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Involvement of *cis*-Zeatin, Dihydrozeatin, and Aromatic Cytokinins in Germination and Seedling Establishment of Maize, Oats, and Lucerne

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Abstract The aims of this study were to monitor endogenous cytokinin levels during germination and early seedling establishment in oats, maize, and lucerne to determine which cytokinin forms are involved in these processes; to quantify the transfer ribonucleic acid (tRNA)-bound cytokinins; and to measure cytokinin oxidase/dehydrogenase (CKX) activity. Cytokinins were identified using UPLC-MS/MS. The predominant free cytokinins present in the dry seeds were dihydrozeatin-type (DHZ) in lucerne and maize and *c*Z-type (*cis*-zeatin) in oats. Upon imbibition, there was a large increase in *c*Z-type cytokinins in lucerne although the *c*Z-type cytokinins remained at high levels in oats. In maize, the high concentrations of DHZ-type cytokinins decreased prior to radicle emergence. Four tRNA-bound cytokinins [*cis*-zeatin riboside (*c*ZR)>N^6-(2-isopentenyl)adenosine (iPR), dihydrozeatin riboside (DHZR), *trans*-zeatin riboside (*t*ZR)] were detected in low concentrations in all three species investigated. CKX activity was measured using an in vitro radioisotope assay. The order of substrate preference was N^6-(2-isopentenyl)adenine (iP)>*trans*-zeatin (*t*Z)>*c*Z in all three species, with activity fluctuating as germination proceeded. There was a negative correlation between CKX activity and iP concentrations and a positive correlation between CKX activity and *O*-glucoside levels. As *O*-glucosides are less resistant to CKX degradation, they may provide a readily available source of cytokinins that can be converted to physiologically active cytokinins required during germination. Aromatic cytokinins made a very small contribution to the total cytokinin pool and increased only slightly during seedling establishment, suggesting that they do not play a major role in germination.

Keywords *cis*-Zeatin · Cytokinin oxidase/dehydrogenase · Dihydrozeatin · Lucerne · Maize · Oats · tRNA degradation

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Introduction

Upon drying and maturation, seeds become quiescent and are maintained in a metabolically inactive state until favorable environmental and/or chemical conditions trigger germination. Germination begins with the uptake of water by the quiescent seed. Upon imbibition, enzymes are activated and many metabolic activities commence, including the repair of damaged membranes and other

organelles, respiratory activity, protein synthesis using both existing and new messenger ribonucleic acid (mRNA), and deoxyribonucleic acid (DNA) repair and synthesis (Bewley 1997). Germination is considered complete once the radicle has elongated so that it is visible. Radicle extension is a turgor-driven process, and cell division may or may not be involved, depending on the species (Bewley 1997; Leubner-Metzger 2006). All subsequent events such as mobilization of storage reserves are linked to seedling growth and establishment (Bewley 1997). These cellular events and metabolic processes are regulated by changing levels of plant hormones and their interactions with one another (Kamínek and others 1997; Leubner-Metzger 2006). Cytokinins play an important role in promoting cell division and elongation in the embryo and providing positional information for the developing embryo (Leubner-Metzger 2006; Singh and Sawhney 1992). They are also involved in post-germination events, regulating storage reserve mobilization, and promoting root and hypocotyl growth, cotyledon expansion, and chlorophyll synthesis (Singh and Sawhney 1992; Villalobos and Martin 1992).

Seeds are a rich source of cytokinins, with evidence suggesting that they are an important site of cytokinin biosynthesis and metabolism (Emery and Atkins 2006). There are many reports of endogenous isoprenoid cytokinins being detected in mature monocotyledonous and dicotyledonous seeds (see Emery and Atkins 2006 for review), with an increasing list of species where *cis*-zeatin (*c*Z) conjugates are the predominant cytokinin forms, including rice (Takagi and others 1985), chickpea (Emery and others 1998), white lupine (Emery and others 2000), and *Pisum sativum* (Quesnelle and Emery 2007; Stirk and others 2008). In addition to isoprenoid cytokinins, a number of aromatic cytokinins have recently been identified in plants (Strnad 1997; Tarkowská and others 2003). Little is known about the occurrence and function of aromatic cytokinins in seeds, but it is possible that they also play a role in seed development (Emery and Atkins 2006).

Previously, we investigated the cytokinin profiles during germination and seedling establishment in two dicotyledonous species, *Tagetes minuta* and *Pisum sativum* (Stirk and others 2005, 2008). In both species, *c*Z derivatives and N^6-benzyladenine (BA) were the main cytokinins detected. In *Tagetes minuta* collected from the wild, a very high concentration of BA was detected in the dry achenes, with levels decreasing rapidly upon imbibition. No interconversion appeared to take place as BA was the only aromatic cytokinin detected (Stirk and others 2005). Shortly after radicle emergence, a transient peak of five *c*Z derivatives [*cis*-zeatin riboside-5′-monophosphate (*c*ZRMP)>*cis*-zeatin riboside (*c*ZR)>*c*Z>*cis*-zeatin-*O*-glucoside (*c*ZOG), and *cis*-zeatin riboside-*O*-glucoside (*c*ZROG)] was also detected, with *c*Z-type being the predominant cytokinin forms (Stirk and others 2005). Similarly in *Pisum sativum* seeds, the only cytokinins detected during germination were aromatic BA and *c*Z-type, with other isoprenoid cytokinins detected only after radicle emergence. BA was detected after 30 min of imbibition and after 5 h, when its levels had more than doubled. BA concentrations slowly decreased following radicle emergence. *meta*-Topolin (*m*T) was the only other aromatic cytokinin detected but it occurred in very low concentrations. After 5 h of imbibition, the contents of *c*ZRMP had greatly increased, with small amounts of other *c*Z derivatives (*c*Z, *c*ZR, *c*ZOG, and *c*ZROG) also being detected. Following radicle emergence, *c*ZRMP, *c*ZR, and *c*ZROG concentrations increased so that *c*Z-type were the predominant cytokinins in the developing radicle (Stirk and others 2008).

In the past, *c*Z-type cytokinins were largely overlooked as they were considered to be either biologically inactive or weakly active. As *c*Z is a normal constituent of transfer ribonucleic acid (tRNA), it was assumed that the presence of *c*Z-type in extracts was an artifact due to tRNA degradation during extraction (Emery and Atkins 2006). Similarly, the presence of BA was also overlooked as it was thought to be fully synthetic. To date, the biosynthetic and degradation pathway of aromatic cytokinins remains unknown (Mok and Mok 2001). With improved analytical methods, these cytokinins are now routinely detected in plant tissues. In an extensive study where the cytokinin profiles in the leaves and shoots of over 150 plant species were analyzed, *c*Z metabolites were found to be ubiquitous throughout the plant kingdom. In many of the monocotyledonous and dicotyledonous taxa, *c*Z-type accounted for more than 50% of the total cytokinin pool, with *c*ZOG and *c*ZROG being the most abundant (Gajdošová and others 2011). The aim of the present study was to determine if *c*Z-type and aromatic cytokinins are common forms in seeds during germination and early seedling establishment. In addition, tRNA-bound cytokinins were also quantified, and the activity and substrate specificity of cytokinin oxidase/dehydrogenase (CKX) were measured to establish if it is correlated to the endogenous cytokinins present.

Materials and Methods

As changes in *c*Z-type and aromatic cytokinins in monocotyledonous seeds have not been analyzed in detail, two important monocotyledonous crops, *Avena sativa* L. (oats cv. Heroes and cv. Witteberg) and *Zea mays* L. (maize cv. Sahara-type), and one dicotyledonous crop, *Medicago sativa* L. (lucerne cv. SA Standard), were selected for this

investigation. Lucerne was germinated in 65-mm plastic petri dishes lined with two layers of Whatman No. 1 filter paper and wetted with 3 ml of distilled water. Oats and maize were germinated in 90-mm petri dishes and wetted with 7 ml of distilled water. Additional distilled water was added as required during the experiment to keep the filter paper moist. Lucerne and maize were incubated in a controlled-environment chamber at 25 ± 1°C and oats at 20 ± 1°C, with a continuous light intensity of 10–20 μmol m^{-2} s^{-1}. Five samples were collected at various times during imbibition and germination to include dry quiescent seeds, a stage prior to radicle emergence, as the first radicles emerged, during radicle emergence (germination), and a few hours after maximum germination was achieved (early seedling establishment). Collection times differed between species and experiments depending on the rates of germination. Samples consisting of the entire seed (endosperm and embryo) were immediately frozen in liquid nitrogen, lyophilized, and then manually ground in a mortar and pestle to achieve a powder.

In the first germination trial, free endogenous cytokinins were quantified in duplicate samples per harvest time. In the second germination trial, both free and tRNA-bound cytokinins were quantified with three replicates per harvest time. Duplicate samples were also analyzed for CKX activity in the dry seeds, as the radicles began to emerge, and after maximum germination was achieved (early seedling establishment). In the first trial, once the radicle had emerged in maize, samples were separated into the root and kernel fractions for cytokinin analysis.

Quantification and Identification of Free Endogenous Cytokinins

Dried samples were analyzed for free endogenous cytokinins using a modified protocol described by Novák and others (2003, 2008). During extraction in 10 ml of Bieleski buffer (60% methanol, 25% $CHCl_3$, 10% HCOOH, and 5% H_2O), a cocktail of 23 deuterium-labeled isoprenoid and aromatic cytokinin standards (Olchemim Ltd, Czech Republic) was added, each at 3 pmol per sample, to check recovery during purification. After overnight extraction, the homogenate was centrifuged (15,000×*g*, 4°C) and the pellets re-extracted in the same way for 1 h. The samples were purified using combined cation (SCX cartridge) and anion [DEAE-Sephadex-C18 cartridge] exchanger and immunoaffinity chromatography based on a generic monoclonal cytokinin antibody (Faiss and others 1997). This procedure resulted in three fractions containing (1) the free bases, ribosides, and *N*-glucosides, (2) ribotides, and (3) *O*-glucosides. These were evaporated to dryness and dissolved in 30 μl of the mobile phase for UPLC-MS/MS analysis.

The samples were analyzed using ultra-performance liquid chromatography (UPLC) (ACQUITY UPLC® System, Waters Corp., Milford, MA, USA) linked to a Xevo® TQ-S triple-quadrupole mass spectrometer (UPLC-MS/MS) equipped with an electrospray interface [ESI(+)] and photodiode array detector (Waters PDA 2996). Samples were injected on a C18 reverse-phase column (Waters ACQUITY UPLC BEH C18; 1.7 μm; 2.1 × 50 mm), and elution was performed with a methanolic gradient composed of 100% methanol (A) and 15 mM formic acid (B) adjusted to pH 4.0 with ammonium. At a flow rate of 250 μl min^{-1}, the following protocol was used: 0 min 10% A + 90% B—8 min linear gradient; 50% A + 50% B then column equilibration. Without post-column splitting, the effluent was introduced into the PDA (scanning range = 210–400 nm with 1.2-nm resolution) with an electrospray source (source block/desolvation temperature = 120/575°C, capillary voltage = + 0.35 kV), and quantitative analysis of the different cytokinins was performed in multireaction monitoring mode with optimized conditions (cone voltage, collision energy in the collision cell, dwell time) corresponding to the exact diagnostic transition for each cytokinin (Novák and others 2008). Quantification was performed by Masslynx software using a standard isotope dilution method. The ratio of endogenous cytokinin to appropriately labeled standard was determined and further used to quantify the level of endogenous compounds in the original extract according to the known quantity of added internal standard (Novák and others 2003, 2008).

Quantification and Identification of tRNA-bound Cytokinins

tRNA was selectively extracted from 2 g DW of plant material using a method based on phenol/m-cresol treatment (Maaß and Klämbt 1981) that had previously been optimized for seed extraction. Due to the high amounts of contaminants such as starch and proteins, it was not possible to determine tRNA levels using spectrophotometry. Instead, formaldehyde agarose gel electrophoresis was run with isolated tRNA aliquots and increasing amounts of yeast tRNA standard. tRNA content was determined by gel densities using the Alpha Digi DOC software based on calibration from yeast tRNA standards (http://www.vtpup.cz/manual/PrF_rusteglad_Alphalnnotech_AlphaDigiDoc_datasheet_EN.pdf).

To quantify the tRNA-bound cytokinins, an aliquot of the isolated tRNA pellet was hydrolyzed overnight in 100 mM NaOH and dephosphorylated by alkaline phosphatase. Internal cytokinin standards were added and the samples purified by immunoaffinity chromatography. In addition, the free cytokinins in these samples were quantified by UPLC-MS/MS as described above.

Determination of CKX Activity and Substrate Specificity

Duplicate dried samples of lucerne, oats, and maize harvested at three time points were extracted and partially purified using the method described by Motyka and others (2003). The CKX activity and substrate specificity were measured using an in vitro radioisotope assay described by Gajdošová, Spíchal, and others (2011). This bioassay is based on the conversion of labeled cytokinins [2-^{3}H]N^{6}-(2-isopentenyl)adenine (iP), [2-^{3}H]*trans*-zeatin (*t*Z), and [2-^{3}H]*c*Z to [^{3}H]adenine. The assay mixture comprised 100 mM TAPS-NaOH buffer with 75 µM 2,6-dichloroindophenol (pH 8.5), 2 µM labeled cytokinin and the enzyme preparation that was optimized for each species (equivalent to 0.3125 mg tissue FW for oats and maize and 40 mg tissue FW for lettuce). Following incubation for 30 min at 37°C, the reaction was terminated and the substrate and product separated by HPLC (Gajdošová, Spíchal, and others 2011). Bovine albumin was used as a standard to measure protein concentrations (Bradford 1976).

Results

Cytokinin yields ranged from 0.28 to 38% DW, with the *O*-glucosides and ribotides having the lowest recovery due to enzymatic cleavage. However, the cytokinin concentrations could be accurately calculated as internal standards were used for all the cytokinin derivatives.

Endogenous Free Cytokinins

In the first germination trial, the cytokinin complement in the dry lucerne seeds was predominantly *c*Z-type cytokinins (76%), especially *c*ZRMP. Very low concentrations of iP-, *t*Z-, dihydrozeatin-type (DHZ), and aromatic cytokinins were measured (Table 1). Within 12 h of imbibition there was a large increase in all *c*Z conjugates, with *c*ZRMP>*c*ZROG>*c*ZR being detected in the highest concentrations. The concentration of the *c*Z-type cytokinins remained high during germination and early seedling establishment. The concentration of iP- and DHZ-type cytokinins increased slightly at the onset of germination, whereas the levels of *t*Z-type cytokinins did not change for the duration of the experiment. Aromatic cytokinin concentrations increased within 12 h due to an increase in *m*T-type cytokinins, especially *m*T and *meta*-topolin riboside-5′-monophosphate (*m*TRMP) and remained at these elevated concentrations for the duration of the experiment. Some BA derivatives were also detected following imbibition (Table 1).

Table 1 Free endogenous cytokinins in germinating lucerne (cv. SA Standard) incubated at 25°C

Cytokinin	Time after imbibition				
	Dry	12 h	24 h	30 h	36 h
Germination	0%	6%	81%	82%	82%
Free cytokinin concentration (pmol g^{-1} DW)					
iP	–	0.21	1.83	2.69	3.89
iPR	3.11	5.92	3.22	2.40	1.82
iPR9G	–	–	0.10	0.21	0.47
iPRMP	4.87	18.06	14.95	12.74	9.18
Total iP	**7.98**	**24.19**	**20.10**	**18.04**	**15.36**
*t*Z	–	0.95	0.72	0.54	0.54
*t*ZR	0.51	0.87	0.85	0.67	0.39
*t*ZOG	–	1.21	–	–	–
Total *t*Z	**0.51**	**3.03**	**1.57**	**1.21**	**0.93**
*c*Z	0.37	1.37	8.20	13.70	17.48
*c*ZR	1.93	32.00	26.7	11.59	11.26
*c*ZOG	0.31	1.98	8.83	19.59	41.26
*c*ZROG	3.65	59.29	29.29	25.83	21.88
*c*Z9G	0.30	0.50	0.65	0.53	1.03
*c*ZRMP	43.55	252.38	163.82	367.54	138.53
Total *c*Z	**50.11**	**347.52**	**237.40**	**438.78**	**231.44**
DHZ	0.18	0.01	0.46	1.35	1.86
DHZR	4.58	8.48	7.48	6.54	5.10
DHZOG	–	–	–	0.02	0.66
DHZROG	1.57	2.25	1.69	1.53	1.22
DHZ9G	0.57	0.32	0.25	0.49	0.43
DHZRMP	–	–	3.93	–	3.62
Total DHZ	**6.90**	**11.06**	**13.81**	**9.93**	**12.89**
Total Isoprenoid	**65.50**	**385.80**	**272.97**	**467.96**	**260.62**
BA	–	9.66	–	–	4.53
BAR	–	0.36	0.88	0.98	2.21
BARMP	–	6.20	3.96	2.41	9.97
*m*T	0.05	47.07	26.66	25.92	47.29
*m*TR	–	5.22	9.41	11.98	17.03
*m*TRMP	–	33.81	46.60	33.51	44.63
*o*T	0.25	0.27	0.30	0.37	0.36
Total Aromatic	**0.30**	**102.59**	**87.81**	**75.17**	**126.02**

"–", below the limit of detection ($n = 2$)

In the second germination trial, the cytokinin complement in the dry lucerne seeds was predominantly DHZ- (46%) and *c*Z-type (27%) cytokinins, with lower concentrations of iP- and *t*Z-type cytokinins measured. The only aromatic cytokinins detected in the dry seeds were N^{6}-benzyladenosine-5′-monophosphate (BARMP) and *meta*-topolin-*O*-glucoside (*m*TOG) occurring at very low concentrations (Fig. 1a; Table 2). The general trend was an increase in the total cytokinin pool following imbibition with a slight decrease during seedling establishment. Within 10 h of imbibition, there was an eightfold increase

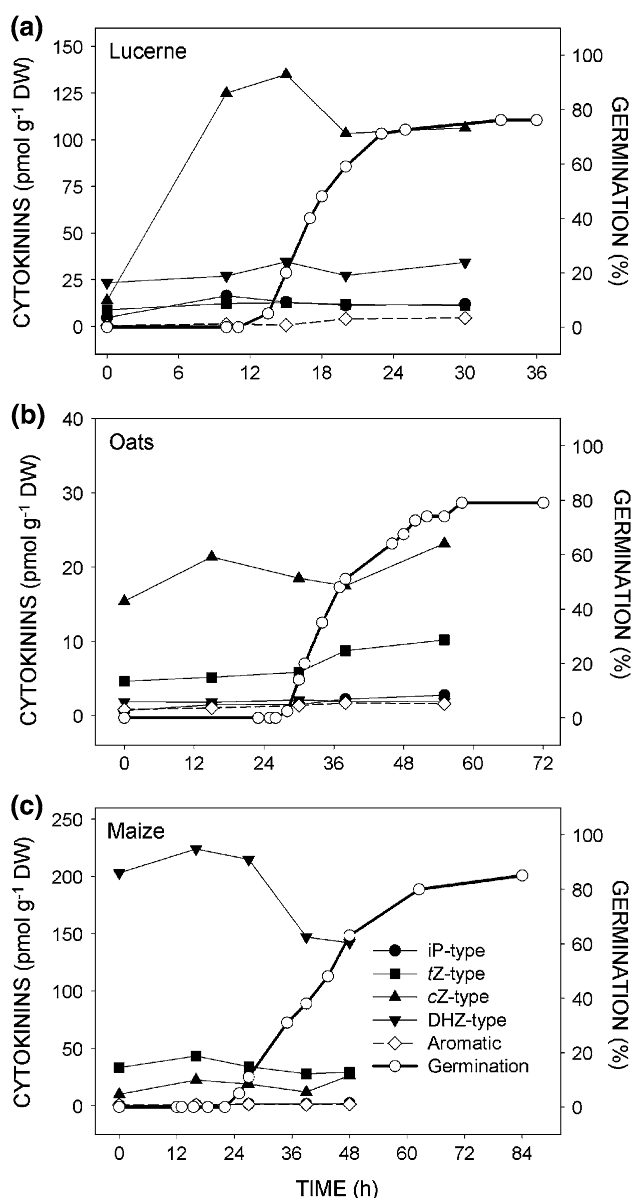

Fig. 1 Germination curves (% radicle emergence) and changes over time in the concentrations of endogenous free cytokinin types detected in germinating **a** lucerne, **b** oats, and **c** maize ($n = 3$)

in the *c*Z derivatives, with *c*ZR>*c*ZRMP>*c*ZROG being detected in the highest concentrations (Table 2). The concentration of the *c*Z-type cytokinins remained high following radicle emergence and decreased slightly during early seedling establishment. The concentration of DHZ- and iP-type cytokinins increased slightly following imbibition whereas the levels of *t*Z-type cytokinins remained very low and did not change for the duration of the experiment. Aromatic cytokinin concentrations increased slightly following imbibition but remained at these low concentrations for the duration of the experiment. The highest concentrations of aromatic cytokinins were measured during seedling establishment (Fig. 1a; Table 2). The *O*-glucosides were the prevalent conjugate type in the dry lucerne seeds. Following imbibition, the % ribosides and ribotides increased whereas the free bases and 9-glucosides contributed only a small percentage to the total cytokinin pool (Fig. 2a).

In the first germination trial with oats (cv. Heroes), *c*Z-type cytokinins made up 93% of the total cytokinin pool in the dry caryopses, with very low concentrations of iP-, *t*Z-type, and aromatic cytokinins. No DHZ-types were detected for the duration of the experiment (Table 3). With the commencement of germination, there was a decrease in *c*Z-type concentrations (12 and 24 h). However, following radicle emergence, the concentration of *c*Z-type cytokinins increased again. After 12 h, the concentration of *t*Z-type had greatly increased but decreased again prior to radicle emergence (24 h) and remained low for the duration of the experiment. iP-Type cytokinins remained at low concentrations during germination but increased slightly during seedling establishment (48 and 60 h), especially N^6-(2-isopentenyl)adenosine-5′-monophosphate (iPRMP). There was an increase in the concentration of *m*T 12 h after imbibition, and its concentration decreased thereafter. BA derivatives were detected only during early seedling establishment (60 h; Table 3).

In the second germination trial with oats (cv. Witteberg), *c*Z-type cytokinins made up 86% of the total cytokinin pool in the dry oat caryopses, with lower concentrations of *t*Z-, DHZ-, and iP-type cytokinins. A number of aromatic cytokinins [BA-, *m*T-, *ortho*-topolin- (*o*T), and *para*-topolin- (*p*T) types] were detected but occurred at very low concentrations (Fig. 1b; Table 4). Cytokinin concentrations increased following imbibition and continued increasing during seedling establishment. This increase was due mainly to fluctuating concentrations of *c*Z-type cytokinins, and by 38 h (during radicle emergence) the concentration of *t*Z-type had also increased. iP-Type cytokinins remained at low concentrations prior to radicle emergence but increased slightly during radicle emergence (30 h) and seedling establishment (38 h and 55 h), especially iPRMP. There was a threefold increase in the concentration of *m*TOG after imbibition whereas the concentration of the numerous other aromatic cytokinins remained very low (Fig. 1b; Table 4). The *O*-glucoside conjugates were the prevalent cytokinin type in the dry oat caryopses and remained so throughout germination and early seedling establishment. Following imbibition, the percentage of ribotides, free bases, and ribosides increased, with the free bases and ribosides decreasing during early seedling establishment. The 9-glucosides contributed only a small percentage to the total cytokinin pool (Fig. 2b).

In the first germination trial, the dry kernels of maize had high levels of aromatic BA and isoprenoid DHZ-type cytokinins (46 and 43%, respectively, of the total cytokinin

Table 2 Free and tRNA-bound cytokinins in lucerne (cv. SA Standard) germinated at 25°C

Cytokinin	Time after imbibition				
	Dry (0 h)	10 h	15 h	20 h	30 h
Germination	0%	0%	20%	71%	76%
Free cytokinin concentration (pmol g^{-1} DW)					
iP	0.34 ± 0.13	1.07 ± 0.24	0.78 ± 0.22	0.52 ± 0.08	1.43 ± 0.09
iPR	2.01 ± 0.46	5.18 ± 0.30	4.40 ± 0.94	2.93 ± 0.41	2.13 ± 0.21
iPR9G	0.09 ± 0.03	0.12 ± 0.02	0.12 ± 0.01	0.14 ± 0.02	0.27 ± 0.06
iPRMP	2.22 ± 0.61	9.97 ± 2.06	7.74 ± 0.99	7.66 ± 1.17	8.06 ± 0.74
Total iP	**4.66**	**16.34**	**13.04**	**11.25**	**11.89**
*t*Z	0.36 ± 0.21	0.80 ± 0.19	0.86 ± 0.19	0.65 ± 0.10	0.68 ± 0.11
*t*ZR	0.83 ± 0.20	2.72 ± 0.50	2.95 ± 0.50	2.58 ± 0.40	2.06 ± 0.29
*t*ZOG	4.02 ± 0.81	3.43 ± 0.84	3.40 ± 0.39	3.05 ± 1.80	3.43 ± 1.79
*t*ZROG	2.33 ± 0.75	2.26 ± 0.09	2.53 ± 0.03	1.92 ± 0.10	1.63 ± 0.05
*t*Z9G	0.43 ± 0.18	0.42 ± 0.16	0.44 ± 0.19	0.32 ± 0.12	0.29 ± 0.10
*t*ZRMP	0.94 ± 0.22	2.55 ± 0.83	2.36 ± 0.85	3.29 ± 1.54	3.05 ± 0.79
Total *t*Z	**8.91**	**12.18**	**12.54**	**11.81**	**11.14**
*c*Z	0.57 ± 0.25	7.90 ± 1.79	6.06 ± 1.55	4.02 ± 0.64	5.50 ± 1.10
*c*ZR	3.09 ± 0.70	51.53 ± 2.78	54.63 ± 12.07	44.05 ± 4.18	38.21 ± 3.00
*c*ZOG	0.64 ± 0.18	4.97 ± 0.60	7.50 ± 1.64	5.78 ± 1.33	9.78 ± 5.89
*c*ZROG	2.63 ± 0.35	17.71 ± 5.32	26.15 ± 7.55	9.82 ± 3.62	15.70 ± 0.93
*c*Z9G	1.99 ± 0.47	3.21 ± 0.41	3.10 ± 0.57	3.40 ± 0.34	3.42 ± 0.22
*c*ZRMP	5.00 ± 0.96	39.63 ± 9.36	37.53 ± 8.64	36.30 ± 19.81	33.79 ± 11.43
Total *c*Z	**13.92**	**124.95**	**134.97**	**103.37**	**106.40**
DHZ	1.48 ± 0.63	2.10 ± 0.14	1.95 ± 0.57	1.59 ± 0.21	1.50 ± 0.14
DHZR	1.17 ± 0.34	3.78 ± 0.21	4.23 ± 1.27	3.40 ± 0.38	3.21 ± 0.17
DHZOG	8.45 ± 3.84	7.92 ± 0.28	13.60 ± 0.72	13.40 ± 1.16	21.19 ± 12.07
DHZROG	11.02 ± 3.12	10.50 ± 0.95	13.00 ± 1.66	6.58 ± 1.26	6.54 ± 0.36
DHZ9G	0.29 ± 0.10	0.34 ± 0.05	0.33 ± 0.05	0.34 ± 0.10	0.26 ± 0.03
DHZRMP	0.91 ± 0.66	2.34 ± 1.63	1.48 ± 0.82	1.90 ± 1.22	1.60 ± 0.76
Total DHZ	**23.32**	**26.98**	**34.59**	**27.21**	**34.30**
Total Isoprenoid	**50.81**	**180.45**	**195.14**	**153.64**	**163.73**
BA	–	–	–	2.74 ± 0.58	3.43 ± 2.22
BARMP	0.09 ± 0.09	0.11 ± 0.09	0.07 ± 0.03	0.08 ± 0.00	0.04 ± 0.03
*m*TOG	–	–	0.15 ± 0.05	1.09 ± 0.11	0.67 ± 0.76
*m*TROG	0.26 ± 0.04	0.25 ± 0.06	0.22 ± 0.07	0.10 ± 0.00	0.10 ± 0.02
*o*T	–	0.86 ± 0.34	0.19 ± 0.04	–	0.21 ± 0.06
*p*T	–	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.01
Total Aromatic	**0.35**	**1.23**	**0.64**	**4.02**	**4.47**
tRNA-bound cytokinin concentration (pmol g^{-1} DW)					
iPR	3.01 ± 0.51	2.24 ± 0.72	1.05 ± 0.20	1.36 ± 0.68	1.55 ± 0.82
*t*ZR	0.05 ± 0.02	0.04 ± 0.02	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
*c*ZR	6.65 ± 1.21	7.10 ± 2.24	4.21 ± 0.82	5.49 ± 2.06	6.15 ± 1.97
DHZR	0.31 ± 0.08	0.39 ± 0.15	0.20 ± 0.05	0.30 ± 0.14	0.35 ± 0.16
Total tRNA-bound	**10.02**	**9.77**	**5.48**	**7.18**	**8.09**
tRNA (μg g^{-1} DW)	114.2 ± 27.6	70.7 ± 53.4	89.38 ± 19.6	114.4 ± 30.1	240.8 ± 89.0

Results are given as mean ± SD ($n = 3$)

"–", below the limit of detection

content). Very low concentrations of the other isoprenoid and aromatic cytokinins were detected (Table 5). The concentration of all isoprenoid cytokinins remained fairly constant during germination. However, following radicle emergence there was a large peak in *c*Z-type cytokinins, due mainly to *c*ZRMP after 48 h in the root fraction. By 55 h, the concentration was decreasing. Similarly, iP- and *t*Z-type cytokinins also showed an increase in

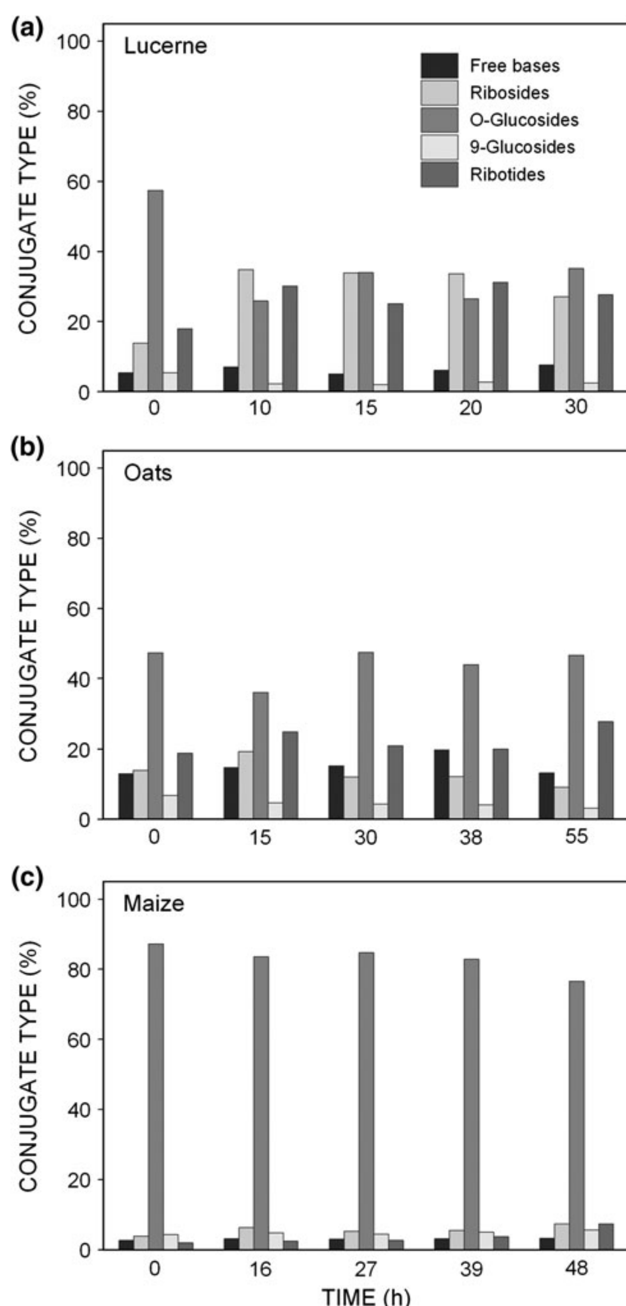

Fig. 2 Ratios of the various cytokinin conjugates in germinating **a** lucerne, **b** oats, and **c** maize ($n = 3$)

concentration following radicle emergence in the root fraction (48 h), whereas the concentrations of these isoprenoid cytokinin types remained steady in the kernel (Table 5). The concentration of the DHZ-type cytokinins did not fluctuate much during the entire experiment, with high concentrations also being detected in the kernel following radicle emergence. BA concentrations decreased following imbibition. Aromatic cytokinins increased in the root fraction following radicle emergence as well as high concentrations of BA detected in the kernel at 55 h (Table 5).

Table 3 Free endogenous cytokinins in germinating oat (cv. Heros) incubated at 20°C

Cytokinin	Time after imbibition				
	Dry	12 h	24 h	48 h	60 h
Germination	0%	0%	2%	60%	63%
Free cytokinin concentration (pmol g^{-1} DW)					
iP	0.02	0.15	0.07	0.26	0.18
iPR	0.01	0.22	0.25	0.93	1.40
iPR9G	–	–	–	0.07	0.38
iPRMP	–	0.34	0.33	2.11	3.31
Total iP	**0.03**	**0.71**	**0.65**	**3.37**	**5.27**
*t*Z	0.01	28.68	0.18	0.13	0.12
*t*ZR	0.05	0.95	0.22	0.26	0.64
*t*ZOG	–	2.12	–	–	–
Total *t*Z	**0.06**	**31.75**	**0.40**	**0.39**	**0.76**
*c*Z	0.34	4.44	0.53	0.56	0.47
*c*ZR	0.71	2.11	2.18	3.34	4.93
*c*ZOG	0.30	2.33	0.27	0.52	0.67
*c*ZROG	0.35	0.92	0.53	1.96	5.16
*c*ZRMP	13.47	–	–	–	–
Total *c*Z	**15.17**	**9.80**	**3.51**	**6.38**	**11.23**
Total Isoprenoid	**15.26**	**42.26**	**4.56**	**10.14**	**17.26**
BA	–	–	–	–	6.27
BAR	–	0.20	–	–	0.27
BA9G	–	–	–	0.09	0.15
*m*T	0.38	3.39	1.41	1.07	0.45
*o*T	0.30	0.35	0.32	0.33	0.38
Total Aromatic	**0.68**	**3.94**	**1.73**	**1.49**	**7.52**

"–", below the limit of detection ($n = 2$)

In the second germination trial, DHZ-type cytokinins (82% of the total cytokinin content) were prevalent in the dry kernels of maize, followed by *t*Z-type cytokinins (13%). Lower concentrations of the other isoprenoid cytokinins (iP- and *c*Z-type) were detected. Aromatic cytokinins were BA and *m*T-type that were present at very low concentrations (Fig. 1c; Table 6). The concentration of all the isoprenoid cytokinins initially increased slightly upon imbibition (16 h) and then decreased, especially the *c*Z-type, prior to radicle emergence. All aromatic cytokinins remained at low concentrations upon imbibition and increased slightly during early seedling establishment (48 h; Fig. 1c; Table 6). The ratio of the different conjugate types remained fairly constant in the dry kernels and during germination and early seedling establishment, with the *O*-glucosides making up over 76% of the total cytokinin pool (Fig. 2c).

Table 4 Free and tRNA-bound cytokinins in oats (cv. Witteberg) germinated at 20°C

Cytokinin	Time after imbibition				
	Dry (0 h)	15 h	30 h	38 h	55 h
Germination	0%	0%	14%	51%	74%
Free cytokinin concentration (pmol g^{-1} DW)					
iP	0.28 ± 0.01	0.29 ± 0.02	0.23 ± 0.00	0.48 ± 0.42	0.39 ± 0.05
iPR	0.19 ± 0.01	0.33 ± 0.01	0.18 ± 0.01	0.38 ± 0.29	0.22 ± 0.01
iPR9G	0.02 ± 0.00	0.02 ± 0.00	–	–	0.16 ± 0.03
iPRMP	0.14 ± 0.09	0.82 ± 0.19	1.08 ± 0.15	1.33 ± 0.15	1.96 ± 0.24
Total iP	**0.63**	**1.46**	**1.49**	**2.19**	**2.73**
*t*Z	0.97 ± 0.33	0.79 ± 0.05	0.70 ± 0.08	0.89 ± 0.29	0.91 ± 0.29
*t*ZR	0.13 ± 0.01	0.18 ± 0.03	0.13 ± 0.01	0.14 ± 0.02	0.19 ± 0.02
*t*ZOG	1.60 ± 0.17	2.08 ± 0.59	3.06 ± 0.80	5.20 ± 0.93	6.47 ± 0.42
*t*ZROG	0.17 ± 0.02	0.19 ± 0.04	0.16 ± 0.03	0.15 ± 0.01	0.25 ± 0.00
*t*Z9G	1.20 ± 0.17	1.06 ± 0.06	0.89 ± 0.10	0.94 ± 0.20	0.68 ± 0.21
*t*ZRMP	0.54 ± 0.44	0.85 ± 0.05	0.88 ± 0.29	1.41 ± 0.53	1.68 ± 1.25
Total *t*Z	**4.61**	**5.15**	**5.82**	**8.73**	**10.18**
*c*Z	1.19 ± 0.22	3.00 ± 0.27	2.83 ± 0.16	3.76 ± 1.46	3.44 ± 0.50
*c*ZR	2.77 ± 0.44	5.23 ± 0.49	3.04 ± 0.29	3.12 ± 0.01	3.09 ± 0.70
*c*ZOG	4.01 ± 0.67	4.64 ± 0.16	6.03 ± 0.42	4.82 ± 0.35	6.84 ± 1.23
*c*ZROG	3.86 ± 1.29	2.68 ± 0.54	2.47 ± 0.61	2.05 ± 0.57	2.60 ± 0.11
*c*Z9G	0.10 ± 00.01	0.10 ± 0.02	0.09 ± 0.03	0.10 ± 0.04	0.13 ± 0.04
*c*ZRMP	3.45 ± 2.26	5.70 ± 3.46	4.03 ± 3.03	3.60 ± 1.86	7.07 ± 0.32
Total *c*Z	**15.38**	**21.35**	**18.49**	**17.45**	**23.17**
DHZ	0.14 ± 0.04	0.16 ± 0.01	0.26 ± 0.04	0.40 ± 0.24	0.22 ± 0.01
DHZR	0.16 ± 0.04	0.19 ± 0.03	0.16 ± 0.01	0.21 ± 0.12	0.12 ± 0.01
DHZOG	0.60 ± 0.12	0.66 ± 0.09	0.99 ± 0.24	0.82 ± 0.26	0.82 ± 0.06
DHZROG	0.57 ± 0.16	0.32 ± 0.11	0.30 ± 0.14	0.19 ± 0.06	0.24 ± 0.04
DHZ9G	0.27 ± 0.03	0.24 ± 0.01	0.28 ± 0.03	0.25 ± 0.01	0.25 ± 0.05
DHZRMP	0.10 ± 0.04	0.21 ± 0.06	0.08 ± 0.07	0.03 ± 0.02	0.19 ± 0.25
Total DHZ	**1.84**	**1.78**	**2.07**	**1.90**	**1.84**
Total Isoprenoid	**22.46**	**29.74**	**27.87**	**30.27**	**37.92**
BAR	–	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
BA9G	–	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	–
BARMP	0.15 ± 0.10	0.10 ± 0.05	0.06 ± 0.03	0.03 ± 0.01	0.09 ± 0.09
*m*T	0.34 ± 0.13	0.25 ± 0.22	0.31 ± 0.20	0.57 ± 0.48	0.22 ± 0.12
*m*TR	–	–	–	0.03	–
*m*TOG	0.21 ± 0.26	0.57 ± 0.17	0.77 ± 0.40	0.75 ± 0.49	1.19 ± 0.57
*m*TROG	–	–	0.08 ± 0.04	0.06 ± 0.00	–
*o*T	0.01 ± 0.01	–	0.02 ± 0.01	0.07 ± 0.09	0.01 ± 0.01
*p*T	0.10 ± 0.01	0.06 ± 0.03	0.08 ± 0.03	0.16 ± 0.14	0.05 ± 0.01
Total Aromatic	**0.81**	**1.00**	**1.34**	**1.69**	**1.57**
tRNA-bound cytokinin concentration (pmol g^{-1} DW)					
iPR	0.03 ± 0.03	0.04 ± 0.02	0.06 ± 0.02	0.07 ± 0.02	0.06 ± 0.04
*t*ZR	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
*c*ZR	1.48 ± 0.97	1.55 ± 0.50	2.09 ± 0.57	2.12 ± 0.44	1.78 ± 0.63
DHZR	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
Total tRNA-bound	**1.54**	**1.61**	**2.19**	**2.23**	**1.87**
tRNA (μg g^{-1} DW)	236.4 ± 165.6	175.3 ± 86.9	214.2 ± 57.8	187.8 ± 69.5	204.8 ± 8.1

Results are given as mean ± SD ($n = 3$)

"–", below the limit of detection

Table 5 Free endogenous cytokinins in germinating maize (cv. Sahara type) incubated at 25°C

Cytokinin	Time after imbibition						
				48 h		55 h	
	Dry	24 h	36 h	Root	Kernel	Root	Kernel
Germination	0%	0%	29%	49%		75%	
Free cytokinin concentration (pmol g^{-1} DW)							
iP	–	–	–	0.57	–	2.73	–
iPR	0.03	–	0.03	6.17	0.05	11.00	0.09
iPR9G	–	–	0.15	22.49	0.25	17.53	0.18
iPRMP	–	–	–	32.77	1.66	33.09	1.63
Total iP	**0.03**	–	**0.18**	**62.00**	**1.96**	**64.35**	**1.90**
*t*Z	1.53	0.11	0.22	3.13	0.44	4.28	0.52
*t*ZR	0.17	0.26	0.32	6.45	0.31	5.98	0.32
*t*ZOG	0.70	0.57	0.93	4.36	1.03	3.15	0.79
*t*Z9G	4.17	2.70	3.55	39.88	3.20	24.34	4.98
Total *t*Z	**6.57**	**3.64**	**5.02**	**53.82**	**4.98**	**37.76**	**6.61**
*c*Z	0.24	0.21	0.22	16.23	0.14	14.76	0.20
*c*ZR	0.20	0.37	0.81	10.20	0.25	8.15	0.33
*c*ZOG	0.33	0.24	0.42	20.60	0.23	11.51	–
*c*ZROG	0.31	0.31	0.50	9.52	–	7.10	0.30
*c*Z9G	–	–	–	0.29	–	0.52	–
*c*ZRMP	–	–	–	465.09	7.96	59.86	–
Total *c*Z	**1.08**	**1.13**	**1.95**	**521.93**	**8.58**	**101.90**	**0.83**
DHZ	3.55	3.08	4.28	3.39	4.29	3.09	4.79
DHZR	10.28	17.73	26.73	16.24	15.43	11.10	23.17
DHZOG	4.87	3.65	5.49	8.69	4.82	2.28	3.99
DHZROG	12.07	9.90	11.76	8.79	8.71	4.21	10.91
DHZ9G	0.74	0.84	1.07	3.50	1.67	2.39	1.50
Total DHZ	**31.51**	**35.20**	**49.33**	**40.61**	**34.92**	**23.07**	**44.36**
Total Isoprenoid	**39.19**	**39.97**	**56.48**	**678.36**	**50.44**	**227.07**	**53.70**
BA	34.15	–	–	–	–	38.54	25.17
BAR	–	–	–	0.74	0.29	1.07	–
*m*T	1.06	8.48	–	1.06	0.05	8.48	0.59
*o*T	0.40	0.09	0.06	0.90	0.16	1.87	0.09
Total Aromatic	**35.61**	**8.57**	**0.06**	**2.70**	**0.50**	**49.96**	**25.85**

Following radical emergence, the samples were divided into the root fraction and remaining kernel fraction

"–", below the limit of detection ($n = 2$)

tRNA-bound Cytokinins

In lucerne, the amount of isolated tRNA initially decreased during germination but more than doubled during early seedling establishment (30 h; Table 2). Four cytokinins [*c*ZR>N^6-(2-isopentenyl)adenosine (iPR)>dihydrozeatin riboside (DHZR)>*trans*-zeatin (*t*ZR)] were detected in the tRNA extracts and these generally occurred in much lower concentrations compared to the free cytokinin forms. The exception was the tRNA-bound iPR which was detected in higher concentrations than the free iPR in the dry lucerne seeds. The contents of tRNA-bound cytokinins did not follow the same trend as the free cytokinins, instead remaining at a fairly constant level for the duration of the experiment (Table 2). This is the first report of DHZR being detected in tRNA.

The amount of tRNA isolated from oats remained at high concentrations for the duration of the experiment (Table 4). As with lucerne, the same four cytokinin ribosides were detected, with *c*ZR being the prevalent form. Concentrations were much lower than those of the free cytokinins and, unlike the free cytokinins, they did not increase during the course of the experiment (Table 4).

The amount of tRNA isolated from maize increased as germination progressed and remained at these elevated levels during early seedling establishment (Table 6). Similar to lucerne and oats, *c*ZR was the prevalent cytokinin, with DHZR, iPR, and *t*ZR detected only in very low

Table 6 Free and tRNA-bound cytokinins in maize (cv. Sahara type) germinated at 25°C

Cytokinin	Time after imbibition				
	Dry (0 h)	16 h	27 h	39 h	48 h
Germination	0%	0%	11%	38%	63%
Free cytokinin concentration (pmol g^{-1} DW)					
iP	0.01 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.03	0.09 ± 0.00
iPR	0.04 ± 0.01	0.05 ± 0.01	0.09 ± 0.01	0.12 ± 0.02	0.28 ± 0.00
iPR9G	0.11 ± 0.03	0.12 ± 0.02	0.11 ± 0.03	0.22 ± 0.03	0.20 ± 0.05
iPRMP	0.11 ± 0.08	0.44 ± 0.08	1.16 ± 0.14	0.94 ± 0.10	1.47 ± 0.29
Total iP	**0.27**	**0.66**	**1.40**	**1.34**	**2.04**
*t*Z	3.06 ± 1.40	2.98 ± 0.83	2.09 ± 0.24	1.56 ± 0.10	1.85 ± 0.13
*t*ZR	0.97 ± 0.11	1.37 ± 0.15	1.25 ± 0.18	1.09 ± 0.12	1.33 ± 0.14
*t*ZOG	12.92 ± 3.48	16.06 ± 5.05	12.15 ± 2.33	10.39 ± 1.54	9.83 ± 3.06
*t*ZROG	6.05 ± 1.35	9.05 ± 1.32	5.82 ± 1.00	4.71 ± 0.63	3.69 ± 1.18
*t*Z9G	9.10 ± 0.95	12.06 ± 2.13	10.43 ± 2.38	7.63 ± 1.15	9.52 ± 1.08
*t*ZRMP	1.05 ± 0.24	1.77 ± 0.80	2.26 ± 0.34	2.43 ± 0.61	3.09 ± 0.59
Total *t*Z	**33.15**	**43.29**	**34.00**	**27.81**	**29.31**
*c*Z	0.26 ± 0.04	1.23 ± 0.18	0.94 ± 0.18	0.50 ± 0.05	1.12 ± 0.09
*c*ZR	1.32 ± 0.47	5.40 ± 0.98	4.48 ± 2.61	2.15 ± 0.46	6.32 ± 2.55
*c*ZOG	2.72 ± 0.35	3.51 ± 0.95	2.74 ± 0.97	2.49 ± 0.27	2.45 ± 0.54
*c*ZROG	4.49 ± 1.39	9.73 ± 3.72	8.78 ± 2.93	4.96 ± 2.48	8.15 ± 4.98
*c*Z9G	0.07 ± 0.01	–	0.03 ± 0.06	0.06 ± 0.01	0.11 ± 0.03
*c*ZRMP	1.01 ± 0.18	2.51 ± 0.84	1.85 ± 0.52	1.77 ± 0.16	8.13 ± 0.94
Total *c*Z	**9.87**	**22.38**	**18.82**	**11.93**	**26.28**
DHZ	3.26 ± 0.55	4.69 ± 0.20	4.93 ± 0.55	3.78 ± 0.50	3.44 ± 0.13
DHZR	7.22 ± 1.36	11.28 ± 1.05	8.30 ± 3.00	6.99 ± 0.68	6.81 ± 1.07
DHZOG	66.32 ± 23.27	64.60 ± 18.53	70.20 ± 7.17	49.58 ± 5.34	55.55 ± 7.70
DHZROG	122.41 ± 46.23	139.71 ± 26.85	128.38 ± 14.63	83.76 ± 25.55	72.98 ± 25.23
DHZ9G	1.36 ± 0.25	1.67 ± 0.10	1.44 ± 0.23	1.47 ± 0.27	1.30 ± 0.10
DHZRMP	2.45 ± 0.68	2.08 ± 0.58	1.55 ± 1.22	1.50 ± 0.29	1.92 ± 1.33
Total DHZ	**203.02**	**224.03**	**214.80**	**147.08**	**142.00**
Total Isoprenoid	**246.31**	**290.36**.	**269.02**	**188.16**	**199.63**
BAR	0.02 ± 0.03	0.00 ± 0.01	–	0.01 ± 0.01	0.01 ± 0.01
BARMP	0.29 ± 0.19	0.25 ± 0.08	0.33 ± 0.08	0.26 ± 0.12	0.15 ± 0.06
*m*TR	0.01 ± 0.01	0.04 ± 0.02	0.03 ± 0.05	–	–
*m*TOG	0.21 ± 0.02	0.22 ± 0.21	0.83 ± 0.72	0.70 ± 0.24	0.96 ± 0.52
*m*TROG	0.04 ± 0.02	0.14 ± 0.11	0.03 ± 0.02	0.08 ± 0.07	0.16 ± 0.12
*o*T	–	–	0.01 ± 0.01	–	–
Total Aromatic	**0.57**	**0.65**	**1.23**	**1.05**	**1.28**
tRNA-bound cytokinin concentration (pmol g^{-1} DW)					
iPR	0.02 ± 0.02	0.01 ± 0.01	0.04 ± 0.02	0.06 ± 0.01	0.25 ± 0.06
*t*ZR	0.04 ± 0.04	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.02	0.03 ± 0.01
*c*ZR	1.56 ± 0.48	1.09 ± 0.11	0.98 ± 0.27	1.19 ± 0.16	2.85 ± 0.60
DHZR	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.04 ± 0.01	0.11 ± 0.02
Total tRNA-bound	**1.66**	**1.14**	**1.06**	**1.31**	**3.24**
tRNA (μg g^{-1} DW)	130.7 ± 71.0	98.5 ± 8.6	319.7 ± 102.8	267.8 ± 135.0	315.3 ± 21.7

Results are given as mean ± SD ($n = 3$)

"–", below the limit of detection

Table 7 Activity and substrate specificity of crude CKX extracts of lucerne, oats, and maize measured in an in vitro radioisotope assay

Species	Time after imbibition	CKX activity (nmol Ade mg^{-1} protein h^{-1})		
		[^{3}H]iP	[^{3}H]*t*Z	[^{3}H]*c*Z
Lucerne	0 h	2.5 ± 0.1	0.6 ± 0.0	0.1 ± 0.0
	13 h	1.5 ± 0.2	0.2 ± 0.1	0.1 ± 0.0
	24 h	2.1 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
Oats	0 h	137.0 ± 23.8	21.5 ± 1.3	3.6 ± 0.0
	30 h	195.5 ± 32.9	22.5 ± 2.1	4.0 ± 0.4
	48 h	161.6 ± 6.0	21.1 ± 2.4	4.0 ± 0.0
Maize	0 h	362.4 ± 26.0	13.0 ± 0.2	1.0 ± 0.1
	27 h	283.8 ± 1.7	15.8 ± 0.9	1.3 ± 0.1
	48 h	242.4 ± 6.9	14.0 ± 2.2	1.0 ± 0.2

Results are presented as mean ± SE ($n = 2$)

amounts. The levels of tRNA-bound cytokinins remained constant during germination but doubled during early seedling establishment (48 h). Overall, concentration of the tRNA-bound cytokinins was much lower compared to the free cytokinins (Table 6).

CKX Activity and Substrate Specificity

The CKX activity was determined using the radioisotope assay based on measuring the formation of [^{3}H]adenine, the degradation product from the breakdown of [2-^{3}H]iP, [2-^{3}H]*t*Z, and [2-^{3}H]*c*Z. For all three plant species investigated, the order of preference of potential CKX substrate was iP>*t*Z>*c*Z. The activity of the CKX enzyme did vary greatly among the species, with activity one to two orders of magnitude lower in lucerne compared to the two monocotyledonous species tested (Table 7). Enzyme activity fluctuated as germination proceeded. In lucerne, CKX activity initially decreased following imbibition (13 h) but increased during early seedling establishment (24 h). In contrast with lucerne, CKX activity increased in the course of germination in oats (30 h) and decreased during early seedling establishment (48 h). In maize, CKX activity was the highest in the dry kernels and gradually decreased as germination proceeded (Table 7).

Discussion

Many similarities in cytokinin profiles were apparent in the three species investigated in the present study. *c*Z-type cytokinins were present in high concentrations in the dry seeds of oats and lucerne, whereas in maize, DHZ-type cytokinins occurred in the highest concentrations. Endogenous cytokinin levels fluctuated during germination and seedling establishment, with an increase in various cytokinin forms with large changes in *c*Z-type cytokinins. The results of the present study add to the increasing list of species whose seeds contain high levels and diversity of *c*Z-type cytokinins. These results are similar to that of *Arabidopsis* where *c*Z forms (*c*ZR and *c*ZRMP) prevailed in the dry seeds, although this profile did not change after 24 h of imbibition. Following germination, *c*Z-type decreased and *t*Z-type dominated during all vegetative stages, with *c*Z-type increasing again with the onset of senescence (Gajdošová, Spíchal, and others 2011). An abundance of *c*Z-type cytokinins are also found in other plant organs such as leaves and shoots in many plants from diverse families (Gajdošová, Spíchal, and others 2011).

Different cytokinins exhibit different biological activities, with *c*Z generally having lower activity in a number of bioassays (Sakakibara 2006) as well as activating specific cytokinin receptors to a lesser extent than *t*Z (Spíchal and others 2004). For example, *c*Z is active but with a lower efficiency (that is, it requires a higher concentration to elicit a biological response) in the soybean callus bioassay (cell division; van Staden and Drewes 1991), the oat leaf senescence (chlorophyll synthesis), and the *Amaranthus* (betacycanin synthesis) bioassays as well as in promoting cell division in the tobacco callus bioassay (Gajdošová, Spíchal, and others 2011). The in vitro zygotic pea embryo bioassay can detect biological activity for a number of cytokinins, including *c*Z-type, in a concentration-dependent manner, with activity of *c*ZR comparable to that of *t*ZR (Quesnelle and Emery 2007). These results provide evidence that in certain systems such as seeds where *c*Z-type are abundant and occur in high concentrations, they may be biologically active to specific types of growth responses.

Although the biological function of *c*Z conjugates is unclear, one possible function of *c*Z-type isomers may be regulating cell division in seeds. Dobrev and others (2002) showed that the ratio of *c*Z:*t*Z is important in regulating the cell cycle in synchronized tobacco cell suspension cultures. Accumulation of cytokinins is often correlated with the onset of cell division (Kamínek and others 1997). In the present study, higher cytokinin concentrations were measured in the root samples of maize compared to the kernel samples collected at the same time (Table 1), suggesting that these cytokinins are associated with root growth during early seedling establishment. Similar to the present study, cytokinin peaks during early seedling establishment were also detected in sorghum (Dewar and others 1998), some monocotyledonous species (Leubner-Metzger 2006), and in chick-pea seeds (Villalobos and Martin 1992). These peaks of post-germination cytokinins were implicated in radicle growth and seedling establishment, with cytokinins playing a role in promoting cell division and mobilization of storage reserves (Dewar and others 1998; Leubner-Metzger

2006). The increased cytokinin levels following radicle emergence in the three species investigated in the present study suggest that they may have a similar function.

Certain tRNAs carry a prenylated adenosine adjacent to the anticodon and, when degraded, can provide a source of *c*Z-type cytokinins (Sakakibara 2006). However, there was little correlation between the tRNA content and the tRNA-bound cytokinins measured in the three species investigated in the present study. Although it is likely that tRNA is the main source of *c*Z-type cytokinins that have been identified in bacteria such as *Rhodococcus facians* (Pertry and others 2009) and in lower-order plants such as the moss *Physcomitrella patens* (von Schwartzenberg and others 2007), it is probably that there is more than one source for *c*Z-type cytokinins in higher-order plants, especially in tissues with high *c*Z levels such as the germinating seeds investigated in the present study. Other possible explanations for the high levels of the various *c*Z-type cytokinins present in the seeds would be either an independent *c*Z biosynthesis pathway (Kasahara and others 2004; Martin and others 2001) or isomerization (Bassil and others 1993). However, it has since been shown that *cis*–*trans* isomerization is unlikely to occur naturally in plants (Gajdošová, Spíchal, and others 2011). The origin of these high levels of *c*Z-type in germinating seeds requires further investigation.

The biosynthesis of aromatic cytokinins has yet to be elucidated but evidence suggests that their de novo synthesis is independent of that of isoprenoid cytokinins (Strnad 1997). This supports the idea that aromatic and isoprenoid cytokinins probably have different physiological functions (Strnad 1997; Tarkowská and others 2003). Based on various bioassays, aromatic cytokinins are thought to have a greater influence on metabolism and growth processes, especially those involving morphogenesis in more mature tissues compared to isoprenoid cytokinins that stimulate, in particular, cell division (Holub and others 1998; Kamínek and others 1987). This may also be the case in the seeds investigated in the present study where aromatic cytokinins were detected in the dry seeds but generally made only a very small contribution to the total cytokinin pool throughout germination and early seedling establishment.

CKX plays an important role in regulating local endogenous isoprenoid cytokinin levels and distribution in plants, being the only known enzyme capable of irreversibly degrading naturally occurring cytokinins (Galuszka and others 2000; Kamínek and others 1997). A number of *CKX* genes have been identified in monocotyledonous plants such as rice and maize and in dicotyledonous plants such as *Arabidopsis* and poplar (Gu and others 2010). CKX shows both spatial and temporal patterns with regard to both different plant tissues and in different cell compartments, with the highest CKX activity generally found in seeds and roots (Galuszka and others 2000). CKX activity was measured in the three species investigated in the present study and was much higher in the two monocotyledonous species compared to the dicotyledonous species (Table 4).

Although the biological properties of CKX are variable, it is highly substrate-specific, catalyzing the cleavage of the N^6-unsaturated isoprene side chain of iP, *t*Z, and, to a lesser extent, *c*Z and their ribosides from the purine ring (Galuszka and others 2000; Kamínek and others 1997; Motyka and others 2003). The same trend in substrate specificity was observed in the three species investigated in the present study (Table 4). Unlike isoprenoid cytokinins, aromatic cytokinins are not susceptible to degradation by CKX, instead favoring glycosylation (Strnad 1997; Tarkowská and others 2003). However, data presented by Frébortová and others (2004) showed that CKX from *Zea mays* (ZmCKX1) is capable of cleaving aromatic cytokinins, albeit at very low rates.

CKX is influenced by a number of regulatory mechanisms that depend on cytokinin concentrations, with CKX activity generally increasing with cytokinin accumulation, whether due to endogenous formation or exogenous application. For example, exogenously applied BA was found to increase the contents of endogenous isoprenoid cytokinins (Z- and DHZ-type) and, consequently, the CKX activity in tobacco cultures (Motyka and others 2003). In maturing maize kernels, cytokinin levels peaked 9 days after pollination and declined rapidly thereafter. The transient cytokinin peak coincided with increased mitotic activity in the endosperm and maximum CKX activity (Dietrich and others 1995). Similar timing of upregulation of the CKX in immature maize kernels was measured where there was a sharp increase in the *Zmckx1* gene expression between 5 and 17 days after pollination (Bilyeu and others 2003). It is thus likely that cytokinin oxidation is an important mechanism in regulating cytokinin levels in seeds (Emery and Atkins 2006). In the present study, there was a negative correlation between CKX activity and iP concentrations and a positive correlation between CKX activity and *O*-glucoside levels. Maize, which had the highest CKX activity (Table 7), had the lowest iP concentrations of the three species analyzed, whereas *O*-glucoside conjugates made up over 75% of the total cytokinin pool (Fig. 2c). In contrast, lucerne, which had the lowest CKX activity of the three species analyzed, had higher iP concentrations (Table 2), whereas *O*-glucoside conjugates contributed only between 25 and 58% of the total cytokinin pool (Fig. 2a). In contrast to free bases and ribosides, *O*-glucosides are resistant to CKX degradation (Armstrong 1994; Galuszka and others 2007) and so may provide a readily available source of cytokinins that can be converted

to physiologically active cytokinins that are required during germination and early seedling establishment.

In conclusion, *c*Z-type cytokinins increased in concentration following imbibition in lucerne so that they were the prevalent form throughout germination and early seedling establishment. This suggests that *c*Z-type cytokinins are probably involved in germination and seedling establishment in lucerne. In oats, the *c*Z-type cytokinins were the prevalent form in the dry seeds as well as throughout germination and early seedling establishment, whereas DHZ-type cytokinins were the prevalent cytokinins in maize. Lower concentrations of tRNA-bound cytokinins were quantified in these three species. CKX activity was much higher in the two monocotyledonous species compared to the dicotyledonous species tested, with maize and oats having the highest ratio of *O*-glucosides. In seeds such as lucerne and oats where *c*Z-type are abundant and occur in high concentrations, they can have an important biological role, especially as they have a higher resistance to CKX degradation. Aromatic cytokinins made only a very small contribution to the total cytokinin pool and only began to increase slightly during seedling establishment. This suggests that aromatic cytokinins do not play a role in germination but could possibly be involved in nutrient mobilization and chlorophyll synthesis as the seedlings mature.

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