

DEVELOPMENT AT A GLANCE

Cytokinin signaling in plant development

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ABSTRACT

The phytohormone cytokinin plays diverse roles in plant development, influencing many agriculturally important processes, including growth, nutrient responses and the response to biotic and abiotic stresses. Cytokinin levels in plants are regulated by biosynthesis and inactivation pathways. Cytokinins are perceived by membrane-localized histidine-kinase receptors and are transduced through a His-Asp phosphorelay to activate a family of transcription factors in the nucleus. Here, and in the accompanying poster, we summarize the current understanding of cytokinin metabolism, transport and signaling, and discuss how this phytohormone regulates changes in gene expression to mediate its pleiotropic effects.

KEY WORDS: Cytokinin, Plant hormones, Cell signaling, Two-component signaling

Introduction

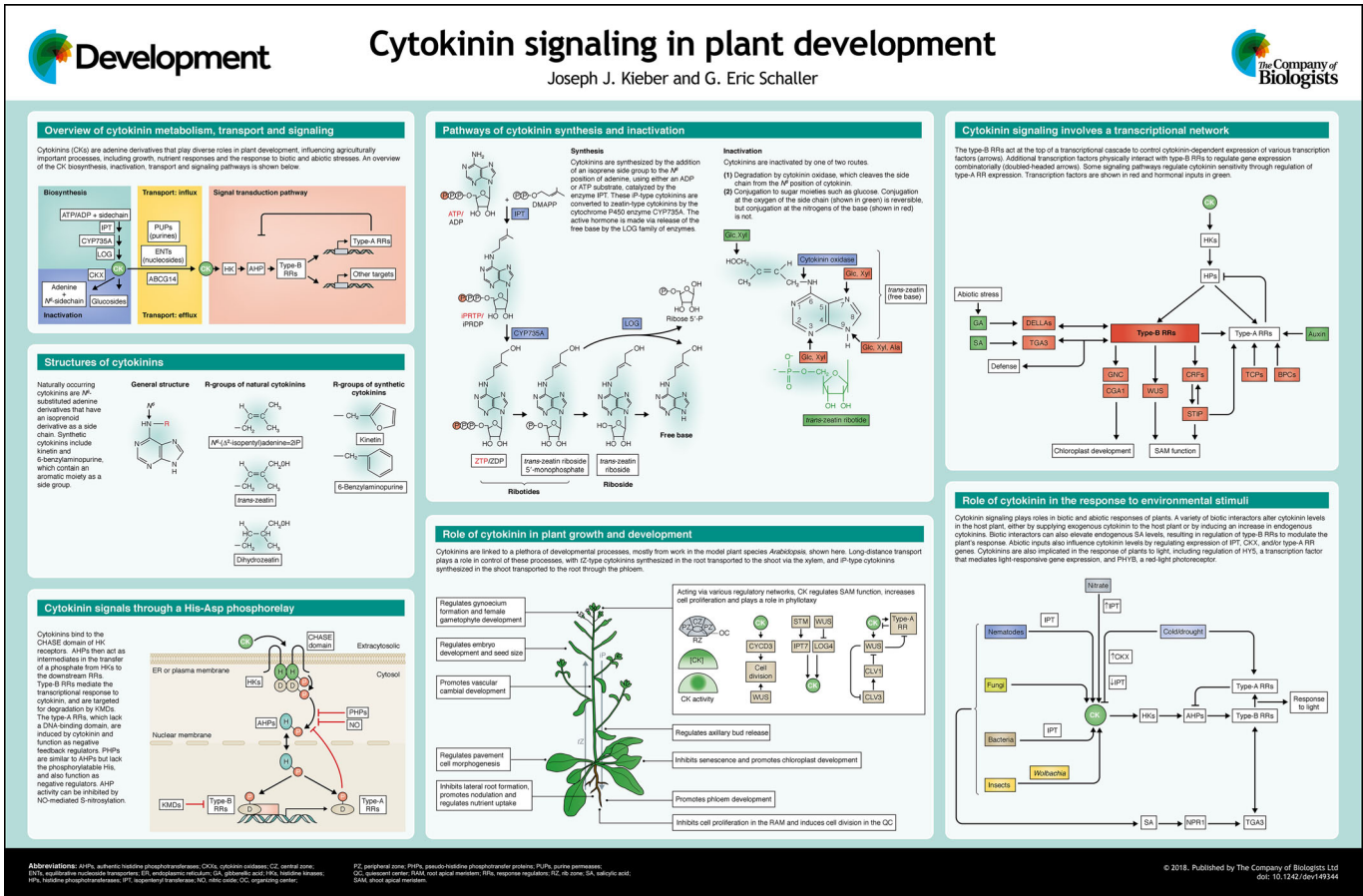
Cytokinins were discovered in a search for factors that promote cell proliferation in cultured plant cells in concert with a second phytohormone, auxin. This search resulted in the identification of the synthetic cytokinin kinetin (Miller et al., 1956, 1955), and subsequent studies identified the cytokinin zeatin as an endogenous plant growth regulator (Letham, 1973). Cytokinins have since been linked to numerous aspects of plant growth and development (Argueso et al., 2012; Kieber and Schaller, 2014; Mok and Mok, 1994).

Here, we provide a brief overview of the metabolic pathways that regulate the levels of this group of plant hormones and highlight recent advances in the understanding of cytokinin transport. We then discuss the mechanisms by which cytokinins signal to influence plant development. We focus on studies in *Arabidopsis*, in which most of the initial discoveries were made.

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The biosynthesis and metabolism of cytokinins

Naturally occurring cytokinins are adenine derivatives with isoprenoid side chains attached to the N^6 position of the adenine ring. Almost all organisms make cytokinins; for example, isopentenyl adenine derivatives are found adjacent to the anticodon loop of a subset of tRNAs in most eukaryotes and bacteria (Persson et al., 1994). However, only in plants and the protist *Dictyostelium* have cytokinins been definitively shown to act in cellular signaling. In higher plants, the most prevalent cytokinin is zeatin, which can occur in both *trans* and *cis* configurations. The *trans* form, *trans*-zeatin (*tZ*), is an active cytokinin in all plant species; in contrast, *cis*-zeatin (*cZ*) is active in only a subset, even though it is present ubiquitously in plants and in some, such as rice, is the major form (Gajdošová et al., 2011). The active species of cytokinins are free bases (Hothorn et al., 2011; Lomin et al., 2015), but cytokinins can also exist in inactive forms as ribosides (in which a ribose sugar is attached to the N^9 nitrogen of the purine ring) and as ribotides (in which the ribose moiety contains a phosphate group).

The biosynthesis of cytokinins begins with the addition of a prenyl group derived from dimethylallyl diphosphate to the N^6 position of ADP/ATP. This is catalyzed by an isopentenyl transferase (IPT) (Sakakibara, 2006), which is encoded by a multigene family in most plants (Kakimoto, 2001; Takei et al., 2001). The resulting iP ribotides can subsequently be converted to *tZ*-type cytokinins via hydroxylation of the isoprenoid side chain in a process that is carried out by the cytochrome P450 enzymes CYP735A1 and CYP735A2 (Takei et al., 2004b). This hydroxylation of the side chain of iP to form *tZ*-type cytokinins plays an important role in promoting shoot growth in *Arabidopsis* (Kiba et al., 2013). In contrast, the biosynthesis of *cZ* is not well understood, but likely involves tRNA-IPTs catalyzing the N^6 -prenylation of adenine on tRNA (Kasahara et al., 2004). The free-base, active forms of cytokinins are synthesized from cytokinin ribotides in a single enzymatic step, which is catalyzed by LONELY GUY (LOG) family cytokinin nucleoside 5'-monophosphate phosphoribohydrolases (Kurakawa et al., 2007; Kuroha et al., 2009).

The levels of active cytokinins can also be modulated via conjugation to a sugar, most commonly glucose, or through irreversible cleavage by cytokinin oxidases (CKXs) (Werner et al., 2006). *O*-glycosylation, which occurs at the oxygen on the side chains of cytokinins, is catalyzed by glucosyltransferases and can be reversed by β -glucosidases (Brzobohaty et al., 1993). In contrast, *N*-glycosylation, which occurs primarily on the N^7 or N^9 of the purine ring, is thought to be irreversible. Glucosyl conjugates are inactive in bioassays, and these conjugated cytokinins also fail to bind to histidine kinase (HK) cytokinin receptors (Spichal et al., 2004). CKXs irreversibly cleave both the free-base and riboside forms of cytokinins at the N^6 -side chains to decrease active cytokinin levels (Werner et al., 2006). Notably, overexpression of CKXs leads to a reduced level of endogenous cytokinin and results in a variety of developmental defects (Werner et al., 2003, 2001). Both *tZ* and iP are cleaved by CKXs, but dihydrozeatin and the synthetic cytokinins kinetin and 6-benzylaminopurine are resistant to cleavage by CKXs (Galuszka et al., 2007; Zalabák et al., 2014).

The synthesis and degradation of cytokinins are regulated by multiple inputs, including endogenous developmental cues as well as biotic and abiotic factors (Sakakibara, 2005; Werner et al., 2006). For example, the LOG genes are differentially expressed in various tissues during plant development, and this is important for regulating the activity of the shoot apical meristem (Chickarmane et al., 2012; Kuroha et al., 2009). Members of the CKX family in *Arabidopsis* also exhibit distinct patterns of expression, intracellular

locations, enzymatic properties and responses to external factors (Galuszka et al., 2007; Kowalska et al., 2010; Schmölling et al., 2003). Furthermore, the expression of several CKX genes is induced by cytokinin, providing a feedback mechanism to dampen cytokinin function. The levels of available macronutrients, such as nitrate and phosphate, also regulate the expression of CKX as well as IPT genes (Argueso et al., 2009). Using publicly available transcriptomic data, the response of all genes involved in cytokinin signaling and metabolism to various environmental cues has been described (Ramireddy et al., 2014). Furthermore, it has been shown that a variety of organisms that interact with plants, including bacteria, fungi, nematodes and insects, can modulate cytokinin levels *in planta*, often by supplying cytokinin that they themselves have synthesized (Naseem and Dandekar, 2012; Shigenaga and Argueso, 2016; Siddique et al., 2015).

Cytokinin transport

It was originally thought that cytokinins were synthesized in the roots and then transported to the shoots, but it is now clear that cytokinins are synthesized in numerous cell types in both roots and shoots, and that both local and long-distance transport occurs (Hirose et al., 2008; Kamada-Nobusada and Sakakibara, 2009; Miyawaki et al., 2004). Cytokinins are transported from roots to shoots via the xylem (primarily as *tZ*-ribosides) and from shoots to roots via the phloem (primarily as iP-type cytokinins) (Hirose et al., 2008; Kudo et al., 2010). Root cytokinin levels rise in response to nitrate, and the transport of this elevated cytokinin could represent a long-distance signal to coordinate shoot and root development (Miyawaki et al., 2004; Takei et al., 2004a). Although *tZ*-riboside is the primary form of cytokinin transported from root to shoot, the active free-base *tZ* is also transported, though at much lower levels (Osugi et al., 2017). The translocated *tZ* plays a role in regulating leaf size, but not meristem activity in the shoot. Interestingly, the relative ratio of *tZ*/*tZ*-ribotide in the xylem sap changes in response to nitrate availability, suggesting that plants modulate the ratio of these cytokinin species translocated from the root in order to fine tune shoot growth in response to varying environmental conditions (Osugi et al., 2017). Shoot-to-root transport of cytokinins takes place through symplastic connections in the phloem and regulates vascular patterning and auxin transport in *Arabidopsis* (Bishopp et al., 2011b), as well as nodulation in legumes (Sasaki et al., 2014).

There is only limited information regarding the mechanisms of cytokinin transport, though potential efflux and influx transporters have been identified (Duran-Medina et al., 2017). As with other phytohormones, ABC transporters are involved in efflux, with ABCG14 mediating cytokinin efflux across the plasma membrane. ABCG14 is expressed primarily in the pericycle and stele of roots and is crucial for loading cytokinin into the xylem sap for transport to the shoot; disruption of ABCG14 results in a 90% reduction in cytokinin levels in the xylem and retardation of shoot growth (Ko et al., 2014; Zhang et al., 2014). Two families of influx carriers have been implicated in cytokinin transport: the PUP family of purine permeases (Bürkle et al., 2003; Gillissen et al., 2000; Zürcher et al., 2016) and the ENT family of nucleoside transporters (Hirose et al., 2005; Sun et al., 2005; Wormit et al., 2004). However, neither are specific for cytokinins; the PUPs can transport purines, such as adenine, in addition to free cytokinins, whereas the ENTs are able to transport nucleosides as well as cytokinin-ribosides. Substrate affinities for these transporters are in the micromolar range, consistent with a general role in purine and nucleoside transport and suggesting that they are able to redistribute cytokinins in bulk with their other more abundant substrates.

Plasma membrane-localized influx carriers might facilitate cytokinin uptake into cells as well as into the phloem for shoot-to-root transport, and, if localized to internal membranes, might act to redistribute cytokinin within the cell. Interestingly, recent studies with PUP14, the most abundant family member, suggest that its influx activity serves to deplete the pool of active cytokinins in the apoplast (Zürcher et al., 2016). However, the relatively low affinity of this transporter for cytokinins (20–40 μM) (Bürkle et al., 2003; Gillissen et al., 2000) compared with the dissociation constant of the HK receptors for cytokinins (1–8 nM) (Lomin et al., 2015, 2011; Romanov et al., 2006) poses a problem for this model. Additional transporters will undoubtedly be discovered as we learn more about the cellular compartmentalization, and hence transport, of the various proteins involved in cytokinin biosynthesis, metabolism and perception.

Cytokinin perception and signal transduction

The cytokinin signal transduction pathway involves a His-Asp phosphorelay similar to that found in bacterial two-component signaling systems, which are the major routes by which bacteria sense and respond to environmental stimuli (Cheung and Hendrickson, 2010; Stock et al., 2000). The two key signaling components in these bacterial systems are a membrane-localized sensor kinase that perceives environmental stimuli, and a response regulator that propagates the signal, often by directly regulating the transcription of target genes. Signal transduction occurs via phosphotransfer between a His residue in the sensor kinase and an Asp residue in the receiver domain of the response regulator. The cytokinin pathway in plants utilizes an extended version of the basic two-component system that typically involves four sequential phosphorylation events alternating between histidine and aspartate residues. This multistep phosphorelay makes use of a ‘hybrid’ HK receptor that contains both histidine kinase and receiver domains, authentic histidine phosphotransferases (AHPs) and separate response regulators (RRs) (Appleby et al., 1996; Schaller et al., 2011). These various cytokinin signaling elements in *Arabidopsis*, rice and other plants are encoded by gene families with overlapping functions (Du et al., 2007; Kieber and Schaller, 2014; Pareek et al., 2006; Pils and Heyl, 2009).

The HK cytokinin receptors have a conserved cytokinin-binding extracytosolic CHASE (cyclases/histidine kinases-associated sensing extracellular) domain, at least two transmembrane domains, and a cytosolic region containing a histidine kinase domain, a canonical receiver domain, and a diverged receiver domain unlikely to function in phosphotransfer (Higuchi et al., 2004; Inoue et al., 2001; Nishimura et al., 2004). The HK receptors primarily localize to the endoplasmic reticulum (ER) membrane, with the CHASE domain oriented into the ER, suggesting that the site of cytokinin binding *in planta* is in the lumen of the ER. This is based on the localization of native and tagged receptors as well as biochemical analyses that have determined which membrane fractions harbor high-affinity cytokinin-binding sites (Caesar et al., 2011; Lomin et al., 2011; Wulfetange et al., 2011). A small portion of HK receptors are found on the plasma membrane (Zürcher et al., 2016) although there is currently debate in the field over the degree to which these are involved in mediating the cytokinin response (Duran-Medina et al., 2017).

Cytokinin binding to the CHASE domain results in activation of the cytosolic histidine-kinase domain and autophosphorylation on the conserved His residue, and this is followed by transfer of the phosphate group to a conserved Asp within the receiver domain (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001;

Yamada et al., 2001). The phosphate is subsequently transferred to the downstream AHP and type-B RR proteins, forming a positive regulatory circuit in which the cytokinin signal eventually results in a transcriptional change in the nucleus (Hwang and Sheen, 2001). Some HK receptors possess both protein kinase and phosphatase activities (Mähönen et al., 2006b), with the switch between these activities being regulated by cytokinin binding.

The AHPs, which act downstream of HK receptors in cytokinin signaling (Hutchison et al., 2006), mediate transfer of a phosphoryl group from the receiver domain of an activated HK receptor to the receiver domain of an RR in the multistep phosphorelay. The AHPs possess no catalytic activity, but rather act essentially as high-energy phospho-donors for the RR autophosphorylation activity. The AHPs are partially redundant positive regulators of cytokinin signaling (Hutchison et al., 2006), but they also act downstream of other plant HKs, such as CKII (Deng et al., 2010; Liu et al., 2017). The AHP proteins have a conserved cysteine residue that is *S*-nitrosylated by nitric oxide *in vitro* and *in vivo*, which inhibits their ability to act as phosphotransfer proteins, reducing both their ability to receive a phosphate from a histidine kinase and to transfer a phosphate to the RRs (Feng et al., 2013). Plants also contain pseudo-histidine phosphotransfer proteins (PHPs), which lack the histidine phosphorylation site (Miyata et al., 1998; Schaller et al., 2008; Suzuki et al., 1998, 2000). These PHPs act as negative regulators of cytokinin signaling and, in *Arabidopsis*, play roles in protoxylem differentiation (Bishopp et al., 2011a; Mähönen et al., 2006a), leaf phyllotaxy (Besnard et al., 2014), and cell patterning during lateral root initiation (Moreira et al., 2013).

There are two types of RRs involved in cytokinin signaling: type-B RRs and type-A RRs. Type-B RRs, which are activated by phosphorylation of the Asp residue in their receiver domain by AHPs, are essential for the initial transcriptional response to cytokinin (Argyros et al., 2008; Ishida et al., 2008). They are characterized by the presence of a receiver domain and a large C-terminal extension that includes a Myb-like DNA-binding domain. The preferred DNA-binding motifs for type-B RRs have been defined, and these motifs are found upstream of many cytokinin-regulated genes (Hosoda et al., 2002; Raines et al., 2016a; Sakai et al., 2000; Zubo et al., 2017). ChIP-seq (chromatin immunoprecipitation combined with sequencing) approaches have revealed that type-B RRs bind to their target sites *in planta* in a cytokinin-dependent manner (Zubo et al., 2017), with binding presumably being activated by phosphorylation of the receiver domain in a manner analogous to that of bacterial response regulators. The rate of turnover varies among members of the type-B RR family, and is regulated at least in part by an E3-ubiquitin ligase complex that includes the KISS ME DEADLY (KMD) F-box proteins (Kim et al., 2013, 2012).

Type-A RRs, by contrast, act as negative-feedback regulators of cytokinin signaling. They contain a receiver domain but, unlike type-B RRs, lack a classic output domain for transcriptional regulation. Type-A RRs are transcriptionally induced in response to cytokinin via direct activation by type-B RRs (Brandstatter and Kieber, 1998; D’Agostino et al., 2000; Taniguchi et al., 1998, 2007), and cytokinin also stabilizes many type-A RRs in a phosphorylation-dependent manner (To et al., 2007). Transcript levels of some type-A RRs are also responsive to various other inputs. For example, *ARR7* and *ARR15* are repressed by auxin in the shoot apical meristem (Leibfried et al., 2005; Wigge et al., 2005), but are induced by auxin in the root apical meristem (Müller and Sheen, 2008). The regulation of type-A RR expression by other signaling pathways might modulate cytokinin sensitivity in various

cell types and/or in response to different environmental cues. The mechanism by which type-A RRs negatively regulate cytokinin signaling is not well understood, but likely involves both competition for phosphotransfer from type-B RRs as well as phospho-dependent interactions with target proteins (To et al., 2007).

The transcriptional response to cytokinins

The treatment of plants with exogenous cytokinin results in substantial changes in gene expression (Bhargava et al., 2013; Brenner and Schmülling, 2015). The basal expression level of many cytokinin-induced genes decreases in cytokinin-insensitive mutants, indicating that they are regulated by endogenous cytokinin in the plant (Argyros et al., 2008). A meta-analysis of multiple microarray experiments performed in *Arabidopsis* has established a set of genes for which expression levels robustly change following treatment with exogenous cytokinin (Bhargava et al., 2013; Brenner and Schmülling, 2015). The transcriptional response to cytokinin in rice shows significant similarity to that of *Arabidopsis*, but substantial differences are also evident; in particular, many more genes that exhibit rapid expression changes are found in rice than in *Arabidopsis* (Raines et al., 2016a). Gene ontology (GO) analyses of type-B RR targets and cytokinin-regulated genes is consistent with the pleiotropic effects of cytokinin, revealing enrichment for hormonal responses, pointing toward the extensive crosstalk that occurs between hormones in the control of growth and development, as well as enrichment for responses to biotic and abiotic stimuli, including light (Bhargava et al., 2013; Zubo et al., 2017). GO analyses also indicate that transcription factors are highly enriched, supporting a model in which type-B RRs act at the top of a transcriptional cascade, with additional transcription factors providing specificity to the downstream responses. Among the targets of type-B RRs is the gene encoding the transcription factor WUSCHEL, a key regulator of shoot meristem activity, thereby providing a direct link between cytokinin signaling and the regulation of shoot growth and development (Meng et al., 2017; Wang et al., 2017; Zhang et al., 2017; Zubo et al., 2017).

Although the transcriptional response to cytokinin requires type-B RR function (Argyros et al., 2008; Yokoyama et al., 2007), there are also other transcription factors that appear to act in concert with type-B RRs to modulate gene expression (Argueso et al., 2010; Raines et al., 2016b). For example, type-B RRs interact with the salicylic acid-regulated transcription factor TGA3 to activate gene expression in response to pathogens (Choi et al., 2010). DELLA proteins, which act as hubs to relay environmental information to various transcriptional circuits, also directly interact with type-B RRs and serve as co-activators for gene targets involved in root growth and photomorphogenesis (Marín-de la Rosa et al., 2015). The cytokinin response factors (CRFs), which are a subclass of AP2 transcription factors induced by cytokinin, modulate the expression of a large number of cytokinin-regulated genes (Raines et al., 2016b; Rashotte et al., 2006). It is likely that other transcription factors act with type-B RRs to provide combinatorial control to enable cytokinins to mediate distinct outputs in various cell types and in response to different environmental conditions.

Cytokinin functions in plant development

Cytokinins play many roles in plant development, often acting in concert with other hormones, most notably auxin, to regulate cell division and differentiation. Below, we briefly summarize the roles of cytokinin in development, but point the reader to several recent

reviews that discuss these functions in more depth (Chandler and Werr, 2015; Kieber and Schaller, 2014; Schaller et al., 2015; Zürcher and Müller, 2016).

One key role of cytokinin is to regulate cell proliferation by influencing cell division and/or differentiation (Schaller et al., 2014). In the shoot, cytokinins promote cell proliferation, including the activity of apical and axillary meristems. Increases in cytokinin levels, in response to reduced expression of a CKX gene, have been linked to increases in grain yield in indica rice varieties (Ashikari et al., 2005), and the disruption of CKX genes in *Arabidopsis* leads to increased seed yield (Bartrina et al., 2011). Cytokinins also play a role in regulating leaf phyllotaxy (Besnard et al., 2014), gynoecium development (Cucinotta et al., 2016; Marsch-Martinez et al., 2012; Müller et al., 2017; Sehra and Franks, 2015), female gametophyte development (Cheng et al., 2013; Yuan et al., 2016) and vascular cambial development (Immanen et al., 2016; Nieminen et al., 2008). In contrast to their role in promoting growth of the shoot, cytokinins inhibit root growth, in part by promoting cell differentiation in the root apical meristem (Dello Ioio et al., 2012; Moubayidin et al., 2010; Werner et al., 2001) and by regulating root branching (Chang et al., 2013, 2015). Cytokinins also regulate root architecture, acting to inhibit lateral root initiation and primary root elongation, and they can modulate root function by regulating the expression of nutrient transport and uptake proteins (Argueso et al., 2009; Werner et al., 2010). Finally, cytokinins act in concert with auxin to regulate vascular development, with cytokinin promoting phloem and auxin xylem development via a pair of mutually inhibitory interactions (Bishopp et al., 2011a; De Rybel et al., 2014; Mähönen et al., 2006a).

Perspectives

We now have a reasonable understanding of the multistep phosphorelay by which cytokinin signals are transduced, starting from membrane-localized HK receptors through to the activation of nuclear-localized type-B RRs. Among the immediate transcriptional targets of the type-B RRs are a set of proteins, the type-A RRs, that act in a negative regulatory feedback loop to dampen the output in response to elevated cytokinin levels. Similarly, cytokinins induce the expression of enzymes (CKXs and glucosyltransferases) that inactivate the signal itself. These negative-feedback pathways indicate that plant cells tightly regulate the output of the cytokinin pathway, which likely reflects its profound effects on plant development and perhaps the common targeting of cytokinin signaling by pathogens. These and other aspects of the pathway provide multiple points of crosstalk with endogenous and environmental signals, reflecting the pleiotropic effects of cytokinin.

Despite the enormous progress made in understanding cytokinin metabolism and signaling, important questions remain. What is the source of *cZ* in plants? Perhaps, as suggested by early cytokinin researchers, tRNA is a source for this *cis* isomer. Are functional cytokinin receptors localized to both the ER membrane and the plasma membrane? What imparts specificity to cytokinin signaling to enable distinct outputs in various cell types? This is likely to involve the particular subsets of cytokinin signaling elements found in a cell as well as different contextual inputs that modulate cytokinin-responsive gene expression, including partner transcription factors that regulate type-B RR target selection. What is the precise mechanism, in addition to phospho-competition, by which the type-A RRs negatively regulate cytokinin signaling? Are there other as-yet-unidentified elements that play a role in cytokinin signaling? What is the role of cytokinin in plants beyond the

Arabidopsis model? As cytokinin has already been linked to important agricultural traits, including grain yield (Jameson and Song, 2016), nodulation and other biotic interactions (Miri et al., 2016; Naseem et al., 2014; O'Brien and Benkova, 2013), and providing resistance to abiotic stress (Bielach et al., 2017), furthering our understanding of cytokinin function is a promising avenue to enhancement of agricultural productivity to meet the increasing demands of a growing world population.

Competing interests

The authors declare no competing or financial interests.

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A high-resolution version of the poster is available for downloading in the online version of this article at <http://dev.biologists.org/content/145/4/dev149344/F1.poster.jpg>.

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