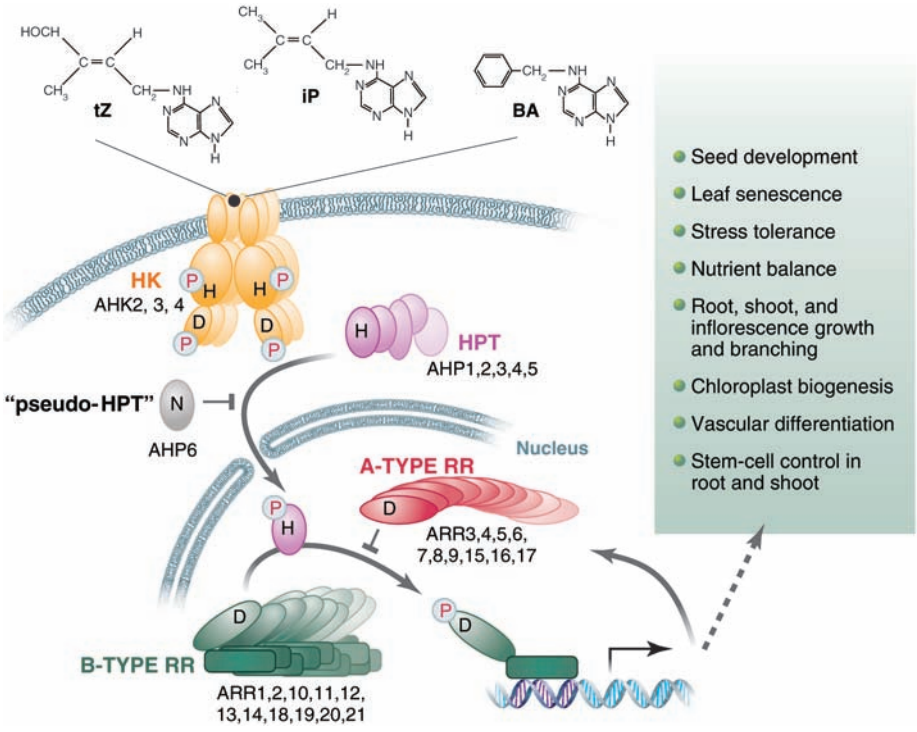


**Fig. 1.** Model for the cytokinin multistep two-component circuitry through histidine (H), and aspartate (D) phosphorelay, involving histidine-kinase receptors (HK), phosphotransfer proteins (HPT), a "pseudo–HPT" with an asparagine (N) instead of the D, and A-type and B-type RRs. Each signaling step is executed by a family of genes that largely act redundantly, as illustrated. *Arabidopsis* genes implicated in signaling are listed as abbreviations (AHK, *Arabidopsis* histidine kinases; AHP, *Arabidopsis* histidine phosphotransfer proteins; ARR, *Arabidopsis* response regulator). Different effects of cytokinin signaling are indicated on the right. The chemical structure of three cytokinins is shown on top: trans-zeatin (tZ), $N^6$-($\Delta^2$-isopentenyl)adenine (iP), and 6-benzylamino purine (BA).

tors. AHP6 inhibits cytokinin signaling, probably by competing with other AHPs for interaction with activated receptors or RRs in *Arabidopsis*. Cytokinin signaling, in turn, represses transcription of *AHP6*. Lack of AHP6 function causes ectopic cytokinin signaling, leading to pattern defects in vascular tissue (*16*). Thus, the presence of AHP6 may limit the number of cells responding to cytokinin and, thereby, may help sharpen and define cell differentiation boundaries.

Plants not only use feedback loops to control cytokinin signaling but selectively allow other factors to influence pathway activity. WUSCHEL, a homeodomain transcription factor required for shoot-stem cell function, directly attenuates transcription of some A-type RR genes, which likely increases cytokinin signaling activity in the shoot-stem cell pool and extends its size (*17*). New transcriptional regulators involved in mediating cytokinin signaling other than the conserved B-type RRs have emerged. Studies of a subset of cytokinin responsive factors (CRFs) have revealed their cytokinin-dependent nuclear translocation. CRFs and B-type RRs share some cytokinin target genes but do not control the most prominent cytokinin-inducible A-type RR genes. The phenotypes of *crf* mutants appear to be complex with both cytokinin-dependent and independent features (*18*).

Is there a common denominator in the seemingly diverse cytokinin responses? Ectopic cytokinin causes cell proliferation and shoot growth in tissue culture. The cell cycle regulator cyclin D3 seems to be an important mediator of this effect. Consistently, cytokinin receptor triple mutants (*2*) or B-type RR triple mutants (*19*) have retarded growth in roots and shoots. However, plants with partially reduced endogenous cytokinin signaling or concentrations display an increase in the size of the root system (*2*, *6*, *20*). A detailed analysis of cytokinin's function in the root supports a role of cytokinin to promote differentiation, which counteracts proliferation (*6*, *20*). One explanation for the opposite effects depending on signaling activity might be that plants with a strong reduction in cytokinin reception and signaling have an impaired vascular system that might limit the potential to grow. It is also possible that different cytokinin threshold levels are required for cell proliferation, elongation, and differentiation (*2*). In fully differentiated leaves, cytokinin's role in inhibiting senescence is unlikely to depend on cell proliferation. Thus, the functions of cytokinin seem to be more diverse and context-dependent than previously anticipated. In accordance with this view, data sets from different microarray experiments aimed at identifying transcriptional targets of cytokinin appear to vary considerably except for their inclusion of the common targets, A-type RR genes. The future challenge is to analyze cytokinin functions at cellular resolution to understand how signaling integrates with the context.

The genetic and molecular characterization of cytokinin signaling began about 10 years ago, and the core signaling circuitry has now been established and verified. However, most functional studies have been based on whole-plant responses to exogenously applied cytokinins. Given the multiple roles and redundancy of the pathway, tailored approaches will be required to study the individual functions of cytokinin in different tissues and cell types at various developmental stages. For example, sensitive cytokinin reporters will facilitate in vivo analyses of the cells that transduce cytokinin signaling. To circumvent redundancy and lethality of classical genetic approaches, inducible transgenes expressing RNA interference constructs, or constitutively active or dominant-negative signaling components, can be used to manipulate the pathway activity in a targeted manner. Understanding how cytokinin signaling integrates with other interacting signals and elucidating its complex biosynthesis pathways and little-known transport systems will bring additional exciting discoveries (*5*). With the increased availability of plant genome sequences and functional analysis systems, it will be interesting to compare the role of phosphorelay signaling among different plant species exhibiting diverse architecture and life cycles and various growth patterns.

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