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Environmental perception avenues: the interaction of cytokinin and environmental response pathways

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ABSTRACT

Cytokinins were discovered in the 1950s by their ability to promote cell division in cultured plant cells. Recently, there have been significant breakthroughs in our understanding of the biosynthesis, metabolism, perception and signal transduction of this phytohormone. These advances, coupled with physiological and other approaches, have enabled remarkable progress to be made in our understanding of the interactions between cytokinin function and environmental inputs. In this review, we first highlight the most recent advances in our understanding of cytokinin biosynthesis, metabolism and signalling. We then discuss how various environmental signals interact with these pathways to modulate plant growth, development and physiology.

Key-words: abiotic stress; light; nutrients; signal transduction.

INTRODUCTION

Cytokinins were first identified as factors that promoted the proliferation of cultured plant cells (Miller *et al.* 1955, 1956). Subsequently, this phytohormone was linked to numerous aspects of plant growth and development, including seed germination, vasculature development, meristem function, apical dominance and leaf senescence (Mok & Mok 2001). Much progress has been made recently in our understanding of cytokinin biosynthesis and signalling, and how environmental cues interact with these components to modulate plant growth, development and physiology. In this review, we will first discuss cytokinin biosynthesis, metabolism and signalling, and with an emphasis on recent findings, we will then discuss the role of cytokinin in the assimilation of various macronutrients, the crosstalk with light signalling and, finally, the interactions of cytokinin with abiotic and biotic factors. For additional information, the reader should see recent detailed reviews of cytokinin biosynthesis and metabolism (Sakakibara 2006; Hirose *et al.* 2008) and signalling (Ferreira & Kieber 2005; Choi & Hwang 2007; Müller & Sheen 2007; To & Kieber 2007).

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CYTOKININ BIOSYNTHESIS

The first committed and rate-limiting step of cytokinin biosynthesis, the transfer of an isopentenyl moiety from dimethylallyl diphosphate (DMAPP) to the N^6 position of ATP/ADP (Fig. 1), is catalysed by the enzyme isopentenyltransferase (IPT) (Kakimoto 2001; Takei, Sakakibara & Sugiyama 2001a; Sakakibara 2006; Hirose *et al.* 2008). The immediate products of the IPT reaction are isopentenyladenine (iP) ribotides, the isoprene side chain of which is subsequently *trans*-hydroxylated by the P450 monooxygenases CYP735A1 and CYP735A2 to yield zeatin ribotides (Takei, Yamaya & Sakakibara 2004b). Cytokinin nucleotides can be converted to their most active free base forms via dephosphorylation and deribosylation. Recently, a gene encoding an enzyme catalysing one such reaction was identified in rice. The *lonely guy* (*log*) mutant was isolated in a screen for rice plants that displayed shoot meristem defects (Kurakawa *et al.* 2007). *LOG* encodes a phosphoribohydrolase that directly and specifically converts cytokinin-5′-monophosphates to the free base form (Fig. 1). *LOG* expression is localized to the tip of shoot meristems, and it likely fine tunes the spatial distribution of bioactive cytokinins to regulate meristem activity.

The first plant *IPTs* were identified *in silico* by their sequence similarity to the *Agrobacterium tumefaciens tmr* gene, and their identity was confirmed by the expression and analysis of the proteins in *Escherichia coli* (Kakimoto 2001; Takei *et al.* 2001a). IPTs are encoded by a small gene family that displays distinct spatial and sub-cellular distributions (Miyawaki, Matsumoto-Kitano & Kakimoto 2004; Takei *et al.* 2004a; Hirose *et al.* 2008). This suggests that cytokinin synthesis is localized in discrete sites throughout the plant, which is at odds with the classic notion that cytokinins are made primarily in roots and transported to shoots. Despite their non-overlapping patterns of expression, single and double loss-of-function mutants of *ipt* are aphenotypic in *Arabidopsis*; however, higher-order loss-of-function *ipt* mutants display reduced rosette development, reduced shoot meristem function and increased root growth (Miyawaki *et al.* 2006), phenotypes similar to transgenic plants engineered to have a reduced cytokinin content via

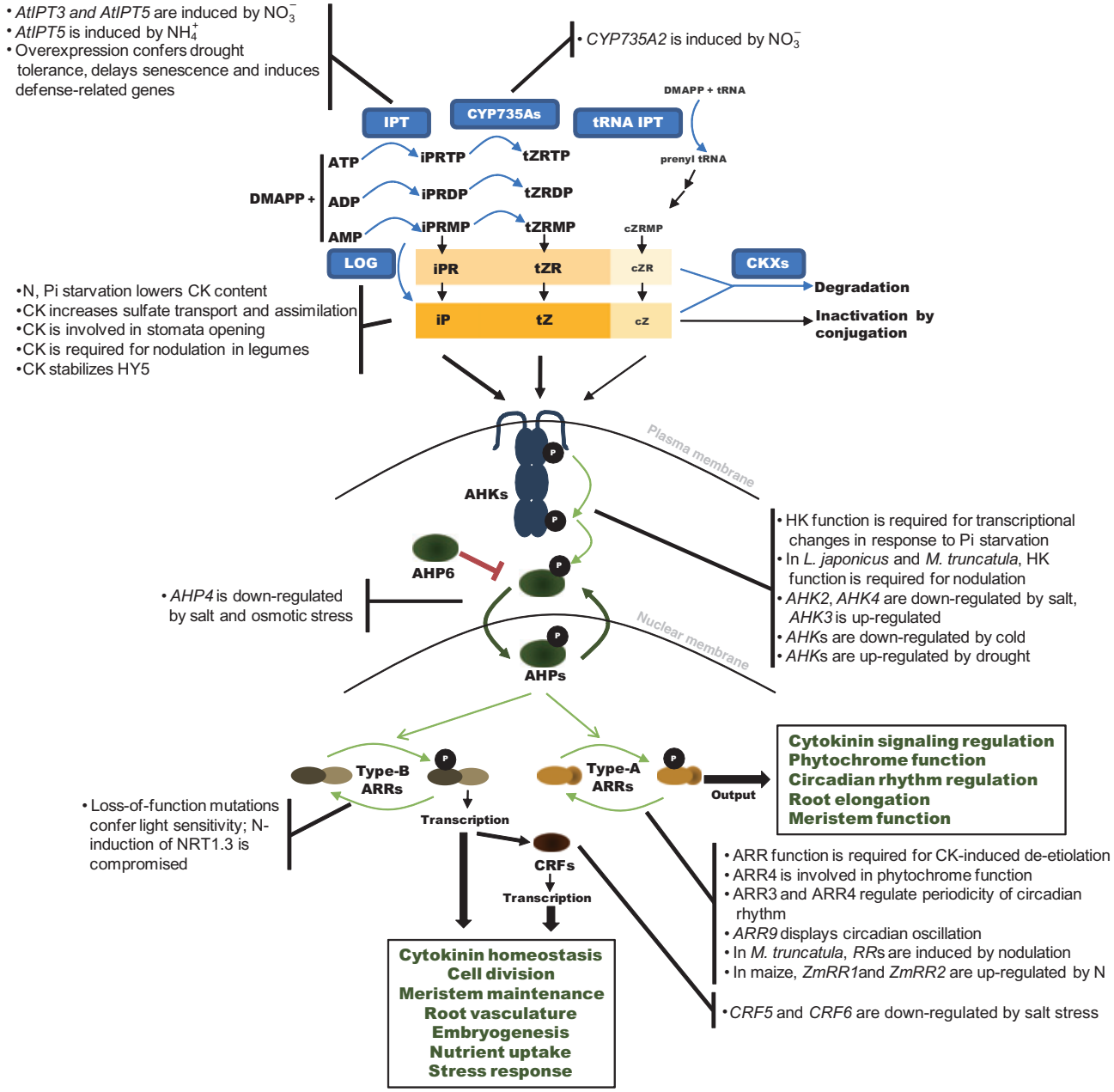

Figure 1. Environmental inputs into cytokinin synthesis and signalling. For the biosynthetic pathway, reactions in which the genes encoding the enzymes have been identified are depicted in blue. Biologically active cytokinins are highlighted in yellow, with the free base cytokinin species being more active than the riboside forms. It is also likely that, at least in some species such as maize, *cis*-zeatin is also an active cytokinin. Cytokinin levels are tightly controlled in plants via inactivation by conjugation to sugar moieties and degradation by cytokinin oxidase/dehydrogenase (CKX). Cytokinin signalling is a multi-step phospho-relay, and phosphoryl transfer is depicted by the light green arrows. Output processes are depicted by the black arrows. See text for additional details regarding the role of the various steps shown. The effect of environmental signals on various genes encoding these processes is depicted. DMAPP, dimethylallyl diphosphate; tZ, *trans*-zeatin; cZ, *cis*-zeatin; iP, N^6-(Δ^2-isopentenyl) adenenine; iPRTP, iP riboside 5′-triphosphate; iPRDP, iP riboside 5′-diphosphate; iPRMP, iP riboside 5′-monophosphate; iPR, iP riboside; tZRTP, tZ riboside 5′-triphosphate; tZRDP, tZ riboside 5′-diphosphate; tZRMP, tZ riboside 5′-monophosphate; tZR, tZ riboside; cZRMP, cZ riboside 5′-monophosphate; cZR, cZ riboside; IPT, isopentenyltransferase; CK, cytokinin; AHKs, *Arabidopsis* hybrid sensor kinase receptors; AHPS, *Arabidopsis* histidine phosphotransfer proteins; ARRs, *Arabidopsis* response regulators; CRFs, cytokinin response factors; HK, hybrid sensor kinase receptor.

overexpression of the cytokinin-degrading enzyme cytokinin oxidase/dehydrogenase (CKX) (Werner *et al.* 2003).

CYTOKININ METABOLISM

In addition to the regulation of its biosynthesis, the levels of active cytokinins are also regulated by conjugation to sugars and by degradation. Cytokinins can be irreversibly inactivated by conjugation to glucose at the N^3, N^7 and N^9 positions of the adenine ring, and can be reversibly modified by conjugation to glucose and, to a lesser extent xylose, to the hydroxyl group of the side chain (Sakakibara 2006). Several of the enzymes catalysing these reactions have been identified (Martin, Mok & Mok 1999a,b; Martin *et al.* 2001). Overexpression of a zeatin *O*-glucosyltransferase in maize leads to phenotypes resembling those of cytokinin-deficient plants, consistent with a role for this enzyme in inactivating cytokinins (Pineda Rodo *et al.* 2008).

Cytokinins with unsaturated isoprenoid side chains (Z- and iP-type cytokinins) can be irreversibly degraded by cleavage of the N^6 side chain by the enzyme CKX. CKX is encoded by a multigene family that is differentially regulated by biotic and abiotic factors (Brugiere *et al.* 2003; Werner *et al.* 2003, 2006). Major quantitative trait loci (QTL) that are responsible for increasing the number of reproductive organs in the inflorescence meristem, and hence grain number, of the *indica* variety of rice were identified as a loss-of-function mutation in a *CKX* gene, *OsCKX2* (Ashikari *et al.* 2005). Reduced expression of this cytokinin oxidase gene causes an increase in the cytokinin content in the inflorescence, which leads to the increase in the number of reproductive organs.

In sum, the steady-state level of active cytokinins is determined by the relative rates of biosynthesis and release of the conjugated forms and the rates of conjugation and degradation.

CYTOKININ SIGNALLING

Elements of the cytokinin signalling pathway share homology to bacterial two-component signalling cascades, by which bacteria perceive extracellular stimuli via a membrane-bound receptor kinase and a cystolic response regulator (RR). In plants, the cytokinin two-component system is a multi-step phospho-relay, consisting of hybrid sensor kinase receptors (HKs), histidine phosphotransfer proteins (HPs) and RRs (Fig. 1) (Ferreira & Kieber 2005; Müller & Sheen 2007; To & Kieber 2007). In *Arabidopsis*, these proteins are referred to as the *Arabidopsis* hybrid sensor kinase receptors (AHKs), *Arabidopsis* histidine phosphotransfer proteins (AHPs) and *Arabidopsis* response regulators (ARRs), respectively.

The cytokinin receptors

The first cytokinin receptor (*AHK4/CRE1/WOL*) was isolated as a mutant that failed to form large green calli on shoot-initiation media and/or whose function in heterologous system was dependent on applied cytokinin (Inoue *et al.* 2001; Suzuki *et al.* 2001a; Ueguchi *et al.* 2001). Two additional cytokinin receptors (AHK2 and AHK3) are present in *Arabidopsis* (Higuchi *et al.* 2004; Nishimura *et al.* 2004). The AHKs bind active cytokinins with high affinity and are able to complement yeast and *E. coli* histidine kinase mutants in a cytokinin-dependent manner (Inoue *et al.* 2001; Suzuki *et al.* 2001a; Yamada *et al.* 2001). The three AHKs display kinase activity when bound to their ligand; however, in the absence of cytokinin, AHK4 (but not AHK2 nor AHK3) displays phosphatase activity (Mähönen *et al.* 2006b). The ability to regulate kinase and phosphatase activities may provide multiple strategies to modulate cytokinin responses.

The AHKs are positive, functionally overlapping regulators of cytokinin signalling (Higuchi *et al.* 2004; Nishimura *et al.* 2004; Riefler *et al.* 2006). The triple receptor mutant is insensitive to cytokinin, and its growth is severely impaired, including reduced leaf size and number, an extremely stunted root and an abbreviated inflorescence that produces a few flowers that are mostly sterile (Nishimura *et al.* 2004; Riefler *et al.* 2006).

The HPs

The AHPs are a small family of proteins that act as intermediates in cytokinin signalling. The AHPs interact directly with various histidine sensor kinases and type-A and type-B RRs (see further discussion) in yeast two-hybrid assays (Urao *et al.* 2000; Suzuki *et al.* 2001b; Tanaka *et al.* 2004; Dortay *et al.* 2006). Genetic analyses indicate that the AHPs are partially redundant positive elements in cytokinin signalling (Hutchison *et al.* 2006). The quintuple *ahp1,2,3,4,5* mutant has a stunted primary root phenotype very similar to that of the triple receptor mutant (Hutchison *et al.* 2006). *AHP6*, a pseudo HP that lacks the His residue that is the target of phosphorylation, acts as an inhibitor of cytokinin signalling (Mähönen *et al.* 2006a) that functions to facilitate protoxylem specification by interfering with cytokinin signalling in a spatially defined manner (Mähönen *et al.* 2006a).

The type-A RRs

The type-A RRs are members of a large gene family whose transcription is rapidly elevated by exogenous cytokinin (Brandstatter & Kieber 1998; D'Agostino, Deruère & Kieber 2000; Asakura *et al.* 2003; Jain, Tyagi & Khurana 2006). In addition to transcriptional regulation, cytokinin treatment also results in an increase in the half-life of a subset of type-A ARR proteins (To *et al.* 2007). Type-A RRs contain a single receiver domain that is phosphorylated on a conserved Asp by the upstream AHPs. Genetic analysis in *Arabidopsis* indicates that type-A ARRs are partially redundant negative regulators of cytokinin signalling (To *et al.* 2004) that act through phospho-dependent interactions with as yet to be identified target proteins (To *et al.* 2007).

Disruption of subsets of type-A *ARR* genes affects multiple aspects of development, including rosette size, root elongation, phyllotaxy, meristem function and embryo development (To *et al.* 2004; Leibfried *et al.* 2005; Müller & Sheen 2008). Four type-A *ARRs* are directly repressed by the meristem identity transcription factor WUSHEL, and the disruption of these type-A ARRs leads to defects in the shoot apical meristem function (Leibfried *et al.* 2005). Similarly, in maize, the disruption of a single type-A RR leads to alterations in leaf phyllotaxy and an increased size of the meristem (Giulini, Wang & Jackson 2004). Two type-A *ARR* genes have recently been shown to be repressed by auxin and to play a role in root stem cell specification in the early embryo (Müller & Sheen 2008).

The type-B RRs

Type-B ARRs contain a receiver domain as well as a C-terminal extension that harbours a DNA-binding GARP domain. A consensus binding sequence for type-B ARRs has been delineated, (G/A)GGAT(T/C), and this sequence is over-represented in the promoters of many of the cytokinin primary response genes, including the type-A *ARRs* (Sakai, Aoyama & Oka 2000; Rashotte *et al.* 2003). Consistent with their role as transcription factors, type-B ARRs localize to the nucleus (Hwang & Sheen 2001; Imamura, Yoshino & Mizuno 2001; Asakura *et al.* 2003; Mason *et al.* 2004). Genetic and molecular analyses indicate that the type-B ARRs are redundant positive elements in cytokinin signalling and are the immediate upstream activators of type-A *ARR* gene expression (Hwang & Sheen 2001; Sakai *et al.* 2001; Mason *et al.* 2005; Argyros *et al.* 2008; Ishida *et al.* 2008b). The *arr1,10,12* triple mutant shows almost complete insensitivity to cytokinin in numerous assays and displays severe developmental defects similar to those observed in the triple *ahk* and quintuple *ahp* mutants (Argyros *et al.* 2008; Ishida *et al.* 2008b).

Other transcription factors involved in cytokinin responses

In addition to the type-B ARRs, there are several other transcription factors that have been implicated in the response to cytokinin. The cytokinin response factors (CRFs) act, along with the type-B ARRs, to mediate the transcriptional response to cytokinin (Rashotte *et al.* 2006). The six CRFs are a subset of the APETALA2 (AP2)-like superfamily. Three of the *CRFs* are induced in response to exogenous cytokinin in a type-B ARR-dependent manner (Rashotte *et al.* 2003, 2006). A subset of cytokinin-regulated genes are misexpressed in *crf* loss-of-function mutants. Recently, a subgroup of the plant-specific transcription factor family GLABROUS1 enhancer-binding protein [GeBP, GPL (GeBP-like)] has been implicated in cytokinin responsiveness (Chevalier *et al.* 2008). The disruption of three closely related members of this family results in an elevated level of type-A *ARR* gene expression and reduced sensitivity to cytokinin specifically in the shoot. The GeBP/GPL proteins likely act to antagonize the negative feedback of the type-A ARRs.

CYTOKININ AND THE ASSIMILATION OF MACRONUTRIENTS: IT'S ELEMENTAL MY DEAR WATSON

Nitrogen

The abilities to sense nitrogen levels and to regulate nitrogen uptake are essential for plant growth and reproduction. Plants obtain nitrogen from inorganic forms present in the soil, the most abundant of which is nitrate (NO_3^-). The perception and assimilation of nitrate is dynamically regulated *in planta* (Stitt 1999). Once absorbed from the soil, nitrate ions are translocated to the shoot, where they are reduced to nitrogen-containing organic compounds, including amino acids and proteins. Nitrate levels regulate the expression of genes involved in its assimilation and reduction, such as nitrate transporters, nitrate reductase, glutamine synthetase and glutamate synthase, as well as the enzyme activities of their encoded proteins (reviewed in Sakakibara, Takei & Hirose 2006). The level of nitrate also correlates with the expression of genes involved in sugar metabolism, sulphur assimilation and secondary metabolism, reflecting the importance of nitrate as a principal regulator of nitrogen assimilation and plant metabolism (Forde 2002).

The level of nitrate available to the plant regulates the endogenous concentration of cytokinins. Plants grown on low levels of nitrogen show reduced levels of cytokinin, and the addition of nitrate leads to an increase in the levels of various cytokinin species (Salama & Waering 1979; Samuelson & Larsson 1993; Takei *et al.* 2001b), which in turn leads to the up-regulation of the cytokinin-responsive type-A RRs (Sakakibara *et al.* 1998; Taniguchi *et al.* 1998; Kiba *et al.* 1999). Nitrate addition up-regulates cytokinin levels in part by inducing expression of cytokinin biosynthetic genes. *AtIPT3* and *AtIPT5* expression are up-regulated after nitrate treatment, predominantly in the roots (Miyawaki *et al.* 2004; Takei *et al.* 2004a). However, the addition of ammonium to N-starved plants leads to an increase in the levels of *AtIPT5* but not *AtIPT3*, indicating that the regulation of cytokinin biosynthesis depends on the forms of nitrogen available to the plant (Takei *et al.* 2004a). The expression of *CYP735A2* (Fig. 1) is also regulated by nitrate levels (Takei *et al.* 2004b; Wang *et al.* 2004).

Recent microarray analyses have revealed that the expression of the nitrate- and cytokinin-inducible low-affinity nitrate transporter gene *NRT1.3* is compromised in a type-B *arr1,10,12* triple mutant (Argyros *et al.* 2008), suggesting that the expression of this nitrate-responsive gene is, in part, regulated by cytokinin signalling components. Genome-wide microarray analysis revealed that treatment with either cytokinin or nitrate induces the expression of various genes involved in primary metabolism (Wang *et al.* 2000, 2003, 2004; Rashotte *et al.* 2003; Brenner *et al.* 2005;

Kiba *et al.* 2005), and a significant overlap among the sets of genes induced by each treatment is observed (Sakakibara *et al.* 2006).

The correlation among nitrate, cytokinin levels and their effects on gene expression has led to the suggestion that cytokinin can act as a root to shoot signal to regulate tissue-specific nitrogen metabolism (Takei *et al.* 2001b, 2002; Gessler, Kopriva & Rennenberg 2004; Sakakibara 2006). Whereas iP is the most abundant cytokinin species present in leaf exudates and in the phloem (Weiler & Ziegler 1981; Emery & Atkins 2002; Corbesier *et al.* 2003), zeatin riboside is transported in the xylem (Beveridge *et al.* 1997; Takei *et al.* 2001b). Along with the localized expression of *AtIPT3* in the phloem (Miyawaki *et al.* 2004) and the high levels of expression of *CYP735A2* in the roots and stems, but low *CYP735A2* levels in the leaves (Takei *et al.* 2004b), these data suggest a model in which increased nitrate in the roots leads to the induction of *AtIPT3* and *CYP735A2* and thus the biosynthesis of zeatin ribosides in the root, which can subsequently be transported to the shoot via the xylem. In the shoot, up-regulation of *AtIPT3* and possibly *AtIPT5* would lead to the accumulation of iP cytokinins, which are translocated via the phloem to other parts of the plant. The balance of different cytokinin species, perceived by two-component elements, would signal the availability of nitrate forms, and would ultimately lead to the expression of cytokinin-responsive and, potentially, also nitrate-responsive genes to regulate nitrogen metabolism in the plant (Sakakibara *et al.* 2006).

Phosphorus

Phosphorus is a macronutrient that is essential for many biochemical reactions, and its availability as orthophosphate (Pi), the form of phosphorus that is preferentially taken up by plants, is limited in most agricultural soils. In response to phosphate starvation, plants undergo various forms of adaptation that are reminiscent of responses to alterations in the levels of cytokinin, including an increase in the number of lateral roots and an increase in the root : shoot ratio (Raghothama 1999).

A role for cytokinins in the Pi-starvation response has been suggested based on evidence that cytokinin levels are reduced in Pi-starved plants (Salama & Waering 1979; Horgan & Waering 1980; Wagner & Beck 1993). Consistent with this, the *Arabidopsis pho1* and *pho2* mutants, which fail to accumulate and hyper-accumulate Pi in shoots, respectively, show altered sensitivity to cytokinin (Lan, Li & Fischer 2006).

Pi starvation leads to complex changes in gene expression (Hammond *et al.* 2003; Wu *et al.* 2003). An initial transient change in the expression of genes encoding general stress response factors is observed, followed by the induction of genes directly involved in the response to Pi starvation (Hammond *et al.* 2003). In general, cytokinins down-regulate Pi starvation-responsive genes (Martin *et al.* 2000; Hou *et al.* 2005). Microarray analysis of rice plants under Pi starvation not only confirmed these results but also identified genes that were up-regulated or unchanged upon cytokinin addition, indicating that the effect of cytokinin in Pi-starvation gene expression is complex (Wang *et al.* 2006).

The *ahk3,4* mutant is defective for the cytokinin repression of gene expression in the local response to Pi starvation but is unaffected in the systemic response (Martin *et al.* 2000; Franco-Zorrilla *et al.* 2005). This suggests that while AHK3 and AHK4 are necessary for the local response, AHK2 may play an important role in the systemic response, perhaps redundantly with AHK3. Consistent with this model, *AHK4* is primarily expressed in roots and *AHK2* and *AHK3* most highly in shoots (Higuchi *et al.* 2004; Nishimura *et al.* 2004). The expression of *AHK4* was found to be repressed by the addition of Pi, indicating a negative feedback regulatory role for cytokinin (Franco-Zorrilla *et al.* 2002).

Sulphur

Sulphur is assimilated by plant roots in its inorganic, anionic sulphate form. Upon assimilation, sulphate is reduced to sulphide in plastids, giving rise to the sulphur-containing amino acid cysteine, which is either directly incorporated into proteins and glutathionine or converted into the amino acid methionine. In addition, sulphur acts as a cofactor for various enzymes, is involved in the modification of proteins, carbohydrates and lipids, and is essential for the formation of secondary metabolites involved in defence responses to pathogens and herbivores (Leustek *et al.* 2000).

Sulphate-responsive genes are up-regulated in response to cytokinin in either sulphur-depleted or non-depleted conditions and are only marginally up-regulated by other plant hormones (Ohkama *et al.* 2002). The expression of *APR1*, which encodes an enzyme involved in the key step of sulphate assimilation, is induced by cytokinin (Ohkama *et al.* 2002). The genes encoding sulphate transporters are also regulated by cytokinin (Maruyama-Nakashita *et al.* 2004), as well as by sucrose and/or nitrate (Ohkama *et al.* 2002; Rouached *et al.* 2008). However, the concentration of cytokinins is not altered after sulphate starvation, and the application of cytokinin does not change the concentration of *O*-acetyl-L-serine (Ohkama *et al.* 2002), a cysteine biosynthetic precursor that acts as a positive regulator of sulphate starvation-responsive genes (Kim *et al.* 1999; Hirai *et al.* 2003), suggesting that the action of cytokinin in the regulation of sulphate uptake and sulphate-responsive genes is most likely indirect.

Iron

Iron is a micronutrient required in small quantities for plant growth. In alkaline soils, iron can become limiting, leading to chlorosis and other defects. Iron deficiency triggers the induction of genes involved in iron uptake, including genes encoding high-affinity iron transporters. The expression of these genes is regulated primarily at the level of transcription, and a basic helix-loop-helix (bHLH) transcription

factor, called *FIT1* in *Arabidopsis*, is involved in this regulation (Briat, Curie & Gaymard 2007). A recent report provides evidence that cytokinins act to negatively regulate the expression of a subset of iron-responsive genes (Séguéla *et al.* 2008). This repression requires the AHK3 and AHK4 receptors but is independent of iron status and of *FIT1*. A transient rise in *AtIPT3* and type-A *ARR* gene expression occurred in response to iron resupply to iron-starved plants (Séguéla *et al.* 2008), which is reminiscent of the induction of these same genes in response to nitrogen resupply. It was found that other factors that inhibit root growth, such as mannitol and NaCl, also repress the iron-starvation response genes (Séguéla *et al.* 2008). The authors propose that cytokinin down-regulates iron-responsive gene expression through a growth-dependent pathway (Séguéla *et al.* 2008), which may underlie the effect of cytokinin on other nutrient assimilation pathways. Further studies that carefully examine the timing of the effects of cytokinin on root growth and nutrient-regulated genes expression, coupled with a better understanding of the transcription circuits regulating iron-responsive gene expression, should help clarify this issue.

THE BRIGHT SIDE OF LIGHT

The response pathways for light and cytokinin are intertwined in several contexts. The profound changes that occur in etiolated seedlings in response to light, called photomorphogenesis, can be partially mimicked by growth of seedlings in the presence of exogenous cytokinin or by elevation of endogenous cytokinin (Chory *et al.* 1994; Lochmanova *et al.* 2008). The cytokinin-induced de-etiolation is absent in a type-B *arr1,10,12* triple mutant (Argyros *et al.* 2008), indicating that the canonical two-component response pathway is necessary for this response. While these studies demonstrate that increased cytokinins are sufficient to partially mimic photomorphogenesis in etiolated seedlings, they do not address if they are involved in other responses to light.

A key mediator of many light responses is the red light photoreceptor phytochrome. Sweere *et al.* found that the type-A RR ARR4 stabilized the active, Pfr form of one phytochrome, PhyB, by reducing the rate of dark reversion, and thus ARR4 acts as a positive regulator of the PhyB function (Sweere *et al.* 2001). This effect required a phosphorylatable form of ARR4 and depended on the AHK cytokinin receptors (Mira-Rodado *et al.* 2007). In contrast, loss-of-function mutations in *arr3*, *arr4*, *arr3,4* and the *arr3,4,5,6* quadruple mutants were more sensitive to red light as compared to wild-type seedlings (To *et al.* 2004), suggesting that these type-A ARRs, including ARR4, are negative regulators of the PhyB function. These conflicting data may reflect differences in the growth conditions used in these two studies, as Mira-Rodado *et al.* found that the *arr4* loss-of-function mutant was hyposensitive to red light in their growth conditions (Mira-Rodado *et al.* 2007). Nevertheless, these results implicate multiple type-A ARRs in the regulation of phytochrome function.

Another point of convergence between light signalling and cytokinin occurs via the HY5 protein. This transcription factor is a positive regulator of photomorphogenesis, acting downstream of multiple families of photoreceptors, including the phytochromes and cryptochromes (Chang *et al.* 2008). Intriguingly, *hy5* mutants are partially insensitive to cytokinin in root elongation and callus initiation assays (Cluis, Mouchel & Hardtke 2004). Recent studies have demonstrated that cytokinin can increase the abundance of HY5 protein but not its transcript levels, suggesting that cytokinin increases HY5 protein stability (Vandenbussche *et al.* 2007). HY5 was also found to be necessary for the induction of anthocyanin in response to cytokinin in blue light but not for the inhibition of hypocotyl elongation by cytokinin in the dark (Vandenbussche *et al.* 2007). These results suggest that a subset of cytokinin responses may be mediated through HY5.

Recently, the regulation of cytokinin degradation by CKX has been linked to the response to shade in *Arabidopsis*. Plants grown under canopy shade receive a low ratio of red/far red light (R/FR), which induces a number of developmental changes including increased hypocotyl elongation and a rapid arrest of leaf primordia growth (Carabelli *et al.* 2007). Carabelli *et al.* found that exposure to low R/FR resulted in a rapid increase in auxin signalling in the leaf primordia, which in turn led to the elevation of expression of *AtCKX6* expression (Carabelli *et al.* 2007). This is hypothesized to result in the reduction of cytokinin levels in the leaf primordia, and hence a reduction in cell proliferation. Consistent with this, a loss-of-function *ckx6* mutant did not display the arrest of leaf primordia growth in response to low R/FR conditions that was observed in wild-type seedlings (Carabelli *et al.* 2007).

Elements of the two-component cytokinin response pathway play a role in the circadian clock, which entrains plants to light/dark diurnal cycles. The first evidence for this came from the genetic analysis of type-A ARR mutants. The *arr3,4* and *arr3,4,5,6* mutants had a longer circadian period and a leading phase change similar to that displayed in *phyB* mutants (Salomé *et al.* 2005). The changes in circadian rhythms were absent in the *arr3* and *arr4* single mutants, suggesting that these two RRs have redundant functions in this response (Salomé *et al.* 2005). Surprisingly, the altered periodicity of the *arr3,4* mutant was suppressed by a reduction in *ARR8* and *ARR9* function, even though no photoperiod phenotype was observed in the *arr8,9* mutant itself. This suggests that these pairs of type-A ARRs can act antagonistically in this response. The finding that the introduction of an *ARR5* genomic transgene into the *arr3,4,5,6* mutant fully rescued the cytokinin hypersensitivity (To *et al.* 2004, 2007), but did not affect the altered periodicity of the circadian rhythm (Salomé *et al.* 2005), suggests that ARR3 and ARR4 act independently of cytokinin in this context. Furthermore, ARR4 displays both PhyB-dependent and -independent effects on the circadian clock (Salomé *et al.* 2005; Hanano *et al.* 2006). Intriguingly, the expression of the *ARR9* gene (but none of the other two-component genes) displays a strong circadian oscillation, and the timing of this expression

is controlled by the major clock components [CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL 1 (LHY1), TIMING OF CAB EXPRESSION 1 (TOC1)] (Ishida, Yamashino & Mizuno 2008a). Cytokinin treatment was found to cause a shift in the phase of the circadian clock (Hanano *et al.* 2006; Zheng *et al.* 2006) in an ARR4- and PhyB-dependent manner (Zheng *et al.* 2006). A mechanistic link for this effect is suggested by the observation that cytokinin induces the expression of *LHY* and *CCA1* genes but represses the expression of *TOC1* (Zheng *et al.* 2006). Thus, there appears to be an interdependent regulatory loop between the clock genes and cytokinin response genes (i.e. *ARR9*). Further studies should help define the mechanisms underlying this intriguing interaction among light, cytokinin and circadian rhythm.

IN TIMES OF TROUBLE

Cytokinin function has been linked to a variety of abiotic stresses (Hare, Cress & van Staden 1997). The examination of public microarray expression data reveals that the genes encoding the proteins in the cytokinin signalling pathway are differentially affected by various abiotic stresses (Fig. 2). For example, cold stress appears to rapidly up-regulate the expression of multiple type-A *ARRs* and conversely to down-regulate the expression of all three cytokinin receptors (Fig. 2). This suggests a role for cytokinin in the response to cold stress, but there are no reports linking cytokinin to a rapid response to cold stress.

The expression of the *AHK2* and *AHK3* genes was found to be induced after dehydration (Tran *et al.* 2007), which is also observed in the public microarray data (Fig. 2). The elevation of these cytokinin receptors could lead to an increase in the sensitivity to cytokinin. The exposure of plants to drought results in a decrease in the level of cytokinins in the xylem sap (Bano *et al.* 1994; Shashidhar, Prasad & Sudharshan 1996). A recent study has confirmed that isoprene-type cytokinins (zeatin and zeatin riboside) are decreased in the xylem in response to drought stress but surprisingly found that the level of the aromatic cytokinin 6-benzylaminopurine (BAP) was elevated (Alvarez *et al.* 2008). The increased level of aromatic cytokinin could

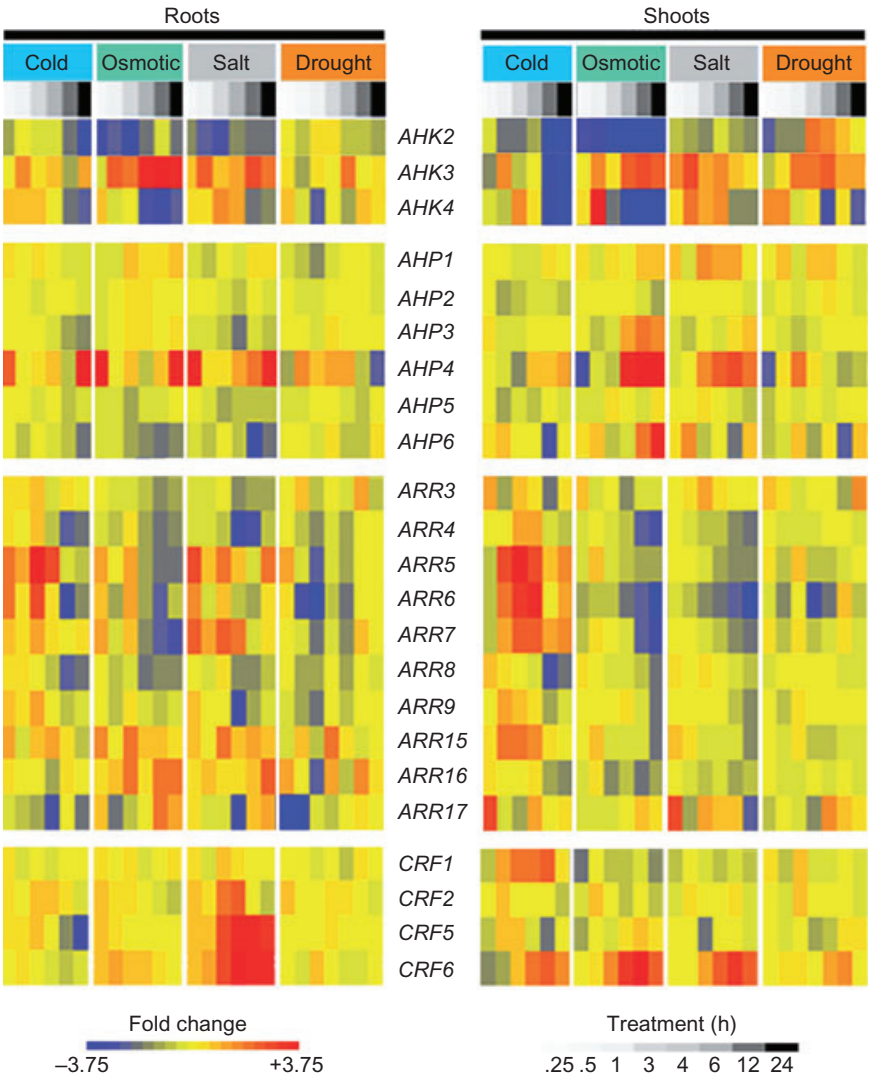

Figure 2. Responsiveness of cytokinin signalling genes to different abiotic stresses in *Arabidopsis*. The expression heat map data are adapted from BIO Array Resource (BAR, http://bar.utoronto.ca/) (Toufighi *et al.* 2005), using the default parameters. The values are log2-transformed ratios with the colour scale as depicted. The times of the experimental treatment for the cold, osmotic and salt conditions are indicated in the bar, lower right. Drought experiments include a 0.25 h treatment.

inhibit leaf senescence during drought conditions and/or might increase the level of proline (Alvarez *et al.* 2008), which acts as an osmotic protectant in response to water stress.

Consistent with the notion that elevated cytokinin levels may promote survival in drought conditions, a recent study found that the expression of *Agrobacterium ipt* from a drought/maturation-induced promoter [*SENESCENCE ASSOCIATED RECEPTOR PROTEIN KINASE* (*SARK*)] resulted in a remarkable tolerance to extreme drought conditions in tobacco (Rivero *et al.* 2007). Transgenic plants showed almost complete recovery following a drought regime that killed wild-type plants. Moreover, no loss in yield was reported under water restriction (Rivero *et al.* 2007). This is consistent with the finding that BAP levels are elevated in conditions of water deficit (Alvarez *et al.* 2008) and suggests that endogenous cytokinin may play a role in conferring drought tolerance.

The treatment of *Arabidopsis* with either osmotic or salt stress has a strong effect on the expression of the *AHK* cytokinin receptors (Fig. 2). *AHK2* and *AHK4* were down-regulated, both in the root and the shoot, in response to osmotic or salt stress. Conversely, *AHK3* was up-regulated in response to these conditions (Fig. 2). Consistent with this, an *AHK3* orthologue from *Medicago sativa* was found to be induced by salt stress (Coba de la Peña *et al.* 2008). This altered suite of *AHK* receptor expression may have important effects on receptor output following exposure to cytokinin. Consistent with an important role of these AHKs in the response to salt stress, the disruption of *AHK2* and/or *AHK3* resulted in increased tolerance to drought and salt stresses (Tran *et al.* 2007). These data suggest that the AHKs may act as negative regulators in stress signalling. Consistent with this, *ahk2,3* double mutants displayed constitutively elevated expression of stress-responsive genes (Tran *et al.* 2007). Interestingly, *AHP4*, but none of the other *AHPs*, is down-regulated in response to salt and osmotic stress (Fig. 2). AHP4 is evolutionarily distinct from the other AHPs, and, in contrast to the other functional HPs in *Arabidopsis*, may play a negative role in cytokinin signalling in some context (Hutchison *et al.* 2006). The expression of several of the *CRF* genes, which were identified as cytokinin-responsive AP2 transcription factors (Rashotte *et al.* 2006), is down-regulated in response to salt stress, especially in roots. These genes may play an important role in mediating the input of cytokinin into the salt stress response pathway.

CYTOKININ AND PATHOGENS: OUT, DAMN'D SPOT!

After pathogen infection, plants initiate a series of defence responses in order to contain the invader. When defence is not possible, disease develops, leading to a complex and intimate association in which plants try to minimize pathogen damage, while pathogens try to obtain nutrients to grow and complete their life cycle.

Several lines of evidence support a possible link between cytokinin and pathogenicity. One line of evidence comes from observations that cytokinin levels in plants are altered after pathogen infection. For example, in beans (*Phaseolus vulgaris*), the levels of active cytokinins are decreased after inoculation with the viral pathogen white clover mosaic potexvirus (Clarke *et al.* 1999). In wheat plants, infection with the fungal pathogen *Tilletia caries* leads to an increase in the cytokinin levels to twice the levels seen in uninfected plants (Maksimov, Ganiev & Khairullin 2002). Moreover, the application of exogenous cytokinin leads to changes in pathogen susceptibility. For example, when bean seedlings are treated with exogenous cytokinin, the level of replication of white clover mosaic potexvirus is reduced, accompanied by an induction of defence-response genes, such as those encoding pathogenesis-related (PR) proteins (Clarke, Burritt & Guy 1998). Up-regulation of stress- and defence-related genes also occurs in cytokinin-treated seedlings and plant cell cultures (Schäfer *et al.* 2000; Rashotte *et al.* 2003; Brenner *et al.* 2005) and in tomato plants overexpressing the *Agrobacterium ipt* gene (Martineau *et al.* 1994). Further evidence of crosstalk between cytokinin and defence-response pathways was provided by the characterization of the *uni1-d* mutant in *Arabidopsis*. *uni1-d* carries a mutation in a Coiled Coil-Nucleotide Binding domain-Leucine-Rich Repeat (CC-NB-LRR) resistance protein and shows increased cytokinin sensitivity and up-regulation of defence-related genes (Igari *et al.* 2008).

While there appears to be a link among cytokinin levels, pathogenicity and defence-related gene expression, the sources and species of cytokinins involved in specific plant–pathogen interactions is uncertain. Several pathogens, such as the bacteria *A. tumefaciens* and the biotrophic actinomycete *Rhodococcus fascians*, are able to synthesize cytokinins, which is important for their pathogenicity (Crespi *et al.* 1992; Sakakibara *et al.* 2005). The analysis of *Arabidopsis* plants overexpressing cytokinin oxidases indicates that both plant- and pathogen-derived cytokinins play a role in disease development in the *R. fascians* interaction (Depuydt *et al.* 2008). Plasmodia of the obligate biotrophic pathogen *Plasmodiophora brassicae*, causal agent of clubroot disease, can produce cytokinin (Muller & Hilgenberg 1986), and the infection of *Arabidopsis* plants with this pathogen leads to the up-regulation of *ARR5* and differential regulation of genes encoding cytokinin oxidases, suggesting that pathogen-derived cytokinins are involved in disease development (Devos *et al.* 2006; Siemens *et al.* 2006). Moreover, transgenic plants overexpressing cytokinin oxidases show decreased disease symptoms indicating a role for cytokinins in *P. brassicae* pathogenicity (Siemens *et al.* 2006).

Several physiological functions attributed to cytokinins may explain their role in plant–pathogen interactions. The interaction of some biotrophic pathogens and their hosts leads to the formation of green bionissia (formerly known as green islands), which are sites of green living tissue surrounding the sites of active pathogen growth (Walters, McRoberts & Fitt 2008). The formation of these green bionissia is correlated with elevated levels of cytokinins in these tissues, as for example, in the interaction of barley and

the fungal pathogen *Blumeria graminis* (Coghlan & Walters 1992). It is commonly thought that cytokinins delay the onset of senescence in green bionissia, allowing pathogens to obtain more nutrients from the plant (Walters *et al.* 2008). The effects of cytokinin in nutrient assimilation and allocation within the plant, previously described in this review and elsewhere (Ashby 2000; Walters & McRoberts 2006), might also play an important role, especially for biotrophic pathogens, which obtain their nutrients from living plant cells.

Nevertheless, necrotrophic pathogens, or those that obtain their nutrients from dead plant cells, are also able to cause the formation of green tissue around the sites of infection, which have been named green necronissia (Walters *et al.* 2008). For instance, the inoculation of the fungal pathogen *Pyrenophora teres* on barley plants can cause the formation of green necronissia, and the application of exogenous cytokinin is able to mimic this effect (Angra & Mandahar 1991). Similar results were observed in maize leaves infected with *Dreschlera maydis* (anamorph of *Cochliobolus heterostrophus*) (Angra-Sharma & Sharma 1999). In contrast to its role in the formation of green bionissia, a role for cytokinin in these interactions might occur through the regulation of cell death (Carimi *et al.* 2003). A specific role for cytokinin in the pathogenicity of necrotrophic pathogens might also be exerted through the control of senescence. Because these pathogens use toxins and cell wall-cleaving enzymes to cause cell damage and death, the control of plant cell senescence might be correlated to the pathogen's ability to cause disease. This could be the case in the relationship between *Arabidopsis* and the necrotrophic fungus *Botrytis cinerea*, which, although unable to synthesize cytokinins, induces senescence in plant cells in a manner that can be reverted by the overexpression of a bacterial *ipt* (Swartzberg *et al.* 2008).

CYTOKININ AND NODULATION (NOD): FIXING TO GROW?

Nitrogen-fixing rhizobacteria play an important role in the control of available nitrogen in the soil. Through the process of nitrogen fixation, these bacteria are able to convert N_2 into organic forms that can be assimilated by the plants. The bacteria obtain dicarboxylic acids from the plant in exchange for ammonia produced during nitrogen fixation, an energetically costly process that is tightly regulated in legume plants.

The process of nitrogen fixation occurs in specialized structures in the legume root called nodules. In the course of nodule organogenesis, the bacteria induce morphogenic alterations in the roots of their legume plant hosts, a process that requires extensive modification of the root epidermis for infection initiation and subsequent activation of cell division leading to nodule formation, and in which Nod factors produced by rhizobial bacteria play a crucial role.

Cytokinins have been suggested to play a role in Nod based on various lines of evidence, including their ability to induce cell division. Some nitrogen-fixating bacteria, such as *Rhizobium leguminosarum* and *Bradyrhizobium japonicum*, produce compounds with cytokinin-like activity that could act to induce cell division in the host during Nod (Phillips & Torrey 1972; Sturtevant & Taller 1989). Application of exogenous cytokinin leads to the induction of cortical cell divisions (Torrey 1961) and up-regulation of early nodulin genes (Dehio & Debruijn 1992; Mathesius *et al.* 2000). The expression of the *Agrobacterium ipt* gene in a *Rhizobium* Nod^- (Nod defective) strain conferred the ability to induce nodule-like structures on alfalfa roots. Moreover, the expression of the same gene in *E. coli* led to the formation of cell division clusters indicative of nodule formation, suggesting that cytokinin is sufficient for some aspects of Nod, at least in some species (Cooper & Long 1994).

The expression of two-component elements involved in cytokinin signalling is correlated to the Nod process. An *ARR5* promoter-GUS reporter was rapidly induced in response to rhizobial infection in *Lotus japonicus* in the deformed root hairs, as well as in nodule primordia, but declined as nodule development progressed (Lohar *et al.* 2004). In *Medicago truncatula*, the expression of *MtCRE1* (an orthologue of CRE1/AHK4) and type-A *MtRR* genes, is elevated upon inoculation in the areas of nodule development (Gonzalez-Rizzo, Crespi & Frugier 2006). Moreover, the disruption of the *MtCRE1* gene results in a failure to initiate cortical cell divisions that are necessary for Nod in *M. truncatula* (Gonzalez-Rizzo *et al.* 2006) and *L. japonicus* (Murray *et al.* 2007), while a gain-of-function mutation in the same receptor leads to the spontaneous formation of root nodules in the absence of rhizobia (Tirichine *et al.* 2007). Together, these studies demonstrate that cytokinin function is necessary and sufficient for Nod.

The mode of action of cytokinin in Nod is still unclear and most likely involves interplay among different factors. Cytokinin could play an important role in the regulation of the transcription of genes involved in the re-differentiation of cortical cells, as genes encoding transcription factors with roles in Nod, such as *NIN* and *NSP2* (Schauser *et al.* 1999; Oldroyd & Long 2003), are regulated by cytokinin, with *NIN* showing a kinetics and spatial pattern of expression similar to that of RRs (Grønlund *et al.* 2005; Gonzalez-Rizzo *et al.* 2006). Another interesting possibility is that cytokinin might play a role in the regulation of the Ca^{++} spiking in response to Nod (Ehrhardt, Wais & Long 1996), possibly through the action of cytokinin-regulated calcium-dependent protein kinases (CDPKs) such as *MtCPK3* in *M. truncatula* (Gargantini *et al.* 2006). Furthermore, cytokinin might also play an important role in regulating the sink/source relationships and nitrogen metabolism in nodules to coordinate the exchange of nutrients between the rhizobial cells within the nodule and the host plant.

CONCLUSIONS

In sum, while it is clear that cytokinin likely plays a role in the response to many environmental signals, much remains to be clarified. Physiological studies have correlated changes in cytokinin levels to responses to environmental

cues. Genome-wide microarray studies reveal overlapping transcriptional responses between cytokinin and various environmental inputs. The components of the cytokinin biosynthetic and signalling pathway are, in turn, transcriptionally altered by environmental perturbations. While many of the links of cytokinin to various environmental stimuli have been simply correlative, recent studies using mutants that alter cytokinin biosynthesis or signalling have begun to demonstrate an important role for cytokinins in these responses. However, because of the wide range of outputs of the cytokinin signalling pathway, dissecting the role of cytokinin in the response to a particular stress remains challenging. For example, ethylene has been implicated in the response to both biotic and abiotic stresses, and cytokinin elevates ethylene biosynthesis (Vogel *et al.* 1998). This is a complicating factor in dissecting the role of cytokinin in the response to a particular stress, as it is unclear if the effects of cytokinin are direct or the result of altered ethylene levels. Nevertheless, it is evident that cytokinin plays an important role in the response to a diverse array of environmental inputs. Continued analysis using the large number of tools now available to alter cytokinin levels and responsiveness will continue to shed light on this subject. As we further our understanding of the circuitry underlying the input of cytokinin into the response to various environmental signals, we should be able to engineer these pathways to produce plants with increased tolerance to biotic and abiotic stresses.

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