The Perception of Cytokinin: A Story 50 Years in the Making[1]

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In 1913, Gottlieb Haberlandt found that phloem exudates from various plant species had the ability to stimulate cell division in wounded potato (*Solanum tuberosum*) tubers (Haberlandt, 1913). These studies implied the existence of a soluble factor(s) that could promote cell division in plant cells. In the 1950s, work primarily by Carlos Miller in Folke Skoog's laboratory identified cytokinin as a factor that, in concert with auxin, promoted cell division in plant cells (Miller et al., 1955, 1956). This role in cell division has since become one of the defining characteristics of cytokinin and an area of intense research interest, particularly in how it relates to the control of meristem activity. However, cytokinins do more than just regulate cell division. Cytokinins have also been implicated in many plant growth and developmental processes, including organogenesis, leaf senescence, vascular differentiation, sink/source relationships, and nutrient acquisition (Mok and Mok, 1994, 2001; Davies, 2004).

Recent decades have seen a flowering of cytokinin research at the molecular level, with key steps in cytokinin biosynthesis, metabolism, and signal transduction having been elucidated. Naturally occurring cytokinins are N^6-substituted adenine derivatives, and the genes encoding biosynthetic enzymes have been identified in plants, as have genes encoding several important cytokinin metabolic enzymes, including cytokinin oxidase, glucosyltransferase, and xylosyltransferase (Martin et al., 1999a, 1999b, 2001; Mok and Mok, 2001; Kakimoto, 2003a). In addition, as shown in Figure 1, a model for cytokinin perception and signaling has emerged that is similar to bacterial two-component phosphorelays (Kieber, 2001; Hutchison and Kieber, 2002; Sheen, 2002; Heyl and Schmulling, 2003; Kakimoto, 2003b; Grefen and Harter, 2004; Mizuno, 2004; Ferreira and Kieber, 2005). In bacteria, His kinase receptors generally perceive an environmental stimulus, which in turn regulates their autophosphorylation on a His residue (Stock et al., 2000). The phosphate group is in turn transmitted to an Asp residue on a response regulator, thus modulating its function, which is often to act as a transcription factor. There is a more complex version of two-component elements that includes two additional phosphotransfers, which, as in simple two-component systems, occur in sequence His-Asp-His-Asp. It is this phosphorelay mechanism that is found in plant and fungal species, including the cytokinin response pathway (Fig. 1).

Although various clues implicated a two-component signaling pathway in cytokinin signaling about 15 years ago, it was not until the turn of the millennium that a cytokinin receptor was definitively identified. Here, we describe how the cytokinin receptor was identified and discuss the implications and future directions enabled by this finding. Readers are referred to other recent reviews for more detailed discussions of the signaling pathway and role of cytokinin in the regulation of plant growth and development (Müller and Sheen, 2007; To and Kieber, 2008; Werner and Schmulling, 2009).

DISCOVERY OF THE CYTOKININ RECEPTORS

The first clue regarding the identity of the cytokinin receptors was the identification of the *CYTOKININ INDEPENDENT1* (*CKI1*) gene, which encodes an integral membrane protein that is similar to prokaryotic two-component His kinases (Kakimoto, 1996). The genetic screen employed in that study was based on the classic cell proliferation response of callus tissue described by Miller and Skoog, where it was found that high cytokinin-auxin ratios induce shoot formation from undifferentiated plant cells in vitro, while high auxin-cytokinin ratios induce root formation (Miller et al., 1955, 1956; Skoog and Miller, 1957). *CKI1* was identified as a gene that, when overexpressed, conferred upon Arabidopsis (*Arabidopsis thaliana*) hypocotyl explants the ability to proliferate and to produce shoots in vitro in the absence of exogenous cytokinin (Kakimoto, 1996). This observation suggested that CKI1 or some other His kinase was a positive regulator of the cytokinin response pathway. This idea was further supported by the subsequent identification of a set of response regulators as cytokinin primary response genes (Brandstatter and Kieber, 1998; Taniguchi et al., 1998; D'Agostino et al., 2000). However, the gain-of-function nature of the *CKI1* allele used in these studies made the link to cytokinin signaling tenuous. Indeed, CKI1 is most likely not a cytokinin receptor, but cytokinin does turn out to be perceived by a family of closely related His kinases.

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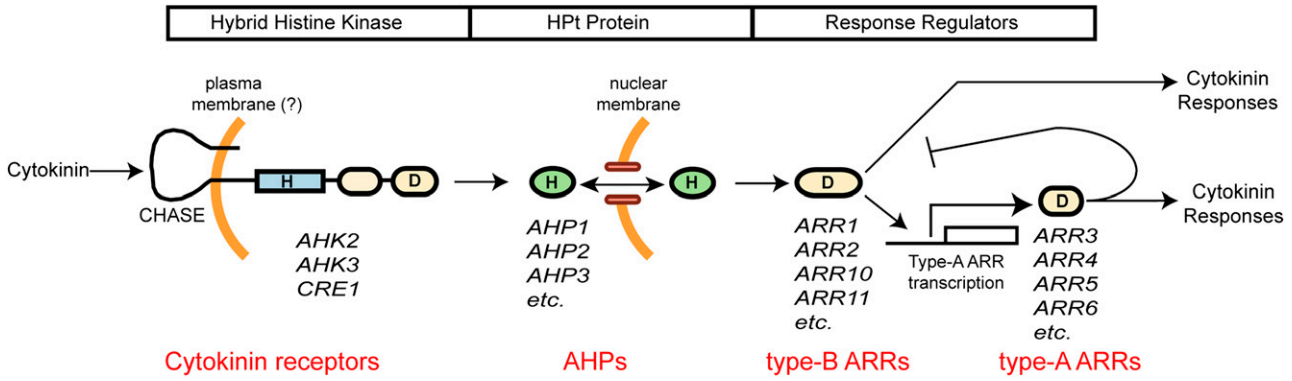

Figure 1. Model of the cytokinin response pathway in Arabidopsis. Cytokinin binds to the extracellular CHASE domain of a cytokinin receptor, initiating a phosphorelay that ultimately results in the phosphorylation of the ARR proteins. The His (H) and Asp (D) residues that become phosphorylated are indicated on each protein. Note that some type A ARRs are also found in the cytoplasm, but for simplicity they are only shown in the nucleus.

The authentic cytokinin receptors were first identified by Kakimoto and coworkers using the converse of the screen they used to identify CKI1; that is, they screened for Arabidopsis mutants whose hypocotyl explants failed to form shoots in conditions (i.e. high cytokinin/auxin medium) that induced shoot formation in the wild type (Inoue et al., 2001). They identified loss-of-function mutations in the *CYTOKININ RESISTANT1* (*CRE1*) gene, which encoded a sensor His kinase related to CKI1. These loss-of-function *cre1* mutants also displayed insensitivity to cytokinin in root elongation assays, which suggests that CRE1 acts as a positive element in cytokinin signaling. The definitive data that CRE1 acts as a cytokinin receptor came from the demonstration that, when expressed in yeast, CRE1 conferred the ability to complement a disruption of an endogenous yeast His kinase, synthetic lethal of *N*-end rule (SLN1), in a cytokinin-dependent manner. This result indicated that the function of CRE1 was activated upon cytokinin binding, a defining feature for a cytokinin receptor. Additional studies published soon thereafter confirmed the identity of CRE1 (also referred to as AHK4 for ARABIDOPSIS HISTIDINE KINASE4 or WOL for WOODEN LEG) as a cytokinin receptor (Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). Subsequent studies confirmed that active cytokinins bind to the extracellular domain of CRE1 and identified two additional genes, *AHK2* and *AHK3*, in the Arabidopsis genome that encoded paralogous cytokinin receptors (Higuchi et al., 2004; Nishimura et al., 2004).

THE CYTOKININ RECEPTORS AND THE HIS-ASP PHOSPHORELAY

The initial discovery that His kinases played a role in cytokinin signaling suggested that other downstream elements of a two-component signaling pathway could mediate transduction of the signal. Fortunately, key genetic tools were being developed concurrently in Arabidopsis, which allowed for this hypothesis to be readily examined. The cDNA and genomic sequencing projects were well under way, and analysis confirmed the presence and repertoire of genes encoding His kinases, His-containing phosphotransfer (HPt) proteins, and response regulators: all the elements that constitute a multistep phosphorelay. Equally important, at that time T-DNA insertional libraries were also being developed for Arabidopsis, a tool that would make it possible to routinely perform reverse genetic approaches (Krysan et al., 1996; Alonso et al., 2003). Indeed, within just a few years, evidence was assembled that placed the cytokinin receptors firmly within the context of a two-component signaling pathway that served to relay the cytokinin signal from receptor to nucleus.

According to the model derived from research conducted over the last decade (Fig. 1), the cytokinin receptors autophosphorylate on a conserved His residue in response to cytokinin binding. The phosphate is passed to an Asp in the receiver domain of the cytokinin receptor and from there to another protein referred to as the HPt protein (Miyata et al., 1998; Hutchison and Kieber, 2007). In the absence of cytokinin, CRE1 acts as a phosphatase to remove phosphate from HPt proteins (Mähönen et al., 2006). The HPt proteins, called AHPs (for ARABIDOPSIS HISTIDINE-CONTAINING PHOSPHOTRANSFER PROTEIN) in Arabidopsis, are in flux between cytosol and nucleus (Punwani et al., 2010). Upon entering the nucleus, the phosphorylated AHPs can pass its phosphate on to members of a transcription factor family called the type B response regulators (referred to in Arabidopsis as type B ARRs, for ARABIDOPSIS RESPONSE REGULATOR), which regulate the plethora of transcriptional changes that characterize the cytokinin response, including both the induction and repression of cytokinin-regulated genes (Argyros et al., 2008). Interestingly, although the receptors were identified almost a decade ago, the subcellular location

at which the cytokinin signal is perceived has not yet been conclusively demonstrated. Initial evidence based on transient expression of an AHK3-GFP fusion in protoplasts suggests that the receptor is mainly localized to the plasma membrane (Kim et al., 2006), but this has yet to be confirmed with protein expressed in plants at native levels.

The primary response pathway for cytokinin is composed of three types of positive regulators (cytokinin receptors, AHP proteins, and type B response regulators) that act within a signaling circuit. But signaling pathways, while requiring a means for activation, also require negative feedback loops to dampen the response upon high or prolonged signal input, to rapidly turn off the pathway once the signal is removed, and/or as a means of cross talk from other signaling pathways. Such negative regulation turns out to be a major role of the type A response regulators, referred to as the type A ARRs in Arabidopsis (To et al., 2007). The genes for the type A response regulators are direct targets of the type B response regulators (Hwang and Sheen, 2001; Sakai et al., 2001) and, as a result, are strongly and rapidly induced in response to cytokinin. Furthermore, the type A response regulator proteins are in general stabilized by cytokinin-induced phosphorylation (To et al., 2007), which acts synergistically with their transcriptional induction to effect a large increase in type A response regulator protein levels upon stimulation of the pathway. Their induction serves to negatively regulate the cytokinin pathway. This negative regulation by the type A response regulators is likely to act in part through phosphorylation-dependent interactions with various target proteins (To et al., 2007), although it may also involve competition with the type B response regulators for phosphorylation by the AHP proteins. What these type A response regulator targets are and how they mediate the negative regulation of the cytokinin response pathway remain open questions.

ANCIENT ORIGINS

Of course, the true history of cytokinin perception goes back much farther than our ability to study it. What are the origins of the cytokinin receptor(s) and the signal response pathway? Or, to put it another way, how did plants acquire the ability to sense and transmit the cytokinin signal in the first place? The available information suggests that the elements of the signaling pathway were acquired very early in the plant lineage, but these did not become adapted for the perception of cytokinin until the advent of land plants.

The key elements in the cytokinin signaling system appear to have been acquired from prokaryotes, based on the finding that cytokinin signaling makes use of the ancient two-component system of His-Asp phosphorelays that is widespread in prokaryotes (Stock et al., 2000) and has also been identified in such eukaryotes as slime molds, fungi, and plants (Wolanin et al., 2002; Schaller et al., 2008). Acquisition of two-component signaling elements by eukaryotes is thought to have occurred primarily through lateral gene transfer, the endosymbiosis of bacteria being a key source for two-component signaling elements (Anantharaman et al., 2007). In the case of plants, endosymbiosis of the cyanobacteria that gave rise to the chloroplast may have been the most significant contributor of these signaling elements.

However, although plants may have acquired the basic elements for a two-component system from bacteria, they did not acquire a system readymade to detect cytokinin itself. Each AHK cytokinin receptor is composed of an extracellular CHASE domain, which binds cytokinin and is flanked by two transmembrane domains, as well as an intracellular portion made up of a His kinase domain and two receiver domains, although only one of the receiver domains contains the conserved Asp phosphorylation target (Fig. 1). The CHASE domain is thus critical to signal input by cytokinin. CHASE domains themselves are extracellular sensing domains found in diverse species, including prokaryotes, slime molds, and plants, and are adaptable to the binding of a variety of ligands, including peptides and adenine derivatives such as cytokinins (Anantharaman and Aravind, 2001; Heyl et al., 2007). Phylogenetic analysis suggests that a cytokinin-binding CHASE domain did not appear in the plant lineage until the advent of land plants, for this domain is present in mosses, lycophytes, and higher plants but appears to be absent in algae (Pils and Heyl, 2009).

The different types of response regulators key to cytokinin signaling appear in the plant lineage at different times (Pils and Heyl, 2009). The type B response regulators are present in algae, indicating a role in plant signaling that predates their function within the cytokinin signaling pathway. It will be interesting to elucidate the ancestral role(s) of the type B response regulators and to determine if this role(s) is retained in higher plants. In contrast, the type A response regulators first appear in land plants, coincident with the appearance of cytokinin receptors. Taken together, the phylogenetic data suggest the use of two-component systems early in the plant lineage, but these were not appropriated for the detection and transmission of the cytokinin signal until the advent of land plants about 725 million years ago.

CONTROL OF CELL DIVISION AND MERISTEM ACTIVITY

Cytokinins were originally identified based on their ability to promote cell division in plant cells. Now, with our knowledge of the genes involved in both cytokinin synthesis and signaling, we are beginning to sort out the extent to which cytokinin regulates cell division, in which tissues cytokinin plays a significant role, and how cytokinin interacts with auxin to control

cell division. Interestingly, cytokinin has differing effects on shoot and root growth, pointing to differing mechanisms for the regulation of cell division at the shoot and root meristems.

Cytokinin is involved in the formation, maintenance, and growth of the shoot apical meristem (Barton, 2001). The classic experiments of Skoog and Miller (1957) demonstrated that cytokinins could stimulate the formation of shoot meristems in plant tissue culture. In addition, application of cytokinin can rescue the *SHOOTMERISTEMLESS* mutant phenotype (Jasinski et al., 2005). Finally, loss of components for either cytokinin synthesis or cytokinin perception results in decreased size of the shoot apical meristem (Higuchi et al., 2004; Nishimura et al., 2004; Kurakawa et al., 2007). Central to the action of cytokinin at the shoot apical meristem is the *WUSCHEL* (*WUS*) transcription factor, which acts to promote stem cell activity (Leibfried et al., 2005; Gordon et al., 2009). *WUS* is activated through the action of cytokinin and the cytokinin receptor-mediated signaling pathway, the cytokinin receptors *AHK2* and *CRE1* both being implicated in the activation of *WUS*. *WUS* in turn represses the type A response regulators. Since the type A response regulators normally inhibit cytokinin signaling, this repressor activity of *WUS* should serve to make the cells more sensitive to cytokinin, thereby creating a positive feedback loop that promotes meristem activity. Additional regulatory loops are also being characterized as well as a potential tie-in to auxin, which can stimulate expression of the cytokinin receptor *CRE1* (Gordon et al., 2007, 2009).

In contrast to its role in the shoot, cytokinin inhibits root growth (Werner et al., 2001). Application of cytokinin results in a decrease in the level of cell division at the root tip, and cytokinin-insensitive mutants display an increase in size of the root meristem (Ioio et al., 2008). How does this work? As with many effects of cytokinin, the model being developed points to cross talk between cytokinin and auxin signaling as being key to the process. A key intermediary in this process is the *SHY2* gene, an auxin repressor of the *Aux/IAA* gene family (Ioio et al., 2008). Cytokinin induces the expression of SHY2 in the root, which in turn downregulates expression of the *PIN* genes for auxin transport, resulting in decreased levels of auxin at the root meristem and a decrease in the rate of cell division.

In addition to the apical meristems, cytokinin is also involved in the regulation of lateral meristems. In *Populus*, orthologs of the AHK cytokinin receptors and type A response regulators are expressed in the cambial zone, and reduction of cytokinin content in the cambium via expression of a cytokinin oxidase gene in transgenic *Populus* caused fewer cell divisions in the vascular cambium and hence a reduction in radial growth (Nieminen et al., 2008). Consistent with a role for cytokinin as a positive regulator of vascular cambium, disruption of a subset of the *IPT* cytokinin biosynthetic genes in Arabidopsis severely reduced cambial activity (Matsumoto-Kitano et al., 2008). Thus, cytokinin regulates cell division in the context of the root, shoot, and lateral meristems, albeit with sometimes differing roles. However, much remains to be learned regarding how cytokinin signaling feeds into cell cycle regulation in different contexts.

HOW DO ONE SIGNAL AND THREE RECEPTORS MEDIATE SO MANY DIVERSE OUTPUTS?

One of the hallmark features of phytohormones, including cytokinins, is their pleiotropic nature. Cytokinin has been implicated in the regulation of a plethora of plant processes, and the question arises of how a single hormone can mediate such a diverse array of outputs. While there have been suggestions of other cytokinin receptors in addition to the AHK sensor kinases, no compelling data implicate any other proteins in cytokinin perception. Furthermore, the absence of all known cytokinin responses examined in the *ahk2/ahk3/cre1* triple mutant suggests that perhaps there are no additional cytokinin receptors. Thus, the diverse processes regulated by cytokinin may all be derived from binding to these few AHK receptors. This is similar to animal signaling systems, in which one signal or signaling system can mediate multiple outputs. For example, the Notch/Delta and EGF signaling systems play roles in a multitude of diverse developmental outcomes (Citri and Yarden, 2006; Ehebauer et al., 2006). As in these systems, the specificity of the response of target cells in plants to cytokinin likely depends on the interaction with other signaling cues received by that cell, including other phytohormonal cues, and the prepatterning of the cell prior to the perception of cytokinin. Thus, one cell could be primed to respond to cytokinin to undergo cell division and another to undergo differentiation. Such a scenario would allow a small number of cytokinin receptors to mediate diverse and distinct outcomes. Furthermore, multiple cytokinin species (e.g. trans-zeatin, cis-zeatin, dihydrozeatin, aromatic cytokinins, etc.) are present in plants, and these bind with distinct affinities to the different cytokinin receptor isoforms (Yonekura-Sakakibara et al., 2004; Romanov et al., 2006). Thus, different forms of cytokinin could have distinct informational content via binding to unique subsets of cytokinin receptors, thus mediating different outputs.

An alternative, but not mutually exclusive, possibility is that there are additional cytokinin receptors present in plants. Several other potential cytokinin receptors have been suggested, including a seven-pass transmembrane protein (Plakidou-Dymock et al., 1998) and several cytokinin-binding proteins (Kulaeva et al., 1998), and in rice (*Oryza sativa*), there is a gene encoding a protein with a predicted CHASE domain fused to a Ser/Thr kinase intracellular domain (Han et al., 2004). However, it remains to be seen if these other proteins serve as authentic cytokinin receptors. The surprising finding that the triple *ahk2/ahk3/cre1*

Arabidopsis mutant is not embryo lethal might indicate the existence of additional cytokinin perception systems, although it is also possible that cytokinin signaling is not essential for plant life. The necessity of cytokinin for plant growth and cell division remains to be determined and will represent an important step in clarifying these questions.

CONCLUSION

The identification of the cytokinin receptor and the subsequent elucidation of the rest of the cytokinin response pathway represent the capstone of over 50 years of cytokinin research. However, these findings are only the beginning of the story, as we are now in a position to dissect the molecular mechanisms by which this fascinating phytohormone mediates its diverse effects on plant growth and development. Historical studies on the effects of cytokinin and more recent studies taking advantage of mutations in the receptors and other signaling components have defined a plethora of plant responses influenced by cytokinin, ranging from senescence to light responses to interactions with biotic and abiotic stimuli. How this simple, ancient signaling mechanism interacts with the rest of the plant signaling network, including other phytohormones, to achieve integrated control of developmental events such as meristem function will also be the focus of future studies. Perhaps we will soon understand at the molecular level the mechanisms by which cytokinin regulates those first effects noted by Miller and Skoog, the regulation of cell proliferation and the induction of shoot organogenesis.

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