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# Cadmium exposure in *Brassica juncea* causes a decline in transpiration rate and leaf expansion without effect on photosynthesis

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## Abstract

***Brassica juncea*** **L. is able to accumulate more than 400 μg $g^{-1}$ DW Cd in the shoot, a physiological trait which may be exploited for the bioremediation of contaminated soils and waters. Cd accumulation is accompanied by metabolic adaptation, in particular, the rapid induction of phytochelatin (PC) biosynthesis. Sequestration of Cd by PCs provides an essential cellular mechanism for Cd detoxification. To address the effects of Cd exposure on leaf physiology as compared to induction of PC synthesis, the accumulation of Cd in relation to growth rate, transpiration rate, $CO_2$ assimilation, and PC synthesis, has been analysed in a time-course study using the same leaf material. Furthermore, expression of the rate-limiting enzyme for glutathione (GSH) synthesis, γ-glutamylcysteine synthetase (γ-ECS), has been assessed by RNA blot, and was compared to expression of metallothionein class 2 (MT2). These results indicate that while photosynthesis was not affected by exposure to 25μM $CdNO_3$, transpiration showed a significant decline, in particular, under lower light conditions (≤300 μmol photons $m^{-2}$ $s^{-1}$), starting 48 h after the onset of Cd exposure. Reduced transpiration correlated with reduced leaf expansion growth, and a decrease in Cd accumulation rate. A quantitative comparison revealed that during the entire time-course, PC content was theoretically sufficient to chelate all Cd taken up. Expression of γ-ECS appeared to correlate closely with Cd accumulation and PC synthesis, whereas transcript amounts for MT2 increased only later. These results suggest that stringent control of Cd detoxification by PCs protects photosynthesis, but does not prevent a decline in transpiration rate.**

Key words: *Brassica juncea*, gas exchange, chlorophyll fluorescence, growth, phytochelatin, γ-ECS, MT2, cadmium.

## Introduction

Recently, novel strategies for bioremediation of heavy metal-polluted soils and waters by phytoextraction and/or phytomining with (hyper)accumulator plants have led to a surge of interest in the physiology of (hyper)accumulating plant species (McGrath *et al*., 1993; Robinson *et al*., 1997). In this context, *Brassica juncea* has been identified as a high biomass-producing crop with the capacity to take up and accumulate heavy metals such as Cd, Cu, Ni, Zn, Pb, and Se (Kumar *et al*., 1995; Salt *et al*, 1995*b*, 1997; Blaylock *et al*., 1997; Raskin *et al*., 1997).

For cadmium (Cd), soil contents between 2 and 6.4 ppm have been reported for several polluted sites in Germany (Hammer and Müller, 1994). To enhance bioavailability of Cd in the soil, and thus to improve bioremediation efficiency, the use of chelating agents has been proposed (Blaylock *et al*., 1997). Upon addition of EDTA, up to 80% of total soil Cd is solubilized and becomes available for phytoremediation (Susanne Lambrecht and Angela Haag-Kerwer, unpublished results). Cd ions are taken up by the root and, to a significant extent, are translocated to the shoot where they finally accumulate in the leaves, reaching the highest concentration in foliar trichomes (Speiser *et al*., 1992; Salt *et al*., 1995*b*). Despite recent progress in understand-

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Abbreviations: GSH, glutathione; NPT, non-protein-thiols; PCs, phytochelatins; MTs, metallothioneins; PFD, photon flux density (400–700 nm); FW, fresh weight; DW, dry weight; γ-ECS, γ-glutamylcysteine synthetase; MT2, metallothionein class 2.

ing individual aspects of Cd accumulation, the cellular and whole plant mechanisms for Cd accumulation are only partially understood. In particular, little is known about the co-ordination of cellular sequestration mechanisms with adaptation of photosynthesis, transpiration and plant growth.

In non-tolerant plant species, Cd is known to accumulate in various parts of the plant, resulting in a reduction of growth (Weigel and Jäger, 1980) and an inhibition of photosynthesis (Bazzaz *et al*., 1974; Hampp *et al*., 1974), and may therefore strongly affect biomass production. For several plants species, it has been shown that light and dark reactions of photosynthesis are inhibited by heavy metals at different target sites (Krupa and Baszynski, 1995), photosystem (PS) II being particularly sensitive (van Assche and Clijsters, 1985; Krupa and Baszynski, 1995, and literature cited therein). Cd is thought to act at PSII on both the oxidizing (donor) and the reducing (acceptor) side. Moreover, PSII reaction centres and PSII electron transport are affected by interaction with Cd, the metal impairing enzyme activity and/or protein structure (van Assche and Clijsters, 1985). A further indirect effect may come from inhibition of chlorophyll synthesis (Padmaja *et al*., 1990). A shortcoming of previous studies has been that Cd effects on photosynthesis were mainly investigated *in vitro*, using isolated chloroplasts, and it is unknown whether these *in vitro* observations are of any relevance for the *in vivo* bioactivity of Cd in the intact leaf of adult plants. In a recent study, seedlings of *Brassica napus* were exposed to Cd under high light intensity and for an extended time period (Larsson *et al*., 1998). These seedlings showed a significant decrease in chlorophyll content and photochemical quantum yield of photosynthesis (Larsson *et al*., 1998).

To maintain biomass production under field conditions on Cd-contaminated sites, *B. juncea* requires efficient cellular detoxification mechanisms in roots and shoots alike. Cd detoxification depends to a significant degree on specific cysteine-rich polypeptides, known as phytochelatins (PCs), which are enzymatically synthesized from glutathione (GSH; Grill *et al*., 1985; Rauser, 1990, 1995; Zenk, 1996, and literature cited therein). Recent work with Cd-sensitive mutants of *Arabidopsis thaliana* has provided compelling evidence for the participation of PCs in the cellular mechanism(s) for protection against Cd (Howden *et al*., 1995*a*, *b*). In particular, two Cd-sensitive mutants were shown to be impaired in the enzyme γ-glutamylcysteine synthetase (γ-ECS), the key enzyme of GSH biosynthesis (Cobbett *et al*., 1998), and phytochelatin synthase (Ha *et al*., 1999), respectively. For *B. juncea*, PCs have been shown to accumulate upon Cd exposure in roots and leaves of fully grown plants (Speiser *et al*., 1992; Salt *et al*., 1995*a*; Schäfer *et al*., 1998; Heiss *et al*., 1999). More recently, molecular analysis has revealed that massive PC synthesis during Cd accumulation apparently depends on a coordinate up-regulation of several genes involved in sulphur assimilation (Heiss *et al*., 1999) and GSH synthesis (Schäfer *et al*., 1998).

At present, little is known about how photosynthesis and transpiration are affected when Cd is accumulated in adult *B. juncea* plants, neither has PC synthesis been linked to the growth rate of leaves accumulating the toxic Cd ion. Thus, the aim of the present study was to compare the temporal pattern of Cd accumulation in the leaves, with effects on photosynthesis, transpiration and growth rate. Simultaneously, leaf GSH and PC contents were monitored, and transcript amounts for γ-ECS assessed by RNA blot analysis. These results provide evidence for a Cd-induced decline of transpiration rate and leaf growth, concomittant with the induction of PC formation, whereas $CO_2$ assimilation was unaffected. As far as is known, this is the first study where ecophysiological parameters and cellular adaptations were compared under indentical conditions for the same plant material.

## Materials and methods

### *Plant material*

Seeds of *Brassica juncea* L. var. Vitasso were germinated on sand and grown in a greenhouse with supplementary light to provide a 16 h light period. After 4 weeks, plants were selected for uniformity and transferred to the hydroponic system. Four plants were fixed on a polystyrol-plate in a pot containing 3.0 l of nutrient solution each. The nutrient solution (pH 5.2) contained 0.5 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 1.5 mM $KNO_3$, 1.0 mM $Ca(NO_3)_2$, 11.5 μM $H_3BO_3$, 125 nM $CuSO_4$, 2 μM $MnCl_2$, 25 nM $Na_2MoO_4$, 200 nM $ZnSO_4$, and 20 μM Fe-EDTA, respectively. Plants were transferred to a growth chamber and were adapted for a further 10 d to a photon flux density (400–700 nm) of 250–300 μmol photons $m^{-2}\,s^{-1}$ (at the top of the plants), a 16 h light period, a day/night temperature and humidity regime of 25/20 °C and 55/75% RH, respectively. The nutrient solution was continuously aerated and changed every second day until the experiment was started. During treatment with 25 μM $CdNO_3$, the nutrient solution was changed daily to avoid depletion of Cd in the hydroponic medium. GSH, PC, Cd content, and transcript amounts for γ-ECS and MT2, respectively, were determined from the same leaf samples which were frozen in liquid nitrogen immediately after harvest. For time-dependent analysis a third and a fourth leaf were harvested and pooled. It was confirmed that third and fourth leaves did not differ significantly ($P<0.05$) in Cd content. Moreover, fresh weight : area and dry weight : area ratios of those leaves were not affected by Cd treatment.

### *Photosynthesis, transpiration and growth*

Light-response curves of gas exchange were measured on the fourth leaf from the top using a steady-state porometer (HCM-1000, Walz, Effeltrich, FRG). Light of increasing intensities was applied using an external light source and measurements of the response curves of treated plants were started 5, 24, 48, 72, and 96 h after exposure to $CdNO_3$. An adaptation of 10–20 min to each light regime prior to measurement allowed $CO_2$ echange and water vapour exchange to reach

a steady state. Net $CO_2$ exchange and transpiration rates were calculated (von Caemmerer and Farquhar, 1981).

In parallel to the gas exchange measurements, a different leaf similar in age and size was used to measure chlorophyll fluorescence using a pulse-amplitude modulation fluorometer (PAM-2000, Fa. Walz, Effeltrich, FRG). The fiberoptic was kept in a constant distance (*c.* 1 cm) and angle (60°) to the leaf by a leafclip. The light intensity was measured close to the leaf surface. To determine maximum fluorescense a saturating light pulse of about 3500 µmol photons $m^{-2} s^{-1}$ and of 800 ms duration was superimposed on the prevailing light levels. This light intensity was checked to be saturating, following the P-peak characteristics of the Kautsky-kinetic. Prior to measurements of the light response curves readings of potential quantum yield of PSII ($F_v/F_m$) were taken following a 20 min dark period. This time interval was checked to be sufficient to relax all fast components of non-photochemical quenching (Keiller *et al.*, 1994). Total non-photochemical quenching ($q_N$) was calculated (van Kooten and Snel, 1990). The electron quantum yield of PSII was calculated as $(F_m'–F)/F_m'$, where $F$ is the steady-state fluorescence in the light and $F_m'$ is the maximum fluorescence in the light (Genty *et al.*, 1989).

Leaf area was determined by measuring length and maximal width of every leaf. Leaf area was calculated thereafter, using a standard curve. For total leaf area four plants were measured continuously over 5 d. Additionally the leaf area of eight leaves at the third and the fourth node were measured continuously.

### *Determination of GSH and phytochelatin contents*

Plant material frozen in liquid nitrogen was extracted in 0.5 M HCl and 100 mM ascorbate (following the procedure of Salt *et al.*, 1995*a*). The extract was passed through a 0.2 µm filter and applied to a C18 reversed phase HPLC column (Kontron Instruments, Munich). Thiols were separated with a gradient of acetonitrile/0.1% TFA over 22 min: 2–5 min 0–10%, 5–12 min 10–12%, 12–22 min 12–20%. GSH and PCs were detected at 412 nm, after post-column derivatization with 75 µM Ellman's reagent at pH 7.6. Identification and quantification was based on standards provided by M Zenk, Munich.

### *Cadmium accumulation in plants*

For the determination of time-dependent accumulation of Cd in *B. juncea* an aliquot of the leaves frozen in liquid nitrogen was dried in a microwave oven. The material was ground to a fine powder and extracted in 3 ml of concentrated $HNO_3$ at 160 °C for about 3 h. Cadmium content was analysed using a flame atomic absorption spectrophotometer (Perkin Elmer, 4100). Certified standard material (Peach leaf, GBW 08501, Fa. Breitländer) was used as a control.

### *Isolation of total RNA and Northern blot analysis*

Isolation of total RNA followed the protocol of Logemann *et al.* (Logemann *et al.*, 1987). The cloning of partial cDNAs for *B. juncea* γ-ECS and MT2 has been described earlier (Schäfer *et al.*, 1997). Alkaline Northern blot analysis, probe labelling by PCR, hybridization, and non-radioactive detection were performed according to Löw and Rausch (Löw and Rausch, 1996). Hybridization temperature was 42 °C for both probes (probe sizes: γ-ECS, 697 bp; MT2, 298 bp). High stringency washes were based on 65% homology.

### *Statistics*

Gas exchange measurements and leaf sampling were performed with different plants from at least three independent experiments. Data are presented as means with standard deviation. Analysis of significance was performed using paired Student's *t*-test (Sigma Blot) with a significance value of $P \leq 0.05$. If no error bars are given data derive from the most complete set of analysis measured in November, 1997.

## Results

### *Cadmium accumulation in leaves saturates after 72 h*

Cadmium content of third and fourth leaves (values for 48 h Cd exposure: $368 \pm 146$ and $377 \pm 109$ µg Cd $g^{-1}$ DW, respectively) did not differ significantly. Thus, to obtain enough leaf material to perform the complete analysis from the same plant material, third and fourth leaves were pooled. As early as 5 h after adding $CdNO_3$ to the hydroponic medium, a significant amount ($19 \pm 11$ µg Cd $g^{-1}$ DW) was translocated to the leaves (Fig. 1). Based on a relative water content of 92%, this equals a leaf sap concentration of about 18 µM, i.e. almost the same concentration as the nutrient solution. Highest Cd uptake rates were observed between 24 h and 48 h after the onset of exposure. During this time interval, Cd content of the leaves increased almost 4-fold, yielding 310 µg Cd $g^{-1}$ DW after 48 h of exposure. This amount corresponded to a more than 10-fold increase as compared to the concentration in the nutrient solution. After 48 h, the Cd accumulation rate levelled off and a maximum of 460 µg Cd $g^{-1}$ DW was found at the end of experiment.

### *Cadmium inhibits leaf expansion growth*

A detailed growth analysis of third and fourth leaves revealed that when *B. juncea* plants were exposed to 25 µM $Cd^{2+}$ in hydroponic culture, growth rate declined only 24 h after the onset of Cd exposure. After 48 h, growth rate was only about 25% of control plants. As shown in Fig. 2, third and fourth leaves of control plants grew with constant rates during the 96 h of experiment, while in response to Cd stress third leaves stopped growing

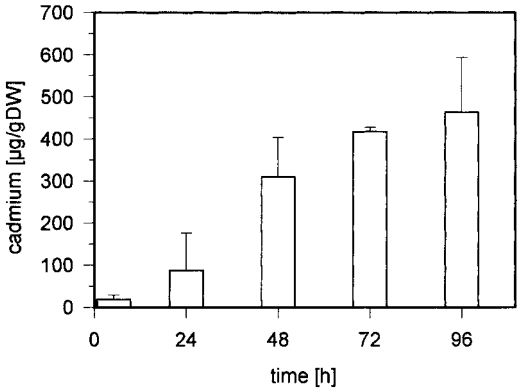


**Fig. 1.** Leaf cadmium content (µg Cd $g^{-1}$ DW] at 5, 24, 48, 72, and 96 h after Cd-application to the nutrient solution. Data are expresed as means ± SD of three independent experiments.

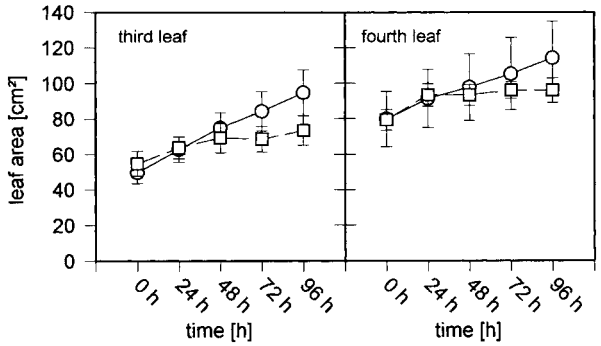


**Fig. 2.** Changes in leaf area ($cm^2$) of third and fourth leaves (from top) of *B. juncea* plants over a 96 h period. Circles, control plants; squares, Cd-treated plants. Data are expressed as means ± SD ($n = 8$).

after 48 h, whereas fourth leaves stopped growing after 24 h. Concomitant with this cessation of growth, a reduction of the transpiration rate was observed (see below). Moreover, older leaves (leaf storey 6–10 from the top) showed signs of wilting, beginning 2–3 d after Cd application (data not shown). The growth analysis of third and fourth leaves was complemented by an assessment of the daily increase of total leaf area per plant, including the youngest leaves which showed the most drastic growth reduction. The results (Table 1) confirm that cessation of growth occurs during the second day after the onset of Cd exposure. After 96 h, increase of total leaf area of Cd-exposed plants was reduced by about 60% as compared to control plants (Table 1), thereby significantly reducing further Cd accumulation.

### *Cadmium affects transpiration but not photosynthesis*

The $CO_2$ assimilation rate over a photon flux density from 15–2000 µmol $m^{-2}$ $s^{-1}$, was not significantly affected by Cd over a 96 h exposure period. To determine maximum photosynthetic capacity, light-response curves of gas exchange were measured over 5–96 h after addition of Cd. Cd treatment did not significantly influence maximum photosynthetic capacity or quantum yield of $CO_2$ uptake as shown by the representative set of data in Figs 3 and 4. Maximum rates of net $CO_2$ fixation under saturating light conditions varied between 22.7 ± 1.6 and 27.3 ± 6.6 µmol $m^{-2}$ $s^{-1}$ for control and between 21.7 ± 0.7 and 24.4 ± 4.9 µmol $m^{-2}$ $s^{-1}$ for Cd-treated plants, respectively, during the 96 h period of analysis. Chlorophyll fluorescence analysis indicated, that neither

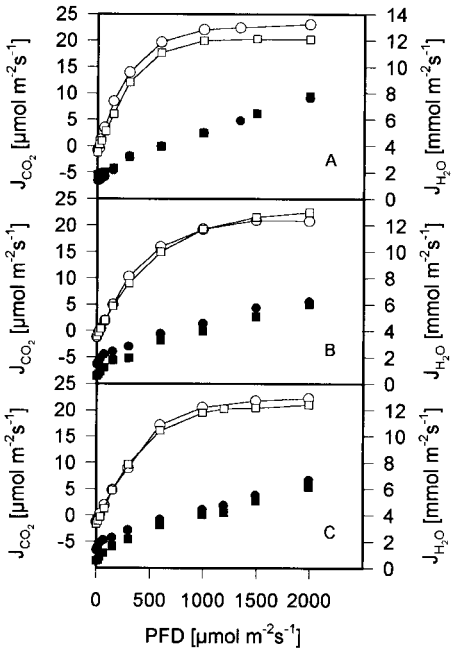


**Fig. 3.** Light-response curves of net $CO_2$ assimilation ($J_{CO2}$ [µmol $m^{-2}$ $s^{-1}$], open symbols) and transpiration rate ($J_{H2O}$ [mmol $m^{-2}$ $s^{-1}$], closed symbols) at 5 h (A), 48 h (B) and 96 h (C) after the onset of Cd exposure. Only fourth leaves were measured. Circles, controls; squares, Cd-treated plants. A representative set of data (Nov. 97) is shown.

non-photochemical quenching ($q_N$) nor the potential electron quantum yield of PSII ($F_v/F_m$) were changed significantly after exposure to Cd. The values of $F_v/F_m$ close to 0.8 (varying between 0.80 and 0.74 for control and 0.79 and 0.73 for Cd-treated plants, respectively) indicate no significant toxic effect of Cd on photosynthesis. The only transient (statistically not significant) decline in maximum photosynthetic capacity of about 10%, observed 5 h after the onset of Cd exposure (Fig. 3), was paralleled by a transient decrease of electron quantum yield of PSII ($\Delta F/F'_m$) by up to 25% under high light intensities (Fig. 4).

In marked contrast to $CO_2$ assimilation, transpiration was clearly affected by Cd exposure when photon flux density was ≤300 µmol $m^{-2}$ $s^{-1}$. After 96 h at lower light regimes, transpiration rates of Cd-treated plants were significantly lower than those of the controls. Conversely, after 5 h (Fig. 5) and even after 24 h (data not shown), transpiration of Cd-treated plants was not different from control plants, indicating a delayed response as compared

**Table 1.** *Daily increase of total leaf area for the first, second, third and fourth day of experiment*

Total leaf area of four plants each was determined continuously throughout the experiment. Values are given as the percentage increase per day. Data are expressed as mean (±SE). Different indices indicate that the data are significantly different at the level of $P \leq 0.05$.

| | 0–24 h | 24–48 h | 48–72 h | 72–96 h | Total |
|---|---|---|---|---|---|
| Control ± SE | 7.7 ± 2.8% a | 9.3 ± 1.8% a | 9.1 ± 3.6% a | 14.2 ± 2.1% a | 46.4 ± 5.2% |
| Cadmium ± SE | 8.2 ± 3.0% a | 5.0 ± 1.3% b | 2.2 ± 3.3% b | 2.7 ± 1.9% b | 19.3 ± 5.9% |

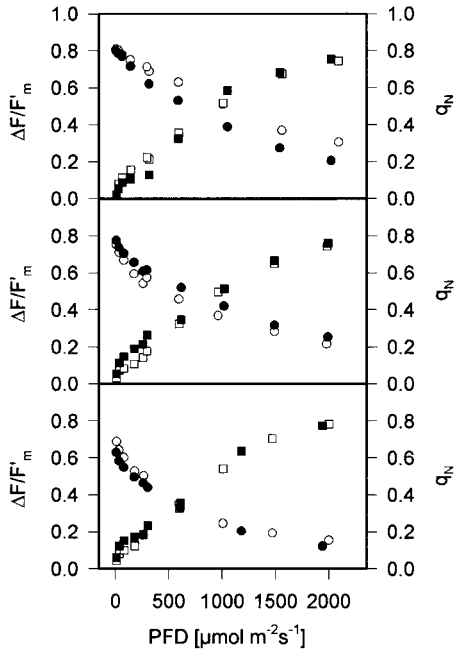


**Fig. 4.** Light-response curves of effective quantum yield of PSII ($\Delta F/F'_m$; circles) and non-photochemical quenching ($q_N$, squares) at 5 h (A), 48 h (B) and 96 h (C) after the onset of Cd exposure. Open symbols, controls; closed symbols, Cd-treated plants. A representative set of data (Nov. 97) is shown.

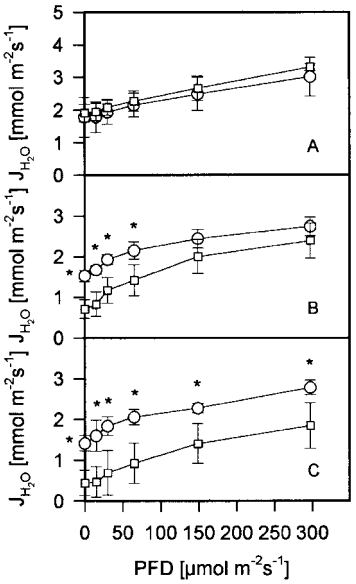


**Fig. 5.** Transpiration rates ($J_{H2O}$ [mmol $m^{-2}s^{-1}$]) at 5 h (A), 48 h (B) and 96 h (C) after the onset of Cd exposure. Data of three independent experiments are shown, including the data of the lower light regime ($\leq$300 μmol photons $m^{-2}$ $s^{-1}$) as depicted in Fig. 3. Data are expressed as means $\pm$ SD ($n>3$). Data points marked with * are statistically significant different ($P\leq0.05$).

to the rapid induction of PC synthesis (see below). As the reduced transpiration rate was observed under the same PFD used for cultivation of plants, the 50% decline is likely to affect plant development. However, the parallel time-course of growth inhibition and decrease of transpiration, both starting 48 h after the onset of Cd exposure, did not permit identification of the nature of the of relationship that exists between both processes. Interestingly, when exposed to light intensities above 300 μmol photons $m^{-2}$ $s^{-1}$, Cd-treated *B. juncea* did not show significant differences in transpiration rates compared to the controls (Fig. 5).

### *Cd-induced phytochelatin formation correlates with increased expression of γ-glutamylcysteine synthetase*

Glutathione (GSH) content in leaves of control plants varied between 100 and 200 nmol $g^{-1}$ FW, with a mean value of 144 $\pm$ 56 nmol GSH $g^{-1}$ FW, whereas phytochelatins (PC) were absent or below the limits of detection. Similar GSH contents were also found in leaves after Cd exposure (Fig. 6); however, 5 h after onset of Cd-exposure PC concentration had already reached about 140 nmol GSH equivalents $g^{-1}$ FW, the predominant species being PC3 and PC4 (Fig. 6B). Following a lag phase of 24 h, the rate of PC synthesis increased dramatically, yielding an almost 3-fold increase of PCs $d^{-1}$ between 24 h and 72 h after the onset of Cd-exposure.

When comparing Cd content (Fig. 1) and PC formation on a fresh weight basis, assuming an average relative water content of 92% (see above), PC:Cd ratios were approximately 3 at 24 h, 48 h and 96 h, but 5.5 at 72 h after the onset of Cd exposure. This transient 'overshoot' of PC formation indicates that between 48 h and 72 h cytosolic Cd remained high enough (despite the levelling off of total Cd content) to keep PC synthase in the activated state. The later decline of PC concentration suggests that after transfer of Cd-PC complexes to the vacuole, free PCs are subject to metabolic turnover.

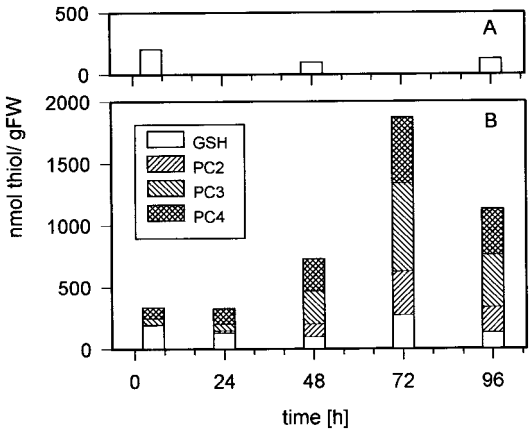


**Fig. 6.** Changes of glutathione (GSH) and phytochelatin concentrations (PC), namely PC2, PC3 and PC4, in leaves of control and Cd-treated plants between 5 h and 96 h after the onset of Cd exposure. Values for controls (A) and Cd-treated plants (B) are expressed as nmol GSH equivalents $g^{-1}$ FW. Data from samples collected in Nov. 97 are presented.

Northern blot analysis (Fig. 7) of transcript amounts for γ-ECS, coding for the regulatory enzyme of GSH synthesis, showed a significant increase immediately after the onset of Cd-exposure. At 48 h, when the high Cd uptake rate in the leaves yielded a more than 3-fold increase in foliar Cd and PC concentrations, the maximum induction of γ-ECS mRNA was observed. After 72 h, transcript levels were lowered again and a new steady-state for γ-ECS transcripts appeared to be established, consistent with the strong decline in further Cd accumulation. Interestingly, transcript amounts for metallothionein class 2 (MT2) were not changed early after the exposure to Cd, and increased only with a significant delay as compared to γ-ECS (Fig. 7). This delayed increase of MT2 transcript levels did not always occur to the same extent in independent experiments.

## Discussion

This study on *B. juncea* provides the first comprehensive analysis of leaf physiology (growth, $CO_2$ assimilation, chlorophyll fluorescence, transpiration) and synthesis of Cd-sequestering phytochelatins (GSH and PC concentrations, expression of γ-ECS) during Cd accumulation under controlled conditions in a time-course study over a period of several days. Since all parameters were determined for the same leaf material, the physiological and biochemical data may be directly compared. PC formation was selected as a biochemical marker for cellular Cd sequestration, because genetic analysis has recently provided direct evidence for PC involvement in Cd detoxification (see Introduction; Cobbett *et al*., 1998; Ha *et al*., 1999). Of course, this does not exclude the participation of other cellular mechanisms for Cd sequestration, such as binding to organic acids as recently suggested (Salt *et al*., 1997). The relatively high concentration of Cd (2.85 ppm) freely available to the plant was chosen to evaluate the ability of *B. juncea* to withstand severe stress. Similar Cd concentrations may occur on field sites (see Introduction), especially when the phytoremediation process is enforced by increasing Cd availability to the plants with artificial chelators such as EDTA or EGTA (Ernst, 1996; Blaylock *et al*., 1997).

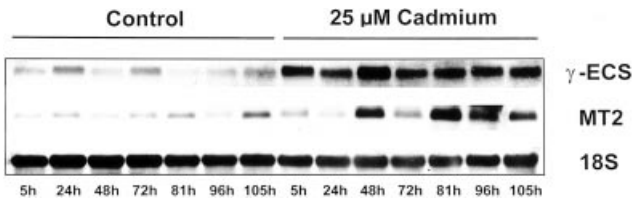


**Fig. 7.** Northern blot analysis of γ-ECS- and MT2-mRNAs in leaves of control and Cd-treated plants. Total RNA was extracted from the same frozen material of controls and stressed leaves as used for Cd and PC analysis. 5 µg of total RNA were loaded per lane. Equal loading was confirmed using a 18S rRNA probe.

The results presented indicate that the exposure of *B. juncea* to high Cd concentrations does not significantly affect photosynthetic activity during a 4 d treatment, as shown by light response curves of $CO_2$-exchange and photosynthetic electron transport at PSII (as summarized in Table 2). This observation is in marked contrast to previous *in vitro* studies which have demonstrated dramatic effects of Cd on photosynthesis at various levels such as (i) direct effects on PSII electron transport (van Assche and Clijsters, 1985; Baszynski, 1986), (ii) decrease of plastoquinone levels in the chloroplast (Krupa *et al*., 1992), (iii) inhibition of the Calvin cycle (Weigel, 1985*a*, *b*), or (iv) failure in chlorophyll biosynthesis (Padmaja *et al*., 1990). The recently reported Cd-induced reduction in growth, leaf chlorophyll content and photochemical quantum yield of photosynthesis for 10-d-old seedlings of *B. napus* grown under high light intensity (Larsson *et al*., 1998) indicates, that *in vivo* Cd effects on photosynthesis depend on plant species, plant age, time of Cd exposure, and also the light regime. The absence of Cd effects on photosynthetic performance *in vivo* as observed in this study with adult *B. juncea* plants most likely results from an effective protection of chloroplasts by cellular detoxification mechanisms, keeping Cd in the cytosol below a critical threshold level. In particular, maximum $CO_2$ assimilation and electron quantum yield at PSII under high light intensities were not significantly changed throughout a 96 h period of Cd exposure, despite considerable Cd accumulation. Moreover, even during the period of first Cd entry into the leaf, and later during the period of most rapid Cd accumulation, $CO_2$ assimilation was not impaired. Also, no significant changes in the chlorophyll content of the third and fourth leaves was observed (data not shown), as described earlier for much lower Cd concentrations (Salt *et al*., 1995*b*). This suggests that the cellular detoxification response is in operation without delay, in agreement with the well-established allosteric activation of constitutively expressed PC synthase (Grill *et al*., 1989). Furthermore, these results indicate that the expression of γ-ECS, the rate-limiting enzyme for GSH biosynthesis, is up-regulated as early as 5 h after Cd addition, in agreement with the reported induction of several other genes required for sulphate assimilation and GSH synthesis (Schäfer *et al*., 1998; Heiss *et al*., 1999). Thus, in addition to activation of PC synthase, leaf cells adapt the expression of genes regulating their sulphur assimilation flux to replenish the rapidly turning over GSH pool during PC accumulation.

A closer inspection of the time-course for Cd accumulation and PC formation reveals that while Cd accumulation clearly levels off after 48 h, the synthesis of PCs continues at a high rate for another day, yielding about a 3-fold increase between 48 h and 72 h. Since PC synthesis is dependent on allosteric activation of PC synthase, the continued PC accumulation after 48 h indicates that not

**Table 2.** *Summary of Cd-effects on leaves of* B. juncea

| | Day 1 (0–24 h) | Day 2 (24–48 h) |
|---|---|---|
| Photosynthesis | Transient, non-significant decline | No effect |
| Transpiration | No effect | 50% lowered |
| Leaf expansion growth | No effect | Strong decline |
| Cd-uptake rate | 3.5 μg $g^{-1}$ DW h | Highest rate: 8.8 μg $g^{-1}$ DW h |
| PC-production | Slight induction | Maximum induction |
| γ-ECS mRNA | Slight induction | Maximum induction |
| MT2 mRNA | No effect | Strong, but variable induction |

all Cd entering leaf cell cytoplasm has been sequestered, and that cytosolic Cd concentrations remain high enough to keep PC synthase in the activated state. Thus, it may be suggested that the transient cytosolic accumulation of Cd may be the cause for inhibition of leaf growth and transpiration (see below).

Interestingly, the maximum Cd concentration found in this study was similar to the concentration found in *B. juncea* plants and seedlings exposed to much lower solution concentrations (0.1 μM Cd; Salt *et al.*, 1995*b*, 1997), indicating an overall limitation in total leaf Cd uptake in *B. juncea*, irrespective of the Cd concentration administered to the plant. Possible reasons could be limitations of root to shoot translocation, and/or a saturable capacity for intracellular detoxification of Cd. $PC_3$ ($\gamma$-$(EC)_3G$) has been proposed to be the most likely candidate for Cd complexation and sequestration (Zenk, 1996). However, a Cd–$S_4$ co-ordination has been observed in extracts of root tissue and a high molecular weight (HMW)-Cd complex described (Salt *et al.*, 1995*b*). This complex has been proposed as the major deposition form of Cd in *B. juncea*. It is composed of PC-Cd and acid-labile sulphur in about equal stoichiometry (Speiser *et al.*, 1992). Based on this information, a PC-derived thiol:Cd ratio of 2 (or maximum 3) would be likely to be sufficient for cellular Cd detoxification, suggesting that, in leaves of *B. juncea*, PC concentration was eventually sufficient for sequestration of all Cd taken up.

Despite maintenance of full photosynthetic activity, leaf expansion growth was strongly affected, albeit with a delay of 24 h. Growth of the third and fourth leaves came to a complete stop within 48 h. Growth reduction in response to Cd-stress was also reported for *Phaseolus vulgaris* (Poschenrieder *et al.*, 1989), and for various *Brassica* species after exposure to excess Zn or Cu (Ebbs and Kochian, 1997). These authors hypothesized that the observed decrease in shoot Fe and Mn contents may significantly contribute to the inhibition of plant growth. Fe and Mn levels were not analysed in this study, however, other data indicate a significant decrease of Mn content in roots and shoots of *B. juncea* in response to Cd (Salt *et al.*, 1995*b*). Interestingly, the present study revealed that the reduction of leaf expansion growth was accompanied by a decrease in transpiration rate of about 50% under moderate light intensities ($\leq$300 μmol photons $m^{-2}$ $s^{-1}$).

Reduction of growth and lowered transpiration are common signs of leaf senescence, being usually correlated with an increased production of abscisic acid (Heaton *et al.*, 1987). However, mediation of Cd effects on growth and transpiration via ABA-mediated signalling does not appear likely because no correlation was found between concentrations of Cd and ABA in roots of Cd-exposed bean seedlings (Poschenrieder *et al.*, 1989).

When compared with γ-ECS transcripts, MT2 mRNA content increased with a significant delay. MT2 is thought to be involved in metal homoeostasis, in particular, when extensive protein degradation occurs, and strong induction of MT2 expression has been observed during leaf senescence (Buchanan-Wollaston, 1997). Although MT2 is known to be copper-inducible in leaves and foliar trichomes of *A. thaliana* (Garcia-Hernandes and Taiz, 1997), there is as yet little evidence for MT2 participation in Cd-detoxification in *B. juncea*, and Cd-MT-complexes have not yet been demonstrated. As no correlation was observed between the MT2 expression and the time-dependent Cd accumulation in the leaves, the late induction of MT2 transcripts might rather indicate a role in secondary effects like Cd-induced senescence.

It is concluded that dramatic physiological responses occur in leaves of *B. juncea* during Cd accumulation despite apparent protection of photosynthesis. In particular, these results suggest that, during continuous exposure of *B. juncea* to elevated Cd concentrations, the inhibition of transpiration (and growth) may limit the potential for phytoremediation. The detailed time-course study indicated that cytosolic Cd concentration remained high enough to keep PC synthase in the activated state even after Cd accumulation had declined. The 24 h delay of growth inhibition renders the involvement of rapid ABA root-shoot signalling unlikely. Rather, Cd appears directly to affect cell division and/or expansion and transpiration in the leaf.

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