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Effect of exogenous cytokinins, auxins and adenine on cytokinin *N*-glucosylation and cytokinin oxidase/dehydrogenase activity in de-rooted radish seedlings

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Abstract

The role of cytokinin *N*-glucosylation and degradation by cytokinin oxidase/dehydrogenase (CKX, EC 1.5.99.12) in response to application of exogenous auxins (2,4-dichlorophenoxyacetic acid [2,4-D] and α-naphthaleneacetic acid [NAA]) and cytokinins (N^6-benzyladenine [BA] and *trans*-zeatin [Z]) was investigated in de-rooted seedlings of *Raphanus sativus* L. cv. Rampouch. Both auxins applied for 24 h at 1 and 10 μM concentration increased *N*-glucosylation of exogenously applied [^{3}H]dihydrozeatin (DHZ) by up to 20%. The level of endogenous 7*N*-glucosides (of Z, isopentenyladenine [iP] and DHZ) was increased by 2,4-D and NAA at 10 μM concentration by 28 and 23%, respectively, the level of Z being decreased by 90 and 59%, respectively. 2,4-D and NAA suppressed CKX activity ca. by half. Exogenous cytokinins Z and BA applied at 1 and 10 μM concentration stimulated 7*N*-glucosylation of [^{3}H]DHZ (by up to 40%). BA both at 1 and 10 μM, increased the level of endogenous Z by up to 35% and that of 7*N*-glucosides by up to 27%. BA application also strongly stimulated CKX activity (by up to 180%). Feeding with 1 and 10 μM Z resulted in ca. 100-fold and 2000-fold increase of Z level, respectively. The main metabolite, Z7G, was increased ca. 6-fold and 60-fold, respectively. Levels of Z 9-glucoside (Z9G), *trans*-zeatin riboside (ZR) and Z *O*-glucoside (ZOG) were elevated to lesser extent. As compared to BA, Z had only negligible effect on CKX activity. Adenine (1–500 μM) was preferentially 7*N*-glucosylated inhibiting competitively 7*N*-glucosylation of [^{3}H]DHZ. At high concentrations (100–500 μM) it increased endogenous levels of active cytokinins, especially of Z, however, it had no effect on CKX activity. Cytokinin *N*-glucosylation proved to be involved in down-regulation of active cytokinins in response to auxin and in the re-establishment of cytokinin homeostasis following application of exogenous cytokinins.

Abbreviations: BA – N^6-benzyladenine; CKX – cytokinin oxidase/dehydrogenase; 2,4-D – 2,4-dichlorophenoxyacetic acid; DHZ – dihydrozeatin; DHZR – dihydrozeatin riboside; DHZ7G – dihydrozeatin 7-glucoside; DHZ9G – dihydrozeatin 9-glucoside; DHZOG – dihydrozeatin *O*-glucoside; iP – N^6-(Δ^2-isopentenyl) adenine; iPR – N^6-(Δ^2-isopentenyl) adenosine; iP7G – N^6-(Δ^2-isopentenyl) adenine 7-glucoside; NAA – α-naphthaleneacetic acid; Z – *trans*-zeatin; ZR – *trans*-zeatin riboside; ZOG – *trans*-zeatin *O*-glucoside; ZROG – *trans*-zeatin riboside *O*-glucoside; Z7G – *trans*-zeatin 7-glucoside; Z9G – *trans*-zeatin 9-glucoside

Introduction

The plant hormones auxin and cytokinin interact in a complex manner to control many aspects of growth and differentiation. The variety of ways in which they regulate physiological responses (e.g., they act synergistically to regulate cell division but antagonistically to control lateral bud or root outgrowth) suggests that their interactions occur at multiple levels. These include mutual regulation of active hormone concentration (i.e., the concentration of structural forms of hormones which exhibit high physiological activity), input in signal transduction pathways, alteration of gene expression, post-translational modifications and direct modulation of enzyme activities (Coenen and Lomax 1997).

Cytokinins and auxin mutually influence their pools. According to several reports, application of exogenous auxin diminishes the level of cytokinins. A decrease of *trans*-zeatin riboside (ZR) in tobacco cell cultures after application of NAA has been reported (e.g., see Hansen et al. 1987). The mechanism of auxin action, however, still remains unclear. Auxins have been reported to stimulate oxidative breakdown (Palni et al. 1988) and glucosylation (Crouch and van Staden 1995) of physiologically active cytokinins in a tissue-dependent manner. In other studies, a decrease of CKX activity in auxin-overproducing tobacco (Eklöf et al. 1997) and an inhibition of β-(9-cytokinin) alanine synthase by IAA (Parker et al. 1986) were found. Zhang et al. (1995) reported that NAA reduced transcription of *ipt* gene, as well as the level of the corresponding protein in *ipt* transformed tobacco tissue. Recent study of *ipt* genes in Arabidopsis have revealed that two genes (*AtIPT5* and *AtIPT7*, both located in roots) are up-regulated by auxin, while the other six are not affected (Miyawaki et al. 2004).

Cytokinins themselves affect their own endogenous pool. Increase in their concentration, resulting from either uptake or intracellular biosynthesis, promotes autoinductive accumulation of cytokinins (with the exception of roots). Subsequent cytokinin accumulation activates the oxidative and/or conjugative pathways of cytokinin degradation/deactivation (Kamínek et al. 1997). In soybean Thomas and Katterman (1986) reported stimulation of ZR production after application of exogenous urea-type cytokinins. Similar result was obtained by Hansen et al. (1987) in tobacco callus tissues upon treatment with aromatic cytokinins. BA partially removed the inhibiting effect of auxin on cytokinin accumulation in *ipt* transformed tobacco callus (Zhang et al. 1995). The positive effect of exogenous cytokinins on the endogenous cytokinin pool correlated well with the growth stimulation in sugar beet cell suspension (Vaňková et al. 1991).

This paper has been aimed to elucidate the possible role of cytokinin *N*-glucosylation and oxidative degradation pathways in the cytokinin–auxin and cytokinin–cytokinin relationships in radish. In this species conjugation to glucose represents the dominant regulatory mechanism in cytokinin metabolism (Parker and Letham 1973; McGaw et al. 1984), while CKX, which catalyzes cytokinin oxidative degradation, exhibits relatively low activity (McGaw and Horgan 1985). De-rooted radish seedlings were used to study the metabolism of radiolabeled DHZ in response to exogenous auxins and cytokinins and their effect on the pool of endogenous cytokinins and activity of CKX.

Materials and methods

Plant material

Seeds of radish *Raphanus sativus* L. cv. Rampouch were sown on perlite supplied with Knop solution. Seedlings were grown for 7 days in a cultivation chamber – 16 h light (16,000 lux)/8 h dark, at 22 °C. Plants were harvested when they had two well-developed cotyledons.

Incubation with [^{3}H]DHZ

De-rooted seedlings were incubated for 24 h in 1 μM or 10 μM aqueous solutions of auxins (2,4-D or NAA) and cytokinins (Z or BA) or 1–500 μM adenine. The substrate [^{3}H]DHZ (1.8 TBq mmol^{-1}, prepared by Dr Jan Hanuš, Institute of Experimental Botany AS CR, Prague, Czech Republic, 4 nmol per sample) was supplied 2 h after the initiation of incubation.

Cytokinin extraction and purification

Cytokinins were extracted and purified according to the method by Dobrev and Kamínek (2002). Samples (ca. 1 g FW) were frozen in liquid nitrogen and extracted overnight with a solution of methanol/water/formic acid (15/4/1, v/v/v, pH∼2.5, −20 °C). In the case of samples for endogenous cytokinin analysis nine deuterium labeled standards ([$^2H_5$]Z, [$^2H_5$]ZR, [$^2H_5$]Z7G, [$^2H_5$]Z9G, [$^2H_5$]ZOG, [$^2H_5$]ZROG, [$^2H_6$]iP, [$^2H_6$]iPA, [$^2H_6$]iP9G; products of Apex Organics, Honiton, UK) were added, each at 100 pmol per sample. The extract was passed through Si–C_{18} columns (SepPak Plus, Waters, Milford, MC, USA) to remove interfering lipophilic substances. After organic solvent evaporation in vacuo, the aqueous residue was applied on an Oasis MCX mixed mode (cation exchange and reverse-phase) column (Waters, 150 mg). Adsorbed cytokinin nucleotides were eluted with 0.35 M ammonium in water, bases and ribosides with 0.35 M ammonium in 60% methanol (v/v). The eluates were evaporated in vacuo. Nucleotide samples were dephosphorylated for 1 h at 37 °C with acid phosphatase (0.6 U per sample). Evaporated samples were re-suspended in 200 μl 20% (v/v) acetonitrile. Samples for the determination of radiolabeled metabolites were subjected to HPLC, samples for analysis of endogenous cytokinins to LC/MS_n.

HPLC of [^3H]DHZ metabolites

Radiolabeled metabolites of [^{3}H]DHZ were analysed using HPLC – Perkin Elmer Series 200 Quaternary Pump coupled to Diode Array Detector (235C, Perkin Elmer, Wellesley, MA, USA) and flow-through radioactivity detector (RAMONA 2000, Raytest, Germany), column: Luna C18(2) (150 mm/4.6 mm/3 μm Phenomenex, Torrance, CA, USA). The sample (10 μl) was eluted at a flow rate 0.6 ml/min at 30 °C and UV detection was performed at 270 nm. Mobile phase: A = 40 mM CH_3COOH + NH_4OH, pH 4.1; B = CH_3OH/CH_3CN = 1/1 (v/v). Linear gradient: 10% B to 20% B in 2 min, to 45% B in 17 min, to 100 %B in 2 min, 100% B for 2 min, 100% B to 10% B in 2 min. The radioactive metabolites were identified on the basis of coincidence of retention times with authentic standards.

LC/mass spectrometry

LC-MS analysis was performed using a Beckman 125-binary HPLC gradient pump and 507 autosampler coupled to an Ion Trap Mass Spectrometer Finnigan MAT LCQ-MS^n equipped with an electrospray interface. Samples (10 μl) were injected on an RP-C_{18} column (Merck; Supersphere RP Select B, 2 × 250 mm, 4 μm) and eluted with a linear gradient of CH_3CN (B) in 0.001%, v/v, CH_3COOH in water (mobile phase, A): 14% B to 20% B in 14 min, to 80% B in 6 min and to 100% B in 6 min, at a flow rate 0.2 ml/min.

Detection and quantification were carried out using a Finnigan LCQ operated in the positive ion, full-scan MS/MS mode using a multilevel calibration graph with deuterated cytokinins as internal standards. The electrospray ionization (ESI) probe was installed with a sheath and auxiliary gasses ran at 96 and 6 units, respectively. The heated metal capillary temperature was maintained at 250 °C and capillary voltage at 2.5 V (Dobrev et al. 2002). The levels of 20 cytokinin derivatives were measured. The detection limit was calculated for each compound as $3.3\sigma/S$, where σ is the standard deviation of the response and S the slope of the calibration curve. Each sample was injected at least twice.

Determination of cytokinin oxidase/dehydrogenase activity

The method described by Motyka et al. (1996) was used to measure CKX activity in de-rooted radish seedlings. CKX was extracted from ca. 3.5 g tissue FW with 100 mM Tris–HCl buffer (pH 7.5) and purified on polyvinylpyrrolidone (Sigma Co., St Louis, USA) 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 sulphate to 80% saturation. Protein content was determined according to the method of Bradford (1976) using BSA as a standard. The assay of CKX activity was based on the conversion of [2-^{3}H]iP (7.4 Bq mol^{-1}, prepared by

Dr Jan Hanuš, Institute of Experimental Botany AS CR, Prague, Czech Republic) to [^{3}H]adenine. Separation of the substrate from the product of the enzyme reaction was achieved by TLC on microcrystalline cellulose plates (Aldrich Co., Milwaukee, USA) developed with the upper phase of the 4:1:2 (v/v/v) mixture of ethylacetate: *n*-propanol:water. The radioactivity of the adenine and iP containing zones was determined by liquid scintillation technique using Packard TRI-CARB 2500 TR scintillation counter.

Results and discussion

The effect of exogenous auxins on cytokinin 7N-glucosylation and CKX activity

The dominant metabolite of [^{3}H]DHZ (1.8 TBq/mmol, 4 nmol) in de-rooted radish seedlings after 24 h incubation was its 7-glucoside (ca. 56% of the total activity) (Figure 1, controls). Minor metabolites were dihydrozeatin 9-glucoside (DHZ9G), dihydrozeatin *O*-glucoside (DHZOG) and dihydrozeatin riboside (DHZR), together accounting for ca. 17%. [^{3}H]DHZ was used as labeled substrate for these studies since it is not degraded by CKX.

Application of 2,4-D or NAA to radish seedlings promoted 7*N*-glucosylation of [^{3}H]DHZ at both tested concentrations (1 and 10 μM, respectively), subsequently reducing the substrate level (Figure 1a, b). The maximal increase was by 20%.

Auxin treatment (with exception of 1 μM NAA) lowered significantly the endogenous levels of biologically active cytokinins, especially that of the predominant Z. Ten micromolar 2,4-D exhibited the strongest inhibitory effect (Table 1). These results agree with the well-documented effect of exogenous auxins on the levels of active cytokinins (Hansen et al. 1987).

The content of the most abundant metabolites in radish – Z7G and iP7G was increased at 10 μM auxin concentration (2,4-D by 28% and NAA by 23%) (Table 1). The amount of DHZ7G was low when compared to the other 7*N*-glucosides, probably due to the minor presence of the corresponding base in the radish species (below the detection limit 0.1 pmol/g FW). The level of 9*N*-glucosides was much lower than that of 7-gluco-

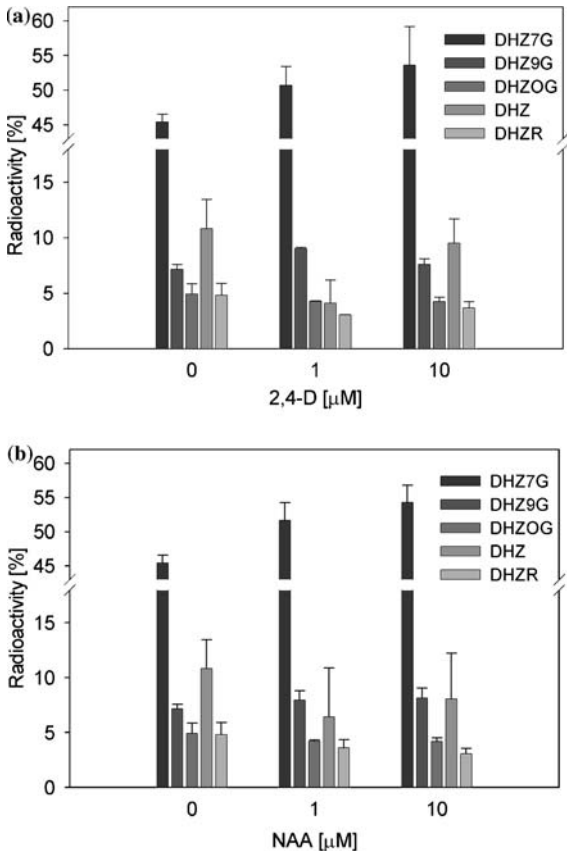

Figure 1. The effect of 2,4-dichlorophenoxyacetic acid (a) and α-naphthaleneacetic acid (b) on the metabolism of [^{3}H]dihydrozeatin in radish seedlings. Values are expressed as percentage of the total applied radioactivity. Means ± SE of three independent experiments are shown.

Table 1. The impact of 2,4-dichlorophenoxyacetic acid or α-naphthaleneacetic acid on the levels of endogenous cytokinins.

	Control	NAA (μM)		2,4-D (μM)	
		1	10	1	10
Z	1.7	1.9	1.0*	0.7*	0.1*
ZR	0.1	0.3	0.4	ND	ND
iP	0.1	0.3	ND	0.2	ND
ZOG	1.7	2.2	1.3	1.3	1.1
Z7G	21.4	17.0	22.1	19.0	26.8*
iP7G	19.1	18.2	28.5*	16.0	32.8*
DHZ7G	1.0	0.6	1.4	0.9	1.5
Z9G	0.9	1.0	0.8	1.1	1.0
iP9G	0.1	0.3	0.1	0.3	0.1
DHZ9G	0.2	0.2	0.4	0.2	0.4

The results presented are based on three independent experiments. Each determination was duplicated. The SE values averaged 12% and did not exceed 20% of the mean.
* Statistically significant difference ($p < 0.05$).

sides and showed no significant differences following the auxin treatment. These results are in accordance with data published by Crouch and van Staden (1995), who found an increase in cytokinin 7-glucosylation of exogenous BA after application of 2,4-D to apex-derived cultures of wild carnation. In cultures derived from seeds N^6-benzyladenine 7-glucoside (BA7G) was the second most abundant after BA riboside.

The activity of the cytokinin degrading enzyme CKX in de-rooted radish seedlings was found to be rather low, as it was also reported by McGaw and Horgan (1985). Application of auxins (both 2,4-D and NAA) to de-rooted radish seedlings caused a decrease of CKX activity (by up to 31% and 54%, respectively, Table 2). The published data on the effect of auxins on the level and activity of CKX are rather contradictory. Eklöf et al. (1997) found that transgenic tobacco leaves over-expressing the auxin biosynthetic genes (*iaaM* and *iaaH*) exhibited in young leaves lower activity of CKX, as well as a decreased level of all endogenous cytokinins, except Z7G. On the other hand, a moderate increase of maize CKX activity *in vitro* was detected after addition of NAA to tobacco pith explants incubated with [^{3}H]ZR (Palni et al. 1988). In our previous experiments we found no significant effects on CKX activity following the application of exogenous auxins to tobacco callus tissue (Motyka et al. 1990). The observed discrepancy may be caused by differences in the relative proportion of the activities of CKX and *N*-glucosyltransferase(s) among the individual species. Cytokinin oxidative break-down may be involved in auxin–cytokinin relationships in species with predominant cytokinin degradation by CKX, while in the others, like radish, the auxin may affect mostly *N*-glucosylation.

Table 2. The effect of auxins, cytokinins and adenine on the activity of cytokinin oxidase/dehydrogenase in radish seedlings.

Sample	Concentration [μM]	Cytokinin oxidase activity [pmol Ade mg^{-1} protein h^{-1}]
Control	0	9.0
2,4-D	1	5.8
	10	4.1
NAA	1	6.2
	10	6.2
Z	1	8.6
	10	7.3
BA	1	18.3
	10	24.7
Ade	1	8.6
	10	8.3
	100	8.3

Cytokinin oxidase/dehydrogenase activity was determined in assay mixture containing 2 μM [2-^{3}H]iP, 100 mM TAPS–NaOH (pH 8.5) and protein preparation equivalent to 675 mg/assay (400 mg/assay for BA 1 and 10 μM) in a total volume of 50 μl. The results of one representative experiment from three independent ones are presented. The given values represent the means of three replicates. The SE values averaged 7% and did not exceed 15% of the mean.

Comparing the total cytokinin content in the control and auxin treated plants, a decrease was observed at 1 μM auxin and an increase at 10 μM auxin (caused predominantly by 7*N*-glucosides). This may indicate an altered rate of cytokinin biosynthesis in presence of auxin or induced cytokinin turnover.

In conclusion, the mechanism by which auxins influence cytokinin levels varies depending on species cytokinin metabolism. Our results indicate that in radish, as species with predominant *N*-glucosylation, this pathway is involved in the antagonistic effect of the auxin on cytokinins.

The effect of exogenous cytokinins on cytokinin 7N-glucosylation and CKX activity

The exogenous supply of BA (1 and 10 μM) to radish seedlings promoted the conversion of [^{3}H]DHZ. The amount of the non-metabolized substrate decreased 2-fold (Figure 2a), while the predominant metabolic route 7*N*-glucosylation was stimulated. [^{3}H]DHZ 9*N*-glucoside and *O*-glucoside levels increased only slightly. Application of Z had the same effects on [^{3}H]DHZ metabolism as BA (Figure 2b).

BA had a positive effect on the level of endogenous Z, increasing it by 11 and 35% at 1 and 10 μM, respectively, and especially on 7*N*-glucosides (by up to 42% for Z7G, Table 3). The amount of ZOG was mildly elevated at 10 μM concentration. The stimulatory effects of exogenous BA on the level of endogenous cytokinins agree to previously published results (see e.g., Hansen et al. 1987; Vaňková et al. 1987).

Incubation with Z had a much more dramatic effect on the endogenous cytokinins than incubation with BA. The endogenous level of Z was increased by uptake from the medium from 1.7 pmol/g FW in the control plants to ca.

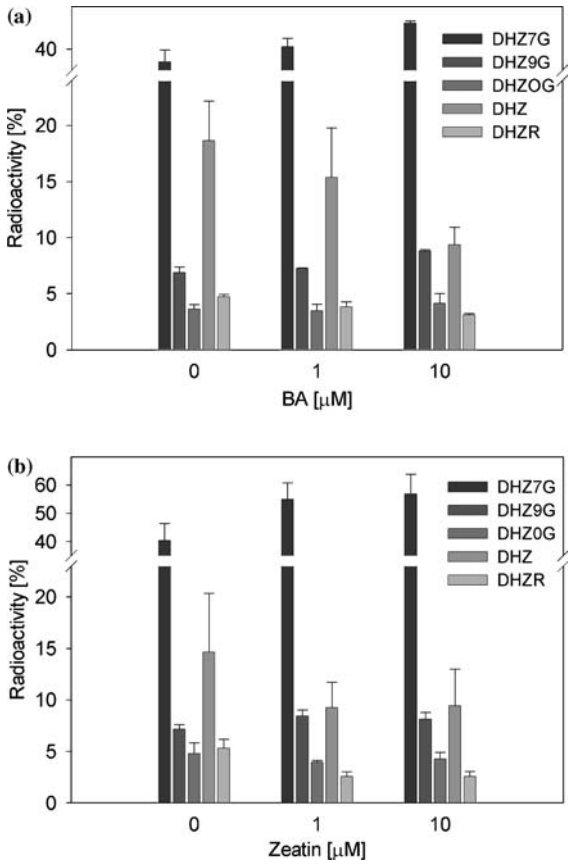

Figure 2. The effect of N^6-benzyladenine (a) and *trans*-zeatin (b) on metabolism of [^{3}H]dihydrozeatin in radish seedlings. Values are expressed as percentage of the total applied radioactivity. Means ± SE of three independent experiments are shown.

Table 3. The impact of *trans*-zeatin (Z) and N^6-benzyladenine (BA) on the levels of endogenous cytokinins.

	Control	Zeatin (μM)		Benzyladenine (μM)	
		1	10	1	10
Z	1.7	97.9*	2552.7*	1.9	2.3*
ZR	0.1	5.8*	97.8*	0.2	0.2
ZOG	1.7	22.6*	341.6*	1.6	2.2
Z7G	23.3	149.8*	1543.3*	30.4*	23.7
iP7G	20.8	21.0	31.0*	27.9*	31.0*
DHZ7G	0.2	0.4	ND	0.3	0.5
Z9G	0.9	9.8*	169.0*	0.66	0.6
iP9G	0.1	0.2	0.2	ND	ND
DHZ9G	0.2	0.4	ND	0.3	0.5

The results are based on three independent experiments. Each determination was duplicated. The SE values averaged 12% and did not exceed 20% of the mean.
* Statistically significant difference ($p < 0.05$).

100 pmol/g FW and 2500 pmol/g FW in seedlings treated with 1 and 10 μM Z, respectively (Table 3). The whole Z metabolism, including 7*N*- and 9*N*-glucosylation, ribosylation and *O*-glucosylation, was greatly stimulated. The main metabolite was Z7G, which was enhanced up to 6-fold and 60-fold, respectively.

Cytokinin glucosylation seems to be an efficient mechanism used by the cells to reduce excess levels of both exogenous and endogenous cytokinins. Our results correspond to those of McGaw et al. (1985) and Letham and Gollnow (1985), who reported massive 7*N*-glucosylation of [^{3}H]Z and [^{3}H]BA in radish cotyledons.

Treatment of radish seedlings with BA, which is not a substrate of CKX, enhanced at both 1 and 10 μM concentration activity of this enzyme by 100 and 180%, respectively (Table 2). Several mechanisms of promotion of CKX activity by non-substrate cytokinins have been proposed (Kamínek et al. 1997). It seems probable that cytokinins that are not substrates of CKX are able to promote accumulation of endogenous cytokinin substrates, which may subsequently induce CKX. Our previous study using cytokinin-overproducing tobacco plants revealed a strong increase in CKX activity as a consequence of elevated cytokinin levels (Motyka et al. 1996; Motyka et al. 2003). Stimulation of CKX activity by endogenous or externally applied (both substrate and non-substrate) cytokinins has also been reported in other plants and plant cultures (Chatfield and Armstrong 1986; Kamínek and Armstrong 1990; Motyka and Kamínek 1990; Zhang et al. 1996; Auer et al. 1999). It is evident that BA, which is intensively glucosylated (data not shown), is capable of stimulating both metabolic processes, i.e., *N*-glucosylation and oxidative breakdown. The relative proportion of these two pathways may depend on the developmental stage of the plant. Letham and Gollnow (1985) found a decline in conversion of Z to its 7-glucoside and an increase in formation of adenosine during senescence.

Surprisingly application of Z, which is a substrate of CKX, had only a negligible effect on CKX activity when determined after 24 h incubation of radish seedlings. The impact of Z was repeatedly and thoroughly checked. The weaker stimulatory effect of Z, in comparison with BA, was reported earlier (Chatfield and Armstrong

1986; Bilyeu et al. 2001). Our results indicate that Z may affect CKX activity by different, so far unknown, mechanism(s) than non-substrate cytokinin BA.

The effect of adenine on cytokinin 7N-glucosylation and CKX activity

The effect of adenine on 7*N*-glucosylation of [^{3}H]DHZ in radish seedlings after 24 h incubation was tested in the concentration range 1–100 μM (Figure 3). 7*N*-glucosylation of [^{3}H]DHZ was inhibited even at 1 μM concentration. Suppression of DHZ 7*N*-glucosylation was accompanied by an increase in the level of the corresponding free base. No substantial changes in the amounts of other DHZ metabolites were observed. This finding corresponds to the fact that cytokinin *N*-glucosyltransferases have rather broad substrate specificity and accept also adenine as a substrate, though at lower affinity (Entsch et al. 1979). The hypothesis that the synergistic effect of adenine towards cytokinins is due to its inhibition of cytokinin deactivation was published by Baumann et al. (1994). Labeled adenine, exogenously added to suspension-cultured coffee cells was readily glucosylated to the corresponding 7*N*-glucoside. Hence, the possible mechanism for the adenine effect towards cytokinins might be a competitive binding to the cytokinin 7*N*-glucosyltransferase. The very early finding of Skoog and Tsui (1948), published before cytokinins were discovered, that

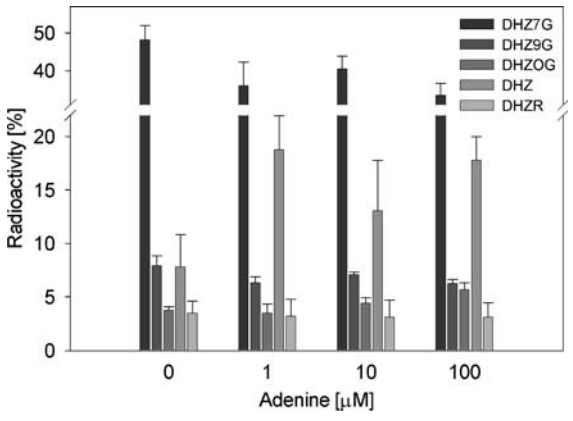

Figure 3. The effect of adenine on metabolism of [^{3}H]dihydrozeatin in radish seedlings. Values are expressed as percentage of the total applied radioactivity. Means ± SE of three independent experiments are shown.

Table 4. The impact of adenine on the levels of endogenous cytokinins.

	Control	Adenine (μM)			
		1	10	100	500
Z	1.7	2.5	1.6	6.0*	11.3*
ZR	0.1	0.2	0.3	0.3	0.6*
iP	0.1	0.2	ND	ND	0.5*
ZOG	1.7	1.6	2.1	1.3	0.9
Z7G	23.3	24.4	17.8*	19.4*	17.9*
iP7G	25.8	39.9*	35.7*	29.0*	34.7*
DHZ7G	4.7	4.9	2.7	3.1	4.7
Z9G	0.9	0.5	0.5	0.6	0.2
iP9G	0.1	0.1	0.4	ND	1.2*
DHZ9G	0.2	0.3	0.4	0.7*	0.5*
cZ	2.7	0.7*	2.6	1.4	1.7
cZR	0.7	0.9	0.5	0.7	0.8

The presented results are based on three independent experiments. Each determination was duplicated. The SE values averaged 12% and did not exceed 20% of the mean.
* Statistically significant difference ($p < 0.05$).

adenine applied at high concentration was capable to induce budding of tobacco stem sections and cell division in tobacco stem pith can be explained on the basis of protection of endogenous cytokinins against their *N*-glucosylation.

Our results on endogenous cytokinin analysis confirmed this hypothesis predicted on the basis of indirect experiments. At high concentrations (100–500 μM) adenine increased the level of active endogenous cytokinins, especially of Z (Table 4). At 1 μM adenine, the level of iP7G was elevated by 55%. Adenine at such a low concentration stimulated the glucosylation apparatus, however, did not saturate it. Adenine applied at 10 and 100 μM concentration decreased the level *N*-glucosides, especially of Z7G and DHZ7G. At 500 μM adenine was toxic to seedlings and they became wilty. The increase of glucoside levels (with exception of Z7G) at this high concentration (500 μM) might be connected with the disturbance of cytokinin homeostasis.

The activity of CKX *in vivo* was not affected by adenine (Table 2). Similar results were reported by Chatfield and Armstrong (1986) and Kamínek and Armstrong (1990).

N-Glucosylation of cytokinins together with their degradation with CKX represent important down-regulation mechanisms, which are indispensable for cytokinin action as a signal. It has been repeatedly reported that exogenously applied

radiolabeled cytokinins are inactivated to a significant extend by their *N*-glucosylation (McGaw et al. 1985; Letham and Gollnow 1985). Exploitation of advanced analytical techniques, namely HPLC/MS^n, allowed us to determine not only the conversion of applied cytokinins to their metabolites, but also their impact on the whole pool of endogenous cytokinins. Moreover, it was possible to specify the effect of exogenous auxin on cytokinin pool. In general, both exogenous cytokinins and auxin stimulate down-regulation of endogenous cytokinins by their conversion to inactive *N*-glucosides and in this way regulate the strength of cytokinin signal. Detail knowledge of cytokinin *N*-glucosylation in various plant species may allow prediction of the fate of different exogenous cytokinins in plant cells. This kind of information can be helpful for selection of appropriate cytokinins for practical applications.

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References

Auer C.A., Motyka V., Brezinova A. and Kaminek M. 1999. Endogenous cytokinin accumulation and cytokinin oxidase activity during shoot organogenesis of *Petunia hybrida*. Physiol. Plant. 105: 141–147.

Baumann T.W., Schulthess B.H., Linden A. and Ruedi P. 1994. Structure and metabolism of 7-glucopyranosyladenine – the product of a cytokinin-sparing reaction? Phytochemistry 36: 537–542.

Bilyeu K.D., Cole J.L., Laskey J.G., Riekhof W.R., Esparza T.J., Kramer M.D. and Morris R.O. 2001. Molecular and biochemical characterization of a cytokinin oxidase from maize. Plant Physiol. 125: 378–386.

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

Chatfield J.M. and Armstrong D.J. 1986. Regulation of cytokinin oxidase activity in callus tissues of *Phaseolus vulgaris* L. cv Great Northern. Plant Physiol. 80: 493–499.

Coenen C. and Lomax T.L. 1997. Auxin–cytokinin interactions in higher plants: old problems and new tools. Trends Plant Sci. 2: 351–356.

Crouch N.R. and van Staden J. 1995. Promotion by 2,4-D of 7-glucosylation of benzyladenine in seed-derived and shoot apex-derived cell cultures of *Dianthus zeyheri*. Physiol. Plant. 93: 645–650.

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

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

Eklöf S., Åstot C., Blackwell J., Moritz T., Olsson O. and Sandberg G. 1997. Auxin–cytokinin interactions in wild-type and transgenic tobacco. Plant Cell Physiol. 38: 225–235.

Entsch B., Letham D.S., Parker C.W. and Summons R.E. 1979. Preparation and characterization, using high-performance liquid chromatography, of an enzyme forming glucosides of cytokinins. Biochim. Biophys. Acta 570: 124–139.

Hansen C.E., Meins F. Jr. and Aebi R. 1987. Hormonal regulation of zeatin–riboside accumulation by cultured tobacco cells. Planta 172: 520–525.

Kaminek M. and Armstrong D.J. 1990. Genotypic variation in cytokinin oxidase from *Phaseolus* callus cultures. Plant Physiol. 93: 1530–1538.

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

Letham D.S. and Gollnow B.I. 1985. Regulators of cell division in plant tissues. XXX. Cytokinin metabolism in relation to radish cotyledon expansion and senescence. J. Plant Growth Regul. 4: 129–145.

McGaw B.A., Heald J.K. and Horgan R. 1984. Dihydrozeatin metabolism in radish seedlings. Phytochemistry 23: 1373–1377.

McGaw B.A. and Horgan R. 1985. Cytokinin metabolism and the control of cytokinin activity. Biol. Plant. 27: 180–187.

McGaw B.A., Horgan R. and Heald J.K. 1985. Cytokinin metabolism and the modulation of cytokinin activity in radish. Phytochemistry 24: 9–13.

Miyawaki K., Matsumoto-Kitano M. and Kakimoto T. 2004. Expression of cytokinin biosynthetic isopentenyltransferase genes in arabidopsis: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant J. 37: 128–138.

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

Motyka V. and Kaminek M. 1990. Regulation of cytokinin catabolism in tobacco callus cultures. In: Nijkamp H.J.J., Van der Plas L.H.W. and Van Aartrijk J. (eds), Plant Cellular and Molecular Biology. Kluwer Academic Publishers, Dordrecht, pp. 492–497.

Motyka V., Vankova R., Capkova V., Petrasek J., Kaminek M. and Schmülling T. 2003. Cytokinin-induced upregulation of cytokinin oxidase activity in tobacco includes changes in

enzyme glycosylation and secretion. Physiol. Plant. 117: 11–21.

Palni L.M.S., Burch L. and Horgan R. 1988. The effect of auxin concentration on cytokinin stability and metabolism. Planta 174: 231–234.

Parker C.W., Entsch B. and Letham D.S. 1986. Inhibitors of two enzymes which metabolise cytokinins. Phytochemistry 25: 303–310.

Parker C.W. and Letham D.S. 1973. Regulators of cell division in plant tissues. XVI. Metabolism of zeatin by radish cotyledons and hypocotyls. Planta 114: 199–218.

Skoog F. and Tsui C. 1948. Chemical control of growth and bud formation in tobacco stem segments and callus cultured *in vitro*. Am. J. Bot. 35: 782–787.

Thomas J. and Katterman F.R. 1986. Cytokinin activity induced by thidiazuron. Plant Physiol. 81: 681–683.

Vankova R., Hsiao K.C., Bornman C.H. and Gaudinova A. 1991. Effects of synthetic cytokinins on levels of endogenous cytokinins and respiration patterns of *Beta vulgaris* cells in suspension. J. Plant Growth Regul. 10: 197–199.

Vankova R., Kaminek M., Eder J. and Vanek T. 1987. Dynamics of production *trans*-zeatin and *trans*-zeatin riboside by immobilized cytokinin-autonomous and cytokinin-dependent tobacco cells. J. Plant Growth Regul. 6: 147–157.

Zhang X.D., Letham D.S., Zhang R. and Higgins T.J.V. 1996. Expression of the isopentenyl transferase gene is regulated by auxin in transgenic tobacco tissues. Transgenic Res. 5: 57–65.

Zhang R., Zhang X., Wang J., Letham D.S., McKinney S.A. and Higgins T.J.V. 1995. The effect of auxin on cytokinin levels and metabolism in transgenic tobacco tissue expressing an *ipt* gene. Planta 196: 84–94.