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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; *cZOG*, *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; *tZ7G*, trans-zeatin *N7*-glucoside; *tZ9G*, *trans* zeatin *N9*-glucoside; *tZOG*, trans-zeatin *O*-glucoside; *tZR*, *trans*-zeatin 9-riboside; *tZRMP*, *trans*-zeatin 9-riboside 5′-monophosphate; *tZRMP*, *trans*-zeatin 9-riboside 5′-monophosphate; *tZROG*, *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 (*tZ*) or isopentenyladenine (iP; [4]). *tZ* 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 (*cZ*), a stereoisomer of *tZ*, is attributed solely to the tRNA decay in Arabidopsis [7]. Nevertheless, there are plant species in which *cZ* derivatives form a majority of detected CKs in contrast to Arabidopsis [8]. Whether *cZ* 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 *cZ* metabolites is also of tRNA origin in this ancestral land plant model [9].

In vitro conversion of *tZ* 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 *tZ* and *cZ* 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 *tZ* riboside (*tZR*; all CK abbreviations are in accordance to Ref. [13]) after treatment of potato tubers with *cZ*, 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 *tZ* 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]. *tZ* and *tZR* 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 *tZ* 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

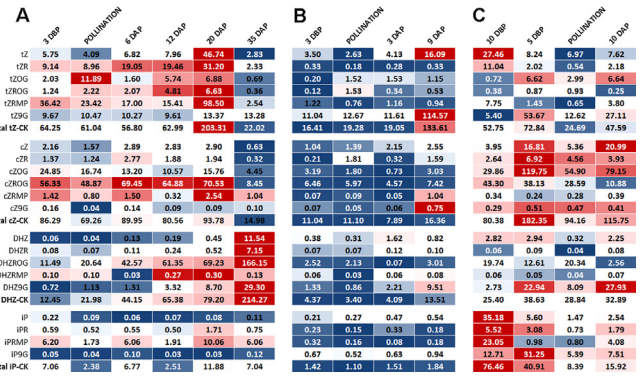

Fig. 1. Cytokinin profiling in maize reproductive organs.
The heat map shows the distribution of cytokinins in maize ovules and kernels (A), silks (B) and tassels (C) before and after pollination. The red and the blue cells correspond to higher and lower concentrations (in pmol per g of fresh weight), respectively; the scale is separate for each metabolite throughout all developmental stages in all three organs together. *tZ*, *trans*-zeatin; *tZR*, *trans*-zeatin riboside; *cZ*, *cis*-zeatin; *cZR*, *cis*-zeatin riboside; iP, isopentenyladenine; iPR, isopentenyladenosine; *tZOG*, *trans*-zeatin *O*-glucoside; *tZROG*, *trans*-zeatin riboside-*O*-glucoside; *tZ9G*, *trans*-zeatin *N9*-glucoside; *tZRMP*, *trans*-zeatin riboside 5′-monophosphate; *cZOG*, *cis*-zeatin *O*-glucoside; *cZROG*, *cis*-zeatin riboside-*O*-glucoside; *cZ9G*, *cis*-zeatin *N9*-glucoside; *cZRMP*, *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.

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 *cZ* and DHZ ribosides was detected, in addition to *tZ*, *tZR* and its monophosphate (*tZRMP*).

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 *tZ* 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