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Cytokinin-induced upregulation of cytokinin oxidase activity in tobacco includes changes in enzyme glycosylation and secretion

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Regulation of cytokinin oxidase (CKX) activity in relation to enzyme glycosylation and secretion was studied in wild-type (WT) and transgenic conditionally isopentenyltransferase gene (*ipt*)-expressing (IPT) tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) cell suspensions, calli and leaves. An increase in endogenous cytokinin content due to the tetracycline (Tc)-induced derepression of the *ipt* gene transcription or surface application of N^6-benzylaminopurine (BA) resulted in significant enhancement of CKX activity in all these plant materials. As revealed by Concanavalin A-Sepharose 4B chromatography the cytokinin-induced enhancement of CKX activity was associated predominantly with the *N*-glycoform of the enzyme (10- to 15-fold increase) in calli and leaves. Application of BA to the culture media of WT and IPT cell suspensions and the derepression of the *ipt* by Tc substantially enhanced endogenous levels of isoprenoid cytokinins and CKX activity in both cells and the culture medium. Most CKX activity in control, BA- and Tc-treated cells was associated with the non-glycosylated form of the enzyme, whereas the majority of CKX activity in the culture media was due to the glycosylated form. The pH optimum of CKX in cells (pH 8.5) differed considerably from that in the culture medium (pH 6.0). No significant differences were found in apparent K_m(iP) values of CKX between control, BA- and Tc-treated IPT cells and media or between purified glycosylated and non-glycosylated CKX. These results suggest that cytokinins induce changes in the proportions of glyco- and non-glycoforms of the enzyme in multicellular calli and leaves, and influence its secretion to the cell exterior.

Introduction

Cytokinins are plant hormones, which influence a wide variety of developmental and physiological processes in plants. Their active pool in plant cells is regulated at different levels including their biosynthesis, uptake from extracellular sources, metabolic interconversions, inactivation and degradation, as well as signal transduction and transport (Zažímalová et al. 1999, Mok and Mok 2001).

Irreversible metabolic degradation of isoprenoid cytokinins by removal of the N^6-side chain is specifically catalysed by cytokinin oxidase (CKX) which is widely distributed in plants (reviewed in Armstrong 1994, Hare and van Staden 1994). Mechanistically, it has been classified until recently as a copper-containing amine oxidase (EC 1.4.3.6., Hare and van Staden 1994) which catalyses the N^6-side-chain cleavage of cytokinins, using molecular oxygen as the oxidant (Laloue and Fox 1989). The CKXs in different plant materials vary markedly in their biochemical properties, however, they are very conservative in their substrate specificity preferring N^6-(Δ^2-isopentenyl)adenine (iP), zeatin (Z) and their ribosides as substrates.

Recently, the CKX gene (*ckx*) from maize kernels was independently cloned and expressed in two laboratories

Abbreviations – BA, N^6-benzylaminopurine; BA-IPT, BA-treated *ipt*-transformed plants (tissues, cells) with an inactive *ipt* gene; BA-WT, BA-treated wild-type plants (tissues, cells); BisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; C-IPT, control *ipt*-transformed plants (tissues, cells) with an inactive *ipt* gene; CKX, cytokinin oxidase; Con A, concanavalin A; C-WT, control wild-type plants (tissues, cells); DHZ, dihydrozeatin; iP, N^6-(Δ^2-isopentenyl)adenine; *ipt*, isopentenyltransferase gene; IPT, *ipt*-transformed plants (tissues, cells); NAA, naphthalene-1-acetic acid; TAPS, *N*-tris (hydroxymethyl)methyl-3-aminopropanesulphonic acid; Tc, tetracycline; Tc-IPT, *ipt*-transformed plants (tissues, cells) with Tc-induced *ipt* gene following Tc application; Tc-WT, wild-type plants (tissues, cells) after Tc application; WT, wild-type plants (tissues, cells); Z, *trans*-zeatin. Abbreviations for natural cytokinins according to Kamínek et al. (2000) are listed as a footnote to Table 1.

using different approaches (Houba-Hérin et al. 1999, Morris et al. 1999) and details of genomic organization of the maize gene (*Zmckx1*) and some of its *Arabidopsis* homologues (*AtCKX1-AtCKX7*) were described (Bilyeu et al. 2001, Werner et al. 2001). Based on the results of very recent experiments, the existing classification of CKX as a copper-containing amine oxidase has been questioned (Rinaldi and Comandini 1999a,1999b, Galuszka et al. 2000, Mok and Mok 2001). At present, two entries about CKX exist in the IUBMB Enzyme Nomenclature database; the first (EC 1.4.3.18) proposes to integrate the CKX under the recommended name 'cytokinin oxidase' to the group of flavin-containing amine oxidases, the other (EC 1.5.99.12) suggests to classify the enzyme as 'cytokinin dehydrogenase' that catalyses oxidation of cytokinins in the absence of oxygen without producing hydrogen peroxide during the catalytic reaction (Galuszka et al. 2001).

In spite of the impressive progress made in CKX research, little is known about the regulation of CKX activity in plant tissues and cells. It is apparent that the CKX in plant cells is subjected to multiple regulatory mechanisms, most of them depending directly on the concentration and/or compartmentation of cytokinins in the cell (reviewed in Armstrong 1994, Jones and Schreiber 1997, Kamínek et al. 1997). Presence of a signal sequence targeting CKX towards secretion which was found in maize CKX and its *Arabidopsis* homologues indicates that secretion of the enzyme to the cell exterior may be a part of mechanism(s) regulating CKX activity within and outside cells (see Haberer and Kieber 2002). It was shown that the activity of CKX was significantly increased in tobacco callus tissues exposed to exogenous (both substrate and non-substrate) cytokinins (Motyka and Kamínek 1990). Our previous study using conditionally cytokinin-overproducing tobacco plants revealed a positive CKX activity response to enhanced cytokinin accumulation (Motyka et al. 1996). Stimulation of CKX activity by endogenous or externally applied cytokinins has also been reported in other plants and plant cultures (Chatfield and Armstrong 1986, Kamínek and Armstrong 1990, Zhang et al. 1995, Redig et al. 1997, Auer et al. 1999).

It is thought that the control of CKX activity is dependent, at least in part, on glycosylation. The CKXs from most plant sources have been shown to be glycoproteins, as evidenced by the affinity of the enzyme for the lectin Concanavalin A (Con A; Chatfield and Armstrong 1988). However, glycosylation is not a global characteristic of the enzyme, since the occurrence of non-glycosylated CKX and/or CKX with a very low degree of glycosylation has been reported for several plants including tobacco (Kamínek and Armstrong 1990, Motyka and Kamínek 1994, Zhang et al. 1995). Two molecular forms of CKX with or without N-glycans were identified in cultured tissues of two different *Phaseolus* species (Kamínek and Armstrong 1990) and indicated diverse intracellular localization of the CKX isoforms.

In this study we investigated whether the regulation of CKX activity by endogenously overproduced and exogenously applied cytokinins is associated with changes in enzyme glycosylation in tobacco cell suspension cultures and tissues exhibiting different degree of organization. We show here that differences in the glycosylation of the enzyme occur following a cytokinin trigger, which may contribute to the regulation of CKX activity in tobacco plants. Furthermore we found changes in the secretion of the CKX protein, possibly as a consequence of the glycosylation, pointing to an apoplastic degradation pathway for cytokinins.

Materials and methods

Chemicals

Unless otherwise stated all chemicals were from Sigma Co., St Louis, USA. Cytokinins were purchased from Apex Organics, Honiton, Devon, UK; [^{3}H]iP was synthesized by Dr Hanuš, Institute of Experimental Botany, Prague, Czech Republic.

Plant material

Wild-type (WT) and transgenic conditionally isopentenyltransferase gene (*ipt*)-expressing (clone 35SoIPT-5/TetR, IPT) leaves, callus and cell cultures of tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) were used in this study. Construction of transgenic plants harbouring the *ipt* gene under transcriptional control of the tetracycline (Tc)-dependent modified 35S promoter (Gatz et al. 1992) as well as criteria for selection of *ipt* transformants have been described elsewhere (Faiss et al. 1997).

The callus cultures and cultivation conditions have been already described (Motyka et al. 1996). If not otherwise stated, 34-day-old calli (in the late exponential growth phase) were used for the investigations.

The WT and IPT cell suspension cultures were established from root-derived calli by transfer of 3-week-old tissue pieces into liquid MS medium supplemented with naphthalene-1-acetic acid (NAA; 1 mg l^{-1}) and N^6-benzylaminopurine (BA; 0.1 mg l^{-1}). Kanamycin monosulphate (100 mg l^{-1}) was added to the medium of IPT cultures. The cells and small cell clusters were cultured in darkness, in 250 ml flasks containing 100 ml of medium at 26°C and continuously shaken (120 r.p.m). For the determination of BA effects and/or the influence of the Tc-dependent *ipt* gene transcription on the CKX activity, small clumps of cells released from the calli during 1 week of cultivation were transferred to the media specified below.

BA treatments of tobacco tissues

The effect of BA application on the CKX activity in vivo was examined in both WT (BA-WT) and IPT (BA-IPT) tobacco leaves, calli and cell cultures. The BA treatment of the callus tissues was performed by a modified procedure described previously (Motyka and Kamínek

1990). An aqueous solution of BA (22.5 mg l^{-1}) was applied directly to the surface of callus pieces at a rate of 0.2 ml g^{-1} tissue FW. Following treatment, the calli were incubated for 24 h (or for specified time periods) at 26°C (16-h/8-h light/dark period).

The detached leaves were partly disrupted on the surface by a razor blade and treated with BA (2.25 mg l^{-1} in 50 m*M* Na-citrate buffer, pH 5.0) using a quick vacuum infiltration. The incubation proceeded for specified periods of time at 26°C (16-h/8-h light/dark period) in 16 cm Petri dishes containing 25 ml of the same BA solution. Distilled water or 50 m*M* Na-citrate buffer (pH 5.0) without BA were applied to the control WT (C-WT) and IPT (C-IPT) calli and leaves, respectively. After incubation, both callus and leaf tissues were immediately frozen in liquid nitrogen and stored at −70°C until analysis.

For the determination of BA effects on the CKX activity the WT and IPT cell cultures were incubated in darkness at 26°C under continuous shaking (120 r.p.m) for 4 days in MS liquid medium supplemented with NAA (1 mg l^{-1}) and BA (0.1 mg l^{-1}) . The control cultures were grown in the same media lacking BA. After incubation, the cells were separated from the medium by filtration and immediately frozen in liquid nitrogen. Aliquots of media were frozen in liquid nitrogen and stored at −70°C for analysis of secreted cytokinins. For determination of CKX activity the proteins from the medium were precipitated by the addition of ammonium sulphate to 80% saturation, kept for 20 h at 4°C and separated by centrifugation. The pellets were frozen by submersion in liquid nitrogen and stored at −70°C.

Derepression of *ipt* gene transcription

Derepression of the *ipt* gene expression in callus cultures by Tc application was carried out as described in Motyka et al. (1996). Tc was applied in water (2 mg l^{-1}) to the surface of calli (0.2 ml g^{-1} tissue FW). In the present study, Tc-derepressed IPT calli (Tc-IPT) were analysed 6 days after the Tc treatment.

The *ipt* gene was induced in detached leaves by a short vacuum infiltration of Tc solution (1 mg l^{-1} in 50 m*M* Na-citrate buffer, pH 5.6) as described in Gatz et al. (1992). The Tc-treated leaves were incubated for specified time intervals under identical conditions as corresponding BA-treated leaves. The tissue treatment with water or 50 m*M* Na-citrate buffer (pH 5.6) without Tc was carried out in the control (C-WT and C-IPT) calli and leaves, respectively. After incubation, callus and leaf tissues were frozen in liquid nitrogen and stored at −70°C.

Cell suspension cultures derived from clumps of cells released from IPT calli during their 1 week cultivation in liquid medium were used for determination of the effect of *ipt* gene expression on CKX activity. Suspensions were grown in liquid MS medium containing NAA (1 mg l^{-1}) and kanamycin monosulphate (100 mg l^{-1}) supplemented with Tc (2 mg l^{-1}). Trypan Blue solution (0.4%, Sigma) was used for the viability test (Phillips 1973). Incubation conditions and subsequent procedures were the same as for the BA-treated cultures.

In all measurements, the Tc-treated non-transformed WT tissues and cultures (Tc-WT) were used as controls to the Tc-IPT material.

Cytokinin analysis

Extraction and purification of cytokinins was performed as described by Dobrev and Kamínek (2002). Cytokinins were extracted with a solvent mixture of methanol:water:formic acid (15:4:1; v/v/v) at 5 ml g^{-1} FW overnight at −20°C. Deuterium-labelled cytokinin standards (50 pmol per sample) were added to the extraction medium. Extracts were centrifuged (15 000 *g*, 4°C, 20 min) and the pellet extracted once more with additional extraction solvent (5 ml) for 30 min. Pooled supernatants were forced through Si-C_{18} cartridge (Waters Co., Milford, MA, USA) and evaporated to near dryness with a rotary vacuum evaporator at 40°C. The residue was dissolved in 5 ml 1 *M* formic acid and applied to Oasis MCX cartridge containing 150 mg reversed-phase ion-exchange sorbent (Waters Co.). The MCX cartridge was washed with 5 ml 1 *M* formic acid followed by 5 ml methanol. Cytokinin nucleotides were eluted by 5 ml 0.35 *M* NH_4OH, whereas cytokinin bases, ribosides and glucosides were released with 5 ml 0.35 *M* NH_4OH in 60% methanol. The eluate containing cytokinin bases, ribosides and glucosides was evaporated to dryness using a Speed-Vac. The residue was dissolved in 100 µl 20% acetonitrile and after filtration through Nylon (0.2 µm) series 8000 microcentrifuge filtration device (Scientific Resources Inc., Eatontown, USA) was analysed by HPLC-MS.

The eluate containing cytokinin nucleotides was evaporated in a Speed-Vac to near dryness and dissolved in 2 ml 50 m*M* ammonium acetate buffer (pH 10). Calf-intestine alkaline phosphatase (Sigma) was added at 0.2 U g^{-1} FW. After incubation (37°C, 1 h) the sample solution was acidified to pH 6–7 (acetic acid), and passed through an Si-C_{18} cartridge. The retained cytokinin ribosides (the dephosphorylation products of cytokinin nucleotides) were eluted with 10 ml 50% (v/v) methanol. Following evaporation, the residue was dissolved in 100 µl 20% acetonitrile and, after filtration, analysed by HPLC-MS.

LC-MS analysis was carried out using Beckman 125-binary HPLC gradient pump and 507 autosampler coupled to Ion Trap Mass Spectrometer Finnigan MAT LCQ-MS^n equipped with an electrospray interface. The sample (10 µl) was injected onto an RP-C8 column (Merck KGaA, Darmstadt, Germany; Supersphere RP Select B, 2 m$M \times 250$ m*M*, 4 µm) and eluted with the linear gradient (mobile phase A: 0.001%, v/v, acetic acid in water, B: acetonitrile): 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

of 0.2 ml min^{-1}. Under these chromatographic conditions all analysed cytokinins including *N*- and *O*-glucosyl conjugates and *cis*Z(R) and Z(R) were separated.

Detection and quantification were performed 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 sheath and auxiliary gasses run at 96 and 6 units, respectively. The heated metal capillary temperature was maintained at 250°C and the capillary voltage at 2.5 V.

Cytokinin oxidase activity

The method of Chatfield and Armstrong (1986), as modified by Motyka and Kamínek (1994) was used to extract and measure the CKX activity. For the extraction of the protein, frozen plant material was homogenized in 0.1 *M* Tris-HCl buffer (pH 7.5). After filtration through a polyvinylpolypyrrolidone (Sigma) column, centrifugation and removal of nucleic acids by polyethyleneimine (Polymin P, Serva Feinbiochemica, Heidelberg, Germany), the proteins were precipitated by the addition of solid ammonium sulphate to 80% saturation. Protein concentrations were 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 to adenine as described elsewhere (Motyka et al. 1996). The reaction mixture contained 100 m*M* *N*-tris(hydroxymethyl)methyl-3-amine propanesulphonic acid (TAPS)-NaOH buffer (pH 8.5), 2 μ*M* [2-^{3}H]iP (7.4 Bq mol^{-1}) and enzyme preparations (equivalent to 0.06–0.27 mg protein g^{-1} FW for tissues and cells or 0.3–2.5 mg protein ml^{-1} for cultivation medium) in a total volume of 50 μl. In the case of the copper-imidazole-sensitized assay (Chatfield and Armstrong 1987), the TAPS-NaOH in the reaction mixture was substituted by 100 m*M* imidazole buffer (pH 6.0) containing 25 m*M* sodium acetate and 5 m*M* $CuCl_2$. After incubation at 37°C, the reaction was stopped by the addition of 120 μl 0.75 m*M* adenine and iP in 95% (v/v) cold ethanol and 10 μl 200 m*M* tetrasodium EDTA. Precipitated protein was removed by centrifugation and adenine and iP were separated by TLC on microcrystalline cellulose plates (Aldrich Co., Milwaukee, WI, 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 using Packard TRI-CARB 2500 TR scintillation counter.

The pH optimum of CKX activity was determined by running the standard enzyme assay in different buffers including 0.1 *M* MES-NaOH (for pH 5.0, 6.0 and 7.0), 0.1 *M* MOPS-NaOH (for pH 6.7, 7.2 and 7.8) and 0.1 *M* TAPS-NaOH (for pH 7.8, 8.5 and 9.0). The apparent K_m(iP) values of CKX preparations were determined using the copper-imidazole-sensitized assay and [2-^{3}H]iP as the substrate. The concentration range of [2-^{3}H]iP in the reaction mixture was 0.3–10 μ*M*. The apparent K_m(iP) values were calculated on the basis of Lineweaver-Burk double reciprocal plot of CKX activity as a function of iP concentration. The CKX activity of enzyme preparations determined by the copper-imidazole-sensitized assay was 4- to 20-fold higher as compared to the standard assay.

Con A-Sepharose 4B column chromatography

Con A-Sepharose 4B chromatography was carried out as described elsewhere (Motyka and Kamínek 1994, Motyka et al. 1996). Enzyme preparations equivalent to 10–30 mg protein were dissolved in 3 ml of 25 m*M* bis(2- hydroxyethyl)iminotris(hydroxymethyl)methane (BisTris)-HCl (pH 6.5) and loaded onto a Con A-Sepharose 4B (Sigma) column (0.75 × 7 cm, 3 ml bed volume) equilibrated with the same buffer containing $(NH_4)_2SO_4$ (200 m*M*), $CaCl_2$ (1 m*M*) and $MnCl_2$ (1 m*M*). The column was washed with 21 ml of 25 m*M* BisTris-HCl (pH 6.5) containing ammonium sulphate (200 m*M*) and eluted with 21 ml of the same solution supplemented with methylmannose (200 m*M*). 3 ml fractions were collected and tested for CKX activity, using the copper-imidazole-sensitized assay. The absorbance at 280 nm (A_{280}) of the fractions was measured using UNICAM 5625 UV/VIS spectrometer. Calibration with egg albumin showed that the capacity of the column was sufficient to bind all tobacco protein applied.

Glycoprotein analysis

Glycoprotein patterns were examined in tobacco tissues, cells and media before and after application of BA or Tc. The glycoprotein analyses were performed either directly upon protein samples extracted in the same way as for measurements of CKX activity or in fractions after Con A-Sepharose 4B affinity chromatography. The extracted proteins were dialysed against 50 m*M* Tris-HCl (pH 6.8), purified by acetone precipitation and solubilized in the standard electrophoretic sample buffer (Laemmli 1970). Protein concentration was measured as described in Schäffner and Weissmann (1973) and preparations equivalent to 10 μg protein were loaded in each lane. Proteins were subjected to 1-D electrophoresis on slabs of 12.5% polyacrylamide gel containing 0.1% (w/v) SDS in a discontinuous system according to Laemmli (1970). For *N*-glycoprotein detection, proteins were transferred by electroblotting from the gel onto a sheet of nitrocellulose and visualized with a Con A-peroxidase assay (Ochiai et al. 1985).

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 three replicates are presented. Statistical variations of results are specified in legends for tables and figures.

Results

Glycosylation of cytokinin oxidase in tobacco calli

The association of CKX activity with the enzyme glycosylation pattern 24 h after the exogenous application of BA to the IPT tobacco calli was determined and compared with that of non-treated control IPT tissue (0 h). The Con A-Sepharose 4B chromatography revealed that the majority of the CKX activity (84 and 59% of the total enzyme activity for C-IPT and BA-IPT derived enzymes, respectively) did not bind to the lectin (Fig. 1). The remaining enzyme activity (16 and 41% of the total for C-IPT and BA-IPT, respectively) was retained on the Con A affinity column and eluted by 200 m*M* methylmannose (Fig. 1). These results demonstrate an 11-fold increase in activity associated with the most frequent *N*-glycoform(s) of the enzyme in response to BA treatment. A similar change in distribution of CKX activity following BA application was found in WT calli (data not shown).

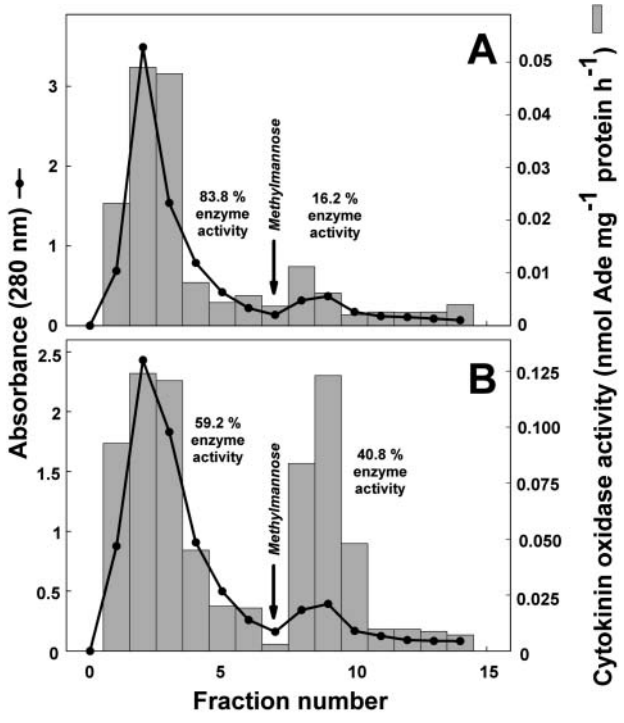

Fig. 1. The effect of exogenous application of BA solution (22.5 mg l^{-1}, 0.2 ml g^{-1} tissue FW) to the surface of *ipt*-transformed (IPT) tobacco callus upon binding of cytokinin oxidase activity to Con A-Sepharose 4B. The enzyme preparations from untreated (A) and BA-treated (B) IPT tobacco calli equivalent to 10 mg protein were dissolved in 3 ml of 25 m*M* BisTris-HCl (pH 6.5) and loaded onto the ConA-Sepharose 4B column (0.75 × 7 cm, 3 ml bed volume). The column was washed with 21 ml of 25 m*M* BisTris-HCl (pH 6.5) containing ammonium sulphate (200 m*M*) and eluted with 21 ml of the same solution supplemented with methylmannose (200 m*M*). Fractions of 3 ml were collected and tested for CKX activity, using the copper-imidazole-sensitized assay as described in Materials and methods.

Dynamics of cytokinin oxidase activity and its relation to glycosylation of the enzyme in tobacco leaves

The dynamics of CKX activity and its relation to level of glycosylated form of the enzyme exhibiting affinity to Con A in response to BA- and Tc-treatment were also determined in tobacco leaves. The aim of this experiment was to find out the effects on enzyme activity when cytokinin is applied exogenously or endogenously produced. It should also contribute to the information whether the same or similar regulatory mechanisms participate in the control of CKX activity in undifferentiated and differentiated tissues.

The effect of BA on CKX activity was determined in both WT and IPT leaves. As similar trends were found in both types of leaves only results for IPT are presented in Fig. 2. The BA treatment induced a considerable and rapid enhancement of CKX activity in IPT leaves. The significant increase was detected already 4 h after BA application (approximately 2.5-fold compared with untreated leaves) and reached the maximum (approximately 7-fold) 24 h after the beginning of treatment (Fig. 2A). A substantial proportion of BA-enhanced CKX activity was associated with the glycosylated form of the en-

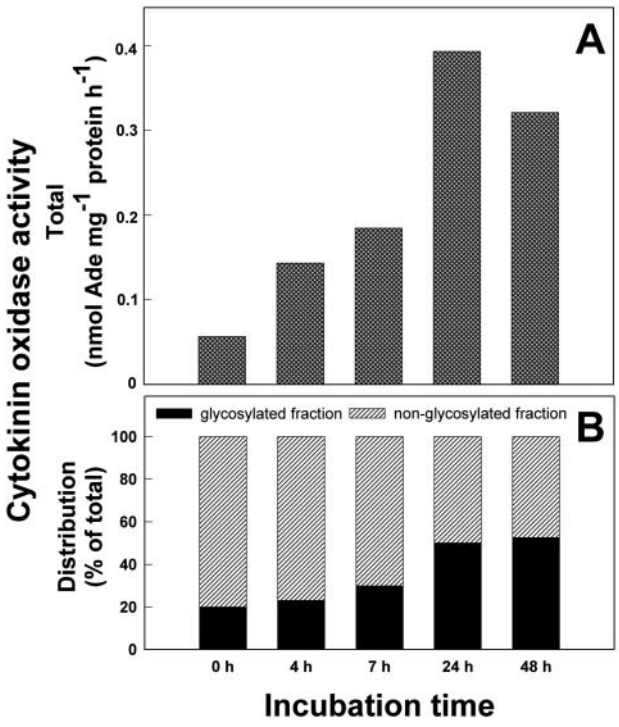

Fig. 2. Time course of cytokinin oxidase activity (A) and its glycosylation pattern (B) in *ipt*-transformed (IPT) tobacco leaves after exogenous application of BA. The BA solution (2.25 mg l^{-1} in 50 m*M* Na-citrate buffer, pH 5.0) was applied by a quick vacuum infiltration to detached leaves. CKX activity was determined by the standard assay, its glycosylation pattern was established after Con A-Sepharose 4B chromatography by the copper-imidazole-sensitized assay as described in Materials and methods. The values represent the means of three replicates. The SD values averaged 7% and did not exceed 16% of the mean.

zyme. The activity associated with the glycosylated fraction relative to the total enzyme activity increased from 20% (0 h) to 53% within the surveyed time interval (48 h). This represents a 15-fold enhancement compared to the 3-fold increase of activity associated with the non-glycosylated form (Fig. 2B). A control treatment of tissues with 50 m*M* Na-citrate buffer (pH 5.0) affected neither the total CKX activity nor its association with particular enzyme glycosylation pattern in both WT and IPT leaves (data not shown).

It was reported that derepression of *ipt* gene transcription significantly increased CKX activity in detached tobacco leaves (Motyka et al. 1996). As is shown in Fig. 3A, CKX activity in tobacco leaves was increased 24 h after Tc-induced *ipt* derepression and continued to rise for another 3 days. Similarly to the BA treatment, the increased CKX activity after the Tc application was associated predominantly with the glycosylated form of the enzyme. Con A-Sepharose 4B chromatography revealed an increase in the enzyme activity from 16% (0 h) to 56% (4 days) of the total (Fig. 3B) associated with the glycosylated fraction (i.e. bound to the lectin). It represents a 10-fold enhancement of the enzyme activity compared to a 1.5-fold increase of activity associated with the non-glycosylated form. Neither the treatment of both WT and IPT leaves with Na-citrate buffer lacking Tc nor the application of Tc to WT leaves had any effect on CKX activity or the CKX glycosylation pattern (data not shown).

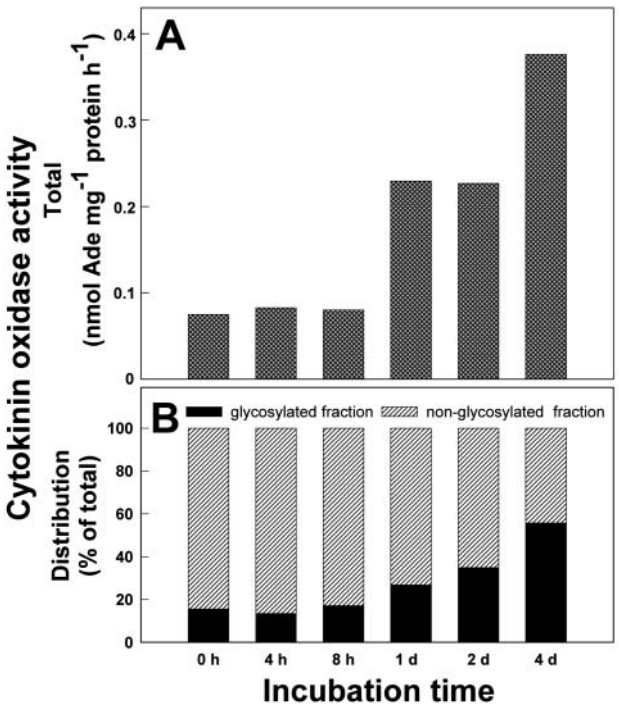

Fig. 3. Time course of cytokinin oxidase activity (A) and its glycosylation pattern (B) in *ipt*-transformed (IPT) tobacco leaves after derepression of the *ipt* gene transcription. Induction of the *ipt* gene was performed by a quick vacuum infiltration of detached leaves with Tc solution (1 mg l^{-1} in 50 m*M* Na-citrate buffer, pH 5.6). CKX activity was determined by the standard assay, its glycosylation pattern was established after Con A-Sepharose 4B chromatography by the copper-imidazole-sensitized assay as described in Materials and methods. The values represent the means of three replicates. The SD values averaged 8% and did not exceed 18% of the mean.

Regulation of cytokinin content and cytokinin oxidase activity in tobacco cell cultures

Changes in the content of endogenous cytokinins and in CKX activity after the exogenous application of BA and/or after the derepression of the Tc-dependent *ipt* gene transcription were determined in WT and IPT tobacco cell suspension cultures. When cultured in liquid MS medium supplemented with BA, both IPT and WT cells exhibited a similar spherical phenotype. Cells forming small clumps at the beginning of the subculture interval gradually divided, grew and finally formed big and tight clusters. The Tc-induced derepression of the *ipt* gene transcription resulted in a distinctive sealing together of the cell clusters that contained smaller individual cells compared to the control with the inactive *ipt* gene. The FW increase after 4 days incubation was approximately 4-times lower in Tc-IPT cells compared to the C-IPT and BA-IPT ones. Testing of cell viability with Trypan Blue showed no differences between individual variants and excluded the possibility of Tc-toxicity at the concentrations used (2 mg l^{-1}, data not shown).

Using a combination of HPLC and mass spectrometry, endogenous cytokinin contents were determined in IPT cell suspension cultures. The levels and abbreviations of 18 isoprenoid cytokinins in cells and media of control as well as of BA- and Tc-treated suspension cultures are presented in Table 1. The application of BA (0.1 mg l^{-1}) to the culture media of IPT cell suspension led to 5-fold enhancement of total cytokinin contents in the cells, however, no increase in total cytokinin levels was found in the medium. Zeatin- and dihydrozeatin (DHZ)-type cytokinins (ZRMP, DHZRMP and, to a lesser extent Z7G, ZR and Z) were particularly responsible for the increase in total cytokinin contents in BA-IPT cells. On the contrary, the level of ZROG was decreased. The levels of analysed cytokinins in BA-treated medium were similar to the controls, being relatively low with a high proportion of Z (Table 1). The application of Tc to the media of the IPT cell suspension resulted in a substantial increase in total cytokinin content in both cells and the media (288-fold and 35-fold, respectively, compared to the control). Following Tc application, Z and DHZ were the major cytokinins detected in the media, while corresponding ribosides (ZR, DHZR), their *O*-glucosides (ZROG, DHZROG), 7-glucosides (Z7G, DHZ7G) and DHZ nucleotide (DHZRMP) accumulated predominantly in cells. The contents of iP-type cytokinins in both cells and media were much lower than those of Z- and DHZ-type (Table 1).

The application of BA to the culture media of both WT and IPT cell suspensions significantly increased

Table 1. Endogenous cytokinin levels in cells and cultivation medium of *ipt*-transformed (IPT) tobacco cell suspension cultures following BA and Tc application. Tobacco cell suspensions were cultivated in liquid MS medium supplemented with NAA (1 mg l^{-1}) and kanamycin monosulphate (100 mg l^{-1}). The BA or Tc solutions were applied to the medium of appropriate variants to final concentrations 0.1 and 2 mg l^{-1}, respectively. The incubation proceeded at 26°C in darkness (shaking at 120 r.p.m) for 4 days. Cytokinin contents were determined by a combination of HPLC-MS as described in Materials and methods. The data represent the means of three replications. The SD values averaged 22% and did not exceed 43% of the mean.

	Cytokinin content					
	in cells (pmol g^{-1} FW)			in medium (pmol ml^{-1})		
Cytokinin	CONTROL	+BA	+Tc	CONTROL	+BA	+ Tc
iP	1.6	1.2	21.3	0.04	0.09	1.75
iPR	ND[a]	ND	14.2	0.01	0.01	0.04
iP7G	81.6	77.7	748.4	0.03	0.09	0.15
iP9G	ND	ND	0.8	ND	ND	ND
iPRMP	3.6	2.8	62.0	0.01	0.02	0.04
Z	4.5	8.0	1476.6	7.05	6.79	204.61
ZR	44.0	157.4	27414.0	ND	0.33	10.64
Z7G	2.0	13.1	9108.0	0.04	0.04	1.96
Z9G	ND	ND	23.5	ND	ND	1.14
ZOG	0.1	1.7	6.3	0.49	ND	ND
ZROG	49.0	5.6	2990.5	ND	0.01	0.17
ZRMP	12.1	755.3	ND	0.67	0.84	10.11
DHZ	1.5	ND	165.0	0.01	ND	59.84
DHZR	1.1	1.6	5456.0	ND	0.04	3.55
DHZ7G	ND	3.0	2251.0	0.02	0.03	0.13
DHZ9G	ND	ND	5.2	ND	ND	0.02
DHZROG	1.5	2.8	2804.0	ND	ND	0.11
DHZRMP	2.3	34.5	6371.5	0.01	0.03	0.32
Total	204.9	1064.7 (*5-fold*[b])	58924.7 (*288-fold*[b])	8.38	8.32 (–[c])	294.59 (*35-fold*[b])

Abbreviations: iP, N^6-(Δ^2-isopentenyl)adenine; iPR, N^6-(Δ^2-isopentenyl)adenine 9-riboside, iP7G, N^6-(Δ^2-isopentenyl)adenine 7-glucoside; iP9G, N^6-(Δ^2-isopentenyl)adenine 9-glucoside; iPRMP, N^6-(Δ^2-isopentenyl)adenine 9-riboside-5′-monophosphate; Z, *trans*-zeatin; ZR, *trans*-zeatin 9-riboside; Z7G, *trans*-zeatin 7-glucoside; Z9G, *trans*-zeatin 9-glucoside; ZOG, *trans*-zeatin *O*-glucoside; ZROG, *trans*-zeatin 9-riboside *O*-glucoside; ZRMP, *trans*-zeatin 9-riboside-5′-monophosphate; DHZ, dihydrozeatin; DHZR, dihydrozeatin 9-riboside; DHZ7G, dihydrozeatin 7-glucoside; DHZ9G, dihydrozeatin 9-glucoside; DHZROG, dihydrozeatin 9-riboside *O*-glucoside; DHZRMP, dihydrozeatin 9-riboside-5′-monophosphate; according to Kamínek et al. (2000).
[a]ND, not detected.
[b]Increase in total cytokinin content compared to the control.
[c]No increase.

CKX activity in both the cells and culture medium (Table 2). The CKX activity in BA-treated cells (both WT and IPT) was enhanced 6-fold compared to the non-treated control, while 14- and 10-fold enhancement of the enzyme activity was found in the media of WT and IPT cultures, respectively (Table 2). Analogous results were obtained following the application of kinetin (0.1 mg l^{-1}) instead of BA (data not shown).

Similarly, the derepression of *ipt* gene transcription after Tc application to the transgenic IPT suspension cultures was followed by a significant increase in CKX activity in both cells (6-fold) and media (15-fold) (Table 2). The Tc-treatment of the non-transformed WT cultures did not significantly affect the CKX activity in either cells or media.

Similar results, including BA- and Tc-induced enhancement of CKX activity in both cells and media of tobacco suspension cultures, were found when a Cu^{2+}-imidazole-sensitized assay was used for the determination of CKX activity (Fig. 4A). As the presence of copper-imidazole resulted in a significant increase of CKX activity (6- and 13-fold in IPT cells and media, respectively), the copper-imidazole-sensitized assay was used to measure the enzyme activity in Con A-Sepharose 4B chromatography fractions. Most of the CKX activity in both IPT control (75%) and BA- and Tc-treated cells (85 and 75%, respectively) was associated with the non-glycosylated form of the enzyme (Fig. 4B). Conversely, the majority of CKX activity in the IPT media was as-

Table 2. Cytokinin oxidase activity in cells and cultivation medium of WT and IPT tobacco cell suspension cultures following BA and Tc application. Tobacco cell suspensions were cultivated in liquid MS medium supplemented with NAA (1 mg l^{-1}) and kanamycin monosulphate (100 mg l^{-1}, only IPT cultures). The BA or Tc solutions were applied to the medium of appropriate variants to final concentrations 0.1 and 2 mg l^{-1}, respectively. The incubation proceeded at 26°C in darkness (shaking at 120 r.p.m) for 4 days. Cytokinin oxidase activity in both cells and medium was determined in the standard assay mixture containing 2 μ*M* [2-^{3}H]iP, 100 m*M* TAPS-NaOH buffer (pH 8.5) and protein preparation in a total volume of 50 μl. The values represent the means of three replicates. The SD values averaged 8% and did not exceed 17% of the mean.

		Cytokinin oxidase activity (nmol Ade mg^{-1} protein h^{-1})	
Plant material	Treatment	Cells	Medium
WT	0 (control)	0.020	0.021
	+ BA	0.121	0.294
	+ Tc	0.021	0.023
IPT	0 (control)	0.021	0.026
	+ BA	0.122	0.261
	+ Tc	0.126	0.389

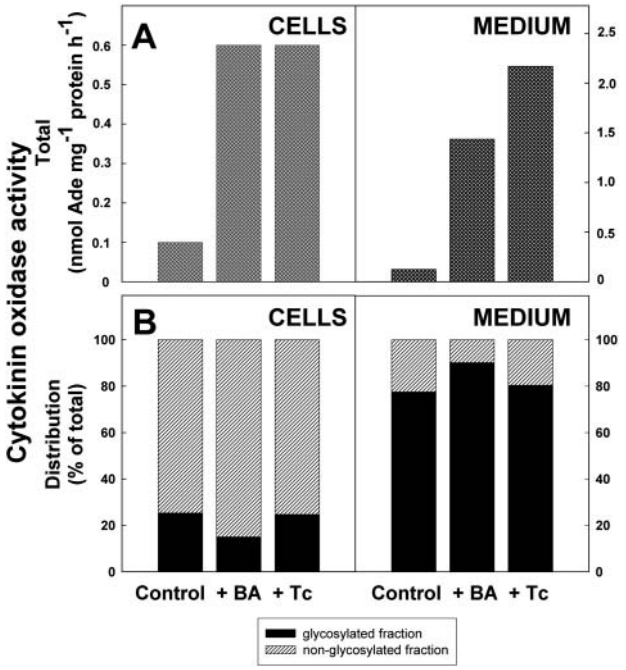

Fig. 4. Activity (A) and glycosylation pattern (B) of cytokinin oxidase in cells and cultivation medium of *ipt*-transformed (IPT) tobacco cell suspension cultures. Cell suspensions were cultivated in liquid MS medium supplemented with NAA (1 mg l^{-1}) and kanamycin sulphate (100 mg l^{-1}) . The BA or Tc solutions were applied to the medium of appropriate variants to final concentrations 0.1 and 2 mg l^{-1}, respectively. The incubation proceeded at 26°C in darkness (shaking at 120 r.p.m) for 4 days. CKX activity and its glycosylation pattern after Con A-Sepharose 4B chromatography in both cells and medium were determined by the copper-imidazole-sensitized assay as described in Materials and methods. The values represent the means of three replicates. The SD values averaged 6% and did not exceed 13% of the mean.

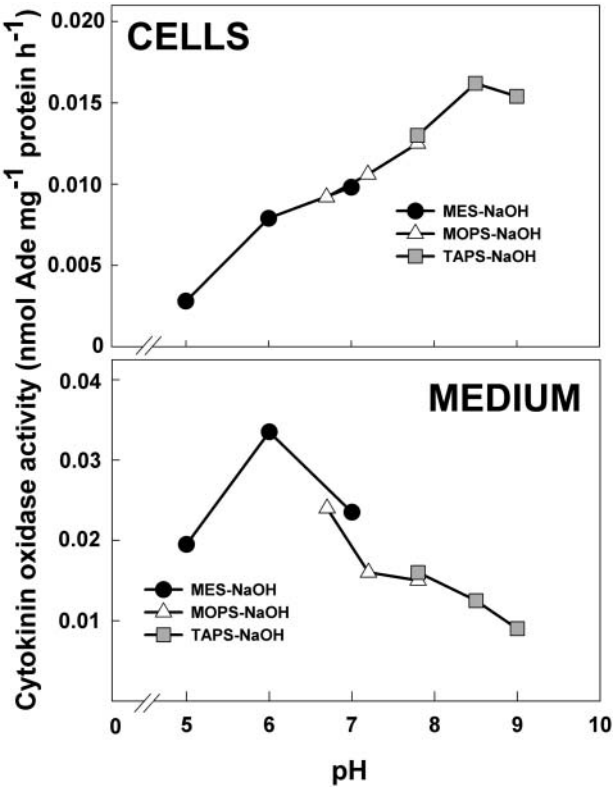

Fig. 5. Effect of pH on the *in vitro* activity of cytokinin oxidase from *ipt*-transformed (IPT) tobacco cells and cultivation medium. The pH optimum for cytokinin oxidase activity was determined by running the standard enzyme assay in different buffers including 0.1 *M* MES-NaOH, 0.1 *M* MOPS-NaOH and 0.1 *M* TAPS-NaOH between pH 5.0 and pH 9.0; other details are described in Materials and methods. The values represent the means of three replicates. The SD values averaged 5% and did not exceed 12% of the mean.

sociated with the glycosylated form of the enzyme (78% in the control and 90 and 81%, respectively, in BA- and Tc-treated media) indicating secretion of the glycoform of CKX from cells to the media (Fig. 4B).

The pH optimum of CKX in cells was relatively high (pH 8.5) and differed considerably from that in the culture medium (pH 6.0) (Fig. 5). On the other hand, no significant differences were found in apparent K_m(iP) values of CKX between IPT cells and media or between corresponding non-glycosylated and glycosylated fractions after Con A-Sepharose 4B chromatography (1–3 μ*M* for iP as substrate). Induction of CKX activity after BA treatment and/or after Tc-induced derepression of the *ipt* gene did not significantly affect the affinity of the enzyme for the substrate iP (Table 3).

Glycoprotein patterns in tobacco leaves, calli and cell cultures

The glycoprotein analysis (1-D SDS-PAGE followed by W-blotting and Con A-peroxidase assay) revealed broad glycoprotein spectra across a range of 20–90 kDa with different proportions of glycoproteins in tobacco leaves, calli, cells and media. Partially quantitatively balanced glycoprotein spectra differed between calli and leaves as well as between cells and the corresponding media (data not shown). Both WT and IPT preparations were found to express the glycoproteins in a specific manner and to exhibit different glycoprotein spectra. However, no new specific glycoprotein was detectable in response to the enhancement of CKX activity after BA or Tc application in leaves, calli, cells or media (data not shown).

Discussion

CKX is the plant enzyme known to inactivate cytokinins by irreversible degradation. This widely distributed enzyme constitutes a crucial point in controlling cytokinin levels in plant cells. Notwithstanding the recent progress in CKX research (Houba-Hérin et al. 1999, Morris et al. 1999, Bilyeu et al. 2001, Galuszka et al. 2001, Werner et al. 2001), present knowledge of the physiological role, function and regulation of CKX in plants is still very limited. The CKX glycosylation pattern and its secretion may be involved in the control of CKX activity and its physiological role in plant cells and tissues.

Table 3. The apparent K_m(iP) values of cytokinin oxidase preparations from *ipt*-transformed (IPT) tobacco cells, cultivation medium and corresponding non-glycosylated and glycosylated fractions separated by Con A-Sepharose 4B chromatography. The Michaelis constants for iP were determined using the copper-imidazole-sensitized assay as described in Materials and methods. The concentration range of [2-^{3}H]iP in the reaction mixture was 0.3–10 μ*M*; three repetitions were performed for each substrate concentration. The SD values averaged 10% and did not exceed 27% of the mean.

		K_m(iP) (μ*M*)		
		CONTROL	+ BA	+ Tc
Cells		2.2	2.3	1.5
Medium		2.5	1.7	1.0
Fractions afer Con A-Sepharose 4B chromatography:				
Cells:	non-glycosylated fraction	1.2	1.7	3.0
	glycosylated fraction	1.0	1.0	2.9
Medium:	non-glycosylated fraction	1.0	2.3	1.7
	glycosylated fraction	2.8	2.2	3.0

Regulation of CKX activity was studied in wild-type and transgenic conditionally *ipt* gene-expressing tobacco leaves, callus and cell suspension cultures. Consistent with our previous data, the exogenous application of BA as well as the increase in endogenous cytokinin content due to the Tc-induced derepression of *ipt* gene transcription significantly enhanced the CKX activity in both tobacco calli and leaves (Figs 1,2A and 3A). A similar effect was found in tobacco cell suspension cultures after the application of BA (in both WT and IPT suspensions) and/or Tc (in IPT suspensions only) to the media (Table 2, Fig. 4). The stimulation of cytokinin degradation in plant cells in vivo after exposure to exogenous cytokinins has been demonstrated in several tissue culture systems (Terrine and Laloue 1980, Palmer and Palni 1987). This phenomenon is thought to be mediated by the promotion of CKX activity in response to both substrate and non-substrate external cytokinins (Chatfield and Armstrong 1986, Kamínek and Armstrong 1990, Motyka and Kamínek 1990, Redig et al. 1997, Auer et al. 1999). Recent studies revealed that CKX activity may also be enhanced by endogenous cytokinins overproduced in plant cells transformed by the cytokinin biosynthetic *ipt* gene having a native (Zhang et al. 1995) or conditionally induced promoter (Motyka et al. 1996, Redig et al. 1997).

We report here a substantial increase in endogenous isoprenoid cytokinins in suspension-cultured tobacco cells after the application of exogenous BA and/or after the Tc-induced derepression of *ipt* gene transcription. Following the Tc-treatment, elevated levels of isoprenoid cytokinin bases were also found in the cultivation media (Table 1) suggesting an increased secretion of cytokinins from cells. This finding corresponds with the recently reported excretion of cytokinins to the cultivation medium by suspension-cultured cytokinin-autonomous tobacco VBI-0 cells (Petrášek et al. 2002) and with the high accumulation of cytokinins in the medium of cytokinin-overproducing *Physcomitrella patens* mutants (Wang et al. 1981, Reutter et al. 1998, Schulz et al. 2001). In both cells and media we detected primarily the Z-type (low affinity to CKX) and the DHZ-type (non-substrates of CKX) cytokinins. A clear difference in the concentration ratios of cytokinin ribosides to the corresponding bases was found for cells versus medium; the Z- and DHZ-ribosides were prevailing forms in the cells while their bases predominated in the medium. This agrees with a presumed translocation of cytokinin bases from cells to the extracellular space across the plasma membrane which is impermeable to the ribosides (Laloue and Pethe 1982, Schulz et al. 2000, 2001). The levels of the iP-type substrate cytokinins were much lower than those of Z- and DHZ-type. The high proportion of iP7G and iPRMP compared to iP and iPR in Tc-treated cells probably resulted from their partial or complete resistance to degradation by CKX. It has been suggested that the induction of CKX activity by both exogenous and endogenous cytokinins contribute to the re-establishment and maintenance of cytokinin homeostasis in plant cells (Kamínek et al. 1997, Zažímalová et al. 1999). On the other hand, there are some indications that the relative importance of cytokinin oxidation in planta may have been overestimated and that the production of cytokinin 7-glucosides may also represent an important metabolic pathway for maintaining balance of biologically active cytokinins in tobacco cells (Eklöf et al. 1996). In this respect it is interesting that we found much higher contents of 7-glucosides of iP, Z and DHZ in both tobacco cells and media compared to corresponding 9-glucosides, which were almost or totally absent. It suggests that cytokinin 7- and 9-glucosides may play different roles in metabolic inactivation of isoprenoid cytokinins in tobacco, however, the physiological significance needs further investigation.

The Con A-Sepharose 4B chromatography showed that the cytokinin-induced increase of CKX activity in both WT and IPT tobacco calli and leaves was predominantly associated with the glycosylated form of the enzyme. A similar pattern of enhancement of CKX activity was found in both types of tissues following the exogenous BA treatment and the endogenous cytokinin overproduction after Tc-induced *ipt* derepression (Figs 1,2B and 3B). Preferred induction of the glycosylated form of CKX compared to the non-glycosylated form was also found to be responsible for the enhancement of the enzyme activity in senescing barley leaves (V. Motyka, unpublished results). These data suggest that the control of CKX activity in response to changes of cytokinin levels is governed in differentiated and undifferentiated plant tissues by similar or identical mechanisms including CKX glycosylation. It looks like that this does not imply to cells suspensions where exists artificially increased imbalance between the size of cell interior and exterior which may significantly affect the enzyme secretion.

Although the CKX proteins are generally classified as glycoproteins (reviewed in Armstrong 1994, Galuszka et al. 2000), the majority of tobacco CKX has no affinity for the lectin Con A (Motyka and Kamínek 1994,

Zhang et al. 1995, Motyka et al. 1996). It indicates that the form prevailing in tobacco is unglycosylated that significantly differs from most other CKXs examined to date. The role and function of the induction of CKX glycosylation by cytokinins is not known. It is possible that the presence of a glycan affects compartmentation of the enzyme in plant cells and, thus, its access to the substrate. Two molecular forms of CKX differing in their glycosylation pattern were identified in cultured tissues of two *Phaseolus* species. It was suggested that these forms had different subcellular compartmentation with the glycosylated form in the cell wall or secreted to the cell exteriors and the unglycosylated form in an internal compartment (Kamínek and Armstrong 1990). The presence of a signal sequence in the recently cloned *ckx* gene (Houba-Hérin et al. 1999, Morris et al. 1999, Bilyeu et al. 2001) targeting the enzyme toward secretion and the high proportion of the glycosylated form of CKX secreted to the cultivation media of tobacco cell suspension reported here supports this hypothesis. It also indicates that at least some tobacco CKXs, which have not been cloned so far, are also targeted towards secretion.

The glycoprotein spectra differed between calli and leaves as well as between cells and the corresponding media. However, no new specific glycoprotein was detected in response to the BA- or Tc-induced enhancement of CKX activity. The analysis of glycoproteins in fractions from the Con A-Sepharose 4B column revealed that the glycosylation of the induced form of CKX is of the *N*-type (data not shown). A close connection between *N*-glycosylation of CKX and its function is obvious. It is known that glycan moieties can affect the biological activity of proteins in many ways – such as protection against proteolysis, directing compartmentalization and translocation, and dictating the function. Removal of the *N*-glycan from ascorbate oxidase (D'Andrea et al. 1993) and *N*-acetylgalactosaminidase (Zhu et al. 1998) was found to significantly affect the enzyme activity. Moreover, mutation of any of the three *N*-glycosylation sites of *N*-acetylgalactosaminidase impaired the expression level and altered subcellular distribution of mutated molecules (Zhu et al. 1998). Similarly, the role of the glycan moiety in the CKX molecule may function in allocating the enzyme to the specific compartment. Large differences in pH optima between glycosylated and non-glycosylated forms of CKX from different *Phaseolus* species (Kamínek and Armstrong 1990) and from tobacco cells and their cultivation media, as reported here (Fig. 5), support this opinion and indicate a close connection between the pH optimum and the glycosylation pattern of the enzyme in plants. On the other hand, no significant dependence was found here between apparent K_m(iP) values of CKX and its glycosylation (Table 3), probably due to the relatively long distance of the active centre of the enzyme from *N*-glycosylation sites.

Exogenous application of BA as well as derepression of the *ipt* gene by Tc enhanced CKX activity not only in cells, but also in the cultivation medium (Table 2, Fig. 4A). Apparently, the stimulatory effect of cytokinin on CKX activity also included enhancement of enzyme secretion to the media. Similarly, high CKX activities were detected in the culture media of *Physcomitrella patens* protoplasts (Houba-Hérin et al. 1999) and yeasts *Pichia pastoris* and *Saccharomyces cerevisiae* (Morris et al. 1999, Bilyeu et al. 2001, Werner et al. 2001). The secretion of CKX suggests an extracellular regulation of cytokinin levels in the course of their apoplastic transport and/or upon their arrival at the plasma membrane. The increase in extracellular CKX activity can be taken as a first hint of an extracellular route(s) in cytokinin signalling. In addition to the reported targeting of CKX towards secretion, CKX might also be targeted to chloroplasts as indicated by the recent detection of CKX activity associated with these organelles (Benková et al. 1999).

Our data suggest that cytokinin-induced regulation of the glycosylation pattern of the CKX protein and consequent changes in subcellular compartmentation and secretion of the enzyme represent an intrinsic mechanism controlling cytokinin pools in plant symplast and apoplast.

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