

# New insights into the signaling and function of cytokinins in higher plants

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## Abstract

Phytohormones (plant hormones) play a role in the regulation of cellular activities including cell division, elongation and differentiation, pattern formation, organogenesis, reproduction, sex determination, and responses to abiotic and biotic stress. The phytohormonal signal transduction pathways operate *via* cytokinin perception by transmembrane receptors consisting of two domains: an extracellular domain responsible for hormone binding and an intracellular domain, sensory His/Asp kinase (HK). Upon phytohormone binding, the receptor undergoes a conformational change which activates its protein kinase activity. The phosphorylated intracellular domain transfers the phosphoryl group to the His residue of the histidine phosphotransfer protein (HPt) which in turn phosphorylates Asp residue in the Response Regulator (RR). The phosphorylated RR protein acts as either a positive or negative transcription factor that interacts with the gene promoter or other target protein. The two-component signaling pathway, operating through the His-Asp phosphorelay, controls many physiological and developmental processes throughout the plant lifecycle (from seed-to-seed). Downstream plant hormone signaling also includes proteolysis of transcriptional regulators that block the activity of transcription factors. Two-component signaling operates more frequently in higher plants. Five classic plant hormones had been discovered by the middle of the twentieth century: auxins, cytokinins, gibberellins, ethylene and abscisic acid. The more recently characterized hormones include brassinosteroids, strigolactones, jasmonates and salicylates. Considering the tremendous work that has been undertaken during the last decades, in this review we decided to concentrate on cytokinin signaling in various plant developmental processes, including responses to biotic and abiotic stresses.

**Key words:** signal transduction, cytokinin, phytohormone, cytokinin receptor, histidine-aspartate phosphorelay, two-component relay system, legume plants, symbiosis

## Introduction

Plant hormones (phytohormones) are small chemical molecules that play a crucial role in plant growth, differentiation and development. They are classified into 9 groups, based on their chemical structure and function in various physiological processes (Table 1).

Cytokinins, a group of cell chemical mediators, play an important role in the regulation of physiological, metabolic, developmental and biochemical processes during plant ontogenesis. Cytokinin action, like that of other phytohormones, is mainly pleiotropic. Its impact depends on the concentration ratio of different plant hormones, as well as the balance and cross-talk between them (Pieterse et al., 2009; Wang and Irving, 2011).

Naturally occurring cytokinins are adenine derivatives. However, synthetic cytokinins, i.e. urea derivati-

ves, show a higher activity in *in vitro* cultures than natural phytohormones (Fig. 1). Natural derivatives of adenine (*trans*-zeatin, 2iP, etc., Table 1) are stored or transported as ribosides *via* membrane channels and finally hydrolyzed to free physiologically active hormones and ribose. They are then recognized by the membrane receptor with an intracellular histidine kinase activity. Hormone recognition is the first step of the signal transduction pathway.

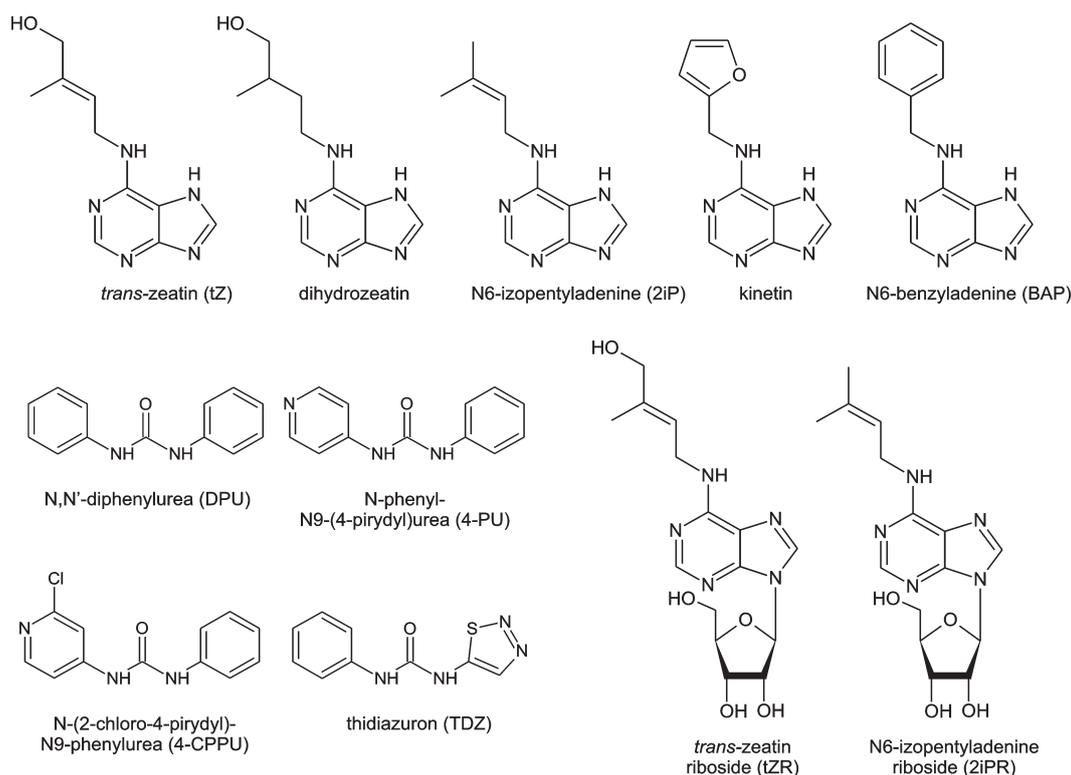
## Synthesis and degradation of cytokinins

Cytokinin homeostasis is partially and temporarily regulated though the balance between its synthesis and degradation processes. Cytokinins (such as 2iP riboside and zeatin riboside) occur in a bound form in the anticodons of tRNA specific for serine, isoleucine, tyrosine

**Table 1.** Classification of phytohormones

Class	Name	Function
I. Auxins	indole-3-acetic acid (IAA) 4-chloroindole-3-acetic acid (4-Cl-IAA) 2-phenylacetic acid (PAA) indole-3-butyric acid (IBA)	Phytohormones of classes I-V regulate vegetative growth: Auxins contribute to developmental patterning, cell elongation, organogenesis and tropic responses
II. Cytokinins	N <sup>6</sup> -furfuryladenine (Kinetin) <i>trans</i> -Zeatin <i>cis</i> -Zeatin 6-benzyladenine (BAP) 2-izopentyl-adenine (2iP) <i>trans</i> zeatin riboside (tZR) 2-izopentyl-adenine riboside (2-iPR)	Cytokinins are involved in maintaining a stem cell population at the shoot apical meristem, promote differentiation at the root apex, nutrient uptake and allocation and leaf senescence
III. Strigolactones (diterpenoid lactones)	(+)-Strigol (+)-Strigyl acetate (+)-Orobanchol (+)-Orobanchyl acetate (+)-5-Deoxystrigol Sorgolactone	Strigolactones are produced in plant roots. They attract mycorrhizal fungi and promote the germination of parasitic plants of the genus <i>Striga</i>
IV. Gibberellins	Gibberellin A1 (GA1) Gibb-3-ene-1,10-dicarboxylic acid; Gibberellic acid (GA3) Gibberellins (GA1-n) (126 identified)	Gibberellins promote seed germination, and, in some seeds, the mobilization of storage reserves
V. Brassinosteroids (polyhydrosteroids)	24-epibrassinolide (24-epiBL) 28-homobrassinolide (28-homoBL)	Brassinosteroids promote cell elongation, and are involved in pollen development
VI. Abscisic acid	Abscisic acid (ABA)	Abscisic acid accumulates during seed maturation and protects the embryo during seed desiccation, and represses germination. Its synthesis is induced by drought or other abiotic stresses, leading to the expression of stress-responsive genes
VII. Ethylene	Ethene (ET)	Ethene promotes senescence and fruit ripening, stress responses and vegetative development
VIII. Jasmonates	Jasmonic acid (JA) Methyl jasmonate (MeJA) as JA conjugated to amino acids	Jasmonates and Salicylates respond to biotic stress
IX. Salicylates	2-Hydroxybenzoic acid (SA) Methyl 2-hydroxybenzoate; Methyl salicylate (MeSA)	

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**Fig. 1.** Structures of natural and synthetic cytokinins and their ribosides. Five adenine-derivatives, four urea-derivatives and two cytokinin ribosides are shown

and cysteine (Armstrong et al., 1969; Barciszewska et al., 1988; Skoog et al., 1966), in rRNA (Murai et al., 1978) and in the vicinity of the mRNA polyadenylate region (e.g. BA riboside) (de la Serve et al., 1984). Currently, two independent pathways of spare cytokinin synthesis are postulated: either by (i) the release of cytokinin during oxidative degradation of nucleic acids (RNA and DNA) or ii) *de novo* synthesis (Schmulling, 2004). *Cis*-zeatin type cytokinins (*cZ*-type) are created during the tRNA. tRNA isopentenyltransferases (tRNA IPT) produce *cis*-hydroxy isopentenyl tRNA which is further degraded to *cis*-zeatin (Hirose et al., 2008; Miyawaki et al., 2006). *Cis*-zeatin undergoes conversion into a biologically active form, i.e. *trans*-zeatin, in a reaction catalyzed by enzymes from the group of *cis*-/*trans*-isomerases. The slow turnover rate of tRNA is, however, insufficient to fulfill the cell requirement for cytokinins (Barciszewski et al., 2000; Czerpak and Piotrowska, 2003; Haberer and Kieber, 2002). The key enzyme in the *de novo* synthesis pathway of cytokinin is adenosine phosphate-isopentenyltransferase (IPT). It uses ATP, ADP or AMP as substrates and catalyzes the first reaction. This reaction is a rate-limiting step in cytokinin biosynthesis. The completion of the *Arabidopsis thaliana* genome enabled

Takei and co-workers (2001) to identify nine genes encoding putative *AtIPT* genes. An analysis of *AtIPT* gene expression profiles indicated not only the root meristem as the place of cytokinin synthesis, but also young leaves, flowers, immature seeds, phloem tissue, embryos and the nodal stem. On the basis of this finding it might be suggested that cytokinins are synthesized all over the plant organism. The expression profiles of *AtIPT* genes, however, exhibit considerable discrimination within the diverse range of plant tissues and organs (Miyawaki et al., 2004; Nordström, 2004). The presence of nitrogen, sulphate and phosphate apparently modulates *IPT* gene expression (Hirose et al., 2008; Sakakibara, 2006). Cytokinins that appear in plant cells undergo various transformations and modifications, including glycosylation, hydroxylation and ribosilation. As a consequence, inactive cytokinins are produced and they may act as a cytokinin store or a transport pool. Inactive cytokinin forms (e.g. nucleotides, nucleosides) may be reconverted into active ones in the reaction with the phosphoribohydrolase enzyme, encoded by the *LOG* gene (LONELY GUY). Neither cytokinin ribosides nor glycosides possess physiological activity *in planta* (Kudo et al., 2010; Kurakawa et al., 2007). Irreversible cytokinin de-

gradation is strictly regulated by cytokinin oxidases/dehydrogenases (CKXs) (Ashikari et al., 2005). They cleave the N6-side chain by oxidation, which yields a corresponding aldehyde and adenine or adenosine (Frebort et al., 2011). The reaction requires an abundance of molecular oxygen as an electron acceptor. *CKXs* gene expression profiles differ between one another depending on the plant organs, the physiological state and environmental conditions (Perilli et al., 2010). Cytokinin degradation processes are significant factors in the regulation of their biological activity.

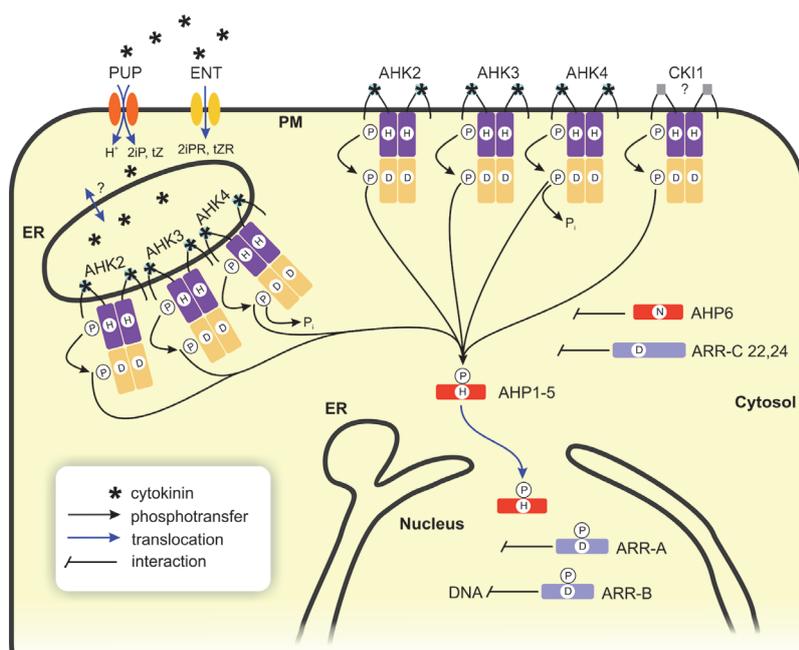
### Cytokinin transport

Cytokinins are mobile plant hormones. As a result, hormone transport mechanisms are involved in the precise induction of cell signaling throughout a plant organism. Radioactively labeled cytokinins applied to leaves were found in other organs, suggesting the existence of some long distance mechanisms of translocation (Foo et al., 2007). Cytokinins synthesized in the root meristem move acropetally (root-to-shoot) through xylem sap by the action of transpiration flow. The major form of cytokinins identified in xylem sap is a *trans*-zeatin nucleoside (tZR) (Hirose et al., 2008; Kudo et al., 2010). Root-to-shoot transport of CKs is controlled by endogenous and environmental stimuli. Supplementation of nitrate increases the flow rate of root-synthesized cytokinins in maize, followed by accumulation of IPT proteins and response regulator (RR) transcripts in leaves (Takei et al., 2001; 2004). This finding implies that tZR acts as a long-distance messenger for nitrate signaling. Bishopp and co-workers (2011) reported that the quantity of cytokinin transported acropetally is low in comparison to basipetal (shoot-to-root) transport through phloem (iP-type cytokinins). Cell-to-cell movement of cytokinin requires movement across the plasma membrane in a proton-coupled manner. For cytokinin nucleobases belonging to a large purine permease family (PUP) cellular uptake is mediated by transporters (Nongpiur et al., 2012), while for purine nucleosides mediation is effected by an equilibrative nucleoside transporter family (ENT) (Fig. 2). Cytokinin translocation by PUP is energy dependent and occurs against the concentration gradient. *Arabidopsis* competition studies have shown that cytokinins use the same transport system as adenine and caffeine. Adenine uptake was competitively inhibited via *trans*-zeatin (Ce-

dzich et al., 2008; Gillissen et al., 2000). The *AtPUP1* gene is expressed in all organs except roots. This suggests that in shoots, root-derived free cytokinins are transported out by phloem.

### Cytokinin signaling

The biological function of cytokinins is well described, although the molecular mechanisms of their action has yet to be fully explained. Despite the fact that cytokinins were discovered over 60 years ago (Skoog and Miller, 1957), it was only a decade ago that their receptor (CRE1/AHK4) was identified in *Arabidopsis thaliana* (Inoue et al., 2001; Suzuki et al., 2001). The gene encoding this histidine kinase is located on chromosome 2 of the *Arabidopsis* genome. The CRE1 protein is composed of (i) an N-terminal signal domain (CHASE, Cyclases/Histidine kinases Associated Sensory Extracellular), responsible for hormone binding, (ii) an endocellular transmitter domain which possesses kinase activity, (iii) two regulatory domains at the C-terminus. The dissociation constant of the CRE1/*trans*-zeatin complex, consistent with the natural cytokinin content, remains at  $\mu\text{M}$  level (Romanov et al., 2006). The structure of *Arabidopsis thaliana* AHK4 was solved by Hothorn (2011). It reveals a similarity to the *Vigna radiata* cytokinin binding protein (CSBP), (Pasternak et al., 2006). Recent studies have demonstrated that CRE1 is located in the ER membrane, which brings new insights to the cytokinin signaling model (Caesar et al., 2011; Wulfetange et al., 2011). It is proposed that cytokinins enter cell cytoplasm using PUP and ENT channels. Subsequently, they are directed to the endoplasmic reticulum and activate cytokinin receptors anchored to the membrane (Heyl et al., 2012; Hothorn et al., 2011; Kudo et al., 2010; Pas et al., 2004). Upon binding the biologically active cytokinin, receptor dimerization and autophosphorylation of conservative His residue occur. The next steps include: (i) translocation of the phosphate group from the histidine residue to the aspartate on the regulatory domain of CRE1, (ii) movement of the phosphate group to the histidine residue of HPT proteins (histidine phosphotransfer proteins) serving as phosphor carrier proteins. HPT are small (app. 16kDa) monomeric proteins linking the initiation of a cytokinin signal in the cell cytoplasm with nuclear gene responses (Tanaka et al., 2004). HPT proteins induce the expression of type-A or type-B response regulators. However, stu-



**Fig. 2.** Cytokinin transduction pathway and active transport. Signaling cascade involves: i) sensory His kinases (AHK2-4) anchored to the plasma membrane (PM) or endoplasmic reticulum (ER); ii) His-containing phosphotransfer protein (AHP1-5) or signal inhibitor (AHP6) and iii) three types of response regulators (ARR-A/B/C). Cytokinin independent kinase (CKI1) is also shown. Active His (H) and Asp (D) residues that transfer phosphate (P) are circled in white. Cytokinins (2iP, tZ) or their ribosides (2iPR, tZR) are transported by two types of channels: purine permeases (PUP) or equilibrative nucleoside transporters (ENT). PUP operates in a proton-coupled manner, while ENT facilitates diffusion of nucleosides according to concentration gradient. Adapted with modifications from (Hwang et al., 2012; Nongpiur et al., 2012)

dies performed by Punwani and co-workers (2010) show that the expression pattern of *hpt* genes is not altered by an exogenously applied cytokinin. It is proposed that Hpt proteins contribute to ethylene signaling by interactions with ER-anchored ethylene receptors (ENR) as well (Nongpiur et al., 2012). Response regulators are composed of an N-terminal receiver domain, with the central aspartate residue necessary for acquiring the phosphate group from Hpt transmitters. B-type response regulators possess an additional output domain with the ability to bind DNA and activate particular cytokinin sensitive genes (Oka et al., 2002). A-type ARRr create a negative feedback under cytokinin responses, whereas B-type ARRr initiate the expression of cytokinin sensitive genes (Kiba et al., 2003; To et al., 2007).

### Cytokinin antagonist

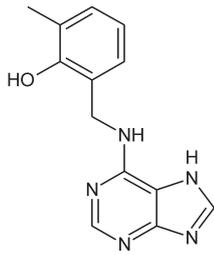
6-(2-hydroxy-3-methylbenzylamino)-purine, known as PI-55 (Fig. 3), is structurally related to natural cytokinin 6-benzylaminopurine (BAP) with -OH group at C2 and -CH<sub>3</sub> group at C3 of the benzyl moiety. PI-55 strongly

decreased cytokinin activity and exhibited antagonistic properties to BAP. PI-55 appeared to be a competitive inhibitor of the natural ligand *trans*-zeatin binding to the *Arabidopsis* cytokinin receptors (cytokinin response 1, CRE1/AHK4) or *Arabidopsis* histidine kinase AHK3 (Spichal et al., 2009).

### Cytokinins in symbiotic interactions

Symbiotic interactions between soil bacteria of *Rhizobium* sp. and a legume plant led to the induction of a root organ: the nitrogen-fixing nodule. Cytokinins appear to be a key differentiation signal of nodule organogenesis by controlling the expression of the *NIN* gene (*Nodule Inception*) and other transcriptional regulators through mechanisms operating both locally and systemically.

The rhizobial nodulation (Nod) factors identified as lipo-chitooligosaccharide have been revealed to be a crucial bacterial signaling molecule in a symbiotic interaction. They promote root infection and nodule formation (Roche et al., 1991). Nevertheless, the implications of cytokinins in symbiotic interactions have been clearly



**Fig. 3.** Structure of cytokinin antagonist PI-55

demonstrated with the use of a *Rhizobium Nod<sup>-</sup>* strain, which is unable to synthesize Nod factors and therefore nodulate the plant host.

When a bacterial *Nod<sup>-</sup>* mutant strain was genetically modified to secrete cytokinin, *transzeatin*, the induction of uncolonized nodule-like structures on *Medicago sativa* roots was observed. This demonstrated that cytokinins mimic some of the morphogenetic effects of Nod factors (Cooper and Long, 1994). However, such empty nodules were not able to fix atmospheric nitrogen. Exogenous cytokinins, in a similar way to rhizobia and Nod factors, induced cortical cell divisions, amyloplast deposition and expression of early nodulin genes, including *ENOD40* and *NIN*, in different legume plants (Dehio and de Bruijn, 1992; Jimenez-Zurdo et al., 2000; Mathesius et al., 2000; Murray et al., 2007; Tirichine et al., 2007; Torrey, 1961). Recent data explicitly show that cytokinins are important signals in symbiotic interactions and nodulation, and act downstream of Nod factor perception (Frugier et al., 2008; Gonzalez-Rizzo et al., 2006).

The Nod factor is recognized by receptors containing peptidoglycan-binding lysin (LysM) domains. Downstream of this perception, the other transduction elements have been defined: (i) a leucine-rich repeat receptor-like kinase, (ii) putative cation channels (CASTOR, and POLLUX), (iii) nucleoporins (NUP85 and NUP133) and (iv) the calcium- and calmodulin-dependent kinase (CCaMK, also known as DMI3) (Andriankaja et al., 2007; Middleton et al., 2007; Oldroyd and Downie, 2006; Saito et al., 2007; Schauser et al., 1999), (v) Nod factor signaling pathways 1 and 2 (NSP1, NSP2) and an ethylene response transcription factor (ERN) and (vi) an NIN protein. These are necessary for the regulation of transcription associated with bacterial infection and nodule organogenesis (Fig. 4).

In a similar fashion to *snf2*, gain-of-function CCaMK alleles also induce the formation of spontaneous nodules in an NSP1-, NSP2- and NIN-dependent manner (Glea-

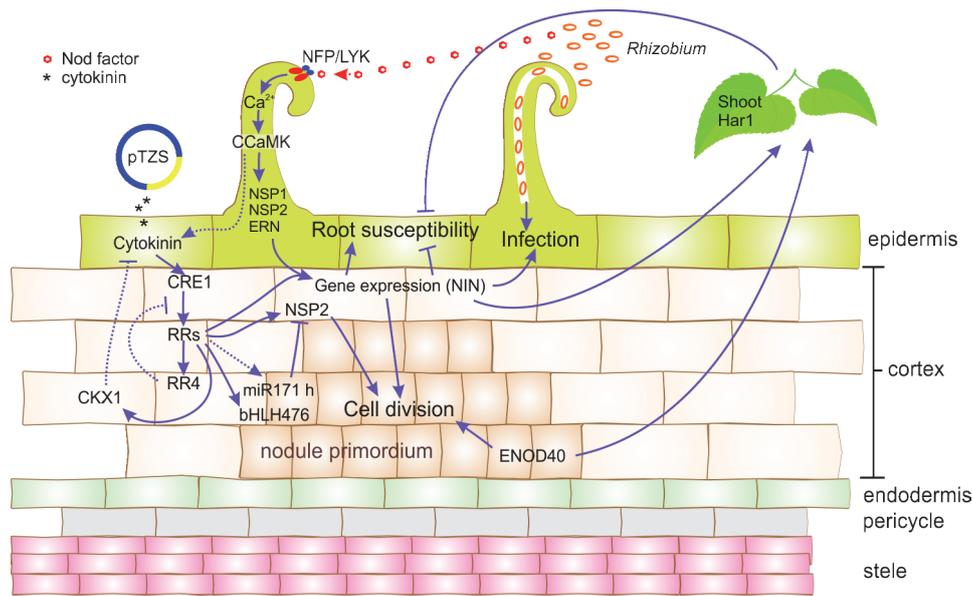
son et al., 2006; Tirichine et al., 2006; Tirichine et al., 2007). These results suggest a linear pathway from Nod factor perception through CCaMK and LHK1 (in *Lotus japonicus*) or MtCRE1 (in *M. truncatula*) leading to nodule organogenesis. Therefore, rhizobial activation of two cytokinin response regulators, MtRR1 and MtRR4, depends on Nod-factor signal transduction components (Gonzalez-Rizzo et al., 2006). A parallel model, in which two separate signaling pathways converge at NSP2 to mediate nodulation, has also been considered (Oldroyd, 2007). It has been found that ERN transcription factors, which belong to the AP2/ERF family, act together with NSP1 and NSP2 to mediate the transcriptional activation required for the formation of infection threads and nodules (Andriankaja et al., 2007; Middleton et al., 2007). It is possible that cytokinins are involved in the regulation of the expression and nuclear location of ERNs including other transcriptional factors required in nodulation (e.g. NSP2). All experimental data clearly demonstrate the role of cytokinins in effective nodules formation.

### Cytokinin receptors in the regulation of nodule organogenesis

Cytokinin signaling genes are upregulated after rhizobial inoculation. In *Medicago truncatula*, *Sinorhizobium meliloti* infection activates a receptor histidine kinase gene, *Mtcre1* and subsequently RRs. The latter are similar to the *Arabidopsis thaliana* cytokinin responsive genes *ARR4-5* and *ARR10-12* (Gonzalez-Rizzo et al., 2006; Lohar et al., 2006).

Under rhizobial infection, the *Mtcre1* gene is expressed in the cortical cells of the Nod factor active zone, followed by development of nodule primordium. Finally, its expression becomes restricted to the nodule meristematic region (Lohar et al., 2006). A decrease in cytokinin accumulation and perception inhibits nodulation. In *Lotus japonicus* overexpression of heterologous cytokinin, oxidase/dehydrogenase genes, involved in cytokinin degradation, resulted in reduced nodulation (Lohar et al., 2004). Likewise, specific downregulation of *Mtcre1* and a loss-of-function mutation in the *L. japonicus* ortholog, *Lhk1* (*hit1-1* allele), led to the formation of cytokinin sensitive roots, which dramatically inhibited nodulation (Gonzalez-Rizzo et al., 2006; Murray et al., 2007).

It was also shown that *L. japonicus* gain-of-function mutation in the cytokinin receptor gene, *LHK1*, induced the spontaneous formation of nodules in the absence of



**Fig. 4.** The role of cytokinin in nodulation. Solid and dotted lines represent proven and putative interactions, respectively. Early nodulation events involve: (i) Nod factor (NF) perception by Nod factor perception/Lys-M kinase receptor (NFP/LYK); (ii) Calcium signaling through a calcium and calmodulin dependent kinase (CCaMK); (iii) activation of Nod factor signaling pathways 1 and 2 (NSP1/2) and ethylene response transcription factor (ERN) and, (iv) nodule inception transcription factor (NIN). Nod factor signaling cascade can be bypassed via cytokinins produced by bacteria (pTZS plasmid) causing NF independent nodulation. In the epidermis NIN is necessary for infection and inhibits root susceptibility to rhizobial signaling. Local and systemic feedback regulation of infection are also cytokinin-dependent. There are also genes that act downstream of the cytokinin pathway: miR171h isoform targeting NSP2 and bHLH476, a direct cytokinin targets, as well as cytokinin oxidase/dehydrogenase (CKX1). Adapted with modifications from (Ariel et al., 2012; Frugier et al., 2008)

rhizobia. Such mutation is called spontaneous nodule formation 2 (*snf2*) (Tirichine et al., 2007).

Studies on genes involved in nodule organogenesis directly regulated by cytokinin-induced signals have led to the identification of the 12-bp RR binding site (RRBS). They were found in promoters of several cytokinin-regulated genes linked to nodulation: *RR4* (a Type-A Response Regulator) (Plet et al., 2011), and the NSP2 transcription factor (Heckmann et al., 2006; Kalo et al., 2005). The involvement of RRBSs in cytokinin-induced expression of nodulation genes allowed the further search for direct targets of cytokinins functioning in the root cortical cell division and nodule formation. The recently described transcription factor has been named the basic Helix-Loop-Helix (bHLH) TF – *bHLH476* (Ariel et al., 2012). Using the RNAi-mediated gene silencing method and an insertional mutagenesis it was also proven that both *NSP2* and *bHLH476* are direct targets of a cytokinin signaling pathway in the development of an effective symbiosis. This definitely indicates that cytokinin signaling is essential to induce cortical cell divisions and nodule organogenesis.

### Cytokinins in the shoot apical meristem

The activity of cytokinins is essential to maintain undifferentiated, pluripotent cells in a shoot apical meristem (SAM). Werner and co-workers (2003; 2001) investigated a transgenic *Arabidopsis thaliana* overexpressing gene encoding CKXs enzyme. In such plants a decrease in active cytokinins was observed, which subsequently resulted in a sharp reduction in the size of SAM. A similar outcome was obtained from the analysis of the *Arabidopsis* triple mutant of genes encoding cytokinin receptors *ahk2 ahk3 ahk4/cre1*. Decreasing cytokinin content resulted in a dramatic reduction in meristem size and leaf-initiation rate. Cytokinins positively affect the shoot apical meristem by upregulating transcription factor *STM* (*SHOOT MERISTEMLESS*). They induce cell division and thus prevent their differentiation (Nishimura et al., 2004; Riefler et al., 2006; Rupp et al., 1999; Shani et al., 2006). Although cytokinins play a crucial role in SAM, their level and activity are under very strict control. The central elements in the control of SAM maintenance are KNOTTED1-like homeobox (*KNOX1*)

proteins. These transcription factors are repressors of cell differentiation via the establishment of a high cytokinin to gibberellin ratio within SAM cells (Di Giacomo et al., 2008). KNOX1 proteins expression is restricted to SAM, where they suppress the *GA20ox* gene responsible for gibberellin synthesis (Kyojuka, 2007). Recently, it has been shown that the *STM* transcription factor also induces *IPT* gene expression (Gordon et al., 2009).

Gordon and co-workers also inferred the impact of cytokinins on *WUSCHEL* (*WUS*) expression pattern. *WUS* is a homeodomain transcription factor, which is expressed in the central zone (CZ) cells of the shoot apical meristem. *WUS* positively regulates stem-cell proliferation by repressing the expression of genes encoding type-A response regulators, such as *ARR5* and *ARR7*. Interactions between type-A *ARR* genes and cytokinins resemble a negative feedback loop. Exogenously applied cytokinins rapidly elevate the expression level of type-A *ARRs* which in turn are negative regulators of cytokinin responses by downregulation of *WUS* expression (Ferreira and Kieber, 2005; Hwang and Sheen, 2001; Sablowski, 2009). Studies on rice have shown that cytokinin content in a shoot apical meristem might be increased thanks to the action of an enzyme with phosphoribohydrolase (*LOG*) activity, which converts inactive cytokinin nucleotides into free base hormones. Rice plant harboring mutation in *LOG* gene demonstrates a remarkable reduction in SAM size (Kurakawa et al., 2007; Kuroha et al., 2009; Perilli et al., 2010).

### Modification of apical dominance

Apical dominance is a special plant feature which determines its architecture. The main shoot growing in the intact plant is shown to be predominant, simultaneously suppressing the outgrowth of axillary buds. The high content of auxins in the top of the shoot is responsible for this phenomenon. Decapitation of the shoot apex induces cytokinin synthesis, which in turn stimulates shoot branching. Shoot branching and the degree of apical dominance differs between plant species, which is manifested in the large variety of plant shapes. Sato and co-workers (2009) proposed a functional model of an auxin-cytokinin interaction which concerns apical dominance and shoot branching. Auxins derived from the apex induce expression of cytokinin degradation genes (*CKX*). They also suppress transcription of the *IPT*

gene. At the same time, the auxin efflux carrier (*PIN*) gene is upregulated, stimulating basipetal polar auxin transport in the stem. As a consequence, the outgrowth of axillary buds is repressed. Once the shoot apex is decapitated, a decreasing content of auxin in the stem is observed. This, in turn, activates the *IPT* gene and *de novo* synthesis of cytokinins in the stem. Cytokinins follow into the dormant axillary bud and promote their outgrowth. Afterwards, *de novo* synthesis of auxin (IAA) is initiated and the *IPT* gene is again repressed (Dun et al., 2006; Foo et al., 2007; Shimizu-Sato et al., 2009).

### Cytokinins and root growth

The root meristem represents a place of continual competition between auxins and cytokinins. The relationship between these hormones reveals a physiological and developmental alteration in the function of a plant organism. It is well known that auxins promote cell division in the CZ of the root meristem. As a consequence, an increase root size is observed. Cytokinins are also involved in the determination of root size, however they do it in a different manner. They promote cell differentiation in the transition zone (TZ) of the root meristem, which in turn diminishes the overall root size (Dello Ioio et al., 2007; Perilli et al., 2010; Werner et al., 2003; Werner et al., 2001). From the molecular point of view, the path from hormone action to root size is not straightforward. Cytokinin mediation of cell differentiation starts with its signal perception by CRE1 followed by phosphorylation and dephosphorylation of protein signal transporters. This signal path activates the type-B response regulator *ARR1* (Taniguchi et al., 2007). The *ARR1* transcription factor acts *in trans* and initializes the expression of the *SHORT HYPOCOTYL2* (*SHY2*) gene. *SHY2* is the auxin-response repressor gene which belongs to the family of 24 auxin-responsive genes (Aux/IAA) and encodes a nuclear protein characterized by a very short lifespan (reaching up to 8 min) (Tian et al., 2002). It was shown that a loss-of-function mutation in the *SHY2* gene results in a larger root meristem size and resistance to cytokinins (Chapman and Estelle, 2009). This gene is expressed in TZ of the root meristem and negatively regulates the *PIN* gene and in parallel downregulates expression of cytokinin synthesis genes (*IPT*). There are eight *PIN* genes in the *Arabidopsis* genome. *PIN* proteins are located asymmetrically on the plasma membrane where they

direct polar auxin transport within the tissue. Attenuation of PIN proteins activity by cytokinins results in a decreasing rate of auxin in root meristem CZ, leading to the initiation of cell differentiation in TZ of the root meristem. The growth of the root is finally retarded. Cytokinins alter PIN protein function at the posttranscriptional stage, but the level of *PIN* transcripts remains unchanged (Zhang et al., 2011). This general signaling model undergoes further control by auxins, which direct degradation of the SHY2 protein and sustention of *PIN* function, remains auxin transport active. An additional aspect of hormone interactions during the establishment of root meristem size is the cross-talk between cytokinins and ethylene. Cytokinins stimulate ethylene biosynthesis, which itself strongly affects root growth and also induces expression of the *PIN* gene (Ruzicka et al., 2009).

### Leaf senescence

Leaf senescence is a programmed aging process, leading to death. It represents the final stage of leaf development. During this highly regulated genetic process a wide variety of physiological conversions take place, ranging from changes in gene expression and cell metabolism to cell structure. Many biotic and abiotic factors influence leaf senescence (e.g. drought, nutrient limitation, temperature, oxidative stress, pathogen infection). The process accelerates in darkness and slows down in the light (Wingler et al., 1998). Overbalanced catabolic processes can be observed in the cells. Carbon assimilation is replaced by chlorophyll degradation, followed by membrane lipid, protein and nucleic acid decay. During leaf senescence, the cytokinin synthesis gene (*IPT*) is downregulated, in contrast to cytokinin *CKXs* genes where the expression level increases (Schippers et al., 2007). Cytokinins delay leaf senescence, *inter alia* by preventing chlorophyll breakdown. Balibrea Lara and co-workers (2004) showed that cytokinins induce the activity of extracellular invertase, ionically anchored to the cell wall. Subsequently, this enzyme is directly involved in hydrolysis of sucrose to hexose monomers previously released from phloem elements by sucrose transporters. Extracellular invertase, together with the hexose transporter activity, promotes a higher uptake of hexose sugars in senescing leaves. The inhibition of extracellular invertase activity results in the retardation of leaf senescence delay mediated by cytokinins. In the senescing

leaf expression of type-A cytokinin signaling genes, ARRs represent a reduced transcription (Buchanan-Wollaston et al., 2005). However, the exogenously applied cytokinin upregulates these response regulators (Rashotte et al., 2003). Moreover, cytokinins induce genes encoding ribosomal proteins (Cerny et al., 2011; Cherepneva et al., 2003; Kimura et al., 2001). Cytokinins inhibit respiration during senescence and prevent oxidation damage in the cell through the control of lipoxygenase and peroxidase expression (downregulation effect), (Boonman et al., 2007; Crowell et al., 1990; Mok and Mok, 2001; Werner et al., 2008).

### Regulation of the cell cycle

Cytokinins were discovered by observation of their ability to stimulate cell division. They promote cytokinesis by participating in cell cycle regulation at both the G1/S phase and G2/M phase progression. Cytokinins increase the amount of the G1 phase CycD3 protein (cyclin 3, type D) required for G1/S transition. Cyclins regulate the substrate specificity of CDK-Cyc (CDK – cyclin dependent kinases) complexes (Dewitte et al., 2003; 2007). Type-D cyclins possess an N-terminal LxCx(D/E) retinoblastoma-binding motif, responsible for interaction with a retinoblastoma-related protein (RBR). RBR further induces elongation factor 2 (E2F), necessary for the promotion of S phase entry (Dewitte et al., 2003; Dewitte et al., 2007; Kim and Park, 2007; Nordström, 2004). The regulatory role of cytokinins in the G2/M transition state is connected with Cyclin dependent serine/threonine kinases (CDK), the main regulatory proteins of the cell cycle. Plants CDKs are divided into seven classes (Doonan and Kitsios, 2009; Vandepoele et al., 2002). CDKs possess a conserved PSTAIRE amino acid sequence located in a non-catalytic domain, which is responsible for binding to a cyclin partner. The CDK/Cyc complex undergoes a post-translational modification of phosphorylation/dephosphorylation. The Cdc25 (cell division cycle 25) factor activates the CDK/Cyc complex through dephosphorylation of conserved amino acid residues – Thr 14 and Thr 15. The activity of Cdc25 itself is upregulated by cytokinins (Lipavska et al., 2011).

### Cytokinins and pathogen responses

Plants interact with living organisms either in a symbiotic or antagonistic way. The pathogenic attack is ef-

fective in the stimulation of the plant immune system. It may be said that “immune reactions are all about hormones talking”. The major conversational partners here are: salicylic acid (SA), jasmonates (JA) and ethylene (ET). However, the more we know about plant defense, the more important cytokinins in these very complex processes become (Jones and Dangl, 2006; Verhage et al., 2010). It is known that some microbial species may synthesize or utilize plant cytokinins during the formation of gall structures. Cytokinin receptors bind both bacterial and plant – derived cytokinins; however, they cause different molecular outputs. After penetration of plant host cells by the below mentioned bacterial species, an abnormal cytokinin synthesis and accumulation occurs, followed by an abnormal cell proliferation. As a consequence, galls form on plant roots (*Agrobacterium thumefaciens*) or leaves (*Rhodococcus fascians*). Stimulation of cytokinin receptors results in a higher expression of genes responsible for control of cell division (CycD3). Studies on *Arabidopsis thaliana* infected by *Pseudomonas syringae* pv. tomato (*Pst*; non-cytokinin producing pathogen) demonstrated the involvement of cytokinins in plant defense reactions in a salicylic acid signaling dependent manner (Argueso et al., 2012; Choi et al., 2011; Choi et al., 2010; Hwang et al., 2012). Plant-derived cytokinins induce resistance to pathogen infections using a two-component signaling system, which begins with their perception by CRE1/AHK4. In consequence, the type-B response regulator (ARR2) becomes activated. ARR2 binds the salicylic acid response factor (TGA3). TAG3 (TGACG motif-binding protein 3) is involved in the hyperactivation of genes encoding SID proteins (SA biosynthesis) and PR (pathogenesis related) class 1, 2 and 5. PR1 genes are molecular markers of the SA-signaling pathway. The ARR2/TGA3 complex interacts with *pr* promoters inducing the transcription of *pr* class 1, 2 and 5 genes. Cytokinins enhance plant immunity by elevation of SA-dependent defense responses. However, SA provides negative feedback in cytokinin signaling as a consequence of their inhibition. In this way a regulatory circle is observed. Surprisingly, tobacco plants infected by *Pseudomonas syringae* pv. tomato exhibit slightly different defense mechanisms. In this case, cytokinins participate in plant immunity, but do not induce salicylic acid signaling. Grosskinsky and co-workers (2011) discovered that cytokinin synthesis induced by pathogen infection in tobacco does not result in an SA

level increase. At the same time, in infected plant cells they observed an accumulation of phytoalexin, such as scopoletin and capsidiol.

Although we have a general insight into the implications of cytokinin in plant defense mechanisms, there is still a great need for further investigation of the plant immune system.

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