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Metabolism of Zeatin-Type Cytokinins in Monocotyledonous Plants

Ph.D. THESIS

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I hereby declare that this thesis has been written solely by myself and that all the sources used in this thesis are cited and included in the References part.

In Olomouc 19th October 2017 …….

Tomáš HLUSKA

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Abstrakt

Cytokininy jsou rostlinné hormony, deriváty adeninu s postranním řetězcem v N^6-poloze. Ovlivňují mnoho fyziologických procesů.

Zatímco metabolismus *trans*-zeatinu a isopentenyladeninu, které jsou považovány za vysoce aktivní cytokininy, byl extenzivně studován, metabolismus dalších, jako *cis*-zeatin, dihydrozeatin, či aromatické cytokininy je opomíjen.

V této práci jsme použili jako model pro studium metabolických přeměn cytokininů, hlavně zeatinů, vyvíjející se kukuřičné zrno. Místo zeatin reduktasy jsme však zjistili novou enzymatickou aktivitu přeměňující *trans*-zeatin na 6-(3-methylpyrrol-1-yl)purin. Enzym se však nepodařilo identifikovat kvůli jeho nestabilitě. Zato se nám podařilo identifikovat enzym odpovědný za *cis-trans* isomeraci zeatinů jako nukleotidpyrofosfatasu/fosfodiesterasu. Potvrdili jsme jeho aktivitu; za substráty preferuje FAD a jiné dinukleotidy. Navrhli jsme několik jeho funkcí *in planta*.

Dále jsme navrhli dvě nové hypotézy týkající se metabolismu a fyziologie cytokininů. První vysvětluje převahu údajně neaktivního *cis*-zeatinu v některých rostlinách. Druhá navrhuje dvourychlostní cytokininový systém.

Klíčová slova	anticytokininy, *cis*-zeatin, cytokininy, flaviny, isomerace, kukuřice, metabolismus, nukleotidpyrofosfatasa/fosfodiesterasa, semena, vývoj semene, zeatin *cis*-*trans*-isomerasa, zeatiny
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Abstract

Cytokinins are plant hormones, derivatives of adenine with side chain at the N^6-position. They are involved in many physiological processes.

While the metabolism of *trans*-zeatin and isopentenyladenine, that are considered to be highly active cytokinins, was extensively studied, the metabolism of minor cytokinins as *cis*-zeatin, dihydrozeatin and aromatic cytokinins is largely neglected.

Here we used maize developing seed as model to study interconversions of cytokinins, mainly zeatins. Instead of zeatin reductase, we have detected novel enzymatic activity converting *trans*-zeatin to 6-(3-methylpyrrol-1-yl)purine. The enzyme was not identified due to its instability. On the other hand, we have identified the causative enzyme of zeatin *cis-trans* isomerization as nucleotide pyrophosphatase/phosphodiesterase. Activity of the enzyme was confirmed; it prefers FAD and other dinucleotides as substrates. We proposed several functions for the enzyme *in planta*.

Further, two novel hypotheses concerning cytokinin metabolism and physiology are presented. The first explains prevalence of *cis*-zeatin, a purportedly inactive cytokinin, in certain species. The second one proposes a two-speed cytokinin system.

Keywords	Anticytokinins, *cis*-Zeatin, Cytokinins, Flavins, Isomerization, Kernels, Maize, Metabolism, Nucleotide Pyrophosphatase/Phosphodiesterase, Seed Development, Zeatin *cis–trans* Isomerase, Zeatins
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AIM OF WORK

⇒ review on cytokinins and their metabolism with focus on *cis*-zeatin and its significance and metabolism in plants

⇒ study of cytokinin metabolism in maize kernels

⇒ identification of novel metabolite of *trans*-zeatin; elucidation of its activity and biosynthesis

⇒ purification and identification of zeatin *cis-trans* isomerase from maize

⇒ characterisation of the responsible protein

INTRODUCTION

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Cytokinins (CKs) are plant hormones involved in many physiological processes. They are considered with auxins as one of the main groups of phytohormones as they together control cell division and hence influence the overall plant's architecture.

First cytokinin was discovered in the mid of 50s of the last century (Miller et al., 1955). The cytokinetic activity was detected in old and autoclaved DNA and the purified compound was named kinetin. This spurted search for compounds, natural and synthetic, with similar activity. Interestingly, the first compound with CK-like activity isolated from a natural source (that is, not degraded DNA) was *N,N'*-diphenylurea (Shantz and Steward, 1955). It was isolated from coconut milk, as that was previously recognized to induce cytokinesis. It was, in fact, used in growth media before the discovery of cytokinins. However, the identified *N,N'*-diphenylurea was probably present due to contamination as the coconuts were processed in a facility where urea herbicides were previously synthesised (Jacobs, 1979). To date, presence of *N,N'*-diphenylurea in coconut milk was not confirmed. On the other hand, presence of the kinetin in coconut milk was validated (Ge et al., 2005).

Thus the first unambiguously identified natural cytokinin was zeatin (Letham, 1963), presumably the *trans* isomer, as the purification was followed by bioassay for cell division (see below).

As a general requirement for cytokinin activity, intact adenine ring and N^6-substituent of moderate size were recognised, with a notable exception of the phenylurea derivatives (Skoog and Armstrong, 1970). Nowadays, CKs are considered as N^6-derivatives of adenine, regardless of their activity in classical cytokinin bioassays.

Isoprenoid and aromatic cytokinins are recognised in accordance to the nature of the side chain (Fig. 1). Modifications of the side chain further define properties of the respective cytokinin types.

The function of CKs can be summarised as trigger of cellular change essential for numerous decisions throughout the plant's life. Both developmental processes and adaptive responses to various abiotic and biotic inputs. The response is further influenced by crosstalk with other signals that vary with cell's history and thus context (Zürcher and Müller, 2016).

cis or *trans* – That's What Matters

The first cytokinin isolated from a natural source was zeatin obtained from immature sweet corn (*Zea mays*, hence the name) seeds (Letham, 1963). As the hydroxymethyl group is positioned at a double bond, zeatin forms two geometric isomers. Quickly

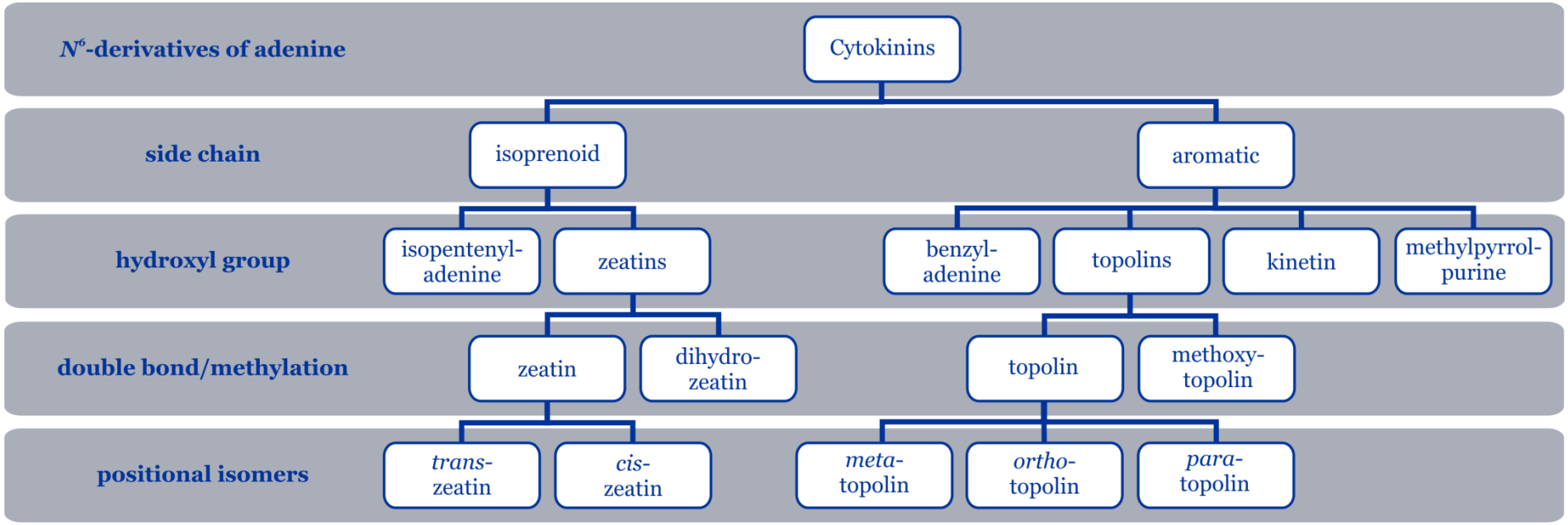

Fig. 1: Cytokinin types. Cytokinins are N^6-derivatives of adenine either with isoprenoid or aromatic side chain. Hydroxyl group further differentiates zeatins from isopentenyladenine and topolins from benzyladenine. Additional aromatic cytokinins exist – kinetin and 6-(3-methylpyrrol-1-yl)purine. Methoxytopolins exist in analogous isomers as topolins. Created by Lucia Gallová.

it was established, that the highly active compound is *trans*-zeatin (*t*Z). Soon, *cis*-zeatin (*c*Z) was identified in tRNA (Hall et al., 1967). However, its activity in the classical bioassays was miniscule (Mok et al., 1978; Schmitz et al., 1972; Vreman et al., 1974). Also the activity of several enzymes of cytokinin metabolism (especially of the catabolic cytokinin oxidase/dehydrogenase) towards *c*Z was often negligible and thus *cis*-zeatin was often neglected.

Over the years, however, the number of reports of plants or plant organs containing predominantly the *cis*-isomer grew. This includes potatoes (Lulai et al., 2016; Mauk and Langille, 1978; Suttle and Banowetz, 2000), unfertilized hop cones (Watanabe et al., 1982), rice (Kudo et al., 2012; Takagi et al., 1985), chickpea and white lupine (Emery et al., 1998, 2000), maize (Veach et al., 2003; Vyroubalová et al., 2009; Zalabák et al., 2014), *Tagetes minuta* (Stirk et al., 2005, 2012a), pea (Quesnelle and Emery, 2007), *Lolium rigidum* (Goggin et al., 2010), lucerne and oats (Stirk et al., 2012b) or oilseed rape (Tarkowská et al., 2012). The large-scale analyses in the plant kingdom (Gajdošová et al., 2011) and in bryophytes (Záveská Drábková et al., 2015) revealed, that the *c*Z-dominance is not a unique trait.

Further, *c*Z shows activity in systems that are relevant to it. Development of pea embryo is promoted by *c*Z (Quesnelle and Emery, 2007). It was also shown to inhibit growth of roots of *c*Z-dominant rice, a trait typical for CKs (Kudo et al., 2012).

Moreover, the low activity in bioassays and low content in plants could suggest that it is indeed only an artefact from tRNA hydrolysis, without physiological significance and that the interaction of enzymes is merely because of low ability to discriminate between the isomers. When *c*Z was found to be active in some bioassay, it was usually explained by isomerization to the *trans*-isomer. Only the discovery of the first enzyme strictly specific towards *c*Z (Martin et al., 2001) unambiguously showed, that its levels must be regulated by the plant and it is thus physiologically important. Also the direct interaction of *c*Z with CK receptors (Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004) showed that it is solely responsible for its activity. And even though majority of CKX enzymes degrade *c*Z inefficiently, there are few isoenzymes that even prefer *c*Z over the other CKs (Gajdošová et al., 2011; Köllmer et al., 2014; Zalabák et al., 2014). Thus if *trans*-zeatin were the big and strong brother of the Zeatins family, the *cis*-zeatin would be the, maybe weaker, yet more resilient brother.

The author's main objection to the proposal, that *c*Z is synthesised mainly or exclusively *via* tRNA was that it would be a waste of energy and resources for the plant (in terms of synthesis of tRNA for production of significant amounts of hormone). However, as the plant tRNA contains mainly *cis*-zeatin (see below and Miyawaki et al.,

2006), which is the main cytokinin involved in stress responses (as reviewed in Schäfer et al., 2015), it is only logical, that a specific compound (i.e. not unmodified nucleotides) released from RNA serves as stress signal because the RNA hydrolysis is stress-sensitive. Moreover, regular RNA turnover keeps the level of *c*Z, which may serve as the maintenance deadpool signal (see below). Further, *c*Z has an advantage to serve as both stress and maintenance signal, because the plant already possesses a response system, that slightly cross-reacts to it, unlike for other modified bases in RNAs.

In that case how about the plants that contain predominantly *c*Z as maize, rice and many others? As we have recently proposed, that is consequence of a random evolutionary chance leading to differential preference of *O*-glucosyltransferases towards zeatin isomers. As will be discussed below, these enzymes temporarily deactivate CKs by attaching a glucose molecule to their side chain. This, however, makes them resistant to removal by catabolic enzyme cytokinin oxidase/dehydrogenase. Thus if this *O*-glucosyltransferase is specific towards *c*Z, it accumulates and becomes the dominant CK-type. But if the *O*-glucosyltransferase prefers the *t*Z, the *trans*-zeatin is the main CK. It may even not discriminate between the isomers well as is the case of *Arabidopsis* enzyme, but the higher production of *t*Z leads to its prevalence over *c*Z.

Dihydrozeatin

The reduced form of zeatin – dihydrozeatin (DHZ) is less active than *trans*-zeatin (Skoog and Armstrong, 1970). Nevertheless, it was first isolated from yellow lupine immature seeds following activity measurement using the tobacco callus bioassay (Koshimizu et al., 1967a, 1967b) and it showed substantial activity also in other works (Mok et al., 1978; Schmitz et al., 1972). As a matter of fact, it was the most active isoprenoid cytokinin in *Phaseolus vulgaris* bioassay (Mok et al., 1978). The carbon bearing methyl and hydroxymethyl is chiral. Although the naturally occurring DHZ was identified as (*S*)-dihydrozeatin (Fujii and Ogawa, 1972), the (*R*)-isomer is more active in the bioassays (Matsubara et al., 1977). Further, it is capable to activate at least some CK receptors (Choi et al., 2012; Kuderová et al., 2015; Lomin et al., 2011; Spíchal et al., 2004).

However, it is omitted from many analyses and hypotheses nowadays (e.g. Kasahara et al., 2004; Miyawaki et al., 2006). That is probably because of its in general low quantities and because of complete lack of knowledge about its metabolism. For example, there are only three publications dealing with the zeatin reductase, the putative DHZ biosynthetic enzyme, published over the course of 35 years (Gaudinová et

al., 2005; Martin et al., 1989; Sondheimer and Tzou, 1971). We even don't know how DHZ is removed from the plant, as it is resistant to degradation (Galuszka et al., 2007). There is a single report of metabolism of DHZ to adenine in maize (Podlešáková et al., 2012) Otherwise, there is no evidence of other conversion besides glycosylations. Thus, DHZ is the addicted relative, nobody is talking about.

Aromatic Cytokinins

Aromatic cytokinins are adenine derivatives with aromatic side chain (Fig. 1). They include N^6-benzyladenine (BAP) and its hydroxy- and methoxyderivatives (Strnad, 1997; Tarkowská et al., 2003), N^6-furfuryladenine (kinetin; Ge et al., 2005) and recently (re-)discovered 6-(3-methylpyrrol-1-yl)purine (MPP) and its derivatives (this work; Haidoune et al., 1990; Sørensen et al., 2017).

Unlike the metabolism of isoprenoid CKs that has been extensively studied, the metabolism of aromatic CKs, mainly their biosynthesis, is largely unknown. This is partly because of their scarce occurrence and hence lack of suitable model. So far, they are known to be very weak substrates of the degradation enzyme (Galuszka et al., 2007; Laloue and Fox, 1989), that is why they are very effective as morphogens in various *in vitro* culture techniques. They are glycosylated in a pattern analogous to recognition by receptors (Mok et al., 2005). Paradoxically, we have unambiguous information about biosynthesis only for the newest aromatic cytokinin – 3-methylpyrrolpurine. It is synthesised directly from *trans*-zeatin, albeit the biosynthetic enzyme was not purified and identified due to its instability (this work).

Natural occurrence of aromatic cytokinins has been confirmed only in few plant, algae and microorganism species (Doležal et al., 2002; Edlund et al., 2017; Stirk et al., 2012a, 2012b; Strnad, 1997; Tarkowská et al., 2003). Moreover, in many cases, their detection in living samples might be the result of labware contamination due to their high stability, adhesiveness to glass surfaces and massive usage in *in vitro* culturing (Ondřej Novák, personal communication). It was hypothesised that the aromatic cytokinins in plants may in fact originate from symbiotic bacteria (Miroslav Strnad, personal communication). That would explain disappearance of BAP and its metabolites from poplar callus over time of several *in vitro* passages (Pavel Jaworek, personal communication) as well as the inconsistency in detection of aromatic cytokinins in various plants (RJ Neil Emery, personal communication).

Cytokinin Biosynthesis

The biosynthesis of cytokinins starts with the addition of a side chain to an adenine either in nucleotide form, or bound in tRNA. Several additional steps are generally included in the CK biosynthesis as hydroxylation of the side chain, removal of phosphates and/or ribose, reduction of the double bond in *trans*-zeatin's side chain (Fig. 2). The biosynthesis of cytokinins was reviewed by author previously (Frébort et al., 2011).

Recently, the research is focused largely on *Arabidopsis* and on iP and *t*Z as the main active cytokinins and thus there is certain bias. As has been mentioned above, the synthesis of DHZ from *t*Z received very little attention so far and also the biosynthesis of aromatic cytokinins remains complete enigma to date. Another example is the assumption of strict preference of precursor use for synthesis of iP and *t*Z or *c*Z, although fungi, animals and many bacteria do not possess the methylerithrytol pathway (see below) purportedly responsible for iP and *t*Z precursor synthesis and/or they lack enzymes for *de novo* CK synthesis.

Isopentenyltransferase

The first step of CK biosynthesis is catalysed by isopentenyltransferases. The correct name is dimethylallyltransferase, as dimethylallyl diphosphate is substrate, rather than isopentenyl diphosphate. However, throughout this work, the commonly used name isopentenyltransferase (IPT) will be used.

The addition of isoprenyl side chain is always referred to as the rate limiting step of CK biosynthesis (Kamada-Nobusada and Sakakibara, 2009). Taking into account that the free bases, that are considered to be the active forms, are usually present in minute quantities, while the ribosides and ribotides are usually present in much higher quantities, it seems probable that other step in the cytokinin biosynthesis is the real bottleneck.

There are currently three classes of isopentenyltransferases recognized to be involved in CK biosynthesis: adenylate dimethylallyltransferase (AMP-dependent; EC 2.5.1.27), tRNA dimethylallyltransferase (EC 2.5.1.75) and adenylate dimethylallyltransferase (ADP/ATP-dependent; EC 2.5.1.112). At this moment, only the tRNA IPTs seem to strictly adhere to the single activity of dimethylallyl pyrophosphate:adenine[37] in tRNA dimethylallyltransferase. The adenylate IPTs, on the other hand, are enzymes utilizing multiple substances both as acceptor and as donor substrates (see below).

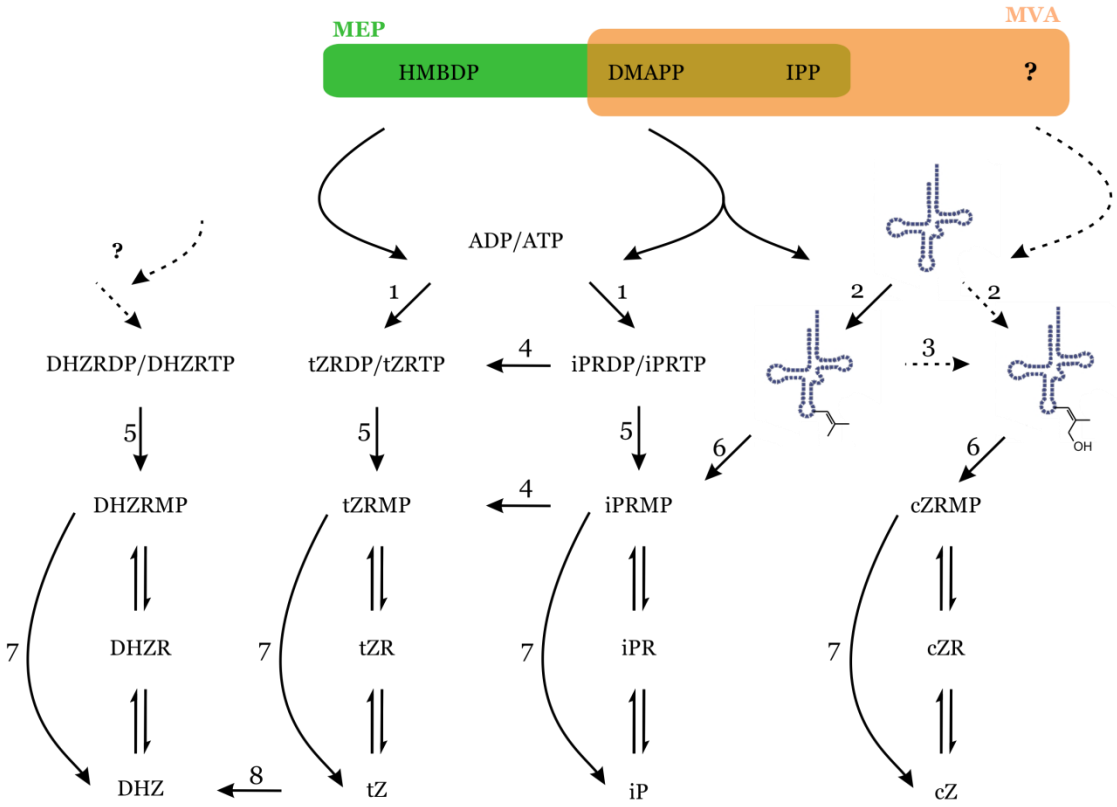

Figure 2: Biosynthesis of cytokinins in plants. Precursors for CK biosynthesis, HMBDP and DMAPP, come either from methylerythritol (MEP) or mevalonate (MVA) pathway. Plant adenylate IPTs (1) utilize mostly ADP or ATP and tRNA IPTs (2) use adenine in position 37 of certain tRNAs as acceptor substrate. *cis*-Zeatin shall originate from tRNA, but its synthesis is unclear, as neither *cis*-hydroxylated precursor, nor *cis*-hydroxylase (3) were hitherto identified in plants. Nucleotides of iP may be hydroxylated by cytochrome P450 (4) to form tZ. Upon hydrolysis of β- and γ-phosphates (5) or tRNA hydrolysis (6) the resulting monophosphates may be activated in one step by CK-specific phosphoribohydrolase named Lonely guy (7). Alternatively, the nucleotides, nucleosides and nucleobases are probably interconverted by enzymes of purine metabolism. Free base of tZ may be reduced to DHZ by zeatin reductase (8). Whether there is any *de novo* biosynthesis of DHZ is currently unknown. Created by Lucia Gallová.

In plants, there are two pathways providing isoprenoid units with distinct localisation and usage of the products. The first one is mevalonate pathway (MVA) that operates in cytosol of plants and is present also in animals, fungi, Archea, some bacteria, *Trypanosoma* and *Leishmania*. The end-product is Δ^3-isopentenyl pyrophosphate (IPP) that can be freely isomerized to dimethylallyl pyrophosphate (DMAPP). No hydroxylated intermediate was found to date in this pathway. The enzymology of this pathway was reviewed (Miziorko, 2011). The second pathway is called methylerythritol pathway (MEP) or non-mevalonate pathway and operates in plastids of plants, in green algae and in majority of bacteria. Series of steps leads to (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBDP), that is ultimately reduced to yield either IPP or DMAPP. There is exchange of IPP and DMAPP in between the

compartments to some extent (Laule et al., 2003). DMAPP and HMBDP serve as side chain donors in iP and *tZ* biosynthesis, respectively (Sakakibara et al., 2005).

In the reports concerned more with the IPT enzymology, than the CKs' function, wider substrate specificity was reported, including mono-, di- and tri- nucleotides of purine as well as pyrimidine bases (Abe et al., 2007; Chu et al., 2010b). Interestingly, the *Hl*IPT from hop is capable to bind diadenosine polyphosphates and use them as substrates. It is even capable of forming diisoprenylated product (Chu et al., 2010a). However, NAD and FAD are not substrates. *Ma*IPT from mulberry prefers the DMAPP as the donor substrate, but it is capable to use also HMBDP, geranyl pyrophosphate (C_{10}) and IPP. Farnesyl pyrophosphate (C_{15}) is not an acceptable substrate for *Ma*IPT (Abe et al., 2007). The *Hl*IPT possesses large cavity, that shall be also able to accommodate geranyl pyrophosphate (Chu et al., 2010b).

Although it was assumed in the past, that CKs are synthesised in the root and transported to the aerial part, expression analysis showed, that *IPT* expression is widespread throughout the whole plant (Chen et al., 2014; Liu et al., 2013; Miyawaki et al., 2004; Sakamoto et al., 2006; Song et al., 2015; Takei et al., 2004a; Vyroubalová et al., 2009; Ye et al., 2006). It is also responsive to other hormones, mostly auxin (Liu et al., 2013; Miyawaki et al., 2004; Tanaka et al., 2006; Ye et al., 2006) and to some nutrients, mostly to nitrate (Miyawaki et al., 2004; Takei et al., 2004a).

Besides differential expression, the proteins are localized to various subcellular compartments. With the exception of cytosol-localized *At*IPT4 and mitochondria-localized *At*IPT7, the rest of *Arabidopsis* adenylate IPTs was shown to be localized to plastids (Kasahara et al., 2004). Among them *At*IPT3 and *At*IPT5 are the mostly expressed ones (Takei et al., 2004a). However, this result was based on C-terminal fusion with green fluorescent protein (GFP). When the GFP was fused to the N-terminus, *At*IPT3 was localized to nucleus due to farnesylation (Galichet et al., 2008). A non-farnesylable C333S mutant was placed in plastids, unless it lacked the chloroplast transit peptide. In that case it was localized in cytoplasm (Galichet et al., 2008). It was hypothesized, that the dual localization may be a regulation dependent on availability of isoprenoid units coming from MVA and MEP pathways.

The Tmr protein with the IPT activity encoded by *Agrobacterium tumefaciens*' Ti-plasmid lacks any transit peptide. It localizes to chloroplasts nevertheless (Sakakibara et al., 2005; Ueda et al., 2012).

Is There an Alternative Pathway for Cytokinin Biosynthesis?

There are few lines of evidence for additional CK biosynthetic pathway. For example, 2-MeS-iP was shown to be synthesised independently of the *fas* operon in *Rhodococcus fascians* (Pertry et al., 2009) and its levels were increased in mutants of all genes in the *fas* operon (Pertry et al., 2010). Indeed, hitherto 2-MeS-CKs are known to be synthesised only in tRNA. But 2-MeS-*c*Z levels were decreased in *ipt* and *cyp450* mutants (Pertry et al., 2010). Furthermore, *c*Z was also decreased in *cyp450* mutant, although it should originate from tRNA hydrolysis (Miyawaki et al., 2006). Additionally, *Physcomitrella patens* bears only *tRNA IPTs* of which only *PpIPT*1 is significantly expressed (Lindner et al., 2014). Upon knock-out of this gene, CKs in tRNA were practically missing and the levels of free *c*Z were greatly reduced. The amount of free iP and *t*Z rose about 4-times though. The authors claim, that no other *IPTs* were significantly upregulated, but it was previously reported that badly designed primers may be responsible for lack of *PpIPTs* expression (Patil and Nicander, 2013). Third, *Leptosphaeria maculans* is a fungi with single *IPT* gene identified (Trdá et al., 2017). Upon silencing to ~10 % of expression, *c*Z levels decreased by ~15 %, but iP-type CKs increased. Note, that in all three instances, iP-type CKs were primarily increased.

So, is there an alternative pathway for CK biosynthesis? As the Betteridge's law rules, the answer is probably no. In the case of *Rhodococcus* and *Physcomitrella*, other *IPT,* specifically *tRNA IPTs*, could be responsible for the increase of cytokinins. The *Leptosphaeria* is supposed not to contain any other *IPT* gene. The mutant still contained almost as much CKs as WT. This could be due to elevated post-transcriptional regulation counter-balancing decreased transcription. However, the changed CK profile (*t*Z and DHZ remained the same, *c*Z decreased and iP increased) suggests that another enzyme may be at play.

Hydroxylation of Isopentenyladenine

In the iP-dependent pathway, zeatins are formed by hydroxylation of the side chain.

This was observed in the cauliflower for the first time (Chen and Leisner, 1984). NADPH-dependent microsomal proteins converted iP riboside (iPR; all CK abbreviations are in accordance to Kamínek et al., 2000) to *t*Z riboside (*t*ZR) and iP to *t*Z. It was deducted, that cytochrome P450 monooxygenase (CYP) is responsible for the reaction, as it was inhibited by metyrapone, ethylene and CO (Chen and Leisner, 1984). Later, two such CYPs were identified in *Arabidopsis* (Takei et al., 2004b). The enzymes utilize nucleotides of iP, mainly mono- and diphosphate. Free base and riboside were very weak substrates. The only products detected *in vitro* were *t*Z-type cytokinins.

Interestingly though, *Saccharomyces cerevisiae* expressing *AtIPT*4 with the cytochromes, contained also DHZ-type cytokinins. The levels reached up to 50 % and 80 % of *t*Z-type in the strain expressing *CYP735A*1 for free base and riboside, respectively.

*CYP735A*1 was expressed the most in roots and flower, but expression of *CYP735A*2 reached about 20-times higher levels in the root and in the stem (Takei et al., 2004b). The expression of *CYP735A*2 is restricted to the columella cells and to the vascular bundle (Kiba et al., 2013). Further, the expression of both *CYPs* is upregulated by iP and *t*Z and downregulated by auxin and abscisic acid. Again, *CYP735A*2 is much more responsive than *CYP735A*1 (Takei et al., 2004b).

It was confirmed, that the CYP735As are major contributors to *trans*-zeatin synthesis, when double knock-out mutant had <5 % of *t*Z-type cytokinins, while iP-type CKs were increased to ~200 % (Kiba et al., 2013). The mutant's phenotype resembled the phenotype of other CK-deficient or CK-insensitive mutants *ipt*3 5 7 and *ahk*2 3. Although the plant's growth was affected mainly in the shoot and barely in the root (Kiba et al., 2013), it showed increased defects in lateral root primordia positioning (Chang et al., 2015). The phenotype could be rescued by supplementation of *t*Z, but not iP or DHZ, thus reflecting the lack of *t*Z as the causative agent of the phenotype (Kiba et al., 2013).

The *cis*-hydroxylase is known to act upon iP in tRNA (Persson and Björk, 1993). The gene was named *Mia*E in *Salmonella typhimurium* and it is not present in *Escherichia coli*, hence absence of *c*Z in *E. coli*'s tRNA. The MiaE protein was identified to be a nonheme diiron monooxygenase (Mathevon et al., 2007). A structural model of MiaE was created; it belongs to ferritin-like fold proteins, suggesting it might form homo- or hetero-oligomers (Kaminska et al., 2008). The gene is in general present only in few species, incl. for example *Nostoc* (Kaminska et al., 2008). But no *Mia*E homologue has been identified up to now in plants. Interestingly, the NCBI database contains sequence of *Mia*E from fungus *Beauveria bassiana* (KGQ13519). This fungus belongs to family *Clavicipitaceae* and is pathogen of various arthropod species. Identical sequence was found in *Klebsiella michiganensis* though. Thus it may be either contamination during sequencing or it was obtained through horizontal gene transfer. Still, this protein and a homologue from *Enterobacter cloacae* (identity 78 % over 96 % of sequence length) contain additional RraB domain. The RraB proteins are found only in *Gammaproteobacteria* and bind to RNase E to regulate its activity (Yeom et al., 2008). The *K. michiganensis* protein does not contain this domain. Thus there is one protein with 100 % identity, but lacking a domain and another protein with the domain, but not identical. This hints on the horizontal gene transfer.

cis-Zeatin – *Unde es*?

Unlike the biosynthesis of *t*Z and iP, the biosynthesis of *cis*-zeatin remains a mystery. Nowadays, it is accepted, that *c*Z originates from tRNA (Miyawaki et al., 2006). However, that doesn't answer the question, where does the *cis*-hydroxylated side chain originate from. There are in principle four possibilities for *cis*-zeatin biosynthesis:

1) isoprenylation of adenine, either free or tRNA-bound, with (*Z*)-4-hydroxy-3-methylbut-2-enyl diphosphate
2) hydroxylation of iP, either free or tRNA-bound
3) isomerization of *t*Z
4) dehydrogenation of dihydrozeatin

The first two options are analogies to *t*Z biosynthesis. However, there is no evidence for either of them to function *in planta* at the moment.

Zeatin *cis-trans* isomerase was described (Bassil et al., 1993), but its existence was recently disproved (this work). Indeed, majority of the *in planta* evidence is against existence of zeatin *cis-trans* isomerase (Gajdošová et al., 2011; Kasahara et al., 2004; Kuroha et al., 2002; Miyawaki et al., 2006; Nandi and Palni, 1997; Yonekura-Sakakibara et al., 2004), with the exception of few extreme conditions (this work; Kudo et al., 2012; Suttle and Banowetz, 2000; Trdá et al., 2017).

The last option is merely hypothetical possibility; no such enzyme has ever been reported. However, dihydrozeatin is resistant to CKX cleavage (Galuszka et al., 2007; Šmehilová et al., 2009; Zalabák et al., 2014), but it disappears during seed imbibition and following seedling growth. Thus it was hypothesised, that DHZ could serve as source of active cytokinins before *de novo* biosynthesis starts (Václavíková et al., 2009). Indeed, author attempted to measure the putative dihydrozeatin oxidase. Although no *t*Z or *c*Z were detected, substantial amount of adenine was formed as a consequence of substrate degradation (Hluska, 2010). Accordingly, Podlešáková et al. (2012) reported conversion of DHZ9G to adenine in maize.

Thus the *cis*-zeatin biosynthesis still remains a big unknown.

Activation of Cytokinins

The cytokinins are synthesized as nucleotides, but free bases and ribosides are the active forms. Thus hydrolysis of phosphates and – optionally – of ribose must precede.

Enzymes with wide substrate specificities as 5'-nucleotidase, or alkaline or acid phosphatase were thought to dephosphorylate the nucleotides. Also the enzymes of

nucleotide salvage pathway may be of importance to CK metabolism as reviewed by Chen (1997).

Nevertheless, a CK-specific phosphoribohydrolase was identified in rice (Kurakawa et al., 2007). Mutant of the corresponding gene in rice was identified based on screen for plants with defects in shoot meristem. Flowers of this particular one often contained sole stamen and no pistil. Hence it was called *lonely guy* (*log*). The defective gene was annotated as lysine decarboxylase, but no lysine decarboxylase activity could be detected with a recombinant protein. Because of clustering of homologues in *Agrobacterium rhizogenes* and *Rhodococcus fascians* to *IPT* genes, its involvement in cytokinin metabolism was elaborated. Indeed, it was shown to activate the cytokinin nucleotide monophosphates in one step. Di- or triphosphates were not substrates, just as AMP or CK nucleosides and free bases (Kurakawa et al., 2007). Similar results were obtained with LOG from pathogenic fungus *Claviceps purpurea* (Hinsch et al., 2015)

Rice and *Arabidopsis* genome contain 11 and 9 *LOG* genes, respectively (Kurakawa et al., 2007; Kuroha et al., 2009). The *Arabidopsis* proteins in general do not discriminate much between respective nucleotide monophosphates of isoprenoid CKs. Activity with BAPRMP ranged approx. from 5 to 75 % of the activity with iPRMP though (Kuroha et al., 2009). Upon *LOG* overexpression, plants showed increased levels of iP, iP7G and iP9G; decreased levels of iPRMP and levels of iPR remained the same. Interestingly though, plants overexpressing *AtLOG*4 had decreased levels of *t*Z and *t*ZOG in the shoots (although they were increased in the roots; Kuroha et al., 2009).

*AtLOG*7 possesses a central role in the CK activation as knockout of this sole gene led to pronounce alterations of CK metabolism. Contrary multiple knock-out lacking all *AtLOGs*, but *AtLOG*6, *AtLOG*7 and *AtLOG*9 (authors of the study did not create mutants of *AtLOG*6 and *AtLOG*9 at all) had CK levels comparable to wild-type (WT) plants (Tokunaga et al., 2012). Remarkably, *At*LOG7 is the only protein with slightly higher activity than the rice LOG protein, with the rest of proteins with much lower activity. Also, *At*LOG7 has the most levelled activity towards all isoprenoid cytokinins. Its expression is rather low, though (Kuroha et al., 2009). On the other hand, the most expressed gene is *AtLOG*8, but the corresponding enzyme has the lowest activity (Kuroha et al., 2009).

A fusion protein of isopentenyltransferase and lonely guy was discovered in *Rhodococcus fascians* (Creason et al., 2014), *Claviceps purpurea* (Hinsch et al., 2015), in *Fusarium pseudograminearum* (Sørensen et al., 2017) and several other *Fusarium* species (Niehaus et al., 2016). Most species of the *Fusarium fujikuroi* species complex

have two genes coding for the protein. *Fusarium proliferatum* ET1 has even three *IPT-LOG* genes (Niehaus et al., 2016). These organisms with IPT-LOG fusion proteins are all pathogens, that need to purge their host with active cytokinins to have it their way, rather than tightly balancing the production of CKs as required by plants. Thus it is advantageous for them to link the production of CKs with their activation, unlike for plants that strictly regulate spatio-temporal distribution of both activities.

All of the fungal fusion genes are in cluster with cytochrome P450 either in head-to-head or head-to-tail orientation. CpIPT-LOG was able to perform both activities. It showed high preference for DMAPP over HMBDP. ADP and ATP could not serve as substrates. (Hinsch et al., 2015).

Still, LOG may provide the more straightforward option for CK activation, but there is some circumstantial evidence indicating the significance of the two-step activation. First, there are considerate amounts of ribosides *in planta*, often at similar, if not higher level than of the nucleotides (e.g. this work; Tarkowská et al., 2012; Zalabák et al., 2014). If LOG activation were the main option, the ribosides would be expected to be present only in minute quantities and the nucleotides to be the major form among the active trinity of nucleobases, nucleosides and nucleotides. Second, the ribosides are the main transport form of CKs (Hirose et al., 2008). The CKs could be dephosphorylated before loading to and phosphorylated after unloading from xylem sap by unspecific phosphatases/kinases. This would ensure effective distinction between the translocation and local forms of CKs, similarly to sugar transport via sucrose. Third, CKs fed to plants are often ribosylated and/or phosphorylated (Sondheimer and Tzou, 1971; Suttle and Banowetz, 2000; Tokunaga et al., 2012). Thus, at least in some instances, the cytokinins are interconverted through the nucleoside. Whether the enzymes are CK-specific or unspecific, remains to be elucidated.

Nevertheless, even when LOG does activate the cytokinins, preceeding hydrolysis of β- and γ-phosphates must occur, as ADP and ATP are the preferred substrates of plant adenylate ITPs (e.g. Kakimoto, 2001; Sakano et al., 2004). It was reported that in some organisms, *LOGs* are organised in operons or as fusions with certain phosphoanhydrases or Nudix hydrolases (Samanovic et al., 2015). Proteins coded by these genes could be responsible for the hydrolysis of CK nucleotides.

Deactivation of Cytokinins

There are several options for CK deactivation, when they are present at higher quantities than needed. The first option is ribosylation, eventually with phosphorylation (Sondheimer and Tzou, 1971; Suttle and Banowetz, 2000; Tokunaga et

al., 2012). The other option is glycosylation at the adenine or at the side chain of zeatins and topolins. The last option is reversible degradation by cytokinin oxidase/dehydrogenase.

Glycosylation

Cytokinins can be glycosylated either at the nitrogens of the purine ring or at the hydroxyl of the zeatins' side chain. Glycosylation of cytokinins on the side chain leads to loss of activity, because the modified side chain cannot be recognized anymore. This *O*-glycosylation is usually reversible though and thus thought of as of a storage form (Sakakibara, 2006). On the other hand, the modification of the adenine ring diminishes binding to CK receptors (Spíchal et al., 2004), but enzymes of CK metabolism, notably the catabolic CKX (Galuszka et al., 2007; Zalabák et al., 2014), and possibly also the transporters may still recognize these metabolites. Thus they are thought of as of the deactivation products. The *N*-glycosides are usually referred to as resistant to hydrolysis, but there are several reports of their hydrolysis. So kinetin *N3*-glucoside is cleaved by β-glucosidase (other *N3*-glucosides were not tested; Brzobohatý et al., 1993) and activity of DHZ3G in bioassays is attributed to hydrolysis (Bajguz and Piotrowska, 2009). Free BAP is released in soybean from its *N9*-tetrahydropyrrol and *N9*-tetrahydrofuran derivatives (Zhang and Letham, 1989) and *N9*-tetrahydropyrrol and *N9*-glucosides of 3-methoxyBAP, BAP and DHZ are hydrolysed in maize (Podlešáková et al., 2012).

Many additional conjugates of CKs have been identified in the past. Besides the canonical glucosides and ribosides also *O*-xylosylderivatives of (dihydro)zeatin (Turner et al., 1987), cytokinins with glucose attached to ribose (Taylor et al., 1984; Zhang et al., 2001) or zeatin riboside with a branched 5-sugar chain on the side chain (Kobayashi et al., 1995). One could include also *O*-acetyl-(dihydro)zeatin identified in yellow lupine (Letham and Zhang, 1989), although that is ester and not glycoside. Nevertheless, significance of these modifications is challenging to estimate as there were hardly ever follow-up studies.

At first, a cytokinin 7-glucosyltransferase was reported (Entsch et al., 1979). The enzyme was localized to radish cotyledons and used preferentially UDP-glucose or TDP-glucose. As acceptor substrates it could use wide range of substances, including adenine or aromatic cytokinins. Of adenines with aliphatic side chain, it used in decreasing order of preference isopentyladenine, dihydrozeatin, propyladenine, *t*Z, *t*ZOG and *c*Z. Thus substances with saturated side chain were preferred. Only traces of *N9*-glucosides were detected with the exception of *t*Z, *c*Z and the aromatic CKs, where

up to half of products were recovered as *N9*-glucosides. Another enzyme responsible for formation of *N9*-glucosides was probably also present in the extracts from radish cotyledons (Entsch and Letham, 1979).

Interesting system for study of CK glycosylation provide common bean (*Phaseolus vulgaris*) and lima bean (*Phaseolus lunatus*) as they are relative, yet differ in ability to glycosylate cytokinins and possibly also to degrade them (as hypothesised in Mok et al., 1978). The respective enzymes were purified (Dixon et al., 1989; Turner et al., 1987). The xylosyltransferase (*t*ZOXT) from *Phaseolus vulgaris* uses exclusively UDP-xylose, while the glucosyltransferase (*t*ZOGT) from *Phaseolus lunatus* is able to use both UDP-glucose and UDP-xylose, albeit the K_m of the later substrate is 10-times higher than of the first one (Dixon et al., 1989). In case the enzyme was provided with both substrates simultaneously, less than 10% of product was recovered as *t*Z *O*-xyloside. On the other hand, the *t*ZOGT was strictly specific towards *t*Z, while the *t*ZOXT could utilize both *t*Z and DHZ. Neither *c*Z, nor *t*ZR were substrates for either of the enzymes. The genes were later identified (Martin et al., 1999a, 1999b). Additional genes of putative cytokinin glycosyltransferases were identified in both beans, but they were not active with cytokinins (Meek et al., 2008).

Interestingly, both cytokinin *O*-glucosyltransferases identified in maize have strict specificity for *cis*-zeatin and were thus designated as *cis*-zeatin *O*-glucosyltransferases (*c*ZOGT; Martin et al., 2001; Veach et al., 2003). They glucosylate *t*Z only very weakly (Veach et al., 2003). The genes showed somewhat differential expression. Both genes were strongly expressed in roots of 2-weeks old plants and *cZOGT*1 showed increasing expression in kernels from week 1 to 3 after pollination.

On the other hand, there were identified 5 CK-specific glucosyltransferases in *Arabidopsis* (Hou et al., 2004) and they do not discriminate between the zeatin isomers so strictly. Of these, two produced specifically *N*-glucosides with preference for *N7*-glucoside, with the exception of BAP. Among the isoprenoid CKs, the activity was in the order DHZ >> iP > *t*Z >> *t*ZOG >> *c*Z. The other three enzymes produced *O*-glucosides, two of them with very low activity though. They were reported to have other substrates later (Gandia-Herrero et al., 2008; Poppenberger et al., 2005). However, UGT85A1 glucosylates *t*Z and *c*Z with similar activity, while DHZ was worse substrate. Interestingly, the *Arabidopsis* proteins were reported not to glucosylate topolins (Hou et al., 2004), even though they are usually metabolised similarly as zeatins (Mok et al., 2005).

It should be noted though, that these five enzymes were identified in screen of 105 glucosyltransferases for ability to glucosylate five CKs excluding *cis*-zeatin. Thus it is

possible that other glycosyltransferases, using other sugar donor and/or *cis*-zeatin as acceptor, exist in *Arabidopsis*. Further, there was no genome or "glucosyltransferase-ome"-wide search for CK-specific enzymes in maize. Therefore, it is still possible there are other glycosyltransferases present in maize.

Nevertheless, the differences in preferences of *O*-glucosyltransferases in these model organisms for *c*Z-type- and *t*Z-type-dominant species, led us to propose a novel hypothesis to explain the prevalence of isomers of zeatins in each of them. Rather than "purposeful" drive towards preference of one isomer over the other, the substrate specificity of the *O*-glucosyltransferases led fairly randomly to the dominance of one or the other. The kingdom-wide analysis of CKs (Gajdošová et al., 2011) showed that preference for either one of zeatin isomers is not restricted to a particular taxon but is in fact a random occurrence. That is compatible with single locus being solely responsible for this rather than a complex network that would require more mutations to switch from one isomer to the other. Further, expression of *t*ZOGT in maize led to equal amounts of *c*Z-type and *t*Z-type cytokinins in mature plants (Pineda Rodó et al., 2008) therefore confirming once again that the selectivity of *O*-glucosyltransferases is responsible for prevalence of either of the isomers.

Rice and maize, both *c*Z-type-dominant plants, were transformed with *c*ZOGT and *t*ZOGT, respectively (Kudo et al., 2012; Pineda Rodó et al., 2008). Predictably, the plants had increased levels of mainly *c*Z(R)OG and *t*ZOG, respectively. Both species had reduced shoot growth (CK-deficient phenotype as seen in Werner et al., 2003), but it was more pronounced in the maize. Unexpectedly both plants had also elevated levels of chlorophyll and delayed senescence. That is usually associated with increased CK levels (Gan and Amasino, 1995). Indeed, the maize plants showed increased levels of *t*Z and iP in the leaves. However, the senescence delay was probably a seasonal trait (Pineda Rodó et al., 2008). Further, roots were longer, although rice had reduced number of crown roots (Kudo et al., 2012). Thus, the phenotypes were in general similar. The only difference was development of sexual organs and seeds, that was highly affected in the maize expressing *t*ZOGT (Pineda Rodó et al., 2008). Mainly the size of tassels was reduced by 75% in heterozygous plants and they were feminized to various degrees in homozygous plants. Again, this was a seasonal trait (Pineda Rodó et al., 2008).

Pineda Rodó et al. (2008) noted, that the over-expression of *t*ZOGT may affect the intracellular distribution of CKs. And indeed, respective CK forms show differential sub-cellular distribution (Benková et al., 1999; Jiskrová et al., 2016). The CK glucosides are localized mainly to apoplast and vacuole. In apoplast they probably originate from deactivation to prevent further activation of the receptors and in vacuoles they are

stored until degraded or re-used. Targeting maize β-glucosidase to vacuoles led to decreased accumulation of *t*ZOG in tobacco grown on medium with *t*Z, while localization to chloroplast (it's natural compartment) increased it (Kiran et al., 2012). Thus the *O*-glucosides are possibly reversibly hydrolysed only in plastids, in the place of CK biosynthesis, and in apoplast. On the other hand, in the vacuoles they accumulate and thus the apparent increase of *O*-glucosides during plant maturation (e.g. Šmehilová et al., 2009).

Cytokinin Oxidase/Dehydrogenase

The enzyme degrading cytokinins is one of the longest known of these involved in CK metabolism and thus best characterized today. The topic of CK degradation has been reviewed previously (Frébort et al., 2011; Schmülling et al., 2003).

The enzyme was first described in the 70s (Pačes et al., 1971) and cloned independently by two groups almost 30 years later (Houba-Hérin et al., 1999; Morris et al., 1999). It is a flavoenzyme with covalently bound FAD using artificial electron acceptors (Galuszka et al., 2001). Putative natural electron acceptors were identified (Frébortová et al., 2010). Because the enzymes efficiently use Q_0, an isoprenoid side chain-deprived analogue of ubiquinone, it was proposed, that the cytokinin oxidase/dehydrogenase (CKX) enzymes may be associated with membranes (Galuszka et al., 2001). Recently, *At*CKX1 was shown to be transmembrane protein localised to endoplasmic reticulum (Niemann et al., to be published).

Cytokinin oxidases/dehydrogenases form small gene families. The respective enzymes differ in subcellular localization, temporal and spatial expression and substrate preferences. The most studied are enzymes from Arabidopsis and maize. Several genes were cloned and heterologously expressed (Frébortová et al., 2007, 2015; Houba-Hérin et al., 1999; Kowalska et al., 2010; Morris et al., 1999; Šmehilová et al., 2009). Characterization of the complete family of CKX enzymes was performed in Arabidopsis and maize (Galuszka et al., 2007; Zalabák et al., 2014).

CKX is able to efficiently degrade all types of natural cytokinins with the exception of resistant DHZ (Galuszka et al., 2007; Zalabák et al., 2014) or aromatic CKs that are very weak substrates (Galuszka et al., 2007; Kowalska et al., 2010). Also *c*Z was considered as weak substrate, but there are several enzymes that cleave it preferentially. Among them *Zm*CKX8, 9 and 10 (Zalabák et al., 2014) or *At*CKX1 and 7 (Gajdošová et al., 2011). And indeed, *Arabidopsis* over-expressing *At*CKX1 or *At*CKX7 had drastically reduced levels of *c*Z-type CKs (Köllmer et al., 2014). Considering that AtCKX7 and *Zm*CKX10 are the only CKX from the respective organisms localized to

cytosol, the preference for *c*Z-type CKs may not be coincidence as it may be involved in removal of tRNA-derived *c*Z.

Besides other effects, alteration of CKX activities affects several important agricultural traits (Ashikari et al., 2005; Pospíšilová et al., 2016; Werner et al., 2010).

Cytokinin Signalling

Transduction of CK signal relies on modular system similar to the one used by bacteria called two-component signalling, as it is composed of hybrid histidine kinase (HK) receptor and response regulator (RR). In plants, additional component is required – histidine-containing phosphotransfer protein (HP) – for transfer of the signal to nucleus (Fig. 3). The CK signal circuity was reviewed in more detail recently (Keshishian and Rashotte, 2015; Zürcher and Müller, 2016).

There are three CK receptors in *Arabidopsis* along other histidine kinases involved in ethylene, light and osmotic sensing (Kakimoto, 2003). Among the CK receptors, AHK4 is unique in several aspects: it is highly specific for free bases of *t*Z and iP (Spíchal et al., 2004), it possesses phosphatase activity in absence of CKs (Mähönen et al., 2006b) and it provokes several-fold higher response than AHK2 and AHK3 (Hwang and Sheen, 2001). AHK3 and maize receptors recognize in general also *c*Z, DHZ and respective ribosides with affinities similar to other isoprenoid cytokinins (Lomin et al., 2011; Romanov et al., 2006; Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004). There is currently discrepancy concerning the subcellular localization of the CK receptors (Caesar et al., 2011; Wulfetange et al., 2011), but recent experiments confirmed that the functional receptors are localized on the plasma membrane (Zürcher et al., 2016).

Upon CK binding, the receptors form dimers and transfer phosphate to HPs. HPs in turn transfer the phosphate to downstream proteins in nucleus (Hutchison et al., 2006). However, it was shown that the localization of HPs is independent of CK perception (Punwani and Kieber, 2010). Unlike the other HPs, the *Arabidopsis* HP6 has the conserved Asp replaced with Asn and thus cannot accept the phosphate. It competes with the other HPs in binding to the receptors and thus attenuates the CK signalling (Mähönen et al., 2006a).

Among the receiver proteins are Response Regulators, that are classified as type-A, -B and -C, based on their domain structure and responsiveness of their expression to CK treatment (Müller, 2011). Type-B RRs are positive regulators of the CK response and they serve as transcription factors directly influencing expression of response genes (Argyros et al., 2008). Among the response genes are also *type-A RRs*

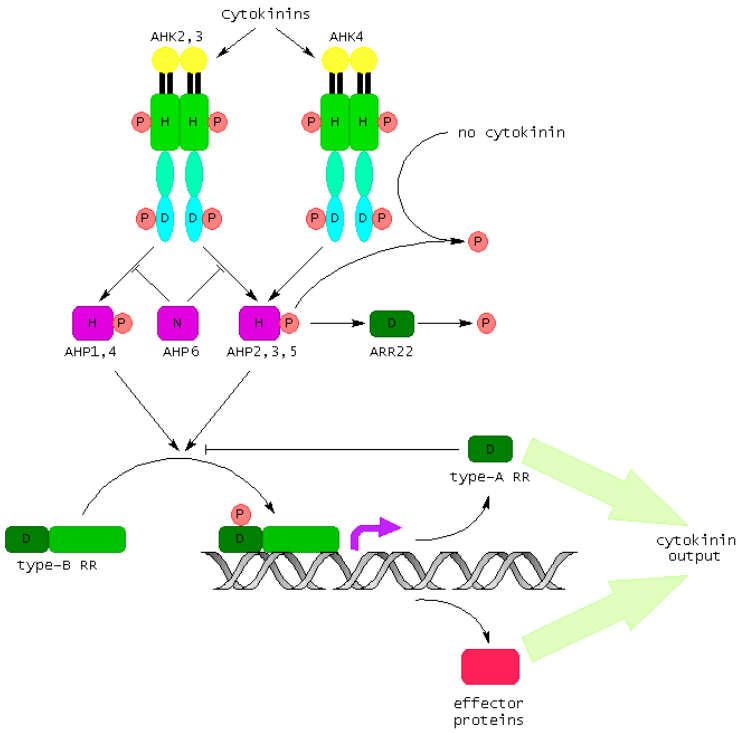

Figure 3: Schematic representation of current state of the art of cytokinin signaling. The cytokinin receptors, the histidine kinases, phosphorylate themselves in presence of cytokinins and transfer further the phosphate to the histidine phosphotransfer proteins (AHPs). However, in absence of cytokinins, AHK4 has phosphatase activity and dephosphorylates all AHPs. The transfer is inhibited by AHP6, which have substituted the conserved Asp for Asn. AHP2,3 and 5 are fastly dephosphorylated by ARR22. The AHPs transfer the phosphate group to type-B response regulators, which work as transcription factors. One of their targets are also type-A response regulators, which inhibit the phosphotransfer from AHPs to type-B RRs. The invariant residues are marked (H - histidine; D - aspartate; N - asparagine). The phosphate group is marked by P in pink circle. The transmembrane domains of the HKs are shown as black dashes, the extracellular CHASE domain in yellow, the transmitter domain is shown in green and receiver and receiver-like domains in light blue with and without marked D, respectively.

(Rashotte et al., 2003) and they serve as negative regulators of the CKs, probably by competing with type-B RRs for the phosphate. Thus they serve as negative feedback loop. The function of type-C RRs is less well characterized (Zürcher and Müller, 2016). Their structure resembles that of type-A RRs, but their expression is not upregulated by CKs (Kiba et al., 2004). Their expression is restricted to reproductive organs (Gattolin et al., 2006; Kiba et al., 2004).

Cytokinin Transport

There are several lines of evidence for CK transport. CKs serve as long-range signal for nitrogen (Takei et al., 2001b, 2004a), *t*Z and iP have been detected in xylem and phloem sap, respectively (Hirose et al., 2008; Weiler and Ziegler, 1981). Experiments with grafted plants have repeatedly shown ability of WT roots or shoots to complement

for a detrimental mutation (Foo et al., 2007; Kiba et al., 2013; Matsumoto-Kitano et al., 2008) and overall, the transport of CKs has been shown (Letham and Palni, 1983; Letham and Zhang, 1989; Zhang et al., 2002). Lastly, the expression sites of *IPT* and *LOG* genes do not overlap with the CK response domains (Zürcher and Müller, 2016). But for a long time, the CK transporters remained elusive.

The first evidence of a particular protein putatively transporting CKs, was inhibition of adenine uptake by then newly identified *Arabidopsis thaliana* purine permease 1 (*At*PUP1) by kinetin and zeatin and to lesser extend by their ribosides (Gillissen et al., 2000). Later, inhibition of adenine uptake by another member of the family, *At*PUP2, by more cytokinins was shown together with direct transport of *t*Z by *At*PUP1 (Bürkle et al., 2003). Rice with mutated *ospup*7, one of the homologues, had about 3-times increased levels of iP and iPR, while levels of *t*Z-type CKs were not affected (Qi and Xiong, 2013). However, the protein was not tested for cytokinin transport, although it was able to transport caffeine.

Other candidates for CK transport are the equilibrative nucleoside transporters (ENT). Adenosine uptake by *Os*ENT2 protein was inhibited by iPR and, to lesser extent by free base of *t*Z (Hirose et al., 2005). K_m values were determined to be 32 and 660 μM for iPR and *t*ZR, respectively. By the means of screen for suppressor of *IPT* overexpression, *At*ENT8 was identified as another potential CK transporter (Sun et al., 2005). Together with *At*ENT3, but not *At*ENT1, they were shown to be involved in transport of cytokinin ribosides *in planta*. Furthermore, adenosine uptake was inhibited mainly by iPR, but also by *t*ZR when measured with *At*ENT3, 6 and 7 (Hirose et al., 2008), with similar K_m values for *At*ENT6, as reported for *Os*ENT2. However, multiple members of the transporter family from *Arabidopsis* were previously characterized and reported not to transport kinetin, zeatin or *t*ZR (Wormit et al., 2004). The discrepancy could be due to use of *t*Z-type CKs, that have much higher K_m, but Hirose et al. (2008) showed reduction of the adenosine uptake by 20 – 30 % even with *t*ZR.

Nevertheless, because of the low affinities towards CKs and comparable or higher affinity of these transporters towards canonical nucleobases or nucleosides, their involvement in CK transport was often disputed (Frébort et al., 2011; Kudo et al., 2010). Recently, direct involvement of two transporters with CKs *in planta* was shown.

The *ATP-binding cassette transporter subfamily G* 14 (*ABCG*14) is co-expressed with *IPTs* and its expression is induced upon CK treatment (Ko et al., 2014) and knock-out mutants' phenotype resembled phenotypes of *ahk*2 3 knock-out and of plants over-expressing *CKX* (Ko et al., 2014; Zhang et al., 2014). The phenotype of

shoots could be rescued by application of *t*Z, but not of iP (Ko et al., 2014), while root elongation was less sensitive to *t*Z application in the mutants than in WT plants (Zhang et al., 2014). The mutants had decreased levels of *t*Z and DHZ in the shoots, while *c*Z levels were increased. In the roots, the opposite was true. Both roots and shoots of the mutants had increased levels of iP. Overall, the CK content in the roots was higher, while it was lower in the mutants' shoots. And indeed, the expression of the responsive genes corresponded accordingly (Ko et al., 2014; Zhang et al., 2014). Grafting experiment showed unequivocally that root-derived *t*Z is crucial for the development of shoots, but shoots do not affect growth of roots (Ko et al., 2014).

*At*PUP14 was shown to be a negative regulator of CK signalling (Zürcher et al., 2016). The cotyledons of *Arabidopsis* heart-staged embryo do not transmit the CK signal, even though *AHK*4 is expressed there and the downstream signalling pathway is functional as shown by expression of *CKI*1 – a constitutively signalling HK. Transport of *t*Z by *At*PUP14 was directly shown. It was higher than by *At*PUP1, and it was ATP-dependent. Transport of radioactive *t*Z was inhibited by free bases of other cytokinins, but not by *t*ZR. Further, cytokinin signalling was diminished in mesophyll protoplast cells upon *At*PUP14 expression or upon presence of CKX enzyme in the "apoplast", but not in the cytosol of the cells (Zürcher and Müller, 2016). This clearly illustrates the importance of matching subcellular localization of cytokinins and CK receptors for triggering of the signalling. Similar results were observed in barley by our group (Pospíšilová et al., 2016). Localization of *At*CKX1 to apoplast was detrimental to plant development, while plants with *At*CKX1 directed to vacuoles showed only weak phenotype and phenotype of plants with cytosolic *At*CKX1 was miniscule.

Physiological Function

Cytokinins affect wide range of physiological processes. They are primarily involved in cell growth and differentiation. Cytokinins shape the overall plant's architecture, they are important for proper functioning of shoot and root apical meristems, they regulate apical dominance and lateral root formation. Cytokinins are crucial during gametophyte development and embryogenesis as evidenced by many detrimental effects due to imbalance in CK signalling. They are involved in many physiological processes as regulation of chlorophyll synthesis, chloroplast maintenance and senescence in general, signalisation of nutrient availability, density and opening/closure of stomata and others. In general they have protective role against many stresses as salt, drought or heat, as well as cold stress. And lastly, but hardly for plant leastly, they are involved in interactions between plants and associated

organisms. Sometimes they are used to alert the defence system, while in other cases the associated organisms hijacked the plant hormones for their own good. Some of the physiological functions were reviewed previously (Schäfer et al., 2015; Werner and Schmülling, 2009; Zürcher and Müller, 2016).

Here is presented only selection of cytokinin physiological functions that are of interest to the author and that are relevant to this thesis.

Cytokinins in tRNA

There is a hypermodified nucleotide in many tRNAs forming A=U pairing at the first position of codon. While the tRNAs recognising UNN codons contain mostly cytokinins or wybutosine – a hypermodified guanine, those recognising ANN codons contain almost exclusively N^6-threonylcarbamoyladenosine (t6A). No such conservation is present in tRNAs binding to CNN or GNN codons (Skoog and Armstrong, 1970).

It is of interest, that iP was discovered in tRNA in the same year (Biemann et al., 1966; Hall et al., 1966), as the free form (Helgeson and Leonard, 1966; Klämbt et al., 1966). It was established soon, that tRNA contains also hydroxylated CKs (Hall et al., 1967) and their 2-methylthioderivatives (Burrows et al., 1970). However, unlike free cytokinins, those bound in tRNA contain the hydroxyl group predominantly in *cis*-position (Hall et al., 1967; Miyawaki et al., 2006).

Cytokinins are present in tRNAs of organisms from all life kingdoms with the exception of Archea. The cytokinin is present at position 37 (A37), next to the anticodon. The modification occurs at the middle one of three adenosines. This is not sole requirement though, as there are other unmodified adenosine triplets. However, CKs are not present in all tRNAs of this type or even consistently in analogous tRNAs from different organisms (Skoog and Armstrong, 1970).

The cytokinins were detected also in rRNA (Taller et al., 1987), despite previous extensive searches were fruitless (Skoog and Armstrong, 1970). However, it was shown also, that BAP and kinetin are incorporated into RNA upon feeding (Murai et al., 1977, 1978; Teyssendier de la Serve and Jouanneau, 1979). Even iP was incorporated into rRNA of tobacco cells (Teyssendier de la Serve et al., 1981 as cited in Taller et al., 1987), but again only after treatment of the cells with iP. This all would suggest that the incorporation is rather unintended, possibly due to error by RNA polymerase using CKs instead of adenine. But definitely the cytokinins are not synthesised in rRNA or mRNA post-transcriptionally as in the case of tRNA. Involvement of putative tRNA:kinetin transglycosylase was proposed (Barciszewski et al., 1992). Analogous enzymes are in

fact known to be involved in incorporation of other modified residues into tRNA (Romier et al., 1996 and references therein).

The t6A resembles cytokinins to some extent. It can be also modified at the *C2*-position with methylthiol group. However, it is not active in tobacco bioassay (Skoog and Armstrong, 1970). Interestingly, although none of the archeal tRNAs contain CK bases, few contain t6A next to the UNN anticodons, for example those of *Halobacterium salinarum* and *H. volcanii* (Gupta, 1984; Nicoghosian et al., 1985).

The modifications in the anticodon loop of tRNA in general serve improved codon-anticodon reading and increased translation efficiency. The hypermodifications of purine at position 37 (N^6-isopentenyl, N^6-threonylcarbamoyl and/or 2-methylthiol groups) facilitate the complementary codon binding (Pongs and Reinwald, 1973). It was thought that is due to enhancing the A=U base-pair stability (McCloskey and Nishimura, 1977). Surprisingly, an UV thermal denaturation analysis showed decreased base stacking in a modified anticodon stem loop of $tRNA^{Phe}$ (Cabello-Villegas et al., 2002). While the stem of the unmodified anticodon stem loop forms A-form helix including base pairs U32=A38 and U33=A37 and the loop contains only the three bases of the anticodon, the isopentenyl side chain of modified A37 disturbs the bonding to U33 and increases the dynamics within the loop region (Cabello-Villegas et al., 2002). It is needed to say though, that the modified anticodon stem loop was prepared by enzymatic addition of the isopentenyl side chain by MiaA and hence did not contain the 2-methylthiol group, neither modifications on other residues as the WT $tRNA^{Phe}$. That way the modification on position 37 orders the anticodon loop and forms the canonical U-turn (Fig. 4). In this way it contributes to the modified wobble hypothesis (Agris et al., 2007).

It was shown, that the modification of A37 greatly reduces +1 frameshifting by slippage (Urbonavičius et al., 2001), but it fails to affect -1 frameshifting (Urbonavičius et al., 2003).

The consequences of missing modifications are often deleterious. The organisms with undermodified tRNA are impaired in their growth. The *mia*E mutant of *Salmonella typhimurium*, that lacks hydroxylation on the isoprenyl chain, is unable to grow on organic acids as carbon source (Persson et al., 1998; Persson and Björk, 1993). Likewise, *Escherichia coli* grown in iron deficient conditions lacks the methylthio modification of iP. Its generation time was extended to 35 mins due to undermodified $tRNA^{Phe}$, $tRNA^{Tyr}$ and $tRNA^{Trp}$ (Griffiths and Humphreys, 1978). Both the iron-depleted *E. coli* and *mia*A mutant have increased uptake of aromatic amino acids (Buck and Griffiths, 1981). It was hypothesised, that this is because of synthesis of enterobactin,

that is required for obtaining iron from environment and its synthesis competes for precursor chorismic acid with the synthesis of aromatic amino acids. Further, the *Physcomitrella patens ipt*1 mutant had reduced growth rate (Lindner et al., 2014). That actually resembles treatment of WT *P. patens* with CKs and indeed, the mutant had unexpectedly increased levels of iP- and *t*Z-type CKs. Similarly, growth reduction was reported for *Claviceps purpurea* with knock-out of the *cptrna ipt* gene (Hinsch et al., 2016), but the results were not much convincing. Also the *Arabidopsis* plants with knock-out of *atipt*9 or the double knock-out *atipt*2 9 were often chlorotic (Miyawaki et al., 2006).

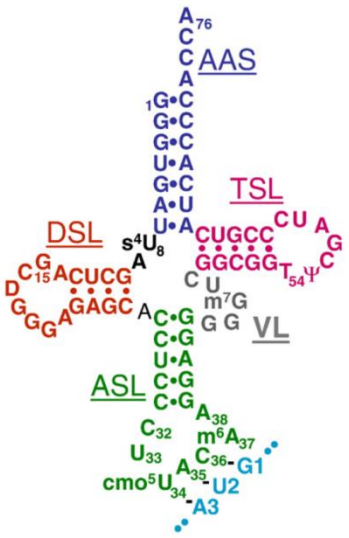

Figure 4: tRNA primary sequence, secondary structure, and codon binding. The sequence and secondary structure of *E. coli* $tRNA^{Val}$. The physical and functional domains of the *E. coli* $tRNA^{Val}_{UAC}$ sequence and secondary structure are the amino acid-accepting stem, AAS (dark blue), the dihydrouridine stem and loop, DSL (red), the anticodon stem and loop, ASL (green), the variable loop, VL (gray), and the thymidine stem and loop, TSL (purple). The modified nucleosides in this tRNA are: s^4U, 4-thiouridine; D, dihydrouridine; cmo^5U, uridine-5-oxyacetic acid; m^6A, N^6-methyladenosine; m^7G, 7-methylguanosine; T, ribothymidine; and Ψ, pseudouridine. Because of the wobble nucleoside modification, cmo^5U34, *E. coli* $tRNA_{UAC}^{Val}$ is capable of decoding all of the fourfold degenerate valine codons. The tRNA is shown binding the cognate codon for valine, GUA, in light blue. The sharp U-turn between U33 and cmo^5U34 is depicted. Taken from Agris et al. (2007).

The only case of improved growth upon removal of cytokinins from tRNA is the *Bradyrhizobium* sp. strain ORS285. This photosynthetic nodule-inducing bacterium contains single *IPT* gene homologous to other *Mia*A genes. The *mia*A mutant lacked all cytokinins, but its growth on minimal medium was faster than that of WT. The mutant retained its fast growth even after complementation with *Tzs* gene from *Agrobacterium tumefaciens*, but not after complementation with the *Mia*A gene. Thus this effect was due to lack of tRNA modifications, and not due to lack of cytokinins (Podlešáková et al., 2013; alternatively, it could be due to lack of specific CKs as *c*Z and/or 2-MeS-CKs).

However, the relevance of tRNA-derived cytokinins is questionable. It was proposed, that CKs were first present in tRNA and only later acquired function as hormones (Kamínek, 1974). Plants needed to avoid interference of their developmental processes with tRNA turnover. They achieved that mainly in three ways (Kamínek, 2015):

I. reduction of tRNA species bearing the cytokinin-like modification and replacement with others, as wybutosine
II. preference for *cis*-zeatin over iP in tRNA
III. modulation of CK perception to prefer *trans*-zeatin

Thus while bacteria contain CKs in most of their tRNAs recognising UNN codons, either iP or *c*Z, depending on presence of *Mia*E gene (*cf. Escherichia coli* and *Salmonella typhimurium*), plants' tRNAs contain relatively little CKs and the majority is present as *c*Z (Miyawaki et al., 2006).

The importance of the tRNA-derived CKs has been demonstrated only recently. The above mentioned *Bradyrhizobium* sp. strain ORS285 *mia*A mutant showed delayed development of nodules on *Aeschynomene* plants (Podlešáková et al., 2013). Similarly, the *cptrna ipt* mutant showed decreased virulence. Formation of sclerotia, dormant stage of the fungus, was retarded and the majority of sclerotia were white and soft (Hinsch et al., 2016).

From Inflorescence to Germinated Seed

Cytokinins guide the plant from development of inflorescence with gametophytes to embryogenesis and germination. Few steps will be discussed here.

The reproductive organs are unique in matter of CK signalling as they rely on CKI1, a histidine kinase working independently of CKs, and type-C response regulators, that resemble negative CK regulators type-A RRs, except their expression is not regulated by CKs. This may indicate that the reproductive organs require CK-like signalling, but do not rely on CKs for it. Yet, cytokinins appear to be crucial for the development as well.

Cytokinin independent 1 (*CKI*1) was identified as putative CK receptor by means of activation T-DNA tagging (Kakimoto, 1996). Overexpression of *CKI*1 led to characteristic effects of CK action (Kakimoto, 1996). However, it does not relay the cytokinin signal (Hwang and Sheen, 2001). It was shown to rely on AHPs though (Deng et al., 2010). The CKI1 was shown to phosphorylate AHP1, 2, 3 and 5 *in vitro* (Mähönen et al., 2006b) and the receiver domain was shown to dephosphorylate AHP1 and AHP2 (Nakamura et al., 1999) similarly to AHK4 (Mähönen et al., 2006b). It is not clear, whether Mähönen et al. (2006b) did not test for or did not observe such activity of CKI1. Also, it may be that only the receiver domain possesses this activity, which is diminished in intact receptor. Bimolecular fluorescence complementation and yeast two-hybrid assay showed direct interaction with AHP2, 3 and 5 and to lesser extent also with AHP1 (Pekárová et al., 2011). Accordingly, the phosphatase activity towards AHP1 reported by Nakamura et al. (1999) was somewhat slower than towards AHP2.

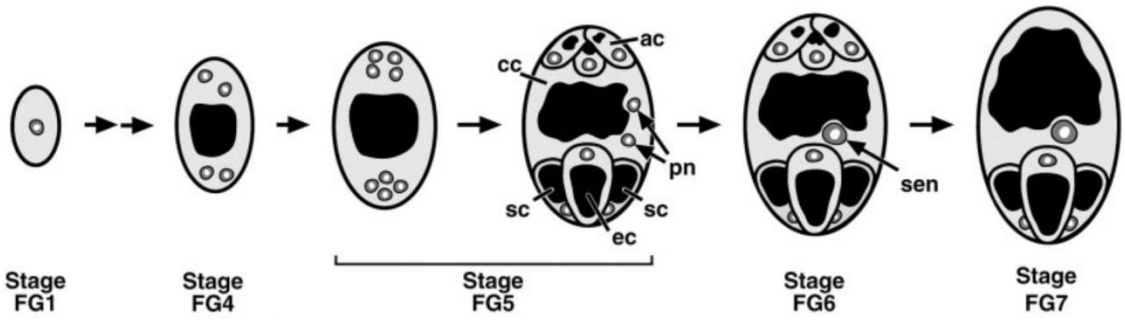

Figure 5: Depiction of female gametophyte development. Megagametogenesis has been described and divided into seven stages (Christensen et al., 1997). Before megagametogenesis, a diploid megaspore mother cell undergoes meiosis and produces four haploid megaspores, three of which degenerate. The surviving megaspore defines stage FG1. The megaspore then undergoes three rounds of mitosis without cytokinesis, giving rise to an eight-nucleate cell (early stage FG5). Immediately following the third mitosis, cell walls form and partition the nuclei into cellular compartments (late stage FG5). The central cell (cc) inherits two nuclei, called the polar nuclei (pn), which fuse to form a homodiploid secondary endosperm nucleus (sen; stage FG6). Finally, the antipodal cells (ac) degenerate. The mature female gametophyte (stage FG7) consists of one central cell, one egg cell (ec), and two synergid cells (sc; Christensen et al., 1998). Taken from Pischke et al. (2002)

Expression analysis and analysis of defective seeds in multiple *ahp* mutants confirmed importance of AHP2, 3 and 5 in CKI1-dependent cascade *in vivo* (Liu et al., 2017). Another proteins functioning downstream of CKI1 may be the type-C RRs. Their domain structure resembles that of type-A RRs, the primary response genes of CKs (Kiba et al., 2004). Their expression is not induced by CKs though; it is rather "cytokinin independent". It is mostly restricted to flowers and siliques (Gattolin et al., 2006; Kiba et al., 2004) similarly to expression of *Cytokinin independent* 1.

The *CKI*1 was shown by two groups independently to be involved in the development of the female gametophyte (Hejátko et al., 2003; Pischke et al., 2002). Both groups determined they are not able to obtain homozygous plants for their respective knock-out alleles and reciprocal (back)crossing showed that defective female gametophyte (also megagametophyte or embryo sac) is responsible, while pollen was viable. In contrast to the naked female gametes of fucoid algae, the female gametophyte of angiosperms is enveloped with a maternal tissue and represents a well organised and polarised structure (Fig. 5). In general, no defects were observed until the FG4 stage, while in the FG5 stage, half of the ovules of the heterozygous plants were defective. The phenotype of the mutant female gametophytes could be divided to two categories. In the first, the megagametophytes were in the process of degeneration. In the second, the embryo sac cavity was filled with a matrix of cytoplasmic strands connecting large number (>100) of small vacuoles as well as greater than usual number of nuclei (>15). Interestingly, only weak signal was obtained in the developing ovule using RNA *in situ* hybridization and β-glucuronidase histochemical assay (Hejátko et al., 2003; Pischke et

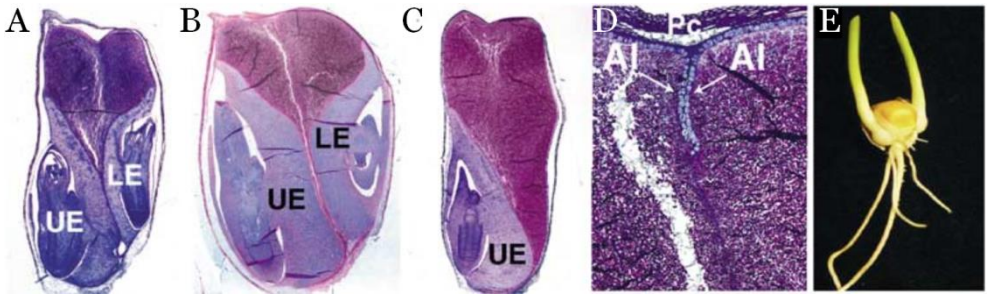

Figure 6: Production of double embryo kernels in maize expressing *IPT* under senescence-induced promoter. Longitudinal section of typical double embryo kernels from transgenic plants (A, B) and of single embryo kernel from WT plant (C). (D) Longitudinal section of the transgenic double embryo kernel showing the junction of the two fused endosperms. Al – aleurone layer; Pc – pericarp. (E) Transgenic double embryo kernel 5 days post germination. Both seedlings grow simultaneously and grow into normal fertile plants. Taken from Young et al. (2004)

al., 2002). Strong expression was observed within the mature megagametophyte, mainly in the nucleus of the central cell and to lesser extent also of the egg cell (Pischke et al., 2002). Sharp peak of expression was observed between 12 and 24 hours post pollination (Hejátko et al., 2003).

Later, expression of *MYB*119 was shown to be directly dependent on *CKI*1 activity (Rabiger and Drews, 2013). *MYB*119 and *MYB*64 are transcription factors responsible for the switch during FG5 stage from nuclear divisions to cellularization-differentiation (Rabiger and Drews, 2013). Although the previous report stated localisation of CKI1-GFP to the plasma membrane, recent report claims it is localised mainly to the endoplasmic reticulum, similarly as CK receptors, and that it becomes polarised when the nuclei at each pole divide to form the four-nucleate FG4 (Yuan et al., 2016). Since then it is localised to the antipodal cells and central cell. It was shown that ectopic expression of *CKI*1 can initiate central cell-program in the egg cell and synergid cells. Such megagametophytes form seeds with two endosperms and lacking embryo after fertilization (Yuan et al., 2016).

Similarly to *cki*1 mutant, triple *ahk*2 3 4 mutant shows female gametophyte lethality as well. Although not all papers report it, it was shown to depend upon strength of the expression down-regulation (Cheng et al., 2013 and references therein). The *CKI*1 expression at the chalazal end is preceded by expression of *AHK*4 strictly localised to chalaza before stage FG1 up to stage FG2 (Cheng et al., 2013). Expression of *AtIPT*1 was also observed. On the other side, *AtCKX*7 was expressed in mature female gametophyte at the egg cell end (Köllmer et al., 2014).

Unlike other members of *Poaceae*, members of the tribe *Maydeae* are monoecious, producing unisexual florets. In maize, both florets are established as hermaphroditic initially. Unisexuality is established through abortion of respective floret organs so that

pistil initials are removed from the tassel florets and stamen initial are eliminated from the florets in the ear inflorescence. Maize plants expressing *IPT* of *Agrobacterium tumefaciens* had two silks emerging from each ear spikelet (Young et al., 2004). This was because the lower floret was not aborted. These plants yielded kernels with two embryos (Fig. 6), but smaller endosperm, because the seeds were of normal size (i.e. part of the endosperm was replaced with the second embryo). Both of these embryos were viable rising two plants after germination (Fig. 6E) and they were genetically distinct (Young et al., 2004).

Unlike *CKI*1, cytokinins were shown to have role also in male gametophyte development. Although the rice anthers contained much less iP than leaves blades, they did contain albeit not much, but consistently higher levels of *t*Z-type CKs (Hirano et al., 2008). Biosynthetic genes *OsIPT*3, *OsIPT*7 and *LOG* were expressed with the genes of signalling pathway mainly at the beginning of anther development followed by expression of *OsCKX*1, 5 and 11. In Arabidopsis, the null mutant of CK receptors *ahk*2 3 4 had smaller anthers that failed to dehisce (Kinoshita-Tsujimura and Kakimoto, 2011). Defects included fewer pollen grains, undeveloped vasculature and general degeneration of the anther structures.

As was mentioned above, maize plants expressing the *t*ZOGT have feminized tassels (Pineda Rodó et al., 2008). Besides *t*ZOG, it had also increased levels of *t*Z and iP free bases, thus it is difficult to estimate, whether this phenotype was caused by insufficient or ample CK signalling. Similarly, maize expressing *ZmCKX*1 under anther- or pollen-specific promoters showed male sterility (Huang et al., 2003). However, the above mentioned maize producing CKs under senescence-driven promoter showed normal tassel development (Young et al., 2004). Either the classical senescence is not involved in unisexual tassel development, or the cytokinins could not overrule it.

Developing seeds are the richest source of cytokinins (Rijavec and Dermastia, 2010). Anthesis is followed by a sharp peak of cytokinin content (Arnau et al., 1999; Brugière et al., 2003; Dietrich et al., 1995; Morris et al., 1993). This increase is of the active forms and occurs at the chalazal end of the seed (Brugière et al., 2003, 2008). This period corresponds to the period of rapid endosperm nuclear and cell division (Dietrich et al., 1995; Morris et al., 1993). Correspondingly, expression of *IPT* was shown in several systems (Brugière et al., 2008; Miyawaki et al., 2004; Song et al., 2015; Vyroubalová et al., 2009). In many pepper cultivars (*Capsicum annuum*) a low night temperature induces parthenocarpy (Tarchoun et al., 2003). These unpollinated fruits were shown to lack the increase of *t*ZR, that is present in the pollinated fruits (Honda et al., 2017). This lack of CK peak in unfertilized ovules was observed also in maize

(Rijavec et al., 2011). Interestingly, unfertilized cones of hop lacked *t*ZR and iPR, but had increased level of *c*ZR (albeit at ~fifth levels of *t*ZR and iPR; Watanabe et al., 1982).

The decline in CK content is accompanied by increase of CKX activity (Brugière et al., 2003; Dietrich et al., 1995; Song et al., 2015). In maize, iPR showed second, smaller peak at 20 days after pollination with additional increase of *ZmCKX*1 transcription (Brugière et al., 2003). The increase of CKX activity is missing in aborting apical kernels of maize (Dietrich et al., 1995).

Often the dormant seeds contain less active cytokinins as *cis*-zeatin (Emery et al., 2000; Gajdošová et al., 2011; Quesnelle and Emery, 2007; Stirk et al., 2012b) or dihydrozeatin (Arnau et al., 1999; Emery et al., 2000; Stirk et al., 2012b; Zalabák et al., 2014). There seems to be a pattern of *t*Z-dominant plants having *c*Z in their seeds and *c*Z-dominant plants having DHZ in their seeds, although more widespread analysis would be necessary. This may suggest that these are favourable because of their resistance to degradation and/or because of low activity that may be advisable at this developmental stage. Because dihydrozeatin is quickly depleted upon seed imbibition (Stirk et al., 2012b; Zalabák et al., 2014), we have previously proposed it may serve as source of active cytokinins upon metabolic interconversion (Frébort et al., 2011). However, there is 7-glucosyltransferase expressed in cotyledons of radish (*Raphanus sativus*; Entsch et al., 1979). This enzyme was reported to use preferentially dihydrozeatin among tested substrates. As *N7*-glucosides are rarely determined in the analyses, DHZ may be in fact converted to DHZ7G and thus "disappear" from the analysis.

Besides the *c*Z and DHZ, plant seeds are often reported to contain also aromatic cytokinins. So is kinetin found in the liquid endosperm of coconut (for review see Yong et al., 2009), 3-methylpyrrolpurine was found in seeds of maize (this work) and BAP and topolins are found in *Tagetes minuta* (Stirk et al., 2005, 2012a), pea (Stirk et al., 2008), in seeds of maize, oat and lucerne (Stirk et al., 2012b) and in many other plant species (unpublished results referenced in Stirk et al., 2008). This suggests that aromatic CKs may perform a common role in seed germination.

We have presented in this work, that MPP is capable to bind both to CK receptors and to CKX, although it neither activates them, nor is substrate of CKX. Thus it has dual role. It decreases response on HKs and by inhibiting CKX it extends the response. As MPPR and MPP9G are present in amounts comparable with other CKs, their effect on CKX may be even more profound as ribosides and *N9*-glucosides are often preferred substrates of CKX. On the other hand, they will not affect the receptors, as those do not bind *N9*-glucosides and only weakly bind ribosides. However, MPPR and MPP9G

probably will not be able to bind to CKX in the unique way as reported for MPP (Fig. 15). Will they be able to inhibit CKX?

Plant-Associated Organisms

The content of cytokinins in the plant-associated bacteria was known since the beginning of cytokinin research. Indeed, the isopentenyladenine was purified for the first time from the bacterium *Rhodococcus fascians* (Helgeson and Leonard, 1966; Klämbt et al., 1966) and the biosynthetic gene IPT was first cloned from *Agrobacterium tumefaciens* (Akiyoshi et al., 1984; Barry et al., 1984).

There seem to be an ambivalent function of cytokinins in the plant-interactor interaction. While many of the interactors produce cytokinins, to form green islands, to produce galls and/or to form local sink, plants use cytokinins to increase their resistance against pathogens. Sometimes, the CKs can have even both functions, as exemplified by *Pseudomonas syringae* activating cytokinins (Hann et al., 2014), but susceptible to immune response induced by high CK levels (Choi et al., 2010; Großkinsky et al., 2011), *Rhodococcus fascians* producing CKs, yet expressing *CKX* (Pertry et al., 2010) or *Heterodera schachtii* requiring just the right level of cytokinins (Shanks et al., 2016). Thus, as always, the right kind and the right amount of cytokinin is necessary for a successful interaction.

The plant's resistance is mainly regulated by ethylene, salicylic acid and/or jasmonic acid. While ethylene and jasmonic acid are mainly involved in the defence response against necrotrophic pathogens, salicylic acid mainly participates in interaction with (hemi)biotrophs (O'Brien and Benková, 2013). However, evidence for the involvement of CKs in the plant immunity was presented recently as well. The mechanism of crosstalk between CKs and salicylic acid was shown for the first time in *Arabidopsis* infected with *Pseudomonas syringae* pv. *tomato* DC3000 (Choi et al., 2010). Applied *trans*-zeatin hyperactivated salicylic acid-dependent defense response through ARR2. The response regulator activated salicylic acid-responsive *Pr1*, but only in interaction with NPR1 and TGA3, the salicylic acid receptor and interacting transcription factor, respectively.

Increased CK levels in tobacco, either by ectopic expression of *IPT* or by exogenous application, also led to resistance towards *P. syringae* pv. *tabaci* (Großkinsky et al., 2011). On the contrary, CK treatment enhanced susceptibility to the necrotroph *Botrytis cinerea*. Increased levels of CKs led to production of phytoalexins sesquiterpene capsidiol and hydroxycoumarin scopoletin (Großkinsky et al., 2011). Abscisic acid, known to repress salicylic acid-dependent defences (de Torres Zabala et

al., 2009; Mohr and Cahill, 2007), also decreased the effect of the cytokinin treatment (Großkinsky et al., 2014).

Although *P. syringae* proliferation was less restricted upon *trans*-zeatin treatment (Großkinsky et al., 2013) than after kinetin treatment (Großkinsky et al., 2011), the symptom development was less profound (i.e. the bacteria grew better, but symptoms were not so serious). Further, *cis*-zeatin did not restrict the growth of the pathogen at all, but the symptom development was less profound than in control (albeit more profound than in *trans*-zeatin treatment; Großkinsky et al., 2013). Similar results were observed for tobacco plants expressing *AtIPT*2 (putatively producing *c*Z; Schäfer et al., 2015). This suggests that plant's aim may not be necessarily to stop the pathogen from growing, but rather to force it to comply primarily with plant's objectives.

However, more interesting is the interactor's ability to hijack plant's system for their own advantage. By using brute force *Agrobacterium tumefaciens* causes crown gall tumours on dicotyledonous plants as well as some gymnosperms (Gelvin, 2012). This bacteria possesses a tumour-inducing (Ti) plasmid. It is solely responsible for bacteria's pathogenicity as was shown by introducing this plasmid into benign bacteria (Broothaerts et al., 2005; Teyssier-Cuvelle et al., 2004). Part of the Ti plasmid, called T-DNA, is transferred into the plant cell in the form of single stranded DNA (Yusibov et al., 1994) and incorporated into host's genome. Interestingly, octopine-type Ti plasmids carry two T-DNA sequences (Zhu et al., 2000). This DNA carries and upon incorporation expresses three genes involved in auxin and cytokinin biosynthesis, while others synthesise opines. Opines are conjugates of pyruvate or α-ketoglutarate with amino acids. These opines cannot be processed by plant cells and thus they are excreted into the extracellular milieu. On the other hand, *Agrobacterium* uptakes them actively, hydrolyses them and uses them as carbon, nitrogen and energy source (Gordon and Christie, 2014).

Agrobacterium with mutation in *tmr* locus, the part of Ti plasmid, caused rooty galls that lacked cytokinins (Akiyoshi et al., 1983). Subsequently, the gene was cloned and expressed in *Escherichia coli* (Akiyoshi et al., 1984; Barry et al., 1984). Later, direct production of *trans*-zeatin by the enzyme was shown by incorporation of labelled precursor. It was shown, that the Tmr protein is localized in plastids, although it lacks apparent plastid-targeting sequence (Sakakibara et al., 2005).

Rhodococcus fascians uses more delicate approach. It contains linear plasmid with *fas* operon that contains 6 genes (Pertry et al., 2010). Homologous operon was described also in mobile pathogenicity island of *Streptomyces turgidiscabies* (Kers et al., 2005). While the *S. turgidiscabies* produces scab on potatoes, it is capable to form

leafy galls on tobacco and *Arabidopsis* (Joshi and Loria, 2007), just like *R. fascians*. The *fas* operon contains complete set of genes for CK production – *IPT*, *CYP450* and *LOG* and the bacteria secretes set of 6 cytokinins – iP, *t*Z, *c*Z and their 2-methylthioderivatives (Pertry et al., 2009). Although the *cis*-zeatin and the 2-methylthioderivatives bind less efficiently to CK receptors and they have low activities in various bioassays, the mixture of six cytokinins is very potent (Pertry et al., 2009). However, the *fas* operon contains also *CKX* with preference towards iP-type CKs. As expected, iP was increased in the *ckx* knock-out mutant. However, levels of *c*Z and *t*Z were unexpectedly decreased. And they were also decreased in the *log* knock-out mutant.

Expectedly, the strains with mutations in *ipt* and *cyp450* were nonvirulent. The *ckx* mutant generated strongly attenuated phenotype with no more than 30 % of plants fully responsive, suggesting important role of the CK degrading enzyme in the infection. The *log* mutant was able to infect the plant, but the symptoms diminished over time. Thus the LOG is probably not required for symptom initiation, but it is essential for symptom maintenance (Pertry et al., 2010).

All the mutants had two things in common: 1) they lacked 2-MeS-*t*Z and 2) level of 2-MeS-iP was increased in all of them. As there is not a significant difference in levels of 2-MeS-iP between plasmid-free and WT bacteria, there must exist other pathway for production of 2-MeS-iP independent of the *fas* operon. Possibly analogous to *Tzs* of *Agrobacterium*, it is not directly involved in plant colonization. It is notable, that also the other cytokinins were not completely diminished in the mutants of *fas* operon genes.

So why are *R. fascians* and *S. turgidiscabies* the only organisms to produce the leafy galls? They contain the *fas* operon that is involved in the CK biosynthesis only, unlike the T-DNA of *Agrobacterium*, which also encodes auxin biosynthetic genes. But *R. fascians* was shown to produce auxins as well (Vandeputte et al., 2005). Thus, the difference is in the control over virulence genes (T-DNA integrated into host's genome vs. *fas* operon operated by the bacteria itself) and the mix of CKs modulated by the CKX expression. *S. turgidiscabies* was reported to have different set of hormones without further details (Stes et al., 2013), so it's not possible to hypothesise on the importance of respective cytokinins for the formation of the leafy galls.

Nevertheless, it was suggested, that the plant's CK homeostasis control is the first response. However, that seems unlikely. Rather, the bacteria flush the plant with mixture of highly active cytokinins, including *t*Z and iP recognized by AHK4. This is sufficient for establishment of novel meristem(s). Approximately 2 days later, the

Table 1: Organisms using cytokinins for their interaction with plant host. Interactors with their basic classification are listed (G- and G+ are Gram-negative and Gram-positive bacteria, respectively). Cytokinins that accumulate the most, or that were shown to be crucial for the pathogenicity (as for *Rhodococcus* and *Claviceps*) and cytokinins that are depleted upon infection are listed. Hosts with affected organ and major cytokinins in the infected organ are listed.

Interactor	Classification	Cytokinins Important for the Interaction	Depleted Cytokinins	Host Organ	Major Cytokinins of Host
Agrobacterium tumefaciens	G- *Rhizobiales*	tZ		wide range of hosts roots	mainly tZ & iP
Rhodococcus fascians	G+ *Actinomycetales*	iP cZ, 2-MeS-CKs (di)methyl-iP	tZ	wide range of hosts shoots	mainly tZ & iP
Claviceps purpurea	Fungi *Ascomycota*	cZ-type		rye and other grasses flowers	zeatins, mainly cZ(R)OG, DHZ(R)OG
Ustilago maydis	Fungi *Basidiomycota*	cZRPs, cZR, tZ	cZ9G, tZ9G	maize, teosinte kernels	cZ9G, tZ9G, DHZ(R)OG
Magnaporthe oryzae	Fungi *Ascomycota*	cZR		rice and other cereals leaves	cZ-type

Table 1: Organisms using cytokinins for their interaction with plant host. Interactors with their basic classification are listed (G- and G+ are Gram-negative and Gram-positive bacteria, respectively). Cytokinins that accumulate the most, or that were shown to be crucial for the pathogenicity (as for *Rhodococcus* and *Claviceps*) and cytokinins that are depleted upon infection are listed. Hosts with affected organ and major cytokinins in the infected organ are listed. (*continued*)

Interactor	Classification	Cytokinins Important for the Interaction	Depleted Cytokinins	Host Organ	Major Cytokinins of Host
Leptosphaeria maculans	Fungi *Ascomycota*	cZR, cZ, iP	tZ-type	*Brassica* crops cotyledons	tZ-type; tZ7G, iP7G
Phtheirospermum japonicum	Plant *Eudicots*	tZ-type		Arabidopsis roots	mainly tZ & iP
Heterodera schachtii	Animal *Nematoda*	iP-type, iP		more than 200 plants including Arabidopsis roots	mainly tZ & iP
Phyllonocrycter blancardella	Animal *Arthropoda*	tZ-type, DHZ-type	cZR, cZR(OG)	apple trees leaves	iPRPs, iPR, tZ
Wolbachia	G- *Rhickettsiales*				

bacterium starts expressing its own *CKX* with preference for iP, effectively removing it from the signal. Thus the additional cytokinins are perceived by AHK3 with wider ligand recognition and the signal attenuates. The plant now has nothing to respond to, but the signal is sufficient to promote the growth of the new meristem(s) into the leafy galls.

Recently, the importance of the CK mix was questioned as novel *Rhodococcus fascians* isolate A21d2 lacking the *fas* operon was identified (Creason et al., 2014). The authors further showed, that only iP-type CKs accumulated upon *fas* induction in the D188 strain (the same strain as used by Pertry et al., 2010). However, it still accumulated *c*Z-type CKs (including 2-MeS-derivative), albeit not in induction-dependent manner. Indeed, that would not contradict the hypothesis proposed in previous paragraph.

Further, two novel cytokinin derivatives were identified in *Rhodococcus* (Radhika et al., 2015). To examine the function of the two methyltransferases located in vicinity of the *fas* operon, authors expressed them with *IPT* from the *fas* operon and detected two novel compounds. They were N^6-(3-methylpent-2-enyl)-adenine [1] and N^6-(3-ethylpent-2-enyl)-adenine. Surprisingly, the enzymes did not use any CK or DMAPP as substrate, but IPP, the positional isomer of DMAPP. The produced methyl- and ethylpentenyl diphosphate are then used by the *IPT* from *Rhodococcus* to produce the novel CKs (Radhika et al., 2015). The *S. turgidiscabies* also possesses these methyltransferases (Joshi and Loria, 2007) and thus it is probable it produces analogous CK derivatives. The *Rhodococcus fascians* isolate A21d2 does not contain analogous methyltransferases, but it does contain protein with radical SAM domain (as the methyltransferases do) and another protein with an alpha/beta hydrolase domain (Creason et al., 2014). Thus it may form other additional CK derivatives.

Although *Pseudomonas syringae* does not produce cytokinins on its own and plants use cytokinins in their defence against it (see above), *Pseudomonas* still manages to hijack plants' hormones for its advantage. Upon infection, *Pseudomonas* injects plant cells with approx. 30 effectors though needle-like structure known as type III secretion system. Their aim is to suppress plant's pattern-triggered immunity through modification of hormone signalling (Hann et al., 2014). One of the effectors is HopQ1, protein with homology to nucleoside hydrolases. Authors showed through several approaches, that the protein is responsible for plant's response attenuation.

[1] This compound has two isomers. Although the authors do not state it explicitly, the compound depicted in the paper has the same configuration as *trans*-zeatin, what may explain its high activity.

Unfortunately, the researchers used a spectroscopic method for nucleoside hydrolase activity measurement instead of available HPLC, to measure the activity of an enzyme with homology to nucleoside hydrolase. They concluded that the enzyme has Lonely Guy-like, i.e. phosphoribohydrolase activity, although the nucleoside hydrolases reportedly did not accept nucleoside phosphates as substrates (Parkin et al., 1991). Although it is true, that one of the conserved aspartates is shifted in the motif, which could explain difference in substrate specificity, the authors omitted to report iPR levels in *Arabidopsis* expressing HopQ1, or to use HPLC for the *in vitro* activity determination, both of which would provide unambiguous hint on the protein's true enzymatic activity.

Similarly *Verticillium* appears to manipulate CK levels to its own advantage as was shown in cotton (Misaghi et al., 1972), tomato (Patrick et al., 1977) and Arabidopsis (Reusche et al., 2013). *Verticillium* is a soil-borne fungus that invades plants through roots, grows through xylem to upper part and eventually proliferates in leaves, where it induces senescence and switches to necrotrophic life style. The infection of *Arabidopsis* by *Verticillium* causes decrease specifically of *trans*-zeatin free base (with slight, albeit insignificant decrease of other *t*Z metabolites) with upregulation of *AtCKX*1, 2 and 3 (Reusche et al., 2013). It wasn't shown, whether the changes are induced directly by the fungus, or whether they are plant's response to infection, but considering that the fungus only profits from the induced senescence, the first option seems plausible.

Knowledge of fungi producing cytokinins dates back to the 60s of last century (Miller, 1967), however, the significance for interaction with plant was shown only recently. Genes for CK *de novo* biosynthesis were identified in *Claviceps purpurea* (Hinsch et al., 2015), several species of the *Fusarium fujikuroi* species complex (Niehaus et al., 2016) and in *Fusarium pseudograminearum* (Sørensen et al., 2017). All of them contain the biosynthetic genes fused to form IPT-LOG protein. Additionally, the corresponding gene is in cluster with *CYP450*, knock-out of which led to depletion of zeatins (Hinsch et al., 2015; Sørensen et al., 2017).

Interestingly, the *cpcyp450* mutant of *C. purpurea* showed a hypersporulation phenotype (Hinsch et al., 2015). The amoeba *Dictyostelium discoideum* is known to produce an sporulation inhibitor discadenine, a CK derivative (Abe et al., 1976). Although both organisms belong to different supergroups (Adl et al., 2012), it would be possible, that they both use similar mechanism for sporulation regulation involving CK derivative.

However, the tRNA-derived cytokinins are crucial for the infection for *Claviceps purpurea* (Hinsch et al., 2016), *Magnaporthe oryzae* (Chanclud et al., 2016) and

Leptosphaeria maculans (Trdá et al., 2017). Deletion of the *tRNA IPT* in these organisms abolished *c*Z production (in *M. oryzae* also production of iP-type CKs) and reduced virulence.

Wheat infected with *F. pseudograminearum* was shown to contain 3-methylpyrrolpurine and two of its derivatives (Sørensen et al., 2017). MPP was first discovered in tobacco crown gall cells (Haidoune et al., 1990) and recently reported in maize (this work). However, the authors did not include mock-treatment control and thus it cannot be concluded that it originates from the *Fusarium* infection and isn't wheat endogenous CK.

The *L. maculans* was reported to possess CK degrading activity that was strictly specific towards iP (Trdá et al., 2017). This is reminiscent of the *Rhodococcus*'s need for CKX activity for the infection development. However, *CKX* gene was not identified. Possibly, it could be adenine deaminase, that was previously reported to degrade CKs (Pospíšilová et al., 2008) and the produced hypoxanthine usually co-elutes with adenine on reversed-phase HPLC. Similarly, maize infected with *Ustilago maydis* displayed shift from glucosides to the active forms (Morrison et al., 2015). This wasn't solely due to novel synthesis as the *N9*-glucosides were largely depleted. The authors argue this could be due to inhibition of *N*-glucosyltransferase or *O*-glucosyltransferase or due to activation of β-glucosidase. The third option would deplete rather the *O*-glucosides that actually piled up. Affecting the activity of *O*-glucosyltransferase would not affect the *N9*-glucosides. A CK *N9*-glucosyltransferase was previously described in maize seedlings (Rijavec et al., 2011). However, its inhibition would rather prevent accumulation of *N9*-glucosides, rather than deplete them. Thus CKX seem to be responsible for the depletion, as *N9*-glucosides are their substrates (Zalabák et al., 2014). It is noteworthy though, that levels of DHZ9G were depleted as well and DHZ-type CKs are resistant to CKX cleavage (Galuszka et al., 2007). However, DHZ-type CKs seemed to be generally decreased in the infected tissues.

Recently, the pathogenic plant *Phtheirospermum japonicum* was shown to employ cytokinins during Arabidopsis invasion (Spallek et al., 2017). Although the biosynthetic genes were not identified, production of *t*Z-type CKs by the parasitic plant was unambiguously shown. Reduction of CK level by *AtCKX*3 expression greatly diminished hypertrophy, although it did not affect the formation of haustoria, a specialized organ with vasculature connection used for feeding.

The importance of nematode-derived CKs for plant colonization has been shown recently as well (Siddique et al., 2015). *Heterodera schachtii* is a cyst nematode that invades plant roots mostly near the tip and proceeds intracellularly toward the vascular

cylinder. A feeding site called syncytium is formed there through reprogramming of chosen cells (Goverse et al., 2000).

The genome of *Heterodera schachtii* contains single *IPT* gene with homology to *tRNA IPTs* (Siddique et al., 2015). An ortholog from *Heterodera glycines* was shown on Affymetrix Soybean GeneChip to be expressed the most at the beginning of the infection and to decline later on. Silencing of the *H. schachtii IPT via* RNAi approach reduced levels of iP free base by 40% in the nematode, but it did not affect the nematode growth, neither establishment of the syncytium. However, syncytium size and number and size of female nematodes were significantly decreased.

The authors argue that the tRNA IPT pathway produces *c*Z-type CKs. However, that is the case for plants, more specifically for *Arabidopsis*. In other plants and moreover in animals, the situation may be different. And indeed, the tRNA sequences from animals compiled in tRNA database (Jühling et al., 2009) contain only (2-MeS-)iP, but no *c*Z. Nevertheless, the amount of *c*Z-type, iP-type and total CKs in the RNAi line was 78%, 80% and 81% of WT. Thus the biosynthesis of both was probably affected in similar way.

Recently, involvement of genes expressed by the host was studied as well (Dowd et al., 2017). In the previous study (Siddique et al., 2015), authors concluded that host-derived cytokinins are important for the interaction as *atipt*3 5 7 mutants had reduced syncytia size. In the new study, they showed that adenylate *AtIPT*1 and *tRNA AtIPT*2 were upregulated, while expression of adenylate *AtIPT*3 and *tRNA AtIPT*9 was downregulated. Still the degradation of cytokinins is important for host colonization as well, as expression of *AtCKX*5, 6 and 7 was upregulated. Indeed, *ckx*5 6 double mutant showed significant reduction of female nematodes (Dowd et al., 2017).

There is discrepancy in which *Arabidopsis* histidine kinase is involved in the nematode-Arabidopsis interaction. Shanks et al. (2016) reported decrease of female nematodes in *ahk*4 and in all *ahk* double mutants to a similar extent. On the other hand, Siddique et al. (2015) reported significant decrease in number of female nematodes only in the *ahk*3 4 double mutant. However, number of male nematodes increased to a similar degree, albeit the difference was not statistically significant. Thus the total number of nematodes infecting *Arabidopsis* roots was similar. Moreover, the syncytium size was decreased in all three double mutants to a similar extent (Siddique et al., 2015).

The involvement of CKs in the formation of green islands by leaf-mining insect has been also suggested (Giron et al., 2007). Interestingly, the greatest changes are

observed in *t*Z- and DHZ-type CKs, while *c*ZR(OG) is completely missing (Zhang et al., 2017). But in that case, bacterial symbionts are most likely involved in the production of the CKs (Kaiser et al., 2010).

At last, an honourable mention of *Mycobacterium tuberculosis*, although it does not interact with plants. However, it is bacterium that uses plant hormones to invade mammals, more specifically humans. More than that, it uses plant hormones to live right in the heart of the enemy – inside of macrophages, the immune cells that shall remove the invading organisms. The success of mycobacterial infection resides on proper levels of Lonely guy homologue (Samanovic et al., 2015). There seem to be increase of CK degradation products in response to LOG accumulation due to dysfunctional homologue of ubiquitin. The degradation products damage cell membranes and confer hypersensitivity to nitric oxide (Samanovic et al., 2015). Interestingly, a distant relative *Rhodococcus equi* uses similar mode of action when infecting mostly young horses or AIDS patients (von Bargen and Haas, 2009). It appears to hold the complete arsenal for CK production as well and thus possibly it uses CKs during infection as well.

The function of CKs in the mycobacterial infection is not known hitherto. It was proposed it may serve the bacteria for their own communication. To date, knowledge about CK perception in mammals is lacking, although CKs are capable to influence cell cycle progression through direct interaction with cyclin-dependent kinases. Moreover, iP is capable to regulate gene expression in mammalian cells (Colombo et al., 2009).

What the interactions have in common is (Table 1):

1) the interactors tend to produce CKs with lower activity as *c*Z-type CKs and/or 2-methylthioderivatives of cytokinins. Possibly, the 2-methylthioderivatives are much more represented in the plant-interactor interactions, as they are generally not reported, rather than reported not to be present
2) the interactors usually produce the active forms – free bases of CKs and thus stall the plant's CK perception with little effort
3) in some instances, the interactor actively degrades particular forms of CKs
4) the production of CKs usually diminishes over time. After the gall or feeding site has been established, the interactor doesn't need to invest as much resources into the production of CKs

Although the past part focused largely on cytokinins, they are not the only phytohormones involved in the plant-interactor interactions as reviewed for plant immunity by Shigenaga and Argueso (2016). Similarly, *Agrobacterium*, *Rhodococcus* and *Claviceps*, as well as *Fusarium* produce auxins. *Fusarium* produces gibberellins as

Figure 7: Hulk and Deadpool of the Marvel Universe. Hulk is strong, using brute force. Deadpool on the other hand is resilient due to his immortality. Are there Hulks and Deadpools in the Cytokinin Universe? Created by David Ocampo.

well. Both auxin and ethylene were proposed to play a role in the establishment of syncytium by the nematodes.

Hulks and Deadpools of the Cytokinin Universe

Throughout the universe of plant cytokinins, there seem to be recursive theme of the strong, but specialized Hulk, versus the less active, but more resilient Deadpool (Fig. 7). For starter, there are the highly active *trans*-zeatin and isopentenyladenine, whose levels are tightly controlled by CKX and other detoxifying enzymes. On the other hand, dihydrozeatin is resistant to CKX cleavage and *cis*-zeatin and aromatic cytokinins are in general weak substrates (Galuszka et al., 2007; Zalabák et al., 2014). But this resistance to deactivation makes their feeble signal persistent (as demonstrated for *Rhodococcus*; Pertry et al., 2009). Similarly the cytokinin receptor AHK4 strictly discriminates for free bases of the highly active *t*Z and iP, while the AHK3 is not able to differentiate respective isoprenoid cytokinins apart very well and it is also able to perceive ribosides

and partly nucleotides (Spíchal et al., 2004). Overall, its response is much lower than that of AHK4 (Hwang and Sheen, 2001; Spíchal et al., 2004). There also appears to be low- and high-affinity transport of cytokinins (Cedzich et al., 2008). Next, the Lonely guy protein is capable to quickly and specifically activate CKs (almost) directly from their biosynthetic form (Kuroha et al., 2009), yet there is the possibility of two-step activation, which may originate simply from plant's inability to regulate the unspecific activity, but more likely it does have a physiological significance, as discussed above. As mentioned also above, *At*LOG8 has the weakest activity among the *Arabidopsis* LOGs, yet its expression is the highest (Kuroha et al., 2009). Unless it is in fact a promiscuous enzyme with both cytokinin phosphoribohydrolase and lysine decarboxylase activities, it must work to substantially activate small amounts of cytokinins globally throughout the plant.

Thus, to expand the previously published hypothesis, that *c*Z is important under growth-limiting conditions (Schäfer et al., 2015), here the author would like to propose an extension of that hypothesis: there are two sets of metabolites, enzymes and perception relays for CKs. While one, including, but not limited to *t*Z, iP, AHK4 and *At*LOG7, is highly active and it has specialized functions in promoting and regulating rapid growth, there is other one, including, but not limited to *c*Z, DHZ, AHK3 and *At*LOG8 that is involved in maintenance growth. After all, cytokinins were discovered for their cytokinetic properties.

Cytokinin Metabolism in Maize: Novel Evidence of Cytokinin Abundance, Interconversions and Formation of a New *trans*-Zeatin Metabolic Product With a Weak Anticytokinin Activity

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Abstract

Cytokinins are an important group of phytohormones. Their tightly regulated and balanced levels are essential for proper cell division and plant organ development. Here we report precise quantification of CK metabolites and other phytohormones in maize reproductive organs in the course of pollination and kernel maturation. A novel enzymatic activity dependent on $NADP^+$ converting *trans*-zeatin to 6-(3-methylpyrrol-1-yl)purine was detected. MPP shows weak anticytokinin properties and inhibition of cytokinin dehydrogenases due to their ability to bind to an active site in the opposite orientation than substrates. Although the physiological significance of *t*Z side chain cyclization is not anticipated as the 3-methylpyrrolpurine occurrence in maize tissue is very low, properties of the novel cytokinin metabolite indicate its potential for utilization in plant *in vitro* tissue culture. Furthermore, feeding experiments with different isoprenoid CKs revealed distinct preferences in glycosylation of *t*Z and *cis*-zeatin. While *t*Z is preferentially glucosylated at the *N9* position, *c*Z forms mainly *O*-glucosides. Since *O*-glucosides, in contrast to *N9*-glucosides, are resistant to irreversible cleavage catalysed by cytokinin dehydrogenases, the observed preference of maize cytokinin glycosyltransferases to *O*-glucosylate zeatin in the *cis*-position might be a reason why *c*Z derivatives are over-accumulated in different maize tissues and organs.

Introduction

Besides auxins, cytokinins (CKs) are major plant hormones regulating cell division and elongation as well as organogenesis and many other physiological processes in plants. Naturally occurring CKs are N^6-isoprenoid derivatives of adenine and its sugar conjugates, which can originate *in planta* by two metabolic pathways.

The first step of the *de novo* pathway employs an activity of adenylate isopentenyltransferase (IPT; EC 2.5.1.112) to conjugate ATP or ADP with dimethylallyl pyrophosphate (Kakimoto, 2001; Takei et al., 2001a); the conjugation product, isopentenyladenosine 5´-di- or 5´-triphosphate, can be further hydroxylated on the isoprenoid side chain by CK specific cytochrome P450 monooxygenases (Takei et al., 2004b). Both hydroxylated and non-hydroxylated CK riboside phosphates are activated by CK-specific phosphoribohydrolases (LOG; EC 3.2.2.n) to form *trans*-zeatin or isopentenyladenine (Kurakawa et al., 2007). *t*Z and iP together with their sugar conjugates form a majority of the CK pool found in vegetative tissues of the model plant *Arabidopsis thaliana*.

The other CK production pathway, based on the decay of prenylated tRNA, exists in all eukaryotic and prokaryotic organisms with the exception of Archaea (Golovko et al., 2002). The role of tRNA prenylation via tRNA isopentenyltransferase (tRNA IPT; EC 2.5.1.75) is to strengthen fidelity of the anticodon reading during translation (Lamichhane et al., 2013). Contribution of tRNA-released CKs to their cellular or tissue pools is unclear, though. Based on the Arabidopsis double *trna ipt* knock-out mutants, an origin of all derivatives of *cis*-zeatin, a stereoisomer of *t*Z, is attributed solely to the tRNA decay in Arabidopsis (Miyawaki et al., 2006). Nevertheless, there are plant species in which *c*Z derivatives form a majority of detected CKs in contrast to *Arabidopsis* (Gajdošová et al., 2011). Whether *c*Z originates by more robust RNA decay in these species or by an alternative pathway has not yet been elucidated. Recently, a work quantifying CKs in *Physcomitrella trna ipt*1 knock-out mutants showed that a majority of *c*Z metabolites is also of tRNA origin in this ancestral land plant model (Lindner et al., 2014).

In vitro conversion of *t*Z to its *cis*-counterpart and *vice versa* was shown with a partially purified protein from the bean endosperm (Bassil et al., 1993). However, a gene encoding the hypothetical isomerase has never been found and feeding experiments with radioactively labeled precursors have showed a distinct origin of the isoprenoid side chain in *t*Z and *c*Z in *Arabidopsis* (Kasahara et al., 2004). Further, there is only a single report of *in vivo* inter-conversion of zeatin stereoisomers. Suttle and Banowetz (2000) reported 5-9 % of recovered radioactivity associated with *t*Z riboside (*t*ZR) after treatment of potato tubers with *c*Z, but all the other feeding experiments reported no isomerization (Kuroha et al., 2002; Nandi and Palni, 1997; Yonekura-Sakakibara et al., 2004).

A reduced form of zeatin, dihydrozeatin (DHZ), was found as a prevalent CK metabolite in some dormant seeds (Stirk et al., 2012a; Zalabák et al., 2014) and in the endosperm of maturing seeds (Mok et al., 1990). NADPH-dependent enzymatic activity reducing *t*Z to DHZ has been detected in extracts from bean embryos (Martin et al., 1989) and pea leaves (Gaudinová et al., 2005). However, a contribution of the activity to the DHZ pool was not confirmed and a gene coding for the zeatin reductase has not yet been identified.

Developing seeds and reproductive organs are considered as tissues with the highest concentration of CKs. The content of various CK types and metabolic enzymes was determined in the maize caryopsis in several independent studies (Brugière et al., 2003; Dietrich et al., 1995; Rijavec et al., 2011; Veach et al., 2003). Dynamic changes in the total content and various CK forms were observed in relation to rapid cell division and cell expansion in certain periods of the caryopsis development (Rijavec and

Dermastia, 2010). *t*Z and *t*ZR were found to be major CKs, whose levels significantly increased and showed the maximum around the 10th day after pollination (DAP). Other types of CKs either did not show significant changes or were not measured (Rijavec et al., 2011). Interestingly, levels of *t*Z increased again when the embryo was fully developed and the endosperm became starchy. Levels of iP riboside (iPR) start to elevate around the 20th DAP predominantly in the maternal tissue (Brugière et al., 2003). A significant difference in the total CK content between the unfertilized cob and kernels 10 to 16 DAP was also observed in the study of Veach et al. (2003), where a dramatic elevation of *O*-glucosylated forms of *c*Z and DHZ ribosides was detected, in addition to *t*Z, *t*ZR and its monophosphate (*t*ZRMP).

An increase in CK content in maize caryopsis is subsequently accompanied by raised activity of CK dehydrogenase (CKX), an enzyme irreversibly cleaving the CK molecule (Bilyeu et al., 2001; Brugière et al., 2003). CK free bases are good substrates of CKX enzymes that may regulate their availability for binding to CK receptors. It was shown that local CK maxima during caryopsis development are at least partially supplied *in situ* by *de novo* biosynthesis. An expression of *ZmIPT*2 reached its maximum in the maternal tissue around the 10th day of the development when the total CK content cumulates (Brugière et al., 2008). Interestingly, transcripts of *tRNA IPT* genes were found more abundant in vascular cells of the maternal-pedicel tissue than in other tissues (Rijavec et al., 2011).

Recently, all isozymes from maize *CKX* gene family were functionally characterized with focus on their substrate specificity (Zalabák et al., 2014). The study was accompanied with detailed changes in profiles of all types of CKs during the early development of maize seedlings. It is obvious that the dormant seed serves as a storage pool of CKs where DHZ-types especially are accumulated. Other isoprenoid CKs are likewise present in higher concentrations than later in emerging radicula and coleoptile. In this work, we bring detailed profiles of all CK derivatives in maize reproductive organs before and after the pollination and during kernel maturation. We also focus on possible inter-conversions among different isoprenoid CKs and bring evidence about a new metabolic product of *t*Z with a weak anticytokinin activity.

Material and Methods

Plant Material

Maize seeds (*Zea mays* 'Cellux'; Morseva, Czech Republic) were imbibed in tap water and germinated in the dark on wetted filter paper. After 2 days, the germinated seedlings were transferred to aerated hydroponic tanks filled with Hoagland nutrient

solution (Vlamis and Williams, 1962) and supplemented with CKs. The plants were grown in a growth chamber with a 16-h light period (250 μE m^{-2} s^{-1}) at 27°C and an 8-h dark period at 20°C. Maturing kernels were excised from cobs in the maternal pedicel zone and cultivated in Murashige and Skoog (M&S) agar (Murashige and Skoog, 1962) supplemented with radioactively labeled CKs. To cultivate plants through to the reproductive phase, germinated seedlings were transferred to pots with a 2:1 mixture of soil and perlite (Perlit Ltd., Czech Republic) and fertilized every 14 days. Plants were kept in controlled greenhouse conditions over a period from spring to summer when day temperatures ranged from 23 to 35°C and night temperatures from 16 to 20°C. Samples of reproductive organs were collected and pooled from three cobs at the same developmental stage. Before pollination and the 1st week after pollination, ovule samples were collected together with the rachis. Later developmental stages were trimmed out of rachis and glumes and 3 to 5 kernels from each cob were pooled. Male reproductive organs were sampled without peduncle as independent tassel branches.

Hormonal Profiling

The analysis of plant hormones was carried out as described previously (Djilianov et al., 2013; Dobrev and Vankova, 2012). The frozen plant material was homogenized in liquid nitrogen by mortar and pestle. An aliquot of about 100 mg fresh weight was transferred into microcentrifuge tube. Mixture of stable isotope labelled internal standards (10 pmol) and 500 μl cold extraction buffer (methanol/water/formic acid, 15/10/5, v/v/v, -20°C) were added to the plant homogenate. The following internal standards: [$^{13}C_6$]indole-3-acetic acid (IAA; Cambridge Isotope Laboratories), [$^{2}H_4$]salicylic acid (SA), [$^{2}H_4$]1-aminocyclopropane-1-carboxylic acid (ACC; both Sigma-Aldrich, U.S.A.), [$^{2}H_5$]jasmonic acid (JA; C-D-N Isotopes Inc.), [$^{2}H_6$]abscisic acid (ABA), [$^{2}H_2$]gibberellin A19 (GA19), [$^{2}H_5$]*t*Z, [$^{2}H_5$]*t*ZR, [$^{2}H_5$]*t*Z7G, [$^{2}H_5$]*t*Z9G, [$^{2}H_5$]*t*ZOG, [$^{2}H_5$]*t*ZROG, [$^{2}H_5$]*t*ZRMP, [$^{2}H_3$]DHZ, [$^{2}H_3$]DHZR, [$^{2}H_3$]DHZ9G, [$^{2}H_6$]iP, [$^{2}H_6$]iPR, [$^{2}H_6$]iP7G, [$^{2}H_6$]iP9G, [$^{2}H_6$]iPRMP (all OlchemIm, Czech Republic). After incubation for 30 min at -20°C, the extract was centrifuged at 17 000*g* and supernatant collected. The second extraction of the residue followed. The two supernatants were pooled and evaporated in a vacuum concentrator. Sample residue was dissolved in 0.1 M formic acid and applied to mixed mode reversed-phase–cation exchange solid-phase extraction (SPE) column (Oasis-MCX, Waters, U.S.A.). Two hormone fractions were sequentially eluted: (1) fraction A eluted with methanol – containing hormones of acidic and neutral character (auxins, ABA, SA, JA), and (2) fraction B eluted with 0.35 M NH_4OH in 70% methanol – containing the hormones of basic character (CKs) and ACC. The fractions were evaporated to dryness in a vacuum concentrator and

dissolved in 30 µl 10% methanol. An aliquot (10 µl) from each fraction was separately analysed on HPLC (Ultimate 3000, Dionex, U.S.A.) coupled to hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems, U.S.A.) set in the selected reaction monitoring mode. The mass spectrometer was run at electrospray ionization mode, negative for fraction A, and positive for fraction B. Ion source parameters included: ion source voltage -4000 V (negative mode) or +4500 V (positive mode), nebulizer gas 50 psi, heater gas 60 psi, curtain gas 20 psi, heater gas temperature 500°C. Quantification of hormones was performed using isotope dilution method with multilevel calibration curves. Data processing was carried out with Analyst 1.5 software (Applied Biosystems). Each sample was run in two technical replicates.

Extraction and Activity Assay of Enzyme Converting *trans*-Zeatin

Plant tissue was mixed with extraction 0.1 M potassium phosphate buffer, pH 7.3, supplemented with 0.5 mM EDTA, 5 mM dithiothreitol, 20% glycerol (v/v), 0.3% Triton X-100 (v/v) and protease inhibitor cocktail from Sigma-Aldrich in 1/1.5 ratio and homogenized using Ultra Turrax homogenizer. Cleared supernatant (20 min, 9600*g*) was used for an activity assay or further purified. Solid ammonium sulfate was added to the supernatant to 20% saturation. After complete dissolution of the sulfate, the extract was centrifuged (30 min, 9600*g*) and loaded onto Octyl-Sepharose 4 Fast Flow column (GE Healthcare Life Sciences, U.K.) equilibrated with the extraction buffer with 20% ammonium sulfate. Unbound proteins were washed from the column by the same buffer and the enzyme activity was eluted by 10% ammonium sulfate in the extraction buffer. The eluted proteins were desalted and concentrated using Amicon Ultra-15 Centrifugal Filter Units with 10 kDa NMWL (Merck Millipore, U.S.A.).

The enzyme assay was set up in 0.1 M potassium phosphate buffer, pH 8.0, with 160 µM *t*Z, 0.25 mM NADPH or $NADP^+$ and 200 µM dithiothreitol and incubated for 1 to 10 h at 28°C. The enzymatic reaction was stopped by the addition of double volume of 96% ethanol, diluted with 3-fold excess of 15 mM ammonium formate, pH 4.0, cleared through 0.22 µm nylon filter and injected onto a reversed-phase column (ZORBAX Eclipse Plus C18, RRHD, 1.8 µm, 2.1x50 mm, Agilent Technologies, U.S.A.) coupled to Ultra performance liquid chromatography Nexera (Shimadzu, Japan). The mobile phase A was 15 mM ammonium formate, pH 4.0, and the mobile phase B was 100% methanol, flow rate was 0.4 ml min^{-1} and column temperature 40°C. The column was eluted with gradient as follows: 5% B for 2 min, 5-70% B for 14 min. Data processing was carried out with LabSolutions software. The activity was calculated

from peak area of *t*Z oxidized product 6-(3-methylpyrrol-1-yl)purine with absorption maximum at 300 nm.

Purification of Unknown Metabolite

To reveal the origin of the oxidized product from *t*Z, an enzymatic reaction with a partially purified enzyme was set up following the description above but with addition of 100 pmol of [^{3}H]*t*Z (592 GBq mmol^{-1}). The reaction was stopped by double volume of ethanol and separated on an reversed-phase column (Luna C18, 3 μm, 4.6x150 mm, Phenomenex, U.S.A.) coupled to an HPLC Series 2000 autosampler and quaternary pump (PerkinElmer, U.S.A.). The mobile phase A was 40 mM acetic acid adjusted with ammonium to pH 4.0 and the mobile phase B was acetonitrile/methanol, 1/1 (v/v); the flow rate was 0.6 ml min^{-1} and the column temperature 25°C. The column was eluted with gradient as follows: 10-15% B for 2 min, 15-20% B for 9 min, 20-34% B for 0.1 min, 34-45% B for 7.9 min, and 45–100% B for 2 min. The compounds were monitored at 270 and 300 nm by a diode array detector (PerkinElmer) and, after online mixing with 3 volumes (1.8 ml min^{-1}) of liquid scintillation cocktail (Flo-Scint III, Packard BioScience Co., U.S.A.), on a Ramona 2000 flow-through radioactivity detector (Raytest, Germany).

Quantitative purification of the unknown metabolite from the reaction mixture was performed on X-Bridge Prep C18 column (5 μm, 10x100 mm; Waters) connected to Waters 1525 binary HPLC pump. The mobile phase A was water and the mobile phase B was methanol; the flow rate was 2.7 ml min^{-1} and the column temperature was 30°C. The column was eluted with a gradient as follows: 5% B for 5 min, 5-20% B for 15 min, 20-80% B for 27 min, 80-100% B for 3 min and 100% B for 3 min. The compounds were monitored at 268 and 300 nm using Waters 2487 dual wavelength absorbance detector (Waters). The fraction containing the metabolite was evaporated to dryness.

Synthesis of 6-(3-methylpyrrol-1-yl)purine

This compound was prepared by oxidation of *t*Z with manganese dioxide according to Letham and Young (1971). Briefly, *t*Z (500 mg), activated MnO_2 (5 g) and dry dimethylformamide (50 ml) were vigorously stirred in the glass Ace pressure tube at 90°C for 90 min. TLC (silica gel; chloroform/methanol/25% aqueous ammonia, 4/1/0.05) revealed two minor components, adenine and trace of zeatin, and a major spot of a product with higher R_f. The reaction mixture was then cooled down to 25°C, MnO_2 was filtered off and the filtrate was evaporated on a vacuum rotary evaporator at 45°C. The residue after evaporation (~ 750 mg) was treated with water (25 ml) to obtain

130 mg of crude product which was purified on silica gel (60 g) using chloroform/methanol/25% ammonia, 4/1/0.05, as a mobile phase. Fractions containing pure compound were collected, evaporated and crystallized from methanol (10 ml) to yield 50 mg product of 98% purity as tested by HPLC/MS. The product was thus further purified by semi-preparative HPLC as described above.

Quantification of 6-(3-methylpyrrol-1-yl)purine and its Derivatives

Fresh maize tissue samples of 150 to 1000 mg were ground to fine consistency and extracted with 1 ml of ice-cold methanol/water/formic acid (15/4/1, v/v/v) overnight at 4°C. [$^{15}N_4$]MPP (3 pmol) was added to each sample as standard. The homogenates were centrifuged at 19 000*g* for 10 min, corresponding supernatants were further purified using strong cation exchange SPE cartridges (100mg/ml Bond Elut SCX, Agilent Technologies) followed by anion-exchange chromatography (DEAE Sephadex, GE Healthcare Life Sciences) in combination with reversed-phase SPE (100mg/ml, Bond Elut C18, Agilent Technologies). Afterwards, the samples were analysed by reversed-phase ultra-high performance chromatography-tandem mass spectrometry (UHPLC-(+)ESI-MS/MS; Micromass, Manchester, U.K.) using CSH column (Acquity UPLC® CSH C18, 1.7µm, 2.1x50mm; Waters) and gradient consisting of methanol (eluent A) and 15 mM ammonium formate, pH 4.0 (eluent B). MPP and its derivatives were detected using multiple-reaction monitoring mode of the transition of the ion $[M+H]^+$ to the appropriate product ion. Masslynx 4.1 software (Waters) was used to analyze the data. The standard isotope dilution method (Romanov et al., 2006) was used to quantify the MPP levels.

Cytokinin Receptor Competition Assay

The binding assay was performed according to the previously described method (Romanov et al., 2006). *Escherichia coli* cultures strain KMI001 (DrcsC, cps::lacZ) bearing *pPIN-III::ZmHK*1 or *pPIN-III::ZmHK*3 vectors (Podlešáková et al., 2012) were grown in liquid M9 medium supplemented with 50 µg ml^{-1} ampicillin and 0.1% (w/v) casamino acids at 25°C overnight, with shaking (200 rpm), to OD600 ~ 1.0. The portions of the cell suspension (1 ml each) were mixed with 3 pmol of [^{3}H]*t*Z (592 GBq $mmol^{-1}$), with or without tested competitors at 10 µM concentrations, by turning the tubes upside down. After 30 to 60 min incubation at 4°C, the suspension was centrifuged (8000*g*, 5 min, 4°C), the supernatant was carefully removed, and the bacterial pellet was resuspended in 1 ml of scintillation cocktail (Beckman, U.S.A.) in an ultrasonic bath. Radioactivity was measured by a Hidex 300 SL scintillation counter

(Hidex, Finland). Non-specific binding was determined using a large excess of unlabeled *t*Z for each compound. To discriminate the specific and non-specific binding of [^{3}H]*t*Z on the bacterial membrane, 10 μM *t*Z was used and this residual value was subtracted from all the data.

Arabidopsis Root Assay

Arabidopsis thaliana seeds (ecotype Columbia-0) were cultivated on vertical M&S agar plates in the presence of different concentrations of the tested compounds or pure solvent (0.05% DMSO) as described in detail in Podlešáková et al. (2012). After 14 days, the plates were photographed and the length of the primary roots was evaluated using

Table 2: Data collection and refinement statistics.

	ZmCKX4a + MPP
Space group	$P4_32_12$
Asymmetric unit	monomer
Unit cell (Å)	
a	80.1
b	80.1
c	203.2
α=β=γ (°)	90.0
Resolution (Å)	49.5-2.00
Observed reflections	326446 (49737)[a]
Unique reflections	44421 (7038)
Completeness (%)	97.1 (97.1)
$I/\sigma(I)$	22.6 (2.9)
$CC_{1/2}$[b]	100.0 (92.9)
R_{sym} (%)	4.8 (45.8)
R_{cryst} (%)	19.7
R_{free} (%)	21.3
RMSD bond lengths (Å)	0.010
RMSD bond angles (°)	0.99
B average value (Å^2)	
Protein	44.8
Ligand	64.9
Solvent	46.0

[a] Numbers in parentheses represent values in the highest resolution shell 2.00-2.12 Å.

[b] $CC_{1/2}$ stands for a percentage of correlation between intensities from random half-dataset (Storoni et al., 2004).

Scion Image software (Scion Corporation, U.S.A.). The number of fully emerged lateral roots was counted under a stereomicroscope. In total, fifty plants on 5 independent plates were analysed for each treatment.

Cytokinin Oxidase/Dehydrogenase Activity Assay

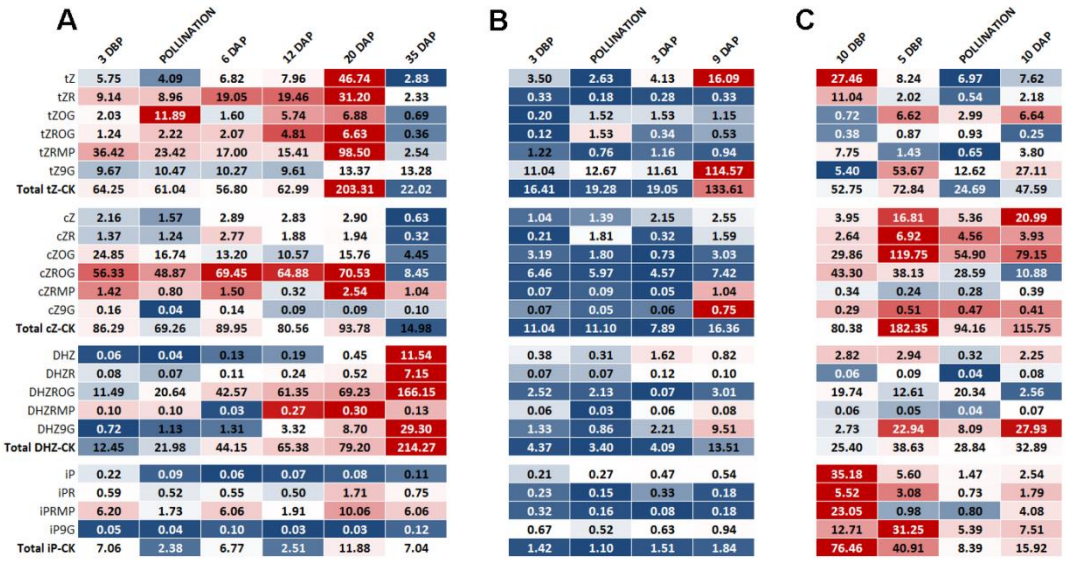
Pure recombinant maize CKX enzymes (Zalabák et al., 2014) were used to test an inhibitory effect of MPP on their activities. A continuous method based on bleaching of 2,6-dichlorophenolindophenol (DCPIP) was used (Laskey et al., 2003). The reaction mixture contained 0.1 mM DCPIP and varying concentrations of iP and inhibitor (5 to 50 μM) in 200 mM Tris/HCl buffer, pH 7.0. The kinetic parameters were calculated using the software GraFit Version 4.0.12 (Erithacus Software, U.K.).

Crystallization and Structure Determination

*ZmCKX*4a ORF, cloned into a *pTYB*12 vector, was expressed in *E. coli* BL21 STAR (DE3) cells. The enzyme was purified by affinity chromatography and ion-exchange chromatography on HighQ column as published previously (Zalabák et al., 2014) and concentrated to 35 mg ml^{-1} by ultrafiltration using an Amicon Ultra-15 Centrifugal Filter Units with 30 kDa NMWL (Merck Millipore). ZmCKX4a in 20 mM Tris/HCl, pH 8.0, and 5% glycerol was crystallized over reservoirs containing 100 mM HEPES, pH 7.5, and 50% 2-methylpentane-2,4-diol. Crystals were infiltrated by 2 mM MPP in DMSO for 1 hour and then they were directly flash frozen in liquid nitrogen. Diffraction data were collected at 100 K on the Proxima 1 beamline at the SOLEIL synchrotron (Saint-Aubin, France) at 2.0 Å resolution. Intensities were integrated using the XDS program (Kabsch, 2010) and data quality was assessed using the correlation coefficient $CC_{1/2}$ (Karplus and Diederichs, 2012). The crystal structures were determined by performing molecular replacement with Phaser (Storoni et al., 2004), using the monomer of ZmCKX4a (PDB ID: 4O95; Kopečný et al., 2015) as search models. Model refinement was performed with BUSTER-TNT (Bricogne et al., 2015). Electron density maps were evaluated using COOT (Emsley and Cowtan, 2004). Refinement statistics are presented in Table 2. Molecular graphics images were generated using PYMOL (www.pymol.org). The atomic coordinates and structure factors have been deposited in the Protein Data Bank (Berman et al., 2000) under accession code 5HHZ.

Endogenous Content and Dynamics of Cytokinin Accumulation in Maize Reproductive Organs

The CK content in maize plants has been studied extensively, but the majority of these studies have focused only on a few derivatives and certain developmental stages. In a recent study, we reported detailed profile and dynamics of CK content in maize seedlings during the first 20 days after the germination (Zalabák et al., 2014). Herein, we focus on dynamics and profiles of all known isoprenoid CK metabolites in reproductive organs of maize (Fig. 8). CK profiles were measured also in selected vegetative tissues for comparison (Fig. 9). Generally, content of all types of *t*Z derivatives is notably higher in reproductive organs than in young seedlings. The ratio of *t*Z to iP-type CKs is strongly in favour of *t*Z in ovules, silks and developing kernels. iP-type CKs are considerably more abundant in tassels where the amount of *t*Z and iP is almost equal. Ovules as well as tassels before pollination seem to be places of vigorous



A

	3 DBP	POLLINATION	6 DAP	12 DAP	20 DAP	35 DAP
tZ	5.75	4.09	6.82	7.96	46.74	2.83
tZR	9.14	8.96	19.05	19.46	31.20	2.33
tZOG	2.03	11.89	1.60	5.74	6.88	0.69
tZROG	1.24	2.22	2.07	4.81	6.63	0.36
tZRMP	36.42	23.42	17.00	15.41	98.50	2.54
tZ9G	9.67	10.47	10.27	9.61	13.37	13.28
Total tZ-CK	64.25	61.04	56.80	62.99	203.31	22.02
cZ	2.16	1.57	2.89	2.83	2.90	0.63
cZR	1.37	1.24	2.77	1.88	1.94	0.32
cZOG	24.85	16.74	13.20	10.57	15.76	4.45
cZROG	56.33	48.87	69.45	64.88	70.53	8.45
cZRMP	1.42	0.80	1.50	0.32	2.54	1.04
cZ9G	0.16	0.04	0.14	0.09	0.09	0.10
Total cZ-CK	86.29	69.26	89.95	80.56	93.78	14.98
DHZ	0.06	0.04	0.13	0.19	0.45	11.54
DHZR	0.08	0.07	0.11	0.24	0.52	7.15
DHZROG	11.49	20.64	42.57	61.35	69.23	166.15
DHZRMP	0.10	0.10	0.03	0.27	0.30	0.13
DHZ9G	0.72	1.13	1.31	3.32	8.70	29.30
Total DHZ-CK	12.45	21.98	44.15	65.38	79.20	214.27
iP	0.22	0.09	0.06	0.07	0.08	0.11
iPR	0.59	0.52	0.55	0.50	1.71	0.75
iPRMP	6.20	1.73	6.06	1.91	10.06	6.06
iP9G	0.05	0.04	0.10	0.03	0.03	0.12
Total iP-CK	7.06	2.38	6.77	2.51	11.88	7.04

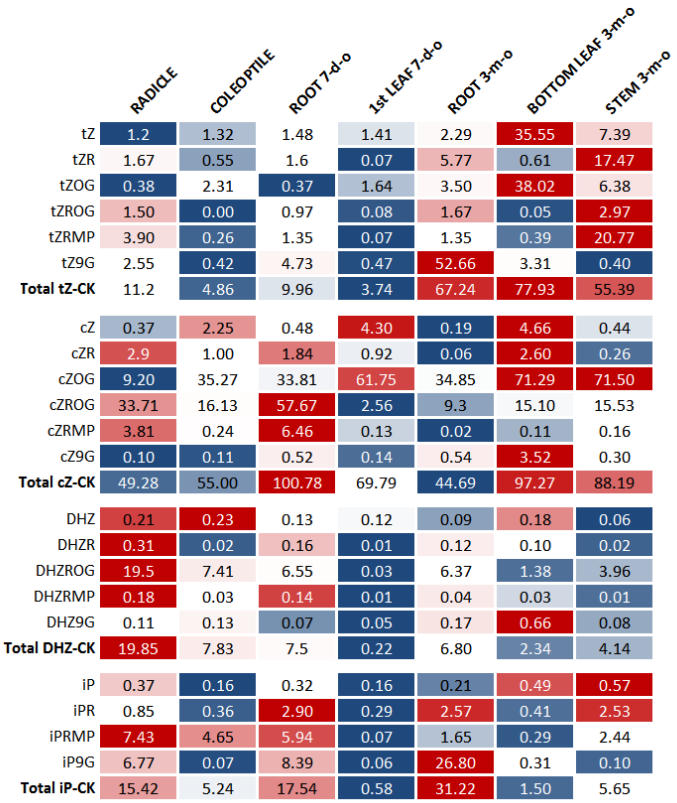
B

3 DBP	POLLINATION	3 DAP	9 DAP
3.50	2.63	4.13	16.09
0.33	0.18	0.28	0.33
0.20	1.52	1.53	1.15
0.12	1.53	0.34	0.53
1.22	0.76	1.16	0.94
11.04	12.67	11.61	114.57
16.41	19.28	19.05	133.61
1.04	1.39	2.15	2.55
0.21	1.81	0.32	1.59
3.19	1.80	0.73	3.03
6.46	5.97	4.57	7.42
0.07	0.09	0.05	1.04
0.07	0.05	0.06	0.75
11.04	11.10	7.89	16.36
0.38	0.31	1.62	0.82
0.07	0.07	0.12	0.10
2.52	2.13	0.07	3.01
0.06	0.03	0.06	0.08
1.33	0.86	2.21	9.51
4.37	3.40	4.09	13.51
0.21	0.27	0.47	0.54
0.23	0.15	0.33	0.18
0.32	0.16	0.08	0.18
0.67	0.52	0.63	0.94
1.42	1.10	1.51	1.84

C

10 DBP	5 DBP	POLLINATION	10 DAP
27.46	8.24	6.97	7.62
11.04	2.02	0.54	2.18
0.72	6.62	2.99	6.64
0.38	0.87	0.93	0.25
7.75	1.43	0.65	3.80
5.40	53.67	12.62	27.11
52.75	72.84	24.69	47.59
3.95	16.81	5.36	20.99
2.64	6.92	4.56	3.93
29.86	119.75	54.90	79.15
43.30	38.13	28.59	10.88
0.34	0.24	0.28	0.39
0.29	0.51	0.47	0.41
80.38	182.35	94.16	115.75
2.82	2.94	0.32	2.25
0.06	0.09	0.04	0.08
19.74	12.61	20.34	2.56
0.06	0.05	0.04	0.07
2.73	22.94	8.09	27.93
25.40	38.63	28.84	32.89
35.18	5.60	1.47	2.54
5.52	3.08	0.73	1.79
23.05	0.98	0.80	4.08
12.71	31.25	5.39	7.51
76.46	40.91	8.39	15.92

Figure 8: Cytokinin profiling in maize reproductive organs. The heat map shows the distribution of cytokinins in maize ovules and kernels (A), silks (B) and tassels (C) before and after pollination. The red and the blue cells correspond to higher and lower concentrations (in pmol per g of fresh weight), respectively; the scale is separate for each metabolite throughout all developmental stages in all three organs together. *t*Z, *trans*-zeatin; *t*ZR, *trans*-zeatin riboside; *c*Z, *cis*-zeatin; *c*ZR, *cis*-zeatin riboside; iP, isopentenyladenine; iPR, isopentenyladenosine; *t*ZOG, *trans*-zeatin *O*-glucoside; *t*ZROG, *trans*-zeatin riboside-*O*-glucoside; *t*Z9G, *trans*-zeatin *N9*-glucoside; *t*ZRMP, *trans*-zeatin riboside 5'-monophosphate; *c*ZOG, *cis*-zeatin *O*-glucoside; *c*ZROG, *cis*-zeatin riboside-*O*-glucoside; *c*Z9G, *cis*-zeatin *N9*-glucoside; *c*ZRMP, *cis*-zeatin riboside 5'-monophosphate; DHZROG, dihydrozeatin riboside-*O*-glucoside; DHZRMP, dihydrozeatin riboside 5'-monophosphate; iP9G, isopentenyladenine *N9*-glucoside; iPRMP, isopentenyladenine riboside 5' -monophosphate. CKs that are not listed were below the detection limit.



	RADICLE	COLEOPTILE	ROOT 7-d-o	1st LEAF 7-d-o	ROOT 3-m-o	BOTTOM LEAF 3-m-o	STEM 3-m-o
tZ	1.2	1.32	1.48	1.41	2.29	35.55	7.39
tZR	1.67	0.55	1.6	0.07	5.77	0.61	17.47
tZOG	0.38	2.31	0.37	1.64	3.50	38.02	6.38
tZROG	1.50	0.00	0.97	0.08	1.67	0.05	2.97
tZRMP	3.90	0.26	1.35	0.07	1.35	0.39	20.77
tZ9G	2.55	0.42	4.73	0.47	52.66	3.31	0.40
Total tZ-CK	11.2	4.86	9.96	3.74	67.24	77.93	55.39
cZ	0.37	2.25	0.48	4.30	0.19	4.66	0.44
cZR	2.9	1.00	1.84	0.92	0.06	2.60	0.26
cZOG	9.20	35.27	33.81	61.75	34.85	71.29	71.50
cZROG	33.71	16.13	57.67	2.56	9.3	15.10	15.53
cZRMP	3.81	0.24	6.46	0.13	0.02	0.11	0.16
cZ9G	0.10	0.11	0.52	0.14	0.54	3.52	0.30
Total cZ-CK	49.28	55.00	100.78	69.79	44.69	97.27	88.19
DHZ	0.21	0.23	0.13	0.12	0.09	0.18	0.06
DHZR	0.31	0.02	0.16	0.01	0.12	0.10	0.02
DHZROG	19.5	7.41	6.55	0.03	6.37	1.38	3.96
DHZRMP	0.18	0.03	0.14	0.01	0.04	0.03	0.01
DHZ9G	0.11	0.13	0.07	0.05	0.17	0.66	0.08
Total DHZ-CK	19.85	7.83	7.5	0.22	6.80	2.34	4.14
iP	0.37	0.16	0.32	0.16	0.21	0.49	0.57
iPR	0.85	0.36	2.90	0.29	2.57	0.41	2.53
iPRMP	7.43	4.65	5.94	0.07	1.65	0.29	2.44
iP9G	6.77	0.07	8.39	0.06	26.80	0.31	0.10
Total iP-CK	15.42	5.24	17.54	0.58	31.22	1.50	5.65

Figure 9: Cytokinin profiling in maize vegetative organs. Red and blue correspond to higher and lower concentrations (in pmol per g of fresh weight), respectively; the scale is separate for each metabolite throughout all samples. *t*Z, *trans*-zeatin; *t*ZR, *trans*-zeatin riboside; *c*Z, *cis*-zeatin; *c*ZR, *cis*-zeatin riboside; iP, isopentenyladenine; iPR, isopentenyladenosine; *t*ZOG, *trans*-zeatin *O*-glucoside; *t*ZROG, *trans*-zeatin riboside-*O*-glucoside; *t*Z9G, *trans*-zeatin *N*9-glucoside; *t*ZRMP, *trans*-zeatin riboside 5'-monophosphate; *c*ZOG, *cis*-zeatin *O*-glucoside; *c*ZROG, *cis*-zeatin riboside-*O*-glucoside; *c*Z9G, *cis*-zeatin *N*9-glucoside; *c*ZRMP, *cis*-zeatin riboside 5'-monophosphate; DHZROG, dihydrozeatin riboside-*O*-glucoside; DHZRMP, dihydrozeatin riboside 5'-monophosphate; iP9G, isopentenyladenine *N*9-glucoside; iPRMP, isopentenyladenine riboside 5' -monophosphate. Cytokinins that are not listed were below the detection limit.

CK biosynthesis as primary biosynthetic products *t*ZR and iPR phosphates are the most abundant CK derivatives there. In tassels, phosphates are accompanied also by high levels of *t*Z and iP free bases, which are the major active CKs mediating sensing on CK receptors (Lomin et al., 2015). In developing kernels, the highest accumulation of free *t*Z and *t*ZR was detected between the 6th and the 20th DAP. At older developmental stages, *t*Z accumulates mainly in a form of *N*9-glucoside, especially in silks and older roots. *t*Z *O*-glucosides accumulate predominantly in older leaves and maturing kernels, thus in tissues where high *t*Z content was detected in general (Fig. 8, 9). In contrast to *t*Z, *c*Z occurs predominantly as the *O*-glucoside and *c*Z9G is present only in negligible amounts in all analysed tissues. The dynamics of *c*Z levels in the course of development appears to be more constant than that of *t*Z and iP with only two exceptions. The first is approximately 2-fold increase observed in tassels 5 days before pollination (DBP) and the second is more than 5-fold decrease in maturing kernels between 20 and 35 DAP.

On the other hand, levels of DHZ metabolites tend to grow in the course of kernel and silk maturation and clearly follow the *t*Z maxima. Contrary to the detected dramatic decrease of *t*Z and *c*Z content, DHZ derivatives accumulate in the desiccating kernel 35 DAP (Fig. 8A).

Content of Other Phytohormones in Reproductive Organs

Other low-molecular-mass compounds, classified as phytohormones, were quantified in the same samples of reproductive organs as CKs and compared with their levels in vegetative organs (Fig. 10). Content of major auxin, IAA, is relatively low and stable in silks. There is a decrease of IAA concentration in the kernel (to levels comparable with those in silks) shortly after pollination. Significantly higher accumulation was observed in tassels in the same period of flowering. Interestingly, IAA level dramatically increased in mature kernels, being the highest amount of detected IAA in all the organs tested. Concentration of gibberellin precursor GA19 in reproductive organs is very low in contrast to elongated coleoptile. Tassels during pollen shedding are the only exception. Similarly, ethylene precursor ACC is unambiguously over-accumulated in tassels during pollination. In developing kernels and silks, the ACC level continuously increases from ovules to milky endosperm stage and then decreases during desiccation (35 DAP). Concentration of ABA reaches maxima in all the organs during pollination

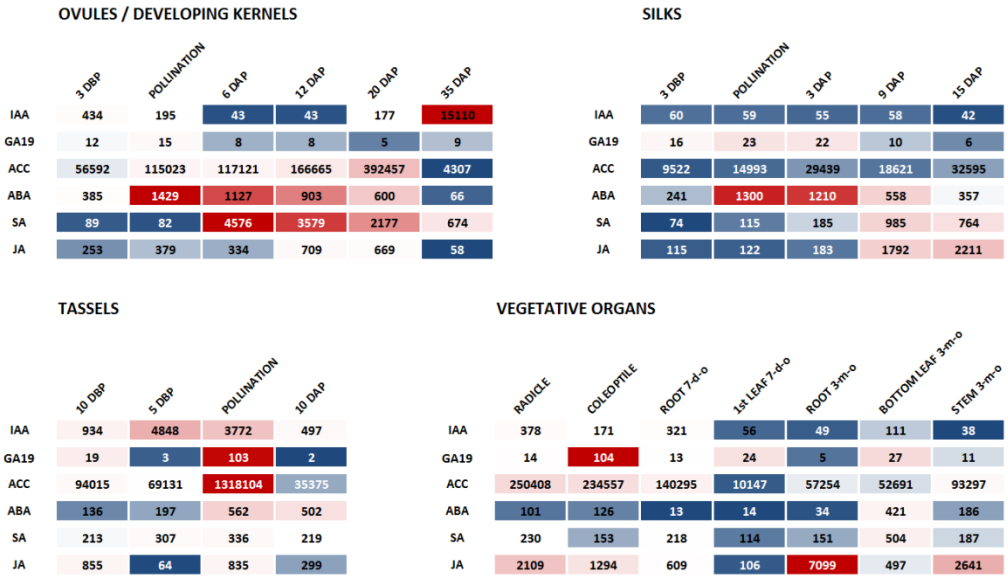


Figure 10: Phytohormonal profiling in the maize reproductive and vegetative organs. The heat map shows the phytohormone distribution, where the red and the blue cells correspond to higher and lower concentrations (in pmol per g of fresh weight), respectively; the scale is separate for each metabolite throughout all developmental stages in all organs together. IAA – indole-3-acetic acid; GA19 – gibberellin A19; ACC – 1-aminocyclopropane-1-carboxylic acid; ABA – abscisic acid; SA – salicylic acid; JA – jasmonic acid.

and then drops down. Nonetheless, levels in tassels are approximately 3-fold lower than in female organs. The highest concentration of SA was measured in silks and developing kernels 6 to 20 DAP, in contrast to its low concentration in ovules before and during pollination. SA concentration is rather low in tassels. JA levels are constant with exception of an enhancement in desiccated silks and decline in desiccated kernels 35 DAP and in tassels 5 DBP. Levels of several precursors and deactivation products of above mentioned phytohormones correlate with amounts of their active forms (data not shown).

Conversions of Exogenously Applied Cytokinins in Maize

Free bases of zeatin-type CKs in 1 and 5 μM concentrations were added to the nutrition solution in which 7-d-old maize seedlings were cultivated. After 3 days, roots and upper parts were collected and CK quantification was performed. In general, *c*Z and DHZ accumulated in one order of magnitude higher amounts than *t*Z (Fig. 11A). The experiment further revealed that applied CK is mainly converted to inactive *N9*-glucoside in the case of *t*Z or to *O*-glucosides in the case of *c*Z and DHZ (Fig. 11B). Besides, additional changes were observed. The levels of CK types, other than those added to the medium, also changed in response to uptake. The most profound effect in the root was found for the uptake of *c*Z, which caused increase of pools of both *t*Z and DHZ. Interestingly, in the case of *t*Z metabolite levels, the rise was caused rather by substantial surge of biosynthetic and active forms (*t*Z, *t*ZR and *t*ZRMP; Fig. 11B), while the rise of inactivation product – *t*Z *N9*-glucoside (*t*Z9G) was less intense (e.g. almost 10- and 100-fold rise of *t*Z after 1 and 5 uM *c*Z feeding, respectively, and only 1.5- and 4-fold increase of *t*Z9G). Uptake of *t*Z caused, apart from an increase of *t*Z metabolites, a 6-fold increase of DHZ metabolites when the higher concentration of *t*Z was applied. The pool of iP-type CKs stayed unchanged in all treatments.

In the shoot, where both long-distance transport and local metabolism play role, the trends were less profound. However, in the case of *t*Z treatment, levels of both *c*Z and DHZ rose. DHZ levels increased also in the case of *c*Z uptake (Fig. 11A). Application of all three CKs induced activity of CKX enzymes (data not shown).

In an additional experiment, radioactively labelled 1 nM *t*Z, *c*Z, DHZ and iP (~150 kBq per ml of agar) were added to the M&S agar medium with excised maturing kernels sampled approximately 20 DAP. The uptake was followed in the range of 5 h to 3 days, then kernel samples were collected, CK fraction extracted and separated by HPLC with a radioactivity detector. Radioactively labeled CKs applied at nanomolar concentrations were not supplemented with non-labeled compounds in order to follow their

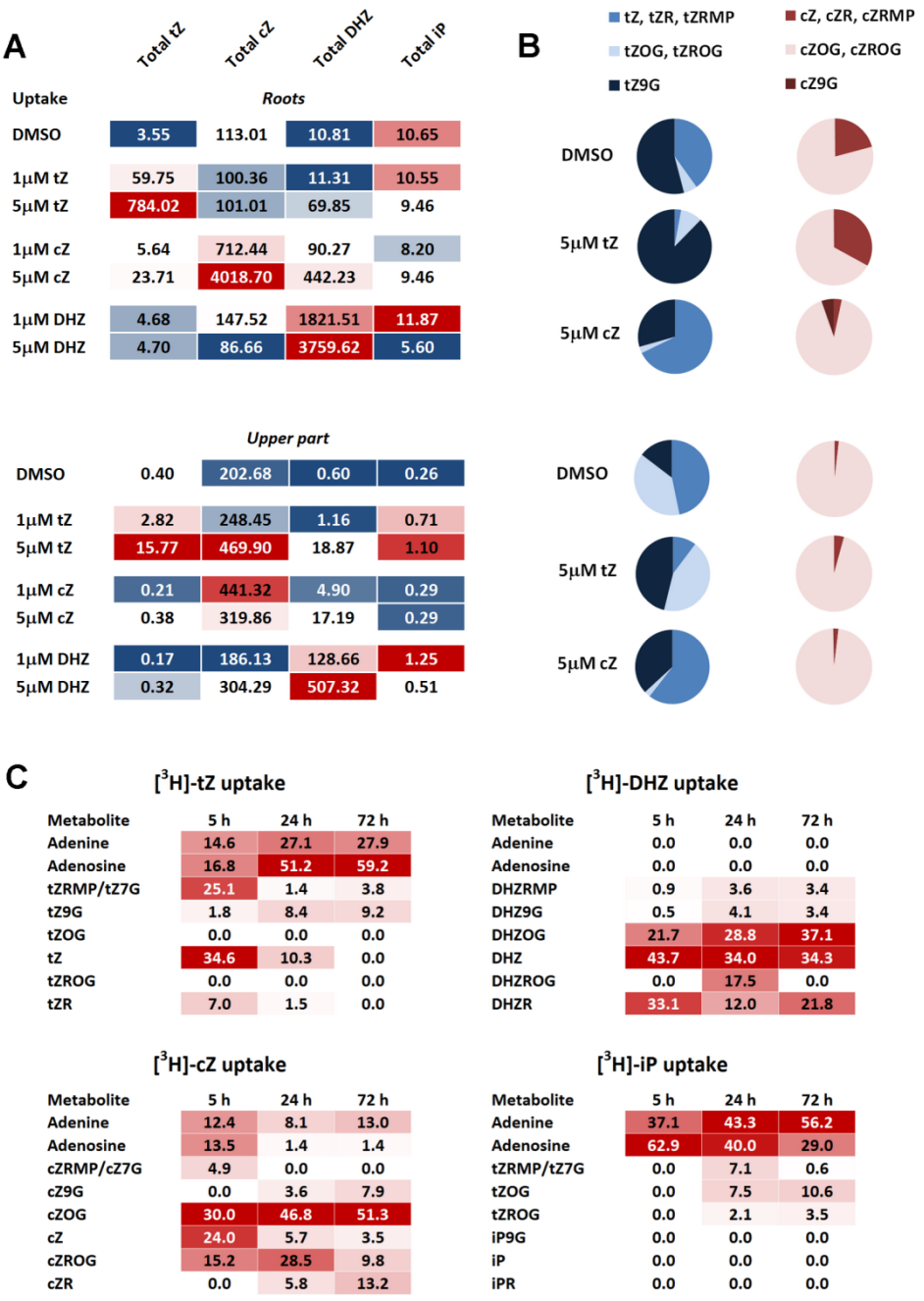


Figure 11: Distribution of cytokinins in maize seedlings upon uptake of different exogenously applied cytokinins. Roots and upper part of 10-d-old maize seedlings cultivated in Hoagland nutrient solution were separately analyzed 3 days after the solution was supplemented with various concentration of zeatin-type CKs. (A) Distribution of cytokinin types is expressed in pmol per gram of fresh tissue. Red and blue colours in the heat map correspond to higher and lower concentrations, respectively. The value in each block represents the summed concentrations of all determined metabolites of each group: *tZ* - *trans*-zeatin, *cZ* - *cis*-zeatin, DHZ - dihydrozeatin, and iP - isopentenyladenine. (B) Ratio among different CK forms in non-treated maize and maize treated with 5 µM *tZ* and *cZ*. Pie charts represent portions of free bases and ribosides; *O*-glucosides and ribosides-*O*-glucosides; and *N9*-glucosides for *tZ* and *cZ* determined separately in roots and upper parts. (C) Distribution of radioactivity after uptake of tritium labeled CKs to maturing (~20 DAP) kernels. The values are percentage of total recovered radioactivity from each sample.

physiological metabolic fate not disturbed by induced non-physiological response, e.g. activation of CKX enzymes. When [^{3}H]*t*Z and [^{3}H]iP were taken up to maturing kernels, the majority of radioactivity was found in degraded products (adenine, adenosine) within 5 h after the application (Fig. 11C). In contrast, [^{3}H]DHZ was converted only to its *O*-glucoside and to a lesser extent to *N9*-glucoside, while no degradation products were detected. Similarly, [^{3}H]*c*Z was predominantly converted to its *O*-glucoside and only 10 to 20 % of radioactivity was detected in degradation products (Fig. 11C). Detectable inter-conversion of [^{3}H]*t*Z to [^{3}H]*c*Z and *vice versa* was not observed in any tested tissue and nor was detected any reduction of the side chain to produce DHZ. Part of the radioactivity detected in kernels fed with [^{3}H]iP was found in peaks corresponding to retention times of *t*Z metabolites, which suggests the presence of a CK-specific P450 monooxygenase.

Novel Enzymatic Activity Converting *trans*-Zeatin *in vitro*

To detect a tissue with the predicted zeatin reductase activity (Martin et al., 1989), extracts made from different maize tissues were tested in *in vitro* reactions with 10 μM *t*Z and 0.25 mM NADPH as described by Gaudinová et al. (2005). Dithiothreitol in 0.2 mM concentration was added to inhibit degradation by CKX. Reduction of *t*Z to DHZ was not catalyzed by protein extracts either from vegetative or reproductive organs. However, when the reactions were analyzed by HPLC, a novel unknown peak was observed. In comparison to all known isoprenoid CKs, the compound had a greater retention time on a reversed-phase HPLC column (i.e. was more hydrophobic) and the absorption maximum shifted from 268 to 300 nm. The rate of production of the unknown metabolite was dependent on amount of crude extract and presence of NADPH. When NADPH was substituted with $NADP^+$, consumption of *t*Z and formation of the unknown metabolite was even faster. *t*ZR and *t*Z9G were also efficiently converted to products with absorption maxima at 300 nm. On the

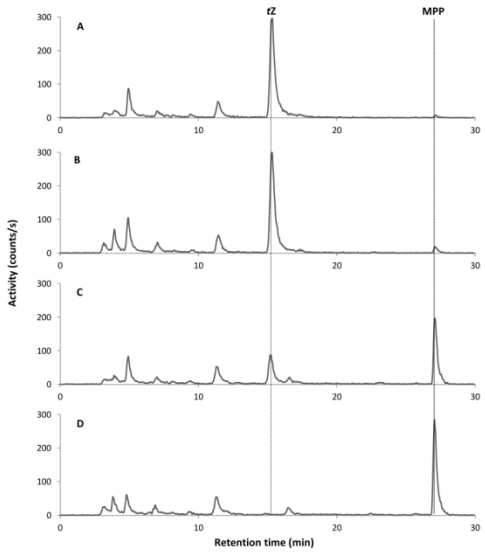


Figure 12: Formation of MPP from [^{3}H]*t*Z. [^{3}H]*t*Z was incubated with maize protein extract in absence (A,B), or presence (C,D) of $NADP^+$, for 5 hours (A,C) or 23 hours (B,D). The metabolites were analyzed by HPLC coupled with online radioactivity detector.

Table 3. The enzymatic activity converting trans-zeatin to MPP in different maize organs. Specific activity was calculated from concentration of formed 6-(3-methylpyrrol-1-yl)purine. The same material was used for phytohormone quantification in two replicates.

Organ	Stage	Specific activity (pkat mg^{-1})	Organ	Stage	Specific activity (pkat mg^{-1})
Ovules	3DBP	1.28 ± 0.32	Tassels	10 DBP	3.69 ± 1.37
Ovules	Pollination	0.21 ± 0.11		5 DBP	6.98 ± 2.90
Kernels	6 DAP	0.39 ± 0.17		Pollination	4.57 ± 2.16
	12 DAP	0.93 ± 0.34		10 DAP	2.32 ± 1.09
	20 DAP	0.12 ± 0.04	Silks	3DBP	0.45 ± 0.17
	35 DAP	0.09 ± 0.02		Pollination	0.12 ± 0.05
Roots	7-d-old	0.22 ± 0.09		3 DAP	7.16 ± 3.20
Upper part	7-d-old	0.06 ± 0.02		9DAP	10.45 ± 4.25

other hand, the other two zeatin forms (*c*Z, DHZ) did not produce similar product with the same absorption maximum and/or similar retention time. To confirm the *t*Z origin of the unknown metabolite, [^{3}H]*t*Z was added to the reaction mixture containing $NADP^{+}$ and analyzed by HPLC coupled to a radioactivity detector (Fig. 12). The enzyme activity converting *t*Z to the unknown metabolite was quantified in different organs. The greatest activity was detected in male reproductive organs and silks after pollination (Table 3), where it was 10 to 100-fold higher than in vegetative organs. The enzyme was partially purified from 100 g of tassels (5 DAP) using ammonium sulfate precipitation and hydrophobic chromatography on Octyl-Sepharose, where it was retained and eluted with decreasing ionic strength of ammonium salt. However, the enzyme was too unstable to proceed with further purification steps and decomposed within a couple of days at 4°C.

Identification and Properties of the Unknown Metabolite

Approximately 5 mg of *t*Z was enzymatically converted with the partially purified enzyme and the product was purified by semi-preparative HPLC. Fractions containing unknown product with an absorption maximum at 300 nm were pooled and evaporated to dryness. The unknown compound was analyzed by nuclear magnetic resonance on a Bruker Advance III 600 MHz instrument. Its structure was identified as 6-(3-methylpyrrol-1-yl)purine (MPP), a cyclic product of *t*Z oxidation (Fig. 13). To verify the structure, MPP was synthesized from *t*Z by MnO_2 catalysis and further purified by semi-preparative HPLC as referred above. Data from the nuclear magnetic resonance were consistent with those published previously (Haidoune et al., 1990).

A series of assays was performed to verify whether MPP was able to bind to and activate a CK receptor (Podlešáková et al., 2012; Romanov et al., 2006). It was found that MPP was not able to activate CK signal transduction via either of two tested maize CK histidine kinase receptors *Zm*HK1 and *Zm*HK3. Nevertheless, MPP was able to compete with *t*Z in binding at least to ZmHK3 (Fig. 14A). The supplementation of M&S media with MPP did not have any distinct inhibitory effect on elongation of the *Arabidopsis* primary root and lateral root branching. On the contrary, MPP slightly increased the length of primary root although not as much as the strong anticytokinin compound LGR-991 (Fig. 14B; Nisler et al., 2010). In conclusion, both types of experiments confirmed weak anticytokinin-like activity of MPP.

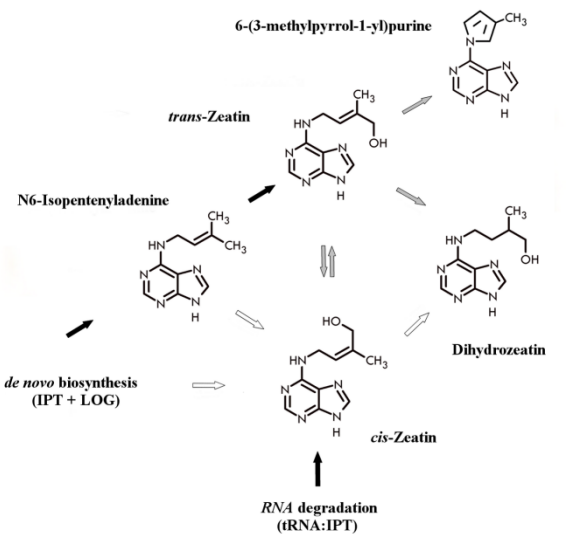


Figure 13: Zeatin inter-conversions in plants. Black arrows indicate enzymatic steps that were confirmed *in planta* and responsible gene(s) are known; grey arrows indicate enzymatic conversions shown only in *in vitro* assays; white arrows indicate hypothetical reactions (conversions) without any proof of evidence.

Furthermore, MPP was tested as a substrate and later on as an inhibitor of different maize CKX enzymes (Zalabák et al., 2014). The 3-methylpyrrol side chain of MPP was not able to be cleaved by any of eight tested CKXs (*Zm*CKX1 to 5, *Zm*CKX8, *Zm*CKX10 and *Zm*CKX12). On the other hand, MPP inhibited at least two CKX enzymes when iP was used as a substrate and DCPIP as an electron acceptor. For *Zm*CKX1, the apparent K_m value for iP and the inhibitory constant K_i for MPP are 3.5 μM and 16.3 μM, respectively, and for *Zm*CKX4a 28.9 μM and 64.9 μM, respectively (Fig. 14C).

Binding of MPP in the active site of CKX, namely *Zm*CKX4a, significantly differed from the binding of natural substrate as observed by X-ray crystallography (Fig. 15). The isoprenoid side-chain of iP or *t*Z lied over the isoalloxazine plane of the FAD cofactor and the N^6 atom was hydrogen-bonded to catalytic aspartate (Malito et al., 2004). The purine ring pointed towards the entrance and its *N9* atom could establish hydrogen bond to glutamate residue or remained unbound depending on CKX isoform. On the contrary, MPP did not bind over the isoalloxazine plane of FAD as deep as the substrates. The nitrogen atom of 3-methylpyrrol side chain of MPP had no free hydrogen for interaction with the catalytic aspartate (D170 in *Zm*CKX4a). Thus, MPP bound in a flipped orientation and its 3-methylpyrrol moiety pointed towards the

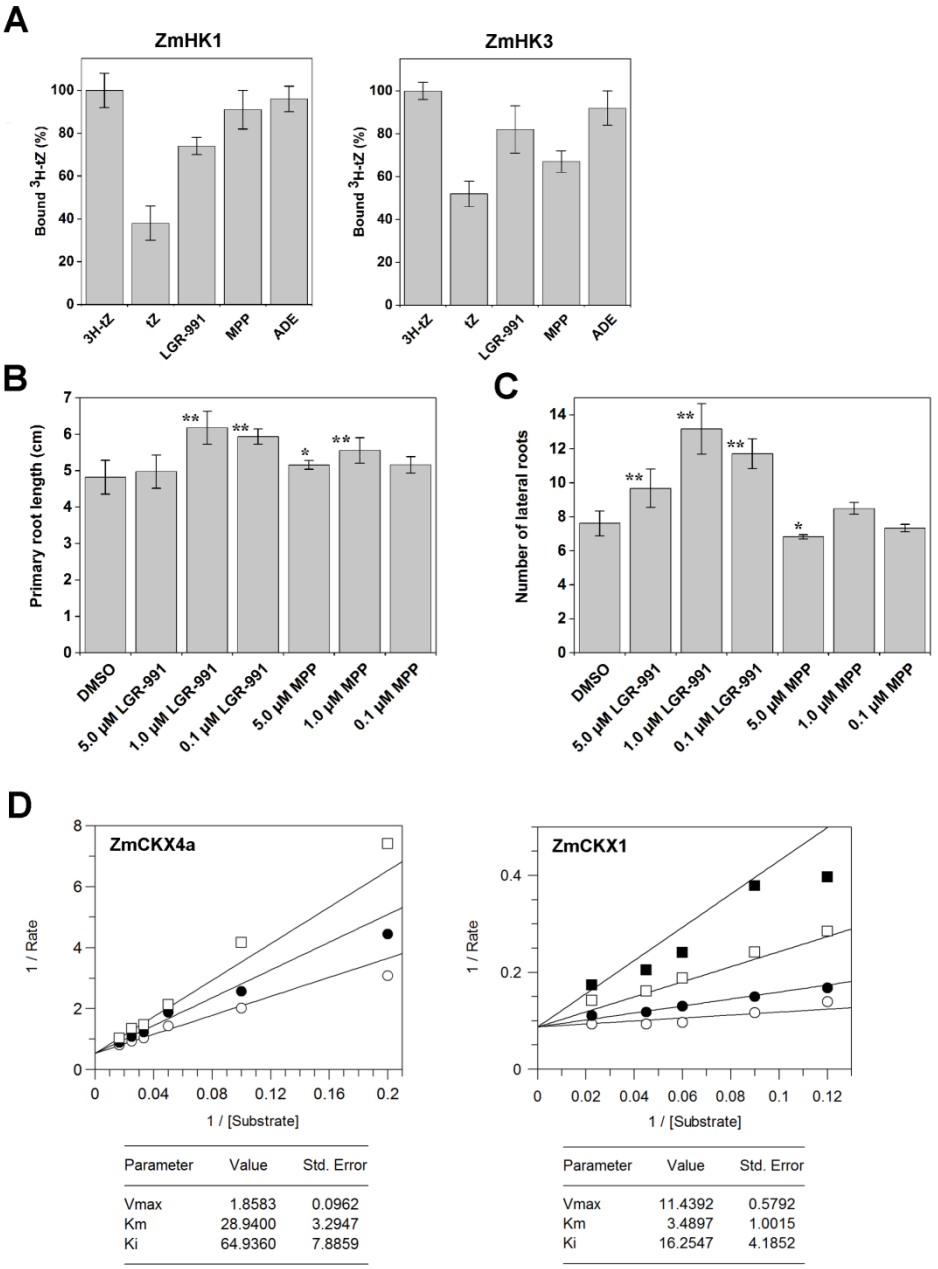


Figure 14: Properties of 6-(3-methylpyrrol-1-yl)purine as an anticytokinin and inhibitor of cytokinin dehydrogenases. (A) Effect of 6-(3-methylpyrrol-1-yl)purine (MPP) and anticytokinin LGR-991 on specific binding of 2 nM [^{3}H]*t*Z in a live-cell binding assay employing *E. coli* expressing cytokinin receptors *Zm*HK1 and *Zm*HK3. Both compounds were used in 20 µM concentration. Adenine (ADE) was used as a negative control. The radioactivity in samples without competing substances was 4844 dpm and 4289 dpm for ZmHK1 and ZmHK3, respectively. (B) Primary root length and (C) number of lateral roots of *Arabidopsis* seedlings grown on media supplemented with MPP and LGR-991 was determined in 14-d-old seedlings; each value is an average ± SD of measurements in at least 80 plantlets grown on 10 independent vertical dishes; ** indicates very significant ($P < 0.01$) or * significant ($P < 0.05$) differences between DMSO-treated and MPP/LGR-911-treated plants (t-test). (D) Lineweaver-Burk plots of *Zm*CKX1 and *Zm*CKX4a activity in the absence or presence of MPP. Degradation rates were determined by continuously measuring the bleaching of DCPIP for different concentrations of isopentenyladenine and MPP. Each combination was repeated at least three times.

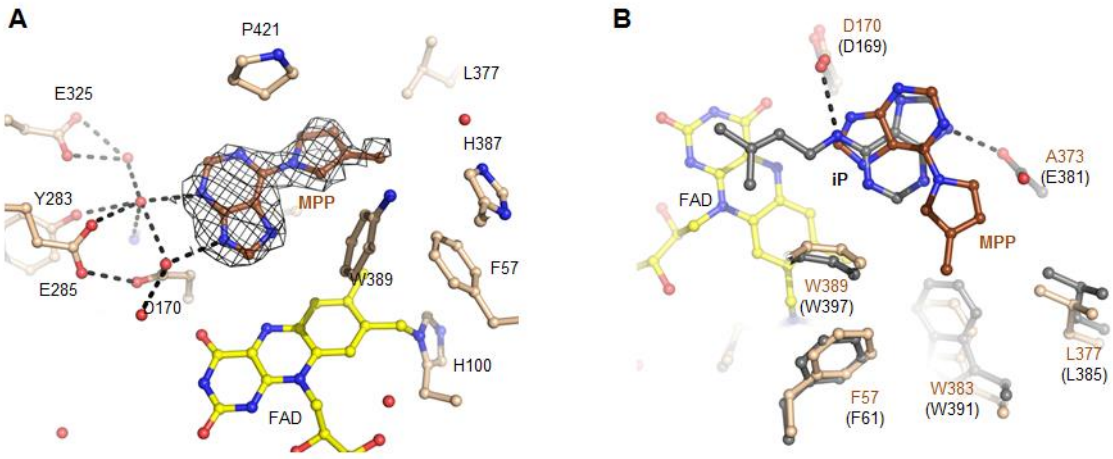


Figure 15: Binding of 6-(3-methylpyrrol-1-yl)purine in the active site of *Zm*CKX4a. (A) 6-(3-methylpyrrol-1-yl)purine (MPP; in Fo-Fc omit map contoured at 3.0 σ), which is colored in brown, is hydrogen bonded via its *N3* and *N9* atoms of the purine ring to the oxygen atom of D170 and to a water molecule, which is in contact with several residues including D170, E285 or Y283. FAD cofactor is yellow coloured and neighbouring residues are labelled. (B) Superposition of bound MPP and natural substrate isopentenyladenine (iP; PDB: 1W1Q; Malito et al., 2004). Oxygen atom of D170 establishes a hydrogen bond with N^6 of iP. Isoprenoid side chain lies over isoalloxazine plane of FAD while the purine ring points towards the entrance. Neighboring residues in *Zm*CKX4a are labelled in brown and those in *Zm*CKX1 are shown in brackets.

entrance, while the *N9* atom of purine ring bound to D170 and the *N3* atom interacted with a water molecule, which was in contact with several residues including D170, E285 or Y283. Taken together, the binding sites of CK substrates and MPP overlapped, and MPP behaved as a weak competitive CKX inhibitor as supported by enzyme kinetics.

Quantification of 6-(3-methylpyrrol-1-yl)purine in Plant Tissues

MPP with incorporated stable ^{15}N isotope(s) was synthesized (Dr. Libor Havlíček, Czech Academy of Science, Prague) and used for quantification in different reproductive and vegetative maize tissues by UHPLC-(+)ESI-MS/MS analysis. Nevertheless, its quantity was almost at the limit of detection and did not exceed a concentration of 100 fg per gram of fresh weight (Table 4), which is about four orders of magnitude less than the amount of its precursor *t*Z. Samples prepared from 1 g of kernels 12 DAP and tassels 5 DBP were screened for possible MPP metabolites. Fragmentation ions of putative MPP *N9*-glucoside (MPP9G) and MPP 9-riboside (MPPR) were obtained from standards prepared by enzymatic conversion of *t*Z9G and *t*ZR and purified by semi-preparative HPLC. Although the quantity of MPP9G, MPPR and MPPR 5´-monophosphate was much higher than that of unconjugated MPP, it did not reach the amount of the respective *t*Z counterparts (Table 4).

Table 4: Quantification of 6-(3-methylpyrrol-1-yl)purine and its metabolites in maize kernels and tassels. Compounds were quantified by UHPLC-(+)ESI-MS/MS using standard isotope dilution method. MPP, 6-(3-methylpyrrol-1-yl)purine; MPPR, 6-(3-methylpyrrol-l-yl)purine *N9*-riboside; *t*Z, *trans*-zeatin; *t*ZR, *t*Z riboside; *t*Z9G, tZ *N9*-glucoside; *t*ZRMP, *t*ZR 5´monophosphate.

metabolite	Tassels (5 DBP)	Kernels (12 DAP)
	(fmol g^{-1} of FW)	
*t*Z	12 984	7 792
MPP	0.2	1.2
*t*ZR	1 136	10 436
MPPR	32	56
*t*Z9G	86 100	6 868
MPP *N9*-glucoside	1 064	1 892
*t*ZRMP	1 508	11 572
MPPR 5´-monophosphate	74	416

Discussion

The precise enzymatic regulation of the CK content is crucial for proper development of cereal kernels, their mass and quantity. The greater number of grains per panicle in Habataki variety of rice is caused by a non-functional allele of *CKX* gene, which is expressed during the establishment of floral primordia (Ashikari et al., 2005). Similarly, silencing of the *HvCKX*1, the closest orthologue of *ZmCKX*1, led to stronger filling of barley grains (Zalewski et al., 2010), most probably due to a regulatory role of CKs in sugar metabolism and translocation (Balibrea Lara et al., 2004). On the contrary, the overexpression of *ZmCKX*1 gene under the control of pollen- or anther-specific promoters in maize gave rise to male-sterile plants (Huang et al., 2003). The male floret development was preliminarily terminated as a result of CK depletion at the apex of the plants. Accordingly, our measurements show that tassels before pollination accumulate both major CKs - iP and *t*Z. In contrast, in the female organs the levels of iP and *t*Z are relatively low and constant before and during pollination and start to increase 12 to 20 DAP. Greater sensitivity of the male than the female inflorescences to CK imbalance can be demonstrated on maize plants overexpressing *t*Z-specific glucosyltransferase (Pineda Rodó et al., 2008). Ubiquitous expression led to abnormalities resembling CK-deficient plants and *inter alia* feminized tassel florets caused by transformation of active *t*Z free base to non-active *O*-glucoside. On the other hand, there were no defects observed in the female inflorescences except reduced rates of kernel filling. The importance of precise regulation of the CK maxima can be further demonstrated on transgenic maize over-expressing *IPT* during gametophyte formation

and development. Enhanced level of CKs in the female gametophyte led to emergence of two functional florets per spikelet and to later formation of double embryo kernels while the male gametophyte was not affected (Young et al., 2004).

Changes in the ratio between two major phytohormones, CKs and auxins, were proposed to be an important regulatory switch in developmental processing taking place in maturing kernels. For instance, IAA concentration abruptly increased and the ratio of CK to auxin declined at the time when cell division was decreasing and nuclear DNA endoreduplication was increasing in the endosperm around 20 DAP (Lur and Setter, 1993). Accordingly, high CK to low auxin content was detected in kernels up to 20 DAP where maximal cell division occurs. Both *t*Z and iP levels accumulated around 20 DAP and then sharply declined, while IAA levels accumulated in large quantities at 35 DAP. Similar turn in IAA to CK levels is observed in ovules several days before pollination. Developing kernel is a place of massive cell division where high pool of active CKs needs to be maintained contrary to ripened tassels, in which levels of active CKs during and after the pollination drop down. Profile of the CK content shown in this study is in accordance with previous study of Brugière et al. (2003) who demonstrated maximum of *t*ZR between 9 to 20 DAP, followed by a sharp increase in iPR concentration at 20 DAP. However, in our study, the 20 DAP increase in iP-type CKs was accompanied also by the accumulation of *t*Z metabolites, which was not the case in the previous study. Discrepancy can be attributed to different maize cultivars used and the fact that the ratio between *t*Z- and iP-type CKs, which is influenced by activity of CK-specific cytochrome P450 monooxygenase, was shown as a variable parameter between low and high yielding barley cultivars (Powell et al., 2013). Accumulation of active CKs and IAA was enormous in tassels 10 DBP. Whereas CK levels decreased and were comparable with levels in ovules, IAA content remained high in tassels reaching levels 20-fold above those detected in ovules at the time of pollination and shortly thereafter. The ethylene precursor, ACC accumulated in maturing kernels 20 DAP similarly to cytokinins. That is in agreement with ethylene biosynthetic genes expression during kernel development and ethylene's function at the onset of endosperm cell death (Gallie and Young, 2004).

The role of ABA in seed (embryo) dormancy is well known. ABA level increased at the time of pollination and remained elevated until 20 DAP to protect the embryo from germination. Interestingly, the ABA concentration declined dramatically from 20 to 35 DAP to a very low level observed in almost desiccated kernels. A similar trend was observed in the previous study of Capelle et al. (2010), in which the authors detected, despite a sharp decline of ABA content in the endosperm, high levels remaining in the embryo till the end of the desiccation process. Low GA levels in kernels were observed

after the pollination due to antagonistic effect of GAs to embryo dormancy (White et al., 2000). In contrast, high GA levels were observed in tissues with strong elongation growth such as silks or coleoptiles a couple of days after germination and tassels during pollination (Fig. 10). An essential role of GAs on pollen formation and shedding has been confirmed in various GA-deficient mutants, which are usually male-sterile (e.g. Huang et al., 2003).

The biosynthetic pathway for the two most active CKs, iP and *t*Z, is well known. Conversely, the origin of the other two isoprenoid CKs, *c*Z and DHZ, is far less clear. In *Arabidopsis*, two tRNA IPTs were shown as the only source of *c*Z (Miyawaki et al., 2006). However, it is not clear whether the much higher *c*Z content detected in other species (Gajdošová et al., 2011), including maize, originates only from RNA decay or if there is another biogenesis pathway. Zeatin isomerase, which was originally predicted based on experiments by Bassil et al. (1993), seems to be an *in vitro* artefact of another enzyme's activity (this work). Additional alternative for the origin of *c*Z-rich species might be the existence of specific family of cytochrome P450 monooxygenases with hydroxylation preference for the *cis*-position or a direct *de novo* production conditioned by the *in vivo* occurrence of a specific precursor, which has not been confirmed up to now.

The uptake experiment revealed that maize roots are effective at converting *t*Z to its *N9*-glucoside, but have very low potency to *O*-glucosylate *t*Z or its riboside (Fig. 11B). On the contrary, *c*Z was present only as *O*-glucosides and only traces of *c*Z *N9*-glucoside were detected in roots after application of high concentration of *c*Z (Fig. 11B).The two CK specific *O*-glucosyltransferases found in maize so far have strict preference only for *c*Z (Martin et al., 2001; Veach et al., 2003). Recent characterization of substrate preferences of all maize CKX enzymes showed that CK *O*-glucosides are resistant to irreversible degradation, but some CKXs are able to degrade *N9*-glucosides of both *t*Z and *c*Z very effectively (Zalabák et al., 2014). Interestingly, two out of five CK specific glucosyltransferases found in the Arabidopsis genome are able to *O*-glucosylate both *t*Z and *c*Z (Hou et al., 2004). The typical CK profile of the Arabidopsis plant (e.g. Wang et al., 2011) differs from that of maize, mainly by the already mentioned lower representation of *c*Z derivatives but also by the higher ratio of *t*ZOG to *c*ZOG. Hence, we can hypothesize that species, which contain prominently more *c*Z than others (e.g. maize versus Arabidopsis), have a battery of *O*-glycosylation enzymes with preferred specificity toward *c*Z. Then even though equal amount of *c*Z originates continuously in all species by the same house-keeping process of tRNA isopentenylation and subsequent RNA decay, *c*Z can preferentially accumulate in species like maize (with *c*Z-specific *O*-glucosyltransferases) as it is protected against degradation by CKX

enzymes, contrary to *t*Z derivatives. This hypothesis is further supported by the approximately one order of magnitude lower accumulation of total *t*Z derivatives in comparison to *c*Z or DHZ when roots were fed with respective CK at 1 μM concentration (Fig. 11A).

CK profiling revealed that the predicted zeatin reductase (Martin et al., 1989) should be most active in maturing kernels between 20 to 35 DAP as a profound shift between *t*Z and DHZ content was observed there (Fig. 8A). However, the feeding experiment with radioactively labelled [^{3}H]*t*Z did not confirm reduction to DHZ in kernels between 20 to 30 DAP, neither did the *in vitro* enzymatic assay of zeatin reductase give positive results (data not shown). On the other hand, a novel enzymatic activity converting *t*Z to MPP was detected in protein extracts from all the tested tissues when $NADP^+$ was present as a cofactor. Cyclization of the *t*Z side chain to form 3-methylpyrrol resulted in a loss of CK-like activity. The compound was still able to bind to the active site of the CK receptor, *Zm*HK3, and to block binding of *t*Z even with greater potency than the anticytokinin LGR-991. However, contrary to LGR-911 it did not bind to the *Zm*HK1 receptor. An ability to act as a weak anticytokinin was also shown in the Arabidopsis root elongation assay. Interestingly, MPPR was isolated from tobacco crown gall cells in 1990 by the group of Professor Laloue (Haidoune et al., 1990) and shown to have no CK activity in a plant cell division assay but inhibitory effect on growth of tobacco cell suspensions at a higher concentration. Their observation can be simply explained by the anticytokinin effect of the aglycone released *in vivo*. Quantification of MPP in different maize tissues reported here demonstrates that the metabolite is present in extremely low amounts and probably does not have any physiological function at least in maize. Analysis of mass spectra revealed fragmentation ions, which can be assigned to MPPR, its monophosphate and MPP *N*9-glucoside, indicated these metabolites were present in much greater amounts. However, a precise analysis could not be performed because of the lack of their internal standards. Potential physiological significance of higher accumulation of MPP glycosides is not expected as any function for CK glycosides, isoprenoid or aromatic, has not been shown to date. Furthermore, uptake of radiolabeled *t*Z by maturing kernels did not confirm the ability of this tissue to convert it to the MPP metabolite effectively.

In conclusion, the observed formation of MPP and its glycosides is most probably an unspecific reaction of some $NADP^+$ dependent dehydrogenase, which primarily prefers other substrate(s). Nevertheless, MPP is the first compound showing, on the one hand, anticytokinin activity and on the other hand an ability to inhibit CKX. Hence, it can be used in the future for structural studies of CK binding or metabolizing proteins or applied for *in vivo* tissue culture studies where the CK effect needs to be suppressed.

PURIFICATION OF MAIZE NUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASE CASTS DOUBT ON THE EXISTENCE OF ZEATIN *cis-trans* ISOMERASE IN PLANTS

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Abstract

Almost 25 years ago, an enzyme named zeatin *cis-trans* isomerase from common bean has been described by Bassil et al. (1993). The partially purified enzyme required an external addition of FAD and dithiothreitol for the conversion of *cis*-zeatin to its *trans*-isomer that occurred only under light. Although an existence of this important enzyme involved in the metabolism of plant hormones cytokinins was generally accepted by plant biologists, the corresponding protein and encoding gene have not been identified to date. Based on the original paper, we purified and identified an enzyme from maize, which shows the described zeatin *cis-trans* isomerase activity. The enzyme belongs to nucleotide pyrophosphatase/phosphodiesterase family, which is well characterized in mammals, but less known in plants. Further experiments with the recombinant maize enzyme obtained from yeast expression system showed that rather than the catalytic activity of the enzyme itself, a non-enzymatic flavin-induced photoisomerization is responsible for the observed zeatin *cis-trans* interconversion *in vitro*. An overexpression of the maize nucleotide pyrophosphatase/phosphodiesterase gene led to decreased FAD and increased FMN and riboflavin contents in transgenic *Arabidopsis* plants. However, neither contents nor the ratio of zeatin isomers was altered suggesting that the enzyme is unlikely to catalyze the interconversion of zeatin isomers *in vivo*. Using enhanced expression of a homologous gene, functional nucleotide pyrophosphatase/phosphodiesterase was also identified in rice.

Introduction

N^6-(4-hydroxy-3-methyl-but-2-enyl)adenine, known as zeatin due to its discovery in maize (*Zea mays*), belongs to an important class of plant hormones called cytokinins that regulate many physiological processes (Werner and Schmülling, 2009). Zeatin is found throughout the plant kingdom in the form of two geometrical isomers denoted as *cis*- and *trans*-zeatin, respectively. *trans*-Zeatin biosynthesis occurs either directly from adenylate and a hydroxylated side chain precursor *(E)*-4-hydroxy-3-methylbut-2-enyl diphosphate by adenylate isopentenyltransferase (EC 2.5.1.27 and EC 2.5.1.112; Kakimoto, 2001) or by further hydroxylation of another cytokinin isopentenyladenine ((γ,γ-dimethylallylamino)purine) by specific cytochrome P450 (Takei et al., 2004b). On the other hand, the only route of the formation of *cis*-zeatin is the hydrolysis of tRNAs that had been previously prenylated on N^6-amino group of certain adenosine phosphate residues by tRNA isopentenyltransferase (EC 2.5.1.75), which was evidenced by the

decrease in *cis*-zeatin levels in *trna ipt* knock-out mutants of *Arabidopsis* (Miyawaki et al., 2006) and *Physcomitrella* (Lindner et al., 2014).

Since *cis*-zeatin is the predominant cytokinin in many plant species (Gajdošová et al., 2011), there are still alternative pathways under consideration. The expression of tRNA isopentenyltransferase genes is rather uniform throughout the plant ontogenesis, not responding to stimuli or any changes in cytokinin content. Nevertheless, an involvement of development- and/or stimuli-dependent RNase(s) liberating *cis*-zeatin cannot be ruled out. In addition, there is still a need for specific hydroxylase forming the *cis*-hydroxylated side chain. Such a *cis*-hydroxylase encoded by *Mia*E gene was found in the bacterium *Salmonella typhimurium* (Persson and Björk, 1993), but no homologue has been identified up to now in plants.

Therefore it was generally accepted that *cis*-zeatin could be produced from its *trans*-isomer by zeatin *cis-trans* isomerase, an enzyme partly purified and reported almost 25 years ago (Bassil et al., 1993). Nevertheless, the existence of such an enzyme has been disputed, as it was never identified; e.g. upon feeding plants with a radioactive cytokinin, no isomerization was observed in maize (Yonekura-Sakakibara et al., 2004). The only exception was 5 to 9 % of radioactivity recovered as *trans*-zeatin upon feeding potato tubers with *cis*-zeatin (Suttle and Banowetz, 2000). However, the radioactive cytokinin was applied in the dark, while the reaction catalyzed by the enzyme *in vitro* was described to require light. Furthermore, *Arabidopsis* multiple knock-out plants in adenylate isopentenyltransferase genes show decreased levels of *trans*-zeatin and isopentenyladenine but not *cis*-zeatin, while tRNA isopentenyltransferase gene knock-out specifically reduces *cis*-zeatin (Miyawaki et al., 2006). Neither of these results would have been plausible, if significant *cis-trans* isomerization of zeatin had occurred *in planta*.

Here we report on the purification of the protein responsible for the *in vitro* zeatin *cis-trans* isomerase activity described earlier. The protein was purified from maize and identified by mass spectrometry as a nucleotide pyrophosphatase/phosphodiesterase. A recombinant enzyme was produced using yeast *Pichia pastoris* expression system and its substrate specificity determined. To assess a possible role of nucleotide pyrophosphatase/phosphodiesterase in cytokinin metabolism, experimental plants with an altered expression of the encoding gene were prepared and characterized.

Materials and Methods

Purification of the Protein with Zeatin *cis-trans* Isomerase Activity

Whole maize cobs with immature seeds (930 g), approximately 10 days after pollination, were collected in the field and homogenized using a food processor with 1 L of 50 mM Tris/HCl, pH 8.0, at 4°C for 1 h. After a centrifugation at 4800*g*, the pellet was re-extracted with the same volume of fresh buffer and centrifuged again. Ballast proteins were precipitated with additions of protamine sulfate (1 g per 10 g of proteins), manganese(II) chloride (final concentration 7.5 mM) and ammonium sulfate (20% saturation, 114 g L^{-1}) according to published protocol (Šebela et al., 2000). After the addition of each precipitant, the solution was stirred for 10 min and then centrifuged at 4800*g* for 10 min. The supernatant was loaded onto a DEAE-Sepharose (GE Life Sciences) 5.0 cm i.d. × 30 cm column equilibrated with 50 mM Tris/HCl, pH 8.0, containing ammonium sulfate at 20% saturation. The flow-through fraction was desalted using a MiniKros® hollow fiber tangential flow filtration system with a 10-kDa polysulfone module (Spectrum Laboratories) and loaded on a High Q (Bio-Rad) 1.5 cm i.d. × 20 cm column equilibrated with 50 mM Tris/HCl, pH 8.0. The bound proteins were eluted with a gradient of KCl from 0 to 1.0 M. Fractions showing the zeatin *cis-trans* isomerase activity were pooled and transferred to 20 mM Tris/HCl buffer, pH 7.4, containing 0.5 M NaCl and 5 mM of each $MgCl_2$, $MnCl_2$, and $CaCl_2$ using Amicon® Ultra centrifugal filters (10 000 NMWL; Merck Millipore). The sample was then loaded on a Concanavalin A-Sepharose (Sigma-Aldrich) 1.0 cm i.d. × 10 cm column equilibrated with the same buffer and eluted with the buffer containing 1 M methyl α-D-mannopyranoside. The sample was then transferred to 50 mM Tris/HCl, pH 8.0 using the above centrifugal filters.

The enzyme was further purified using an FPLC BioLogic DuoFlow 10 system (Bio-Rad). The sample was first loaded onto a Resource Q 6 mL column (GE Life Sciences) equilibrated with 50 mM Tris/HCl, pH 8.0, and eluted with a linear gradient up to 0.5 M KCl. Active fractions were pooled, transferred to 5 mM K-phosphate, pH 7.0, containing 0.1 M NaCl and 0.5 mM $CaCl_2$. The recovered proteins were loaded onto a Bio-Scale CHT5-I column (Bio-Rad) and eluted with 0.75 M K-phosphate buffer, pH 7.0. Active fractions were pooled and transferred to 50 mM Tris/HCl, pH 8.0. Concentrated enzyme solution was loaded onto a HiTrap Blue HP column (GE Life Sciences) and eluted with 2 M KCl.

Finally, the purified enzyme was applied to Novex® isoelectric focusing electrophoresis gels (ThermoFisher Scientific) with pH 3-10 buffers. The gels were run

at a constant voltage of 100 V for 1 h, then at 200 V for 1 h and finally at 400 V for an additional hour. The margin lines were excised and silver-stained. Protein bands were excised from the rest of the gel slab in accordance to the staining and crushed by pressing through a syringe several times. Afterwards it was extracted overnight with 100 µL of McIlvaine buffer pH 7.5 at 4°C.

Protein Identification by Mass Spectrometry

After SDS-PAGE followed by Coomassie staining, protein bands of interest were excised from the gel slab and processed for in-gel digestion by a modified trypsin (Šebela et al., 2006). Peptides from the digests were desalted using ZipTip C18 pipette tips (Merck Millipore Ltd.) according to manufacturer's instructions and finally reconstituted in 10 µl of 0.1% (v/v) trifluoroacetic acid (TFA). Prior to MALDI mass spectrometry (MS) and tandem mass spectrometry (MS/MS), α-cyano-4-hydroxy-cinnamic acid (a matrix compound) was dissolved to 0.7 mg ml^{-1} in a solvent mixture containing 85% (v/v) acetonitrile, 15% (v/v) water, 0.1% (v/v) TFA and 1 mM $NH_4H_2PO_4$. An aliquot (0.5 µl) of the sample solution corresponding to an initial protein amount of around 200 ng was spotted onto the target (MTP AnchorChipTM 384 BC; Bruker Daltonik), immediately mixed with 0.5 µl of the matrix solution and left to dry at laboratory temperature.

MS and MS/MS analyses were performed on an ultrafleXtreme MALDI-TOF-TOF instrument equipped with a LIFT cell and Smartbeam-II laser operating at a repetition rate up to 2 kHz (Bruker Daltonik). All mass spectra were obtained in the reflectron positive ion mode. The mass spectrometer was controlled by flexControl 3.3 software for acquisition and flexAnalysis 3.3 for spectra processing. The accelerating voltages in the ion source for MS and MS/MS analyses were 25 kV and 7.5 kV, respectively. In MS/MS mode (no collision gas was used), an accelerating potential of 19 kV was applied to fragments (coming from a timed ion gate) in the LIFT cell. The instrument was calibrated externally using peptide standards supplied by the manufacturer. Manual mass spectra acquisitions were done from 2000 laser shots in the MS mode and 5000-10 000 shots in the MS/MS mode. The following settings were applied for MS/MS in an AutoXecute method: primary choice mass range of precursors: 750-3000; number of precursor masses: 15; peak intensity: >800; peak quality factor: >30; signal/noise: >7; FAST minimal fragment mass: 250; LIFT: measure fragments only.

Combined MS/MS datasets were processed by flexAnalysis 3.3, uploaded to ProteinScape 3.0 (Bruker Daltonik) and searched against the NCBI non-redundant

database with the Mascot 2.2 search engine (Matrix Science, London, UK). The search parameters were as follows: Viridiplantae (green plants) were set as a taxonomy; trypsin was set as a protease with 1 missed cleavage allowed; carbamidomethylation of cysteine was set as a fixed modification and methionine oxidation as a variable modification; +1 was set as a peptide charge; monoisotopic masses were considered; other settings: instrument - MALDI-TOF-TOF, significance threshold - $p < 0.05$, peptide mass tolerance - 25 ppm, MS/MS fragment mass tolerance - 0.7 Da.

Design and Synthesis of *ZmNPP* Gene

A maize nucleotide pyrophosphatase/phosphodiesterase (*ZmNPP*) gene was synthesized by GeneArt service of ThermoFisher Scientific. Minor alterations from the native sequence were implemented as follows: a CAT triplet was placed before the start codon to form an *Nde*I restriction site, the sequence stretch GCCTCC from the position 107 was changed to GCTAGC to form an *Nhe*I restriction site, which allows to clone *ZmNPP* fragment without predicted signal sequence, and the triplet CTT at the position 901 was changed to TTG to remove a *Hind*III restriction site. Neither of these changes led to an amino acid substitution. The TGA stop codon was changed to TAA to form a new *Hind*III restriction site and an adjacent *Xho*I site was added to yield a sequence TAAGCTTCTCGAG. All changes were made in order to clone the gene into plasmids for expression in *E. coli*. Gateway *att*B1 and *att*B2 sequences were added up- and down-stream of the coding sequence, respectively.

Preparation of Recombinant ZmNPP from *Pichia pastoris* Expression System

The *ZmNPP* gene was amplified from synthesized DNA with the following primers: P1_fw (5'-G*GAATTC*ATGGCGTCTCCGCCCCACTC-3') and P2_rev (5'-GCCG*CTCGAG* TTTTGTTCGGCAACAGAATCGTGCC-3') with *Eco*RI and *Xho*I restriction sites, respectively, shown underlined in italics. After restrictions with the respective endonucleases, the gene was cloned into a vector pPICZα (ThermoFisher Scientific). The plasmid was then linearized with *Mss*I. Chemically induced transformation to *Pichia pastoris* X-33 was performed in accordance with the manufacturer's protocol.

Transformed yeasts were cultivated in a bioreactor Biostat® Cplus (Sartorius). The vessel was filled with water up to 8.5 L containing 134 g of yeast nitrogen base without amino acids (ThermoFisher Scientific), 400 mL of glycerol and 2 mL of antifoam A (Sigma-Aldrich) and steam sterilized. After cooling down, 1 L of 1 M potassium phosphate, pH 7.0, and 50 mL of filter-sterilized PTM_1 Trace Salts (Pichia

Fermentation Process Guidelines, ThermoFisher Scientific) were added and the conditions were set as follows: agitation 400 rpm, air flow 2 vvm, temperature 28°C, and pH 7.0. After 3 h, 0.5 L of an overnight preculture of *Pichia pastoris* harboring *pPICZa::ZmNPP* was added and agitation was set to keep the concentration of dissolved oxygen above 20% of saturation. After 24 h of fermentation, 50% glycerol containing 5% PTM_1 Trace Salts was fed continuously to the bioreactor at the rate of 2 mL min^{-1}. After another 24 h, an induction of protein expression with methanol started, increasing the rate up to 3 mL min^{-1}, while the feeding with glycerol was decreased linearly. The methanol was fed to the bioreactor for 3 more days.

Yeast cells were then collected by a centrifugation at 4800*g* for 30 min. The medium was conditioned with $^1/_{40}$ volume of 1 M Tris and 2 M NaCl, run through a DEAE-Sepharose 5.0 cm i.d. × 30 cm column, concentrated and conditioned to 20 mM Tris/HCl, pH 8.0, on a SartoJet pump with Sartocon® Slice Ultrasart polyethersulfone 10-kDa NMWCO cassettes (Sartorius). The enzyme was further purified on the High Q 1.5 cm i.d. × 20 cm and HiTrap Blue HP columns as described above.

Enzyme Activity Assays

The interconversion of zeatin isomers was measured with 0.2 mM *cis*-zeatin in 100 mM McIlvaine buffer containing 20 mM $MgCl_2$, 0.1 mM FAD and 0.8 to 2.0 mM dithiothreitol. Upon mixing, the samples were incubated at 37°C under white fluorescent light (500 μE m^{-2} s^{-1}). The reaction was stopped by the addition of two volumes of methanol after 1 h. For each assay, a control reaction was set-up, where the enzyme sample was boiled for 5 min prior to addition of the other components and the enzymatic activity was calculated from the difference in the concentration of *trans*-zeatin between the two reactions. Zeatin content was analyzed on a Nexera ultra fast liquid chromatography (UFLC; Shimadzu) equipped with a Zorbax RRHD Eclipse Plus C18 column, 1.8 μm, 2.1×50 mm (Agilent) thermostated at 40°C. Zeatin isomers were eluted with 15 mM formic acid, set up with ammonium hydroxide to pH 4.0 at the flow rate of 0.4 mL min^{-1} with gradient of methanol as follows (min/%): 0.0/22; 3.0/22; 4.0/90; 5.5/90; 6.0/22; 8.0/22 and their content determined from peak areas at 268 nm using standard compounds. A typical chromatogram of the reaction is shown in Figure 16A.

The assay of nucleotide pyrophosphatase/phosphodiesterase activity was done in identical manner, but without *cis*-zeatin and the reaction mixture was kept in dark. Moreover, dithiothreitol was omitted in the assays with purified recombinant protein as no longer needed. The samples were analyzed on the same UFLC system and conditions

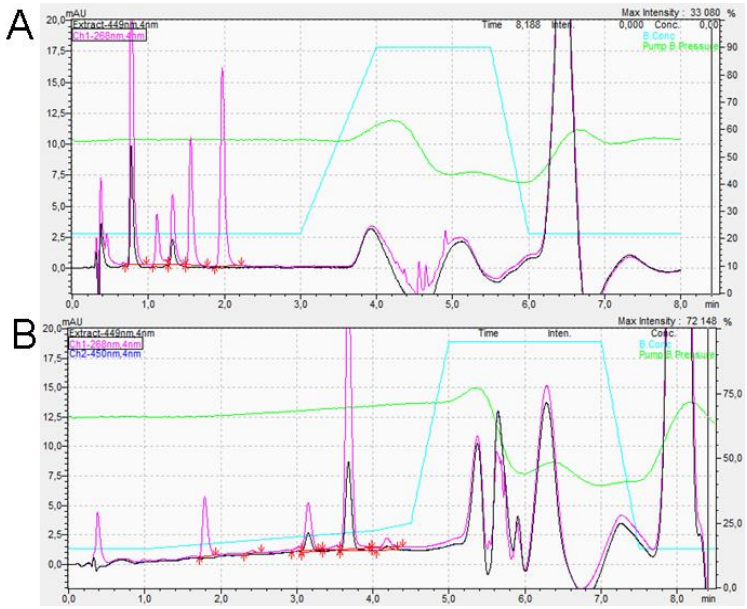


Figure 16: Typical UFLC chromatograms of enzymatic reactions. (A) shows the reaction of cis-zeatin isomerization with FAD at 0.75 min, FMN at 1.30 min, tZ at 1.55 min and cZ at 1.95 min. (B) shows typical chromatogram of FAD hydrolysis with FMN peak at 3.15 min and FAD at 3.65 min. Blue line indicates gradient of methanol, green line pressure in UFLC system, purple and black line absorbance at 268 and 449 nm, respectively.

as above, but eluted with 20 mM phosphoric acid (set up to pH 6.5 with ammonium hydroxide) using the following methanol gradient (min/%): 0.0/15; 1.0/15; 4.0/22; 4.5/25; 5.0/95; 7.0/95; 7.5/15; 10.0/15 and monitored at 449 nm. A typical chromatogram of the reaction is shown in Figure 16B. In plant samples, extracted flavins were analyzed by the same procedure but quantified on a FP-2020 Plus fluorescence detector (JASCO) with excitation at 265 nm and emission at 530 nm using standard compounds.

To determine the nucleotide pyrophosphatase/phosphodiesterase activity with various substrates, the same reaction was set up with 0.5 mM substrate and with an appropriate amount of the purified recombinant enzyme hydrolyzing maximally 40 % of the substrate. The reaction was stopped by pipetting aliquots at time-points 0, 10, 20 and 30 min into two volumes of methanol. The samples were all centrifuged at 21 000*g* for 10 min and mixed with 15 mM triethylamine set with phosphoric acid to pH 6.0 in such a ratio to bring methanol to 5% and filtered using 0.22 µm Costar® Spin-X® centrifuge tube filters (Corning Inc.) at 10 000*g* for 3 min. The samples were then analyzed by the UFLC equipped with a Polaris 180Å C18-A, 3 µm, 2.0x150 mm (Agilent) thermostated at 35°C using a methanol gradient in 15 mM triethylamine (set up to pH 6.0 with phosphoric acid) at the flow rate of 0.2 mL min^{-1}. The gradient was set up as follows (min/%): 0.0/10; 1.5/10; 4.0/40; 4.5/60; 7.0/60; 7.5/10; 10.0/10. The analytes were quantified using UV-Vis detector using standard compounds.

To determine enzyme specific activity, protein concentration was estimated using a Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) with bovine serum albumin as a calibration standard with linearization (Ernst and Zor, 2010).

Expression of the *ZmNPP* Gene in Tomato Hairy Roots

The gene was cloned into pGWB17 vectors (35S promoter, kanamycin and hygromycin resistance) using Gateway protocol (ThermoFisher Scientific) and electroporated into *Agrobacterium rhizogenes* strain 15834, which was used to transform tomato as described previously (Šmehilová et al., 2009). The main root was used for propagation; 2-cm long tips of lateral roots were used for the determination of gene expression and activity level and the rest was used for genotyping. Gene expression was determined as described previously (Šmehilová et al., 2009) with primers ZmNPP_fw (5'-CCCCAACCACTACTCCATCGT-3') and ZmNPP_rev (5'-TCGTGGTTTTTCATGGTGA AGT-3').

Expression of the *ZmNPP* gene in *Arabidopsis*

Arabidopsis plants expressing maize nucleotide pyrophosphatase/phosphodiesterase gene cloned into the pGWB17 vector were prepared from *Arabidopsis thaliana* ecotype Col-0 using a floral-dip method (Clough and Bent, 1998). Homozygous plants were selected by PCR detection of the inserted *ZmNPP* gene. A plant was considered homozygous when at least 100 offspring plants carried the insert and at the same time there was no negative offspring plant. The plants were grown for 4 weeks in an environmental chamber (16 h fluorescence light intensity of 150 μE m^{-2} s^{-1}/8 h dark, 22°C, 55% relative humidity) in the soil. Third to sixth leaves were collected and pooled from 4 plants into each sample.

Cultivation of Rice Plants with Enhanced Expression of Nucleotide Pyrophosphatase/Phosphodiesterase

Rice (*Oryza sativa japonica*) T-DNA insertional line PFG_2B-60145.L with the corresponding wild type Hwayoung were purchased from POSTECH, South Korea. The line has an insertion in the promoter sequence of the gene LOC_Os01g10020, 300 bp upstream of the start codon, which contains the promoter sequence of *OsTubA*1 gene. Rice seeds were germinated in Agroperlit (Perlit); after 2-3 weeks the seedlings were planted into the soil and grown for 2 weeks in an environmental chamber (12 h fluorescence light intensity of 250 μE m^{-2} s^{-1}, 28°C/12 h dark, 25°C, 65% relative humidity). Then shoots were collected from each plant separately with the exception of the albino plants that were pooled from two plants into one sample.

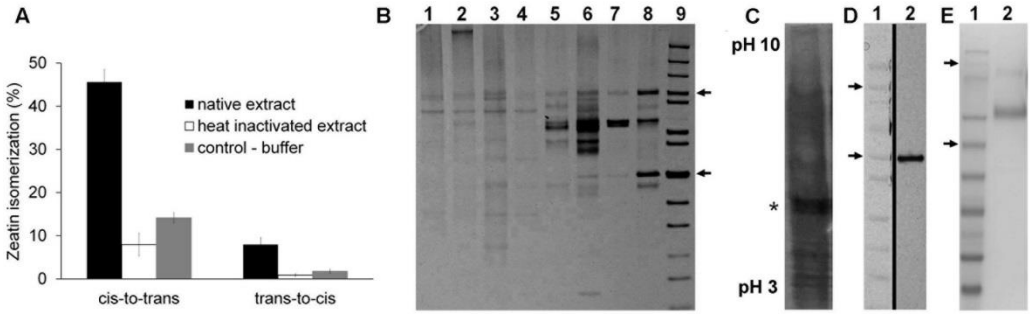


Figure 17: Purification and identification of the protein with zeatin *cis-trans* isomerase activity. (A) Typical conversion of zeatin isomers (0.2 mM) by maize kernel extracts after incubated in 100 mM McIlvaine buffer containing 20 mM $MgCl_2$, 0.1 mM FAD and 2.0 mM dithiothreitol, at 37°C under white fluorescent light for 2 hours; determined by UFLC; graph depicts average of three technical replicates with standard deviation (B) SDS-PAGE of the individual protein purification steps: 1, crude extract; 2, extract after protein precipitation; 3, fraction from DEAE-Sepharose; 4, High Q; 5, Concanavalin A; 6 Resource Q; 7 Bio-Scale CHT5-I; 8 HiTrap Blue HP; 9, MW marker, 100 and 50 kDa indicated by arrows. (C) Isoelectric focusing gel with the sample after HiTrap Blue HP (silver-stained lane); the rest of the gel slab was sliced, proteins extracted and activity detected in the band indicated by the asterisk. (D) SDS-PAGE of the isolated active protein: 1, MW marker, 100 and 50 kDa indicated by arrows; 2, purified active protein; the vertical black line denotes combining of two image parts. (E) SDS-PAGE of the isolated recombinant *Zm*NPP protein: 1, MW marker; 2, purified enzyme from the yeast growth medium.

Analysis of Transgenic Plants

Enzymatic activity and protein content were assayed as above using samples prepared from 0.1 g of the leaves that were crushed in liquid nitrogen and extracted with 0.2 mL of 50 mM Tris/HCl, pH 8.0. Total flavins were first extracted with methanol/methylene chloride (9:10) from 5 mg of starting material as described previously (Hiltunen et al., 2012) then the sample was extracted again with 10 mM sodium phosphate containing 10% (v/v) acetonitrile. The whole procedure was conducted in a dim light. Finally, 10 µL of a mixture of both extracts (1:1) was mixed with 50 µL of 20 mM ammonium phosphate, pH 6.5, and analyzed using UFLC with fluorescence detector as above on the same type of column, but 150 mm long. Chlorophyll content was determined using published method (Porra et al., 1989). Cytokinins in *Arabidopsis* plants were determined using published method (Svačinová et al., 2012).

Results

Purification of the Protein with Zeatin *cis-trans* Isomerase Activity

When we examined extracts from several plant species including maize, *Arabidopsis*, common bean, wheat and rice, the highest activity was found in maize, detectable in all developmental stages and organs (results not shown). Maize immature kernels were

Table 5: Purification of the protein with zeatin *cis-trans* isomerase activity from maize. Maize cobs 10 day after pollination (930 g) were used as a starting material; the activity was determined using UFLC as a production of trans-zeatin and protein content by linearized Bradford method (see Materials and Methods).

Purification step	Total activity (nkat)	Total proteins (mg)	Specific activity (nkat mg^{-1})	Purification grade (-fold)	Yield (%)
Crude extract	475	11 900	0.04	1.0	100.0
Precipitation	658	12 400	0.05	1.3	138.3
DEAE-Sepharose	724	3 110	0.23	5.8	152.2
High Q	788	1 006	0.78	19.6	165.6
Concanavalin A	162	86.20	1.89	47.1	34.1
Resource Q	145	19.00	7.66	191	30.6
Hydroxyapatite	68.1	5.00	13.6	340	14.3
HiTrap Blue HP	45.2	0.71	64.5	1 610	9.5

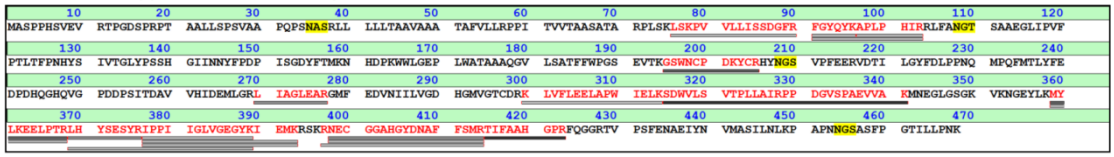
therefore chosen as the starting material for enzyme purification. The rate of conversion of *cis*- to *trans*-zeatin was about 5-fold higher than the opposite reaction (Fig. 17A), both with a relatively high background after boiling that reached up to 25 % of the rate with native extracts.

The whole purification procedure was quite tedious and required a series of six chromatographic steps to obtain 0.7 mg of a final enzyme preparation from almost one kilogram of maize cobs with kernels at the stage of liquid endosperm. The progress of individual purification steps with 10 % activity yield resulted in 1600-fold purified enzyme with the specific activity of 65 nkat mg^{-1} (Table 5). However, several protein bands were still detectable on an SDS-PAGE gel (Fig. 17B). To identify a particular protein showing the isomerase activity, the active fraction from HiTrap Blue was further separated using isoelectric focusing (Fig. 17C). The gel slab was then sliced, separated proteins extracted and tested for the enzymatic activity. The zeatin *cis-trans* isomerase activity was found in the fraction corresponding to a 50 kDa band on the original SDS-PAGE gel (Fig. 17D).

Identification of the Purified Enzyme

MALDI-TOF/TOF MS and MS/MS allowed assigning the purified protein band with *in vitro* isomerase activity to the accession number NP_001146857 in the NCBInr database. The peptide mass fingerprinting data allowed assigning 19 peptides with *m/z* values in the range of 805-2816 Da providing a probability-based score of 131 and sequence coverage of 32.3% (Fig. 18). The MS/MS-based data provided much

Protein 1:	LOC100280465 [Zea mays]		
Accession:	gi|226531542	**Score:**	131.00
Database:	NCBInr	**MW [kDa]:**	51.10
Seq. Coverage [%]:	32.30 %	**pI:**	6.39
		No. of Peptides:	19
Modification(s):	Carbamidomethyl, Oxidation		

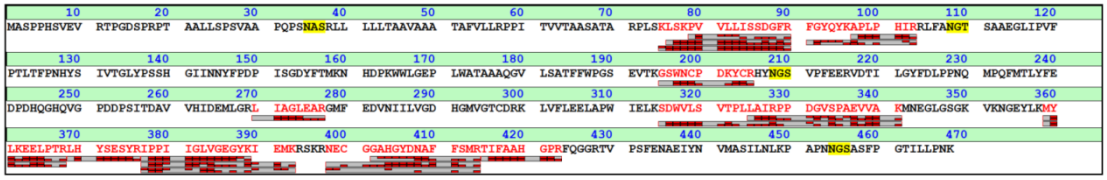


m/z meas.	Δ m/z [ppm]	z	P	Range	Sequence	Modification
1630.9577	3.102	1	0	76-90	LSKPVVLLISSDGFR	
805.3849	-3.721	1	0	91-96	FGYQYK	
1589.8623	2.282	1	1	91-103	FGYQYKAPLPHIR	
803.4890	0.4263	1	0	97-103	APLPHIR	
1442.5965	1.727	1	1	195-205	GSWNCPDKYCR	Carbamidomethyl: 5, 10
842.5132	4.535	1	0	270-277	LIAGLEAR	
1828.0556	-3.451	1	1	300-314	KLVFLEELAPWIELK	
2816.5555	-0.07056	1	0	315-341	SDWVLSVTPLLAIRPPDGVSPAEVVAK	
1279.6700	-1.122	1	1	359-368	MYLKEELPTR	
1295.6715	3.933	1	1	359-368	MYLKEELPTR	Oxidation: 1
1054.4965	1.202	1	0	369-376	LHYSESYR	
2391.2725	0.7624	1	1	369-389	LHYSESYRIPPIIGLVGEGYK	
1355.7942	0.6609	1	0	377-389	IPPIIGLVGEGYK	
1857.0556	0.1071	1	1	377-393	IPPIIGLVGEGYKIEMK	
1873.0493	-0.5566	1	1	377-393	IPPIIGLVGEGYKIEMK	Oxidation: 16
2088.8738	-1.189	1	1	397-414	RNECGGAHGYDNAFFSMR	Carbamidomethyl: 4
1932.7761	0.4818	1	0	398-414	NECGGAHGYDNAFFSMR	Carbamidomethyl: 3

m/z meas.	Δ m/z [ppm]	z	P	Range	Sequence	Modification
1948.7639	-3.170	1	0	398-414	NECGGAHGYDNAFFSMR	Carbamidomethyl: 3; Oxidation: 16
969.5245	-1.992	1	0	415-423	TIFAAHGPR	

Figure 18: Results of MALDI-TOF/TOF MS identification of protein band with zeatin *cis-trans* isomerase activity.

Protein 1:	LOC100280465 [Zea mays]		
Accession:	gi|226531542	**Score:**	1345.46
Database:	NCBInr	**MW [kDa]:**	51.10
Seq. Coverage [%]:	29.10 %	**pI:**	6.39
		No. of Peptides:	19
Modification(s):	Carbamidomethyl, Oxidation		



Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	P	Range	Sequence	Modification
	1	1759.0511	1.99	1		130.9	0	75-90	S.KLSKPVVLLISSDGFR.F	
	1	1630.9552	1.57	1		131.8	0	76-90	K.LSKPVVLLISSDGFR.F	
	1	1302.7463	3.59	1		37.6	0	79-90	K.PVVLLISSDGFR.F	
	1	1589.8526	-3.87	1		23.3	0	91-103	R.FGYQYKAPLPHIR.R	
	1	803.4888	0.19	1		43.7	0	97-103	K.APLPHIR.R	
	1	1442.5953	0.92	1		57.6	0	195-205	K.GSWNCPDKYCR.H	Carbamidomethyl: 5, 10
	1	842.5075	-2.30	1		58.0	0	270-277	R.LIAGLEAR.G	
	1	2816.5538	-0.66	1		112.1	0	315-341	K.SDWVLSVTPLLAIRPPDGVSPAE VVAK.M	
	1	1718.9823	1.36	1		73.0	0	325-341	L.LAIRPPDGVSPAEVVAK.M	
	1	1279.6730	1.17	1		66.8	0	359-368	K.MYLKEELPTR.L	
	1	1295.6672	0.60	1		51.0	0	359-368	K.MYLKEELPTR.L	Oxidation: 1
	1	1054.4941	-1.05	1		68.9	0	369-376	R.LHYSESYR.I	

Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	P	Range	Sequence	Modification
	1	1355.7946	0.94	1		90.9	0	377-389	R.IPPIIGLVGEGYK.I	
	1	1873.0452	-2.73	1		37.7	0	377-393	R.IPPIIGLVGEGYKIEMK.R	Oxidation: 16
	1	1857.0562	0.43	1		74.5	0	377-393	R.IPPIIGLVGEGYKIEMK.R	
	1	1932.7772	1.05	1		109.7	0	398-414	R.NECGGAHGYDNAFFSMR.T	Carbamidomethyl: 3
	1	1948.7717	0.80	1		45.7	0	398-414	R.NECGGAHGYDNAFFSMR.T	Carbamidomethyl: 3; Oxidation: 16
	1	1472.6410	2.31	1		46.2	0	402-414	G.GAHGYDNAFFSMR.T	
	1	969.5270	0.52	1		70.2	0	415-423	R.TIFAAHGPR.F	

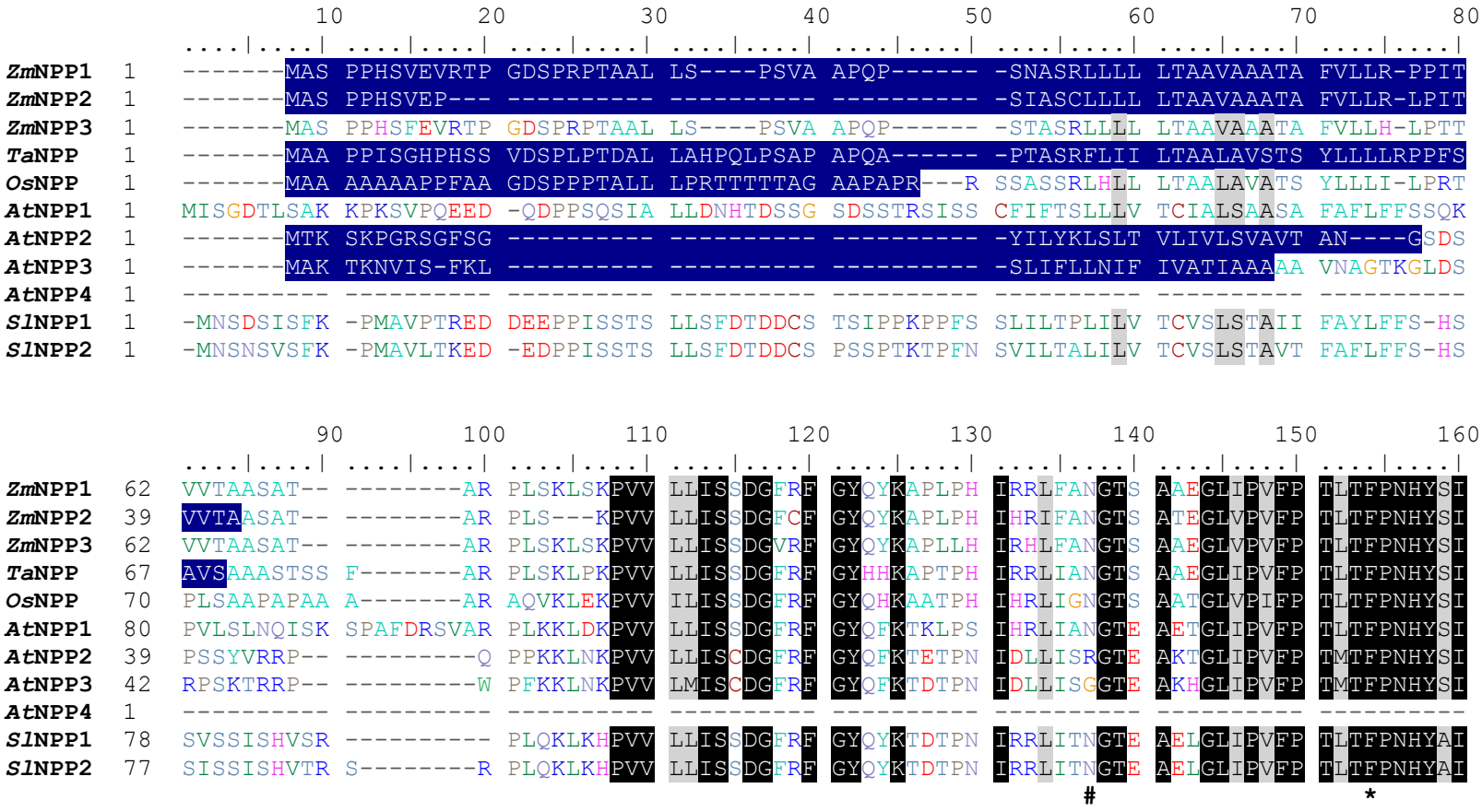
Figure 19: Results of MALDI-TOF-TOF MS/MS identification of protein band with zeatin *cis-trans* isomerase activity.

more convincing score value of 1345 (Fig. 19). This accession is annotated as a nucleotide pyrophosphatase/phosphodiesterase from *Zea mays* (*Zm*NPP; LOC100280465; Zm00001d039454_P001). The protein consists of 468 amino acids with a theoretical molecular mass of 51.2 kDa, which well corresponds to the results obtained by SDS-PAGE, and pI 6.36 determined by Compute pI/Mw (Bjellqvist et al., 1993).

Characterization of Plant Nucleotide Pyrophosphatases/Phosphodiesterases

A BLAST analysis against translated plant genomes using *Zm*NPP sequence as a query returned only a few results per plant. At the time of ZmNPP identification, the database of maize genome assembly contained an additional gene with a high homology in a stretch of 160 amino acids, while the rest of the gene encoded Sir2 domain that possesses a NAD-hydrolysis-dependent deacetylase activity. However, at the time of completing this paper (July 2017), the current maize genome assembly (AGPv4) contains three annotated *NPP* genes with similar length and only minor alterations (indels and several amino acid substitutions, Fig. 20). Because we were not aware of the other two *Zm*NPPs, we have focused in our work only on the identified enzyme. For the same reason, we refer to the enzyme as *Zm*NPP, without the number. Nevertheless, the mass spectrometry analysis (Fig. 18, 19) unambiguously confirmed that a protein present in the purified fraction is *Zm*NPP1 (Zm00001d039454_P001) and not *Zm*NPP2 (Zm00001d047948_P001) or *Zm*NPP3 (Zm00001d044311_P001). A single gene LOC_Os01g10020 was found in rice and four in *Arabidopsis*, whose genes are located on chromosome 4, next to each other (At4g29680, At4g29690, At4g29700, and At4g29710).

Unlike human nucleotide pyrophosphatase/phosphodiesterase genes that contain a large number of introns and undergo an alternative splicing, all plant genes, interestingly with the exception of the additional two *ZmNPPs* (*ZmNPP*2 and *ZmNPP*3), contain a single exon. As shown in Figure 21, plant NPP proteins cluster separately from animal enzymes in the phylogenetic tree. Mostly, when there is more than one encoding gene in the genome (e.g. in *Arabidopsis* and tomato), the paralogs are located sequentially on the same chromosome. This all suggests that before the evolutional divergence of animals and plants, there was only a single precursor gene. While there were multiple gene duplications in animals before the divergence of clades (Zimmermann et al., 2012), the genes in plants duplicated relatively recently, after the speciation.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ZmNPP2 1 -------MAS PPHSVEP--- ---------- ---------- ---------- -SIASCLLLL LTAAVAAATA FVLLR-LPIT
ZmNPP3 1 -------MAS PPHSFEVRTP GDSPRPTAAL LS----PSVA APQP------ -STASRLLLL LTAAVAAATA FVLLH-LPTT
TaNPP 1 -------MAA PPISGHPHSS VDSPLPTDAL LAHPQLPSAP APQA------ -PTASRFLII LTAALAVSTS YLLLLRPPFS
OsNPP 1 -------MAA AAAAAPPFAA GDSPPPTALL LPRTTTTTAG AAPAPR---R SSASSRLHLL LTAALAVATS YLLLI-LPRT
AtNPP1 1 MISGDTLSAK KPKSVPQEED -QDPPSQSIA LLDNHTDSSG SDSSTRSISS CFIFTSLLLV TCIALSAASA FAFLFFSSQK
AtNPP2 1 -------MTK SKPGRSGFSG ---------- ---------- ---------- -YILYKLSLT VLIVLSVAVT AN----GSDS
AtNPP3 1 -------MAK TKNVIS-FKL ---------- ---------- ---------- -SLIFLLNIF IVATIAAAAA VNAGTKGLDS
AtNPP4 1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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SlNPP2 1 -MNSNSVSFK -PMAVLTKED -EDPPISSTS LLSFDTDDCS PSSPTKTPFN SVILTALILV TCVSLSTAVT FAFLFFS-HS
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TaNPP 67 AVSAAASTSS F-------AR PLSKLPKPVV LLISSDGFRF GYHHKAPTPH IRRLIANGTS AAEGLIPVFP TLTFPNHYSI
OsNPP 70 PLSAAPAPAA A-------AR AQVKLEKPVV ILISSDGFRF GYQHKAATPH IHRLIGNGTS AATGLVPIFP TLTFPNHYSI
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AtNPP3 42 RPSKTRRP-- ---------W PFKKLNKPVV LMISCDGFRF GYQFKTDTPN IDLLISGGTE AKHGLIPVFP TMTFPNHYSI
AtNPP4 1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
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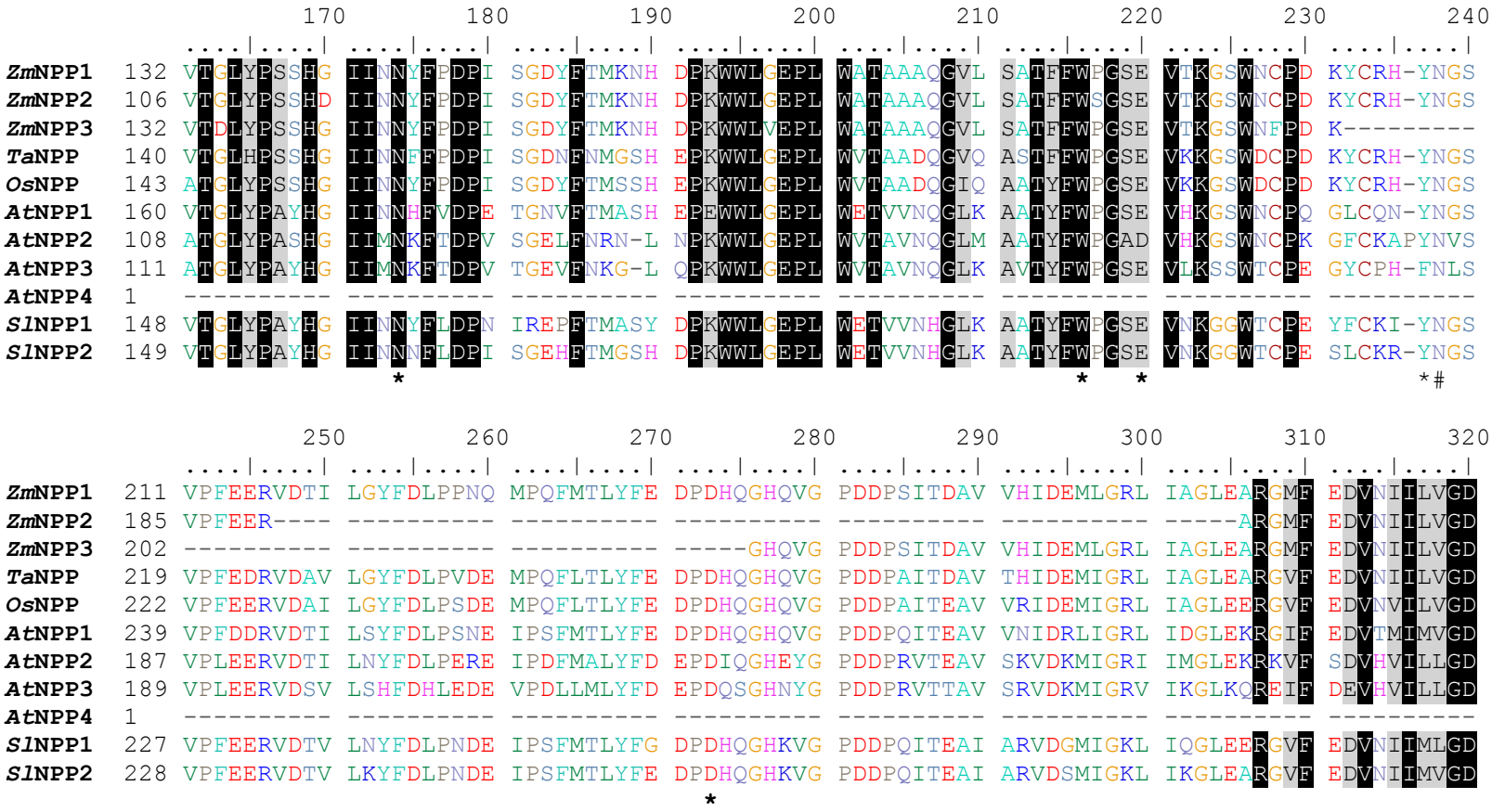
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ZmNPP3 132 VTDLYPSSHG IINNYFPDPI SGDYFTMKNH DPKWWLVEPL WATAAAQGVL SATFFWPGSE VTKGSWNFPD K---------
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* * * *#
250 260 270 280 290 300 310 320
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ZmNPP2 185 VPFEER---- ---------- ---------- ---------- ---------- ---------- -----ARGMF EDVNIILVGD
ZmNPP3 202 ---------- ---------- ---------- -----GHQVG PDDPSITDAV VHIDEMLGRL IAGLEARGMF EDVNIILVGD
TaNPP 219 VPFEDRVDAV LGYFDLPVDE MPQFLTLYFE DPDHQGHQVG PDDPAITDAV THIDEMIGRL IAGLEARGVF EDVNIILVGD
OsNPP 222 VPFEERVDAI LGYFDLPSDE MPQFLTLYFE DPDHQGHQVG PDDPAITEAV VRIDEMIGRL IAGLEERGVF EDVNVILVGD
AtNPP1 239 VPFDDRVDTI LSYFDLPSNE IPSFMTLYFE DPDHQGHQVG PDDPQITEAV VNIDRLIGRL IDGLEKRGIF EDVTMIMVGD
AtNPP2 187 VPLEERVDTI LNYFDLPERE IPDFMALYFD EPDIQGHEYG PDDPRVTEAV SKVDKMIGRI IMGLEKRKVF SDVHVILLGD
AtNPP3 189 VPLEERVDSV LSHFDHLEDE VPDLLMLYFD EPDQSGHNYG PDDPRVTTAV SRVDKMIGRV IKGLKQREIF DEVHVILLGD
AtNPP4 1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
SlNPP1 227 VPFEERVDTV LNYFDLPNDE IPSFMTLYFG DPDHQGHKVG PDDPQITEAI ARVDGMIGKL IQGLEERGVF EDVNIIMLGD
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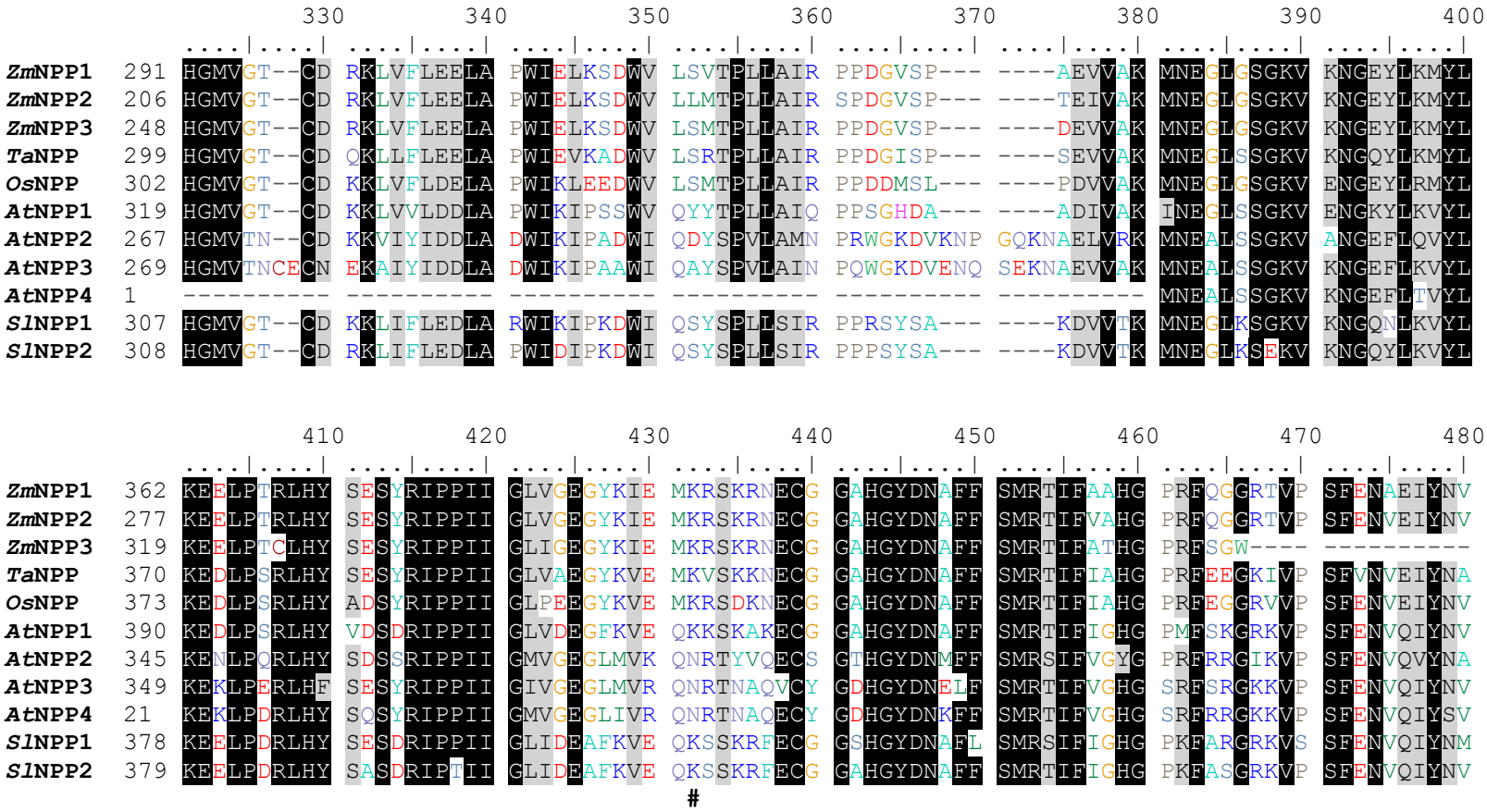
*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TaNPP 299 HGMVGT--CD QKLLFLEELA PWIEVKADWV LSRTPLLAIR PPDGISP--- ----SEVVAK MNEGLSSGKV KNGQYLKMYL
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ZmNPP3 319 KEELPTCLHY SESYRIPPII GLIGEGYKIE MKRSKRNECG GAHGYDNAFF SMRTIFATHG PRFSGW---- ----------
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AtNPP2 345 KENLPQRLHY SDSSRIPPII GMVGEGLMVK QNRTYVQECS GTHGYDNMFF SMRSIFVGYG PRFRRGIKVP SFENVQVYNA
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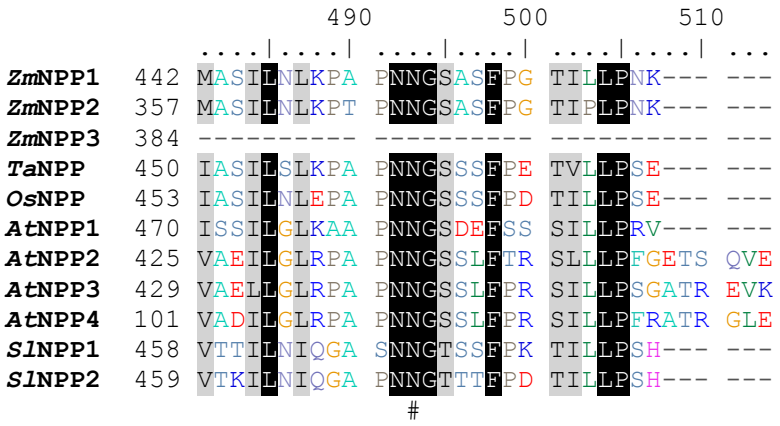

Figure 20: Alignment of plant nucleotide pyrophosphate/phosphodiesterase protein sequences. Identical and similar amino acids are shaded in black and gray, respectively. Amino acids important for substrate binding are marked with * (numbering as in *Zm*NPP1): Phe 125, Asn 145, Trp 187 (part of WPG motif), Glu 191 (that is homologous to Asp 308 in mouse NPP1), Tyr 207 and Asp 243. Asparagine residues predicted to be glycosylated are marked with #: Asn 108, Asn 208, Asn 454 and asparagine in *At*NPP2 to 4 corresponding to Lys 393; asparagine residues 35, 33 and 34 in *Zm*NPP1, *At*NPP1 and *At*NPP2 are not marked. Predicted signal peptides giving a reliable prediction are shaded with dark blue.

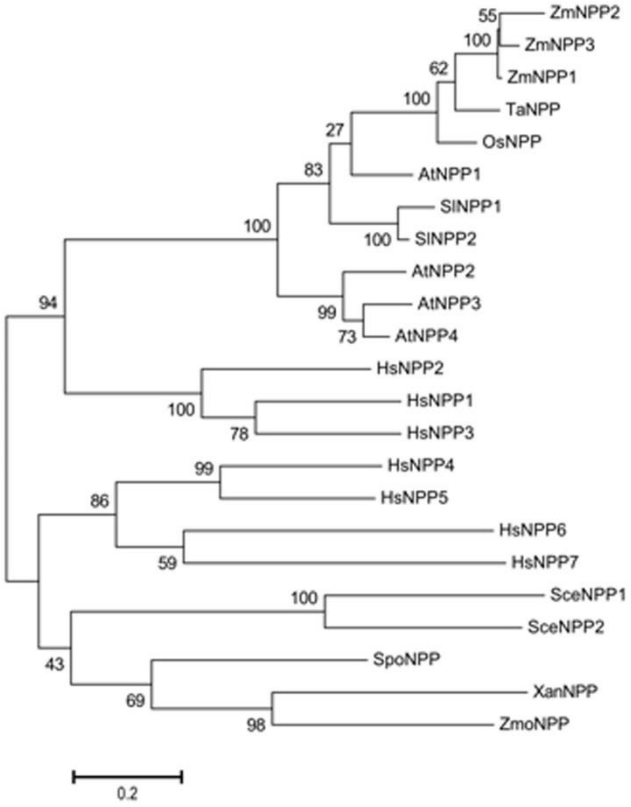

Figure 21: Phylogenetic analysis of nucleotide pyrophosphatases/phosphodiesterases. The following sequences were collected: *Xanthomonas Xan*NPP (WP_011051855.1), *Zymomonas mobilis Zmo*NPP (WP_011241358.1), *Schizosaccharomyces pombe Spo*NPP (O94323.1), *Saccharomyces cerevisiae Sce*NPP1 (P25353.2), *Sce*NPP2 (P39997.1), *Zea mays Zm*NPP1 (Zm00001d039454_P001), *Zm*NPP2 (Zm00001d047948_P001), *Zm*NPP3 (Zm00001d044311_P001), *Oryza sativa Os*NPP (LOC_Os01g10020.1), *Arabidopsis thaliana At*NPP1 (AT4G29680.1), *At*NPP2 (AT4G29690.1), *At*NPP3 (AT4G29700.1), *At*NPP4 (AT4G29710.1), *Triticum aestivum Ta*NPP (ADK32530.1), *Solanum lycopersicum Sl*NPP1 (Solyc07g037950.1), *Sl*NPP2 (Solyc07g037960.1), *Homo sapiens Hs*NPP1 (P22413.2), *Hs*NPP2 (Q13822.3), *Hs*NPP3 (O14638.2), *Hs*NPP4 (Q9Y6X5.3), *Hs*NPP5 (Q9UJA9.1), *Hs*NPP6 (Q6UWR7.2) and *Hs*NPP7 (Q6UWV6.3). The sequences were aligned using CLUSTAL W method (Thompson et al., 1994). The phylogenetic tree was built by the MEGA 6.0 software by the maximum likelihood method (Tamura et al., 2013). To estimate evolutionary distance, the proportions of amino acid differences were computed using Poisson Correction Distance. The reliability of different phylogenetic clusters was evaluated by the bootstrap test (1000 bootstrap replications).

The prediction of plant NPPs' subcellular localization by TargetP (Emanuelsson et al., 2007) is mostly ambiguous. Two exceptions are *Zm*NPP2 and *At*NPP3, both with high confidence to be secreted proteins. Both of them have also predicted one trans-membrane helix by TMHMM server (Krogh et al., 2001), but the program is known to predict signal peptides as trans-membrane helices sometimes. The other proteins' localization is predicted with lower confidence with reliability class usually 4 or 5.

The plant NPPs in general contain all the amino acids that were shown to be crucial for the catalysis in animals (Kato et al., 2012) with two notable exceptions: Lys 277 from mouse NPP1 does not align to any specific amino acid in the plant NPPs and the Asp 308 that is crucial for substrate binding *via* water molecule is substituted with glutamate (Glu 191 in *Zm*NPP1) as in *Xanthomonas* enzyme. The plant enzymes are predicted to be glycosylated by NetNGlyc (Gupta and Brunak, 2002) at several asparagine residues. Without ambiguity, all enzymes shall be glycosylated at residues corresponding to Asn 108 and 208 of *Zm*NPP1 with the exception of *At*NPP2 and *At*NPP3 that have residue corresponding to Asn 108

substituted for Arg and Gly, respectively. The three *Arabidopsis* proteins that have asparagine at the position corresponding to *Zm*NPP1's Lys 393 shall be glycosylated there. The last position undergoing glycosylation is the one corresponding to Asn 454 of *Zm*NPP1. At this position, about half of proteins are weakly predicted to be glycosylated, while the rest not to be glycosylated. Lastly, ZmNPP1, *At*NPP1 and *At*NPP2 shall be glycosylated at positions 35, 33 and 34, respectively. An alignment of all aforementioned plant NPP enzymes with indicated putative glycosylation sites, conserved amino acids and predicted signal peptides is shown in Figure 20.

Substrate Specificity of Recombinant Maize Nucleotide Pyrophosphatase/Phosphodiesterase

To study the catalytic reaction, heterologous expression was preferred to purification from maize kernel extract for obtaining sufficient amount of *Zm*NPP. First attempts using *Escherichia coli* did not lead to successful protein production. Alternatively, the expression was successful using a methanol-inducible expression in the methylotrophic yeast *Pichia pastoris* with the expression vector pPICZα that fuses a signal peptide to the N-terminus of the recombinant protein thus driving its secretion into the growth medium. The recombinant protein was then concentrated and purified to homogeneity (Figure 17E) using three relatively simple chromatographic steps as described in Materials and Methods. The size of the recombinant protein was around 60 kDa due to the presence of His-tag and a linker (3.5 kDa) and probably due to a heavier glycosylation pattern in yeast.

The purified recombinant protein showed the zeatin *cis-trans* isomerase reaction in the presence of FAD and light. The enzyme does not significantly speed up *cis-trans* zeatin conversion in the presence of FMN; however, a non-enzymatic conversion in the

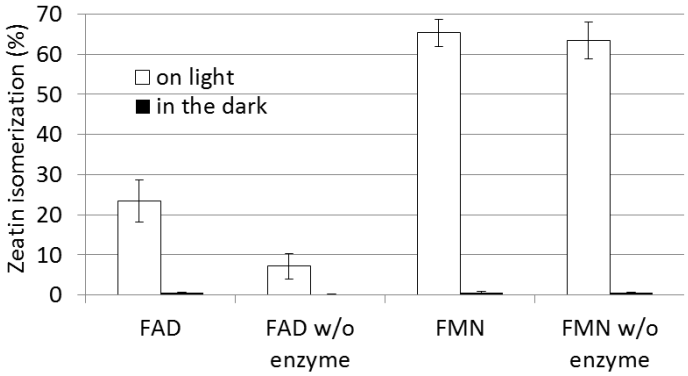

Figure 22: Comparison of *cis*-to-*trans* conversion of zeatin isomers (0.2 mM) in a presence of FAD and FMN. Reactions were set up with or without purified recombinant ZmNPP enzyme and incubated in 100 mM McIlvaine buffer containing 20 mM $MgCl_2$, 0.1 mM FAD/FMN, at 37°C under white fluorescent light or in the dark for 20 minutes; determined by UFLC; graph depicts average of three technical replicates with

presence of FMN is much faster than in the presence of FAD (Figure 22). The obtained recombinant *Zm*NPP was also readily able to hydrolyze a range of typical nucleotide pyrophosphatase/phosphodiesterase substrates as shown in Figure 23 and none of these reactions required light. With the best substrate FAD, enzyme exhibited a specific activity of 496.8 nkat mg protein^{-1} and K_m of 32.7 µM (Figure 23). In general, dinucleotides (FAD, diadenosine polyphosphates, NADP$^+$, and UDP-Glc) were better substrates than mononucleotides. The hydrolytic reaction produced AMP from FAD, NADP$^+$, ATP, and diadenosine tetra- and pentaphosphate. Further hydrolysis of produced adenosine polyphosphates was observed only after a prolonged incubation. Hydrolysis of other nucleoside triphosphates also led to monophosphate products.

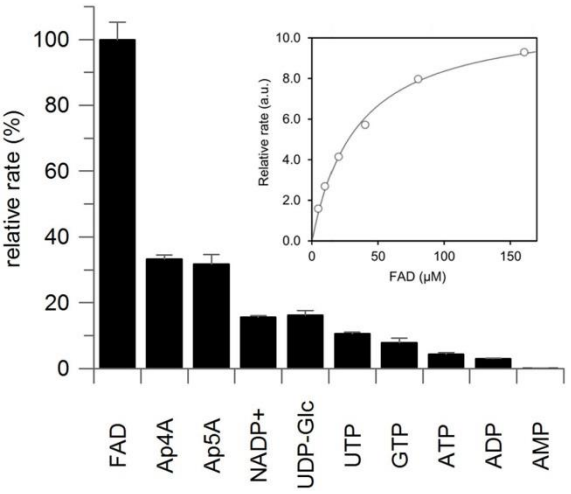

Figure 23: Substrate specificity of recombinant maize nucleotide pyrophosphatase/phosphodiesterase.
Substrates (0.5 mM) were incubated with the purified enzyme in 100 mM McIlvaine buffer containing 20 mM $MgCl_2$ at 37°C for the period of 30 min, during which both the decrease in substrate concentration and increase in the product concentration were monitored by UFLC and the tangent was used to calculate the reaction rate. Graph depicts average of three technical replicates with standard deviation. The insert shows the saturation curve of FAD hydrolysis with maximal velocity on y-axis expressed in arbitrary units (a.u.), from which K_m of 32.7±3.0 µM was calculated by GraFit software.

Expression of Maize Nucleotide Pyrophosphatase/Phosphodiesterase in Plant Systems

In order to quickly verify *Zm*NPP activity *in planta*, the *ZmNPP* gene was first expressed in a tomato hairy root system (Collier et al., 2005) and several independent transgenic lines were propagated by main root excision. The *ZmNPP* expressing lines showed 2 to 10 times higher activity of nucleotide pyrophosphatase/phosphodiesterase with FAD (assayed as the production of FMN) compared to wild type and heat inactivated samples. Similarly, zeatin *cis*-to-*trans* isomerization rate measured *in vitro* in the extracts from various transgenic lines was higher as shown in Figure 24A. There was a strong correlation (R^2 = 0.9655) between zeatin isomerization and nucleotide pyrophosphatase/phosphodiesterase activity, which was, at the same time, independent of the presence of *cis*-zeatin (Fig. 24B).

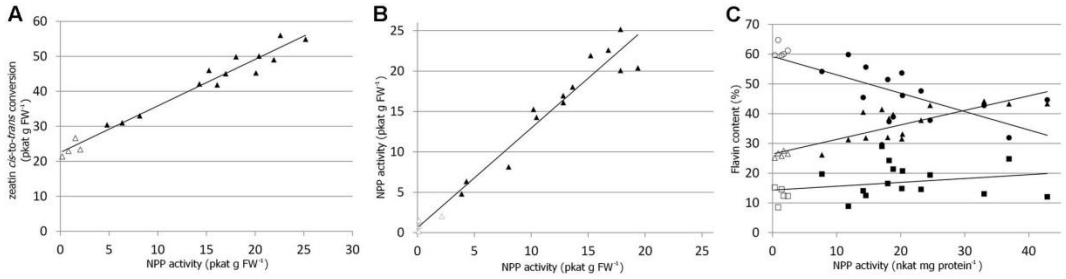

Figure 24: Elevated nucleotide pyrophosphatase/phosphodiesterase (NPP) activity in transgenic plants. (A) NPP activity assayed as the production of FMN in the extracts from various tomato hairy root lines expressing *ZmNPP* and correlation to zeatin *in vitro cis*-to-*trans* conversion per 1 g of fresh weight (FW). (B) Activity of NPP (as the production of FMN) in the presence (y-axis) and absence (x-axis) of *cis*-zeatin in various tomato hairy root lines. The reaction was set up for each sample twice identically except of the presence/absence of *cis*-zeatin. (C) Relation of endogenous contents of FAD (circles), FMN (triangles) and riboflavin (squares) towards the activity of nucleotide pyrophosphatase/phosphodiesterase in 4-week-old *Arabidopsis* plants expressing *ZmNPP*. Samples were taken from four independent lines. Open and solid symbols represent wild type and transgenic lines, respectively.

Further, *Arabidopsis* plants expressing the *ZmNPP* gene under 35S promoter were prepared. The specific activity of nucleotide pyrophosphatase/phosphodiesterase with FAD as the substrate increased in the extracts from leaves of 4-week-old *Arabidopsis* plants by an order of one magnitude compared to wild type (Table 6). Accordingly, the transgenic plants had decreased levels of endogenous FAD and increased levels of FMN and riboflavin (Figure 24C). However, no significant changes in the levels of endogenous zeatin isomers were observed. The levels of zeatins' free bases in green and senescent leaves of *Arabidopsis* are depicted in Figure 25. The plants also did not show any visible phenotypic changes or changes in the chlorophyll content (Table 6).

To assess the function of putative rice nucleotide pyrophosphatase/phosphodiesterase LOC_Os01g10020, 2-week-old rice plants in which the expression of encoding gene was enhanced by disrupting a native promoter of the gene with the promoter sequence of α-tubulin gene *OsTubA1* were examined. Originally, this only available T-DNA insertion mutant of *NPP* gene in monocot species was purchased in order to study loss-of-function phenotype; however, it later turned out that the insertion of T-DNA to the promotor region led to its overexpression rather than disruption. Approximately one fourth of the obtained plants were albinos, in which chlorophyll content was below the detection limit and the nucleotide pyrophosphatase/phosphodiesterase activity increased about 50-times compared to the wild type, while green mutant plants showed only about 10-times activity increase. The total flavin content decreased about 3-times in albinos, but remained unchanged in green mutant plants (Table 6).

Table 6: Characteristics of *Arabidopsis* and rice plants with altered NPP activity. Extracts from the leaves of 4-week-old *Arabidopsis* expressing *ZmNPP* gene (LOC100280465) and 2-week-old rice plants with enhanced expression of putative *OsNPP* gene (LOC_Os01g10020) were analyzed as described in Materials and Methods. NPP activity was measured as FAD hydrolysis. Results are given as mean values ± standard deviation; p-value was determined using Student's t-test against t wild type lines with number of biological replicates indicated for each line in the table; < LOD – below limit of detection; n.d. - not determined.

Plant	***Arabidopsis***				**Rice**				
Line	wild type	#5	#8	#15	wild type	#1	#2	#3	#4 (albino)
Biological replicates	5	5	4	5	9	16	3	5	3
Plants per replicate	4	4	4	4	1	1	1	1	2
Specific activity (nkat mg^{-1})	0.07 ± 0.04	1.26 ± 0.30	1.65 ± 0.61	0.86 ± 0.24	0.01 ± 0.01	0.09 ± 0.02	0.12 ± 0.02	0.05 ± 0.02	0.57 ± 0.12
p-value		*1.29E-03*	*0.021*	*2.53E-03*		*1.39E-10*	*4.41E-03*	*0.029*	*0.022*
Total flavins (pmol)	2.4 ± 0.5	2.3 ± 0.5	2.3 ± 0.3	2.1 ± 0.6	5.6 ± 1.5	5.8 ± 1.5	4.5 ± 1.2	6.0 ± 2.4	1.8 ± 0.2
p-value		*0.94*	*0.74*	*0.56*		*0.78*	*0.35*	*0.77*	*7.59E-05*
FAD (%)	61.1 ± 1.9	44.1 ± 6.0	42.5 ± 8.8	45.3 ± 8.5	51.2 ± 0.9	51.5 ± 1.5	49.5 ± 2.6	52.0 ± 1.3	55.1 ± 0.5
p-value		*3.20E-03*	*0.033*	*0.019*		*0.56*	*0.45*	*0.30*	*5.9E-4*
FMN (%)	26.3 ± 0.8	38.3 ± 4.1	40.3 ± 4.9	34.6 ± 5.7	46.7 ± 0.6	45.8 ± 1.3	47.7 ± 2.3	45.3 ± 1.3	34.4 ± 1.4
p-value		*3.57E-03*	*0.015*	*0.042*		*0.040*	*0.58*	*0.11*	*5.09E-03*
Riboflavin (%)	12.6 ± 2.3	17.6 ± 4.4	17.2 ± 5.3	20.0 ± 5.1	2.2 ± 0.5	2.7 ± 0.4	2.8 ± 0.3	2.7 ± 0.3	10.5 ± 2.0
p-value		*0.090*	*0.23*	*0.041*		*7.29E-03*	*0.046*	*0.063*	*0.026*
Chlorophyll *a* (µg mg^{-1})	0.71 ± 0.10	0.65 ± 0.11	0.70 ± 0.11	0.76 ± 0.12	1.09 ± 0.19	1.02 ± 0.12	1.07 ± 0.04	1.33 ± 0.24	< LOD
p-value		*0.45*	*0.91*	*0.46*		*0.38*	*0.80*	*0.19*	*n.d.*
Chlolophyll *b* (µg mg^{-1})	0.24 ± 0.03	0.22 ± 0.03	0.23 ± 0.04	0.25 ± 0.04	0.28 ± 0.05	0.27 ± 0.03	0.28 ± 0.02	0.37 ± 0.06	< LOD
p-value		*0.43*	*0.83*	*0.51*		*0.37*	*0.92*	*0.082*	*n.d.*
Chlorophyll *a*/*b*	3.01	3.00	3.05	3.04	3.85	3.86	3.84	3.59	n.d.
p-value		*0.73*	*0.052*	*0.13*		*0.96*	*0.93*	*0.021*	*n.d.*

Discussion

In the original paper by Bassil et al. (1993), the zeatin *cis-trans* isomerase activity detected in immature seeds of common bean had following attributes: (i) the conversion required FAD or FMN, light and a reducing agent such as dithiothreitol; (ii) both zeatin isomers were substrates, although the rate of conversion of *cis*- to *trans*-zeatin was higher, and (iii) there was also a noticeable background conversion when the enzyme preparation was inactivated by boiling. Purification of a protein with the zeatin *cis-trans* isomerase activity from maize kernels led to the identification of the nucleotide pyrophosphatase/phosphodiesterase, which hydrolyzes FAD without any light requirement. NPP family of 7 members has been well known in humans. Most of human enzymes contain a single transmembrane domain residing in the plasma membrane and hydrolyze mostly nucleotides and their analogues or phospholipids (Stefan et al., 2005). Plant NPPs are less characterized. The situation is complicated by the lack of sequence determination and an overlap of the catalyzed reaction with other enzyme families i.e. purple acid phosphatases (PAP; Li et al., 2002; Olczak et al., 2003) and Nudix hydrolases (**nu**cleoside **di**phosphate compounds linked to a moiety, **X**; Maruta et al., 2012), which catalyze hydrolytic breakdown of pyrophosphate and phosphodiester bonds of numerous nucleotide sugars. Chloroplast localized ADP-Glc pyrophosphatase that belongs to purple acid phosphatase family, mis-annotated as NPP, has been found to negatively regulate starch biosynthesis in rice and barley (Kaneko et al., 2014; Nanjo et al., 2006). The only plant enzyme characterized to date resembling the mammalian ones has been found in wheat (Joye et al., 2010).

The amino acid sequence of *Zm*NPP shows the highest similarity to the families 1 to 3 of human nucleotide pyrophosphatase/phosphodiesterases and also to the enzyme from wheat, but it lacks the transmembrane domain, which is consistent with the enzyme's purification as a soluble protein. Correspondingly, it has been shown that

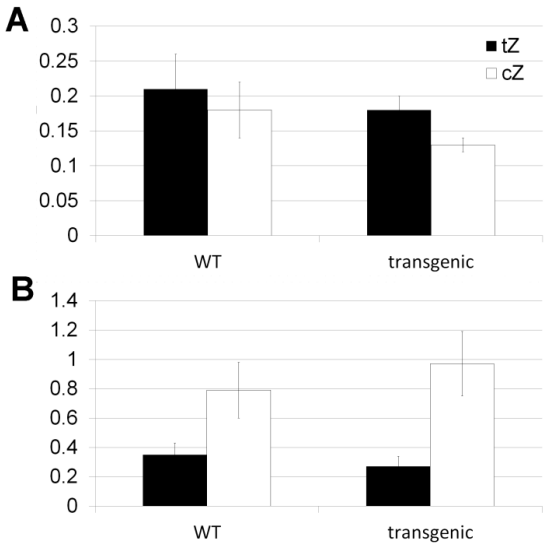

Figure 25: Level of *trans*-zeatin and *cis*-zeatin in green (A) and senescent (B) leaves of *Arabidopsis* of wild type or expressing *Zm*NPP. None of the results was significantly different (Student's t-test; $p < 0.05$; $n = 4$) between the wild type and *Zm*NPP plants.

some human enzymes (Belli et al., 1993; Sakagami et al., 2005; Wu et al., 2004) as well as the wheat enzyme (Joye et al., 2010) may exist in a soluble form. Accordingly to the mammalian and wheat enzymes, which are *N*-glycosylated, the *Zm*NPP is probably also glycosylated as predicted by NetNGlyc (Gupta and Brunak, 2002), evidenced by binding to Concanavalin A-Sepharose and a hyper-glycosylation observed on the recombinant *Zm*NPP protein. To determine the substrate specificity, recombinant *Zm*NPP was prepared in *Picchia pastoris* and purified from the culture medium. With the partially purified enzyme, the zeatin isomerization reaction proceeded even in the absence of dithiothreitol needed to inhibit the breakdown of zeatin to adenine in plant extracts that probably occurred due to the activity of cytokinin dehydrogenase (EC 1.5.99.12; Galuszka et al., 2001).

Flavins, in general, have been known to possess rich chemistry, which is exploited by nature in many enzymatic systems. However, FMN and FAD are also known to form singlet and triplet excited states upon illumination and induce non-enzymatic flavin sensitized photoisomerism of e.g. retinol, bilirubin and stilbenes by direct energy transfer (Heelis, 1982). Flavin induced photoisomerisation of bilirubin is used for a treatment of neonatal jaundice (Knobloch et al., 1991). FMN is more potent in photoisomerisation induction. It is therefore very likely that *in vitro Zm*NPP hydrolysis of FAD to FMN and AMP induces a non-enzymatic photoisomerization of zeatin (Fig.

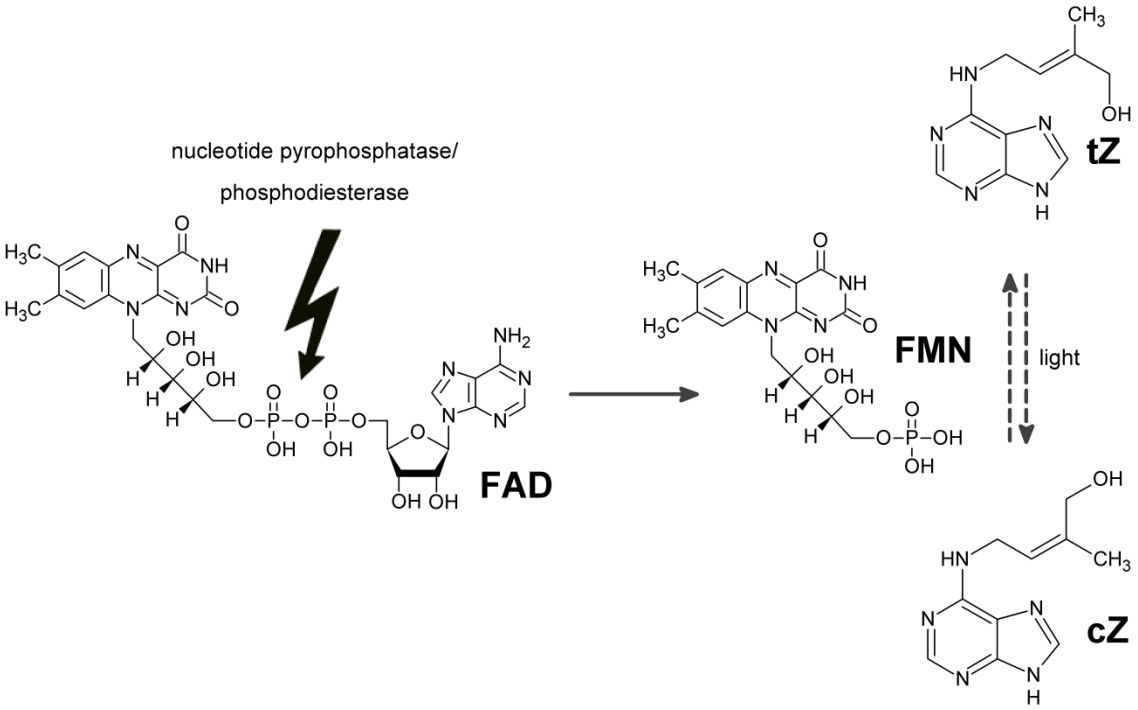

Figure 26: Proposed mode of action of "zeatin *cis-trans* isomerase". Instead of catalyzing the isomerization using FAD and light as cofactors, the enzyme hydrolyses FAD and formed FMN is more efficient non-enzymatic catalyst of the isomerization. Thus the apparent increase in zeatin isomerization rate upon enzyme addition was observed.

26). Photoisomerization of zeatin with FAD then occurred also with the heat-inactivated enzyme, albeit at a lower rate. The conversion of zeatin isomers found *in vitro* is reminiscent of an older paper reporting on *trans*-to-*cis* isomerization of geraniol and geranyl phosphate to nerol and neryl phosphate, respectively, by cell-free extracts of carrot and peppermint, in the presence of FAD or FMN, a thiol or sulfide and light (Shine and Loomis, 1974), which may have also happened non-enzymatically by flavin sensitization.

There is not enough supporting evidence for zeatin isomerization *in vivo*. Upon feeding potato tubers with radioactively labeled *cis*-zeatin, only 5 to 9 % of the label was found in *trans*-zeatin (Suttle and Banowetz, 2000). Recently, *cis-trans* isomerization was reported in pathogenic fungus *Leptosphaeria maculans* (Trdá et al., 2017). First, the authors observed an increase in *cis*- or *trans*-zeatin, when the fungus was fed with the other isomer. We have observed similar occurrence ourselves when we treated maize with micromolar concentrations of zeatins, but it was not confirmed with radiolabeled cytokinins at physiological levels (this work). Further, Trdá et al. fed the fungus with zeatins and observed accumulated isomers in the medium. Similarly to our work reported here, the *cis*-to-*trans* isomerization was approximately 3-times faster than the other way. Also, when the fungus was not added, or it was boiled prior to incubation, the conversion was smaller (Trdá et al., 2017). Thus, one can hypothesize, that nucleotide pyrophosphatase/phosphodiesterase is at play here. On the contrary, the *Arabidopsis* knock-out plants deficient in biosynthesis of either of the zeatin isomers were not able to complement its loss by isomerization (Miyawaki et al., 2006). We, therefore, prepared overexpression plants to assess whether altered nucleotide pyrophosphatase/phosphodiesterase activity affects the content or ratio of zeatin isomers *in vivo*. The FAD hydrolyzing activity in the lines expressing maize enzyme was increased 12 to 23-times. In these plants, FAD levels were decreased as expected (Table 6). However, besides the increase in FMN, the direct product of FAD hydrolysis, part of FMN was probably converted to riboflavin. *Arabidopsis* leaves expressing *ZmNPP* did not show any alteration in the ratio or the total content of two zeatin isoforms (Fig. 25). This should not be surprising considering the plants are able to suppress non-enzymatic conversion of zeatin isomers. Otherwise, the plants would have to have *trans* to *cis* ratio at the equilibrium. This may be due to several reasons, possibly because of low concentrations in the plant and limited amount of light intracellularly. Further, flavins and cytokinins are each localized to different subcellular compartments. While flavins are mainly in mitochondria and nucleus (Giancaspero et al., 2013), cytokinins are predominantly localized to apoplast (Jiskrová et al., 2016). No visible phenotype alterations were observed on these plants. Similarly, no phenotype

change was observed in AtNUDX23 overexpressing plants with about 20% decrease in flavin content (Maruta et al., 2012).

To shed more light on the physiological function of plant nucleotide pyrophosphatase/phosphodiesterase, rice plants, in which the expression of *OsNPP* encoding gene was enhanced by disrupting its native promoter with a strong and constitutive promoter of α-tubulin, were examined. Approximately one fourth of the obtained plants were albinos with much higher increase in the nucleotide pyrophosphatase/phosphodiesterase activity than was observed in remaining green mutant plants. Except for a significant decrease in total flavin content in the albino plants, there was a significant shift from FMN to riboflavin (Table 6). We cannot currently speculate about the lack of chlorophyll, NPP activity and flavin content, what is the cause and what is the consequence, or whether there is any causation at all. However, considering the fact we observed no albino plants of wild type origin, we can hypothesize that in some rice plants the *OsNPP* upregulation is so strong, it leads to severe alterations in flavin content ultimately causing chlorophyll depletion. These data are in agreement with previous observations that the reduction in the flavin content to less than 50% leads to a stunted growth and chlorosis of plants (Hedtke et al., 2011; Ouyang et al., 2010). The authors conclude, that increased photooxidative damage and down-regulated FAD-dependent cytokinin oxidase/dehydrogenase, respectively are responsible for the observed phenotype.

As we have no hints on a physiological function yet, we can only hypothesize based on the substrate preferences about the following functions of NPP *in planta*:

I deactivation of signaling molecules – several of the substrates serve as signaling molecules. In particular, extracellular ATP is known to play a crucial role in plant growth, development and stress responses. Recently, a receptor was identified in Arabidopsis (Choi et al., 2014). It is still not clear, whether dinucleotide polyphosphates Ap_nA do have a physiological function or whether they are rather toxic side products of the metabolism (McLennan, 2000). However, they have been shown to accumulate upon a heavy metal- or heat-stress, functioning as alarmones, or to induce activity of the phenylpropanoid pathway (Pietrowska-Borek et al., 2011). Nucleotide pyrophosphatase could serve as a deactivating agent for these signaling molecules. However, low levels of these compounds *in vivo* (e.g. diadenosine tri- and tetraphosphate are present in nanomolar concentrations; Andrzej Guranowski, personal communication) and high K_m and/or low activity of *Zm*NPP for these compounds suggest this enzyme would not be very efficient in terminating their signal.

II pathogen defense – riboflavin has been reported to induce a resistance against several pathogens in plants, including *Botrytis cinerea* (Azami-Sardooei et al., 2010). The expression of *AtNPP*3 is increased about 7-fold upon *B. cinerea* inoculation as can be seen in the Genevestigator database (Hruz et al., 2008). Since riboflavin is an indirect product of NPP reaction and it accumulates upon NPP overexpression, it is plausible, that NPP may function in the overall defense mechanism against *B. cinerea*. Moreover, soluble sugars are known to induce resistance against a wide range of pathogens including *B. cinerea* (Derridj et al., 2012) and Arabidopsis NPPs are in general upregulated upon a sugar treatment as shown in the Genevestigator database (Hruz et al., 2008). Thus NPP could represent a step in this resistance induction cascade. However, while sugar induces resistance against *B. cinerea* both in bean and tomato, riboflavin is effective only in bean. Thus either tomato uses a different mechanism, or other than here proposed cascade exists. Furthermore, *Pseudomonas syringae* was shown to require guanosine tetra- and pentaphosphates for the colonization of plants (Chatnaparat et al., 2015). Although these compounds have not yet been tested for hydrolysis by NPP enzymes, it's likely they do serve as substrates. Thus by hydrolyzing a crucial metabolite, the NPP enzyme would limit the pathogen spreading. And indeed, the expression of *AtNPP*3 is shown by the Genevestigator database (Hruz et al., 2008)to be in general increased after inoculation with *P. syringae*.

III redirection of carbon flux – NDP-saccharides are substrates for the formation of polysaccharides such as starch and cellulose. It has been shown that the purple acid phosphatase with ADP-glucose pyrophosphatase activity diminishes the starch formation (Kaneko et al., 2014; Rodríguez-López et al., 2000). The expression of *AtNPPs* after imbibition suggests they may be involved in storage mobilization through seed germination. During their expression in siliques they may regulate the cell wall formation in endosperm. Furthermore, pyrophosphate seems to play a regulatory role in starch synthesis (Farré et al., 2000). NPP could produce this pyrophosphate and thus promote the starch synthesis in the siliques during seed germination, similarly as mammalian NPPs are known to regulate bone mineralization by the release of pyrophosphate from NTPs (Kato et al., 2012).

IV regulation of availability of redox cofactors – it has been proposed, that the *Opuntia* nucleotide pyrophosphatase/phosphodiesterase regulates the

availability of redox cofactors during fruit ripening (Spanò et al., 2011). A similar function may be proposed for the NPPs studied here, as the maize enzyme prefers redox cofactors, mainly FAD over other substrates. In addition, there is an increased expression during seed maturation.

V regulation of light perception – flavins are cofactors in three out of five classes of photoreceptors in *Arabidopsis* (Christie et al., 2015). All three classes are specifically involved in blue light perception. Thus by adjusting the availability of a particular flavin species, NPP may regulate the blue light perception.

Because of the wide substrate specificity of plant NPPs overlapping with other enzymes, the disclosure of their physiological function will require an integrative approach and research involving all these enzymes.

From the data presented in this work, it appears highly unlikely that nucleotide pyrophosphatase/phosphodiesterase participates in zeatin *cis-trans* isomerization *in vivo* as there is no significant change in the ratio of the zeatin isomers in plants with altered activity of this enzyme.

CONCLUSIONS

The biosynthesis of cytokinins has been described well in *Arabidopsis*. The side chain – hydroxylated or not – is transferred to ADP or ATP. If iPR phosphate is formed, it may be hydroxylated to form tZR phosphate. After partial dephosphorylation to nucleotide monophosphates, the phosphoribosyl moiety is removed by a CK-specific hydrolase providing the active cytokinin.

However, the research focused on *tZ*-dominant *Arabidopsis* and slight of old research leads to several yet unanswered questions. Thus the importance of such enzymes as zeatin reductase or zeatin *O*-xylosyltransferase cannot be estimated. The recent re-discovery of "novel" cytokinins hints that there may be more diversity to CKs and their metabolism as is currently reflected by the research topics.

Here we attempted to elaborate on some of the "old truths" of the cytokinin field. We chose the maize developing seed as working material, because it is rich in cytokinins and many of the interconversions were described to occur in immature endosperm. Also maize is *cis*-zeatin dominant plant and we have shown previously that the dormant seeds are rich for dihydrozeatin. This all makes the maize kernel a compelling model for cytokinin metabolism studies.

By cytokinin profiling we confirmed the typical peak in cytokinin content upon pollination followed by decrease of the unsaturated zeatins, while dihydrozeatin reached maximum at 35 days after pollination. The search for zeatin reductase that would yield the dihydrozeatin led to discovery of novel enzymatic activity producing "novel" aromatic cytokinin 6-(3-methylpyrrol-1-yl)purine. The responsible enzyme was not identified though because of its instability.

We had more luck with detection of the zeatin *cis-trans* isomerase activity. However, the causative enzyme was identified to be nucleotide pyrophosphatase/phosphodi-esterase. Its activity was confirmed both *in vitro* and *in vivo*. The enzyme prefers dinucleotides, such as FAD. We have proposed several physiological functions of the enzyme, but more research is necessary to decipher its true relevance/significance.

Besides the results of wet lab work, two novel hypotheses are presented. The first explains the prevalence of reputedly inactive zeatin isomer in many species. We propose this is result of zeatin *O*-glucosyltransferase's substrate preference, rather than "higher intention" because of certain advantage due to *cis*-zeatin abundance. The second proposes that there are in fact two distinct CK systems. While one aims for the

strong signal leading to fast and distinguished growth, the second one is involved in maintenance growth.

ABBREVIATIONS

ABA	abscisic acid
ABCG	ATP-binding cassette transporter subfamily G
ACC	1-aminocyclopropane-1-carboxylic acid
ADP	adenosine diphosphate
ARR	response regulator from Arabidopsis
ATP	adenosine triphosphate
BAP	N^6-benzyladenine (for 6-benzylaminopurine)
CK	cytokinin
CKX	cytokinin oxidase/dehydrogenase
CYP	cytochrome P450 monooxygenase
*c*Z	*cis*-zeatin
*c*ZOGT	*cis*-zeatin *O*-glucosyltransferase
DAP	day(s) after pollination
DBP	day(s) before pollination
DCPIP	2,6-dichlorophenolindophenol
DHZ	dihydrozeatin (N^6-(4-hydroxy-3-methylbutyl)adenine)
DMAPP	dimethylallyl pyrophosphate
dpi	days post infection
ENT	equilibrative nucleoside transporter
FAD	flavin adenine dinucleotide
GA19	gibberellin A19
GFP	green fluorescent protein
HMBDP	(*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate
HK	histidine kinase
HP	histidine-containing phosphotransfer protein
IAA	indole-3-acetic acid
iP	N^6-(Δ^2-isopentenyl)adenine
IPP	Δ^3-isopentenyl pyrophosphate
IPT	isopentenyl transferase

JA	jasmonic acid
LOG	Lonely Guy (cytokinin-specific phosphoribohydrolase)
M&S	Murashige and Skoog (medium or agar)
MEP	methylerythritol phosphate (pathway)
MPP	6-(3-methylpyrrol-1-yl)purine
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MVA	mevalonate (pathway)
PAGE	polyacrylamide gel electrophoresis
PUP	purine permease
Q_0	2,3-dimethoxy-5-methyl-1,4-benzoquinone
RNAi	RNA interference
RR	response regulator
SA	salicylic acid
SPE	solid-phase extraction
t6A	N^6-threonylcarbamoyladenosine
TFA	trifluoroacetic acid
*t*Z	*trans*-zeatin
*t*ZOGT	*trans*-zeatin *O*-glucosyltransferase
*t*ZOXT	*trans*-zeatin *O*-xylosyltransferase
UFLC	ultra fast liquid chromatography
WT	wild-type (organism; i.e. non-mutated)

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RÉSUMÉ

Tomáš HLUSKA

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https://www.researchgate.net/profile/Tomas_Hluska

Work Experience:

Crop Research Institute — **Olomouc, The Czech Rep.**
research assistant — *03/2017–08/2017*

- successfully cloned poplar cytokinin receptors to expression vector, despite previous troubles with the cloning
- expression analysis of poplar IPTs in transgenic Arabidopsis plants

Faculty of Science, Palacký University — **Olomouc, The Czech Rep.**
student research assistant — *09/2008–06/2016*

- work in molecular biology laboratory
- plant cultivation (Arabidopsis, maize, rice) at various conditions (incl. hydropony)
- analyses of various types of compounds with electrophoresis, blotting, liquid chromatography (incl. HPLC)

Research Internships abroad:

Angewandte Genetik, Freie Universität — **Berlin, Germany**
total 6 months — *2012 & 2014*

- Tomáš Werner's lab

RIKEN Plant Science Center — **Yokohama, Japan**
total 2.5 months — *2009*

- prof. Sakakibara's lab

Danmarks Tekniske Universitet — **Kgs. Lyngby, Denmark**
total 10 months — *2007–2008*

- project at CBS

Research Interests:

liquid chromatography – purification of LMW substances, as well as proteins; HPLC
cytokinins – *cis*-zeatin and its biosynthesis and role in plants, isopentenyl transferases, CK modifying enzymes
nucleotide pyrophosphatases/phosphodiesterases – their role in plants
protein chemistry – purification, heterologous expression, enzymatic kinetics

Teaching Experience:

Faculty of Science, Palacký University **Olomouc, The Czech Rep.**
teaching Cloning and Genetic Engineering *2016*
- course for MSc. students (majors Biochemistry, Biotechnology)
- lectures about modern methods from in vitro procedures to applications in agriculture or gene therapies

Back to School event **Jednov, The Czech Rep.**
Chemistry laboratory *2013–2016*
- chemical experiments with active participation of beholders

Faculty of Science, Palacký University **Olomouc, The Czech Rep.**
advisor to students *2010–2015*
- 3 BSc. and 1 MSc. student

teaching Laboratory Technique for Biochemists
- introduction for freshmen of Bachelor majors Biochemistry and Biotechnology and Genetic Engineering with work in biochemical laboratory
- training of basic chemical methods, as well as more advanced procedures used in biochemical laboratories
- establishment of new microscopy protocol

Faculty of Science, Palacký University **Olomouc, The Czech Rep.**
Summer biotechnology project 1 and 2 *2012–2014*
- mentored students working in molecular biology labs and oversaw their subsequent report writing in English

Faculty of Science, Palacký University **Olomouc, The Czech Rep.**
establishment of new protocol for Molecular Biology lab course *2009*
- development of own protocol for detection of pathogens in food with qPCR

Preparation of scholars for Chemistry Olympiad

Education:

Faculty of Science, Palacký University **Olomouc, The Czech Rep.**
PhD. major Biochemistry *currently*
Dissertation title: Metabolism of Zeatin-Type Cytokinins in Monocots

MSc. major Biochemistry – *Passed with Honours* *2010*
Master thesis title: On the Hunt for Zeatin *cis-trans* Isomerase

BSc. major Biochemistry – *Passed with Honours* *2008*
Bachelor thesis title: Expression and Characterization of Plant Adenylate Isopentenyltransferase

Danmarks Tekniske Universitet **Kgs. Lyngby, Denmark**
student exchange program Erasmus *09/2007–06/2008*

PC skills:

OS Windows
MS Office 2003 and newer

ACDLabs ChemSketch 10.0
BioEdit v. 7.0
Cytoscape v. 2_6_1
PyMOL v. 0.99
ViM v. 7.4
UNIX, Perl and R

Language skills:

English – level C
German – level B2

Interests:

reading, photography, sport (cycling, volleyball), desk games, tourism and travelling, cooking, foreign languages

Publications:

Sum of Times Cited without self-citations (08/2017): 212
h-index: 3

- Niemann MCE, Weber H, **Hluska T**, Leonte G, Anderson SM, Senes A, Werner T (submitted) Cytokinin-degrading CKX1 from *Arabidopsis* is a membrane-bound protein requiring homooligomerization in the endoplasmic reticulum for its cellular aktivity. *Plant Phys.*
- **Hluska T**, Šebela M, Lenobel R, Frébort I and Galuszka P (2017) Purification of Maize Nucleotide Pyrophosphatase/Phosphodiesterase Casts Doubt on the Existence of Zeatin *Cis–Trans* Isomerase in Plants. *Front. Plant Sci.* 8, 1473
- **Hluska T**, Dobrev PI, Tarkowská D, Frébortová J, Zalabák D, Kopečný D, Plíhal O, Kokáš F, Briozzo P, Zatloukal M, Motyka V and Galuszka P (2016) Cytokinin metabolism in maize: Novel evidence of cytokinin abundance, interconversions and formation of a new trans-zeatin metabolic product with a weak anticytokinin activity. *Plant Sci.* 247, 127-137
- Frébort I, Kowalska M, **Hluska T**, Frébortová J and Galuszka P (2011) Evolution of cytokinin biosynthesis and degradation. *J. Exp. Bot.* 62, 2431-2452
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Selected Personally Attended Conferences (⇒ presentation):

- **Hluska T**, Baková M, Strouhal O, Lenobel R, Šebela M and Galuszka P (2013) 20 Years Later, Mystery Resolved: Nucleotide Pyrophosphatase is Responsible for *In Vitro* Zeatin *Cis-Trans* Isomerase Activity. *GRC Conference Enzymes, Coenzymes and Metabolic Pathways*, Waterville Valley, USA
- **Hluska T**, Strouhal O, Plíhal O, Dobrev P, Motyka V, Kuzma M, Šebela M and Galuszka P (2012) Metabolism of Zeatin-type Cytokinins in Monocots. *2nd Meeting on Metabolism, Signaling and Function of Cytokinin*, Berlin, Germany
- **Hluska T**, Baková M, Šebela M, Lenobel R and Galuszka P (2011) Purification and Properties of Zeatin *Cis-Trans* Isomerase from Maize, Tobacco and Arabidopsis. *25th Annual Symposium of The Protein Society*, Boston, USA
- **Hluska T**, Pospíšilová H, Mrízová K, Ohnoutková L, Ryparová O and Galuszka P (2010) Alteration of plant growth by modification of cytokinin metabolism during late seed and root development. *International Symposium on Plant Productivity*, Peterborough, Canada

⇒ **Hluska T**, Ryparová O, Václavíková V, Klásková J, Švehlová L, Lenobel R, Šebela M and Galuszka P (2010) On the Hunt for Zeatin *Cis-Trans* Isomerase. *Conference of experimental plant biology*, Prague, Czech Rep.

- Václavíková K, **Hluska T**, Floková K, Slováková K, Galuszka P and Tarkowski P (2009) The cytokinin metabolism in maize. *Internanional Symposium "Auxins and Cytokinins in Plant Development"*, Prague, Czech Rep.

SUPPLEMENTS

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Cytokinin metabolism in maize: Novel evidence of cytokinin abundance, interconversions and formation of a new *trans*-zeatin metabolic product with a weak anticytokinin activity

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ABSTRACT

Cytokinins (CKs) are an important group of phytohormones. Their tightly regulated and balanced levels are essential for proper cell division and plant organ development. Here we report precise quantification of CK metabolites and other phytohormones in maize reproductive organs in the course of pollination and kernel maturation. A novel enzymatic activity dependent on $NADP^+$ converting *trans*-zeatin (*tZ*) to 6-(3-methylpyrrol-1-yl)purine (MPP) was detected. MPP shows weak anticytokinin properties and inhibition of CK dehydrogenases due to their ability to bind to an active site in the opposite orientation than substrates. Although the physiological significance of *tZ* side-chain cyclization is not anticipated as the MPP occurrence in maize tissue is very low, properties of the novel CK metabolite indicate its potential for utilization in plant *in vitro* tissue culture. Furthermore, feeding experiments with different isoprenoid CKs revealed distinct preferences in glycosylation of *tZ* and *cis*-zeatin (*cZ*). While *tZ* is preferentially glucosylated at the *N9* position, *cZ* forms mainly *O*-glucosides. Since *O*-glucosides, in contrast to *N9*-glucosides, are resistant to irreversible cleavage catalyzed by CK dehydrogenases, the observed preference of maize CK glycosyltransferases to *O*-glycosylate zeatin in the *cis*-position might be a reason why *cZ* derivatives are over-accumulated in different maize tissues and organs.

Abbreviations: ABA, abscisic acid; ACC, aminocyclopropane-1-carboxylic acid; CK, cytokinin; CKX, cytokinin oxidase/dehydrogenase; *cZ*, *cis*-zeatin; *cZ*OG, *cis*-zeatin *O*-glucoside; DAP, days after pollination; DBP, days before pollination; DCPIP, 2,6-dichlorophenolindophenol; DHZ, dihydrozeatin; DHZ9G, dihydrozeatin *N9*-glucoside; DHZR, dihydrozeatin 9-riboside; dpm, disintegration per minute; GA19, gibberellin 19; HK, histidine kinase; IAA, indole-3-acetic acid; iP, isopentenyladenine; iP7G, isopentenyladenine *N7*-glucoside; iP9G, isopentenyladenine *N9*-glucoside; iPR, isopentenyladenosine; iPRMP, isopentenyladenosine 5′-monophosphate; IPT, isopentenyl transferase; JA, jasmonic acid; LOG, lonely guy; MPP, 3-methylpyrrolpurine; MPPR, 3-methylpyrrolpurine 9-riboside; NMWL, nominal molecular weight limit; SA, salicylic acid; SPE, solid phase extraction; *tZ*, *trans*-zeatin; *tZ*7G, trans-zeatin *N7*-glucoside; *tZ*9G, *trans* zeatin *N9*-glucoside; *tZ*OG, trans-zeatin *O*-glucoside; *tZ*R, *trans*-zeatin 9-riboside; *tZ*RMP, *trans*-zeatin 9-riboside 5′-monophosphate; *tZ*RMP, *trans*-zeatin 9-riboside 5′-monophosphate; *tZ*ROG, *trans*-zeatin 9-riboside *O*-glucoside.

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1. Introduction

Besides auxins, cytokinins (CKs) are major plant hormones regulating cell division and elongation as well as organogenesis and many other physiological processes in plants. Naturally occurring CKs are N^6-isoprenoid derivatives of adenine and its sugar conjugates, which can originate *in planta* by two metabolic pathways.

The first step of the *de novo* pathway employs an activity of adenylate isopentenyl transferase (IPT; EC 2.5.1.112) to conjugate ATP or ADP with dimethylallyl pyrophosphate [1,2]; the conjugation product, isopentenyladenosine 5′-di- or triphosphate, can be further hydroxylated on the isoprenoid side chain by CK specific cytochrome P450 monooxygenases [3]. Both hydroxylated and non-hydroxylated CK riboside phosphates are activated by CK-specific phosphoribohydrolases (LOG; EC 3.2.2.n) to form *trans*-zeatin (*t*Z) or isopentenyladenine (iP; [4]). *t*Z and iP together with their sugar conjugates form a majority of the CK pool found in vegetative tissues of the model plant *Arabidopsis thaliana*.

The other CK production pathway, based on the decay of prenylated tRNA, exists in all eukaryotic and prokaryotic organisms with the exception of Archaea [5]. The role of tRNA prenylation via tRNA:isopentenyl transferase (tRNA:IPT; EC 2.5.1.75) is to strengthen fidelity of the anticodon reading during translation [6]. Contribution of tRNA-released CKs to their cellular or tissue pools is unclear, though. Based on the Arabidopsis double *trna:ipt* knock-out mutants, an origin of all derivatives of *cis*-zeatin (*c*Z), a stereoisomer of *t*Z, is attributed solely to the tRNA decay in Arabidopsis [7]. Nevertheless, there are plant species in which *c*Z derivatives form a majority of detected CKs in contrast to Arabidopsis [8]. Whether *c*Z originates by more robust RNA decay in these species or by an alternative pathway has not yet been elucidated. Recently, a work quantifying CKs in *Physcomitrella trna:ipt1* knock-out mutants showed that a majority of *c*Z metabolites is also of tRNA origin in this ancestral land plant model [9].

In vitro conversion of *t*Z to its *cis*-counterpart and *vice versa* was shown with a partially purified protein from the bean endosperm [10]. However, a gene encoding the hypothetical isomerase has never been found and feeding experiments with radioactively labelled precursors have showed a distinct origin of the isoprenoid side chain in *t*Z and *c*Z in Arabidopsis [11]. Further, there is only a single report of *in vivo* inter-conversion of zeatin stereoisomers. Suttle and Banowetz [12] reported 5–9% of recovered radioactivity associated with *t*Z riboside (*t*ZR; all CK abbreviations are in accordance to Ref. [13]) after treatment of potato tubers with *c*Z, but all the other feeding experiments reported no isomerization [14–16].

A reduced form of zeatin, dihydrozeatin (DHZ), was found as a prevalent CK metabolite in some dormant seeds [17,18] and in the endosperm of maturing seeds [19]. NADPH-dependent enzymatic activity reducing *t*Z to DHZ has been detected in extracts from bean embryos [20] and pea leaves [21]. However, a contribution of the activity to the DHZ pool was not confirmed and a gene coding for the zeatin reductase has not yet been identified.

Developing seeds and reproductive organs are considered as tissues with the highest concentration of CKs. The content of various CK types and metabolic enzymes was determined in the maize caryopsis in several independent studies [22–25]. Dynamic changes in the total content and various CK forms were observed in relation to rapid cell division and cell expansion in certain periods of the caryopsis development [26]. *t*Z and *t*ZR were found to be major CKs, whose levels significantly increased and showed the maximum around the 10th day after pollination (DAP). Other types of CKs either did not show significant changes or were not measured [25]. Interestingly, levels of *t*Z increased again when the embryo was fully developed and the endosperm became starchy. Levels of iP riboside (iPR) start to elevate around the 20th DAP predominantly in the maternal tissue [23]. A significant difference in the

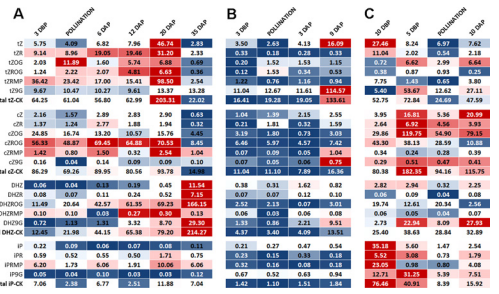

Fig. 1. Cytokinin profiling in maize reproductive organs.
The heat map shows the distribution of cytokinins in maize ovules and kernels (A), silks (B) and tassels (C) before and after pollination. The red and the blue cells correspond to higher and lower concentrations (in pmol per g of fresh weight), respectively; the scale is separate for each metabolite throughout all developmental stages in all three organs together. *t*Z, *trans*-zeatin; *t*ZR, *trans*-zeatin riboside; *c*Z, *cis*-zeatin; *c*ZR, *cis*-zeatin riboside; iP, isopentenyladenine; iPR, isopentenyladenosine; *t*ZOG, *trans*-zeatin *O*-glucoside; *t*ZROG, *trans*-zeatin riboside-*O*-glucoside; *t*Z9G, *trans*-zeatin *N*9-glucoside; *t*ZRMP, *trans*-zeatin riboside 5′-monophosphate; *c*ZOG, *cis*-zeatin *O*-glucoside; *c*ZROG, *cis*-zeatin riboside-*O*-glucoside; *c*Z9G, *cis*-zeatin *N*9-glucoside; *c*ZRMP, *cis*-zeatin riboside 5′-monophosphate; DHZROG, dihydrozeatin riboside-*O*-glucoside; DHZRMP, dihydrozeatin riboside 5′-monophosphate; iP9G, isopentenyladenine *N*9-glucoside; iPRMP, isopentenyladenine riboside 5′ -monophosphate. CKs that are not listed were below the detection limit.

total CK content between the unfertilized cob and kernels 10–16 DAP was also observed in the study of Veach et al. [24], where a dramatic elevation of *O*-glucosylated forms of *c*Z and DHZ ribosides was detected, in addition to *t*Z, *t*ZR and its monophosphate (*t*ZRMP).

An increase in CK content in maize caryopsis is subsequently accompanied by raised activity of CK dehydrogenase (CKX), an enzyme irreversibly cleaving the CK molecule [23,27]. CK free bases are good substrates of CKX enzymes that may regulate their availability for binding to CK receptors. It was shown that local CK maxima during caryopsis development are at least partially supplied *in situ* by *de novo* biosynthesis. An expression of *ZmIPT2* reached its maximum in the maternal tissue around the 10th day of the development when the total CK content cumulates [28]. Interestingly, transcripts of *tRNA:IPT* genes were found more abundant in vascular cells of the maternal-pedicel tissue than in other tissues [25].

Recently, all isozymes from maize *CKX* gene family were functionally characterized with focus on their substrate specificity [18]. The study was accompanied with detailed changes in profiles of all types of CKs during the early development of maize seedlings. It is obvious that the dormant seed serves as a storage pool of CKs where DHZ-types especially are accumulated. Other isoprenoid CKs are likewise present in higher concentrations than later in emerging radicula and coleoptile. In this work, we bring detailed profiles of all CK derivatives in maize reproductive organs before and after the pollination and during kernel maturation. We also focus on possible inter-conversions among different isoprenoid CKs and bring evidence about a new metabolic product of *t*Z with a weak anticytokinin activity.

2. Material and methods

2.1. Plant material

Maize seeds (*Zea mays* ‘Cellux’; Morseva, Czech Republic) were imbibed in tap water and germinated in the dark on wetted filter paper. After 2 days, the germinated seedlings were transferred to aerated hydroponic tanks filled with Hoagland nutrient solution [29] and supplemented with CKs. The plants were grown in a growth chamber with a 16-h light period (250 $\mu E\,m^{-2}\,s^{-1}$) at 27 °C

and an 8-h dark period at 20 °C. Maturing kernels were excised from cobs in the maternal pedicel zone and cultivated in Murashige and Skoog (MS) agar [30] supplemented with radioactively labelled CKs. To cultivate plants through to the reproductive phase, germinated seedlings were transferred to pots with a 2:1 mixture of soil and perlite (Perlit Ltd., Czech Republic) and fertilized every 14 days. Plants were kept in controlled greenhouse conditions over a period from spring to summer when day temperatures ranged from 23 to 35 °C and night temperatures from 16 to 20 °C. Samples of reproductive organs were collected and pooled from three cobs at the same developmental stage. Before pollination and the 1st week after pollination, ovule samples were collected together with the rachis. Later developmental stages were trimmed out of rachis and glumes and 3–5 kernels from each cob were pooled. Male reproductive organs were sampled without peduncle as independent tassel branches.

2.2. Hormonal profiling

The analysis of plant hormones was carried out as in Refs. [31,32]. The frozen plant material was homogenized in liquid nitrogen by mortar and pestle. An aliquot of about 100 mg fresh weight was transferred into microcentrifuge tube. To the plant homogenate 500 μl cold extraction buffer (methanol/water/formic acid, 15/10/5, v/v/v, −20 °C) and a mixture of stable isotope labelled internal standards (10 pmol) were added. The following internal standards: [$^{13}C_6$]indole-3-acetic acid (IAA; Cambridge Isotope Laboratories), [$^2H_4$]salicylic acid (SA), [$^2H_4$]1-aminocyclopropane-1-carboxylic acid (ACC; both Sigma-Aldrich, U.S.A.), [$^2H_5$]jasmonic acid (JA; C-D-N Isotopes Inc.), [$^2H_6$]abscisic acid (ABA), [$^2H_2$]gibberellin A19 (GA19), [$^2H_5$]*tZ*, [$^2H_5$]*tZR*, [$^2H_5$]*tZ*7G, [$^2H_5$]*tZ*9G, [$^2H_5$]*tZ*OG, [$^2H_5$]*tZ*ROG, [$^2H_5$]*tZ*RMP, [$^2H_3$]DHZ, [$^2H_3$]DHZR, [$^2H_3$]DHZ9G, [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]iP7G, [$^2H_6$]iP9G, [$^2H_6$]iPRMP (all OlchemIm, Czech Republic). After incubation for 30 min at −20 °C, the extract was centrifuged at 17 000*g* and supernatant collected. The second extraction of the residue followed. The two supernatants were pooled and evaporated in a vacuum concentrator. Sample residue was dissolved in 0.1 M formic acid and applied to mixed mode reversed phase–cation exchange solid-phase extraction (SPE) column (Oasis-MCX, Waters, U.S.A.). Two hormone fractions were sequentially eluted: (1) fraction A eluted with methanol–containing hormones of acidic and neutral character (auxins, ABA, SA, JA), and (2) fraction B eluted with 0.35 M NH_4OH in 70% methanol–containing the hormones of basic character (CKs) and ACC. The fractions were evaporated to dryness in a vacuum concentrator and dissolved in 30 μl 10% methanol. An aliquot (10 μl) from each fraction was separately analysed on HPLC (Ultimate 3000, Dionex, U.S.A.) coupled to hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems, U.S.A.) set in the selected reaction monitoring mode. The mass spectrometer was run at electrospray ionization mode, negative for fraction A, and positive for fraction B. Ion source parameters included: ion source voltage −4000 V (negative mode) or +4500 V (positive mode), nebulizer gas 50 psi, heater gas 60 psi, curtain gas 20 psi, heater gas temperature 500 °C. Quantification of hormones was performed using isotope dilution method with multilevel calibration curves. Data processing was carried out with Analyst 1.5 software (Applied Biosystems). Each sample was run in two technical replicates.

2.3. Extraction and activity assay of enzyme converting trans-*zeatin*

Plant tissue was mixed with extraction 0.1 M potassium phosphate buffer, pH 7.3, supplemented with 0.5 mM EDTA, 5 mM dithiothreitol, 20% glycerol (v/v), 0.3% Triton X-100 (v/v) and protease inhibitor cocktail from Sigma-Aldrich in 1/1.5 ratio and homogenized using Ultra Turrax homogenizer. Cleared supernatant (20 min, 9600 × g) was used for an activity assay or further purified. Solid ammonium sulfate was added to the supernatant to 20% saturation. After complete dissolution of the sulfate, the extract was centrifuged (30 min, 9600*g*) and loaded onto Octyl-Sepharose 4 Fast Flow column (GE Healthcare Life Sciences, U.K.) equilibrated with the extraction buffer with 20% ammonium sulfate. Unbound proteins were washed from the column by the same buffer and the enzyme activity was eluted by 10% ammonium sulfate in the extraction buffer. The eluted proteins were desalted and concentrated using Amicon Ultra-15 Centrifugal Filter Units with 10 kDa NMWL (Merck Millipore, U.S.A.).

The enzyme assay was set up in 0.1 M potassium phosphate buffer, pH 8.0, with 160 μM *tZ*, 0.25 mM NADPH or $NADP^+$ and 200 μM dithiothreitol and incubated for 1–10 h at 28 °C. The enzymatic reaction was stopped by the addition of double volume of 96% ethanol, diluted with 3-fold excess of 15 mM ammonium formate, pH 4.0, cleared through 0.22 μm nylon filter and injected onto a reverse-phase column (ZORBAX Eclipse Plus C18, RRHD, 1.8 μm, 2.1 × 50 mm, Agilent Technologies, U.S.A.) coupled to Ultra performance liquid chromatography Nexera (Shimadzu, Japan). The mobile phase A was 15 mM ammonium formate, pH 4.0, and the mobile phase B was 100% methanol, flow rate was 0.4 ml min^{-1} and column temperature 40 °C. The column was eluted with gradient as follows: 5% B for 2 min, 5–70% B for 14 min. Data processing was carried out with LabSolutions software. The activity was calculated from peak area of *tZ* oxidized product 6-(3-methylpyrrol-1-yl)purine (MPP) with absorption maximum at 300 nm.

2.4. Purification of unknown metabolite

To reveal the origin of the oxidized product from *tZ*, an enzymatic reaction with a partially purified enzyme was set up following the description above but with addition of 100 pmol of [^{3}H]*tZ* (592 GBq $mmol^{-1}$). The reaction was stopped by double volume of ethanol and separated on an ODS column (Luna C18 3 μm, 4.6 × 150 mm, Phenomenex, U.S.A.) coupled to an HPLC Series 2000 autosampler and quaternary pump (PerkinElmer, U.S.A.). The mobile phase A was 40 mM acetic acid adjusted with ammonium to pH 4.0 and the mobile phase B was acetonitrile/methanol, 1/1 (v/v); the flow rate was 0.6 ml min^{-1} and the column temperature 25 °C. The column was eluted with gradient as follows: 10–15% B for 2 min, 15–20% B for 9 min, 20–34% B for 0.1 min, 34–45% B for 7.9 min, and 45–100% B for 2 min. The compounds were monitored at 270 and 300 nm by a diode array detector (PerkinElmer) and, after online mixing with 3 volumes (1.8 ml min^{-1}) of liquid scintillation cocktail (Flo-Scint III, Packard BioScience Co., U.S.A.), on a Ramona 2000 flow-through radioactivity detector (Raytest, Germany).

Quantitative purification of the unknown metabolite from the reaction mixture was performed on X-Bridge Prep C18 column (10 × 100 mm, 5 μm; Waters) connected to Waters 1525 binary HPLC pump. The mobile phase A was water and the mobile phase B was methanol; the flow rate was 2.7 ml min^{-1} and the column temperature was 30 °C. The column was eluted with a gradient as follows: 5% B for 5 min, 5–20% B for 15 min, 20–80% B for 27 min, 80–100% B for 3 min and 100% B for 3 min. The compounds were monitored at 268 and 300 nm using Waters 2487 dual wavelength absorbance detector (Waters). The fraction containing the metabolite was evaporated to dryness.

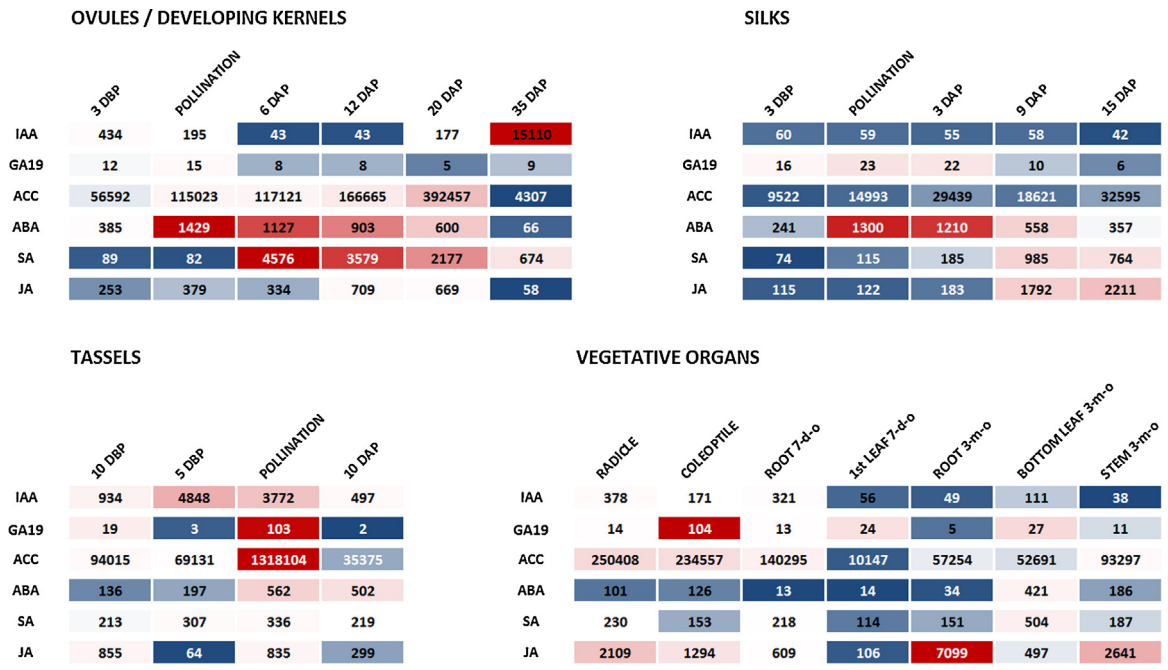

Fig. 2. Phytohormonal profiling in the maize reproductive and vegetative organs.
The heat map shows the phytohormone distribution, where the red and the blue cells correspond to higher and lower concentrations (in pmol per g of fresh weight), respectively; the scale is separate for each metabolite throughout all developmental stages in all organs together. IAA—indole-3-acetic acid; GA19—gibberellin A19; ACC—1-aminocyclopropane-1-carboxylic acid; ABA—abscisic acid; SA—salicylic acid; JA—jasmonic acid.

2.5. Synthesis of 6-(3-methylpyrrol-1-yl)purine

This compound was prepared by oxidation of *tZ* with manganese dioxide according to Ref. [33]. Briefly, *tZ* (500 mg), activated MnO_2 (5 g) and dry dimethylformamide (50 ml) were vigorously stirred in the glass Ace pressure tube at 90 °C for 90 min. TLC (silica gel; chloroform/methanol/25% aqueous ammonia, 4/1/0.05) revealed two minor components, adenine and trace of zeatin, and a major spot of a product with higher R_f. The reaction mixture was then cooled down to 25 °C, MnO_2 was filtered off and the filtrate was evaporated on a vacuum rotary evaporator at 45 °C. The residue after evaporation (~750 mg) was treated with water (25 ml) to obtain 130 mg of crude product which was purified on silica gel (60 g) using chloroform/methanol/25% ammonia, 4/1/0.05, as a mobile phase. Fractions containing pure compound were collected, evaporated and crystallized from methanol (10 ml) to yield 50 mg product of 98% purity as tested by HPLC/MS. The product was thus further purified by semi-preparative HPLC as described above.

2.6. Quantification of 6-(3-methylpyrrol-1-yl)purine and its derivatives

Fresh maize tissue samples of 150–1000 mg were ground to fine consistency and extracted with 1 ml of ice-cold methanol/water/formic acid (15/4/1, v/v/v) overnight at 4 °C. [$^{15}N_4$]MPP (3 pmol) was added to each sample as standard. The homogenates were centrifuged at 19 000*g* for 10 min, corresponding supernatants were further purified using strong cation exchange SPE cartridges (100 mg/ml Bond Elut SCX, Agilent Technologies) followed by anion-exchange chromatography (DEAE Sephadex, GE Healthcare Life Sciences) in combination with reversed-phase SPE (100 mg/ml, Bond Elut C18, Agilent Technologies). Afterwards, the samples were analyzed by reversed-phase ultra-high performance chromatography-tandem mass spectrometry (UHPLC-(+)ESI-MS/MS; Micromass, Manchester, U.K.) using CSH column (Acquity UPLC® CSH C18, 2.1 × 50 mm, 1.7 μm; Waters) and gradient consisting of methanol (eluent A) and 15 mM ammonium formate, pH 4.0 (eluent B). MPP and its derivatives were detected using multiple-reaction monitoring mode of the transition of the ion $[M+H]^+$ to the appropriate product ion. Masslynx 4.1 software (Waters) was used to analyze the data. The standard isotope dilution method [34] was used to quantify the MPP levels.

2.7. Cytokinin receptor competition assay

The binding assay was performed according to the previously described method [34]. *Escherichia coli* cultures strain KMI001 (DrcsC, cps:lacZ) bearing *pPIN-III:ZmHK1* or *pPIN-III:ZmHK3* vectors [35] were grown in liquid M9 medium supplemented with 50 μg ml^{-1} ampicillin and 0.1% (w/v) casamino acids at 25 °C overnight, with shaking (200 rpm), to OD600 ~1.0. The portions of the cell suspension (1 ml each) were mixed with 3 pmol of [^{3}H]*tZ* (592 GBq $mmol^{-1}$), with or without tested competitors at 10 μM concentrations, by turning the tubes upside down. After 30–60 min incubation at 4 °C, the suspension was centrifuged (8000*g*, 5 min, 4 °C), the supernatant was carefully removed, and the bacterial pellet was resuspended in 1 ml of scintillation cocktail (Beckman, U.S.A.) in an ultrasonic bath. Radioactivity was measured by a Hidex 300 SL scintillation counter (Hidex, Finland). Non-specific binding was determined using a large excess of unlabeled *tZ* for each compound. To discriminate the specific and non-specific binding of [^{3}H]*tZ* on the bacterial membrane, 10 μM *tZ* was used and this residual value was subtracted from all the data.

2.8. Arabidopsis root assay

Arabidopsis thaliana seeds (ecotype Columbia-0) were cultivated on vertical MS agar plates in the presence of different concentrations of the tested compounds or pure solvent (0.05% DMSO) as described in detail in Ref. [35]. After 14 days, the plates were photographed and the length of the primary roots was evaluated using

Scion Image software (Scion Corporation, U.S.A.). The number of fully emerged lateral roots was counted under a stereomicroscope. In total, fifty plants on 5 independent plates were analyzed for each treatment.

2.9. Cytokinin dehydrogenase activity assay

Pure recombinant maize CKX enzymes [18] were used to test an inhibitory effect of MPP on their activities. A continuous method based on bleaching of 2,6-dichlorophenolindophenol (DCPIP) was used [36]. The reaction mixture contained 0.1 mM DCPIP and varying concentrations of iP and inhibitor (5 to 50 μM) in 200 mM Tris/HCl buffer, pH 7.0. The kinetic parameters were calculated using the software GraFit Version 4.0.12 (Erithacus Software, U.K.).

2.10. Crystallization and structure determination

ZmCKX4a ORF, cloned into a pTYB12 vector, was expressed in *E. coli* BL21 STAR (DE3) cells. The enzyme was purified by affinity chromatography and ion-exchange chromatography on HighQ column as published previously [18] and concentrated to 35 mg ml^{-1} by ultrafiltration using an Amicon Ultra-15 Centrifugal Filter Units with 30 kDa NMWL (Merck Millipore). ZmCKX4a in 20 mM Tris/HCl, pH 8.0, and 5% glycerol was crystallized over reservoirs containing 100 mM HEPES, pH 7.5, and 50% 2-methyl-2,4-pentanediol. Crystals were infiltrated by 2 mM MPP in DMSO for 1 h and then they were directly flash frozen in liquid nitrogen. Diffraction data were collected at 100 K on the Proxima 1 beamline at the SOLEIL synchrotron (Saint-Aubin, France) at 2.0 Å resolution. Intensities were integrated using the XDS program [37] and data quality was assessed using the correlation coefficient $CC_{1/2}$ [38]. The crystal structures were determined by performing molecular replacement with Phaser [39], using the monomer of ZmCKX4a (PDB ID: 4O95, [40]) as search models. Model refinement was performed with BUSTER-TNT [41]. Electron density maps were evaluated using COOT [42]. Refinement statistics are presented in Supplementary Table 1. Molecular graphics images were generated using PYMOL (www.pymol.org). The atomic coordinates and structure factors have been deposited in the Protein Data Bank (www.rcsb.org[43]) under accession code 5HHZ.

3. Results

3.1. Endogenous content and dynamics of cytokinin accumulation in maize reproductive organs

The CK content in maize plants has been studied extensively, but the majority of these studies have focused ononly a few derivatives and certain developmental stages. In a recent study, we reported detailed profile and dynamics of CK content in maize seedlings during the first 20 days after the germination [18]. Herein, we focus on dynamics and profiles of all known isoprenoid CK metabolites in reproductive organs of maize (Fig. 1). CK profiles were measured also in selected vegetative tissues for comparison (Supplementary Fig. 1). Generally, content of all types of *t*Z derivatives is notably higher in reproductive organs than in young seedlings. The ratio of *t*Z to iP-type CKs is strongly in favor of *t*Z in ovules, silks and developing kernels. iP-type CKs are considerably more abundant in tassels where the amount of *t*Z and iP is almost equal. Ovules as well as tassels before pollination seem to be places of vigorous CK biosynthesis as primary biosynthetic products *t*ZR and iPR phosphates are the most abundant CK derivatives there. In tassels, phosphates are accompanied also by high levels of *t*Z and iP free bases, which are the only active CKs mediating sensing on CK receptors [44]. In developing kernels, the highest accumulation of free *t*Z and *t*ZR was detected between the 6th and the 20th DAP. At older developmental stages, *t*Z accumulates mainly in a form of *N9*-glucoside, especially in silks and older roots. *t*Z *O*-glucosides accumulate predominantly in older leaves and maturing kernels, thus in tissues where high *t*Z content was detected in general (Fig. 1, Supplementary Fig. 1). In contrast to *t*Z, *c*Z occurs predominantly as the *O*-glucoside and *c*Z9G is present only in negligible amounts in all analyzed tissues. The dynamics of *c*Z levels in the course of development appears to be more constant than that of *t*Z and iP with only two exceptions. The first is approximately 2-fold increase observed in tassels 5 days before pollination (DBP) and the second is more than 5-fold decrease in maturing kernels between 20 and 35 DAP. On the other hand, levels of DHZ metabolites tend to grow in the course of kernel and silk maturation and clearly follow the *t*Z maxima. Contrary to the detected dramatic decrease of *t*Z and *c*Z content, DHZ derivatives accumulate in the desiccating kernel 35 DAP (Fig. 1A).

3.2. Content of other phytohormones in reproductive organs

Other low-molecular-mass compounds, classified as phytohormones, were quantified in the same samples of reproductive organs as CKs and compared with their levels in vegetative organs (Fig. 2). Content of major auxin, IAA, is relatively low and stable in silks. There is a decrease of IAA concentration in the kernel (to levels comparable with those in silks) shortly after pollination. Significantly higher accumulation was observed in tassels in the same period of flowering. Interestingly, IAA level dramatically increased in mature kernels, being the highest amount of detected IAA in all the organs tested. Concentration of gibberellin precursor GA19 in reproductive organs is very low in contrast to elongated coleoptile. Tassels during pollen shedding are the only exception. Similarly, ethylene precursor ACC is unambiguously over-accumulated in tassels during pollination. In developing kernels and silks, the ACC level continuously increases from ovules to milky endosperm stage and then decreases during desiccation (35 DAP). Concentration of ABA reaches maxima in all the organs during pollination and then drops down. Nonetheless, levels in tassels are approximately 3-fold lower than in female organs. The highest concentration of SA was measured in silks and developing kernels 6–20 DAP, in contrast to its low concentration in ovules before and during pollination. SA concentration is rather low in tassels. JA levels are constant with exception of an enhancement in desiccated silks and decline in desiccated kernels 35 DAP and in tassels 5 DBP. Levels of several precursors and deactivation products of above mentioned phytohormones correlate with amounts of their active forms (data not shown).

3.3. Conversions of exogenously applied cytokinins in maize

Free bases of zeatin-type CKs in 1 and 5 μM concentrations were added to the nutrition solution in which 7-d-old maize seedlings were cultivated. After 3 days, roots and upper parts were collected and CK quantification was performed. In general, *c*Z and DHZ accumulated in one order of magnitude higher amounts than *t*Z (Fig. 3A). The experiment further revealed that applied CK is mainly converted to inactive *N9*-glucoside in the case of *t*Z or to *O*-glucosides in the case of *c*Z and DHZ (Fig. 3B). Besides, additional changes were observed. The levels of CK types, other than those added to the medium, also changed in response to uptake. The most profound effect in the root was found for the uptake of *c*Z, which caused increase of pools of both *t*Z and DHZ. Interestingly, in the case of *t*Z metabolite levels, the rise was caused rather by substantial surge of biosynthetic and active forms (*t*Z, *t*ZR and *t*ZRMP; Fig. 3B), while the rise of inactivation product – *t*Z *N9*-glucoside (*t*Z9G) was less intense (e.g. almost 10- and 100-fold rise of *t*Z after 1 and 5 uM *c*Z feeding, respectively, and only 1.5- and 4-fold increase of *t*Z9G).

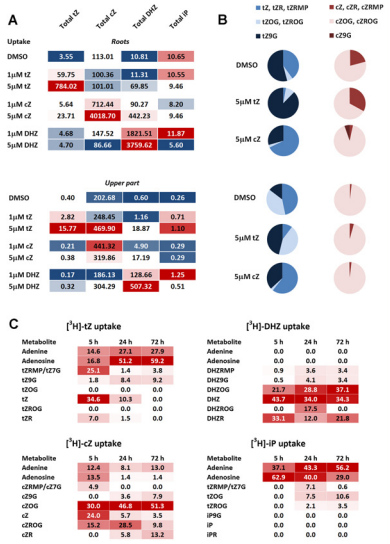

Fig. 3. Distribution of cytokinins in maize seedlings upon uptake of different exogenously applied cytokinins.
Roots and upper part of 10-d-old maize seedlings cultivated in Hoagland nutrient solution were separately analyzed 3 days after the solution was supplemented with various concentration of zeatin-type CKs. (A) Distribution of cytokinin types is expressed in pmol per gram of fresh tissue. Red and blue colours in the heat map correspond to higher and lower concentrations, respectively. The value in each block represents the summed concentrations of all determined metabolites of each group: *tZ*—*trans*-zeatin, *cZ*—*cis*-zeatin, DHZ—dihydrozeatin, and iP—isopentenyladenine. (B) Ratio among different CK forms in non-treated maize and maize treated with 5 μM *tZ* and *cZ*. Pie charts represent portions of free bases and ribosides; *O*-glucosides and ribosides-*O*-glucosides; and *N9*-glucosides for *tZ* and *cZ* determined separately in roots and upper parts. (C) Distribution of radioactivity after uptake of tritium labelled CKs to maturing (~20 DAP) kernels. The values are percentage of total recovered radioactivity from each sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Uptake of *tZ* caused, apart from an increase of *tZ* metabolites, a 6-fold increase of DHZ metabolites when the higher concentration of *tZ* was applied. The pool of iP-type CKs stayed unchanged in all treatments.

In the shoot, where both long-distance transport and local metabolism play role, the trends were less profound. However, in the case of *tZ* treatment, levels of both *cZ* and DHZ rose. DHZ levels increased also in the case of *cZ* uptake (Fig. 3A). Application of all three CKs induced activity of CKX enzymes (data not shown).

In an additional experiment, radioactively labelled 1 nM *tZ*, *cZ*, DHZ and iP (~150 kBq per ml of agar) were added to the MS agar medium with excised maturing kernels sampled approximately 20 DAP. The uptake was followed in the range of 5 h to 3 days, then kernel samples were collected, CK fraction extracted and separated by HPLC with a radioactivity detector. Radioactively labelled CKs applied at nanomolar concentrations were not supplemented with non-labelled compounds in order to follow their physiological metabolic fate not disturbed by induced non-physiological response, *e.g.* activation of CKX enzymes. When [^{3}H]*tZ* and [^{3}H]iP were taken up to maturing kernels, the majority of radioactivity was found in degraded products (adenine, adenosine) within 5 h after the application (Fig. 3C). In contrast, [^{3}H]DHZ was converted only to its *O*-glucoside and to a lesser extent to *N9*-glucoside, while no degradation products were detected. Similarly, [^{3}H]*cZ* was predominantly converted to its *O*-glucoside and only 10–20% of radioactivity was detected in degradation products (Fig. 3C). Detectable inter-conversion of [^{3}H]*tZ* to [^{3}H]*cZ* and *vice versa* was not observed in any tested tissue and nor was detected any reduction of the side chain to produce DHZ. Part of the radioactivity detected in kernels fed with [^{3}H]iP was found in peaks corresponding to retention times of *tZ* metabolites, which suggests the presence of a CK-specific P450 monooxygenase.

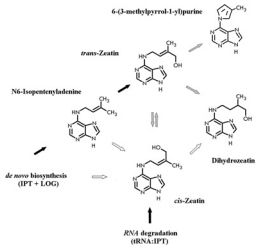

Fig. 4. Zeatin inter-conversions in plants.
Black arrows indicate enzymatic steps that were confirmed *in planta* and responsible gene(s) are known; grey arrows indicate enzymatic conversions shown only in *in vitro* assays; white arrows indicate hypothetical reactions (conversions) without any proof of evidence.

3.4. *Novel enzymatic activity converting* trans*-zeatin in vitro*

To detect a tissue with the predicted zeatin reductase activity [20], extracts made from different maize tissues were tested in *in vitro* reactions with 10 μM *tZ* and 0.25 mM NADPH as described in Ref. [21]. Dithiothreitol in 0.2 mM concentration was added to inhibit degradation by CKX. Reduction of *tZ* to DHZ was not catalyzed by protein extracts either from vegetative or reproductive organs. However, when the reactions were analyzed by HPLC, a novel unknown peak was observed. In comparison to all known isoprenoid CKs, the compound had a greater retention time on a reverse-phase HPLC column (i.e. was more hydrophobic) and the absorption maximum shifted from 268 to 300 nm. The rate of production of the unknown metabolite was dependent on amount of crude extract and presence of NADPH. When NADPH was substituted with $NADP^+$, consumption of *tZ* and formation of the unknown metabolite was even faster. *tZR* and *tZ9G* were also efficiently converted to products with absorption maxima at 300 nm. On the other hand, the other two zeatin forms (*cZ*, DHZ) did not produce similar product with the same absorption maximum and/or similar retention time. To confirm the *tZ* origin of the unknown metabolite, [^{3}H]*tZ* was added to the reaction mixture containing $NADP^+$ and analyzed by HPLC coupled to a radioactivity detector (Supplementary Fig. 2). The enzyme activity converting *tZ* to the unknown metabolite was quantified in different organs. The greatest activity was detected in male reproductive organs and silks after pollination (Table 1), where it was 10–100-fold higher than in vegetative organs. The enzyme was partially purified from 100 g of tassels (5 DAP) using ammonium sulfate precipitation and hydrophobic chromatography on Octyl-Sepharose, where it was retained and eluted with decreasing ionic strength of ammonium salt. However, the enzyme was too unstable to proceed with further purification steps and decomposed within a couple of days at 4 °C.

3.5. *Identification and properties of the unknown metabolite*

Approximately 5 mg of *tZ* was enzymatically converted with the partially purified enzyme and the product was purified by semi-preparative HPLC. Fractions containing unknown product with an absorption maximum at 300 nm were pooled and evaporated to dryness. The unknown compound was analyzed by nuclear magnetic resonance (NMR) on a Bruker Advance III 600 MHz instrument. Its structure was identified as 6-(3-methylpyrrol-1-yl)purine (MPP), a cyclic product of *tZ* oxidation (Fig. 4). To verify the structure, MPP was synthesized from *tZ* by MnO_2 catalysis and further purified by semi-preparative HPLC as referred above. NMR data were consistent with those published in Ref. [45].

Table 1
The enzymatic activity converting *trans*-zeatin to 6-(3-methylpyrrol-1-yl)purine in different maize organs.

Organ	Stage	Specific activity (pkat mg^{-1})	Organ	Stage	Specific activity (pkat mg^{-1})
Ovules	3DBP	1.28 ± 0.32	Tassels	10 DBP	3.69 ± 1.37
Ovules	Pollination	0.21 ± 0.11		5 DBP	6.98 ± 2.90
Kernels	6 DAP	0.39 ± 0.17		Pollination	4.57 ± 2.16
	12 DAP	0.93 ± 0.34		10 DAP	2.32 ± 1.09
	20 DAP	0.12 ± 0.04	Silks	3DBP	0.45 ± 0.17
	35 DAP	0.09 ± 0.02		Pollination	0.12 ± 0.05
Roots	7-d-old	0.22 ± 0.09		3 DAP	7.16 ± 3.20
Upper part	7-d-old	0.06 ± 0.02		9DAP	10.45 ± 4.25

Specific activity was calculated from concentration of formed 6-(3-methylpyrrol-1-yl)purine. The same material was used for phytohormone quantification in two replicates.

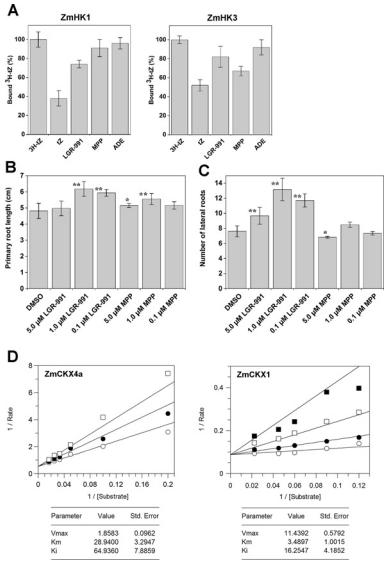

Fig. 5. Properties of 6-(3-methylpyrrol-1-yl)purine as an anticytokinin and inhibitor of cytokinin dehydrogenases.
(A) Effect of 6-(3-methylpyrrol-1-yl)purine (MPP) and anticytokinin LGR-991 on specific binding of 2 nM [^{3}H]*trans*-zeatin in a live-cell binding assay employing *E. coli* expressing cytokinin receptors ZmHK1 and ZmHK3. Both compounds were used in 20 μM concentration. Adenine (ADE) was used as a negative control. The radioactivity in samples without competing substances was 4844 dpm and 4289 dpm for ZmHK1 and ZmHK3, respectively. (B) Primary root length and (C) number of lateral roots of Arabidopsis seedlings grown on media supplemented with MPP and LGR-991 was determined in 14-d-old seedlings; each value is an average ± SD of measurements in at least 80 plantlets grown on 10 independent vertical dishes; ** indicates very significant ($P < 0.01$) or * significant ($P < 0.05$) differences between DMSO-treated and MPP/LGR-911-treated plants (t-test). (D) Lineweaver-Burk plots of ZmCKX1 and ZmCKX5 activity in the absence or presence of MPP. Degradation rates were determined by continuously measuring the bleaching of DCPIP for different concentrations of isopentenyladenine and MPP. Each combination was repeated at least three times.

A series of assays was performed to verify whether MPP was able to bind to and activate a CK receptor [34,35]. It was found that MPP was not able to activate CK signal transduction via either of two tested maize CK histidine kinase receptors ZmHK1 and ZmHK3. Nevertheless, MPP was able to compete with tZ in binding at least to ZmHK3 (Fig. 5A). The supplementation of MS media with MPP did not have any distinct inhibitory effect on elongation of the Arabidopsis primary root and lateral root branching. On the contrary, MPP slightly increased the length of primary root although not as much as the strong anticytokinin compound LGR-991 (Fig. 5B; [46]). In conclusion, both types of experiments confirmed weak anticytokinin-like activity of MPP.

Furthermore, MPP was tested as a substrate and later on as an inhibitor of different maize [18]. The 3-methylpyrrol side chain of MPP was not able to be cleaved by any of eight tested CKXs (ZmCKX1 to 5, ZmCKX8, ZmCKX10 and ZmCKX12). On the other hand, MPP inhibited at least two CKX enzymes when iP was used as a substrate and DCPIP as an electron acceptor. For ZmCKX1, the apparent K_m value for iP and the inhibitory constant K_i for MPP are 3.5 μM and 16.3 μM, respectively, and for ZmCKX4a 28.9 μM and 64.9 μM, respectively (Fig. 5C).

Binding of MPP in the active site of CKX, namely ZmCKX4a, significantly differed from the binding of natural substrate as observed by X-ray crystallography (Fig. 6). The isoprenoid side-chain of iP or *tZ* lied over the isoalloxazine plane of the FAD cofactor and the N10 atom was hydrogen-bonded to catalytic aspartate [47]. The purine ring pointed towards the entrance and its *N9* atom could establish hydrogen bond to glutamate residue or remained unbound depending on CKX isoform. On the contrary, MPP did not bind over the isoalloxazine plane of FAD as deep as the substrates. The nitrogen atom of 3-methylpyrrol side chain of MPP had no free hydrogen for interaction with the catalytic aspartate (D170 in ZmCKX4a). Thus, MPP binded in a flipped orientation and its 3-methylpyrrol moiety pointed towards the entrance, while the *N9* atom of purine ring binded to D170 and the *N3* atom interacted with a water molecule, which was in contact with several residues including D170, E285 or Y283. Taken together, the binding sites of CK substrates and MPP overlapped, and MPP behaved as a weak competitive CKX inhibitor as supported by enzyme kinetics.

Table 2
Quantification of 6-(3-methylpyrrol-1-yl)purine and its metabolites in maize kernels and tassels.

Metabolite	Tassels (5 DBP)	Kernels (12 DAP)
	(fmol g^{-1} of FW)	
tZ	12 984	7 792
MPP	0.2	1.2
tZR	1 136	10 436
MPPR	32	56
tZ9G	86 100	6 868
MPP *N9*-glucoside	1 064	1 892
tZRMP	1 508	11 572
MPPR 5′-monophosphate	74	416

Compounds were quantified by UHPLC-(+)ESI-MS/MS using standard isotope dilution method. MPP, 6-(3-methylpyrrol-1-yl)purine; MPPR, 6-(3-methylpyrrol-1-yl)purine N9-riboside; tZ, *trans*-zeatin; tZR, tZ riboside; tZ9G, tZ N9-glucoside; tZRMP, tZR 5′monophosphate.

3.6. Quantification of 6-(3-methylpyrrol-1-yl)purine in plant tissues

MPP with incorporated stable ^{15}N isotope(s) was synthesized (Dr. Libor Havlíček, Czech Academy of Science, Prague) and used for quantification in different reproductive and vegetative maize tissues by UHPLC-(+)ESI-MS/MS analysis. Nevertheless, its quantity was almost at the limit of detection and did not exceed a concentration of 100 fg per gram of fresh weight (Table 2), which is about four orders of magnitude less than the amount of its precursor *tZ*. Samples prepared from 1 g of kernels 12 DAP and tassels 5 DBP were screened for possible MPP metabolites. Fragmentation ions of putative MPP *N9*-glucoside and MPP 9-riboside (MPPR) were obtained

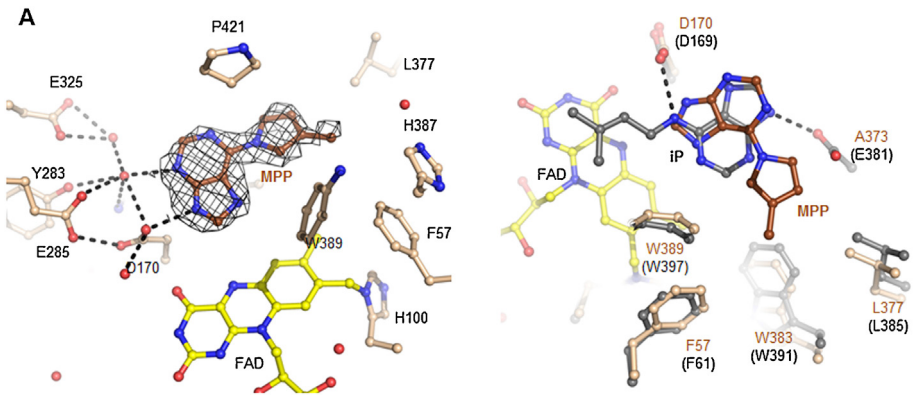

Fig. 6. Binding of 6-(3-methylpyrrol-1-yl)purine in the active site of ZmCKX4a.
(A) 6-(3-methylpyrrol-1-yl)purine (MPP; in Fo-Fc omit map contoured at 3.0 σ), which is colored in brown, is hydrogen bonded via its *N3* and *N9* atoms of the purine ring to the oxygen atom of D170 and to a water molecule, which is in contact with several residues including D170, E285 or Y283. FAD cofactor is yellow coloured and neighbouring residues are labelled. (B) Superposition of bound MPP and natural substrate isopentenyladenine (iP; PDB: 1W1Q; [47]). Oxygen atom of D170 establishes a hydrogen bond with N10 of iP. Isoprenoid side-chain lies over isoalloxazine plane of FAD while the purine ring points towards the entrance. Neighboring residues in ZmCKX4a are labelled in brown and those in ZmCKX1 are shown in brackets. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

from standards prepared by enzymatic conversion of *t*Z9G and *t*ZR and purified by semi-preparative HPLC. Although the quantity of MPP *N9*-glucoside, MPPR and MPPR 5′-monophosphate was much higher than that of unconjugated MPP, it did not reach the amount of the respective *t*Z counterparts (Table 2).

4. Discussion

The precise enzymatic regulation of the CK content is crucial for proper development of cereal kernels, their mass and quantity. The greater number of grains per panicle in Habataki variety of rice is caused by a non-functional allele of *CKX* gene, which is expressed during the establishment of floral primordia [48]. Similarly, silencing of the *HvCKX1*, the closest orthologue of *ZmCKX1*, led to stronger filling of barley grains [49], most probably due to a regulatory role of CKs in sugar metabolism and translocation [50]. On the contrary, the overexpression of *ZmCKX1* gene under the control of pollen- or anther-specific promoters in maize gave rise to male-sterile plants [51]. The male floret development was preliminarily terminated as a result of CK depletion at the apex of the plants. Accordingly, our measurements show that tassels before pollination accumulate both major CKs—iP and *t*Z. In contrast, in the female organs the levels of iP and *t*Z are relatively low and constant before and during pollination and start to increase 12–20 DAP. Greater sensitivity of the male than the female inflorescences to CK imbalance can be demonstrated on maize plants overexpressing *t*Z-specific glucosyltransferase [52]. Ubiquitous expression led to abnormalities resembling CK-deficient plants and *inter alia* feminized tassel florets caused by transformation of active *t*Z free base to non-active *O*-glucoside. On the other hand, there were no defects observed in the female inflorescences except reduced rates of kernel filling. The importance of precise regulation of the CK maxima can be further demonstrated on transgenic maize overexpressing *IPT* during gametophyte formation and development. Enhanced level of CKs in the female gametophyte led to emergence of two functional florets per spikelet and to later formation of double embryo kernels while the male gametophyte was not affected [53].

Changes in the ratio between two major phytohormones, CKs and auxins, were proposed to be an important regulatory switch in developmental processing taking place in maturing kernels. For instance, IAA concentration abruptly increased and the ratio of CK to auxin declined at the time when cell division was decreasing and nuclear DNA endoreduplication was increasing in the endosperm around 20 DAP [54]. Accordingly, high CK to low auxin content was detected in kernels up to 20 DAP where maximal cell division occurs. Both *t*Z and iP levels accumulated around 20 DAP and then sharply declined, while IAA levels accumulated in large quantities at 35 DAP. Similar turn in IAA to CK levels is observed in ovules several days before pollination. Developing kernel is a place of massive cell division where high pool of active CKs needs to be maintained contrary to ripened tassels, in which levels of active CKs during and after the pollination drop down. Profile of the CK content shown in this study is in accordance with previous study of Brugière et al. [23] who demonstrated maximum of *t*ZR between 9–20 DAP, followed by a sharp increase in iPR concentration at 20 DAP. However, in our study, the 20 DAP increase in iP-type CKs was accompanied also by the accumulation of *t*Z metabolites, which was not the case in the previous study. Discrepancy can be attributed to different maize cultivars used and the fact that the ratio between *t*Z- and iP-type CKs, which is influenced by activity of CK-specific cytochrome P450 monooxygenase, was shown as a variable parameter between low and high yielding barley cultivars [55]. Accumulation of active CKs and IAA was enormous in tassels 10 DBP. Whereas CK levels decreased and were comparable with levels in ovules, IAA content remained high in tassels reaching levels 20-fold above those detected in ovules at the time of pollination and shortly thereafter. The ethylene precursor, ACC accumulated in maturing kernels 20 DAP similarly to cytokinins. That is in agreement with ethylene biosynthetic genes expression during kernel development and ethylene's function in onset of endosperm cell death [56].

The role of ABA in seed (embryo) dormancy is well known. ABA level increased at the time of pollination and remained elevated until 20 DAP to protect the embryo from germination. Interestingly, the ABA concentration declined dramatically from 20 to 35 DAP to a very low level observed in almost desiccated kernels. A similar trend was observed in the previous study of Capelle et al. [57], in which the authors detected, despite a sharp decline of ABA content in the endosperm, high levels remaining in the embryo till the end of the desiccation process. Low GA levels in kernels were observed after the pollination due to antagonistic effect of GAs to embryo dormancy [58]. In contrast, high GA levels were observed in tissues with strong elongation growth such as silks or coleoptiles a couple of days after germination and tassels during pollination (Fig. 2). An essential role of GAs on pollen formation and shedding has been confirmed in various GA-deficient mutants, which are usually male-sterile (e.g. [51]).

The biosynthetic pathway for the two most active CKs, iP and *t*Z, is well known. Conversely, the origin of the other two isoprenoid CKs, *c*Z and DHZ, is far less clear. In Arabidopsis, two tRNA:IPTs were shown as the only source of *c*Z [7]. However, it is not clear whether the much higher *c*Z content detected in other species [8], including maize, originates only from RNA decay or if there is another biogenesis pathway. Zeatin isomerase, which was originally predicted based on experiments by Bassil et al. [10], seems to be an *in vitro* artefact of another enzymeís activity (Hluska et al., manuscript in preparation). Additional alternative for the origin of *c*Z-rich species might be the existence of specific family of cytochrome P450 monooxygenases with hydroxylation preference for the *cis*-position or a direct *de novo* production conditioned by the *in vivo* occurrence of a specific precursor, which has not been confirmed up to now. The uptake experiment revealed that maize roots are effective at converting *t*Z to its *N9*-glucoside, but have very low potency to *O*-glucosylate *t*Z or its riboside (Fig. 3B). On the contrary, *c*Z was present only as *O*-glucosides and only traces of *c*Z *N9*-glucoside were detected in roots after application of high concentration of *c*Z (Fig. 3B).The two CK specific *O*-glucosyltransferases found in maize so far have strict preference only for *c*Z [24,59]. Recent characterization of substrate preferences of all maize CKX enzymes showed that CK *O*-glucosides are resistant to irreversible degradation, but some CKXs are able to degrade *N9*-glucosides of both *t*Z and *c*Z very effectively [18]. Interestingly, two out of five CK specific glucosyltransferases found in the Arabidopsis genome are able to *O*-glucosylate both *t*Z and *c*Z [60]. The typical CK profile of the Arabidopsis plant (e.g. [61]) differs from that of maize, mainly by the already mentioned lower representation of *c*Z derivatives but also by the higher ratio of *t*ZOG to *c*ZOG. Hence, we can hypothesize that species, which contain prominently more *c*Z than others (e.g. maize versus Arabidopsis), have a battery of *O*-glycosylation enzymes with preferred specificity toward *c*Z. Then even though equal amount of *c*Z originates continuously in all species by the same house-keeping process of tRNA isopentenylation and subsequent RNA decay, *c*Z can preferentially accumulate in species like maize (with *c*Z-specific *O*-glucosyltransferases) as it is protected against degradation by CKX enzymes, contrary to *t*Z derivatives. This hypothesis is further supported by the approximately one order of magnitude lower accumulation of total *t*Z derivatives in comparison to *c*Z or DHZ when roots were fed with respective CK at 1 μM concentration (Fig. 3A).

CK profiling revealed that the predicted zeatin reductase [20] should be most active in maturing kernels between 20–35 DAP as a profound shift between *t*Z and DHZ content was observed there (Fig. 1A). However, the feeding experiment with radioactively labelled [^{3}H]*t*Z did not confirm reduction to DHZ in kernels between 20–30 DAP, neither did the *in vitro* enzymatic assay of zeatin reductase give positive results (data not shown). On the other hand, a novel enzymatic activity converting *t*Z to MPP was detected in protein extracts from all the tested tissues when $NADP^+$ was present as a cofactor. Cyclization of the *t*Z side chain to form 3-methylpyrrol resulted in a loss of CK-like activity. The compound was still able to bind to the active site of the CK receptor, ZmHK3, and to block binding of *t*Z with even greater potency than the anticytokinin LGR-991. However, contrary to LGR-911 it did not bind to the ZmHK1 receptor. An ability to act as a weak anticytokinin was also shown in the Arabidopsis root elongation assay. Interestingly, MPPR was isolated from tobacco crown gall cells in 1990 by the group of Professor Laloue [45] and shown to have no CK activity in a plant cell division assay but inhibitory effect on growth of tobacco cell suspensions at a higher concentration. Their observation can be simply explained by the anticytokinin effect of the aglycone released *in vivo*. Quantification of MPP in different maize tissues reported here demonstrates that the metabolite is present in extremely low amounts and probably does not have any physiological function at least in maize. Analysis of mass spectra revealed fragmentation ions, which can be assigned to MPPR, its monophosphate and MPP *N9*-glucoside, indicated these metabolites were present in much greater amounts. However, a precise analysis could not be performed because of the lack of their internal standards. Potential physiological significance of higher accumulation of MPP glycosides is not expected as any function for CK glycosides, isoprenoid or aromatic, has not been shown to date. Furthermore, uptake of radiolabeled *t*Z by maturing kernels did not confirm the ability of this tissue to convert it to the MPP metabolite effectively.

In conclusion, the observed formation of MPP and its glycosides is most probably an unspecific reaction of some $NADP^+$ dependent dehydrogenase, which primarily prefers other substrate(s). Nevertheless, MPP is the first compound showing, on the one hand, anticytokinin activity and on the other hand an ability to inhibit CKX. Hence, it can be used in the future for structural studies of CK binding or metabolizing proteins or applied for *in vivo* tissue culture studies where the CK effect needs to be suppressed.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2016.03.014.

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Purification of Maize Nucleotide Pyrophosphatase/Phosphodiesterase Casts Doubt on the Existence of Zeatin *Cis–Trans* Isomerase in Plants

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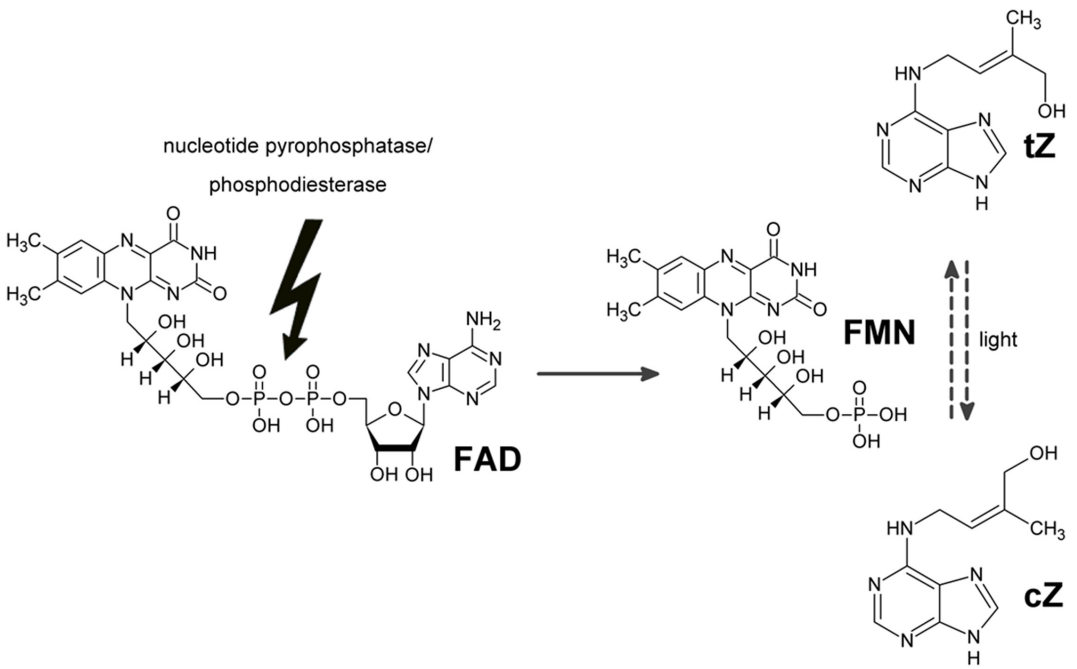
Almost 25 years ago, an enzyme named zeatin *cis–trans* isomerase from common bean has been described by Bassil et al. (1993). The partially purified enzyme required an external addition of FAD and dithiothreitol for the conversion of *cis*-zeatin to its *trans*- isomer that occurred only under light. Although an existence of this important enzyme involved in the metabolism of plant hormones cytokinins was generally accepted by plant biologists, the corresponding protein and encoding gene have not been identified to date. Based on the original paper, we purified and identified an enzyme from maize, which shows the described zeatin *cis–trans* isomerase activity. The enzyme belongs to nucleotide pyrophosphatase/phosphodiesterase family, which is well characterized in mammals, but less known in plants. Further experiments with the recombinant maize enzyme obtained from yeast expression system showed that rather than the catalytic activity of the enzyme itself, a non-enzymatic flavin induced photoisomerization is responsible for the observed zeatin *cis–trans* interconversion *in vitro*. An overexpression of the maize nucleotide pyrophosphatase/phosphodiesterase gene led to decreased FAD and increased FMN and riboflavin contents in transgenic *Arabidopsis* plants. However, neither contents nor the ratio of zeatin isomers was altered suggesting that the enzyme is unlikely to catalyze the interconversion of zeatin isomers *in vivo*. Using enhanced expression of a homologous gene, functional nucleotide pyrophosphatase/phosphodiesterase was also identified in rice.

Keywords: zeatin, isomerization, maize, nucleotide pyrophosphatase/phosphodiesterase, flavins



GRAPHICAL ABSTRACT | Proposed mode of action of "zeatin *cis-trans* isomerase". Instead of catalyzing the isomerization using FAD and light as cofactors, the enzyme hydrolyses FAD and formed FMN is more efficient non-enzymatic catalyst of the isomerization. Thus the apparent increase in zeatin isomerization rate upon enzyme addition was observed.

INTRODUCTION

N^6-(4-hydroxy-3-methyl-but-2-enyl)adenine, known as zeatin due to its discovery in maize (*Zea mays*), belongs to an important class of plant hormones called cytokinins that regulate many physiological processes (Werner and Schmülling, 2009). Zeatin is found throughout the plant kingdom in the form of two geometrical isomers denoted as *cis*- and *trans*-zeatin, respectively. *trans*-Zeatin biosynthesis occurs either directly from adenylate and a hydroxylated side chain precursor *(E)*-4-hydroxy-3-methyl-but-2-enyl diphosphate) by adenylate dimethylallyltransferase (EC 2.5.1.27; Kakimoto, 2001) or by further hydroxylation of another cytokinin isopentenyladenine [(γ,γ-dimethylallylamino)purine] by specific cytochrome P450 (Takei et al., 2004). On the other hand, the only route of the formation of *cis*-zeatin is the hydrolysis of tRNAs that had been previously prenylated on N^6-amino group of certain adenosine phosphate residues by tRNA dimethylallyltransferase (EC 2.5.1.75), which was evidenced by the decrease in *cis*-zeatin levels in *trna:ipt* knock-out mutants of *Arabidopsis* (Miyawaki et al., 2006) and *Physcomitrella* (Lindner et al., 2014).

Since *cis*-zeatin is the predominant cytokinin in many plant species (Gajdošová et al., 2011), there are still alternative pathways under consideration. The expression of tRNA dimethylallyltransferase genes is rather uniform throughout the plant ontogenesis, not responding to stimuli or any changes in cytokinin content. Nevertheless, an involvement of development- and/or stimuli-dependent RNase(s) liberating *cis*-zeatin cannot be ruled out. In addition, there is still a need for specific hydroxylase forming the *cis*-hydroxylated side-chain. Such a *cis*-hydroxylase encoded by *miaE* gene was found in the bacterium *Salmonella typhimurium* (Persson and Björk, 1993), but no homologue has been identified up to now in plants.

Therefore it was generally accepted that *cis*-zeatin could be produced from its *trans*- isomer by zeatin *cis–trans* isomerase, an enzyme partly purified and reported almost 25 years ago (Bassil et al., 1993). Nevertheless, the existence of such an enzyme has been disputed, as it was never identified; e.g., upon feeding plants with a radioactive cytokinin, no isomerization was observed in maize (Yonekura-Sakakibara et al., 2004). The only exception was 5–9% of radioactivity recovered as *trans*-zeatin upon feeding potato tubers with *cis*-zeatin (Suttle and Banowetz, 2000). However, the radioactive cytokinin was applied in the dark, while the reaction catalyzed by the enzyme *in vitro* was described to require light. Furthermore, *Arabidopsis* multiple knock-out plants in adenylate dimethylallyltransferase genes show decreased levels of *trans*-zeatin and isopentenyladenine but not *cis*-zeatin, while tRNA dimethylallyltransferase gene knock-out specifically reduces *cis*-zeatin (Miyawaki et al., 2006). Neither of these results would have been plausible, if significant *cis–trans* isomerization of zeatin had occurred *in planta*.

Here we report on the purification of the protein responsible for the *in vitro* zeatin *cis–trans* isomerase activity described earlier. The protein was purified from maize and identified by mass spectrometry as a nucleotide pyrophosphatase/phosphodiesterase. A recombinant enzyme was produced using yeast *Pichia pastoris* expression system and its substrate specificity determined. To assess a possible role

of nucleotide pyrophosphatase/phosphodiesterase in cytokinin metabolism, experimental plants with an altered expression of the encoding gene were prepared and characterized.

MATERIALS AND METHODS

Purification of the Protein with Zeatin *Cis–Trans* Isomerase Activity

Whole maize cobs with immature seeds (930 g), approximately 10 days after pollination, were collected in the field and homogenized using a food processor with 1 L of 50 mM Tris/HCl, pH 8.0, at 4°C for 1 h. After a centrifugation at 4,800*g*, the pellet was re-extracted with the same volume of fresh buffer and centrifuged again. Ballast proteins were precipitated with additions of protamine sulfate (1 g per 10 g of proteins), manganese(II) chloride (final concentration 7.5 mM) and ammonium sulfate (20% saturation, 114 g L^{-1}) according to published protocol (Šebela et al., 2000). After the addition of each precipitant, the solution was stirred for 10 min and then centrifuged at 4,800 *g* for 10 min. The supernatant was loaded onto a DEAE-Sepharose (GE Life Sciences) 5.0 cm i.d. × 30 cm column equilibrated with 50 mM Tris/HCl, pH 8.0, containing ammonium sulfate at 20% saturation. The flow-through fraction was desalted using a MiniKros® hollow fiber tangential flow filtration system with a 10-kDa polysulfone module (Spectrum Laboratories) and loaded on a High Q (Bio-Rad) 1.5 cm i.d. × 20 cm column equilibrated with 50 mM Tris/HCl, pH 8.0. The bound proteins were eluted with a gradient of KCl from 0 to 1.0 M. Fractions showing the zeatin *cis–trans* isomerase activity were pooled and transferred to 20 mM Tris/HCl buffer, pH 7.4, containing 0.5 M NaCl and 5 mM of each $MgCl_2$, $MnCl_2$, and $CaCl_2$ using Amicon® Ultra centrifugal filters (10,000 NMWL; Merck Millipore). The sample was then loaded on a Concanavalin A-Sepharose (Sigma–Aldrich) 1.0 cm i.d. × 10 cm column equilibrated with the same buffer and eluted with the buffer containing 1 M methyl α-D-mannopyranoside. The sample was then transferred to 50 mM Tris/HCl, pH 8.0 using the above centrifugal filters.

The enzyme was further purified using an FPLC BioLogic DuoFlow 10 system (Bio-Rad). The sample was first loaded onto a Resource Q 6 mL column (GE Life Sciences) equilibrated with 50 mM Tris/HCl, pH 8.0, and eluted with a linear gradient up to 0.5 M KCl. Active fractions were pooled, transferred to 5 mM K-phosphate, pH 7.0, containing 0.1 M NaCl and 0.5 mM $CaCl_2$. The recovered proteins were loaded onto a Bio-Scale CHT5-I column (Bio-Rad) and eluted with 0.75 M K-phosphate buffer, pH 7.0. Active fractions were pooled and transferred to 50 mM Tris/HCl, pH 8.0. Concentrated enzyme solution was loaded onto a HiTrap Blue HP column (GE Life Sciences) and eluted with 2 M KCl.

Finally, the purified enzyme was applied to Novex® isoelectric focusing electrophoresis gels (ThermoFisher Scientific) with pH 3-10 buffers. The gels were run at a constant voltage of 100 V for 1 h, then at 200 V for 1 h and finally at 400 V for an additional hour. The margin lines were excised and silver-stained. Protein bands were excised from the rest of the gel slab in accordance to the staining and crushed by pressing through a syringe several times. Afterward it was extracted overnight with 100 μL of McIlvaine buffer pH 7.5 at 4°C.

Protein Identification by Mass Spectrometry

After SDS-PAGE followed by Coomassie staining, protein bands of interest were excised from the gel slab and processed for in-gel digestion by a modified trypsin (Šebela et al., 2006). Peptides from the digests were desalted using ZipTip C18 pipette tips (Merck Millipore Ltd.) according to manufacturer's instructions and finally reconstituted in 10 μl of 0.1% (v/v) trifluoroacetic acid (TFA). Prior to MALDI mass spectrometry (MS) and tandem mass spectrometry (MS/MS), α-cyano-4-hydroxycinnamic acid (a matrix compound) was dissolved to 0.7 mg ml^{-1} in a solvent mixture containing 85% (v/v) acetonitrile, 15% (v/v) water, 0.1% (v/v) TFA and 1mM $NH_4H_2PO_4$. An aliquot (0.5 μl) of the sample solution corresponding to an initial protein amount of around 200 ng was spotted onto the target (MTP AnchorChipTM 384 BC; Bruker Daltonik), immediately mixed with 0.5 μl of the matrix solution and left to dry at laboratory temperature.

MS and MS/MS analyses were performed on an ultrafleXtreme MALDI-TOF-TOF instrument equipped with a LIFT cell and Smartbeam-II laser operating at a repetition rate up to 2 kHz (Bruker Daltonik). All mass spectra were obtained in the reflectron positive ion mode. The mass spectrometer was controlled by flexControl 3.3 software for acquisition and flexAnalysis 3.3 for spectra processing. The accelerating voltages in the ion source for MS and MS/MS analyses were 25 and 7.5 kV, respectively. In MS/MS mode (no collision gas was used), an accelerating potential of 19 kV was applied to fragments (coming from a timed ion gate) in the LIFT cell. The instrument was calibrated externally using peptide standards supplied by the manufacturer. Manual mass spectra acquisitions were done from 2,000 laser shots in the MS mode and 5,000–10,000 shots in the MS/MS mode. The following settings were applied for MS/MS in an AutoXecute method: primary choice mass range of precursors: 750–3000; number of precursor masses: 15; peak intensity: >800; peak quality factor: >30; signal/noise: >7; FAST minimal fragment mass: 250; LIFT: measure fragments only.

Combined MS/MS datasets were processed by flexAnalysis 3.3, uploaded to ProteinScape 3.0 (Bruker Daltonik) and searched against the NCBI non-redundant database with the Mascot 2.2 search engine (Matrix Science, London, United Kingdom). The search parameters were as follows: Viridiplantae (green plants) were set as a taxonomy; trypsin was set as a protease with 1 missed cleavage allowed; carbamidomethylation of cysteine was set as a fixed modification and methionine oxidation as a variable modification; +1 was set as a peptide charge; monoisotopic masses were considered; other settings: instrument – MALDI-TOF-TOF, significance threshold – $p < 0.05$, peptide mass tolerance – 25 ppm, MS/MS fragment mass tolerance – 0.7 Da.

Design and Synthesis of *ZmNPP* Gene

A maize nucleotide pyrophosphatase/phosphodiesterase (*ZmNPP*) gene was synthesized by GeneArt service of

ThermoFisher Scientific. Minor alterations from the native sequence were implemented as follows: a CAT triplet was placed before the start codon to form an *Nde*I restriction site, the sequence stretch GCCTCC from the position 107 was changed to GCTAGC to form an *Nhe*I restriction site, which allows to clone *ZmNPP* fragment without predicted signal sequence, and the triplet CTT at the position 901 was changed to TTG to remove a *Hind*III restriction site. Neither of these changes led to an amino acid substitution. The TGA stop codon was changed to TAA to form a new *Hind*III restriction site and an adjacent *Xho*I site was added to yield a sequence TAAGCTTCTCGAG. All changes were made in order to clone the gene into plasmids for expression in *E. coli*. Gateway *att*B1 and *att*B2 sequences were added up- and down-stream of the coding sequence, respectively.

Preparation of Recombinant ZmNPP from *Pichia pastoris* Expression System

The *ZmNPP* gene was amplified from synthesized DNA with the following primers: P1_fw (5′-G*GAATTC*ATGGCGTCTCCGCC CCACTC-3′) and P2_rev (5′-GCCG*CTCGAG*TTTTGTTCG GCAACAGAATCGTGCC-3′) with *Eco*RI and *Xho*I restriction sites, respectively, shown underlined in italics. After restrictions with the respective endonucleases, the gene was cloned into a vector pPICZα (ThermoFisher Scientific). The plasmid was then linearized with *Mss*I. Chemically induced transformation to *Pichia pastoris* X-33 was performed in accordance with the manufacturer's protocol.

Transformed yeasts were cultivated in a bioreactor Biostat® Cplus (Sartorius). The vessel was filled with water up to 8.5 L containing 134 g of yeast nitrogen base without amino acids (ThermoFisher Scientific), 400 mL of glycerol and 2 mL of antifoam A (Sigma–Aldrich) and steam sterilized. After cooling down, 1 L of 1 M potassium phosphate, pH 7.0, and 50 mL of filter-sterilized PTM_1 Trace Salts (Pichia Fermentation Process Guidelines, ThermoFisher Scientific) were added and the conditions were set as follows: agitation 400 rpm, air flow 2 vvm, temperature 28°C, and pH 7.0. After 3 h, 0.5 L of an overnight preculture of *Pichia pastoris* harboring pPICZα::ZmNPP was added and agitation was set to keep the concentration of dissolved oxygen above 20% of saturation. After 24 h of fermentation, 50% glycerol containing 5% PTM_1 Trace Salts was fed continuously to the bioreactor at the rate of 2 mL min^{-1}. After another 24 h, an induction of protein expression with methanol started, increasing the rate up to 3 mL min^{-1}, while the feeding with glycerol was decreased linearly. The methanol was fed to the bioreactor for 3 more days.

Yeast cells were then collected by a centrifugation at 4,800*g* for 30 min. The medium was conditioned with $^1/_{40}$ volume of 1 M Tris and 2 M NaCl, run through a DEAE-Sepharose 5.0 cm i.d. × 30 cm column, concentrated and conditioned to 20 mM Tris/HCl, pH 8.0, on a SartoJet pump with Sartocon® Slice Ultrasart polyethersulfone 10-kDa NMWCO cassettes (Sartorius). The enzyme was further purified on the High Q 1.5 cm i.d. × 20 cm and HiTrap Blue HP columns as described above.

Enzyme Activity Assays

The interconversion of zeatin isomers was measured with 0.2 mM *cis*-zeatin in 100 mM McIlvaine buffer containing 20 mM $MgCl_2$, 0.1 mM FAD and 0.8 to 2.0 mM dithiothreitol. Upon mixing, the samples were incubated at 37°C under white fluorescent light (500 μE m^{-2} s^{-1}). The reaction was stopped by the addition of two volumes of methanol after 1 h. For each assay, a control reaction was set-up, where the enzyme sample was boiled for 5 min prior to addition of the other components and the enzymatic activity was calculated from the difference in the concentration of *trans*-zeatin between the two reactions. Zeatin content was analyzed on a Nexera UFLC (Shimadzu) equipped with a Zorbax RRHD Eclipse Plus C18 column, 2.1 mm i.d. × 50 mm, 1.8 μm (Agilent) thermostated at 40°C. Zeatin isomers were eluted with 15 mM formic acid, set up with ammonium hydroxide to pH 4.0 at the flow rate of 0.4 mL min^{-1} with gradient of methanol as follows (min/%): 0.0/22; 3.0/22; 4.0/90; 5.5/90; 6.0/22; 8.0/22 and their content determined from peak areas at 268 nm using standard compounds. A typical chromatogram of the reaction is shown in Supplementary Figure S1A.

The assay of nucleotide pyrophosphatase/phosphodiesterase activity was done in identical manner, but without *cis*-zeatin and the reaction mixture was kept in dark. Moreover, dithiothreitol was omitted in the assays with purified recombinant protein as no longer needed. The samples were analyzed on the same UFLC system and conditions as above, but eluted with 20 mM phosphoric acid (set up to pH 6.5 with ammonium hydroxide) using the following methanol gradient (min/%): 0.0/15; 1.0/15; 4.0/22; 4.5/25; 5.0/95; 7.0/95; 7.5/15; 10.0/15 and monitored at 449 nm. A typical chromatogram of the reaction is shown in Supplementary Figure S1B. In plant samples, extracted flavins were analyzed by the same procedure but quantified on a FP-2020 Plus fluorescence detector (JASCO) with excitation at 265 nm and emission at 530 nm using standard compounds.

To determine the nucleotide pyrophosphatase/phosphodiesterase activity with various substrates, the same reaction was set up with 0.5 mM substrate and with an appropriate amount of the purified recombinant enzyme hydrolyzing maximally 40% of the substrate. The reaction was then stopped by pipetting aliquots at time-points 0, 10, 20, and 30 min into two volumes of methanol. The samples were all centrifuged at 21,000 *g* for 10 min and mixed with 15 mM triethylamine set with phosphoric acid to pH 6.0 in such a ratio to bring methanol to 5% and filtered using 0.22 μm Costar® Spin-X® centrifuge tube filters (Corning Inc.) at 10,000*g* for 3 min. The samples were then analyzed by the UFLC equipped with a Polaris 180Å C18-A, 2.0 mm i.d. × 150 mm, 3 μm (Agilent) thermostated at 35°C using a methanol gradient in 15 mM triethylamine (set up to pH 6.0 with phosphoric acid) at the flow rate of 0.2 mL min^{-1}. The gradient was set up as follows (min/%): 0.0/10; 1.5/10; 4.0/40; 4.5/60; 7.0/60; 7.5/10; 10.0/10. The analytes were quantified using UV-Vis detector using standard compounds.

To determine enzyme specific activity, protein concentration was estimated using a Bio-Rad Protein Assay Dye Reagent

Concentrate (Bio-Rad) with bovine serum albumin as a calibration standard with linearization (Ernst and Zor, 1996).

Expression of the *ZmNPP* Gene in Tomato Hairy Roots

The gene was cloned into pGWB17 vectors (35S promoter, kanamycin and hygromycin resistance) using Gateway protocol (ThermoFisher Scientific) and electroporated into *Agrobacterium rhizogenes* strain 15834, which was used to transform tomato as described previously (Šmehilová et al., 2009). The main root was used for propagation; 2-cm long tips of lateral roots were used for the determination of gene expression and activity level and the rest was used for genotyping. Gene expression was determined as described previously (Šmehilová et al., 2009) with primers ZmNPP_fw (5′-CCCCAACCACTACTCCATCGT-3′) and ZmNPP_rev (5′-TCGTGGTTTTTCATGGTGAAGT-3′).

Expression of the *ZmNPP* gene in *Arabidopsis*

Arabidopsis plants expressing maize nucleotide pyrophosphatase/phosphodiesterase gene cloned into the pGWB17 vector were prepared from *Arabidopsis thaliana* ecotype Col-0 using a floral-dip method (Clough and Bent, 1998). Homozygous plants were selected by PCR detection of the inserted *ZmNPP* gene. A plant was considered homozygous when at least 100 offspring plants carried the insert and at the same time there was no negative offspring plant. The plants were grown for 4 weeks in an environmental chamber (16 h fluorescence light intensity of 150 $\mu E\ m^{-2}\ s^{-1}$/8 h dark, 22°C, 55% relative humidity) in the soil. Third to sixth leaves were collected and pooled from 4 plants into each sample.

Cultivation of Rice Plants with Enhanced Expression of Nucleotide Pyrophosphatase/Phosphodiesterase

Rice (*Oryza sativa japonica*) T-DNA insertional line PFG_2B-60145.L with the corresponding wild type Hwayoung were purchased from POSTECH, South Korea. The line has an insertion in the promoter sequence of the gene LOC_Os01g10020, 300 bp upstream of the start codon, which contains the promoter sequence of *OsTubA1* gene. Rice seeds were germinated in Agroperlit (Perlit); after 2–3 weeks the seedlings were planted into the soil and grown for 2 weeks in an environmental chamber (12 h fluorescence light intensity of 250 $\mu E\ m^{-2}\ s^{-1}$, 28°C/12 h dark, 25°C, 65% relative humidity). Then shoots were collected from each plant separately with the exception of the albino plants that were pooled from two plants into one sample.

Analysis of Transgenic Plants

Enzymatic activity and protein content were assayed as above using samples prepared from 0.1 g of the leaves that were crushed in liquid nitrogen and extracted with 0.2 mL of 50 mM Tris/HCl, pH 8.0. Total flavins were first extracted with methanol/methylene chloride (9:10) from 5 mg of starting material as described previously (Hiltunen et al., 2012) then the sample was extracted again with 10 mM sodium phosphate containing 10% (v/v) acetonitrile. The whole procedure was conducted in a dim light. Finally, 10 μL of a mixture of both extracts (1:1) was mixed with 50 μL of 20 mM ammonium phosphate, pH 6.5, and analyzed using UFLC with fluorescence detector as above on the same type of column, but 150 mm long. Chlorophyll content was determined using published method (Porra et al., 1989). Cytokinins in *Arabidopsis* plants were determined using published method (Svačinová et al., 2012).

RESULTS

Purification of the Protein with Zeatin *Cis–Trans* Isomerase Activity

When we examined extracts from several plant species including maize, *Arabidopsis*, common bean, wheat and rice, the highest activity was found in maize, detectable in all developmental stages and organs (results not shown). Maize immature kernels were therefore chosen as the starting material for enzyme purification. As shown in **Figure 1A**, the rate of conversion of *cis*- to *trans*-zeatin was about 5-fold higher than the opposite reaction, both with a relatively high background after boiling that reached up to 25% of the rate with native extracts.

The whole purification procedure was quite tedious and required a series of six chromatographic steps to obtain 0.7 mg of a final enzyme preparation from almost one kilogram of maize cobs with kernels at the stage of liquid endosperm. The progress of individual purification steps with 10% activity yield resulted in 1600-fold purified enzyme with the specific activity of 65 nkat mg^{-1} as summarized in **Table 1**. However, several protein bands were still detectable on an SDS-PAGE gel (**Figure 1B**). To identify a particular protein showing the isomerase activity, the active fraction from HiTrap Blue was further separated using isoelectric focusing (**Figure 1C**). The gel slab was then sliced, separated proteins extracted and tested for the enzymatic activity. The zeatin *cis–trans* isomerase activity was found in the fraction corresponding to a 50 kDa band on the original SDS-PAGE gel (**Figure 1D**).

Identification of the Purified Enzyme

MALDI-TOF/TOF MS and MS/MS allowed assigning the purified protein band with *in vitro* isomerase activity to the accession number NP_001146857 in the NCBInr database. The peptide mass fingerprinting data allowed assigning 19 peptides with *m/z* values in the range of 805–2816 Da providing a probability-based score of 131 and sequence coverage of 32.3% (Supplementary Figure S2). The MS/MS-based data provided much more convincing score value of 1345 (Supplementary Figure S3). This accession is annotated as a nucleotide pyrophosphatase/phosphodiesterase from *Zea mays* (ZmNPP; LOC100280465; Zm00001d039454_P001). The protein consists of 468 amino acids with a theoretical molecular mass of 51.2 kDa, which well corresponds to the results obtained by SDS-PAGE, and pI 6.36 determined by Compute pI/Mw (Bjellqvist et al., 1993).

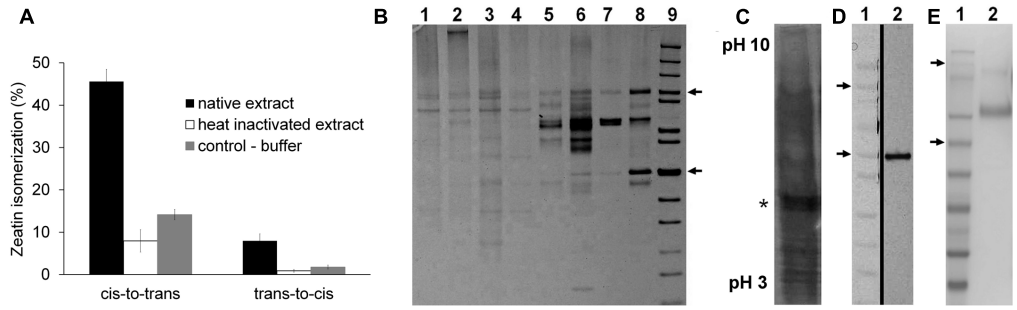


FIGURE 1 | Purification and identification of the protein with zeatin *cis–trans* isomerase activity. **(A)** Typical conversion of zeatin isomers (0.2 mM) by maize kernel extracts after incubated in 100 mM McIlvaine buffer containing 20 mM $MgCl_2$, 0.1 mM FAD and 2.0 mM dithiothreitol, at 37°C under white fluorescent light for 2 h; determined by UFLC; graph depicts average of three technical replicates with standard deviation. **(B)** SDS-PAGE of the individual protein purification steps: 1, crude extract; 2, extract after protein precipitation; 3, fraction from DEAE-Sepharose; 4, High Q; 5, Concanavalin A; 6 Resource Q; 7 Bio-Scale CHT5-I; 8 HiTrap Blue HP; 9, MW marker, 100 and 50 kDa indicated by arrows. **(C)** Isoelectric focusing gel with the sample after HiTrap Blue HP (silver-stained lane); the rest of the gel slab was sliced, proteins extracted and activity detected in the band indicated by the asterisk. **(D)** SDS-PAGE of the isolated active protein: 1, MW marker, 100 and 50 kDa indicated by arrows; 2, purified active protein; the vertical black line denotes combining of two image parts. **(E)** SDS-PAGE of the isolated recombinant ZmNPP protein: 1, MW marker; 2, purified enzyme from the yeast growth medium.

TABLE 1 | Purification of the protein with zeatin *cis–trans*-isomerase activity from maize.

Purification step	Total activity (nkat)	Total proteins (mg)	Specific activity (nkat mg^{-1})	Purification grade (-fold)	Yield (%)
Crude extract	475	11 900	0.04	1.0	100.0
Precipitation	658	12 400	0.05	1.3	138.3
DEAE-Sepharose	724	3 110	0.23	5.8	152.2
High Q	788	1 006	0.78	19.6	165.6
Concanavalin A	162	86.20	1.89	47.1	34.1
Resource Q	145	19.00	7.66	191	30.6
Hydroxyapatite	68.1	5.00	13.6	340	14.3
HiTrap Blue HP	45.2	0.71	64.5	1 610	9.5

Maize cobs 10 day after pollination (930 g) were used as a starting material; the activity was determined using UFLC as a production of trans-zeatin and protein content by linearized bradford method (see Materials and Methods).

Characterization of Plant Nucleotide Pyrophosphatases/Phosphodiesterases

A BLAST analysis against translated plant genomes using ZmNPP sequence as a query returned only a few results per plant. At the time of ZmNPP identification, the database of maize genome assembly contained an additional gene with a high homology in a stretch of 160 amino acids, while the rest of the gene encoded Sir2 domain that possess a NAD-hydrolysis-dependent deacetylase activity. However, at the time of completing this paper (July 2017), the current maize genome assembly (AGPv4) contains three annotated *NPP* genes with similar length and only minor alterations (indels and several amino acid substitutions, Supplementary Figure S4). Because we were not aware of the other two ZmNPPs, we have focused in our work only on the identified enzyme. For the same reason, we refer to the enzyme as ZmNPP, without the number. Nevertheless, the mass spectrometry analysis (Supplementary Figures S2, S3) unambiguously confirmed that a protein present in the purified fraction is ZmNPP1 (Zm00001d039454_P001) and not ZmNPP2 (Zm00001d047948_P001) or ZmNPP3 (Zm00001d044311_P001). A single gene LOC_Os01g10020 was found in rice and four in *Arabidopsis*, whose genes are located on chromosome 4, next to each other (At4g29680, At4g29690, At4g29700, and At4g29710).

Unlike human nucleotide pyrophosphatase/phosphodiesterase genes that contain a large number of introns and undergo an alternative splicing, all plant genes, interestingly with the exception of the additional two *ZmNPPs* (*ZmNPP2* and *ZmNPP3*), contain a single exon. As shown in **Figure 2**, plant NPP proteins cluster separately from animal enzymes in the phylogenetic tree. Mostly, when there is more than one encoding gene in the genome (e.g., in *Arabidopsis* and tomato), the paralogs are located sequentially on the same chromosome. This all suggests that before the evolutional divergence of animals and plants, there was only a single precursor gene. While there were multiple gene duplications in animals before the divergence of clades (Zimmermann et al., 2012), the genes in plants duplicated relatively recently, after the speciation.

The prediction of plant NPPs' subcellular localization by TargetP (Emanuelsson et al., 2007) is mostly ambiguous. Two exceptions are ZmNPP2 and AtNPP3, both with high confidence to be secreted proteins. However, both of them

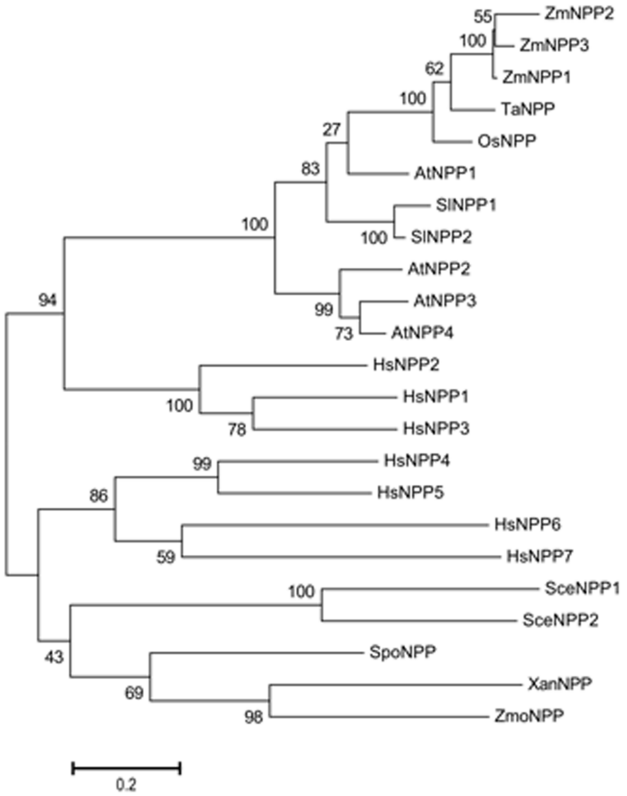


FIGURE 2 | Phylogenetic analysis of nucleotide pyrophosphatases/phosphodiesterases. The following sequences were collected: *Xanthomonas* XanNPP (WP_011051855.1), *Zymomonas mobilis* ZmoNPP (WP_011241358.1), *Schizosaccharomyces pombe* SpoNPP (O94323.1), *Saccharomyces cerevisiae* SceNPP1 (P25353.2), SceNPP2 (P39997.1), *Zea mays* ZmNPP1 (Zm00001d039454_P001), ZmNPP2 (Zm00001d047948_P001), ZmNPP3 (Zm00001d044311_P001), *Oryza sativa* OsNPP (LOC_Os01g10020.1), *Arabidopsis thaliana* AtNPP1 (AT4G29680.1), AtNPP2 (AT4G29690.1), AtNPP3 (AT4G29700.1), AtNPP4 (AT4G29710.1), *Triticum aestivum* TaNPP (ADK32530.1), *Solanum lycopersicum* SlNPP1 (Solyc07g037950.1), SlNPP2 (Solyc07g037960.1), *Homo sapiens* HsNPP1 (P22413.2), HsNPP2 (Q13822.3), HsNPP3 (O14638.2), HsNPP4 (Q9Y6X5.3), HsNPP5 (Q9UJA9.1), HsNPP6 (Q6UWR7.2) and HsNPP7 (Q6UWV6.3). The sequences were aligned using CLUSTAL W method (Thompson et al., 1994). The phylogenetic tree was built by the MEGA 6.0 software by the maximum likelihood method (Tamura et al., 2013). To estimate evolutionary distance, the proportions of amino acid differences were computed using Poisson Correction Distance. The reliability of different phylogenetic clusters was evaluated by the bootstrap test (1000 bootstrap replications).

have also predicted one trans-membrane helix by TMHMM server (Krogh et al., 2001). The other proteins' localization is predicted with lower confidence with reliability class usually 4 or 5.

The plant NPPs in general contain all the amino acids that were shown to be crucial for the catalysis in animals (Kato et al., 2012) with two notable exceptions: Lys 277 from mouse NPP1 does not align to any specific amino acid in the plant NPPs and the Asp 308 that is crucial for substrate binding *via* water molecule is substituted with glutamate (Glu 191 in ZmNPP1) as in *Xanthomonas* enzyme. The plant enzymes are predicted to be glycosylated by NetNGlyc (Gupta and Brunak, 2002) at several asparagine residues. Without ambiguity, all enzymes shall be glycosylated at residues corresponding to Asn 108 and 208 of ZmNPP1 with the exception of AtNPP2 and AtNPP3 that have residue corresponding to Asn 108 substituted for Arg and Gly, respectively. The three *Arabidopsis* proteins that have asparagine at the position corresponding to ZmNPP1's Lys 393 shall be glycosylated there. The last position undergoing glycosylation is the one corresponding to Asn 454 of ZmNPP1. At this position, about half of proteins are weakly predicted to be glycosylated, while the rest not to be glycosylated. Lastly, ZmNPP1, AtNPP1 and AtNPP2 shall be glycosylated at positions 35, 33 and 34, respectively. An alignment of all aforementioned plant NPP enzymes with indicated putative glycosylation sites, conserved amino acids and predicted signal peptides is shown in Supplementary Figure S4.

Substrate Specificity of Recombinant Maize Nucleotide Pyrophosphatase/ Phosphodiesterase

To study the catalytic reaction, heterologous expression was preferred to purification from maize kernel extract for obtaining sufficient amount of ZmNPP. First attempts using *Escherichia coli* did not lead to successful protein production. Alternatively, the expression was successful using a methanol inducible expression in the methylotrophic yeast *Pichia pastoris* with the expression vector pPICZα that fuses a signal peptide to the N-terminus of the recombinant protein thus driving its secretion into the growth medium. The recombinant protein was then concentrated and purified to homogeneity (**Figure 1E**) using three relatively simple chromatographic steps as described in Section "Materials and Methods." The size of the recombinant protein was around 60 kDa due to the presence of His-tag and a linker (3.5 kDa) and probably due to a heavier glycosylation pattern in yeast.

The purified recombinant protein showed the zeatin *cis–trans* isomerase reaction in the presence of FAD and light. The enzyme does not significantly speed up *cis–trans* zeatin conversion in the presence of FMN; however, a non-enzymatic conversion in the presence of FMN is much faster than in the presence of FAD (Supplementary Figure S5). The obtained recombinant ZmNPP was also readily able to hydrolyze a range of typical nucleotide pyrophosphatase/phosphodiesterase substrates as shown in **Figure 3** and none of these reactions required light. With the best substrate FAD, enzyme exhibited a specific activity of 496.8 nkat mg protein^{-1} and K_m of 32.7 μM (**Figure 3**). In general, dinucleotides (FAD, diadenosine polyphosphates, $NADP^+$, and UDP-Glc) were better substrates than mononucleotides. The hydrolytic reaction produced AMP from FAD, $NADP^+$, ATP, and diadenosine tetra- and pentaphosphate. Further hydrolysis of produced adenosine polyphosphates was observed only after a prolonged incubation. Hydrolysis of other nucleoside triphosphates also led to monophosphate products.

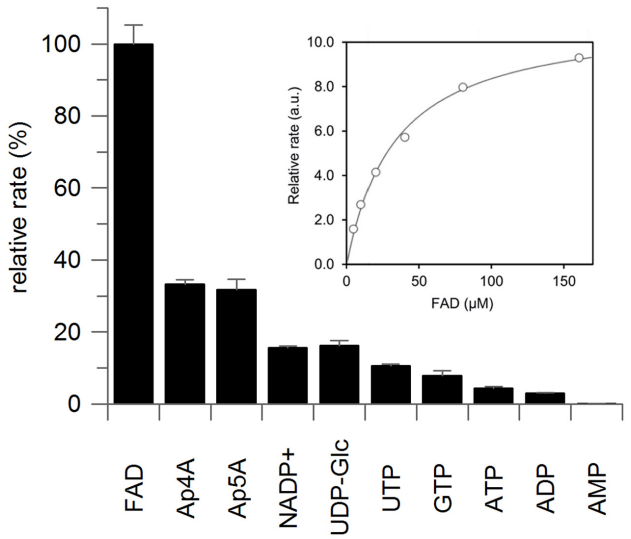


FIGURE 3 | Substrate specificity of recombinant maize nucleotide pyrophosphatase/phosphodiesterase. Substrates (0.5 mM) were incubated with the purified enzyme in 100 mM McIlvaine buffer containing 20 mM $MgCl_2$ at 37°C for the period of 30 min, during which both the decrease in substrate concentration and increase in the product concentration were monitored by UFLC and the tangent was used to calculate the reaction rate. Graph depicts average of three technical replicates with standard deviation. The insert shows the saturation curve of FAD hydrolysis with maximal velocity on *y*-axis expressed in arbitrary units (a.u.), from which K_m of 32.7 ± 3.0 μM was calculated by GraFit software.

Overexpression of Maize Nucleotide Pyrophosphatase/Phosphodiesterase in Plant Systems

In order to quickly verify ZmNPP activity *in planta*, the *ZmNPP* gene was first expressed in a tomato hairy root system (Collier et al., 2005) and several independent transgenic lines were propagated by main root excision. The ZmNPP expressing lines showed 2–10 times higher activity of nucleotide pyrophosphatase/phosphodiesterase with FAD (assayed as the production of FMN) compared to wild type and heat inactivated samples. Similarly, zeatin *cis*-to-*trans* isomerization rate measured *in vitro* in the extracts from various transgenic lines was higher as shown in **Figure 4A**. There was a strong correlation ($R^2 = 0.9655$) between zeatin isomerization and nucleotide pyrophosphatase/phosphodiesterase activity, which was, at the same time, independent of the presence of *cis*-zeatin (**Figure 4B**).

Further, *Arabidopsis* plants overexpressing the *ZmNPP* gene under 35S promoter were prepared. The specific activity of nucleotide pyrophosphatase/phosphodiesterase with FAD as the substrate increased in the extracts from leaves of 4-week-old *Arabidopsis* plants by an order of one magnitude compared to wild type (**Table 2**). Accordingly, the transgenic plants had decreased levels of endogenous FAD and increased levels of FMN and riboflavin (**Figure 4C**). However, no significant changes in the levels of endogenous zeatin isomers were observed. The levels of zeatins' free bases in green and senescent leaves of *Arabidopsis* are depicted in Supplementary Figure S6. The plants also did not show any visible phenotypic changes or changes in the chlorophyll content (**Table 2**).

To assess the function of putative rice nucleotide pyrophosphatase/phosphodiesterase LOC_Os01g10020, 2-week-old rice plants in which the expression of encoding gene was enhanced by disrupting a native promoter of the gene with the promoter sequence of α-tubulin gene *OsTubA1* were examined. Originally, this only available T-DNA insertion mutant of *NPP* gene in monocot species was purchased in order to study loss-of-function phenotype; however, it later turned out that the insertion of T-DNA to the promotor region led to its overexpression rather to disruption. Approximately one fourth of the obtained plants were albinos, in which chlorophyll content was below the detection limit and the nucleotide pyrophosphatase/phosphodiesterase activity increased about 50-times compared to the wild type, while green mutant plants showed only about 10-times activity increase. The total flavin content decreased about 3-times in albinos, but remained unchanged in green mutant plants (**Table 2**).

DISCUSSION

In the original paper by Bassil et al. (1993), the zeatin *cis–trans* isomerase activity detected in immature seeds of common bean had following attributes: (i) the conversion required FAD or FMN, light and a reducing agent such as dithiothreitol; (ii) both zeatin isomers were substrates, although the rate of conversion of *cis*- to *trans*-zeatin was higher, and (iii) there was also a noticeable background conversion when the enzyme preparation was inactivated by boiling. Purification of a protein with the zeatin *cis–trans* isomerase activity from maize kernels led to the identification of the nucleotide pyrophosphatase/phosphodiesterase, which hydrolyzes FAD without any light requirement. NPP family of seven members has been well known in mammals. Most of human enzymes contain a single transmembrane domain residing in the plasma membrane and hydrolyze mostly nucleotides and their analogs or phospholipids (Stefan et al., 2005). Plant NPPs are less characterized. The situation is complicated by the lack of sequence determination and an overlap of the catalyzed reaction with other enzyme families, i.e., purple acid phosphatases (PAP; Li et al., 2002; Olczak et al., 2003) and Nudix hydrolases (**nu**cleoside **di**phosphate compounds linked to a moiety, X; Maruta et al., 2012), which catalyze hydrolytic breakdown of pyrophosphate and phosphodiester bonds of numerous nucleotide sugars. Chloroplast localized ADP-Glc pyrophosphatase that belongs to purple acid phosphatase family has been found to negatively regulate starch biosynthesis in rice and barley (Nanjo et al., 2006; Kaneko et al., 2014). The only plant enzyme characterized to date resembling the mammalian ones has been found in wheat (Joye et al., 2010).

The amino acid sequence of ZmNPP shows the highest similarity to the families 1 to 3 of human nucleotide pyrophosphatase/phosphodiesterases and also to the enzyme

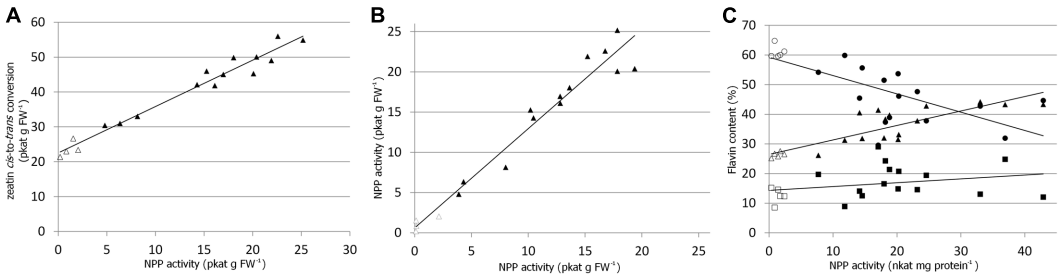


FIGURE 4 | Elevated nucleotide pyrophosphatase/phosphodiesterase (NPP) activity in transgenic plants. **(A)** Nucleotide pyrophosphatase/phosphodiesterase activity assayed as the production of FMN in the extracts from various tomato hairy root lines overexpressing *ZmNPP* and correlation to zeatin *in vitro cis*-to-*trans* conversion per 1 g of fresh weight (FW). **(B)** Activity of NPP (as the production of FMN) in the presence (*y*-axis) and absence (*x*-axis) of *cis*-zeatin in various tomato hairy root lines. The reaction was set up for each sample twice identically except of the presence/absence of *cis*-zeatin. **(C)** Relation of endogenous contents of FAD (circles), FMN (triangles) and riboflavin (squares) toward the activity of nucleotide pyrophosphatase/phosphodiesterase in 4-week-old *Arabidopsis* plants overexpressing *ZmNPP*. Samples were taken from four independent lines. Open and solid symbols represent wild type and transgenic lines, respectively.

TABLE 2 | Characteristics of *Arabidopsis* and rice plants with altered nucleotide pyrophosphatase/phosphodiesterase activity.

Plant	***Arabidopsis***				**Rice**				
Line	**Wild type**	**#5**	**#8**	**#15**	**Wild type**	**#1**	**#2**	**#3**	**#4 (albino)**
Biological replicates	5	5	4	5	9	16	3	5	3
Plants per replicate	4	4	4	4	1	1	1	1	2
Specific activity (nkat mg^{-1})	0.07 ± 0.04	1.26 ± 0.30	1.65 ± 0.61	0.86 ± 0.24	0.01 ± 0.01	0.09 ± 0.02	0.12 ± 0.02	0.05 ± 0.02	0.57 ± 0.12
p-value		*1.29E-03*	*0.021*	*2.53E-03*		*1.39E-10*	*4.41E-03*	*0.029*	*0.022*
Total flavins (pmol)	2.4 ± 0.5	2.3 ± 0.5	2.3 ± 0.3	2.1 ± 0.6	5.6 ± 1.5	5.8 ± 1.5	4.5 ± 1.2	6.0 ± 2.4	1.8 ± 0.2
p-value		*0.94*	*0.74*	*0.56*		*0.78*	*0.35*	*0.77*	*7.59E-05*
FAD (%)	61.1 ± 1.9	44.1 ± 6.0	42.5 ± 8.8	45.3 ± 8.5	51.2 ± 0.9	51.5 ± 1.5	49.5 ± 2.6	52.0 ± 1.3	55.1 ± 0.5
p-value		*3.20E-03*	*0.033*	*0.019*		*0.56*	*0.45*	*0.30*	*5.9E-4*
FMN (%)	26.3 ± 0.8	38.3 ± 4.1	40.3 ± 4.9	34.6 ± 5.7	46.7 ± 0.6	45.8 ± 1.3	47.7 ± 2.3	45.3 ± 1.3	34.4 ± 1.4
p-value		*3.57E-03*	*0.015*	*0.042*		*0.040*	*0.58*	*0.11*	*5.09E-03*
Riboflavin (%)	12.6 ± 2.3	17.6 ± 4.4	17.2 ± 5.3	20.0 ± 5.1	2.2 ± 0.5	2.7 ± 0.4	2.8 ± 0.3	2.7 ± 0.3	10.5 ± 2.0
p-value		*0.090*	*0.23*	*0.041*		*7.29E-03*	*0.046*	*0.063*	*0.026*
Chlorophyll *a* (μg mg^{-1})	0.71 ± 0.10	0.65 ± 0.11	0.70 ± 0.11	0.76 ± 0.12	1.09 ± 0.19	1.02 ± 0.12	1.07 ± 0.04	1.33 ± 0.24	< LOD
p-value		*0.45*	*0.91*	*0.46*		*0.38*	*0.80*	*0.19*	*n.d.*
Chlolophyll *b* (μg mg^{-1})	0.24 ± 0.03	0.22 ± 0.03	0.23 ± 0.04	0.25 ± 0.04	0.28 ± 0.05	0.27 ± 0.03	0.28 ± 0.02	0.37 ± 0.06	< LOD
p-value		*0.43*	*0.83*	*0.51*		*0.37*	*0.92*	*0.082*	*n.d.*
Chlorophyll *a/b*	3.01	3.00	3.05	3.04	3.85	3.86	3.84	3.59	n.d.
p-value		*0.73*	*0.052*	*0.13*		*0.96*	*0.93*	*0.021*	*n.d.*

Extracts from the leaves of 4-week-old Arabidopsis overexpressing ZmNPP gene (LOC100280465) and 2-week-old rice plants with enhanced expression of putative nucleotide pyrophosphatase/phosphodiesterase OsNPP gene (LOC_OS01G10020) Were analyzed as described in Section "Materials and Methods." Nucleotide pyrophosphatase/phosphodiesterase activity was measured as fad hydrolysis. results are given as mean values ± standard deviation; p-value was determined using student's t-test against the wild type lines with number of biological replicates indicated for each line in the table; <LOD, below limit of detection; N.D., not determined.

from wheat, but it lacks the transmembrane domain, which is consistent with the enzyme's purification as a soluble protein. Correspondingly, it has been shown that some human enzymes (Belli et al., 1993; Wu et al., 2004; Sakagami et al., 2005) as well as the wheat enzyme (Joye et al., 2010) may exist in a soluble form. Accordingly to the mammalian and wheat enzymes, which are *N*-glycosylated, the ZmNPP is probably also glycosylated as predicted by NetNGlyc (Gupta and Brunak, 2002), evidenced by binding to Concanavalin A-Sepharose and a hyper-glycosylation observed on the recombinant ZmNPP protein. To determine the substrate specificity, recombinant ZmNPP was prepared in *Picchia pastoris* and purified from the culture medium. With the partially purified enzyme, the zeatin isomerization reaction proceeded even in the absence of dithiothreitol needed to inhibit the breakdown of zeatin to adenine in plant extracts that probably occurred due to the activity of cytokinin dehydrogenase (EC 1.5.99.12; Galuszka et al., 2001).

Flavins, in general, have been known to possess rich chemistry, which is exploited by nature in many enzymatic systems. However, FMN and FAD are also known to form singlet

and triplet excited states upon illumination and induce non-enzymatic flavin sensitized photoisomerism of e.g., retinol, bilirubin and stilbenes by direct energy transfer (Heelis, 1982). Flavin induced photoisomerization of bilirubin is used for a treatment of neonatal jaundice (Knobloch et al., 1991). It is therefore very likely that *in vitro* ZmNPP hydrolysis of FAD to FMN and AMP induced a non-enzymatic photoisomerization of zeatin as FMN is more potent in such a process. Photoisomerization of zeatin with FAD then occurred also with the heat-inactivated enzyme, albeit in a lower rate. The conversion of zeatin isomers found *in vitro* is reminiscent of an older paper reporting on *trans*-to-*cis* isomerization of geraniol and geranyl phosphate to nerol and neryl phosphate, respectively, by cell-free extracts of carrot and peppermint, in the presence of FAD or FMN, a thiol or sulfide and light (Shine and Loomis, 1974), which may have also happened non-enzymatically by flavin sensitization.

There is not enough supporting evidence for zeatin isomerization *in vivo*. Upon feeding potato tubers with radioactively labeled *cis*-zeatin, only 5% to 9% of the label was found in *trans*-zeatin (Suttle and Banowetz, 2000). Recently, *cis–trans* isomerization was reported in pathogenic fungus *Leptosphaeria maculans* (Trdá et al., 2017). First, the authors observed an increase in *cis*- or *trans*-zeatin, when the fungus was fed with the other isomer. We have observed similar occurrence ourselves when we treated maize with micromolar concentrations of zeatins, but it was not confirmed with radiolabeled cytokinins at physiological levels (Hluska et al., 2016). Further, Trdá et al. (2017) fed the fungus with zeatins and observed accumulated isomers in the medium. Similarly to our work reported here, the *cis*-to-*trans* isomerization was approximately 3-times faster than the other way. Also, when the fungus was not added, or it was boiled prior to incubation, the conversion was smaller (Trdá et al., 2017). Thus, one can hypothesize, that nucleotide pyrophosphatase/phosphodiesterase is at play here. On the contrary, the *Arabidopsis* knock-out plants deficient in biosynthesis of either of the zeatin isomers were not able to complement its loss by isomerization (Miyawaki et al., 2006). We, therefore, prepared overexpression plants to assess whether altered nucleotide pyrophosphatase/phosphodiesterase activity affects the content or ratio of zeatin isomers *in vivo*. The FAD hydrolyzing activity in the lines expressing maize enzyme was increased 12–23-times. In these plants, FAD levels were decreased as expected. However, besides the increase in FMN, the direct product of FAD hydrolysis, part of FAD was probably converted to riboflavin. *Arabidopsis* leaves overexpressing *ZmNPP* did not show any alteration in the ratio or the total content of two zeatin isoforms. This should not be surprising considering the plants are able to suppress non-enzymatic conversion of zeatin isomers. Otherwise, the plants would have to have *trans* to *cis* ratio at the equilibrium. This may be due to several reasons, possibly because of low concentrations in the plant and limited amount of light intracellularly. Further, flavins and cytokinins are each localized to different subcellular compartments. While flavins are mainly in mitochondria and nucleus (Giancaspero et al., 2013), cytokinins are predominantly localized to apoplast (Jiskrová et al., 2016). No visible phenotype alterations were observed on these plants. Similarly, no phenotype change was observed in AtNUDX23 overexpressing plants with about 20% decrease in flavin content (Maruta et al., 2012).

To shed more light on the physiological function of plant nucleotide pyrophosphatase/phosphodiesterase, rice plants, in which the expression of *OsNPP* encoding gene was enhanced by disrupting its native promoter with a strong and constitutive promoter of α-tubulin, were examined. Approximately one fourth of the obtained plants were albinos with much higher increase in the nucleotide pyrophosphatase/phosphodiesterase activity than was observed in remaining green mutant plants. Except for a significant decrease in total flavin content in the albino plants, there was a significant shift from FMN to riboflavin. We cannot currently speculate about the lack of chlorophyll, NPP activity and flavin content, what is the cause and what is the consequence, or whether there is any causation at all. However, considering the fact we observed no albino plants of wild type origin, we can hypothesize that in some rice plants the OsNPP upregulation is so strong, it leads to severe alterations in flavin content ultimately causing chlorophyll depletion. These data are in agreement with previous observations that the reduction in the flavin content to less than 50% leads to a stunted growth and chlorosis of plants (Ouyang et al., 2010; Hedtke et al., 2011). The authors conclude, that increased photooxidative damage and down-regulated FAD-dependent cytokinin dehydrogenase, respectively are responsible for the observed phenotype.

Because of the wide substrate specificity of nucleotide pyrophosphatases/phosphodiesterases that overlaps with purple acid phosphatases and Nudix hydrolases, understanding their physiological function will require an integrative approach and research involving all these enzymes. The best understood so far is the role of purple acid phosphatase with ADP-Glc pyrophosphatase activity that diminishes the starch formation (Rodríguez-López et al., 2000; Kaneko et al., 2014). The increased expression of putative nucleotide pyrophosphatases/phosphodiesterases from *Arabidopis thaliana* after seed imbibition as can be seen in the Genevestigator database (Hruz et al., 2008) suggests they may be involved in a storage mobilization through seed germination. Very likely, the product of enzymatic hydrolysis pyrophosphate also plays a regulatory role in starch synthesis in potato tubers (Farré et al., 2000).

So far there are indices that nucleotide pyrophosphatases/phosphodiesterases are involved in regulation of availability of redox cofactors as it has been proposed for the enzyme from *Opuntia* during fruit ripening (Spanò et al., 2011). Another role could be in pathogen defense, where the follow-up product of nucleotide pyrophosphatase/phosphodiesterase reaction riboflavin has been reported to induce a resistance against several pathogens in plants, including *Botrytis cinerea* (Azami-Sardooei et al., 2010). Indeed, the expression data for the putative nucleotide pyrophosphatase/phosphodiesterase encoded by the At4g29700 gene available from the Genevestigator database (Hruz et al., 2008) show about seven-fold increase upon *B. cinerea* inoculation. Furthermore, *Pseudomonas syringae* was shown to require guanosine tetra- and pentaphosphates

for the colonization of plants (Chatnaparat et al., 2015), availability of which may be controlled by nucleotide pyrophosphatase/phosphodiesterase thus limiting the pathogen spreading.

CONCLUSION

From the data presented in this work, it appears highly unlikely that nucleotide pyrophosphatase/phosphodiesterase participates in zeatin *cis–trans* isomerization *in vivo* as there is no significant change in the ratio of the zeatin isomers in plants with altered activity of this enzyme. Recently, we have postulated a hypothesis that the high levels of *cis*-zeatin type cytokinins in certain species such as maize may be a combined effect of different substrate specificity of cytokinin-specific glycosyltransferases and the ability of cytokinin dehydrogenase to preferentially degrade cytokinin *N*-glucosides (Hluska et al., 2016). While *Arabidopsis* has only a limited ability to *O*-glucosylate *cis*-zeatin (Hou et al., 2004), there are two cytokinin-specific glycosyltransferases known in maize that both prefer *cis*-zeatin to its *trans*-isomer (Veach et al., 2003). As a result, *cis*-zeatin *O*-glucosides that are resistant to the cleavage by cytokinin dehydrogenase accumulate in maize, whereas *N*-glucosides produced in *Arabidopsis* are readily degraded by the enzyme (Galuszka et al., 2007).

AUTHOR CONTRIBUTIONS

PG and TH designed research; TH, MŠ, and RL performed all experiments and data collection; TH, PG, and IF analyzed data; TH, IF, and PG wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01473/full#supplementary-material

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Faculty of Science

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Metabolism of Zeatin-Type Cytokinins in Monocotyledonous Plants

Tomáš HLUSKA

Summary of Ph.D. Thesis

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This thesis has been completed within the framework of the Ph.D. study program P1406 Biochemie guaranteed by the Department of Biochemistry, Faculty of Science, Palacký University in Olomouc in the period 2010 – 2018.

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SOUHRN

Cytokininy jsou rostlinné hormony, deriváty adeninu s postranním řetězcem v N^6-poloze. Ovlivňují mnoho fyziologických procesů.

Zatímco metabolismus *trans*-zeatinu a isopentenyladeninu, které jsou považovány za vysoce aktivní cytokininy, byl extenzivně studován, metabolismus dalších, jako *cis*-zeatin, dihydrozeatin, či aromatické cytokininy je opomíjen.

V této práci jsme použili jako model pro studium metabolických přeměn cytokininů, hlavně zeatinů, vyvíjející se kukuřičné zrno. Místo zeatin reduktasy jsme však zjistili novou enzymatickou aktivitu přeměňující *trans*-zeatin na 6-(3-methylpyrrol-1-yl)purin. Enzym se však nepodařilo identifikovat kvůli jeho nestabilitě. Zato se nám podařilo identifikovat enzym odpovědný za *cis-trans* isomeraci zeatinů jako nukleotidpyrofosfatasu/fosfodiesterasu. Potvrdili jsme jeho aktivitu; za substráty preferuje FAD a jiné dinukleotidy. Navrhli jsme několik jeho funkcí *in planta*.

Dále jsme navrhli dvě nové hypotézy týkající se metabolismu a fyziologie cytokininů. První vysvětluje převahu údajně neaktivního *cis*-zeatinu v některých rostlinách. Druhá navrhuje dvourychlostní cytokininový systém.

SUMMARY

Cytokinins are plant hormones, derivatives of adenine with side chain at the N^6-position. They are involved in many physiological processes.

While the metabolism of *trans*-zeatin and isopentenyladenine, that are considered to be highly active cytokinins, was extensively studied, the metabolism of minor cytokinins as *cis*-zeatin, dihydrozeatin and aromatic cytokinins is largely neglected.

Here we used maize developing seed as model to study interconversions of cytokinins, mainly zeatins. Instead of zeatin reductase, we have detected novel enzymatic activity converting *trans*-zeatin to 6-(3-methylpyrrol-1-yl)purine. The enzyme was not identified due to its instability. On the other hand, we have identified the causative enzyme of zeatin *cis-trans* isomerization as nucleotide pyrophosphatase/phosphodiesterase. Activity of the enzyme was confirmed; it prefers FAD and other dinucleotides as substrates. We proposed several functions for the enzyme *in planta*.

Further, two novel hypotheses concerning cytokinin metabolism and physiology are presented. The first explains prevalence of *cis*-zeatin, a purportedly inactive cytokinin, in certain species. The second one proposes a two-speed cytokinin system.

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AIM OF WORK

⇒ review on cytokinins and their metabolism with focus on *cis*-zeatin and its significance and metabolism in plants

⇒ study of cytokinin metabolism in maize kernels

⇒ identification of novel metabolite of *trans*-zeatin; elucidation of its activity and biosynthesis

⇒ purification and identification of zeatin *cis-trans* isomerase from maize

⇒ characterisation of the responsible protein

INTRODUCTION

Cytokinins (CKs) are plant hormones involved in many physiological processes. They are considered with auxins as one of the main groups of phytohormones as they together control cell division and hence influence the overall plant's architecture. Isoprenoid and aromatic cytokinins are recognised in accordance to the nature of the side chain. Modifications of the side chain further define properties of the respective cytokinin types.

The function of CKs can be summarised as trigger of cellular change essential for numerous decisions throughout the plant's life. Both developmental processes and adaptive responses to various abiotic and biotic inputs. The response is further influenced by crosstalk with other signals that vary with cell's history and thus context (Zürcher and Müller, 2016).

cis or *trans* – That's What Matters

The first cytokinin isolated from a natural source was zeatin obtained from immature sweet corn (*Zea mays*, hence the name) seeds (Letham, 1963). As the hydroxymethyl group is positioned at a double bond, zeatin forms two geometric isomers. Quickly it was established, that the highly active compound is *trans*-zeatin (*t*Z). Soon, *cis*-zeatin (*c*Z) was identified in tRNA (Hall et al., 1967). However, its activity in the classical bioassays was miniscule (Mok et al., 1978; Schmitz et al., 1972; Vreman et al., 1974). Also the activity of several enzymes of cytokinin metabolism (especially of the catabolic cytokinin oxidase/dehydrogenase) towards *c*Z was often negligible and thus *cis*-zeatin was mostly neglected.

Over the years, however, the number of reports of plants or plant organs containing predominantly the *cis*-isomer grew. The large-scale analyses in the plant kingdom (Gajdošová et al., 2011) and in bryophytes (Záveská Drábková et al., 2015) revealed, that the *c*Z-dominance is not a unique trait. Further, *c*Z shows activity in systems that are relevant to it. Development of pea embryo is promoted by *c*Z (Quesnelle and Emery, 2007). It was also shown to inhibit growth of roots of *c*Z-dominant rice, a trait typical for CKs (Kudo et al., 2012). Direct interaction of *c*Z with CK receptors (Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004) showed that its activity in bioassays is not due to isomerization to *t*Z.

The reduced form of zeatin – dihydrozeatin (DHZ) was first isolated from yellow lupine immature seeds following activity measurement using the tobacco callus bioassay (Koshimizu et al., 1967a, 1967b) and it showed substantial activity also in other works (Mok et al., 1978; Schmitz et al., 1972). As a matter of fact, it was the most active isoprenoid cytokinin in *Phaseolus vulgaris* bioassay (Mok et al., 1978). Further, it is capable to activate at least some CK receptors (Choi et al., 2012; Kuderová et al., 2015; Lomin et al., 2011; Spíchal et al., 2004).

However, it is omitted from many analyses and hypotheses nowadays (e.g. Kasahara et al., 2004; Miyawaki et al., 2006). That is probably because of its in general low quantities and because of complete lack of knowledge about its metabolism. For example, there are only three publications dealing with the zeatin reductase, the putative DHZ biosynthetic enzyme, published over the course of 35 years (Gaudinová et al., 2005; Martin et al., 1989; Sondheimer and Tzou, 1971). We even don't know how DHZ is removed from the plant, as it is resistant to degradation (Galuszka et al., 2007). There is a single report of metabolism of DHZ to adenine in maize (Podlešáková et al., 2012) Otherwise, there is no evidence of other conversion besides glycosylations.

Often the dormant seeds contain less active cytokinins as *cis*-zeatin (Emery et al., 2000; Gajdošová et al., 2011; Quesnelle and Emery, 2007; Stirk et al., 2012b) or dihydrozeatin (Arnau et al., 1999; Emery et al., 2000; Stirk et al., 2012b; Zalabák et al., 2014). There seems to be a pattern of *t*Z-dominant plants having *c*Z in their seeds and *c*Z-dominant plants having DHZ in their seeds, although more widespread analysis would be necessary. This may suggest that these are favourable

because of their resistance to degradation and/or because of low activity that may be advisable at this developmental stage.

Aromatic Cytokinins

Natural occurrence of aromatic cytokinins has been confirmed only in few plant, algae and microorganism species (Doležal et al., 2002; Edlund et al., 2017; Stirk et al., 2012a, 2012b; Strnad, 1997; Tarkowská et al., 2003). Moreover, in many cases, their detection in living samples might be the result of labware contamination due to their high stability, adhesiveness to glass surfaces and massive usage in *in vitro* culturing (Ondřej Novák, personal communication). It was hypothesised that the aromatic cytokinins in plants may in fact originate from symbiotic bacteria (Miroslav Strnad, personal communication). That would explain disappearance of BAP and its metabolites from poplar callus over time of several *in vitro* passages (Pavel Jaworek, personal communication) as well as the inconsistency in detection of aromatic cytokinins in various plants (RJ Neil Emery, personal communication).

Besides the *c*Z and DHZ, plant seeds are often reported to contain also aromatic cytokinins. So is kinetin found in the liquid endosperm of coconut (for review see Yong et al., 2009), 3-methylpyrrolpurine was found in seeds of maize (this work) and BAP and topolins are found in *Tagetes minuta* (Stirk et al., 2005, 2012a), pea (Stirk et al., 2008), in seeds of maize, oat and lucerne (Stirk et al., 2012b) and in many other plant species (unpublished results referenced in Stirk et al., 2008). This suggests that aromatic CKs may perform a common role in seed germination.

Cytokinin Biosynthesis

The biosynthesis of cytokinins starts with the addition of a side chain to an adenine either in nucleotide form, or bound in tRNA. Several additional steps are generally included in the CK biosynthesis as hydroxylation of the side chain, removal of phosphates and/or ribose or reduction of the double bond in *trans*-zeatin's side chain.

The first step of CK biosynthesis is catalysed by isopentenyltransferases. The addition of isoprenyl side chain is always referred to as the rate limiting step of CK biosynthesis (Kamada-Nobusada and Sakakibara, 2009). Taking into account that the free bases are usually present in minute quantities, while the ribosides and ribotides are usually present in much higher quantities, it seems probable that other step in the cytokinin biosynthesis is the real bottleneck.

There are currently three classes of isopentenyltransferases recognized to be involved in CK biosynthesis: adenylate dimethylallyltransferase (AMP-dependent; EC 2.5.1.27), tRNA dimethylallyltransferase (EC 2.5.1.75) and adenylate dimethylallyltransferase (ADP/ATP-dependent; EC 2.5.1.112). At this moment, only the tRNA IPTs seem to strictly adhere to the single activity of dimethylallyl pyrophosphate:adenine[37] in tRNA dimethylallyltransferase. The adenylate IPTs, on the other hand, are enzymes utilizing multiple substances both

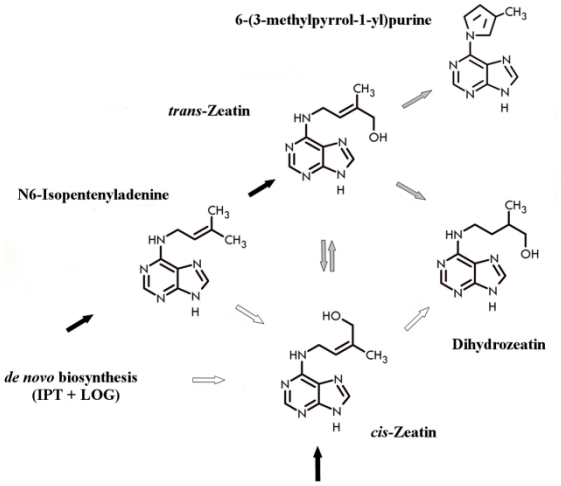

Figure 1: Zeatin inter-conversions in plants. Black arrows indicate enzymatic steps that were confirmed *in planta* and responsible gene(s) are known; grey arrows indicate enzymatic conversions shown only in *in vitro* assays; white arrows indicate hypothetical reactions (conversions) without any proof of evidence.

as acceptor and as donor substrates. Depending on the donor substrate, iP-type or *t*Z-type cytokinin may be produced.

In the iP-dependent pathway, zeatins are formed by hydroxylation of the side chain. This was observed in the cauliflower for the first time (Chen and Leisner, 1984). Later, two cytochromes P450 forming *trans*-zeatin were identified in *Arabidopsis* (Takei et al., 2004). The enzymes utilize nucleotides of iP, mainly mono- and diphosphate. The only products detected *in vitro* were *t*Z-type cytokinins. Interestingly though, *Saccharomyces cerevisiae* expressing *AtIPT*4 with the cytochromes, contained also DHZ-type cytokinins. It was confirmed, that the CYP735As are major contributors to *trans*-zeatin synthesis, when double knock-out mutant had <5 % of *t*Z-type cytokinins, while iP-type CKs were increased to ~200 % (Kiba et al., 2013). The mutant's phenotype resembled the phenotype of other CK-deficient or CK-insensitive mutants *ipt*3 5 7 and *ahk*2 3.

The *cis*-hydroxylase is known to act upon iP in tRNA (Persson and Björk, 1993). The gene is in general present only in few bacterial species, incl. for example *Nostoc* (Kaminska et al., 2008). But no homologue has been identified up to now in plants.

Unlike the biosynthesis of *t*Z and iP, the biosynthesis of *cis*-zeatin in plants remains a mystery. Nowadays, it is accepted, that *c*Z originates from tRNA (Miyawaki et al., 2006). However, that doesn't answer the question, where does the *cis*-hydroxylated side chain originate from. There are in principle four possibilities for *cis*-zeatin biosynthesis:

1) isoprenylation of adenine, either free or tRNA-bound, with (*Z*)-4-hydroxy-3-methylbut-2-enyl diphosphate
2) hydroxylation of iP, either free or tRNA-bound
3) isomerization of *t*Z
4) dehydrogenation of dihydrozeatin

Hitherto these options have not been proven to operate *in planta*.

Deactivation of Cytokinins

There are several options for CK deactivation, when they are present at higher quantities than needed. The first option is ribosylation, eventually with phosphorylation (Sondheimer and Tzou, 1971; Suttle and Banowetz, 2000; Tokunaga et al., 2012). The other option is glycosylation at the adenine or at the side chain of zeatins and topolins. The last option is reversible degradation by cytokinin oxidase/dehydrogenase.

Cytokinins can be glycosylated either at the nitrogens of the purine ring or at the hydroxyl of the zeatins' side chain. Glycosylation of cytokinins on the side chain leads to loss of activity, because the modified side chain cannot be recognized anymore. This *O*-glycosylation is usually reversible though and thus thought of as of a storage form (Sakakibara, 2006). However, *O*-glucosides tend to accumulate during plant's development (e.g. this work; Šmehilová et al., 2016) and thus the cleavability is probably organelle-dependent. On the other hand, the modification of the adenine ring diminishes binding to CK receptors (Spíchal et al., 2004), but enzymes of CK metabolism, notably the catabolic CKX (Galuszka et al., 2007; Zalabák et al., 2014), and possibly also the transporters may still recognize these metabolites. Thus they are thought of as of the deactivation products. The *N*-glycosides are usually referred to as resistant to hydrolysis, but there are several reports of their hydrolysis.

Interestingly, both cytokinin *O*-glucosyltransferases identified in maize have strict specificity for *cis*-zeatin and were thus designated as *cis*-zeatin *O*-glucosyltransferases (Martin et al., 2001; Veach et al., 2003). They glucosylate *t*Z only very weakly (Veach et al., 2003). On the other hand, there were identified 5 CK-specific glucosyltransferases in *Arabidopsis* (Hou et al., 2004) and they do not discriminate between the zeatin isomers so strictly. Of these, two produced specifically *N*-glucosides. The other three enzymes produced *O*-glucosides, two of them with very low activity

though. They were reported to have other substrates later (Gandia-Herrero et al., 2008; Poppenberger et al., 2005). However, UGT85A1 glucosylates *t*Z and *c*Z with similar activity, while DHZ was worse substrate.

The enzyme degrading cytokinins is one of the longest known of these involved in CK metabolism and thus best characterized today. The cytokinin oxidase/dehydrogenase (CKX) was first described in the 70s (Pačes et al., 1971) and cloned independently by two groups almost 30 years later (Houba-Hérin et al., 1999; Morris et al., 1999). It is a flavoenzyme with covalently bound FAD using artificial electron acceptors (Galuszka et al., 2001). Putative natural electron acceptors were identified (Frébortová et al., 2010).

Hulks and Deadpools of the Cytokinin Universe

Throughout the universe of plant cytokinins, there seem to be recursive theme of the strong, but specialized Hulk, versus the less active, but more resilient Deadpool. Thus, to expand the previously published hypothesis, that *c*Z is important under growth-limiting conditions (Schäfer et al., 2015), here the author would like to propose an extension of that hypothesis: there are two sets of metabolites, enzymes and perception relays for CKs. While one, including, but not limited to *t*Z, iP, AHK4 and *At*LOG7, is highly active and it has specialized functions in promoting and regulating rapid growth, there is other one, including, but not limited to *c*Z, DHZ, AHK3 and *At*LOG8 that is involved in maintenance growth. After all, cytokinins were discovered for their cytokinetic properties.

Purification and Identification of Unknown Metabolite

The analysis of plant hormones was carried out as described previously (Djilianov et al., 2013; Dobrev and Vankova, 2012).

Quantitative purification of the unknown metabolite from the reaction mixture was performed on semi-preparative HPLC with the mobile phase A water and the mobile phase B methanol. Purified substance was analysed by nuclear magnetic resonance by Dr. Marek Kuzma (Institute of Microbiology, Czech Academy of Science, Prague). The identified compound was prepared by oxidation of *t*Z with manganese dioxide according to Letham and Young (1971).

Testing of cytokinin activity

The binding assay was performed according to the previously described method (Romanov et al., 2006).

Arabidopsis thaliana seeds (ecotype Columbia-0) were cultivated on vertical M&S agar plates in the presence of different concentrations of the tested compounds or pure solvent (0.05% DMSO) as described in detail in Podlešáková et al. (2012).

Activity and Inhibition of CKX

Pure recombinant maize CKX enzymes (Zalabák et al., 2014) were used to test an inhibitory effect of MPP on their activities. A continuous method based on bleaching of 2,6-dichlorophenolindophenol (DCPIP) was used (Laskey et al., 2003).

Crystals of *Zm*CKX4a were infiltrated by 2 mM MPP in DMSO for 1 hour and then they were directly flash frozen in liquid nitrogen. Diffraction data were collected at 100 K on the Proxima 1 beamline at the SOLEIL synchrotron (Saint-Aubin, France) at 2.0 Å resolution. The atomic coordinates and structure factors have been deposited in the Protein Data Bank (Berman et al., 2000) under accession code 5HHZ.

Purification of the Protein with Zeatin *cis-trans* Isomerase Activity

Whole maize cobs with immature seeds (930 g), approximately 10 days after pollination, were collected in the field and extracted with 1 L of 50 mM Tris/HCl, pH 8.0. Ballast proteins were precipitated according to published protocol (Šebela et al., 2000). The protein was purified on BioLogic LP and BioLogic DuoFlow systems with several columns.

Finally, the purified enzyme was applied to Novex® isoelectric focusing electrophoresis gels (ThermoFisher Scientific) with pH 3-10 buffers. The gels were crushed by pressing through a syringe several times. Afterwards it was extracted overnight with 100 µL of McIlvaine buffer pH 7.5 at 4°C. Protein identification was achieved using tryptic in-gel digestion after SDS-PAGE followed by tandem mass spectrometry (MS/MS) of the obtained peptides on an ultrafleXtreme MALDI-TOF/TOF instrument (Bruker Daltonik, Germany) using the published method (Dyčka et al., 2015).

Preparation of Recombinant *Zm*NPP in yeast

A maize nucleotide pyrophosphatase/phosphodiesterase (*ZmNPP*) gene was synthesized by GeneArt service of ThermoFisher Scientific with minor alterations from the native sequence. The gene was cloned into pPICZα vector (ThermoFisher Scientific) and transformed into *Pichia pastoris* X-33. The protein was produced in Biostat® Cplus (Sartorius) bioreactor and purified on DEAE-Sepharose and HiTrap Blue HP columns.

Enzyme Activity Assays

The interconversion of zeatin isomers was measured as described previously (Bassil et al., 1993) with 0.2 mM *cis*-zeatin. For each assay, a control reaction was set-up, where the enzyme sample was boiled for 5 min prior to addition of the other components and the enzymatic activity was calculated from the difference in the concentration of *trans*-zeatin between the two reactions. The reaction mixture was analysed on reversed-phase HPLC with 15 mM formic acid, set up with ammonium hydroxide to pH 4.0 at the flow rate of 0.4 mL min^{-1} with gradient of methanol.

The assay of nucleotide pyrophosphatase/phosphodiesterase activity was done in identical manner, but without *cis*-zeatin and the reaction mixture was kept in dark. The samples were analysed on the same UFLC system and conditions as above, but eluted with 20 mM phosphoric acid (set up to pH 6.5 with ammonium hydroxide) and methanol gradient. In plant samples, extracted flavins were analysed by the same procedure but quantified on a FP-2020 Plus fluorescence detector (JASCO) with excitation at 265 nm and emission at 530 nm using standard compounds.

To determine the nucleotide pyrophosphatase/phosphodiesterase activity with various substrates, the same reaction was set up with 0.5 mM substrate and with an appropriate amount of the purified recombinant enzyme hydrolyzing maximally 40 % of the substrate. The reaction was stopped at several time points and analysed on reversed-phase UFLC with 15 mM triethylamine set with phosphoric acid to pH 6.0 and methanol.

To determine enzyme specific activity, protein concentration was estimated using a Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) with bovine serum albumin as a calibration standard with linearization (Ernst and Zor, 2010).

Analysis of *NPPs* in plants

The gene was cloned into pGWB17 vectors using Gateway protocol (ThermoFisher Scientific) and electroporated into *Agrobacterium rhizogenes* strain 15834, which was used to transform tomato as described previously (Šmehilová et al., 2009).

Arabidopsis plants expressing *Zm*NPP gene cloned into the pGWB17 vector were prepared from *Arabidopsis thaliana* ecotype Col-0 using a floral-dip method (Clough and Bent, 1998). Third to sixth leaves from 4 plants grown in soil were collected and pooled into each sample.

Rice (*Oryza sativa japonica*) T-DNA insertional line PFG_2B-60145.L with the corresponding wild type Hwayoung were purchased from POSTECH, South Korea. Rice seeds were germinated in Agroperlit (Perlit) for 2 weeks. Then shoots were collected from each plant separately with the exception of the albino plants that were pooled from two plants into one sample.

Enzymatic activity and protein content were assayed as above. Total flavins were extracted by previously reported method with minor modifications (Hiltunen et al., 2012). Chlorophyll content was determined using published method (Porra et al., 1989) and so were the cytokinins in *Arabidopsis* plants (Svačinová et al., 2012).

Cytokinins in Maize

In this work, we have profiled all known isoprenoid CK metabolites in reproductive organs. CK profiles were measured also in selected vegetative tissues for comparison. Other substances classified as phytohormones were quantified in the same samples as well. Generally, content of all types of *t*Z derivatives is notably higher in reproductive organs than in young seedlings. The ratio of *t*Z to iP-type CKs is strongly in favour of *t*Z in ovules, silks and developing kernels. iP-type CKs are considerably more abundant in tassels where the amount of *t*Z and iP is almost equal. Ovules as well as tassels before pollination seem to be places of vigorous CK biosynthesis as primary biosynthetic products *t*ZR and iPR phosphates are the most abundant CK derivatives there. In tassels, phosphates are accompanied also by high levels of *t*Z and iP free bases, which are the major active CKs mediating sensing on CK receptors (Lomin et al., 2015). In developing kernels, the highest accumulation of free *t*Z and *t*ZR was detected between the 6th and the 20th DAP. At older developmental stages, *t*Z accumulates mainly in a form of *N9*-glucoside, especially in silks and older roots. *t*Z *O*-glucosides accumulate predominantly in older leaves and maturing kernels, thus in tissues where high *t*Z content was detected in general. In contrast to *t*Z, *c*Z occurs predominantly as the *O*-glucoside and *c*Z9G is present only in negligible amounts in all analysed tissues. The dynamics of *c*Z levels in the course of development appears to be more constant in general. On the other hand, levels of DHZ metabolites tend to grow in the course of kernel and silk maturation and clearly follow the *t*Z maxima. Contrary to the detected dramatic decrease of *t*Z and *c*Z content, DHZ derivatives accumulate in the desiccating kernel 35 DAP.

Profile of the CK content shown in this study is in accordance with previous study of Brugière et al. (2003) who demonstrated maximum of *t*ZR between 9 to 20 DAP, followed by a sharp increase in iPR concentration at 20 DAP. However, in our study, the 20 DAP increase in iP-type CKs was accompanied also by the accumulation of *t*Z metabolites, which was not the case in the previous study. Discrepancy can be attributed to different maize cultivars used and the fact that the ratio between *t*Z- and iP-type CKs, which is influenced by activity of CK-specific cytochrome P450 monooxygenase, was shown as a variable parameter between low and high yielding barley cultivars (Powell et al., 2013).

When free bases of zeatin-type CKs in 1 and 5 μM concentrations were added to the nutrition solution to 7-d-old maize seedlings, *c*Z and DHZ accumulated in general in one order of magnitude higher amounts than *t*Z (Fig. 2A). The experiment further revealed that applied CK is mainly converted to inactive *N9*-glucoside in the case of *t*Z or to *O*-glucosides in the case of *c*Z and DHZ (Fig. 2B). Additionally, DHZ accumulated upon application of both *c*Z and *t*Z. On the other hand, the pool of iP-type CKs stayed unchanged in all treatments.

When radioactive cytokinins were applied to excised maturing kernels at concentrations more approaching the physiological levels, majority of radioactivity was recovered in the degradation products in case of *t*Z and iP, while DHZ and *c*Z were converted mainly to their *O*-glucosides with minority of radioactivity recovered in *N9*-glucoside or degradation product. Neither *cis-trans* isomerization, nor reduction to DHZ were observed. iP was hydroxylated to *t*Z though.

The differences in metabolism of *t*Z and iP vs. *c*Z and DHZ led us to propose a novel hypothesis. In agreement with our results, maize *O*-glucosyltransferases are known to use preferentially *c*Z as substrate (Veach et al., 2003). On the other hand, the only *Arabidopsis* CK-specific *O*-glucosyltransferase does not discriminate between the isomers (Hou et al., 2004). As *O*-glucosides are resistant to CKX degradation, the ability of plants to *O*-glucosilate respective zeatin isomers with different efficiency leads to observed differences in ratio of the respective isomers *in planta*. This hypothesis is further supported by the approximately one order of

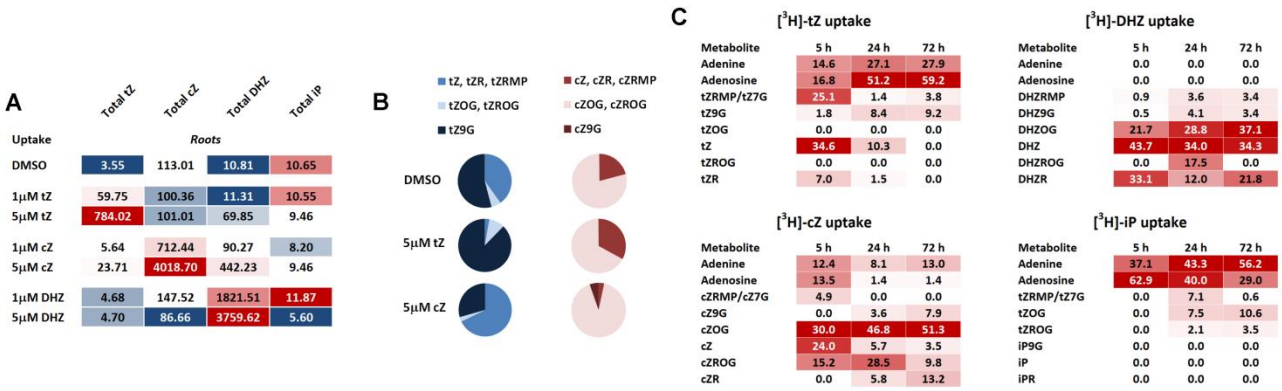

Figure 2: Distribution of cytokinins in maize seedlings upon uptake of different exogenously applied cytokinins. Roots of 10-d-old maize seedlings cultivated in Hoagland nutrient solution were separately analyzed 3 days after the solution was supplemented with various concentration of zeatin-type CKs. (A) Distribution of cytokinin types is expressed in pmol per gram of fresh tissue. Red and blue colours in the heat map correspond to higher and lower concentrations, respectively. The value in each block represents the summed concentrations of all determined metabolites of each group. (B) Ratio among different CK forms in non-treated maize and maize treated with 5 µM *t*Z and *c*Z. Pie charts represent portions of free bases and ribosides; *O*-glucosides and ribosides-*O*-glucosides; and *N*9-glucosides for *t*Z and *c*Z determined separately in roots. (C) Distribution of radioactivity after uptake of tritium labelled CKs to maturing (~20 DAP) kernels. The values are percentage of total recovered radioactivity from each sample.

magnitude lower accumulation of total *t*Z derivatives in comparison to *c*Z or DHZ when roots were fed with respective CK at 1 µM concentration (Fig. 2A). Results of previously published experiments also support this hypothesis.

Identification and Properties of 6-(3-methylpyrrol-1-yl)purine

CK profiling revealed that the predicted zeatin reductase (Martin et al., 1989) should be most active in maturing kernels between 20 to 35 DAP as a profound shift between *t*Z and DHZ content was observed there. However, the feeding experiment with radioactively labelled [^{3}H]*t*Z did not confirm reduction to DHZ in kernels between 20 to 30 DAP, neither did the *in vitro* enzymatic assay of zeatin reductase give positive results. On the other hand, an unknown peak was observed during HPLC analyses, when we attempted to detect zeatin reductase activity. In comparison to all known isoprenoid CKs, the compound had a greater retention time on a reversed-phase HPLC column (i.e. was more hydrophobic) and the absorption maximum shifted from 268 to 300 nm. The reaction was NADP(H)-dependent and *t*ZR and *t*Z9G could serve as substrates as well, while *c*Z and DHZ could not.

To identify the novel substance, *t*Z was enzymatically converted with partially purified enzyme and the substance was refined by semi-preparative HPLC and identified by NMR as 6-(3-methylpyrrol-1-yl)purine (MPP). To verify the structure, MPP was chemically synthesized from *t*Z. Data from the nuclear magnetic resonance were consistent with those published previously (Haidoune et al., 1990). Purification of the biosynthetic enzyme was attempted, but it was not successful due to its poor stability.

A series of assays was performed to test cytokinin activity of MPP. It was found that MPP was not able to activate CK signal transduction via either of two tested maize CK histidine kinase receptors *Zm*HK1 and *Zm*HK3. Nevertheless, MPP was able to compete with *t*Z in binding at least to *Zm*HK3. The supplementation of growth media with MPP did not have any distinct inhibitory effect on elongation of the *Arabidopsis* primary root and lateral root branching. On the contrary, MPP slightly increased the length of primary root. In conclusion, both types of experiments confirmed weak anticytokinin-like activity of MPP.

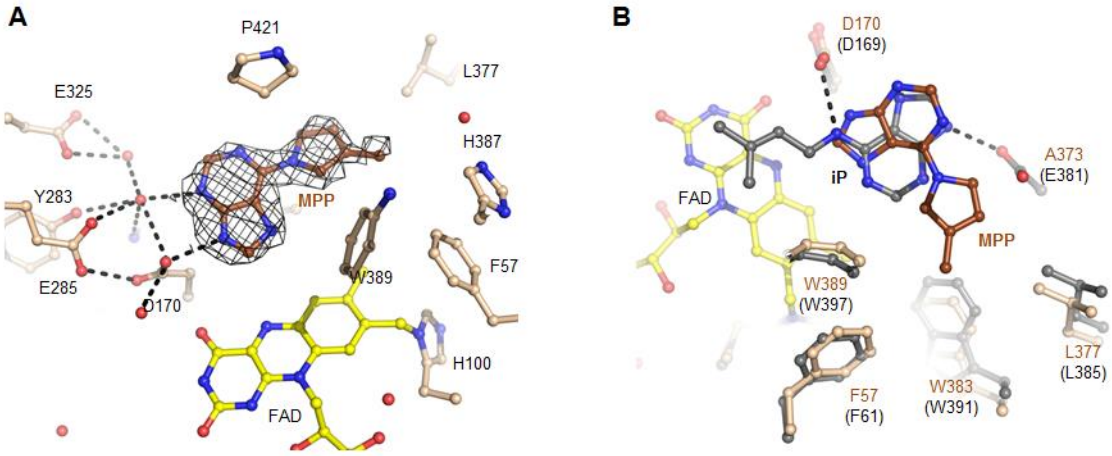

Figure 3: Binding of 6-(3-methylpyrrol-1-yl)purine in the active site of *Zm*CKX4a. (A) 6-(3-methylpyrrol-1-yl)purine (MPP; in Fo-Fc omit map contoured at 3.0 σ), which is coloured in brown, is hydrogen bonded via its *N3* and *N9* atoms of the purine ring to the oxygen atom of D170 and to a water molecule, which is in contact with several residues including D170, E285 or Y283. FAD cofactor is yellow coloured and neighbouring residues are labelled. (B) Superposition of bound MPP and natural substrate isopentenyladenine (iP; PDB: 1W1Q; Malito et al., 2004). Oxygen atom of D170 establishes a hydrogen bond with N^6 of iP. Isoprenoid side chain lies over isoalloxazine plane of FAD while the purine ring points towards the entrance. Neighbouring residues in *Zm*CKX4a are labelled in brown and those in *Zm*CKX1 are shown in brackets.

Expectedly, the CKX enzymes were not able to degrade MPP. On the other hand, MPP inhibited at least *Zm*CKX1 and *Zm*CKX4a when iP was used as a substrate and DCPIP as an electron acceptor. Binding of MPP in the active site of *Zm*CKX4a significantly differed from the binding of natural substrates as observed by X-ray crystallography (Fig. 15). It did not bind over the isoalloxazine plane of FAD as deep as the substrates. The nitrogen atom of the 3-methylpyrrol side chain of MPP had no free hydrogen for interaction with the catalytic aspartate (D170 in *Zm*CKX4a). Thus, MPP bound in a flipped orientation and its 3-methylpyrrol moiety pointed towards the entrance. Taken together, the binding sites of CK substrates and MPP overlapped, and MPP behaved as a weak competitive CKX inhibitor as supported by enzyme kinetics.

MPP was detected in maize tissues, but its quantity was almost at the limit of detection and did not exceed a concentration of 100 fg per gram of fresh weight (Table 1), which is about four orders

Table 1: Quantification of 6-(3-methylpyrrol-1-yl)purine and its metabolites in maize kernels and tassels. Compounds were quantified by UHPLC-(+)ESI-MS/MS using standard isotope dilution method. MPP, 6-(3-methylpyrrol-1-yl)purine; MPPR, 6-(3-methylpyrrol-l-yl)purine *N9*-riboside; *tZ*, *trans*-zeatin; *t*ZR, *t*Z riboside; *t*Z9G, tZ *N9*-glucoside; *t*ZRMP, *t*ZR 5´monophosphate.

metabolite	Tassels (5 DBP)	Kernels (12 DAP)
	(fmol g^{-1} of FW)	
*t*Z	12 984	7 792
MPP	0.2	1.2
*t*ZR	1 136	10 436
MPPR	32	56
*t*Z9G	86 100	6 868
MPP *N9*-glucoside	1 064	1 892
*t*ZRMP	1 508	11 572
MPPR 5´-monophosphate	74	416

of magnitude less than the amount of its precursor *t*Z. Its metabolites – glucoside and nucleoside and nucleotide, were also identified and their levels were much higher than of the free base, but still lower than of the respective *t*Z counterparts (Table 1).

Zeatin *cis-trans* Isomerase: The End of an Old Story

Additionally, we detected zeatin *cis-trans* isomerase activity in seeds of maize. Attributes of the detected activity were in agreement with the previous report (Bassil et al., 1993), namely (i) the conversion required FAD or FMN, light and a reducing agent such as dithiothreitol; (ii) both zeatin isomers were substrates, although the rate of conversion of *cis*- to *trans*-zeatin was higher, and (iii) there was also a noticeable background conversion when the enzyme preparation was inactivated by boiling.

The activity could be detected in most developmental stages of maize and in many additional plant species. The protein was purified from immature maize kernels (Table 2) until the activity could be assigned to a single band on SDS-PAGE. MALDI-TOF/TOF MS and MS/MS allowed assigning the purified protein band to the accession number NP_001146857 in the NCBInr database. This accession is annotated as a nucleotide pyrophosphatase/phosphodiesterase from *Zea mays* (*Zm*NPP; LOC100280465; Zm00001d039454_P001).

The family of nucleotide pyrophosphatases/phosphodiesterases is well described in mammals. There are several reports of plant enzymes classified as NPP, but they are typically mis-classified, as other enzyme families catalyse the same reactions. Only the wheat enzyme is unambiguously NPP (Joye et al., 2010).

Characterization of Plant NPPs

A BLAST analysis against translated plant genomes using *Zm*NPP sequence as a query returned only a few results per plant. At the time of *Zm*NPP identification, the database of maize genome assembly contained an additional gene coding for fused protein of NPP and Sir2 domains. Thus we have focused in our work only on the identified enzyme. For the same reason, we refer to the enzyme as *Zm*NPP, without the number. Currently, three *NPP* genes with similar length and only minor alterations are present in the maize genome database. A single gene LOC_Os01g10020 was found in rice and four in *Arabidopsis*, whose genes are located on chromosome 4, next to each

Table 2: Purification of the protein with zeatin *cis-trans* isomerase activity from maize. Maize cobs 10 day after pollination (930 g) were used as a starting material; the activity was determined using UFLC as a production of trans-zeatin and protein content by linearized Bradford method (see Materials and Methods).

Purification step	Total activity (nkat)	Total proteins (mg)	Specific activity (nkat mg^{-1})	Purification grade (-fold)	Yield (%)
Crude extract	475	11 900	0.04	1.0	100.0
Precipitation	658	12 400	0.05	1.3	138.3
DEAE-Sepharose	724	3 110	0.23	5.8	152.2
High Q	788	1 006	0.78	19.6	165.6
Concanavalin A	162	86.20	1.89	47.1	34.1
Resource Q	145	19.00	7.66	191	30.6
Hydroxyapatite	68.1	5.00	13.6	340	14.3
HiTrap Blue HP	45.2	0.71	64.5	1 610	9.5

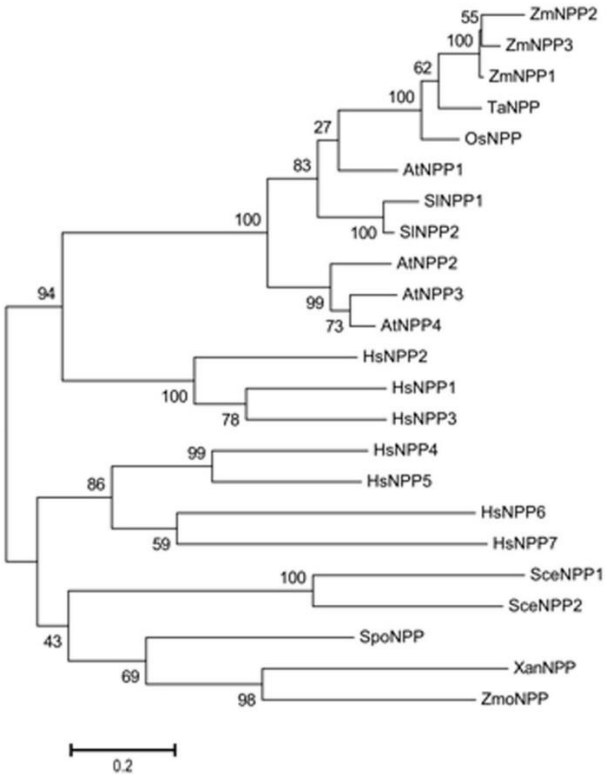

Figure 4: Phylogenetic analysis of nucleotide pyrophosphatases/phosphodiesterases. The following sequences were collected: *Xanthomonas Xan*NPP (WP_011051855.1), *Zymomonas mobilis Zmo*NPP (WP_011241358.1), *Schizosaccharomyces pombe Spo*NPP (O94323.1), *Saccharomyces cerevisiae Sce*NPP1 (P25353.2), *Sce*NPP2 (P39997.1), *Zea mays Zm*NPP1 (Zm00001d039454_P001), *Zm*NPP2 (Zm00001d047948_P001), *Zm*NPP3 (Zm00001d044311_P001), *Oryza sativa Os*NPP (LOC_Os01g10020.1), *Arabidopsis thaliana At*NPP1 (AT4G29680.1), *At*NPP2 (AT4G29690.1), *At*NPP3 (AT4G29700.1), *At*NPP4 (AT4G29710.1), *Triticum aestivum Ta*NPP (ADK32530.1), *Solanum lycopersicum Sl*NPP1 (Solyc07g037950.1), *Sl*NPP2 (Solyc07g037960.1), *Homo sapiens Hs*NPP1 (P22413.2), *Hs*NPP2 (Q13822.3), *Hs*NPP3 (O14638.2), *Hs*NPP4 (Q9Y6X5.3), *Hs*NPP5 (Q9UJA9.1), *Hs*NPP6 (Q6UWR7.2) and *Hs*NPP7 (Q6UWV6.3). The sequences were aligned using CLUSTAL W method (Thompson et al., 1994). The phylogenetic tree was built by the MEGA 6.0 software by the maximum likelihood method (Tamura et al., 2013). To estimate evolutionary distance, the proportions of amino acid differences were computed using Poisson Correction Distance. The reliability of different phylogenetic clusters was evaluated by the bootstrap test (1000 bootstrap replications).

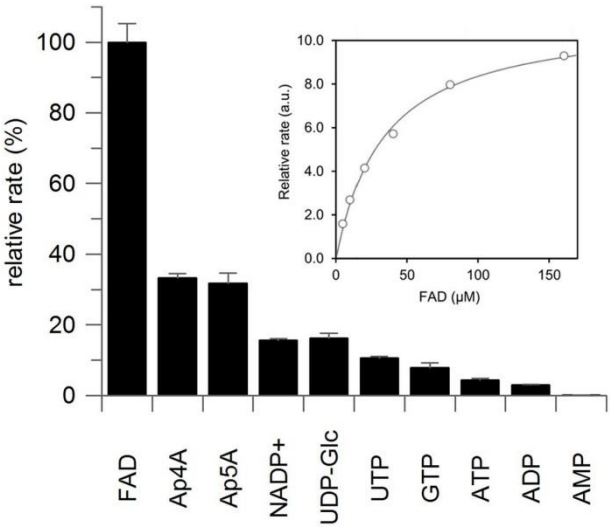

Figure 5: Substrate specificity of recombinant maize nucleotide pyrophosphatase/phosphodiesterase. The insert shows the saturation curve of FAD hydrolysis with maximal velocity on y-axis expressed in arbitrary units (a.u.), from which K_m of 32.7±3.0 μM was calculated by GraFit software.

other (At4g29680, At4g29690, At4g29700, and At4g29710).

Unlike human *nucleotide pyrophosphatase/phosphodiesterase* genes that contain a large number of introns and undergo an alternative splicing, all plant genes, interestingly with the exception of the additional two *ZmNPPs* (*ZmNPP2* and *ZmNPP3*), contain a single exon. As shown in Figure 4, plant NPP proteins cluster separately from animal enzymes in the phylogenetic tree. Mostly, when there is more than one encoding gene in the genome (e.g. in *Arabidopsis* and tomato), the paralogs are located sequentially on the same chromosome. This all suggests that before the evolutional divergence of animals and plants, there was only a single precursor gene. While there were multiple gene duplications in animals before the divergence of clades (Zimmermann et al., 2012), the genes in plants duplicated relatively recently, after the speciation.

Substrate Specificity of Recombinant *Zm*NPP

The maize nucleotide pyrophosphatase/phosphodiesterase was successfully produced in the methylotrophic

yeast *Pichia pastoris* with methanol-inducible expression and protein secreted to media. The purified recombinant protein showed the zeatin *cis-trans* isomerase reaction in the presence of FAD and light. However, the reaction was much faster, when FMN was used, but there was no difference between reaction with native enzyme and control with boiled enzyme.

Flavins are known to form excited states upon illumination and to induce non-enzymatic photoisomerism. This is used in treatment of neonatal jaundice (Knobloch et al., 1991). Similar mechanism works in zeatin isomerisation. As FMN is more potent in photoisomerisation induction, the NPP reaction appears to accelerate the *cis-trans* isomerisation of zeatins (Fig. 6).

As the protein was established to be truly NPP, additional compounds were tested as substrates. *Zm*NPP was able to efficiently hydrolyze wide range of typical NPP substrates (Fig. 5). In general, dinucleotides were better substrates than mononucleotides and FAD was the best substrate among tested substances with a specific activity of 496.8 nkat mg protein^{-1} and K_m of 32.7 μM (Fig. 5).

Expression of *Zm*NPP in Plant Systems

In order to quickly verify *Zm*NPP activity *in planta*, the *ZmNPP* gene was first expressed in a tomato hairy root system (Collier et al., 2005). The *ZmNPP* expressing lines showed 2 to 10 times higher activity of nucleotide pyrophosphatase/phosphodiesterase with FAD (assayed as the production of FMN) compared to wild type and heat inactivated samples. Similarly, zeatin *cis*-to-*trans* isomerization rate measured *in vitro* in the extracts from various transgenic lines was higher. There was a strong correlation (R^2 = 0.9655) between zeatin isomerization and nucleotide pyrophosphatase/phosphodiesterase activity, which was, at the same time, independent of the presence of *cis*-zeatin.

Further, *Arabidopsis* plants expressing the *ZmNPP* gene under 35S promoter were prepared. The specific activity of nucleotide pyrophosphatase/phosphodiesterase with FAD as the substrate increased in the extracts from leaves of 4-week-old *Arabidopsis* plants by an order of one magnitude compared to wild type. Accordingly, the transgenic plants had decreased levels of endogenous FAD and increased levels of FMN and riboflavin. However, no significant changes in the levels of endogenous zeatin isomers were observed. Neither did the plants show any visible phenotypic changes or changes in the chlorophyll content.

To assess the function of putative *Os*NPP LOC_Os01g10020, 2-week-old rice plants in which the expression of encoding gene was enhanced by disrupting a native promoter of the gene with the promoter sequence of α-tubulin gene *OsTubA1* were examined. The insertion of T-DNA to the promotor region led to its overexpression rather than disruption. Approximately one fourth of the obtained plants were albinos, in which chlorophyll content was below the detection limit and the nucleotide pyrophosphatase/phosphodi-esterase activity increased about 50-times compared to the wild type, while green mutant plants showed only about 10-times activity increase. The total flavin content decreased about 3-times in albinos, but remained unchanged in green mutant plants.

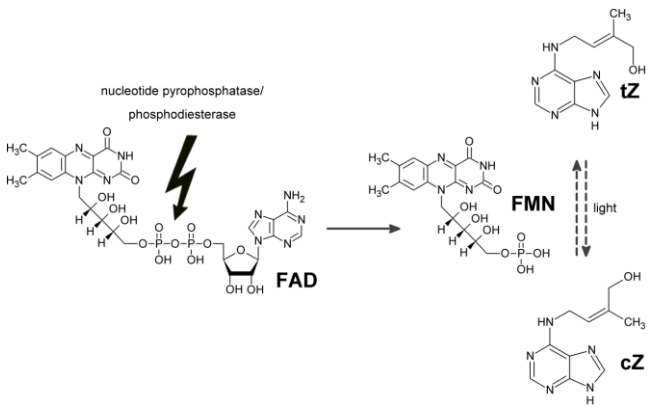

Figure 6: Proposed mode of action of "zeatin *cis-trans* isomerase".

We have proposed several possible functions for NPPs *in planta*. However, because of the wide substrate specificity of plant NPPs overlapping with other enzymes, the disclosure of their physiological function will require an integrative approach and research involving all these enzymes.

CONCLUSIONS

Here we studied the interconversions of cytokinins in maize. Maize is an interesting model, as it contains *c*Z, reportedly inactive substance, as the main cytokinin. The developing seed is the richest source of cytokinins and many of the enzymatic reactions were reported to occur there.

We have extensively profiled isoprenoid cytokinins and additional phytohormones in the reproductive organs as well as cytokinins in the vegetative organs. The results were in general in agreement with previously observed trends of peak of *t*Z content at the first third of seed development and accumulation of DHZ in desiccating seed.

Despite the observed exchange between *t*Z and DHZ as the main cytokinins at the final stage of seed development, we have observed no zeatin reductase activity, neither after feeding with radioactively labelled *t*Z, nor *in vitro*. Instead we have detected novel enzymatic activity leading to 6-(3-methylpyrrol-1-yl)purine. This substance is the first compound showing, on the one hand, anticytokinin activity and on the other hand an ability to inhibit CKX. Its binding to CKX is unique though. Hence, it can be used in the future for structural studies of CK binding or metabolizing proteins or applied for *in vivo* tissue culture studies where the CK effect needs to be suppressed. The biosynthetic enzyme was not identified because of its instability.

We had more luck with detection of the zeatin *cis-trans* isomerase activity. However, the causative enzyme was identified to be nucleotide pyrophosphatase/phosphodiesterase. Its activity was confirmed both *in vitro* and *in vivo*. The enzyme prefers dinucleotides, such as FAD. We have proposed several physiological functions of the enzyme, but more research is necessary to decipher its true relevance/significance.

There is not enough supporting evidence for zeatin isomerization *in vivo*. Upon feeding potato tubers with radioactively labeled *cis*-zeatin, only 5 to 9 % of the label was found in *trans*-zeatin (Suttle and Banowetz, 2000). Recently, *cis-trans* isomerization was reported in pathogenic fungus *Leptosphaeria maculans* (Trdá et al., 2017). First, the authors observed an increase in *cis*- or *trans*-zeatin, when the fungus was fed with the other isomer. We have observed similar occurrence ourselves when we treated maize with micromolar concentrations of zeatins, but it was not confirmed with radiolabeled cytokinins at physiological levels. Similarly to our work reported here, the *cis*-to-*trans* isomerization was approximately 3-times faster than the other way. Also, when the fungus was not added, or it was boiled prior to incubation, the conversion was smaller (Trdá et al., 2017). Thus, one can hypothesize, that nucleotide pyrophosphatase/phosphodiesterase is at play here. On the contrary, other *in vivo* experiments failed to show any zeatin isomerisation (Gajdošová et al., 2011; Kasahara et al., 2004; Miyawaki et al., 2006; Tokunaga et al., 2012; Yonekura-Sakakibara et al., 2004).

Besides the results of wet lab work, two novel hypotheses are presented. The first explains the prevalence of reputedly inactive zeatin isomer in many species. We propose this is result of zeatin *O*-glucosyltransferase's substrate preference, rather than "higher intention" because of certain advantage due to *cis*-zeatin abundance. The second proposes that there are in fact two distinct CK systems. While one aims for the strong signal leading to fast and distinguished growth, the second one is involved in maintenance growth.

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