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ORIGINAL ARTICLE

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Cytokinin effects on tetrapyrrole biosynthesis and photosynthetic activity in barley seedlings

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Abstract Cytokinin promotes morphological and physiological processes including the tetrapyrrole biosynthetic pathway during plant development. Only a few steps of chlorophyll (Chl) biosynthesis, exerting the phytohormonal influence, have been individually examined. We performed a comprehensive survey of cytokinin action on the regulation of tetrapyrrole biosynthesis with etiolated and greening barley seedlings. Protein contents, enzyme activities and tetrapyrrole metabolites were analyzed for highly regulated metabolic steps including those of 5-aminolevulinic acid (ALA) biosynthesis and enzymes at the branch point for protoporphyrin IX distribution to Chl and heme. Although levels of the two enzymes of ALA synthesis, glutamyl-tRNA reductase and glutamate 1-semialdehyde aminotransferase, were elevated in dark grown kinetin-treated barley seedlings, the ALA synthesis rate was only significantly enhanced when plant were exposed to light. While cytokinin do not stimulatorily affect Fe-chelatase activity and heme content, it promotes activities of the first enzymes in the Mg branch, Mg protoporphyrin IX chelatase and Mg protoporphyrin IX methyltransferase, in etiolated seedlings up to the first 5 h of light exposure in comparison to control. This elevated activities result in stimulated Chl biosynthesis, which again parallels with enhanced photosynthetic activities indicated by the photosynthetic parameters F_V/F_M, J_{CO2max} and J_{CO2} in the kinetin-treated greening seedlings during the first hours of illumination. Thus, cytokinin-driven acceleration of the tetrapyrrole metabolism supports functioning and assembly of the photosynthetic complexes in developing chloroplasts.

Keywords Tetrapyrrole · Chlorophyll · Photosynthesis · Phytohormones · Metabolism

Abbreviations ALA: 5-Aminolevulinic acid · ALAD: 5-Aminolevulinic acid dehydratase · Chl: Chlorophyll · Fe-chelatase: Ferrochelatase · GluTR: Glutamyl-tRNA reductase · GSAT: Glutamate 1-semialdehyde aminotransferase · Mg-chelatase: Magnesium protoporphyrin IX chelatase · MgPMT: Magnesium protoporphyrin IX methyltransferase · MgProto: Magnesium protoporphyrin IX · MgProtoMME: Magnesium protoporphyrin IX monomethylester · PChlide: Protochlorophyllide · PPFD: Photosynthetic photon fluence density · Proto: Protoporphyrin IX · POR: NADPH-protochlorophyllide oxidoreductase · ZnProto: Zinc protoporphyrin IX

Introduction

Cytokinins are plant hormones influencing a large number of developmental and physiological processes in plants including cell division, organ formation and regeneration, apical dominance, chloroplast differentiation, de-etiolation, leaf senescence, response to pathogens and nutrient metabolism (Mok and Mok 2001; Kakimoto 2003). In the last years, rapid progress has been achieved in the elucidation of cytokinin metabolism, signal perception and transduction (Mok and Mok 2001; Haberer and Kieber 2002; Kakimoto 2003).

Numerous reports have emphasized that cytokinin promotes the development of light-exposed plants and affect the efficiency of the photosynthetic apparatus

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(Kraepiel and Miginiac 1997; Thomas et al. 1997). The expression of many photosynthesis-associated nuclear and chloroplastic genes is induced by light and cytokinin. Current models propose that cytokinin and light might act through common signal transduction intermediates to control the same downstream responses (Chory et al. 1994). Recent studies suggest that cytokinin and light signaling converge at the level of ARR4 and phytochrome B (Hwang and Sheen 2001; Sweere et al. 2001).

However, experimental evidence was presented in several reports, that cytokinin also strongly promotes photomorphogenetic plant development of etiolated seedlings by increasing levels of nuclear and plastid-encoded chloroplast proteins. Treatment of etiolated cucumber and maize seedlings with cytokinins activates photosynthetic enzymes in the dark and enhances the photosynthetic activity during subsequent illumination of plants (Harvey et al. 1974; Caers and Vendrig 1986).

It is well documented that stimulation of chlorophyll (Chl) synthesis during greening of etiolated leaves is due to an increased synthesis of the first tetrapyrrole precursor, 5-aminolevulinic acid (ALA; Fletcher and McCullagh 1971; Fletcher et al. 1973; Lew and Tsuji 1982). Application of cytokinin to cucumber and barley increased the levels of plastid $tRNA^{glu}$ (Masuda et al. 1994) and of transcripts encoding glutamyl-$tRNA^{glu}$ reductase (GluTR) participating in ALA synthesis (Masuda et al. 1995; Bougri and Grimm 1996). Stimulatory effect of cytokinin on the activity of ALA dehydratase (ALAD) was reported in senescent leaves of barley (Stobart et al. 1972; Hukmani and Tripathy 1994). Cytokinins strongly increased the level of mRNA encoding NADPH-protochlorophyllide oxidoreductase (POR) and the content of this enzyme in cucumber and lupine cotyledons (Kuroda et al. 1996; Kusnetsov et al. 1998; Kuroda et al. 2001).

However, coherent survey is missing about the contribution of cytokinin on the regulation of the expression and activities of enzymes of the entire tetrapyrrole biosynthetic pathway. We were encouraged to extend our knowledge on cytokinin control of tetrapyrrole biosynthesis and present effects of cytokinin on the contents and activities of key enzymes of this pathway in etiolated and greening barley seedlings. Secondly, we assessed the influence of cytokinin on photosynthetic processes by measuring Chl fluorescence and the CO_2 gas exchange and correlated the cytokinin-effects on expression and activities of certain enzymatic steps in the Chl metabolism with those on photosynthetic activities.

Materials and methods

Plant material and growth conditions

Barley seedling of wild type (*Hordeum vulgare* L. cv. Gonar, obtained from Institute of Arable Farming and Plant Breeding, National Academy of Sciences of Belarus, Zhodino, Belarus) and the mutant *albostrians* (*Hordeum vulgare* L. cv. Haisa; Hagemann and Scholz 1962) were grown at 23°C in continuous darkness. Starting from the fourth day, seedlings were sprayed three times per day with a 93 μM solution of kinetin or water (as control) under dim green light. Seven days after sowing, seedlings were exposed to illumination at photosynthetic photon fluence density (PPFD) of 80 μmol photons m^{-2} s^{-1} (PAR: 400–700 nm). Dark-grown or illuminated white leaves of *albostrians* displayed the mutant phenotype. Yellow etiolated or greening seedlings were considered to be the control. Harvest of white and yellow *albostrians* mutant leaves in darkness were described before (Yaronskaya et al. 2003).

Western-blot analysis

Extraction of total leaf protein, SDS-PAGE and immunoblotting were performed as described (Kruse et al. 1995). Antisera against recombinant tobacco proteins were prepared at the IPK, Gatersleben, Germany, by Dr. R. Manteuffel, with the exception of antisera against barley GluTR and pea CHL D (kindly provided by Dr. Gamini Kannangara, former Carlsberg Laboratories, Copenhagen, Denmark, and Dr. John Weinstein, former Clemson University, USA, respectively).

Determination of ALA-synthesizing capacity and of some enzyme activities of tetrapyrrole biosynthesis

Leaf segments (0.3 g) were incubated with 50 mM levulinic acid (LA) in 0.1 M Tris–HCl buffer (pH 7.2) in light or in darkness for 5 h. ALA determination was carried out as previously described (Yaronskaya et al. 2003). Isolation of plastids, stromal fractions and assay of the rate of ALA synthesis in stromal fractions were done according to (Masuda et al. 1994, 1995). Magnesium protoporphyrin IX chelatase (Mg-chelatase), magnesium protoporphyrin IX methyltransferase (MgPMT) and ferrochelatase (Fe-chelatase) assays were performed as described earlier (Yaronskaya et al. 2003). Fractions of crude chloroplast extracts, which were assayed for enzyme activity of Mg-chelatase, MgPMT and Fe-chelatase, were extracted twice with acetone: 0.1 N NH_4OH (9:1 v/v) for the analysis of their reaction products. The amounts of magnesium protoporphyrin IX (MgProto) (λ_{ex} 420 nm/λ_{em} 595 nm) and zinc protoporphyrin IX (ZnProto) (λ_{ex} 416 nm/λ_{em} 589 nm) in hexane-washed water-acetone extracts were spectrofluorometrically measured and quantified by calibration with standard porphyrins. The Mg-chelatase activity was expressed as pmol MgProto mg^{-1} protein h^{-1}. The calculated amount of ZnProto yielded from subtraction of the ZnProto amount detected in the heat control. The Fe-chelatase activity was expressed as pmol ZnProto mg^{-1} protein h^{-1}. The Mg porphyrins of the MgPMT assay were quantitatively transferred to diethyl ether, separated by thin

layer chromatography (Duggan and Gassman 1974) and extracted from silica gel into hexane-washed acetone. The amounts of magnesium protoporphyrin IX monomethylester (MgProtoMME) (λ_{ex} 420 nm/λ_{em} 595 nm) were spectrofluorometrically measured and quantified by calibration with standard porphyrin. The activity of MgPMT was expressed as pmol MgProtoMME mg^{-1} protein h^{-1}.

Pigment and heme analysis

Pigments were extracted from leaves with acetone: 0.1 N NH_4OH (9:1 v/v). The amounts of protochlorophyllide (Pchlide) (λ_{ex} 450 nm/λ_{em} 636 nm) in hexane-washed water-acetone extracts were spectrofluorometrically measured and quantified using authentic standards. The content of non-covalently bound heme was essentially determined after removal of free heme and Chl from the leaf sample (0.3 g) with acetone: 0.1 N NH_4OH (9:1 v/v; Weinstein and Beale 1984; Yaronskaya et al. 2003).

Analysis of photosynthetic activity

The Chl fluorescence measurements were carried out on attached primary leaves directly under the respective greening conditions using a portable Chl fluorometer (PAM 2000, Walz, Effeltrich, Germany). For determination of F_0 and F_M the leaves were dark-adapted for 30 min with leaf clips. The F_V/F_M was calculated by $(F_M\text{-}F_0)/F_M$. CO_2 gas exchange measurements were carried out on attached primary leaves in an open CO_2/H_2O gas exchange cuvette system using an infrared gas analyzer (LI−6400, Fa. LI-COR Biosciences, Inc., Lincoln, Nebraska, USA) at a temperature of 22°C, a relative humidity of ca. 50%, a cuvette air flow rate of 300 µmol s^{-1} and an external CO_2 concentration of 360 µl l^{-1}. Irradiance during the measurement was provided by an LED source (6400-02B) with 10% blue and 90% red light. Light-dependent CO_2 exchange rates (light saturation curves) were surveyed by changing PPFD from 1,500 to 20 µmol photons m^{-2} s^{-1}. Each light saturation curve was continued with analysis of dark respiration rates of the leaves after 5 min dark adaptation. The calculation of the gas exchange parameters is based on the equations described by von Caemmerer and Farquhar (1981). The modeling of the light saturation curves of CO_2 uptake rates and the calculation of the maximum CO_2 uptake rates (J_{CO2max}) as well as the net CO_2 uptake rates at growth conditions (J_{CO2}) are described in detail before (Poers 1999).

Miscellaneous and statistical evaluation

The data on enzyme activities and contents of Pchlide and heme represent the means of atleast three independent experiments, with three replicates each time. The data of Chl fluorescence and CO_2 gas exchange were statistically checked by the *t* test upon accomplishing the *F* test.

Results

Cytokinin-dependent changes of Pchlide levels and ALA-synthesizing capacity in dark-incubated greening barley seedlings

In pre-experiments, we proved that application of kinetin in the concentration range between 46 and 460 µM stimulated Pchlide synthesis of green barley leaves during dark incubation. In principle, use of 93 µM kinetin in our experiments appeared to be applicable and is consistent with the application of similar amounts of this phytohormone published previously (Chory et al. 1994; Kuroda et al. 1996; Kusnetsov et al. 1998). The kinetin treatment of etiolated seedlings led only to a marginal increase of the Pchlide content (8% compared to control) (data are not shown). These results are in agreement with previous observations in etiolated cucumber cotyledons indicating no kinetin effect on Pchlide amounts (Fletcher and McCullagh 1971). But after light exposure of etiolated leaves for 2 and 5 h, their Pchlide levels were elevated in the first 30 min of dark incubation (Fig. 1a). The stimulatory kinetin effect on PChlide accumulation was persistent only in the first hours of darkness. After a 24 h dark period, Pchlide accumulation was similar in kinetin-treated and control seedlings (Fig. 1a).

Kinetin-stimulated Pchlide accumulation during the first period of dark incubation may result from light-induced cytokinin-promoted formation of the first specific tetrapyrrole precursor, ALA. Thus, etiolated barley leaves were exposed to light for different time periods from 2 min to 8 h, before their ALA synthesis rate was determined during a 5 h dark incubation. Interestingly, a 2 min light pulse stimulated ALA synthesis in the following dark incubation. The ALA synthesis rate in darkness was elevated upon kinetin treatment after a light exposure of up to 5 h, in comparison to control conditions, before the stimulatory effect of cytokinin expired (Fig. 1b). The light-dependent but transient positive effect of cytokinin on ALA formation becomes apparent, since kinetin did not increase the ALA-synthesizing capacity of seedlings which were completely kept in darkness.

Then, the potential capacity of ALA synthesis was determined from stromal protein fractions of isolated plastids of kinetin-treated and kinetin-untreated etiolated barley leaves. ATP and NADPH were added, because ALA formation of the stromal protein fraction could not be detected without these additional components. Leaf incubation with kinetin increased the rate of ALA synthesis by 55% (0.822 ± 0.075 nmol ALA mg^{-1} protein h^{-1}) in comparison to the control seedlings (0.529 ± 0.068 nmol ALA mg^{-1} protein h^{-1}).

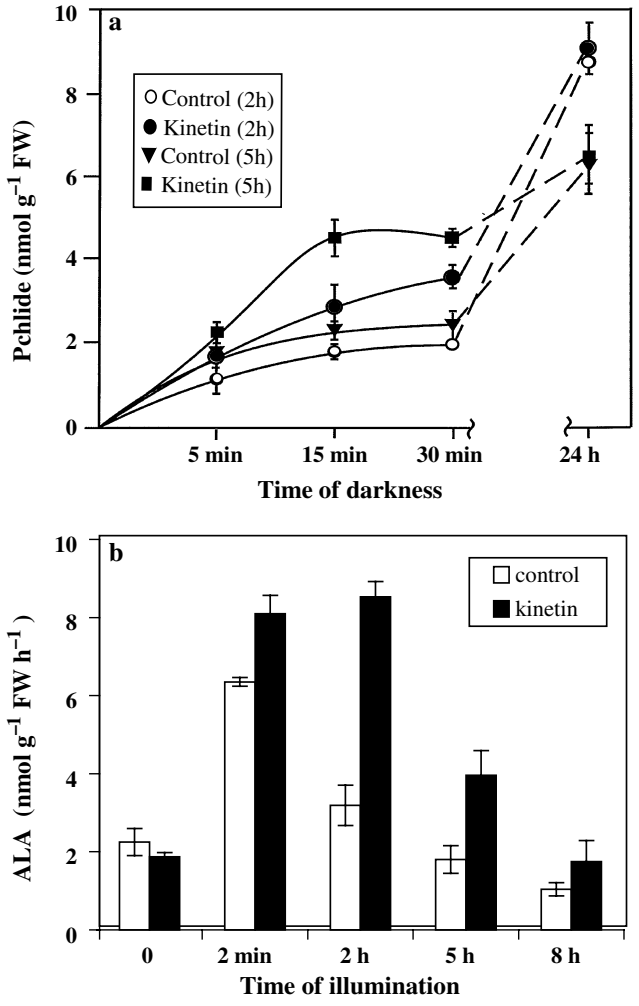

Fig. 1 a, b Effects of cytokinin on protochlorophyllide (*Pchlide*) synthesis (**a**) and ALA-synthesizing capacity (**b**). **a** Etiolated barley seedlings treated with water (control) or kinetin were illuminated for 2 or 5 h (80 µmol photons m^{-2} s^{-1}). After illumination, seedlings were transferred back to darkness for 24 h. PChlide accumulation was determined in leaf samples after the first minutes during dark incubation and compared with the value of samples harvested after 24 h darkness. **b** Etiolated barley seedlings incubated in water (control) or additionally with kinetin were illuminated (80 µmol photons m^{-2} s^{-1}) for different time points. After illumination, leaves were incubated for 5 h in the dark in 50 mM LA. The data are mean ± SD from three experiments, each of which was performed in triplicate

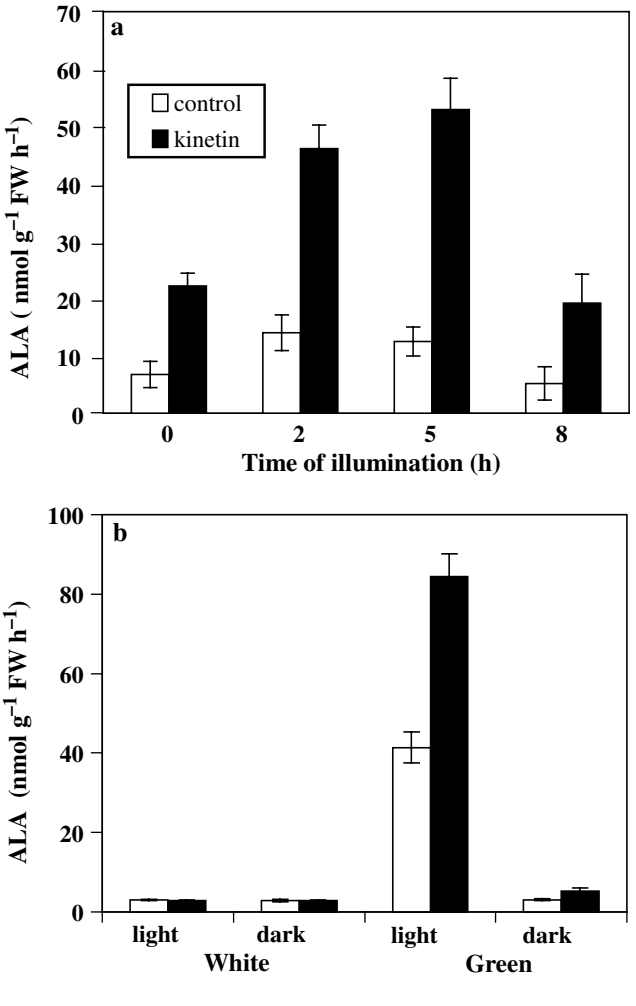

Fig. 2 a, b Effects of cytokinin on ALA-synthesizing capacity of wild-type and *albostrians* barley seedlings. **a** Etiolated wild-type barley seedlings sprayed with water (control) or kinetin solution were illuminated (80 µmol photons m^{-2} s^{-1}) for 24 h. Then leaf samples were taken at different time points and incubated for 5 h in light in 50 mM LA. **b** Etiolated *albostrians* mutant seedlings were incubated with water or with kinetin and exposed to light (80 µmol photons m^{-2} s^{-1}) for 3 h. After illumination, leaves were incubated for 5 h in 50 mM LA in light or in darkness. *White* white mutant leaves; *Green* green mutant leaves. The data are mean ± SD from three experiments, each of which was performed in triplicate

Stimulatory effect of cytokinin on ALA-synthesizing capacity is enhanced during light incubation of greening barley seedlings

A more than threefold increase of ALA-synthesis rates of kinetin-treated etiolated leaves was observed during light exposure (Fig. 2a) in comparison to the rates of control leaves. These results confirm previous reports on kinetin-stimulated ALA synthesis (Fletcher and McCullagh 1971; Fletcher et al. 1973; Lew and Tsuji 1982).

The steady-state levels of the two enzymes involved in the rate-limiting synthesis of ALA, GluTR and glutamate 1-semialdehyde aminotransferase (GSAT) were analyzed in kinetin-treated etiolated and greening barley leaves. Kinetin substantially increased the level of GluTR in etiolated leaves (Fig. 3a). During illumination, the GluTR content increased more drastically and continued in kinetin-treated leaves up to 5 h after illumination start, in comparison to control leaves. The GSAT content was also elevated in etiolated kinetin-treated leaves compared to control leaves (Fig. 3a). But in illuminated seedlings the GSAT levels did not differ in treated and non-treated plants.

Stimulating kinetin effects on ALAD activity have been previously reported (Stobart et al. 1972; Hukmani and Tripathy 1994). But under our experimental conditions, cytokinin application to etiolated barley seedlings with kinetin did not affect the activity (data not shown) and the protein level of ALAD (Fig. 3a) both in dark-grown and greening leaves.

It is tempting to compare the effect of cytokinin treatments on tetrapyrrole biosynthesis of wild-type barley seedlings with the mutant *albostrians*. *Albostrians* carries a nuclear-gene-induced plastome mutation that causes a block in chloroplast development (Hagemann and Scholz 1962). Lack of the translational apparatus in mutant plastids can lead to undifferentiated white seedlings which are not photosynthetically active (Boerner et al. 1976). While Chl formation is not detectable in white *albostrians* seedlings, the heme levels of light and dark-grown mutant seedlings are only reduced to 38 and 55% of the values of green seedlings, respectively. The activity of ALA synthesis of white seedlings gave rise to 25% of that of green seedlings (Yaronskaya et al. 2003). It was proposed that synthesis of ALA intended for Chl formation must be blocked in white tissue, while the remaining ALA synthesis provides precursors for heme synthesis. Thus, white *albostrians* seedlings are useful for studying kinetin effects on those part of ALA formation that mainly contributes to heme biosynthesis.

Etiolated leaves of *albostrians* mutant and control seedlings treated with and without kinetin were illuminated for 3 h, before ALA-synthesizing capacity was determined in light and darkness. While the twofold increase of ALA synthesis rate in kinetin-treated control seedlings is confirmative (Fig. 2b vs. Figs. 1b and 2a), cytokinin did not stimulate activities for ALA formation

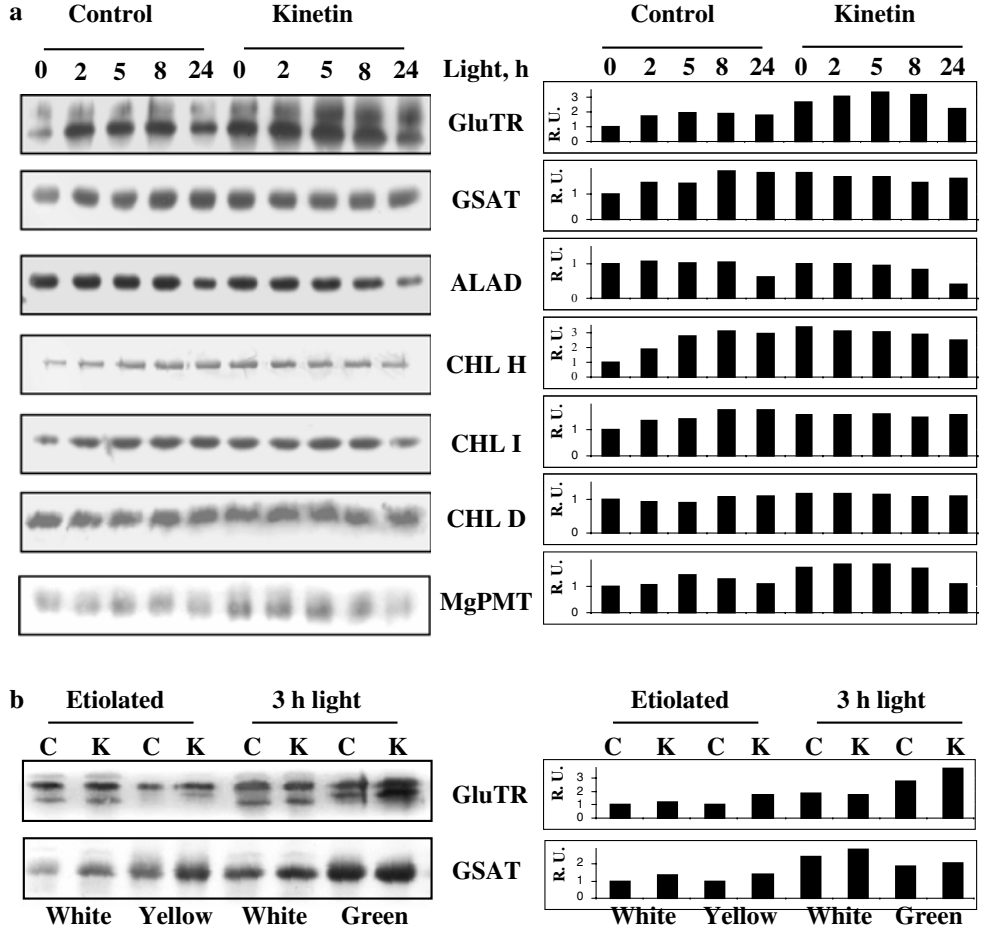

Fig. 3 **a**, **b** Western-blot analysis of etiolated and greening wild-type and *albostrians* mutant barley seedlings and quantification of immunoblots. **a** Etiolated wild-type barley seedlings were sprayed with water (control) or kinetin solution and illuminated for 2, 5, 8 and 24 h (80 µmol photons m^{-2} s^{-1}). Antisera against the following proteins were applied for glutamyl-tRNA reductase (*GluTR*), glutamate 1-semialdehyde aminotransferase (*GSAT*), 5-aminolevulinate dehydratase (*ALAD*), the three subunits of Mg-chelatase (*CHL H*, *CHL I*, *CHL D*) and Mg protoporphyrin IX methyltransferase (*MgPMT*). **b** Etiolated mutant plants were treated with water (*C*) or cytokinin (*K*) and were illuminated for 3 h (80 µmol photons m^{-2} s^{-1}). Antisera against the following proteins were applied for GluTR and GSAT. *W* white mutant leaves after dark or light incubation; *Y* yellow mutant leaves after dark incubation; *G* green mutant leaves after light incubation. Amounts of immune-reacting proteins were quantified by using the TotalLab v2.01 software. Values obtained from each Western blot were normalized to the value for the immune-reacting protein band of the etiolated untreated plant sample

in the white mutant seedlings. This is in agreement with cytokinin-non-responsive levels of GluTR and GSAT in etiolated and illuminated white mutant seedlings which were sprayed with cytokinin solution (Fig. 3b). In conclusion, the rate-limiting ALA biosynthesis of *albostrians* cannot be affected by kinetin.

Activities of enzymes in the Mg branch are modified in response to cytokinin treatment, but not Fe-chelatase

The Mg-chelatase activity increased during illumination of etiolated barley seedlings, reaching a maximum after 2 h (upon kinetin treatment) and 8 h (under control conditions) (Fig. 4a) and declined to the dark level after 24 h of illumination. The results with etiolated control seedlings correspond to previous observations on light induction of Mg-chelatase activity during greening (Jensen et al. 1996). Almost a threefold higher Mg-chelatase activity was demonstrated in kinetin-treated etiolated leaves compared to control (Fig. 4a). After a 2 h light exposure, the activity of Mg-chelatase in kinetin-treated leaves exceeded that of control leaves by 57% and after 5 and 8 h of continuous illumination by 28 and 18%, respectively. The kinetin stimulus on Mg-chelatase was completely abolished during a longer light period.

Comparison of the amounts of the three Mg-chelatase subunits in kinetin-treated and control seedlings revealed that, the level of the subunit CHL H was nearly three times higher upon kinetin treatment in darkness in comparison to the control condition. During light exposure of 24 h, CHL H converged to similar amounts under both treatments (Fig. 3a). A stimulatory kinetin effect during dark incubation on the amount of the CHL D and CHL I subunits could not be observed. Thus, the enhanced Mg-chelatase activity parallels the CHL H levels. The protein patterns of the Mg-chelatase subunits in greening control barley seedlings was similar as previously presented (Petersen et al. 1999).

MgPMT activity transiently increased during illumination of etiolated barley seedlings, reaching a maximum after 5 h in light, and decreased to dark levels after 8 and 24 h of light exposure. Kinetin application of etiolated leaves led to 63% more MgPMT activity (Fig. 4b) and an elevated MgPMT protein content in comparison to control plants (Fig. 3a).

Over a period of 24 h in light, the Fe-chelatase activity doubled in control seedlings, while the enzyme activity remained constant upon kinetin treatment (Fig. 4c). The increase of Fe-chelatase activity during greening corresponds to light-induced expression of one of the two genes encoding Fe-chelatase in photosynthetic tissues of cucumber (Suzuki et al. 2002). Non-covalently bound heme slightly accumulated during irradiation of etiolated seedlings independent from kinetin application. The water control leaves contained 8.02 ± 0.28 nmol heme g^{-1}FW in the etiolated state and 11.72 ± 0.89 nmol heme g^{-1}FW after a 24 h light expo-

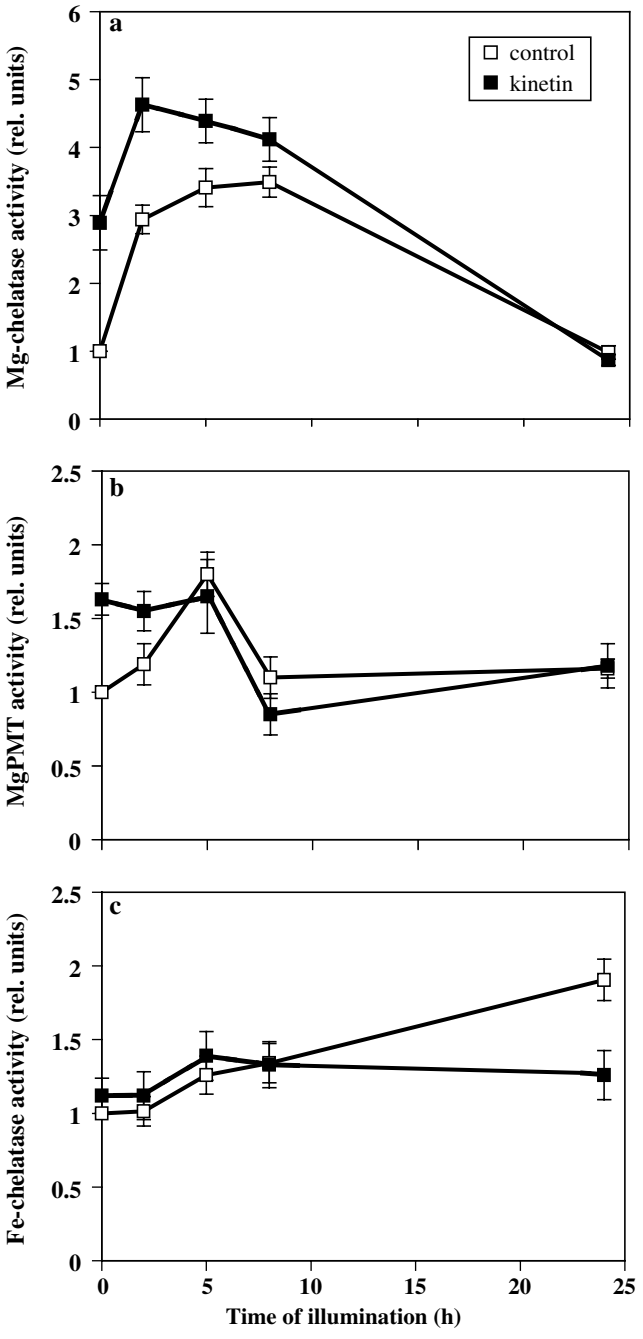

Fig. 4 a–c Effects of cytokinin on the activities of Mg protoporphyrin IX chelatase (*Mg-chelatase*), Mg protoporphyrin IX methyltransferase (*MgPMT*) and ferrochelatase (*Fe-chelatase*) in etiolated and greening barley seedlings. Etiolated barley seedlings sprayed with water (control) or kinetin solution were illuminated for 2, 5, 8 and 24 h (80 µmol photons m^{-2} s^{-1}). **a**, **b** Activities of Mg-chelatase and MgPMT were measured in crude chloroplasts isolated from etiolated and greening seedlings. **c** Fe-chelatase activity was determined in plastid extracts obtained from etiolated and greening barley seedlings. The data represent the ratios of enzyme activity of each sample to the activity of etiolated control seedlings. The activities of Mg-chelatase, MgPMT and Fe-chelatase in etiolated control seedlings were 620 ± 40 pmol MgProto mg^{-1} protein h^{-1}, 950 ± 81 pmol MgProtoMME mg^{-1} protein h^{-1} and $1{,}120 \pm 93$ pmol ZnProto mg^{-1} protein h^{-1}, respectively. The data are mean ± SD from three separate experiments performed in triplicate

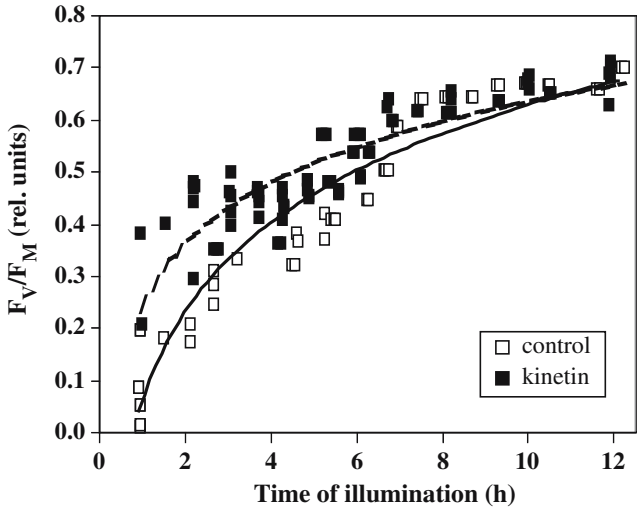

Fig. 5 Effects of cytokinin on F_V/F_M in etiolated and greening barley seedlings. Etiolated barley seedlings sprayed with water (control) or kinetin solution were illuminated for 12 h (80 µmol photons m^{-2} s^{-1}). Values were measured under greening conditions after 30 min dark adaptation of the primary leaves. Ten seedlings were individually analyzed. The data represent the average value validated by the *t* test

sure, and the cytokinin-treated leaves contained 7.98 ± 0.49 nmol heme g^{-1}FW in the etiolated state and 11.69 ± 0.86 nmol heme g^{-1}FW after a 24 h light exposure. This indicates that kinetin does not primarily affect the Fe branch.

The effect of cytokinin on the photosynthetic activity in greening barley seedlings

The kinetin effect was assessed on primary photosynthetic processes and the activity of the Calvin cycle. The maximum quantum yield of seedlings in the dark-adapted state (F_V/F_M) was measured as the maximum efficiency of excitation energy transfer from the antennae in open photosystem II reaction centres (Fig. 5). During the first 6 h of illumination, the kinetin-treated seedlings expressed a significantly higher F_V/F_M than the control seedlings. The F_V/F_M values of the kinetin-treated seedlings were two times higher than the control values after the first 2 h of light exposure. At the end of the 12 h greening, F_V/F_M values of both control and kinetin variants converged to values close to 0.8, which would reflect unstressed green leaves. Moreover, during the 12 h light period the F_0 values of the kinetin-treated barley seedlings were significantly higher than those of the control plants (18 to 104% higher).

The capacity of the photosynthetic dark reaction during greening was analyzed by gas exchange measurements. The maximum net CO_2 uptake rates under saturated light conditions and the net CO_2 uptake rates at greening light conditions are shown in Fig. 6. In general, etiolated seedlings initially released CO_2 in the first hours of illumination. The first net CO_2 uptake was determined under light saturation earlier in the kinetin-treated barley seedlings (2 h after onset of light vs. 4 h in control leaves) and remained higher in the first 10 h during greening in comparison to control seedlings (Fig. 6a). At ambient PPFD during growth of barley seedlings, the kinetin-treated seedlings showed again the first significant net CO_2 uptake rates 6 h after onset of light, whereas the control plants required at least 8 h for a positive CO_2 exchange rate (Fig. 6b). Moreover, during the first 12 h of illumination, kinetin-treated seedlings displayed always a higher net CO_2 uptake rate at a given time than the control seedlings, indicating a stimulated CO_2 assimilation.

The dark respiration rates of the kinetin-treated and control seedlings were very similar during the greening period examined (control: 1.71 ± 0.52 µmol CO_2 m^{-2} s^{-1}; cytokinin: 1.75 ± 0.60 µmol CO_2 m^{-2} s^{-1}).

Discussion

We compared the effects of cytokinin on protein levels and activities of key steps of tetrapyrrole biosynthesis of etiolated and greening barley seedlings including those of ALA synthesis and enzymes at the metabolic branch point directing Proto to Chl and heme. In dark-grown kinetin-treated barley seedlings, the ALA synthesis rate was not increased (Fig. 1b), although levels of GluTR and GSAT were elevated (Fig. 3a). As a consequence of non-modulated ALA synthesis, Pchlide content remained stable in kinetin-treated etiolated leaves (Fig. 1a). It is suggested that suppression of ALA synthesis in the dark compromise the cytokinin effect. The negative regulator FLU interacts with GluTR and functions in a feedback-controlled circuit from the Mg branch on ALA synthesis (Meskauskiene et al. 2001). Light releases the repression of ALA formation. Consequently, additional cytokinin stimulates ALA synthesis rate already after a 2 min light pulse. The kinetin-triggered stimulation of ALA synthesis continues for at least 8 h during greening, in comparison to control, before the effect is attenuated (Fig. 2a). Cytokinin stimulation on ALA formation in light is consistent with elevated levels of GluTR in barley seedlings (Fig. 3a) and with kinetin-induced accumulation of *HemA* mRNA in light- and dark-grown cucumber cotyledons (Masuda et al. 1995) and of *hemA1* mRNA in etiolated barley leaves (Bougri and Grimm 1996). Benzyladenine had no effect on the expression of GSAT in cucumber cotyledons (Masuda et al. 1994), but *gsa* expression was activated in kinetin-treated etiolated barley seedlings (Bougri and Grimm 1996). Increased levels of endogenous $tRNA^{glu}$ upon cytokinin application (Masuda et al. 1995) can also contribute to kinetin-stimulated ALA-synthesizing capacity in light-exposed seedlings.

Light induction of Mg-chelatase and MgPMT activities after transition from dark to light in greening barley

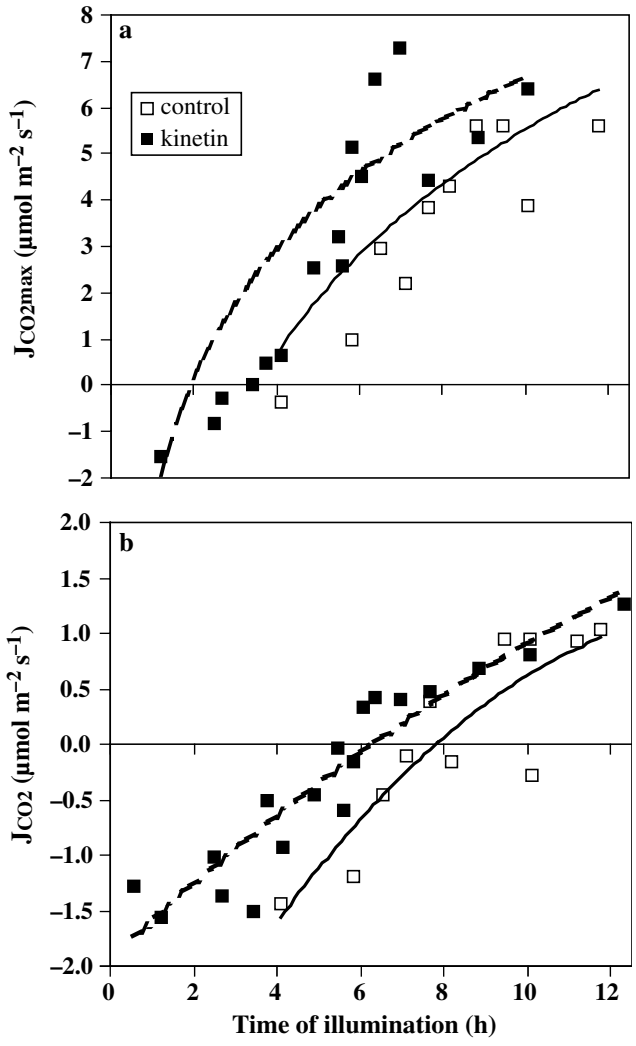

Fig. 6 **a**, **b** Effects of cytokinin on maximum net CO_2 uptake rates under light saturation (**a**) and net CO_2 uptake rates at growth of PPFD (**b**) in etiolated and greening barley seedlings. Etiolated barley seedlings treated with water (control) or kinetin were illuminated for 12 h (80 µmol photons m^{-2} s^{-1}). Values were calculated from light saturation curves of primary leaves measured under greening conditions. Ten seedlings were individually analyzed. The data represent the average value validated by the *t* test

seedlings (Fig. 4a, b) was also previously demonstrated in other plant species (Papenbrock and Grimm 2001; Alawady and Grimm 2005). The transient light-stimulated increase of the two enzyme activities is also detectable in kinetin-treated barley seedlings. But, the activities of both enzymes are already 63% (for MgPMT) and 200% (for Mg-chelatase) higher in etiolated seedlings in comparison to control seedlings. The promoting effects of kinetin on the activities of Mg-chelatase and MgPMT are observed within 2–5 h of illumination and correlate to the enhanced Chl accumulation after kinetin treatment (data not shown). Enhanced Mg-chelatase and MgPMT activities are accompanied by higher contents of the CHL H subunit and MgPMT (Fig. 3a). It is assumed that increased protein contents in kinetin-treated seedlings are due to stimulated gene expression of the respective genes, as it was previously demonstrated for the *HemA* (see above) and the *POR* gene (Kuroda et al. 1996; Kusnetsov et al. 1998). A transient reporter assay revealed *cis*-acting elements for kinetin response in the 5′-upstream region of the cucumber *POR* gene (Kuroda et al. 2001).

We assume that cytokinin mainly promotes enzymatic steps of tetrapyrrole biosynthesis for the synthesis of Chl. In this context, the results obtained with the white leaves of the barley *albostrians* mutant, which has an entire block in chloroplast development, are sensible. The white mutant seedlings do not accumulate Chl but contain reasonable amounts of heme (Yaronskaya et al. 2003). ALA synthesis as well as GluTR and GSAT contents was not enhanced upon cytokinin application (Figs. 2b, 3b). As this part of the tetrapyrrole pathway which provides metabolites for Chl formation is abolished, the remaining metabolic activities are insensitive to cytokinin signaling. Intact chloroplasts are required for normal response to cytokinin (Kulaeva et al. 2002) and plastids of *albostrian*s mutant presumably lose certain sensitivity to respond on cytokinin. Our results reflect the modified tetrapyrrole metabolism in response to impaired plastid development in *albostrians*. The pathway is regulated on the demands for heme rather than for Chl synthesis.

The rapid kinetin-promoted ALA synthesis as well as the Mg-chelatase and MgPMT activity coincide with accelerated photosynthetic activity in the beginning of illumination of etiolated seedlings. Stimulated Chl synthesis parallels enhanced photosynthetic activity indicated by values for F_V/F_M, J_{CO2max} and J_{CO2} in the kinetin-treated greening seedlings during the first hours of illumination. It is assumed that both the light-driven part and the biochemical part of photosynthesis are accelerated by additional kinetin supply (Figs. 5, 6). Several plastid-localized, in particular photosynthetic, enzymes were shown to be activated by cytokinin in dark-grown seedlings, like ribulose 1,5-bisphosphate carboxylase/oxygenase (Feierabend 1969; Harvey et al. 1974), NADP-glyceraldehydephosphate dehydrogenase (Feierabend 1969; Harvey et al. 1974), ATP synthase (Kasten et al. 1997; Kusnetsov et al. 1999; Sherameti et al. 2004), nitrate reductase (Rao et al. 1984) and phosphoglycerate kinase (Kasten et al. 1997). More recently, many genes were found to be cytokinin-induced in light-grown seedlings (Hoth et al. 2003). All in all, stimulated chloroplast development and the accelerated formation of photosynthetic units upon kinetin treatment is not only promoted by induced gene activation or repression (Hoth et al. 2003) and stimulated protein (Schmülling et al. 1997) and galactolipid biosynthesis (Usciati et al. 1974; Yamaryo et al. 2003), but is consequently explained by enhanced Chl content.

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