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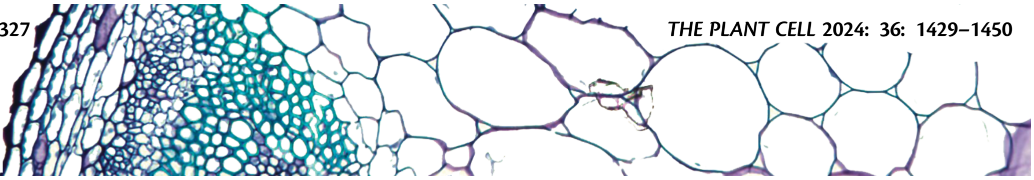

Cytokinin: From autoclaved DNA to two-component signaling

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Abstract

Since its first identification in the 1950s as a regulator of cell division, cytokinin has been linked to many physiological processes in plants, spanning growth and development and various responses to the environment. Studies from the last two and one-half decades have revealed the pathways underlying the biosynthesis and metabolism of cytokinin and have elucidated the mechanisms of its perception and signaling, which reflects an ancient signaling system evolved from two-component elements in bacteria. Mutants in the genes encoding elements involved in these processes have helped refine our understanding of cytokinin functions in plants. Further, recent advances have provided insight into the mechanisms of intracellular and long-distance cytokinin transport and the identification of several proteins that operate downstream of cytokinin signaling. Here, we review these processes through a historical lens, providing an overview of cytokinin metabolism, transport, signaling, and functions in higher plants.

Introduction

In the early part of the 20th century, the Austrian botanist Gottlieb Haberlandt, the "father of plant tissue culture," postulated the existence of "growth enzymes," substances that are released from one set of cells to stimulate the growth and development of other cells (Haberlandt 1902, 1913). In the 1950s, cytokinin was identified as one such factor because it promoted cell division and shoot initiation in cultured cells in concert with another phytohormone, auxin (Miller et al. 1955, 1956). In recent decades, remarkable progress has been made in our understanding of the biology of cytokinin. Here, we highlight recent findings as well as the major breakthroughs that led to our understanding of how plants synthesize, degrade, and transport cytokinins, the signaling pathway underlying the perception of cytokinin, and the myriad roles that this fascinating phytohormone plays in plant growth and development and responses to biotic and abiotic stress. We also discuss how modulation of cytokinin content has been used to improve various aspects of different crops, including enhanced drought tolerance, and yield.

Cytokinin biosynthesis and metabolism

Cytokinins were first identified by Carlos Miller and others in Folke Skoog's laboratory in a search for factors that could promote the growth of plant cells in culture (for an excellent review of the discovery of cytokinin, see Amasino 2005). High activity was found in an old sample of herring sperm DNA, and subsequent work using autoclaved DNA identified the active component as an adenine derivative (6-furfural-aminopurine) that is a degradation product of DNA, which they named kinetin (Miller et al. 1955; Miller et al. 1956). Similar to other cytokinins, kinetin is an N^6-substituted adenine derivative (Fig. 1). In addition to its ability to promote cell division in concert with auxin, different ratios of kinetin to auxin in media were found to promote shoot or root organogenesis, or undifferentiated growth (Skoog and Miller 1957). The first endogenous cytokinin from plants was identified from immature corn kernels and named zeatin (Letham et al. 1964; Jameson 2023). Trans-zeatin has an isoprene derivative as the N^6 side chain and is the most common naturally occurring cytokinin in

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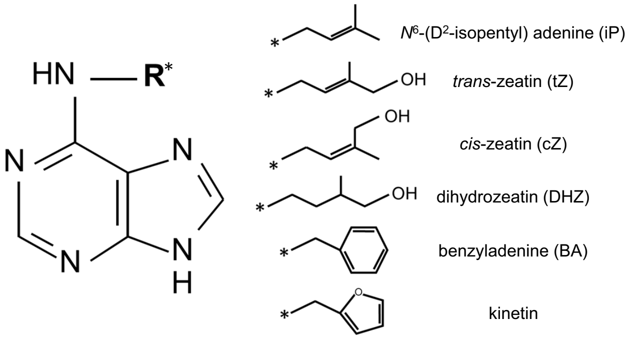

Figure 1. Structures of various cytokinins. Cytokinins are adenine derivatives. The general structure of the adenosine backbone of cytokinin is indicated to the left. Various sides chains, shown on the right, can be attached at the N^6 position of the adenine ring (indicated by an R*), leading to cytokinin species with differing biological activity. Note that BA and kinetin are not naturally occurring cytokinins.

higher plants, which also contain N^6-(Δ2-isopentenyl) adenine (iP) and dihydrozeatin (DHZ), as well as various derivatives of these (Fig. 1). Cytokinins with aromatic sides chains, including kinetin, have also been identified as minor cytokinins from some plant species (Strnad 1997). The various forms of cytokinin differ in their in planta stability, their transport characteristics throughout the plant, and their binding affinity for different cytokinin receptors (Kamada-Nobusada and Sakakibara 2009; Hluska et al. 2021).

tRNA from most organisms, except Archea, contain cis-zeatin as a modified base (Lindner et al. 2014). It was thus postulated that breakdown of this prenylated tRNA was the primary source of cytokinin in plants. However, recent work has shown that plants lack the ability to convert cis-zeatin to trans-zeatin (Hošek et al. 2020), and in many plants, cis-zeatin is not an active cytokinin (Gajdošová et al. 2011). Thus, tRNA is most likely not a significant source of cytokinin in such plants. However, in some plants, including many crop species (e.g. rice, maize, pea, and potato), the cis form of zeatin is found at high levels (Gajdošová et al. 2011; Kudo et al. 2012; Hluska et al. 2021) and is capable of binding to and activating a subset of cytokinin receptors in some species (e.g. Yonekura-Sakakibara et al. 2004; Lomin et al. 2011). The source of this cis-zeatin in these species may be breakdown of tRNA, though an enzyme involved in the breakdown of prenylated tRNA has not yet been identified. Alternatively, there may be a distinct biosynthetic pathway for cis-zeatin.

For most cytokinins, the first step in the biosynthetic pathway involves the linking of an isoprene moiety from dimethylallyl pyrophosphate (DMAPP) to the N^6 position of adenosine to form N^6-(Δ^2-isopentenyl) adenosine (iP) (Fig. 2). The first enzyme (isopentyl transferase [IPT]) catalyzing such a reaction was found in *Dictyostelium discoideum* (Taya et al. 1978), and, subsequently, a gene present on the transfer-DNA from *Agrobacterium tumefaciens* was shown to encode an enzyme with similar activity (Akiyoshi et al. 1984). In 2001, two groups identified nine *IPT* genes in the Arabidopsis genome and found that in addition to the tRNA-IPTs, plants possess a unique clade of *IPT* genes that the research groups subsequently demonstrated were involved in cytokinin biosynthesis (Kakimoto 2001; Takei et al. 2001) (see Table 1 for a list of genes relevant to cytokinin function). An important difference between the plant and bacterial enzymes is that plant IPTs use ATP/ADP rather than AMP as the adenosine source in the reaction. A recent phylogenetic analysis suggests that the tRNA-IPTs can be traced back to the last common ancestor of eukaryotes and that the IPTs involved in cytokinin biosynthesis in plants are derived from tRNA-IPTs (Wang 2020c).

Isotope-labeling experiments in Arabidopsis demonstrated that the primary source of the DMAPP used as the prenyl donor for the IPT reaction comes from the methylerythritol phosphate pathway (Kasahara et al. 2004), which is localized to plastids. Consistent with this, four of the Arabidopsis IPTs involved in cytokinin biosynthesis are localized to plastids, though two others are localized to the cytosol (IPT4) and mitochondria (IPT7) (Miyawaki et al. 2004; Takei et al. 2004a). A minor portion of DMAPP for cytokinin biosynthesis likely also comes from mevalonic acid because lovastatin, an inhibitor of mevalonic acid biosynthesis, can reduce cytokinin content (Laureys et al. 1999). The crystal structures for both a plant and an *A. tumefaciens* IPT have been solved, and this analysis revealed that the prenylation reaction proceeds via an SN2-reaction mechanism (Sugawara et al. 2008; Chu et al. 2010). Interestingly, the plant enzyme has two basic residues (Lys275 and Lys220) that interact with ATP to neutralize the charge of the β- and γ-phosphate, but these residues are replaced by acidic amino acids (Asp221 and Asp171) in bacterial IPTs, which likely explains the difference in the adenosine utilized in bacterial vs plant IPTs.

The products of the IPT reaction are iP ribosides, which are converted to trans-zeatin ribosides by hydroxylation of the prenyl side chain, a reaction catalyzed by the CYP735A cytochrome P450 monooxygenases (Takei et al. 2004b). Disruption of the genes encoding these CYP735As in Arabidopsis and rice eliminates trans-zeatin biosynthesis, allowing one to define specific roles of trans-zeatin type cytokinins in regulating growth and development. In the case of Arabidopsis, the *cyp735a1/2* double mutant shows reduced shoot growth but little effect on root growth (Kiba et al. 2013). This may reflect the observation that ARABIDOPSIS HISTIDINE KINASE3 (AHK3) is the cytokinin receptor most highly expressed in shoots (Higuchi et al. 2004; Nishimura et al. 2004), and it has a higher affinity for trans-zeatin than iP (Romanov et al. 2006; Stolz et al. 2011; Lomin et al. 2012). Disruption of the CYP735As in rice resulted in decreased growth of both roots and shoots, with substantial reductions in leaf size and rate of leaf formation, fewer tillers, delayed flowering, and highly reduced inflorescences (Kiba et al. 2023). This suggests that in rice, trans-zeatin is a positive regulator of shoot and inflorescence meristem function, consistent with analyses of cytokinin receptor mutants in rice (Burr et al. 2020). The enzyme responsible for the conversion

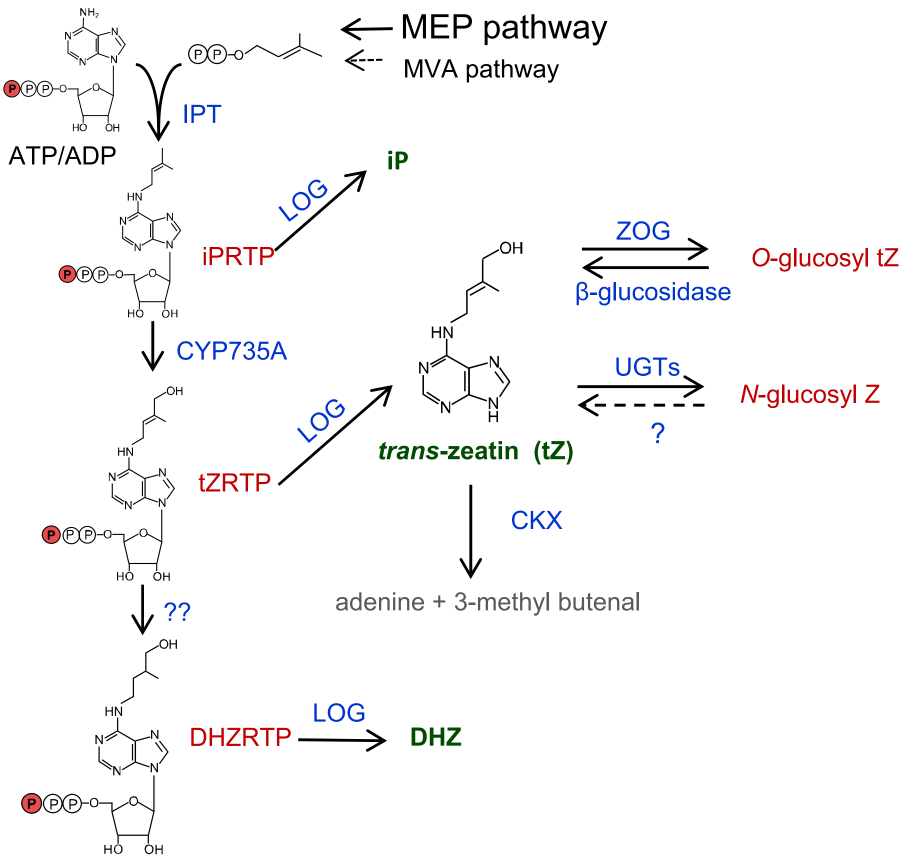

Figure 2. Generalized pathway for cytokinin biosynthesis and catabolism. Enzymes catalyzing each step are shown in blue. Inactive cytokinin ribosides are shown in red, and the active free base forms indicated in green. The source of the dimethylallyl pyrophosphate (DMPP) side chain of cytokinin is primarily from the methylerythritol phosphate (MEP) pathway, with minor contributions from mevalonic acid (MVA) pathway. The structure of the free base form of only zeatin is indicated; for the structures of iP and DHZ, see Fig. 1. Further, the conjugation and degradation of only trans-zeatin is indicated; both iP and DHZ type cytokinin are also subject to these processes to differing extents. The formation of cis-zeatin is not shown. See text for additional details.

of trans-zeatin to dihydrozeatin has not yet been identified in plants.

Biologically active cytokinins are produced by the removal of the riboside groups from iP riboside 5′-monophosphate, trans-zeatin riboside 5′-monophosphate, or dihydrozeatin riboside 5′-monophosphate to form the active, free-base cytokinins, a reaction catalyzed by the LONELYGUY (LOG) family of enzymes (Chen et al. 2022). LOG was first identified in a screen for rice mutants that failed to maintain shoot meristem function (Kurakawa et al. 2007). The LOG name comes from the phenotype of the spikelets in the mutants, which often contain only a single stamen and no pistil (Kurakawa et al. 2007). LOG is present as a small gene family in most angiosperms, as well as in moss (*Physcomitrium patens*) and liverworts (*Selagenella moellendorffii*), and are also found as single copy genes in green algae (Chen et al. 2022). Disruption of multiple *LOG* genes in Arabidopsis leads to severe retardation of shoot and root growth and defects in the maintenance of the apical meristems, with *LOG4* playing a major role in the shoot apical meristem (SAM) and *LOG3* and *LOG4* in the root (Tokunaga et al. 2012). *LOG4* expression is restricted to the L1 layer of the SAM and may supply apically derived cytokinin to underlying cell layers of the SAM to help pattern the expression of WUSCHEL (WUS), a key regulator of stem cell function within the stem cell niche (Chickarmane et al. 2012). Overexpression of *OsLOGL5* in rice reduced primary root growth, tiller number, and yield (Wang et al. 2020a). In contrast, mutations that alter the C-terminal domain of *OsLOGL5* did not significantly affect the growth or morphology of the plants but led to significantly increased yield in the field (Wang et al. 2020a), though the effects of these C-terminal mutations on *OsLOGL5* function have not been examined.

Cytokinin levels can be decreased through either conjugation to sugars, especially glucose, or irreversible cleavage by cytokinin oxidases. In terms of conjugation, glucose can be linked to the nitrogen at primarily the N^7 or N^9 position of the purine ring or to the oxygen on the side chains of iP, DHZ, and zeatin (both cis and trans) (Chen et al. 2021). Glucosyl conjugates are generally inactive in bioassays and fail to bind to cytokinin receptors (Spichal et al. 2004). Conjugations at the *N* positions were thought to be irreversible, but recent findings are changing that notion as metabolic studies on Arabidopsis seedlings have shown that trans-zeatin

Table 1. List of important genes in cytokinin metabolism, signaling, and transport

Gene/Protein	Name	Type	Function
HK	HISTIDINE KINASE	Signaling	Cytokinin receptors
HP/HPT	HISTIDINE PHOSPHOTRANSFER PROTEINS	Signaling	Phosphorelay proteins from receptors to RRs
PHP	PSEUDO- HISTIDINE PHOSPHOTRANSFER PROTEINS	Signaling	Negative regulators; lack conserved His that is target of phosphorylation
Type-B RR	TYPE-B RESPONSE REGULATOR	Signaling	Primary transcription factors; positive elements in the cytokinin response
Type-A RR	TYPE-A RESPONSE REGULATOR	Signaling	Rapidly induced by cytokinin; act as negative regulators of the cytokinin response
IPT	ISOPENTENYL TRANSFERASE	Biosynthesis	Biosynthetic enzyme; links isoprene moiety to N^6 position of adenosine
CYP735A	CYTOCHROME P450 MONOOXYGENASES	Biosynthesis	Conversion of iP ribosides to trans-zeatin
LOG	LONELY GUY	Biosynthesis	Cytokinin riboside 5′-monophosphate phosphoribohydrolase; activity generates active free-base forms of cytokinins
CKX	CYTOKININ OXIDASE	Degradation	FAD-dependent amine oxidases, cleavage of N^6-side chains; degrades cytokinins
UGT76C1 and 2	UDP-GLUCOSYL TRANSFERASE 76C1 and 2	Inactivation	*N*-glycosyltransferases
OsZOG1	ZEATIN O-GLUCOSYLTRANSFERASE 1	Inactivation	*O*-glycosyltransferases
ENT	EQUILIBRATIVE NUCLEOSIDE TRANSPORTERS	Transport	Cytokinin transporter
ABCGs	ATP-BINDING CASSETE TRANSPORTER G	Transport	Cytokinin transporter
PUPs	PURINE PERMEASES	Transport	Cytokinin transporter
HvSWEET11	SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS	Transport	Cytokinin and sugar transporter
AZG	AZAGUANINE PURINE TRANSPORTER	Transport	Purine and cytokinin transporter

N-glucosides (but not those of iP) are rapidly converted to trans-zeatin (Hošek et al. 2020). The *O*-glycosylated forms can also be converted into active cytokinins by β-glucosidases (Brzobohaty et al. 1993). Genes encoding enzymes catalyzing *N* or *O* (or both) glycosylation of cytokinin have been identified (Chen et al. 2021). In Arabidopsis, the glycosyltransferases UGT76C1 and UGT76C2 can glucosylate multiple different cytokinins at the N^7 or N^9 positions, and UGT85A1 glycosylates cytokinin at the oxygen on the side chain. As expected, disruption of *UGT76C2* led to a significant decrease in cytokinin *N*-glucoside levels, and overexpression had the opposite effect (Wang et al. 2011). The lines with altered *UGT76C2* function did not result in any obvious morphological alterations in plants, though there were some modest changes in the expression of cytokinin-regulated genes (Wang et al. 2011). The lack of effect on growth and development may be due to compensatory changes in cytokinin metabolism in these lines that keep the active, free-base forms within a physiologically suitable range. In contrast, knockdown of a rice cytokinin O-glucosyltransferase (OscZOG1) had substantial effects on growth and development, including increased lateral roots, tillering, panicle branching, grain number per panicle, and seed size; overexpression of OscZOG1 had the opposite effects, concomitant with an increased level of cis-zeatin O-glucoside (Shang et al. 2016). These results are consistent with the idea that conjugation of cytokinin plays an important role in regulating active cytokinin levels in vivo.

A major regulator of cytokinin content in plant tissues is its irreversible degradation by cytokinin oxidases (CKX), which are FAD-dependent amine oxidases that cleave the N^6-side chains from a subset of cytokinins (Werner et al. 2006). Cytokinin oxidase was first cloned from *Zea mays* kernels (Houba-Hérin et al. 1999; Morris et al. 1999) and has since been identified in all land plants. Phylogenetic analysis suggests that the *CKX* genes from land plants are derived from a single chlamydial ancestral gene (Wang et al. 2020b). The substrate preferences vary among CKX isoforms, but in general the free bases and their ribosides are the preferred substrates; dihydrozeatin and aromatic cytokinins such as 6-benzylaminopurine (BA) are generally resistant to cleavage (Galuszka et al. 2007). The expression of multiple *CKX* genes is induced in response to elevated cytokinin, acting as a negative feedback mechanism to reduce cytokinin levels (Bhargava et al. 2013; Polko et al. 2021).

Alteration of CKX function has substantial potential to improve various agronomic traits (reviewed in Jameson and Song 2016; Jameson and Song 2020) and, indeed, various *CKX* genes have been disrupted in multiple crop species using overexpression, RNAi, TILLING, or, more recently, CRISPR/Cas9 (Mandal et al. 2022). Species targeted include rice, wheat, barley, rapeseed, and potato (Jameson and Song 2016). In general, CKX loss-of-function lines tend to show increased yields, while lines overexpressing CKX often have larger root systems and are more drought tolerant. For example, in a recent study in rapeseed (an allotetraploid), disruption of six of the eight copies of two *CKX* genes by TILLING resulted in increased seed yield from both greenhouse and field-grown plants, likely as a result of a more active inflorescence meristem leading to more flowers (Schwarz et al. 2020). Disruption of *Gn1a*, which encodes a CKX enzyme, in the indica subspecies of rice results in a substantial increase in grain yield (Ashikari et al. 2005; Li et al. 2016). Interestingly, overexpression of CKX in multiple crops leads to a higher content of various micro- and macro-elements in the shoots, likely due at least in part to increased size of the root systems. For example, the concentration of zinc, which is deficient in the diet of nearly 25% of the world's population, was significantly increased in the seeds of transgenic barley plants overexpressing CKX (Ramireddy et al. 2018). Likewise, in rapeseed, overexpression of a *CKX* gene resulted in plants that displayed an increase in the content of multiple macro- and microelements in shoots (Nehnevajova et al. 2019). In maize, overexpression of a *CKX* gene specifically in roots resulted in a larger root system without affecting the size of the shoot, and an increase in the content of various minerals, including K, P, and Zn in the shoot and in some lines, resulted in an increase in Cu, Zn, and Mn in seeds (Ramireddy et al. 2021). Similar results were also obtained with overexpression of a *CKX* gene in barley (Ramireddy et al. 2018).

Transport of cytokinins

Cytokinins are synthesized in both roots and shoots and then can be transported short distances to neighboring cells or long distances to other tissues (Sakakibara 2006; Zhang et al. 2023). Trans-zeatin and trans-zeatin riboside cytokinins are generally synthesized in the roots and transported to shoots through the xylem, whereas iP and *cis*-zeatin type cytokinins are mostly synthesized in the shoots and transported to the roots through the phloem (Sakakibara 2006). Although the systemic, mobile nature of cytokinins was known for many years, the identity of the transporters involved in their translocation have only recently come to light.

In the early 2000s, a series of genetic and biochemical studies identified members of the EQUILIBRATIVE NUCLEOSIDE TRANSPORTERS (ENT) family as nucleoside transporters with broad substrate specificity, with some members being able to transport cytokinins (Möhlmann et al. 2001; Hirose et al. 2005). ENT proteins typically localize to the plasma membrane and can transport nucleosides down their concentration gradients (Hyde et al. 2001). A mutation in *ENT8* was identified as a suppressor of an Arabidopsis *IPT* overexpression line, and disruption of this gene resulted in hyposensitivity to exogenous isopentenyladenine and trans-zeatin ribosides, as well as a reduced uptake of isopentenyladenine riboside (Sun et al. 2005). In rice, OsENT2 was shown to transport cytokinin ribosides when expressed in yeast, suggesting it may also participate in cytokinin riboside uptake, though no in planta transport assays have been done for this ENT (Hirose et al. 2008). These studies suggest that a subset of ENTs is likely involved in the long-distance translocation from roots to shoots of the riboside forms of isopentenyladenine and trans-zeatin.

Another class of transporters acting in the long-distance movement of cytokinin belongs to the ATP-BINDING CASSETTE TRANSPORTER G (ABCG) family. Disruption of *ABCG14* resulted in phenotypes consistent with reduced cytokinin function, suggesting that it might act as a cytokinin transporter (Zhang et al. 2014). ABCG proteins transport a variety of substrates, including auxin. Through genetic and biochemical analyses, ABCG14 was shown to be a PM-localized protein that functions in the uptake of trans-zeatin and DHZ-type cytokinins (Ko et al. 2014; Zhang et al. 2014). ABCG14 is highly expressed in roots, and quantification of cytokinins in both shoot and roots of the *abcg14* mutant showed a hyperaccumulation of cytokinins in roots compared with wild-type plants, suggesting a function in the root to shoot transport of cytokinin (Ko et al. 2014; Zhang et al. 2014). Grafting experiments confirmed that ABCG14 functions as a cytokinin transporter between roots and shoots (Ko et al. 2014). ABCG14 plays a role in both the loading of cytokinin into the xylem in the roots (Ko et al. 2014; Zhang et al. 2014) and the unloading of cytokinin from the phloem in shoots via an apoplastic pathway (Zhao et al. 2021). In rice, OsABCG18 plays a similar role in root to shoot transport of cytokinin (Kim et al. 2020). Another ABCG transporter, ABCG11, has been proposed as a cytokinin transporter due to cytokinin-associated phenotypes displayed by an *abcg11* loss-of-function mutant (Yang et al. 2022); however, biochemical evidence of transporter activity is lacking. Likewise, disruption of the genes encoding 3 non-intrinsic ABC transporters (ABCI19, ABCI20, and ABCI21) resulted in cytokinin hypersensitivity in Arabidopsis (Kim et al. 2020). These ABCI proteins were localized to the ER and found to be induced by light, though, like ABCG11, these have not yet been shown to transport cytokinin.

Another class of cytokinin transporters belongs to the PURINE PERMEASES (PUPs) family, which likely reflects the fact that cytokinins are purine derivatives. There are 21 *PUP* genes in Arabidopsis (Jelesko 2012), of which *AtPUP1* was the first shown to encode a transporter of cytokinin (Gillissen et al. 2000). A survey of the other *PUP* genes led to the identification *PUP14* as the member with highest expression in a variety of plant tissues and strongest genetic effect on cytokinin responses (Zürcher et al. 2016). PUP14 was localized to the PM, and its activity was inversely correlated with cytokinin content in the apoplast, thus leading to the hypothesis that its physiological function was to attenuate cytokinin signaling by transporting cytokinins from the apoplast into cells, reducing the signal available for PM localized cytokinin receptors (Zürcher et al. 2016). Given the observations that the affinity of this transporter is significantly lower than that of the receptors for cytokinin, that the bulk of the cytokinin receptors are found in the ER, and that a rice PUP cytokinin transporter (OsPUP1) has been localized to the ER (Xiao et al. 2019), a counter-hypothesis is that the PUPs function by transporting cytokinins intracellularly between ER, the cytosol, and the apoplast, thus regulating cytokinin signaling by depleting cytokinin from ER-localized receptors (Romanov et al. 2018).

Recently, a member of the SWEET (SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS) family of sugar transporters in barley (*Hv*SWEET11b) was shown to function as a dual transporter of both sugars and cytokinins in developing barley grains (Radchuk et al. 2023). Given that cytokinin regulates sink-source relationships, which involves sugar transport, this directly links the transport of cytokinins to one of the physiological processes they control (Radchuk et al. 2023).

Finally, two transporters in the azaguanine purine transporter family (AZG) family have been linked to cytokinin transport. AZG2 transported purines and cytokinin with high affinity (Tessi et al. 2021) and localized to both the ER and the plasma membrane. AZG2 plays a role in lateral root development. Interestingly, AZG1, which was also shown to transport cytokinin, was identified as a protein that co-purified with the PIN1 auxin transporter in native gels, indicating that it may play a role in the interaction between auxin and cytokinin (Tessi et al. 2023).

Deciphering the signaling pathway

Starting in the mid-1970s, multiple groups reported the biochemical purification of cytokinin binding proteins (Takegami and Yoshida 1975; Polya and Davis 1978; Sussman and Kende 1978; Moore 1979; Keim and Fox 1980), including binding to isolated plant ribosomes (Fox and Erion 1975), but these approaches failed to identify the molecular nature of the cytokinin receptor. Recent decades have seen remarkable progress in our understanding of the molecular mechanisms underlying cytokinin perception and signaling. In the mid-1990s, 2 independent lines of investigation converged on the same signaling pathway. The CKI1 histidine kinase was identified in a screen for genes that when overexpressed in cultured cells conferred the ability to form shoots in the absence of exogenous cytokinin (Kakimoto 1996). In parallel, two genes encoding response regulators (ARR5 and ARR7) were identified in a screen for cytokinin primary response genes (Brandstatter and Kieber 1998). Histidine Kinases (HKs) and Response Regulators (RRs) comprise the elements of two-component signaling pathways in prokaryotic organisms (Stock Stock and Mottonen 1990; Stock Robinson and Goudreau 2000). In their simplest form, two-component systems are comprised of an often membrane-localized sensor HK that perceives some environmental stimuli (e.g. phosphate levels). Upon perception of the signal, the HK autophosphorylates on a His residue and then in turn transfers the phosphate to an Asp residue within the receiver domain of an RR protein. Phosphorylation of the RR regulates its activity, which most often involves direct regulation of transcription of target genes. Connecting both a HK (CKI1) and two RRs (ARR5 and ARR7) to the response to cytokinin strongly suggested that cytokinin perception involves a two-component–like pathway, which subsequent studies confirmed. However, in the case of cytokinin (and other eukaryotic two-component pathways), the response pathway is actually similar to phosphorelays, which involve an extra pair of phosphotransfers between His and Asp residues (Swanson et al. 1994; Appleby et al. 1996; Schaller et al. 2011). In these systems, there is a receiver domain fused to the HK receptor protein, and when the HK is activated, an Asp residue within this receiver domain accepts the transferred phosphate in an intramolecular reaction. A small histidine-containing phosphotransfer protein then shuttles the phosphate from the HK receiver domain to an Asp residue on the downstream RR proteins (Fig. 3).

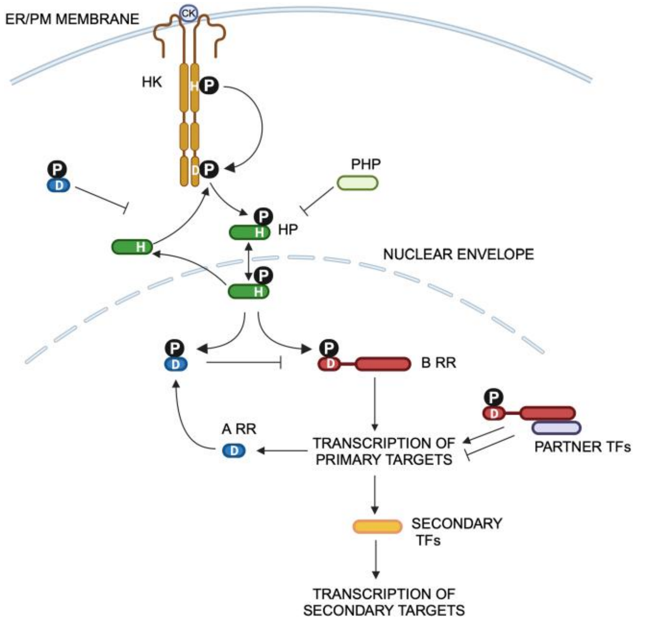

Figure 3. Canonical cytokinin signaling pathway. Cytokinin binds to the CHASE domain in the HK receptors and initiates a phosphorelay as indicated by the P. See text for additional details.

While CKI1 was the first HK to be linked to cytokinin signaling, it lacks a cytokinin binding domain and does not act as a cytokinin receptor, though it may feed into the downstream signaling elements (Dobisova et al. 2017). Authentic cytokinin HK receptors were identified in genetic screens for loss-of-function mutants impaired in cytokinin responses in cultured cells [e.g. shoot formation in tissue culture (Inoue et al. 2001)], by reverse genetic analysis of HK homologs in Arabidopsis (Ueguchi et al. 2001), by transient expression in Arabidopsis protoplasts (Hwang and Sheen 2001), by using bioassays in *Escherichia coli* or yeast, and/or by direct binding assays (Inoue et al. 2001; Suzuki et al. 2001; Ueguchi et al. 2001; Yamada et al. 2001). Plant cytokinin HK receptors are generally encoded by small gene families (e.g. three in Arabidopsis and four in rice) and share a similar structure, with two or three transmembrane domains flanking the cytokinin binding domain, a cytosolic HK domain fused to both a functional receiver domain as well as a degenerate receiver domain. Genetic analysis has demonstrated that the His kinase activity of AHK4 in Arabidopsis is required for cytokinin signaling (Mähönen et al. 2006a), as is the Asp residue that receives the phosphate within the functional receiver domain (Hodgens et al. 2020). Interestingly, multiple missense mutations that alter residues within or near the first transmembrane domain of AHK4 also eliminate its function, which may reflect disruption of its membrane insertion (Hodgens et al. 2020).

Cytokinin binds to a CHASE (cyclases/histidine kinases associated sensor extracellular) domain in these HK receptors, an evolutionarily ancient domain that binds a wide variety of ligands, including other adenine derivatives and peptides (Anantharaman and Aravind 2001). Cytokinin-binding, CHASE domain–containing HK cytokinin receptors have been identified in a bryophyte (*P. patens*), a lycophyte (*S. moellendorffii*), a gymnosperm (*Picea abies*), and all angiosperms examined (Lomin et al. 2021), which suggests that the ability to perceive cytokinin arose early in the evolution of land plants. In the case of *P. patens*, the CHASE domain HKs are essential for the response to exogenous cytokinin (von Schwartzenberg et al. 2016), even though *P. patens* contains only a tRNA-IPT and thus these receptors may reflect a response to endogenous cytokinin derived solely from breakdown of prenylated tRNA. The various cytokinin HK receptors in higher plants have different spatial patterns of expression and bind to different cytokinin species with distinct affinities (Spichal et al. 2004; Romanov et al. 2006; Stolz et al. 2011; Lomin et al. 2012, 2021). Thus, although the cytokinin receptors display varying degrees of genetic redundancy in the species that have been examined, they also likely have distinct functionalities.

An unresolved question in the field is the subcellular localization of these receptors. Early studies using analysis of AHK fusions to fluorescent proteins suggested a plasma membrane localization (Kim et al. 2006). However, subsequent biochemical analysis indicated that all three AHK cytokinin receptors localize to the endoplasmic reticulum (ER) (Wulfetange et al. 2011). This was based on analysis of high-affinity cytokinin binding to membrane fractions purified by two-phase partitioning in Arabidopsis lines harboring a single functional AHK receptor, as well as localization of fluorescent fusion proteins in transiently transformed tobacco leaves. Furthermore, aqueous two-phase partitioning of microsomes revealed that epitope-tagged AHK2 and AHK3 colocalized with ER membranes (Wulfetange et al. 2011). Likewise, a second study confirmed that fluorescently labeled AHK3 and AHK4 localized to the ER in both tobacco leaves and stably transformed Arabidopsis, and the authors showed that these fusion proteins complemented an *ahk2 ahk3* loss-of-function mutant (Caesar et al. 2011). Likewise, the maize ZmHK1 protein was also localized to the ER (Lomin et al. 2011). Finally, the pH optima of cytokinin binding to the Arabidopsis receptors is more consistent with binding in the ER lumen than in the apoplast. This issue was extensively discussed in an excellent review (Romanov et al. 2018). However, recent results indicate that a subset of the cytokinin receptors may actually reside in the plasma membrane (Antoniadi et al. 2020). These authors found that immobilized cytokinin (i.e. covalently linked to beads), which cannot enter cells, was capable of activating all three AHK receptors in Arabidopsis protoplasts as measured by both activation of a cytokinin-responsive reporter (Liu and Müller 2017) and induction of Cytokinin Response Factors (*CRF*) transcript levels (Rashotte et al. 2006). Further, super-resolution microscopy of AHK4-GFP and AHK3-GFP fusion proteins indicated that a fraction of the signal is present at the cell surface (Antoniadi et al. 2020). Finally, more recent analysis of cytokinin receptors across the plant kingdom has revealed a wide range of pH optima for cytokinin binding, consistent with different subcellular localizations (Lomin et al. 2021). Thus, it seems that cytokinin receptors in higher plants are primarily localized to the ER with cytokinin binding occurring in the ER lumen, but a fraction of the functional receptors may be at the plasma membrane, binding cytokinin in the apoplast.

Histidine-containing phosphotransfer proteins

The Authentic Histidine-containing Phosphotransfer proteins (AHPs) act downstream of the cytokinin receptors. AHPs are found in unicellular algae, moss, lycophytes, gymnosperms, and angiosperms, with the AHPs from land plants forming a distinct phylogenic clade from those in algae (Pils and Heyl 2009; Rashotte 2021). The AHPs are often found as small gene families in higher plants (e.g. five in Arabidopsis and two in rice), and at least in Arabidopsis, these AHPs have overlapping functions as positive elements in cytokinin signaling (Hutchison et al. 2006). These proteins shuttle between the cytosol and the nucleus to mediate the transfer of a phosphoryl group from the receiver domain of the activated HK receptors to the receiver domain of an RR protein (Punwani et al. 2010). Their phosphotransferase activity is negatively regulated by nitric oxide–induced S-nitrosylation, suggesting a link between redox signaling and cytokinin function (Feng et al. 2013). In addition to their role in cytokinin signaling, the AHPs also act downstream of other HKs, including CKI1 and likely AHK1 in Arabidopsis. For example, disruption of multiple AHPs results in loss of central cell and antipodal cell fates coupled with a gain of egg cell or synergid cell attributes during female gametophyte development in Arabidopsis (Liu et al. 2017), similar to the phenotypes of *cki1* loss-of-function mutations (Yuan et al. 2016).

The AHPs contain an essential His that is the target of phosphotransfer from the upstream AHKs. In addition to the AHPs, plant genomes also encode proteins similar to AHPs that lack the His residue required for phosphorelay. These so-called Pseudo Histidine Phosphotransfer proteins (PHPs) are negative regulators of cytokinin signaling, likely acting in a dominant negative manner to block AHP phosphotransfer activity (Mähönen et al. 2006b). AHP6 (an Arabidopsis PHP) mediates cross talk between auxin and cytokinin in multiple developmental events, including root vascular patterning, the formation of passage cells, lateral root organogenesis, proliferation of the inflorescence meristem, shoot phyllotaxy, and gynoecium development

(Mähönen et al. 2006b; Moreira et al. 2013; Besnard et al. 2014; Müller et al. 2017; Andersen et al. 2018). For example, in vascular development, bisymmetric domains of auxin and cytokinin ultimately lead to the formation of phloem and xylem (Mähönen et al. 2006b) (see below). Similar to this, in the shoot apical meristem, leaves initiate at positions of auxin maxima, and the robustness of this phyllotaxy is enhanced by a spatio-temporal pattern of AHP6 that regulates fields of cytokinin signaling (Besnard et al. 2014).

Phylogenic analysis indicates that the PHPs are present in gymnosperms and early diverging angiosperms but not in bryophytes (Vaughan-Hirsch et al. 2021). The monocot and dicot PHPs fall into two distinct clades, with the monocots having a Gln at the position of the conserved His residue and dicots having an Asn at this position. This suggests that PHPs evolved twice independently from AHPs: one event in monocots event giving rise to a to a PHP(Gln) class and a second in eudicots giving rise to the PHP(Asn) class. Consistent with their independent evolutionary origin, rice PHPs appear to have functions distinct from their Arabidopsis counterparts (Vaughan-Hirsch et al. 2021).

Response regulators

The final step of the cytokinin-regulated phosphorelay is the transfer of the phosphate from the AHPs to an Asp residue on the receiver domain of response regulators (RRs). There are two main types of RRs in plants. The type-B RRs are characterized by the presence of a receiver domain and a large C-terminal extension harboring a Myb-like DNA binding domain. Type-B RRs are generally not transcriptionally regulated by cytokinin and act as positive regulators of cytokinin signaling. In contrast, type-A RRs, which form a distinct phylogenetic clade from the type-B RRs, lack a DNA binding domain, are generally transcriptionally induced by cytokinin and act as negative regulators of cytokinin signaling. Most phylogenetic analyses suggest that type-A and type-B RRs originated in the Charophyte algae and are found throughout the land plant lineage, from the liverwort *Marchantia polymorpha* and the moss *Physcomitrella patens*, through Gymnosperms and Angiosperms (Pils and Heyl 2009; Rashotte 2021).

Type-B RRs are DNA-binding transcription factors that mediate the initial transcriptional response to cytokinin (Sakai et al. 2000; Hwang and Sheen 2001; Sakai et al. 2001; Imamura et al. 2003; Mason et al. 2005; Argyros et al. 2008; Hill et al. 2013). As with other elements in the cytokinin signaling pathway, type-Bs are generally encoded by a small gene family in higher plants (e.g. 11 in Arabidopsis and 13 in rice, though not all have been shown to be involved in cytokinin signaling). There is functional overlap in the gene family in Arabidopsis; single mutants have at most modest effects, but disruption of multiple type-B RRs leads to cytokinin insensitivity, effects on growth and development similar to disruption of the cytokinin HK receptors, and, in the highest combination mutants, the near absence of a transcriptional response to cytokinin (Mason et al. 2005; Argyros et al. 2008). The receiver domain of the type-B RRs negatively regulates their transcriptional activity (Sakai et al. 2001; Liang et al. 2012), and phosphorylation of this domain at the conserved Asp residue results in increased binding of type-B RRs to their genomic targets (Zubo et al. 2017; Xie et al. 2018). Type-B RRs were found to bind upstream of genes that were both activated and repressed by cytokinin, and disruption of type-B RRs block the cytokinin regulation of both classes of genes (Mason et al. 2005; Argyros et al. 2008), suggesting that type-B RRs are necessary for both gene activation and repression in response to cytokinin. Type-B RR protein stability is regulated via the KISS ME DEADLY F-box proteins (Kim et al. 2013).

A large number of transcription factors are regulated in response to cytokinin (Argueso et al. 2010; Brenner et al. 2012; Bhargava et al. 2013), suggesting that type-B RRs operate at the top of a transcriptional cascade, with the succeeding waves of transcription being regulated by these inducible transcription factors (Fig. 3). Various other transcription factors act in concert with type-B RRs and contribute to differences in transcriptomic responses to cytokinin (Leuendorf and Schmülling 2021). These partner transcription factors likely modulate the target genes to which activated type-B RRs bind, either through direct or indirect interaction. Many such type-B RR interacting partners have been identified, including CRFs, DELLA, BPCs, NPR1, TCPs, HY5, TGA3, HD-ZIP IIIs, and EIN3 (Rashotte et al. 2006; Choi et al. 2010; Efroni et al. 2013; Marín-de la Rosa et al. 2015; Raines et al. 2016b; Shanks et al. 2016; Yan et al. 2017; Zhang et al. 2017; Hodgens et al. 2020).

The type-A RRs contain a receiver domain but, unlike the type-B RRs, lack a DNA binding domain and most are transcriptionally induced in response to cytokinin (D'Agostino et al. 2000; Tsai et al. 2012). Genetic analyses indicate that ten type-A RRs in Arabidopsis function as negative regulators of cytokinin signaling, thus acting as a negative feedback loop to dampen cytokinin signaling (Kiba et al. 2003; To et al. 2004; Leibfried et al. 2005; Lee et al. 2007; To et al. 2007). The mechanism by which type-A RRs negatively regulate cytokinin signaling is not fully understood but likely involves both competition with the type-B RRs for phosphotransfer from the upstream AHPs and phospho-specific interactions with regulatory proteins (To et al. 2007; Shanks et al. 2018).

The stability of type-A RR proteins also plays an important role in their regulation. In response to elevated cytokinin, type-A RRs are phosphorylated, and this inhibits their degradation by the 26S proteosome (To et al. 2007). This stabilization acts synergistically with the transcriptional upregulation of type-A RRs to highly increase RR protein levels in response to elevated cytokinin. Intriguingly, in contrast to unphosphorylated type-A RRs, the phosphorylated forms are targeted for degradation by autophagy in an EXO70D-dependent manner (Acheampong et al. 2020). The relative contributions of 26S proteome vs autophagic protein turnover is likely influenced by multiple regulatory inputs and differs across the type-A RR family members. These processes likely complement each other to optimize type-A RR protein levels, and hence cytokinin responsiveness, in response to various developmental and environmental cues.

Another negative feedback loop in cytokinin signaling in addition to the type-A RRs is provided by the cytokinin induction of the EAR motif-containing transcriptional repressor TIE1/TIE2 (He et al. 2022). ARR1 induces transcription of *TIE1* and *TIE2* by directly binding to their regulatory regions in the genome. TIE1/2 also interact directly with type-B RRs to represses transcription of their target genes in Arabidopsis roots, thus dampening the response to elevated cytokinin.

A number of cytokinin-responsive reporter transgenes have been constructed based on the transcriptional induction of the type-A RRs. The first were fusions of the promoter of the Arabidopsis *ARR5* gene to either a GUS or GFP reporter (D'Agostino et al. 2000; Lohar et al. 2004). Although these reporters are useful, they suffer from lack of specificity and universality because ARR5 is likely regulated by other regulatory inputs and is not induced in all cell types. A synthetic reporter was developed comprised of concatemerized type-B binding sites fused to a GFP reporter called *TCS::GFP* (Müller and Sheen 2008). Although useful for reporting cytokinin signaling, *TCS::GFP* showed little or no expression in developmental context known to involve cytokinin (e.g. the shoot apical meristem [SAM]) and was found to rapidly silence in transgenic plants. An improved version, called *TCSn::GFP*, was developed with more robust expression that is more consistent with known cytokinin functions in Arabidopsis and which is stable over several generations (Zürcher et al. 2013). *TCSn::GFP* is a valuable tool to querying the endogenous state of cytokinin signaling, and it or derivatives have been used in multiple plant species, including Arabidopsis (Zürcher et al. 2013), rice (Tao et al. 2017), tomato (Steiner et al. 2020), and *Medicago truncatula* (Fonouni-Farde et al. 2017).

Role of cytokinin in plant development and responses to stress

Cytokinin exerts a profound influence on nearly all aspects of plant development, orchestrating intricate processes that determine growth, differentiation, and overall morphogenesis (Fig. 4). In addition, cytokinin also plays a pivotal role in regulating several physiological processes that help maintain optimal plant development in response to environmental cues. These processes were extensively reviewed previously (Argueso et al. 2009; Hwang et al. 2012; Kieber and Schaller 2014; Schaller et al. 2015; Kieber and Schaller 2018; Wybouw and De Rybel 2019; Liu et al. 2020), and so here we simply highlight a subset of the various responses regulated by cytokinin, focusing on seminal work that elucidated these responses, the more recent findings, and the molecular mechanisms at play.

Cell division

Cytokinin plays a crucial role in cell division and cell cycle control, orchestrating the growth and development of plants (Schaller et al. 2014). After this hormone was discovered by its ability to promote cell division, studies showed that oscillations in cytokinin levels accompanied the progression of the cell cycle, peaking at phase transitions (Hartig and Beck 2005), suggesting a link between cytokinin and cell cycle control. Further work demonstrated that cytokinin regulates the expression of CYCD3 D-type cyclins (Riou-Khamlichi et al. 1999), which in turn regulate the transition from the G_1 to the S phase of the cell cycle. Cytokinin-induced shoot regeneration in vitro was also found to be dependent on cytokinin-regulated CYCD3s (Dewitte et al. 2007). The bHLH transcription factor CYTOKININ-RESPONSIVE GROWTH REGULATOR (CKG) was recently identified as a mediator of the G_1/S transition in Arabidopsis (Park et al. 2021). *CKG* is transcriptionally induced by cytokinin, and *ckg* mutants show altered expression of cell cycle regulators and display a delay in the G_1/S transition. Although the studies described above indicated a role for cytokinin in the G_1/S transition via the action of CYCD3s and CKG, evidence for a role for cytokinin in the G_2/M transition also exists. Treatment of synchronized BY-2 tobacco cell cultures with the cytokinin biosynthesis inhibitor lovastatin blocks the G_2/M transition (Hartig and Beck 2005), and *ahk2*, *ahk3*, *ahk4* triple mutants show a delayed G_2/M transition in roots (Higuchi et al. 2004).

Cell cycle control by cytokinin regulates the activity and maintenance of meristems, ensuring sustained growth and proper development. SAM size is directly correlated to cytokinin content (Kurakawa et al. 2007; Bartrina et al. 2011), and the spatial distribution of cytokinin is highly regulated within the SAM (Gordon et al. 2009; Chickarmane et al. 2012). Cytokinin also directly regulates the activity of the homeodomain transcription factor WUS, which controls stem cell fate and maintenance in the SAM (Lopes et al. 2021). *WUS* expression is induced by cytokinin, and *WUS* is a direct transcription target of type-B ARR1, ARR10, and ARR12 (Meng et al. 2017; Zhang et al. 2017; Zubo et al. 2017; Xie et al. 2018). In turn, WUS represses type-A *RR* transcription (Leibfried et al. 2005) and cytokinin biosynthesis by downregulating *LOG4* (Chickarmane et al. 2012). This feedback loop between WUS and cytokinin signaling has been hypothesized to control cytokinin content and signaling in the SAM and therefore SAM activity. The mechanisms by which cytokinin regulates cell division in the SAM involve CYCD3, which is a direct transcriptional target of the cytokinin-regulated SAM transcription factor SHOOT MERISTEMLESS (Scofield et al. 2013), and MYB3R4 transcription factor, which promotes the G_2/M transition in dividing cells of the SAM (Yang et al. 2021a).

The role of cytokinin in the root apical meristem (RAM) is opposite of that in the SAM. Increased cytokinin content leads to a smaller RAM, and mutants with reduced cytokinin

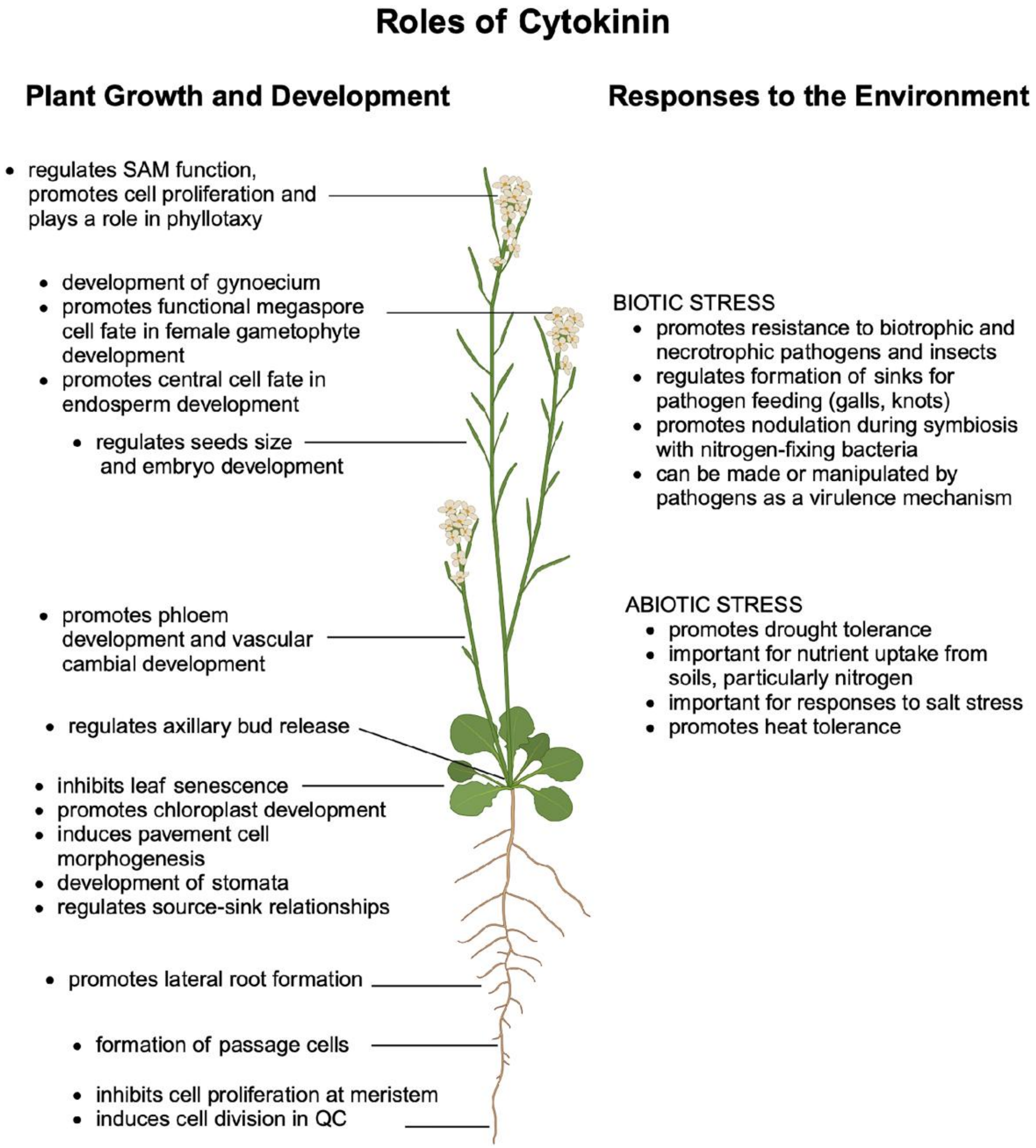

Figure 4. Roles of cytokinins in plant growth and responses to the environment. See text for additional details.

content (e.g. *ipt3*, *ipt5*, *ipt7* triple mutant) have an enlarged RAM (Dello Ioio et al. 2007). Cytokinin reduces RAM size by promoting the exit of cells from the meristem, acting via an incoherent regulatory loop between IPT7 and the HD-ZIPIII transcription factor PHABULOSA (Dello Ioio et al. 2012). This negative effect of cytokinin on the RAM is in part mediated by its antagonistic interaction with auxin, via transcriptional upregulation of auxin signaling repressors (Dello Ioio et al. 2008; Moubayidin et al. 2010; Perilli et al. 2013) and altered regulation of auxin efflux and influx carriers (Marhavý et al. 2011; Zhang et al. 2013), preventing auxin-induced cell division. Cytokinin can directly regulate cell division in the RAM independently of auxin. This is mediated by the CCS52A1 E3 ligase, whose gene expression is directly regulated by ARR2 and whose activity induces endoreplication by degradation of mitotic cyclins (Takahashi et al. 2013).

After cells exit the RAM in the Arabidopsis root, they stop dividing and rapidly elongate in a region called the elongation zone and then finally stop growing and mature into their final cell forms in the differentiation zone (Petricka et al. 2012). In addition to its role in inducing the exit of cells from the root meristem, cytokinin also promotes cessation of cell growth in the distal elongation zone at least in part by increasing the rigidity of the cell wall (Liu et al. 2022).

In rice, disruption of cytokinin function by disruption of cytokinin biosynthesis (LOG genes) or perception (HK cytokinin receptors) results in a drastic effect on both the inflorescence and the spikelet meristems (Kurakawa et al. 2007; Burr et al. 2020). In Arabidopsis, disruption of all three *HK* cytokinin receptors or of all seven *LOG* genes results in a substantial reduction in the size of the SAM (Higuchi et al. 2004; Nishimura et al. 2004; Riefler et al. 2006; Tokunaga et al. 2012), though this may be at least partially a secondary consequence of defects in the vasculature in these mutants. Further, unlike in rice, there are few, if any, effects on the floral meristem in cytokinin signaling mutants in Arabidopsis. In terms of type-A RRs, disruption of a single type-A RR gene (*ABPHYL1*) results in an enlarged meristem in maize (Jackson and Hake 1999), but no such effect is observed even in multiple type-A RR mutants in Arabidopsis (To et al. 2004). Overall, the results suggest that cytokinin may play a more pronounced role in regulating SAM function in monocots compared with dicots.

Vascular and cambium development

The plant vascular system is essential for the survival of plants on land. The main vascular tissues, namely xylem and phloem, originate from procambial stem cells that differentiate into mature vascular tissue. A role for cytokinin in vascular morphogenesis was first revealed by the identification of the *wooden leg* allele of *AHK4* (Scheres et al. 1995), which displays a pronounced reduction in the number of cell files in the vascular bundles, a phenotype that is even more pronounced in *ahk2,3,4* triple mutants (Riefler et al. 2006). Vascular morphogenesis and differentiation require precise control of cytokinin function in concert with auxin. A feedback loop that helps define the cellular zones of auxin/cytokinin function in the developing vasculature involves the auxin transcription factor MONOPTEROS/AUXIN RESPONSE FACTOR 5, which increases the expression of *AHP6*, a negative regulator of cytokinin signaling. This leads to the inhibition of cytokinin signaling in specific domains of the vascular system, regulating meristematic activity of the procambium and the formation of xylem and phloem (Mähönen et al. 2006a, 2006b). Cytokinins also induce a bisymmetric distribution of the PIN-FORMED (PIN) auxin efflux proteins, resulting in accumulation of auxin in the central domain of protoxylem (Bishopp et al. 2011). In addition to its effects on AHP6, MP can also directly activate the expression of the transcription factor TARGET OF MONOPTEROS 5 (TOM5), which together with the transcription factor LONESOME HIGHWAY (LHW), regulates the expression of *LOG3* and *LOG4*, locally increasing cytokinin levels in the protoxylem, and regulating vascular morphogenesis and growth (De Rybel et al. 2013). TOM5 and LHW also regulate the cell mobile transcription factor SHORTROOT, which can induce *CKX3* expression and further regulate vascular development through localized cytokinin signaling domains (Yang et al. 2021b).

As plants grow, their stems and roots thicken, a process known as secondary growth, which results from cambial meristem activity and cell proliferation. Studies in Arabidopsis and poplar demonstrated that *ipt* mutants have decreased root and stem thickening, as well as reduced cambial activity, establishing a role for cytokinin in promoting cambium development (Matsumoto-Kitano et al. 2008; Nieminen et al. 2008). The genes encoding the transcription factor AINTEGUMENTA and the cyclin CYCD3;1 are both transcriptionally upregulated by cytokinin in the cambium (Randall et al. 2015). Both genes are required for proper root and stem thickening, implicating them in a conserved regulatory module controlling vascular development (Randall et al. 2015). Recently, a study in Arabidopsis identified members of the transcription factor family LATERAL ORGAN BOUNDARIES DOMAIN (LBD) as regulators of secondary growth initiation (Ye et al. 2021). *LBD3* and *LBD4* are transcriptionally induced in response to cytokinin and initiate activation of the cambium meristematic cells. Other members, *LBD1* and *LBD11*, are transcriptionally induced by cytokinin later and together contribute to further radial growth and cambial stem cell maintenance. These LBD transcription factors were also determined genetically to inhibit cytokinin signaling, forming a feedback loop that regulates secondary growth (Ye et al. 2021). THE AT-HOOK MOTIF CONTAINING NUCLEAR LOCALIZED 15 transcription factor can also induce cytokinin biosynthesis locally and stimulate cambial activity (Rahimi et al. 2022).

Female gametophyte

During female gametogenesis in plants, the haploid female gametophyte (FG) develops within the ovule, surrounded by the sporophyte, to eventually generate megaspore cells. The development of the FG follows a spatial orientation along the chalazal/micropylar axis of the sporophyte. Cytokinin signaling has been shown to be essential in both the sporophyte (Kinoshita-Tsujimura and Kakimoto 2011) and in the gametophyte (Cheng et al. 2013) for female megaspore formation. In strong alleles of *ahk* receptor triple mutants, FG development is impaired, and the functional megaspore fails to form (Cheng et al. 2013). The effect of cytokinin on FG development was demonstrated to be associated with altered cell cycle regulation (Zhang et al. 2022). Interestingly, even though the requirement for cytokinin signaling through HK receptors for FG development has been demonstrated, cytokinin-independent signaling through two-component elements mediated by CKI1 and multiple AHPs is also required for FG development as *cki1* mutant plants display specific defects in FG development (Hejátko et al. 2009) that can be phenocopied by an *ahp2, ahp3, ahp5* triple mutant (Liu et al. 2017). CKI is also involved in central cell specification that generates the endosperm (Yuan et al. 2016) through a mechanism that appears to involve activation of the cytokinin signaling pathway via the AHPs (Liu et al. 2017), ultimately activating multiple type-B RRs (Zhu et al. 2022).

Leaf senescence

One of the most well-known effects of cytokinin is its anti-senescence activity. During developmental leaf senescence, which is age dependent and genetically programmed, as well as stress-induced senescence, nutrients are remobilized from leaves to other parts of the plant, where they can be reutilized for growth or storage. Levels of senescence are antagonistically correlated with levels of cytokinin content and signaling (Zwack and Rashotte 2013).

Application of cytokinin to plants delays leaf senescence (Gan and Amasino 1996). In addition, increasing endogenous cytokinin levels through transgenic approaches can also reduce senescence when properly regulated (Kant et al. 2015; Glanz-Idan et al. 2022). This was elegantly demonstrated using the promoter of a senescence-associated gene, *SAG12*, to drive the expression of an *IPT* gene in tobacco plants (Gan and Amasino 1995). Using this transgenic approach, cytokinin levels were increased specifically in cells undergoing onset of senescence, and transgenic plants expressing *SAG12::IPT7* showed decreased developmental as well as stress-induced senescence (Gan and Amasino 1995).

At the level of signaling, a genetic screen for mutants with delayed senescence identified a gain-of-function allele of *AHK3* as an important regulator of senescence in Arabidopsis (Kim et al. 2006). AHK3 functions in senescence by the activation of the type-B ARR2, whose transcriptional function is required for the anti-senescence activity (Kim et al. 2006). Downstream of type-B RR function, the CRF transcription factors were also found to have a role in senescence regulation. Higher-order mutant *crf1,3,5,6* plants display faster developmental senescence (Raines et al. 2016a), while overexpression of *CRF6* delays stress-related senescence (Zwack and Rashotte 2013).

The mechanisms underlying the reduction of leaf senescence by cytokinin are relatively unknown. The strongest association has been with cell wall-invertases (CWINV). CWINVs function by cleaving sucrose molecules into glucose and fructose, which then become available for metabolic processes associated with plant growth. A delay in developmental or stress-induced senescence is associated with increased CWINV enzymatic activity and gene expression (Gan and Amasino 1995). Moreover, expression of a *CWINV* driven by a cytokinin inducible promoter also correlates with decreased senescence, and that can be reverted by the use of a chemical inhibitor of CWINV activity (Balibrea Lara et al. 2004).

Biotic stress

A role for cytokinin in plant-pathogen interaction was long proposed, especially in the relationship between plants and biotrophic pathogens in which exchange of nutrients is required (Walters and McRoberts 2006). This is the case in the formation of the so-called "green islands," which are formed around sites of biotrophic pathogen colonization, an area of reduced senescence that supports pathogen growth. Further studies in Arabidopsis showed that application of high concentrations of cytokinin, as well as genetic approaches increasing cytokinin content and signaling (Choi et al. 2010; Argueso et al. 2012), do not activate defense directly but rather prime the plants for stronger defense in a variety of plant-pathogen systems (Albrecht and Argueso 2017). This priming effect by cytokinin is also observed in biocontrol agents such as rhizobacteria, which produce cytokinin to activate plant defense and reduce disease (Großkinsky et al. 2016). Molecular mechanisms of immunity activation against biotrophs by cytokinin include formation of a transcriptional complex between the type-B ARR2, the salicylic acid co-receptor NPR1, and the TGA3 transcription factor (Choi et al. 2010). Type-A RRs also participate by negatively regulating immunity in a phosphorylation-dependent manner (Argueso et al. 2012). Stomatal immunity is also partially controlled by cytokinin via regulation of peroxidases (Arnaud et al. 2017).

Apart from a role in immunity, cytokinin is also important in interactions of susceptibility between plants and pathogens. In such cases, increased cytokinin production or signaling initiated by pathogens leads to localized regions of cell proliferation, which become the galls, tumors, and knots that are often symptoms of plant diseases (McIntyre et al. 2021). The crown gall disease caused by *A. tumefaciens* is a classic example of this type of interaction (Akiyoshi et al. 1984), but many other pathogens such as fungi (Malinowski et al. 2016), nematodes (Siddique et al. 2015), and even parasitic plants (Spallek et al. 2017) can also manipulate cytokinin biosynthesis or signaling to create regions of cell division at sites of infection. Such regions of cell proliferation function as sinks, to which metabolites are rerouted to, away from source leaves, aiding in pathogen growth and multiplication in a process involving sugars and amino acid transporters that are also regulated by cytokinin (McIntyre et al. 2021).

Recently, a function for cytokinin in immunity against necrotrophic pathogens was also revealed (Gupta et al. 2020; Li et al. 2021; Liu et al. 2023). In tomato, application of micromolar amounts of cytokinin had a protective effect against the necrotrophic fungal pathogen *Botrytis cinerea*, through mechanisms that include the expression of defense marker genes as well as production of ethylene (Gupta et al. 2020). Interestingly, cytokinin can also be produced and sensed by *Botrytis*, having a role in fungal development and energy utilization, which underscores the complex relationship of this hormone in plant-pathogen interactions (Gupta et al. 2021).

Finally, cytokinin also plays a role in symbiotic interactions, particularly in the interactions of legume plants and rhizobia bacteria during nodule formation for nitrogen fixation. Evidence supporting a major role of cytokinin in nodulation comes from the fact that *Medicago truncatula* and *Lotus japonicus* mutants impaired in cytokinin signaling display reduced nodule formation in response to rhizobia infection (Plet et al. 2011; Held et al. 2014), while gain-of-function mutations in these genes leads to nodulation in the absence of rhizobia. Nodule formation is initiated by perception of bacteria-secreted nodulation (Nod) factors by plant cell surface LysM-type receptor kinases. After Nod factor perception, cytokinin biosynthesis and signaling are increased locally in the root, mostly in the root cortex (Lohar et al. 2004). This localized cytokinin accumulation induces the expression of key nodulation transcription factors, including NODULATION SIGNALING PATHWAY (NSP) 1 and 2, by binding of type-B RRs to their promoters to induce their transcription (Ariel et al. 2012). NSP1 and NSP2 then regulate genes that lead to cortical cell dedifferentiation and proliferation for nodule formation, such as *NODULE INCEPTION 1*. Interestingly, legumes also inhibit nodulation in order to balance the carbon costs of nodule formation with their needs for nitrogen fixation, and this suppression of nodulation is also mediated by type-B RRs (Chen et al. 2022). In soybeans the type-B RR GmRRB1d can physically interact with NSP2, interfering with its transcriptional activity, resulting in reduced nodulation (Chen et al. 2022).

Drought and salt tolerance

Cytokinin regulates a myriad of responses to abiotic stress (Argueso et al. 2009; Zwack and Rashotte 2015; Mandal et al. 2022), including the responses to drought and elevated salinity. Pioneering studies from the Blumwald laboratory demonstrated that increased levels of endogenous cytokinin via a transgene harboring an *IPT* gene under the control of a senescence/stress-inducible promoter could confer drought tolerance in tobacco (Rivero et al. 2007) and other plant species (Peleg et al. 2011; Reguera et al. 2013, Décima Oneto et al. 2016; Beznec et al. 2021). The drought tolerance in these lines may be mediated by the increased expression and activity of antioxidant enzymes, which may act to suppress the accumulation of reactive oxygen species during drought stress (Xu et al. 2016). In contrast to these findings, Arabidopsis *ipt* mutants show increased drought tolerance (Tran et al. 2007), and similar results are observed in plants overexpressing *CKX* genes (Lubovská et al. 2014). These seemingly opposing roles for cytokinin in drought tolerance maybe be due to different mechanisms of action and specific cellular contexts. In Arabidopsis, a negative role for cytokinin in drought tolerance occurs through a crosstalk with abscisic acid signaling (Huang et al. 2018). SnRK2 kinases, which participate in abscisic acid signaling, phosphorylate type-A RRs, stabilizing their protein levels and thus inhibiting cytokinin signaling. Conversely, type-B RRs physically interact with SnRK2 kinases to inhibit their function (Huang et al. 2018). Through this mutual interaction, these hormones act to fine tune growth and drought responses.

Cytokinin also appears to affect tolerance to salinity, though this also appears to be a complex interaction. In Arabidopsis, *ahk2* and *ahk3* mutants were found to be more salt tolerant than the wild type (Tran et al. 2007). Consistent with this, Arabidopsis lines with reduced cytokinin content (*CKX* overexpression or *ipt* loss-of-function) displayed enhanced salt tolerance (Nishiyama et al. 2011), and elevation of endogenous cytokinin via inducible expression of an *IPT* gene resulted in hypersensitivity to salt (Wang et al. 2015). In tomato, elevation of cytokinin biosynthesis in roots resulted in enhanced salt tolerance (Ghanem et al. 2011). These results suggest that cytokinin negatively regulates salt tolerance. However, a different study reported that *ahk2* mutants, but not mutations in the other 2 AHK receptors, displayed hypersensitivity to salt stress (Kumar and Verslues 2015). Likewise, in rice, reduced expression of *AHPs* via RNAi resulted in hypersensitivity to elevated salinity (Sun et al. 2014), and disruption of 2 type-A RR genes in rice displayed increased salinity tolerance (Wang et al. 2019), suggesting that cytokinin positively regulated salinity tolerance in rice. Consistent with this, reduced expression of *OsCKX2* (via RNAi) resulted in increased endogenous cytokinin and strong salt tolerance in vegetative shoots, which lead to a significant increase in yield in the presence of high salinity (Joshi et al. 2018). In contrast, overexpression of a cytokinin glucosyltransferase in rice, which would decrease active cytokinin levels, also enhanced salt tolerance in adult plants, though it led to increased salt sensitivity in young seedlings (Li et al. 2020). Modulation of cytokinin redistribution in rice such that there were elevated levels of cytokinin specifically in roots resulted in increased salt tolerance (Yin et al. 2020). Overall, these and other studies suggest a complex relationship between cytokinin function and salinity tolerance, with perhaps differing effects depending on the tissues in which cytokinin function is altered and on the particular plant species examined.

A recent study revealed that salt stress induced degradation of multiple type-B RRs in Arabidopsis via phosphorylation by 2 MAP kinases (MPK3/6) (Yan et al. 2021), suggesting that these MAPKs modulate salt sensitivity by reducing cytokinin sensitivity by targeted degradation of type-B RRs.

Nutrient uptake

Cytokinin influences the uptake and distribution of essential nutrients, such as nitrogen, phosphorus, and potassium (Pavlů et al. 2018). This can enhance nutrient acquisition efficiency, critical for sustaining growth during environmentally stressful conditions. Of these essential nutrients, nitrogen, in the form of nitrate, is one of the most growth limiting nutrients and is tightly linked to cytokinin levels and signaling. In response to nitrate supply, the expression of *IPT* genes is increased (Miyawaki et al. 2004), leading to the accumulation of cytokinin in the roots via the action of *IPT3* (Takei et al. 2004a). The increased cytokinin levels in the roots results in changes in root architecture (Ruffel et al. 2011) and also changes in expression of nitrate transporters (Lezhneva et al. 2014), fine-tunning root growth and nitrate uptake to the environmental conditions present. Cytokinins produced in the roots are then translocated to shoots via the xylem, mainly via the ABCG14 cytokinin transporter (Poitout et al. 2018). In the shoot, cytokinin influences multiple aspects of plant growth, including glutamine/glutamate metabolism (Poitout et al. 2018) and SAM activity (Landrein et al.

2018). Thus, levels of cytokinin within the plant function as a long-distance systemic signal of the nitrogen status, modulating plant growth. A possible mechanism mediating this long-distance signal has been recently elucidated: NPL7, a master transcriptional regulator of nitrate signaling, binds to the promoters of the cytokinin transporters *ABCG14*, *PUP14* and *PUP18* (Alvarez et al. 2020), inducing cytokinin transport to shoots. NLP7 is also required for the cytokinin-dependent expression of *CRFs* in the shoot, which in turn regulate expression of *PIN* auxin efflux transporters that mediate shoot growth in response to nitrate (Abualia et al. 2022).

Conclusions

Cytokinin plays pleiotropic roles throughout plant development, with often very distinct roles and effects in different tissues, at different times in development, and in response to various biotic and abiotic factors. An unresolved question remains: What is the molecular basis for the specificity of cytokinin? That is, how does this particular phytohormone have such distinct effects on different cells and tissues throughout the plant and across development? Differences in the cellular context in which the cytokinin signal is perceived likely account for the specificity. The cellular context is defined by the suite of cytokinin signaling elements expressed in a target cell (multiple paralogs of each signaling element in most plants), the activity of various partner transcription factors (multiple such factors identified as described above), the epigenetic state of the genome, and other interacting signaling pathways impinging on the cell. Differences in chromatin accessibility have been linked to differences in the effect of cytokinin on particular target genes in roots and shoots, and cytokinin also alters the accessibility of chromatin throughout the Arabidopsis genome in a type-B RR-dependent manner (Potter et al. 2018). Single cell approaches may shed light on the specifics of various interactions and specificity involved in cytokinin signaling.

Much of this review has focused on results in the model plant Arabidopsis, with some brief highlights from other species. Cytokinin function is being analyzed in many other plant species, including monocots such as maize and rice, as well as the lower plant *Marchantia polymorpha* (Aki et al. 2019). The future will likely reveal new aspects of cytokinin function beyond the Arabidopsis paradigm.

There are undoubtably additional inputs into cytokinin signaling yet to be identified, as well as missing components involved in cytokinin metabolism (e.g. what is the enzyme involved in the biosynthesis of dihydrozeatin? What is the source of cis-zeatin?). Further, we are only just beginning to understand cytokinin transport throughout the plant and within the cell. Finally, the modulation of cytokinin function to improve various aspects of agriculture is just starting. Although the past decades have seen remarkable progress in our understanding of cytokinin biology, the future should continue to reveal the secrets of this remarkable signaling molecule.

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