J Plant Growth Regul (2005) 24:188–200
DOI: 10.1007/s00344-005-0043-9

J P G R
Journal of Plant Growth Regulation

The Involvement of Cytokinin Oxidase/Dehydrogenase and Zeatin Reductase in Regulation of Cytokinin Levels in Pea (*Pisum sativum* L.) Leaves

Alena Gaudinová,[1] Petre I. Dobrev,[1] Blanka Šolcová,[1] Ondřej Novák,[2] Miroslav Strnad,[2] David Friedecký,[3] and Václav Motyka[1]*

[1]*Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 135, CZ-16502 Prague 6, Czech Republic;* [2]*Laboratory of Growth Regulators, Palacký University & Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Šlechtitelů CZ-78371 Olomouc, Czech Republic;* [3]*Laboratory for Inherited Metabolic Disorders, Department of Clinical Biochemistry, University Hospital, I. P. Pavlova 6, CZ-77520 Olomouc, Czech Republic*

Abstract

Cytokinin metabolism in plants is very complex. More than 20 cytokinins bearing isoprenoid and aromatic side chains were identified by high performance liquid chromatography-mass spectrometry (HPLC-MS) in pea (*Pisum sativum* L. cv. Gotik) leaves, indicating diverse metabolic conversions of primary products of cytokinin biosynthesis. To determine the potential involvement of two enzymes metabolizing cytokinins, cytokinin oxidase/dehydrogenase (CKX, EC 1.5.99.12) and zeatin reductase (ZRED, EC 1.3.1.69), in the control of endogenous cytokinin levels, their *in vitro* activities were investigated in relation to the uptake and metabolism of [2−^{3}H]*trans*-zeatin ([2−^{3}H]Z) in shoot explants of pea. *Trans*-zeatin 9-riboside, *trans*-zeatin 9-riboside-5′-monophosphate and cytokinin degradation products adenine and adenosine were detected as predominant [2−^{3}H]Z metabolites during 2, 5, 8, and 24 h incubation. Increasing formation of adenine and adenosine indicated extensive degradation of [2−^{3}H]Z by CKX. High CKX activity was confirmed in protein preparations from pea leaves, stems, and roots by *in vitro* assays. Inhibition of CKX by dithiothreitol (15 mM) in the enzyme assays revealed relatively high activity of ZRED catalyzing conversion of Z to dihydrozeatin (DHZ) and evidently competing for the same substrate cytokinin (Z) in protein preparations from pea leaves, but not from pea roots and stems. The conversion of Z to DHZ by pea leaf enzyme was NADPH dependent and was significantly inhibited or completely suppressed *in vitro* by diethyldithiocarbamic acid (DIECA; 10 mM). Relations of CKX and ZRED in the control of cytokinin levels in pea leaves with respect to their potential role in establishment and maintenance of cytokinin homeostasis in plants are discussed.

Key words: Aromatic cytokinin; *cis*-zeatin; Cytokinin; Cytokinin oxidase/dehydrogenase; Dihydrozeatin; Metabolism; Pea; *trans*-zeatin; Zeatin reductase

Received: 14 April 2005; accepted: 16 June 2005; online publication 31 October 2005.
**Corresponding author; e-mail:* vmotyka@ueb.cas.cz

INTRODUCTION

Cytokinins are plant hormones that are involved in regulation of the cell cycle and affect many aspects of plant development such as cell and plastid differentiation, seed development and germination, apical dominance, flowering, fruit development, and leaf senescence (Mok 1994; Werner and others 2003). All native cytokinins are derivatives of adenine bearing an isoprenoid, isoprenoid-derived, or aromatic substituent at the N^6-position of the purine ring. For many years, research has concentrated on *trans*-zeatin (Z)-, N^6-(Δ^2-isopentenyl)adenine (iP)- and dihydrozeatin (DHZ)-type cytokinins (especially cytokinin free bases and ribosides). Recently, because of the introduction of advanced analytical methods, namely high performance liquid (gas) chromatography/mass spectrometry (HPLC (GC)/MS), a wide spectrum of other natural cytokinins, including cytokinin *N*-, *O*-glucosides and nucleotides (for example, Corbesier and others 2003; Novák and others 2003; Boiten and others 2004), *cis*-zeatin (*cis*Z) derivatives (for example, Dobrev and others 2002; Veach and others 2003), and aromatic cytokinins (Tarkowská and others 2003; for review see Strnad 1997), has been identified and quantified in plants. Regardless of the numerous possible modifications of the purine ring and/or the side chain in the cytokinin molecule, the presence of an N^6-substituent is believed to be necessary for expression of biological activity in cytokinins (Letham and Palni 1983; Mok and Mok 2001).

The pool of active cytokinins in plant cells is regulated at different levels via biosynthesis, uptake from extracellular sources, metabolic interconversions, inactivation and degradation, as well as signal transduction (Kamínek and others 1997; Zažímalová and others 1999; Mok and Mok 2001; Haberer and Kieber 2002; Schmülling 2002; Sakakibara 2004).

The main objective of the present work was to determine the potential involvement of two cytokinin metabolizing enzymes, cytokinin oxidase/dehydrogenase (CKX, EC 1.5.99.12) and zeatin reductase (ZRED, EC 1.3.1.69), in the regulation of endogenous cytokinin levels and their role in the establishment and maintenance of cytokinin homeostasis in pea as a crop plant of high economic importance. For this purpose, identification and quantification of endogenous cytokinin forms, investigation of uptake and metabolism of [2−^{3}H]*trans*-zeatin ([2−^{3}H]Z), and characterization of CKX and ZRED enzyme activities *in vitro* in pea plants were performed as partial tasks.

The irreversible cytokinin degradation catalyzed by CKX and the conversion of Z-type cytokinins to DHZ and/or its derivatives catalyzed by ZRED represent two closely related metabolic pathways involved in regulation of endogenous cytokinin levels. The CKX activity cleaves the isoprenoid N^6-side chain of iP- and Z-type cytokinins, converting them to adenine or adenosine and the corresponding side chain aldehyde(s) (for review see Armstrong 1994). On the other hand, ZRED activity catalyzes reduction of the N^6-side chain double bond of Z or its metabolites, yielding products (DHZ-type cytokinins) that are resistant to CKX (for review see Armstrong 1994; Hare and van Staden 1994). In this respect, the conversion of Z- to DHZ-type cytokinins may preserve cytokinin biological activity, especially in tissues containing highly active CKX(s). Cytokinins of the DHZ-type evidently contribute to the total cytokinin pool in plants; however, there is no evidence that certain physiological processes are predominantly and expressly regulated by them.

Impressive progress in CKX research has recently been achieved involving cloning, expression, and characterization of the CKX gene(s) (Houba-Hérin and others 1999; Morris and others 1999; Bilyeu and others 2001, 2003; Galuszka and others 2001, 2004; Werner and others 2001, 2003; Yang and others 2002a, 2002b; Massonneau and others 2004). To date, the sequences of 17 fully annotated *CKX* genes are known, including two prokaryotic genes (for review see Schmülling and others 2003). However, the data concerning ZRED are still very limited. Except for articles by Mok's group (Martin and others 1989, Mok and others 1990), there is no other work describing the detection and partial characterization of ZRED activity *in vitro* in protein preparations extracted and partially purified from plant material.

This article adds new data to the present knowledge of the occurrence and metabolism of cytokinins in plants, with particular emphasis on metabolic pathways catalyzed by CKX and ZRED. We show that the two pathways and their corresponding enzymes are involved in regulation of the endogenous cytokinin pool and may contribute to the establishment and maintenance of cytokinin homeostasis in pea. Detection and characterization of both CKX and ZRED enzyme activities in cell-free preparations from pea leaves are described. We also report on the presence of aromatic N^6-benzyladenine (BA)- and *cis*Z-type cytokinins in pea.

Table 1. The HPLC-MS Analysis of Endogenous Cytokinins in Pea (*Pisum sativum* L. cv. Gotik) Leaves

Cytokinin	Cytokinin content (pmol g^{-1} FW)
iP	0.993 ± 0.255
iPR	1.834 ± 0.300
iPRMP	13.712 ± 2.232
Z	0.326 ± 0.057
ZR	0.250 ± 0.036
Z9G	0.052 ± 0.010
ZOG	0.655 ± 0.095
ZROG	0.444 ± 0.091
ZRMP	0.645 ± 0.080
cisZ	0.067 ± 0.018
cisZR	1.902 ± 0.246
cisZOG	0.077 ± 0.026
cisZROG	0.659 ± 0.127
cisZRMP	3.699 ± 0.938
DHZ	0.238 ± 0.043
DHZR	0.391 ± 0.067
DHZOG	0.756 ± 0.214
DHZROG	0.360 ± 0.082
DHZRMP	1.382 ± 0.294
BA	13.784 ± 3.198
BAR	2.893 ± 0.941
Total	45.119 ± 9.350

Cytokinin levels were determined by a combination of HPLC-MS as described in Materials and Methods. The results of one representative experiment from three independent ones are presented. The SD values averaged 21% and did not exceed 35% of the mean.

*Abbreviations: iP, N^6-(Δ^2-isopentenyl)adenine; iPR, N^6-(Δ^2-isopentenyl)adenine 9-riboside; iPRMP, N^6-(Δ^2-isopentenyl)adenine 9-riboside-5′-monophosphate; Z, *trans*-zeatin; ZR, *trans*-zeatin 9-riboside; Z9G, *trans*-zeatin 9-glucoside; ZOG, *trans*-zeatin *O*-glucoside; ZROG, *trans*-zeatin 9-riboside O-glucoside; ZRMP, *trans*-zeatin 9-riboside-5′-monophosphate; *cis*Z, *cis*-zeatin; *cis*ZR, *cis*-zeatin 9-riboside; *cis*ZOG, *cis*-zeatin *O*-glucoside; *cis*ZROG, *cis*-zeatin 9-riboside *O*-glucoside; *cis*ZRMP, *cis*-zeatin 9-riboside-5′-monophosphate; DHZ, dihydrozeatin; DHZR, dihydrozeatin 9-riboside; DHZOG, dihydrozeatin *O*-glucoside; DHZROG, dihydrozeatin 9-riboside *O*-glucoside; DHZRMP, dihydrozeatin 9-riboside-5′-monophosphate; BA, N^6-benzyladenine; BAR, N^6-benzyladenine 9-riboside; according to Kamínek and others (2000).

Materials and Methods

Chemicals

Unless otherwise stated, all chemicals were from Sigma Co. (St. Louis, MO). Radiolabeled cytokinins [2−^{3}H]Z and [2−^{3}H]iP (specific activity 1300 TBq mol^{-1} each) were synthesized by Dr. Hanuš (Institute of Experimental Botany AS CR, Prague, Czech Republic). Deuterium-labeled cytokinins (for abbreviations see Table 1) [$^2H_5$]Z, [$^2H_5$]ZR, [$^2H_5$] Z9G, [$^2H_5$]ZOG, [$^2H_5$]ZROG, [$^2H_5$]ZRMP, [$^2H_3$]DHZ, [$^2H_3$]DHZR, [$^2H_6$]iP, [$^2H_6$]iPR, and [$^2H_6$]iPRMP were from Olchemim Ltd. (Olomouc, Czech Republic); [$^2H_7$]BA and [$^2H_7$]BAR were synthesized by Dr. Doležal (Institute of Experimental Botany AS CR, Olomouc, Czech Republic).

Plant Material

Seeds of pea (*Pisum sativum* L. cv. Gotik and cv. Canis), bean (*Phaseolus vulgaris* L. cv. Gama), and soybean (*Glycine max* (L.) Merr. cv. Lek-2760/98) were obtained from Agritec Ltd. (Šumperk, Czech Republic), SEMO Ltd. (Smržice, Czech Republic), and the Faculty of Horticulture-Mendeleum, Mendel University of Agriculture and Forestry (Lednice, Czech Republic), respectively. The plants were grown in a greenhouse at the Institute of Experimental Botany AS CR (Prague, Czech Republic) in soil under natural light conditions (ca. 16 h light/8 h dark cycle and 22°/25°C) for 4 weeks. For the uptake experiments, they were de-rooted and the rootless plants were used for incubation with [2−^{3}H]Z as specified below. For determination of endogenous cytokinin levels and enzyme activities, the seedlings were divided into leaves, stems, and roots immediately frozen in liquid nitrogen and stored at –70°C until analyses.

Endogenous Cytokinin Analysis

Endogenous cytokinins were purified from plant tissue extracts by solid-phase, ion-exchange, and immunoaffinity chromatography (IAC) using polyspecific monoclonal antibodies against cytokinins and analyzed by HPLC-MS as described by Novák and others (2003). Three different fractions after purification were obtained and measured for cytokinin contents separately: bases (including also ribosides and *N*-glucosides), *O*-glucosides, and nucleotides. The latter two fractions were treated by β-glucosidase and alkaline phosphatase, respectively, before the HPLC-MS analysis. The LC-MS analysis was carried out using the HPLC Alliance 2690 Separations Module (Waters, Milford, MA) linked to PDA 996 (Waters) and ZMD 2000 single quadrupole mass spectrometer equipped with an electrospray interface [LC(+)ESI-MS] (Micromass, Manchester, UK). The samples (25 μL) were injected onto a C18 reversed-phase column (Waters; Symmetry; 5 μm; 150 mm × 2.1 mm) and eluted with the methanolic gradient (phase A: 100% CH_3OH, B: 15 mM $HCOONH_4$, pH 4.0, flow rate 0.25 mL min^{-1}): 0 min, 10% A and 90% B; 25 min, 50% A and 50% B; 30 min, 50% A and 50% B. Using a post column split of 1:1, the effluent was simultaneously introduced into the diode array detector and the electrospray source (source block temperature 100 °C, desolving temperature 250 °C, capillary voltage +3.0 V, cone voltage 20 V). Under these conditions, quantitative analyses of the different cytokinins were performed in selective ion-recording mode.

All studied cytokinins were determined in methanolic gradient as dominant quasi-molecular ions of [*M* +H]+ with limits of detection ranging between 10 and 50 fmol. For routine analysis, a linearity range between 25 (75) fmol and 100 pmol was reached (Novák and others 2003). Quantification of endogenous cytokinin metabolites was performed using internal deuterium-labeled cytokinin standards. All data were processed by Masslynx software (version 3.5, Micromass, Manchester, UK).

Uptake and Metabolism of [^{3}H]*trans*-Zeatin

The uptake and metabolism of [2–^{3}H]Z were investigated in de-rooted pea plants incubated for 2, 5, 8, and 24 h in 1 ml water solution containing 40 kBq [2-^{3}H]Z. Extraction and purification of cytokinins were performed as described by Dobrev and Kamínek (2002). The radioactive metabolites of [2-^{3}H]Z were identified by HPLC consisting of Series 2000 autosampler and quaternary pump (both PerkinElmer, Wellesley, MA), column Luna C18(2) (Phenomenex, 150 × 4.6 mm, 3 μm) heated at 35 °C and two detectors coupled in series; 235C diode array detector (PerkinElmer) and Ramona 2000 flow-through radioactivity detector (Raytest, Straubenhardt, Germany). Two solvents (A: 40 mM CH_3COOH adjusted with NH_4OH to pH 4.0 and B: CH_3CN/CH_3OH, 1/1, v/v) were used at flow rate of 0.6 mL min^{-1} for the linear gradient 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 column eluate was monitored at 270 nm by the diode array detector, and after online mixing with 3 volumes (1.8 ml min^{-1}) of liquid scintillation cocktail (Flo-Scint III, Packard BioScience Co., Meriden, CT) it was monitored by a Ramona 2000 radioactivity detector. Under these conditions the sensitivity limit of the detection was 30 Bq, and the linear range of response was between 100 Bq and 650 kBq. The radioactive metabolites of [2-^{3}H]Z were identified on the basis of comparison of their retention times with authentic standards.

Determination of Cytokinin Oxidase/ Dehydrogenase Activity *In Vitro*

The CKX activity was determined *in vitro* by the radioisotopic method based on the enzymatic degradation of [2-^{3}H]iP or [2-^{3}H]Z to [^{3}H]adenine (Motyka and others 1996). For the extraction of protein, frozen plant tissues were homogenized in 0.1 M Tris-HCl buffer (pH 7.5) and purified on a polyvinylpolypyrrolidone column. After centrifugation and removal of nucleic acids by Polymin P (1%, v/v, Serva Feinbiochemica, Heidelberg, Germany), the proteins were precipitated by the addition of solid ammonium sulfate to 80% saturation. Protein contents were determined according to the method of Bradford (1976) using bovine serum albumin as standard. The assay mixture contained 100 mM TAPS-NaOH (pH 8.5) or MES-NaOH (pH 6.0), 2 μM [2-^{3}H]iP (7.4 TBq mol^{-1}), and enzyme preparations (equivalent to 100–500 mg of fresh tissue). In a few cases, 3 μM [2-^{3}H]Z (8.3 TBq mol^{-1}) was used instead of [2-^{3}H]iP. After incubation at 37 °C, the reaction was terminated by the addition of 95% cold ethanol (120 μl) and 200 mM Na_4EDTA (10 μl). Radiolabeled products of the enzyme reaction were separated and quantified using the HPLC system as described above for the detection of [2-^{3}H]Z metabolites, with the following modifications: samples were injected on a Luna C8 column (Phenomenex, 50 × 4.6 mm, 3 μm) and eluted at a flow rate of 0.6 ml min^{-1} at 35 °C with the gradient (mobile phase A: 40 mM CH_3COOH + NH_4OH, pH 5.0; B: CH_3OH/CH_3CN, 1:1, v/v) 1%–70% B for 1 min; 70% B for 1 min; 70%–1% B for 1 min, and equilibration 1% B for 10 min.

The pH optimum for CKX activity was determined as described by Motyka and others (2003). Details of concanavalin A (Con A)–Sepharose 4B chromatography and substrate competition assays were published previously (Motyka and Kamínek 1994; Motyka and others 1996, 2003).

Determination of Zeatin Reductase Activity *In Vitro*

Zeatin reductase activity was analyzed *in vitro* in crude protein preparations on the basis of [2-^{3}H]Z conversion to [^{3}H]DHZ as described by Martin and others (1989). Plant tissues were ground under liquid nitrogen by a pestle in a mortar and homogenized in 2:1 parts (v/w) of cold extraction buffer (0.1 M potassium phosphate, pH 7.3) containing 5 mM dithiotheitol (DTT) and 0.5 mM EDTA using a Vibracell device equipped with an ultrasonic CV18 processor. After centrifugation (17,000 × *g*, 20 min, 4 °C), proteins in the supernatant were fractionated by solid ammonium sulfate. The fraction precipitated between 30% and 60% ammonium sulfate was collected, redissolved in 1–2 ml extraction buffer, and used for the enzyme assay. The standard reaction mixture contained 15 mM DTT, 0.25 mM NADPH, 3 μM [2-^{3}H]Z (8.3 TBq mol^{-1}) and enzyme extract (equivalent to 400–800 mg of fresh tissue) in

0.1 M potassium phosphate buffer, pH 8.0 (total volume 300 µl). In some experiments, a chelating agent of diethyldithiocarbamic acid (DIECA, 10 mM) was added to the assay. After 16 h incubation at 26 °C, the reaction was stopped by addition of 1 mL of cold ethanol, the mixture was placed for 30 min at 4 °C, and then the precipitated proteins were removed by centrifugation (18,000 × *g*, 30 min). The supernatant was concentrated *in vacuo* (Hetovac VR I Concentrator, Heto) to approximately 100 µL. Radiolabeled products of the enzyme reaction were separated and quantified by HPLC coupled to a flow-through radioactivity detector as described above for the detection of [2-^{3}H]Z metabolites.

Presentation of the Results

If not otherwise stated, each experiment was repeated two or three times. As the repeated experiments showed similar tendencies, the results of one representative experiment of two or three replicates are presented. Statistical variations of results are expressed as the average ± SD values in the tables and figures and/or specified in their legends.

Results and Discussion

Cytokinin Analysis

Using a combination of HPLC and mass spectrometry, the levels of endogenous cytokinins including bases, ribosides, nucleotides, *N*- and *O*-glucosides were measured in pea (*Pisum sativum* L. cv. Gotik). The contents and abbreviations of detected cytokinins in leaves are presented in Table 1. More than 20 cytokinins bearing isoprenoid and aromatic side chains were identified, indicating their diverse metabolic conversions.

Cytokinin nucleotides (iPRMP, *cis*ZRMP) and aromatic cytokinins (BA, BAR) were the major cytokinins found in pea leaves in concentrations ranging from 2.9 to 13.8 pmol g^{-1} FW (Table 1). It is probable that their high levels are related to total or partial resistance to degradation by CKX (Armstrong 1994). Although aromatic cytokinins (long considered to be purely synthetic derivatives) have already been identified in several plant tissues (for review see Strnad 1997; Taylor and others 2003), according to our knowledge they were detected in pea for the first time here. Interestingly, BA and BAR were the only aromatic cytokinins present in pea; neither BA nucleotide nor BA(R) hydroxylated derivatives (topolins) were found in detectable quantities in pea leaves, stems, and roots (data not shown).

Also, the finding of relatively high levels of *cis*Z derivatives (especially *cis*ZRMP and, to a lesser extent, *cis*ZR and *cis*ZROG) in pea is novel and corresponds well with increasing evidence of the presence and potential function of free *cis*Z-type cytokinins in plants (for review see Mok and Mok 2001) including legumes (Emery and others 1998, 2000). In general, our results support the suppositions that *cis*Z derivatives are more prevalent and probably more relevant to cytokinin biology than previously thought, having unique functions in plant tissues and being synthesized in plant cells in (a) distinct way(s) compared to their corresponding *trans* isomers (Kasahara and others 2004). The recent finding of genes and enzymes specific for *cis*Z-type cytokinins (Martin and others 2001; Veach and others 2003), as well as recognition of *cis*Z by cytokinin-responsive His-protein kinases (Yonekura-Sakakibara and others 2004), affirms such assumptions. Besides, the abundance of *cis*Z-type cytokinins in plants may also be due to their weaker affinity for CKX compared to *trans*-zeatins (Pačes and Kamínek 1976; Chatfield and Armstrong 1986; Bilyeu and other 2001).

On the basis of data from other authors (referred to above), as well as our own results, BA-type aromatic cytokinins as well as *cis*Z derivatives may be widespread in plant tissues but were not found before because of less sensitive detection techniques. In this regard, plants analyzed in early experiments have generally not been re-examined for the occurrence of these cytokinins using more sophisticated techniques.

The levels of other cytokinin derivatives were considerably lower, ranging from approximately 0.1 to 1.9 pmol g^{-1} FW (Table 1). The total quantities of DHZ-type cytokinins, including the base, the riboside, and the *N*- and *O*-glucosides (1.75 pmol g^{-1} FW), were similar to the Z derivatives (1.73 pmol g^{-1} FW) and slightly lower than levels of the corresponding iP-type cytokinins (2.83 pmol g^{-1} FW). Davies and others (1986) reported derivatives of Z (Z, ZR, ZOG) and DHZ (DHZ, DHZR) as major cytokinins in vegetative shoots of pea. The occurrence of DHZ-type cytokinins (non-substrates of CKX), together with cytokinins of Z-type (CKX substrates), suggests a potential involvement of ZRED activity in cytokinin metabolism in pea plants. Although the iP- and Z-type cytokinins can be easily degraded by CKX attack (Armstrong 1994), a conversion of Z-type cytokinins by reduction of their N^6-side chain to DHZ and/or its derivatives catalyzed by ZRED may represent a mechanism of preservation of cytokinin activity, especially in tissues containing high concentrations and/or activities of CKXs, such as legumes.

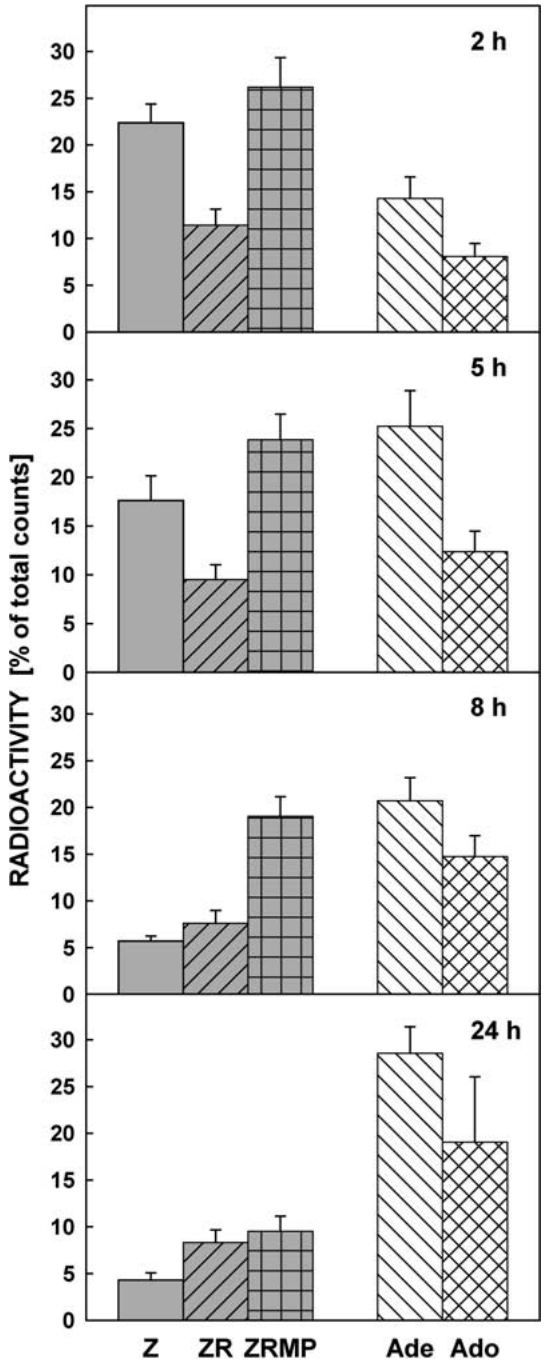

Figure 1. Distribution of radioactivity detected in de-rooted pea (*Pisum sativum* L. cv. Gotik) plants 2, 5, 8, and 24 h after application of [2-^{3}H]Z. Products of [2-^{3}H]Z metabolism were analyzed using HPLC coupled to an on-line radioactivity detector. Values are expressed as percentage of the total extracted radioactivity taken up by plants. The results of one of two experiments showing the same trends are presented. Each determination was duplicated. The SD values averaged 9% and did not exceed 19% of the mean. (Ade, adenine; Ado, adenosine; Z, *trans*-zeatin; ZR, *trans*-zeatin 9-riboside; ZRMP, *trans*-zeatin 9-riboside-5′-monophosphate).

Comparable levels of total endogenous cytokinins with spectra of individual metabolites similar to those presented for leaves were also found in pea stems and roots. As in pea leaves, iPRMP was the major cytokinin derivative found in stems and roots (data not shown).

Uptake and Metabolism of [2-^{3}H]*trans*-zeatin

To acquire more information about cytokinin interconversions, the uptake and metabolic fate of [2-^{3}H]Z were investigated in de-rooted pea plants by means of HPLC coupled to on-line radioactivity detector. The rootless plants were incubated for 2, 5, 8, and 24 h in water containing [2-^{3}H]Z. After 24 h, up to 71% (16.9 kBq g^{-1} FW) of the initial radioactivity was taken up by plants. From the total radioactivity taken up within 24 h, 35% (5.3 kBq g^{-1} FW) was extracted at 2 h, 28% (4.9 kBq g^{-1} FW) at 5 h, 22% (4.1 kBq g^{-1} FW) at 8 h, and 15% (2.6 kBq g^{-1} FW) at 24 h.

Most of [2-^{3}H]Z taken up by plants within 2 h was metabolized to corresponding 9-riboside (ZR, 11 %) and 9-riboside-5′-monophosphate (ZRMP, 26 %), whereas only 22% of extracted radioactivity was retained in unmetabolized [2-^{3}H]Z (Figure 1). The rest of the radioactivity was associated with cytokinin degradation products (adenine 14%, adenosine 8%) and unidentified metabolites (probably related to *cis*Z and Z-*N*-glucosides; none of them exceeding 5% of total radioactivity). A gradual decrease of [2-^{3}H]Z and its derivatives during prolonged incubation (from 59% of total counts after 2 h to 21% after 24 h) was accompanied by a corresponding increase in radioactivity associated with adenine and adenosine (Figure 2), indicating extensive degradation of Z by CKX. A slight decline of total radioactivity associated with Z-type cytokinins together with adenine and adenosine in the course of incubation (not exceeding 15% of the total counts; Figure 2) was probably due to a weak conversion of [^{3}H]Z to other metabolites that were not followed in these experiments (presumably *cis*Z and Z-*N*-glucosides; data not shown).

Our results using labeled Z indicated that de-rooted pea plants have the ability to metabolize this cytokinin extensively. From the high level of radioactivity associated with adenine and/or adenosine, it is obvious that the N^6-side chain cleavage catalyzed by CKX is the dominant form of [2-^{3}H]Z metabolism in pea. Similar data arising from feeding experiments with labeled Z or ZR were obtained in various plant tissues, including legumes (for example, Parker and others 1978; Palni and others 1984; Van Staden and Forsyth 1986; Zhang and Letham

Table 2. Cytokinin Oxidase/Dehydrogenase (CKX) Activity in Isolated Organs of Pea (*Pisum sativum* L. cv. Gotik)

	CKX activity (nmol Ade mg^{-1} protein h^{-1})	
Organ	TAPS-NaOH buffer (pH 8.5)	MES-NaOH buffer (pH 6.0)
Leaf	0.037 ± 0.001	0.014 ± 0.001
Stem	0.081 ± 0.003	0.016 ± 0.001
Root	0.049 ± 0.008	0.028 ± 0.002

The CKX activity was determined in the assay mixture containing 2 μM [2-^{3}H]iP (7.4 TBq mol^{-1}), 100 mM TAPS-NaOH (pH 8.5), or MES-NaOH (pH 6.0) buffer and protein preparation equivalent to 100 mg $assay^{-1}$ (leaf, stem) or 250 mg $assay^{-1}$ (root) for TAPS-NaOH buffer and 250 mg $assay^{-1}$ (leaf, stem) or 500 mg $assay^{-1}$ (root) for MES-NaOH in a total volume of 50 μL. The results of one representative experiment from three independent ones are presented. Each determination was duplicated. The SD values averaged 7% and did not exceed 16% of the mean.

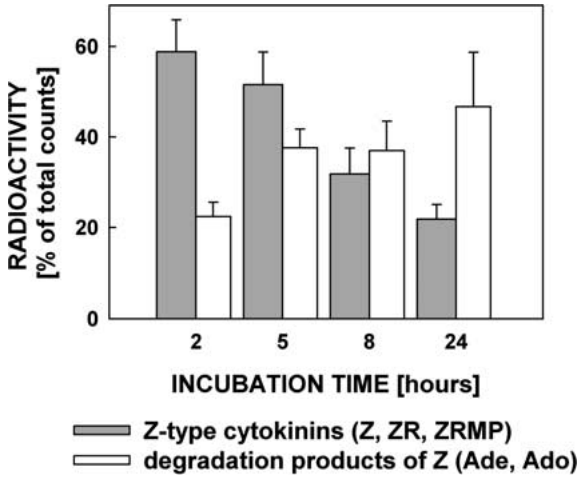

Figure 2. Proportion of radioactivity associated with Z-type cytokinins and their degradation products in de-rooted pea (*Pisum sativum* L. cv. Gotik) plants 2, 5, 8, and 24 h after application of [2-^{3}H]Z. Other details are as described in Figure 1.

1990; Singh and others 1992). Astonishingly, neither DHZ nor its derivatives were found after [2-^{3}H]Z feeding to de-rooted pea plants in our study, although an enzyme activity catalyzing conversion of Z to DHZ was detected *in vitro* in protein preparations from pea leaves (see below). The failure to detect radiolabeled DHZ-type cytokinins as products of [2-^{3}H]Z conversion could be due to the preferential degradation of [2-^{3}H]Z by CKX in these experiments. Our data contradict results of other authors showing extensive *in vivo* formation of DHZ derivatives in incubation studies with radiolabeled Z and/or ZR in different plant species, including legumes (Sondheimer and Tzou 1971; Singh and others 1988; Zhang and others 2002). The discrepancy also indicates that the metabolism of exogenously applied cytokinins in isolated plant parts may differ considerably from that of endogenous cytokinins in intact plants, as previously reported by Letham and Palni (1983) and Jameson (1994).

Activity of Cytokinin Oxidase/Dehydrogenase *In Vitro*

Enzyme preparations from pea leaves exhibited relatively high CKX activity in *in vitro* assays based on the conversion of [2-^{3}H]Z or [2-^{3}H]iP (Table 2) to adenine. As iP was determined to be the predominant substrate of CKX from pea leaves in substrate competition assays (Table 3), it has been used preferentially in most CKX analyses. In agreement with published data (for review see Armstrong 1994), Z weakly inhibited degradation of [2-^{3}H]iP in competition assays, whereas DHZ and BA had no competitive effects on [2-^{3}H]iP degradation at all (Table 3).

Higher CKX activity than in leaves was detected in crude preparations from pea stems (1.1- to 2.2-fold) and roots (1.3- to 2-fold; Table 2). These data correlate with the similar distribution of CKX activity (roots > stems > leaves) in tobacco (Motyka and others 1996), as well as with reports of considerably higher CKX activity in roots relative to shoots of maize and barley (Jones and Schreiber 1997; Bilyeu and others 2001; Galuszka and others 2004). Vaseva-Gemisheva and others (2004) reported more than 90% of the CKX activity located in roots of two pea cultivars during their vegetative development. Studies of [^{3}H]iP metabolism *in vivo* revealed the involvement of an enzymatic system capable of degrading endogenous iP-type cytokinins to adenine and/or adenosine in pea isolated organs (King and Van Staden 1987, 1990). Besides, the occurrence of CKX activity *in vitro* in other legumes, such as bean and soybean, has been reported (Chatfield and Armstrong 1986; Kamínek and

Table 3. Effect of Unlabeled Cytokinins on the *in vitro* Degradation of [2-^{3}H]iP by Cytokinin Oxidase/Dehydrogenase (CKX) from Pea (*Pisum sativum* L. cv. Gotik) Leaves

	Concentration of unlabeled cytokinin	
Unlabeled cytokinin	2 μM	20 μM
	CKX activity (% of control)[a]	
iP	52 ± 3	12 ± 3
Z	91 ± 4	40 ± 8
DHZ	101 ± 22	97 ± 9
BA	104 ± 9	98 ± 13

[a]*The CKX activity of control assayed without unlabeled cytokinins was 0.033 nmol Ade mg^{-1} protein h^{-1} (100 %). Unlabeled cytokinins were added to the standard assay mixture containing 2 μM [2-^{3}H]iP (7.4 TBq mol^{-1}) and 100 mM TAPS-NaOH buffer (pH 8.5) at concentrations equal to that of the labeled substrate (2 μM) and in 10-fold excess (20 μM). The results of one representative experiment from three independent ones are presented. Each determination was duplicated. The SD values averaged 12% and did not exceed 22% of the mean. For abbreviations see Table 1.*

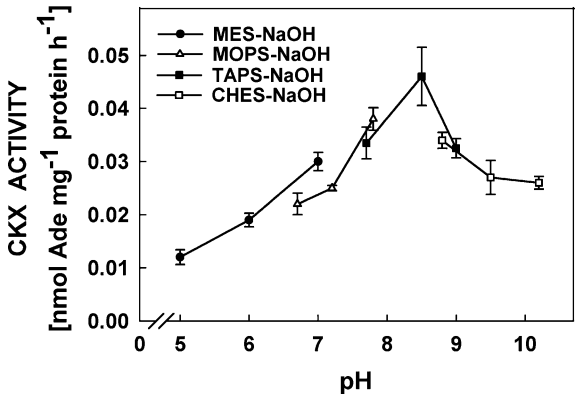

Figure 3. Effect of pH on the *in vitro* activity of cytokinin oxidase/dehydrogenase (CKX) from pea (*Pisum sativum* L. cv. Gotik) leaves. The pH optimum of CKX activity was determined by running the standard enzyme assay in different buffers including 0.1 M MES-NaOH, 0.1 M MOPS-NaOH, 0.1 M TAPS-NaOH, and 0.1 M CHES-NaOH between pH 5.0 and pH 10.2. The values represent the means of three replicates. The SD values averaged 7% and did not exceed 12% of the mean.

Armstrong 1990; Hare and Van Staden 1994; Jäger and others 1997).

A considerably higher level of CKX activity was detected in TAPS-NaOH (pH 8.5) than in MES-NaOH (pH 6.0) buffer (2.6-, 5.1- and 1.8-fold, respectively, for leaves, stems, and roots; Table 2), indicating a high pH optimum for the pea enzyme. A detailed analysis of pH dependence of CKX from pea leaves examined in four buffers over the pH range 5.0–10.2 revealed maximum enzyme activity at pH 8.5 (Figure 3). The relatively high pH optimum also reported for CKXs in other plant species (Motyka and Kamínek 1994; Motyka and others 2003) including legumes (Kamínek and Armstrong 1990) suggested the presence of a non-glycosylated CKX isoform or an isoform with a very low degree of glycosylation in pea. Indeed, the Con A–Sepharose 4B chromatography revealed that most of the CKX activity from pea leaves (96%) did not bind to lectin, which confirmed the occurrence of a non-glycosylated form of the enzyme in this tissue (Figure 4). The remaining portion of the CKX activity (4%) was retained on the Con A affinity column and eluted only after addition of methyl-mannose. Our results demonstrate a close connection between a high pH optimum and a low proportion of glycosylated isoform(s) of CKX, as was also reported for other plants (Kamínek and Armstrong 1990; Motyka and others 2003). The presence of non-glycosylated CKX and/or CKX with a very low degree of glycosylation is rather unusual in plants, and its high levels in pea leaves, as found here, and in *Phaseolus lunatus* calli (Kamínek and Armstrong 1990) suggests a possible function in some legume genotypes. The reported differences in glycosylation and pH optima of the pea leaf enzyme from CKX(s) in most of the other plant tissues may be associated with its (their) different distribution in cell compartments. As no CKX gene has been identified in pea until now, it is, however, unclear whether the enzyme activity measured in this work resulted from one or more form(s) of CKX.

The degree of protein glycosylation might govern activity, stability, and localization of the enzyme and thus add a supplementary mechanism of CKX regulation in plants (Armstrong 1994). The fact that all CKX enzymes known so far contain between one and eight predicted glycosylation sites (for review see Schmülling and others 2003) makes this form of CKX regulation even more complex.

Activity of Zeatin Reductase *In Vitro*

On the basis of our endogenous cytokinin analysis revealing the occurrence of DHZ-type cytokinins, as well as the literature data concerning the detection of DHZ derivatives in *Pisum* vegetative parts (Davies and others 1986), the potential existence of ZRED activity catalyzing conversion of Z to DHZ has been assumed in this species. Until now, the ZRED activity has been isolated and characterized only in soluble fractions of immature seeds of *Phaseolus* (Martin and others 1989; Mok and others 1990).

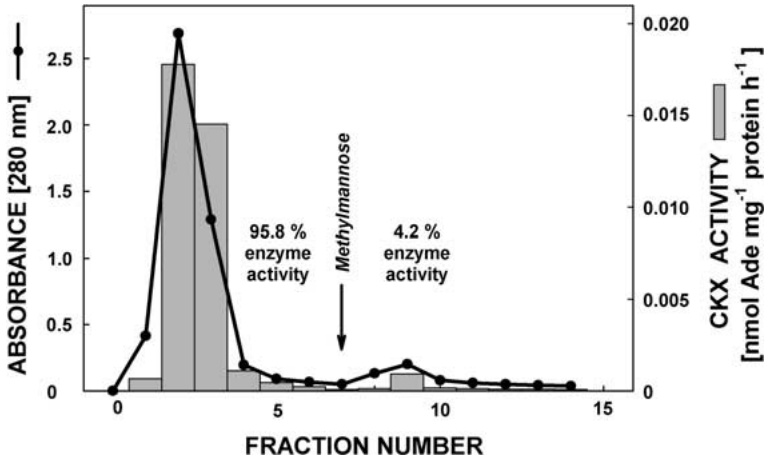

Figure 4. Separation of glycosylated and non-glycosylated cytokinin oxidase/dehydrogenase (CKX) isoforms from pea (*Pisum sativum* L. cv. Gotik) leaves by concanavalin A (Con A)–Sepharose 4B column chromatography. The enzyme preparation equivalent to approximately 5 mg protein was loaded onto the Con A–Sepharose 4B column (0.75 × 7 cm, 3 ml bed volume). The non-glycosylated CKX was washed by 21 ml of 25 mM BisTris-HCl (pH 6.5) containing $(NH_4)_2SO_4$ (200 mM); the glycosylated fraction was eluted with 21 ml of the same solution supplemented with methylmannose (200 mM). Fractions of 3 ml were collected and assayed for CKX activity.

Substantial variations of ZRED activities *in vitro* were estimated between *Phaseolus* species, with high activity in *P. vulgaris* and *P. coccineus* embryos but only marginally detectable activity in *P. lunatus* (Mok and others 1990).

Using a radioisotopic method with [2-^{3}H]Z as a substrate of the enzyme reaction in this study, relatively high ZRED activity was detected in crude protein preparations from pea leaves after partial inhibition of CKX by dithiothreitol (15 mM; Figure 5). The conversion of Z → DHZ mediated by pea leaf enzyme was NADPH-dependent. Under the chosen NADPH concentration (0.25 mM) approximately 30% of radiolabeled DHZ was formed from [2-^{3}H]Z by ZRED action (Figure 5), which appeared to be an optimal ratio for Z → DHZ conversion in *in vitro* assays. For the conversion of Z to DHZ by pea leaf enzyme presented in Figure 5 (26%), the ZRED activity of 0.019 nmol DHZ mg^{-1} protein h^{-1} (compared to the CKX activity of 0.029 nmol Ade mg^{-1} protein h^{-1}) was calculated.

Similar to our data, Martin and others (1989) reported a strong NADPH-dependence of the enzyme reaction catalyzed by ZRED from bean embryos with the highest Z → DHZ conversion (45%–55%) at 0.0625–0.25 mM NADPH. NADPH was the only cofactor required for the bean enzyme activity and could not be substituted by NADH (Martin and others 1989). On the other hand, the ZRED activity in enzyme preparations from pea leaves was significantly inhibited or completely suppressed in our experiments by the addition of a chelating agent, DIECA (10 mM), to the reaction mixture (Figure 6), which suggests that a metal cofactor is necessary in the oxidoreductase mechanism of Z → DHZ conversion catalyzed by the enzyme.

Only very weak or no enzymatic conversion of Z to DHZ was detected under the same experimental conditions with ZRED preparations from pea stems and roots (Figure 5 and data not shown). As endogenous DHZ was found to occur also in pea stems and roots (data not shown), it is possible that the method we used was not sensitive enough for detection of ZRED activity in these organs or that DHZ was imported and/or originated by some alternative means. Although ZRED activity was detected in leaves of other pea cultivars as demonstrated for cv. Canis, it was not found in leaves of other legumes (bean, soybean) (Table 4). This finding supports the assumption that ZRED activities may vary substantially between different legume species, which corresponds to the suggested genotypic variations of ZRED activities in *Phaseolus* embryos (Mok and others 1990).

Using [2-^{3}H]Z as a substrate of the enzyme reaction, we compared the ZRED activity with that of CKX (both enzyme activities expressed in nmol of formed product [that is, DHZ and Ade, respectively] mg^{-1} protein h^{-1}) in pea leaves. Based on the data presented in Figure 5, Figure 6, and Table 4, the proportion of ZRED to CKX activities ranged from 0.7 to 1.9.

Summarizing our results, it can be concluded that the presented experiments opened new views on cytokinin homeostasis in pea as a crop plant of high economic significance. They provided original data

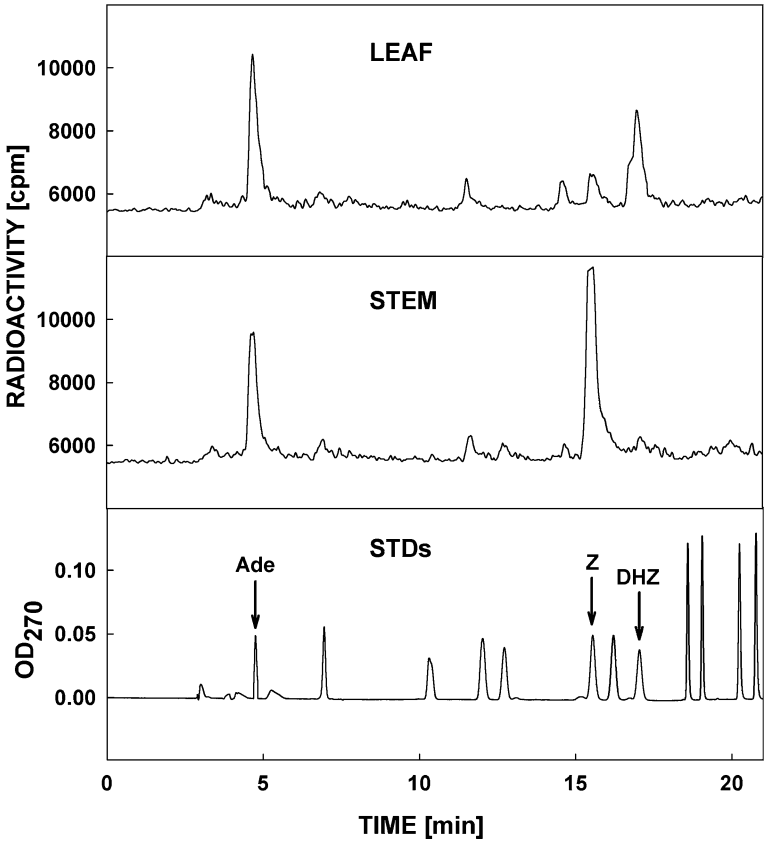

Figure 5. HPLC analysis of products of [2-^{3}H]Z conversion *in vitro* by protein preparations from pea (*Pisum sativum* L. cv. Gotik) leaves and stems in the presence of NADPH (0.25 mM) in the assay mixture demonstrating CKX (both leaves and stems) and ZRED (leaves only) activities. The ZRED activity was determined in the assay mixture containing 3 μM [2-^{3}H]Z (8.3 TBq mol^{-1}), 100 mM potassium phosphate buffer (pH 8.0) containing 15 mM dithiothreitol + 0.25 mM NADPH and protein preparation (equivalent 400 to 800 mg of fresh tissue) in a total volume of 300 μL. The results of one of three experiments showing the same trends are presented. The data represent the means of three replications. The SD values averaged 12% and did not exceed 22% of the mean. (STDs, standards; Ade, adenine; Z, *trans*-zeatin; DHZ, dihydrozeatin).

Table 4. Comparison of Zeatin Reductase (ZRED) and Cytokinin Oxidase/Dehydrogenase (CKX) Activities in Protein Preparations from Pea (*Pisum sativum* L. cv. Gotik and cv. Canis), Bean (*Phaseolus vulgaris* L. cv. Gama), and Soybean (*Glycine max* (L.) Merr. cv. Lek-2760/98) Leaves

Plant material (leaves)	ZRED activity (nmol DHZ mg^{-1} protein h^{-1})	CKX activity (nmol Ade mg^{-1} protein h^{-1})
Pisum sativum L. cv. Gotik	0.043 ± 0.011	0.023 ± 0.002
Pisum sativum L. cv. Canis	0.027 ± 0.003	0.032 ± 0.004
Phaseolus vulgaris L.	n.d.	0.031 ± 0.001
Glycine max (L.) Merr.	n.d.	0.013 ± 0.001

The enzyme activities were determined in the assay mixture containing 3 μM [2-^{3}H]Z (8.3 TBq mol^{-1}), 100 mM potassium phosphate buffer (pH 8.0) containing 15 mM dithiothreitol + 0.25 mM NADPH, and protein preparation (equivalent 400 to 900 mg of fresh tissue) in a total volume of 300 μl. The results of one of three experiments showing the same trends are presented. The data represent the means of three replications. The SD values averaged 13% and did not exceed 25% of the mean.

to the present knowledge of the occurrence and metabolism of cytokinins in plants, especially in legumes, with particular respect to metabolic pathways catalyzed by CKX and ZRED. Simultaneous determination of CKX and ZRED activities in pea leaves allowed comparisons of the actual roles of the

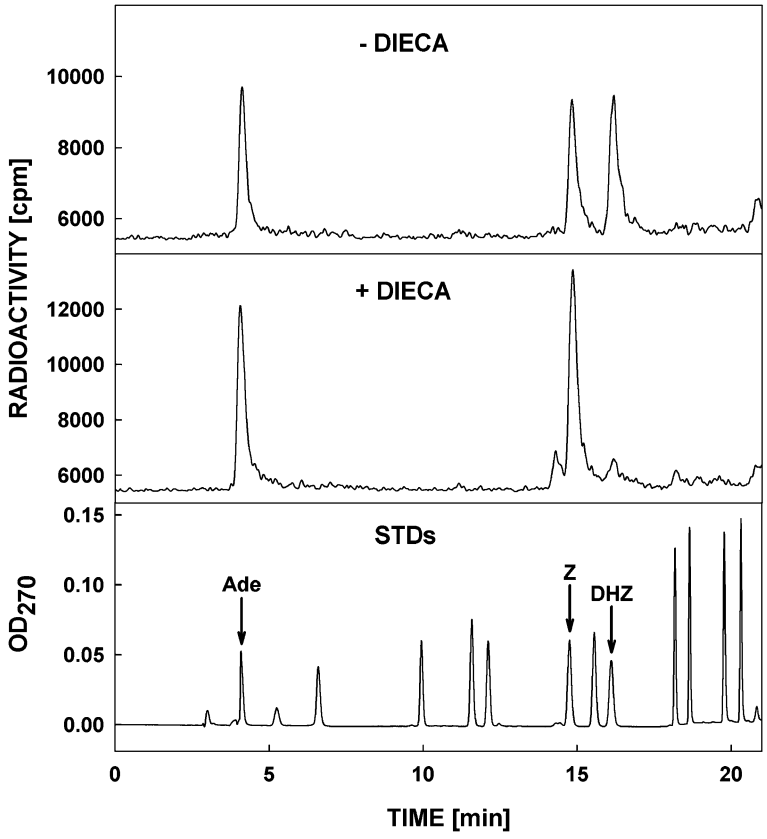

Figure 6. Effect of diethyldithiocarbamic acid (DIECA) on the conversion of [2-^{3}H]Z *in vitro* by protein preparations from pea (*Pisum sativum* L. cv. Gotik) leaves. The ZRED activity was determined as described in Figure 5 in the presence or absence of DIECA (10 mM). The data represent the means of three replications. The SD values averaged 8% and did not exceed 17% of the mean. (STDs, standards; Ade, adenine; Z, *trans*-zeatin; DHZ, dihydrozeatin).

two enzymes in control of cytokinin levels in plants, which represents a novel approach toward the investigation of the mechanisms maintaining hormonal homeostasis. However, it should be taken into account that CKX downregulates not only the contents of Z but also those of DHZ at the level of their common precursor.

Although various Z- and DHZ-type cytokinins were identified by HPLC-MS in pea leaves, indicating their diverse metabolic conversions — including those catalyzed by CKX and ZRED — particularly remarkable was the finding of relatively high contents of non-hydroxylated aromatic cytokinins (BA, BAR) and *cis*Z derivatives. Even though the endogenous cytokinin analysis was not intended as the main task of this study, the presence of BA- and *cis*Z-type cytokinins in pea leaves is interesting, and their identification supported increasing evidence of a more widespread occurrence and importance of these cytokinin forms in the plant kingdom.

Characterization of CKX activity revealed the existence of a non-glycosylated CKX isoform with relatively high pH optimum (pH 8.5) in pea leaves. The presence of non-glycosylated CKX and/or CKX with a very low degree of glycosylation is generally rather uncommon in plants, and the finding in pea suggests a more abundant occurrence as well as possible relevance and function in some legume genotypes. The data presented here confirmed that nonglycosylated or slightly glycosylated CKXs exhibit high pH optima (Kamínek and Armstrong 1990; Motyka and others 2003). Also, the detection of ZRED activity in leaves (that is, vegetative organs) of pea is novel, because so far this enzyme activity had been isolated only from *Phaseolus* embryos (that is, generative organs; Martin and others 1989; Mok and others 1990). Although the proportion of ZRED to CKX activities was found to vary in a relatively broad range in pea leaves, a close relationship between conversions of Z-type cytokinins catalyzed by ZRED and their degradation by CKX is obvious. The ZRED and CKX activities evidently compete in plants for the same substrate cytokinin, Z. The conversion of Z to DHZ by ZRED activity is probably functional, especially in plants rich in cytokinins of the DHZ-type, such as legumes.

As DHZ-type cytokinins are resistant to the attack by CKX, the potency of ZRED may contribute to the preservation of cytokinin activity in these plants.

The involvement of CKX in regulation of cytokinin levels in plants is well known (for review see Armstrong 1994; Kamínek and others 1997). The fact that ZRED activity converts cytokinins to forms protected from breakdown by CKX underlines a potential role of ZRED in cytokinin homeostasis. In this respect, the demonstration of ZRED activity *in vitro* in this work could facilitate the appreciation of functioning of this enzyme in plants and the identification and cloning of corresponding genes from pea or other plant species.

ACKNOWLEDGMENTS

This work was supported by the Grant Agency of the Czech Republic (206/03/0313). The authors thank Dr. Miroslav Kamínek for critical reading of manuscript and Vanda Lacmanová and Marie Korecká for excellent technical assistance.

REFERENCES

Armstrong DJ. 1994. ''Cytokinin oxidase and the regulation of cytokinin degradation'' In: Mok DWS, Mok MC, editors. *Cytokinins: Chemistry, Activity, and Function*. Boca Raton, FL, USA, CRC Press. pp 139–154.

Bilyeu KD, Cole JL, Laskey JG, Riekhof WR, Esparza TJ, and others. 2001. Molecular and biochemical characterization of a cytokinin oxidase from maize. Plant Physiol 125:378–386.

Bilyeu KD, Laskey JG, Morris RO. 2003. Dynamics of expression and distribution of cytokinin oxidase/dehydrogenase in developing maize kernels. Plant Growth Regul 39:195–203.

Boiten H, Azmi A, Dillen W, De Schepper S, Debergh P, and others. 2004. The *Rg-1* encoded regeneration capacity of tomato is not related to an altered cytokinin homeostasis. New Phytol 161:761–771.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Chatfield JM, Armstrong DJ. 1986. Regulation of cytokinin oxidase activity in callus tissues of *Phaseolus vulgaris* L. cv Great Northern. Plant Physiol 80:493–499.

Corbesier L, Prinsen E, Jacqmard A, Lejeune P, Van Onckelen H, and others. 2003. Cytokinin levels in leaves, leaf exudate and shoot apical meristem of *Arabidopsis thaliana* during floral transition. J Exp Bot 54:2511–2517.

Davies PJ, Horgan R, Heald JK, McGaw BA. 1986. Endogenous cytokinins in vegetative shoots of peas. Plant Growth Regul 4:311–323.

Dobrev PI, Kamínek M. 2002. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. J Chromatogr A 950:21–29.

Dobrev P, Motyka V, Gaudinová A, Malbeck J, Trávníčková A, and others. 2002. Transient accumulation of *cis*- and *trans*-zeatin type cytokinins and its relation to cytokinin oxidase activity during cell cycle of synchronized tobacco BY-2 cells. Plant Physiol Biochem 40:333–337.

Emery RJN, Leport L, Barton JE, Turner NC, Atkins CA. 1998. *Cis*-isomers of cytokinins predominate in chickpea seeds throughout their development. Plant Physiol 117: 1515–1523.

Emery RJN, Ma Q, Atkins CA. 2000. The forms and sources of cytokinins in developing white lupin seeds and fruits. Plant Physiol 123:1593–1604.

Galuszka P, Frébort I, Šebela M, Sauer P, Jacobsen S, and others. 2001. Cytokinin oxidase or dehydrogenase? Mechanism of cytokinin degradation in cereals. Eur J Biochem 268:450–461.

Galuszka P, Frébortová J, Werner T, Yamada M, Strnad M, and others. 2004. Cytokinin oxidase/dehydrogenase genes in barley and wheat. Cloning and heterologous expression. Eur J Biochem 271:3990–4002.

Haberer G, Kieber JJ. 2002. Cytokinins. New insights into a classic phytohormone. Plant Physiol 128:354–362.

Houba-Hérin N, Pethe C, d' Alayer J, Laloue M. 1999. Cytokinin oxidase from *Zea mays*: purification, cDNA cloning and expression in moss protoplasts. Plant J 17:615–626.

Jäger AK, Stirk WA, Van Staden J. 1997. Cytokinin oxidase activity in habituated and non-habituated soybean callus. Plant Growth Regul 22:203–206.

Jameson PE. 1994. ''Cytokinin metabolism and compartmentation'' In: Mok DWS, Mok MC, editors. *Cytokinins: Chemistry, Activity, and Function* Boca Raton, FL, USA: CRC Press. pp 113–128.

Jones RJ, Schreiber BMN. 1997. Role and function of cytokinin oxidase in plants. J Plant Growth Regul 23:123–134.

Kamínek M, Armstrong DJ. 1990. Genotypic variation in cytokinin oxidase from *Phaseolus* callus cultures. Plant Physiol 93:1530–1538.

Kamínek M, Březinová A, Gaudinová A, Motyka V, Vaňková R, and others. 2000. Purine cytokinins: a proposal of abbreviations. Plant Growth Regul 32:253–256.

Kamínek M, Motyka V, Vaňková R. 1997. Regulation of cytokinin content in plant cells. Physiol Plant 101:689–700.

Kasahara H, Takei K, Ueda N, Hishiyama S, Yamaya T, and others. 2004. Distinct isoprenoid origins of *cis*- and *trans*-zeatin biosyntheses in *Arabidopsis*. J Biol Chem 279:14049–14054.

King RA, Van Staden J. 1987. The metabolism of N^6-(Δ^2-isopentenyl)[^{3}H]adenine by isolated organs of *Pisum sativum*. J Plant Physiol 131:181–190.

King RA, Van Staden J. 1990. The metabolism of N^6(Δ^2-isopentenyl)[^{3}H]adenine by different stem sections of *Pisum sativum*. Plant Growth Regul 9:237–246.

Letham DS, Palni LMS. 1983. The biosynthesis and metabolism of cytokinins. Annu Rev Plant Physiol 34:163–197.

Martin RC, Mok MC, Habben JE, Mok DWS. 2001. A maize cytokinin gene encoding an *O*-glucosyltransferase specific to *cis*-zeatin. Proc Natl Acad Sci USA 98:5922–5926.

Martin RC, Mok MC, Shaw G, Mok DWS. 1989. An enzyme mediating the conversion of zeatin to dihydrozeatin in *Phaseolus* embryos. Plant Physiol 90:1630–1635.

Massonneau A, Houba-Hérin N, Pethe C, Madzak C, Falque M, and others. 2004. Maize cytokinin oxidase genes: differential expression and cloning of two new cDNAs. J Exp Bot 55:2549–2557.

Mok DWS, Mok MC. 2001. Cytokinin metabolism and action. Annu Rev Plant Physiol Plant Mol Biol 52:89–119.

Mok DWS, Mok MC, Shaw G, Dixon SC, Martin RC. 1990. ''Genetic differences in the enzymatic regulation of zeatin metabolism in Phaseolus embryos'' In: Pharis RP, Rood SB, editors. *Plant Growth Substances 1988* Berlin, Germany: Springer-Verlag. pp 267–274.

Mok MC. 1994. Cytokinins and plant development—an overview In: Mok DWS, Mok MC, editor. *Cytokinins: Chemistry, Activity, and Function.* Boca Raton, FL, USA: CRC Press. pp 155–166.

Morris RO, Bilyeu KD, Laskey JG, Cheikh NN. 1999. Isolation of a gene encoding a glycosylated cytokinin oxidase from maize. Biochem Biophys Res Commun 255:328–333.

Motyka V, Faiss M, Strnad M, Kamínek M, Schmülling T. 1996. Changes in cytokinin content and cytokinin oxidase activity in response to derepression of *ipt* gene transcription in transgenic tobacco calli and plants. Plant Physiol 112:1035–1043.

Motyka V, Kamínek M. 1994. Cytokinin oxidase from auxin- and cytokinin-dependent callus cultures of tobacco (*Nicotiana tabacum* L.). J Plant Growth Regul 13:1–9.

Motyka V, Vaňková R, Čapková V, Petrášek J, Kamínek M, and others. 2003. Cytokinin-induced upregulation of cytokinin oxidase activity in tobacco includes changes in enzyme glycosylation and secretion. Physiol Plant 117:11–21.

Novák O, Tarkowski P, Tarkowská D, Doležal K, Lenobel R, and others. 2003. Quantitative analysis of cytokinins in plants by liquid chromatography—single-quadrupole mass spectrometry. Anal Chim Acta 480:207–218.

Pačes V, Kamínek M. 1976. Effect of ribosylzeatin isomers on the enzymatic degradation of N^6-(Δ^2-isopentenyl)adenosine. Nucleic Acids Res 3:2309–2314.

Palni LMS, Palmer MV, Letham DS. 1984. The stability and biological activity of cytokinin metabolites in soybean callus tissue. Planta 160:242–249.

Parker CW, Letham DS, Gollnow BI, Summons RE, Duke CC, and others. 1978. Regulators of cell division in plant tissues. XXV. Metabolism of zeatin by lupin seedlings. Planta 142: 239–251.

Sakakibara H. 2004. ''Cytokinin biosynthesis and metabolism'' In: Davies PJ, editor. *Plant Hormones. Biosynthesis, Signal Transduction, Action*! Dordrecht The Netherlands: Kluwer Academic Publishers. pp 95–114.

Schmülling T. 2002. New insights into the functions of cytokinins in plant development. J Plant Growth Regul 21:40–49.

Schmülling T, Werner T, Riefler M, Krupková E, Manns IBY. 2003. Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, *Arabidopsis* and other species. J Plant Res 116:241–252.

Singh S, Letham DS, Jameson PE, Zhang R, Parker CW, and others. 1988. Cytokinin biochemistry in relation to leaf senescence. IV. Cytokinin metabolism in soybean explants. Plant Physiol 88:788–794.

Singh S, Palni LMS, Letham DS. 1992. Cytokinin biochemistry in relation to leaf senescence. 5. Endogenous cytokinin levels and metabolism of zeatin riboside in leaf discs from green and senescent tobacco (*Nicotiana rustica*) leaves. J Plant Physiol 139:279–283.

Sondheimer E, Tzou D-S. 1971. The metabolism of hormones during seed germination and dormancy. II. The metabolism of 8-^{14}C-zeatin in bean axes. Plant Physiol 47:516–520.

Strnad M. 1997. The aromatic cytokinins. Physiol Plant 101:674–688.

Tarkowská D, Doležal K, Tarkowski P, Åstot C, Holub J, and others. 2003. Identification of new aromatic cytokinins in *Arabidopsis thaliana* and *Populus* × *canadensis* leaves by LC-(+)ESI-MS and capillary liquid chromatography/frit-fast atom bombardment mass spectrometry. Physiol Plant 117:579–590.

Taylor NJ, Stirk WA, van Staden J. 2003. The elusive cytokinin biosynthetic pathway. South Afr J Bot 69:269–281.

Van Staden J, Forsyth C. 1986. The metabolism of adenine and zeatin in immature caryopses of maize. J Plant Physiol 124:299–308.

Vaseva-Gemisheva I, Lee D, Alexieva V, Karanov E. 2004. Cytokinin oxidase/dehydrogenase in *Pisum sativum* plants during vegetative development. Influence of UV-B irradiation and high temperature on enzymatic activity. Plant Growth Regul 42:1–5.

Veach YK, Martin RC, Mok DWS, Malbeck J, Vankova R, and others. 2003. *O*-Glucosylation of *cis*-zeatin in maize. Characterization of genes, enzymes, and endogenous cytokinins. Plant Physiol 131:1374–1380.

Werner T, Motyka V, Strnad M, Schmülling T. 2001. Regulation of plant growth by cytokinin. Proc Natl Acad Sci USA 98:10487–10492.

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, and others. 2003. Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15:2532–2550.

Yang SH, Yu H, Goh CJ. 2002a. Functional characterisation of a cytokinin oxidase gene *DSCKX1* in *Dendrobium* orchid. Plant Mol Biol 51:237–248.

Yang SH, Yu H, Goh CJ. 2002b. Isolation and characterization of the orchid cytokinin oxidase *DSCKX1* promoter. J Exp Bot 53:1899–1907.

Yonekura-Sakakibara K, Kojima M, Yamaya T, Sakakibara H. 2004. Molecular characterization of cytokinin-responsive histidine kinases in maize. Differential ligand preferences and response to cis-zeatin. Plant Physiol 134:1654–1661.

Zažímalová E, Kamínek M, Březinová A, Motyka V. 1999. ''Control of cytokinin biosynthesis and metabolism'' In: Hooykaas PJJ, Hall MA, Libbenga KR, editors. *Biochemistry and Molecular Biology of Plant Hormones* Amsterdam, The Netherlands: Elsevier Science B.V. pp 141–160.

Zhang R, Letham DS. 1990. Cytokinin translocation and metabolism in lupin species. III. Translocation of xylem cytokinin into the seeds of lateral shoots of *Lupinus angustifolius*. Plant Sci 70:65–71.

Zhang R, Letham DS, Willcocks DA. 2002. Movement to bark and metabolism of xylem cytokinins in stems of *Lupinus angustifolius*. Phytochemistry 60:483–488.