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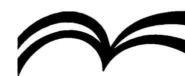
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RESEARCH PAPER

Altered cytokinin metabolism affects cytokinin, auxin, and abscisic acid contents in leaves and chloroplasts, and chloroplast ultrastructure in transgenic tobacco

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Abstract

Cytokinins (CKs) are involved in the regulation of plant development including plastid differentiation and function. Partial location of CK biosynthetic pathways in plastids suggests the importance of CKs for chloroplast development. The impact of genetically modified CK metabolism on endogenous CK, indole-3-acetic acid, and abscisic acid contents in leaves and isolated intact chloroplasts of *Nicotiana tabacum* was determined by liquid chromatography/mass spectrometry and two-dimensional high-performance liquid chromatography, and alterations in chloroplast ultrastructure by electron microscopy. Ectopic expression of *Sho*, a gene encoding a *Petunia hybrida* isopentenyltransferase, was employed to raise CK levels. The increase in CK levels was lower in chloroplasts than in leaves. CK levels were reduced in leaves of tobacco harbouring a CK oxidase/dehydrogenase gene, *AtCKX3*. The total CK content also decreased in chloroplasts, but CK phosphate levels were higher than in the wild type. In a transformant overexpressing a maize β-glucosidase gene, *Zm-p60.1*, naturally targeted to plastids, a decrease of CK-*O*-glucosides in chloroplasts was found. In leaves, the changes were not significant. CK-*O*-glucosides accumulated to very high levels in leaves, but not in chloroplasts, of plants overexpressing a *ZOG1* gene, encoding *trans*-zeatin-*O*-glucosyltransferase from *Phaseolus lunatus*. Manipulation of the CK content affected levels of indole-3-acetic and abscisic acid. Chloroplasts of plants constitutively overexpressing *Sho* displayed ultrastructural alterations including the occasional occurrence of crystalloids and an increased number of plastoglobuli. The other transformants did not exhibit any major differences in chloroplast ultrastructure. The results suggest that plant hormone compartmentation plays an important role in hormone homeostasis and that chloroplasts are rather independent organelles with respect to regulation of CK metabolism.

Key words: Abscisic acid, auxin, chloroplast ultrastructure, cytokinin metabolism, cytokinin oxidase/dehydrogenase, β-glucosidase, isopentenyltransferase, *Nicotiana tabacum*, *Sho*, zeatin-*O*-glucosyltransferase.

Introduction

The plant hormones cytokinins (CKs) are known to be involved in many processes related to plastid development and functions. Within 10 years of the discovery of kinetin (6-furfurylaminopurine) as a plant growth regulator CK (Miller *et al.*, 1956) in cultured tobacco tissue, it was

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Abbreviations: ABA, abscisic acid; BA, benzyladenine; CaMV, cauliflower mosaic virus; CK, cytokinin; DMSO, dimethylsulphoxide; FW, fresh weight; IAA, indole-3-acetic acid; IPT, isopentenyltransferase; LCMS, liquid chromatography/mass spectrometry; WT, wild type.

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shown that kinetin could stimulate chloroplast differentiation from proplastids, including grana formation (Stetler and Laetsch, 1965). Kinetin was also found to almost double chloroplast number per cell in etiolated tobacco leaf discs incubated in the light (Boasson and Laetsch, 1969).

It has been demonstrated that exogenously applied CKs induce partial development of chloroplasts from proplastids, amyloplasts, and etioplasts, especially in darkness (Chory *et al*., 1994; Kusnetsov *et al*., 1994). For example, benzyladenine (BA) treatment of excised watermelon cotyledons accelerated degradation of reserve material and differentiation of plastids with a more developed inner membrane system, as well as increased levels of plastid pigments and enzymes (Longo *et al*., 1979). Moreover, applied BA accelerated the redevelopment of grana and stroma in regreening plastids, the increase of reappeared NADPH-protochlorophyllide oxidoreductase, and the contents of chlorophyll and protein in regreening tobacco leaves (Zavaleta-Mancera *et al*., 1999*a*, *b*).

The action of CKs in plastids seems to be mediated by the stimulation of the expression of several plastid-related genes, of both nuclear and plastid origin, in particular that of the nuclear gene for chlorophyll *a*/*b*-binding polypeptide of the light-harvesting complex and the gene coding for the small subunit of Rubisco in *Nicotiana tabacum* (Abdelghani *et al*., 1991), *Arabidopsis* (Chory *et al*., 1994), or *Dianthus caryophyllus* (Winiarska *et al*., 1994). For extensive reviews, see Schmülling *et al*. (1997) or Parthier (1989).

Recently, Brenner *et al*. (2005) identified by genome-wide expression profiling five rapidly CK-induced plastid transcripts in *Arabidopsis* seedlings, indicating a fast transfer of the CK signal to plastids or its direct perception there. The CK effect on gene expression may be mediated via the hormone interaction with specific proteins. In the presence of *trans*-zeatin, a 64 kDa chloroplast zeatin-binding protein was found to activate the chloroplast but not the nuclear genome (Lyukevich *et al*., 2002).

The occurrence of endogenous CKs in plastids has been proven. CKs are present in chloroplast tRNAs (Vreman *et al*., 1978). Zeatin-type CKs were detected in isolated spinach chloroplasts by paper chromatography and soybean callus bioassay (Davey and Van Staden, 1981). Immunolocalization of CKs revealed low labelling in the stroma of plastids of *Tilia cordata* embryo cotyledons and roots (Kärkönen and Simola, 1999). Eventually, CK occurrence in chloroplasts was rigorously confirmed by LCMS (liquid chromatography/mass spectrometry) which allows identification of a whole range of CK metabolites with high accuracy (Benková *et al*., 1999).

The importance of CKs for plastid development and function may be deduced from the partial localization of the CK biosynthetic pathway to this compartment. Kasahara *et al*. (2004) showed that the prenyl group of CKs may be synthesized in plastids by the methylerythritol phosphate pathway and that four isopentenyltransferases (IPTs) are localized to plastids in *Arabidopsis* cells. Unexpectedly, Sakakibara *et al*. (2005) have found that *Agrobacterium* IPT functions in plastids, even though it lacks a typical plastid-targeting sequence, and it prefers a substrate different from that of genuine plant IPTs. It was previously shown that the enhanced content of CKs in chloroplasts of tobacco plants overexpressing the *A. tumefaciens ipt* gene driven by a light-inducible promoter of a *Pisum sativum* small subunit of Rubisco (P*ssu-ipt*) correlated with highly anomalous chloroplast ultrastructure (Synková *et al*., 2006). Interestingly, although a clear increase in CKs was observed in chloroplasts, it remained about an order of magnitude lower than the levels found in whole leaf extracts.

In the present study, the impact of manipulation of CK metabolism on the endogenous CK pool in leaves and isolated intact chloroplasts, and on chloroplast ultrastructure in tobacco (*Nicotiana tabacum* L.) was evaluated. CK-overproducing plants (expressing the *Sho* gene coding for a *Petunia hybrida* IPT), plants with decreased CK levels (overexpressing CK oxidase/dehydrogenase *AtCKX3* from *Arabidopsis thaliana*), and plants with altered CK glucoconjugation (overexpressing maize β-glucosidase *Zm-p60.1* or zeatin-*O*-glucosyltransferase *ZOG1* from *Phaseolus lunatus*) were compared. The simplified model of CK metabolism with highlighted enzymes involved in this study is presented in Fig. 1. Taking into account the immense cross-talk among plant hormones, the contents of auxin (IAA) and abscisic acid (ABA) were also determined.

Materials and methods

Plant materials and growth conditions

Samples of mature leaves from four different types of transgenic tobacco plants with altered CK metabolism and the corresponding wild types (WTs), all in the vegetative stage, collected imediately after the dark period, were used.

1. Tobacco *in vitro* lines expressing an *IPT* homologue *Sho* (*Shooting*) gene from *P. hybrida* under the control of a constitutive cauliflower mosaic virus (CaMV) 35S promoter or construct with four 35S enhancer elements (Zubko *et al*., 2002) and WT *N. tabacum* L. cv. Petit Havana SR1 cultivated on solid hormone-free MS medium (Murashige and Skoog, 1962) supplemented with sucrose (30 g l^{-1}). To achieve inducible *Sho* expression, a dexamethasone-inducible gene expression system (pOp6/LhGR; Šámalová *et al*., 2005) was employed. *Sho* expression was induced in 8–9-week-old transgenic plants with 20 μM dexamethasone [in 0.05% dimethylsulphoxide (DMSO), 5×50 ml per plant watered for 13 d]. As controls, transgenic plants treated with water or 0.05% DMSO and WT SR1 plants treated with water, 0.05% DMSO, or 20 μM dexamethasone in 0.05% DMSO were used. The plants were cultivated in a growth chamber (16/8 h photoperiod at 130 μE m^{-2} s^{-1}, day/night temperature of 25/23 °C and relative humidity ~80%) for 5 weeks and then transferred, before *Sho* induction, to a greenhouse (in summer, without additional light supplementation).

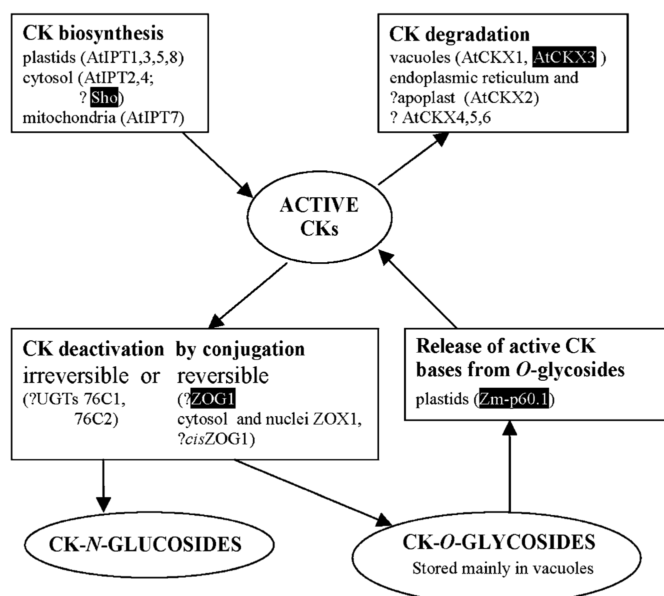

Fig. 1. Simplified model of CK metabolism including known localization of examples of involved enzymes proved by *in planta* experiments. The enzymes employed in this study are highlighted. AtCKX, *Arabidopsis* CK oxidase/dehydrogenase (Werner *et al.*, 2003); AtIPT, *Arabidopsis* isopentenyltransferase (Kasahara *et al.*, 2004); Sho, petunia isopentenyltransferase (Zubko *et al.*, 2002); UGT, *Arabidopsis N*-glucosyltransferase (Hou *et al.*, 2004); Zm-p60.1, maize β-glucosidase (Kristoffersen *et al.*, 2000); ZOG1, bean *trans*-zeatin-*O*-glucosyltransferase (Martin *et al.*, 2001*a*); *cis*ZOG, maize *cis*-zeatin-*O*-glucosyltransferase (Martin *et al.*, 2001*b*); ZOX1, bean zeatin-*O*-xylosyltransferase (Martin *et al.*, 1993); ?, unknown or uncertain localization. The interconversions among iP-, *trans*Z-, *cis*Z-, and DHZ-type CKs and their bases, ribosides, and phosphates, CK transport and tissue localization are not depicted. For more details see Sakakibara (2006).

All the other plants were cultivated in a soil substrate in a growth chamber (16/8 h photoperiod at 150 μE m^{-2} s^{-1}, 26/20 °C and relative humidity ~80%).:

2. Eleven-week-old tobacco plants (*35S:AtCKX3*) overexpressing a gene for CK oxidase/dehydrogenase from *A. thaliana* under a constitutive CaMV 35S promoter (Werner *et al.*, 2001) and 9-week-old WT *N. tabacum* L. cv. Samsun NN.

3. Eight- to 9-week-old tobacco plants (*35S:P60*) overexpressing a maize β-glucosidase *Zm-p60.1* naturally targeted to plastids under a CaMV 35S promoter (Kiran *et al.*, 2006) and WT *Nicotiana tabacum* L. cv. Petit Havana SR1.

4. Eight- to 9-week-old tobacco plants (*35S:ZOG1*) harbouring a *trans*-zeatin-*O*-glucosyltransferase gene from *P. lunatus* under a constitutive CaMV 35S promoter (Martin *et al.*, 2001*a*) and WT *N. tabacum* L. cv. Wisconsin 38.

Chloroplast isolation

Intact chloroplasts were isolated and purified as described by Kiran *et al.* (2006). The chloroplast fraction was recovered from the homogenate of deribbed leaves in homogenization medium [0.33 M sorbitol; 50 mM TRIS–HCl, pH 7.8; 0.4 mM KCl; 0.04 mM Na_2EDTA; 0.1% (w/v) bovine serum albumin; 1% (w/v) polyvinylpyrrolidone; 5 mM isoascorbic acid] by centrifugation, layered on a Percoll density gradient [40% and 80% (v/v) Percoll solution in resuspension medium (0.33 M sorbitol; 2 mM Na_2EDTA; 1 mM $MgCl_2$; 1 mM $MnCl_2$; 50 mM HEPES, pH 7.6)] and centrifuged (1000 *g*; 15 min; 4 °C). Intact chloroplasts were collected at the interface of the gradient, diluted with medium, and centrifuged (1000 *g*; 2 min; 4 °C). The pellet was resuspended in medium. All the procedures were done at 4 °C.

Chlorophyll determination

Chlorophyll was extracted into 80% (v/v) acetone. The total chlorophyll content (*a*+*b*) was calculated from the absorbance at 652 nm of the clear extract after centrifugation (500 *g*, 5 min) according to Arnon (1949).

Chloroplast integrity

The integrity of chloroplasts was determined by the latency of glyceraldehyde-3-phosphate dehydrogenase according to Latzko and Gibbs (1968). Chloroplasts were incubated in a reaction mixture (0.33 M TRIS–HCl, pH 8.5; 17 mM $Na_2HAsO_4{\cdot}7H_2O$; 4 mM cysteine; 20 mM NaF; 40 μM $NADP^+$). The reaction was initiated with 0.02 M glyceraldehyde-3-phosphate. Reduction of $NADP^+$ was followed at 340 nm for 5 min. The same assay was run with chloroplasts disrupted with 0.01 M $MgCl_2$. The percentage of intact chloroplasts was calculated from the difference between the original and disrupted sample.

Extraction and purification of IAA, ABA, and CK

The detailed procedure for hormone extraction, purification, and quantification has been described in Kiran *et al.* (2006). IAA, ABA, and CKs were extracted overnight at –20 °C with Bieleski solvent (Bieleski, 1964). [^{3}H]IAA and [^{3}H]ABA (Sigma, USA) and 12 deuterium-labelled CKs ([$^2H_5$]*t*-Z, [$^2H_5$]*t*-ZR, [$^2H_5$]*t*-Z7G, [$^2H_5$]*t*-Z9G, [$^2H_5$]*t*-ZOG, [$^2H_5$]*t*-ZROG, [$^2H_3$]DHZ, [$^2H_3$]DHZR, [$^2H_6$]iP, [$^2H_6$]iPR, [$^2H_6$]iP7G, and [$^2H_6$]iP9G; Apex Organics, UK) were added as internal standards. The extracts were purified using Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA) and an Oasis MCX mixed mode, cation exchange, reverse-phase column (150 mg, Waters) (Dobrev and Kamínek, 2002). After a wash with 1 M HCOOH, IAA and ABA were eluted with 100% MeOH and evaporated to dryness. Further, CK phosphates were eluted with 0.34 M NH_4OH in water, and CK bases, ribosides, and glucosides were eluted with 0.34 M NH_4OH in 60% (v/v) MeOH. Phosphates were converted to ribosides with alkaline phosphatase. IAA and ABA were separated and quantified by two-dimensional high-performance liquid chromatography (2D-HPLC) according to Dobrev *et al.* (2005). Purified CK samples were analysed by an LCMS system consisting of an HTS PAL autosampler (CTC Analytics, Switzerland), Rheos 2000 quaternary pump (FLUX, Switzerland) with Csi 6200 Series HPLC oven (Cambridge Scientific Instruments, UK), and an LCQ Ion Trap mass spectrometer (Finnigan, USA) equipped with an electrospray. A 10 μl aliquot of sample was injected onto a C18 column (AQUA, 2 mm×250 mm×5 μm, Phenomenex, USA) and eluted with 0.0005% acetic acid (A) and acetonitrile (B). The HPLC gradient profile was as follows: 5 min 10% B, then increasing to 17% for 10 min, and to 46% for a further 10 min at a flow rate of 0.2 ml min^{-1}. The column temperature was kept at 30 °C. The effluent was introduced into a mass spectrometer being operated in the positive ion, full-scan MS/MS mode. Quantification was performed using a multilevel calibration graph with deuterated CKs as internal standards. Plant hormone content is usually presented in picomoles per gram fresh weight (FW) of plant tissue. Here, hormone concentration is also expressed per unit of chlorophyll, allowing the comparison with hormone levels in isolated chloroplasts. The differences were estimated according to the value of the standard error.

Transmission electron microscopy

Leaf blade segments (2 mm×2 mm×leaf thickness) were fixed for 2 h with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer,

pH 7.2, followed by 2 h in 2% (w/v) osmic acid. After dehydration in a graded ethanol series up to 100% ethanol, samples were embedded via propylene oxide in low viscosity Spurr's resin. The ultrastructure was evaluated on transverse ultrathin sections of embedded objects contrasted with a saturated solution of uranyl acetate in 70% (v/v) aqueous ethanol, followed by a lead citrate solution treatment according to Reynolds (1963) using a transmission electron microscope (Philips Morgani) at an operating voltage of 70 kV.

Results

Hormone analyses

In order to investigate the role of hormone compartmentation and to study the interactions among plant hormones, the contents of CKs, free IAA, and ABA were determined in mature leaves and in isolated intact chloroplasts of four different types of transgenic tobacco with altered CK metabolism and in the corresponding WTs during the vegetative stage of plant development. The integrity of the analysed chloroplasts immediately after their isolation was >90%, as checked by the latency of the activity of the stromal enzyme glyceraldehyde-3-phosphate dehydrogenase.

Plants expressing isopentenyltransferase Sho

The total CK content in leaves after *Sho* induction by dexamethasone was eight times that in the corresponding WT (SR1) (Fig. 2A). The most elevated CK metabolites were phosphates (predominantly iPMP) and *N*-glucosides (especially iP7G). The level of physiologically active CK bases and ribosides (predominantly of iP and iPR) increased to a lesser extent. No significant difference in the content of CK-*O*-glucosides was observed. As CK levels did not differ significantly among WT plants treated with water, with dexamethasone in DMSO, or with DMSO solution or non-induced *Sho* gene-carrying plants, only the CK content of WT plants treated with water is shown. The CK elevation in induced *Sho* leaves was lower when the CK content was expressed per mg of chlorophyll, as the transgenics had a higher chlorophyll content than the WT (Fig. 2B). The difference between *Sho* transformants and the WT was much lower in isolated chloroplasts (Fig. 2C) than in leaves. Indeed, except for CK-*O*-glucosides, the percentage of leaf CKs sequestered in chloroplasts was lower in *Sho* compared with WT (see supplementary Table 1 available at *JXB* online). Nevertheless, the CK content of chloroplasts from plants expressing *Sho* was more than twice as high as that of the WT. The contents of individual CKs in leaves and isolated chloroplasts are given in supplementary Table 2 available at JXB online.

On a FW basis, the higher CK content in *Sho* leaves compared with the WT was accompanied by a slightly higher content of IAA and ABA (Table 1). These differences were not significant when expressed per unit of chlorophyll. Chloroplasts of *Sho* plants had lower IAA, but higher ABA content than chloroplasts of WT plants.

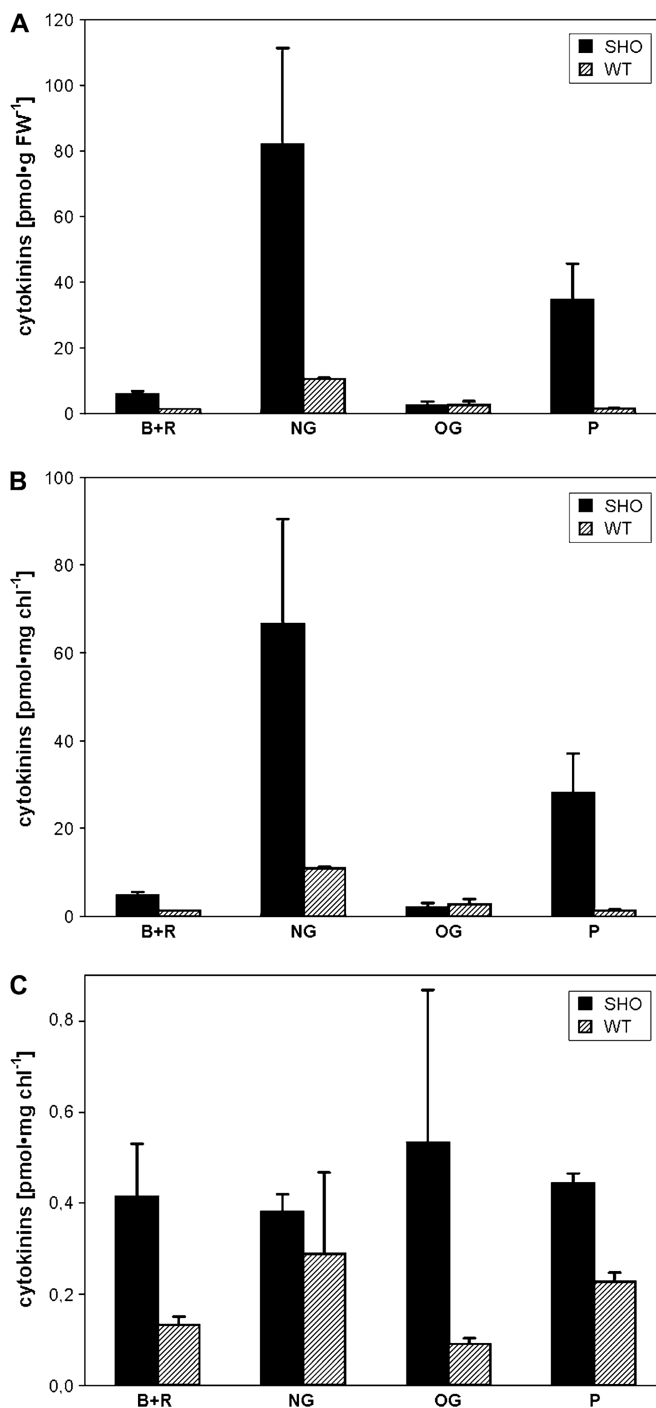

Fig. 2. Cytokinin content (B+R, free bases and ribosides; NG, *N*-glucosides; OG, *O*-glucosides; P, phosphates) in leaves (per fresh weight A and per unit of chlorophyll B) and in isolated intact chloroplasts (C) of transgenic dexamethasone-induced *Sho* (SHO, black) and control SR1 (WT, striped) tobacco. Error bars display the SE.

Plants expressing CK oxidase/dehydrogenase AtCKX3

The constitutive overexpression of *AtCKX3* significantly delayed development of transgenic plants; consequently, hormone contents and chloroplast ultrastructure were

Table 1. *Endogenous contents of free IAA and ABA in leaves and in isolated intact chloroplasts of transgenic dexamethasone-induced* Sho *and WT (SR1) tobacco*

Values represent the mean of two replicates (individual measurements are shown in parentheses).

	Sho	WT (SR1)
IAA		
Leaves (pmol g^{-1} FW)	90.7 (117.9; 63.5)	71.2 (81.1; 61.3)
Leaves (pmol mg^{-1} chlorophyll)	73.7 (95.8; 51.6)	74.2 (84.4; 63.9)
Chloroplasts (pmol mg^{-1} chlorophyll)	2.5 (2.8; 2.2)	3.4 (2.8; 4.0)
ABA		
Leaves (pmol g^{-1} FW)	453.5 (749.1; 157.8)	360.0 (628.1; 91.9)
Leaves (pmol mg^{-1} chlorophyll)	368.7 (609.0; 128.3)	375.0 (654.3; 95.7)
Chloroplasts (pmol mg^{-1} chlorophyll)	16.8 (18.7; 14.9)	12.8 (13.3; 12.2)

analysed in transgenic plants that were older than the corresponding WT (SNN) plants. The total CK content was reduced in leaves (Fig. 3A) to about one-half of that in the WT. Particularly altered were the levels of CK-*N*-glucosides and, to a lesser extent, bases and ribosides. The chlorophyll content was reduced in transgenic plants, so that the differences between transgenic and WT tobacco are reduced when expressed per unit of chlorophyll (Fig. 3B). The CK content was changed to a lesser extent in isolated intact chloroplasts (Fig. 3C). Further, when *AtCKX3* and WT were compared, the percentage of leaf CK bases and ribosides remained almost unchanged, whilst those of phosphates sequestered in chloroplasts increased; the values for CK-glucosides dropped significantly (see supplementary Table 1 available at *JXB* online).

The content of IAA was not significantly changed in *AtCKX3* leaves (Table 2) compared with WT when expressed per FW, but it was higher when expressed per unit of chlorophyll. The ABA level was lower in leaves of transgenic tobacco. No significant changes of IAA or ABA levels were observed in chloroplasts (Table 2).

Plants expressing β*-glucosidase* Zm-p60.1

Compared with WT (SR1) leaves, a trend towards an increase in CK bases and ribosides and CK-*N*-glucosides was observed in leaves (Fig. 4A, B) of tobacco over-expressing maize β-glucosidase *Zm-p60.1* targeted to plastids. A dramatic decrease in the levels of CK-*O*-glucosides remained restricted to the CK pool found in chloroplasts (Fig. 4C). Interestingly, a moderate decrease was also observed for CK-*N*-glucosides in the chloroplast fraction. The trend towards an increase in CK bases and ribosides observed in whole leaf extracts was also seen in the chloroplast fraction. Accordingly, the percentage of leaf CK-*O*- and *N*-glucosides sequestered in chloroplasts was significantly lower in the transgenic plants than in WT plants, while the values changed only marginally for

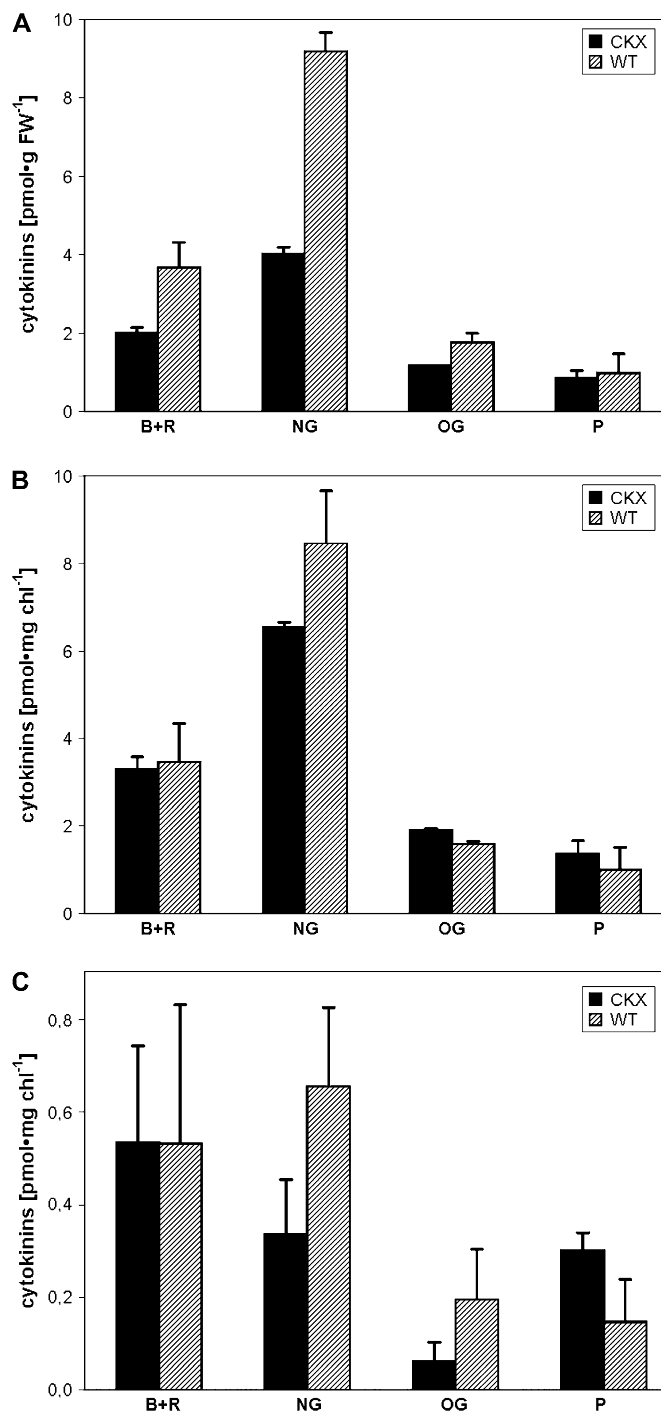

Fig. 3. Cytokinin content (B+R, free bases and ribosides; NG, *N*-glucosides; OG, *O*-glucosides; P, phosphates) in leaves (per fresh weight A and per unit of chlorophyll B) and in isolated intact chloroplasts (C) of transgenic *35S:AtCKX3* (CKX, black) and control SNN (WT, striped) plants. Error bars display the SE.

CK bases and ribosides, and for phosphates (see supplemetary Table 1 available at *JXB* online).

The IAA measurements revealed a significantly lower level of IAA in leaves and in chloroplasts of transgenic plants compared with the WT IAA level (Table 3). The

Table 2. *Endogenous contents of free IAA and ABA in leaves and in isolated intact chloroplasts of transgenic* 35S:AtCKX3 *and WT (SNN) tobacco*

Values represent the mean of two replicates (individual measurements are shown in parentheses).

	35S:AtCKX3	WT (SNN)
IAA		
Leaves (pmol g^{-1} FW)	125.0 (177.4; 72.6)	122.1 (147.3; 96.9)
Leaves (pmol mg^{-1} chlorophyll)	200.5 (279.1; 121.8)	114.4 (151.6; 77.2)
Chloroplasts (pmol mg^{-1} chlorophyll)	5.2 (4.3; 6.1)	5.2 (9.9; 0.4)
ABA		
Leaves (pmol g^{-1} FW)	359.5 (447.7; 271.3)	1102.3 (822.9; 1381.6)
Leaves (pmol mg^{-1} chlorophyll)	579.9 (704.4; 455.4)	974.1 (847.2; 1100.9)
Chloroplasts (pmol mg^{-1} chlorophyll)	68.0 (44.0; 92.0)	58.8 (106.6; 10.9)

ABA content was reduced in *Zm-p60.1* leaves compared with WT (Table 3). In chloroplasts, the difference in ABA level was less pronounced.

*Plants expressing zeatin-*O*-glucosyltransferase* ZOG1

When a *ZOG1* gene was constitutively expressed in tobacco, CK-*O*-glucosides accumulated in leaves (Fig. 5A). The level of *t*-ZOG was two orders of magnitude higher in leaves of transformants than in those of WT (W38). No significant difference in the content of CK-*N*-glucosides, and only marginal reduction of the remaining CK forms was observed. These differences reduced further when the CK content was expressed per unit of chlorophyll (Fig. 5B), while that of CK-*O*-glucosides was more profound, as the chlorophyll content was lower in *ZOG1* than in WT. By contrast, in chloroplasts, very low levels of CK-*O*-glucosides were found (Fig. 5C). The predominant CK forms were bases and ribosides, the contents of which, as well as that of CK phosphates, were higher in *ZOG1* chloroplasts than in WT chloroplasts. Indeed, out of the transgenic plants analysed, only in the plants overexpressing *ZOG1* was the percentage of leaf CK bases and ribosides sequestered in chloroplasts significantly higher in the transgenic plants than in WT plants (see supplementary Table 1 available at *JXB* online).

In leaves of transgenic plants, a 2-fold higher IAA content was detected (Table 4). The ABA level was not significantly affected in *ZOG1* leaves on a FW basis, but was higher when expressed per unit of chlorophyll. IAA and ABA contents were higher in *ZOG1* chloroplasts than in WT chloroplasts.

Ultrastructure observations

The most striking anomaly in chloroplast ultrastructure was the occasional occurrence of crystalloid structures in chloroplasts of *in vitro*-cultivated plants harbouring the *Sho* gene under transcriptional control of four 35S

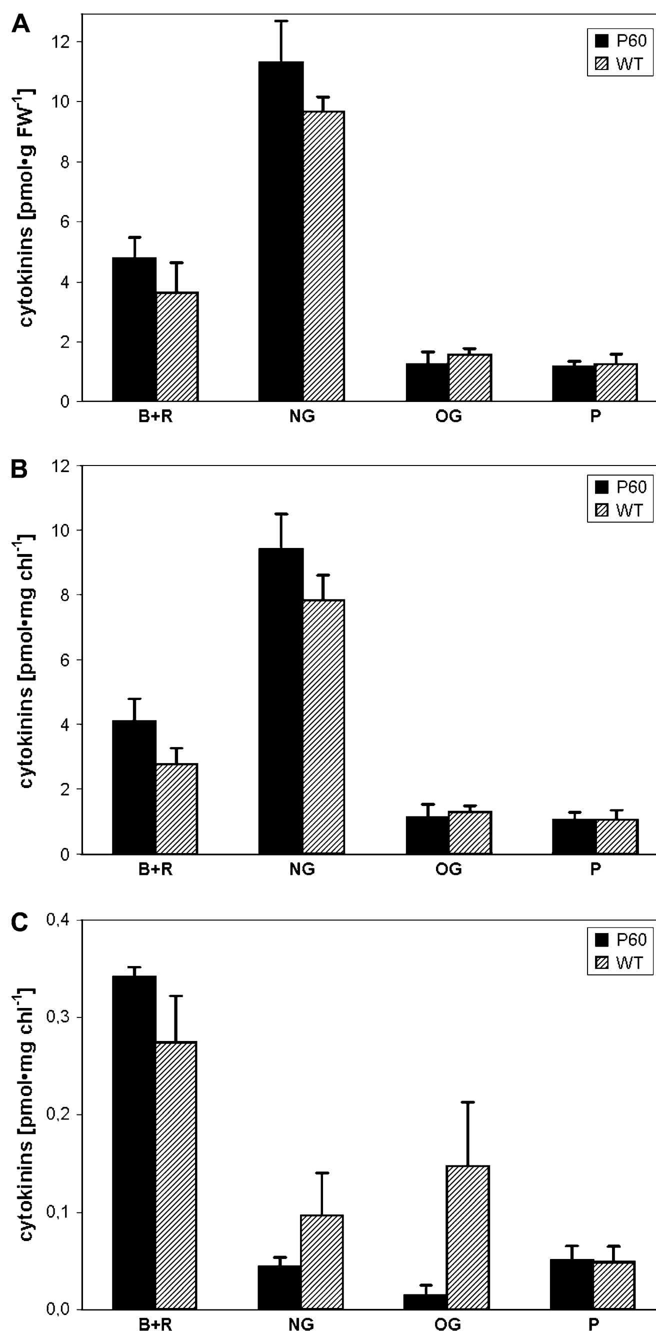

Fig. 4. Cytokinin content (B+R, free bases and ribosides; NG, *N*-glucosides; OG, *O*-glucosides; P, phosphates) in leaves (per fresh weight A and per unit of chlorophyll B) and in isolated intact chloroplasts (C) of transgenic *35S:Zm-p60.1* (P60, black) and control SR1 (WT, striped) tobacco. Error bars display the SE.

enhancers (Fig. 6A, detailed view in Fig. 6B) as well as the 35S promoter. However, some chloroplasts from these plants did not show apparent changes in the structure compared with the WT (Fig. 6C). Chloroplasts from *Sho*-overexpressing plants contained more plastoglobuli than WT chloroplasts (Fig. 6D). The crystalloids were never present in chloroplasts of WT plants cultivated *in vitro*.

Table 3. *Endogenous contents of free IAA and ABA in leaves and in isolated intact chloroplasts of transgenic* 35S:Zm-p60.1 *and WT (SR1) tobacco*

Values represent the mean of two replicates (individual measurements are shown in parentheses).

	35S:Zm-p60.1	WT (SR1)
IAA		
Leaves (pmol g^{-1} FW)	85.7 (95.1; 76.2)	144.0 (146.5; 141.5)
Leaves (pmol mg^{-1} chlorophyll)	60.3 (66.9; 53.6)	135.2 (137.5; 132.8)
Chloroplasts (pmol mg^{-1} chlorophyll)	0.8 (1.4; 0.2)	1.2 (2.1; 0.3)
ABA		
Leaves (pmol g^{-1} FW)	481.6 (486.9; 476.3)	839.0 (785.9; 892.1)
Leaves (pmol mg^{-1} chlorophyll)	338.7 (342.4; 334.9)	787.5 (737.6; 837.4)
Chloroplasts (pmol mg^{-1} chlorophyll)	23.7 (21.5; 25.8)	26.3 (40.4; 12.1)

After induction of the *Sho* gene expression, no crystalloids were found, only an increased grana stacking was observed (Fig. 6E). The crystalloids did not appear in chloroplasts of WT plants treated either with water (Fig. 6F), with dexamethasone in DMSO, or with DMSO solution, or in chloroplasts of non-induced plants carrying the *Sho* gene.

Representative chloroplasts from the other types of transgenic tobacco and corresponding WTs are shown in Fig. 7. The most obvious feature observed was the different starch accumulation. The chloroplasts of tobacco overexpressing *AtCKX3* (Fig. 7A) and *Zm-p60.1* (Fig. 7C) have less starch inclusions than chloroplasts of WT SNN (Fig. 7B) and SR1 (Fig. 7D), respectively. In contrast, the chloroplasts of *ZOG1* transformants (Fig. 7E) contained more starch inclusions than WT W38 chloroplasts (Fig. 7F). With increasing starch content, the shape of chloroplasts altered from lens-shaped to more loaf-like.

Discussion

Hormone analyses

In order to elucidate the degree of chloroplast CK autonomy, the impact of altered CK metabolism on the CK pool in isolated intact chloroplasts and in bulk leaf tissue was compared. Bearing in mind hormone cross-talk, the effect of altered CK metabolism on IAA and ABA levels was also followed. The presence of these hormones in chloroplasts was demonstrated in earlier studies through the use of immunocytochemical (Sossountzov *et al.*, 1986; Ohmiya and Hayashi, 1992; Kärkönen and Simola, 1999) or fractionation techniques, followed by HPLC or LCMS analysis (Sandberg *et al.*, 1990; Benková *et al.*, 1999). Chloroplasts were found to be the compartment where most of the ABA in leaf tissue is formed by the methylerythritol phosphate pathway (Milborrow and Lee, 1998).

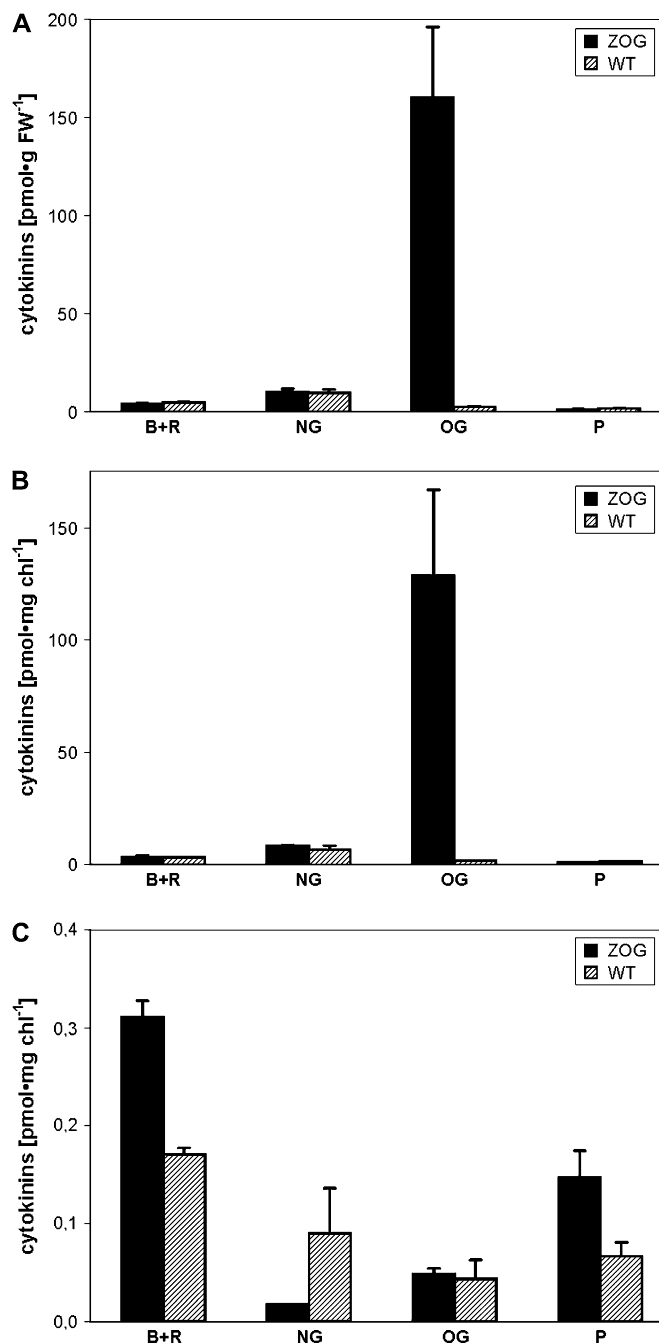

Fig. 5. Cytokinin content (B+R, free bases and ribosides; NG, *N*-glucosides; OG, *O*-glucosides; P, phosphates) in leaves (per fresh weight A and per unit of chlorophyll B) and in isolated intact chloroplasts (C) of transgenic *35S:ZOG1* (ZOG, black) and control W38 (WT, striped) tobacco. Error bars display the SE.

This pathway also provides most of the prenyl group of CKs (Kasahara *et al.*, 2004). Nordström *et al.* (2004) demonstrated *de novo* CK synthesis in *Arabidopsis* shoots and tobacco leaves, and suggested that the presence of chloroplasts might be a prerequisite for the iPMP-independent pathway of CK biosynthesis.

Plants expressing isopentenyltransferase Sho

Numerous studies of plants transformed with the *ipt* gene, encoding an IPT from *Agrobacterium tumefaciens*, have demonstrated enhanced biosynthesis of endogenous CKs, especially of the Z-type (Redig *et al*., 1996; Motyka *et al*., 2003). Recently, genuine plant *IPT* genes (*AtIPT1–9*) were identified in the *A. thaliana* genome (Kakimoto, 2001; Takei *et al*., 2001) and their homologue *Sho* in *P. hybrida* (Zubko *et al*., 2002). The overexpression of the plant homologue *AtIPT8* (Sun *et al*., 2003), as well as *Sho* (Zubko *et al*., 2002), in contrast to the bacterial *ipt*, leads to profound accumulation of the iP-type of CKs. A dexamethasone-inducible gene expression system (pOp6/LhGR; Šámalová *et al*., 2005) was used to drive *Sho* expression in transgenic tobacco. Following *Sho* induction in leaf extracts, major increases were observed in iP7G and iPMP, which is in line with previous results reported for petunia and tobacco overexpressing *Sho* constitutively (Zubko *et al*., 2002). The CK content was also increased in chloroplasts, although to a relatively lower extent. This is in accordance with preliminary results of feeding experiments (data not shown) showing that this IPT preferentially utilizes mevalonate as a substrate, indicating its cytosolic localization. In a recent analysis of P*ssu-ipt* tobacco (Synková *et al*., 2006), similarly to *Sho* plants,

Table 4. *Endogenous contents of free IAA and ABA in leaves and in isolated intact chloroplasts of transgenic* 35S:ZOG1 *and WT (W38) tobacco*

Values represent the mean of two replicates (individual measurements are shown in parentheses).

	35S:ZOG1	WT (W38)
IAA		
Leaves (pmol g^{-1} FW)	88.8 (102.1; 75.5)	44.2 (38.4; 50.0)
Leaves (pmol mg^{-1} chlorophyll)	77.2 (88.8; 65.6)	24.0 (20.8; 27.1)
Chloroplasts (pmol mg^{-1} chlorophyll)	1.5 (1.8; 1.1)	1.0 (0.5; 1.4)
ABA		
Leaves (pmol g^{-1} FW)	528.1 (662.4; 393.8)	523.3 (566.9; 479.6)
Leaves (pmol mg^{-1} chlorophyll)	459.2 (575.9; 342.4)	284.0 (307.7; 260.3)
Chloroplasts (pmol mg^{-1} chlorophyll)	13.1 (20.9; 5.2)	10.1 (15.0; 5.2)

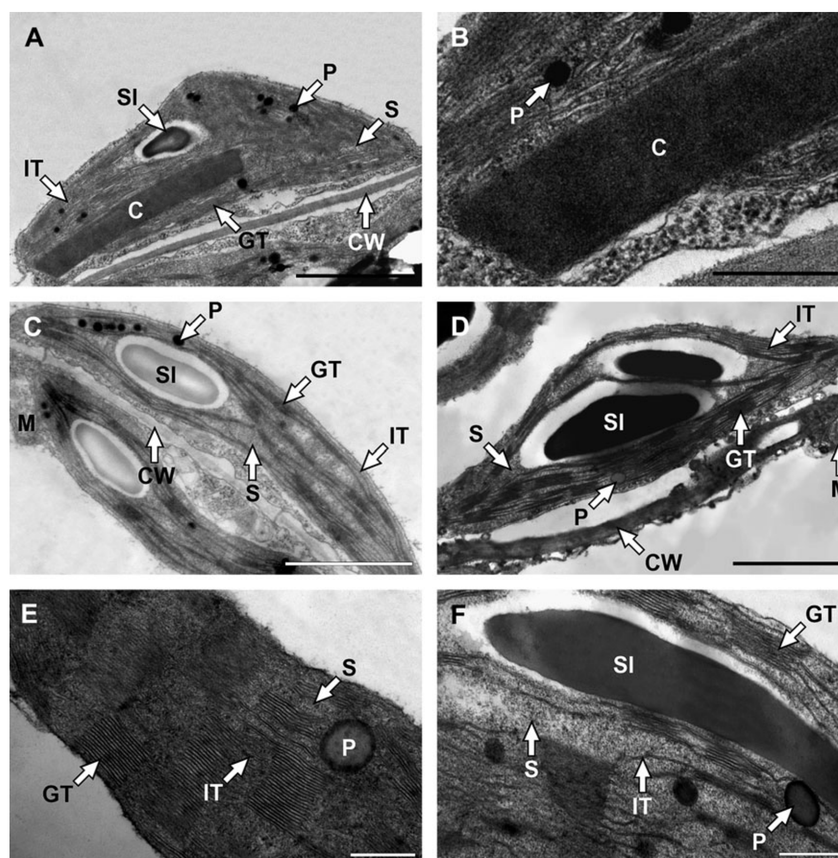

Fig. 6. Transmission electron micrographs of representative chloroplast cross-sections taken from the intact leaves of tobacco expressing the *Sho* gene and four 35S enhancer elements (A, C) with detail of crystalloid (B) and control SR1 (D) tobacco growing under sterile conditions; and chloroplast cross-section detail taken from the leaf of transgenic dexamethasone-induced *Sho* (E) and control SR1 (F) tobacco cultivated in a greenhouse. C, crystalloid; CW, cell wall; GT, granal thylakoids; IT, intergranal thylakoids; M, mitochondrion; P, plastoglobuli; S, stroma; SI, starch inclusion. Bar=2 μm in A, C and D; and 500 nm in B, E and F.

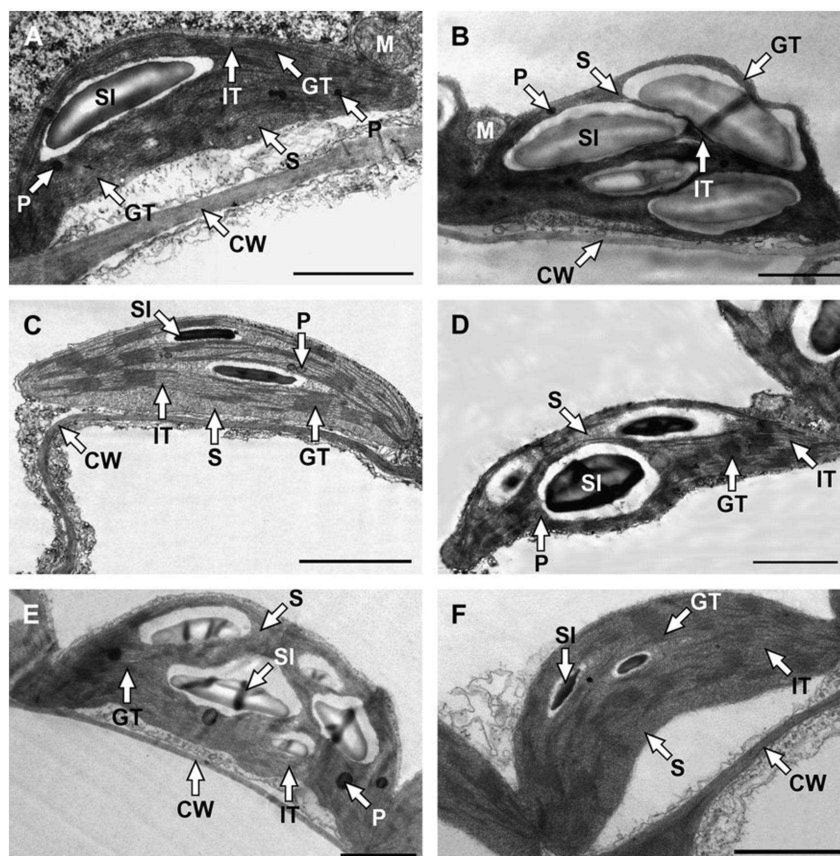

Fig. 7. Transmission electron micrographs of representative chloroplast cross-sections taken from the intact leaves of transgenic *35S:AtCKX3* (A) and control SNN (B), transgenic *35S:Zm-p60.1* (C) and control SR1 (D), transgenic *35S:ZOG1* (E) and control W38 (F) tobacco. CW, cell wall; GT, granal thylakoids; IT, intergranal thylakoids; M, mitochondrion; P, plastoglobuli; S, stroma; SI, starch inclusion. Bar=2 μm.

CK levels increased in chloroplasts to much lower levels compared with leaf extracts, indicating that the main CK pool accumulates outside chloroplasts in both Z- and iP-type CK-overproducing plants.

The content of IAA in leaves of *Sho*-expressing plants did not differ significantly from that in WT, whereas the chloroplasts contained a lower level of IAA than WT chloroplasts. In *ipt* tobacco, Eklöf *et al.* (1997, 2000) found lower levels of IAA and reduced rates of IAA synthesis and turnover. This has led to the suggestion that CKs might down-regulate IAA levels. Recently, a decrease in auxin biosynthesis following an increase in CK levels achieved by induction of *ipt* was observed in *Arabidopsis*, however, with a significant delay. Thus, the decrease was hypothesized to be mediated through an altered development rather than through a direct CK–auxin cross-talk (Nordström *et al.*, 2004).

A higher ABA content was found in chloroplasts and in leaves (when expressed per FW) of *Sho* plants compared with WT. After overexpression of bacterial *ipt*, both a decrease (Synková *et al.*, 1999; Chang *et al.*, 2003) and an increase (Macháčková *et al.*, 1997) in ABA content was reported.

Plants expressing CK oxidase/dehydrogenase AtCKX3

The CK oxidase/dehydrogenase selectively cleaves unsaturated N^6 side chains of Z, iP, and their corresponding ribosides, while CK phosphates, *O*-glucosides, and CKs with saturated side chains are not substrates for CK oxidase/dehydrogenase (Armstrong, 1994). It was shown that overexpression of *AtCKX* genes in tobacco and *Arabidopsis* plants resulted in a reduced content of endogenous CKs and a strongly altered phenotype, with dwarfed shoot habit, enhanced root growth, and delayed flowering and senescence (Werner *et al.*, 2001, 2003). More abundant Z-derived CKs were more strongly reduced than were iP derivatives in *35S:AtCKX1* and *35S:AtCKX2 Arabidopsis*, in contrast to *35S:AtCKX1* and *35S:AtCKX2* tobacco where the more profound changes were in iP-type CKs. To our knowledge, the CK content has never been measured in any *35S:AtCKX3* transgenic plant. These plants are phenotypically identical to *35S:AtCKX1* and the enzyme is ultimately targeted to the same compartment, the vacuole, as proved by *in planta* experiments exploring an AtCKX–green fluorescent protein (GFP) fusion. Nevertheless, the apparent K_m value for

AtCKX3 against iP was 14 times higher than that for AtCKX1 (Werner *et al*., 2003). It has been shown that the ectopic expression of *AtCKX3* reduced the CK levels in leaves of adult tobacco plants and also influenced the CK levels in chloroplasts. In leaves, the most strongly reduced CK metabolites were *N*-glucosides. It is obvious that after a tremendous increase of the activity of an important CK catabolic pathway (side chain cleavage by oxidase/dehydrogenase), the activity of the other CK deactivation pathway (*N*-glucosylation) was suppressed. In young tobacco seedlings with very low *N*-glucosylation activity, oxidase/dehydrogenase overexpression had only a marginal effect on the level of *N*-glucosides (Werner *et al*., 2001). This discrepancy could be explained by the different developmental stages of the analysed tobacco plants. In *35S:AtCKX1* and *35S:AtCKX2 Arabidopsis* seedlings, Z9G and iPG were reduced compared with WT.

No significant differences were observed between the IAA levels, expressed on a FW basis, in leaves of adult *AtCKX3* and WT tobacco plants. This finding contrasts with the results of Werner *et al*. (2003), who found reduced levels of IAA in *35S:AtCKX1* and *35S:AtCKX2 Arabidopsis* seedlings. However, the authors speculate that CKs need not directly regulate IAA metabolism, but that the different tissue composition, i.e. reduced size of the shoot apical meristem with fewer meristematic cells and reduced cell production in leaves, might lead to a lowering of IAA-producing shoot tissue in transgenic plants.

A lower ABA level was found in *AtCKX3* leaves compared with WT leaves, which might be linked to prolonged life span and retarded senescence of transgenic plants. Brugière *et al*. (2003) demonstrated induction of maize *CKX1* gene expression in leaf discs by ABA, suggesting a role for this hormone in lowering CK concentrations under different abiotic stresses.

Plants expressing β-glucosidase Zm-p60.1

Maize β-glucosidase Zm-p60.1 was the first plant enzyme shown to release active CKs from CK-*O*- and *N3*-glucosides *in vivo* (Brzobohatý *et al*., 1993). Recently, it was shown that ectopic overexpression of *Zm-p60.1* can perturb CK homeostasis in transgenic tobacco (Kiran *et al*., 2006). Higher levels of CK bases and ribosides in upper leaves and internodes of transgenic plants were found. The present results are consistent with those of the previous report. Moreover, it was found that a tendency to higher accumulation of CK bases and ribosides is also apparent in chloroplasts. The dramatic decrease in CK-*O*-glucoside levels reported here for chloroplasts isolated from *Zm-p60.1* plants corresponded well with the plastid/chloroplast location of Zm-p60.1 (Kristoffersen *et al*., 2000) and a strong decrease in apparent K_m for *t*-ZOG when the values obtained for the enzyme purified *in vitro* and present in isolated chloroplasts were compared (Kiran *et al*., 2006).

Lower IAA levels were determined in leaves as well as isolated chloroplasts of *Zm-p60.1* plants compared with WT. This agrees with previous results, where a steeper fall in IAA gradient from high to low from youngest leaves downward, and a decreased IAA content in the apex and first internodes in *Zm-p60.1* tobacco plants was found (Kiran *et al*., 2006). CK effects might be partly mediated by elevation of ethylene biosynthesis (Genkov *et al*., 2003). Ethylene treatment was shown to stimulate the oxidative decarboxylation of IAA (Winer *et al*., 2000). Thus, the elevated contents of active CKs in *Zm-p60.1* plants could lead to lower levels of free IAA.

ABA measurements revealed a lower ABA content in *Zm-p60.1* leaves than in WT. This finding is in contrast to those of a previous study (Kiran *et al*., 2006), when the tendency of increased ABA accumulation especially in older leaves was observed.

Plants expressing trans*-zeatin-*O*-glucosyltransferase* ZOG1

Tobacco transformed with the *ZOG1* gene from *P. lunatus* under the control of the CaMV 35S promoter was constructed by Martin *et al*. (2001*a*). They found a strongly elevated *t*-ZOG content with no substantial changes in the level of other CK metabolites. In the present study, an order of magnitude higher accumulation of ZOG in *ZOG1* leaves was detected, which may have been caused by the analysis of older material in the present case and different cultivation conditions. The elevated CK-*O*-glucoside concentrations in bulk leaf tissue did not significantly influence the content of these metabolites in intact chloroplasts isolated from the transgenic plants. This indicates that CK-*O*-glucosides are accumulated mainly outside chloroplasts, which agrees with the previous observation that transgenic plants overexpressing *Zm-p60.1* can accumulate high levels of *t*-ZOG when grown on a medium supplemented with *t*-Z despite high Zm-p60.1 β-glucosidase activity in chloroplasts (Kiran *et al*., 2006). The vacuole has been proposed as the main storage compartment for CK-*O*-glucosides, based on feeding experiments in *Chenopodium rubrum* (Fußeder and Ziegler, 1988).

A 2-fold higher IAA content was observed in *ZOG1* leaves compared with WT. Martin *et al*. (2001*a*) reported that IAA does not serve as a substrate for zeatin-*O*-glucosyltransferase and therefore excluded changes in IAA glucosylation as triggering the observed variations in morphology of transformants (e.g. aerial roots, multiple shoots, more compact stature than WT).

Ultrastructure observations

Considerable changes were observed in the chloroplast ultrastructure of tobacco constitutively expressing the *Sho* gene, including an increased number of plastoglobuli and the appearance of crystalloids in plants grown *in vitro*.

These crystalloids were not found in other CK metabolism transformants analysed in this work and their corresponding WTs. In another type of transgenic tobacco, with strongly elevated endogenous CKs, P*ssu-ipt*, anomalies in chloroplast ultrastructure together with crystalloid occurrence were also found, and the distinct crystalline structures were hypothesized to be formed by 2D crystals of light-harvesting complex proteins (Synková *et al.*, 2006).

When dexamethasone-induced *Sho* plants were analysed, increased grana stacking was noticed. Likewise, an increase in grana stacking was observed in chloroplasts of transgenic *ipt* tobacco (Čatský *et al.*, 1993) and after CK treatment (Wilhelmová and Kutík, 1995; Salopek-Sondi *et al.*, 2002).

Except for *Sho* tobacco, marked changes in the ultrastructure of chloroplasts were not found in the other CK transformants analysed. Only a difference in starch accumulation, associated with a change in chloroplast shape, was perceived. This finding is in accordance with data showing that exogenous CK application may increase starch accumulation in chloroplasts (Wilhelmová and Kutík, 1995; Stoynova *et al.*, 1996). The involvement of endogenous CKs in starch formation and amyloplast development was proved by Miyazawa *et al.* (2002) in tobacco BY-2 cells.

In conclusion, a highly non-uniform distribution of CKs was found between chloroplasts and bulk leaf tissue in transgenic tobacco lines with several distinct alterations of CK metabolism, indicating that chloroplasts are relatively independent organelles with respect to the regulation of CK metabolism. Thus, it is evident that the estimation of overall CK content in plant tissue is not sufficient to assess its level at a particular site of action and that different compartmentation of individual CK metabolites should be taken into account. CK glucosides do not usually accumulate in chloroplasts, while active forms of CKs, bases, and ribosides are often found in them. In line with previous reports, the present data suggest a complex nature of mutual interactions between CKs, IAA, and ABA that apparently cannot be described by a simple model based on the current state of the art in this field. Thus, Werner *et al.* (2003) and Nordström *et al.* (2004) suggested that the effect of CKs on IAA levels might be mediated through altered development. Very variable data can be found in the literature on the interaction of CKs and ABA. CKs are known to counteract many processes induced by ABA (e.g. senescence or stomata closure). Elevated CK content thus might be balanced by higher ABA levels, which is also the case for *Sho* plants. However, the CK–ABA cross-talk does not seem to be straightforward. It probably strongly depends on the actual physiological conditions of the studied material including environmental factors and/or tissue specificity.

Supplementary data

Supplementary data available at JXB online are Table 1 showing the percentage of leaf CKs sequestered to chloroplasts and Table 2 listing the CK contents in leaves and isolated chloroplasts.

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