The Cytosolic O-Acetylserine(thiol)lyase Gene Is Regulated by Heavy Metals and Can Function in Cadmium Tolerance*

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Regulation of the expression of the cytosolic O-acetylserine(thiol)lyase gene (Atcys-3A) from Arabidopsis thaliana under heavy metal stress conditions has been investigated. Northern blot analysis of Atcys-3A expression shows a 7-fold induction after 18 h of cadmium treatment. Addition of 50 μM CdCl₂ to the irrigation medium of mature Arabidopsis plants induces a rapid accumulation of the mRNA throughout the leaf lamina, the root and stem cortex, and stem vascular tissues when compared with untreated plants, as observed by in situ hybridization. High pressure liquid chromatography analysis of GSH content shows a transient increase after 18 h of metal treatment. Our results are compatible with a high cysteine biosynthesis rate under heavy metal stress required for the synthesis of GSH and phytochelatins, which are involved in the plant detoxification mechanism. Arabidopsis-transformed plants overexpressing the Atcys-3A gene by up to 9-fold show increased tolerance to cadmium when grown in medium containing 250 μM CdCl₂, suggesting that increased cysteine availability is responsible for cadmium tolerance. In agreement with these results, exogenous addition of cystine can, to some extent, also favor the growth of wild-type plants in cadmium-containing medium. Cadmium accumulates to higher levels in leaves of tolerant transformed lines than in wild-type plants.

In recent years, contamination of soils and waters by toxic heavy metals and organic pollutants represents a major environmental and human health problem. The possibility of the removal of these toxic heavy metals by phytoextraction technology has increased interest in the knowledge of the physiological and molecular mechanisms of plant adaptation to heavy levels of heavy metals. Several heavy metals such as cadmium and copper are tolerated to certain low levels through the synthesis of small peptides named phytochelatins, which form a tight complex with the metal ion to inactivate and store it in the vacuole (1). Phytochelatins are enzymatically synthesized from glutathione as a response of the plant to toxic levels of the heavy metals that produce a transient decrease of GSH content (2). Glutathione is synthesized by a two-step ATP-dependent reaction catalyzed by the γ-glutamylcysteine synthetase and glutathione synthetase. These reactions seem to be highly regulated at the transcriptional and translational levels, as well as by feed-back control of the γ-glutamylcysteine synthetase by GSH. In Arabidopsis, a coordinated response of the glutathione biosynthesis genes to heavy metals has been reported (3). One of the factors that also regulates GSH biosynthesis is cysteine availability, because exogenous addition of cysteine has been shown to increase the GSH content (4). However, little work has been carried out on the regulation of cysteine biosynthesis under heavy metal stress or environmental conditions.

The last step of cysteine biosynthesis is catalyzed by the O-acetylserine(thiol)lyase enzyme (OASTL) that incorporates sulfide into the O-acetyl-L-serine molecule. This molecule is produced by an acetylation reaction of L-serine catalyzed by the serine acetyltransferase enzyme. Both serine acetyltransferase and OASTL activities have been demonstrated to be localized in the three cellular compartments involved in protein synthesis, cytosol, chloroplast, and mitochondrion (5). However, the contribution of each OASTL and serine acetyltransferase isoform to a particular metabolic pathway is unknown (6, 7). Recently, the Atcys-3A gene coding for the cytosolic OASTL isoform from Arabidopsis has been shown to be regulated by salt stress and the hormone abscisic acid (8). In this work, we describe the Cd induction of the cytosolic OASTL from Arabidopsis thaliana and the involvement of this enzyme in cadmium tolerance, by overexpression of the corresponding cDNA in transformed plants.

EXPERIMENTAL PROCEDURES

Plant Material, Growth, and Treatments—Wild-type A. thaliana (ecotype Columbia) plants were grown on moist vermiculite supplemented with Hoagland medium at 20 °C in the light and 18 °C in the dark, under a 16-h white light/8-h dark photoperiod. Cadmium chloride treatments were performed by addition to the Hoagland medium of CdCl₂ to 50 μM final concentration, unless otherwise indicated. For the tolerance test, seeds were surface-sterilized and germinated on solid MS medium with and without CdCl₂, at indicated concentrations, after cold treatment for 1 day to improve germination. The plants were grown in a growth chamber under the same conditions described above. For circadian experiments plants were grown on soil under a 16-h white light/8-h dark photoperiod for 3 weeks, before harvesting every 4 h for 36 h. After this treatment, plants were shifted to constant light and harvested every 4 h during 1 day.

In Vivo Growth Measurements—Seeds were germinated on solid MS medium for 5 days on vertical Petri dishes and transferred onto medium containing different concentrations of CdCl₂. Plants were weighed after 6 days of further growth. Total seedling fresh weight was determined after gently drawing the root out of the agar medium.

Analytical Determination of Cysteine, Glutathione, and O-Acetylserine(thiol)lyase Activity—Cysteine and glutathione were extracted and subsequently quantified by reverse-phase HPLC after derivatization with monobromobimane (Molecular Probes) following the methods described by Rauser et al. (9). Plant tissues were homogenized in cold

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1 The abbreviations used are OASTL, O-acetylserine(thiol)lyase; PC, phytochelatin; HPLC, high pressure liquid chromatography; CHES, 2-(cyclohexylamino)ethanesulfonic acid.
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extraction buffer (4 ml/g fresh weight) containing 0.1 N HCl and 1 mM EDTA using a mortar and pestle with liquid nitrogen. Homogenates were centrifuged at 15,000 × g for 15 min in the cold. Thióles were reduced at 4 °C for 15 min by mixing 400 μl of extracted samples with 600 μl of 200 mM CHES (pH 9.2) and 100 μl of 250 mM NaBH₃CN, 350-μl aliquots were derivatized in the dark for 15 min by adding 200 μl 150 mM monobromobimane. The reaction was stopped by adding 250 μl of 0.25% (v/v) methanesulfonic acid at room temperature. Derivatized thiols were separated and quantified by reverse-phase HPLC (10). O-Acetylsérine(thiolo)lyase activity was measured in crude extracts from wild-type or transformed Arabidopsis plants following a previously described method (11).

Cadmium Analysis—Three-week-old plants were irrigated for 14 days with Hoagland medium supplemented with 250 μM CdCl₂, final concentration. The leaves were collected and dried at 65 °C during 5 h. The dried material was wet-ashed in HNO₃-HClO₃ (7:1) (v/v) at 65 °C until the volume of the solution was reduced from 20 to 2 ml. Finally, the samples were analyzed by inductively coupled plasma atomic emission spectrometry using a Fisons-ARL 3410 sequential multielement instrument.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from leaves, and Northern blot analysis was performed as previously described (11). The blots were sequentially hybridized with a gene-specific Atcys-3A probe and then with maize 17 S DNA for detection of Arabidopsis 18 S rRNA (12). Hybridization with CCA1 gene probe was used as a positive control of plant circadian oscillator (13). For quantifying mRNA levels, radioactivity distributed on the blots was imaged by an InstantImager electronic autoradiographer (Packard Instrument Co.). The level of Atcys-3A mRNA was normalized to the level of the 18 S rRNA obtained for each sample.

In Situ Hybridizations—Probes for in situ hybridization were labeled with uridine 5′-[(35)S]triphosphate. The Atcys-3A cDNA insert (11) subeloned into pBlueScriptII KS was amplified by polymerase chain reaction using standard M13 reverse and forward primers. About 1 μg of the polymerase chain reaction product was used as template to synthesize [35S]labeled RNA using T7 RNA polymerase (antisense probe) or T3 RNA polymerase (sense probe).

Mature Arabidopsis plants grown under standard conditions or treated with cadmium chloride (50 μM) for 18 h were used for in situ hybridization. Leaves, stems, and roots were cut into small pieces, fixed in 4% formaldehyde, embedded in wax, and sections were processed and hybridized under conditions as described previously (12). For autoradiography, slides were coated with Amersham Pharmacia Biotech Hypercoat LM-1 nuclear emulsion and exposed for 11 days at 4 °C. The samples were then developed in Eastman Kodak Co. D19 developer prechilled at 14 °C and were fixed in 30% sodium thiosulfate for 5 min. After developing, the tissues were stained with 0.05% toluidine blue in water for 0.5 min, rinsed, dehydrated, and air-dried.

For purposes of comparison, the tissue sections from control and stressed plants were fixed onto a single glass slide and hybridized with the same labeled probe. Tissue sections were observed in an Olympus BX50 microscope attached to a JVC TKC-1381 digital CCD color video camera for image capture. Images were processed and mounted by using the Olympus MicroImage analysis program and Adobe Photoshop software, respectively.

Arabidopsis Plant Transformation—For plant transformation, the β-glucuronidase gene in the binary vector pBI121 (CLONTECH), under the control of the cauliflower mosaic virus 35 S promoter, was replaced by the full-length Atcys-3A CDNA (11). A 260-base pair fragment containing the polycladenylation signal from the nopaline synthase gene of Agrobacterium Ti plasmid was placed downstream of the Atcys-3A gene. The resulting plasmid, named pBIOAS, was transformed into Agrobacterium tumefaciens strain CV50. A. thaliana (ecotype Columbia) was transformed by using the vacuum infiltration method described by Bechtold et al. (14). Transformed seeds were tested for kanamycin resistance on solid MS medium containing 50 mg l⁻¹ kanamycin. Integration into the nuclear genome of the plant was analyzed by genomic Southern blot analysis. T₅ or subsequent generations of transgenic plants not exposed to Cd, using as a positive control the 18 S rRNA obtained for each sample.

To gain insight into this response, the effect of cadmium chloride on Atcys-3A expression was investigated at the tissue level by in situ hybridization. In roots, the transcript of the O-acetylsérine(thiolo)lyase Atcys-3A gene was localized in the cortex, but the amount of detected signal was higher in cadmium-treated plants as compared with nontreated plants (Fig. 2, C and D). In stem sections, the level of expression of Atcys-3A was almost undetectable above background in control plants (Fig. 2, E and F). However, it was possible to detect signal in the cortex and the vascular tissue of cadmium-stressed plants (Fig. 2, G and H). In leaf, the basal level of expression observed in all cell types was increased throughout the leaf lamina after cadmium treatment (Fig. 2, L–O). Although the in situ hybridization technique is not a quantitative method, for purposes of comparison we hybridized the tissue sections on the same slide accumulation after 1 h was observed. Concomitant with the increasing mRNA levels, OASTL enzyme activity increased by 2.5-fold after 18 h of exposure to metal (Fig. 1C). The time course of changes in Atcys-3A transcript and OASTL enzyme activity levels could reflect a diurnal rhythm of this gene. To check this possibility, circadian experiments were performed on plants not exposed to Cd, using as a positive control the CCA1 (circadian clock-associated 1) gene (13). We were unable to observe any fluctuation of Atcys-3A gene expression over a 60-h period, suggesting that the pattern of induction of Atcys-3A mRNA is specific to the heavy metal treatment (data not shown).

RESULTS

Effect of Cadmium Treatment on Atcys-3A Transcript Abundance—Northern analysis showed a 7-fold induction of Atcys-3A transcript level in leaves in response to cadmium chloride treatment when compared with nontreated plants (Fig. 1, A and B). Although the maximum level of induction was observed 18 h after treatment, a significant increase in RNA accumulation after 1 h was observed. Concomitant with the increasing mRNA levels, OASTL enzyme activity increased by 2.5-fold after 18 h of exposure to metal (Fig. 1C). The time course of changes in Atcys-3A transcript and OASTL enzyme activity levels could reflect a diurnal rhythm of this gene. To check this possibility, circadian experiments were performed on plants not exposed to Cd, using as a positive control the CCA1 (circadian clock-associated 1) gene (13). We were unable to observe any fluctuation of Atcys-3A gene expression over a 60-h period, suggesting that the pattern of induction of Atcys-3A mRNA is specific to the heavy metal treatment (data not shown).

FIG. 1. Regulation of the cytosolic O-acetylsérine(thiolo)lyase transcript abundance and enzyme activity in leaves of cadmium-treated Arabidopsis plants. CdCl₂ (50 μM) was added to the irrigation medium of 3 week-old Arabidopsis plants at the indicated times. Atcys-3A mRNA accumulation in leaf extracts was determined by Northern blot analysis. The experiment was repeated three times, and a representative hybridized blot is shown (A). The level of Atcys-3A mRNA and 18 S rRNA was quantified for the representative hybridized blot shown in A as indicated under “Experimental Procedures,” and the level of Atcys-3A was normalized to the level of the 18 S rRNA obtained for each sample (B). O-acetylsérine(thiolo)lyase activity. The values are averages of at least three independent experiments, and the error bars represent the standard deviations. The symbol * indicates significant differences (p < 0.05) from the data at t = 0 (C).
and therefore processed them under the same conditions. The high level of Atcys-3A mRNA detected in trichomes of untreated plants (12, 15) was maintained or even increased in cadmium-treated Arabidopsis (data not shown).

Effect of Cadmium Treatment on Cysteine and Glutathione Content in Leaf Tissues—Other workers have demonstrated that cadmium induces the biosynthesis of phytochelatin peptides from GSH. To investigate whether cadmium treatment induces the biosynthesis of the precursor molecules, cysteine and GSH, we determined by HPLC analysis the content of both compounds in leaf tissues from cadmium-treated Arabidopsis. It was clearly observed that the GSH content increased about 2-fold after 18 h of treatment with 50 μM cadmium and dropped to the basal level within 24 h of treatment. However, the level of cysteine was not significantly increased. Similarly to the mRNA accumulation, a rapid increase that peaks after 1 h was also observed (Table I). However, after exposure of higher concentrations of cadmium for 24 h, a depletion of the cysteine and GSH levels was observed. At 100 μM Cd, the level of cysteine declined 35% with respect to untreated plants, and a decrease of 30% was observed in the GSH level. Larger decreases in either cysteine (43% depletion) or GSH (34% depletion) levels were detected at the highest concentration of 500 μM Cd used (data not shown). These results are in concordance

Fig. 2. Effect of heavy metal on the expression of the cytosolic O-acetylserine(thiol)lyase gene in Arabidopsis tissues. Transverse sections (10 μm) of A. thaliana leaves were hybridized in situ with 35S-labeled antisense (A–O) or sense (P–R) RNA probes. A and B, bright- and dark-field micrographs, respectively, of untreated roots, hybridized with antisense Atcys-3A 35S-RNA. C and D, bright- and dark-field micrographs, respectively, of roots treated with 50 μM CdCl₂ for 18 h, hybridized with antisense Atcys-3A 35S-RNA. E and F, bright- and dark-field micrographs, respectively, of untreated stems, hybridized with antisense Atcys-3A 35S-RNA. G and H, bright- and dark-field micrographs, respectively, of stems treated with 50 μM CdCl₂ for 18 h, hybridized with antisense Atcys-3A 35S-RNA. I and R, bright- and dark-field micrographs, respectively, of untreated leaf, hybridized with antisense Atcys-3A 35S-RNA. L and M, bright- and dark-field micrographs, respectively, of leaf treated with 50 μM CdCl₂ for 18 h, hybridized with antisense Atcys-3A 35S-RNA. N and O, magnification (× 20) of the images in L and M, respectively. P and R, bright- and dark-field micrographs, respectively, of untreated leaf, hybridized with sense Atcys-3A 35S-RNA.
with the behavior of the GSH content upon Cd treatment of *Arabidopsis* also observed by other authors (3). Circadian fluctuation of cysteine or GSH levels (without Cd treatment) was not observed (data not shown).

**Cadmium Tolerance in Transformed *Arabidopsis* Plants Overexpressing the Atcys-3A Gene**—We have produced *Arabidopsis*-transformed plants overexpressing the Atcys-3A gene via Agrobacterium-mediated transformation. Full-length Atcys-3A cDNA was fused in sense orientation to the cauliflower mosaic virus 35 S S promoter to obtain constitutive expression of the gene. Six independent transformed lines, tested by Southern blot, were used for further analysis. Northern blot analysis of the transformed plants showed up to a 9-fold increase in Atcys-3A mRNA accumulation in the pBIOAS-10 line compared with control-transformed plants expressing the pBI121 plasmid (lines pBI121-C1, -C3, -C5, and -C7) (Fig. 3A). The transformed plants also showed a concomitant increase in OASTL activity in leaf extracts (Fig. 3B).

We have tested the effect of the cadmium ion on the transformed plants by growing seeds on solid MS medium containing 250 μM CdCl₂. The transformed lines showing the higher levels of overexpression of the Atcys-3A gene were able to germinate and grow on this medium (Fig. 3C). Wild-type or transformed pBI121-C1 control plants germinate poorly in the presence of the metal, and those plants that did develop cotyledons did not produce leaves and died after 5–7 days (Fig. 3C).

The high cadmium resistance shown by the transformed lines overexpressing Atcys-3A could suggest that cysteine availability is a main factor for tolerance. In this sense, we investigated whether exogenous addition of cysteine to pBI121 control seedlings is sufficient to mimic the cadmium resistance observed in the Atcys-3A-overexpressing transformed seedlings. To avoid reaction in the medium between the heavy metal and the thiol group of cysteine, we used exogenous addition of cystine, which lacks the free thiol group. Addition of 200 μM cystine to control plants was sufficient to support their growth in the presence of 250 μM cadmium, although not to the extent observed in the transformed lines (Fig. 4).

Cadmium analysis by atomic emission spectrometry of mature transformed Atcys-3A plants growing for 14 days on cadmium-containing media showed an increase of cadmium accumulation in leaves compared with control plants (Table II). Cadmium accumulation in different lines correlates with mRNA and OASTL activity levels, with line pBIOAS-10 having the highest level of cadmium accumulation, a 72% higher cadmium concentration than control plants.

**Characterization of the Cadmium Tolerance of Transformed pBIOAS Line 10**—Two different Cd resistance tests were performed. In the first test, transformed seeds were germinated in the presence of a range of Cd concentrations and compared with wild-type. Transformed seeds were able to germinate in the presence of CdCl₂ up to 400 μM, producing green leaves. By contrast, wild-type seedlings either failed to germinate or germinated but were unable to produce leaves, at 250 μM CdCl₂ and bleached white and died at a cadmium concentration of 400 μM (Fig. 5A). In the other test, we studied the inhibition of growth of wild-type and line pBIOAS-10 seedlings upon Cd treatment. pBIOAS-10 was able to survive over 300 μM concen-
transformation, whereas wild-type died. Dead seedlings showed a fresh weight of around 2 mg (Fig. 5B).

The glutathione level was also determined in the transformed pBIOAS line 10 and compared with the level in wild-type plants. In the absence of cadmium, the concentrations of GSH in the transformed line and wild-type were similar. However, treatment with 250 μM CdCl₂ for 18 h produced opposite effects in wild-type and the transformed line, a 30% decrease of the GSH content in wild-type, and an increase of 54% in the line 10 (Fig. 6).

**DISCUSSION**

Heavy metals such as cadmium, mercury, lead, and arsenic are highly reactive with sulfydryl groups and can affect a vast array of biochemical processes. It is now well established that the thiolate peptides, the phytochelatins, play an essential role in the detoxification of cadmium and some other heavy metals. Thus, the Arabidopsis cad1 mutant deficient in PC synthase is highly sensitive to cadmium ion (16). Arabidopsis plants treated with heavy metals such as cadmium and copper respond by increasing transcription of genes involved in GSH synthesis and reduction (gsh1, gsh2, and gr1) (3). Furthermore, increased levels of γ-glutamylcysteine synthetase and glutathione synthetase have also been observed in cadmium-treated species such as maize (17), tobacco, and tomato cells (18, 19) and Brassica juncea (20). Because phytochelatin synthesis produces a detectable depletion in GSH content (3), it is expected that the GSH precursor thiol molecule, cysteine, has to be produced at higher rates to support GSH biosynthesis under cadmium treatment.

The data reported here clearly show a transient increase of the O-acetylseryine(thiol)lyase upon cadmium treatment, and

**FIG. 4. Effect of cystine feeding on cadmium tolerance.** Seeds from control pBII121 lines C1 (A and E), C3 (B and F), C5 (C and G), and C7 (D and H) were plated on solid MS media containing 250 μM CdCl₂ in the presence (A–D) or absence (E–H) of 200 μM cystine. Seedlings were visualized after 15 days of growth with an Olympus SZ4045TR stereo microscope.

**TABLE II**

Cadmium concentration in leaves of transformed Arabidopsis lines overexpressing the Atcys 3A gene

Mature Arabidopsis plants were incubated with 250 μM CdCl₂ for 14 days, and leaves were then collected from different pots of the same plant line for Cd determination, as described under “Experimental Procedures.” Values ± S.E. are shown from five independent measurements. The symbol * indicates significant differences (p < 0.05) from the control plants. DW, dried weight.

| Plant line | Cadmium concentration mg Cd/g DW |
|------------|---------------------------------|
| Control    | 0.39 ± 0.02                     |
| pBIOAS-1   | 0.49* ± 0.06                    |
| pBIOAS-2   | 0.45* ± 0.05                    |
| pBIOAS-3   | 0.34 ± 0.03                     |
| pBIOAS-4   | 0.47* ± 0.03                    |
| pBIOAS-5   | 0.37 ± 0.03                     |
| pBIOAS-10  | 0.67* ± 0.06                    |

**FIG. 5. Tolerance of transformed pBIOAS line 10 to cadmium.** A, sensitivity of wild-type and transformed pBIOAS line 10 seedlings to Cd. Seeds were germinated on solid MS media containing different concentrations of CdCl₂ as indicated and were grown for 15 days. Seedlings were then visualized with an Olympus SZ4045TR stereo microscope. B, growth of wild-type and transformed pBIOAS line 10 seedlings upon Cd treatment. Five-day-old seedlings germinated on Cd-free MS medium were transferred to medium containing CdCl₂ at the indicated concentrations. Total seedling fresh weights were determined after an additional 6 days of growth. Each data point represents the mean of five independent measurements, in which ten seedlings were weighed. Error bars represent the S.D. Curve fitting showing the tendency of the data (dashed lines, wild-type in red and line 10 in blue) was calculated by nonlinear regression using the program Microsoft Excel 98.
Cd.

Mature plants of PbIOAS line 10 leaves upon cadmium treatment were incubated in the absence (−Cd) or the presence of 250 μM CdCl$_2$ (+Cd) for 18 h, and leaves were collected for GSH determination. Error bars represent the standard deviations. The symbol * indicates significant differences ($p<0.05$) from data of the same plant in absence of Cd.

This effect is observed at the transcriptional and enzymatic level. Although the increase in OASTL activity is only 2.5-fold, we have to point out that these measurements represent the sum of the three OASTL isoforms identified in the cell. In situ hybridization data also show that the transcript is significantly induced in the whole leaf lamina and in the cortex and the vascular tissue of root and stem. In B. juncea, coordinate changes of expression for several sulfur assimilation enzymes, including OASTL, have been observed in response to cadmium treatment (20, 21). All these data support the idea that plants respond to cadmium toxicity by inducing the genes required for phytochelatin synthesis, the genes involved in cysteine, and in glutathione biosynthesis (3).

In addition, because cadmium and other heavy metals are very reactive with thiol molecules, the reduced availability of free cysteine for GSH biosynthesis can significantly reduce and limit the amount of phytochelatin synthesis. Addition of exogenous cystine supports plant growth in cadmium-containing media. In fact, in transformed plants overexpressing the Atcys-3A gene, the tolerance to cadmium ion also increases, up to 400 μM CdCl$_2$, in the case of the line pBIOAS-10. By increasing the OASTL mRNA and enzyme level, the cysteine synthesis machinery within the transformed plants seems to be able to supply the required precursor for GSH synthesis and therefore for PC synthesis. Although the level of O-acetylserine(thiol)lyase gene expression and, therefore, cysteine biosynthesis in transformed plants enhances the capacity of plants for toxic metal sequestration, which may be useful for phytoremediation of heavy metal-contaminated environments. Overexpression of PC synthase in yeast leads to increased tolerance to heavy metals, but no data have been reported about tolerance in plants (22, 23). It will be interesting to determine whether manipulation of the PC synthase in Arabidopsis is sufficient to support metal tolerance or whether its precursor, cysteine, is the limiting step in the process. However, the limiting step could be different between species; thus in B. juncea GSH biosynthesis and not cysteine availability seems to be the limiting step, although an increase in OASTL expression is also observed on cadmium treatment (20). Overexpression of γ-glutamylcysteine synthetase in this species enhances cadmium tolerance to the same level (24) as that reported here. To conclude, in Arabidopsis, cysteine availability seems to be one of the limiting steps for GSH and, therefore, for PC biosynthesis.

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**Fig. 6.** Glutathione content in wild-type and transformed PbIOAS line 10 leaves upon cadmium treatment. Mature plants were incubated in the absence (−Cd) or the presence of 250 μM CdCl$_2$ (+Cd) for 18 h, and leaves were collected for GSH determination. Error bars represent the standard deviations. The symbol * indicates significant differences ($p<0.05$) from data of the same plant in absence of Cd.