

Cytokinin Complexity: Insights into Plant Growth Regulation

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Abstract

This book chapter provides a comprehensive examination of the versatile roles played by cytokinin, a crucial plant hormone, in shaping various facets of plant growth and development. Focusing on its impact on root and shoot architecture, female gametophyte development, vascular tissue differentiation, and root nodule formation, the chapter elucidates the intricate molecular mechanisms and signaling pathways through which cytokinin orchestrates these intricate processes. The interplay between cytokinins and other plant hormones, such as auxins, helps orchestrate developmental processes like cell division, shoot and root formation, and vascular differentiation. By unraveling the molecular cross-talk between cytokinin and key developmental events, this chapter aims to offer a deeper understanding of the hormone's regulatory functions, providing valuable insights for researchers, educators, and students engaged in unraveling the complexities of plant hormone signaling networks. The exploration of cytokinin's diverse functions underscores its central role in coordinating plant growth and development at multiple levels.

Keywords: Biosynthesis, Cytokinin, Developmental mechanism, Plant hormone, Signaling pathways, Trans-zeatin.

1. Introduction

Cytokinin, recognized as a fundamental plant growth phytohormone, plays a pivotal role in fostering both cell division and cell differentiation within plants. These adenine derivatives feature a variable side chain at the N6 position of the purine (Sakakibara 2006). Operating as a key growth-promoting phytohormone, cytokinin exerts its influence across various facets of plant growth and development (Fahad et al. 2015). The signaling pathway of cytokinin is facilitated by a two-component system that orchestrates the sequential transfer of phosphoryl groups from receptors to downstream effectors. In *Arabidopsis*, upon cytokinin binding, receptors belonging to a small class of His kinases undergo autophosphorylation at a highly conserved histidine residue. Subsequently, the phosphoryl group is translocated to PHOSPHOTRANSFER PROTEINS and further to RESPONSE REGULATORS. This cascade ultimately activates the transcription of downstream effector genes, thereby initiating the signaling pathway. This chapter primarily consolidates recent advancements and our present comprehension of cytokinin metabolism, translocation, signal transduction, regulatory mechanisms, and the diverse physiological roles cytokinin plays in the intricate tapestry of plant growth and development (Kakimoto 2001).

2. Discovery

Cytokinins, classified as phytohormones, play a crucial role in promoting cell division and cell differentiation in plants (Haberer and Kieber 2002). These adenine derivatives carry either an isoprene-derived or an aromatic side chain at the N6 position of the purine (Sakakibara 2006). The discovery of cytokinins can be traced back to the 1950s when Folke Skoog

and colleagues isolated the first cytokinesis-promoting factor, kinetin, from autoclaved herring sperm DNA (Feng et al. 2017). Subsequently, other growth-promoting factors similar to kinetin were identified in various plants. The first naturally occurring cytokinin, trans-zeatin (tZ), was isolated from maize endosperm. Over the years, researchers have identified compounds with cytokinin activities from numerous plant species, with isopentenyladenine (iP) and tZ being the most prevalent and extensively studied natural cytokinins. The discovery of cytokinins not only advanced plant tissue culture methods but also significantly influenced studies in plant biology. Cytokinins are involved in regulating diverse processes in plant growth and development, encompassing aspects such as female gamete and embryo development, seed germination, vascular development, shoot apical meristem development, photomorphogenesis, leaf senescence, and floral development (Chen and Leisner 1984)). Additionally, cytokinins play a crucial role in regulating adaptive responses to environmental stresses (Bielach et al. 2017). In recent decades, the identification of enzymes controlling the modification and activity of cytokinins has provided valuable insights into the fundamental molecular mechanisms of cytokinin biosynthesis (Shakakibara 2006).

3. Structure and types of cytokinin

Cytokinins, as adenine derivatives, exhibit structural diversity based on the configuration of the N6-side chain, leading to the classification of isoprenoid cytokinins and aromatic cytokinins. Isoprenoid cytokinins, such as N6-(Δ^2 -isopentenyl)-adenine (iP) and zeatin, feature an isopentenyl side chain, while aromatic cytokinins, like kinetin, N6-benzyl adenine (6-BA), and topolin, possess a benzyl or hydroxybenzyl group at the N6 position. The trans- and cis-configurations of the hydroxylated side chain result in trans-

zeatin (tZ) and cis-zeatin (cZ), respectively. Aromatic cytokinins, though present in lower quantities, include ortho-topolin (oT), meta-topolin (mT), and methoxy derivatives of 6-BA (Feng et al. 2017). These cytokinins often occur as nucleobases or in conjugation with various moieties, such as nucleosides, nucleotides, and glycosides. The nucleobases typically serve as the active form, while the nucleosides act as reservoirs for cytokinin storage.

Beyond naturally occurring cytokinins, synthetic derivatives, including phenylurea-type, thidiazuron, and adenine-type kinetin, have been generated. The biological activities of cytokinins vary across plant species, tissues, and developmental stages, with different cytokinins exhibiting distinct affinities for receptors. For instance, cytokinin receptors generally show high affinity for free bases and low affinity for riboside derivatives. Specific cytokinin receptors in plants, such as *Arabidopsis* AHK3 and maize ZmHK1 and ZmHK2, display preferences for particular ligands, underscoring the specificity of cytokinin signaling in diverse plant systems (Kieber and Schaller 2018).

4. Cytokinin biosynthesis

Cytokinins, integral to plant growth regulation, undergo intricate biosynthetic processes involving the *de novo* synthesis and tRNA degradation pathways, contributing to the diversity of cytokinin types in plants (Frebort 2011). In the *de novo* synthesis pathway, the rate-limiting step is the transfer of the prenyl moiety from dimethylallyl diphosphate (DMAPP) to the N6 position of ATP, ADP, or AMP. This pivotal reaction is catalyzed by adenylate isopentenyltransferase (IPT), and in *Arabidopsis*, the genome contains nine IPT genes, including AtIPT1 to AtIPT9. Notably, AtIPT4 and AtIPT8 (PGA22) overexpression results in increased iP levels

and a typical cytokinin response, highlighting their significant roles. Furthermore, the preference of Arabidopsis IPTs for ADP or ATP leads to the production of iP ribosides, mainly iPRDP and iPRTP. Subsequent hydrolytic reactions release iP riboside (iPR) and free base iP.

The tRNA degradation pathway involves tRNA isopentenyl transferase (tRNA-IPT) enzymes, such as AtIPT2 and AtIPT9 in Arabidopsis, which transfer an isopentenyl unit from DMAPP to the N6 position of the nucleotide adjacent to the 3'-end of the tRNA anticodon. A loss-of-function mutant of both AtIPT2 and AtIPT9 results in a significant reduction in cZR and cZRMP contents, emphasizing the crucial role of tRNA degradation as the primary source for cZ-type cytokinins (Feng et al. 2017). Biochemical studies have revealed two possible pathways for trans-zeatin (tZ) biosynthesis: an iPRMP-dependent pathway, catalyzed by cytochrome P450 monooxygenase CYP735A1 and CYP735A2, and an iPRMP-independent pathway, proposing zeatin synthesis from AMP and side chain precursor by IPT (Sakakibara 2006). The complex biosynthetic pathways of cytokinins involve multiple enzymes and precursor molecules, resulting in the production of diverse cytokinin types. These pathways play pivotal roles in regulating various aspects of plant growth and development, illustrating the intricate interplay of cytokinins in the dynamic physiology of plants (Fig. 1) (Sakakibara 2006).

4.1 Activation of cytokinins

The two proposed pathways for cytokinin activation, namely the one-step and two-step reaction pathways, provide insights into the dynamic processes governing the conversion of inactive riboside forms to active cytokinins in plants. The two-step pathway, considered the predominant

route, involves sequential cleavage of the phosphate and sugar moieties from nucleotides, ultimately yielding the active nucleobases iP and tZ. This pathway has been extensively studied, with the identification of key enzymes such as nucleoside N-ribohydrolase (NRH) responsible for the second hydrolytic step. In contrast, the one-step pathway, exemplified by the action of LONELY GUY (LOG) proteins, facilitates direct conversion of cytokinin nucleotides to their free-base forms, bypassing the intermediate nucleoside stage. The identification of LOG homologs in various plant species, including the nine LOG genes in Arabidopsis, suggests a conserved mechanism across monocots and dicots. The coexistence of these two pathways highlights the intricacies of cytokinin activation, reflecting the adaptability of plants to different environmental cues and growth conditions.

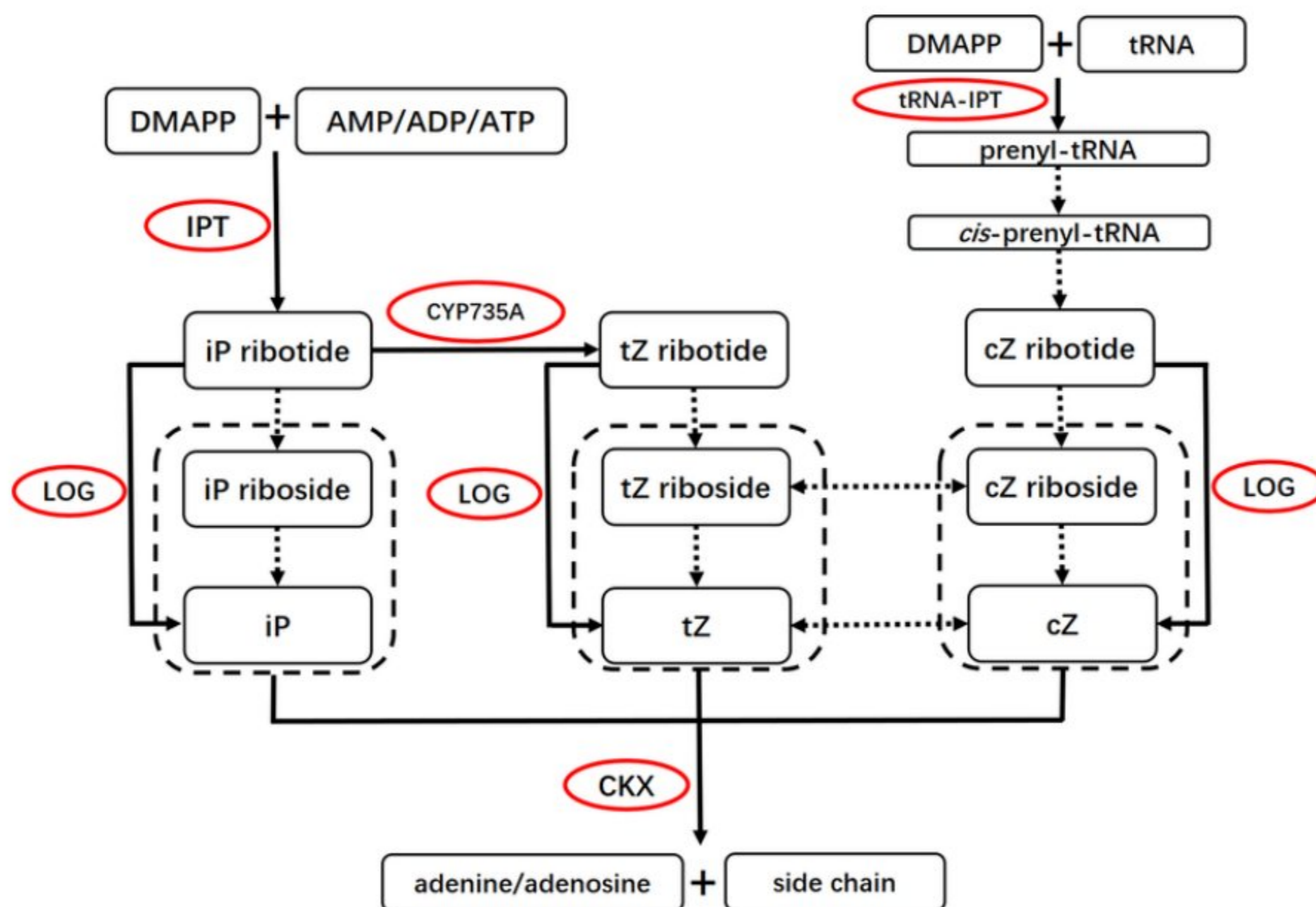


Figure 1. Basic scheme for the cytokinin biosynthesis and degradation pathways. Solid arrows indicate pathways with genes that are known, and

dotted arrows indicate pathways with genes that remain to be identified. The enzymes are marked by red frames. The iP, Z and their ribosides inside the dotted boxes could be degraded by CKX. cZ, cis-zeatin; DMAPP, dimethylallyldiphosphate; CKX, cytokinin oxidase/dehydrogenase; cZ, cis-zeatin; DMAPP, dimethylallyl diphosphate; iP, N 6-(Δ^2 -isopentenyl) adenine; IPT, adenosine phosphate-isopentenyltransferase; LOG, LONELY GUY; tRNA-IPT, tRNAisopentenyltransferase; tZ, trans-zeatin; Z, zeatin. This figure was modified and redrawn from reference (Kudo et al. 2010).

4.2 Sites of cytokinin biosynthesis

Earlier studies proposed that cytokinin biosynthesis primarily takes place in roots; however, recent investigations indicate a more widespread distribution throughout the entire plant. AtIPT genes, key players in cytokinin biosynthesis, exhibit expression in various shoot tissues, including leaves, stems, flowers, and siliques. The specific AtIPT gene expression patterns further highlight the spatial differentiation in cytokinin biosynthesis. For instance, AtIPT1 is predominantly expressed in the vascular stele of roots, leaf axils, ovules, and immature seeds, while AtIPT3 is observed in the phloem companion cells. AtIPT4 and AtIPT8 show expression in immature seeds, particularly in the chalazal endosperm, and AtIPT5 is present in lateral root primordia, root-cap columella, upper parts of young inflorescences, and fruit abscission zones. AtIPT7 exhibits expression in the endodermis of the root elongation zone, trichomes on young leaves, and pollen tubes, indicating diverse sites of cytokinin biosynthesis within the plant. Additionally, the differential expression of LOG genes, which play a role in cytokinin activation, across various tissues during plant development underscores the varied distribution of cytokinin types within different plant tissues (Takei et al. 2001).

Interestingly, the distribution of cytokinins is not uniform across plant tissues. Xylem sap is characterized by predominantly containing trans-zeatin (tZ)-type ribosides, while phloem sap is enriched in isopentenyladenine (iP) and cis-zeatin (cZ)-type ribosides. The dominance of tZ-type cytokinins in xylem sap aligns with the expression patterns of CYP735A genes, responsible for the hydroxylation of the isopentenyl side chain, primarily occurring in roots. This spatial differentiation in cytokinin distribution and biosynthetic gene expression suggests a finely tuned regulatory mechanism that adapts to the distinct physiological needs of different plant tissues (Choudhary 2013).

4.3 Regulation of cytokinin biosynthesis

The synthesis of cytokinins is intricately regulated by a variety of factors, encompassing hormonal signals and macronutrient availability. In *Arabidopsis*, cytokinins play a crucial role in promoting cell division by counteracting the effects of auxin. Specifically, auxin induces the expression of AtIPT5 and AtIPT7, while cytokinins exert a repressive influence on AtIPT1, AtIPT3, AtIPT5, and AtIPT7 in the shoot meristem. This dynamic interplay between cytokinins and auxin highlights their antagonistic relationship in regulating cellular processes. Additionally, auxin negatively regulates cytokinin biosynthesis in the nodal stem of pea plants by suppressing the expression of the PsIPT gene (Muller 2011).

Furthermore, macronutrients contribute to the intricate regulation of cytokinin biosynthesis. Nitrate, a key macronutrient, has been shown to enhance the accumulation of various cytokinins in maize and trans-zeatin (tZ)-type cytokinins in *Arabidopsis* roots. This effect is attributed to the induction of AtIPT3 expression, as evidenced by a significant reduction in

nitrate-dependent cytokinin accumulation in an *ipt3* null mutant. Other macronutrients, including sulfate and phosphate, also play regulatory roles in modulating AtIPT3 expression.

Beyond transcriptional regulation, posttranslational modifications also play a pivotal role in fine-tuning cytokinin biosynthesis. For instance, AtIPT3 contains characteristic CaaX boxes—short cysteine-containing motifs recognized by farnesyl transferase. Farnesylation directs the subcellular localization of AtIPT3, with nonfarnesylated AtIPT3 primarily localized in plastids, while farnesylated AtIPT3 is found in the nucleus and cytoplasm. This dual-layered regulation, involving both transcriptional and posttranslational mechanisms, highlights the sophisticated control mechanisms that govern cytokinin biosynthesis in response to various environmental and developmental cues (Ahmad et al. 2023).

4.4 Cytokinin degradation

In addition to cytokinin biosynthesis and activation, the degradation of cytokinins is a crucial regulatory mechanism that helps maintain optimal cytokinin levels in plants. Cytokinin oxidase/dehydrogenase (CKX) enzymes, classified as oxidoreductases with a flavin adenine dinucleotide (FAD)-binding motif, play a central role in this process. These enzymes irreversibly degrade endogenous cytokinins with an unsaturated side chain, including isopentenyladenine (iP), cis-zeatin (cZ), and trans-zeatin (tZ) (Feng et al. 2017).

Maize, for instance, possesses a family of 13 ZmCKX genes expressed in various tissues such as grains, roots, leaves, and immature ears. Among these, ZmCKX1 and ZmCKX3 have been demonstrated to exhibit

enzymatic activity (Vyroubalova et al. 2009). Similarly, Arabidopsis harbors seven CKX genes (AtCKX1 to AtCKX7), each displaying distinct expression patterns across different tissues. AtCKX1 is primarily expressed in the shoot apex, young floral tissues, and the root-hypocotyl junction, while AtCKX2 is found in the shoot apex and stipules. AtCKX4 is predominantly expressed in trichomes, stipules, and root caps, and AtCKX5 expression is confined to the edges of the youngest emerging leaves and in shoot and root meristems. AtCKX6, on the other hand, is expressed in the vasculature.

The subcellular localization of CKX enzymes further contributes to their functional specificity. AtCKX1 and AtCKX3 are found in the plant vacuole, while AtCKX2 localizes in the endoplasmic reticulum. Altering the expression of CKX genes results in significant phenotypic changes in various plant species. Overexpression of AtCKX1 to AtCKX4 in Arabidopsis, driven by the 35S promoter, leads to reduced cytokinin levels and cytokinin-deficient phenotypes in shoots and roots. Similarly, overexpressing the orchid DSCKX1 gene in Arabidopsis results in developmental phenotypes due to decreased cytokinin levels (Wang et al. 2020).

Interestingly, manipulating CKX gene expression can have profound effects on crop yield. For instance, in rice, the OsCKX2 gene, encoding a cytokinin oxidase, is linked to a quantitative trait locus (GN1A) controlling grain yield. Transgenic rice plants expressing antisense constructs of OsCKX2 show elevated cytokinin levels, leading to increased inflorescence meristems and reproductive organs, ultimately enhancing grain productivity. In other cases, such as with AtCKX3 and AtCKX5 in Arabidopsis, root-specific overexpression of CKX genes promotes root growth, contributing to

improved drought tolerance and nutrient absorption. These findings underscore the significance of CKX enzymes as pivotal targets for molecular strategies aimed at enhancing crop yields through the precise modulation of cytokinin levels (Chen et al. 2022).

4.5 Cytokinin transport

The transport of different types of cytokinins in plants has been revealed through studies employing reciprocal grafting with *ipt1*, 3, 5, 7 mutants and wild-type *Arabidopsis* plants. These investigations indicate that isopentenyladenine (iP) is predominantly transported from shoots to roots, while trans-zeatin (tZ) might primarily move from roots to shoots. The importance of root-to-shoot transport of tZ for shoot development has been underscored by analyzing the phenotype of the *cyp735a1 cyp735a2* double mutant. This highlights the necessity of an efficient transport system for cytokinins to reach their target cells. Three main types of proteins contribute to cytokinin transport: purine permeases (PUPs), nucleoside transporters (equilibrative nucleoside transporters, ENTs), and ATP-binding cassette (ABC) transporters.

Arabidopsis has two identified PUP genes, AtPUP1 and AtPUP2, which encode transporters localized to the plasma membrane, facilitating cytokinin uptake into cells. These PUPs are primarily expressed in phloem, suggesting their involvement in the uptake of cytokinins from xylem sap during long-distance transport. AtPUP1 and AtPUP2 have demonstrated the ability to transport adenine and nucleobase cytokinin derivatives in yeast and *Arabidopsis* cells. However, with more than 20 PUP genes in the

Arabidopsis genome, further investigations are needed to elucidate the specific functions of each.

In higher plants, the translocation of cytokinin ribosides appears to be mediated by ENTs. Arabidopsis AtENT8 (also known as SOI33) and AtENT3 have been identified as mediators of iP uptake in yeast. In rice, OsENT2 is implicated in the selective transport of cytokinin nucleosides into vascular tissues. Furthermore, studies have identified the involvement of the Arabidopsis ABC transporter subfamily G14 (AtABCG14) in loading tZ-type cytokinins into the xylem for transport from roots to shoots. The expression of AtABCG14 is observed in the pericycle and stellar cells of the root, and loss of its function severely impairs the translocation of tZ-type cytokinins, impacting overall plant growth and development (Nedvĕd 2021).

5. Cytokinin signal transduction

The discovery of cytokinins in the 1950s marked the beginning of extensive research on their physiological roles and metabolism. However, understanding cytokinin signal transduction lagged behind, and until the late 1990s, efforts to unravel the signaling mechanisms faced challenges. Initial hypotheses proposed the involvement of calmodulin and a G-protein-linked receptor in cytokinin signaling, but direct evidence was lacking (Suzuki et al 2001). Genetic screens for Arabidopsis mutants with altered responses to exogenously applied cytokinins did not yield significant results due to the challenges posed by the strong influence of cytokinins on the ethylene response and the redundancy of cytokinin signaling components (Hwang et al. 2012).

In the mid-1990s, breakthroughs occurred when Tatsuo Kakimoto and colleagues identified key regulators of cytokinin signaling through genetic screens for mutants with constitutive or impaired cytokinin responses. The positive regulators, CYTOKININ INDEPENDENT 1 (CKI1) and CYTOKININ RESPONSE 1 (CRE1), were identified, along with the discovery of cytokinin-inducible genes, type-A Arabidopsis response regulators (ARRs). These findings paved the way for a more comprehensive understanding of the cytokinin signal transduction cascade (Pischke et al. 2002).

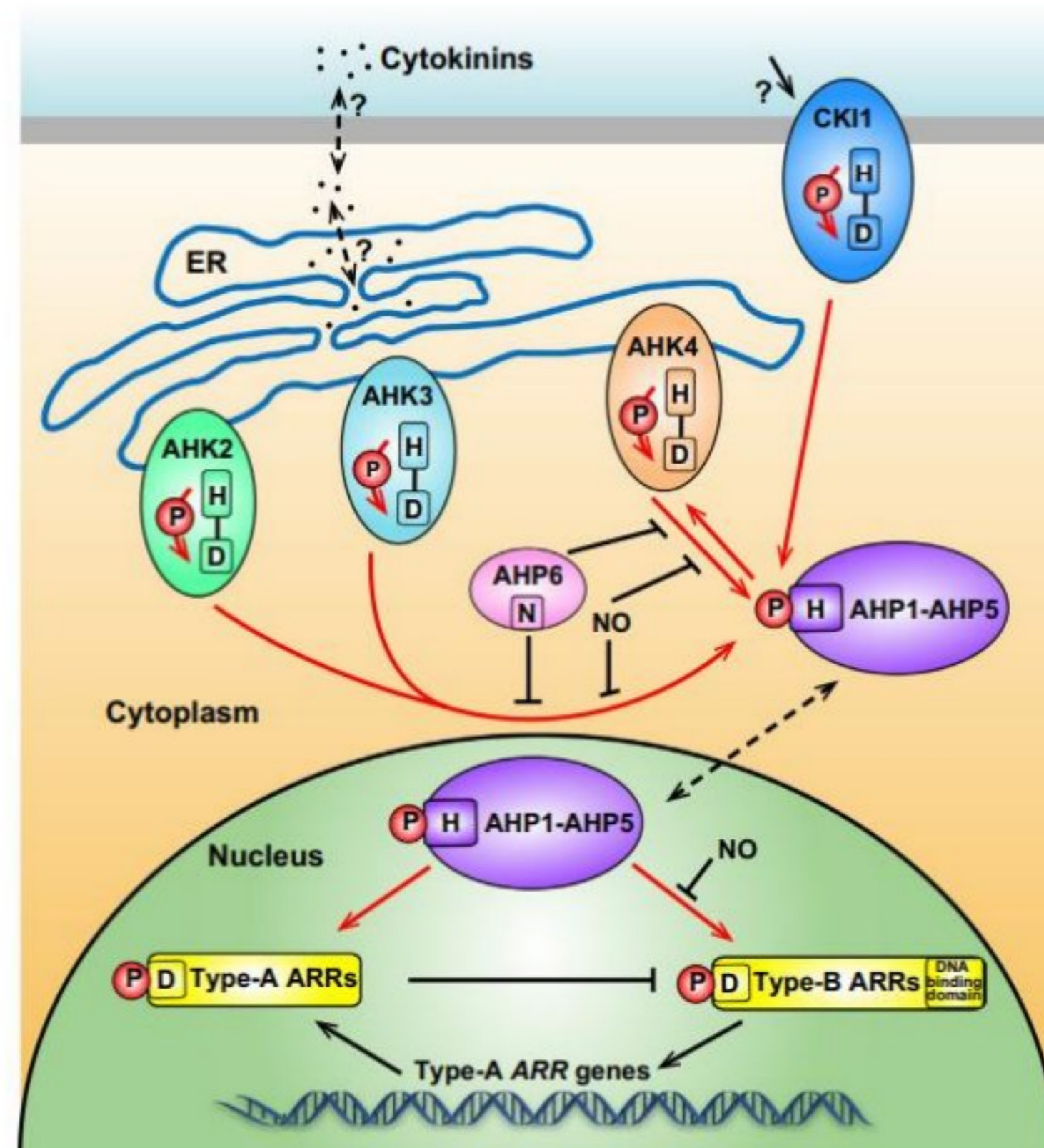


Figure 2. Core steps of the cytokinin signaling pathway. The cytokinin signaling cascade is initiated by cytokinin binding to the cytokinin receptors AHK2, AHK3, and AHK4 within the lumen of the endoplasmic reticulum (ER). After binding to cytokinins, the cytokinin receptors are autophosphorylated at conserved histidine residues in the kinase domain. The

phosphate groups are then transferred to the conserved aspartic acid residues in the receiver domain of the receptors, and then are likely transferred to the histidine residues of AHPI-AHPS in the cytoplasm. The phosphorylated AHPs translocate into the nucleus by an unknown mechanism and transfer the phosphate groups to the conserved aspartic acid residues in the receiver domain of type-A ARR or type-B ARR. In the absence of cytokinins, AHK4/CRE1/WOL removes phosphate groups from AHPs. CK/1 also mediates phosphorylation of AHPs in a cytokinin-independent manner. The stability of type-A ARR proteins may be regulated by phosphorylation. The phosphorylated type-B ARRs activate the expression of downstream genes, regulating plant growth and development. Type-B ARRs activate the expression of downstream genes, regulating plant growth and development. Type-B ARRs activate the expression of type-A ARRs, and type-A ARRs, in turn, act to repress the activity of type-B ARRs by a negative feedback mechanism. P denotes the phosphate group; H and D indicate histidine and aspartic acid; black solid circles indicate cytokinins (Feng et al. 2017).

Cytokinin signaling involves a two-component system with a phosphorelay mechanism, mediated by three histidine kinases (AHK2, AHK3, and AHK4) as cytokinin receptors. Upon cytokinin binding, receptor activation involves autophosphorylation and subsequent phosphorylation of downstream components, including Arabidopsis type-B and type-A response regulators (ARRs). The phosphorylated type-B ARRs, which are MYB-class transcription factors, activate the expression of type-A ARR genes and other downstream targets, leading to cytokinin responses. Type-A ARR proteins, in turn, negatively regulate phosphorelay activity, forming a feedback loop. The bidirectional phosphorelay is regulated by the cytokinin receptors and various negative regulators (Tran et al. 2010). This signaling pathway,

composed of AHK receptors, AHPs, and ARR2s, has been identified not only in *Arabidopsis* but also in other plant species like rice, *Marchantia polymorpha*, and *Physcomitrella patens*, indicating the evolutionary conservation of cytokinin signaling components. The elucidation of the cytokinin signal transduction pathway has provided valuable insights into the molecular mechanisms governing plant growth and development (Fig. 2) (Feng et al. 2017).

6. Functions

Over the last decade, significant strides have been made in unraveling the intricacies of cytokinin, a plant hormone, encompassing its biosynthesis, perception, and signaling pathways. The realization that any disruption in these processes profoundly affects various stages of growth and development has sparked a renewed surge of interest in comprehending the impact of cytokinin signaling on developmental mechanisms. Consequently, recent efforts have yielded fresh insights into the role of cytokinin signaling and its downstream targets in pivotal developmental processes such as shoot apical meristem, flower formation, female gametophyte development, stomatal regulation, and vascular growth. This review aims to provide a comprehensive overview of the latest discoveries regarding how cytokinin influences plant growth and development, shedding light on emerging areas for future research.

6.1 Shoot development

Over the past decade, the exploration of cytokinins in plant biology has revealed their pivotal role in shaping plant growth, particularly when in conjunction with auxin. The collaboration of these hormones, specifically

auxin and cytokinin, has been a central focus of research, unveiling their profound influence on plant development. Experimental observations have demonstrated that plants exposed to heightened levels of auxin and cytokinin undergo extensive proliferation and dedifferentiation, ultimately leading to the formation of callus—a mass of undifferentiated cells. Notably, the cultivation of callus under conditions of elevated cytokinin levels has been linked to the induction of shoot regeneration, firmly establishing cytokinins as key players in the intricate orchestration of shoot development (Su et al. 2011).

The significance of cytokinin signaling extends into the realm of shoot apical meristem (SAM) development, where it has been demonstrated to possess the capability to induce shoot formation in callus. Remarkably, under specific conditions, exogenous cytokinins have been shown to trans-differentiate lateral root primordia into a SAM. This intriguing capability suggests that cytokinins can exercise their influence to specify shoot cell fate across diverse cell types. In the natural context, studies have emphasized the crucial role of cytokinins in SAM formation, as reduced cytokinin levels or compromised signaling pathways result in a discernibly smaller SAM (Kean-Galeno et al. 2024). Transport mechanisms further contribute to the nuanced regulation of cytokinins. *ARABIDOPSIS* *ATP-BINDING CASSETTE G14* (*ABCG14*) has emerged as a key player in modulating root-derived cytokinin transport. Additionally, the involvement of *PURINE PERMEASE 14* (*PUP14*) transporters has been suggested in mediating the confinement of cytokinins within the SAM. These transport processes underscore the intricacies involved in the spatial regulation of cytokinins, emphasizing their importance in finely tuning developmental processes.

Delving into the molecular intricacies within the SAM, cytokinins act as positive regulators of WUSCHEL (WUS), a gene crucial for stem cell niche maintenance. Recent observations have shed light on the direct binding of B-type ARR_s to the promoter region of WUS, thereby promoting its expression. The interplay between WUS and cytokinin signaling emerges as indispensable during shoot specification and regeneration. Mutant explants lacking WUS or specific ARR genes exhibit compromised shoot regeneration capabilities, highlighting the synergistic nature of these pathways (Jha et al. 2020).

The dynamic relationship between cytokinins and WUS unfolds as a finely tuned interdependence, where the expression of WUS precedes morphological changes into a SAM. This suggests that WUS may serve as a direct mediator of cytokinin-induced shoot specification downstream of the cytokinin response pathway. The necessity of normal cytokinin response for proper WUS expression is evident, as disruptions in this pathway result in deficient shoot regenerative capacities (Argueso et al. 2010).

6.2 Flower development

Cytokinins play a crucial role in various stages of flower development, exerting their influence on processes such as carpel regeneration and determinacy, as well as gynoecium development. In loss-of-function lines of B-type ARR_s, specifically ARR1 and ARR10, carpel regeneration from callus was impaired. These ARR_s were found to bind the AGAMOUS (AG) promoter region, inducing the expression of the carpel identity-defining gene AG. Carpel regeneration was also hindered in AG amiRNA lines, reinforcing the essential role of AG in carpel formation. These findings, although conducted in a carpel-inducing system, suggest that

cytokinin-dependent control of AG expression through ARR1 and ARR10 may also be functional in normal carpel development (Brecht and Rybel 2019).

Unlike the shoot apical meristem (SAM) that continuously divides to generate new tissues, flower meristems have a defined number of flowers before terminating growth. The determinacy of flower meristems is compromised in *ag-10* mutants when treated with exogenous cytokinins, resulting in additional tissues within the carpels. ETTIN/AUXIN RESPONSE FACTOR 3 (ARF3) normally restricts cytokinin signaling by repressing IPT, LOG, and AHK genes. Prolonged WUS expression in the *arf3-29* mutant suggests that ETTIN/ARF3 repression of cytokinin signaling is crucial for flower determinacy, potentially by limiting WUS expression (Chang et al. 2020).

In gynoecium development, the maximum expression of TCS indicates the involvement of cytokinin signaling. The *arr1/10/12* mutant displays fewer ovules, defects in septum fusion, and reduced transmitting tract tissues. Conversely, elevated cytokinin levels lead to over-proliferation of medial tissues. The bHLH transcription factor SPATULA (SPT) influences cytokinin signaling, directly binding to the ARR1 promoter and inducing cytokinin signaling in medial tissues. Cytokinin signaling is confined within medial tissues by inducing the cytokinin inhibitor AHP6, while AG represses cytokinin signaling by inducing A-type ARRs (Hwang et al. 2012).

This intricate interplay of cytokinins, ARRs, and other regulatory factors underscores their multifaceted roles in flower development, not only in the model plant *Arabidopsis* but also in other species such as *Actinidia*,

This intricate interplay of cytokinins, ARRs, and other regulatory factors underscores their multifaceted roles in flower development, not only in the model plant *Arabidopsis* but also in other species such as *Actinidia*, where a male sex-determining gene, *SHY GIRL*, encodes a C-type ARR that negatively regulates cytokinin signaling, leading to dioecious flowers. The broad implications of cytokinins in gynoecium development highlight their significance across the plant kingdom (Hong and Fletcher 2023).

6.3 Female gametophyte development

Cytokinins play a pivotal role in the development of ovules within the carpels of the gynoecium, particularly during female gametophyte development. Several cytokinin-associated genes, including cytokinin-insensitive (*cki*) single mutants, *arr7/arr15* double mutants, and *ahp2-2/ahp3/ahp5-2* triple mutants, exhibit lethality in female gametophytes. *CKI*, encoding a histidine kinase activating cytokinin response in the absence of cytokinins, is essential for proper cell fate specification (Brecht and Rybel 2019). In *CKI/cki* loss-of-function mutants, antipodal and central cells adopt egg cell fate, emphasizing the role of cytokinin signaling. *TCS* expression is reduced or absent in these cells, further linking cytokinin signaling to cell fate specification. Downstream components *AHP1*, *AHP2*, and *AHP5* are implicated in the standard cytokinin signaling pathway controlling cell fate.

Contrastingly, *CKI* overexpression leads to ectopic *TCS* expression and the specification of the egg cell into a central cell. This misspecification results in the development of a diploid endosperm instead of an embryo upon fertilization. *CKI* is crucial for providing antipodal and central cell fate, while its repression is necessary for synergid and egg cell specification.

However, the specific mechanisms through which CKI determines these cell fates remain unknown (Yuan et al. 2016).

While this female gametophyte development is not conserved across all plants, as evidenced by gymnosperms lacking central cells and endosperm, the presence of a CKI ortholog suggests its importance in different species. In *Ginkgo biloba*, the CKI ortholog partially rescues the *Arabidopsis cki* mutant but fails to confer central cell specification, indicating potential neofunctionalization of CKI during angiosperm evolution, facilitating the formation of central cells and the establishment of endosperm (Brecht and Rybel 2019).

6.4 Root development

Cytokinins, known primarily for their role in shoot development, also play a significant role in root development, as evident from a range of root-related phenotypes observed in biosynthesis, perception, and signaling mutants. The *Arabidopsis* root exhibits clear bilateral symmetry within its vascular tissues, with a central xylem axis flanked by two phloem poles and intervening procambium cells. This bilateral character arises from the tight interplay between auxins and cytokinins. High auxin signaling in xylem cells induces AHP6, repressing cytokinin signaling, while cytokinin signaling in procambial cells affects auxin efflux through PIN-FORMED (PIN) protein expression and localization. Mathematical modeling supports the idea that this interplay is sufficient for achieving bilateral symmetry within the vasculature.

Bilateral symmetry is particularly evident in the pericycle, where lateral roots develop from sets of xylem-pole pericycle cells with high auxin

signaling. AHP6 is implicated in repressing cytokinin signaling in this context. Cytokinins also negatively affect lateral root initiation and organization by disturbing PIN protein localization, thus perturbing local auxin accumulation. This repression of lateral root initiation is crucial for maintaining regular spacing between lateral root primordia (Jing and Strader 2019).

Surprisingly, evidence suggests that bilateral symmetry extends beyond the pericycle into the ground tissue. Endodermal cells at the xylem pole, characterized by higher cytokinin levels, exhibit increased division rates and shorter lengths compared to those neighboring the phloem pole. AHP6 repression of cytokinin signaling in the protoxylem pole endodermis contributes to this bilateral symmetry, guiding correct tissue patterning in collaboration with auxins. In summary, cytokinins play a multifaceted role in root development by influencing vascular tissue symmetry, lateral root initiation, and overall tissue patterning in the root system (Wybouw and De Rybel 2019).

6.5 Cytokinin controls cell divisions in leaf epidermis

Cytokinins play a crucial role in vascular development, as evidenced by classical mutants in the signaling pathway like wooden leg (*ahk4/cre1/wol*) and *ahp6*, known for their vascular defects. Recent years have highlighted the significance of the bHLH transcription factor complex, TMO5/LHW, in regulating vascular proliferation, with loss-of-function resulting in diminished vascular cell files and ectopic expression increasing their numbers. This complex binds to the promoter regions of cytokinin biosynthesis genes LOG3 and LOG4 in the xylem axis, suggesting a direct role in radial expansion. Exogenous cytokinin application rescues vascular

bundle size, indicating the essentiality of cytokinins in radial expansion. Perturbations in cytokinin biosynthesis, transport, or signaling lead to reduced vascular cell file numbers, and the induction of *CYCLIND3;1* and *AINTEGUMENTA* is identified as a direct link between cytokinins and cell division. Moreover, cytokinins influence patterning, as mutants exhibit protoxylem identity in all vascular cells, contrasting with *TMO5/LHW* mutants. The intricate involvement of cytokinins in radial proliferation, patterning, and xylem differentiation in vascular tissues underscores their significance, although the molecular mechanisms underlying these processes remain to be fully elucidated (Wybouw and De Rybel 2019).

6.6 Root nodule formation

The intricate relationship between plants of the Fabaceae family and nitrogen-fixing bacteria is marked by the formation of specialized plant structures known as root nodules, a phenomenon tightly connected to cytokinin signaling. In species like *Medicago truncatula* and *Lotus japonica*, the inoculation of nitrogen-fixing bacteria triggers an upregulation in cytokinin biosynthesis and signaling within the affected roots. Notably, in *Lotus japonica*, triple receptor mutants (*lhk1-1 lhk1a-1 lhk3-1*) exhibit hyperinfection following *Mesorhizobium loti* inoculation, yet the infection threads fail to progress into nodule primordia. This observation suggests that cytokinins may not be heavily involved in the initial infection event but rather play crucial roles during the subsequent development of root nodules.

Evidence supporting the importance of cytokinin signaling in nodulation is further underscored by the induction of nodulation factors like *MtNSP* downstream of cytokinins. This implies that cytokinin signaling is a requisite factor for the nodulation process. Additionally, experiments

involving the overexpression of cytokinin biosynthesis genes demonstrate that root nodule formation can occur even in the absence of nitrogen-fixing bacteria. This finding strongly suggests that cytokinin signaling is not only necessary but also sufficient for the normal development of root nodules, shedding light on the pivotal role played by cytokinins in orchestrating the intricate dance between plants and nitrogen-fixing bacteria in the Fabaceae family (Gamas et al. 2017).

6.7 Vascular development

Cytokinins play a crucial role in vascular development, as evidenced by classical mutants in the signaling pathway like wooden leg (*ahk4/cre1/wol*) and *ahp6*, known for their vascular defects (Wybouw and De Rybel 2019). Recent years have highlighted the significance of the bHLH transcription factor complex, TMO5/LHW, in regulating vascular proliferation, with loss-of-function resulting in diminished vascular cell files and ectopic expression increasing their numbers. This complex binds to the promoter regions of cytokinin biosynthesis genes LOG3 and LOG4 in the xylem axis, suggesting a direct role in radial expansion. Exogenous cytokinin application rescues vascular bundle size, indicating the essentiality of cytokinins in radial expansion (Smet et al. 2019). Perturbations in cytokinin biosynthesis, transport, or signaling lead to reduced vascular cell file numbers, and the induction of *CYCLIND3;1* and *AINTEGUMENTA* is identified as a direct link between cytokinins and cell division. Moreover, cytokinins influence patterning, as mutants exhibit protoxylem identity in all vascular cells, contrasting with TMO5/LHW mutants. The intricate involvement of cytokinins in radial proliferation, patterning, and xylem differentiation in vascular tissues underscores their significance, although the

molecular mechanisms underlying these processes remain to be fully elucidated (Campbell and Turner 2017).

7. Conclusion

In conclusion, cytokinins play a pivotal role as plant hormones, exerting significant influence on various physiological processes crucial for plant growth and development. The journey of cytokinins encompasses their synthesis, signal transduction, and diverse functions within the plant system. Cytokinins are primarily synthesized in the roots and transported upwards to different plant organs. The biosynthesis involves isoprenoid or adenylate-type pathways, resulting in the production of various cytokinin forms, including zeatin, isopentenyladenine, and others. Upon synthesis, cytokinins initiate a complex signal transduction pathway, involving receptors, histidine kinases, and phosphorelays. This process triggers downstream responses, influencing gene expression and cellular activities. The functions of cytokinins extend beyond cell division; they participate in leaf senescence, nutrient mobilization, and stress responses. Cytokinins act as key regulators in maintaining the balance between shoot and root growth, ensuring optimal plant architecture. Moreover, they contribute to the coordination of various developmental stages, from seed germination to flower and fruit development. In summary, the multifaceted roles of cytokinins make them indispensable players in the intricate network of plant growth regulation. Their involvement in diverse physiological processes underscores their importance in shaping the overall growth, development, and adaptive responses of plants in their dynamic environment. As our understanding of cytokinins advances, so does the potential for harnessing their regulatory properties to enhance crop yield, stress tolerance, and overall plant productivity.

References

- Ahmad N, Jiang Z, Zhang, L, Hussain I, Yang X (2023) Insights on phytohormonal crosstalk in plant response to nitrogen stress: A focus on plant root growth and development. *Int J Mol Sci* 24(4): 3631.
- Argueso CT, Raines T, Kieber JJ (2010) Cytokinin signaling and transcriptional networks. *Curr Opin Plant Biol* 13(5): 533–539.
- Bielach A, Hrtyan M, Tognetti VB (2017) Plants under stress: involvement of auxin and cytokinin. *Int J Mol Sci* 18(7): 1427.
- Brecht W, Rybel BD (2019) Cytokinin—a developing story. *Trends Plant Sci* 24(2): 177–185.
- Bürkle L, Cedzich A, Döpke C, Stransky H, Okumoto S, Gillissen B, Frommer WB (2003) Transport of cytokinins mediated by purine transporters of the PUP family expressed in phloem, hydathodes, and pollen of *Arabidopsis*. *Plant J* 34(1): 13–26.
- Campbell L, Turner S (2017) Regulation of vascular cell division. *J Exp Bot* 68(1): 27–43.
- Chang W, Guo Y, Zhang H, Liu X, Guo L (2020) Same actor in different stages: genes in shoot apical meristem maintenance and floral meristem determinacy in *Arabidopsis*. *Front Ecol Evol* 8: 89.
- Chen CM, Leisner SM (1984) Modification of cytokinins by cauliflower microsomal enzymes. *Plant Physiol* 75: 442–446.
- Chen KL, Xu MX, Li GY (2006) Identification of AtENT3 as the main transporter for uridine uptake in *Arabidopsis* roots. *Cell Res* 16: 377–388.
- Chen S, Lou S, Zhao X, Zhang S, Chen L, Huang P, Li G, Li Y, Liu Y, Chen Y (2022) Ectopic expression of a male fertility gene, LOGL8,

represses LOG and hinders panicle and ovule development. *J Crop Sci* 10(6): 1665–1673.

Chaudhary, N. (2013). The interaction of cytokinin and strigolactone in controlling shoot branching in *Arabidopsis thaliana* (Doctoral dissertation, Imperial College London).

Fahad S, Hussain S, Bano A, Saud S, Hassan S, Shan D, Ahmed F, Khan F, Chen Y, Wu C, Tabassum M, Chun M, Afsal M, Jan A, Tariq M, Huang J (2015) Potential role of phytohormones and plant growth-promoting rhizobacteria in abiotic stresses: consequences for changing environment. *Environ Sci Pollut Res Int* 22: 4907–4921.

Feng J, Shi Y, Yang S, Zuo J (2017) Cytokinins. In: Li J, Li C, Smith SM (eds.) *Hormone metabolism and signaling in plants*. Academic Press, pp 77–106.

Frebort, I, Kowalska, M, Hluska, T, Frébortová, J, Galuszka, P (2011) Evolution of cytokinin biosynthesis and degradation. *J Exp Bot* 62(8): 2431–2452.

Gamas P, Brault M, Jardinaud M. F, Frugier F (2017) Cytokinins in symbiotic nodulation: when, where, what for? *Trends Plant Sci* 22(9): 792–802.

Haberer G, Kieber JJ, (2002) Cytokinins. New insights into a classic phytohormone. *Plant Physiol* 128: 354–362.

Hong L, Fletcher JC (2023) Stem cells: Engines of plant growth and development. *Int J Mol Sci*, 24(19): 14889.

Hwang I, Sheen J, Müller B (2012) Cytokinin signaling networks. *Annu Rev Plant Biol* 63: 353–380.

Jha P, Ochatt SJ, Kumar V (2020) WUSCHEL: a master regulator in plant growth signaling. *Plant Cell Rep* 39: 431–444.

- Jing H, Strader LC (2019) Interplay of auxin and cytokinin in lateral root development. *Int J Mol Sci* 20(3): 486.
- Kakimoto T (2001) Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyl transferases. *Plant Cell Physiol* 42: 677–685.
- Kean-Galeno T, Lopez-Arredondo D, Herrera-Estrella L (2024) The shoot apical meristem: an evolutionary molding of higher plants. *Int J Mol Sci* 25(3): 1519
- Kieber JJ, Schaller GE (2018) Cytokinin signaling in plant development. *Development* 145(4): 149344.
- Kudo T, Kiba T, Sakakibara H (2010) Metabolism and long-distance translocation of cytokinins. *J Integr Plant Biol* 52(1): 53–60.
- Müller B (2011) Generic signal-specific responses: cytokinin and context-dependent cellular responses. *J Exp Bot* 62(10): 3273–3288.
- Müller D, Leyser O. (2011). Auxin, cytokinin and the control of shoot branching. *Ann Bot* 107(7): 1203–1212.
- Nedvěd D (2021) Transport and metabolism of radio-labelled cytokinins in plant cells and tissues. *Int J Mol Sci*. 22(7): 3428.
- Pischke MS, Jones LG, Otsuga D (2002) An *Arabidopsis* histidine kinase is essential for megagametogenesis. *Proc Natl Acad Sci USA* 99: 15800–15805.
- Sakakibara H (2006) Cytokinins: activity, biosynthesis, and translocation. *Annu Rev Plant Physiol* 57: 431–449.
- Su YH, Liu YB, Zhang XS (2011) Auxin–cytokinin interaction regulates meristem development. *Mol Plant* 4(4): 616–625.
- Suzuki T, Miwa K, Ishikawa K (2001) The *Arabidopsis* sensor His-kinase, AHK4, can respond to cytokinins. *Plant Cell Physiol* 42: 107–113.

- Takei K, Sakakibara H, Sugiyama T (2001) Identification of genes encoding adenylate iso pentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. *J Biol Chem* 276: 26405–26410.
- Tran LSP, Shinozaki K, Yamaguchi-Shinozaki K (2010) Role of cytokinin responsive two-component system in ABA and osmotic stress signalings. *Plant Signal Behav* 5(2): 148–150.
- Wang X, Ding J, Lin S, Liu D, Gu T, Wu, H, Li Y (2020) Evolution and roles of cytokinin genes in angiosperms 2: Do ancient CKXs play housekeeping roles while non-ancient CKXs play regulatory roles? *J Hortic Res* 7: 29.
- Wybouw B, De Rybel B (2019) Cytokinin—a developing story. *Trends Plant Sci* 24(2): 177–185.
- Yuan L, Liu Z, Song X, Johnson C, Yu X, Sundaresan V (2016) The CKI1 histidine kinase specifies the female gametic precursor of the endosperm. *Dev Cell* 37(1): 34–46.