Review

Cytokinins and their Function in Developing Seeds

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Abstract

Cytokinins are a major group of plant hormones that control various processes in plant growth and development. Chemically they are N^6-substituated adenine derivatives, including their respective ribotides, ribosides and glucosides. The inter-conversion between different cytokinins metabolites represents the transition between active, inactive, storage and transport forms. This process is *in vivo* rather dynamic and rapid. We have finally begun to understand the role of cytokinins in plant development through the identification of genes for the first plant enzyme in the biosynthetic pathway, isopentenyl transferases, less than 10 years ago, in addition to research of other enzymes and their corresponding genes that are involved in cytokinin metabolism and signal transduction. This review focuses on the recent findings on cytokinins with an emphasis on their role during the development of seedsfrom which the first natural cytokinin was isolated more that half a century ago.

Keywords: Biosynthesis, cytokinin, metabolism, physiological role, seed, signal transduction

1. Introduction

The term "cytokinin" generally refers to both naturally occurring and synthetic compounds with hormonal activities that promote growth in plant tissue cultures[1]. The first characterized compound with cytokinin activity was kinetin, isolated from autoclaved herring sperm DNA[2]. However, the first natural occurring cytokinin, zeatin, was isolated from immature maize one-seeded fruit – caryopsis[3,4] and since its discovery it has been shown to be ubiquitous in different plant species and plant tissues. To date, many other naturally occurring cytokinin species have been discovered. Therefore, cytokinins are the largest and most chemically diverse group of plant hormones and are involved in a wide spectrum of unrelated physiological and developmental processes in a variety of plants and plant tissues. The processes range from promotion of cell division, regulation of apical dominance, shoot and root development, leaf expansion and delay of senescence, stomatal opening, seedling germination, circadian rhythms, nodulation in legumes, to promotion of chlorophyll synthesis and regulation of interactions with pathogens [5–14].

The least understood aspect of cytokinins remains their exact role in plant growth and development. Experimental data often imply only a vague association between cytokinins and certain physiological processes, while the exact modes of cytokinin action remain elusive and hard to characterize[15]. The reason why such questions continue to be unresolved is due to the fact that cytokinin biosynthesis in plants remained unclear for more than four decades after the discovery of cytokinin activity in the 1950s by Skoog and Miller[2]. The failure to find genes and proteins involved in cytokinin biosynthesis even provoked a suggestion that cytokinins might not be synthesized by plants themselves, but rather by external symbiotic organisms[16]. However, the improvement of methods for cytokinin isolation and identification[17] and the application of molecular approaches to cytokinin research have proved plant cytokinin biosynthesis and revealed cytokinin biosynthetic genes[18, 19]. This discovery has opened up new areas of cytokinin research in plants, especially resolution of the molecular basis for cytokinin biosynthesis[20] and cytokinin signaling pathways[21–26].

The aim of the current review is to update the progress in the biosynthesis, metabolism and signaling of cytokinins by emphasizing their role in developing seeds. Seeds have played an important role for the research of these plant hormones since the first naturally occurring cytokinin, zeatin, was isolated from the maize caryopsis.

2. Cytokinin Structure and Biological Activity

Cytokinins are N^{-6}-substituated adenines. Based on the N^6 side chain, they can be distinguished into two separate groups, isoprenoid and aromatic. Both the adenine ring and the side chain can be subject to further chemical modifications (Figure 1). The most common modifications on the purine ring involve conjugation with sugars (glucosylation, xylosilation, ribosylation), sugar phosphates (ribotydilation) or aminoacids (alanylation). Chemical modifications greatly affect the chemical and physiological properties of a cytokinin molecule. In fact, free bases are cytokinin species that most strongly bind to cytokinin receptors[27, 28]. The binding is associated with physiological responses, thus these forms are considered as biologically active. Cytokinin conjugates, on the other hand, bind to cytokinin receptors only weakly or not at all[28] and are regarded as biologically inactive. Of the well characterized conjugates, ribosides are considered to be transport forms, since they are the predominating forms found in the plant transport systems of xylem and phloem[19, 29]. Among glucosides, *O*-glucosides are reversibly inactive forms of cytokinins and might serve for cytokinin storage, whereas 7- and 9-glucosides are presumed to be irreversibly inactive end products of cytokinin metabolism, because the enzymes for the cleavage of sugars moieties from cytokinin 7- and 9-glucosides are not known in plants[30].

Based on the N^6 side chain, cytokinins are divided into isoprenoid and aromatic. Both, the purine ring and the side chain, are subject to different biochemical modification, like glucosylation, ribosylation, hydrohylation and isomerisation.

For common usage the cytokinin nomenclature proposed by Letham[118] was adopted instead of the formal IU-

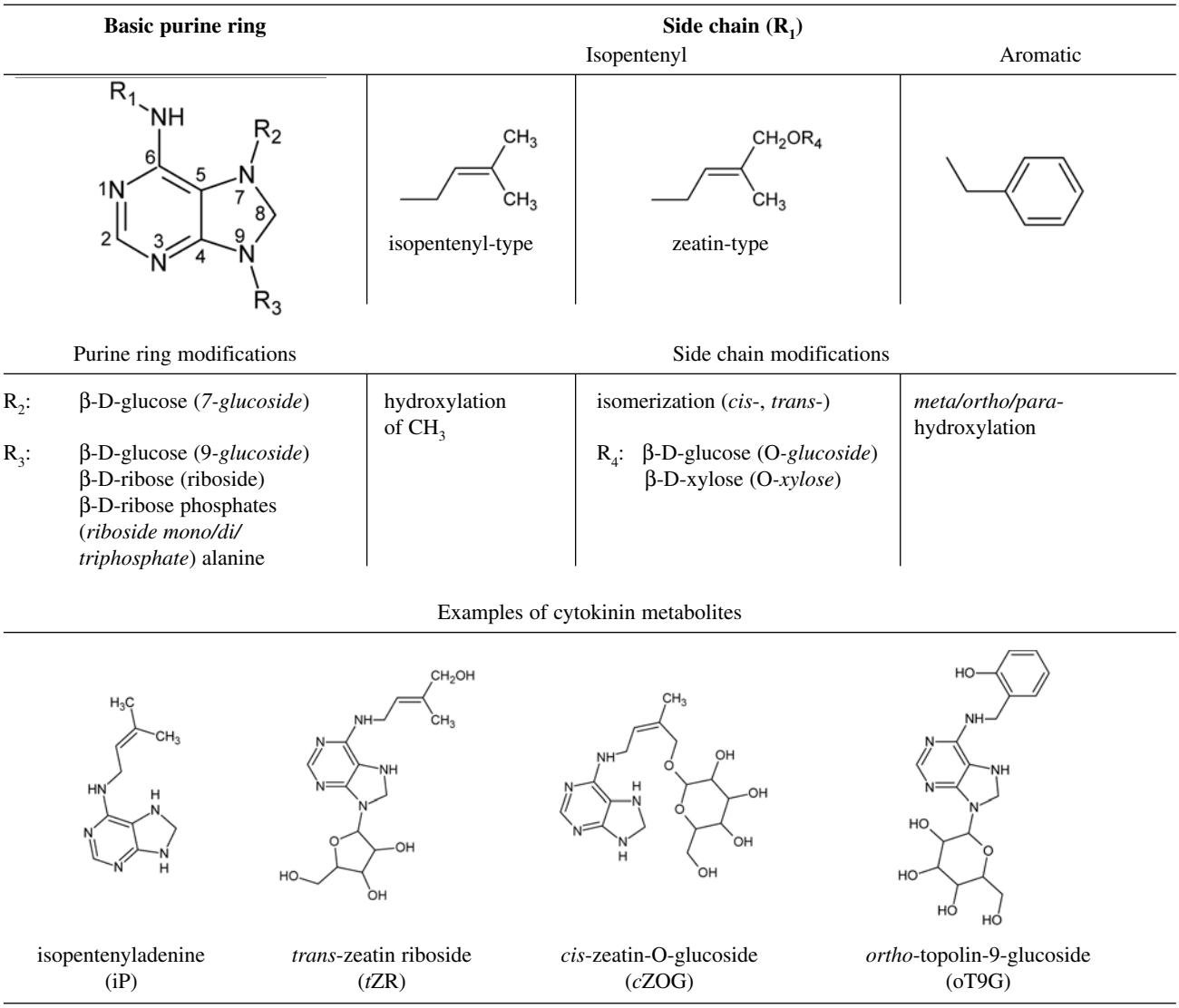

Figure 1: Structural diversity of cytokinin metabolites.

PAC one. Substitutions at the basic isoprenoid cytokinins zeatin (Z) and isopentenyladenine (iP) are denoted with either square brackets, for substitutions on the purine ring, or round brackets, for substitutions on the side chain, e.g. [9G]Z for zeatin 9-glucoside or (diH)[9R]Z for dihydrozeatin riboside, respectively. At present, an even simpler naming system is being used by most researchers, where the abbreviations are just a short version of the longer trivial name, e.g. DHZR for dihydrozeatin riboside.

3. Cytokinin Biosynthesis and Chemical Conversions

3. 1. Key Cytokinin Biosynthetic Enzymes are Isopentenyl Transferases

Cytokinin biosynthesis has not been fully characterized. In particular, biosynthesis and degradation of aromatic cytokinins remains to be elucidated[31] but it probably differs from that of the isoprenoid type. In addition, the aromatic cytokinins have not been identified in many plants and it is unclear whether they are synthesized ubiquitously throughout the plant kingdom. Isoprenoid cytokinins, on the other hand, exist in all plants studied until now. For their biosynthesis, two pathways have been identified: synthesis *de novo* or their release from a prenylated tRNA. The key enzymes in both pathways are isopentenyl transferases, which can use either a free adenosine phosphate (AMP, ADP or ATP) or the adenosine moiety in a t-RNA molecule, respectively.

Adenosine isopentenyltransferase (IPT; EC 2.5.1.27) that catalyzes the N^6-prenylation of adenosine phosphates uses either dimethylallyl diphosphate (DMAPP) or hydroxymethylbutenyl diphospahe (HMBDP). IPT enzymes have been well characterized in the slime mold *Dictyostelium discoideum* and the plant-associated bacterium *Agrobacterium tumefaciens*. IPT from *D. discoideum* uses AMP or ADP[32], but not ATP, whereas IPT from *A. tumefaciens* uses solely AMP[33]. The IPTs identified in plants are somewhat different from the previous two. Studies on recombinant enzymes have shown that plant IPTs have different substrate preferences[18, 19]. The prenyl-moiety, either DMAPP or HMBDP, can have different metabolic origins. HMBDP is a metabolic intermediate of the methylerythritol phosphate (MEP) pathway, which occurs in bacteria and plant plastids, whereas DMAPP is synthesized *via* the MEP pathway and the mevalonate (MVA) pathway, which is commonly found in the cytosol of eukaryotes. Both HMBDP and DMAPP, along with one of the adenine phosphates, form an iP-type of isoprenoid cytokinins. Zeatin type cytokinins can be formed through the hydroxylation of the isoprenoid side chain. An alternative pathway has been proposed[34], in which an unknown precursor of the MVA pathway, together with one of the adenine phosphates, directly forms a *trans*-zeatin-type (*t*Z) of cytokinin. As in previous cases, the reaction is catalyzed by IPT. The search for an alternative explanation of direct zeatin synthesis arose from experiments with labeled precursors, where the rate of *t*Z-type of cytokinins was much greater than that of the iP-type[34]. Further research is needed to confirm whether there is in fact a third precursor, alongside HMBDP and DMAPP, or if the high rates of *trans*-zeatin riboside mono phosphate (*t*ZRMP) synthesis are the result of *cis*-zeatin (*c*Z) to *t*Z conversion, where *c*Z is the final product of the tRNA degradation pathway. tRNA-isopentenyltransferase (tRNA-IPT; EC 2.5.1.8), the key enzyme of this pathway, like IPT, uses DMAPP for the prenylation of adenosine in a tRNA molecule. Afterwards, the prenyl-moiety gets hydroxylated by an unknown enzyme[31] and because the prenyl-moiety of tRNA finally contains a *cis*-hydroxylated group, the *cis* form of zeatin gets released after tRNA degradation. The two cytokinin biosynthetic pathways show that plants can modulate the levels of *cis*- and *trans*-zeatin independently. Because calculations on the rates of tRNA turnover and cytokinin production show tRNA breakdown can account for only 40–50% of present cytokinins, tRNA is not the major pathway of cytokinin biosynthesis[35]. Nevertheless, it should not be regarded as unimportant as some plant species, such as maize and rice, contain substantial amounts of *c*Z-type of cytokinins[36–39] that can be transformed to *t*Z by *cis-trans* isomerase[40].

Because there are multiple IPT genes in plant genomes, it raises a question about how their expression is regulated. In Arabidopsis (*Arabidopsis thaliana* L.), where all nine *AtIPT* genes have been characterized, a comprehensive study on their expression patterns has been made[41]. Results clearly showed that Arabidopsis IPT genes are highly tissue specific and that their expression is regulated by different developmental factors.

3. 2. Cytokinin Oxidase/Dehydrogenase Irreversibly Degrades Cytokinins

The key enzyme of cytokinin degradation is cytokinin oxidase/dehydrogenase (CKX; EC 1.5.99.12), which irreversibly inactivates cytokinins by cleaving the bond between the purine ring and the side chain. Its substrates are free cytokinin bases and ribosides with an unsaturated side chain (i.e. iP, iPR, *t*Z and *t*ZR). Hydroxylated forms (DHZ-type), nucleotides and *O*-glucosydes cannot be degraded by CKX. In the case of *N*-glucosides, the data are somewhat contradictory[42, 43] and it is not yet unequivocally clear whether or not cytokinin *N*-glucosides are degraded by CKX.

The products of iP or iPR degraded by CKX are 3-methyl-2-butenal and adenine, or adenine riboside respectively. Interestingly, the name of the enzyme might be inappropriate, because it seems that CKX is not a copper-containing enzyme, but a flavoprotein that does not requi-

re oxygen for its activity. Therefore, dehydrogenase is a more appropriate description of this enzyme[44].

There is evidence of tissue specific expression of different *CKX* genes[45], indicating that degradation of cytokinins, like their biosynthesis, is a well-regulated process.

3. 3. Several Enzymes Catalyze Modification of the Purine Ring

5'-nucleotide phosphohydrolase or 5'-nucleotidase (EC 3.1.3.5) catalyzes the conversion of cytokinin nucleotides to nucleosides (Figure 2), as shown for the enzymes isolated from wheat germ[46]. This chemical conversion can additionally be catalyzed by non-specific phosphatases, which generally cleave phosphate groups from different phosphorylated chemical compounds. In the case of cytokinin, all kinds of phosphate cleavage are possible. Accordingly, the physiological contribution of cytokinin specific 5-nucleotidase is yet to be assessed[31]. Adenosine nucleosidase (EC 3.2.2.7) catalyzes the conversion of cytokinin ribosides to free bases (Figure 2) and, like the 5'-nucleotidase, has a lower K_m for cytokinin ribosides compared to the K_m of adenosine[47]. This means it has a higher affinity to cytokinin than other purine metabolites and implies that cytokinin metabolic conversions are catalyzed by specific enzymes. Adenosine phosphorylase (EC 2.4.2.1) catalyzes the conversion of cytokinin free bases to ribosides (Figure 2)[48]. The K_m values for cytokinins free bases are higher compared to the K_m values for adenine, which, in contrast to 5'-nucleotidase and adenosine nucleotidase, supports the idea that the catalysis of reactions involving cytokinins is not the primary function of purine metabolic enzymes. Adenosine kinase (EC 2.7.1.20) can catalyze the formation of cytokinin nucleotides from the corresponding nucleoside (Figure 2). The K_m values for cytokinins ribosides are higher than the K_m values for adenosine[49]. Adenine phosphoribosyltransferse (EC 2.4.2.7) is the enzyme that catalyzes the conversion of free bases into nucleotides (Figure 2) and has been characterized in the Arabidopsis model plant system[50, 51]. Interestingly, some Arabidopsis adenine phosphoribosyltransferases have a higher affinity to the synthetic free base benzyladenine (BA) than to adenine[51], which supports the idea that these enzymes only participate in cytokinin metabolism and are not specific for it. The converse reaction is catalyzed by the enzyme riboside 5'-monophosphate phosphoribohydrolase, which directly releases cytokinin free bases from their corresponding nucleotides (Figure 2)[52]. *N*-glucosyltransferse (EC 2.4.1.118) can theoretically catalyze the formation of 3-, 7- or 9-glucosydes (Figure 2), but thus far, 3-glucosylation has not been characterized in plants[31]. 7- and 9- glucosylation make the active free bases irreversibly inactive. The enzyme uses UDP- or TDP-glucose and a cytokinin free base as substrates and prefers 7-glucosylation, at a rate 10-fold greater than 9-glucosylation[53]. N-alanyltransferse (EC 4.2.99.13) catalyzes the conversion of cytokinin free bases into 9-alanyl derivative (Figure 2)[54]. The physiological significance of alanylation is not well understood[31], but it probably represents a process of cytokinin inactivation since the alanine conjugates are also biologically inactive and stable[55].

3. 4. Modification of the Side Chain

There are several modifications of the isoprenoid side chain. *Cis-trans* isomerase catalyzes the conversion of *cis*- to *trans*- zeatin and *vice versa* (Figure 2). This way it greatly enhances the otherwise spontaneous *in vitro* chemical conversion. The enzyme favors *cis* to *trans* conversion and requires a flavin molecule (FAD or FMN), light and dithiothreitol[56]. The physiological significance of this conversion is not fully understood, but a link between *t*Z synthesis and tRNA degradation pathway might be the main physiological function[31]. For some reason *c*Z is found in substantial quantities in the tissues of some plant species, such as chickpea[36], potato[39] and maize[37] and it has been reported that a maize cytokinin receptor ZmHK1 could respond to *c*Z with a sensitivity comparable to that of *t*Z[57].

Zeatin *O*-glucosyltransferase catalyzes the conversion of *t*Z or *c*Z to the respective *O*-glucosides (Figure 2)[58]. Physiologically, this conversion can be of great importance, since it is reversible, and the *O*-glucosides therefore represent a temporary storage form of cytokinins. *O*-glucosyltransferase uses UDP-glucose and one of the cytokinin free bases as substrates. Interestingly, the *O*-glucoyltransferases that have been isolated in plants so far show a higher affinity to either one of *t*Z or *c*Z, and are at the same time highly tissue specific. *Trans*-zeatin *O*-glucosyltransferase 1 (ZOG1) from *Phaseolus lunatus* localizes to immature seeds and is only faintly expressed in roots and leaves[59], whereas *cis*-zeatin *O*-glucosyltransferases 1 and 2 (*cis*ZOG1 and *cis*ZOG2) expresses mostly in the roots. The reverse reaction of *O*-glucosylation, which results in the release of an active free base, is catalyzed by β-glucosidase (Figure 2), which has not been found in plants yet, but is well known in plant associated bacteria, like *Agrobacterium rhizogenes*[60]. *O*-xylosiltransferase (EC 2.4.1.204) catalyzes the conversion of free bases to cytokinin-*O*-xylosides (Figure 2). So far, the enzyme zeatin-*O*-xylosiltransferase 1 has only been found in *Phaseolus vulgaris*[61] and like ZOG1 it localized to immature seeds, but not to roots and leaves. The enzyme uses UDP-xylose and *t*Z or DHZ as substrates. It is structurally closely related to ZOG1 and shows a 90% sequence identity.

Zeatin reductase (EC 1.3.1.69) catalyzes the irreversible conversion of *t*Z to DHZ (Figure 2). This conversion stabilizes the physiological activity of cytokinin free bases, since DHZ is not a substrate for cytokinin oxidase and therefore does not get degraded[44]. In *Phaseolus vulgaris* embryos, the enzyme specifically catalyzes the conversion of *t*Z, but does not use *c*Z, iP or any of the ribosides as substrates[62]. It requires NADPH as a cofactor.

Cytokinin hydroxylase, so far only partially characterized in cauliflower[63], catalyzes the conversion of iP-type cytokinins to *t*Z-type (Figure 2). It seems unclear how common and important this enzyme actually is for this chemical conversion. That is why cytochrome P450 has been implicated in this same conversion, since the reaction is dependent on NADPH and can be limited by inhibitors of cytochrome P450 activity[30].

part of other pathways (e.g. a purine metabolic pathway), but are also involved in cytokinin conversions[30]. In plant tissues, cytokinin free bases, nucleosides and nucleotides appear to interconvert quite rapidly[64]. Since the K_m values of the enzymes involved are often smaller for adenine, adenine riboside or adenine monophosphate compared to isopentenyl adenine (iP) and its corresponding derivatives, it is more probable that the conversions are mediated

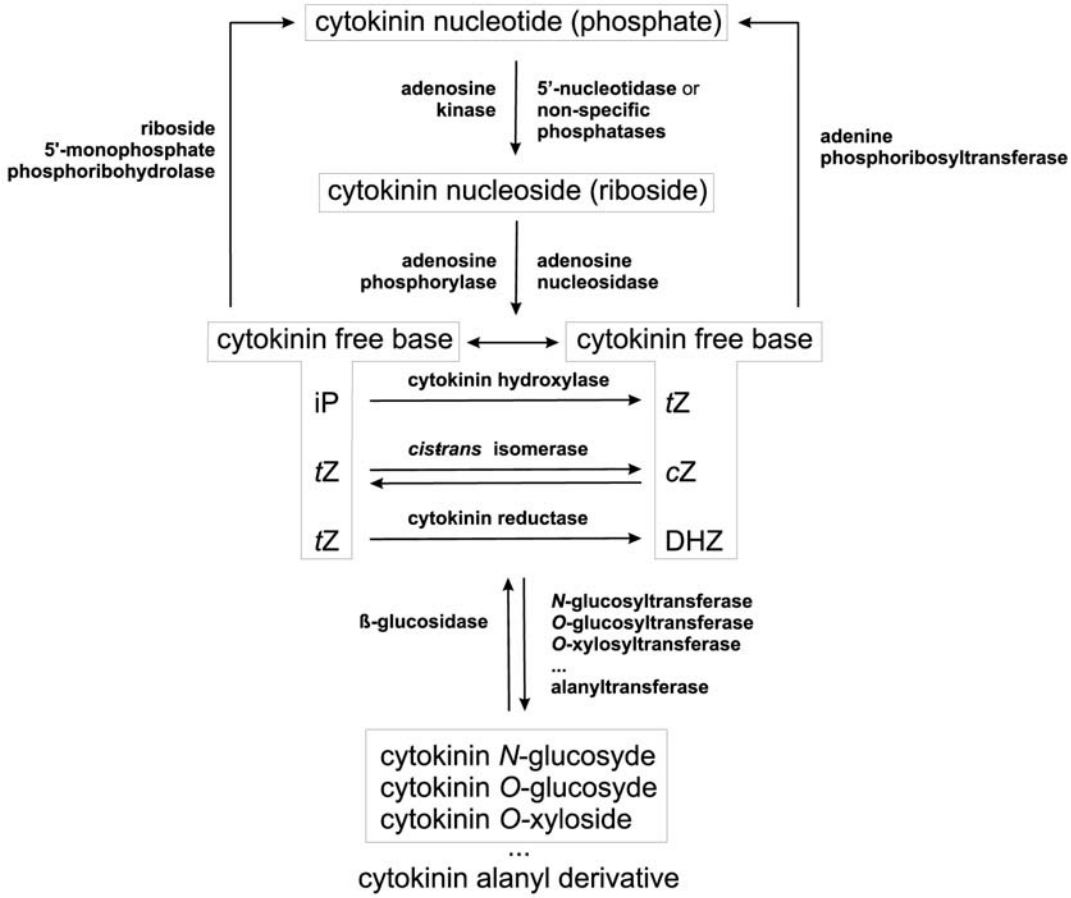

Figure 2: Enzymes involved in cytokinin interconversions.
Enzymes catalyzing the interconversions of cytokinin metabolite are shown in bold. Cytokinin metabolite groups are denoted in boxes. iP – isopentenyl adenine, *t*Z – *trans*-zeatin, *c*Z – *cis*-zeatin, DHZ – dihydrozeatin

3. 5. Cytokinin Metabolic Pathway is Not Clear

The currently proposed cytokinin metabolic pathway (Figure 3) has been constructed on the basis of feeding experiments where metabolites are determined after the application of radio labeled cytokinin standards[30]. Enzymes catalyzing metabolic conversions of cytokinins can be broadly classified into two groups. The first group comprises enzymes which catalyze modifications of the purine ring, and in the second one are those which modify the side chain. It is still not clear at this point whether these enzymes are actually cytokinin specific or are merely a

by the common enzymes of purine metabolism[30]. In any case, further research is needed to evaluate whether there are specific enzymes responsible solely for cytokinin metabolic conversions in plants.

4. How do Cytokinins Affect the Cell – Their Perception and Signal Transduction to the Genes

Cytokinin signal transduction in plants is a variant of the bacterial two component system[22, 26] and is unique

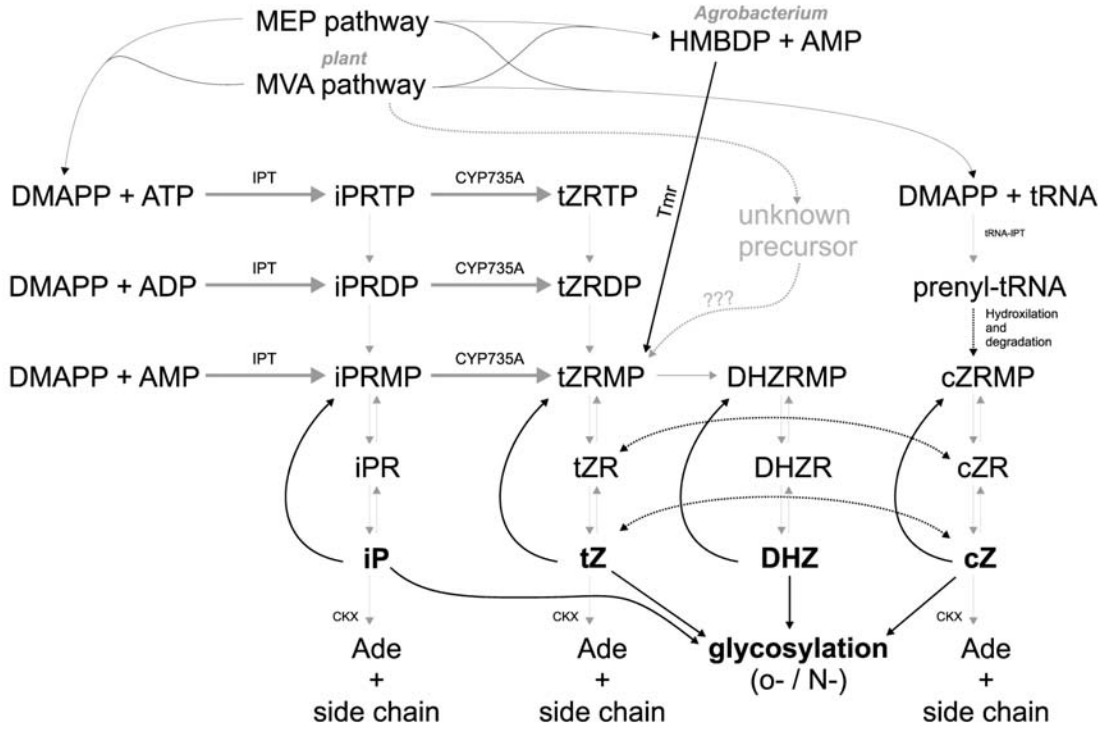

Figure 3: Cytokinin metabolic conversions[30, 66].
MEP – methylerythritol, MVA – mevalonate, DMAPP – dimethylallyl pyrophosphate, HMBDP – hydroxymethylbutenyl diphosphate, Tmr – adenine isopentenyl of *A. tumefaciens*, ATP – adenosine triphosphate, ADP – adenosine diphosphate, AMP – adenosine monophosphate, iPRTP – isopentenyl riboside triphosphate, iPRDP – isopentenyl riboside diphosphate, iPRMP – isopentenyl riboside monophosphate, *t*ZRTP – *trans*-zeatin riboside triphosphate, *t*ZRDP – *trans*-zeatin riboside diphosphate, *t*ZRMP – *trans*-zeatin riboside monophosphate, cZRMP – *cis*-zeatin riboside monophosphate, DHZRMP – dihydrozeatin riboside monophosphate, iPR – isopentenyl riboside, *t*ZR – *trans*-zeatin riboside, *c*ZR – *cis*-zeatin riboside, DHZR – dihydrozeatin riboside, iP – isopentenyl adenine, *t*Z – *trans*-zeatin, DHZ – dihydrozeatin, *c*Z – *cis*-zeatin, Ade – adenine.

to plants among higher eukaryotes[24, 65]. The current model of this cytokinin signaling system predicts a transmembrane histidine kinase receptor (HK), a nuclear response regulator (RR) and an intermediate histidine phosphotransfer protein (HP) that transfers the signal from the transmembrane receptor to the nucleus (Figure 4). The RRs mediate the signal further, either as transcription factors, where they promote the expression of cytokinin-response genes, or as protein kinases that activate other downstream effectors[21, 23, 66]. The cytokinin response factors in Arabidopsis (CRFs) have recently been identified[67], which, together with RRs, induce the expression of cytokinin response genes (Figure 4).

The actual molecular signal that is transferred from a cytokinin molecule to the final effector is a phosphate group. The membrane receptor, the phosphotransfer protein and the response regulator all have either a conserved histidine (His) or aspartic acid (Asp) in their receiver or kinase domain and the phosphate is alternately transferred between His and Asp to the final phosphorylation site, which activates the response protein. The response protein is a protein kinase or a transcription factor (Figure 4).

4. 1. Histidine Kinases are Transmembrane Receptors

It has been proposed that cytokinin histidine kinases (HKs) are transmembrane receptors that bind cytokinin on the outer side of the cell's membrane and transduce the signal across the membrane to the inner side (Figure 4)[66]. A receptor molecule consists of several domains that are important for its function. The transmembrane domains are important for protein anchoring in the membrane. The input domain is essential for cytokinin binding. The histidine kinase domain contains a conserved His and is important for activation through autophosphorylation. The receiver domain contains a conserved Asp and is important for the transfer of the phosphate group from HK to HP[23]. After the binding of a cytokinin molecule, the receptor dimerizes and autophosphorylates itself. Afterwards, the phosphate is transferred from His in the kinase domain to Asp in the receiver domain, and further to a conserved His on an HP[65]. When cytokinin is not bound to the receptor, the latter is not active and a reverse phosphorylation can occur, where the phosphate group is transferred from an HP to the HK. This step suppresses further cytokinin signaling[23]. Different cytokinin receptors have different affinities for particular

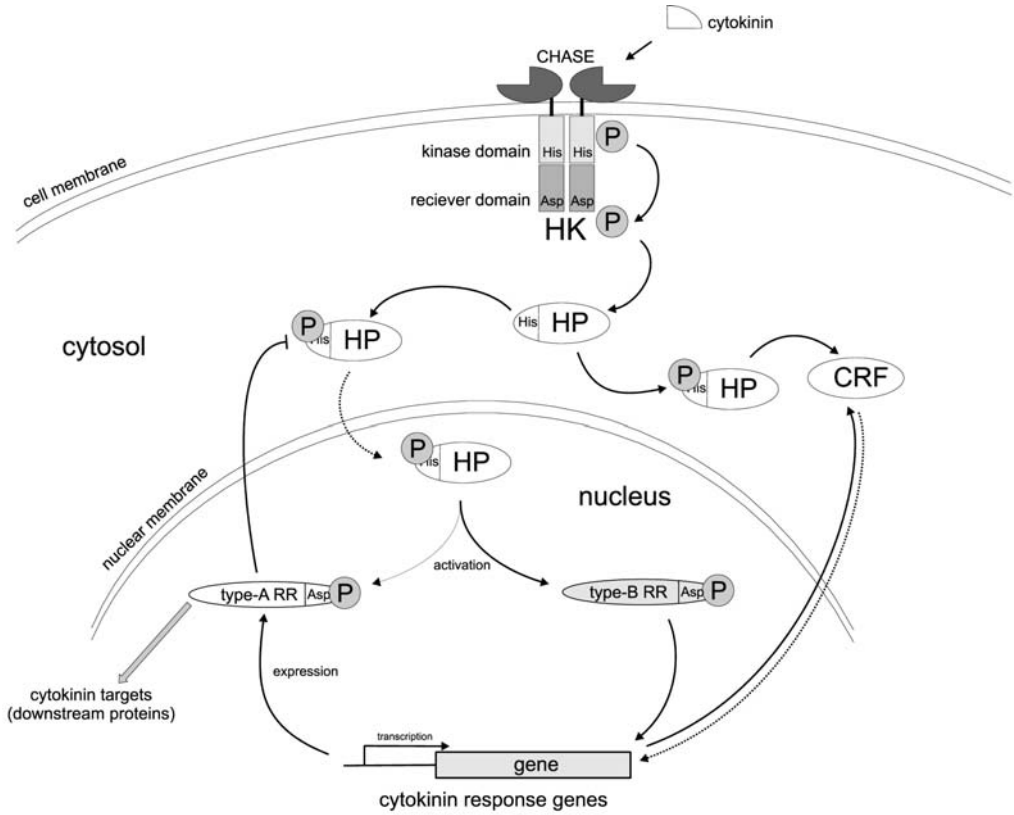

Figure 4: Cytokinin signaling pathway[21, 23, 66]
Cytokinin is perceived by the cytokinin histidine kinase receptor (HK) at the plasma membrane. The binding of a cytokinin molecule to the cytokinin-binding domain (CHASE) of the receptor activates dimerization of the receptor and autophosphorylation on the conserved His on the kinase domain. The phosphate group is transferred *via* a conserved Asp on the receiver domain to a His phosphotransfer protein (HP) in the cytosol, which translocates it to the nucleus. There the phosphate is transferred to a conserved Asp on the receiver domain of either a type-A or type-B response regulator (RR). Phosphorylation of type-B RRs induces transcription of cytokinin primary response genes, type-A RRs and cytokinin response factors (CRF). CRFs are activated by HPs in the cytoplasm and move to the nucleus where they induce transcription of cytokinin-response genes along side type-B activators. Type-A RRs, on the other hand, are not transcription factors, but act as kinases to activate downstream proteins involved in cytokinin regulated processes. Also, they act as feedback regulators of cytokinin signaling.

cytokinin free bases[28, 57]. They can prefer the iP-type, the *t*Z-type or the hydroxylated DHZ-type of free bases or they can bind more than one cytokinin free base. For example, receptors like Arabidopsis AHK3 receptor or ZmHK receptors can bind *t*Z as well as *cZ*[57, 68]. This kind of redundancy is interesting, since a strong overlap is also observed for receptor downstream targets and is clearly visible as phenotype aberrations only in receptor triple mutants[13].

4. 2. Histidine Phosphotransfer Proteins

HPs transfer a phosphate *via* the conserved His from the membrane receptor to the response regulators in the nucleus[23] (Figure 4). HPs show a high level of redundancy, which can clearly be seen in Arabidopsis[69], where single and double mutants do not show any visible abnormalities. Only triple, quadruple and especially quintuple mutants show noticeable major developmental abnormalities respectively, which means the function of one HP can easily be replaced by another.

4. 3. Response Regulators Mediate Cytokinin Function

The response regulators (RRs) are the effectors that mediate cytokinin function[26] (Figure 4). They either regulate the expression of cytokinin response genes as transcription factors or activate different downstream proteins as protein kinases. In Arabidopsis[70] and rice[71], where they have been fully characterized, response regulators fall into four distinct groups: type-A, type-B, type-C and a group of pseudo response regulators (PRRs). The main part in RR action is played by the type-B response regulators, which possess a DNA binding domain and are transcription factors that activate the expression of cytokinin primary response genes[72]. Among these are type-A RRs and CRFs. Type-A RRs do not have a DNA binding domain. They cannot bind DNA and therefore do not act as transcription factors. On the contrary, their targets are still undetermined downstream proteins involved in different physiological processes[23, 26]. Also, in Arabidopsis type-A RRs provide a negative feedback loop in cytokinin signa-

ling[73]. The mechanism how this is achieved still needs to be clarified.

4. 4. Cytokinin Response Factors

Cytokinin response factors are novel components of cytokinin response and were identified in microarray experiments as targets of cytokinin regulated transcription[67]. They belong to the larger *APETALA2*-like class of transcription factors and are in fact related to ethylene RRs. Their activation is dependent only on HPs and not the RRs, but the mechanism of activation still remains to be elucidated[23] (Figure 4). When activated, CRFs move from the cytoplasm to the nucleus, where they regulate transcription of cytokinin response genes[74]. CRF gene targets are somewhat different from type-B RRs, because CRF mutants display different phenotypes that cannot be observed in any of the cytokinin signaling element mutants[74]. Like HKs, HPs and RRs, CRFs show high redundancy and only higher order mutant combinations show visible developmental morphological aberrations[74].

4. 5. Molecular Mechanisms for Regulation of Physiological Process by Cytokinins

Molecular mechanisms by which cytokinins regulate a particular physiological process are not well understood. Genome-wide expression profiling has revealed some putative molecular targets of cytokinin signaling. In the model plant Arabidopsis, cytokinin targets are of two types, immediate-early and delayed response genes[75]. Of the immediate-early response genes, type A RRs, transcription factors and genes associated with development, signal transduction and control of protein turnover are most notable. Of the delayed response genes, the list includes: signaling genes, transcriptional regulators, stress-related genes, cell wall modifying genes, genes involved in hormonal regulation, photosynthesis, carbohydrate metabolism, secondary metabolism and lipid metabolism[75].

5. Cytokinins in Developing Seeds

5. 1. Seed is a Dispersal Mode Used by Plants

The seed is central to several of the many dispersal modes used by plants and is a unique structure of flowering plants (Angiosperms) and Gymnosperms. Seeds contain the plant embryo and the endosperm, which sustain embryo development and germination. In double fertilization of flowering plants, the ovule consists of the embryo sac, enclosed by the nucellus and integuments. The products of double fertilization are a diploid zygote and a triploid primary endosperm cell. The latter develops into a storage endosperm tissue that is structurally adapted to ensure efficient translocation of nutrients to the developing embryo. In grasses, including all cereal plants, the seed is a part of a special fruit called the caryopsis, which is composed of maternal tissue – pericarp, and filial tissues of embryo and endosperm.

5. 2. Cytokinins are Synthesized and Localized in Specific Plant Tissues

It was presumed for a long time that cytokinin biosynthesis occurred only in roots[76]. In addition, cytokinins were clearly demonstrated in the chickpea and lupin xylem exudates[34, 77], as well as in phloem exudates[78], indicating their translocation to the other plant parts. At the same time, however, studies on seeds of *Lupinus angustifolius* showed that cytokinin translocation was insufficient in explaining high quantities of cytokinins in seeds and therefore their local seed biosynthesis was suggested[79, 80] in *Sechium edule* seeds[81] as well as in cell free extracts of immature maize caryopsis[82]. Recently, the expression of the cytokinin biosynthetic gene, IPT, was quantified and localized to Arabidopsis seeds[41] and to the immature maize caryopsis[83]. In maize, the expression of *ZmIPT2* gene and localization of its encoding protein suggest cytokinin biosynthesis in the developing endosperm. Additional transcription studies of *ZmIPT2* and other maize *ZmIPT* genes also show the possibility of cytokinin biosynthesis in the embryo and caryopsis pedicel[83, 84] (T.R. unpublished data). However, at the moment it is not known if the resulting cytokinins are transported to the developing tissues or have another role in the pedicel.

5. 3. Developing Seeds are the Richest Source of Cytokinins

In developing seeds, concentrations of cytokinins exceeded their concentration in other tissues by an order of magnitude[77, 85]. In addition, the seed cytokinins are subjected to significant temporal fluctuations. Cytokinin metabolism has been investigated in seeds of peach[86], kiwi[87], tobacco[88], white lupin[77, 89], chickpea[36], chayote[81], soybean[90], Christmas rose[10,91], pea[38], rice[92, 93], wheat[94–97], maize[98–103] and Arabidopsis[13, 29].

The endospermic fluid of white lupine seeds has the highest cytokinin concentrations ever reported for any plant species. They range up to about 0.6 μmol g^{-1} tissue fresh weight[77, 89] and have been criticized in the past as being artifacts of bioassay techniques[79]. In maize caryopsis the highest cytokinin concentrations were detected between 6 and 10 days after pollination, especially in the basal part of the caryopsis[98, 104]. The concentration was 9000 and 16500 pmol g^{-1} tissue dry weight in the upper and basal part, respectively[98], which concurs with data from previous studies using different measuring techniques[99, 104]. The steep drop in cytokinins to less than 2000 pmol g^{-1} tissue dry weight after 10 days after pollination is very significant[99, 104]. Similarly extreme, is the tempo-

ral differences in cytokinin dihydrozeatin riboside (DHZR) in peach pericarp and seed[86]. Its quantity changes from ~13000 to ~0.5 pmol g^{-1} tissue fresh weight and from ~4 to 57000 pmol g^{-1} tissue fresh weight, respectively.

5. 4. Seed Cytokinin Diversity

Although a wide spectrum of cytokinins exists in plant tissues, only one cytokinin type or form is predominant in one plant species and tissue at one time. In white lupine seeds and maize kernels more than 15 isoprenoid cytokinin metabolites could be detected[37, 77]. It is not clear yet, what are the functions of such diversity and future work needs to address this issue in greater detail. While for some cytokinin metabolites it seams obvious that the regulation of processes like cell division is their main function, it is often difficult to contribute any such roles to other cytokinin metabolites[77]. *Trans*-zeatin-type cytokinins are the predominant type in rice seeds and are therefore more likely to be involved in the regulation of early seed development[93]. In particular, *t*Z and *t*ZR abundance obviously correlates with the rate of endosperm cell division, whereas iP and iPR abundance does not. This is in agreement with observations in seeds of other cereals, maize[98, 99, 105] and wheat[97]. In contrast to monocots, seeds of eudicots harbor large quantities of *cis*-type cytokinins[36, 38, 77]. In pea and lupine seeds for example, developmental processes are regulated by cytokinin *cis*-zeatin-type phosphates and ribosides, which are also the predominant forms.[36,38,77,79]. In peach seeds, phosphates and ribosides of the dihydrozeatin and isopentenyl type are predominant cytokinins s and functions like embryo formation and growth, division of endosperm primary nucleus, endosperm cellularization and endosperm cell division have been contributed to them[86]. On the other hand, the abundance of *t*ZR, which was the only *trans*-zeatin-type cytokinin present, temporally did not correlate with any of the developmental processes during fruit setting.

6. The Physiological Role of Cytokinins in Seed Development

For normal seed development, several sequential events have to be completed. They involve cell division and cell expansion, which both also allow an accumulation of storage material. The accomplishment of these processes is associated with sink strength. The sink strength is determined by the size of the storage organ and its association with the number of cells that comprise the organ, and by the efficiency of unloading photosynthate from the conducting elements to be imported into the storage organ for the synthesis of the final storage products.

Morphological development of the embryo and storage tissues, as well as sink strength establishment are under way at the time of early seed development[106], which temporally correlates with the highest levels of cytokinins [77, 98, 105, 106]. Transcriptome analysis of proliferating Arabidopsis endosperm reveals that several genes involved in cytokinin signaling (i.e. HK receptors, CRF proteins, type-B RRs) are up regulated[107]. Many temporally coinciding processes in developing seeds- specifically cell divisions in the storage tissues, phloem unloading, assimilate uptake and degeneration of nucellar tissue- are probably affected by cytokinin signaling. So far, it has not been proven unequivocally that cytokinins regulate these processes in seeds[38], but evidence from other systems is already available. For example, cell division is stimulated by cytokinins in Arabidopsis tissue culture and *in planta* by up-regulation of proteins regulating cell cycle checkpoints, e.g. D-type cyclins and cyclin dependent kinases[108–111]. Likewise, phloem unloading and sugar uptake are promoted by cytokinins in *Chenopodium rubrum* suspension cultures. There, cytokinins promote the expression and activity of cell wall invertase and hexose transporters, enzymes involved in sugar metabolism and transport[112].

7. Cytokinins and Development of Maize Endosperm

Maize endosperm development is reflected in rapid growth (Figure 5), which is a result of rapid cell division and cell enlargement. At the beginning of sugar import, cells of the central region are the first to stop dividing and begin to differentiate into storage cells. At the time of extensive starch synthesis, cell size decreases from the central region of the so-called starchy endosperm to the periphery (Figure 5), where small, yet still dividing cells support the inner expanding tissue. There is growing evidence that elevated cytokinin content temporally coincides with the process of endosperm cell division[86, 93, 96, 99, 105] or even precedes it[98]. The first peak of active cytokinin forms (i.e. free bases and ribosides) is often present before the rapid increase in cell number. It is followed by a smaller peak, which is associated with numerous cell divisions in the peripheral tissues and with cell expansion in the central part of the endosperm (Figure 5). It seems possible therefore, that cytokinins affect endosperm final size through their effect on cell division[113]. Based on collective evidence a model has been proposed about maize endosperm linking together cytokinin action, sugar metabolism and uptake, and the cell cycle[98]. As proposed in the model, cytokinins affect sink strength and consequently seed size, directly, by up-regulating cell cycle related genes, or indirectly, through the action of sugar signaling. Additionally, through enzymatic activity of cell wall invertase, cytokinins might enhance phloem unloading and sugar import into the endosperm, where sugar monomers are available for degradation and cell wall and starch biosynthesis.

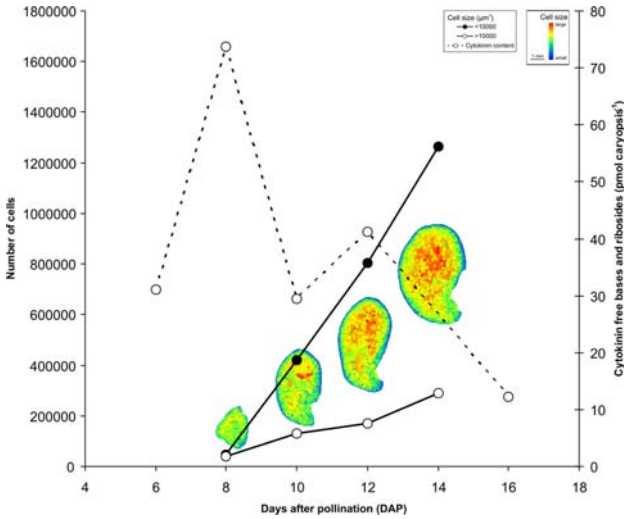

Figure 5: Correlation of maize endosperm development and cytokinin level.
The endosperm pictures show the distribution of cells of different size classes measured on the longitudinal sections of caryopsis collected at different days after pollination (DAP). Because the cell size was not measured in all endosperm cells, the cell size classes were assigned to all endosperm cells using the nearest neighbor method from the 3D model of the endosperm[119]. Growth of maize endosperm 8 to 14 DAP is the time of fastest growth, due to the underlying processes of cell division and expansion[98]. At 14 DAP the small dividing cells (indicated by blue color) can be seen in the peripheral parts of the endosperm, whereas the central part consists of expanding storage cells that accumulate starch. The increase in cell number (solid lines) is preceded by peaks in cytokinin free bases and ribosides (dotted line).

The important mechanism by which plants modulate cytokinin homeostasis is their degradation or inactivation. Several maize *CKX* genes and their corresponding proteins, which irreversibly cleave the N^6-side chain from adenine ring, are localized to the caryopsis pedicel region, endosperm and embryo[43, 84, 114, 115]. The reversible inactivation of *c*Z by glucosylation at the *O* of the side chain is a natural metabolic process in maize caryopsis[37]. In addition, there is evidence for the importance of N^9-glucosylation in maize caryopsis, especially in the developmental phase that follows intense mitotic activity[98].

8. Conclusion

Despite some recent discoveries on cytokinin biosynthesis, catabolism and signal transduction, further research of cytokinin metabolism is required. Almost half a decade since cytokinins were first discovered, we are slowly working our way up to manipulating plant development using the available knowledge on cytokinins. It is becoming clear that cytokinin signaling is very complex, because of its interconnections with other developmental signals, e.g. other hormone signals like auxins and ethylene. For more details readers are referred to the reviews dealing with these topics[116, 117]. How these factors crosstalk amongst each other should be clarified next. Complex developmental frameworks should be described in detail and understood comprehensively. Only then our knowledge of regulatory mechanisms will enable us to manipulate the progression of seed developmental processes to our needs.

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10. References

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Povzetek

Citokinini so ena glavnih skupin rastlinskih hormonov, ki nadzoruje različne procese rasti in razvoja rastlin. Skupaj s svojimi ribotidi, ribozidi in glukozidi so citokinini po kemijski strukturi *N6*-substituirani derivati adenina. Pretvorbe med različnimi kemijskimi oblikami citokininov predstavljajo tudi njihovo prehajanje med aktivnimi, neaktivnimi, založnimi in transportnimi oblikami. V razmerah *in vivo* je ta proces dinamičen in hiter. Z identifikacijo prvega encima v biosintezni poti, izopentenil transferaze, pred manj kot 10 leti, smo končno začeli spoznavati vlogo citokininov v razvoju rastlin. K razumevanju citokininov prispevajo tudi sočasne raziskave encimov in njihovih odgovarjajočih genov, vključenih v citokininsko presnovo in signalno transdukcijo. Pričujoči pregled je osredotočen na najnovejša spoznanja o citokininih s poudarkom na njihovi vlogi med razvojem semen, iz katerih je bil pred več kot pol stoletja izoliran prvi naravni citokinin.