Cytokinin Signaling Networks

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Annu. Rev. Plant Biol. 2012. 63:353–80

The *Annual Review of Plant Biology* is online at plant.annualreviews.org

This article's doi: 10.1146/annurev-arplant-042811-105503

1543-5008/12/0602-0353$20.00

Keywords

auxin, embryo, meristem, vasculature, nodulation, immunity, stress, senescence

Abstract

Despite long-standing observations on diverse cytokinin actions, the discovery path to cytokinin signaling mechanisms was tortuous. Unyielding to conventional genetic screens, experimental innovations were paramount in unraveling the core cytokinin signaling circuitry, which employs a large repertoire of genes with overlapping and specific functions. The canonical two-component transcription circuitry involves His kinases that perceive cytokinin and initiate signaling, as well as His-to-Asp phosphorelay proteins that transfer phosphoryl groups to response regulators, transcriptional activators, or repressors. Recent advances have revealed the complex physiological functions of cytokinins, including interactions with auxin and other signal transduction pathways. This review begins by outlining the historical path to cytokinin discovery and then elucidates the diverse cytokinin functions and key signaling components. Highlights focus on the integration of cytokinin signaling components into regulatory networks in specific contexts, ranging from molecular, cellular, and developmental regulations in the embryo, root apical meristem, shoot apical meristem, stem and root vasculature, and nodule organogenesis to organismal responses underlying immunity, stress tolerance, and senescence.

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INTRODUCTION

The Path to Cytokinin and Auxin Discovery

The Greek philosopher Aristotle, a pioneer in natural science, defined four causes for the existence of biological forms. The efficient cause, or moving principle, represents "that thing as a result of whose presence something first comes into being" (7, book 5, section 1013a), and Aristotle thus anticipated the existence of form-promoting substances. In the nineteenth century, von Sachs (135) connected to this concept by suggesting the existence of organ-forming substances that were made by plants and that moved to different parts to control growth and development. At the same time, Darwin (23) postulated a moving substance to explain the phototropism of coleoptiles. Eventually, a bioassay based on Darwin's observations was used to identify a growth substance of low molecular weight, the plant hormone auxin (140). Auxin was later chemically characterized as indole-3-acetic acid (IAA) (129). Auxin's addition to culture media was "the touchstone of success" (128)

for the establishment of plant tissue cultures, as its activity in promoting cell division prevented the cultures from dying prematurely.

Van Overbeek (134) discovered in 1941 that, besides auxin, coconut milk promotes proliferation of plant tissue cultures; this was reminiscent of much earlier reports by Wiesner (144), who observed secreted substances that induced cell proliferation in wounded tissue, and by Haberlandt (42), who presented experimental evidence for cell-division-inducing substances and noted that nondividing potato parenchyma cells would revert to actively dividing ones in the presence of phloem sap. The efforts to pinpoint the cell-division activity of coconut milk culminated in the isolation of the first cytokinin, kinetin, in 1955 (84). *trans*-Zeatin was the first cytokinin to be isolated from an endogenous source, corn endosperm, in 1961 (82). Other cytokinins followed, purified from various plant species (86). Collectively, biologically active cytokinins represent a heterogeneous class of small, N6-substituted adenine derivatives with either an isoprene-derived or an aromatic side chain (50, 113, 117).

Diverse Cytokinin Functions

Since the initial discovery, a plethora of cytokinin biological functions have been discovered. Early observations of cytokinin functions included de novo organ formation from cultured tissues (118), stimulated leaf expansion and seed germination (83), delayed senescence in detached leaves (103), and release from apical dominance in shoots (143) and roots (13). In many cases, a relationship with auxin was found, giving the cytokinin-auxin relation classical status. Molecular mechanisms underlying these interactions have recently begun to be elucidated (11, 12, 26, 28, 40, 65, 67, 77, 88, 91, 97, 110, 150, 151) and are discussed in detail below.

Advanced molecular, genetic, biochemical, and genomic approaches have uncovered the diverse roles of cytokinin signaling in cell proliferation and differentiation, nodulation, nutrient status, circadian clocks, light responses, transitions to flowering, immunity, stress, and senescence (4, 5, 10, 19, 47, 86, 89, 90, 96, 115, 137, 142) (**Figure 1**). The diverse and specific expression patterns of genes involved in cytokinin biosynthesis and metabolism, such as cytokinin biosynthesis isopentenyltransferase (*IPT*), cytokinin nucleoside 5′-monophosphate phosphoribohydrolase (*LOG*), and cytokinin-degrading cytokinin oxidase/dehydrogenase (*CKX*), independently suggested a wide range of cytokinin functions from the ovule, embryo, primary and lateral root primordia, shoot meristem, and veins to flowers (50, 66, 85, 141). Specifically, the *Arabidopsis ipt1,3,5,7* mutant demonstrates cytokinins' function in cambial activity of the stem and root (80); the *log3,4,7* mutant exhibits reduced inflorescence size and flower numbers but enhanced lateral and adventitious roots (66), resembling *CKX* overexpression (141), whereas the *ckx3,5* mutant shows increased flower organ size and ovule numbers and consequently increased seed yield (9). Interesting and extensive connections between cytokinins and various nutrients, as well as the specificity of long-distance cytokinin transport through the xylem and phloem, are also emerging (4, 9, 50, 113, 142). Many microbes can manipulate plant cytokinin levels, which contributes to plant growth, pathogenesis, and immunity (4, 19, 137, 142).

THE CANONICAL CYTOKININ SIGNALING CIRCUITRY

Elucidating Cytokinin Signaling

Unlike other plant hormones, classical genetic screens based on plant growth phenotypes did not yield prominent mutants in cytokinin signaling for decades. A combination of gene-activation tagging strategy and large-scale tissue transformation facilitated the identification of *CYTOKININ-INDEPENDENT1* (*CKI1*), which conferred constitutive shoot regeneration (61). The CKI1 protein signatures are typical of a hybrid His kinase, comprising both His kinase–containing and response regulator–containing domains and suggesting CKI1's function in a phosphorelay system.

Cytokinin oxidase/dehydrogenases (CKXs): enzymes that irreversibly degrade active cytokinins into adenine or adenosine and side chains

CYTOKININ-INDEPENDENT1 (CKI1): hybrid His kinase of *Arabidopsis* that can autonomously activate cytokinin signaling; it is involved in female gametophyte and vascular cambium development

Response regulators: two-component signaling proteins that are receivers of the His-to-Asp phosphorelay from His kinases in bacteria, yeast, and plants

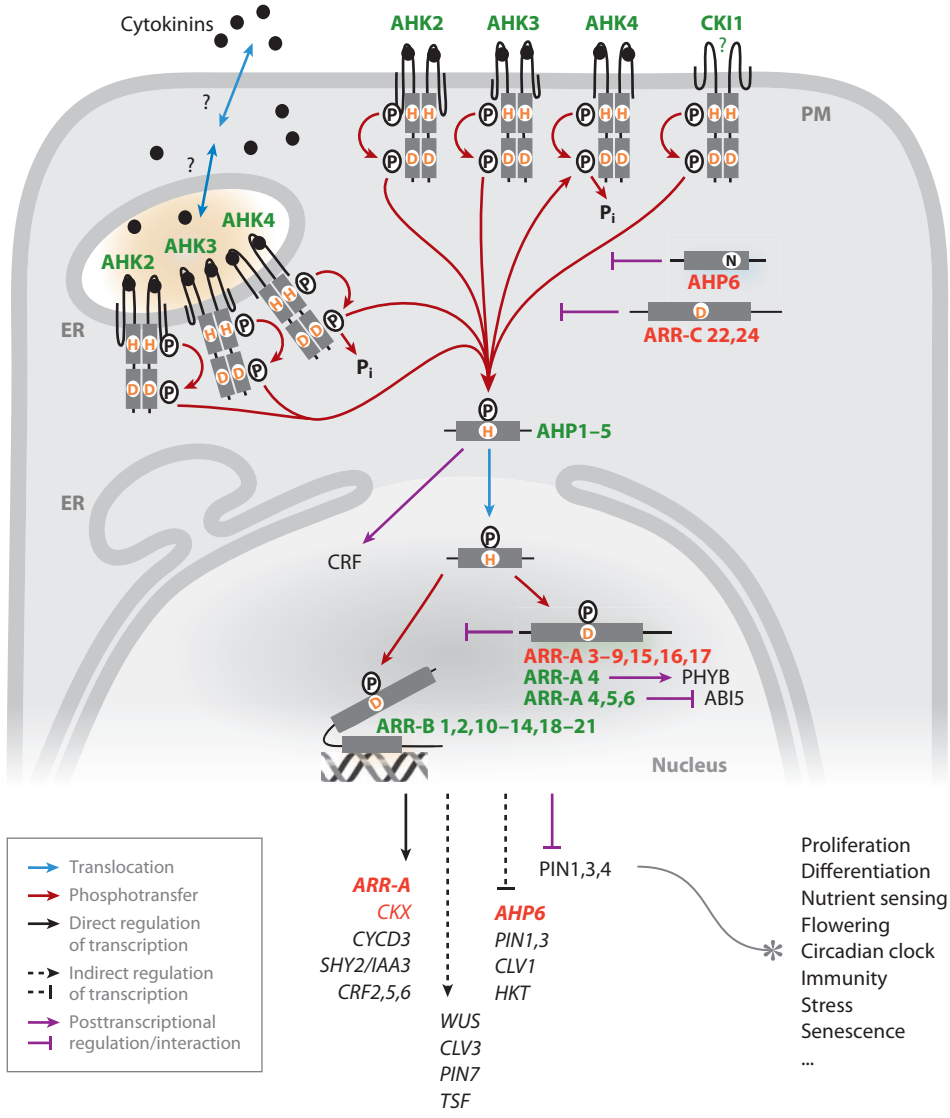


Figure 1

The core cytokinin signaling circuitry, showing *Arabidopsis* His kinases (AHKs), *Arabidopsis* His phosphotransfer proteins (AHPs), and *Arabidopsis* response regulators (ARRs) in a model cell. Conserved His and Asp residues, which accept a phosphoryl group (P), are indicated by orange H and D letters, respectively. Boldface indicates core signaling components, green indicates positive regulators of cytokinin signaling, and red indicates negative regulators. Selected connections to other signals and genes are indicated. Additional abbreviations: ARR-A/B/C, type-A/B/C *Arabidopsis* response regulator; ER, endoplasmic reticulum; CRF, cytokinin response factor; PM, plasma membrane.

This 1996 breakthrough reported by Kakimoto (61) initiated the molecular elucidation of the cytokinin signal transduction pathway in the following years.

Phosphorelay systems (also called two-component signaling systems) are prevalent in bacteria. In the simplest form, they consist of two conserved proteins: a His kinase sensor

and a response regulator protein that are phosphorylated at conserved His and Asp residues, respectively (54). Diverse signals triggering His kinase autophosphorylation and phosphotransfer from the His kinase to the response regulator result in activation of the latter and generation of the output responses. More complex versions of this two-component phosphotransfer involve hybrid His kinases and multiple phosphotransfer steps and often more than two proteins (5, 54, 89, 96, 142).

Further evidence supporting the use of a His-to-Asp phosphorelay system for cytokinin signaling came with the identification of additional *Arabidopsis* genes encoding conserved His kinase, His-containing phosphotransfer, and Asp-containing response regulator domains, such as *Arabidopsis* His kinases (AHKs), *Arabidopsis* His phosphotransfer proteins (AHPs), and *Arabidopsis* response regulators (ARRs) (54, 55, 86, 121). Notably, *Arabidopsis* and maize type-A response regulator genes were functionally characterized as primary cytokinin signaling targets (14, 114, 126). A genetic screen using the shoot-inducing activity of cytokinins in cultured tissue led to identification of the *cytokinin response1-1* (*cre1-1*) mutation, allelic to the previously characterized *woodenleg* (*wol*) mutation, which causes exclusive xylem differentiation defects without affecting other cell types in the root vasculature (57, 75). The affected gene codes for a cytokinin receptor, ARABIDOPSIS HISTIDINE KINASE4 (AHK4), that could respond to cytokinins in a heterologous system (47, 57, 122).

The completion of the *Arabidopsis* genome sequence allowed the systematic compilation of potential phosphorelay signaling components based on characteristic domain signatures (54). Functional demonstration of the cytokinin signaling circuitry in an *Arabidopsis* cellular system established the core logic of the pathway (55): Pathway activation is initiated by autophosphorylation at a conserved His residue of the hybrid His kinases in the N-terminal sensor-kinase domain, which is subsequently carried over to a conserved Asp of the C-terminal receiver domain (**Figure 1**). AHK2, AHK3, and AHK4/CRE1/WOL are activated by cytokinins via specific ligand binding to their transmembrane CHASE domains upstream of the His kinase domain. Plasma membrane–associated CKI1 possesses constitutive His kinase activity in plant cells, and its overexpression is sufficient to activate the entire cytokinin signaling pathway in cells and in planta (**Figure 1**). The signals converge on the AHPs (AHP1–5) to mediate the cytoplasmic-to-nuclear signal transfer (53–55, 61, 122) (**Figure 1**). The nuclear type-B ARRs (ARR1,2,10–14,18–21) as DNA-binding transcription factors directly promote the expression of nuclear type-A ARRs (ARR3–9,15–17) as primary cytokinin target genes and negative-feedback regulators (5, 53–55, 90, 111, 112) (**Figure 1**). Understanding the molecular details for ligand-sensor interactions, hybrid His kinase activation, cytoplasmic-to-nuclear translocation of AHPs, and diverse ARR actions will require advanced structural and detailed mutagenesis-functional studies in vitro and in vivo.

Two-Component Signaling Circuitry

Comprehensive genetic, transgenic, and biochemical analyses firmly established the two-component circuitry in cytokinin signaling in the past decade (**Figure 1**). Most single mutants in the two-component signaling circuitry cause no overt morphological phenotypes (4, 5, 89, 142), although the null *cki1* mutant is lethal in *Arabidopsis* (29, 45, 46, 99). Extensive analyses of the *ahk2*, *ahk3*, and *ahk4* mutants revealed their overlapping as well as specific functions in regulation of shoot, root, and embryo growth and of senescence. The *ahk2,3,4* triple mutants are viable with severe growth retardation and large seeds, but some of these *ahk* mutants are not truly null (47, 48, 63, 64, 89, 94, 104, 120). Two other *ahk2,3,4* triple-mutant allelic combinations with stronger phenotypes never set seeds, owing to the indispensable sporophytic roles that these receptors play in support of anther dehiscence, pollen maturation, and female gametophyte formation and

Arabidopsis His kinases (AHKs): hybrid His kinases that sense cytokinins via the cytokinin-binding CHASE domain

Arabidopsis His phosphotransfer proteins (AHPs): phosphorelay proteins connecting AHKs to ARRs by mediating His-to-Asp phosphotransfer from the cytoplasm to the nucleus

***woodenleg* (*wol*):** dominant-negative *AHK4* allele that causes exclusive xylem differentiation without other cell types in the root vasculature

CHASE domain: conserved domain implicated in cytokinin binding; the name is an abbreviation for cyclases/histidine kinases associated sensory extracellular

Type-B _Arabidopsis_ response regulators: An *ARR* gene family (*ARR1,2,10–14,18–21,23*) encoding DNA-binding transcription factors that mediate cytokinin-dependent transcriptional activation

Type-A _Arabidopsis_ response regulators: An *ARR* gene family (*ARR3–9,15–17*) encoding ARRs that are induced by cytokinins and function as negative regulators to form feedback regulatory loops

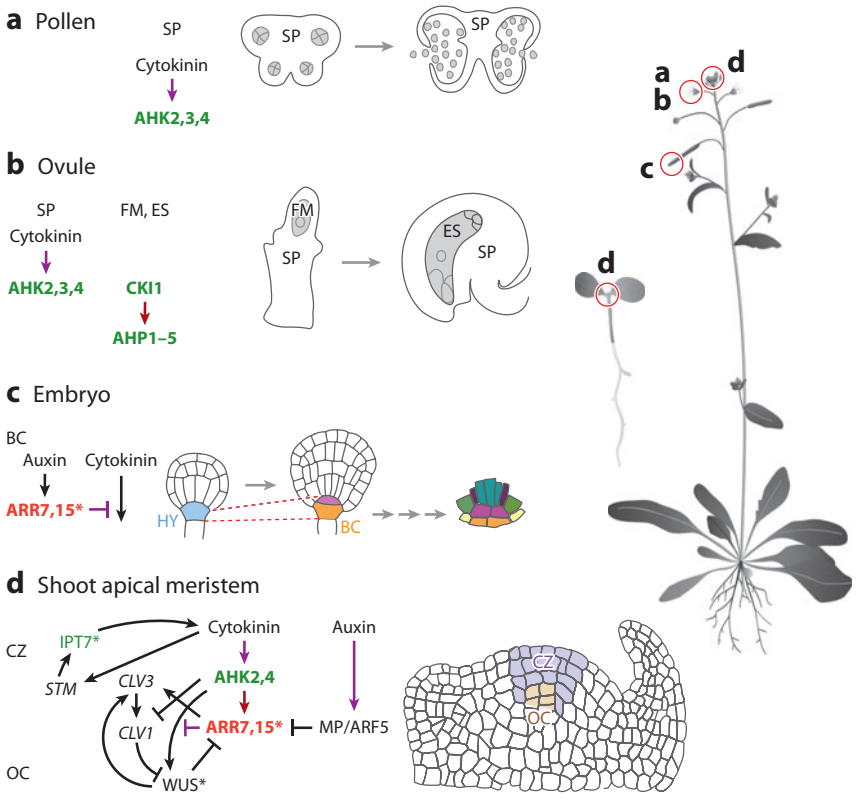


Figure 2

Developmental context of cytokinin functions and interactions with auxin: (*a*) pollen development, (*b*) ovule development, (*c*) embryo development, and (*d*) shoot apical meristem homeostasis. Boldface indicates core signaling components, green indicates positive regulators of cytokinin signaling, and red indicates negative regulators; black lines indicate transcriptional regulation or other interaction, purple indicates posttranscriptional interaction, brown indicates phosphorelay signaling, and gray indicates developmental process influenced. An asterisk indicates that both the transcription and protein function of a given gene are regulated, depending on specific interaction. Both an early and a very late stage of pollen (panel *a*) and embryo sac (ES; panel *b*) development are shown. The left side of each panel shows cytokinin signaling genes identified by genetic analysis. Panel *c* indicates auxin-dependent suppression of cytokinin output in the basal cell (BC) lineage after asymmetrical division of the hypophysis (HY); this suppression is required for correct establishment of the root meristem, as shown on the right, with different colors denoting the distinct stem-cell fates and precursor cells. Panel *d* indicates a vegetative or flower apical meristem with a central zone (CZ; *purple*) and organizing center (OC; *orange*). The left side of this panel shows the complex interaction network between cytokinins and auxin. Other abbreviations: AHK, *Arabidopsis* His kinase; AHP, *Arabidopsis* His phosphotransfer protein; ARR, *Arabidopsis* response regulator; FM, functional megaspore (haploid); SP, sporophytic parental tissue (diploid).

maturation (64). CKI1 function is essential for megagametogenesis (46, 99) (**Figure 2**). More detailed insights that support the physiological functions of CKI1 in mediating constitutive cytokinin signaling as well as cytokinin signaling during sporophyte development were recently provided by studies of an intriguing conditional *CKI1* allele, *cki1-8*; new RNA interference (RNAi) lines; and *CKI1* expression patterns (29, 45) (**Figure 2**). The emerging view is that AHKs and CKI1 contribute independently to eventual cytokinin signaling outputs in specific *Arabidopsis* organs. It will be important to define the regulatory inputs into

CKI1 expression and the role of its putative extracellular sensing domain to elucidate the origin of this novel signaling input.

AHK receptors have different ligand-binding affinities and expression patterns in *Arabidopsis* and maize, potentially contributing to their functional specificity (47, 63, 73, 89, 120). Indeed, genetic analyses confirmed specific functions for AHK3 and AHK4 in senescence and root development (27, 63, 77). However, no link between the genetic observations and a molecular mechanism has been established. Although CKI1–green fluorescent protein (GFP) is mainly localized to the plasma membrane (45, 55), recent reports suggest that *Arabidopsis* and maize His kinase–GFPs are also localized in the endoplasmic reticulum (17, 73, 145). Subcellular fractionation analyses of His kinase proteins and the association of specific cytokinin binding with endomembranes further support the endoplasmic reticulum locales for cytokinin receptors (**Figure 1**). This novel twist modifies previous signaling models and raises questions about how active ligands gain access to the intracellularly located sensing domains of the receptors and hormonal crosstalks (17, 73, 145). Future investigation of functional receptor locales and actions and of cytokinin transport, synthesis, and degradation in different subcellular compartments holds great promise for the discovery of previously unrecognized regulatory mechanisms (50, 113, 141).

Analyses of phosphorelay signaling, protein interactions, and higher-order loss-of-function mutations have shown that AHPs largely function redundantly and interact promiscuously with the receptors (29, 32, 53, 121). Phosphotransfer can be bidirectional. AHK4's intrinsic phosphatase activity, which predominates over kinase activity in the absence of a ligand, has been shown to hydrolyze phosphoryl groups on its receiver domain, depleting the circuitry of those groups (76). Of the six AHPs, AHP6 stands out for its lack of the conserved His residue; it is thus unable to accept a phosphoryl group and has been called pseudo-AHP. It negatively interferes with pathway activity, most likely by competing with AHP1–5 for interaction with the activated receptors. Because AHP6 expression is negatively regulated by cytokinin signaling, its function may contribute to the generation of sharper signaling boundaries within a tissue (76). From the AHPs, the phosphoryl group is passed over to the nuclear ARRs (**Figure 1**).

Transient expression analyses showed that type-B ARRs encode transcriptional activators, whereas type-A ARRs negatively interfere with pathway activity. Transcription of type-A ARRs is directly induced by the activated type-B ARRs, which establishes a negative-feedback loop to the pathway (55, 111, 112). This basic model was validated and extended by generating and analyzing higher-order loss-of-function mutations of the type-A ARRs (4, 5, 89, 131, 132, 142) and type-B ARRs (4–6, 58, 79, 89, 111, 142). These studies were complemented by overexpression experiments (62, 102, 125, 131) (**Figure 1**). The plants were analyzed primarily at the organismal level through use of morphological and physiological assays, which confirmed that type-B ARRs are positive regulators and type-A ARRs are negative regulators in cytokinin signaling.

Limitations and Complexity

Notwithstanding the great value of various genetic studies, including forward, reverse genetics, and activation tagging experiences, cytokinin signaling also revealed the limitations of these approaches. First, owing to redundancy, higher-order loss-of-function mutants have to be generated. Such an approach is not always practical, for example, owing to the absence of null mutants (29, 64), the large number of involved genes, or these genes' close linkage on the genome (4, 54, 89, 142). Second, the degree of genetic deficiency may manifest distinct phenotypes in different contexts, complicating data interpretation; in addition, using mutations that manifest their mutant defects starting from the gamete or the zygote may result in early lethality and unpredictable long-term effects or may trigger the activation of alternative developmental programs to

compensate for the perturbations. Third, although the core logic of the cytokinin signaling network has been elucidated and appears simple, the operating phosphorelay network reaches a dazzling complexity because of the multiple family members that each exert both redundant and specific functions (4, 5, 89, 142) and the numerous modes of crosstalk. For example, on the level of functional hybrid His kinases without CHASE domains (54), the putative osmosensing AHK1 (133), the cytokinin-independent CKI1 (29, 45, 46, 99) and CKI2/AHK5 (31, 59, 61), and the ethylene receptor family member ETR1 (43, 54) may feed the downstream system with activating phosphoryl groups and thus contribute to compensatory strategies to overcome restrictions imposed by mutations in the cytokinin core signaling components.

Transcriptional activation mediated by type-B ARRs is not the sole output of cytokinin signaling. For example, AHPs also directly interact with TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) transcription factors (123). Transcription-independent cytokinin responses can occur via ARR4–phytochrome B interaction, ARR5,7,15 stability, or regulation of PIN-FORMED (PIN) auxin efflux carriers (4, 5, 77, 102, 124, 131, 142, 150). The distinct C-type ARR22 is cytoplasmic and its transcription is not inducible by cytokinins, but it shows a stronger capacity than type-A ARRs to block cytokinin signaling (52, 62). Further complexity is added by the cytokinin response factors (CRFs), which were found to represent a branch of signaling parallel to that of type-B ARRs and to modulate overlapping target genes (5, 22, 101). Microarray analyses using wild-type and mutant seedlings, or cultured tissues, revealed multiple layers of complexity in the kinetics and tissue specificity of genes modulated by cytokinins (4, 5, 35, 49, 62, 101, 127, 142, 148) (**Figure 1**).

To increase resolution, many recent studies focused on a specific function of cytokinin signaling in particular cells, tissues, or organs during development. The inclusion of additional functional strategies to interfere with signaling—such as inducible transgenes to generate conditional mutants, dominantly acting signaling components, or pharmacological treatments—circumvents issues of lethality and pleiotropy. The following sections of this review focus on recent molecular and mechanistic findings that describe how the core cytokinin regulatory circuitry integrates into the signaling networks to function in controlling the development of diverse organs as well as in immunity, stress tolerance, and senescence.

PIN-FORMED (PIN): auxin efflux carrier protein; expression and subcellular localization of PIN proteins determine auxin distribution and regulate auxin-dependent developmental processes

Hypophysis: basal cell–derived founder cell of the primary root meristem

CYTOKININ AND AUXIN CROSSTALK IN EMBRYOGENESIS

Cytokinin Signaling in Early Embryogenesis

Based on classical experiments in cultured tissue, a relative abundance of auxin is associated with de novo development of root identity, whereas an excess of cytokinin promotes shoot development (118). Similarly, the first establishment of root identity during embryogenesis requires auxin signaling (87). However, the lack of overt patterning defects in *ahk2,3,4* mutant embryos suggested that cytokinins are dispensable for the development of the apical basal axis during early development (48, 94, 104).

To address the potential role of cytokinin signaling during embryogenesis, Müller & Sheen created a generic GFP-based two-component sensor (*TCS::GFP*) to monitor the transcriptional output of the cytokinin signaling circuitry in planta (91). The first distinct GFP signal appears at the 16-cell stage in the founder of the root stem cells, the hypophysis. By the transition stage, the asymmetrical division of the hypophysis has generated the apical lens-shaped cell and a basal cell, which abolishes *TCS::GFP* expression. A separate phosphorelay output occurs during the heart stage at the shoot stem-cell precursors. A transcriptional profile of all putative phosphorelay signaling components at the early embryonic heart stage revealed expression of *AHK4*, *AHP2,3,5*, and type-A and

type-B *ARR* genes. These findings provide the first evidence for potential cytokinin signaling during early embryogenesis (91).

Cytokinin and Auxin Signaling Crosstalk in Embryonic Root Stem-Cell Specification

Among the cytokinin signaling components, a nuclear repressor of cytokinin signaling and well-established immediate-early target gene, *ARR7*, is strongly expressed in early embryos based on *ARR7::GFP* reporter gene expression and mRNA in situ hybridizations. Unexpectedly, the *ARR7::GFP* expression pattern after the hypophysis division is reminiscent of the auxin signaling domain revealed by the activity of the *DR5::GFP* synthetic reporter, but not that of *TCS::GFP*. Expression of *ARR15*, the closely related sister gene of *ARR7*, shows a similar pattern, detected by *ARR15::GFP* in the embryo. Further analyses revealed the surprising induction of *ARR7* and *ARR15* by auxin to attenuate cytokinin output in the basal cell of the embryonic root. Promoter mutant characterization allows the uncoupling of cytokinin- and auxin-mediated activation of *ARR7* and *ARR15* in the embryo. Conditionally eliminating *ARR7* and *ARR15* functions during a critical period in the ontogenesis of the root stem-cell system—i.e., when the hypophysis undergoes an asymmetric cell division—results in ectopic cytokinin signaling in the auxin domain and consequently a defective stem-cell system with aberrant expression of the transcription factor marker genes *SCARECROW*, *PLETHORA1*, and *WUSCHEL RELATED HOMEOBOX5* (91). Thus, auxin signaling antagonizes cytokinin signaling in a temporally and spatially defined domain by inducing type-A ARR negative regulators, which suppresses the cytokinin output (**Figure 2*c***). The characterization of the various *arr7,15* double mutants with different degrees of genetic deficiency remains to be clarified (69, 150, 151).

A similar connection between auxin signaling and type-A response regulators has also been documented in rice roots, where auxin induces *OsRR1* via the AP2/ERF transcription factor CROWN ROOTLESS5 to allow the initiation of crown root primordia in rice seedlings (65). The antagonistic relation between cytokinins and auxin in organ formation, as initially reported (118), appears recapitulated during endogenous development: Root development requires that auxin actively repress cytokinin output at a specific stage. In this model, eliminating positive regulators of signaling, such as AHK receptors or AHPs, is not expected to have dramatic consequences. Nevertheless, the embryonic root patterning phenotype in the true null *ahk2,3,4* and *ahp1,2,3,4,5* mutants, if viable, deserves further investigation (29, 64). It is still unclear whether cytokinin signaling exerts a positive function in specifying the embryonic shoot apical meristem, as could be expected in analogy with cytokinins' role in promoting ectopic shoot formation in cultured tissue (18, 118). Based on the *TCS::GFP* expression pattern, cytokinin signaling appears after the key marker genes in shoot meristem specification—i.e., *WUSCHEL* (*WUS*) or *SHOOTMERISTEMLESS* (*STM*)—are expressed (1). Cytokinins may be required mainly for shoot apical meristem homeostasis and/or maintenance, as it alone cannot trigger ectopic shoot formation in cultured tissue, but requires auxin above a critical threshold as demonstrated in tissue culture experiments (18, 61, 97, 118).

DISTINCT CYTOKININ AND AUXIN INTERACTIONS IN THE ROOT MERISTEM

Cytokinin and Auxin Interactions in the Primary Root Apical Meristem

After the initial establishment of the primary root stem-cell system during embryogenesis, complex gene networks ensure the operation of the root apical meristem in postembryonic root growth and development. The proximal root meristem, derived from stem cells, proliferates and expands rapidly in the first 5 days after germination to reach a steady-state meristem size by balancing distinct activities; these activities

are confined to morphologically distinguishable zones, namely the proliferating proximal meristem, the transition zone, and the elongation/differentiation zone (**Figure 3*a***). Comprehensive genetic, phenotypic, and molecular analyses in *Arabidopsis* revealed that cytokinins and auxin play key roles in the control of cell division and differentiation in the primary root apical meristem (10, 96).

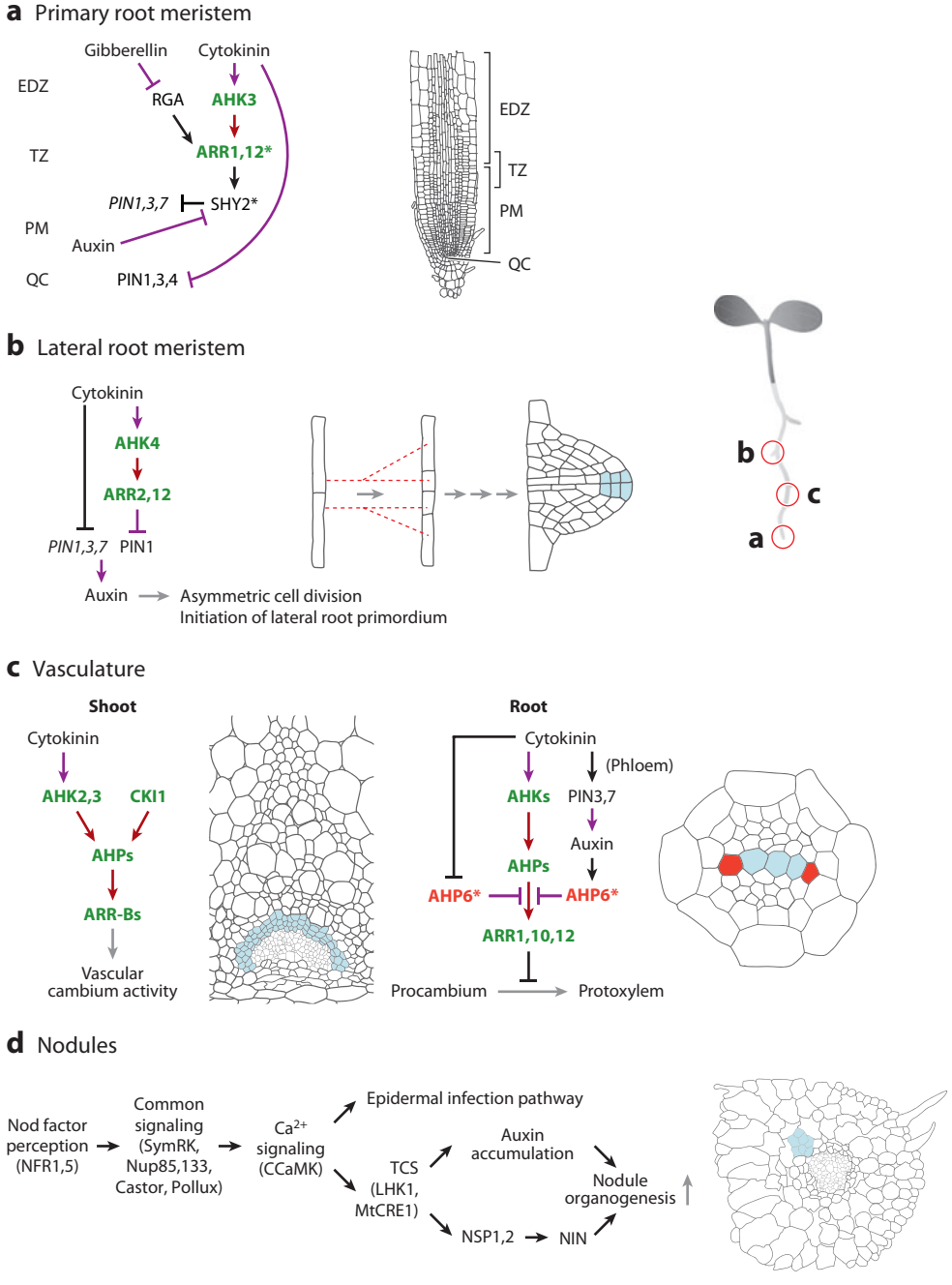
Exogenously applied cytokinins reduce the meristem size and the primary root growth, whereas the cytokinin synthesis (*ipt3*,*5*,*7*) and signaling (*ahk3*, *arr1*,*12*) mutants and ectopic CKX expression to enhance cytokinin catabolism in the root transition zone promote longer primary roots. In contrast, auxin can exert a positive influence at very low concentrations on primary root growth (27, 110, 141). A molecular connection between cytokinins and auxin has emerged with the identification of IAA3/SHY2, a negative regulator of auxin signaling, as a direct transcriptional target of AHK3-ARR1,12 cytokinin signaling (28, 127). Thus, via IAA3/SHY2 upregulation, cytokinin signaling attenuates auxin signaling and hence cell proliferation. However, during the root meristem expansion phase, gibberellin represses *ARR1* expression via REPRESSOR OF GA1, which in turn limits *IAA3/SHY2* expression and increases auxin signaling levels (88). Consequently, cytokinin signaling suppresses auxin signaling, which alters the expression of the auxin efflux carrier PIN genes—*PIN1*, *PIN3*, and *PIN7*—to control cell-to-cell auxin transport, redistribution, and downstream signaling (28, 110) (**Figure 3*a***).

Auxin-induced organogenesis in vitro is also modulated by cytokinins, via differential regulation of *PIN* genes and proteins (97). A recent study (150) with the *Arabidopsis* octuple *arr3*,*4*,*5*,*6*,*7*,*8*,*9*,*15* mutant, which has partial defects in 8 out of 10 type-A ARR genes encoding nuclear repressors and results in elevated endogenous cytokinin signaling, showed reduced PIN1-GFP, PIN3-GFP, and PIN4-GFP fusion protein levels in the

Figure 3

The roles of cytokinins in organ proliferation and differentiation: (*a*) primary root meristem development, (*b*) lateral root meristem initiation, (*c*) vasculature development, and (*d*) nodule organogenesis. Panel *a* shows the regulatory relationships between cytokinins and auxin or gibberellin. During lateral root meristem initiation (panel *b*), the asymmetric cell divisions of pericycle-derived founder cells represent the critical phase, during which ectopic cytokinin signaling abolishes auxin-dependent establishment of a lateral root primordium (*right side of figure*) with an auxin signaling maximum at the tip of the primordium (*blue shading*). During root vasculature development (panel *c*), cytokinin signaling is required for maintenance of procambial cells and suppresses the expression of the cytokinin signaling inhibitor *AHP6* (*red*) in the procambial cells flanking the xylem axis (*blue*). Phloem-transported cytokinins direct auxin flow into the xylem axis by modulating the distribution of PIN3 and PIN7. A high auxin level promotes expression of *AHP6* (*red*) at the xylem axis, which specifies the differentiation of the protoxylem. In the inflorescence stem (panel *c*), the cytokinin signaling from constitutively active CKI1 and cytokinin-activated AHK2 and AHK3 are integrated into the phosphorelay cascades to maintain the activity of the vascular (pro)cambium (*blue*). During nodule organogenesis (panel *d*), Nod factor–activated NFR1 and NFR5 in the epidermis initiate the shared signaling cascade via CCaMK. The epidermal infection pathway then diverges from the cortex organogenic pathway, which is mediated by cytokinin signaling and progresses through the transcriptional factors NSP1, NSP2, and NIN in delimited cortex cells (*blue*). Cytokinin signaling suppresses polar auxin transport, resulting in auxin accumulation to promote nodule organogenesis. Boldface indicates core signaling components, green indicates positive regulators of cytokinin signaling, and red indicates negative regulators; black lines indicate transcriptional regulation or other interaction, purple indicates posttranscriptional interaction, brown indicates phosphorelay signaling, and gray indicates developmental process influenced. An asterisk indicates that both the transcription and protein function of a given gene are regulated, depending on specific interaction. Abbreviations: AHK, *Arabidopsis* His kinase; AHP, *Arabidopsis* His phosphotransfer protein; ARR, *Arabidopsis* response regulator; EDZ, elongation/differentiation zone; PM, proximal meristem; QC, quiescent center; TZ, transition zone.

seedling root tip. The authors observed a similar effect after treatment of wild-type roots with exogenous cytokinins; however, transcript levels of *PIN1*, *PIN3*, and *PIN4* were not strongly affected. Thus, it is suggested that cytokinins alter PIN abundance at the posttranscriptional level and affect auxin signaling maxima in the quiescent center (150), which is spatially uncoupled from *IAA3/SHY2* transcription repression in the upper meristem,



Quiescent center: four cells that represent the stem-cell niche of the root meristem

transition zone, and elongation/differentiation zone (28) (**Figure 3*a***). Understanding the precise functional correlations of the complex spatiotemporal patterns and dynamics of *PIN* gene and protein regulation by cytokinins will require future efforts (28, 97, 110, 150).

Cytokinin Regulation of Lateral Root Initiation

Lateral root development involves de novo establishment of a meristem from root pericycle founder cells adjacent to the xylem poles. Targeted elevation of cytokinin levels in these cells perturbs their asymmetric cell division and consequently the establishment of an auxin gradient, which disrupts lateral root initiation. Interestingly, ectopic cytokinin signaling at later stages does not abolish lateral root development (67). Thus, there is a parallel with the embryonic establishment of the primary root meristem, where ectopic cytokinin signaling interferes with the asymmetrical division of the hypophysis, which aborts further development of the meristem, whereas ectopic cytokinins at later stages are tolerated (91). The mechanisms of cytokinin-auxin interaction are different, however. During lateral root initiation, cytokinins perturb auxin partly by altering *PIN1,3,7* transcription, which affects PIN-dependent lateral root initiation (67). In addition, recent findings show that cytokinin signaling mediated by the receptor AHK4/CRE1/WOL and the type-B ARR2 and ARR12 also control PIN1 localization. Perturbation of cytokinin perception leads to an altered endocytic trafficking of PIN1 in pericycle cells, as excess cytokinin depletes active PIN1 by redirecting it for lytic degradation in vacuoles. Interestingly, the effect persists in the presence of pharmacological transcription inhibition, and thus reveals a novel activity of cytokinin output by an unknown mechanism that specifically requires ARR2 and ARR12 but is independent of their well-documented activity as transcriptional activators (77) (**Figure 3*b***). The finding may be reminiscent of cytokinins' effect on PIN proteins in the primary root (150) (**Figure 3*a***) and could thus represent a more widespread model of how cytokinins antagonize auxin function.

COMPLEX CYTOKININ SIGNALING IN THE SHOOT MERISTEM

Multiple Layers of Cytokinin Regulation in the Shoot Apical Meristem

Conceptually similar to the root apical meristem, the shoot apical meristem is a dynamic structure with a stable organization, depending on an intricate balance of self-renewal to maintain a population of stem cells and cell recruitment out of the meristem into developing organs. In the central zone, the expression domain of the homeodomain transcription factor *WUS* defines the organizing center, which is functionally equivalent to the quiescent center of the root apical meristem and promotes stem-cell identity in overlying cells. This stem-cell population in turn restricts the *WUS*-expressing domain in a negative-feedback loop via the CLAVATA1,2 (CLV1/2) receptor signaling pathways to maintain cell populations in both the organizing center and *CLV3*-expressing stem cells (1) (**Figure 2*d***).

A role for cytokinins in shoot development was anticipated based on the capacity of exogenous cytokinins to induce ectopic shoots from cultured tissue (18, 118). In agreement with these early observations, experiments reveal that loss of endogenous cytokinin signals and signaling correlates with a reduced meristem size, whereas enhanced cytokinin action stimulates meristem activity. For example, the *Arabidopsis ahk2,3,4* receptor mutants exhibit a reduced shoot apical meristem size (48, 94), whereas the *ckx3,5* mutants increase cytokinin levels and form larger shoot apical meristems (9). Cytokinins' proliferation-inducing effect in the shoot apical meristem is in agreement with its classical role in tissue culture, and is associated with an upregulation of cell-cycle-promoting genes such as *CYCLIN D3* (105).

Differential changes in cytokinin signaling can also affect phyllotaxis, the regular arrangement of lateral organs around the main axis (37, 69, 151).

Details on how cytokinins integrate with the key genes operating in the shoot apical meristem have started to emerge. WUS represses transcription of several type-A *ARR*s, enhancing cytokinin perception in its domain (69). In addition, cytokinins induce *WUS* expression via AHK2 and AHK4 but repress *CLV1*, which encodes a receptor kinase for CLV3 peptide signaling to suppress *WUS* expression in the shoot apical meristem (9, 40). As the regulation of *WUS* occurs via both the CLV-dependent and independent pathways (40), multiple feedback loops through cytokinin signaling are installed to reinforce *WUS* expression and cytokinin output in the organizing center. Indeed, *TCS::GFP* expression, indicative of cytokinin signaling activity, peaks in the *WUS* expression domain (40). A positive-feedback loop also exists between cytokinins and *Arabidopsis STM*, which is expressed throughout the shoot apical meristem and prevents the cells from differentiating prematurely. STM promotes cytokinin biosynthesis by inducing transcription of *IPT*7, thus enhancing cytokinin signaling (147). This in turn further activates *STM* transcription (109) (**Figure 2*d***).

Cytokinin and Auxin Interplays in the Shoot Apical Meristem

A direct link between cytokinin and auxin signaling in the inflorescence apical meristem is established by auxin repression of *ARR7* and *ARR15* transcription via direct MONOPTEROS (MP)/AUXIN RESPONSE FACTOR 5 (ARF5) binding to the promoter in the central zone, leading to an increase in cytokinin signaling (151). As ARR7 and ARR15 are required for *CLV3* expression, cytokinins and auxin act together via distinct mechanisms to promote *WUS* expression in the inflorescence apical meristem (151). Investigations with maize shoots suggest that auxin or its transport is required for the expression of the maize type-A response regulator gene *ABERRANT PHYLLOTAXY1* (*ABPH1*). ABPH1 has distinct roles as a negative regulator of shoot apical meristem size and as a positive regulator of *PIN1* expression and auxin levels (68). These studies reveal complex cytokinin and auxin interactions in various shoot apical meristem niches in response to different cues (**Figure 2*d***).

Auxin response factor (ARF): gene family encoding DNA-binding transcription factors that mediate auxin-dependent transcriptional activation or repression

DYNAMIC CYTOKININ SIGNALING IN VASCULAR MORPHOGENESIS

Dual Cytokinin Signaling Inputs from Distinct His Kinases

Cytokinins have recently reemerged as key regulators for vasculature development in procambium maintenance and protoxylem differentiation (2, 45, 75, 80, 93). Reduction of endogenous cytokinins in *Arabidopsis* and *Populus* by ectopic expression of *CKX* genes to promote cytokinin catabolism results in the exclusive formation of protoxylem in root vascular bundles and abnormal development of shoot vascular tissue (45, 76, 80, 93). Furthermore, the *Arabidopsis ipt1,3,5,7* mutant lacking four cytokinin biosynthesis genes does not form cambia and exhibits reduced radial thickness of the root and stem, which can be rescued by exogenous *trans*-zeatin to reactivate the cambium in a dose-dependent manner (80).

The *wol* mutant, a dominant-negative allele of *AHK4/CRE1* with a point mutation in the CHASE domain presumably blocking signaling from multiple His kinases, has a reduced number of procambial cells. Moreover, the vascular cylinder of its primary root consists solely of protoxylem vessels (75, 76). These phenotypes are also observed in the receptor *ahk2,3,4* mutant and the type-B ARR *arr1,10,12* signaling mutant, supporting the essential roles of cytokinin signaling in early procambial cell divisions and differentiations during vascular morphogenesis (75, 148). The *ahp6* mutant, a suppressor of *wol*, partially restores the *wol* defects in vascular bundle

development (74). *AHP6* acts as a cytokinin signaling inhibitor without the His residue by competing with AHP1–5 to prevent transferring phosphoryl groups from His kinases to type-B ARRs, and its expression is suppressed by cytokinins (**Figure 1**). It is evident that the balance between positive and negative regulators is required for the proper patterning of protoxylem vessel formation and maintenance of procambial cell identity. In general, it is postulated that cytokinins promote the proliferation of vascular cambial cells and maintenance of their identities, but suppress protoxylem differentiation in roots (**Figure 3*c***).

Functional analysis of the unique His kinase *CKI1* in shoots further confirms the crucial role of cytokinin signaling in vascular morphogenesis (45). Although null *cki1* mutants are lethal, early clues from transgenic *CKI1* overexpression suggest that its constitutive His kinase activity promotes constitutive activation of cytokinin signaling, such as enhancing proliferation and greening of hypocotyl callus and delaying leaf senescence in the absence of exogenous cytokinins (55, 61). Interestingly, *CKI1* is specifically expressed in the procambium cells of inflorescence stems, and this expression pattern is similar to that of *AHK2* and *AHK3* (45, 94). The loss-of-function *cki1*, *ahk2*, and *ahk3* mutants (but not the *ahk4* mutant) consistently display abnormality in the procambium cell files of inflorescence stems. Furthermore, ectopic expression of CKI1 could partially rescue the defects in growth and vascular development of *ahk2,3* mutants (45). CKI1-mediated signaling output appears to integrate with the AHK2,3-mediated cytokinin signaling pathway, and both are necessary for the proliferation and maintenance of procambial cells (**Figure 3*c***).

Cytokinin and Auxin Antagonism in Xylem and Phloem Differentiation

The mutually inhibitory interactions between cytokinins and auxin have been considered a homeostatic regulatory mechanism for multiple plant organogenesis processes, including vascular morphogenesis. Some of these physiological interactions appear to be caused by the long-distance and cell-to-cell unidirectional transports of cytokinins and auxin at specific developmental stages (10, 11, 27, 28, 67, 91, 96). Recent findings using novel approaches and tools support the indispensable role of long-distance basipetal transport of cytokinins through the phloem in bisymmetric vascular pattern formation via control of polar auxin transport in roots (11, 12). Although exogenous cytokinins reduce *PIN1* and *PIN3* expression, and PIN1-GFP and PIN4-GFP proteins in the root meristem near the root tip, the expression patterns of the PIN3-GFP reporter are more complex (11, 12, 28, 110, 150). Surprisingly, expression of PIN3-GFP or PIN7-GFP is barely detected in the dominant-negative *wol* mutant, which suppresses cytokinin signaling and therefore lacks phloem and nonprotoxylem cells. Exogenous cytokinin or constitutive cytokinin signaling from CKI1 enhances PIN7-GFP levels in the intervening procambial cells and phloem initials and expands its expression to the protoxylem. Careful high-resolution and cell-specific examinations have uncovered the requirement of cytokinin signaling for the precise and distinct radial patterning of PIN1, PIN3, and PIN7. Thus, cytokinins alter the distinct bisymmetric distribution patterns of PIN3 and PIN7 to channel auxin toward a central domain in the root. The mutually inhibitory feedback loop between cytokinins and auxin sets distinct boundaries of hormonal output and phloem and xylem differentiation in the root meristem toward the elongation and differentiation zones. Higher auxin signaling at the xylem axis suppresses cytokinin signaling via activation of *AHP6* expression as a negative regulator, and the lowered cytokinin signaling output specifies the protoxylem identity. The dominant auxin-insensitive mutant *axr3* consistently lacks protoxylem (11, 12) (**Figure 3*c***). The molecular basis underlying the differential roles of AHK4/CRE1/WOL and AHK3 in cytokinin signaling and PIN regulation in the controls of root meristem size and vascular patterns deserves further investigation (11, 12, 27, 28, 110).

THE PIVOTAL ROLE OF CYTOKININ SIGNALING IN NODULE ORGANOGENESIS

Cytokinin Induction of Root Nodule Primordia in the Root Cortex

Cytokinins are key signaling molecules for morphogenesis of nitrogen-fixing nodules in symbiotic interactions with rhizobium bacteria. One of the earliest pieces of evidence for cytokinin action in nodule development was the morphogenetic rescue of nodule formation with nonsymbiotic bacteria carrying the cytokinin biosynthesis *IPT* gene of *Agrobacterium tumefaciens* in *Medicago sativa* (21). The *Arabidopsis* cytokinin primary responsive *ARR5* promoter is activated during nodulation processes, and overexpression of *CKX* to promote cytokinin catabolism results in decreased nodule organogenesis in *Lotus japonicus* (72). Recent genetic studies further support the functional role of cytokinins as positive regulators in nodulation. The suppression of *Medicago truncatula* CRE1 (MtCRE1) His kinase results in severely defective nodule formation (39). Loss-of-function *hyperinfected1* (*hit1*) mutations in lotus His kinase 1 (LHK1), a cytokinin receptor of *Lotus japonicus*, completely abolish nodule primordium development but do not affect infection thread formation (92). The gain-of-function *spontaneous nodules formed2* (*snf2*) mutant of LHK1 develops root nodules spontaneously in the absence of rhizobia and exhibits constitutive cytokinin signaling responses (130). Exogenous cytokinins also induce cortical cell division and activate expression of nodulation genes (21, 25). These findings collectively indicate that cytokinin signaling is essential to initiate nodule organogenesis.

How is cytokinin signaling integrated into the Nod factor signaling pathway for the regulation of nodule development? Nodulation signaling is completely blocked in Nod factor receptor *nfr1* and *nfr5* mutants, the *symbiosis receptor kinase* (*symrk*) mutant lacking Ca^{2+} spiking, the Ca^{2+}/calmodulin-dependent protein kinase *ccamk* mutant, and transcriptional factor *nodule inception* (*nin*) and *nodulation signaling pathway2* (*nsp2*) mutants of *Lotus japonicus* (34). Introducing the constitutive cytokinin signaling *snf2* mutation into the *nfr1*, *nfr5*, *symrk*, or *ccamk* background rescues aborted nodulation and leads to spontaneous nodule formation. However, the *snf2* mutation does not recover defective nodulation in the *nin* or *nsp2* background (130). The expression of critical early-nodulation gene *NIN* is induced by cytokinins and Nod factor perception and upregulated in *snf2* but is completely blocked in the His kinase mutants *cre1* of *Medicago truncatula* and *hit1* of *Lotus japonicus*, respectively (39, 92, 100, 130). Exogenous cytokinins trigger the nodule organogenic pathway in wild-type lotus and *nfr1*, *nfr5*, *symrk*, *nucleoporin85* (*nup85*), *nup133*, *castor*, *pollux*, and *ccamk* mutants, but fail to induce nodule primordia in *nin*, *nsp1*, *nsp2*, and *hit1* mutants (44). These results support a signaling model leading from NFR1-NFR5-mediated Nod factor perception and calcium signature decoding through CCaMK to a cytokinin-receptor-mediated cortex-dividing signaling cue through the NIN and NSP transcriptional regulators. However, downstream of the shared CCaMK, the cytokinin-mediated organogenic pathway in cortex cells acts in parallel with the epidermal infection thread pathway to coordinate bacterial nodule infection by unknown cross-signaling mechanisms (44, 130) (**Figure 3*d***).

Cytokinin and Auxin Synergism in Nodule Proliferation

The MtCRE1 His kinase regulates the expression of a subset of *MtPIN*s encoding auxin efflux carriers, resulting in cytokinin-dependent auxin accumulation in the developing nodule primordium in *Medicago truncatula*. It has been known that bacterial Nod factors induce auxin accumulation by inhibiting polar auxin transport in dividing pericycle and cortex cells, and the latter is critical for nodule initiation (51, 100, 139). Treatments of auxin transport inhibitors also lead to pseudonodule formation and nodulation gene expression (51). Cytokinins appear to act synergistically with

Infection thread: host-driven structure that guides the entry of rhizobia into the root cortex

Nod factor: signaling molecule secreted by rhizobia during an early stage of nodule symbiosis, usually composed of lipochitin oligosaccharides

Nod factor receptor 1 and 5 (NFR1/5): LysM-type Ser/Thr receptor-like kinases that perceive Nod factors

Symbiosis receptor kinase (SYMRK): leucine-rich-repeat receptor-like kinase that functions downstream of NFR1

Ca^{2+}/calmodulin-dependent protein kinase (CCaMK): Ca^{2+}/calmodulin-regulated Ser/Thr protein kinase that relays calcium spiking to downstream signaling cascade

CASTOR and POLLUX: cation channels required for symbiosis locating in the nuclear envelope membrane

the auxin signaling pathway to promote nodule organogenesis instead of lateral root formation (**Figure 3**). Elucidation of the cytokinin origin, spatiotemporal regulation of cytokinin signaling, and integration with other hormonal controls and long-distance signals during symbiotic interactions remain the next challenges.

Salicylic acid: monohydroxybenzoic acid produced by plants that plays an indispensable role in immune response against biotrophic pathogens

***PATHOGENESIS-RELATED (PR)* genes:** heterogeneous group of genes induced in plants by pathogen infection and exogenous chemicals

NOVEL CYTOKININ FUNCTIONS IN IMMUNITY

Besides their critical functions in plant growth and development, cytokinins also play pivotal roles in plant defense and stress responses. Many biotrophic pathogens induce green bionissia in leaves, or dedifferentiation of infected cells to form gall-like structures. These green islands, or dedifferentiated and proliferating cells, have strong sink activity to support pathogen growth. Gall-forming pathogens such as *Rhodococcus fascians*, *Agrobacterium tumefaciens*, and *Plasmodiophora brassicae* produce cytokinins or utilize plant cytokinins to generate gall structures, which are indispensable for their pathogenicity (4, 19, 113, 119, 142). It has been suggested that cytokinins suppress plant immunity to biotrophic pathogens (137). However, recent studies indicate that auxin, rather than cytokinins, might be critical for the suppression of plant defense response (30).

What is the role of plant-derived cytokinins in general plant immunity beyond the specialized biotrophic pathogens? A recent study elucidated a direct effect of cytokinins on defense response by employing *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), a bacterial pathogen that does not secrete cytokinins (20). In this system, endogenous cytokinins perceived by AHK2 and AHK3 receptors promote salicylic acid signaling through ARR2 activation and association with the promoters of *PATHOGENESIS-RELATED* (*PR*) genes, which lead to enhanced plant immunity. However, cytokinin-induced defense response requires active salicylic acid signaling, as the salicylic acid–activated transcription factor TGA1A-related gene 3 (TGA3) specifically interacts with ARR2 and recruits ARR2 to the promoter of defense genes. Overexpression of ARR2 results in the activation of not only *PR* genes but also major regulators of salicylic acid and effector-triggered immune response, such as the salicylic acid biosynthetic gene *SALICYLIC ACID INDUCTION–DEFICIENT 2* (*SID2*), the *WRKY DNA-BINDING PROTEIN 18* (*WRKY18*) transcription factor, and the lipase-like *PHYTOALEXIN-DEFICIENT 4* (*PAD4*), which function upstream of salicylic acid accumulation during effector-triggered immune response (20) (**Figure 4**).

In the *uni-1D* gain-of-function mutant of a putative disease-resistance-related gene, salicylic acid–dependent *PR* gene induction is correlated with elevated cytokinin content, which implies that cytokinins have a role in activating effector-triggered immune response and salicylic acid signaling (56) (**Figure 4**). In cytokinin-regulated immune response against the biotrophic pathogen *Hyaloperonospora arabidopsidis* isolate Noco2, the susceptibility varies depending on exogenous cytokinin levels, and type-A ARRs are involved in determining the dose-dependent effect of cytokinins on plant immunity (J.J. Kieber, personal communication). Interestingly, the cytokinin-induced AP2/ERF-type transcription factor CRF5 also regulates the expression of *PR* genes, and *CRF5*-overexpressing plants show enhanced resistance to *P. syringae* (70) (**Figure 4**). These studies suggest the important role of cytokinins in transcriptional regulation during plant defense response. Cytokinins are enriched in the shoot apical meristem, maintenance of which is critical for plant development. Cytokinin-induced defense mechanisms may have evolved to protect these tissues and maintain their proliferation potential at the same time.

DUAL ROLES OF CYTOKININS IN ABIOTIC STRESS

Cytokinin homeostasis and signaling are rapidly altered under various water-deficit conditions. For example, studies have shown that cytokinin contents and transport are reduced by drought and/or salinity in various plant species

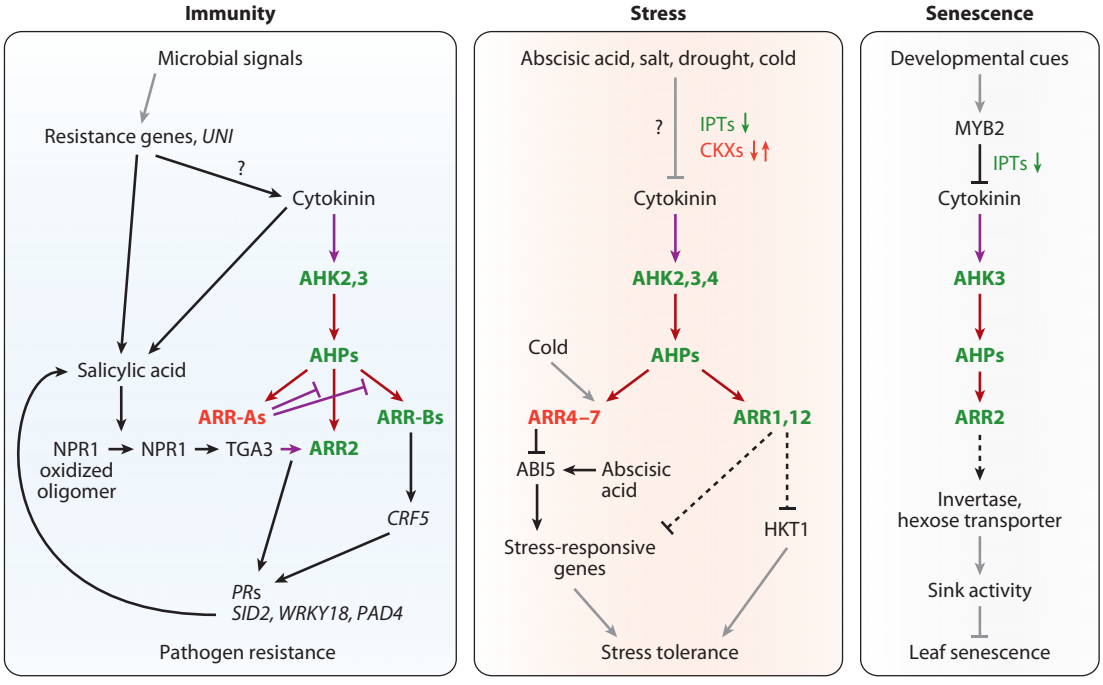


Figure 4

Cytokinin actions in plant immunity, stress tolerance, and senescence. Boldface indicates core signaling components, green indicates positive regulators of cytokinin signaling, and red indicates negative regulators; black lines indicate transcriptional regulation or other interaction, purple indicates posttranscriptional interaction, brown indicates phosphorelay signaling, and gray indicates developmental process influenced. Activation of resistance genes (e.g., *UNI*) may lead to the accumulation of cytokinins, which in turn enhance salicylic acid production and the expression of defense-related genes via interaction between ARR2 and NPR1-activated TGA3 transcription factors. Environmental stresses and abscisic acid may suppress cytokinin contents and signaling. Two-component signaling cascades negatively regulate stress adaptation, acting through a set of type-A *Arabidopsis* response regulators (ARRs), which interact directly with ABI5 to negatively control abscisic acid signaling. ARR1 and ARR12 attenuate salt stress tolerance by suppressing the potassium transporter gene *HKT1*. Different developmental cues regulate the level of endogenous cytokinins through MYB2 repression of the cytokinin biosynthesis *IPT* genes. Specific activation of AHK3 by cytokinins mediates delay of leaf senescence via activation of ARR2. Cytokinins enhance sink activity by regulating invertase and hexose transporter activity. Other abbreviations: AHK, *Arabidopsis* His kinase; AHP, *Arabidopsis* His phosphotransfer protein; ARR-A, type-A *Arabidopsis* response regulator; ARR-B, type-B *Arabidopsis* response regulator.

(4, 95, 116). Cytokinin deprivation in shoots under the stressed conditions may be due to *IPT1,3,5* repression and/or *CKX1,3,6* activation in *Arabidopsis* (95) (**Figure 4**) and decreased transport of root-borne cytokinins in the xylem (24). In the xylem sap, under stressed conditions, the ratio between abscisic acid (ABA) and cytokinin might potentially modulate the various stress and/or developmental processes as long-distance signals (116). In addition, a study has suggested that *O*-glucosylation is involved in the rapid homeostasis of cytokinin metabolites under various physiological stimuli (136). However, low temperature does not alter the cytokinin contents in *Arabidopsis* (60).

The physiological roles of altered cytokinin homeostasis in stress responses are largely unclear. However, cytokinins are known to be antagonistic to ABA responses, especially in stomata closure, senescence, and photosynthesis (98). Recent characterization of *Arabidopsis CKX* overexpression and the *ipt1,3,5,7* mutant plants with reduced endogenous cytokinin levels revealed a strong

Abscisic acid (ABA): a plant stress hormone that functions in abiotic stress resistance, stomata closure, germination, and flowering

ABA INSENSITIVE 5 (ABI5): transcription factor mediating ABA responses, belonging to the family of ABA response element binding factors

stress-tolerant phenotype that was associated with increased cell membrane integrity and ABA hypersensitivity (95). Overexpression of the *Arabidopsis* cytokinin biosynthesis gene *IPT8* confers ABA insensitivity and prevents *ABA INSENSITIVE 1* (*ABI1*) and *ABI5* induction in seedlings, whereas the *ipt8* mutant exhibits ABA hypersensitivity. Conversely, ABA represses the *Arabidopsis* His kinase gene *AHK4/CRE1* and *IPT8* expression (138). Unexpectedly, the type-A ARR *arr3,4,5,6* mutant is as hypersensitive to ABA as the *ipt8* and His kinase *ahk2,3* mutants (133, 138). It has been suggested that ARR4,5,6 interact directly with ABI5 to negatively control ABA signaling (138). Interestingly, cold induces *ARR5,6,7,15* expression, and the *arr5*, *arr6*, and *arr7* mutants exhibit enhanced freezing tolerance and ABA sensitivity similar to *ahk2,3* and *ahk3,4* mutants (60). Thus, specific type-A ARRs appear to mediate a novel cytokinin-ABA signaling interaction unrelated to their negative-feedback functions (**Figures 1** and **4**).

Besides ABA and cold, several abiotic stresses also modulate the expression of cytokinin signaling components. However, detailed expression profiling revealed complex regulations of different family members under various abiotic stresses (4, 133). For example, *AHK2* and *AHK4* were upregulated by dehydration, salinity, or cold stresses in one study (133) but downregulated in another study (4). It was unclear what caused the discrepancies, and more careful examination and comparison of the experimental conditions will be required. *AHK3* expression was consistently and significantly upregulated by multiple abiotic stresses in both studies. Expression of type-A and type-B *ARR* genes were also regulated by various stresses, but the patterns varied significantly among *ARR* members (4, 60). This transcriptional regulation of cytokinin signaling components under unfavorable environmental conditions could reflect the plant's ability to dynamically adjust cytokinin sensitivity to cope with stress.

Intriguingly, it is well known that exogenous application of cytokinins is effective in delaying leaf senescence and results in increased heat tolerance (146). Transgenic tobacco plants expressing a cytokinin biosynthesis *IPT* gene under the control of the *senescence associated receptor kinase* (*SARK*) promoter exhibit enhanced drought survival without yield loss (106, 107). Elevated cytokinin levels are considered to be involved in accumulation of osmolytes (3), photorespiration (107), sugar allocation, nitrogen partitioning (149), root viability, and maintenance of water use efficiency (81), suggesting that cytokinins affect diverse processes of plant physiology and metabolism for stress tolerance in a complex manner. It is worth considering that *CKX*-overexpression, *ipt1,3,5,7*, and *ahk2,3* mutant plants, which exhibit reduced cytokinin levels or cytokinin signaling, cause development and metabolic abnormality, which may affect stress tolerance not identical to wild-type plants (95, 133). For example, many ABA- and stress-inducible genes are already upregulated in the *ahk2,3* mutant (133); this raises the possibility that retarded shoot growth and development of the *CKX*-overexpression, *ipt1,3,5,7*, and *ahk2,3* plants may turn on endogenous stress-responsive systems, which may mimic acclimation to stresses independent of cytokinins. Alternatively, cytokinin reduction in roots can cause enhanced root growth and drought tolerance (142). Cytokinin signaling mediated by type-B ARR1 and ARR12 appears to repress the expression of *high-affinity potassium transporter 1* (*HKT1*) in roots, which results in increased sodium accumulation in the shoots (78) (**Figure 4**). It is still important to elucidate how cytokinins and signaling components modulate stress tolerances, especially those associated with ABA. In general, the output degree of cytokinin actions might determine the range and magnitude of crosstalks with other regulatory circuits involved in stress responses.

CYTOKININ REGULATION IN LEAF SENESCENCE

Senescence is the genetically programmed developmental process leading to chlorophyll

degradation, photosynthetic activity decrease, macromolecule hydrolysis, and eventually cell death (71). A classical cytokinin action is to negatively control leaf senescence (36, 71, 103). During the senescence process, the cytokinin level is reduced and the exogenous application of cytokinins delays the senescence (103). Many, but not all, biotrophic pathogens synthesize cytokinins. Cytokinin secretion to host plant tissues during infection induces green islands—areas remaining green due to chlorophyll retention—at the infection site even though the noninfected regions are senescing (137). Multiple lines of evidence from genetically manipulated plants further support the involvement of cytokinins in senescence.

Early key findings were generated from analyses of transgenic tobacco plants expressing the cytokinin biosynthesis *IPT* gene driven by the promoter of *SENESCENCE-ASSOCIATED GENE 12* (*SAG12*), a representative senescence marker gene (36). In this study, overproduced cytokinins specifically targeted senescing leaves without affecting other developmental processes via an autoregulated senescing program. The *SAG12-IPT* transgenic plants exhibited remarkably delayed senescence. Similarly, when *IPT* expression was driven by a stress- and maturation-induced promoter in tobacco, drought-induced leaf senescence was delayed and, furthermore, transgenic plants became much more tolerant to drought stress (106).

These physiological observations support the idea that cytokinins control plant senescence programs, and the underlying mechanisms are likely conserved among plant species. Recent genetic, molecular, and biochemical studies provide strong evidence for the direct involvement of cytokinin signaling components in the regulation of leaf senescence in *Arabidopsis*. The *ore12-1* mutant displaying delayed leaf senescence has been identified as a gain-of-function mutation in the His kinase gene *AHK3*. In *ore12-1*, the expression of type-A *ARRs* is upregulated and ARR2 is constitutively activated by phosphorylation in the absence of cytokinins (63). ARR2 overexpression also consistently extends leaf longevity (63), whereas the loss-of-function *arr2* mutant slightly facilitates leaf senescence. The loss-of-function *ahk3* mutant (but not the *ahk2* or *ahk4* mutants) confers reduced sensitivity to cytokinins in the leaf senescence assay. Interestingly, overexpression of *AHK2* or *AHK4* with the corresponding *ore12-1* mutation does not promote delayed leaf senescence. These results support the idea that the specific AHK3-ARR2 phosphorelay plays a major regulatory role in cytokinin-dependent leaf longevity by modulating downstream targets implicated in the senescence program (63) (**Figure 4**).

Systemic approaches aimed at uncovering the cytokinin-mediated molecular characteristics of senescence have been carried out in *Arabidopsis* (15, 16). Cytokinin signaling and homeostasis genes have been shown to be differentially regulated during the natural senescence process. Expression of type-A *ARR*s and *IPT* is downregulated, but expression of *CKX* is upregulated. *Arabidopsis* MYB2 attenuates expression of *IPT1,4,5,6,8* at the late stage of development, leading to suppression of axillary bud outgrowth, a part of plant senescence (41). The regulation of cytokinin-synthesizing or cytokinin-degrading enzymes implies that endogenous cytokinin levels are tightly controlled during the senescence process and directly link to cytokinin signaling regulatory circuitry to control plant senescence (**Figure 4**).

Little is known about the crosstalk between cytokinins and other hormonal signals or about downstream molecular links influencing senescence. Unexpectedly, a study of cytokinins and primary metabolism has suggested a direct link between cytokinin-induced senescence delay and a phloem unloading pathway (8). The extracellular invertase and hexose transporters play a crucial role in supplying carbohydrates to sink tissues, and are therefore considered central modulators of sink activity (108). Cytokinins coinduce extracellular invertase and hexose transporters (33, 38), which are functionally linked to phloem unloading and sink activity. When extracellular invertase is expressed under the control of the

senescence-induced *SAG12* promoter, senescence is clearly delayed, mimicking the cytokinin effect (8). These results strongly suggest that cytokinin-mediated senescence delay is caused by increased sink activity via the direct activation of extracellular invertase activity (**Figure 4**).

CONCLUSIONS

Cytokinin functions are tightly integrated into numerous developmental processes and responses to environmental stimuli throughout the plant life cycle. Although the core signaling circuitry appears simple, its specific implementation in the developmental context is complex. Recent progress in the field has advanced at a rapid pace. A comprehensive understanding of cytokinin signaling networks will require elucidation of the single-cell-based, genome-wide cytokinin responses by integrating transcriptome, proteome, interactome, and metabolome in kinetics and physical contexts. The focus on a specific context also requires using targeted approaches, including life-imaging systems for tracking specific developmental processes, conditional mutants, temporally and spatially confined overexpression systems, and pharmacological approaches for precise functional manipulations. In parallel, determining the functional significance of the canonical signaling components in physiological contexts will require thorough characterizations of true null mutants, with thoughtful use of physiological concentrations of exogenous cytokinins.

Our knowledge will soon allow us to build models of how cytokinins integrate with gene regulatory networks within a defined context. Computational models to simulate such networks will become more important, as complexity is expected to overwhelm intuitive understanding. Important questions also need to be addressed regarding pathway mechanistics. For example, what are the molecular mechanisms of cytokinins' transcription-independent functions? How is ligand access granted to functional receptors residing in the endoplasmic reticulum? The combination of relevant questions and powerful tools ensures exciting times ahead.

SUMMARY POINTS

1. Following the discovery of cytokinins and auxin as growth-promoting plant hormones, innovative tissue culture and cell-based functional assays revealed the molecular mechanisms of cytokinin signaling.
2. Comprehensive genomic and genetic analyses have revealed the extensive redundant nature of every cytokinin signaling component in the canonical two-component signaling circuitry, from the hybrid His kinases and His-containing phosphotransfer proteins to type-A and type-B response regulators, which modulate a plethora of primary and secondary target genes with distinct response kinetics.
3. CKI1 is a constitutive His kinase with specific physiological functions in female gametophyte and vasculature development that converge with the functions of other His kinase cytokinin receptors.
4. Branch signaling pathways have been discovered that overlap with or are uncoupled from type-A or type-B response regulators and control protein stability, protein interactions, and auxin efflux carrier PIN1 trafficking.
5. Prevailing and complex cytokinin and auxin interactions specify the stem-cell niche in early embryogenesis and the shoot apical meristem, the transition between proliferation and differentiation in the root apical meristem, and the vascular patterns in roots and shoots.

6. Pivotal roles of cytokinin signaling in nodulation and immunity are emerging.
7. Diverse and complex roles of cytokinin signaling in stress tolerance are mediated by direct and novel interactions with the stress hormone ABA signaling pathway and indirect influence on plant development.
8. Control of sink strength and metabolic activities may underlie the molecular mechanisms of cytokinin regulation of senescence.

FUTURE ISSUES

1. Determining the functional significance of the canonical signaling components in physiological contexts will require thorough characterizations of true null mutants, with thoughtful use of physiological concentrations of exogenous cytokinins.
2. The generation and creative application of conditional mutants and the development of new chemical regulators will circumvent lethality limitations to provide sophisticated tools for probing cytokinin signaling *in planta*.
3. It is essential to determine the subcellular and functional relevance of cytokinin metabolic enzymes and signaling components and to dissect the underlying regulatory mechanisms.
4. Distinguishing the functional importance of local and long-distance transported cytokinins will be future challenges.
5. New tools and concepts will be developed to explore the functions and regulatory modes for cytokinin transporters.
6. Future research will help define the spatiotemporal interactions between cytokinin signaling and other hormonal and signal transduction pathways to illuminate diverse cytokinin functions.
7. The comprehensive understanding of cytokinin signaling networks will require elucidation of the single-cell-based, genome-wide cytokinin responses by integrating transcriptome, proteome, interactome, and metabolome in kinetics and physical contexts.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The authors wish to thank Yan Xiong for his time and expertise in supporting experiments, and Tatsuo Kakimoto, Thomas Schmülling, Ykä Helariutta, Eva Benková, Jianru Zuo, Joe Kieber, David Jackson, Sabrina Sabatini, Jens Stougaard, and Florian Frugier for communication and sharing of new information and ideas. Support from the Advanced Biomass R&D Center of the Korean Ministry of Education, Science, and Technology and the Technology Development Program of the Korean Ministry for Food, Agriculture, Forestry, and Fisheries to I.H.; the Canton of Zurich, Switzerland, and the International Human Frontier Science Program to B.M.; and

the National Science Foundation and the National Institutes of Health to J.S. are gratefully acknowledged.

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Annual Review of Plant Biology
Volume 63, 2012

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