



Cross tolerance to heavy-metal and cold-induced photoinhibition in leaves of *Pisum sativum* acclimated to low temperature

Peter Streb¹, Serge Aubert², Elisabeth Gout³, Jürgen Feierabend⁴ and Richard Bligny³

¹Université Paris-Sud, Laboratoire Ecologie Systématique et Evolution, UMR8079, Bâtiment 362, 91405 Orsay, France

²Laboratoire d'Ecologie Alpine (LECA) UMR 5553 CNRS/Université Joseph Fourier BP 53 38041 Grenoble Cedex 9, France

³Laboratoire de Physiologie Cellulaire Végétale... Génétique Moléculaire des Plantes, UMR5575, CNRS, Université Joseph Fourier, BP53X, Grenoble Cedex 9, France

⁴Fachbereich Biowissenschaften, Goethe-Universität, D-60054 Frankfurt am Main, Germany

ABSTRACT

Under high light intensity, low temperatures as well as heavy metals induce photoinhibition of PSII and oxidative stress in leaves. Since cold acclimation of leaves ameliorates their capacity of antioxidative defence, cross tolerance between cold-induced and heavy metal-induced photoinhibition was investigated in pea leaves grown at either 22 °C or 6 °C. The experimental conditions were chosen to induce a uniform level of short-term photoinhibition at low temperature or in the presence of CuSO₄ or CdCl₂ in leaves grown at 22 °C. Under all conditions photoinhibition of PSII was lower in cold-acclimated (6°C-grown) than in non-acclimated (22°C-grown) pea leaves. In darkness PSII was not affected by all treatments. Other parameters like catalase activity, chlorophyll content and metabolite contents were most sensitive to CuSO₄, but less affected by CdCl₂ and low temperature treatments. Strong oxidation of ascorbate and concomitant loss of catalase activity showed the enhanced oxidative stress in CuSO₄-treated leaves. Generally, all measured parameters were less affected in cold-acclimated leaves than in non-acclimated leaves under all experimental conditions. Cold-acclimated pea leaves contained higher levels of ascorbate and particularly of glutathione and a higher capacity to keep the primary electron acceptor of PSII more oxidised. Incubation with heavy metals caused a nearly complete loss of reduced glutathione. It is suggested that reduced glutathione served as a source for phytochelatin synthesis. The extraordinarily high glutathione content in cold-acclimated pea leaves might therefore increase their ability to chelate heavy metals and thus to protect leaves from heavy-metal induced damage. [*Physiol. Mol. Biol. Plants* 2008; 14(3) : 185-193] E-mail : peter.streb@u-psud.fr

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Many environmental stresses affect plant performance particularly when they are simultaneously exposed to light exceeding the level that the plants received during growth and overloading their photosynthetic capacity (Aro *et al.* 1993, Polle 1997). The energy of excessive light absorbed by the leaves must be either dissipated or consumed by metabolic reactions (Foyer 1997, Huner *et al.* 1998, Ort & Baker 2002). In case that harmless dissipation or utilisation is overloaded, reactive oxygen species are formed leading to general destruction of membranes, proteins and pigments (Asada 1999). Early targets of light-induced destruction under several

different stress conditions in leaves are the photoinhibition of PSII and the photoinactivation of the peroxisomal protein catalase (Streb *et al.* 1993, Aro *et al.* 1993). Prolonged stress exposure at high light leads to more general oxidative cellular damage.

One common stress factor in the northern hemisphere during winter and early spring is low temperature, particularly when combined with high irradiation. Several plant species, including *Pisum sativum*, can acclimate to these conditions. Acclimated plants show less photoinhibition of PSII and of catalase (Feierabend *et al.* 1992, Huner *et al.* 1998, Streb *et al.* 1999), as compared to non-acclimated or low-land tropical plants. Acclimation to low temperature often depends on a cool

Correspondence and Reprint requests : Peter Streb

growth temperature and is accompanied by an increase of the capacity of leaves to scavenge reactive oxygen species as indicated by higher activities of antioxidative enzymes and higher contents of antioxidants, such as ascorbate, glutathione and carotenoids (Streb *et al.* 1999, 2003, Adams III *et al.* 2002). Furthermore, the harmless dissipation of excess light energy is enhanced in these plants, as indicated by more rapid and increased formation of zeaxanthin and a more efficient non-photochemical fluorescence quenching (qN) as well as by a higher capacity to keep the reduction state of the photosynthetic electron transport chain more oxidised (Streb *et al.* 1999, Huner *et al.* 1998, Adams III *et al.* 2002). Cold-acclimated plants also increase their photosynthetic capacity, due to higher activities of Calvin cycle enzymes and higher rates of sucrose synthesis which serve as electron sinks (Huner *et al.* 1993).

The improved protection of rye leaves acclimated to low temperature is illustrated by their cross-tolerance to oxidative damage caused by the herbicide paraquat (Streb *et al.* 1999).

One direct or indirect consequence of heavy metal action in plants is the increased production of reactive oxygen species (Dietz *et al.* 1999). At high irradiance, the two heavy metal salts CdCl₂ and CuSO₄ induce strong photoinhibition of PSII and of catalase as well as bleaching of chlorophyll in rye leaves (Streb *et al.* 1993). Similar inhibitory effects of Cu and Cd were reported for various other plant species, although the role of light was not specifically investigated (e.g. Prasad & Strzalka 1999, Dietz *et al.* 1999, Chugh & Sawhney 1999, Schützendübel & Polle 2002, Tewari *et al.* 2006). This raises the question of whether cold-acclimation can also increase heavy metal tolerance and allow acclimated leaves to attenuate the inhibition of photosynthetic metabolism.

In the present contribution the effects of treatments with CdCl₂ and CuSO₄ were compared to low temperature stress in pea leaves grown at ambient (22°C-grown leaves) and low (6°C-grown leaves) temperature, after exposure to high irradiance. Pea leaves are relatively cold-tolerant with respect to photoinhibition of PSII and of catalase, as compared to plants from low-land tropical origin, but they are much more chilling-sensitive than e.g. alpine plants (Feierabend *et al.* 1992, Streb *et al.* 2003). The tolerance to photoinhibition was markedly increased in cold-acclimated pea leaves (Streb *et al.* 2003). The aim of the present study was to investigate whether cold-acclimation ameliorates heavy metal

tolerance at high irradiation and to compare the effects of heavy metal and cold-stress on the antioxidant defence system and on carbon metabolism.

MATERIAL AND METHODS

Plant material and growing conditions

P. sativum plants were grown under controlled conditions (14 h light, 10 h dark) in white light at 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PAR (photosynthetic active radiation) in Vermiculite moistened with tap water as described (Streb *et al.* 2003). Plants were either grown at a temperature of 22 °C (day) and 18 °C (night) for 10 to 12 days or at a constant temperature of 6 °C for 16 weeks. At this stage 22 °C-grown plants and 6 °C-grown plants had similar leaf numbers and chlorophyll contents on a fresh weight basis (Streb *et al.* 2003).

Experimental treatment

Intact fully developed leaves were detached and floated in plastic boxes or petri dishes (250 ml for 10 g leaves used for NMR-analysis or 10 ml for 0.3 g leaf material used for HPLC analysis, chlorophyll, catalase, and Fv/Fm measurements) on tap water in the presence or absence of 10 mM CuSO₄ or 5 mM CdCl₂ at 25 °C or on ice-cooled water at approximately 5 °C to 7 °C under white light of 1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PAR (4 x 100 W low-voltage halogen lamps). Control leaves were kept in darkness. For the chlorophyll fluorescence measurement of 1-qP and qN attached leaves were used. Leaves were either fixed in Petri dishes floating on water at 25 °C in the presence or absence of 10 mM CuSO₄ or 5 mM CdCl₂ or on ice cooled water. Leaves were dark acclimated for 2 h and illuminated afterwards at 1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PAR. Measurements of 1-qP and qN were made directly after a 1 h illumination period.

Fluorescence measurements

A Mini-PAM (Waltz, Germany) was used for the chlorophyll-fluorescence measurements. The ratio Fv/Fm (variable to maximum fluorescence) in 30 min dark-adapted samples, the non-photochemical fluorescence quenching (qN) and the relative redox-state of the primary electron acceptor of PSII Q_A (1-qP) were calculated as described (Streb *et al.* 1998).

Enzyme assays and analytical methods

For the estimation of catalase activity and of contents of antioxidants and pigments, approximately 0.3 g fresh weight of leaf material was used. Extraction and

estimation of catalase activity and chlorophyll contents from 80 % (v/v) acetone extracts were described by Streb *et al.* (1998). The antioxidants ascorbate, dehydroascorbate, reduced and oxidised glutathione were extracted in 1 % metaphosphoric acid and assayed by HPLC as described by Streb *et al.* (1998).

For NMR-analysis leaf material of 10 g fresh weight was frozen in liquid nitrogen and stored at -20°C . The extraction and determination of metabolite contents by NMR were described in Streb *et al.* (2003). Extracts were measured with a NMR-spectrometer (AMX 400, Bruker, Billerica, MA) in a 10 mm multinuclear tube tuned at 100.6 MHz for ^{13}C -NMR. The resonance signal of $^2\text{H}_2\text{O}$ was used as a lock signal. Data acquisition conditions were exactly as described by Streb *et al.* (2003). ^{13}C -NMR-data were recorded with 225 scans.

The peaks in the NMR-spectra were identified by comparison to standard solutions with known compounds reported in previous publications (Streb *et al.* 2003).

For the estimation of metabolite contents the relative peak response on the same fresh weight basis was calculated. Individual peak responses of free amino acids were combined in order to calculate the total free amino acid content.

Statistical significant differences were calculated with the Sigma Plot program. The p values and the number of experiments are indicated in the legends for the figures. Mean values and the standard errors are shown in the graphics.

RESULTS

When pea leaves grown at 22°C were exposed to an irradiation 10 times higher than growth light either at low temperature (5°C - 7°C) or at 25°C in the presence of CdCl_2 or CuSO_4 photoinhibition of PSII, indicated by the decline of the F_v/F_m ratio, was induced rapidly (Fig. 1A). After 6 h exposure a 50 % inhibition was measured in all treatments, except for the control kept on water at 25°C . High concentrations of CdCl_2 and CuSO_4 were applied in order to induce approximately the same extent of photoinhibition as observed at low temperature (Fig. 1A). In all treatments PSII was not affected in darkness. Cold-acclimated 6°C -grown leaves were markedly more resistant to photoinhibition than 22°C -grown leaves (Fig. 1B). Notably, the extent of photoinhibition induced by Cd and Cu in 6°C -grown leaves was similarly diminished as in the low temperature treatment.

The fluorescence parameters $1-qP$ and qN were determined after 1 h illumination at high light in order to estimate the relative reduction state of the primary electron acceptor Q_A of PSII and the harmless dissipation of excess energy as heat. The relative Q_A reduction state was highest after cold treatments. Both heavy metals increased Q_A reduction in 22°C -grown leaves but decreased Q_A reduction in 6°C -grown leaves, relative to the untreated control. Generally, Q_A reduction was higher in 22°C -grown than in 6°C -grown leaves. In leaves from both growth conditions qN was not substantially affected by any of the experimental treatments (Fig. 2).

Catalase activity was significantly lower in 6°C -grown than in 22°C -grown leaves, while the chlorophyll contents were similar (Fig. 3). During subsequent incubations in light catalase activity increased in control leaves but decreased in darkness (Fig. 3A + B). Exposure to low temperature or CdCl_2 caused declines of catalase activity. This effect was more pronounced in 22°C -grown than in 6°C -grown leaves. In the presence of CuSO_4 catalase was almost completely inactivated in plants from both growth temperatures, however, only when leaves were exposed to light. The chlorophyll content decreased only slightly in CuSO_4 -treated 22°C -grown leaves in high light but was very similar under all other conditions.

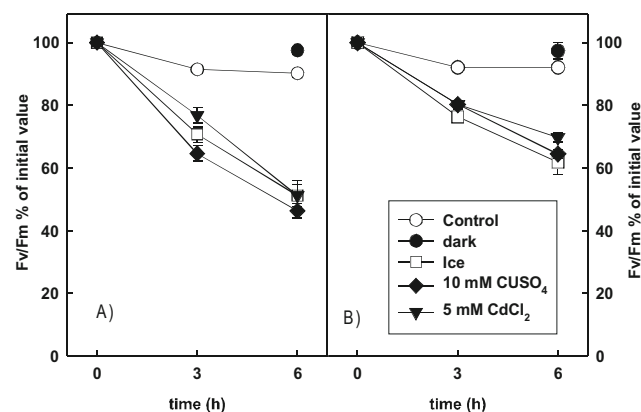


Fig. 1. Changes of the fluorescence ratio F_v/F_m in dark-acclimated pea leaves grown at 22°C for 10-12 days (A), or at 6°C for 16 weeks (B) in % before treatment. Leaves were incubated at $1000\ \mu\text{mol m}^{-2}\text{s}^{-1}$ PAR in water (control, \circ) or solutions of 10 mM CuSO_4 (\blacklozenge) or 5 mM CdCl_2 (\blacktriangledown) at 25°C or on ice cooled water of $5-7^{\circ}\text{C}$ (\square). The mean F_v/F_m value of dark treatments from all incubation conditions is shown (\bullet). The standard error is indicated ($n = 4$). 6°C -grown leaves were significantly less photoinhibited than 22°C -grown leaves in response to heavy metal treatment ($p < 0.01$) and cold treatment ($p < 0.05$) after 6 h incubation time.

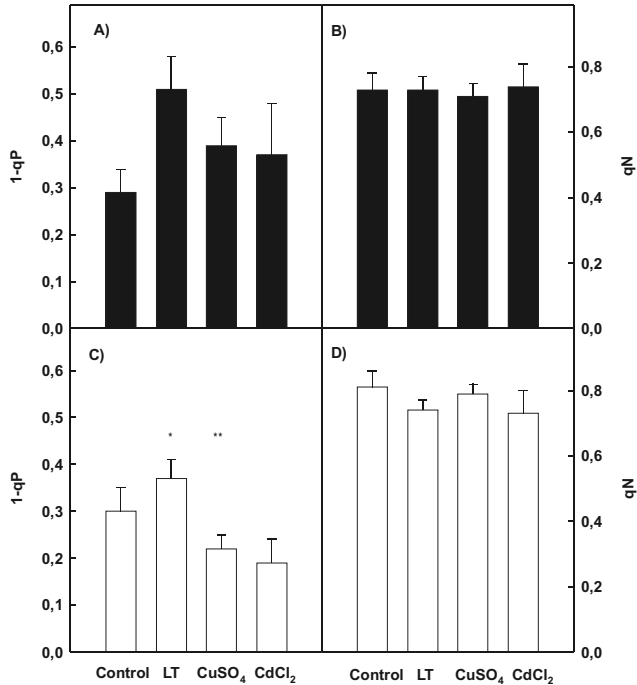


Fig. 2. Fluorescence parameters 1-qP (A, C) as a relative measure of the Q_A reduction state of PSII, and non-photochemical fluorescence quenching qN (B, D) in pea leaves grown at 22° C for 10–12 days (A, B, black bars), or at 6° C for 16 weeks (C, D, white bars). Leaves were kept attached to the plants and fixed in petri dishes, preincubated for 2 h in darkness at 25 °C in the presence or absence (control) of 10 mM CuSO_4 or 5 mM CdCl_2 or in ice cooled water (cold). Subsequently, leaves were illuminated with white actinic light at 1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PAR. 1-qP and qN were determined after 60 min illumination followed by a saturating flash and the switching off of actinic light in order to measure F_o' . Mean values with standard errors are indicated ($n = 4$). Significant differences between 22°C-grown and 4°C-grown leaves are indicated by * ($p < 0.1$) or ** ($p < 0.05$).

Ascorbic acid contents were slightly higher in 6°C-grown than in 22°C-grown leaves (Fig. 4). During incubation in light total ascorbate contents increased slightly in 22°C-grown as well as in 6°C-grown leaves without changes in the redox state. Similarly, exposures to low temperature or CdCl_2 caused some changes of the total ascorbate contents in 22°C-grown and 6°C-grown leaves without affecting the ratio of reduced to total ascorbate. The ascorbate contents remained always higher in 6°C-grown than in 22°C-grown leaves. However, CuSO_4 treatment decreased the proportion of reduced ascorbate, particularly in light (Fig. 4).

Glutathione contents were twice as high in 6°C-grown as in 22°C-grown leaves. Exposure to high light at 25°C

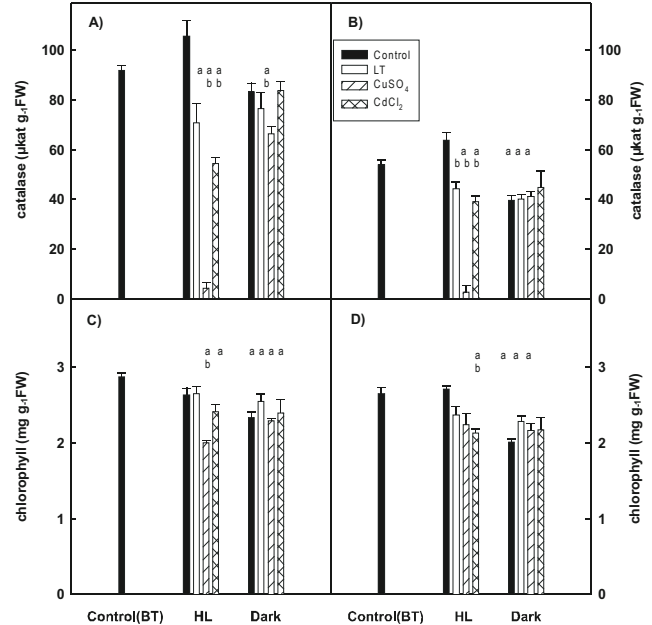


Fig. 3. Catalase activity in $\mu\text{kat g}^{-1}\text{FW}$ (A, B) and chlorophyll content in $\text{mg g}^{-1}\text{FW}$ (C, D) in dark-acclimated pea leaves grown at 22° C for 10–12 days (A, C), or at 6° C for 16 weeks (B, D) before (Control Before Treatment) and after a 6 h treatment of leaves in light of 1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PAR (HL) or in darkness (dark). Leaves were incubated on water (control), or on solutions of 10 mM CuSO_4 or 5 mM CdCl_2 at 25 °C, or at low temperature (LT). Significant differences were calculated and indicated by: a, significant difference as compared to the untreated control or b, significant difference as compared to the control treatment in water at the $p < 0.01$ level ($n = 4$).

increased both the reduced and total glutathione contents markedly in leaves grown at both temperatures but, nevertheless, the redox state was shifted in favour of the oxidised form. At low temperature glutathione contents increased only in 6°C-grown leaves. In the presence of both heavy metals glutathione became largely oxidised in light as well as in darkness. Total glutathione contents decreased, particularly in darkness (Fig. 5).

To further investigate the effects of heavy metal treatments the major soluble metabolites, sugars, malate and total free amino acids, were analysed by NMR. The contents of sucrose and total free amino acids were similar, those of glucose and malate were higher and the fructose contents were lower in 22°C-grown than in 6°C-grown leaves (Fig. 6).

During exposure to high light at 25°C sucrose contents increased markedly in leaves from both growth

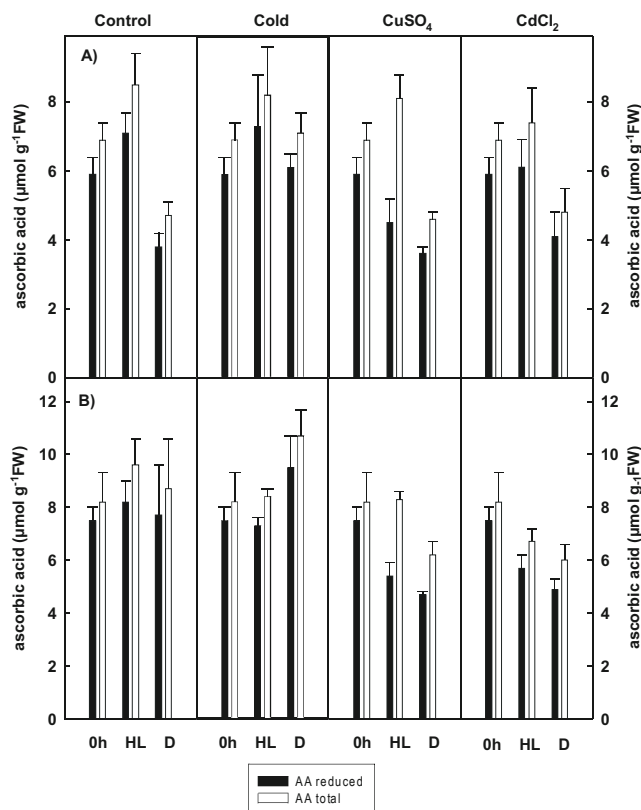


Fig. 4. Contents of reduced (black bars) and total (white bars) ascorbic acid in $\mu\text{mol g}^{-1}\text{FW}$ in pea leaves grown at 22°C for 10–12 days (A), or at 6°C for 16 weeks (B). Assays were performed before incubation (0h) or after 6 h treatment in light of $1000 \mu\text{mol m}^{-2}\text{s}^{-1}$ PAR (HL) or in darkness (D). Leaves were incubated at 25°C on water (Control), on ice cooled water (Cold) or at 25°C on solutions of 10 mM CuSO_4 or 5 mM CdCl_2 . Note that different scales were used for 22°C -grown and 6°C -grown leaves. The ratio ascorbic acid reduced/ascorbic acid total is significantly lower in leaves treated with CuSO_4 in light as compared to all other light treatments at $p < 0.01$ for 22°C -grown leaves and at $p < 0.05$ for 6°C -grown leaves. There is no significant difference between 22°C -grown leaves and 6°C -grown leaves ($n = 3$).

temperatures, those of fructose only slightly. The glucose contents decreased, however, in 22°C -grown leaves. The sucrose contents increased also during high light incubation at low temperature, particularly in 22°C -grown leaves. However, in 22°C -grown leaves this increase was accompanied by declines of glucose and fructose while the latter remained unchanged in 6°C -grown leaves. In the presence of CuSO_4 sucrose contents were not changed and the lowest contents of fructose and glucose were observed in 22°C -grown leaves. In 6°C -grown leaves sucrose contents increased but this was accompanied by a decrease in glucose and

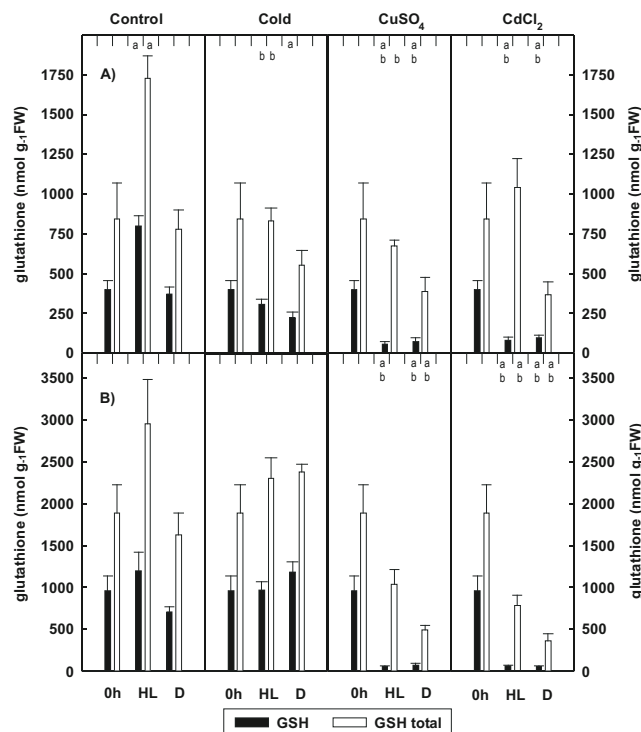


Fig. 5. Contents of reduced (black bars) and total (white bars) glutathione in $\text{nmol g}^{-1}\text{FW}$ in pea leaves grown at 22°C for 10–12 days (A), or grown at 6°C for 16 weeks (B). Assays were performed before incubation (0) or after 6 h treatment in light of $1000 \mu\text{mol m}^{-2}\text{s}^{-1}$ PAR (HL) or in darkness (D). Leaves were incubated at 25°C on water (Control), on ice cooled water (Cold) or at 25°C on solutions of 10 mM CuSO_4 or 5 mM CdCl_2 . Note that different scales were used for 22°C -grown and 6°C -grown leaves. Glutathione content in 22°C -grown leaves was significantly lower than in 6°C -grown leaves at $p < 0.01$ for reduced glutathione and $p < 0.05$ for total glutathione. Significant differences were calculated and indicated by: a, significant difference as compared to the untreated control or b, significant difference as compared to the control treatment in water at the $p < 0.01$ level ($n = 3$).

fructose in the presence of CuSO_4 . After incubation with CdCl_2 , sugar contents remained higher than in CuSO_4 -treated leaves from both growth temperatures but lower than after cold treatments.

During illumination at low temperature malate and free amino acid contents decreased strongly in 22°C -grown leaves while the malate contents increased in 6°C -grown leaves. Heavy metal treatments caused striking declines of the malate content which became undetectable in the presence of CuSO_4 , both in leaves grown at 22°C and at 6°C . The contents of total free

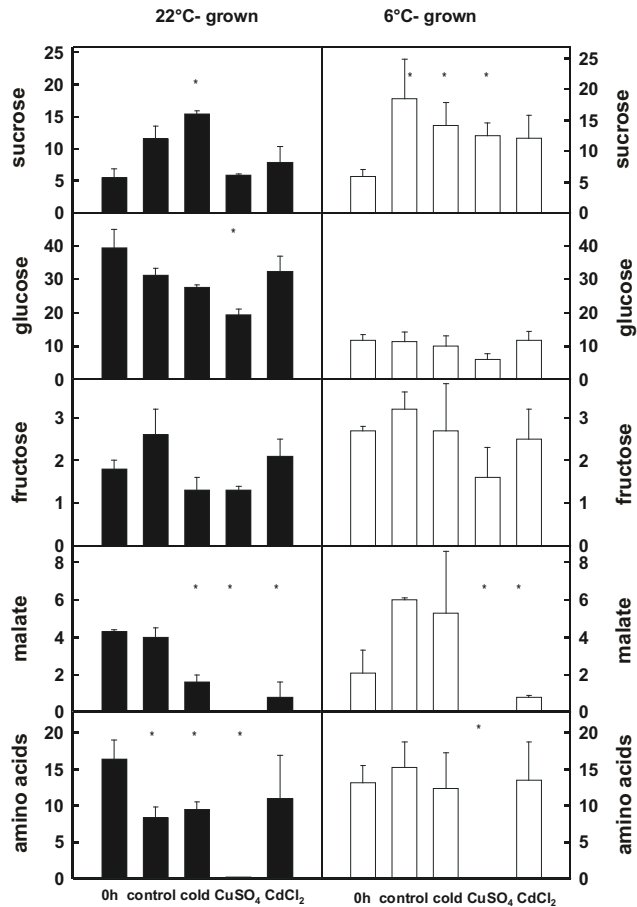


Fig. 6. Contents of metabolites (sucrose, glucose, fructose, malate and total free amino acids) in relative units (peak response on the same fresh weigh basis) in pea leaves grown at 22° C for 10-12 days (left), or at 6° C for 16 weeks (right). Assays were performed before incubation (0h) or after 6 h treatment in light of 1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PAR. Leaves were incubated at 25 °C on water (Control), on ice cooled water (Cold) or at 25 °C on solutions of 10 mM CuSO_4 or 5 mM CdCl_2 . Glucose was significantly higher and fructose significantly lower in 22 °C-grown leaves than in 6 °C-grown leaves ($p < 0.05$). Significant differences to the untreated control at the $p < 0.05$ level are indicated by asterisks ($n = 2$).

amino acids were also strongly diminished by CuSO_4 treatments but were much less affected in the presence of CdCl_2 . The total free amino acid contents remained higher in cold-acclimated than in non-acclimated leaves.

DISCUSSION

Cold-acclimation increases resistance to photo-inhibition under heavy metal stress

Similar to other plant species also pea leaves can

acclimate to low temperature stress in high light when plants were grown at low temperature (Streb *et al.* 1999, 2003). This is evident from the lower photoinhibition of PSII during illumination at low temperature (Fig. 1).

The present results show that cold-acclimated pea leaves were also more resistant to Cu- and Cd- induced photoinhibition of PSII than 22°C-grown leaves.

Cold-acclimated leaves of many plant species showed higher rates of harmless energy dissipation, as indicated by higher rates of non-photochemical fluorescence quenching and are able to keep their photosynthetic electron transport chain in a more oxidised state during light exposure than non-acclimated leaves (Huner *et al.* 1998, Streb *et al.* 1999, Adams III *et al.* 2002). However, non-photochemical fluorescence quenching was only slightly higher in cold- acclimated versus non-acclimated pea leaves under all experimental conditions applied. This suggests that dissipation of excess excitation energy as heat did not significantly contribute to protection of cold-acclimated pea leaves against either low temperature- or heavy metal-induced stress in light. Nevertheless, cold-acclimated leaves kept the relative reduction state of Q_A more oxidised, when leaves were illuminated at low temperature or in the presence of heavy metals. Assuming that the connectivity between light-harvesting complexes and the reaction centre is similar in cold-acclimated and non-acclimated leaves, the fluorescence parameter $1-qP$ may serve as a relative measure of Q_A reduction (Streb *et al.* 2005). The strongest increase of Q_A reduction was observed when leaves were illuminated at low temperature. This may be a consequence of the retardation of the enzymatic reactions of the Calvin cycle at low temperature (Huner *et al.* 1998). Notably, during incubation at low temperature in high light the sucrose contents increased in 22°C-grown leaves as well as in cold-acclimated leaves to even higher levels than after a 25°C-exposure, indicating that sucrose synthesis per se was not affected by the low temperature in 22°C-grown leaves. In the 22°C-grown leaves the increase of sucrose contents was, however, accompanied by a decrease of glucose and fructose (Fig. 6) and may therefore not result from higher photosynthetic activity under these conditions.

In the presence of both heavy metals Q_A remained more oxidised than at low temperature. Cu was shown to act preferentially at the donor side of PSII (Yruela *et al.* 2000, Pätsikkä *et al.* 2001) but Cd appears to have no direct effect on the photosynthetic machinery (Barylá *et al.* 2001). Nevertheless, since sucrose synthesis was higher and Q_A reduction was lower in cold-acclimated

than in non-acclimated leaves, cold-acclimated leaves were obviously able to maintain a higher photosynthetic performance in the presence of both heavy metals.

Responses of other light-sensitive parameters differed after Cu or Cd treatments of pea leaves. Generally, the effect of CuSO₄ was more destructive than that of CdCl₂. Irrespective of the growth conditions, catalase was almost completely inactivated in the presence of CuSO₄ in illuminated leaves but not in darkness, showing that this loss of catalase activity was mainly mediated by light, as previously described for rye leaves (Streb *et al.* 1993). As described for many other plant species treated with Cu or Cd (Dietz *et al.* 1999, Schützendübel & Polle 2002, Mishra *et al.* 2006), a minor decrease of catalase activity occurred also in CuSO₄-treated 22°C-grown leaves in darkness suggesting some direct inactivation (Fig. 3). Such a direct catalase inactivation in darkness was not observed in cold-acclimated leaves. Catalase photoinactivation was also visible in the presence of CdCl₂ but less marked than in the presence of CuSO₄ and again 22°C-grown leaves were more sensitive than 6°C-grown leaves.

Furthermore, CuSO₄ induced also some slight chlorophyll bleaching in 22°C-grown but not in 6°C-grown leaves. Altogether, the results demonstrate that cold-acclimation ameliorated the tolerance for heavy metal-induced stress.

Stress-induced changes of antioxidants and their redox state

Similar to several other cold-acclimated plants (Schöner & Krause 1990, Streb *et al.* 1999, 2003) 6°C-grown pea leaves had also higher ascorbate and glutathione contents than 22°C-grown leaves (Figs. 4 and 5). Particularly the glutathione content was elevated by cold-acclimation. While the total ascorbate contents declined in some heavy metal treatments, particularly in darkness, the redox state did not change markedly, except after CuSO₄-treatment in light. Ascorbate oxidation was slightly but not significantly more pronounced in 22°C-grown leaves and correlated well with the marked decrease of catalase activity, which was not observed in the other treatments. This observation suggests that the lack of catalase activity caused oxidative stress and ascorbate oxidation. Copper-induced ascorbate oxidation was, however, also observed in mulberry plants, although their catalase activity was increased (Tewari *et al.*, 2006).

Total glutathione contents increased in high light at 25°C, but not under all other conditions. In the presence

of both heavy metals glutathione was almost completely oxidised and the total glutathione contents declined. Glutathione is the most important source of phytochelatin synthesis (Rauser 1990, Prasad 1999, Cobbett & Goldsbrough 2002, Sarry *et al.* 2006). It is most strongly induced by Cd and also by Cu and was shown to occur in pea leaves. Plants with enhanced glutathione contents showed higher Cd tolerance (Schützendübel & Polle 2002, Mishra *et al.* 2006). The strong decrease of reduced glutathione in heavy metal-treated leaves therefore suggests a role in phytochelatin synthesis. Because glutathione contents were twice as high in cold-acclimated as in non-acclimated leaves the metal complexing ability of cold-acclimated leaves could be expected to be markedly higher. Their improved antioxidative protection and their superior ability for heavy metal detoxification by phytochelatin synthesis presumably represent the main properties responsible for the observed cross-tolerance of cold-acclimated leaves to heavy metal stress.

Stress-induced changes of metabolite contents

The analysis of some major metabolites gave an impression of the strength and complexity of the stress responses induced by CuSO₄ and CdCl₂. Illumination in the absence of heavy metals induced marked increases of sucrose contents in 22°C- and 6°C-grown leaves, indicating their photosynthetic activity. However, during exposure to light at low temperature this sucrose accumulation in 22°C-grown leaves was accompanied by a decrease of glucose and fructose and may therefore only partially result from photosynthetic activity. The presence of CuSO₄ prevented sucrose synthesis in 22°C-grown leaves and caused marked declines of glucose and fructose. In cold-acclimated leaves, however, sucrose contents increased even in the presence of CuSO₄, providing further evidence that metabolism was less impaired than in non-acclimated leaves. Free total amino acids, malate (Fig. 6) and other metabolites (not shown) declined below the detection limit in CuSO₄-treated leaves, indicating a complex and strong interference with metabolic reactions in both acclimated and non-acclimated leaves. Metabolite changes in CdCl₂-treated leaves were similar as, but less striking than, in CuSO₄-treatments. Sucrose formation was higher in 6°C-grown than in 22°C-grown leaves. Contents of glucose, fructose and total free amino acids remained constant in 6°C-grown leaves but declined in 22°C-grown leaves.

The increase of the malate contents observed in 6°C-grown leaves may result from the activity of the malate valve which can consume photosynthetically

generated reductants in the chloroplast and thus decrease oxidative stress (Scheibe 2004). While the malate content increased strongly in 6°C-grown leaves even at low temperature it decreased in 22°C-grown leaves. Oxidative stress induced by both heavy metals together with the concomitant consumption of reactive oxygen scavengers and reductants may compete with electron transfer to malate and thus cause its substantial decline or even elimination.

CONCLUSION

In cold-acclimated pea leaves heavy metal tolerance is ameliorated, as reflected by a higher resistance to photoinhibition of PSII, a more oxidised electron transport chain, less oxidative damage and less impaired metabolite synthesis than in non-acclimated leaves. Nevertheless, both heavy metals affect metabolic reactions strongly also in cold-acclimated leaves. The higher tolerance of cold-acclimated leaves to heavy metal stress may result from their improved antioxidative protection, particularly their very high glutathione content relative to non-acclimated leaves. Glutathione is the substrate for phytochelatin synthesis (Rauser 1990, Prasad 1999, Cobbett & Goldsbrough 2002, Sarry *et al.* 2006). Plants with higher capacities to synthesise glutathione are more tolerant to Cd treatment (Zhu *et al.* 1999a, 1999b, Mishra *et al.* 2006) and can accumulate higher cadmium concentrations in the tissues (Arisi *et al.* 2000). Since glutathione contents and synthesis are often elevated in cold-acclimated leaves (Streb *et al.* 1999, Kocsy *et al.* 2001) low temperature acclimation might induce increased heavy metal tolerance due to an increased heavy metal complexing capacity.

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