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Increase in activity, glycosylation and expression of cytokinin oxidase/dehydrogenase during the senescence of barley leaf segments in the dark

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The aim of the study was to elucidate relations between senescence and cytokinin oxidase/dehydrogenase (CKX, EC 1.4.3.18/1.5.99.12). Segments derived from first foliage leaves of *Hordeum vulgare* L. cv. Alexis were put with their bases into water and kept in darkness. Their senescence was characterized, e.g. by a 60% decline in chlorophyll within 5 days. During this time, the in vitro activity of CKX increased fast and markedly, e.g. 14-fold. Application of 10^{-4} *M* kinetin (Kin), which slightly retarded the loss of chlorophyll, multiplied the enhancement of CKX activity strongly. Both in the presence and in the absence of Kin, the proportion of glycosylated to non-glycosylated CKX increased during senescence. By hybridization with an antisense RNA probe derived from a fragment of the CKX gene *Zmckx1* of maize, an increase of the corresponding transcript of the barley gene *Hvckx1* in segments incubated without Kin was shown. The content of base and riboside cytokinins slowly declined in such segments, which argues against triggering but for facilitating senescence processes by CKX.

Introduction

In the last years, impressive progress has been made in the molecular biology and biochemistry of cytokinin oxidase/dehydrogenase (CKX, EC 1.4.3.18/1.5.99.12), which catalyses the degradation of plant hormones of the class of cytokinins. It concerns the identification and gain of insight into the expression of CKX genes of various plants (Bilyeu et al. 2001, Brugière et al. 2003, Galuszka et al. 2004, Houba-Hérin et al. 1999, Morris et al. 1999, Werner et al. 2003, Yang et al. 2003) as well as the establishment of a detailed reaction mechanism (Frébortová et al. 2004). On the other hand, there are still many open questions with regard to the role of CKX in developmental physiology. So far, only few, shortly described examples were known in which an enhancement of CKX activity during a developmental event is associated with a decline in the content of endogenous cytokinins (Dietrich et al. 1995, development of maize caryopses; Kaminek et al. 1997a, senescence of oat leaf segments; Conrad et al. 1999, senescence of discs of young kohlrabi tubers). One aim of the present study was to look for such a connection in detail in senescing barley leaf segments. This subject is one of the first in which a retardation of yellowing by kinetin (Kin) was shown (Atkinson 1958 in Strong) and is used for cytokinin assays based on conservation of chlorophyll (cf. Munsche et al. 1968).

Abbreviations – CKX, cytokinin oxidase/dehydrogenase; iP, N^6-(Δ^2-isopentenyl)adenine; Kin, kinetin; PCR, polymerase chain reaction; Z, *trans*-zeatin.

The primary task was the daily determination of the in vitro activity resulting from the concentration(s) and activity(ies) of (iso)CKX molecules and of the cytokinin content during senescence of darkened barley leaf segments. In case of an activity rise, it should be studied furthermore whether this could be because of an enhancement of enzyme glycosylation (cf. Motyka et al. 2003) and/or an increased gene expression. For the detection of corresponding mRNA, the derivation of a probe from the maize gene *Zmckx1* was tried. This CKX gene is the first one sequenced with knowledge of its function (Houba-Hérin et al. 1999, Morris et al. 1999, GenBank accession No. AF044603). Finally, causal relations between CKX, cytokinins and the senescence of leaf segments are discussed.

Materials and methods

Culture of barley seedlings

Caryopses of *Hordeum vulgare* L. cv. Alexis were soaked for 2 h in water and then germinated for 2 days in the dark on moist filter paper. Thereafter, seedlings were grown at 21°C for 8 days in a mixture of leaf mould and sand (3:1) or in PERLITE® supplied with Knop solution (Table 1) at a daily light/dark regime of 18 h white light [35 or 130 μmol photons m^{-2} s^{-1} (Table 1)] and 6 h darkness. The lamps were made by Osram (München, Germany; Radium NL 58 W/21840, Spectralux Plus White) and Toshiba (Beijing, China; ty FL40SS.W/37), respectively.

Incubation of leaf segments

Apical segments were excised from first foliage leaves 7 cm from the tips. Each 12 of such segments were transferred in to 25-ml glass beakers. These they stood with their bases approximately, 1.4 cm deep in water containing 0.05% sodium penicillin G, which was changed daily. In some cases, Kin (Serva, Heidelberg, Germany) was added to the medium. After dark incubation for 0–6 days at 21°C or 26°C (Table 1), the leaf segments were stored at −80°C.

Determination of the contents of pigments and soluble protein

Chlorophylls and carotenoids were extracted from leaf segments with methanol containing 0.05% (w/v) ammonia at 63.5°C and quantified spectrophotometrically according to Wellburn (1994). The content of proteins extracted with 1 *M* NaOH at 80°C was determined spectrophotometrically after complex formation with Coomassie Brilliant Blue G-250 (cf. Motyka et al. 2003).

Determination of CKX activity

The CKX activities shown in Fig. 1 were obtained as follows. After grinding in liquid nitrogen, the leaf material was gently stirred for 1 h with the following mixture (g $FW)^{-1}$: 15 ml 0.2 *M* phosphate buffer (pH 6.5)/0.6 *M* L(+)-ascorbic acid and 1 g (DW) hydrated, acid-activated polyvinylpolypyrrolidone for absorption of phenolic and polyphenolic compounds. The polyvinylpolypyrrolidone and insoluble leaf substances were removed by centrifugation. Nucleic acids were precipitated by addition of approximately 0.02% (w/v) polyethyleneimine. After centrifugation, the supernatant was saturated to 80% by $(NH_4)_2SO_4$ to precipitate proteins including CKX. The protein pellet was dissolved in 500 μl water (g $FW)^{-1}$ of leaf segments and frozen at −80°C.

The CKX activity was assayed by the method of Libreros-Minotta and Tipton (1995), which is based on the detection of 3-methyl-2-butenal resulting from N^6-(Δ^2-isopentenyl)adenine (iP) cleavage by CKX. After formation of a Schiff base with *p*-aminophenol, the aldehyde is determined spectrophotometrically at 352 nm. Extract portions equivalent to 25–80 mg FW were incubated at 37°C in 500 μl final volume of a solution consisting of 13 μ*M* iP (Sigma, St Louis, MO), 100 m*M* imidazole/HCl (pH 6.5) and 10 m*M* $CuCl_2$. The reaction time was chosen between 1.5 and 20 h so that the substrate concentration did not decline below 50% of the initial value. By addition of 300 μl 40% (w/v) TCA, the reaction was terminated. Each extract was assayed five-fold.

In some experiments (Table 1), CKX was extracted, purified and assayed according to Motyka et al. (1996, 2003). Per assay, extracts equivalent to 0.5–750 mg FW in 50 μl of 100 m*M* MOPS/NaOH buffer (pH 7.2) containing 75 μ*M* 2,6-dichlorophenolindophenol were used. [2-^{3}H]N^6-Δ^2-isopentenyladenine synthesized by Dr Hanuš, Institute of Experimental Botany, Prague, served as a substrate, and the radioactive cleavage product adenine was detected. Each assay was carried out at least twice.

Separation of non-glycosylated and glycosylated CKX

Purified extracts of CKX were loaded onto a column of Concanavalin A-Sepharose™ 4B (Sigma). While the non-glycosylated CKX fraction was washed by bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane/HCl buffer (pH 6.5) containing $(NH_4)_2SO_4$, the glycosylated one was eluted by the same solution supplemented with methylmannose. Details were described by Motyka et al. (1996).

Northern blot analysis with a digoxigenin-labelled antisense RNA probe derived from *Zmckx1*

The preparation of a probe for CKX mRNA started from the DNA of seedlings of *Zea mays* L. cv. Brussol, extracted and purified according to Doyle JJ and Doyle LL (1990). It was used for polymerase chain reaction (PCR) with the *Zmckx1*-specific primers 5′-GGCCACGCTCAACTACGACAA-3′ (21mer) and 5′-AACATGCATGGGCTATCATCATCA-3′ (24mer) synthesized by MWG Biotech (Ebersberg, Germany). The amplification was carried out in 50 μl with 50 ng DNA, 1 μ*M* primers, 200 μ*M* deoxynucleoside triphosphates, 0.5 units *Taq* DNA polymerase (GibcoBRL, Karlsruhe, Germany), 1× reaction buffer supplied with the enzyme and 1.5 m*M* $MgCl_2$ for 35 cycles consisting of 1 min at 95°C, 1 min at 62.7°C and 1 min at 72°C. The PCR product was cloned by means of the phagemide vector p Bluescript II KS (Stratagen, Heidelberg, Germany) and *Escherichia coli* (DH5α) and was sequenced as described by Scharrenberg et al. (2003). Using the DIG Northern Starter Kit (Roche Molecular Biochemicals, Mannheim, Germany), the non-template strand of the insert was transcribed to digoxigenin-11-UMP-containing antisense RNA.

This probe was applied in Northern blot analyses with mRNA of barley leaf segments. By means of the PolyATtract® Isolation System III (Promega, Mannheim, Germany), poly(A)+ RNA was isolated from total RNA, which was extracted from the leaf material according to Chomczynski and Sacchi (1987). It was electrophoretically separated in an agarose (1.2%, w/v) gel containing 4.6% (w/v) formaldehyde and transferred onto a positively charged nylon membrane (Roche Molecular Biochemicals). Then, the hybridization procedure was carried out at 63°C with the digoxigenin-labelled probe and the immunological-chemoluminographic check, as recommended in the Instruction Manual of the DIG Northern Starter Kit.

Cytokinin analysis

Cytokinins were extracted from 10 to 30 g leaf segments with ethanol (approximately 80% final concentration, v/v) or methanol/chloroform/7 *M* formic acid (12:5:3, v/v/v) and were partitioned between water and 1-butanol. After purification of the 'butanol-soluble' cytokinins by a phosphocellulose column and by an RP 18 column (Baker, Gross-Gerau, Germany), cytokinins were further separated by chromatography on Sephadex™ LH-20 (Amersham Pharmacia Biotech, Freiburg, Germany) with aqua bidest. The detection was performed by ELISAs, with specific antibodies directed towards *trans*-zeatin (Z) riboside and iP riboside (Conrad et al. 1992). In some cases, aliquots of selected eluate fractions were rechromatographed by HPLC on a LiChrospher 100 RP-18, 5 μm column (Merck, Darmstadt, Germany) with methanol/10 m*M* NH_4 formiate buffer, pH 3.7 (23:77, v/v), as the eluent. The *Amaranthus* assay (Köhler and Conrad 1966) was used for evaluation of paper chromatograms of 'butanol-soluble' cytokinins obtained with the developing solvent ethyl acetate/1-propanol/H_2O (4:1:2, v/v/v, upper phase).

Results

Senescence of darkened segments of barley leaves and its retardation by Kin

Experiments were performed with segments encompassing the upper 7-cm part of the first foliage leaves of 8-day-old barley seedlings. The leaves had finished their prolongation growth. During 4 days of dark incubation at 21°C, 5 × 12 segments together with 5.09 g FW increased their FW by 4%.

Changes in the contents of chloroplast pigments of the segments per gram of initial FW are shown in Fig. 1. While the carotenoid content remained almost constant during 5 days of dark incubation at 21°C, the contents of Chl *a* and Chl *b* decreased considerably. The daily decreases in both chlorophylls were significant as tested by the Wilcoxon assay ($\alpha = 0.05$, $P < 0.02$). Within 4 days, the Chl *a* content declined to 50%, the Chl *b* content to 56% and the molar Chl *a*/Chl *b* ratio from 2.8 to 2.5. Obviously, rapid senescence occurred.

This was confirmed by detection of a decrease in soluble protein of the segments from 14 to 5.6 mg (g initial FW)$^{-1}$, i.e. to 40%, in 4 days (36 segments per variant). Kin at a concentration of 10^{-4} *M* diminished the loss of soluble protein during this time by 19% of the initial content (24 segments per variant). The retardation of senescence by 10^{-4} *M* Kin was also reflected by a diminution in the decrease of Chl *a* and Chl *b* in 4 days by 9% and 8% of initial content, respectively (24 segments per variant, cf. also Table 1).

Increase in CKX activity and its acceleration by cytokinin

In the course of senescence of darkened barley leaf segments at 21°C, the in vitro activity of CKX was determined daily by colorimetric measurement of the degradation of iP at pH 6.5 (Fig. 1). For the calculation of the activities, the initial FW was used. While immediately after the excision of the segments, 0.34 nmol iP were cleaved per gram FW and hour, after 2 days 390%

Table 1. Changes in the total CKX activity and in the activity share of glycosylated CKX during dark incubation of barley leaf segments without and with Kin. For CKX extraction approximately 6 g FW were used per variant. As an indirect parameter for senescence, the absorbance of ethanolic extracts of 20 segments each at 665 nm is given as a measure of chlorophyll content

Incubation at 26°C (d)	A_{665} (% of 0-day control)	CKX activity [nmol iP (g final FW)$^{-1}$ h^{-1}]			Share of glycosylated CKX in total activity (%)
		Total	Non-glycosylated fraction	Glycosylated fraction	
0	100	0.169	0.092	0.077	46
2	33	1.71	0.444	1.26	74
4	6	2.59	0.492	2.10	81
10^{-4} *M* Kin					
2	47	12.6	3.17	9.42	75
4	11	18.9	2.28	16.6	88

and after 4 days 710% of this value were reached. The increases during the first, fourth and fifth day were significant as tested by the Wilcoxon assay ($\alpha = 0.05$, $P < 0.02$). A 15-fold enhancement of CKX activity in 4 days is documented in Table 1, which involves data for leaf segments incubated at 26°C and for iP cleavage per gram final FW and hour measured at pH 7.2 by the radioactive method.

Table 1 also shows that application of 10^{-4} *M* Kin during 4 days of incubation enhanced the CKX activity 7.3-fold compared with the 4-day control value. In three similar experiments involving together approximately 280 segments per variant, a 34-fold increase was found on average. The assay was carried out there without 2,6-dichlorophenolindophenol.

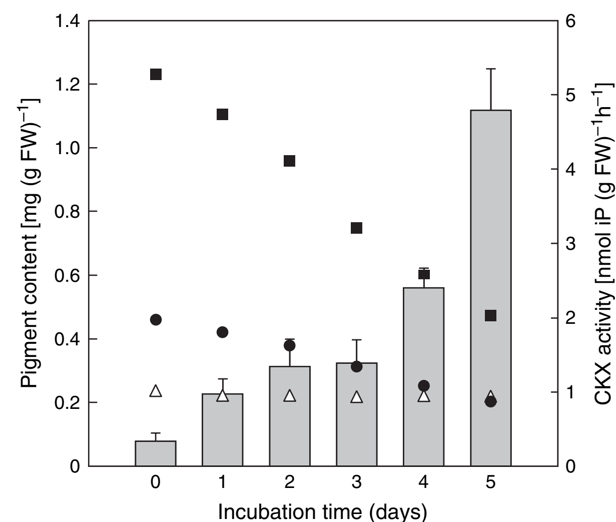

Fig. 1. Changes in the contents of Chl *a* (■), Chl *b* (●) and carotenoids (△) as well as in CKX activity (columns) during dark incubation of barley leaf segments. The points represent content means of 14 samples derived from four segments each and the columns, activity means of nine extracts from 12 segments each. Bars indicate ± SE.

Enhancement of the CKX glycosylation

After separation of CKX into a non-glycosylated and a glycosylated fraction, the degradation of radioactive iP per gram final FW and hour by both forms was measured. The pH value of 7.2 was chosen for the activity assay because it was presumed to lie between the pH optima of the enzyme fractions (Kamínek and Armstrong 1990, Motyka et al. 2003). In Table 1, the representative results of one of two series of analyses are listed. From extracts of approximately 70 leaf segments each, portions equivalent to approximately 3 g FW were fractionated. Already in the first 2 days of dark incubation at 26°C, the activity proportion between non-glycosylated and glycosylated CKX changed from 1:0.8 to 1:2.8 and reached 1:4.3 in the next 2 days. While the activity of the non-glycosylated fraction enhanced only 5.3-fold during 4 days, the activity of the glycosylated one enhanced 27-fold. Incubation with 10^{-4} *M* Kin possibly slightly increased the activity shares associated with glycosylated CKX in comparison to the water controls.

Expression of a CKX gene

A DNA fragment was obtained by PCR using maize DNA and a pair of specific primers, which after cloning and sequencing was revealed as a fragment of the gene *Zmckx1* (Fig. 2). From this 728-bp-long fragment,

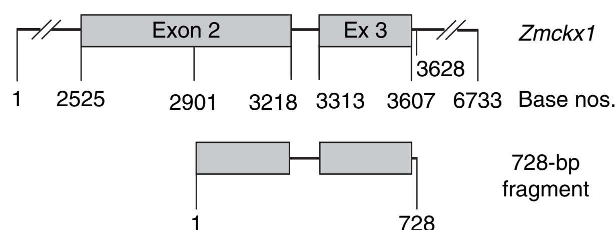

Fig. 2. Position of the 728-bp DNA fragment from which an antisense RNA probe was derived within the gene *Zmckx1*.

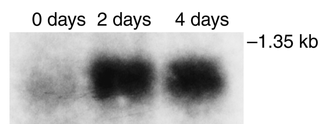

Fig. 3. Northern blot analysis of the expression of a CKX gene in barley leaf segments. Poly(A)+ RNA was isolated from segments incubated for 0, 2 and 4 days in darkness.

applying in vitro transcription of the non-template strand, a digoxigenin-labelled antisense RNA probe was generated.

The probe was used in Northern blots with mRNA of barley leaf segments. From 10 segments each, poly(A)+ RNA was obtained, of which approximately 5 μg (equivalent to 0.4 g FW) were put onto the electrophoresis gel. As Fig. 3 shows, a hybridization product with barley mRNA was detected. This CKX mRNA was faintly present before cutting and increased clearly in the first 2 days of dark incubation at 21°C. Two repetitions yielded similar results. The antisense RNA probe also hybridized with poly(A)+ RNA of leaves of *Phragmites australis* (Cav.) Steud.

Dynamics of cytokinins

By means of two ELISAs and the *Amaranthus* bioassay, cytokinins were detected in barley leaf segments, which in Sephadex LH-20 column chromatography, paper chromatography and HPLC behaved like Z and Z riboside (together with a similar compound: Z-type cytokinins) as well as iP and iP riboside (iP-type cytokinins). In addition to these 'free' cytokinins, cytokinin glucosides and nucleotides were also found.

As shown in Table 2, both groups of 'free' cytokinins quantified by ELISAs decreased rather late during dark incubation of barley leaf segments at 21°C. While 2 days after cutting, no lower mean values were detected than those prior to incubation, until the end of the fourth day, a decrease of nmol Z riboside equivalents per kilogram initial FW by 32% was occurred. For iP-type cytokinins, a clear deficiency was found after 6 days. The *Amaranthus* assay combined with paper chromatography indicated much lower contents of 'free' cytokinins in 4-day samples than in fresh leaf segments in two experiments.

Table 2. Changes in cytokinin contents during the senescence of barley leaf segments. Starting values per kg FW were, e.g. 0.70 nmol Z riboside equivalents of Z and a similar compound + 0.55 of Z riboside = 1.25 for Z-type cytokinins as well as 0.30 nmol iP riboside equivalents of iP + 0.91 of iP riboside = 1.21 for iP-type cytokinins. Percentages were calculated with initial FW

Incubation at 21°C (d)	Cytokinin content in % of starting value	
	Z-type cytokinins	iP-type cytokinins
2	102 (four samples)	104 (four samples)
3	91	99
4	66 (three samples)	104 (two samples)
4, HPLC	73	—
6	88 (two samples)	22
6, HPLC	56	—

Discussion

In barley leaf segments kept with their bases in water in the dark, a clear decrease of chlorophylls and soluble protein indicated senescence. This decline could be moderately retarded by application of Kin. The senescence was induced primarily by the isolation of the segments from the plantlets. Kept under the same light (35 μmol photons m^{-2} s^{-1}) regime as plantlets, segments did not lose chlorophylls significantly more slowly than in darkness (Schlüter 2003). Becker and Apel (1993) stated a stronger expression of a senescence-associated gene in darkened barley leaf segments than in lighted ones. They found its transcript also in naturally senescing attached leaves contrary to two dark- and wound-related transcripts that accumulated in segments. Dark-induced senescence of attached *Hordeum* leaves was reported by Kleber-Janke and Krupinska (1997).

The in vitro activity of CKX of barley leaf segments was low immediately after cutting and increased rapidly in the dark for at least 5 days. During this time, at least a 14-fold increase was reached. Under the light regime, the enhancement was considerably greater (Schlüter 2003). In attached first foliage leaves senescing 'naturally' under these light conditions, the CKX activity also increased. An enhancement of CKX activity in darkened segments of oat leaves was mentioned without providing quantitative data by Kamínek et al. (1997a).

The increase in the CKX activity of barley leaf segments was strongly multiplied by the application of Kin. It seems strange that Kin, which retarded the yellowing, did not delay but accelerated the enhancement in comparison with the untreated controls. However, an increase in CKX activity caused by high concentrations of synthetic or endogenous cytokinins is a well-known phenomenon (Kamínek et al. 1997a, 1997b). In detached, lighted tobacco leaves, such an increase was demonstrated by Motyka et al. (2003, 1996). In their experiments, cytokinins enhanced the share of glycosylated CKX in the total enzyme activity, while the share remained constant in the controls. The present study also shows a preferred increase in the activity of the glycosylated enzyme form in darkened barley leaf segments without cytokinin application. The enhancement in the activity

share of glycosylated CKX in the water control, e.g. from 46 to 81% in 4 days, was nearly as high as that reached in the presence of 10^{-4} *M* Kin. An increase in glycosylation capacity may be a cause for increasing glycosylation degree. If glycosylation activates CKX molecules and/or protects them against proteolysis (cf. Motyka et al. 2003), it contributes to the enhancement of total CKX activity. Recently, Kopečný et al. (2005) found that incomplete deglycosylation of recombinant ZmCKX1 decreased its activity to about 90%. An increase in the share of glycosylated CKX in senescing barley leaf segments may also originate in preferred formation of glycosylable CKX isoenzymes.

Without and especially with cytokinin application, obviously a strong enhancement of CKX formation takes place in the segments. In the first 2 days of incubation, this may be partly because of the increase in the mRNA that hybridizes with the antisense RNA probe derived from the maize gene *Zmckx1*. The increase in this mRNA was confirmed by reverse transcription of poly(A)+ RNA from 0- and 2-day-incubated segments followed by PCR of the cDNA with the *Zmckx1*-specific primers mentioned and by hybridization of the product with ^{32}P-labelled 728-bp DNA derived from *Zmckx1* (Fig. 2, T. Schlüter 2003). A direct hybridization of the 728-bp fragment with a *Hordeum* transcript was stated in run-on experiments with isolated barley nuclei (Conrad et al. 2001). In 2001, P. Galuszka, J. Frébort, M. Šebela, M. Yamada and P. Peč published a sequence of 413 bp encoding a part of a CKX mRNA of barley (GenBank accession no. AF362472). From it, 409 bp correspond to 85% to similar parts of the 728-bp DNA fragment. Obviously, the not-further-sequenced CKX mRNA reported by Galuszka et al. is identical with the mRNA detected with the antisense RNA probe derived from the 728-bp fragment. Evidence that the corresponding protein really functions as CKX is still outstanding. The corresponding gene was named *HvCKX1* by Galuszka et al. (2004). These authors found further CKX genes in barley, sequences of which did not show significant similarities to the 728-bp fragment of *Zmckx1* in database analyses. An increased expression of such genes may be responsible for the continuing enhancement of the CKX activity in leaf segments after the second day of incubation. In de- and attached senescing leaves of *Arabidopsis thaliana*, Buchanan-Wollaston et al. (2005) stated an enhancement of the expression of a putative CKX gene. Brugière et al. (2003) demonstrated a strong promotion of the transcription of *Zmckx1* by application of cytokinins in discs of maize leaves.

In barley leaf segments, almost the same endogenous cytokinins were found as Galuszka et al. (2004) listed for barley seedlings. The increase in CKX activity did not result in a prompt reduction of the content of base and riboside cytokinins which are CKX substrates. It scarcely decreased until the end of the third day of incubation, while the enzyme activity exceeded the starting value 4.1-fold. However, the decrease in the nmol Z riboside equivalents to 68% until the end of the fourth day and the later decrease in iP-type cytokinins show that the activity enhancement may yet have an effect. Kamínek et al. (1997a) stated a decrease in the level of iP and Z in oat leaf segments to 48% during 4 days. Such changes may also be caused, however, by cytokinin conjugation and/or diminishing of cytokinin biosynthesis. On the other hand, an even greater effect of CKX could be superimposed by formation of 'free' cytokinins, mainly at the basal wound (cf. Conrad 1977). Yang et al. (2003) found in attached leaves of the wild-type of *Dendrobium* Sonia 52% of the level of iP-type cytokinins [nmol (kg FW)$^{-1}$] occurring in those of a *DSckx1* antisense transformant, which possessed 28% of the CKX activity of wild-type leaves. Similarly, inverse proportions between CKX activity and cytokinin content were demonstrated in *ckx*-transformed *Nicotiana* and *Arabidopsis* plants and their wild-types (Kopečný et al. 2006, Werner et al. 2001, 2003). The fact that the decrease in the content of 'free' cytokinins reported here for barley leaf segments developed only slowly may be connected with a rather different distribution of the CKX forms and substrates. It was hypothesised that glycosylated CKX is accumulated preferentially in the cell wall or plasmalemma (Kamínek and Armstrong 1990, Motyka et al. 2003).

The delay of cytokinin decline demonstrated in Table 2 argues against a causal involvement of cytokinin shortage in the decrease in chlorophyll and protein contents in the first incubation days and against a role of the fast increase of CKX activity in triggering senescence. Subsequent decrease in the content of 'free' cytokinins may favour processes resulting in death. This conjecture is supported by a just-detectable chlorophyll-conserving effect of a 10 n*M* Z solution in leaf segments of *H. vulgare* cv. Alexis under special conditions (cf. Munsche et al. 1968). Hence, the increase in CKX activity some time after excision may promote indirectly senescence.

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