Changes in Cytokinin Content and Cytokinin Oxidase Activity in Response to Derepression of ipt Gene Transcription in Transgenic Tobacco Calli and Plants

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Metabolic control of cytokinin oxidase by its substrate was investigated in planta using wild-type (WT) and conditionally ipt gene-expressing transgenic (IPT) tobacco (Nicotiana tabacum L.) callus cultures and plants. The derepression of the tetracycline (Tc)-dependent ipt gene transcription was followed by a progressive, more than 100-fold increase in total cytokinin content in IPT calli. The activity of cytokinin oxidase extracted from these calli began to increase 16 to 20 h after gene derepression, and after 13 d it was 10-fold higher than from Tc-treated WT calli. An increase in cytokinin oxidase activity, as a consequence of elevated cytokinin levels, was found in detached leaves (8-fold after 4 d) and in roots of intact plants (4-fold after 3 d). The partially purified cytokinin oxidase from WT, repressed IPT, and Tc-derepressed IPT tobacco calli exhibited similar characteristics. It had the same broad pH optimum (pH 6.5–8.5), its activity in vitro was enhanced 4-fold in the presence of copper-imidazole, and the apparent $K_m$ values were in the range of 3.1 to 4.9 μM. The increase in cytokinin oxidase activity in cytokinin-overproducing tissue was associated with the accumulation of a glycosylated form of the enzyme. The present data indicate the substrate induction of cytokinin oxidase activity in different tobacco tissues, which may contribute to hormone homeostasis.

Cytokinins are a class of plant hormones that are able to trigger cell division in plant tissue culture in the presence of auxin (Skog and Miller, 1957) and are also able to promote or inhibit different growth and developmental processes in whole plants, such as growth of axillary meristems or leaf senescence (Mok, 1994). Understanding of the metabolic regulation of the cytokinin content in plant cells should contribute to a better understanding of regulatory circuits involved in the equilibration of hormone levels as well as corresponding physiological or developmental effects.

There are two main pathways for irreversible metabolic inactivation of cytokinins in plant cells: formation of N-conjugates by specific glucosyltransferases and the oxidative N-side-chain cleavage by the cytokinin oxidase (Letham and Palni, 1983; Kamínek, 1992; Brzobohatý et al., 1994). Cytokinins are known as the key enzyme of cytokinin degradation in plants. It catalyzes the oxidation of cytokinins bearing unsaturated isoprenoid side chains, using molecular oxygen as the oxidant (Armstrong, 1994; Hare and Van Staden, 1994). Cytokinin oxidases from different plant species exhibit the same substrate specificity; however, they differ in their molecular weights, pH optima, kinetic constants, and the degree to which their activity is stimulated by the copper-imidazole complex in vitro (Kamínek, 1992; Armstrong, 1994). These differences may be due in part to a different degree of protein glycosylation, which may affect compartmentation of the enzyme in plant cells and, therefore, their access to the substrate. Variation in molecular forms of cytokinin oxidase was found in closely related genotypes (Kamínek and Armstrong, 1990; Motyka et al., 1994).

Indications that the regulatory mechanisms controlling cytokinin degradation and cytokinin oxidase activity in plant tissues are influenced by the hormone have been

Abbreviations: BAP, N6-benzylaminopurine; C-IPT, control ipt-transformed plants (tissues) with an inactive ipt gene (i.e. after water application); Con A, concanavalin A; C-WT, control wild-type plants (tissues) after water application; DHZ, dihydrozeatin; DHZOG, dihydrozeatin O-glucoside; DHZRP, dihydrozeatin riboside; DHZ9G, dihydrozeatin 9-glucoside; DHZROG, dihydrozeatin riboside O-glucoside; DHZRP, dihydrozeatin riboside 5'-monophosphate; IAC, immunoaffinity chromatography; iP, N6-(Δ2-isopentenyl)adenine; iPRG, N6-(Δ2-isopentenyl)adenine 9-glucoside; iPRP, N6-(Δ2-isopentenyl)adenine riboside; iPRP, N6-(Δ2-isopentenyl)adenine riboside 5'-monophosphate; ipt, isopentenytransferase gene; IPT, ipt-transformed plants (tissues); NAA, naphthalene-1-acetic acid; TAPS, N-tris(hydroxymethyl)-methyl-3-aminopropylsulfonic acid; Tc, tetracycline; Tc-IPT, ipt-transformed plants (tissues) with Tc-induced ipt gene (i.e. after Tc application); Tc-WT, wild-type plants (tissues) after Tc application; WT, wild-type plants (tissues); Z, trans-zeatin; ZOG, zeatin O-glucoside; ZR, zeatin riboside; ZROG, zeatin riboside O-glucoside; ZRG, zeatin 9-glucoside; ZRP, zeatin riboside 5'-monophosphate.

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been employed in a number of studies on cytokinin metabolism and action in various tissues and at different stages of the gene, inducible or tissue-specific promoters have caused pleiotropic physiological and developmental changes (see Klee, 1994). To overcome problems that are caused by a constitutive expression of the gene, inducible or tissue-specific promoters have been employed in a number of studies on cytokinin metabolism and action in various tissues and at different developmental stages (for reviews, see Binns, 1994; Brzobohaty et al., 1994; Gaudin et al., 1994).

In this study we used transformed tobacco harboring the ipt gene under the transcriptional control of the Tc-dependent modified 35S promoter (Gatz et al., 1992). The aim of our study was to answer two questions: (a) does cytokinin oxidase from the nontransformed and transformed plant tissues expressing the ipt gene exhibit the same biochemical characteristics? and (b) is the enhanced accumulation of endogenous cytokinins in transformed plant tissues after expression of the ipt gene accompanied by changes of cytokinin oxidase activity? We report here on the regulation of cytokinin oxidase activity under conditions of enhanced endogenous cytokinin production, resulting from the Tc-induced derepression of the ipt gene in undifferentiated callus tissue as well as different tissues of transgenic tobacco plants. The results suggest a very distinct role for the cytokinin oxidase in regulation of cytokinin content in plant cells.

**MATERIALS AND METHODS**

WT and IPT (clone 35SolPT-5/TetR) plants and callus cultures of tobacco (*Nicotiana tabacum* L. cv Wisconsin 38) were used in this study. Construction of transgenic plants and growth conditions for the selection of ipt transformants will be specified elsewhere (M. Faiss, J. Zalubilová, M. Strnad, and T. Schmülling, unpublished data).

Both WT and IPT callus cultures originated from root tissues. The ipt-transformed calli were derived from F1 plants of a segregating population selected on medium containing kanamycin sulfate (100 mg L⁻¹) and hygromycin (15 mg L⁻¹) and were screened for an in vitro plant phenotype similar to the WT. The callus tissues were cultivated on solid medium (Murashige and Skoog, 1962) supplemented with Suc (30 g L⁻¹), NAA (1 mg L⁻¹), and kinetin (0.2 mg L⁻¹). Kanamycin sulfate (100 mg L⁻¹) and cefotaxime (Duchefa, Haarlem, The Netherlands) (250 mg L⁻¹) were added to the medium of IPT calli. The callus cultures were grown at a 16-h light/8-h dark period at 26°C and subcultured at 4- to 5-week intervals. For investigations we used 34-d-old cultures, which were in the late exponential growth phase.

**Derepression of ipt Gene Transcription**

Regulation of ipt gene expression in callus cultures was examined after applying Tc solution (2 mg L⁻¹ water) in a final volume of 3 mL to the surface of Tc-WT and Tc-IPT tissues 34 d after subculturing. Distilled water without Tc was applied to the control C-WT and C-IPT calli. After incubation (26°C, 16-/8-h light/dark period) for specified periods of time, the tissues were immediately frozen by submersion in liquid nitrogen and stored at -70°C until analysis.

Gene induction in detached leaves was performed by a short vacuum infiltration of Tc solution (1 mg L⁻¹) in 50 mM citrate buffer (pH 5.6) according to Gatz et al. (1992). Induction of the ipt gene in adult plants grown under hydroponic conditions was performed by application of Tc (1 mg L⁻¹) in liquid Hoagland solution (Draper et al., 1988). Different plant tissues were harvested from the whole plants immediately after Tc treatment and 3 d after derepression.

**Cytokinin Analysis**

Cytokinin extraction and purification were carried out by a method that will be described elsewhere (M. Faiss, J. Zalubilová, M. Strnad, and T. Schmülling, unpublished data). After homogenization of the sample in a modified Bieleski solution (Bieleski, 1964), removal of the debris by centrifugation, and removal of the pigments by passage through a C₁₈ cartridge (Waters), the samples were evaporated in vacuo and separated by DEAE-cellulose chromatography into two fractions: fraction B, containing cytokinin bases, nucleosides, and 3-, 9- and O-glucosides, and fraction NT, containing nucleotides. Both fractions were loaded directly onto a monoclonal- and polyclonal-based IAC column, respectively. The O-glucosides present in fraction B were not retained on the monoclonal IAC column and were purified on IAC after hydrolysis by β-glucosidase treatment. After the IAC columns were washed, the cytokinins were eluted with methanol and separated by reverse-phase HPLC. Individual HPLC fractions were analyzed by ELISA (Strnad et al., 1990, 1992). Cytokinin concentrations higher than 1 to 3 pmol g⁻¹ fresh weight were confirmed by photodiode-array HPLC detection.
tion and the respective cytokinin metabolites were identified by GC-mass spectrometry. Electron-impact mass spectra were obtained at an electron energy of 70 eV (Strnad et al., 1994).

Northern Blot Analysis

Total RNA was extracted from calli at different times after Tc application according to Verwoerd et al. (1989). Fifty micrograms of RNA was separated in a 1.5% denaturing agarose-formaldehyde gel, blotted onto a Hybond N membrane (Amersham), and hybridized with a radioactively labeled ipt-specific probe. The lowest-stringency wash was with 0.1× SSC, 0.1% SDS at 65°C. As a control for loading, the blot was rehybridized with a 25S rDNA probe.

Cytokinin Oxidase Activity

The cytokinin oxidase activity was determined using the method of Chatfield and Armstrong (1986) as modified by Motyka and Kamíněk (1994). For the extraction of cytokinin oxidase activity, the plant tissues were homogenized in 100 mM Tris-HCl buffer (pH 7.5) and immediately mixed with the acid-treated PVP. After filtration through a PVP column, centrifugation, and removal of nucleic acids by Polymin P (Serva, Heidelberg, Germany) (1% [v/v], pH 7.5), proteins were precipitated by 80% saturation of ammonium sulfate and separated by centrifugation. The pellets were frozen by submersion in liquid nitrogen and stored at −70°C. For enzyme assays, protein preparations were dissolved in distilled water and the concentration was determined according to the method of Bradford (1976) using BSA as a standard.

The assays of cytokinin oxidase activity were based on the measurement of the rate of conversion of [2-3H]iP to adenine. The assay mixture (50 μL final volume) contained 100 mM TAPS-NaOH buffer (pH 8.5), 2 μM [2-3H]iP (200 μCi/μmol), and enzyme preparations equivalent to 200 to 1200 μg of fresh tissue. All other procedures were performed in the same manner as in the standard cytokinin oxidase assay.

The substrate specificity was determined by a competition assay that was based on the addition of unlabeled cytokinins to the standard assay mixture at concentrations equal to the substrate (2 μM) and in 10-fold excess (20 μM). The pH optimum of the cytokinin oxidase activity was determined by running the assay in four buffers (Mes-NaOH, Mops-NaOH, TAPS-NaOH, and 2-(N-cyclohexylaminoo)ethanesulfonic acid-NaOH, 100 mM each), covering the range between pH 5.5 and 10.0.

Con A-Sepharose 4B chromatography was performed as described elsewhere (Motyka and Kamíněk, 1994). The enzyme preparations, equivalent to 8 to 12 g of callus fresh tissue, were dissolved in 3 mL of 25 mM bisTris-HCl buffer (pH 6.5) and loaded onto a Con A-Sepharose 4B column (0.75 × 7 cm, 3-mL bed volume) equilibrated with the same buffer containing (NH₄)₂SO₄ (200 mM), CaCl₂ (1 mM), and MnCl₂ (1 mM). The column was washed with 21 mL of 25 mM bisTris-HCl (pH 6.5) containing (NH₄)₂SO₄ (200 mM) and eluted with 21 mL of the same solution supplemented with methylmannose (200 mM). Fractions of 3 mL were collected and assayed for cytokinin oxidase activity, using the copper-imidazole-enhanced assay. The A₅₉₀ of the fractions was measured. Calibration with egg albumin showed sufficient capacity of the Con A column to bind all tobacco proteins applied.

RESULTS

Plant Material and ipt Gene Induction

The influence of an enhanced endogenous cytokinin content on cytokinin oxidase activity was investigated in ipt-inducible transgenic tobacco callus cultures and plants (IPT), using corresponding untransformed plant material (WT) as controls. For the present work we first established the root-derived tobacco callus cultures. Figure 1 shows that WT calli did not grow on hormone-free medium and grew slowly when cytokinin was omitted. The addition of auxin (0.6 mg L⁻¹ NAA) and cytokinin (0.2 mg L⁻¹ kinetin) to the Murashige and Skoog medium was required for optimal continuous growth, and the addition of Tc to this medium (1 mg L⁻¹) had no significant effect on the growth of WT calli (Fig. 1). The growth characteristics of callus cultures that were derived from the roots of F₁ plants of the IPT clone were similar to those of cytokinin-free medium containing only NAA or fully complemented medium containing NAA and kinetin (Fig. 1, and data not shown). Their improved ability to grow on cytokinin-free medium
indicates that the activity of the modified 35S promoter was not completely repressed, and that the product of very low amounts of ipt transcripts are sufficient to trigger cell division. A similar observation has been made in transgenic tobacco calli containing a temperature-controlled ipt gene (Schmülling et al., 1989). The simultaneous addition of Tc (1 mg L\(^{-1}\)) to auxin and cytokinin containing solid medium led to the formation of compact tissue in clone IPT5, which is typical for growth on high cytokinin concentrations (Fig. 1). The direct application of Tc in liquid solution to calli had more drastic effects. Callus growth was improved until supraoptimal conditions were reached. Under these conditions, the calli apparently produced high cytokinin levels, turned brownish after a prolonged incubation, and eventually died (results not shown).

To confirm the induction of the ipt gene after Tc treatment of IPT tobacco calli, we carried out a northern blot analysis. Figure 2 shows that no ipt-specific transcript was detectable without the addition of Tc to the culture medium. On the contrary, high levels of ipt-specific steady-state mRNA were detected no later than 2 h after derepression of the ipt gene promoter by Tc (Fig. 2). The inducibility of the ipt gene and the consequences of its expression in whole tobacco plants and detached leaves will be described elsewhere (M. Faiss, J. Zalubilova, M. Strnad, and T. Schmülling, unpublished data).

**Endogenous Cytokinin Content in Tobacco Callus Cultures**

The content of endogenous cytokinins, including bases, ribosides, nucleotides, and N- and O-glucosides, was measured after derepression of the ipt gene transcription in transgenic tobacco calli. Cytokinin analyses were performed from tissues harvested immediately after the application of Tc solution to the callus surface (0 h) and at seven additional points within an interval of 4 h and 11 d after Tc application. No significant changes occurred in Tc-WT tissue during this period (data not shown). Results for Tc-IPT calli are listed in Table I for 16 different cytokinin metabolites and five time points. No increases were found in iP-type cytokinin levels following ipt gene derepression.

![Northern blot analysis showing derepression of ipt gene transcription after addition of Tc to IPT calli. Total RNA was isolated 0 h (lane 1), 2 h (lane 2), 4 h (lane 3), 8 h (lane 4), and 24 h (lane 5) after applying 3 mL of Tc (2 mg L\(^{-1}\)) containing water to the callus surface. The blot was hybridized with a \(^{32}\)P-labeled ipt-specific probe and a 25S rDNA probe as a control for loading.](image)

The earliest increase in the content of the zeatin-type cytokinins was detectable 4 h after Tc application for ZR, which was about 10-fold higher than background after 8 h (Table I, and data not shown). The increase of free cytokinins culminated 4 d after derepression when, compared with the repressed IPT tissues, the level in transgenic calli was elevated about 100-fold for Z, 300-fold for ZR, and about 20-fold for ZRP. The increases in the conjugate forms of ZOG and ZROG were later than those for the corresponding free base and the riboside, and at d 8 it reached about 30-fold and more than 200-fold higher concentrations, respectively, compared with repressed IPT calli. The content of DHZROG increased 20-fold (Table I). An even higher content of the O-glucosides was determined at later times (data not shown), and no appreciable differences were detected in the contents of DHZ, DHZOG, and DHZRP. Only low amounts of cytokinin 9-glucosides were detected in both the control and ipt-transformed calli (Table I).

**Characterization of Cytokinin Oxidase Activity in Tobacco Callus Cultures**

Several biochemical parameters of cytokinin oxidase activity were determined in enzyme preparations extracted from tobacco callus cultures 6 d after surface application of water (C-WT and C-IPT) or Tc (Tc-WT and Tc-IPT).

Based on competition assay analysis, iP and its riboside appeared to be the preferred substrates of the enzyme preparations extracted from tobacco calli of all the investigated types of tissues (results not shown).

All cytokinin oxidase preparations from C-WT, Tc-WT, C-IPT, and Tc-IPT calli exhibited the same pH optimum in a wide range between pH 6.5 and 8.5 without any significant peak of the enzyme activity. Cytokinin oxidase activities from individual tobacco tissues, assayed in reaction mixtures containing either Mes-NaOH (pH 6.7) or TAPS-NaOH (pH 8.5) buffers (both 100 mM), did not show any pronounced differences (results not shown).

The apparent \(K_m\) (iP) values of cytokinin oxidases from C-WT, Tc-WT, C-IPT, and Tc-IPT calli calculated on the basis of double-reciprocal plots were 3.1, 3.4, 3.3, and 4.9 \(\mu\)M, respectively. The conversion of iP to adenine was catalyzed by each of the tobacco enzyme preparations of C-WT, Tc-WT, and C-IPT with a \(V_{max}\) of 0.05 nmol mg\(^{-1}\) protein h\(^{-1}\). The \(V_{max}\) of iP degradation catalyzed by enzyme derived from Tc-IPT tissue was 0.35 nmol mg\(^{-1}\) protein h\(^{-1}\).

The cell-free preparations of cytokinin oxidase from all investigated tobacco calli exhibited an increased activity when the copper-imidazole complex was added to the reaction mixture (Fig. 3). For all enzyme preparations that were tested, the pH optimum of the cytokinin oxidase reaction in the presence of copper-imidazole was shifted to a lower value (pH 6.0) compared with the standard assay conditions (results not shown). The optimal CuCl\(_2\) concentration for the enhancement of cytokinin oxidase from all investigated tobacco calli ranged between 5 and 15 mM. When assayed at 10 mM CuCl\(_2\), the activity of the C-WT, C-IPT, and Tc-IPT tobacco enzyme was enhanced 5.2-, 3.6-, and 5.6-fold, respectively (Fig. 3). Under the same condi-
Table 1. The dynamics of the content of cytokinin metabolites in WT and transgenic IPT tobacco calli after derepression of ipt gene transcription

Tobacco calli were grown in vitro on solidified Murashige and Skoog medium supplemented with 1 mg L⁻¹ NAA and 0.2 mg L⁻¹ kinetin. For ipt gene derepression, Tc containing water was directly applied to the callus surface. Cytokinin concentrations were determined by a combination of HPLC and immunoassays as described in "Materials and Methods." The data are a representative example of three biological replications. Concentrations are in pmol/g fresh weight.

<table>
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<th>IPT 0 h</th>
<th>IPT 8 h</th>
<th>IPT 1 d</th>
<th>IPT 4 d</th>
<th>IPT 8 d</th>
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a Increase in IPT calli at d 8 compared with d 0. b −, No increase.

Dynamics of Cytokinin Oxidase Activity in Tobacco Calli after Derepression of the ipt Gene

The effect of enhanced cytokinin levels on cytokinin oxidase activity in tobacco callus cultures was assayed in enzyme preparations that were extracted from both WT and IPT calli immediately after Tc treatment (0 h) and at seven additional times within an interval of 4 h and 13 d after ipt gene derepression.

The Tc solution treatment induced a significant increase in cytokinin oxidase activity in IPT callus cultures, whereas it had no significant effect on the enzyme activity in WT calli (Fig. 5). The enhancement of cytokinin oxidase activity in IPT tissues after Tc treatment was not detectable before d 1 (Fig. 5). During further incubation (d 1–13), the enzyme activity in IPT calli progressively increased, with enhance-
ment from 2-fold (d 1) up to 10-fold (d 13) compared with the Tc-WT tissues. The tissue treatment with water instead of Tc did not significantly affect the cytokinin oxidase activity in either WT or IPT calli (Fig. 5).

The significant enhancement of cytokinin oxidase activity in IPT calli was found after their continuous cultivation on medium containing Tc (1 mg L\(^{-1}\)). Under these conditions, the specific activity of the enzyme extracted from IPT calli cultured for 34 d on Tc-containing medium was approximately 4-fold higher compared with that from C-IPT, C-WT, and Tc-WT tissues (results not shown).

Dynamics of Cytokinin Oxidase Activity in Detached Tobacco Leaves after Derepression of the \(ipt\) Gene

To test whether the cytokinin oxidase of fully differentiated tissues can also respond to changes in cytokinin content, we performed similar induction experiments in detached tobacco leaves. Tc-induced derepression of the \(ipt\) gene transcription can be achieved in this tissue by a short vacuum infiltration of Tc solution (1 mg L\(^{-1}\)). A significant, more than 50-fold increase of endogenous cytokinin levels, resulting from the \(ipt\) gene derepression in these transgenic leaves, was measured (M. Faiss, J. Zalubilová, M. Strnad, and T. Schmülling, unpublished data).

Cytokinin oxidase activity was determined in both WT- and IPT-detached leaves immediately after Tc application (0 h) and at six additional times between 2 h and 4 d after derepression. Figure 6 shows that the Tc treatment did not affect the cytokinin oxidase activity in WT leaves and resulted in a considerable increase in the enzyme activity in leaves of IPT plants. Similarly, as was found for the calli, the earliest increase of the activity of cytokinin oxidase in

**Figure 4.** Con A-Sepharose 4B chromatography of cytokinin oxidase from nontransformed (WT) and \(ipt\)-transformed (IPT) tobacco callus tissues. The enzyme preparations equivalent to 8 to 12 g of fresh tissue were dissolved in 3 mL of 25 mM bisTris-HCl buffer (pH 6.5) and loaded onto a Con A-Sepharose 4B column (0.75 X 7 cm, 3-mL bed volume). The column was washed with 21 mL of 25 mM bisTris-HCl (pH 6.5) containing (NH\(_4\))\(_2\)SO\(_4\) (200 mM) and eluted with 21 mL of the same solution containing methylmannose (200 mM). Fractions of 3 mL were collected and assayed for cytokinin oxidase activity using the copper-imidazole-enhanced assay as described in “Materials and Methods.” A, Control WT tissues 6 d after water application (C-WT); B, control IPT tissues with a repressed \(ipt\) gene 6 d after water application (C-IPT); C, derepressed IPT tissue 6 d after Tc application (Tc-IPT).

**Figure 5.** Time course of cytokinin oxidase activity in nontransformed (WT) and \(ipt\)-transformed (IPT) tobacco callus tissues after derepression of \(ipt\) gene transcription. Cytokinin oxidase activity was examined at different times after Tc application to the surface of calli (Tc-WT and Tc-IPT). Distilled water without Tc was applied to the control (C-WT and C-IPT) calli. The values represent the means of three replicates. The SD values averaged 8% and did not exceed 18%. Open bars, C-WT; striped bars, Tc-WT; hatched bars, C-IPT; closed bars, Tc-IPT.

**Figure 6.** Time course of cytokinin oxidase activity in nontransformed (Tc-WT) and \(ipt\)-transformed (Tc-IPT) tobacco leaves after derepression of \(ipt\) gene transcription. Induction of the \(ipt\) gene was performed by feeding Tc solution in 50 mM citrate buffer (pH 5.6) by a short vacuum infiltration to detached leaves. The values represent the means of three replicates. The SD values averaged 7% and did not exceed 15%. Striped bars, Tc-WT; closed bars, Tc-IPT.
IPT leaves was detected 24 h after the ipt gene derepression (approximately 2-fold). The maximum enhancement (approximately 8-fold) in the enzyme activity was reached 4 d after Tc application (Fig. 6). The treatment of both WT and IPT leaves with water instead of Tc did not exhibit any effects on cytokinin oxidase activity within the surveyed time interval (results not shown).

Cytokinin Oxidase Activity in Intact Tobacco Plants after Derepression of the ipt Gene

The changes in cytokinin oxidase activity after the derepression of the ipt gene in different tissues were studied in adult WT and IPT tobacco plants. These plants were grown in a hydroponic solution, which could be supplemented with Tc to achieve gene induction. Tc feeding through roots leads to a full derepression of ipt gene transcription in roots and a partial derepression in stems, resulting in increased cytokinin levels in these plant parts. Under these conditions derepression was not effective in the leaves, and the cytokinin levels remain unchanged in this tissue (M. Faiss, J. Zalubilová, M. Strnad, and T. Schmülling, unpublished data).

The cytokinin oxidase activity was determined in different plant tissues of both WT and IPT plants harvested before and after 3 d of Tc feeding through roots, and Table II summarizes the results of these measurements. Elevated endogenous cytokinin levels enhanced the activity of the enzyme significantly only in the roots of Tc-treated IPT plants (approximately four times compared with the non-induced IPT roots). No significant changes were found after Tc treatment in stems of IPT plants, and the cytokinin oxidase activity in leaves remained unchanged after ipt gene derepression, corresponding to the lack of cytokinin increase. In addition to the roots, the highest enzyme activity in WT plants as well as in induced and uninduced IPT plants was found in young leaves, indicating developmental differences (Table II).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cytokinin Oxidase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT 0 d</td>
</tr>
<tr>
<td>Roots</td>
<td>0.029</td>
</tr>
<tr>
<td>Young leavesa</td>
<td>0.028</td>
</tr>
<tr>
<td>Middle leavesb</td>
<td>0.011</td>
</tr>
<tr>
<td>Apical stemc</td>
<td>0.016</td>
</tr>
<tr>
<td>Middle stemc</td>
<td>0.016</td>
</tr>
<tr>
<td>Lower stemc</td>
<td>0.019</td>
</tr>
</tbody>
</table>

a Young leaves were about 10 cm long from the apical stem.
b Middle leaves were fully developed and harvested from the stem about 30 cm above the ground. c Apical, middle, and lower stems were 5-cm-long parts of the stem from the most apical region, from the middle part 30 cm above ground, and the basal region, respectively.

DISCUSSION

The accumulation of endogenous cytokinins in plants because of the expression of the ipt gene from A. tumefaciens has already been reported (for reviews, see Brzobohatý et al., 1994; Gaudin et al., 1994). Despite this accumulation, the elevated contents of endogenous cytokinins in transgenic plant cells is controlled by various homeostatic mechanisms such as conjugation or oxidation, which keep the hormone concentration below a lethal level. Indications that these mechanisms might be regulated by the hormone come from reports of enhanced cytokinin oxidase activity in various undifferentiated tissues or cell cultures after exogenous addition of cytokinins (Chatfield and Armstrong, 1986; Kamínek and Armstrong, 1990; Motyka and Kamínek, 1990). In the present investigation we studied the behavior of cytokinin oxidase, the key cytokinin-degrading enzyme, during conditional overproduction of endogenous cytokinins, which was made possible via the regulated Tc-dependent expression of the ipt gene in transgenic tobacco tissues. The biochemical properties of cytokinin oxidase that was extracted from the root-derived undifferentiated callus tissues were determined. Regulation of cytokinin oxidase activity was investigated in callus cultures as well as in different tissues of the whole plants.

Derepression of the ipt gene in transgenic calli was followed by a rapid increase in cytokinin content that culminated 4 d after Tc application. The cytokinins, which are either resistant to degradation by cytokinin oxidase (cytokinin O-glucosides, nucleotides, DHZ-type cytokinins) or exhibit a low affinity to this enzyme (Z-type cytokinins) (Armstrong, 1994), were responsible for the increase. In accordance with other published data (Zhang et al., 1995), no significant enhancement was found in iP-type cytokinin levels. This indicates, as has been suggested previously, a very rapid conversion of iP-type cytokinins, which are the primary and/or early products of cytokinin biosynthesis (McGaw and Burch, 1995; Morris, 1995) and prefered substrates of cytokinin oxidase (Armstrong, 1994), to accumulating Z-type cytokinins. Low levels of cytokinin N-glucosides in both control and transformed calli also correspond to other reports (e.g. Zhang et al., 1995). It could be that either N-glucosylation does not play an important role in metabolic inactivation of isoprenoid cytokinins in tobacco tissues, or that they are rapidly broken down by the cytokinin oxidase for which they are substrates (Armstrong, 1994). Cytokinin O-glucosides represent the major conjugated forms of cytokinins in both control and ipt-transformed tobacco calli. It is noteworthy that their levels are increased somewhat later than those of free cytokinins. This could mean that specific O-glucosyltransferases become activated only after exposure to elevated cytokinin levels. However, so far nothing is
known, to our knowledge, about the regulation of these enzymes.

Several biochemical parameters that are characteristic of cytokinin oxidase were investigated to test whether new isozymes of the enzyme are formed in transformed tissues, and correspond to those previously reported for the enzyme extracted from auxin- and cytokinin-dependent calli of the same tobacco cultivar grown under different conditions (Motyka and Kaminek, 1994) and are similar for both transformed and nontransformed callus tissue.

The substrate specificity of cytokinin oxidase, which exhibits a definite preference for iP and its riboside as substrates (Kaminek, 1992; Armstrong, 1994; Hare and Van Staden, 1994), was unchanged after induction. Similarly, the pH optimum of the enzyme was not considerably changed following ipt gene derepression.

The stimulation of cytokinin oxidase activity in vitro by the copper-imidazole complex represents another specific characteristic of the enzyme (Chatfield and Armstrong, 1987; Armstrong, 1994; Hare and Van Staden, 1994) that remained unchanged after induction. The activity of cytokinin oxidase from nontransformed, ipt-transformed, and Tc-induced tobacco calli was increased more than four times in the presence of the same copper-imidazole concentrations (Fig. 3).

The ratio of activities of cytokinin oxidase from Tc-induced ipt-transformed tissue and from the other two control tissues (WT and C-IPT) remained constant over the broad range of tested copper concentrations (Fig. 3).

The $K_m$ values reported for cytokinin oxidase from different plant sources and iP as a substrate vary by two orders of magnitude (Armstrong, 1994). Only small differences in the apparent $K_m$ (iP) values in a range from 3.1 to 4.9 μM were found for the enzyme preparations extracted from nontransformed and ipt-transformed tobacco calli, suggesting that derepression of the ipt gene had a minimal effect, if any, on the $K_m$ of the enzyme. These apparent $K_m$(iP) values are very close to those reported for cytokinin oxidase in tobacco and poplar calli (Motyka and Kaminek, 1994), which probably indicates that the enzyme can be induced by its substrate(s).

The effect of increased levels of endogenous cytokinins on cytokinin oxidase activity was similar to that reported for the application of exogenous cytokinins to different callus tissues, including tobacco (Chatfield and Armstrong, 1986; Kaminek and Armstrong, 1990; Motyka and Kaminek, 1990). It is interesting that the increase in cytokinin oxidase activity in derepressed IPT tissues did not prevent the accumulation of zeatin. One possible explanation is that Z is a poor substrate for cytokinin oxidase in tobacco calli (Motyka and Kaminek, 1994), which probably regulates the cytokinin content on the iP-type cytokinin level. Other cytokinins that are not substrates for the oxidase (ZROG, DHZR, and DHZROG) accumulated in ipt transgenic tissue.

The cytokinin oxidase activity was enhanced after Tc-induced derepression of the ipt gene only in the roots of transgenic plants. We did not detect an increase of cytokinin-oxidase activity in stems, despite the fact that they contain higher than normal levels of the hormone (M. Faiss, J. Zalubilová, M. Strnad, and T. Schmuling, unpublished data). It is possible that stem tissue lacks the regulatory components to respond to an increase in cytokinin content, or that only a subset of cells can do so, and the differences were blurred in our assay by dilution.

It is clear that we employed an experimental system that challenges the homeostatic system with differences in endogenous cytokinin concentrations that probably do not occur in nature. However, these endogenous regulatory mechanisms are difficult to detect because changes are small or are paired with variations occurring within tissues. The possibility that the results given here could reflect processes that occur during plant development represents the positive correlation of cytokinin content and cytokinin oxidase activity that has been reported recently by Jones et al. (1992) and Dietrich et al. (1995) for developing maize kernels.

We have expanded the concept of substrate induction for the cytokinin oxidase to differentiated plant tissues and have shown for the first time the dynamics in the response of cytokinin oxidase to conditionally elevated endogenous cytokinin levels. The differences detected between leaf tissues of different developmental stages suggest a possible role of this enzyme in adjusting the cytokinin level with...
respect to physiological processes. An example is the determination of leaf sink strength, a trait that can be influenced by cytokinins and may determine harvest indices.

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