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

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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

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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.

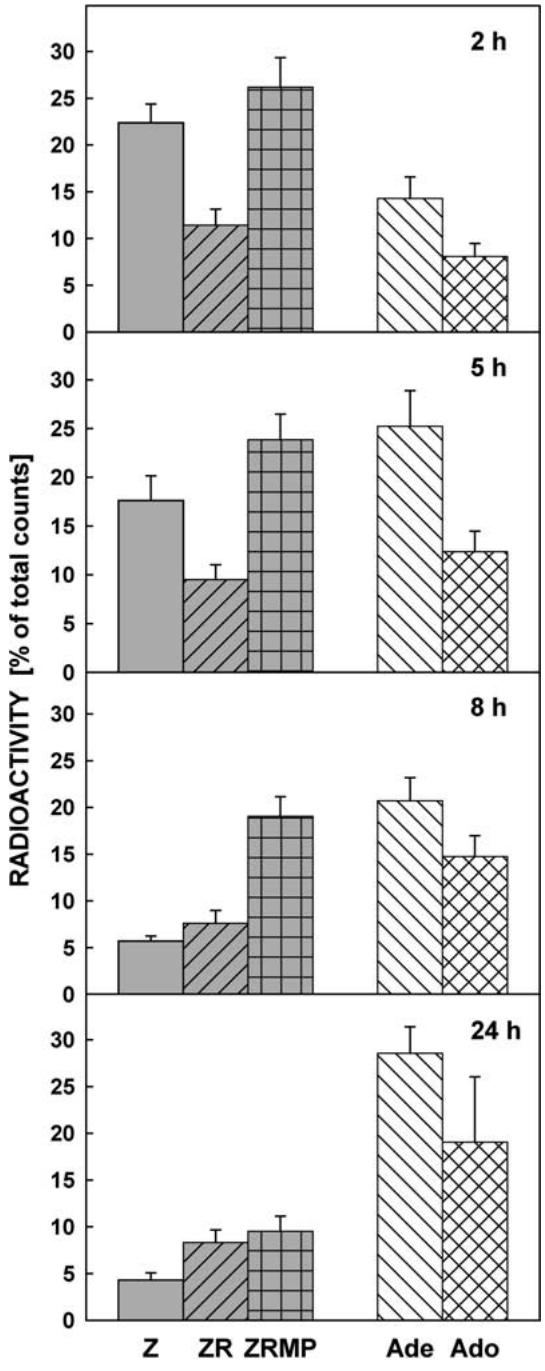

Figure 1. Distribution of radioactivity detected in de-rooted pea (*Pisum sativum* L. cv. Gotik) plants 2, 5, 8, and 24 h after application of [2-^{3}H]Z. Products of [2-^{3}H]Z metabolism were analyzed using HPLC coupled to an on-line radioactivity detector. Values are expressed as percentage of the 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.

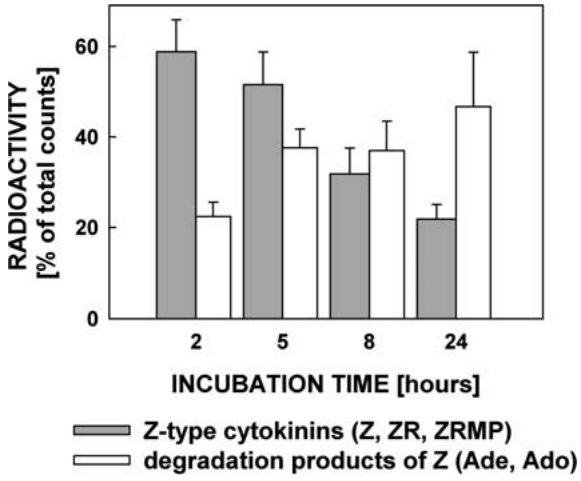

Figure 2. Proportion of radioactivity associated with Z-type cytokinins and their degradation products in de-rooted pea (*Pisum sativum* L. cv. Gotik) plants 2, 5, 8, and 24 h after application of [2-^{3}H]Z. Other details are as described in Figure 1.

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.*

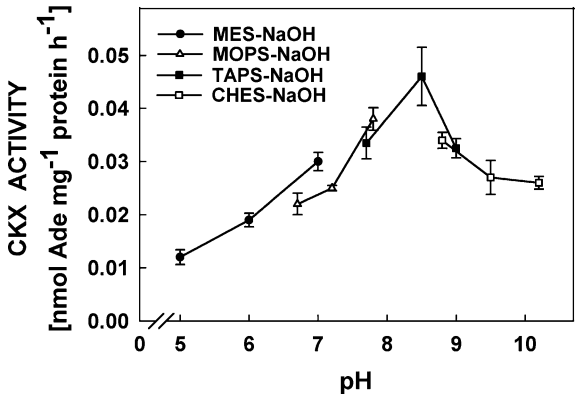

Figure 3. Effect of pH on the *in vitro* activity of cytokinin oxidase/dehydrogenase (CKX) from pea (*Pisum sativum* L. cv. Gotik) leaves. The pH optimum of CKX activity was determined by running the standard enzyme assay in different buffers including 0.1 M MES-NaOH, 0.1 M MOPS-NaOH, 0.1 M TAPS-NaOH, 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).

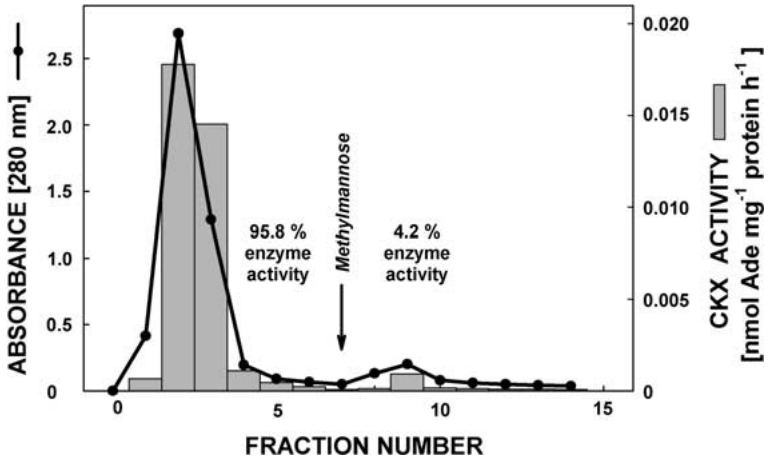

Figure 4. Separation of glycosylated and non-glycosylated cytokinin oxidase/dehydrogenase (CKX) isoforms from pea (*Pisum sativum* L. cv. Gotik) leaves by concanavalin A (Con A)–Sepharose 4B column chromatography. The enzyme preparation equivalent to approximately 5 mg protein was loaded onto the Con 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

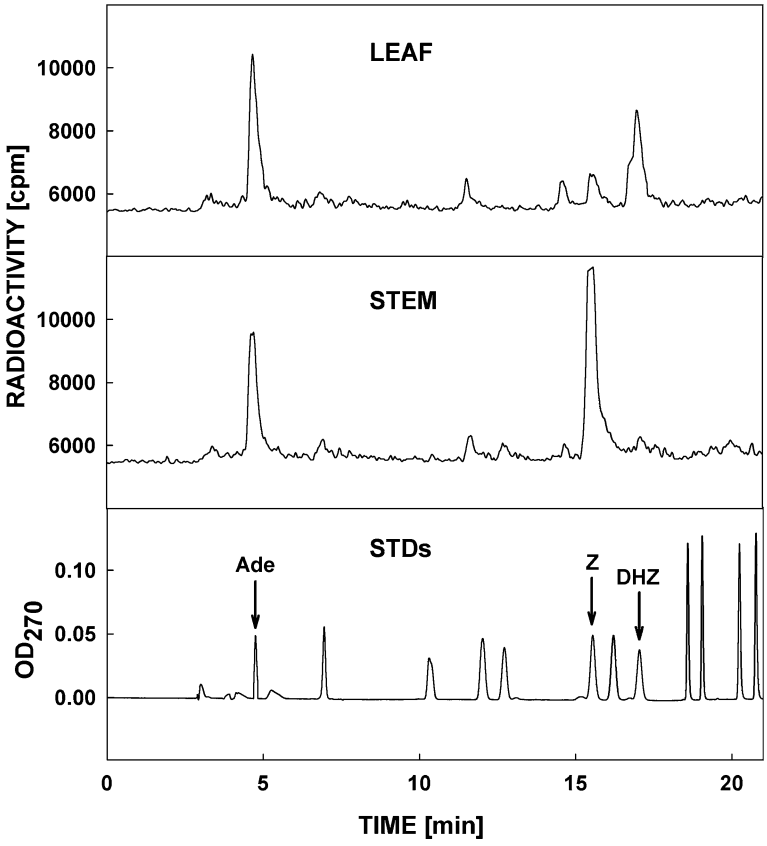

Figure 5. HPLC analysis of products of [2-^{3}H]Z conversion *in vitro* by protein preparations from pea (*Pisum sativum* L. cv. Gotik) leaves and stems in the presence of NADPH (0.25 mM) in the assay mixture demonstrating CKX (both leaves and stems) and ZRED (leaves only) activities. The ZRED activity 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

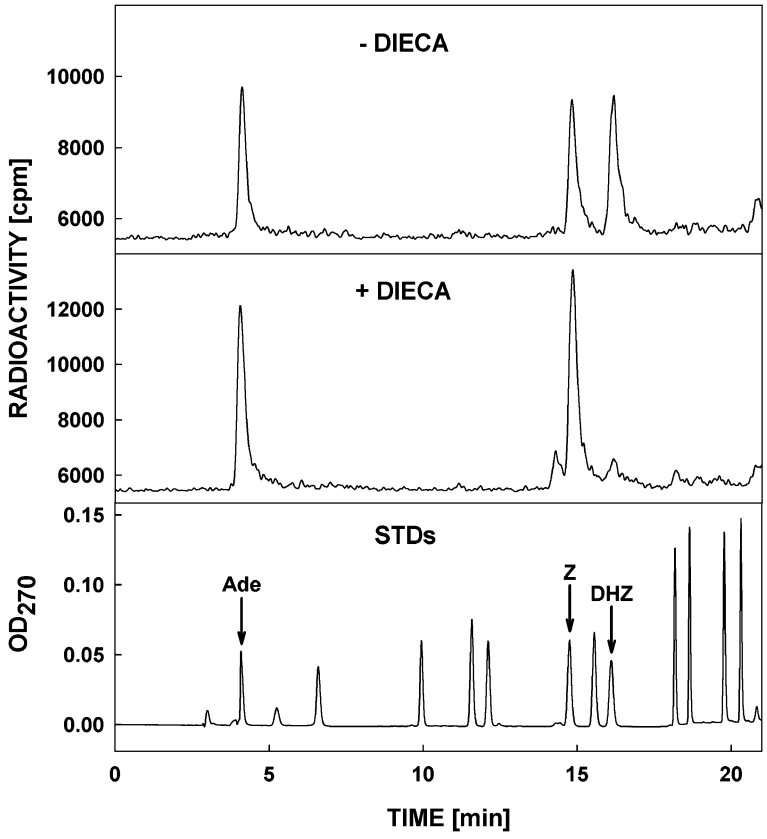

Figure 6. Effect of diethyldithiocarbamic acid (DIECA) on the conversion of [2-^{3}H]Z *in vitro* by protein preparations from pea (*Pisum sativum* L. cv. Gotik) leaves. The ZRED activity was determined as described in Figure 5 in the presence or absence of DIECA (10 mM). The data represent the means 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.

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Efficiency of different methods of extraction and purification of cytokinins

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Abstract

The increasing use of advanced methods, such as mass spectrometry, for the determination of cytokinins has raised special requirements for the extraction and purification of this class of plant hormones. Extraction of *Arabidopsis thaliana* plants with three different solvents, [80% (v/v) MeOH, Bieleski's MCF-7, and modified Bieleski's] provided similar yields of most analyzed cytokinins determined by high-performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS). However, the extraction with a modified Bieleski's solvent ($MeOH–HCO_2H–H_2O$ [15:1:4, v/v/v]) gave the highest responses of deuterated cytokinins (used as test compounds) in plant extracts as compared to the responses of pure deuterated standards (relative internal standard response, RISR). Purification of cytokinins using Oasis MCX sorbent with reversed-phase and cation-exchange characteristics, in comparison to the DEAE Sephadex RP-C_{18} method, provided higher levels of zeatin riboside monophosphate and similar levels of cytokinin bases, ribosides and glucosides. Using this method the content of UV-absorbing contaminates was decreased by about 90% and the RISR values of all tested cytokinin standards but riboside monophosphates were increased about two-fold. The former method provided preparations more suitable for HPLC/MS/MS analysis with respect to simplicity and sample purity.

Keywords: *Arabidopsis thaliana*; Cytokinin extraction; Cytokinin purification; Plant hormones; Solid-phase extraction; HPLC/MS/MS

1. Introduction

Cytokinins are a class of plant hormones that in cooperation with auxin play unique role in the control of developmental processes in plants such as cell division and differentiation, formation and growth of roots and shoots, apical dominance and senescence. Natural cytokinins are 6-*N*-substituted purine derivatives. Those which occur in plants as free bases are supposed to be the biologically active compounds. Glycosidic conjugates of cytokinins are transport, storage or inactivated forms of cytokinins; while cytokinin riboside phosphates predominantly represent the primary products of cytokinin biosynthesis. More than 40 natural cytokinins have been identified in plant tissues (Zažímalová et al., 1999). The structures of those cytokinins analyzed in the present report are shown in Fig. 1. Their occurrence in minute amounts in non-transformed plants ($<10^{-8}$ M), in the presence of structurally related compounds and enzymes catalyzing metabolic conversions and the degradation of cytokinins (Mok and Mok, 2001), complicates both their purification and determination (Horgan and Scott, 1987; Jones et al., 1996). Increasing use of mass spectrometry for the detection and determination of a wide spectrum of cytokinins requires both the operational as well as dependable extraction and purification techniques which prevent metabolic conversions during extraction, and also provide samples of sufficient purity for mass spectrometry.

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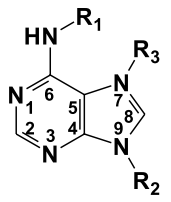
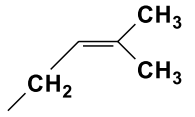
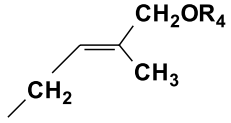


R_1	R_2	R_3	R_4	Common name	Abbreviation
	H	-	-	6-*N*-(2-isopentenyl)adenine	iP
	R	-	-	6-*N*-(2-isopentenyl)adenosine	iPR
	RP	-	-	6-*N*-(2-isopentenyl)adenosine 5′-monophosphate	iPRMP
	-	G	-	6-*N*-(2-isopentenyl)adenine 7-glucoside	iP7G
	G	-	-	6-*N*-(2-isopentenyl)adenine 9-glucoside	iP9G
	H	-	H	*trans*-zeatin	Z
	R	-	H	*trans*-zeatin riboside	ZR
	RP	-	H	*trans*-zeatin riboside 5′-monophosphate	ZRMP
	-	G	H	*trans*-zeatin 7-glucoside	Z7G
	G	-	H	*trans*-zeatin 9-glucoside	Z9G
	H	-	G	*trans*-zeatin *O*-glucoside	ZOG
	R	-	G	*trans*-zeatin riboside *O*-glucoside	ZROG

H, hydrogen; R, ß-D-ribofuranosyl; RP, ß-D-ribofuranosyl-5′-monophosphate; G,ß-D-glucopyranosyl.

Fig. 1. Structures, names and abbreviations of cytokinins investigated.

A wide array of solvents, particularly MeOH, EtOH, perchloric acid and their mixtures, have been used for the extraction of cytokinins (Laloue et al., 1974; Horgan and Scott, 1987; Crouch and Van Staden, 1992). Attention has been paid to the inactivation of phosphatases catalyzing the hydrolysis of cytokinin riboside phosphates during extraction. Bieleski (1964) developed MCF-7 solvent consisting of MeOH–$CHCl_3$–HCO_2H–H_2O (12:5:1:2, v/v/v/v) that was found to inactivate phosphatases in various plant materials. This solvent has also been widely used for the extraction of cytokinins.

As was shown in the model experiments of Horgan and Scott (1987), the extraction of soybean callus tissue with Bieleski's solvent minimizes hydrolysis of AMP. However, preservation of cytokinin riboside phosphates during extraction of the plant material with this solvent has not been directly tested yet. Analyzing soybean callus tissue after feeding with [^{14}C]benzyladenine, which was metabolically converted in vivo to the corresponding riboside monophosphate, Crouch and Van Staden (1992) found no substantial differences in the proportions of riboside monophosphate and the corresponding dephosphorylated product (riboside), when freeze-dried tissue was extracted with either 80% (v/v) EtOH or with Bieleski's solvent. Moreover, the presence of $CHCl_3$ in the Bieleski's solvent was reported to increase the extraction of lipophilic material that complicates further purification (Laloue et al., 1974; Horgan and Scott, 1987). The present availability of labeled cytokinin riboside monophosphates may allow accurate comparisons of different solvents, with respect to protection of cytokinin riboside phosphates.

Anion-exchange column chromatography provides an efficient step for the separation of cytokinin riboside phosphates from cytokinin bases, ribosides and glucosides. DEAE-cellulose and DEAE-Sephadex have been successfully used for this purpose. Cytokinin riboside phosphates, applied in neutral aqueous solution, are retained on such sorbents and can be eluted with an ammonium salt (~1 M) or 6% formic acid. Cytokinin bases, ribosides and glucosides, which are not bound to anion-exchange sorbents, can be retained either on a reversed-phase (RP)-C_{18} or on a cation-exchange column, attached in tandem with the anion-exchange column (MacDonald and Morris, 1985; Redig et al., 1996; Prinsen et al., 1995).

New solid-phase extraction (SPE) sorbents bearing both the RP and cation-exchange characteristics greatly increase and simplify the purification of cytokinins and allow separation of different plant hormones (auxin, abscisic acid, and cytokinins) on a single column, after their simultaneous extraction. Moreover, using MCX RP cation-exchange sorbent, cytokinin riboside monophosphates were separated from cytokinin bases, ribosides and glucosides at high recoveries by simple step elution, with solvents of increasing pH and concentration of MeOH (Dobrev and Kamínek, 2002). Another method using mixed mode reversed-phase anion-exchange SPE prior to two-dimensional HPLC was recently developed for purification of auxin and abscisic acid (Dobrev et al., 2005).

Purification of cytokinins using immunoaffinity chromatography allows very powerful purification of most cytokinins (MacDonald and Morris, 1985; Vaňková et al., 1998; Corbesier et al., 2003). Antibodies of low specificity allowing binding of a wide spectrum of cytokinins have been

successfully used for this purpose. However, even these antibodies do not bind cytokinin *N*-7- and *O*-glucosides (Banowetz, 1994; Jordi et al., 2000) that need to be analyzed separately (Corbesier et al., 2003). Cytokinin *O*-glucosides washed out from immunoaffinity columns can be purified on the same type of column after their hydrolysis by β-glucosidase (Werner et al., 2003; Novák et al., 2003).

The identification and determination of cytokinins by high-performance liquid chromatography-mass spectrometry (HPLC/MS/MS) is increasingly used in plant hormone research. In addition to the more rigorous identification of different cytokinins, these methods also allow the simultaneous quantification of a very wide spectrum of cytokinins in a single run. For these and other reasons, they are becoming a standard in the analysis of cytokinins in plant materials (Redig et al., 1996; Prinsen et al., 1998; Nordström et al., 2004). In contrast to immunological methods (RIA and ELISA), mass spectrometry is also suitable for the determination of cytokinin *N*7- and *O*-glucosides which are not well recognized by antibodies raised against the corresponding cytokinin ribosides (compare Sáenz et al., 2003 and Novák et al., 2003). Because of the high selectivity of mass spectrometry, different cytokinins can be detected and determined in partially purified samples.

The aim of this paper is to compare the efficiency of different methods of cytokinin extraction and purification, with respect to both the prevention of hydrolysis of the cytokinin riboside phosphates, as well as sufficient sample purity for cytokinin determination using HPLC/MS/MS.

2. Results and discussion

The comparison of efficiencies of the different extraction and purification methods is based on the determination of cytokinin levels and relative internal standard responses (RISR) of deuterated cytokinins, expressing the ratio of HPLC/MS/MS chromatogram peak areas of deuterated cytokinins recorded in plants extracts with those of the corresponding pure deuterated standards. The RISR characterizes both the losses during the extraction and purification, as well as the matrix effects of the mass spectrometric analysis.

2.1. Testing of different extraction solvents

In comparing three different solvents (a) 80% (v/v) MeOH, (b) Bieleski's MCF-7, and (c) modified Bieleski's, the deuterated cytokinins were added at the beginning of the extraction, and the extracts were purified using the mixed-mode-SPE as described in Section 4.3. As shown in Fig. 2A, the tested solvents gave similar yields of analyzed cytokinins. Small differences in levels of endogenous cytokinins confirm the efficacy of the internal standard calibration method. However, the modified Bieleski's solvent provided the highest RISR values for almost all cytokinins tested, but riboside monophosphates that were determined after enzymatic hydrolysis as corresponding ribosides. As described in Section 4.6. the hydrolysate was purified on Sep-Pak Plus †C_{18} column where, evidently, contaminants affecting RISR were removed. The results indicate that the solvents differ in their extraction efficiency of compounds other than cytokinins, which are not sufficiently removed during purification and may influence the sensitivity, accuracy and reliability of the determination of cytokinins by HPLC/MS/MS.

Differences in RISR values, between the extractions with Bieleski's and modified Bieleski's solvent, show that $CHCl_3$ in the Bieleski's solvent significantly enhances the extraction of compounds, decreasing the RISR of all tested cytokinins but Z where the difference was not statistically significant. The fact that differences in RISR values in plant material extracted with 80% MeOH and modified Bieleski's solvent are statistically significant only for few cytokinins (iP, iPR and iP7G) supports such opinion. The RISR values exceeding 1.0 (extraction of iPR with 80% MeOH and modified Bieleski's) indicate an increase of the signal due to the matrix effect.

Several authors used Bieleski's solvent without $CHCl_3$ to reduce extraction of contaminants, avoid the problems of handling and disposal of $CHCl_3$-containing extracts (Wang et al., 1995; Jones et al., 1996; Lighfoot et al., 1997). However, the effect of this on the extraction and determination of cytokinins has not been assessed. According to the results presented here, the modified Bieleski's solvent appeared to be the most suitable of the tested solvents for the extraction of cytokinins, prior to their determination using HPLC/MS/MS.

Cytokinin riboside phosphates can be hydrolyzed to the corresponding ribosides by phosphatases during plant tissue extraction (Horgan and Scott, 1987; Crouch and Van Staden, 1992; Redig et al., 1996). Conversions of ZRMP and iPRMP to the corresponding ribosides during extraction with 80% MeOH, Bieleski's MCF-7 and modified Bieleski's solvents were tested. Two hundred picomol of [$^2H_5$]ZRMP and [$^2H_6$]iPRMP were added separately to the frozen powder of plant material at the beginning of the extraction, and the deuterated products were determined using HPLC/MS/MS after extraction and purification by mixed-mode-SPE. Both riboside monophosphates were hydrolyzed almost exclusively to the corresponding ribosides, and further hydrolysis of the ribosides to the corresponding bases was very low ($\leqslant$1.5%, results not shown). Among all of the tested solvents, iPRMP appeared to be much more sensitive to hydrolysis than ZRMP undergoing nearly 60% conversion during extraction with 80% (v/v) MeOH, as compared to 12% and 18% following extraction with Bieleski's and modified Bieleski's solvents, respectively. Hydrolysis of ZRMP was also the highest in 80% (v/v) MeOH (13%), as compared to Bieleski's and modified Bieleski's solvents (both 3%) (Fig. 3). Correspondingly, Horgan and Scott (1987) reported that AMP as a model riboside monophosphate was also hydrolyzed to adenosine

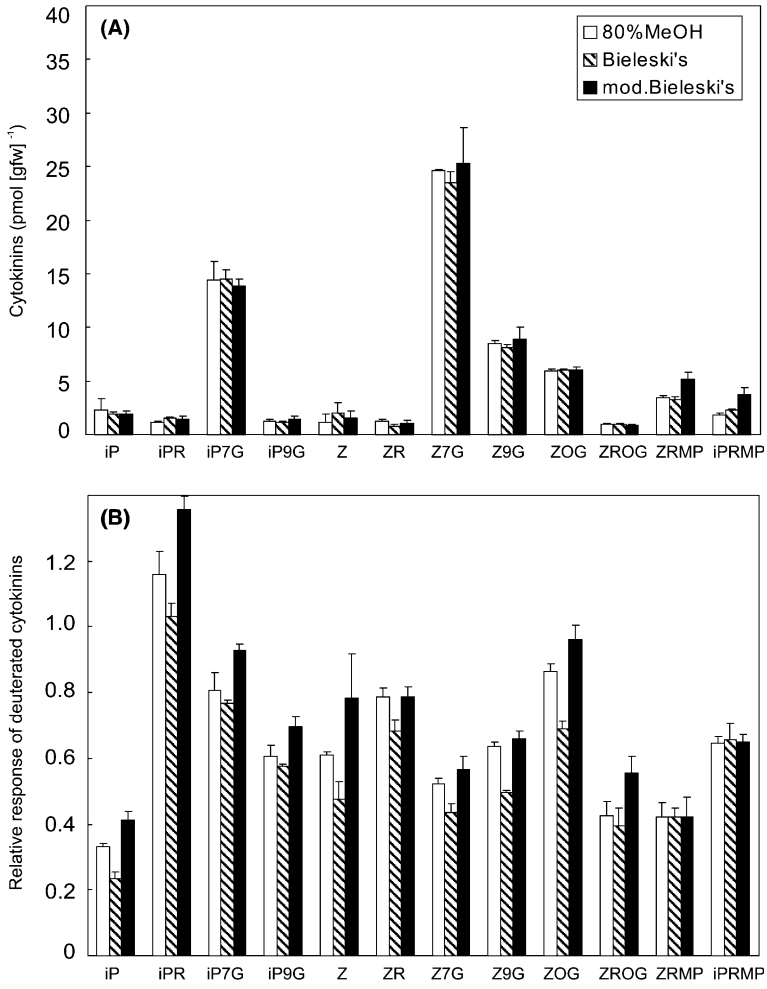


Fig. 2. The effect of three different extraction solvents on cytokinin levels (A) and RISRs, representing the ratio of HPLC/MS/MS responses of deuterated cytokinins from plant extracts to the responses of pure cytokinin standards (B). The plant material was extracted as described in Section 4.2, and the cytokinins were purified as specified in Section 4.4. Results are averages of the analyses of three independent samples. Vertical bars indicate SD.

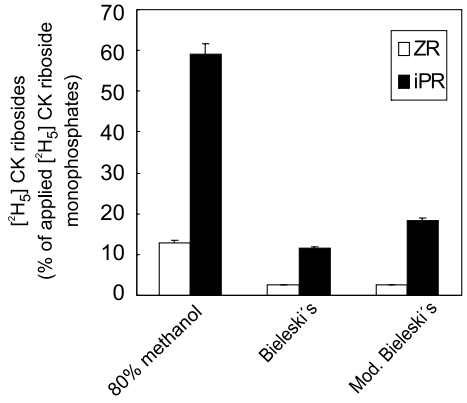


Fig. 3. Conversion of cytokinin (CK) riboside monophosphates ([$^2H_5$]ZRMP and [$^2H_6$]iPRMP) to corresponding CK ribosides following extraction of *Arabidopsis* plants with different solvents. The plant material was supplied with 200 pmol of [$^2H_5$]ZRMP and [$^2H_6$]iPRMP and extracted as described in Section 4.2. Cytokinins were purified as specified in Section 4.2. Results are expressed as a percentage of the cytokinin riboside monophosphates converted to the corresponding ribosides, and represent averages of the analyses of three independent plant samples. Vertical bars indicate SD.

more readily during extraction of soybean callus tissue with 80% MeOH than with Bieleski's solvent.

Evidently, the activity of phosphatases is sufficiently suppressed when plant tissue is powdered in liquid nitrogen and extracted at −20 °C with diluted MeOH acidified with HCO_2H (modified Bieleski's solvent). Moreover, according to Bieleski (1964), extraction in acid solvents at −25 °C speeded-up the deactivation of phosphatases. Unfortunately, he did not test the extraction efficiency of acid solvents lacking $CHCl_3$. As shown in Fig. 3, the omission of $CHCl_3$ from the Bieleski's MCF-7 solvent in the modified Bieleski's solvent did not affect hydrolysis of ZRMP, and only slightly increased hydrolysis of iPRMP.

2.2. Comparison of purification methods

Two different purification procedures, (a) DEAE Sephadex RP-C_{18} and (b) mixed-mode-SPE were compared, using extracts of plant material with modified Bieleski's solvent. Both methods provided similar yields of analyzed cytokinins but iPR and ZRMP where the latter method

provided significantly higher yields (Fig. 4A). The two methods differed significantly in RISR of cytokinins that were almost twice as high in the case of mixed-mode-SPE, as compared to the DEAE Sephadex RP-C_{18} (Fig. 4B). The ratios of RISRs, recorded by the mixed-mode-SPE and DEAE Sephadex RP-C_{18} varied between 1.25 (iP) and 2.3 (Z9G). Values of RISRs for some cytokinins exceeded 1.0, with a maximum of 1.1 (iP7G), most probably due to the increase of the signal by a matrix effect. These results indicate that the DEAE Sephadex RP-C_{18} method was much less efficient in the removal of those contaminants suppressing RISRs. This view is supported by the comparison of total ion current (TIC) of the MS analysis. TIC values were approximately twice as high during the whole interval of elution of cytokinins when plant samples were purified by DEAE Sephadex RP-C_{18}, as compared to mixed-mode-SPE. Moreover, extracts purified by the DEAE Sephadex RP-C_{18} exhibited a much greater variation of TIC profiles of the samples from parallel extractions, than those purified by mixed-mode-SPE (Fig. 5).

It is possible that the differences in RISRs of the tested cytokinins, following their purification using the DEAE Sephadex RP-C_{18} and dual-mode-SPE, are predominantly due to the different recoveries of the deuterated standards. Moreover, the recoveries may be concentration-dependent. To determine the recoveries of cytokinins achieved by the two purification procedures, independent of mass spectrometric analysis, plant extracts corresponding to 1 g of plant material were supplied with [2-^{3}H]Z and [2-^{3}H]ZR, representing cytokinin base and riboside, just before their application onto DEAE Sephadex and dual-mode SPE columns. So as to know if recovery is affected by cytokinin concentration, a separate set of plant extracts was supplied with 40 pmol of the corresponding cold cytokinins, in addition to those labeled [2-^{3}H]. This concentration exceeds the endogenous concentrations of Z and ZR in non-transformed plants (Corbesier et al., 2003; Werner et al., 2003; Ananieva et al., 2004). The recoveries of both [^{3}H]Z and [^{3}H]ZR were very high ($\geqslant$90%) and statistically significant differences were found neither between the two purification procedures nor between samples with and without the addition of cold cytokinins. However, the differences (1.4–2.9%) were not statistically significant, showing that within the tested concentration range recoveries are not dependent on cytokinin concentration (results not shown). This strongly indicates that differences in the levels of tested deuterated cytokinins in plant extracts, purified by

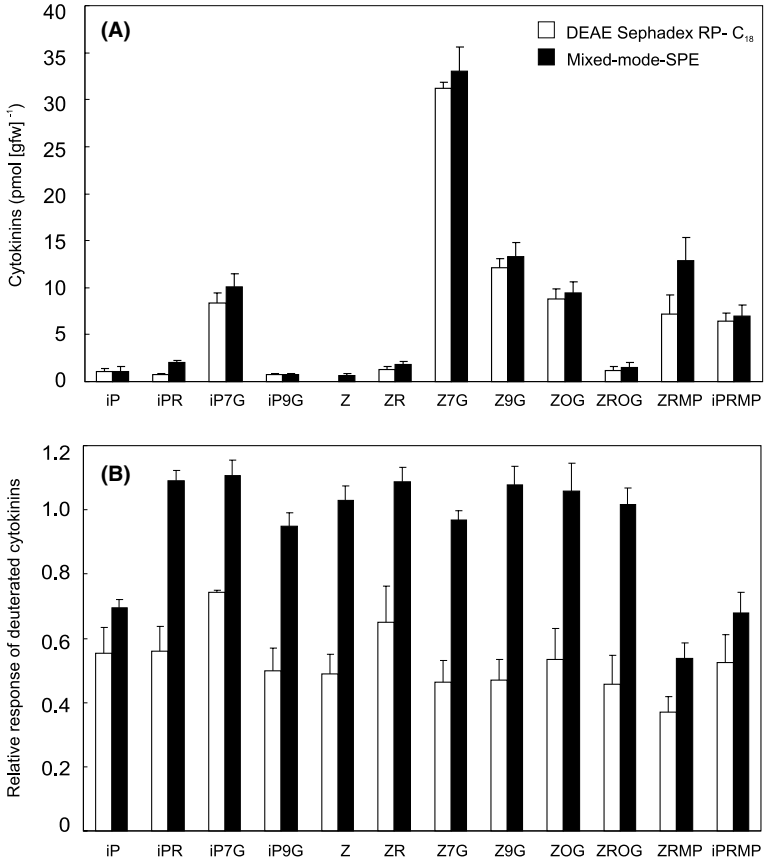


Fig. 4. The effect of purification *of Arabidopsis* plant extracts with DEAE Sephadex RP-C_{18} and mixed-mode-SPE on cytokinin levels (A) and RISRs, representing the ratio of HPLC/MS/MS responses of deuterated cytokinins from plant extract to the responses of pure cytokinin standards (B). The plant material was extracted with modified Bieleski's solvent, as described in Section 4.2, and the cytokinins were purified, as specified in Sections 4.3 and 4.4. Results are averages of the analysis of three independent plant samples. Vertical bars indicate SD.

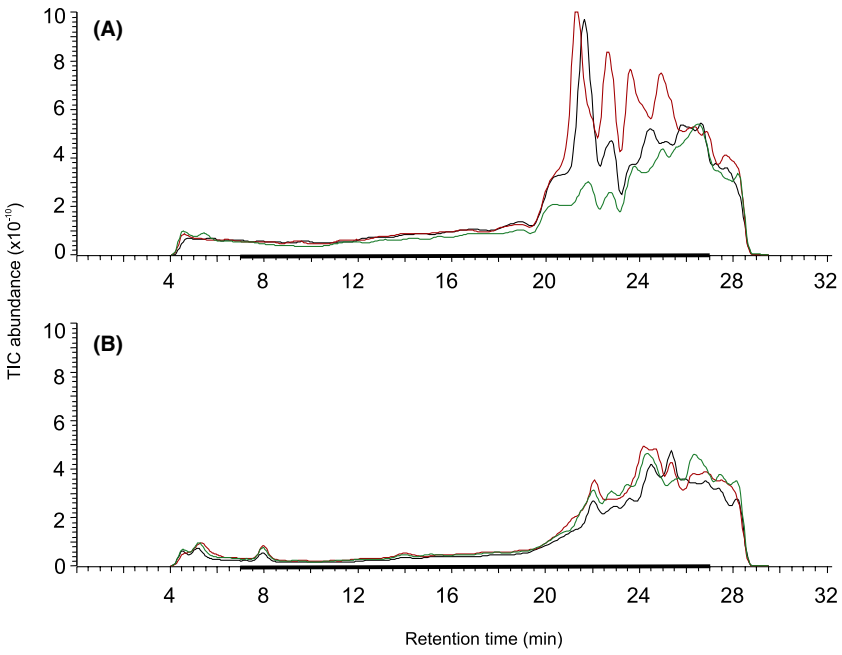


Fig. 5. MS total ion current profiles (TIC) of *Arabidopsis* plant extracts purified with DEAE Sephadex RP-C_{18} (A) and mixed-mode-SPE (B), expressed in arbitrary units. The plant material was extracted with modified Bieleski's solvent, as described in Section 4.2, and the cytokinins were purified as specified in Sections 4.3 and 4.4. Each curve corresponds to one separate analysis. Cytokinins were eluted at retention time from 7 min (Z7G) to 27 min (iPR) as marked by bold lines on the time scales.

the two methods, are caused by the different efficiency of removal of those contaminants interfering with MS analysis, rather than by their differential recoveries.

Purification of cytokinins using dual-mode-SPE is apparently extremely efficient in the removal of UV absorbing contaminants, decreasing the content of UV absorbing material by 90%, as compared to DEAE Sephadex RP-C_{18} (Fig. 6). This demonstrates its potential suitability for the purification of plant extracts prior to the determination of cytokinins by methods other than HPLC/MS/MS, such as ELISA and RIA.

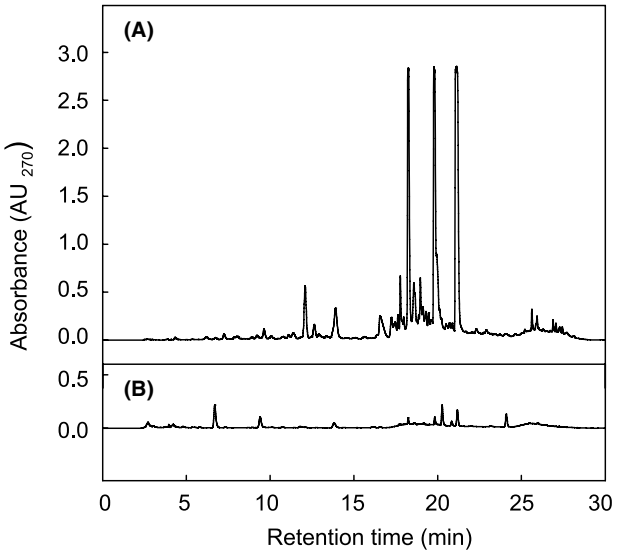


Fig. 6. HPLC UV traces of *Arabidopsis* plant extracts, purified with DEAE Sephadex RP-C_{18} (A) and mixed-mode-SPE (B). The plant material was extracted with modified Bieleski's solvent, as described in Section 4.2, and the cytokinins were purified, as specified in Sections 4.3 and 4.4. No internal standards were added.

3. Conclusion

The modified Bieleski's, as compared to two other tested extraction solvents, sufficiently suppressed dephosphorylation of cytokinin riboside monophosphates and reduced the extraction of compounds decreasing the RISRs of tested deuterated cytokinins. This solvent, lacking $CHCl_3$, is easier and safer to handle and appeared to be the most suitable for extraction of cytokinins. Purification of cytokinins using mixed-mode-SPE, as compared to DEAE Sephadex RP-C_{18} method, was powerful in the removal UV absorbing contaminants providing preparations exhibiting high RISRs of deuterated counterparts of natural cytokinins. This method was found simpler, faster and more operational. It also allows more complex plant hormone analysis by providing a partially purified fraction containing auxin and abscisic acid.

4. Experimental

4.1. Plant material and chemicals

Arabidopsis thaliana (ecotype Columbia) plants were grown in a greenhouse under 16 h light/8 h dark at 23 °C/20 °C daylight cycle. Leaf rosettes of 55-day old plants were frozen in liquid nitrogen and stored at −80 °C.

Deuterium-labeled cytokinins were purchased from Apex (Honiton, Devon, UK). [2-^{3}H]Z and [2-^{3}H]ZR (both 0.59 TBq mmol^{-1}) were obtained from the Isotope Laboratory of the Institute of Experimental Botany, Prague, Czech Republic. EtOH of spectrophotometric grade, MeOH, HCO_2H, NH_4OH, NH_4HCO_3, all of analytical grade, were supplied by Lachema a.s. (Neratovice, Czech Republic). Calf intestine alkaline phosphatase (4U/mg) was a product of Sigma–Aldrich (St. Louis, USA). Sep-Pak Plus †C_{18} cartridges (containing 400 mg sorbent of C_{18} phase bonded on silica gel matrix), and Oasis MCX columns (containing 150 mg sorbent of a sulfonated copolymer, capacity 1 mequiv g^{-1}, particle diameter 30 μm) were obtained from Waters (Milford, MA, USA). DEAE Sephadex A-25, capacity 3.5 mequiv g^{-1} and particle size 40–120 μm was product of Pharmacia (Uppsala, Sweden). Syringes (20 ml) with frits (porosity 20 μm) were purchased from Isolute SPE Accesories (Alltech, UK). The SPE procedures were performed using a vacuum manifold equipped with vacuum control valve that allowed the concurrent processing of 12 samples (Supelco's Visiprep, Sigma, St. Louis, USA).

4.2. Extraction of cytokinins

Three different extraction solvents: (a) 80% (v/v) MeOH, (b) Bieleski's MCF-7 solvent (MeOH–$CHCl_3$–HCO_2H–H_2O 12:5:1:2, v/v/v) and (c) modified Bieleski's solvent (MeOH–HCO_2H–H_2O; 15/1/4, v/v/v) were compared. *Arabidopsis* leaf rosettes were frozen by dipping in liquid nitrogen and then homogenized to a powder with a pestle in a ceramic mortar in liquid nitrogen. One gram plant powder aliquots were distributed to 50 ml polypropylene centrifuge tubes. Ten millilitre aliquots of cold (−20 °C) tested solvents were immediately added together with a mixture of the following deuterium-labeled cytokinin standards (50 pmol each in total volume of 50 μl of 50% MeOH, v/v): [^{2}H$_6$]iP), [^{2}H$_5$]Z, [^{2}H$_3$]DHZ), [^{2}H$_6$]iPR, [^{2}H$_5$]ZR, [^{2}H$_3$]DHZR, [^{2}H$_6$]iP7G, [^{2}H$_6$]iP9G, [^{2}H$_5$]Z7G, [^{2}H$_5$]Z9G, [^{2}H$_5$]ZOG, [^{2}H$_5$]ZROG, [^{2}H$_6$]iPRMP, [^{2}H$_5$]ZRMP and [^{2}H$_3$]DHZRMP. After overnight extraction at −20 °C solids were removed by centrifugation (13,000*g*, 20 min, 4 °C) and re-extracted with 5 ml of corresponding extraction solvent by vortexing for 30 s, followed with standing at −20 °C for 1 h. Pooled extracts were passed through Sep-Pak Plus †C_{18} to remove pigments and lipids and evaporated under vacuum at 40 °C near to dryness prior purification.

4.3. Purification of cytokinins using DEAE Sephadex RP-C_{18}

The method was adapted according to MacDonald and Morris (1985) and Redig et al. (1996). Plant extracts containing deuterated cytokinin standards were diluted with 10 ml water and alkalized with ammonium to pH 7.2–7.5. The solution was applied to a column of DEAE Sephadex (10 ml, HCO_3^- form) connected in tandem with a Sep-Pak Plus †C_{18}. The column was washed with 20 ml of distilled water. At this point, the Sep-Pak Plus †C_{18} column (containing cytokinin bases, ribosides and glucosides) and the DEAE Sephadex column (with entrapped cytokinin riboside phosphates) were disconnected and each column was separately washed with 10 ml water. Cytokinins from Sep-Pak Plus †C_{18} were eluted with 5 ml of 80% (v/v) MeOH and solvent was evaporated under vacuum at 40 °C. The DEAE Sephadex column was connected with a new Sep-Pak Plus †C_{18} column, and the CK riboside phosphates were eluted from the former column into Sep-Pak Plus †C_{18} with 10 ml 1 M NH_4HCO_3. Finally, cytokinin riboside phosphates were eluted from the Sep-Pak Plus †C_{18} with 5 ml of 80% (v/v) MeOH. The solution was evaporated under vacuum at 40 °C to water phase, and the riboside phosphates were hydrolyzed to the corresponding ribosides, as described in Section 4.6.

4.4. Purification of cytokinins mixed-mode-SPE

The method of Dobrev and Kamínek (2002) was used. Plant extracts were diluted with 5 ml of 1 M formic acid and applied on an Oasis MCX column. The column was washed with 5 ml 1 M formic acid, and plant hormones auxin (IAA) and abscisic acid (ABA) were eluted with 5 ml MeOH. Subsequently, the cytokinin riboside phosphates were eluted with 5 ml 0.35 M ammonia in water, and hydrolyzed by alkaline phosphatase, as described in Section 4.6. Cytokinin bases, ribosides and glucosides were eluted in the next step with 5 ml 0.35 M ammonia in 60% (v/v) MeOH. Solvents were evaporated at 40 °C under vacuum.

4.5. Preservation of cytokinin riboside phosphates

Preservation of cytokinin riboside monophosphates during extraction was tested using three different solvents: (a) 80% (v/v) MeOH, (b) Bieleski's MCF-7 and (c) modified Bieleski's solvent. *Arabidopsis* leaf rosettes were frozen and extracted as described in Section 4.2. Two hundred pmol of [^{2}H$_5$]ZRMP and [^{2}H$_6$]iPRMP were added to 1 g aliquots of frozen plant powder at the beginning of extraction, instead of the mixture of deuterated cytokinin standards. Extracted [^{2}H$_5$]ZRMP and [^{2}H$_6$]iPRMP, as well as their ribosides and bases released during extraction, were purified using mixed-mode-SPE (as described in Section 4.4), and determined by HPLC/MS/MS using the standard addition method. The corresponding deuterated ribosides were the only clearly quantified products. The results were expressed as the percentage of deuterated riboside phosphates converted to the corresponding deuterated ribosides.

4.6. Hydrolysis of cytokinin riboside monophosphates

Cytokinin riboside monophosphates were determined as cytokinin ribosides after their dephosphorylation, prior to

HPLC/MS/MS analysis. The MCX column fractions containing cytokinin riboside monophosphates were evaporated to water phase and supplied with 2 M $CH_3CO_2NH_4$ to final 100 mM concentration. After addition of alkaline phosphatase (0.3 U), the solution was incubated at 37 °C for 1.5 h, and then neutralized with formic acid to pH 5–6. The solution was passed through a Sep-Pak Plus †C_{18} column and entrapped cytokinin ribosides were eluted with 5 ml 80% (v/v) MeOH.

4.7. Recoveries of [^{3}H] cytokinin standards and content of contaminants

To determine how the purification procedures affect cytokinin recovery, independent of the cytokinin extraction and MS analysis, *Arabidopsis* leaf rosettes were powdered in liquid nitrogen and extracted with modified Bieleski's solvent under conditions specified in Section 4.2 without the addition of deuterated cytokinin standards. Prior to purification of the cytokinins using DEAE Sephadex RP-C_{18} and mixed-mode-SPE (as described in Sections 4.3 and 4.4, respectively), 4 kBq of [2-^{3}H]Z and [2-^{3}H]ZR were separately added to extract aliquots corresponding to 1 g of plant material. To determine the potential effect of cytokinin concentration on recovery, a separate set of extract aliquots was supplied with 40 pmol of cold cytokinins (Z and ZR), in addition to [^{3}H] cytokinins. The radioactive compounds were separated by HPLC using a series 200 autosampler and quaternary pump (both Perkin–Elmer, Wellesley, MA, USA), a Luna C_{18} (2) column (150 × 4.6 mm, 3 μm, Phenomenex, Torrance, CA, USA) maintained at 35 °C, and two detectors coupled in series: a 235 C diode array detector (Perkin–Elmer, Wellesley, MA, USA), and a Ramona 2000 flow-through radioactivity detector (Raytest, Straubenhardt, Germany). Two solvents (A: 40 mM CH_3CO_2H adjusted with NH_4OH to pH 4 and B: CH_3CN/CH_3OH, 1/1, v/v) were used at a flow rate of 0.6 ml min^{-1} with linear gradients of 10–15% B in 2 min, 15–20% B in 9 min, 20–34% B in 0.1 min, 34–45% B in 7.9 min, 45–100% B in 2 min, 100% B for 2 min, and 100–10% B in 2 min. The column eluate was monitored at 270 nm on the diode array detector and, after on-line mixing with three volumes of liquid scintillation cocktail Flo-Scint III (Packard BioScience, Meriden, CT, USA), on the Ramona 2000 radioactivity detector. The radioactive analytes were identified on the basis of coincidence of their retention times with authentic standards.

4.8. HPLC/MS/MS analysis

The analyses were carried out on a HPLC/MS system, consisting of a PAL HTS autosampler (CTC Analytics, Zwingen, Switzerland) and quaternary HPLC pump Rheos 2000 (Flux Instruments, Basel, Switzerland) connected to an Ion-Trap mass spectrometer LCQ (Finnigan, San Jose, CA, USA) equipped with an electrospray interface (ESI).

Table 1
MS/MS transitions for cytokinin quantitation

Cytokinin	Parent ion	Product ion
ZxG-d5 ($x = 7,9,O$)	387	225
ZROG-d5	519	387
trans-Z-d5	225	207
ZR-d5	357	225
IPxG-d6 ($x = 7,9$)	372	210
IPR-d6	342	210
IP-d6	210	137 + 148
ZxG ($x = 7,9,O$)	382	220
ZROG	514	382
trans-Z	220	202
ZR	352	220
IPxG ($x = 7,9$)	366	204
IPR	336	204
IP	204	136 + 148

Dry samples of purified cytokinins were dissolved in 20 μl 50% (v/v) acetonitrile. Solutions were diluted by the addition of 80 μl H_2O and passed through Micro-Spin centrifuge nylon filters (0.2 μm, Alltech, Deerfield, IL, USA). The filtrate aliquots of 5 μl were injected onto a C_{18} HPLC column (AQUA 250 mm × 2.0 mm, 5 μm, Phenomenex, Torrance, CA, USA) and eluted at a flow rate of 0.2 ml min^{-1} using a linear gradient of acetonitrile (B) in 0.0005% (v/v) acetic acid in water (A): 10% B for 5 min, to 17% B in 10 min; then to 50% B in 11 min; and finally increased to 90% B and maintained for 5 min. The column was equilibrated by the starting composition of the mobile phase for 20 min before each analytical run. MS data were collected in positive MS/MS product ion mode for quantification and the TIC was recorded in a MS full scan (50–2000 amu) mode.

Endogenous natural cytokinins were determined by the same HPLC/MS/MS method using the internal standard calibration and the corresponding deuterated cytokinins as internal standards. The measured masses (m/z) are shown in Table 1. The relative responses of internal standards (RISR) were used to characterize the effectiveness of extraction and purification procedures (recovery) as well as the effectiveness in removal of residual contaminants affecting the MS signal (matrix effect). RISRs are expressed as the ratio of MS/MS responses of deuterated standards (used as test compounds) added to plant samples at the beginning of extraction and responses of corresponding pure deuterated standards.

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Running head:

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Title of article:

Cytokinin oxidase/dehydrogenase activity in oat xylem sap and its regulation by exogenous nitrate

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Abbreviations: *cis*-zeatin, c-Z; *cis*-zeatin riboside, c-ZR; iP, N^6-(2-isopentenyl)adenine; IPT, isopentenyltransferase; CKX, cytokinin oxidase/dehydrogenase; NR, nitrate reductase; t-Z, *trans*-zeatin; t-ZR, *trans*-zeatin riboside;

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Abstract:

There are conflicting views on the functioning of root-sourced cytokinins in root to shoot signaling. Nevertheless, modulation of flux of cytokinins from roots to shoots via xylem flow in response to environmental signals has been repeatedly reported. The concentration of cytokinins in apoplast can be potentially modulated by extracellular cytokinin metabolism. In addressing this issue we found the cytokinin oxidase/dehydrogenase (CKX) activity in xylem sap of oat (*Avena sativa* L.) plants. The enzyme exhibited pH optimum at 8.5 and its activity was associated with glycosylated protein. Since the pH of root-sourced xylem sap is much lower (6.1) the activity of the CKX leaving the roots is suppressed protecting the co-transported cytokinins from degradation. The potential role of CKX in control of the cytokinin concentration in xylem sap in response to environmental signals was tested by the exposure of 12 d old plants for 48 h to nutrient solutions differing in NO_3^- concentration (16-1000 μM). The flux of the root-sourced CKX activity was increased with the increasing NO_3^- supply up to 7-fold correlating well with the increasing flux of cytokinins (*trans*-zeatin riboside, *trans*-zeatin and N^6-(2-isopentenyl)adenine). The flux of corresponding *cis*-isomers of zeatin was, with exception of *cis*-zeatin *O*-glucoside, not affected by the exogenous NO_3^- indicating different regulation of biosynthesis of these two isomers by nitrate. The results are discussed in relation to recently reported alkalization of xylem sap during its flow from roots to shoots suggesting the potential involvement of root-sourced CKX in down-regulation of co-transported cytokinins in the leaf apoplast.

Text:

Since the discovery of occurrence of plant hormone cytokinin in the xylem sap (Weiss and Vaadia, 1965) and detection of cytokinin biosynthesis in roots (Van Staden and Smith, 1978; Chen et al., 1985) the role of root-born cytokinins in the control of plant development has often been questioned (see Dodd and Beveridge, 2006 and references therein). It has been believed that cytokinins synthesized in root tips (Koda and Okazawa, 1978; Miyawaki et al., 2004, Aloni et al., 2005) represent the main source of the hormone for leaves (Van Staden et al.,1988) and that cytokinin trafficking from roots via the xylem to shoots serves not only as a machinery for cytokinin delivery but also as a pathway transferring physiological signals (Beck, 1996). The functioning of xylem sap cytokinins as a root-sourced signal has been impugned by Faiss et al. (1997) who demonstrated that developmental consequences of conditional and dramatic overproduction of cytokinins in tobacco plants harboring the *Agrobacterium tumefaciens* isopentenyltransferase gene (*IPT*) remained restricted to the site of hormone production. Alternatively, copper application to the roots of transgenic tobacco plants harboring the *ipt* gene under the copper-inducible promoter led to the suppression of auxin-dependent apical dominance and an induction of growth of stem lateral buds (McKenzie et al., 1998).

The dilemma of functioning of cytokinins in long-distance and/or paracrine signaling has accompanied the cytokinin research from its early beginning (compare Engelbrecht, 1972 and Mothes et al., 1961). However, the issue is even more complicated owing to the fact that cytokinin biosynthesis is not limited to roots only. Tissues of some organs including immature seeds, young leaves and shoot organs were also reported to produce cytokinins (Chen and Petschow, 1978; Chen et al., 1985; for review see Schmülling, 2002). Recent identification of several cytokinin biosynthesis genes in *Arabidopsis thaliana* encoding adenylate isopentenyltransferases (*AtIPT*) (Kakimoto, 2001; Takei et al, 2001a) that exhibit spatially specific expression patterns (Miyawaki et al., 2004) supports the idea that localized cytokinin biosynthesis might be decisive for the control of specific developmental processes in different plant parts. However, it is not clear if cytokinin biosynthesis in vegetative plant parts is essential for control of shoot development or if serves only as an emergency source of the hormone when cytokinin biosynthesis in roots is suppressed.

Involvement of cytokinins in long distance communication has been suggested in the signaling of root nitrogen nutritional status to shoots (reviewed in Beck, 1996). The amount of total cytokinins exported from roots of *Urtica dioica* and *Plantago major* correlated well with

supply of roots with mineral nitrogen (Wagner and Beck, 1993 and Kuiper and Staal, 1987, respectively). Similarly, NO_3^- application to hydroponically-grown tomato triggered oscillation of leaf growth which was associated with corresponding changes in bioactive forms of cytokinins in the xylem exudates (Rahayu et al., 2005).

An analysis of a sequence of events following nitrate re-supply to nitrogen-depleted maize roots revealed an early accumulation of the primary product of cytokinin biosynthesis ribotide of isopentenyladenine in roots (Takei et al., 2001b; 2002). Moreover, a profiling of the spatial expression pattern of three different *AtIPT*s in *Arabidopsis* plants showed a rapid NO_3^--dependent induction of *AtIPT3* expression suggesting that up-regulation of *AtIPT3* contributes mainly to the cytokinin accumulation in roots in response to changes in NO_3^- supply (Takei et al., 2004). Following accumulation of iP ribotide and *trans*-zeatins in maize roots, the concentration of cytokinins in xylem exudate and subsequently in leaves was increased. In detached maize leaves *trans*-zeatin (t-Z), *trans*-zeatin riboside (t-ZR) and t-Z ribotide induced accumulation of transcripts of *ZmRR1* and *ZRR2* genes that function as nitrogen-responsive regulators (Takei et al., 2001b; 2002).

All these data are in support the functioning of cytokinins in signaling of nitrate availability (reviewed in Sakakibara et al., 2006). However, the existing information is based mostly on correlations and sequences of investigated events that need careful interpretation. Experiments with pea stem branching mutants differing in cytokinin concentration in xylem exudates demonstrated that high shoot auxin levels and low root sap cytokinin levels are not necessarily correlated with apical dominance (Beveridge et al, 1997). Moreover, xylem sap collected by pressurizing of de-topped root systems of pea plants grown at two different N supply levels suggested that N status was more important in regulating leaf expansion than the xylem-sourced cytokinins (Dodd et al., 2004). Taking together, the available evidence indicate that cytokinins participate in some way in signaling of N status of roots acting probably in a concert with some other so far unidentified signals as shown in the involvement of auxin and cytokinins in control of shoot branching (Dun et al., 2006).

The strength of cytokinin signal transferred via xylem is dependent on the cytokinin concentration and the xylem sap flux. Calculations of cytokinin deliveries from roots to shoots based on the determination of these two parameters do not involve the potential control of cytokinin concentration directly in xylem sap. Levels of individual cytokinins in plant cells are metabolically regulated and it is possible that cytokinins in xylem sap are exposed to enzymes that might catalyze their metabolism. Cytokinin oxidase/dehydrogenase (CKX, EC 1.5.99.12), firstly detected in tobacco callus tissue (Pačes et al., 1971), is a good candidate in

this respect. The enzyme catalyzes irreversible removal of unsaturated N^6 side chain of isoprenoid cytokinins producing inactive adenine or adenosine and aldehyde of the corresponding side chain. The preferred CKX substrates are bases of isoprenoid cytokinins (iP, *t*-Z and *cis*-Z) and their ribosides. Cytokinin *O*-glucosides that represent a storage form of cytokinins are resistant to the enzyme attack (reviewed in Armstrong, 1994; Hare and Van Staden, 1994; Schmülling et al, 2003). The enzyme was independently isolated from maize kernels, cloned and expressed in moss protoplasts or recombined yeast (Houba-Hérin et al., 1999 and Morris et al., 1999, respectively). Seven distinct *CKX* genes (*AtCKX1-AtCKX7*) have been identified in *Arabidopsis* genome (Bilyeu at al., 2001) and subsequently a number of CKX homologs have been detected in some other plant species (reviwed in Schmülling et al., 2003; Werner et al., 2006) including cereals (Galuszka et al., 2004). A majority of fully annotated members of the CKX family carry N-terminal sequences predicting ambiguous targeting the enzyme toward secretion (Bilyeu et al., 2001; reviewed in Schmülling et al., 2003). Similarly, CKX activity was detected in media of the moss *Physcomitrella* protoplasts (Houba-Hérin et al., 1999) and the yeast *Pichia* and *Saccharomyces* (Morris et al., 1999 and Werner et al., 2001, respectively) expressing heterologous CKX. Expression of *CKX* transcripts (Brugière et al.. 2003; Werner et al., 2006) as well as CKX activity (reviewed in Armstrong, 1994) is inducible by natural and synthetic cytokinins. Accordingly, the CKX activity was increased in culture medium of transgenic tobacco cell suspension in response to the derepression of bacterial *ipt* gene or application of an exogenous cytokinin (Motyka et al., 2003).

In recent study of Kopečný et al. (2006) constitutive overexpression of secreted native form of *ZmCKX1* in *Arabidopsis* plants was associated with a much higher depletion of zeatin-type compared to iP-type cytokinins than overexpression of engineered *CKX* lacking the predicted signal sequence targeting the enzyme toward secretion. This suggests that zeatin derivatives represent the major transport forms of cytokinins in the apoplast.

In maize, *ZmCKX1* gene was expressed in vasculature of different organs namely in differentiating xylem tissues indicating its possible functioning in down regulation of cytokinin levels transiting in the xylem (Brugière et al., 2003). Similarly, expression of *AtCKX6* gene in *Arabidopsis* was reported to be associated with the vascular system of developing cotyledons, leaves and roots (Werner et al., 2003). Secretion of some CKXs may be a part of the regulatory network controling access of cytokinins to cytokinin plasma membrane receptors. The corresponding sensor histidine kinase receptors have already been

identified in *Arabidopsis* (Inoue et al., 2001; reviewed in Ferreira and Kieber, 2005). Recent identification of purine transporters (AtPUP) capable to mediate uptake of t-Z suggests that PUP-mediated cytokinin uptake might also participate in control of extracellular cytokinin levels (Bürkle et al, 2003).

Despite of wealth of information concerning transport of cytokinins via xylem sap and their potential role in root to shoot signaling, much less is known about potential metabolic control of cytokinins during their long-distance translocation. Data available so far concerning secretion of enzymes involved in metabolism of cytokinins are limited to secretion of CKX from cultured cells and protoplasts into a medium suggesting functioning of secreted enzyme in control of cytokinin concentration in cell proximity. No information is available about the possible occurrence of CKX in xylem sap and its potential functioning in the control of cytokinin levels during their passage from roots to shoots or at the sites of cytokinin destination. As far as our knowledge activity of any enzyme in xylem sap has not been reported so far. To address this issue we detected the CKX activity in xylem sap of oat seedlings and characterized some of biochemical properties of the enzyme. We also proved that flux of both cytokinins and CKX activity via the xylem respond to the supply of roots with nitrate and that this response also affects nitrate reductase (NR) activity that is known to be induced by both nitrate and cytokinins.

RESULTS

Occurrence of CKX activity in xylem sap

Xylem sap collected from 12 d old oat plants grown in perlite saturated with nutrient solution was tested for CKX activity. For the detection of activity of very diluted enzyme we used an assay based on determination of conversion of [^{3}H]isopentenyladenine (iP) to [^{3}H]adenine in presence of 2,6-dichloroindophenol as an electron acceptor. This assay is very specific and allows degradation of substrate cytokinins at concentrations close to the corresponding K_m values (K_m for iP 2.8 μM, Bilyeu et al., 2001) and to the actual concentrations of cytokinins in plants. Moreover, in contrast to the spectrophotometric methods this assay provided reliable results even with crude protein preparations (results not shown). Enzyme activity corresponding to the conversion of 66 pmol iP ml^{-1} h^{-1} at pH 8.5 was detected in non-purified xylem sap. With respect to a very low concentration of proteins in xylem sap (0.1 – 0.9 mg ml^{-1}) there were serious losses of activity following enzyme

purification by precipitation with ammonium sulphate, dialysis or removal of phenolic compounds on a column of polyvinylpyrrolidone even after addition of an inactive protein carrier (bovine serum albumine). This together with very low concentration of interfering substances in xylem sap and limited amount of xylem sap issuing from the detopped root (7.7 - 15 µl plant^{-1} h^{-1}) prevented further purification of the enzyme specifically for studies of responses of enzyme activity to external physiological signals. Xylem sap contains low amounts of interfering compounds. Potential influence of electron acceptors that could be present in xylem sap and enhance CKX activity was eliminated by performing the assay at saturation 2,6-dichloroindophenol concentration (75 µM). In addition to oat plants an apparent but ca 3-fold lower activity of CKX was found in xylem sap of wheat seedlings grown under the same conditions (results not shown).

CKX pH optimum and protein glycosylation

When assayed in buffers covering pH range from 3.5 to 10 the CKX from oat xylem sap exhibited highest activity at pH 8.5 (Fig. 1). Liquid chromatography of CKX on Con A-Sepharose 4B revealed that majority of enzyme activity (87 % of total) was associated with glycosylated form (Fig. 2). Interestingly, the pH of the root-sourced xylem sap was 6.1.

Effect of nitrate on cytokinin levels and CKX activity

The potential involvement of CKX in control of cytokinin levels in xylem sap was tested by following responses of oat plants to changes in nitrate supply. Plants were grown in nutrient solution containing 250 µM of NO_3^-. Under these conditions the NO_3^- uptake was characterized by V_{max} = 9.3 ± 0.5 µmol NO_3^- g^{-1} root FW h^{-1} and K_m = 26.1 ± 0.9 µM NO_3^- corresponding to high affinity NO_3^- uptake. At age of 12 d plants were transferred to nutrient solutions differing in nitrate concentration (16 µM, 62 µM, 250 µM and 1000 µM). To allow plant acclimation, adjustment of the rate of NO_3^- uptake and endogenous NO_3^- levels the xylem exudates were collected 48 h after the transfer. These relatively short alternations in nitrate supply had no significant effect on shoot dry and fresh weight. The fresh and dry weight of roots was decreased inversely to the nitrate concentration shifting the shoot/root ratio from 2.94 (16 µM NO_3^-) to 4.1 (1000 µM NO_3^-) and from 1.66 to 2.37, respectively.

There was a 1.7-fold increase in nitrate concentration in the xylem sap of plants grown in nutrient solution containing 16 µM NO_3^- comparing to 1000 µM NO_3^- (Fig. 3A) and 1.9-fold increase in NO_3^- content in both shoots and roots, respectively (Fig. 3B). However, the

accumulation of NO_3^- was much more pronounced in leaves (increase from 12.9 µmol NO_3^- to 24.7 µmol NO_3^- per shoot) comparing to roots (increase from 9.1 µmol NO_3^- to 11.8 µmol NO_3^- per root). The nitrate reductase (NR) activity was about 10-fold higher in leaves than in roots and the enzyme activity was much more increased in leaves than in roots in response to the increase in NO_3^- supply (Fig. 3C).

Changes in NO_3^- supply had different effects on contents of preponderant bioactive cytokinins (t-ZR and t-Z) and corresponding *cis*-isomers which are known to exhibit very low cytokinin activity. While the content of t-ZR and t-Z in both roots and leaves was enhanced with increasing NO_3^- concentration in nutrient solution the content of *cis*-zeatin (c-Z) and *cis*-zeatin riboside (c-ZR) displayed the opposite trend (Table 1).

The root pressure exudation rates of cytokinins were expressed as cytokinin flux corresponding to the amount of cytokinin in the exudate produced by one g of root FW within 1h. Six cytokinins (iP, t-Z, t-ZR, c-ZR and O-glucosides of c-Z and c-ZR) were found in xylem sap in amounts allowing precise quantitation. The exudation rate of bioactive cytokinins (iP, t-Z and t-ZR) was gradually increasing with increasing NO_3^- supply. The highest, nearly 4-fold increase was recorded for t-ZR which was the foremost cytokinin of this class in the exudate. Interestingly, c-ZR and its derivatives were present in significant amounts but did not respond to changes in NO_3^- supply with exception of c-ZOG the exudation rate of which was slightly increased in plants grown in nutrient solution containing the highest tested NO_3^- concentration (Fig. 4A).

The flux of CKX activity, representing the enzyme activity in the xylem exudate corresponding to 1 g of root FW collected within 1 h, positively correlated with NO_3^- concentration in nutrient solution. The increase was very steep between 16 µM NO_3^- and 250 µM of NO_3^- (6.3-fold) and slowed down at 1000 µM of NO_3^- in nutrient solution (Fig. 4B).

DISCUSSION

The aim of this study has been to analyze xylem sap of oat plants for potential CKX activity and if present to characterize some of its biochemical properties and eventual responses to an environmental signal that is known to affect cytokinin levels.

CKX activity in xylem sap

For our experiments we used oat plants grown in perlite saturated with nutrient solution or in hydropony and collected root pressure xylem sap from de-topped roots. CKX activity far above the detection limit was found in the root pressure xylem sap. Somewhat lower CKX activity was also detected in root pressure xylem sap from wheat grown under same conditions (results not shown). The enzyme exhibited activity maximum at pH 8.5 and the activity was associated predominantly with *N*-glycosylated protein. Different CKXs that characterized so far exhibited pH optima either between 4.5 and 6.0 or above 8.0. Whenever determined the pH optimum of glycosylated CKX was around 6 and the activity associated with this form was secreted to the cultivation medium (see review of Armstrong, 1994 and later data of Motyka et al., 2003 and Galuszka et al., 2004). Evidently, the pH optimum of glycosylated CKX in oat xylem sap reported here is substantially different from other so far reported glycosylated CKXs which are secreted to the cell exterior. The pH of root pressure xylem sap collected from the de-topped oat plants slightly varied around 6.1 and under such conditions cytokinins under the transport via xylem are protected against the attack of co-transported CKX. Moreover, glycosylation can protect CKX against proteolysis and extend its life span (Turner et al., 2006). Taking together, the *N*-glycosylation of CKX with low pH optimum could support delivery of both cytokinins and CKX intact from roots to shoots.

Analysis of cytokinins by their immunolocalization and expression of free-cytokinin responsive *ARR5::GUS* in *Arabidopsis* plants allowed Aloni et al. (2005) monitoring of cytokinin distribution patterns in *Arabidopsis*. In plants completely protected from any air movement, the strongest *ARR5:GUS* expression was spotted in root caps spreading upwards in the root vascular cylinder congruently with cytokinin immunolocalization. Shoots of these plants displayed either no or only low *ARR5::GUS* expression in vascular bundles and acropetally decreased staining. Promotion of transpiration by exposure of plants to a gentle wind caused a considerable increase of *ARR5::GUS* expression in shoots and strongest label in the vascular bundles of stems, leaves, and buds. Expression pattern of *ARR5:GUS* in shoot has suggested accumulation of cytokinins at sites of highest transpiration and also release of cytokinins in leaves from the bluntly vessels of the xylem. If similar scenario occurs also in oat plants one can speculate that not only cytokinins but also root-sourced CKX activity accumulates at the sites of intensive transpiration. Moreover, accumulated cytokinins can antagonize ABA-induced stomatal closure (Tanaka et al., 2006) and stimulate stomatal opening as reported for many monocotyledonous species (reviewed in Dodd, 2003). In this way the transpiration rate as well as delivery of cytokinins and CKX from roots to leaf apoplast can be increased. In *Arabidopsis*, the *AtCKX6:GUS* was highly expressed in the most

apical part of the growing and the *AtCKX6* promoter was active in maturating stomatal guard cells (Werner et al., 2003). In oat degradation of cytokinins by co-delivered CKX would require either lowering of enzyme pH optimum potentially by CKX deglycosylation and/or increasing of pH of apoplast in the vicinity of sites exhibiting high transpiration. Using new techniques allowing determination of pH in microvolumes of xylem sap and in leaf apoplast Jia and Davies (2006) found that as xylem sap leaves the roots and moves to the shoot it becomes more alkaline. The gradual increase of pH can be caused by removal of protons from the xylem stream presumably by ATPases associated with the xylem parenchyma. A comparison of the xylem sap pH between stem base and leaf blade registered difference approximately 1.5 for sunflower. Moreover, the increase in the pH was inversely proportional to the xylem flow responding to the changes in transpiration rates. An increase in pH of xylem sap from 5.0 to 8.0 in response to soil drying was also reported for tomato plants (Wilkinson et al., 1998).

These data allow us to hypothesize that activity of CKX leaving the roots is suppressed by low pH of xylem sap protecting the co-transported cytokinins against the enzyme attack. Increasing pH of xylem sap during its upward flow could create more favorable conditions for CKX-catalyzed cytokinin degradation in leaf apoplast. Moreover, the indirect dependence of alkalization of xylem sap on xylem flow suggests a reciprocal dependence of CKX activity in leaf apoplast on the rate of transpiration. In this system the CKX-catalyzed cytokinin degradation could influence the concentration ratio of cytokinins and co-transported ABA and thus influence stomatal opening (Fig. 5).

Recent report about the expression of high-affinity purine transporters *AtPUP1* and *AtPUP2* mediating also uptake of cytokinins suggests their potential involvement in control of cytokinin levels in the apoplast. In view of accumulation of cytokinins at sites of high transpiration, the expression of PUP1 promoter in the epithem cells of hydatodes is interesting (Bürkle et al., 2003). It implies a potential down-regulation of cytokinins in the apoplast by cytokinin uptake and by root-sourced CKX catalyzed cytokinin degradation at sites of high transpiration. Interestingly, different purine and nucleoside transporters recognizing cytokinins were found to be highly expressed in vasculature of *Arabidopsis* and rice plants (Bürkle et al., 2003; Hirose et al., 2005). Expression of different IPTs, CKXs and purine transporters controlling cytokinin biosynthesis, degradation and uptake, respectively, in the xylem suggests localization of a sophisticated network controlling cytokinin levels during their transport in the vasculature and/or in its near vicinity.

Changes in cytokinin and CKX fluxes in response to NO_3^- supply

A well-known response of xylem sap cytokinins to NO_3^- supply was chosen for characterization of dependence of CKX activity on cytokinin flux in root xylem sap exudate. Oat plants were grown for 12 days in nutrient solution containing a near to optimum concentration of NO_3^- and then transferred for 48 h to nutrient solutions differing in NO_3^- concentration. A nitrate influx into plant roots has been kinetically defined as four additive fluxes. At an external NO_3^- concentration up to 1 mM constitutive high-affinity influx and nitrate-inducible high affinity-influx are characterized by Michaelis - Menten kinetics. Constitutive low-affinity influx and inducible low–affinity influx that occur at high nitrate concentrations (>1 mM) are linearly concentration-dependent (Crawford and Glass, 1998; Forde, 2000; Okamoto et al, 2006). The range of tested NO_3^- concentrations (16 μM to 250 μM) corresponds to the high affinity NO_3^- uptake while the highest tested concentration (1000 μM) lays at the transition to low affinity NO_3^- influx.

The differential NO_3^- supplies for 48 h affected NO_3^- concentration in xylem sap and NO_3^- content and NR activity in leaves and roots (Fig. 3). As has been reported, cytokinins are capable to induce NR activity independently of NO_3^- in several plant species including cereals (Gaudinová, 1990). In light-grown wheat plants the shoot application of benzyladenine failed to enhance the NR activity but when applied to roots the NR activity was increased in both etiolated and light-grown plants (Banowetz 1992). However, in contrast to NO_3^- the induction of NR by cytokinins is not light-dependent (Lips and Roth-Bejerano, 1969), suggesting their ability to stimulate NO_3^- reduction in roots in response to elevated NO_3^- uptake also in dark. In split root cultures of barley the NR-mRNA level, NR activity and content of t-ZR were increased in response to the enhanced local NO_3^- supply (Samuelson and Larsson, 1993). A similar effect displayed different cytokinins, namely t-ZR and t-Z, when applied to the nutrient solution (Samuelson et al., 1995). As reported by Lexa *et al*. (2002) the activity of NR is suppressed after long-term enhancement of endogenous cytokinin levels in transgenic tobacco following *ipt* expression. In experiments described here the exposure of plants for 48 h to different NO_3^- concentrations was not inhibitory.

In our experiments the content of predominating bioactive cytokinins (t-ZR and t-Z) in roots of plants transferred for 48 h into nutrient solution containing high concentration of nitrate (1000 μM) was increased 2.3- and 2.1-fold, respectively, comparing to roots of plants poorly supplied with nitrate (16 μM NO_3^-). This increase could be due to enhancing of expression of *IPT* by nitrate in roots as reported for *AtIPT3* in *Arabidopsis* (Takei et al., 2004; Miyawaki et al., 2004). Contrariwise the content of corresponding *cis*-zeatins, which exhibit

very low cytokinin activity (Kamínek et al., 1979), was decreased 1.5- and 1.4-fold, respectively. Similar trends were found in shoots where content of t-Z and t-ZR was increased 1.5- and 1.8-fold, respectively, while that of c-Z and c-ZR was lowered 1.8- and 1.6-fold, respectively, in plants exposed to a high (1000 μM) as compared to low (16 μM) NO_3^- concentration in the nutrient solution (Table 1). These differences probably reflect the different responses of tRNA-independent and tRNA-dependent cytokinin biosynthetic pathways leading to *trans*- and *cis*-zeatins, respectively, to the nitrate (Myiawaki et al., 2006). Conversion of t-Z and t-ZR to corresponding *cis* isomers by zeatin *cis-trans* isomerase in response to NO_3^- could also be responsible for these differential shifts in cytokinin accumulation (Bassil et al., 1993).

Correspondingly to Badenoch-Jones et al. (1996), we found t-ZR and t-Z as the most abundant bioactive cytokinins in oat root pressure xylem sap. DHZ was not detected in amounts allowing precise quantification but iP was present in low but measurable quantities. Interestingly, *cis*-zeatins, which are only weak cytokinins, were found in xylem sap in both laboratories in concentrations comparable to those of *trans*-zeatins (Fig. 4). Unfortunately, only few monocot species were analyzed for xylem sap cytokinins. Cytokinins of c-Z type were detected in rice, wheat and oat plants. In contrast to dicotyledonous plants rice was the only monocot plant where a cytokinin ribotide (zeatin ribotide) was unequivocally determined (reviewed in Badenoch-Jones et al., 1996).

According to many reports the accumulation of cytokinins in plant tissues is associated with corresponding increase of CKX activity (reviewed in Armstrong, 1994). In tobacco cell suspensions, calli and plants the increase in *ipt* gene transcription was followed by accumulation of endogenous cytokinins and subsequently enhancement of CKX activity that was in case of cell suspensions secreted to culture medium (Motyka et al., 1996; Motyka et al., 2003). Similarly, expression of CKX1 in maize roots was induced by synthetic and natural cytokinins (Brugière et al., 2003). Accordingly, the up-regulation of cytokinin biosynthesis in roots is most probably responsible for enhancement of CKX activity efflux in xylem sap in response to NO_3^- supply (Fig. 4). This view is supported also by about 30-fold higher CKX activity that we registered in oat roots as compared to shoots (results not shown).

In summary, root pressure xylem sap from de-topped oat plants contains CKX activity that is associated with a glycosylated protein. The pH optimum of the enzyme (8.5) is much higher than pH of root xylem sap (6.1) indicating suppression of cytokinin degradation by the enzyme during its transport via the xylem flow. Reported alkalization of the xylem sap in leaf apoplast and its enhancement in response to NO_3^- and water availability may create favorable

conditions for metabolic degradation of co-transported cytokinins and thus decrease of cytokinin/ABA ratio at sites of high transpiration.

MATERIALS AND METHODS

Plant material and growth conditions

For characterization of CKX biochemical properties oat (*Avena sativa* L., cv. Abel) seeds were soaked for 14 h with distilled water under stirring and then sown on perlite saturated with nutrient solution. Plants were grown for 12 d in a controlled climate growth chamber (Sanyo MLE-350H) at 20ºC/18ºC day/night temperature, 80% air humidity and 16 h photoperiod with a photon flux density 300 μmol m^{-2} s^{-1}.

To determine the effect of NO_3^- supply on plant nitrogen status and cytokinin and CKX flux plants were grown hydroponically for 12 d in a growth room with 21°C/15°C day/night air temperature and 16 h photoperiod (photon flux of 400 μmol m^{-2} s^{-1}). Continuously aerated nutrient solution contained $Ca(NO_3)_2$ (158 μM), KNO_3 (70.8 μM), KH_2PO_4 (52.5 μM), $MgSO_4$ (41.3 μM), KCl (47.5 μM), H_3BO_3 (2.5 μM), Fe-EDTA (2 μM), $ZnSO_4$ (0.2 μM), $MnSO_4$ (0.2 μM), $CuSO_4$ (0.05 μM) and $(NH_4)_6Mo_7O_{24}$ (0.01 μM).

To minimize fluctuation in NO_3^- concentration due to the NO_3^- uptake large volumes of nutrient solution were used for each treatment (200 l for each treatment corresponding to 2 L $plant^{-1}$). The NO_3^- concentration of weekly changed nutrient solution was controlled and adjusted each second day. Two days before collection of xylem sap and a sampling of shoots and roots plants were transferred to fresh daily changed nutrient solutions with 16 μM, 62 μM, 250 μM and 1000 μM NO_3^-. Missing Ca^{2+} and K^+ were supplied in form of $CaCl_2$ and KCl.

Collection of xylem sap and sampling of roots and shoots

Root xylem sap for characterization of CKX biochemical properties was collected from 12 old plants grown in perlite. Plants at this stage had two fully developed leaves and the third leaf was just emerging. For determination of the effects of different NO_3^- supply on plant nitrogen status, cytokinin and CKX activity flux root pressure xylem exudate was collected for 2 h beginning 48 h after the transfer of plants to nutrient solutions differing in NO_3^- concentration (4 h after the end of dark period). Shoots were cut-off using a razor blade approximately 0.5 cm above the shoot to root transition. The portion of xylem sap released

during the first 15 min was discarded. The drops of sap were frequently collected for 2 h using a micropipette, immediately cooled down by transfer to a pre-weighted eppendorf tube kept on ice. The tubes were immediately closed to prevent evaporation and finally frozen in liquid nitrogen and stored at -80^{o}C. Shoots and roots were frozen in liquid nitrogen and stored under same conditions.

Measurement of net nitrate influx

Net NO_3^- influx was measured at 250 μM external nitrate concentration. Groups of four intact plants were transferred into 300 mL well aerated fresh nutrient solution. After a lag period of 30 min 5mL samples of the nutrient solution were collected at 20 min intervals to measure the NO_3^- concentration. The net NO_3^- influx was determined from the dynamics of NO_3^- depletion and expressed as μmol g^{-1} root FW h^{-1}. The I_{max} and K_m were determined using Enzfiter programme (Biosoft, Cambridge, UK).

Determination of NO_3^- content

Frozen samples of leaves and roots (1 g FW) were homogenized in liquid nitrogen, extracted with distilled water for 30 min at 90^{o}C and filtered. The NO_3^- concentration in plant extracts, nutrient solutions and xylem sap was determined spectrofotometrically using Skalar San plus analyzer (Breda, the Netherlands). The samples were passed through a column of granulated copper – cadmium to reduce NO_3^- to NO_2^-. The nitrite was estimated spectrophotometrically by measurement of conversion of sulphanilamide and α-naphthylehthylenediamine dihydrochloride to azo dye at 540nm.

Determination of nitrate reductase activity

Leaf and root samples (1 g FW) were homogenized in liquid nitrogen and extracted in 5 mL of 50 mM Tris-HCl buffer (pH 8,0) containing 3% (w/v) bovine serum albumine at 4°C for 30 min. Insoluble material was removed by centrifugation (1500 g, 30 min). The NR activity was determined by an *in vitro* assay as described by Gaudinová (1983). The assay mixture contained in 900 μL final volume sodium phosphate buffer, pH 7.5 (55 mM), KNO_3 (10 mM), enzyme extract (150 μL) and $NADH^+$ (0.45 mM). After incubation for 10 min at 25^{o}C the reaction was terminated by addition of 0.1 mL 0.03M oxaloacetic acid. Formation of azo dye after addition of sulphanilamide and α-naphthylehthylenediamine dihydrochloride was monitored at 540 nm. NR activity was expressed as a rate of generated NO_2^- (μmol g^{-1}FW min^{-1}).

Measurement of cytokinin oxidase/dehydrogenase activity

The cytokinin oxidase/dehydrogenase (CKX) activity was measured by *in vitro* assay based on the conversion of [2-^{3}H]iP, (prepared by Dr. Jan Hanuš, Isotope Laboratory, Institute of Experimental Botany AS CR, Prague, Czech Republic) to [^{3}H]adenine as described by Motyka et al. (2003) with slight modifications. For the enzyme assay, the xylem exudate (20 µl per assay) was used without previous purification. The protein concentration in the xylem sap was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

The assay mixture (50 µL final volume) included TAPS-NaOH buffer (pH 8.5) at final concentration of 100 mM, 75 µM 2,6-dichloroindophenol (pH 8.5), 2 µM substrate ([2-^{3}H]iP, 7.4 Bq mol^{-1}) and the xylem sap (corresponding to 0.4-0.7 mg protein mL^{-1}). After incubation (4 or 16 h, 37^{o}C) the reaction was terminated by adding of 10 µL Na_4EDTA (200 mM) and 120 µL 95% (v/v) ethanol. The substrate was separated from the product of the enzyme reaction by HPLC using Series 200 HPLC Quaternary Pump (Perkin Elmer, Wellesley, MA, USA) coupled to 235C Diode Array Detector (Perkin Elmer) and RAMONA 2000 flow-through radioactivity detector (Raytest, Straubenhart, Germany) on the column Luna C_{18}(2) (50 mm/4.6 mm/3µm) (Phenomenex, Torrance, CA, USA) as described elsewhere (Gaudinová et al., 2005).

The pH optimum of the CKX activity was determined by running the standard enzyme assay in different buffers including 0.1 M *β, β´*-dimethylglutaric acid-NaOH (for pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5), 0.1 M MOPS-NaOH (for pH 6.7, 7.2 and 7.8), 0.1 M TAPS- NaOH (for pH 7.8, 8.5 and 9.0) and 0.1 M CHES-NaOH (for pH 8.8, 9.5 and 10.0).

Determination of glycosylation patterns of cytokinin oxidase/dehydrogenase

The glycosylation pattern of CKX activity in xylem exudate was determined on the basis of Concanavalin A-Sepharose 4B chromatography as described elsewhere (Motyka et al. 1996). Briefly, the collected root pressure xylem sap (pH 6.1) equivalent to 3 mL was applied onto a Con A-Sepharose-4B column (0.75 x 7.00 cm) equilibrated with 25 mM bisTris-HCl (pH 6.5) buffer supplemented with $(NH_4)_2SO_4$ (200 mM), $CaCl_2$ (1 mM) and $MnCl_2$ (1 mM). After washing with 21 mL of 25 mM bisTris-HCl (pH 6.5) containing $(NH_4)_2SO_4$ (200 mM), glycoproteins were eluted with the same buffer supplemented with methylmannose (200 mM). Fractions of 3 mL were collected at flow rate 0.4 mL min^{-1} and CKX activity was determined by radioisotope assay as described above.

Cytokinin analysis

Cytokinins were purified using dual mode solid phase extraction (Dobrev and Kamínek, 2002) and determined by LC/MS/MS. Briefly, the xylem sap (1 mL) was acidified with 5 mL 1M HCOOH, supplied with internal deuterated cytokinin standards, filtered through Sep-Pack †C_{18} cartridge (Waters, Milford, MA, USA) and applied to an Oasis MCX (150 mg) reverse-phase cation-exchange column (Waters). After washing with 5 mL of 1M formic acid and 5 mL of methanol cytokinin nucleotides were eluted with 5 ml 0.35 M NH_4OH and cytokinin bases, ribosides and glucosides with 5 mL 0.35 M NH_4OH in 60% (v/v) methanol. Separated cytokinin nucleotides were dephosphorylated by incubation with calf-intestine alkaline phosphatase (Sigma) and determined as corresponding nucleosides

Detection and quantitation were carried out using LC/MS/MS system consisting of HTS-Pal auto-sampler with cooled sample stack (CTC Analytics, Zwingen, Switzerland), quaternary HPLC pump Rheos 2200 (Flux Instruments, Basel, Switzerland), Delta Chrom CTC 100 Column oven (Watrex, Praha, CR) and TSQ Quantum Ultra AM triple-quad high resolution mass spectrometer (Thermo Electron, San Jose, USA) equipped with an electrospray interface. Dried MCX column eluates were dissolved in 100 µL 10% (v/v) acetonitrile and filtered. A 5 µL aliquot was injected on C_{18} HPLC column Synergy Hydro-RP, 250×2 mm, 4 µm (Phenomenex, Torrance, USA) and analyzed using ternary gradient elution (water/acetonitrile/0.01% acetic acid) starting at 8% of acetonitrile. The concentration of acetonitrile was increased after 5 min to 15% during 10 min and then to 50% during 11 min. The portion of 0.01% acetic acid was maintained at 25% through the whole analysis. Rest of sample was removed out of column by increasing content of acetonitrile to 90% for 9 min, and then was the column equilibrated at 8% of acetonitrile for 20 min period prior next injection. Mass spectrometer was operated in the positive SRM (single reaction monitoring) mode with monitoring of 2 to 4 transitions for each compound. The most intensive ion was used for quantification, the others for identity confirmation. Cytokinins were quantified using multilevel calibration graph with [^{2}H] labeled cytokinins as internal standards. Detection limits of different cytokinins varied from 0.05 to 0.10 pmol/sample. Results represent averages of analyses of two independent samples and of two LC/MS/MS injections for each sample.

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FIGURE LEGENDS:

Figure 1. The effect of pH on the *in vitr*o activity of cytokinin oxidase/dehydrogenase from root xylem sap collected from de-topped 12 d old oat (*Avena sativa* L. cv. Abel) plants grown in perlite saturated with nutrient solution. Root pressure xylem sap was collected for 2 h after excision of shoots. The enzyme activity was determined by an assay based on conversion of [2-^{3}H]iP to [^{3}H]adenine in presence of 2,6-dichloroindophenol. The assay solution was buffered with indicated buffers at 100 μM concentrations. The values represent the means of three replicates. The SD values averaged 7 % and did not exceed 22 % of the mean.

Figure 2. Concanavalin A-Sepharose 4B chromatography of cytokinin oxidase/dehydrogenase from root xylem sap collected from de-topped 12 d old oat (*Avena sativa* L. cv. Abel) plants grown in perlite saturated with nutrient solution. Root pressure xylem sap was collected as described in Fig. 1. Collected xylem sap (pH 6.1) equivalent to 3 mL was applied onto a Con A-Sepharose 4B column (0.75 x 7.00 cm). After washing with 21 mL of 25 mM bisTris-HCl (pH 6.5) containing $(NH_4)_2SO_4$ (200 mM), glycoproteins were eluted with the same buffer supplemented with methylmannose (200 mM). Cytokinin oxidase/dehydrogenase activity in each fraction was measured as specified in Fig. 1.

Figure 3. The effect of NO_3^- supply on NO_3^- concentration in root pressure xylem sap of oat (*Avena sativa* L. cv. Abel) (A), NO_3^- content (B) and nitrate reductase activity (NRA) (C) in shoot and roots. Plants were grown for 2 weeks in growth room with day/night cycle of 16/8 h and 21°C/15°C in nutrient solution with 386 μM NO_3^-. Two days before collection of xylem sap and sapling of shoots and roots plants were transferred for 48 h to fresh nutrient solutions with 16 μM, 62 μM, 250 μM and 1000 μM NO_3^-. Root pressure xylem sap was collected as described in Fig. 1. The NO_3^- concentration in xylem sap and NO_3^- content in shoots and roots were determined spectrophotometrically after reduction to NO_2^-. Error bars represent SE, $n \geq 3$.

Figure 4. The effect of NO_3^- supply on oat (*Avena sativa* L. cv. Abel) root pressure flux of cytokinins (A) and cytokinin oxidase/dehydrogenase (CKX) activity (B). Plants were grown for 2 weeks in growth room with day/night cycle of 16/8 h and 21°C/15°C in nutrient solution with 386 μM μM NO_3^-. Two days before collection of xylem sap plants were transferred for

48 h to fresh nutrient solutions with 16 μM, 62 μM, 250 μM and 1000 μM NO_3^-. Root pressure xylem sap was collected as described in Fig. 1. Cytokinins were extracted and purified using dual mode solid phase extraction (Dobrev and Kamínek, 2002) and determined by HPLC-MS. The CKX activity was measured by *in vitro* assays based on the conversion of [2-^{3}H]iP, to [^{3}H]adenine. Values represent means of two independent samples with two HPLC-MS injections for each sample (A) and means of three independent samples (B). Error bars correspond to SE.

Fig. 5. Schematic showing the proposed protection of cytokinins (CK) against a CKX attack in the xylem sap leaving the roots and activation of the enzyme in the leaf apoplast. Accumulation of cytokinin in the roots, e.g., in response to the increased nitrate supply, enhances CKX activity. Both cytokinins and CKX are co-transported from the roots through the xylem sap flow to the shoots. The low pH value xylem sap leaving the roots suppresses the CKX activity and protects cytokinins against metabolic degradation. Increasing the pH of xylem sap during its upward flow creates conditions favorable for CKX-catalyzed cytokinin degradation in the leaf apoplast. Cytokinins which are accumulated at sites of high transpiration are degraded by co-accumulated CKX. (Plant for displayed illustration, *Echinochloa crus galli* L., painting of Otto Ušák, 1964).

Table I. Content of *trans*- zeatin and *trans*- zeatin riboside and corresponding *cis*- isomers in roots and shoots of oat (*Avena sativa* L. cv. Abel) as affected by NO_3^- supply. Plants were grown for 12 d in growth room with day/night cycle of 16/8 h and 21°C/15°C in nutrient solution with 386 µM NO_3^-. Two days before collection of xylem sap and sampling of shoots and roots the plants were transferred for 48 h to fresh daily changed nutrient solutions with 16 µM, 62µM, 250 µM and 1000 µM NO_3^-. Data are mean values ± SE, n ≥3.

Plant part	NO_3^- concentration (µ*M*)	Cytokinin content (pmol g^{-1} FW)			
		t-Z	t-ZR	c-Z	c-ZR
Root	16	0.20 ± 0.07	0.91 ± 0.08	0.39 ± 0.08	1.18 ± 0.13
	1000	0.46 ± 0.02	1.93 ± 0.18	0.26 ± 0.07	0.87 ± 0.04
Shoot	16	0.84 ± 0.12	0.77 ± 0.14	1.22 ± 0.20	1.90 ± 0.14
	1000	1.23 ± 0.04	1.35 ± 0.11	0.42 ± 0.06	1.15 ± 0.11

FIG. 1

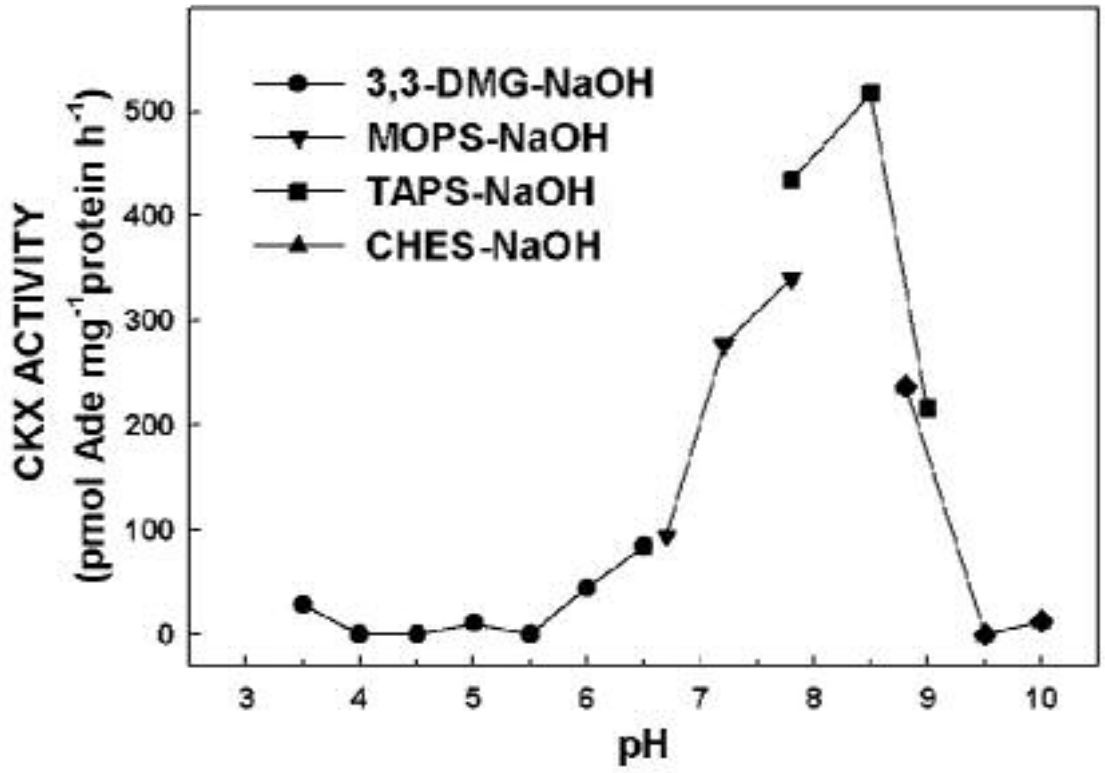

3,3-DMG-NaOH
MOPS-NaOH
TAPS-NaOH
CHES-NaOH
CKX ACTIVITY
(pmol Ade mg-1protein h-1)
0
100
200
300
400
500
3
4
5
6
7
8
9
10
pH

FIG. 2

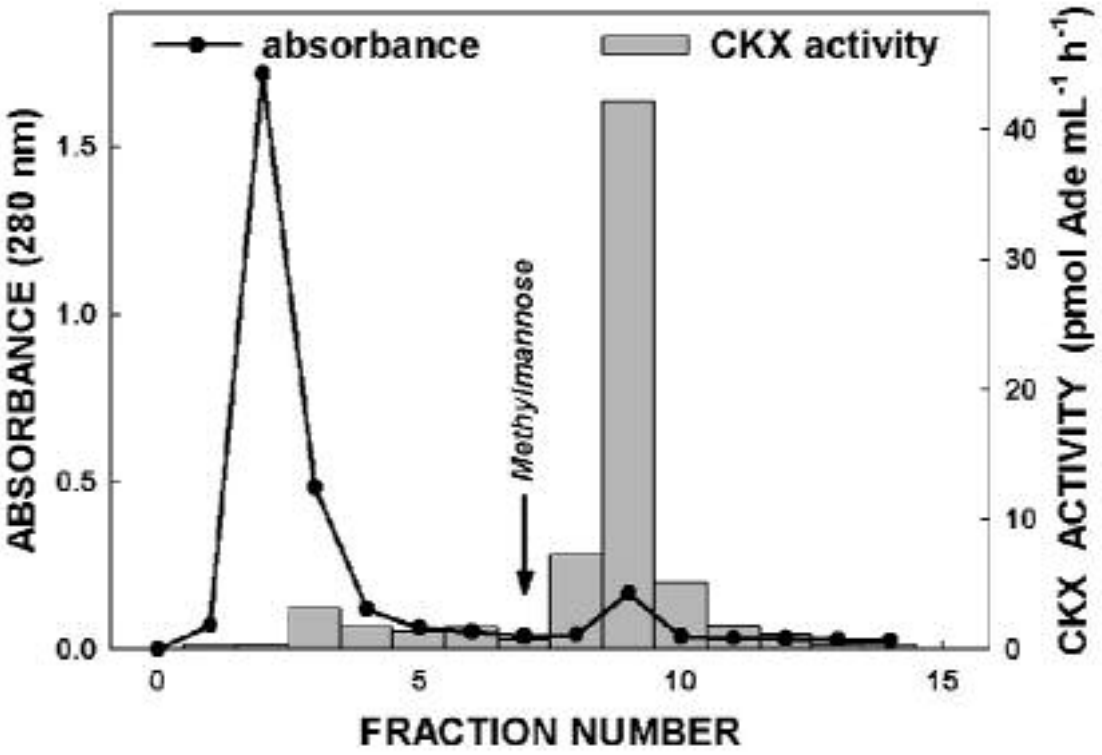

absorbance
CKX activity
ABSORBANCE (280 nm)
CKX ACTIVITY (pmol Ade mL-1 h-1)
Methylmannose
FRACTION NUMBER
0.0
0.5
1.0
1.5
0
10
20
30
40
0
5
10
15

FIG. 3

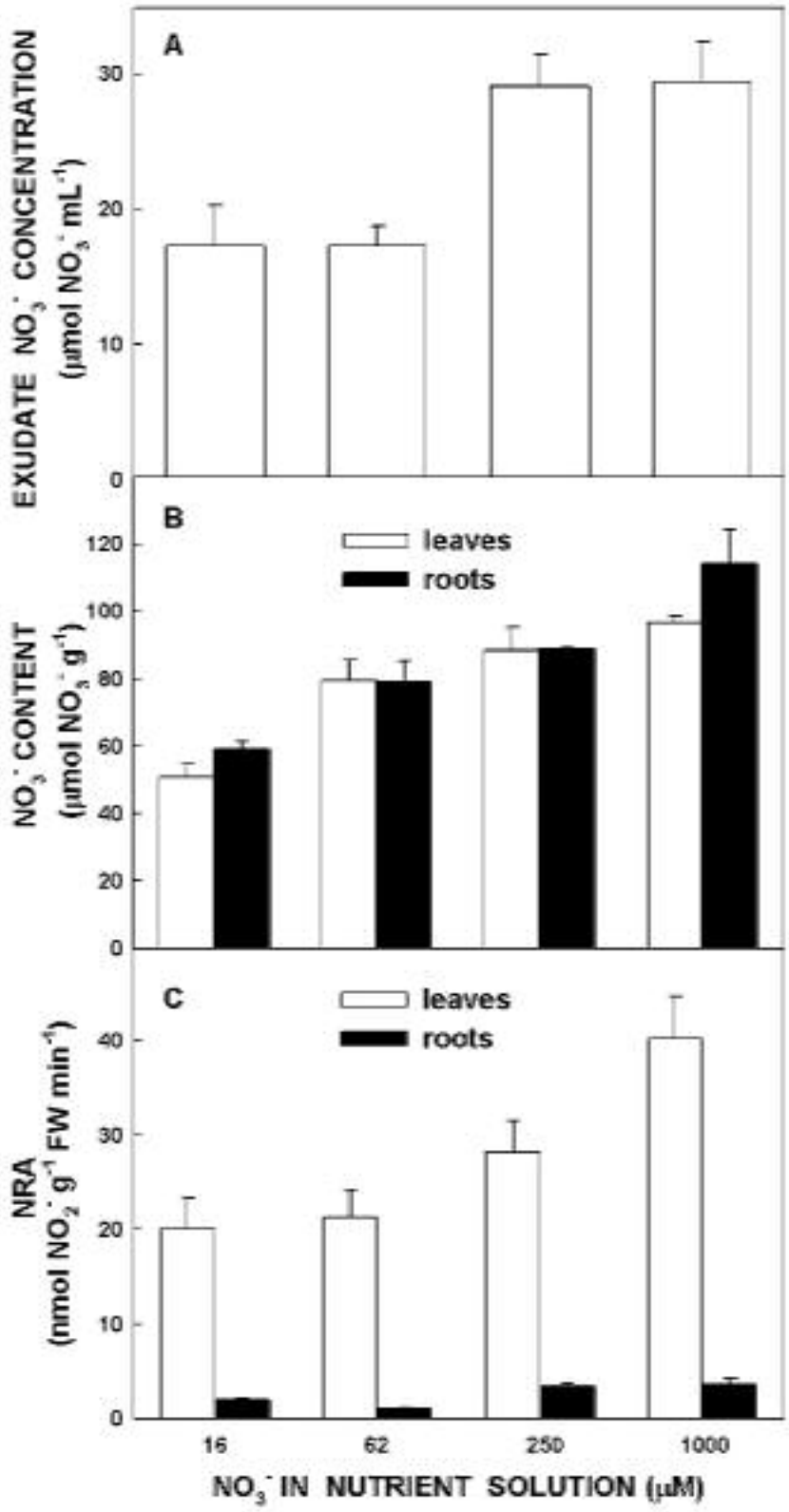

A
EXUDATE NO3- CONCENTRATION (µmol NO3- mL-1)
0
10
20
30
B
leaves
roots
NO3- CONTENT (µmol NO3- g-1)
0
20
40
60
80
100
120
C
leaves
roots
NRA (nmol NO2- g-1 FW min-1)
0
10
20
30
40
16
62
250
1000
NO3- IN NUTRIENT SOLUTION (µM)

FIG.4

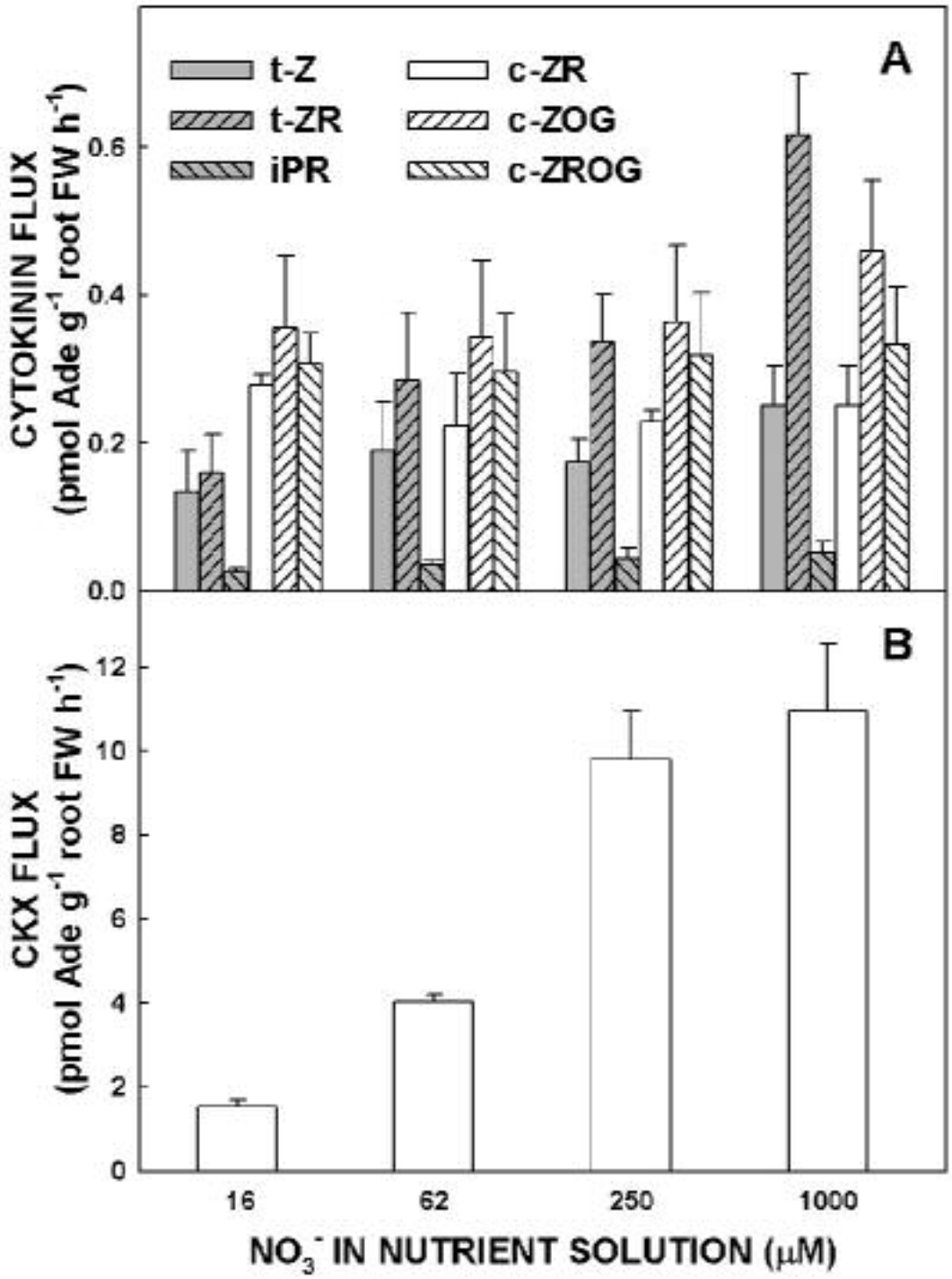
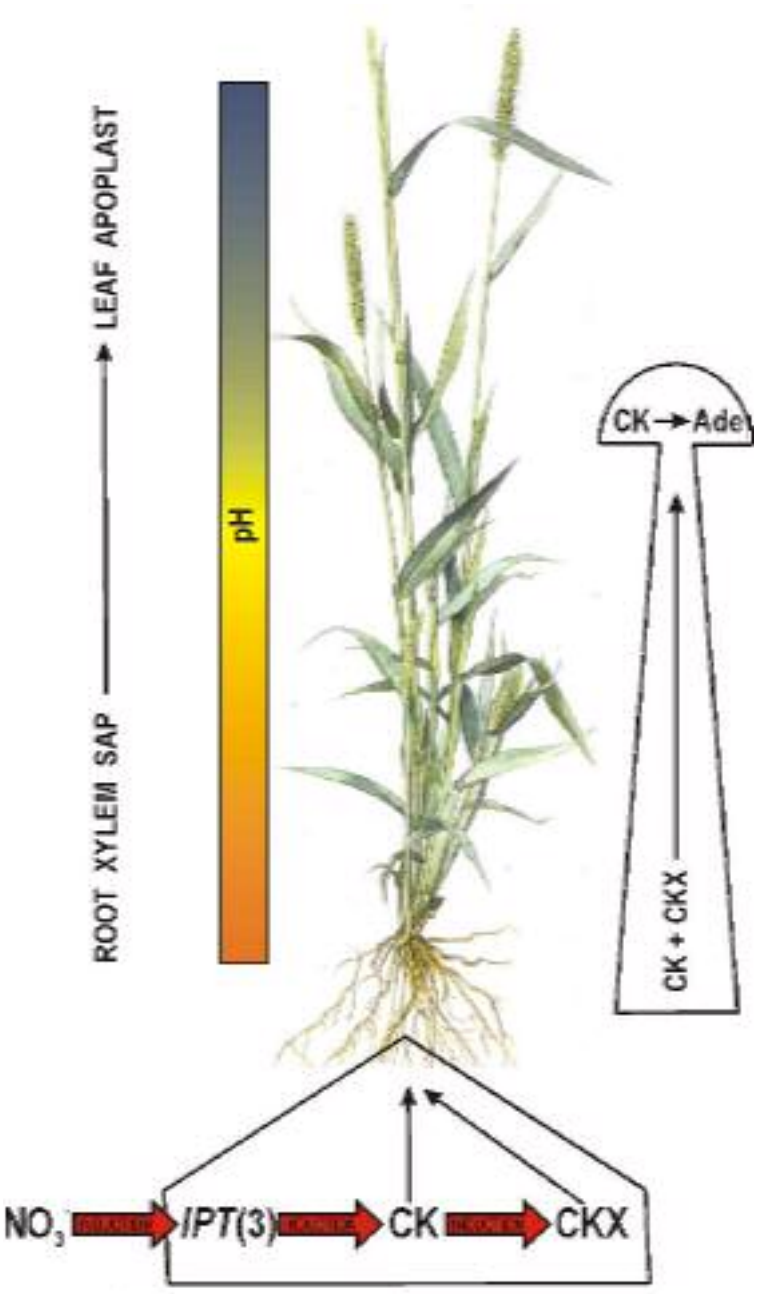

A
t-Z
t-ZR
iPR
c-ZR
c-ZOG
c-ZROG
CYTOKININ FLUX
(pmol Ade g-1 root FW h-1)
0.0
0.2
0.4
0.6
B
CKX FLUX
(pmol Ade g-1 root FW h-1)
0
2
4
6
8
10
12
16
62
250
1000
NO3- IN NUTRIENT SOLUTION (μM)

FIG. 5



Increase in sensitivity of cytokinin oxidase/dehydrogenase radioisotope assay by introducing electron acceptor(s) to the reaction solution containing crude enzyme preparations from pea (*Pisum sativum* L. cv. Gotik) leaves

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Abbreviations:

CKX, cytokinin oxidase/dehydrogenase; DPIP, 2,6 – dichloroindophenol; iP, N^6-(Δ^2-isopentenyl)adenine; MTT, methylthiazolyldiphenyltetrazolium bromide; PMS, phenazine methosulfate; t-Z, *trans*-zeatin

ABSTRACT:

Cytokinin oxidase/dehydrogenase (CKX, EC 1.4.3.18/1.5.99.12) is the key enzyme catalyzing cytokinin degradation in plants. The CKX activity in plant samples can be measured *in vitro* either by radioisotope or spectrophotometric methods. We report here modification of CKX radioisotope method by introducing 2,6-dichloroindophenol (DPIP) or other artificial electron acceptors to the assay mixture and its application for determining the CKX activity in crude plant extracts. The addition of phenazine methosulfate, DPIP or Toluidine Blue O (30, 75 and 300 μM) was found to enhance significantly (ca. 20- to 50-fold) the sensitivity of the radioisotope assay for the crude CKX preparation from pea (*Pisum sativum* L. cv. Gotik) leaves. The *in vitro* degradation of [^{3}H]iP to adenine by the pea leaf enzyme proceeded in a time-dependent manner in the presence of DPIP with optimal conversion (20-40%) achieved after 30 to 70 min incubation. The analysis of pH dependence of CKX revealed no shifts in the pH optima of the enzyme (pH 8.5-9.0) in response to DPIP. The HPLC procedure was optimized allowing reliable separation of the radiolabelled substrate [^{3}H]iP from the product of the CKX catalysed reaction proceeding in the presence of DPIP. It can be concluded from our data that the elevated sensitivity of the CKX assay allows easy and reliable detection of the enzyme activity even in crude extracts from various plant materials.

INTRODUCTION:

Cytokinins are a class of phytohormones that affect a wide variety of developmental and physiological processes in plants. The pool of bioactive cytokinins in plant cells and tissues is regulated at different levels including their biosynthesis, uptake from extracellular sources, metabolic interconversions, inactivation and degradation as well as signal transduction and transport (Mok and Mok 2001, Haberer and Kieber 2002). Irreversible metabolic degradation of cytokinins in plants is specifically catalysed by cytokinin oxidase/dehydrogenase(s) (CKX, EC 1.4.3.18/1.5.99.12) that selectively cleave unsaturated cytokinin isoprenoid side chains resulting in the formation of adenine/adenosine and the corresponding side chain aldehyde (Armstrong 1994). The rate of this reaction can be measured *in vitro* either by radioisotope or spectrophotometric (colorimetric) methods. The radioisotope methods are based on the measurement of conversion of purine ring-radiolabelled cytokinins N^6-(Δ^2-isopentenyl)adenine (iP), *trans*-zeatin or their ribosides as substrates in assays in which adenine or adenosine are separated from unreacted cytokinin substrates by appropriate chromatographic procedures. The colorimetric techniques rely on spectrophotometric determination of changes in UV absorption associated with cytokinin degradation (Libreros-Minotta and Tipton 1995, Kulkarni et al. 2001, Frébort et al. 2002). Poor reliability and relatively low sensitivity of spectrophotometric measurements of CKX activity have been recently improved by introducing a variety of artificial electron acceptors such as 2,6-dichloroindophenol (DPIP) to the reaction (Bilyeu et al. 2001, Galuszka et al. 2001, Frébort et al. 2002, Frébortová et al. 2004). However, DPIP spectrophotometric assay requires sufficiently purified enzyme preparations (Laskey et al. 2003), which makes the procedure time consuming and could cause certain losses of CKX activity during purification. Moreover, the concentration of substrate cytokinins for spectrophotometric detection of products considerably exceeds their physiological level.

In this work, we studied effects of DPIP and other artificial electron acceptors on the CKX activity in pea leaves using the standard radioisotope assay (Motyka et al. 2003) and

[^{3}H]iP as the substrate with the aim to find out and optimize conditions for increasing the sensitivity of the assay and its applicability for determining the CKX activity even in crude plant extracts.

MATERIAL AND METHODS:

Plant material

Seeds of pea (*Pisum sativum* L. cv. Gotik) were obtained from Agritec Ltd. (Šumperk, Czech Republic). The growing conditions as well as a procedure for collecting the pea leaves for CKX analyses were reported previously (Gaudinová et al. 2005).

Determination of cytokinin oxidase/dehydrogenase activity

The crude CKX preparations were extracted and partially purified as described by Gaudinová et al. (2005). The frozen pea leaves 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. Proteins content was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

The standard radioisotope assay for determination of CKX activity in plant extracts based on conversion of [^{3}H]iP to adenine (Motyka et al. 2003) was modified by introducing 2,6-dichloroindophenol (DPIP) or other electron acceptors such as phenazine methosulfate (PMS), Basic blue 24, Coenzym Q_0, Toluidine Blue O and methylthiazolyldiphenyltetrazolium bromide (MTT) to the reaction. The enzyme preparations corresponding to 40-50 mg leaf FW (equivalent of 31-40 μg protein) were incubated in an assay mixture (total volume 50 μl) containing 100 mM TAPS-NaOH (pH 8.5), the electron acceptor at concentrations 30, 75 or 300 μM and 2 μM substrate [2-^{3}H]iP (7.4 Bq mol^{-1}). After incubation (1 h) at 37^{o}C, the reaction was stopped by the addition of 120 μl 95% cold ethanol and 10 μl 200 mM Na_2EDTA.

Separation of the substrate from the product of the enzyme reaction was optimized by HPLC using the Series 200 HPLC Quaternary Pump (Perkin Elmer) coupled to 235°C Diode Array Detector (Perkin Elmer) and Ramona 2000 flow-through radioactivity detector (Raytest) on the column Luna C_{18} (50 mm/4.6 mm/3 µm) connected with two C_{18} guard cartridges. The sample (50 µl) was eluted at a flow rate 0.6 ml min^{-1} and UV detection was performed at 270 nm. Two solvents, A (40 mM CH_3COOH + NH_4OH, pH 5.0) and B (CH_3OH/CH_3CN, 1:1, v/v), were used to obtain the linear gradient 1-70% B in 1 min, 70% for 1 min, 70-1% B in 1 min. For the *on line* radioactivity detection the column eluate was mixed with three volumes of liquid scintillation cocktail (Flo-Scint III, Packard Bioscience). The radioactive iP and adenine were identified on the basis of co-incidence of their retention times with authentic standards.

RESULTS AND DISCUSSION:

The sensitivity of the CKX radioisotope assay was significantly elevated by introducing DPIP, PMS or other electron acceptors to the reaction (Table 1). For the used concentrations (30, 75 and 300 µM), the enhancement of the assay sensitivity as well as the optimal concentration of the electron acceptor differed depending on the particular type of artificial acceptor. The highest increase in the sensitivity of the CKX radioisotope assay compared to the control was found for PMS (30- to 48-fold) followed by DPIP (21- to 31-fold) and Toluidine Blue O (26- to 29-fold), while other tested electron acceptors (Basic Blue 24, Coenzym Q_0 and MTT) were less effective (Table 1). Similarly, also our other data obtained for enzyme preparations from different plant tissues such as developing wheat grains, radish seedlings and tobacco calli revealed a significant enhancement (ca. 20 to 40-fold) of the sensitivity of the CKX radioisotope assay by introducing DPIP or PMS to the reaction solution (Šolcová et al. 2004 and data not shown).

On the basis of these results as well as the literature data obtained for spectrophotometric determinations of CKX activity (Frébort et al. 2002), DPIP was chosen as a useful and a strongly effective electron acceptor in our other investigations. The *in vitro*

degradation of [^{3}H]iP to adenine by CKX preparation from pea leaves was found to proceed in a time-dependent manner in the presence of DPIP with optimal conversion (20-40%) achieved after 30 to 70 min incubation (Fig.1, for comparison see also Fig.3).

The CKX preparation from pea leaves exhibited relatively high pH optima in the range between pH 8.5 and 9.0 when assayed both with and without DPIP (Fig.2). The high pH optimum (pH 8.5) found without DPIP corresponds to that reported by Gaudinová et al. (2005) for the CKX in pea leaves and suggests a presence of a non-glycosylated CKX isoform or an isoform with a very low degree of glycosylation in pea. Evidently, our analysis of pH dependence revealed no shift in the pH optimum of the pea leaf enzyme in response to the presence of DPIP in the reaction mixture. Similarly, no differences between the pH optima were found for CKX in tobacco calli assayed either in the presence or the absence of DPIP (data not shown). These findings are in contrast with the previously reported stimulation of sensitivity of the CKX radioisotope assay by copper-imidazole complexes (Chatfield and Armstrong 1987) and indicate that DPIP does not affect the pH optimum of CKX. Thus, the DPIP sensitized assay allows degradation of substrate cytokinins *in vitro* under conditions similar to those in plants.

As a part of this study, the HPLC method for separation of the radiolabelled substrate from the product of the CKX catalysed reaction proceeding in the presence of DPIP was optimized (Fig. 3). This HPLC procedure (see Material and Methods) allowed reliable separation of adenine from iP with retention times 3.3 and 6.3 min, respectively, and the total duration of one analysed sample not exceeding 13 min.

An increase of sensitivity and reliability of colorimetric techniques for CKX detection by introducing artificial electron acceptors including DPIP was already reported (Bilyeau et al. 2001, Galuszka et al. 2001, Frébort et al. 2002). However, the DPIP spectrophotometric assays require sufficiently purified enzyme preparations (Laskey et al. 2003) and relatively high concentrations of substrate cytokinins exceeding considerably their physiological levels. The DPIP sensitized radioisotope assay described here is very specific and allows

degradation of substrate cytokinins at concentrations close to the corresponding K_m values (K_m for iP 2.8 μM, Bilyeau et al. 2001) and to the actual concentrations of cytokinins in plants. Moreover, in contrast to colorimetric techniques this assay provided reliable results even with crude protein preparations. Thus, the DPIP sensitized radioisotope assay represents a suitable tool for easy detection of the CKX activity in crude extracts from various plant materials.

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Šolcová B, Hoyerová K, Dobrev PI, Kamínek M, Motyka V (2004) Determination of cytokinin oxidase/dehydrogenase activity in crude plant extracts using 2,6-dichloroindophenol or phenazine methosulfate sensitized radioisotope assay. Acta Physiol Plant **26:** (3, Suppl.), abstr. PG-014, p.21.

FIGURE LEGENDS:

Fig.1 The effect of incubation time on [^{3}H]iP degradation *in vitro* by CKX preparation from pea leaves (*Pisum sativum* L. cv. Gotik) in the presence of DPIP in the reaction mixture.

The enzyme preparation was incubated in the assay mixture (total volume 50 µl) containing 100 mM TAPS-NaOH (pH 8.5), 75 µM DPIP and 2 µM [2-^{3}H]iP (7.4 Bq mol^{-1}) for indicated time periods; other details are described in Material and Methods.

Fig.2 The effect of pH on the CKX activity from pea (*Pisum sativum* L. cv Gotik) leaves in the absence and presence of DPIP.

The pH optimum for CKX was determined by running the assay in 100 mM MES-NaOH (pH 5.0, 6.0 and 7.0), MOPS-NaOH (pH 6.7, 7.2 and 7.8), TAPS-NaOH (pH 7.8, 8.5 and 9.0) and CHES-NaOH (pH 8.8, 9.5 and 10.0) buffers lacking and containing DPIP (75 µM); other details are described in Material and Methods.

Fig.3 The separation of [^{3}H]iP and [^{3}H]adenine by HPLC coupled to on-line radioactivity detector.

The radiolabelled substrate (iP) and product (adenine) of the enzyme reaction catalysed by CKX from pea (*Pisum sativum* L. cv Gotik) leaves were separated by HPLC as described in Material and Methods (A = signal from radioacivity detector, B = signal from Diode Array Detector).

Table 1. The effects of different electron acceptors on the *in vitro* CKX activity from pea (*Pisum sativum* L. cv Gotik) leaves.

The effects of electron acceptors applied in three concentrations (30, 75 and 300 μM) on the *in vitro* CKX activity from pea leaves were assayed as described in Material and Methods. *(DPIP = 2,6-dichloroindophenol; PMS = phenazine methosulfate; BB 24 = Basic Blue 24; Coenzyme Q_0 = 2,3-dimethoxy-5-methyl-1,4-benzoquinone; BB 17 = Toluidine Blue O; MTT = methylthiazolyldiphenyltetrazolium bromide).*

		CKX ACTIVITY	
Electron acceptor	**Concentration (μM)**	**(nmol Ade.mg^{-1} protein.h^{-1})**	**Increase**
0 (Control)	**---**	0.037	---
DPIP	30	1.134	*30.7 x*
	75	1.048	*28.3 x*
	300	0.791	*21.4 x*
PMS	30	1.125	*30.4 x*
	75	1.327	*35.8 x*
	300	1.771	*47.8 x*
BB 24	30	0.134	*3.6 x*
	75	0.322	*8.7 x*
	300	0.057	*1.5 x*
Coenzyme Q_0	30	0.342	*9.2 x*
	75	0.722	*19.5 x*
	300	1.030	*27.8 x*
Toluidine Blue O	30	1.077	*29.1 x*
	75	1.053	*28.5 x*
	300	0.952	*25.7 x*
MTT	30	0.126	*3.4 x*
	75	0.135	*3.7 x*
	300	0.367	*9.9 x*

Fig. 1

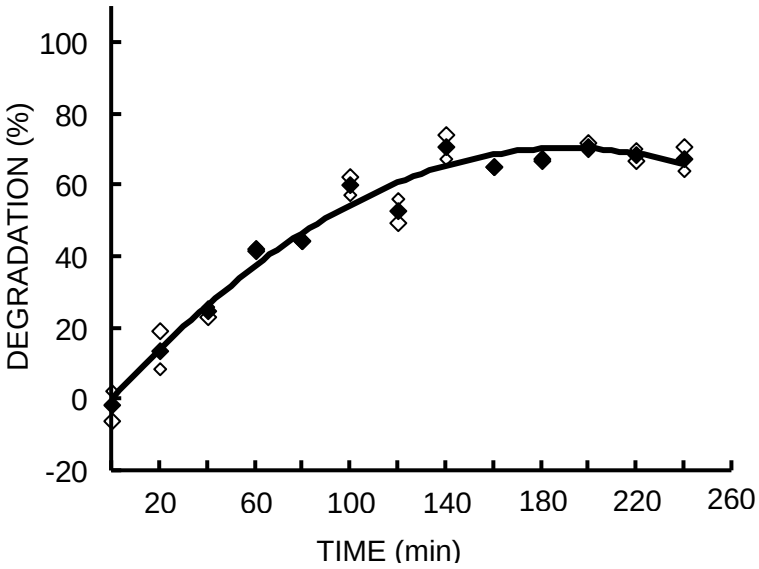

DEGRADATION (%)
100
80
60
40
20
0
-20
20
60
100
140
180
220
260
TIME (min)

Fig. 2

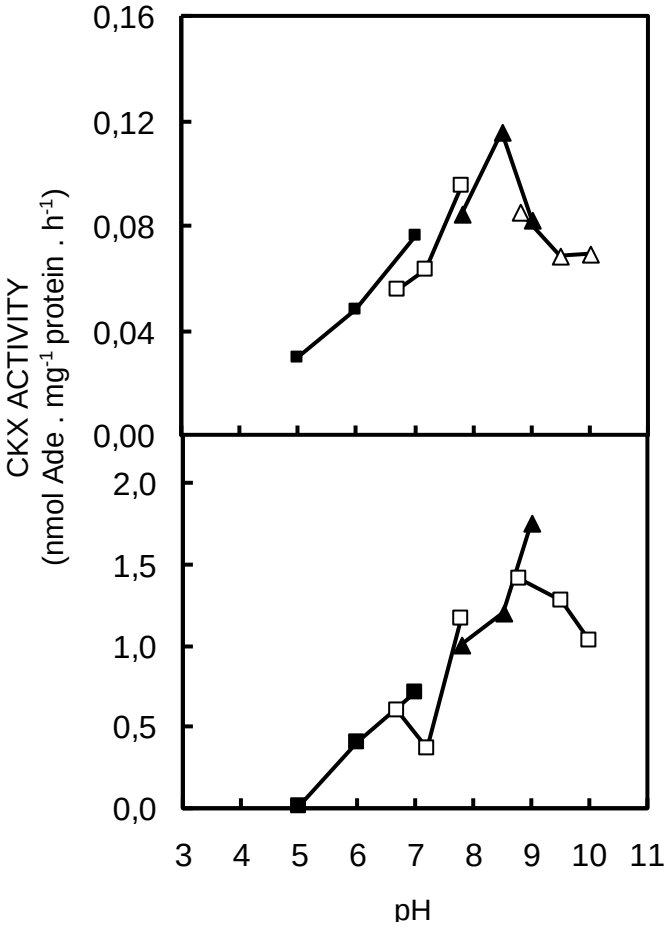
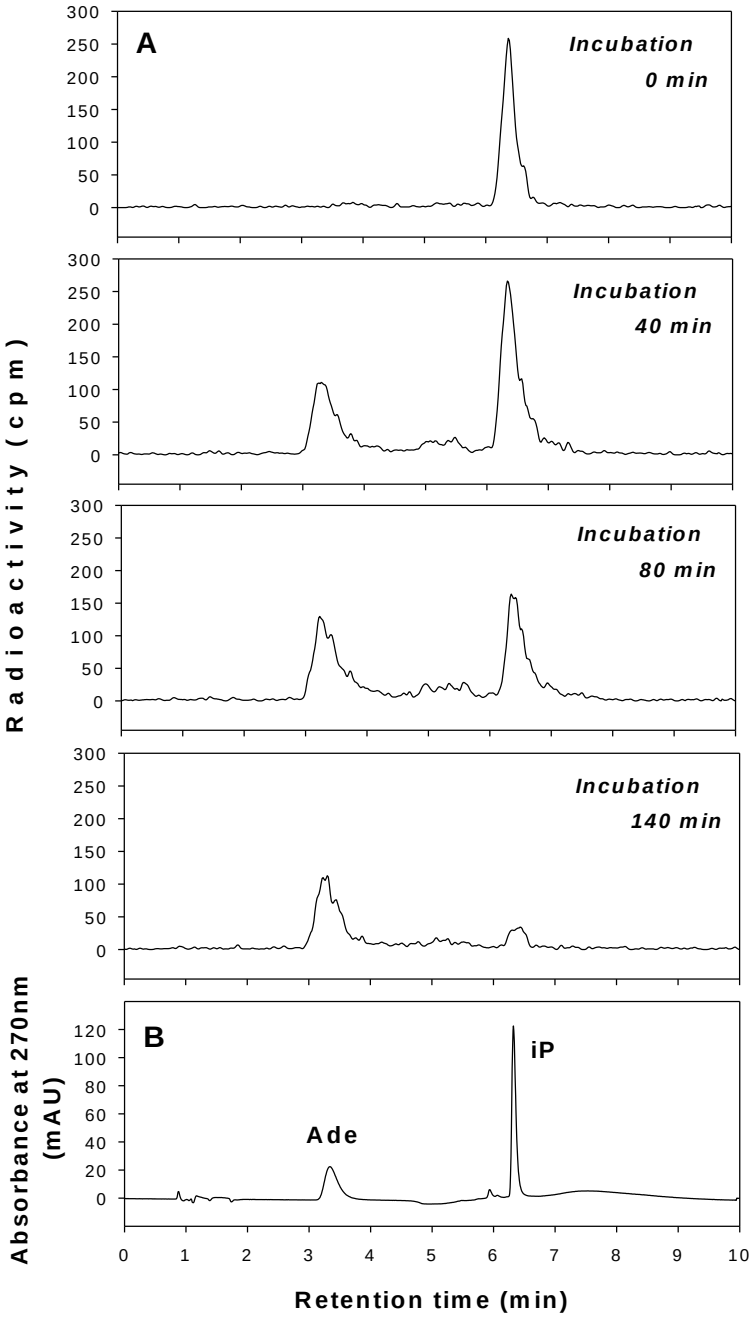

CKX ACTIVITY
(nmol Ade . mg-1 protein . h-1)
0,16
0,12
0,08
0,04
0,00
2,0
1,5
1,0
0,5
0,0
3
4
5
6
7
8
9
10
11
pH

Fig. 3


A
Incubation
0 min
Incubation
40 min
Incubation
80 min
Incubation
140 min
B
iP
Ade
Radioactivity (cpm)
Absorbance at 270nm (mAU)
Retention time (min)

Factors affecting delay of chlorophyll degradation by cytokinins in senescing oat and wheat leaves

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Abbreviations: 3OHBAR, N^6-(3-hydroxybenzyl)adenosine ("meta-topolin riboside"); BA, N^6-benzyladenine; CKX, cytokinin oxidase/dehydrogenase; c-ZOG, *cis*-zeatin *O*-glucoside; c-ZROG, *cis*-zeatin riboside *O*-glucoside; DHZ, dihydrozeatin; DHZ9G, dihydrozeatin 9 *N*-glucoside; DHZOG, dihydrozeatin *O*-glucoside; DHZR, dihydrozeatin riboside; DHZROG, dihydrozeatin riboside *O*-glucoside; DMF, N,N-dimethylformamide; HPLC/MS/MS, high performance liquid chromatography + mass spectrometry; iP, N^6-(2-isopentenyl)adenine; iPR, N^6-(2-isopentenyl)adenosine; t-Z, *trans*-zeatin; t-Z9G; *trans*-zeatin 9 *N*-glucoside; t-ZOG, *trans*-zeatin *O*-glucoside; t-ZR, *trans*-zeatin riboside; t-ZROG, *trans*-zeatin riboside *O*-glucoside; t-Z7G, *trans*-zeatin 7 *N*-glucoside.

ABSTRACT:

It has been frequently argued that the physiological effects of exogenously applied as well as of locally synthesized cytokinins *in planta* are influenced by the cytokinin uptake (influx), translocation and metabolism and may differ between plant species. In spite of these vague predications a complex study assessing the influence of these factors on the final cytokinin activity is missing. In this report we evaluated the effects of these factors on the retention of chlorophyll enhanced by *trans*-zeatin riboside (t-ZR) and N^6-(3-hydroxybenzyl)adenosine (3OHBAR) as representatives of isoprenoid and aromatic cytokinins, respectively. The efficiency of the two cytokinins in preservation of chlorophyll differed in the oat and wheat depending on local or whole adaxial leaf surface application. The oat and wheat leaf pieces differed in the rates of uptake and the dynamics of [^{3}H]t-ZR and [^{3}H]3OHBAR translocation which was very fast in wheat and slower but steadily increasing with time in oat. The oat and wheat leaf apices also differed in the metabolism of applied t-ZR namely in its conversion to the storage dihydrozeatin-*O*-glucoside prevailing in oat and to the inactive t-Z-*N9*-glucoside prevailing in wheat. The t-Z was more efficient in stimulation of cytokinin oxidase/dehydrogenase (CKX) than 3OHBAR and the activity of the enzyme was more increased by the two cytokinins in oat than in wheat leaf apices. The difference in activities of both cytokinins in retention of chlorophyll in wheat and oat can be minimized by their pulse application followed by incubation of leaf apices floating on water.

Key words: cytokinin, zeatin, cytokinin oxidase/dehydrogenase, cytokinin uptake and translocation, wheat, oat.

INTRODUCTION:

Development of specific bioassays has been the basic presumption for discoveries of different plant hormones, including cytokinins. Using this tool the structure-activity relationships of hundreds of natural and synthetic compounds manifesting cytokinin activity has been characterized (Skoog and Abdul Ghani, 1981; Skoog *et al*., 1961; Iwamura *et al.,* 1980). Application of compounds exhibiting cytokinin activity to plants and plant parts has also been very helpful for identification and characterization of biological processes which are regulated either by cytokinins alone or more frequently by interaction/co-operation of cytokinins with other plant hormones including the control of cell division (Miller *et al.,* 1955), organ differentiation (Skoog and Miller, 1957), apical dominance (Wickson and Thimann, 1958), biosynthesis of plant pigments (Biddington and Thomas, 1973; Fletscher and McCullagh, 1971), enlargement of cotyledons (Letham, 1971) and plastid proliferation and differentiation (Yagisawa *et al*., 2003; Luštinec *et al*., 1984).

It has been repeatedly stressed that determination of biological activities of plant hormones using various bioassays provides only relative results which are affected by many endogenous factors including the hormone uptake and translocation in plant tissues as well as the reversible or irreversible metabolic inactivation (Armstrong, 1994; Mok and Mok, 2001; Hou *et al*., 2004). Nevertheless, the involvement of cytokinins in control of specific physiological and developmental processes which were revealed by exogenous cytokinin application has been confirmed by modulation of endogenous cytokinin levels by experimentally controlled expression of cytokinin biosynthetic and metabolic genes namely of isopentenyl transferase and cytokinin oxidase/dehydrogenase, respectively (e.g. Kakimoto, 2001; Myiawaki *et al*., 2004; 2006; Werner *et al*., 2001, 2003).

The identification of cytokinin receptors and *in vitro* characterization of their ligand specificity has provided an efficient tool for comparison of activities of cytokinins at molecular level (Kakimoto, 2001; Inoue *et al*., 2001a; Suzuki *et al*., 2001). Interestingly, the *in vitro* assays using cytokinin receptors heterologously expressed in *Escherichia coli* and yeast mostly confirmed the relative activities of different groups of cytokinins (cytokinin bases,

ribosides, *O*- and *N*-glucosides) characterized earlier by *in vivo* bioassays. However, as reported by Spíchal *et al*. (2004) two cytokinin receptors CRE1/AHK4 and AHK3 differ in recognition of some cytokinins (cytokinin ribosides and ribotides, *cis*-zeatin and dihydrozeatin [DHZ]) suggesting that the *in vitro* binding of cytokinins to cytokinin receptors provides results which cannot be generalized. The various responses of different receptors to different cytokinins can be partially responsible for different activities of individual cytokinins at different plant sites and in different plant species (Skoog and Abdul Ghani, 1981). Dependence of cytokinin activities on hormone translocation and metabolic stability may affect resulting activities not only of exogenously applied cytokinins but also activities of cytokinins synthesized *in planta*. Cytokinins transported via the xylem and phloem to control physiological processes at distant sites from the site(s) of cytokinin biosynthesis can be targets of such regulations.

The chlorophyll retention bioassay has been found suitable for studies of effects of cytokinin uptake, translocation and metabolism on activity of exogenously applied cytokinins. The ability of cytokinins to delay senescence of detached leaves was described early after their discovery (Richmond and Lang, 1957) and used as a fast and simple bioassay for determination of cytokinin activity (Thimann and Sachs, 1966; Kamínek and Luštinec, 1978). The uptake and translocation of locally applied cytokinins can be easily monitored. Moreover, the activities of different cytokinins in suppression of leaf senescence mostly corresponds to those determined by the classical time requiring tobacco callus assay (Kamínek *et al.,* 1979).

Addressing this issue we compared the activities of an isoprenoid and an aromatic cytokinin, N^6-(2-isopentenyl)adenosine (iPR) and N^6-(3-hydroxybenzyl)adenosine ("meta-topolin riboside", 3OHBAR), respectively, in two chlorophyll retention bioassays differing in the way of cytokinin application. We also characterized the differences in uptake and metabolism of the two cytokinins and their potential impact on retention of chlorophyll in detached leaves of two closely related plant species, oat and wheat which were found to differ in responses to cytokinins bearing isoprenoid and aromatic side chain.

MATERIAL AND METHODS

Plant material

Oat (*Avena sativum* L. cv. Abel) and wheat (*Triticum aestivum* L., cv. Munk) seeds were soaked in distilled water overnight and plants were grown in perlite saturated with nutrient solution for 10 d in a growth cabinet at 18h/6h photoperiod, (20°C/18°C) and 90% RH. The first fully developed leaves were excised from 10 d old plants when the second leaf just started to develop.

Chlorophyll content

Collected leaves were frozen in liquid nitrogen and stored at -70°C. Chlorophyll was extracted according to Lichtenthaller (1987) using dimehylformamide (DMF) Frozen leaves (till 1 g) were homogenized in liquid nitrogen and extracted in darkness and cold (+4°C) in DMF (10 ml) over night. The samples were measured by spectrophotometer Unicam 5625 at A_{647}, A_{664} and A_{750}. The chlorophyll content was count according to rovnice from Lichtenthaller (1987).

Different ways of cytokinin application

Two ways of cytokinin application to wheat and oat leaf apices were compared. In so called “test tube” assay cytokinins were applied locally by submersion of the bases of apical leaf apices in 1.5 ml of cytokinin solution or water in a test tube (3 leaf apices in each parallel) and incubated for 5 d in a growth chamber at 18h/6h (20°C/18°C) photoperiod and 90% RH. In the second so called “Petri dish” assay cytokinins were applied to the whole adaxial surface of leaf apices floating on water or cytokinin solution (3 leaf apiece per 5 ml of solution). The incubation started and was terminated 3 h after the beginning of light period.

Translocation of [^{3}H]3OHBAR and [^{3}H]t-ZR

For monitoring translocation of cytokinins 7 cm long apical leaf apices of oat and wheat were submerged with their basal ends in 0.5 ml of incubation solution containing either of

[^{3}H]3OHBAR or [^{3}H] t-ZR (2x10^{-5} M and 4500 dpm µl^{-1} each). The leaves were incubated for 1h, 2h, 4h or 16 h in darkness at 25^{o}C and 100% RH. After incubation the residual cytokinin solution on the leaf base was wiped with filter paper and the leaves were divided into four segments numbered from the leaf base 1 (0-1 cm), 2 – 4 (2 cm each). Collected segments were weighted frozen and kept at -70^{o}C. Radioactivity was extracted with 80% (v/v) methanol by standing at 4^{o}C overnight followed with wortexing and centrifugation. Radioactivity was measured by scintillation counting using Packard TRI-CARB 2500 TR scintillation counter.

Changes in content of endogenous cytokinins during incubation in 3OHBAR and t-ZR solutions

The apical leaf apices 3.5 cm long were submerged by their bases into 1.5 ml of 10^{-4} M solutions of t-ZR, 3OHBAR water and incubated under 18 h/6 h (20^{o}C/18^{o}C) photoperiod. Samples for cytokinin analysis were collected after 3 d and 5 d of incubation 3 h after the beginning of the day period, weighted, frozen in liquid nitrogen and stored at -80^{o}C until analysis.

Cytokinins were extracted and purified using dual-mode solid phase extraction (Dobrev and Kamínek, 2002) and determined by HPLC/MS/MS as described elsewhere (Hoyerová *et al.*, 2006). Briefly, leaf apices were homogenized and extracted overnight in cold methanol:water:formic acid (15:4:1, v/v/v) . Following centrifugation (15,000 g, 4^{o}C, 20 min) part of lipids was removed from the supernatant by filtration through Sep-Pack †C18 cartridge. After evaporation to near dryness, the residue was dissolved in 5 ml 1 M formic acid and applied to an Oasis MCX column (150 mg reverse-phase cation-exchange sorbent). After washing the column with 5 ml of formic acid and 5 ml of methanol cytokinin nucleotides were eluted with 5 ml 0.35 M NH_4OH. Cytokinin bases, ribosides and glucosides were eluted with 5 ml 0.35 M NH_4OH in 60% (v/v) methanol. The eluate containing cytokinin bases, ribosides and glucosides was evaporated to dryness using a Speed-Vac. The eluate containing cytokinin nucleotides was evaporated in the same way to 2-3 ml to remove the

ammonia. The cytokinin nucleotides were dephosphorylated to nucleosides by incubation with calf-intestine alkaline phosphatase (Sigma).

The cytokinins were quantified by HPLC linked to spectrometer using a linear gradient of acetonitrile (B) in 0.0005%, v/v, and acetic acid in water (A): 10% B for 5 min, to 17% B in 15 min and to 50% B in 35 min was used at flow rate of 0.2 ml min-1. Detection and quantification were carried out using mass spectrometer Finnigan LCQ operated in the positive ion, full-scan MS/Ms mode using a multilevel calibration graph with [2H] labeled CKs as internal standards. Each analysis was repeated twice. The results presented here are the mean values of two different experiments.

Measurement of cytokinin oxidase/dehydrogenase activity

The cytokinin oxidase/dehydrogenase (CKX) activity was measured by *in vitro* assays based on the conversion of [2-^{3}H]iP to [2-^{3}H]adenine as described elsewhere (Gaudinová *et al*., 2005) with slight modifications. Briefly, frozen leaf apices incubated with t-ZR, 3OHBAR or water in the "test tube" assay were homogenized in 0.1 M tris-HCl buffer (pH 7.5) and phenolic substances were removed by passing the extract through a column of acid-treated polyvinylpolypyrrolidine. Following precipitation of nucleic acids and nucleoproteins with Polymin P (1%, v/v, pH 7.5; Serva, Heidelberg, Germany) proteins were precipitated by addition of solid ammonium sulphate to 80% saturation. Concentration of proteins was determined by the method of Bradford (1976) using bovine serum albumin as a standard. The CKX activity was determined using the assay mixture (50 µL final volume) containing TAPS-NaOH buffer (pH 8.5) at final concentration of 100 mM, substrate ([2-^{3}H]iP, 7.4 Bq mol^{-1}, 2 µM) and enzyme preparation equivalent to 0.02-0.25 mg protein mL^{-1}). After incubation (1h – 26 h in dependence on % of degradation of CK to adenine, 37^{o}C) the reaction was terminated by adding of 10 µl Na_4EDTA (200 mM) and 120 µl 95% (v/v) ethanol. The substrate was separated from the product of the enzyme reaction by HPLC on the column Luna C_{18}(2) (50 mm/4.6 mm/3µm) (Phenomenex, Torrance, CA, USA) using Series 200 HPLC Quaternary Pump (Perkin Elmer, Wellesley, MA, USA) coupled to 235C Diode Array

Detector (Perkin Elmer) and RAMONA 2000 flow-through radioactivity detector (Raytest, Straubenhart, Germany).

Pulse application of 3OHBAR and t-ZR on chlorophyll retention

Leaf segments (2 cm long) were excised between the second and the fourth cm from the first leaf apex of 10 d old oat and wheat seedlings. The segments were incubated by floating on water and solutions of t-ZR or 3OHBAR ($2x10^{-5}$ M each) for 24 h in darkness (25^{o}C) and then washed on a linen screen with distilled water under stirring to remove soluble cytokinins from the surface of leaf segments. Further incubation proceeded on water only. The segments were collected at 24 h intervals for 5 d, frozen in liquid nitrogen and stored at -70^{o}C for determination of chlorophyll. Each of three replicates contained 10 leaf segments floating on 10 ml of water or cytokinin solution in Petri dish.

RESULTS

For study of senescence-related responses of detached leaf apices to exogenous isoprenoid and aromatic cytokinins the senescence-susceptible cultivars of oat and wheat, cv. Abel and cv. Munk, respectively, were selected. From four tested isoprenoid and five aromatic cytokinins the t-ZR in wheat and 3OHBAR in oat, respectively, exhibited the highest chlorophyll retention activity (Tab. 1).

Retention of chlorophyll depends on the way of cytokinin application

Retention of chlorophyll was significantly affected by the way of cytokinin application. In oat leaf apices the 3OHBAR was much more active than t-ZR when cytokinins were applied locally (“test tube” assay). The residual content of chlorophyll was significantly higher in 3OHBAR-treated leaf apices than in those incubated with t-ZR beginning from $4x10^{-6}$ M. At $2x10^{-5}$ M cytokinin concentration the 3OHBAR-treated apices the retained 3-fold higher amount of chlorophyll comparing to leaf pieces incubated with t-ZR solution (after subtraction of water control values). In contrast, there was no difference in the activity of both cytokinins when they were applied to whole leaf apices surface in “Petri dish” assay (Fig. 1).

Interestingly, the two cytokinins exhibited very different chlorophyll retention activities in wheat leaf apices. The difference between the activityof 3OHBAR and t-ZR in “test tube” assay was significantly reduced while in the “Petri dish” assay the activity of 3OHBAR was much higher than that of t-ZR (4-fold and 2.6-fold at $4x10^{-6}$ M and 2.10^{-5} M cytokinin concentration, respectively).

Translocation and accumulation of [^{3}H]3OHBAR and [^{3}H] t-ZR

The amount of radioactivity of applied [^{3}H]3OHBAR and [^{3}H]t-ZR in the first basal 1 cm leaf segment of 7 cm long leaf apices of oat and wheat submerged to cytokinin solution was progressively increasing with time of incubation. While the first leaf segment of oat accumulated less 3OHBAR and t-ZR than wheat during the first 4 h of incubation it exceeded wheat in accumulation of 3OHBAR but not of t-ZR after 16 h of incubation (Fig. 2). Oat and wheat differ in the dynamics of translocation of radioactivity taken up with t-ZR and [^{3}H]3OHBAR. The acropetal translocation was very fast in wheat leaves being the highest in the apical (fourth) leaf segment already after 1 h of incubation as compared to other leaf sections. The distribution of radioactivity between the leaf segments becomes more equal with time of incubation. Different dynamics of translocation of radioactivity of/from both cytokinins was found in oat leaves where it was mostly steadily increasing in all leaf segments with time of incubation.

The total radioactivity taken up with both cytokinins in the course of incubation and accumulated in the whole leaf apices was higher and nearly constant in wheat while lower and progressively increasing in oat suggesting that the saturation of wheat leaf apices was reached already after 1 h of incubation while it was still progressing in oat within 16 h of incubation.

Changes in content of endogenous cytokinins during incubation of leaf apices in solutions of 3OHBAR and t-ZR

The application of t-ZR and 3OHBAR solution to the basal sites of leaf apices in the "test tube" assay significantly affected the content of endogenous cytokinin in the course of incubation. The following cytokinins were found in wheat and oat leaf extracts in amounts allowing their quantification: t-Z, t-ZR, t-ZOG, t-ZROG, DHZROG, t-Z9G, c-ZOG and c-ZROG. Some other cytokinins occurred only in trace amounts (iP, iPR, DHZ, DHZR, DHZ9G and Z7G). In leaf apices incubated for 5 d in water the content of bioactive t-Z declined 6- and 28-fold in oat and wheat, respectively, while the content of t-ZR slightly declined only in oat and was not changed in wheat leaf apices (Fig. 3). The content of storage cytokinins i.e. t-ZOG and t-ZROG was steadily declining during incubation in water. This decline was more pronounced in oat (4.3-fold) than in wheat (1.6-fold). The decline in bioactive cytokinins was accompanied with an increased content of biologically inactive t-Z9G in wheat but not in oat leaf apices. Interestingly, leaves of both oat and wheat contained very high amounts of *cis*-zeatins represented mainly by their storage *O*-glucosides (c-ZOG and c-ZROG). The content of *cis*-zeatins was 17-fold and 15-folf higher (!) than the sum of all other cytokinins in oat and wheat, respectively.

Incubation of leaf apices in 10^{-4} M solution of t-ZR resulted in a progressive accumulation of high amounts of t-Z and t-ZR in leaf apices of both oat and wheat. The increase of t-Z was almost exponential in wheat and somewhat less pronounced in oat leaf apices. As expected the t-ZR, i.e. cytokinin which was present in the incubation solution, was progressively accumulated in very high amounts in oat and somewhat less in wheat leaf apices. The tZR taken up from the incubation solution was accompanied with accumulation of storage *O*-glucosides (t-ZOG and t-ZROG) and also of DHZOG. Part of taken up t-ZR was evidently metabolized to physiologically inactive *N*-glucosides (t-Z9G and DHZ9G). While the wheat leaf apices accumulated more t-Z9G than oat an opposite was found for DHZ9G. Application of exogenous t-ZR did not significantly affect content of *cis*-zeatins (with the exception of oat where *cis*-zeatins level was slightly increased after 5 d incubation).

In contrast to t-ZR the incubation of leaf apices in solution of 3OHBAR had different effects on the content of isoprenoid cytokinins in oat and wheat leaf apices. While in wheat

the content of bioactive t-Z and t-ZR was increased nearly 5- and 4-fold, respectively, after 5 d incubation it was decreased to one half in oat. Similarly, the application of 3OHBAR increased the content of all other cytokinins including t-Z9G, DHZ9G and t-ZOG + t-ZROG and, interestingly, also that of *cis*-zeatins.

Activity of cytokinin oxidase/dehydrogenase

The activity of CKX was slightly higher in oat comparing to wheat leaf apices. However, the difference was statistically significant only in some experiments. The enzyme activity was increasing with time of incubation reaching the maximum after 3 d in oat and 5 d in wheat, respectively (Fig. 4). Both cytokinins were very potent inducers of CKX activity which was steadily increasing with the incubation time. After 5 d of incubation the CKX activity was increased by 265-fold in oat and 185-fold in wheat leaf apices incubated in 10^{-4} M t-ZR, respectively. Similarly, the application of 3OHBAR was more effective in enhancement of CKX activity in oat than in wheat increasing the CKX activity 175-fold and 84-fold, respectively when compared to the water controls.

The effect of t-ZR, DHZR and 3OHBAR on retention of chlorophyll

Testing the activity of CKX substrate (t-ZR) and non-substrate (3OHBAR and DHZR) cytokinins in retention of chlorophyll in oat and wheat leaf apices in the “test tube” assay revealed that the non-substrate cytokinins (3OHBAR and to a lesser extent also DHZR) were more active in retention of chlorophyll in oat while in wheat leaf apices these differences were much less pronounced (Fig. 5).

The effect of a pulse application of 3OHBAR and t-ZR on chlorophyll retention

Chlorophyll retention in oat and wheat leaf segments pre-incubated for 24 h in water and $2x10^{-5}$M solutions of t-ZR and 3OHBAR was steadily decreasing during the following incubation by floating on water in darkness. The rate of decrease was the same in wheat segments pre-incubated in solutions of both cytokinins. However, it was different in oat

where beginning from the third day of incubation the retention of chlorophyll in leaf segments pre-incubated in t-ZR solution was decreasing more rapidly than in segments pre-incubated in 3OHBAR. Regardless to plant species the pre-treatment of leaf segments with both cytokinins slowed-down the degradation of chlorophyll comparing to the pre-treatment with water.

DISCUSSION

The ability of cytokinins to delay senescence of detached leaves was originally detected in *Xanthium* (Richmon and Lang, 1957). However, it was early recognized that leaves of cereal species react more specifically to cytokinins (Kende, 1964; Thimann and Sachs, 1966). Interestingly, the cytokinins bearing an aromatic N^6-side chain were reported to be more active in retention of chlorophyll than the isoprenoid ones (Varga and Bruinsma, 1973). However, the relative activities of these two classes of cytokinins can be modulated by modification of assay parameters (Kamínek and Luštinec, 1978).

As we report here the leaf apices of two cereal species, oat and wheat, exhibit different susceptibility to aromatic and isoprenoid cytokinins. Comparing chlorophyll retention activities of five aromatic and four isoprenoid cytokinins in the “test tube” assay the chlorophyll retention activity ratios of cytokinins applied to the oat and wheat leaf apices varied between 0.58 and 1.04 for isoprenoid and between 1.24 and 2.48 for aromatic cytokinins (Table 1). This difference suggests that the aromatic cytokinins are more active than isoprenoid cytokinins in oat and somewhat less active in wheat when applied locally to the base of leaf apices. The 3OHBAR and t-ZR which were the most active aromatic and isoprenoid cytokinins in oat and wheat, respectively, were used in further experiments as representatives of the two classes of cytokinins.

Dependence of cytokinin activity on the way of cytokinin application

The 3OHBAR and t-ZR exhibited very different order of chlorophyll retention activities in oat and wheat leaf apices depending on the way of cytokinin application. While in the oat

leaf apices 3OHBAR was much more active than t-ZR when applied locally ("test tube" assay) and it was equally active in the "Petri dish" assay the two cytokinins showed nearly opposite activity ratios in wheat leaf apices (Fig. 1). This difference could be caused not only by the different susceptibility of oat and wheat leaves to the two cytokinins. Differences in cytokinin uptake, translocation and metabolic stability could also influence the delivery of hormonal signal in the site of its action. As reported elsewhere the different activity ratios of t-Z and BA in retention of chlorophyll in response to the application of a single droplet of cytokinin solution to the adaxial surface of detached oat leaves was decreasing with the increasing length of the leaf apices and the volume of applied cytokinin solution (Kamínek and Luštinec, 1978). This suggests that the availability of larger leaf area for translocation of cytokinin allows the more mobile cytokinin(s) to preserve chlorophyll in distant parts of the leaf from the site of cytokinin application. In this particular case the highest actual activity of N^6-benzyladenine (BA) on site of cytokinin application was compensated by the higher mobility of t-Z resulting in preservation of chlorophyll on larger leaf area. Indeed, the local application of t-Z resulted in preservation of chlorophyll over a larger leaf area comparing that of BA (results not shown).

Translocation of [^{3}H]3OHBAR and [^{3}H] t-ZR

The influx of cytokinin may play an important role in control in endogenous cytokinin levels in the competent cells responding to the cytokinin signal. Different dynamics of accumulation of exogenously applied [^{3}H]t-ZR and [^{3}H]3OHBAR in wheat and oat leaf apices (Fig. 2) suggests that t-ZR is taken up more readily by both oat and wheat leaf apices and that there is a fast uptake of both cytokinins by wheat leaf apices reaching a saturation level already after 1 h of incubation. It has been suggested that exogenous cytokinins are passively taken up by different plant organs such as e.g. by watermelon cotyledons (Fantelli *et al*., 1982). The recent identification of purine transporters in *Arabidopsis* (*AtPUP1*, *AtPUP2* and *SOI33*, which also recognize cytokinins, suggests an active influx of cytokinins from the leaf apoplast (Bürkle *et al*., 2003; Hirose *et al.,* 2005; Sun *et. al*., 2005). It is reasonable to

assume that active and potentially regulated cytokinin influx participates on equilibration of intracellular cytokinin levels. Moreover, both passively and actively taken up cytokinins can be entrapped in plant cells by their metabolic conversion to very polar cytokinin ribotides (Laloue and Pethe, 1982; Kamínek *et al*., 1997b).

Higher rate of uptake of [^{3}H]t-ZR and [^{3}H]3OHBAR by wheat comparing to oat leaf apices results in faster saturation of wheat leaf tissues with taken up cytokinins. However, the total amount of taken up [^{3}H]t-ZR remained higher in wheat even after 16 h of incubation while more [^{3}H]3OHBAR was accumulated in oat leaf apices during the same period of time. The faster uptake and higher accumulation of [^{3}H]t-ZR may be partly responsible for relatively higher activity of the t-ZR in wheat comparing to oat leaf apices. Comparing the rates of uptake of [^{3}H]BA by water melon cotyledons of different age Fantelli *et al*. (1982) found that in spite of slower rate of cytokinin uptake by older cotyledons the contents of cytokinins reached nearly the same level after prolonged incubation regardless of the cotyledon age.

Changes in content of endogenous cytokinins during incubation of oat and wheat leaf apices in solutions of 3OHBAR and t-ZR

The decrease in content of bioactive cytokinins (t-Z and t-ZR) as well as in that of their storage *O*-glucosides in oat and wheat leaf apices incubated in water corresponds to the inverse correlation between the endogenous cytokinin levels and the progress of senescence in different plant tissues and plant species (Van Staden *et al.,* 1988; Gan and Amasino, 1996; Conrad *et al.,* 2007). Moreover, the senescence was associated with conversion of t-ZR to biologically inactive t-Z9G and DHZ9G in both wheat and oat leaf apices (Fig. 3). The leaves of wheat and oat contained very high levels of *cis*-zeatins which were decreased in oat but was not changed in wheat leaf apices after 5 days of incubation in water. However, comparing to the *trans*-zeatins the *cis*-zeatin is very potent inhibitor of leaf senescence (Kamínek *et al.,* 1979) in spite of its recognition by cytokinin receptors (Spíchat *et al.,* 2004).

Incubation of leaf apices in 10^{-4}M t-ZR ("test tube" assay) was accompanied with progressively increasing accumulation of this cytokinin in oat while its accumulation was much lower in the wheat leaf apices where it reached the saturation level already at the third day of incubation. This could be due to the higher activity of adenine nucleosidase converting t-ZR to the corresponding base (see Kamínek, 1992; Jameson, 1994) in the wheat comparing to the oat leaf apices. Relatively high amounts of taken up t-ZR were converted to DHZOG in oat while in wheat conversion to the inactive t-Z9G prevailed. These conversions suggest presence of relatively high activity of zeatin reductase and zeatin glucosyl *O*-transferase in oat and of *N*-glucosyl transferase in wheat leaves (Mok and Mok, 2001; Hou *et al*., 2004).

Because of lack of appropriate deuterium-labeled standards of aromatic cytokinins we did not follow the metabolism of applied 3OHBAR which should be mainly metabolized to the inactive *N*-glucosides and storage *O*-glucoside. However, there was a significant progressive increase of accumulation of t-Z in wheat but not oat leaf apices suggesting that accumulation of this isoprenoid cytokinin may have a positive effect on retention of chlorophyll in wheat leaves exposed to 3OHBAR. The levels of other isoprenoid cytokinins were fluctuating around those found in the leaf apices incubated in water and apparently their effect of retention of chlorophyll was only very marginal.

Activity of cytokinin oxidase/dehydrogenase

Enhancement of CKX activity in senescing leaves of oat and wheat incubated in water suggests that *CKX* behaves like a senescence associated gene (SAG). The SAGs are activated at the onset of senescence and participate on recycling of metabolites and nutrients from senescing to developing plant tissues (reviewed in Quirino *at al*., 2000). Degrading the bioactive cytokinins the CKX can act in a co-operative manner with the SAGs preventing stabilization of chloroplasts by cytokinins. It is reasonable to assume that the increase of CKX activity is at least partially responsible for the down-regulation of t-Z and t-ZR which serve as substrate for the enzyme (Fig. 4; Armstrong, 1994). The decline of the

non-substrate cytokinin *O*-glucosides (t-ZOG and t-ZROG) during the senescence may reflect an increasing conversion of these storage cytokinin forms to the bioactive t-Z and t-ZR (Fig.3). Correspondingly, the accumulation of the cytokinin *N*-glucosides which are not converted back to the bioactive cytokinins and which are not substrates of CKX (our unpublished data) or exhibit only weak affinity for CKX (Armstrong, 1994), were either increased (wheat) or not affected (oat) during progressing leaf senescence.

It has been repeatedly reported that CKX activity is up-regulated by both substrate and non-substrate cytokinins in different plant materials (reviewed in Armstrong, 1994) including senescing segments of darkened wheat, oat and barley leaves (Kamínek *et al*. 1997a, Conrad *et al*. 2007). Such up-regulation of CKX activity by kinetin in senescing barley leaf segments has been recently shown to be associated predominantly with the glycosylated CKX isoform (Conrad *et al*. 2007). In our experiments, the CKX activity was dramatically increased during incubation of leaf apices in solutions of both t-ZR and 3OHBAR. Higher increase of CKX activity in response to the exogenous t-ZR and 3OHBAR in oat may be partly responsible for lower activity of t-ZR in retention of chlorophyll in the "test tube" assay where the supply of cytokinins is limited comparing to the "Petri dish" assay (Fig. 1).

Assuming that the higher induction of CKX activity in oat than in wheat leaf apices with exogenous cytokinins could affect the level of endogenous cytokinins the activity of cytokinins which either are or are not substrates of the enzyme was determined in the "test tube" assay using the oat and wheat leaf apices. Indeed, the non-substrate 3OHBAR and DHZR were more efficient than the substrate t-ZR in retention of chlorophyll in the oat comparing the wheat leaf apices (Fig. 5).

The effect of a pulse application of 3OHBAR and t-ZR on chlorophyll retention

The results described above support the view that activity of cytokinins in retention of chlorophyll is dependent on the cytokinin uptake, metabolic interconversions and on the enhancement of CKX activity by the applied cytokinins. The effect of these factors can be reduced by a pulse application of cytokinins and subsequent interruption of cytokinin import

(uptake + influx) by washing-out of the free soluble cytokinins and further incubation of leaf segments floating on water. Under such condition the long-term effects of constantly imported cytokinins as well as their effects on induction of CKX activity can be minimized. It would be ideal if only cytokinins attached to the corresponding cytokinin receptors would remain to affect the chlorophyll retention. Nevertheless, following the pulse application the differences in the activity the t-ZR and 3OHBAR were significantly reduced in oat and completely eliminated in wheat leaf apices (Fig. 6).

In conclusion, the activity of exogenously applied cytokinins in retention of chlorophyll is affected by the cytokinin uptake and translocation, metabolic conversions of applied cytokinins to their bioactive, storage and inactive forms as well by the stimulation of CKX activity by imported cytokinins. The affect of these factors on the final cytokinin activity can differ in different plant species as demonstrated here for wheat and oat.

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FIGURE LEGENDS:

Fig. 1 The effect of different ways of cytokinin application on retention of chlorophyll in oat (cv. Abel) and wheat (cv. Munk) leaf apices: (A) local application by submersion of leaf apiece base into cytokinin solution and (B) application of cytokinin solution to the whole adaxial surface by floating of leaf apices on cytokinin solution.

Incubation proceeded for 120 h under 18h/6h h photoperiod.

Fig. 2 Uptake and translocation of [^{3}H]t-ZR and [^{3}H]3OHBAR in detached oat (cv. Abel) and wheat (cv. Munk) leaves.

Apical leaf apices 7 cm long were submerged with their basal ends in 0.5 ml solutions containing $2x10^{-5}$ M (4500 dpm μl^{-1}) of tested cytokinin and incubated for the indicated time intervals in darkness. Following incubation the radioactivity was determined in leaf segments numbered from the leaf base: 1st = 1 cm segment that was submerged in cytokinin solution and in 2 cm long upward segments 2nd to 4th).

Fig. 3 The effect of exogenous t-ZR and 3OHBAR on content of endogenous cytokinins in oat (cv. Abel) and wheat (cv. Munk) leaf apices during their incubation in 10^{-4} M t-ZR and 3OHBAR solutions ("test tube" assay).

Fig. 4 The effect of exogenous t-ZR and 3OHBAR on activity of cytokinin oxidase/dehydrogenase in oat (cv. Abel) and wheat (cv. Munk) leaf apices during their incubation in 10^{-4} M t-ZR and 3OHBAR solutions ("test tube assay").

Fig. 5. Chlorophyll retention activity of t-ZR, 3OHBAR and DHZR in oat (cv. Abel) and wheat (cv. Munk) leaf apices ("test tube assay").

Fig. 6 Retention of chlorophyll in oat (cv. Abel) and wheat (cv. Munk) leaf segments during their incubation in water after 24 h pulse incubation in water and in $2x10^{-5}$M solutions of t-ZR and 3OHBAR.

Tab. 1. Activity of different cytokinins in retention of chlorophyll in 3.5 cm long apical apices of leaves of oat (cv. Abel) and wheat (cv. Munk).

Leaf apices were incubated in 1.5 ml solutions for 120 h in darkness.

Cytokinin (10^{-4}M)	Retention of chlorophyll (% of initial)		Oat/wheat activity ratio
	Oat (cv. Abel)	Wheat (cv. Munk)	
Control (water)	10.3±3.5	9.3±3.2	1.11
iP	31.5 ±12.5	41.8±4.3	0.75
iPR	31.1±11.5	53.5±5.3	0.58
t-Z	55.3±14.1	59,5±6.0	0.92
t-ZR	72.3±4.2	69.5±2.4	1.04
BA	79.8±8.5	47.5±2.0	1.68
BAR	83.7±4.3	53.9±6.2	1,55
Kin	79.0±1.9	48.9±2.9	1.62
KinR	76.3±8.5	61.7±7.6	1.24
3OHBAR	88.3±9.5	35.6±6.1	2.48

Fig. 1

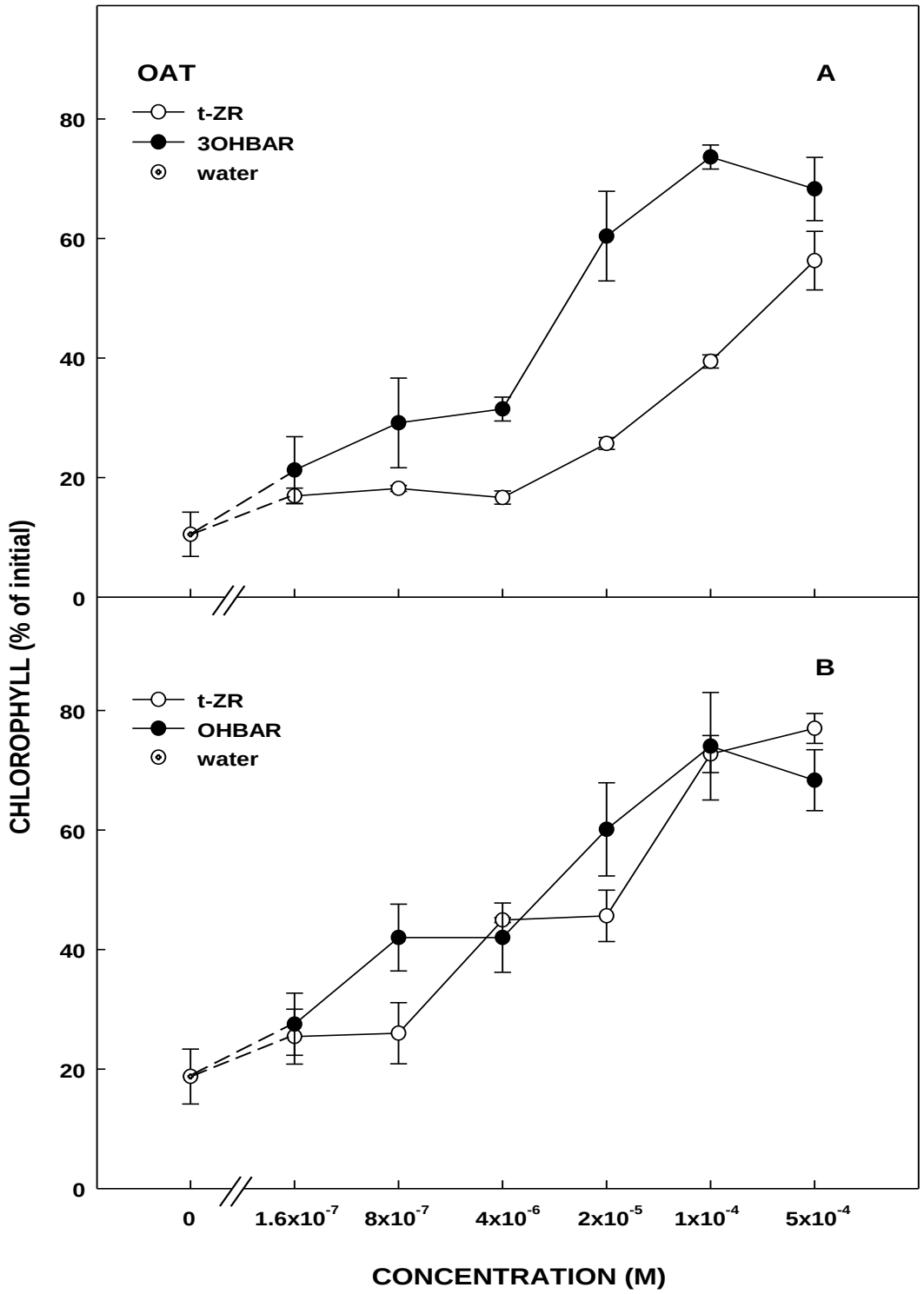

OAT
A
t-ZR
3OHBAR
water
80
60
40
20
0
B
t-ZR
OHBAR
water
80
60
40
20
0
CHLOROPHYLL (% of initial)
0
1.6x10-7
8x10-7
4x10-6
2x10-5
1x10-4
5x10-4
CONCENTRATION (M)

Fig. 2

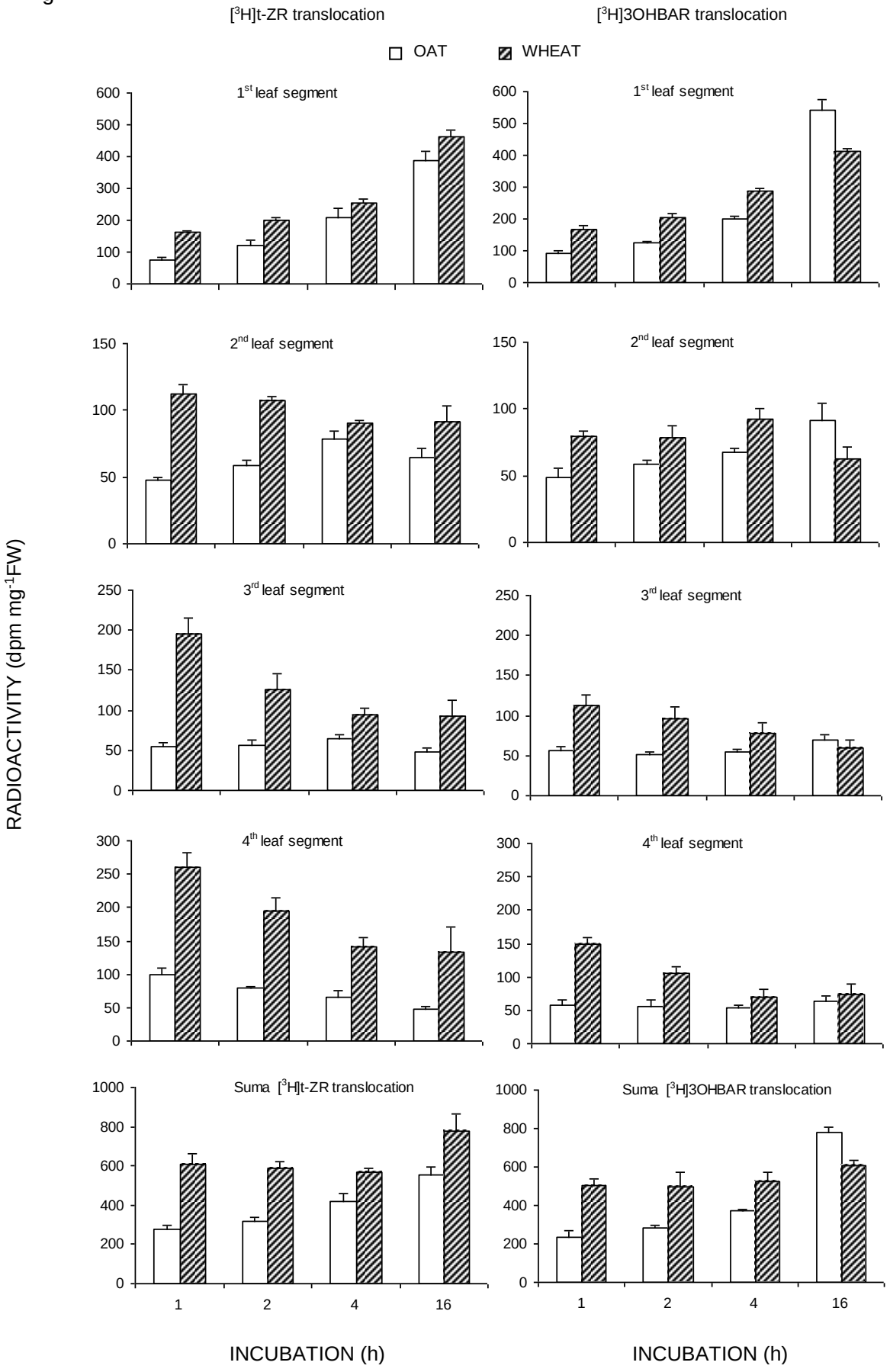

[3H]t-ZR translocation
[3H]3OHBAR translocation
OAT
WHEAT
1st leaf segment
2nd leaf segment
3rd leaf segment
4th leaf segment
Suma [3H]t-ZR translocation
Suma [3H]3OHBAR translocation
RADIOACTIVITY (dpm mg-1FW)
INCUBATION (h)
INCUBATION (h)
1
2
4
16

Fig. 3

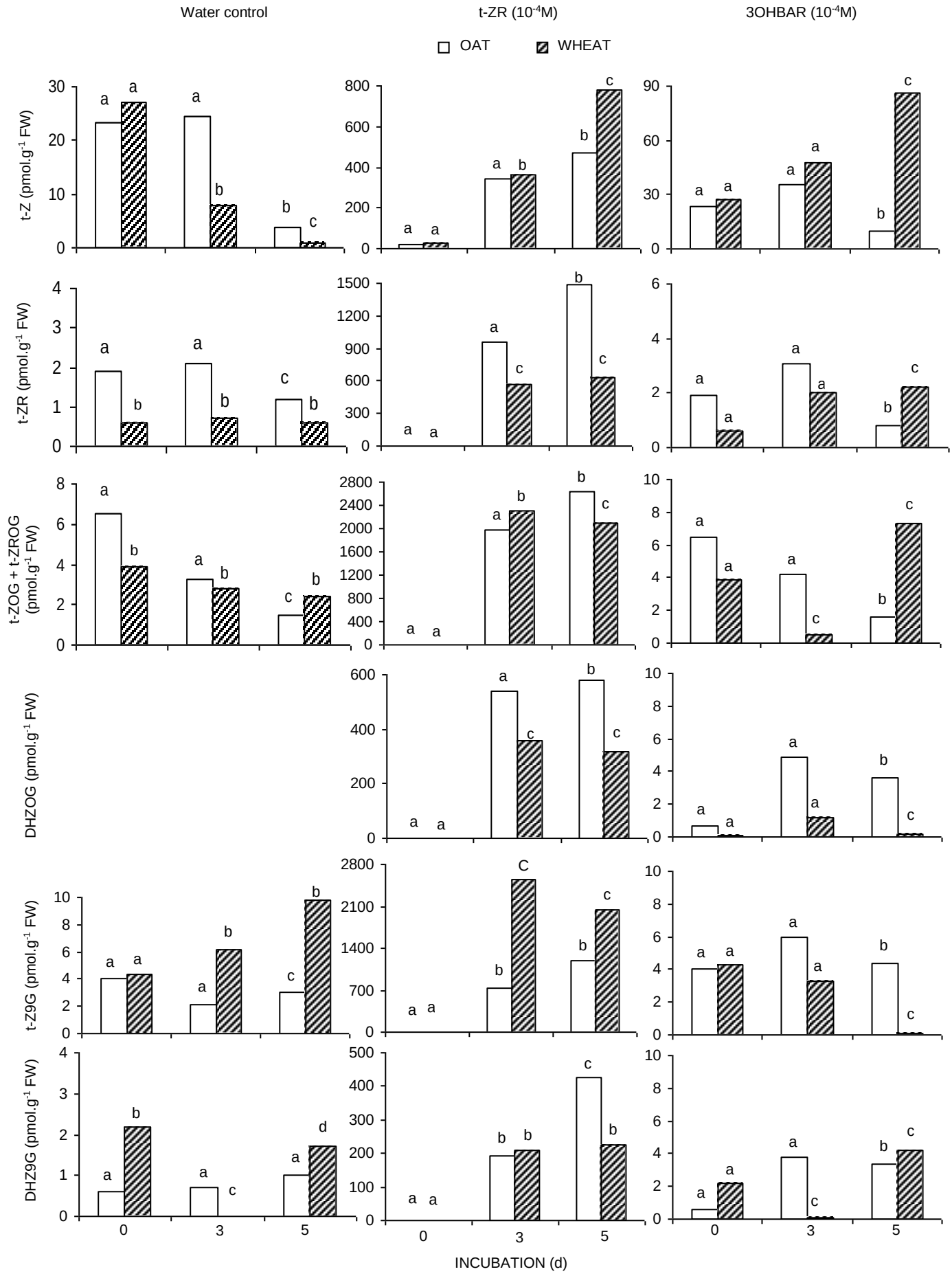

Water control
t-ZR (10-4M)
3OHBAR (10-4M)
OAT
WHEAT
t-Z (pmol.g-1 FW)
t-ZR (pmol.g-1 FW)
t-ZOG + t-ZROG (pmol.g-1 FW)
DHZOG (pmol.g-1 FW)
t-Z9G (pmol.g-1 FW)
DHZ9G (pmol.g-1 FW)
INCUBATION (d)

Fig. 3 - cont.

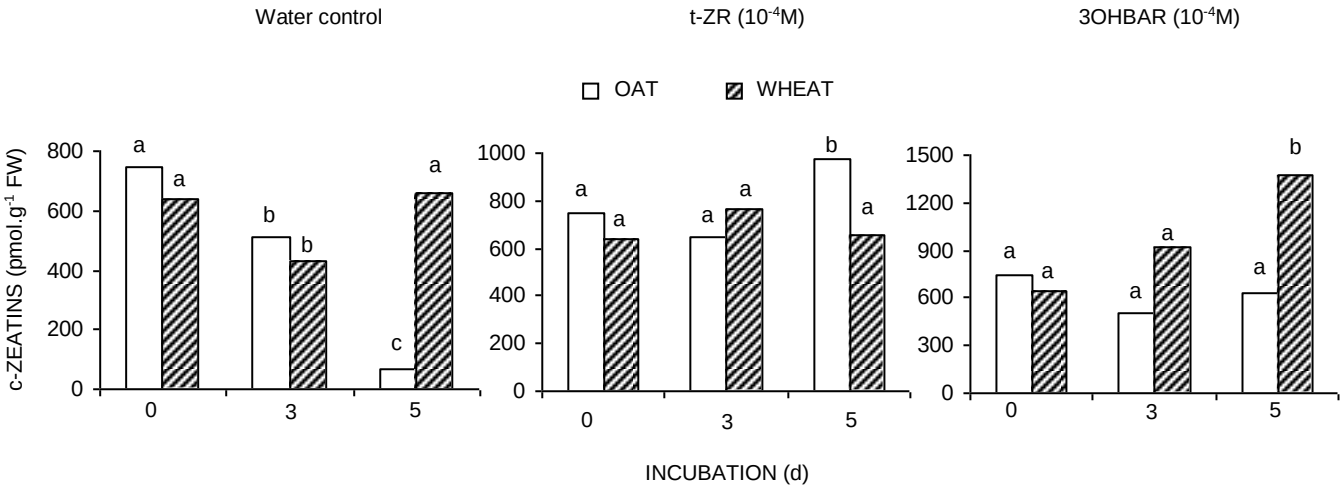

Water control
t-ZR (10-4M)
3OHBAR (10-4M)
OAT
WHEAT
c-ZEATINS (pmol.g-1 FW)
INCUBATION (d)

Fig. 4

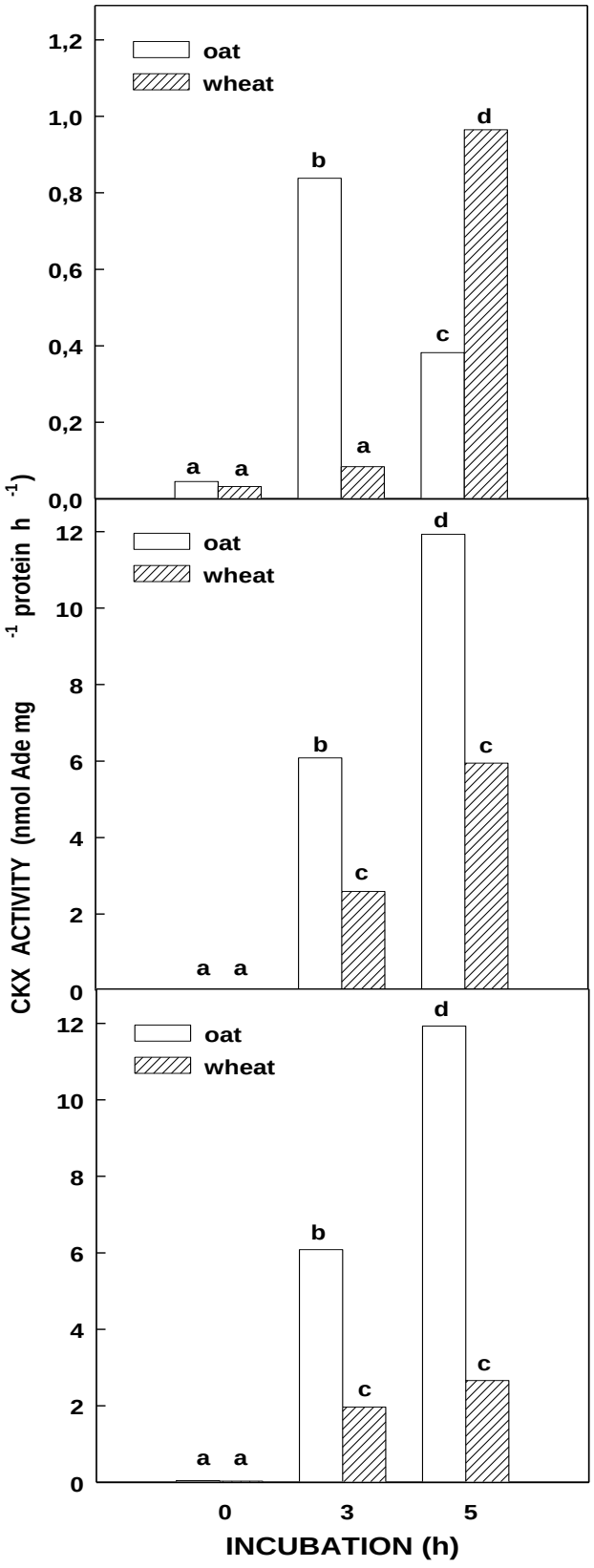

oat
wheat
1,2
1,0
0,8
0,6
0,4
0,2
0,0
a
a
b
a
c
d
12
10
8
6
4
2
0
CKX ACTIVITY (nmol Ade mg -1 protein h -1)
0
3
5
INCUBATION (h)

Fig. 5

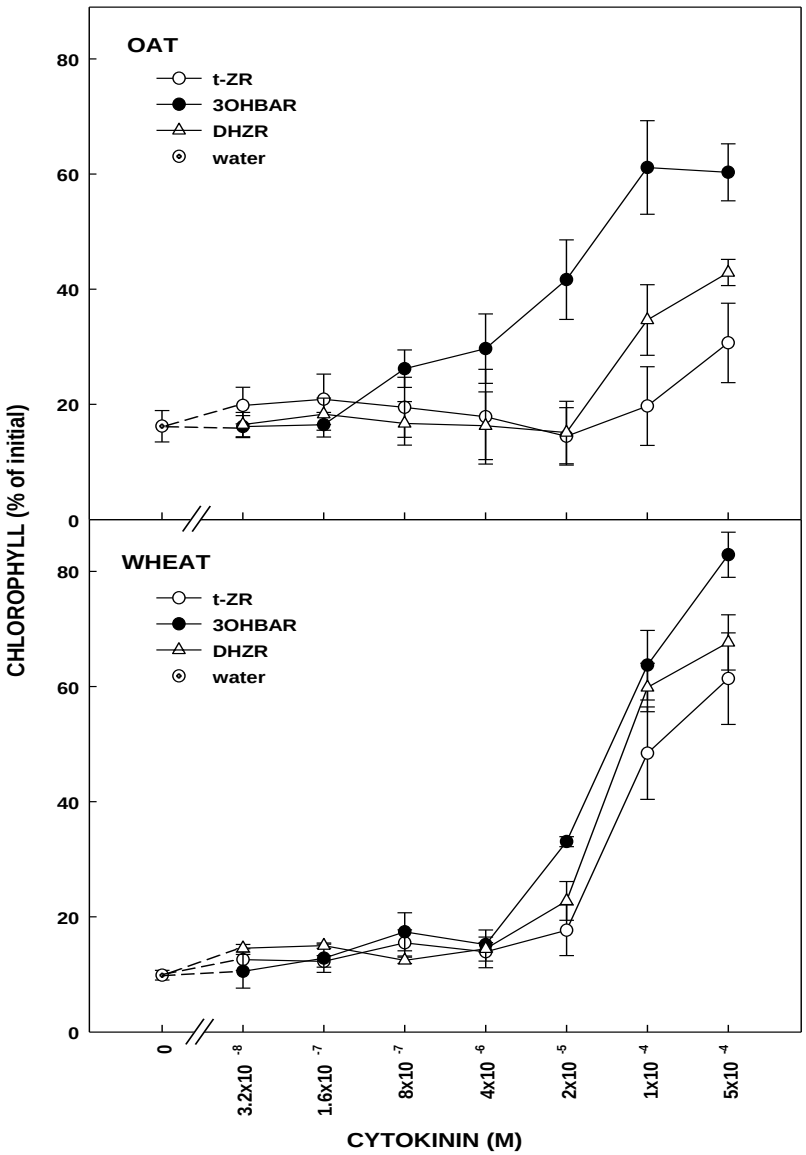
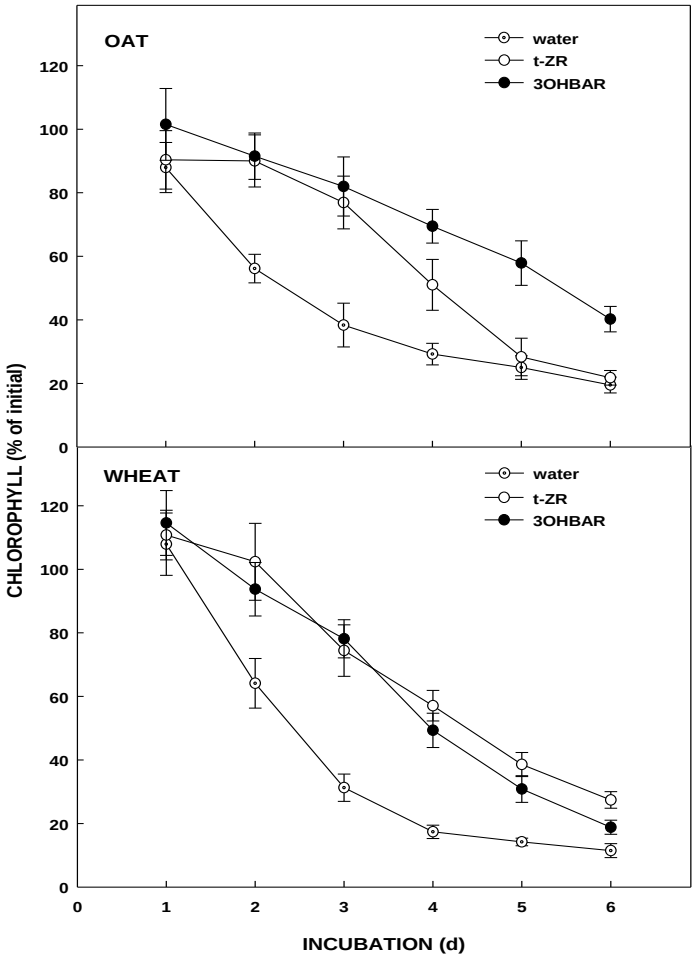

OAT
t-ZR
3OHBAR
DHZR
water
WHEAT
t-ZR
3OHBAR
DHZR
water
CHLOROPHYLL (% of initial)
0
20
40
60
80
0
3.2x10 -8
1.6x10 -7
8x10 -7
4x10 -6
2x10 -5
1x10 -4
5x10 -4
CYTOKININ (M)

Fig. 6


OAT
water
t-ZR
3OHBAR
WHEAT
water
t-ZR
3OHBAR
CHLOROPHYLL (% of initial)
INCUBATION (d)
0
20
40
60
80
100
120
0
1
2
3
4
5
6

The Effect of Senescence-induced *ipt* Expression on Cytokinin Levels, Nitrate Uptake and Allocation and Grain Yield in Wheat

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Abbreviations: c-ZOG, *cis*-zeatin *O*-glucoside; c-ZROG, *cis*-zeatin riboside *O*-glucoside; DAA, days after anthesis; DHZ, dihydrozeatin; DHZ9G, dihydrozeatin 9 *N*-glucoside; DHZOG, dihydrozeatin *O*-glucoside; DHZROG, dihydrozeatin riboside *O*-glucoside; iP, N^6-(2-isopentenyl)adenine; iPR, N^6-(2-isopentenyl)adenosine; *ipt*, cytokinin biosynthesis gene from *Agrobacterium tumefaciens*; *IPT*, higher plant cytokinin biosynthesis gene; LN, low concentration of nitrogen; MS, main stem; ON, near to optimum concentration of nitrogen; *SAG*, senescence associated gene; t-Z, *trans*-zeatin; t-ZOG, *trans*-zeatin *O*-glucoside; t-ZR, *trans*-zeatin riboside; t-ZROG, *trans*-zeatin riboside *O*-glucoside; t-Z7G, *trans*-zeatin 7 *N*-glucoside.

ABSTRACT

Control of leaf senescence using the expression of the cytokinin biosynthetic *ipt* gene under the control of senescence-specific promoter (*SAG12::ipt*) represents an efficient tool for prolongation of leaf photosynthetic activity (Gan and Amassino, Science 270, 186-188, 1995). It has been successfully used for enhancement of productivity of several crop plants. We tested if this approach is efficient also in wheat (*Triticum aestivum* L.) as a strictly monocarpic plant. The *ipt* expression under the senescence-specific *SAG12* promoter in response to the onset of leaf senescence resulted in delay of chlorophyll degradation only in leaves of plants grown under limited N supply. The effect was more apparent in the firs (flag) leaf which contained 10 DAA by 78% higher level of chlorophyll, about 3- fold higher levels of bioactive cytokinins while there was no significant difference in the content of abundant *cis*-zeatins comparing to the leaves of WT plants. There was a progressive increase in nitrate influx in *SAG12::ipt* plants which was associated with increased activity of nitrate reductase. However, the *SAG12::ipt* plants did not differ from WT plants in grain yield components including the number of grains and spike grain weight. Results suggest that delay of leaf senesce of wheat plants expressing the *ipt* also delays translocation of nutrients and metabolites from leaves to grains after anthesis and in this way interferes with the reproductive strategy wheat as strictly monocarpic plant which is based on a programmed fast translocation of metabolites and nutrients from senescing leaves to the reproductive sinks shortly after anthesis.

INTRODUCTION

Leaf senescence is a programmed process representing the final phase of leaf development. In addition to chloroplast disintegration, decline in photosynthesis, proteins and nucleic acids it also includes mobilization and recycling of nutrients and organics from senescing leaves to young plant organs (Thomas and Stoddart, 1980; Buchanan-Wollaston, 1997; Gan and Amasino, 1999; Himelblau and Amasino, 2001). Supporting the target organs the recycling function may play an important role in preventing their premature death (Wingler *at al*., 2005). Both developmental and dark-induced senescence can be reverted by rejuvenation and exposure to light, respectively, as it was shown in zucchini seedlings (Ananieva, *et al.* 2004).

Like many other developmental processes senescence is, at least in part, under hormonal control. While ethylene, abscisic acid and methyl jasmonate promote leaf or cotyledon senescence in many plants species (Noodén and Leopold, 1988; Ueda *et al*., 1981, Naik et al., 2002; Ananieva *et al*., 2004) cytokinins delay senescence-associated processes including degradation of chlorophyll and chloroplast proteins in various plant species (Van Staden *et al.*, 1988; Jordi *et al*., 2000; McCabe *et al*., 2001; Chang *et al*., 2003). Using loss-of-function mutants Riefler *et al*. (2006) showed that sensor histidine kinases, namely AHK3, are involved in mediating cytokinin-dependent chlorophyll retention in *Arabidospis* leaves. Long-term over-expression of *Agrobacterium tumefaciens* gene responsible for cytokinin biosynthesis, adenylate isopentenyl transferase (*ipt*), not only delayed senescence but also revealed developmental abnormalities in tobacco and potato plants (Li et al., 1992; Galis *et al*., 1995). These abnormalities most probably reflected long-lasting disruption of cytokinin homeostasis and shifted the concentration ratios of cytokinins and other plant hormones, namely auxin. A transient increase of cytokinin content following overexpression of *ipt* under the control of heat-inducible promoter also delayed leaf senescence but had less dramatic effect on plant development (Smart *et al.*, 1991). By contrast, the decrease in cytokinin content in *Arabidopsis* plants constitutively overexpressing cytokinin oxidase/dehydrogenase accelerated senescence neither of attached nor detached leaves (Werner *et al.*, 2003).

Senescence is an active energy requiring process (Noodén, 1988) that involves preferential expression of senescence-associated genes (*SAG*s) (Lohman *et al.*, 1994; Buchanan-Wollaston 1997; Quirino *et al*., 2000). Considerable number of *SAG*s have been identified in variety of plant species and used as senescence molecular markers (Weaver *et al.*, 1998; Noh and Amasino, 1999; Pic *et al.*, 2002). The *Arabidospsis SAG12* that encodes Cys protease displays highly senescence-specific expression (Lohman *et al.*, 1994). The *SAG12* promoter was found ubiquitously active in senescing tobacco leaves; however, specific *SAG12* expression domains were detected also in flowers (Grbić, 2002).

Senescence-specific promoter studies identified promoter elements responsible for the senescence-specific activity that are conserved at least in two different species (Noh and Amasino, 1999). Transforming tobacco plants with *ipt* driven *by SAG12* promoter Gan and Amasino (1995) developed a negatively auto-regulated system for senescence-controlled cytokinin biosynthesis. In this system expression of *ipt* is induced at the onset of senescence and subsequently switched off when further progress of senescence is ceased by accumulated cytokinins thus preventing cytokinin overproduction. Using this approach the cytokinin content was increased and leaf senescence was delayed in ageing leaves and flowers of various plant species (McCabe *et al*., 2001; Chang *et al*., 2003; Cowan *et al.*, 2005). The delay of senescence was associated with an increase of photosynthesis rate and life span of the older *SAG12::ipt* tobacco leaves (Wingler *et al*., 1998; Jordi *et al.*, 2000).

Leaf senescence can be triggered by a high availability of carbon relative to nitrogen. Glucose in combination with low nitrogen supply induced yellowing and changes in gene expression in *Arabidopsis* leaves that are characteristics of developmental senescence (Pourtau *et al*., 2004; Pourtau *et al*., 2006). This indicates that leaf senescence of plants grown under limited N supply may respond more readily to the increase in endogenous cytokinin levels or exogenous cytokinin application as it was shown for tobacco and wheat (Jordi *et al.,* 2000; Kamínek *et al*., 2003). Indeed, following cytokinin application to wheat at the beginning of intensive stem elongation the rate of net NO_3^- uptake was increased only in plants grown under limited N supply in comparison with plants grown in nutrient solution containing near to optimum concentration of N (Trčková and Kamínek, 2000). In *SAG12::ipt* transformed tobacco plants the allocation of N was increased in favor of old senescing leaves resulting in inverted vertical N profile. This inversion was achieved not only by increasing the allocation of N to older leaves but also by even more pronounced reduction of N content in young leaves that represent the main source of N and assimilates. Feeding experiments showed that this inversion was reflected in different accumulation of currently up taken ^{15}N (Jordi *et al*., 2000).

It seems reasonable that expression of *ipt* under the *SAG12* promoter may allow potential weakening of wholesale foliar senescence by prolonging span of activity of photosynthetic apparatus (Gan and Amasino, 1995) and in this way increase plant productivity. However, application of this approach for enhancement of seed formation and yield particularly in strictly monocarpic plants may interfere with their reproductive strategy. This strategy is based on programmed fast translocation of metabolites from senescing leaves, where majority of relatively easily accessible N is invested (Hörtensteiner, 2006), to reproductive sinks following development of reproductive structures (Noodén, 1988; Miceli *et al*., 1995). Actually, most if not all N utilized by developing seeds of wheat is mobilized from other plant parts while the plant net NO_3^- uptake capacity sharply declines after anthesis

(Trčková and Kamínek, 2000). The delay of leaf senescence in response to the removal of reproductive sinks in monocarpic soybean and pea (Noodén and Letham, 1993; Pic *et al.*, 2000) as well as to prevention of pollination of tobacco plants (Wingler *et al.*, 2005) corresponds to this concept. While in monocarpic plants the reproductive structures often govern senescence of the whole plant this pattern does not occur in all monocarpic plant species including *Arabidopsis*. Neither dysfunction nor removal of reproductive structures of *Arabidopsis* plants significantly increased longevity of individual rosette leaves but prolonged plant life through production of new leaves and stems with inflorescence (Noodén and Penny, 2001). However, cereals belong to that majority of monocarpic plants which adapted the strategy of replacement of vegetative growth reciprocally to reproductive growth following pollination (Humbeck *et al.,* 1996; Trčková and Kamínek, 2000). Similarly to a differential allocation of N, the young leaves of *SAG::ipt* tobacco plants grown under growth-limiting N supply contained lower amounts of chlorophyll, protein and Rubisco and exhibited lower maximum photosynthetic capacity than corresponding young leaves of WT plants (Jordi *et al.*, 2000). In comparison with WT plants the *SAG12::ipt* plants were slower to in adjusting biomass allocation and stress-induced changes in root/shoot ratio (Cowan *et al.*, 2005). Nevertheless, the total accumulation of dry mass and yield of seeds was increased (Gan and Amasino, 1995). However, the extensive analysis of relationships between photosynthesis and yield of different plant species has indicated that improvement of seed yield by enhancement of single-leaf photosynthesis is possible (Nelson, 1988).

Against this background we examined potentials of senescence-induced auto-regulated *ipt* expression to affect cytokinin levels and progress of senescence as well as yield-related physiological and structural parameters of wheat that has exercised strictly monocarpic reproductive strategy. We were interested to learn if this strategy, that has been acquired during wheat plant phylogenies and further strengthened by breeding, may interfere with manipulated delay of leaf senescence.

MATERIAL AND METHODS

Plant transformation and culture conditions

Wheat (*Triticum aestivum* L. cv. Scamp) plants were grown in a greenhouse at ca 22^{o}C/16^{o}C 16h/8h photoperiod. Immature embryos were excised from seeds 15 days after anthesis (DAA) under aseptic conditions and cultivated for a week on MSO medium (Becker *et al.*, 1994). The transformation was performed by a direct gene delivery method. The plasmid pDB1 containing the *uidA* gene under control of the actin1 promoter and the *bar* gene under control of the CaMV35S promoter (Becker and Loerz, 1993) was delivered to embryos together with either pSG516 –*Arabidopsis thaliana* SAG12 promoter::

Agrobacterium tumefaciens ipt gene, or pSG506 - Arabidopsis thaliana SAG12 promoter::gusA gene (Gan and Amasino, 1995) using a BioRad PDS 1000/He Biolistic Particle Delivery System (Fig.1). The bombarded explants were cultured for 2 weeks on MSI medium at 26^{o}C in darkness and then transferred for another two weeks to regeneration medium (MSI without plant hormones and without casein and yeast extract) supplemented with a selective agent (4mg/l bialaphos). Plantlets were rooted on the same medium with a reduced amount of bialaphos (1 mg/l) and then transferred to the soil and grown in a greenhouse.

The presence of genomic *Agrobacterium ipt* in each transformed plant involved in the experiment was checked by PCR. DNA was extracted from the second leaf shortly after its appearance using Plant DNAzol (Invitrogen, CA, USA) and the bacterial *ipt* cDNA was amplified by PCR using the primers ipt-For (5'-GACGCAAATATGGAAGGTAAGT -3'), *ipt*-Rev (5'-GAATTTCTGTTCCTGTTG-3'). Transcripts of *ipt* were detected in first leaves of transgenic plants 10 to 13 DAA by RT-PCR (Oligotex mRNA Kit, Omniscript RT Kit, Qiagen, Hilden, Germany.)

Cultivation of plants

The wheat *SAG12::ipt* and WT plants selected for further experiments were grown hydroponically in a growth room with day/night cycle of 16/8 h (photon flux of 400 µmol m^{-2} s^{-1}) and 21°C/15°C air temperature. Continuously aerated nutrient solution with 773 µM of N ("low N"; LN) contained $Ca(NO_3)_2$ (316 µM), KNO_3 (141 µM), KH_2PO_4 (105 µM), $MgSO_4$ (82.5 µM), KCl (95 µM), H_3BO_3 (2.5µM), Fe-EDTA (2 µM), $ZnSO_4$ (0.2 µM), $MnSO_4$ (0.2 µM), $CuSO_4$ (0.05 µM) and $(NH_4)_6Mo_7O_{24}$ (0.01 µM). In addition to LN solution nutrient solution containing near to optimum concentration of N (1158 µM N; ON) was used in one designated experiment (Fig. 3) where levels of chlorophyll in leaves of WT and *SAG12::ipt* plants were compared. The concentration nutrients in weekly changed nutrient solution was controlled and adjusted each second day.

Determination of chlorophyll content

Collected leaves were frozen in liquid nitrogen and stored at -70°C. Chlorophyll was extracted according to Lichtenthaller (1987). Frozen leaves (≈1 g) were homogenized in liquid nitrogen and extracted in darkness and cold (+4°C) in dimethylformamide (10 ml) over night. The samples were measured by spectrophotometer Unicam 5625 at A_{647}, A_{664} and A_{750}. The chlorophyll content was calculated according to Lichtenthaller (1987).

Analysis of cytokinins

Collected leaves were frozen in liquid nitrogen and stored at -70°C. Cytokinins were extracted and purified using the method of Dobrev and Kamínek, (2002). Briefly, frozen leaves (1 g) were homogenized in liquid nitrogen extracted in cold methanol/water/formic acid (15:4:1, v/v/v). A mixture of deuterium-labeled internal cytokinin standards (Apex, Honiton, Devon, UK), were added at the beginning of extraction. After overnight extraction at -20°C and sonication solids were separated by centrifugation (15,000 g, 4°C, 20 min) and re-extracted for 30 min with an additional 5 ml extraction mixture. After passing the pooled extracts through a Sep-Pak Plus †C_{18} cartridge (Waters, Mildford, MA, USA) to remove pigments and part of lipids the effluent was evaporated to near dryness using rotatory vacuum evaporator and the residue was redissolved in 5 ml 1 M formic acid. Cytokinins were entrapped on an Oasis MCX cation-exchange, reverse-phase column (150 mg, Waters). After washing the column with 5 ml of methanol followed by 5 ml 1M formic acid cytokinin riboside monophosphates were eluted with 5 ml 0.35 M NH_4OH in water. Cytokinin bases, ribosides and glucosides were subsequently eluted with 5 ml 0.35 M NH_4OH in 60% (v/v). Cytokinin riboside phosphates were dephosphorylated by alkaline phosphatase and determined as corresponding ribosides.

Cytokinins were determined using HPLC/MS/MS. Dry samples of purified cytokinins were dissolved in 20 µl 50% (v/v) acetonitrile, diluted by addition of 80 µl distilled water and passed through Micro-Spin centrifuge nylon filters (0.2 µm, Alltech, Deerfield, IL, USA). Five µl filtrate aliquots were injected onto a RP HPLC column (2 mm x 250 mm, 5 µm, AQUA, Phenomenex, Torrance, CA, USA) linked to a LCQ Ion Trap Mass Spectrometer (Finnigan, San Jose, CA, USA) equipped with an electrospray interface. Cytokinins were separated by HPLC using a linear gradient of acetonitrile (B) in 0.0005% (v/v) acetic acid in water (A): 10% B for 5 min, to 17% B in 10min and to 50% B in 11 min at a flow rate of 0.2 ml min^{-1}. Detection limits of different cytokinins were between 0.5 and 1.0 pmol/sample (i.e. 25 to 50 ? pmol in injected volume) and the linear concentration dependence covered the range from 2 to 500 pmol/sample (0.1 to 25 pmol in injected volume). Results represent averages of analyses of two to four independent samples with two LC/MS injections for each sample.

Nitrate uptake and nitrogen allocation

Net NO_3^- influx was measured in depletion experiments. Depending on of stage of development and plants size each selected intact plant was transferred into 300 – 1000 ml of well aerated fresh nutrient solution (250 µM NO_3^-). After a lag period of 30 min 5ml samples of the nutrient solution were collected at 30 min intervals for 4 - 6 h to measure the NO_3^- concentration. The NO_3^- concentration was determined after reduction to NO_2^- by passing

through a granulated cooper – cadmium column. The nitrite was estimated spectrophotometrically by measurement of conversion of sulphanilamide and α-naphthylehthylenediamine dihydrochloride to azo dye at 540 nm using Skalar San plus analyzer (Breda, Netherlands). The net NO_3^- influx was determined from the dynamics of NO_3^- depletion and expressed as μmol g^{-1} root FW h^{-1}.

The allocation of currently taken up N was measured at flag leaf sheath extension corresponding to DC 41, stage of development as defined by Tottman and Makepeace, (1979). Selected intact wheat plants were transferred for 24 h into a well aerated complete nutrient solution supplied with 46,8 μM $^{15}NO_3^-$ 3.9 mg Ca $(NO_3)_2$ per plant). At the end of feeding the roots were rinsed with nutrient solution containing unlabeled N and plants were transferred back into the fresh nutrient solution. The ^{15}N labelled plants were harvested either 4 d after the end of feeding or at full maturity. Shoots and roots were separately weighed, dried and ground to a fine powder. Total N and ^{15}N content in the samples was determined using EA Eurovector – IRMS IsoPrime (Micromass, UK). The amount of ^{15}N allocated to different plant parts was expressed as % of taken up N.

Determination of nitrate reductase activity

Leaf and root samples (1 g FW) were homogenized in liquid nitrogen and extracted in 5 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 3% (w/v) bovine serum albumine at 4°C for 30 min. Insoluble material was removed by centrifugation (1500 g, 30 min). The NR activity was determined by an *in vitro* assay as described by Gaudinová, (1983). The 0.9 ml reaction mixture consisted from 0.5 ml 0.1 M phosphate buffer pH 7.5, 0.1 M KNO_3, 0.15 ml enzyme extract, and 0.15 ml 0.2 % (w/v) $NADH^+$. After incubation for 10 min at 25°C the reaction was terminated by addition of 0.1 mL 0.03M oxalacetic acid. Formation of azo dye was monitored at 540 nm. NR activity was expressed as the rate of generated NO_2^- (μmol g^{-1}FW min^{-1}).

Statistical analysis

The experiments were three times repeated. Each analysis was performed from two to four independent samples. Significance of differences between the *SAG12::ipt* and WT means was determined using the t-test ($P_{0.05}$).

RESULTS

Plant transformation and ipt expression

A genotypic analysis revealed 26 independent transgenic wheat lines that carry *SAG12::ipt*. Based on distinct phenotypic changes, we chose transgenic line C8/2 for further experiments.

The presence of genomic *ipt* and corresponding transcript in all plants selected for experiments was confirmed by PCR and RT-PCR, respectively (Figs 2A and 2B).

Chlorophyll content

The content of chlorophyll a and b was determined in leaves of plants grown under ON and LN supply at 10 DAA. Differences in chlorophyll content between the *SAG12::ipt* and WT plants were found only in the leaves of plants grown under LN supply (Fig. 3). Chlorophyll content in first (flag) leaves of *SAG12::ipt* plants was higher by 32% while only 10% increase was recorded in the respective second leaves as compared to WT plants. The content of chlorophyll was higher in the first leaves than in the second leaves of the main stem (MS) irrespective of N supply. As compared to WT plants the difference was the highest in *SAG12::ipt* grown under LN supply (increase by 78% and 49% in the first and second leaf, respectively) indicating that the physiological effects of *ipt* expression are preferentially targeted to the flag leaves. There were no statistically significant differences between leaves of *SAG12::ipt*- and WT plants in chlorophyll a/b ratios which varied between 1.8 and 2.1.

Altered cytokinin content in SAG12::ipt transgenic wheat

Cytokinins were determined in the first and second leaves on the main stem and first tiller collected at 2 DAA and 10 DAA. There were not visible symptoms of leaf senescence at 2 DAA but visible senescence of leaves as well as lowered content chlorophyll occurred at 10 DAA (Fig. 2).

Twenty two different cytokinins were identified in the first and second leaves of *SAG12::ipt* and WT plants in amounts allowing precise quantification. For easy functional interpretation of large amount of data the analyzed cytokinins were divided according their structure and biological activity into five groups: (1) bioactive cytokinin bases and ribosides that exhibit high activity in bioassays (Skoog and Ghani, 1981) and most of them is also recognized by cytokinin receptors (Yonekura-Sakakibara *et al*., 2004; Spíchal *et al.*, 2004): (*trans*-zeatin [t-Z], *trans*-zeatin riboside [t-ZR], dihydrozeatin [DHZ], isopentenyladenine [iP] and isopentenyladenosine [iPR], (2) storage *O*-glucosides: t-Z *O*-glucoside [t-ZOG], t-Z riboside *O*-glucoside [t-ZROG], DHZ *O*-glucoside [DHZOG], DHZ riboside *O*-glucoside [DHZOG], (3) irreversibly inactive *N*-glucosides (Letham *et al.,* 1983): [t-Z 7-glucoside [t-Z7G], t-Z 9-glucoside [t-Z9G], DHZ 7-glucoside [DHZ7G], DHZ 9-glucoside [DHZ9G], iP 7-glucoside [iP7G], iP9-glucoside [iP9G], (4) ribotides, representing the first products of cytokinin biosynthesis, that were determined after the enzymatic hydrolysis to corresponding ribosides: ribotides of t-Z and iP, and (5) *cis*-zeatins [c-Zs] that exhibit very low cytokinin activity (Kamínek *et al.,* 1979; Skoog and Ghani, 1981): *cis*-zeatin [c-Z], c-Z riboside [c-ZR], c-Z *O*-glucoside [c-ZOG], c-Z riboside *O*-glucoside [c-ZROG] and c-Z 9-glucoside [c-Z9G].

Both transgenic and control plants contained much higher amounts of c-Zs than was the sum of all other cytokinins (5 to 17- fold). This difference was higher at 2 DAA than at 10 DAA.

Leaves of *SAG12::ipt* harvested at 2 DAA did not differ much in cytokinin content from the controls (results not shown). Much higher differences in cytokinin content were recorded in leaves collected at 10 DAA. With exception of *cis*-Z type cytokinins (c-Zs) there were about two-fold higher levels of the sum of all other cytokinins in the first and the second leaves of MS of *SAG12::ipt* plants with respect to the controls. The highest difference was found in bioactive cytokinin bases and ribosides that were over three-fold higher in leaves of the MS of transgenic plants. Interestingly, the cytokinin content ratios of transgenic and control leaves were very similar in the first and the second leaves. However, the first leaves of the MS, that represent the main source of chemical energy and metabolic carbon-building blocks, contained over five-fold higher levels of bioactive cytokinin bases and ribosides than the corresponding second leaves. By contrast, there were no statistically significant differences in levels of c-Zs between the transgenic and control plants in both the first and the second leaves of the MS. The only difference in content of c-Zs and non-c-Zs were recorded in the first leaves of the tiller where the former prevail (Fig. 4).

Unexpectedly, MS and tillers of transgenic plants did not differ in levels of cytokinin ribotides. However, the first leaves of the main stem and tiller contained nearly two-fold higher levels of cytokinin ribotides than the corresponding second leaves. With exception of the first leaves of tillers the leaves of *SAG12::ipt* and control plants contained similar concentrations of c-Zs.

Nitrate influx, ^{15}N allocation and NR activity

The influx of nitrate expressed per g FW was slightly higher in *SAG12::ipt* plants than that in controls beginning the stage of rapid flag leaf sheet extending (DC43) but appeared to be statistically highly significant as late as at 15 DAA when increased to 85% over the control (Fig. 5). Similar difference was found when the nitrate influx was expressed per plant (results not shown). The of ^{15}N from currently taken up $[^{15}N]O_3^-$ prior anthesis (DC 43 stage of plant development) was slightly more (by 13%) allocated in spikes of control than in those of transgenic plants. The differences ^{15}N allocation to grains, chaff and leaves of maturated transgenic and control plants were not statistically significant (Fig. 6).

Interestingly, the NR activity in the flag leaf of *SAG12::ipt* plants was higher that in corresponding leaves of controls by 37% and 82% at anthesis and at 10 DAA, respectively. However, no significant differences were found between transgenic and control plants in older leaves (Fig. 6).

Analysis of grain yield components

No statistically significant differences were found in analyzed grain yield components which were very similar in *SAG12:ipt* and WT plants including number of spikelets per spike (25.3 and 24.7), number of grains per spike (52.4 and 54.1) weight of spike (2.6 g and 2.9 g), grain weight per spike (2.1 g and 2.2 g), grain weight (38.9 mg and 41.1 mg) and number of tillers (7.8 and 6.7, respectively).

DISCUSSION

Chlorophyll content

Pollination seems to generate a strong signal for onset of leaf senescence and recycling of nutrients and metabolites from leaves to generative organs in monocarpic plants. One of possible responses to this signal(s) is reduction of cytokinin biosynthesis in roots (Nooden and Letham, 1993). In barley photosynthetic capacity of leaves was decreased already 8 DAA followed by decrease of chlorophyll, photosystem II efficiency and levels of cytochrome f and the large subunit of Rubisco (Humbeck *et al.,* 1996). Accordingly, we found no visible symptoms of senescence at anthesis while significant differences in chlorophyll content between the *SAG12::ipt* and WT plants occurred already 9 d later but only under LN supply. Increase of abundance of *ipt* transcripts following pollination which was accompanied with increased cytokinin accumulation and delay of floral senescence was also found in *SAG12:ipt* transformed *Petunia* flowers. Simultaneously the flowers of *SAG12:ipt* plants were less sensitive to ethylene and contained lower amounts of ABA which are known to accelerate senescence (Chang *et al.*, 2003).

Cytokinin content

As reported for leaves and some other plant organs of several plant species the cytokinin content starts to decline with the onset of senescence (Ananieva *et al.,* 2004) and the progress of senescence is accelerated by limited N supply. It is known that changes in the N availability could affect the plant cytokinin status (Sattelmacher and Marschner, 1978; Takei *et al*., 2001; 2002; reviewed in Sakakibara *et al.,* 2006). A positive correlation between the nitrogen supply and delivery of cytokinins from roots to shoots via xylem was reported for several plant species (Samuelson *et al.,* 1992; Wagner and Beck, 1993; Rahayu *et al*., 2005). Testing of the effects of *ipt* expression on cytokinin levels in plants poorly supplied with N allowed us to exploit the advantage of enhancing of senescence progression in LN plants and lowering of expression of interfering plant isopentenyltransferases in leaves which are induced by the nitrate (Takei *et al.,* 2004).

Under LN supply we found only a slight, mostly statistically insignificant differences in cytokinin levels between leaves of transgenic and WT plants shortly after anthesis (2 DAA)

(Fig. 4). The differences became more apparent with progressing leaf senescence at 12 DAA confirming that the levels of non-c-Z-type cytokinins are indeed higher in the transgenic plants (Fig. 4). One would expect that the cytokinin levels due to the senescence-induced *ipt* expression should be higher in the older more senescent second leaf than in the first leaf. However, event though that the two leaves differ in the cytokinin content the effect of *ipt* expression on cytokinin levels in the first and second leaves on the MS and tiller was very similar (compare Figs. 4A and 4B) . This could be caused by very dominant function of the flag leaf in supply of plant with photosyntates (Inoue *et al.,* 2005) and/or by a loss-off function of *SAG12::ipt* in rapidly ageing and dying older wheat leaves. In analyzing the cytokinin content in leaves of tobacco plants from different zones of the shoot Cowan *et al*., 2005) found that the effect of *ipt* expression in *SAG12::ipt* plants is not restricted to the old senescing leaves from the morphological base of the stem indicating that the expression of *ipt* resulted in an increase in cytokinin content and weakening but not reversion of the base to apical cytokinin gradient. Competency of young leaves to synthesize their own cytokinins (Smart, 1991 Miyawaki *et al.*, 2004) may also hamper the reversion of cytokinin gradient due to the senescence-dependent *ipt* expression in transgenic plants.

We found high levels of c-Z-type cytokinins in leaves of both the transgenic and WT plants. The c-Z-type cytokinins were also found in *SAG12::ipt* and WT tobacco plants in amounts equal or lower than the sum iP- and t-Z-type cytokinins. While amounts of c-Z-type cytokinins were very similar the leaves of transgenic plants contained higher amounts of this type cytokinins than leaves of WT plants namely at the bottom of the shoot indicating that the expression of *ipt* resulted in increase in cytokinin content and in weakening but not reversion of the base-apical cytokinin gradient.

Nitrate influx,

The nitrate influx in the present experiment was slightly higher in *SAG12::ipt* beginning from the pre-anthesis (DC43 stage of development) but become statistically highly significant at 15 DAA (Fig. 5) indicating that the *SAG12::ipt* imposed delay of the leaf senesce also delays the drop in cytokinin uptake. As we reported elsewhere the net NO_3^- uptake in wheat plants sharply declined after anthesis and the exogenous application of cytokinin at anthesis to plants grown under LN supply significantly increased NO_3^- uptake rates and overall plant uptake capacity of (Trčková and Kamínek, 2000). This contrasts with the reported reduction of lowering of N uptake by roots in the *SAG12::ipt* tobacco plants which was attributed to reduced C availability to the roots of (Jordi *et al.,* 2000). This difference could be caused by the fact that in our experiments the senescence of the highly photosynthetically active flag leaf only of *SAG12::ipt* plants grown under LN supply was delayed (Fig. 3) while in tobacco plants the senescence much less productive old leaves on the bottom of the stem

senescence of the old leaves on stem bottom was suppressed. Correspondingly, for the same reason tobacco WT plants compared to *SAG12::ipt* plants were apparently slower to adjust biomass allocation in response to stress-induced increase in root:shoot ratio most probably due to the reduced mobilization of nutrients from the older source leaves (Jordi *et al.,* 2000; Cowan *et al.,* 2005). Moreover, the limited expression of *ipt* in non-senescing leaves could stimulate chloroplast development and enhance the photosynthetic activity in flag leaf even before the onset of senescence.

^{15}N allocation

As reported for tobacco the rate of currently taken up ^{15}N by the whole plants was decreased in *SAG12::ipt* plants comparing to WT plants and was preferentially accumulated in senescing leaves (Jordi *et al.,* 2000). We found no difference between the *SAG12::ipt* and WT wheat plants in uptake of ^{15}N from temporary applied $^{15}NO_3^-$ in maturated wheat plants where, as expected, most of absorbed ^{15}N was allocated in grains. There was a slightly higher amount of ^{15}N allocated in spikes of WT plants prior anthesis, which could be result of a limited pre-anthesis expression of *ipt* and attraction of nutrients to flag leaf as indicated in Fig. 6.

NR activity

Activity of NR is known to be induced independently by both NO_3^- and cytokinins (Boris, 1967). Regulation of NR activity by endogenous cytokinin levels seems to reflect changes in plant development (Banowetz, 1992). The enhancement of NR activity by exogenous cytokinin was reported also for wheat and barley seedlings (Gaudinová, 1983; Trčková and Kamínek, 2000). In our experiments NR activity in flag leaf of in *SAG12::ipt* at anthesis was higher by 37% comparing to WT plants. The activity of NR was decreased with grain development. Its activity in flag leaf of WT plants was about two-fold lower at 10 DAA but the drop was less pronounced in *SAG12::ipt* plants where the NR activity was by 82% higher than that in the WT plants (Fig. 6). Accumulation of bioactive cytokinins in flag leaves of *SAG12::ipt* plants (Fig. 4) could be partially responsible for the slowing down of NR activity drop.

Grain yield parameters

We did not find difference in grain yield parameters between the *SAG12::ipt* and WT wheat plants. Interestingly, as reported elsewhere the application of exogenous cytokinin to the whole aerial part wheat plants including the ears increased the grain number per plant (Kamínek *et al.,* 2003). Injection feeding of maize stems under the ears with cytokinin at pollination, which did not affect the leaf senescence, also increased the number of seeds per

year and grain yield (Dietrich *et al.,* 1995). All these data indicate that the targeted suppression of the onset of leaf senescence in *SAG12::ipt* wheat plants delayed recirculation of nutrients and organics from the leaves to the ears and in this way the cytokinin stimulation of sink capacity in developing grains was reduced. Increase of both chlorophyll and cytokinins levels in flag leaves of *SAG12::ipt* of wheat plants supports such opinion.

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Legends to figures:

Fig.1. Schematic representation of the plasmids used in transformation experiments. Abbreviations: SAG12 -promoter of *A. thaliana* SAG12 gene; actin1 - *O. sativa* L. actin1 promoter; 35S - cauliflower mosaic virus 35S promoter; nos - *Agrobacterium. tumefaciens* nopaline synthase gene terminator; ip*t* - *A. tumefaciens* isopentenyl transferase gene; *uidA* - *Escherichia coli* beta-D-glucuronidase gene; *bar* - phosphinothricin acetyl transferase gene; T4E7 - T4 phage endonuclease VII gene; rad52 - *H. sapiens* rad52 gene; NLS - *Pisum sativum* L. nuclear localization sequence.

Fig. 2. PCR and RT-PCR analysis of genomic DNA and total RNA, respectively. DNA was extracted from ca 100 mg of the first leaves of the main stem 20 days old plants (A). Samples of the second leaves (ca 100 mg) were removed from transgenic plants at 10 DAA for RNA extraction (B). The k+ and k- represent positive and negative controls; numbers correspond to different plants analyzed.

Fig. 3. Content of chlorophyll in the first and second leaves of the main stems and tillers collected at 10 DAA from *PSAG12::ipt* and control (WT) plants grown in nutrient solutions containing suboptimum (LN) and near to optimum (ON) concentrations in of NO_3^- (773 µM and 1158 NO_3^- µM, respectively). Differences between columns marked with different letters are statistically significant ($P \leq 0.05$).

Fig. 4. Content of cytokinins in the first and second leaves of main stems (A) and tillers (B) collected at 10 DAA from *PSAG12::ipt* and control (WT) plants grown in nutrient solution containing suboptimum (LN) concentration (773 µM) of NO_3^-. Symbols: B+R, sum of cytokinin bases and ribosides of all cytokinins but *cis*-zeatins; O-G, cytokinin *O*-glucosides; N-G, cytokinin 7- and 9-glucosides; *cis*-Zs, the sum of *cis*-Z derivatives. Differences between columns marked with different letters are statistically significant ($P \leq 0.05$).

Fig. 5. Nitrate influx by *PSAG12::ipt* and control plants at different stage of development calculated from the kinetics of N depletion from the nutrient solution containing 250 µM NO_3^-. Differences between columns marked with different letters are statistically significant ($P \leq 0.05$).

Fig. 6. Allocation of currently taken up N in *PSAG12::ipt* and control plants following 24 h feeding with complete nutrient solution supplemented with ^{15}N shortly prior anthesis (DC43 stage of development; Tottman and Makepeace, 1979).

Fig. 7. Nitrate reductase (NR) activity in extracts from different leaves of *PSAG12::ipt* and control plants. Differences between columns marked with different letters are statistically significant ($P \leq 0.05$).

Fig. 1.

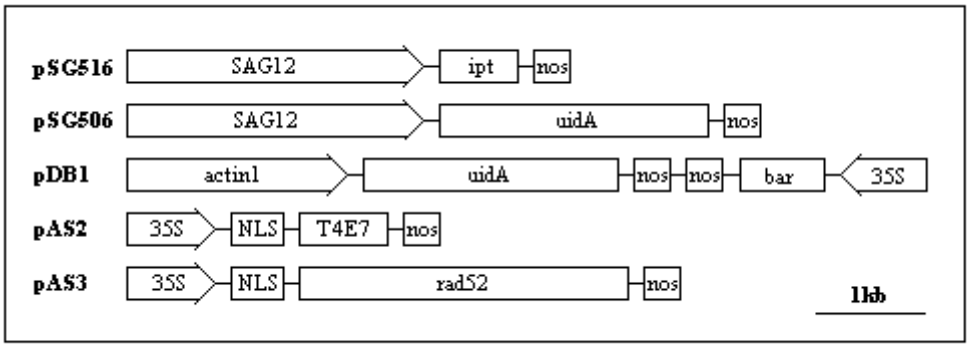
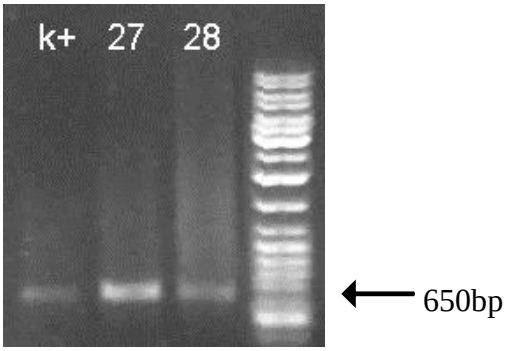


Fig. 2.

A

PCR



B

RT PCR

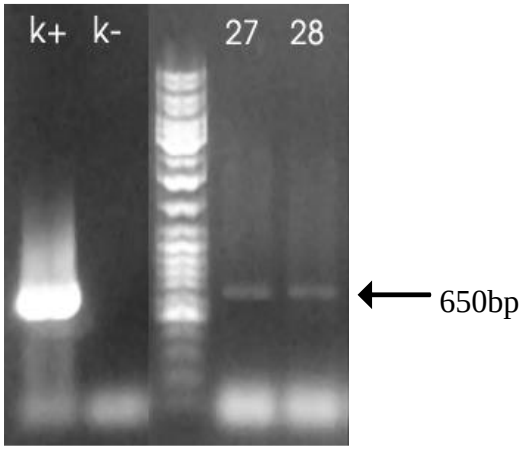


Fig. 3.

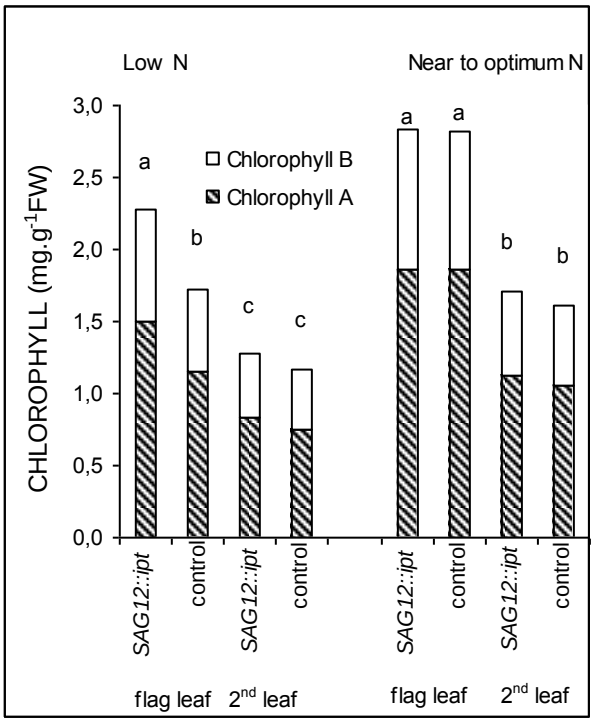

Low N
Near to optimum N
CHLOROPHYLL (mg.g-1FW)
Chlorophyll B
Chlorophyll A
SAG12::ipt
control
flag leaf
2nd leaf

Fig. 4

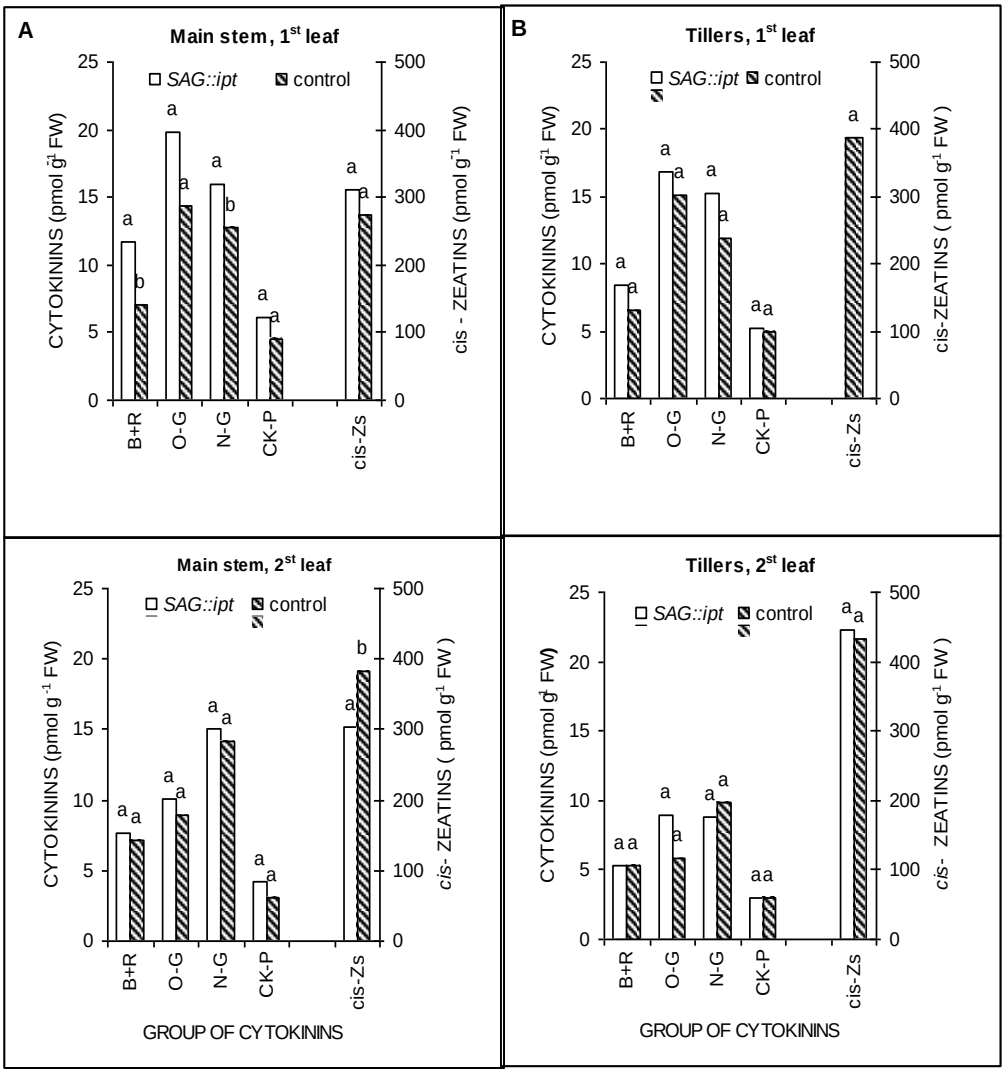

A
Main stem, 1st leaf
B
Tillers, 1st leaf
Main stem, 2st leaf
Tillers, 2st leaf
SAG::ipt
control
CYTOKININS (pmol g-1 FW)
cis - ZEATINS (pmol g-1 FW)
cis-ZEATINS (pmol g-1 FW)
B+R
O-G
N-G
CK-P
cis-Zs
GROUP OF CYTOKININS

Fig. 5

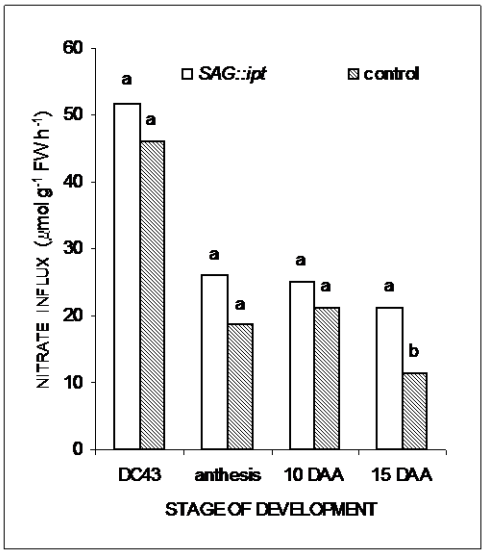

SAG::ipt
control
NITRATE INFLUX (µmol g-1 FW h-1)
60
50
40
30
20
10
0
a
a
a
a
a
a
a
b
DC43
anthesis
10 DAA
15 DAA
STAGE OF DEVELOPMENT

Fig. 6

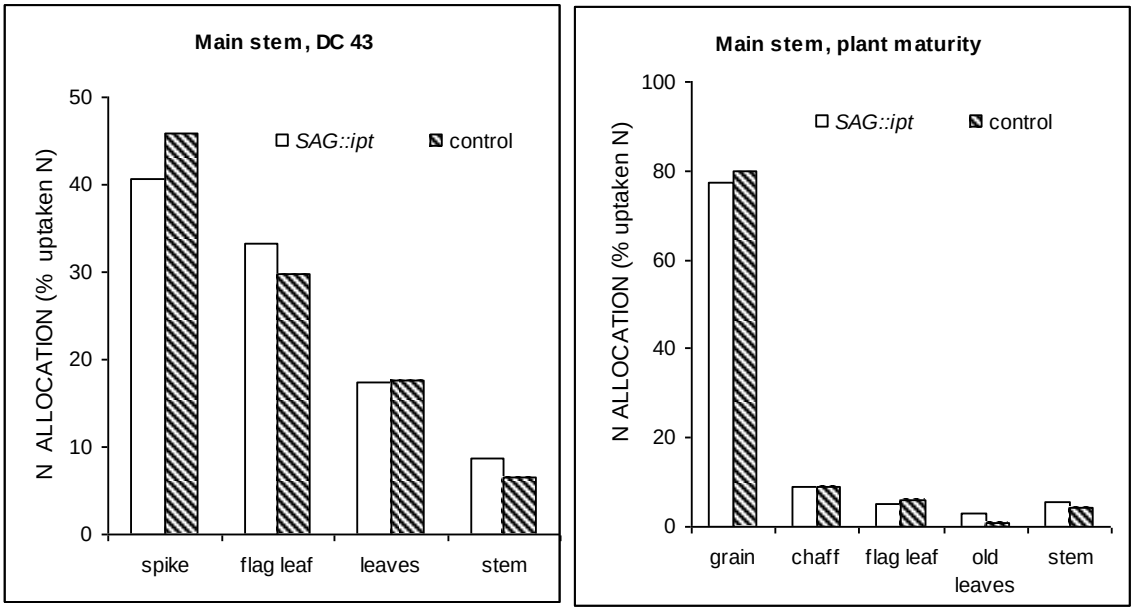
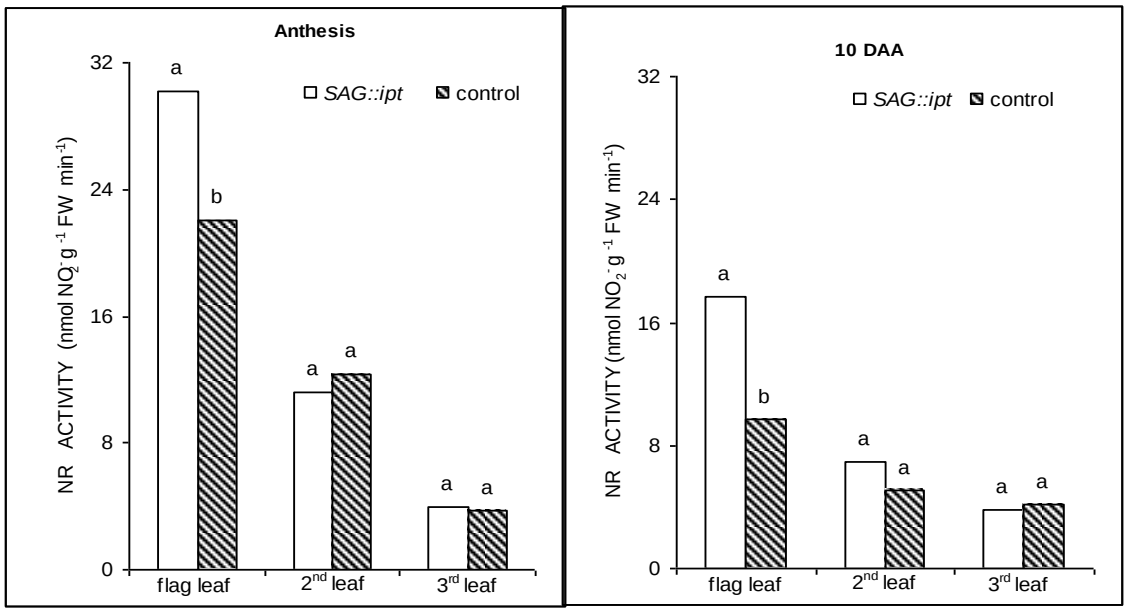

Main stem, DC 43
N ALLOCATION (% uptaken N)
50
40
30
20
10
0
SAG::ipt
control
spike
flag leaf
leaves
stem
Main stem, plant maturity
N ALLOCATION (% uptaken N)
100
80
60
40
20
0
SAG::ipt
control
grain
chaff
flag leaf
old leaves
stem

Fig. 7


Anthesis
SAG::ipt
control
NR ACTIVITY (nmol NO2- g -1 FW min-1)
32
24
16
8
0
a
b
a
a
a
a
flag leaf
2nd leaf
3rd leaf
10 DAA
SAG::ipt
control
NR ACTIVITY (nmol NO2- g -1 FW min-1)
32
24
16
8
0
a
b
a
a
a
a
flag leaf
2nd leaf
3rd leaf