DARWIN REVIEW

Evolution of cytokinin biosynthesis and degradation

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Abstract

Cytokinin hormones are important regulators of development and environmental responses of plants that execute their action via the molecular machinery of signal perception and transduction. The limiting step of the whole process is the availability of the hormone in suitable concentrations in the right place and at the right time to interact with the specific receptor. Hence, the hormone concentrations in individual tissues, cells, and organelles must be properly maintained by biosynthetic and metabolic enzymes. Although there are merely two active cytokinins, isopentenyladenine and its hydroxylated derivative zeatin, a variety of conjugates they may form and the number of enzymes/isozymes with varying substrate specificity involved in their biosynthesis and conversion gives the plant a variety of tools for fine tuning of the hormone level. Recent genome-wide studies revealed the existence of the respective coding genes and gene families in plants and in some bacteria. This review summarizes present knowledge on the enzymes that synthesize cytokinins, form cytokinin conjugates, and carry out irreversible elimination of the hormones, including their phylogenetic analysis and possible variations in different organisms.

Key words: Cytokinin, cytokinin biosynthesis, cytokinin degradation, cytokinin dehydrogenase, cytokinin metabolism, isopentenyl transferase.

Introduction

Plant hormones

The bases of the concept of plant hormones as substances regulating plant growth in small concentrations were laid down at the end of 19th century (Darwin and Darwin, 1880; von Sachs, 1887). The first proof of the existence of low molecular weight substances regulating plant growth was the famous experiment by Fritz Went, in which he put a coleoptile on an agar block for a period of time and, after transferring this block onto decapitated oat plants, he observed promoted growth, eventually asymmetrical, if the block was put on one side (Went, 1928). Many substances from diverse sources have been examined for plant growth-promoting activity in the same manner. The original substance was finally identified as indolyl-3-acetic acid (Haagen-Smith et al., 1946), which had been previously isolated from human urine (Kögl and Kostermans, 1934). Today, the plant hormones are defined as naturally occurring substances operating at low concentrations, which are in most cases able to translocate within the plant body and bind to a specific receptor protein. This definition fits with the term plant growth regulators, which can also be synthetic substances with phytohormone-like activity. There is no clear border between these two terms as some substances have debatable classification and their occurrence in plant tissues is presumed but not confirmed yet. However, based on chemical structure, the basic phytohormone groups are specified as auxins, cytokinins, gibberellins, ethylene, abscisic acid, polyamines, brassinosteroids, jasmonates, salicylic acid, and newly identified strigolactones.

Abbreviations: CKX, cytokinin dehydrogenase; DMAPP, dimethylallyl pyrophosphate; HMBDP, (E)-4 hydroxy-3-methyl-but-2-enyl diphosphate; IPT, isopentenyl transferase; LOG, cytokinin phosphoribohydrolase ‘Lonely guy’; Tmr gene, ‘tumour morphology root’ gene of Agrobacterium tumefaciens (encoding Tmr isopentenyl transferase); Tzs gene, ‘trans-zeatin synthesising’ gene of Agrobacterium tumefaciens (encoding Tzs isopentenyl transferase).
Cytokinins—occurrence in plants

In 1955, a new compound named kinetin was isolated from old or autoclaved (but not fresh) DNA from herring sperm and calf thymus (Miller et al., 1955). The name kinetin was chosen because this substance could promote cell division—cytokinesis. This ability also gave the name to the brand new class of hormones—cytokinins. Cytokinins are defined as substances, which promote cell division and exert other growth regulatory functions in the same manner as kinetin (Skoog and Armstrong, 1970). However, nowadays this term describes all molecules with a similar structure, no matter how active they are. Chemically, the cytokinins are $N^6$ derivatives of adenine, but cytokinin activity is also shown by some phenylurea derivatives.

Naturally occurring cytokinins are adenines, which possess either an isoprenoid or aromatic side chain at the $N^6$ position. The isoprenoid side chain of isopentenyladenine ($N^6$-(Δ$^2$-isopentenyl)adenine) can be hydroxylated in either the cis- or trans-terminal position forming zeatin named according to its first discovery in maize (Zea mays L.; Letham, 1963). The double bond of zeatin is reduced in dihydrozeatin ($N^6$-(4-hydroxy-3-methylbutyl)adenine), but a reduced equivalent of isopentenyladenine has not been detected in planta to date. The isoprenoid cytokinins are widespread in nature; in contrast, the aromatic cytokinins were thought for a long time to be solely synthetic artefacts.

Although kinetin was the first cytokinin identified, it took >40 years until it was found in plant material, such as coconut chunks (Barciszewski et al., 1996) as well as fresh coconut endosperm (Ge et al., 2005), and also in root nodules of Australian pine (Casuarina equisetifolia) infected by the bacterium Frankia (Raman and Elumalai, 1996). On the other hand, derivatives of benzyladenine had already been found in nature in the mid-1970s (Horgan et al., 1973, 1975). Their presence in Populus species eventually provided them with the name topolins from the Czech word ‘topol’ for poplar. Since that time, they have been discovered in increasing numbers of species, for example in tomato crown gall tumours (Nandi et al., 1989), Arabidopsis (Tarkowska et al., 2003), and pea (Gaudinova et al., 2005).

The presence of the respective cytokinin species can vary greatly between plant species, tissues (Hirose et al., 2008), developmental stage (Emery et al., 1998; Quesnelle and Emery, 2007), and environmental conditions (e.g. Takei et al., 2001; Vryouvalova et al., 2009). Recently, the evidence regarding cytokinin occurrence in various organisms and materials has been thoroughly reviewed (Stirk and van Staden, 2010).

Trans-zeatin and isopentenyladenine-type cytokinins were thought to be the predominant cytokinins, with the cis-isomer being present only in minor quantities with low or no activity (Schmitz and Skoog, 1972; Mok et al., 1978). This was reflected by neglecting the cis-isomer in cytokinin analysis and by referring to the trans-isomer as zeatin in general. On the other hand, there are a growing number of reports on cis-zeatin being the dominant cytokinin species in various plants such as potatoes (Mauk and Langille, 1978; Suttle and Banowetz, 2000), unfertilized hop cones (Watanabe et al., 1982), rice (Takagi et al., 1985), maize (Veach et al., 2003; Vryouvalova et al., 2009), and several species of legumes (Emery et al., 1998, 2000; Quesnelle and Emery, 2007). Dihydrozeatin occurs predominantly in dormant seeds and apical buds, and it seems to be produced in liquid endosperm of bean (Martin et al., 1989; Mok et al., 1990). These are all storage organs, suggesting a specialized function of the reduced cytokinin. Since it is resistant to degradation by cytokinin dehydrogenase (CKX; Galuszka et al., 2007) it may serve as a source of active cytokinins before the acceleration of de novo biosynthesis after germination.

Low levels of trans-zeatin can also be found in tRNA, but in ~40 times lower amounts than cis-zeatin (Vreman et al., 1978). However, tRNA isolated from green pea shoots (Pisum sativum L. var. Alaska) contains both isomers in amounts of the same order based on UV detection on thin-layer chromatography (Vreman et al., 1972).

Cytokinins exist in plants not only as free bases but also in the form of nucleosides and nucleotides. The cytokinin bases can be further conjugated to glucose at $N^3$, $N^7$, and $N^9$ of the adenine ring and at the hydroxyl of the side chain. The hydroxyl group of the side chain can be also conjugated to xylose (e.g. Turner et al., 1987). The $N^9$ can be modified with l-alanine (Entsch et al., 1983) and $C^2$ with a 2-methylthio group (Persson et al., 1994; Pertry et al., 2009).

Cytokinins combining the two above-mentioned modifications can occur in plants, and also cytokinins with two sugar moieties attached to each other were found in Monterey pine (Pinus radiata; Taylor et al., 1984; Zhang et al., 2001). The compound called ‘gazer’, isolated from coconut milk, has been identified as 14-O-(3-O-[β-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl-(1→3)-α-L-arabinofuranosyl]-4-O-(α-L-arabinofuranosyl)-β-D-galacto-pyranosyl)-trans-zeatin riboside (Kobayashi et al., 1995).

Cytokinin signalling and function

The cytokinins act, as do other signalling molecules, at very low concentrations (1–50 pmol g$^{-1}$ of fresh weight; Galuszka et al., 2008). They act as both a paracrine local signal in meristematic tissues and as distal signals, for example for signalling of availability of nutrition (Sakakibara, 2006; Werner and Schmülling, 2009). Biased distribution of trans-zeatin-type cytokinins in the xylem and of isopentenyladenine-type cytokinins in phloem saps implies the involvement of selective transport systems (Hirose et al., 2008). Previous studies have shown that some of the purine permeases (PUPs) function as transporters for cytokinin nucleotides (Bürkle et al., 2003; Czedzich et al., 2008), though having a $K_m$ comparable with cellular concentrations of purine that are much higher than those of cytokinins these proteins can hardly transport cytokinins efficiently. Specific equilibrative nucleoside transporters (ENTs) for cytokinin nucleosides were found in rice (Hirose et al., 2005) and Arabidopsis (Hirose et al., 2008).
The cytokinin signal is conveyed by a two-component signalling system, also called the histidyl-to-aspartyl system or His–Asp phosphorelay, which is found in bacteria as well as in plants and fungi, but not in animals. The first component of the cytokinin signalling cascade is the membrane-spanning histidine kinase (Higuchi et al., 2004) containing an extracellular CHASE (cyclases/histidine kinases-associated sensing extracellular) domain (Mougel and Zhulin, 2001), which passes the phosphate to the histidine phosphotransfer proteins that further convey the signal into the nucleus to the last component: type B response regulators. These work as transcription regulators and affect expression of the cytokinin target genes including type A response regulators (To et al., 2007), which inhibit the phosphotransfer from histidine phosphotransfer proteins to the type B response regulators and thus work as a negative feedback loop.

The cytokinin signalling machinery including its variation in different plant species together with evolutionary aspects were comprehensively reviewed recently (Pils and Heyl, 2009). Evolution of cytokinin signal transduction became important to plants during the conquest of land. The moss Physcomitrella patens is the most basal plant species containing all the components necessary for cytokinin signalling. Although found to contain cytokinins (Stirk et al., 2003), algae such as Chlamydomonas reinhardtii only contain a few of the type B response regulators, but not the cytokinin receptor (Pils and Heyl, 2009). The only known non-plant eukaryote encoding the CHASE domain-containing proteins is the social amoeba Dictostelium discoideum that apparently uses isopentenyladenine and its 3-(3-amino-9-pentyl)-adenine either in its nucleotide forms or bound to RNA. The isoprenoid side chains donors found to date are dimethylallyl pyrophosphate (DMAPP) and (E)-4-hydroxy-3-methyl-but-2-enyl diposphat (HMBDP; Krall et al., 2002; Sakakibara et al., 2005). In the case of DMAPP attachment and formation of isopentenyladenine-type cytokinin, the side chain can be further hydroxylated by cytochrome P450 monooxygenase (Takei et al., 2004a). The cytokinin nucleotides (eventually after release from tRNA) are then hydrolysed to free bases.

The first cytokinin biosynthetic activity was detected in the slime mould D. discoideum (Taya et al., 1978), where the activity is important for production of discadencine, a spore germination inhibitor. Interestingly, discadencine shows cytokinin activity in assays of tobacco callus growth (Nomura et al., 1977). The first cytokinin biosynthetic gene was identified in the plant pathogenic bacterium Agrobacterium tumefaciens. The gene Tmr (tumour morphology root) located in the T-DNA (transfer) region on the Tj plasmid (tumour-inducing) was shown to be able to induce tumorgenesis independently of other T-DNA located genes (Lichtenstein et al., 1984). In 1984, the adenylate isopentenyl transferase (IPT) activity of the Tmr gene product was confirmed (Akiyoshi et al., 1984; Barry et al., 1984). The $K_m$ values for both AMP and DMAPP were low ($85.7$ nM and $8.28$ μM, respectively), but the $k_{cat}/K_m$ ratio was also low ($4.7\times10^5$ s$^{-1}$ M$^{-1}$). However, even lower catalytic efficiency was reported for other IPTs studied to date (e.g. Takei et al., 2001; Kakimoto, 2001; Sakano et al., 2004; Abe et al., 2007). In the case of Arabidopsis IPTs (AtIPTs), the $K_m$ values for AMP were found to be much higher than those for ADP and ATP. Furthermore, the cellular level of AMP is low, so AMP is not an important substrate for AtIPTs (Kakimoto, 2001). Agrobacterium tumefaciens contains an additional IPT gene Tzs (trans-zeatin synthesizing) that is not, unlike Tmr, transferred to the plant genome, but rather is expressed in the bacterium itself (Morris et al., 1993).

Interestingly, IPT from mulberry (Morus alba) can also accept dATP, dADP, CDP, and GDP. In the case of GDP, the isopentenyl moiety was transferred to the exocyclic N$^2$Pernisová et al., 2009) and ethylene (Růžička et al., 2007, 2009).

The content of endogenous cytokinins depends on the balance between de novo synthesis, the import and export rate, interconversion of distinct forms, transient inactivation by conjugation (mainly glucosylation), and catabolic reactions resulting in a complete loss of biological activity (Sakakibara, 2006).

### Enzymes of cytokinin metabolism

#### Isopentenyl transferases—the first step in cytokinin biosynthesis

The key biosynthetic and metabolic pathways of cytokinins are summarized in Fig. 1. The biosynthesis of isoprenoid cytokinins starts with transfer of the isoprenoid moiety to adenine either in its nucleotide forms or bound to RNA. The biosynthesis of cytokinins starts with transfer of the isoprenoid moiety to adenine either in its nucleotide forms or bound to RNA.
group, rather than the endocyclic $N^1$, which is closer to $C^6$ (Abe et al., 2007). Although it was originally described that the IPT from hop (Humulus lupulus) does not accept GMP, IMP, CMP, or UMP (Sakano et al., 2004), a recent report concerning the crystal structure describes binding affinity for nucleotides in the order ATP > dATP > ADP > GTP > CTP > UTP (Chu et al., 2010).

There are two types of IPTs, adenylate IPT (EC 2.5.1.27; Blackwell and Horgan, 1993; Kakimoto, 2001; Takei et al., 2001) that adds an isopentenyl group to the $N^6$ atom of AMP, ADP, or ATP (but not adenosine nor adenine) and tRNA IPT (EC 2.5.1.8) acting in the same way on adenine in tRNA. It has been proposed (Xie et al., 2007) and later shown (Zhou and Huang, 2010) that the target adenine is flipped out of the tRNA structure similarly to when it is modified by other nucleic acid editing enzymes (e.g. Xie et al., 2003). This type of IPT is present in almost all living organisms including bacteria (Caillet and Droogmans, 1988), yeast (Yevdakova et al., 2007), animals (Golovko et al., 2000), and plants (Golovko et al., 2002; Miyawaki et al., 2006), but not Archaea. The isopentenylation of adenine in tRNA influences translational efficiency and fidelity (reviewed by Persson et al., 1994). It was shown that this improves translational proofreading by decreasing misreading at the first position of the codon. The cytokinins were also found in rRNA (Taller et al., 1987), but their function is unclear. tRNA was originally proposed to be the source of cytokinins; however, calculations showed that they could
Two possible biosynthetic pathways are proposed for trans-zein-type cytokinins, an isopentenyladenine-dependent pathway, where an isopentenyladenine nucleotide is produced first and is later hydroxylated by cytochrome P450 monooxygenases (Takei et al., 2004a), and an isopentenyladenine-independent pathway, where trans-zeatin-type cytokinin is produced directly by transferring a hydroxylated side chain from the proposed precursor HMBDP to the adenine ring (A˚stot et al., 2000; Sakakibara et al., 2005).

Auxin has been shown to modulate zeatin levels both by decreased expression of the CYP735As (Takei et al., 2004a) and by inhibition of the isopentenyladenine-independent pathway (Nordström et al., 2004). Both de novo biosynthetic pathways date have been found to be relevant only for trans-zeatin.

The question of which pathway is more relevant relates to the origin of the side chain donor. In plants, there are two pathways for isoprenoid production. The first one, the mevalonate pathway, which also operates in animals, fungi, Archaea, and a few bacteria, is located in the cytosol and mitochondria and produces precursors for biosynthesis of sterols, certain sesquiterpenes, and the side chain of ubiquinone. The second one, the methylerythritol phosphate pathway, localized in plastids, was discovered in 1993 and is responsible for production of monoterpenes, some sesquiterpenes, diterpenes, carotenoids, and the side chains of chlorophylls and plastoquinone (Rohmer et al., 1993; Lichtenhaler, 1999; Laule et al., 2003).

The mevalonate pathway starts with condensation of three acetyl-CoA molecules and a subsequent reduction to mevalonate. This reduction is directly controlled by isoprenoid levels and is inhibited by statins (e.g. lovastatin or mevastatin). The mevalonate is then phosphorylated twice to produce mevalonate-5-diphosphate, and another phosphorylation with subsequent decarboxylation-driven dephosphorylation yields isopentenyl diphosphate, which can be isomerized to DMAPP, serving as a precursor for cytokinins.

The methylerythritol phosphate pathway starts with condensation of pyruvate and glyceraldehyde 3-phosphate producing 1-deoxy-d-xylulose-5-phosphate. It is then converted to methylerythritol phosphate. This step is performed by reductosorase, which is inhibited by phosphodiamycin (Proteau, 2004). Methylerythritol phosphate is conjugated with the cytidylphosphate moiety, which is followed by another phosphorylation, release of CMP, and cyclizing. Reduction yields HMBDP, which can be further reduced to isopentenyl pyrophosphate and to a lesser extent to DMAPP. Both HMBDP and DMAPP can work as cytokinin precursors, but the level of HMBDP in plastids is ~5-fold higher (Sakakibara et al., 2005).

It has been shown that Tzs (protein encoded by the Tzs gene from A. tumefaciens) is capable of directly synthesizing zeaatin monophosphate from AMP and HMBDP (Krall et al., 2002). Zeatin-type cytokinin levels increased in Arabidopsis plants overexpressing Tmr, while isopentenyladenine-type cytokinin levels remained low (Astot et al., 2000). On the other hand, Escherichia coli expressing Tmr secreted predominantly isopentenyladenine-type cytokinins (Takei et al., 2001). Since the $K_m$ values of Tmr for both HMBDP and DMAPP are similar (Sakakibara et al., 2005), the preference for either precursor may be determined by their availability. The zeatin-type cytokinin levels in Arabidopsis overexpressing Tmr increased even after addition of metyrapone, an inhibitor of cytochrome P450 (Astot et al., 2000). The direct biosynthesis was also confirmed by labelling experiments (Astot et al., 2000; Sakakibara et al., 2005). This implies the major involvement of the plastidic methylerythritol phosphate pathway to the zeatin-type cytokinin pool in agreement with localization of Tmr in plastids. On the other hand, AtIPT1 as the main cytokinin-producing enzyme in Arabidopsis cannot use HMBDP as a substrate (Sakakibara et al., 2005). The involvement of the mevalonate pathway in cytokinin synthesis has been traditionally considered low or none (Piaggesi et al., 1997). A dual labelling study of incorporation of intermediates from both pathways into cytokinins has revealed an almost exclusive contribution from the methylerythritol pathway to trans-zeatin and isopentenyladenine-type cytokinins (Kasahara et al., 2004). Only a minor input from the mevalonate pathway was observed, which could be explained either by an exchange between the pools of both pathways (e.g. Laule et al., 2003) or by a low rate of utilization of mevalonate pathway precursors by cytosolic AtIPT4 and AtIPT7 (Kasahara et al., 2004). Since to date a cis-hydroxylase has not been identified in plants, nor has an isoprenoid precursor with a hydroxyl group in the cis-position been found in nature, tRNA degradation is the only widely accepted source of cis-zeatin.

In either of the atipt2 or atipt9 mutants, cis-zeatin-type cytokinins were decreased, while being undetectable in the atipt2-9 double mutant. This clearly suggests that tRNA IPTs are indispensable for cis-zeatin production (Miyawaki et al., 2006).

‘Lonely guy’ and phosphatases activate cytokinin nucleotides and nucleosides

Cytokinins are de novo synthesized as low-active nucleotide mono-, di-, or triphosphates; alternatively, the release of cytokinins from tRNA leads to nucleotide monophosphates. Historically it was considered that the interconversions of the nucleotides, nucleosides, and free bases were performed by enzymes of general adenine metabolism. The cytokinin nucleotide phosphates can be in vivo dephosphorylated by phosphatases with broad substrate specificity, such as 5’-nucleotidase (EC 3.1.3.5; Chen and Kristopeit, 1981), or by any abundant alkaline (EC 3.1.3.1) and acid
phosphatase (EC 3.1.3.2). Deribosylation of the cytokinin nucleoside can be mediated by adenosine nucleosidase (EC 3.2.2.7). However, the low affinity of these enzymes toward cytokinins may be insufficient to perform the activation due to physiological concentrations of cytokinins in plant tissues that are up to 6-fold lower than those of adenine derivatives (Galuszka et al., 2008). Arabidopsis mutants deficient in adenosine nucleosidase accumulated more cytokinin nucleotides and nucleosides compared with wild-type plants (Auer, 2002). Recently, a novel uridine ribohydrolase URH1 has been characterized in Arabidopsis (Jung et al., 2009), which also processes isopentenyladenosine, with a $K_m$ about half of that for uridine and adenosine; however, the $k_{cat}$ is four orders of magnitude lower.

It has been demonstrated many times that exogenously applied free cytokinin bases are rapidly metabolized into the corresponding nucleosides and nucleotides (e.g., Sondheimer and Tzou, 1971; Letham and Zhang, 1989; Suttle and Banowetz, 2000). Reverse phosphorylation of nucleotides to nucleosides could be catalyzed by adenosine kinase (EC 2.7.1.20), as was shown for the recombinant enzyme from the moss P. patens (von Schwartzenberg et al., 1998). Adenosine kinase isolated from tobacco cells also shows high affinity for cytokinins (Kwade et al., 2005). Direct conversion of cytokinin free bases to nucleotides can be provided by an adenine phosphoribosyltransferase activity (EC 2.4.2.7; Schnorr et al., 1996; Allen et al., 2002). In contrast to the wild type, Arabidopsis male-sterile mutants deficient in this enzyme were not able to convert exogenously applied benzyladenine to the corresponding ribotide (Moffatt et al., 1991). Interestingly, one of the five Arabidopsis isoforms identified later shows higher affinity for benzyladenine than for adenine (Schnorr et al., 1996). In general, high concentrations of nucleotides and nucleosides are found in young seedlings and developing organs, probably due to an increased de novo synthesis. Therefore, the demand for deactivation of free bases by their conversion back to nucleotides in planta is still questionable, notwithstanding that the above-mentioned enzymes are often constitutively expressed without the possibility of pronounced regulation.

However, the recent discovery of cytokinin-specific phosphoribohydrolase ‘Lonely guy’ (LOG; Kurakawa et al., 2007) suggests that the other reactions may also be performed by cytokinin-specific, yet unidentified enzymes. The LOG was identified in rice plants with altered shoot meristems, reduction in panicle size, and abnormal branching patterns as a consequence of loss-of-function mutation (Kurakawa et al., 2007). Originally, it was annotated as lysine decarboxylase, but the co-occurrence of LOG homologues in operons with cytokinin biosynthetic genes in some bacteria indicated involvement in cytokinin metabolism (Pertry et al., 2010).

Cytokinin dehydrogenase causes irreversible degradation of cytokinins

Oxidative cleavage of cytokinins was first demonstrated in a crude tobacco culture by Paèes et al. (1971), and later by Whitty and Hall (1974) named the enzyme cytokinin oxidase. Cytokinin degradation is achieved through oxidative cleavage of its $N^6$ side chain, resulting in the formation of adenine (or its corresponding derivative for $N^6$-substituted cytokinins) and a side chain-derived aldehyde (Brownlee et al., 1975). The reaction proceeds via cytokinin dehydrogenation to an imine intermediate when an electron acceptor withdraws two electrons from the enzyme’s flavin cofactor. For years it was assumed that molecular oxygen was essential for CKX activity, but a variety of electron acceptors other than oxygen, especially quinone types such as 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Qa), function more efficiently (Galuszka et al., 2001; Frébortová et al., 2004). Therefore, the enzyme was reclassified as a dehydrogenase (cytokinin dehydrogenase; CKX, EC 1.5.99.12).

The maize CKX, known as ZmCKX1, has been localized to the aleurone layer in kernels and to companion phloem cells in stems. Co-localization with laccases suggests that endogenously produced phenolics could serve as a precursor of a CKX electron acceptor (Galuszka et al., 2005). Recently, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), an abundant compound in maize tissues, its oxidative cleavage product 4-nitrosoresorcinol-l-monomethylether (coniferron), and potentially also their free radicals have been shown to serve as very potent natural electron acceptors of CKX (Frébortová et al., 2010).

CKX is the only known enzyme shown to catalyze irreversible inactivation of cytokinins, and its activity has been reported in many plant species and in a few lower organisms, namely the moss Funaria hygrometrica (Gerhäuser and Bopp, 1990), the slime mould D. discoideum (Armstrong and Firtel, 1989), and the yeast Saccharomyces cerevisiae (Van Kast and Laten, 1987). However, the presence of endogenous CKX in the two latter organisms is doubtful (Schmülling et al., 2003). No matching sequence for a cytokinin dehydrogenase gene can be found by BLAST search (tblastn) in the S. cerevisiae genome.

Due to a low concentration of CKX in plant tissues, the isolation of pure protein is extremely difficult. The breakthrough came with the cloning of a gene encoding CKX from maize (Houba-Hérin et al., 1999; Morris et al., 1999), enabling identification of CKX genes in other species and their expression in heterologous hosts or overexpression in plants. The common feature of all known plant CKX enzymes is the presence of a covalently bound FAD molecule. The cofactor is linked through the 8-methyl group of the isoalloxazine ring to a histidine residue of a well-conserved GHS motif in the enzyme’s N-terminal half (Malito et al., 2004). Covalent binding of FAD to protein facilitates redox catalysis and the oxidative power of flavin (Galuszka et al., 2008).

Mature CKX enzymes consist of two domains for FAD and substrate binding, respectively. About one-third of all amino acids are highly conserved among different CKX proteins. The FAD-binding domain contains a large amount of conserved regions, but short highly conserved sequences are also located at the N- and C-termini of this motif. Other examples include the WevPHPWLNl sequence found at around position 390 or the PGQxIF signature at
the C-terminal ends of the proteins (Schmülling et al., 2003). Conservation of amino acid residues suggests their possible functional importance in substrate recognition and electron transport.

**Glucosyl transferases inactivate cytokinins by conjugation**

The levels of biologically active cytokinins must be precisely maintained. Besides irreversible side chain cleavage, the cytokinins can be glucosylated at the N\(^7\) and N\(^8\) positions. N-Glucosyltransferase (EC 2.4.1.118) producing cytokinin N\(^7\)- and N\(^8\)-glucosides was isolated from radish (Raphanus sativus) cotyledons (Entch and Letham, 1979; Entch et al., 1979). The enzyme uses several adenine derivatives as bases from their glucosyl transferases (EC 2.4.1.118) producing cytokinin N\(^7\)- and N\(^8\)-glucosides was isolated from radish (Raphanus sativus) cotyledons (Entch and Letham, 1979; Entch et al., 1979). The enzyme uses several adenine derivatives as substrates, the highest activity being with isoprenoid. Two genes coding for cytokinin N-glucosyltransferases that transfer the glucosyl moiety from uridine 5'-diphosphoglucose to N\(^7\) and N\(^8\) atoms of both isoprenoid and aromatic cytokinins were found in Arabidopsis (Hou et al., 2004).

The N-glucosides show generally low activity in bioassays (Letham et al., 1983; Spichal et al., 2004) and are only weakly metabolized (Parker and Letham, 1973; Parker et al., 1978). N-Glucosides showed indifference to metabolic conversion in radish tissues and thus N-glucosylation is considered as an irreversible deactivating process (McGaw et al., 1984, 1985). However, recombinant AtCKX proteins showed the ability to degrade N\(^2\)-glucosides, and two of them even prefer isopentenyladenine-9-glucoside as a substrate in an acidic environment (Galuszka et al., 2007).

Although N\(^7\)-glucosides have been detected in several plants (Letham et al., 1975), their biosynthetic enzymes have not yet been detected. Dihydrozeatin and its O-glucoside, but not zeatin and its O-glucoside, were metabolized to their N\(^3\)-glucosides in derooted radish seedlings (McGaw et al., 1985). In contrast to N\(^2\) - and N\(^8\)-glucosides, kinetin-N-glucoside can be deglycosylated together with O-glucosides by maize \(\beta\)-glucosidase (Brzobohaty et al., 1993). The only \(\beta\)-glucosidase able to release active free cytokinin bases from their N\(^9\)-glucosides is the enzyme encoded by the rolC oncogene, which is integrated into plant genomes after infection by Agrobacterium rhizogenes causing hairy root syndrome (Estruch et al., 1991). Nevertheless, the specificity towards N\(^8\)-glucosides was confirmed only in vitro with recombinant protein, and release of cytokinin aglycones has never been detected in rolC-infected tissues (Faiss et al., 1996). Exogenous application of cytokinin N\(^9\) glucosides does not usually alter the growth of plant tissues in a cytokinin-dependent manner. However, it was observed that application of N\(^9\)-glucosides to certain tissues can alter development even in nanomolar concentrations in the same way as the corresponding free bases do (P Galuszka et al., unpublished; M Kaminek and V Motyka, personal communication). Thus, the irreversibility of deactivation of the main accumulated cytokinin metabolites is still unclear and knowledge is limited by the small number of studies concerning the activity of various plant \(\beta\)-glucosidases with different cytokinin glucosides.

Cytokinins with a hydroxyl group on the side chain are capable of undergoing O-glycosylations. The genes coding for glucosyltransferases capable of glucosylating zeatins and topolins have been identified and enzymes have been characterized in many plants, for example in Arabidopsis, bean, maize, and soybean (Martin et al., 1999a, b, 2001; Veach et al., 2003; Hou et al., 2004; Mok et al., 2005; Meek et al., 2008). The maize enzymes have been shown to utilize only cis-zeatin (Martin et al., 2001; Veach et al., 2003), so they are distinguished as cis-zeatin-O-\(\beta\)-D-glucosyltransferases (EC 2.4.1.215). The other group, trans-zeatin-O-\(\beta\)-D-glucosyltransferases (EC 2.4.1.203), is also capable of transferring the xylosyl moiety from UDP-Xyl (Martin et al., 1999a, b). Nevertheless, the Arabidopsis enzymes did not discriminate between both isomers and were able to glycosylate both zeatin isomers efficiently (Hou et al., 2004). Extensive O-glycosylations occur in Phaseolus, and many cytokinin O-glycosyltransferases have been identified. One of them is narrowly specific to only xylose conjugation (zeatin O-xylosyltransferase; EC 2.4.2.40; Turner et al., 1987). The phenotype of maize plants, which generally accumulate predominantly cis-zeatin forms (Veach et al., 2003; Vyroubalova et al., 2009), overexpressing a trans-zeatin-specific enzyme from Phaseolus (Pineda Rodó et al., 2008) resembled that of plants overexpressing CKX in terms of a dwarfish shoot and increased root growth (e.g. Werner et al., 2001). The hydroxylated aromatic cytokinins and topolins, as well as hydroxylated thidiazuron, have been shown to be substrates of O-glucosyltransferases (Mok et al., 2005).

Zeatin O-glycosyl derivatives are resistant to the cytokinin-degrading enzyme CKX (Laloue and Pethe, 1982) and are considered to be storage forms as they can be cleaved by \(\beta\)-glucosidase (EC 3.2.1.21; Brzobohaty et al., 1993). Both zeatin O-glycosyltransferase and \(\beta\)-glucosidase are thought to play important roles in maintaining appropriate levels of active cytokinins, as the biosynthetic enzymes work slowly (Takei et al., 2001; Kakimoto, 2001; Sakano et al., 2004; Abe et al., 2007). \(\beta\)-Glucosidase is also present around vascular bundles, where it can participate in cytokinin transport (Kristoffersen et al., 2000). Although most \(\beta\)-glucosidases show broad substrate specificity, the enzyme isolated from rape (Brassica napus) showed significant activity only towards zeatin O-glucoside (Falk and Rask, 1995).

Lupinic acid, the conjugate of zeatin and alanine, is produced in lupin seeds (Lupinus luteus) by zeatin 9-amino-carboxyethyltransferase (EC 2.5.1.50). The alanyl moiety donor is O-acetyl-L-serine, and various N\(^7\)-derivatives of adenine can be the acceptor, but zeatin is the best one (Entsch et al., 1983). It was shown that the enzyme is inhibited by certain urea derivatives, but also by indole-3-acetic acid (auxin) and similar compounds (Parker et al., 1986).

**Zeatin interconversion enzymes—zeatin isomerase and zeatin reductase**

Apart from tRNA degradation, another predicted source of cis-zeatin is the activity of zeatin cis→trans isomerase. It was first described in and partially isolated from immature seeds.
of Phaseolus vulgaris (Bassil et al., 1993). The enzyme required flavin and light as well as dithiothreitol for the reaction. However, no further characterization of the enzyme has been performed and there are only scarce reports of zeatin cis–trans isomerase activity found in vivo. For instance, 4 d and 7 d after application of radioactively labelled cis-zeatin to potato (Solanum tuberosum L.) tubers, ~10% of recovered radioactivity was associated with trans-zeatin (Suttle and Banowetz, 2000). In contrast, there are other reports where no isomerization was detected (Nandi and Palni, 1997; Kuroha et al., 2002; Yonekura-Sakakibara et al., 2004).

The origin of dihydrozeatin is also unclear. The only source found to date is a putative zeatin reductase (EC 1.3.1.69). The conversion of zeatin to dihydrozeatin was first described in excised bean axes (P. vulgaris; Sondheimer and Tzou, 1971). Later, it was detected in leaves of pea (P. sativum) and embryos of Phaseolus coccineus and P. vulgaris, but not of P. lunatus (Mok et al., 1990). In contrast, no conversion of zeatin was observed in leaves of P. vulgaris and soybean (Glycine max; Gaudinová et al., 2005). The enzyme was partially purified and characterized (Martin et al., 1989). The reaction was NADPH dependent, but NADH did not work as a cofactor (Martin et al., 1989). The bean enzyme did not require ATP or cations (Mok et al., 1990), but the pea enzyme was inhibited by addition of diethylthiocarbamate, a chelating agent, suggesting that a metal cofactor may be needed (Gaudinová et al., 2005).

Other possibly cytokinin-related proteins

Several cytokinin-binding proteins were isolated from various plant species (reviewed in Barciszewski et al., 2007), but their function remains largely unknown. Plant pathogenesis-related (PR) proteins that occur in the cytosol may act as a reservoir of cytokinin molecules. Crystal structures of proteins from Vigna radiata (Pasternak et al., 2006) and L. luteus (Fernandes et al., 2008) with trans-zeatin as a ligand have been described. Upon crystal structure determination, it has been suggested that mistletoe (Viscum album) uses absorption of trans-zeatin with lectin to diminish its sensitivity to the host hormone level and therefore to adapt as a parasite (Meyer et al., 2008). Cytokinin-binding protein CBP70 from maize and barley may serve as a plastid transcription elongation factor or a modulator of plastid elongation factor activity (Brovko et al., 2010).

Finding key components of cytokinin metabolism in the plant genetic model Arabidopsis thaliana

Isopentenyl transferases of Arabidopsis

Identification of the first plant IPTs was enabled when the sequencing of the first plant genome of A. thaliana was completed. The AtIPTs were identified by BLAST search against bacterial IPT genes, and it was concluded that there were nine genes (Kakimoto, 2001; Takei et al., 2001). Two of them are putative tRNA IPTs (AtIPT2 and AtIPT9). The activity of AtIPT9 has not been detected yet, but AtIPT2 has been confirmed as a tRNA IPT (Golovko et al., 2002). The supernatants of bacteria expressing all other AtIPTs did secrete cytokinins, indicating their cytokinin biosynthetic activity.

Plastid localization was confirmed for AtIPT1, AtIPT3, and AtIPT5 in leaf cells by fusion with green fluorescent protein (GFP), while AtIPT8 exhibited the same pattern as AtIPT1 and AtIPT3 in root cells (Kasahara et al., 2004). Recently, it was found that AtIPT3 contains a CaaX motif in its C-terminus that is farnesylated. This farnesylation drives translocation to the nucleus in onion (Allium cepa L.) epidermal cells (Galichet et al., 2008), where it could serve as a local source of cytokinins for cell cycle progression. This could seem contradictory to a previous report by Kasahara et al. (2004); however, the authors used GFP fused to the C-terminus, which would block farnesylation and thus the translocation to the nucleus. AtIPT4 and tRNA AtIPT2 were localized in the cytosol and AtIPT7 signal peptide-driven fluorescence was found in mitochondria (Kasahara et al., 2004). Noticeably, the A. tumefaciens Tmr protein is also localized to plastids, although it is missing the N-terminal extension (Sakakibara et al., 2005). The cellular localization of biosynthetic and degradation enzymes in A. thaliana is depicted in Fig. 2.

The expression pattern of individual AtIPT genes is differential (Takei et al., 2004b; Miyawaki et al., 2004).
AtIPT3, AtIPT5, and AtIPT7 are expressed in all examined tissues, with AtIPT3 being the most abundant gene. AtIPT6 and AtIPT1 were expressed in siliques and AtIPT1 additionally in flowers. However, AtIPT6 is a pseudogene in *A. thaliana* ecotype Wassilewskija, and no expression is detected therein (Kakimoto, 2001).

The expression of IPTs was shown to respond to the phytohormone content. AtIPT3, AtIPT5, and AtIPT7 are up-regulated by auxin in roots, unless cytokinins are present (Miyawaki et al., 2004), while in decapitated pea shoots the expression of PsIPT1 and PsIPT2 is up-regulated in the absence of auxin (Tanaka et al., 2006). In soybean (*G. max* L.), GmIPT1 expression was only slightly up-regulated after treatment with auxin (Ye et al., 2006), but treatment with gibberellins resulted in a 2.5-fold increase in expression. The *Arabidopsis* IPT genes were also shown to respond to nutrient status (Miyawaki et al., 2004; Takei et al., 2004b; Hirose et al., 2008). Expression of AtIPT5 in roots was positively correlated with ammonia and nitrate levels in the medium, whereas in shoots it was decreased, as were the transcript levels of AtIPT1 and AtIPT7 (Takei et al., 2004b). AtIPT3 levels decreased in roots, when grown on ammonia, whereas in nitrogen-limited *Arabidopsis* AtIPT3 transcript quickly increased after nitrate supply in both roots and shoots (Miyawaki et al., 2004; Takei et al., 2004b). The level of AtIPT3 is also dependent on the availability of other nutrients such as sulphate, phosphate, and iron (Hirose et al., 2008). Similarly, in maize leaves, levels of ZmIPTs increased 72 h after the stress treatment (Vyroubalová et al., 2009). The transcript level of soybean *GmIPT1* was increased 1.6- and 1.9-fold after 5 h treatment with cold and salt, respectively (Ye et al., 2006). Although tRNA IPTs are generally assumed to be expressed constitutively, without being affected by plant hormones or nutrient status (Miyawaki et al., 2004), the expression of maize ZmIPT10 is increased after longer exposure to a stress environment predominantly in leaves (Vyroubalová et al., 2009).

**LOGs of Arabidopsis**

Interestingly, the structures of AtLOG3 and AtLOG8 had been determined as putative lysine decarboxylases, before their cytokinin phosphoribohydrolase activity was recognized (Jeon et al., 2006). Eleven and nine LOG genes were identified in the rice and *Arabidopsis* genomes, respectively (Kurakawa et al., 2004; Takei et al., 2004b). The level of AtIPT3 is also dependent on the availability of other nutrients such as sulphate, phosphate, and iron (Hirose et al., 2008). Similarly, in maize leaves, levels of ZmIPTs increased 72 h after the stress treatment (Vyroubalová et al., 2009). The transcript level of soybean GmIPT1 was increased 1.6- and 1.9-fold after 5 h treatment with cold and salt, respectively (Ye et al., 2006). Although tRNA IPTs are generally assumed to be expressed constitutively, without being affected by plant hormones or nutrient status (Miyawaki et al., 2004), the expression of maize ZmIPT10 is increased after longer exposure to a stress environment predominantly in leaves (Vyroubalová et al., 2009).

**Cytokinin dehydrogenases of *A. thaliana***

A family of seven homologous genes coding for CKX is present in the *A. thaliana* genome. Two genes are located on chromosome 2 (*AtCKX1* and *AtCKX2*), two on chromosome 5 (*AtCKX3* and *AtCKX7*), and single genes are found on chromosomes 4, 1, and 3 (*AtCKX4*, *AtCKX5*, and *AtCKX6*, respectively). The genomic organization of all sequences is highly conserved, with five exons and four introns (Bilyeu et al., 2001).

The predicted proteins are of a similar size (~60 kDa), but their amino acid identity is between 34.3% (*AtCKX4* and *AtCKX7*) and 65.9% (*AtCKX2* and *AtCKX4*). Sequence alignment of CKXs from known sources indicates conservation of the FAD-binding site and the presence of several small domains (Gu et al., 2010). The divergences in amino acid sequences contribute to functional diversification of AtCKX isozymes. Except for AtCKX7, all proteins have a hydrophobic N-terminal signal peptide that targets them either to vacuoles (*AtCKX1* and *AtCKX3*) or to the apoplast (*AtCKX2*, *AtCKX4*, *AtCKX5*, and *AtCKX6*) (Fig. 2). Vacuolar localization of *AtCKX1* and *AtCKX3* was confirmed by GFP fusion as well as secretion of *AtCKX2* (Werner et al., 2003). The lack of signal peptide in the case of *AtCKX7* indicates its cytosolic localization, which is in agreement with the intracellular expression of recombinant protein in *E. coli* and *Pichia pastoris* (Kowalska et al., 2010). Such a cytosolic CKX was also isolated from maize and characterized (Šmehilová et al., 2009).

*AtCKX* proteins are post-translationally modified by glycosylation since they contain several consensus N-glycosylation sites, NXS/T. Such modifications may contribute to regulation of enzymatic activity (different pH optimum), translocation, and protein stability (Schmülling et al., 2003). Also, the occurrence of both glycosylated and non-glycosylated isoforms of CKX was confirmed in various plant sources and cultured tobacco callus, with higher activity of the glycosylated enzyme (Kaminek and Armstrong, 1990; Motyka et al., 2003).
Functional diversification of *A. thaliana* CKX isozymes is also manifested through different gene expression patterns and biochemical properties. The expression of individual *AtCKX* genes at different developmental stages and in different plant tissues was confirmed using fusion constructs of *CKX* promoter regions and β-glucuronidase (GUS; Werner et al., 2003). *AtCKX1* showed prominent expression in the vascular cylinder of lateral roots, whereas *AtCKX2* showed strongest expression in the shoot apex, and *AtCKX3* was expressed in the young shoot tissues. *AtCKX4* had an interesting pattern of expression in developing trichomes, stamens, petals, and stigmas, as well as a root cap—mostly regions of high mitotic activity. Besides differential patterns of expression in developing shoots and roots, *AtCKX5* showed strong expression in stamens, primordial, and developing pollen, while *AtCKX6* was expressed in the gynoecium at various stages of development.

Constitutive expression of *AtCKX* genes in tobacco and *A. thaliana* resulted in cytokinin-deficient plants (30–60% of the wild type cytokinin content) that typically exhibited stunted shoots with a smaller apical meristem, prolonged plastochrone, slower leaf cell production, and excessive root system development (Werner et al., 2001, 2003). Phenotypes of plants overexpressing individual *AtCKX* genes differ to some extent; in particular, the overexpression of vacuolar proteins caused a stronger cytokinin deficiency syndrome than the overexpression of apoplastic enzymes. Taken together, these facts indicate that specific developmental and physiological functions are fulfilled by each gene and that the tissue-specific regulation of the endogenous cytokinin content is important for ensuring the proper regulation of cytokinin functions in plants (Werner et al., 2003).

The protein structure, first determined for maize CKX (ZmCKX1), displays a two-domain folding topology typical for the members of the vanillyl-alcohol oxidase family (Malito et al., 2004). The ADP moiety of the cofactor is embedded in the FAD-binding domain while its isoalloxazine ring is located at the interface of the two domains. The 8-methyl group of the flavin is covalently linked to the histidine residue in the conserved GHS motif. The CKX active site consists of an internal cavity lined by the flavin ring and a funnel-shaped region on the protein surface. The aliphatic side chain of the substrate is sealed in a solvent-inaccessible cavity in the proximity of FAD. The catalytic reaction starts with transfer of a hydride ion from the α-carbon of the cytokinin to the N^6^ atom of FAD, which is followed by proton abstraction from the N^6^ atom of the cytokinin by an aspartate residue serving as a catalytic base to form a cytokinin imine product that hydrolyses to adenine and the side chain aldehyde (Popelková et al., 2006). The funnel-shaped region functions as a binding site for the adenine ring of cytokinin that is sandwiched between non-polar amino acid residues, but its edge is exposed near the protein surface to the solvent (Malito et al., 2004).

Subtle variation in the amino acid residues at the entrance to the substrate funnel possibly contributes significantly to the substrate binding and turnover rates of individual substrates. The substitution of a glutamate residue found in ZmCKX1 (Glu381) that forms a H-bond to N^9^ of the cytokinin substrate with alanine or serine causes less tight binding of free cytokinin bases; that is, lower activity with isopentenyladenine. Indeed, *AtCKX2* and *AtCKX4*, that possess the glutamate residue, are the most active enzymes and in neutral or slightly basic pH prefer free cytokinin bases as substrates (Galuszka et al., 2007), while *AtCKX1* (alanine instead of glutamate) prefers cytokinin nucleotides over free bases, and *AtCKX7* (serine instead of glutamate) prefers cytokinin N^9^-glucosides. Substrate specificity is also thought to be closely related to the enzyme’s subcellular localization. Preferences of vacuolar enzyme *AtCKX1* for cytokinin nucleotides and cytoplasmic *AtCKX7* for cytokinin N^9^-glucosides that were shown recently (Kowalska et al., 2010) correspond to the presumed occurrence of particular cytokinin derivatives within each intracellular compartment, illustrating that cytokinin levels are actively managed throughout the cell as well as throughout the plant.

Surprisingly, in the active site of CKX there is no room for an electron acceptor to bind in the vicinity of FAD. Therefore, it is assumed that electrons are transferred from the flavin through the protein matrix to an electron acceptor bound on the protein surface. CKX is able to use oxidation products of several phenolic compounds (Galuszka et al., 2005) that comprise a major group of plant secondary metabolites, and thus can serve as a pool of electron acceptors required for proper CKX function. Recently, DIMBOA was isolated from maize phloem sap as a compound enhancing the degradation of isopentenyladenine by ZmCKX1, after oxidative conversion by either laccase or peroxidase that generate transitional free radicals used by CKX as highly effective electron acceptors (Frébortová et al., 2010).

As well as that of ZmCKX1, the crystal structure of AtCKX7 was also determined (Bae et al., 2008). Although the proteins share only 39.4% amino acid identity, the structures of both enzymes are very similar, showing a highly conserved active site.

**Variability of cytokinin-associated genes within the plant kingdom**

Shortly after completion of sequencing of the *Arabidopsis* genome, genome draft sequences of other plants across the whole kingdom have appeared. The prediction and annotation of gene orthologues to *Arabidopsis* cytokinin-associated gene families revealed a certain level of consistency, but also some adaptational diversity. Larger cereal genomes of rice and maize score a higher level of recent evolutionary duplication events; thus, for instance, the maize genome contains, in contrast to *Arabidopsis*, four pairs of CKX paralogous genes with very high homology (up to 90%; Vyroulabalová et al., 2009). Protein products of the paralogues in some of those pairs were described to be functional or have a different pattern of expression (Massonneau et al., 2004; Vyroulabalová et al., 2009). Interestingly, every genome
described so far contains just one copy of a conserved CKX with cytosolic compartmentation, including simple models of the first land plants Physcomitrella patens and Selaginella moellendorfii (Gu et al., 2010). This gene may have a unique role in cytokinin homeostasis due to the opposite effect on growth compared with other CKXs after overexpression in roots (Šmeihlová et al., 2009). Up to the present time, only the Arabidopsis CKX enzyme family has been completely functionally described. Particular members of the family show diverse catalytic efficiency toward differently substituted cytokinins and positional isomers of zeatin, but cis-zeatin can be effectively cleaved only by AtCKX1 (Petrey et al., 2010). On the other hand, all maize CKXs characterized to date are able to degrade cis-zeatin with the same or better efficiency than trans-zeatin (Šmeihlová et al., 2009; P Galuszka et al., unpublished). Considering cis-zeatin and its derivatives as the major cytokinins in maize tissues, such substrate specification can be the result of an evolutionary adaptation. Likewise, specificity for cis-zeatin has been observed for all known cytokinin-specific O-glycosyltransferases from maize (Veach et al., 2003). Other functionally described O-glycosyltransferases from bean have a preference for trans-zeatin, and three enzymes characterized in Arabidopsis, where cis-zeatin content is low, do not distinguish between the isomers (Hou et al., 2004). Nevertheless, phylogenetic analysis of several plant species showed that cytokinin-specific glucosyltransferases cannot be predicted on the basis of sequence similarity with already annotated genes as they clustered independently on several branches of the cladogram of numerous glucosyltransferase gene families. Moreover, some of those genes that cluster together with the defined genes were cloned and functionally tested without any activity toward cytokinins being found (Meek et al., 2008).

An evolutionary rigidity apparent among tRNA IPTs implies functional significance, where, in all genomes annotated to date, there are just two highly conserved genes. The first of them has a prokaryotic origin (AtIPT9 in Arabidopsis) and clusters together with the characterized bacterial genes; however, the second one (AtIPT2) evolved in higher plants later as it forms an independent clade on the phylogenetic tree close to IPT genes identified in human or Saccharomyces (Kakimoto, 2001) (Fig. 3). Surprisingly, only tRNA IPTs of prokaryotic origin were found in genomes of the basal land plant models Physcomitrella and Selaginella (Yevdakova and Schwartzenberg, 2007). Release of cytokinins as the first agents regulating cell division and other physiological processes was most probably fully dependent on decomposition of tRNA molecules during the conquest of land by the first simple plants. Divergence of de novo cytokinin biosynthesis took place later during the evolution of flowering plants most probably due to a demand for higher amounts of these growth regulators as degradation rates of RNA became a limiting factor. This is more intriguing in the light of the fact that a de novo adenylate IPT gene was found in some cyanobacteria; nevertheless, it has not been functionally tested yet. Hence, genesis of de novo cytokinin biosynthesis seems to be the last important step in development of a fully functioning perception system as we know it today (Pils and Heyl, 2009).

**Plant parasitic bacteria—cytokinin as a tool for invasion**

NCBI BLAST of all bacterial protein sequences (blastp) did not produce any significant hits showing conserved cytokinin-binding residues (Heyl et al., 2007) of the plant cytokinin receptor CHASE domain.

Some bacteria, however, produce cytokinins and use them mainly as a chemical signal either for communicating with the plant or as a tool for invading the plant host. Indeed, the first cytokinin biosynthetic gene, Tnr, was identified in the tumour-inducing plant pathogenic bacterium A. tumefaciens. The molecular structure of a protein encoded by Tzs, the other IPT gene of this bacterium, in complex with substrates was recently determined (Sugawara et al., 2008). Tmr and Tzs are very homologous proteins, but their amino acid residues crucial for substrate recognition differ from those of plant adenylate IPTs (Chu et al., 2010). Similar genes are found in other bacteria of the genus Agrobacterium, A. vitis and A. rhizogenes (Takei et al., 2001), and in other plant pathogenic bacteria (for a review, see Kakimoto, 2003).

Hypothetical genes for adenylate IPT were also found in Pantoea agglomerans that has been transformed from a common bacterium associated with many plants into a host-specific gall-forming pathogen by acquiring a plasmid-borne pathogenicity island (Barash and Manulis-Sasson, 2007),Ralstonia solanacearum causing bacterial wilt of Solanaceae, and Pseudomonas syringae (Takei et al., 2001).

T-DNA from A. rhizogenes contains three unique onogenes named rolA, rolB, and rolC. Due to the observed uncontrolled growth phenotypes after infection of plants, they were predicted to act as glucosidases releasing free active auxins and cytokinins from their storage forms (Estruch et al., 1991; Brzobohatý et al., 1993). However, more recent works suggested that the activity toward auxin and cytokinin glucosides observed in vitro is in fact an unspecified reaction without any physiological importance, and that rolC protein acts in vivo in stimulation of some alkaloid metabolic pathways rather than in cytokinin deglycosylation (Faiss et al., 1996; Bulgakov, 2008).

The interaction of a bacterial pathogen with the plant host in relation to cytokinins was studied in detail for the phytopathogenic actinomycete Rhodococcus fascians strain D188 (Petrey et al., 2009). The bacterium relies entirely on the linear plasmid-encoded fas operon for its virulence, secreting six cytokinin bases that synergistically redirect the developmental programme of the plant to stimulate proliferation of young shoot tissue, thus establishing a niche. The enzymatic activities of FasD (IPT), FasE (cytokinin dehydrogenase), and FasF (phosphoribohydrolase) proteins have recently been demonstrated (Petrey et al., 2010). In addition, it is likely that FasA is a putative P450 monoxygenase, which hydroxylates isopentenyladenine to form
Unrooted tree of selected bacterial and plant adenylate and tRNA isopentenyl transferases. All tRNA IPTs form a separate cluster with two branches, one including bacterial IPTs and the other plant and moss IPTs of prokaryotic origin; plant tRNA IPTs of eukaryotic origin form an independent branch in the adenylate IPTs cluster. There are only three prokaryotic adenylate IPTs included, one functionally described from the *Rhodococcus fas* operon and two hypothetical forms from the cyanobacteria *Anabaena* and *Nostoc*. Protein origin description: *Arabidopsis thaliana*, AtIPT1–AtIPT9, all functionally confirmed (Miyawaki et al., 2006); *Zea mays*, ZmIPT1–ZmIPT10, confirmed and predicted (Brugière et al., 2008; Vyroubalová et al., 2009); *Physcomitrella patens*, PpIPT1 (ABP88738), PpIPT2 (XP_001771407), PpIPT3 (XP_001780492), PpIPT4 (XP_001763870), PpIPT5 (XP_001754594), and PpIPT6 (XP_001782787); *Selaginella moellendorffii*, SmIPT (XP_002985201); *Rhodococcus fascians*, RfIPT, functionally confirmed (P46376); *Rhodococcus jostii*, RjIPT (YP_706705); *Anabaena variabilis* ATCC 29413, AvIPT1 (YP_323219) and AvIPT2 (YP_323028); *Nostoc sp. PCC7120*, NoIPT1 (BAB77744) and NoIPT2 (NP_489306); *Acaryochloris marina* MBIC11017, AmIPT (YP_001518792); *Microcoleus chthonoplastes* PCC 7420, McIPT (ZP_05024550); *Nodularia spumigena* CCY9414, NsIPT (ZP_01631398). *Except for the fas operon, there is not enough DNA sequence data for the R. fascians genome; however, three other species of the genus *Rhodococcus* have been completely sequenced, each containing one predicted tRNA IPT gene. The putative tRNA IPT, RjIPT, from *R. jostii*, the closest relative to *R. fascians*, was included in the tree to cover the predicted IPT family of the genus *Rhodococcus*. Protein sequences were processed using the ClustalW interface in BioEdit 7.0.5.3 (Hall, 1999) and the tree was visualized in TreeView 1.6.6 (Page, 1996).
trans- and/or cis-zeatin. Products of the other two fas open reading frames (ORFs) are also only putative; FasB is similar to ferredoxin/pyruvate decarboxylase subunit α and FasC is similar to pyruvate decarboxylase subunit β, both having a binding site for the cofactor thiamine pyrophosphate (Crespi et al., 1994). It is speculated that these two ORFs most probably code for subunits of αβ2 protein that participates in C–C bond cleavage and decarboxylation or two-carbon transfer (as a transketolase). If this protein is related to the metabolism of cytokinins, it may participate in the conversion of the unsaturated aldehyde resulting from the N6-isoprenoid side chain of cytokinin after cleavage with CKX. It also remains to be solved which enzymes are involved in the methylthiolation of cytokinins to yield the 2-methylthio derivatives abundantly produced by this bacterium (Pertry et al., 2009). A similar reaction mechanism applies to the Fas proteins of Streptomyces turgidiscabies, a scab-causing phytopathogen and to date the only other organism known to carry a fas operon (Joshi and Loria, 2007). In addition to six genes homologous to those of R. fascians, the operon, besides several other genes, contains two ORFs coding for methyltransferases (mtr-1 and mtr-2) that may be involved in the production of methylthiolated cytokinins. Related genes in R. faciens may be localized on the att operon; for example, the protein encoded by attC is annotated as a formyltransferase (Maes et al., 2001).

The 2-methylthio derivatives of cytokinins are often found in tRNA, where they are synthesized by the miaB gene (for a review, see Persson et al., 1994). The product of the gene, MiaB, is a bifunctional protein involved in both thiolation and methylation of isopentenyladenosine in tRNA (Pierrel et al., 2004). Another gene, miaE, encoding tRNA 2-methylthio-N6-isopentenyladenosine cis-hydroxylase, was found in Salmonella typhimurium (Persson and Björk, 1993), but it is a rare gene, present neither in E. coli (Persson and Björk, 1993) nor in eukaryotes (our BLAST search). The gene product was later characterized as a non-haem di-iron monooxygenase (Mathevon et al., 2007).

Putative proteins similar to R. fascians CKX (FasE) were identified in many other bacterial species such as Streptoluteichus hindustanensis, Myxococcus xanthus, Saccharopolyspora erythraea, Herpetosiphon aurantiacus, Stigmatella aurantiaca, and Streptomyces pristinae spiralis, but since their functional data are not available and no putative adenylate IPT genes were found in the genomes of these organisms, their function remains highly hypothetical (Pertry et al., 2010). Sequences related to CKX are also present in the genomes of several species of Legionella.

Cytokinin manipulation by other parasitic organisms

There are some other reports suggesting the role of cytokinins in the interaction of plants with other organisms such as insects or fungi, but these views are not supported by current genetic evidence. Leaf-mining herbivorous insects association with bacterial endosymbionts such as Wolbachia are thought to impact green-island induction on leaves through the manipulation of cytokinin levels (Kaiser et al., 2010), but no related sequences of the genes coding for IPT (adenylate or tRNA) or CKX were found. Similarly to plants, cytokinin-specific glucosyltransferases or glucosidases cannot be easily predicted by using sequence similarity.

It has been reported recently that the fungal phytopathogen Usitilago maydis produces cytokinins and abscisic acid for potential regulation of tumour formation in maize (Bruce et al., 2010). This observation contradicts the fact that an NCBI genomic BLAST (tblastn) over U. maydis and other available fungal genomes does not show any sequences similar to adenylate IPT from A. tumefaciens (YP_001967412). The same probably applies to the speculations that cytokinins might be involved in nutrient mobilization and green-island formation in plants infected with biotrophic fungi (reviewed in Walters and McRoberts, 2006).

Has cytokinin metabolism in plants evolved from cyanobacteria?

According to numerous reports, cyanobacteria are able to synthesize various classes of plant hormones, such as auxins, cytokinins, gibberellins, abscisic acid, ethylene, and jasmonates (Hussain et al., 2010). Although the presence of cytokinins in cyanobacteria was indicated (Stirk et al., 1999), definite proof of this and their quantification in isolates of Synechocystis, Chroococcidiopsis, Anabaena, Phormidium, and Oscillatoria by UPLC/MS were published only recently (Hussain et al., 2010). Analyses showed various contents of trans- and cis-zeatin, zeatin riboside, zeatin-O-glucoside, and dihydrozeatin riboside, with cis-zeatin often being the most abundant, except for in Anabaena, where it was not detected at all.

Shortly after the discovery of IPTs in Arabidopsis, the presence of highly homologous genes was found in cyanobacterial genomes. Nostoc sp. strain PCC 7120 (often also called Anabaena sp. PCC 7120) contains one gene each for adenylate IPT and tRNA IPT (Kakimoto, 2005). It was proposed that plant genes might have been acquired through lateral transfer from bacteria to plants via the chloroplast, which is of cyanobacterial origin (Schmittling et al., 2003).

Nostoc sp. PCC 7120 also contains a gene coding for CKX-like protein all0324 (NP_484368), which has homologous counterparts in other cyanobacteria such as Anabaena variabilis ATCC 29413 (YP_325209), Acaryochloris marina MBIC 11017 (YP_001515138), Microcoleus chthonoplastes PCC 7420 (ZP_0027841), and Nodularia spumigena CCY 9414 (ZP_01632120). The latter three species, however, do not contain a gene for the cytokinin biosynthetic enzyme, adenylate IPT, but only the one for the common bacterial tRNA IPT (see Fig. 3).

In the CKX structure, the FAD cofactor is linked to the histidine residue of the conserved GHS motif (Malito et al., 2004). However, the putative Nostoc CKX contains the motif GYT in the same position (starting from Gly67), in
Fig. 4. Alignment of cyanobacterial CKX-like proteins with CKXs from *Arabidopsis thaliana*. Protein origin description: *Arabidopsis thaliana*, AtCKX1–AtCKX7 (Bileyu et al., 2003); *Nostoc* sp. PCC 7120, NoCKX (NP_484368); *Anabaena variabilis* ATCC 29413, AvCKX (YP_325209); *Acaryochloris marina* MBIC11017, AmCKX, (YP_001515138); *Microcoleus chthonoplastes* PCC 7420, McCKX (ZP_05027841); *Nodularia spumigena* CCY9414, NsCKX (ZP_01632120). The position of the motif for covalent binding of FAD cofactor (GHS, where H is the binding residue in all plant CKXs) is indicated. Protein sequences were aligned using the ClustalW interface in BioEdit 7.0.5.3 (Hall, 1999). Amino acid residues predicted based on the structure of ZmCKX1 (Malito et al., 2004) to bind the cytokinin substrate are marked with an asterisk. The cytokinin N9 hydrogen-bonding residue that affects the substrate specificity (Galuszka et al., 2007) is marked with an arrow. Extending C-terminal sequences of NsCKX and McCKX were truncated by 16 and 37 amino acids, respectively.
Fig. 5. Unrooted tree of selected bacterial and plant cytokinin dehydrogenases. *Rhodococcus fascians* CKX separates the group of structurally conserved plant proteins from more variable CKX-like proteins of cyanobacteria. Protein origin description: *Arabidopsis thaliana*, AtCKX1–AtCKX7 (Bileyu et al., 2003), all functionally confirmed (Galuszka et al., 2007); *Zea mays*, ZmCKX1–ZmCKX12 (Výroubalová et al., 2009), confirmed and predicted; *Physcomitrella patens*, PpCKX1–PpCKX6, putative; *Selaginella moellendorffii*, SmCKX1–1–SmCKX2–2, putative; *Rhodococcus fascians*, RfCKX (CAA82745), functionally confirmed (Pertry et al., 2010); CKX-like proteins of cyanobacteria: *Anabaena variabilis* ATCC 29413, AvCKX (YP_325209); *Acaryochloris marina* MBIC11017, AmCKX (YP_001515138); *Microcoleus chthonoplastes* PCC 7420, McCKX (ZP_05027841); *Nostoc* sp. PCC 7120, NoCKX (NP_484368); *Nodularia spumigena* CCY9414, NsCKX (ZP_01632120). Protein sequences were processed using the ClustalW interface in BioEdit 7.0.5.3 (Hall, 1999) and the tree was visualized in TreeView 1.6.6 (Page, 1996).
which FAD might be linked to tyrosine (J Frébortová et al., unpublished data). The Nostoc protein also shows differences in the region around the substrate-binding residue, Asp169, in ZmCKX1 (Malito et al., 2004), and other regions well conserved in plant CKX proteins. These regions are also variable in other cyanobacterial CKX-like proteins, as shown in Fig. 4. In a phylogenetic tree (Fig. 5), all these cyanobacterial CKX analogues cluster independently of plant CKX proteins and show great diversity. It remains to be resolved whether they really function in cytokinin metabolism.

Concerning the cytokinin-related genes, it seems that some cyanobacteria contain the minimal genetic apparatus needed for the management of biosynthesis and metabolic control of the cytokinin level, which later evolved into a more complex system that we see in today’s plants. On the other hand, cytokinin signal transduction machinery that became important to plants during the conquest of land (Pils and Heyl, 2009) is not found in cyanobacteria. The typical CHASE sequence, which contains the key cytokinin-binding residues of the receptor histidine kinase found in plants (Heyl et al., 2007), could not be identified. NCBI BLAST (tblastn) with the AHK4 CHASE domain (Heyl et al., 2007) over the available sequence databases of cyanobacteria did not produce any significant homologies in Nostoc sp. or other cyanobacteria. Even though it has the CHASE-like domain, the regulatory protein sl0267 from Synechocystis sp. PCC 6803 (Mougel and Zhulin, 2001) does not contain the key cytokinin-binding residues as well. Accordingly, no sequences similar to cytokinin type B response regulators were found.

Cyanobacteria, on the other hand, show responses to external addition of cytokinins such as growth promotion (Kapoor and Sharma, 1981; Suzuki et al., 2006) and enhanced transcription (Selivankina et al., 2006). It is therefore reasonable to expect that there exists a signalling pathway different from the one known in plants.

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