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An *Arabidopsis* mutant *atcsr-2* exhibits high cadmium stress sensitivity involved in the restriction of H_2S emission*

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Abstract: The gene *AtCSR* encodes peptidyl-prolyl cis/trans isomerases (PPIases) that accelerate energetically unfavorable cis/trans isomerization of the peptide bond preceding proline production. In our studies, we found that *AtCSR* was associated with cadmium (Cd)-sensitive response in *Arabidopsis*. Our results show that *AtCSR* expression was triggered by Cd-stress in wild type *Arabidopsis*. The expression of some genes responsible for Cd^{2+} transportation into vacuoles was induced, and the expression of the iron-regulated transporter 1 (*IRT1*) related to Cd^{2+} absorption from the environment was not induced in wild type with Cd^{2+} treatment. The expression of Cd-transportation related genes was not in response to Cd-stress, whereas *IRT* expression increased dramatically in *atcsr-2* with Cd^{2+} treatment. The expression of glutathione 1 (*GSH1*) was consistent with GSH being much lower in *atcsr-2* in comparison with the wild type with Cd^{2+} treatment. Additionally, malondialdehyde (MDA), hydrogen peroxide, and Cd^{2+} contents, and activities of some antioxidative enzymes, differed between the wild type and *atcsr-2*. Hydrogen sulfide (H_2S) has been confirmed as the third gas-transmitter over recent years. The findings revealed that the expression pattern of H_2S-releasing related genes and that of Cd-induced chelation and transportation genes matched well in the wild type and *atcsr-2*, and H_2S could regulate the expression of the Cd-induced genes and alleviate Cd-triggered toxicity. Finally, one possible suggestion was given: down-regulation of *atcsr-2*, depending on H_2S gas-transmitter not only weakened Cd^{2+} chelation, but also reduced Cd^{2+} transportation into vacuoles, as well as enhancing the Cd^{2+} assimilation, thus rendering *atcsr-2* mutant sensitive to Cd-stress.

Key words: *AtCSR*, H_2S gas-transmitter, Glutathione, Cadmium stress sensitivity
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1 Introduction

Cadmium (Cd^{2+}) is a heavy metal that exerts a detrimental effect on plants. It can be taken up by roots and transferred into aerial organs, where it accumulates preferentially in leaves (Salt *et al*., 1995). Cd^{2+} exposure in vivo or in vitro also leads to cellular energy disturbances due to limiting mitochondrial function (Byczkowski and Sorenson, 1984; Dorta *et al*., 2003; Sokolova, 2004) and increased oxidative stress (Valko *et al*., 2005; Cherkasov *et al*., 2007). Cd^{2+} also affects gene expression in multiple cellular pathways (Marie *et al*., 2006; Sanni *et al*., 2008). Recently, several studies have focused on plant response mechanisms to Cd^{2+}. Plants may develop a host of mechanisms to protect themselves from Cd toxicity. One mechanism, detoxification, may transfer cytoplasmic Cd^{2+} into the vacuole, and thus lessen

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toxic action. Multiple genes have been reported to respond to Cd stress through this process. The structure, function, and biosynthesis of phytochelatins (PC) have been extensively reported (Ruegsegger *et al*., 1990; Steffens, 1990). PC synthesized from the substrate glutathione (GSH) (Thangavel *et al*., 2007) can integrate with cytoplasmic Cd^{2+} and are both transferred into the vacuole (Clemens, 2001). Additionally, GSH can also facilitate Cd^{2+} sequestration into vacuoles (Guo *et al*., 2008). The two adenosine triphosphate (ATP)-dependent enzymes, γ-glutamylcysteine synthetase (GSH1) and glutathione synthetase (GSH2), catalyze GSH synthesis (Yadav, 2010). ATP-binding cassette (ABC) transporters may mediate transportation of glutamine synthetase-conjugated Cd^{2+} into the mitochondria with Cd^{2+} treatment (Li *et al*., 1997). Multidrug resistance-associated protein 3 (MRP3) is an ABC transporter, and expression of *MRP3* increases after Cd^{2+} treatment (Bovet *et al*., 2003; Zientara *et al*., 2009). Plants over-expressing iron-regulated transporter 1 (*IRT1*) also accumulate higher concentrations of Cd and zinc (Zn) in comparison with wild type under iron (Fe)-deficient conditions, showing an additional role in transporting these metals (Connolly *et al*., 2002). Several *Arabidopsis thaliana* cation exchangers (CAXs) encode tonoplast-localized transporters that appear to be major contributors to vacuolar sequestration of Cd^{2+}. *CAX2* and *CAX4* had high transportation and selection of Cd^{2+} in tonoplast vesicles (Koren′kov *et al*., 2007). It is well known that Cd-stress can generate cellular oxidative stress responses, and may explain cellular toxicity (Romero-Puertas *et al*., 2004). Hydrogen peroxide (H_2O_2) accumulation and lipid peroxidation can indicate oxidative stress caused by Cd^{2+} exposure in plant cells (Wang *et al*., 2011). Plants have developed effective defense systems against H_2O_2 accumulation, which include limiting H_2O_2 formation and development, and H_2O_2 removal under conditions of environmental stress. Antioxidative enzymes, such as catalase (CAT), peroxidases (POD), and ascorbate peroxidase (APX) play a key role in those processes. CAT and POD function in detoxification of H_2O_2, thereby preventing the formation of ·OH radicals (Sandalio *et al*., 2001). APX is an important component of the ascorbate-glutathione cycle responsible for the removal of H_2O_2 in plants (Jimenez *et al*., 1997).

Nitric oxide (NO) and carbon monoxide as two major gas-transmitters are well known and much studied in animals and plants. NO is reportedly beneficial for Cd toxicity in *Arabidopsis* by up-regulating genes related to Fe uptake and promoting Cd^{2+} accumulation in roots (Besson-Bard *et al*., 2009). Hydrogen sulfide (H_2S), regarded as the third gas-transmitter, has been studied in animals, but there are few related reports in plants. It has been illustrated that L-cysteine desulfhydrase (LCD) could catalyze the decomposition of cysteine to pyruvate, ammonia and H_2S (Bloem *et al*., 2004). In addition, D-cysteine desulfhydrase (*DCD*) encodes a DCD that decomposes D-cysteine into H_2S (Riemenschneider *et al*., 2005). In this paper, we screened a Cd-sensitive *Arabidopsis* mutant, identified the mutational site and found a new gene involved in Cd-sensitive response. We further studied the reason for Cd-sensitivity of *atcsr-2*.

2 Materials and methods

2.1 Plant materials and growth conditions

Arabidopsis (Col-0) seeds were surface sterilized by 75% ethanol for 45 s and 5% (50 g/L) sodium hypochlorite (NaClO) for 7 min under sterile conditions, and washed three times with sterile water. The plates were stored for 3 d in darkness at 4 °C. They were then grown on 1/2 Murashige and Skoog (MS) media containing 0.7% (7 g/L) agar and 1% (10 g/L) sucrose under 23 °C, 100 μmol/(m^2·s) light intensity and standard long-day conditions (i.e., 16 h/8 h of light/dark).

2.2 Cd-stress, exogenous H_2S treatment

For Cd-stress treatment, *atcsr-2* seeds were first surface sterilized, then scattered on 1/2 MS media containing 90 μmol/L cadmium chloride ($CdCl_2$) solution which was added into 1/2 MS media before solidifying. After 3 d in darkness at 4 °C, seeds were incubated at 23 °C, with 100 μmol/(m^2·s) light intensity and standard long-day conditions; wild type seeds were treated similarly to the controls. For exogenous H_2S treatment, disinfected seeds, after 3 d in darkness at 4 °C, were preincubated for 12 d on 1/2 MS media under 23 °C, 100 μmol/(m^2·s) light intensity, and standard long-day conditions; under sterile condition,

closed culture dishes were opened before being transferred carefully into closed containers to make seedlings in an airtight atmosphere. Lastly, solutions with 10, 30, or 50 μmol/L sodium hydrosulfide (NaHS) were introduced into the closed containers.

2.3 MDA, H_2O_2, GSH, Cd^{2+} contents, antioxidase activity, and H_2S production rate assays

Malondialdehyde (MDA) content was determined according to published method of Heath and Packer (1968). A total weight of 0.3 g of 12-d-old seedlings were grinded with 0.05 mol/L phosphate buffer (pH 7.8), and diluted with buffer to 3 ml; the solution was centrifuged at 5000×*g* for 5 min under 4 °C, and the supernatant was used to determine MDA content. The detection of H_2O_2 was carried out using 3,3′-diaminobenzidine (DAB) (Sigma, MO, USA) (Thordal-Christensen *et al*., 1997). The 12-d-old seedlings were soaked by 1 mg/L DAB for 1 h in dark conditions, and transferred into light conditions for 12 h; after flushing with water three times, leaves were faded by 95% ethanol in order to locate H_2O_2. GSH content was assayed using a GSH assay kit (Nanjing Jicheng Bioengineering Institute, China). CAT, POD, and APX activities were assayed according to published methods (Chance and Maehly, 1955; Gupta *et al*., 1993). The extraction buffer for the CAT assay contained 100 mmol/L phosphate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1% (10 g/L) polyvinylpolypyrrolidone (PVPP), and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) (pH 7.8), and the assay mixture 50 mmol/L phosphate and 11.6 mmol/L H_2O_2 (pH 6.9). The extraction buffer for POD contained 100 mmol/L phosphate, 1 mmol/L PMSF, 1% (10 g/L) PVPP, and 1 mmol/L EDTA (pH 7.8), and the assay mixture 50 mmol/L phosphate, 10 mmol/L guaiacol, and 100 mmol/L H_2O_2 (pH 6.4). The extraction buffer for APX contained 50 mmol/L phosphate, 0.5 mmol/L ascorbate, 1% (10 g/L) PVPP, and 0.1 mmol/L EDTA (pH 7.0), and the assay mixture 50 mmol/L phosphate, 0.5 mmol/L ascorbate, 0.1 mmol/L EDTA, and 0.1 mmol/L H_2O_2 (pH 7.0). Cd^{2+} content was estimated by atomic absorption spectrophotometer (Perkin-Elmer, Foster City, CA, USA). A total weight of 1 g of dry seedlings nitrified by concentrated nitric acid was used for assay Cd^{2+} content. Determination of H_2S production rate was referred to published measure (Jin *et al*., 2011). Twelve-day-old seedlings were used to extract total protein.

2.4 Analysis of transcript levels

The total RNA was extracted from 12-d-old seedlings. The complementary DNA (cDNA) was synthesized using a reverse transcription system kit (TaKaRa, Japan) and oligo(dT) primers. The cDNA products were standardized for reverse transcription polymerase chain reaction (RT-PCR) using *β-actin* as the internal control. The sequence-specific primers were designed as follows. *AtCSR*: 5′-AACT GCAGATGTCGGCAAGACCTGAAGGAAGTC-3′, 5′-CCCAAGCTTTCAATCGATCACTTTGGTCCTT AGA-3′; *GSH1*: 5′-GGGTCAGTTCGAGCTTAGTG G-3′, 5′-GTGCGGTCCTTGTCAGTGTCT-3′; *GSH2*: 5′-TTCCCTCACATCATCTCCATA-3′, 5′-AAGCAG TCGCAGTGGTTTATT-3′; *MRP3*: 5′-TACAATCT TCCCGACACTATCTC-3′, 5′-ATCACCGAATGAA AGTATCTCCA-3′; *IRT3*: 5′-CAGCTAGAAGATC CACGAGTGCC-3′, 5′-CCCGCAAATGATGTTACC TTACC-3′; *CAX1*: 5′-CGAAGAAACAGATGGCA ATAATGAA-3′, 5′-TGATGACGATGTGGAGCAA GA-3′; *CAX2*: 5′-GACCCATCATCCATCCAATC A-3′, 5′-CCCTGTCAAGGTTCAGCAGTT-3′; *CAX4*: 5′-GGTTTCGGTTGAGGGTGAGTT-3′, 5′-CCGTG ATTGGAATGTGGAGTG-3′; *CAX11*: 5′-ACAAGA ACAAAGCAGCAATCA-3′, 5′-AATGGCGGCGT CATCAACTAT-3′; heavy metal ATPases (*HMA2*): 5′-CATTGGATGGCTTGACTTGCT-3′, 5′-GATTGC GTGGTTGCGAAGATG-3′; *HMA4*: 5′-CTCACAGT TTCCATCCACCAC-3′, 5′-CGGCTTACTTCTCCT CCTATC-3′; *LCD*: 5′-CATGCCATGGCAATGGA GGCGGGAGAGCGGCGCAATG-3′, 5′-CGGGAT CCCTACAATGCAGGAAGGTTTTGACAAG-3′; *DCD*: 5′-CATGCCATGGCAATGAGAGGACGAA GCTTGACACTCTC-3′, 5′-CGGGATCCCTAGAAC ATTTTCCCAACACCATCTT-3′.

3 Results

3.1 Transfer (T)-DNA insertion site identification

In previous studies, our laboratory established the *Arabidopsis* mutant pool using ethyl methanesulfonate mutagenic methods. In the selfed progenies, we screened a Cd-sensitive mutant. Tail-PCR results show that the mutant site was located in *At2g36130*.

To validate that *AtCSR* was related to Cd-sensitive stress response, we obtained *atcsr-2* (SALK_051078C). *AtCSR* encodes peptidyl-prolyl cis/trans isomerases (PPIases), which are enzymes that energetically accelerate unfavorable cis/trans isomerization of the peptide bond preceding proline production (Kiefhaber *et al*., 1990; Hunter, 1998). PPIases include four structurally distinct subfamilies: cyclophilins, FK506-binding proteins, parvulins, and PP2A phosphatase activator (Lu *et al*., 2007). We further detected the transcription expression level of *AtCSR* and, in contrast to wild type, it was significantly lower in *atcsr-2* (Fig. 1).

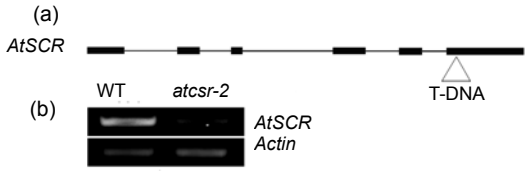

Fig. 1 Isolation of T-DNA insertional mutant

(a) Schematic representation of T-DNA insertion site in the knock-down mutant. The thick lines and thin lines represent exons and introns, respectively. The position of the T-DNA insertion in *atcsr-2* is indicated by a triangle (not to scale). (b) RT-PCR analysis of expression of *AtCSR* transcripts in wild type (WT) and *atcsr-2*. Expression of *actin* was analyzed as a loading control

3.2 Cd-sensitivity of *atcsr-2*

To examine the degree of Cd-sensitivity, the root length was determined in the wild type and *atcsr-2*: this was about 2.2 cm in the wild type and 1.1 cm in *atcsr-2* (Fig. 2). Furthermore, to genetically characterize *atcsr-2*, F_1 progenies from crosses between the wild type and *atcsr-2* were generated. All F_1 plants did not display Cd-sensitive phenotypes. In the F_2 generation, plants exhibited a 1:3 segregation ratio of mutant to the wild type phenotypes and all plants showing the mutant phenotype were *atcsr-2* homozygous.

3.3 More Cd^{2+} accumulation and less GSH generation by *atcsr-2*

GSH also plays a critical role in detoxification of heavy metals because of its capacity to chelate heavy metals and act as a substrate of PC. To contrast differences in GSH content, we measured levels in the wild type and *atcsr-2* following Cd treatment; and found levels were lower in the latter (Fig. 3a).

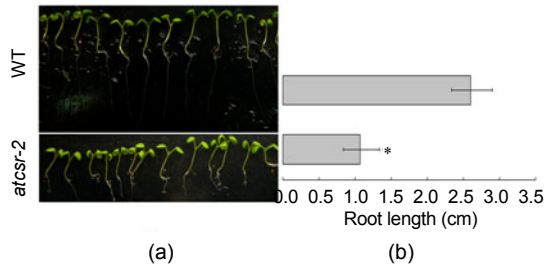

Fig. 2 Identification of Cd-sensitive phenotype of *atcsr-2*

The seeds were scattered on 1/2 MS with 90 μmol/L $CdCl_2$ for 12 d. (a) Representative images of both wild type (WT) and *atcsr-2* seedlings; (b) Root length of WT and *atcsr-2*. Values are expressed as mean±standard error (SE) (n=10), * P<0.05

Additionally, Cd^{2+} content was examined, and, as expected *atcsr-2* accumulated much more Cd^{2+} in plant cells (Fig. 3b).

3.4 Higher oxidative stress and lower antioxidative ability in *atcsr-2*

MDA is regarded as an indicator of lipid peroxidation caused by oxidative stress (Cho and Seo, 2005). MDA content and H_2O_2 accumulation were monitored to show Cd-induced oxidative stress in *atcsr-2* and the wild type—they were both higher in *atcsr-2* (Figs. 3c and 3d). H_2O_2 scavenging is accomplished by various antioxidant enzymes, and thus the activities of the major enzymes involved in H_2O_2 metabolism in seedlings exposed to Cd^{2+} treatment were determined, and found to be lower in *atcsr-2* compared to the wild type (Fig. 3e).

3.5 Expression detection of Cd-response related genes in the wild type and *atcsr-2* with or without Cd^{2+} treatment

To investigate the role of *AtCSR* in responding to Cd-stress, we determined the *AtCSR* expression in the wild type and mutant with or without Cd^{2+} treatment. The *AtCSR* expression dramatically increased with Cd^{2+} treatment in the wild type; however, it sustained a relatively low level with Cd^{2+} treatment in *atcsr-2* (Fig. 4a). To further determine the role of *AtCSR* in Cd^{2+} chelation, we focused on expressions of *GSH1* and *GSH2*. With Cd-stress, *GSH1* expression increased in the wild type; however, it was not induced in *atcsr-2* (Fig. 4b). Differing from *GSH1*, the *GSH2* expression was not regulated by Cd^{2+} treatment. Similarly, expressions of some genes involved in

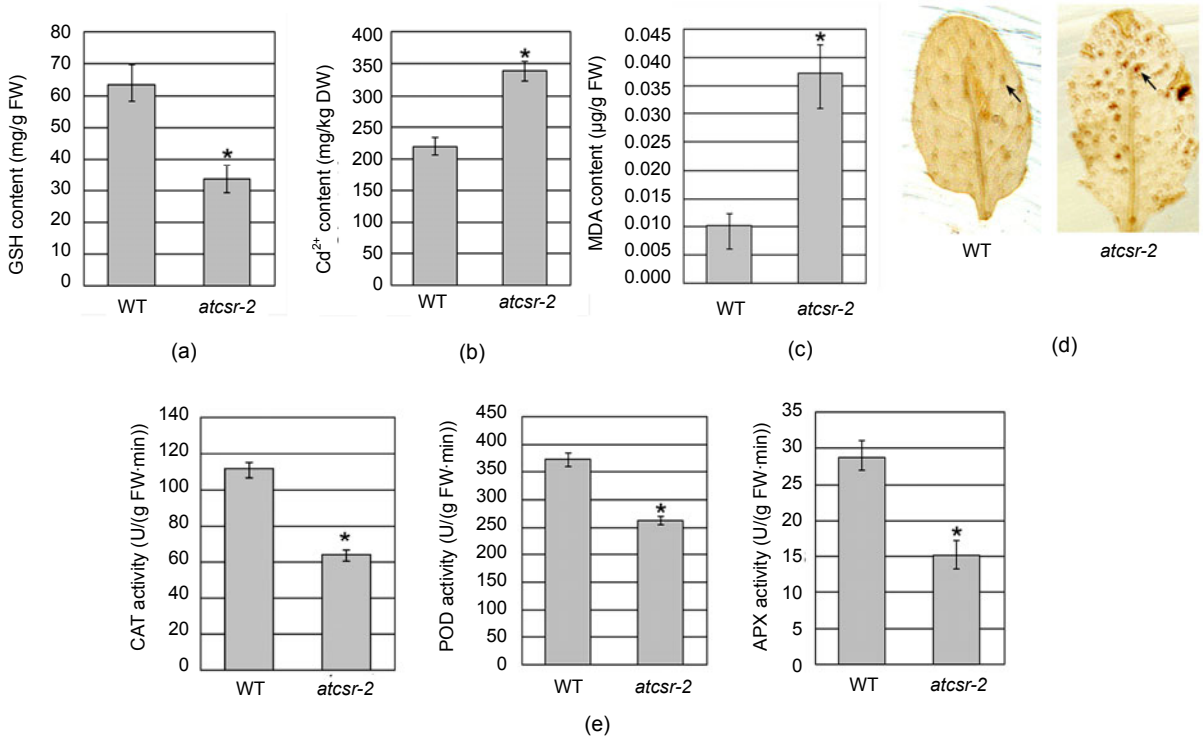

Fig. 3 Determination of MDA, H_2O_2, GSH, and Cd^{2+} contents and antioxidative enzyme activities

Seeds of wild type (WT) and *atcsr-2* were scattered on 1/2 MS with 30 μmol/L $CdCl_2$ for 12 d; (a) GSH content. (b) Total Cd^{2+} content; (c) MDA content; (d) H_2O_2 accumulation (reddish-brown polymer representing the H_2O_2 distribution is shown by full arrows); (e) Antioxidative enzyme activities (Values are means±SE (n=3), $^*P$<0.05). FW: fresh weight; DW: dry weight; GSH: glutathione; MDA: malondialdehyde; CAT: catalase; POD: peroxidases; APX: ascorbate peroxidase

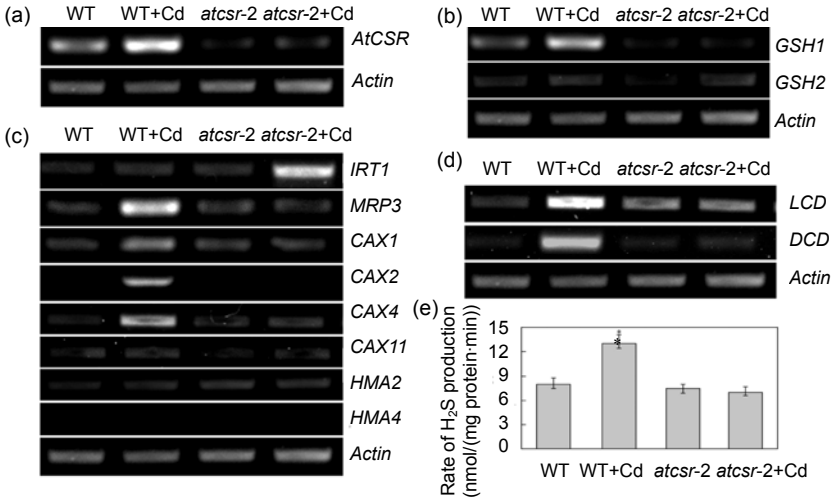

Fig. 4 Expression analyses of Cd-response and H_2S-emission related genes in wild type (WT) and *atcsr-2*

Seeds of WT and *atcsr-2* were scattered on 1/2 MS with 30 μmol/L $CdCl_2$ or without $CdCl_2$ treatment for 12 d, and then seedlings were used in this experiment. (a) Expression analysis of *AtCSR*; (b) Expression analysis of GSH-synthesis related genes; (c) Expression analysis of Cd^{2+}-transportation related genes; (d) Expression analysis of H_2S-emission related genes (*actin* was used as a loading control); (e) Determination of H_2S production rate (Values are expressed as mean±SE (n=3), $^*P$<0.05)

Cd^{2+} transportation were studied in the wild type and mutant with or without Cd^{2+} treatment. The expressions of *MRP3*, *CAX1*, *CAX2*, and *CAX4* were intensely up-regulated, whereas there were no strong inductions in *atcsr-2*. *IRT1* expression did not change in the wild type with or without Cd-stress, but greatly increased in *atcsr-2* with Cd-stress. The expression levels of *CAX11* and *HMA2* were not induced by Cd-stress and *HM4* was not detected; these gene expression levels and trends were the same in *atcsr-2* as in the wild type (Fig. 4c).

3.6 Expression analysis of genes associated with H_2S release and H_2S production rate assay in the wild type and *atcsr-2* with or without Cd^{2+} treatment

Our laboratory is studying the function of H_2S as the third gas-transmitter in a variety of physiological actions. Specifically, our attention has focused on some genes encoding cysteine desulfydrase that are related to H_2S emission in plants. To determine whether H_2S is related to Cd-stress response in *Arabidopsis*, the expressions of H_2S-releasing related genes were determined in the wild type and *atcsr-2* with or without Cd^{2+} treatment. The expression pattern of genes related to H_2S release was the same as that of genes induced by Cd-stress. The expressions of *LCD* and *DCD* markedly increased with Cd^{2+} treatment in the wild type, but were not triggered in *atcsr-2* (Fig. 4d). It has been demonstrated that LCD plays a far more important role in H_2S release (Jin *et al*., 2011). We assayed H_2S production rate using LCD as substrate, and founded that H_2S production rates in the wide type with Cd-stress increased dramatically in comparison to the wide type without Cd-stress, while in *atcsr-2* there was no increase with Cd-stress (Fig. 4e).

3.7 Effects of H_2S on expression of Cd-induced genes and Cd-triggered toxicity

Previous studies demonstrated that Cd-induced genes sufficiently matched with genes involved in H_2S generation in the wild type and *atcsr-2*. We proposed that H_2S as a gas-transmitter regulated the expression of Cd-induced genes, and to test this hypothesis we treated *Arabidopsis* with different H_2S physiological concentrations and monitored expression of Cd-induced genes. The expressions of *GSH1*, *MRP3*, *CAX1*, *CAX2*, and *CAX4* were all up-regulated and *IRT1* was down-regulated by different H_2S physiological concentrations (Fig. 5). Since H_2S could regulate the expression of Cd-chelation and Cd-transportation related genes, we examined whether it could alleviate the Cd-triggered toxicity. The root length of *atcsr-2* with H_2S fumigation was longer than that of *atcsr-2* without H_2S fumigation under conditions of Cd stress, but was shorter than that of *atcsr-2* without Cd stress (Fig. 6).

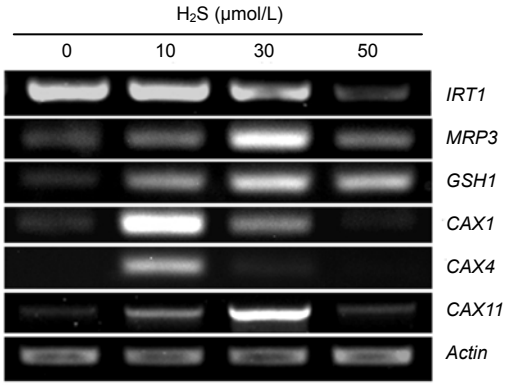

Fig. 5 Effect of H_2S on the expression of GSH-synthesis and Cd-transportation related genes

Wild type seeds were scattered on 1/2 MS for 12 d, and then fumigated with different concentrations of H_2S (10, 30, or 50 μmol/L) for 5 h. The seedlings were then used for RT-PCR analysis of gene expression

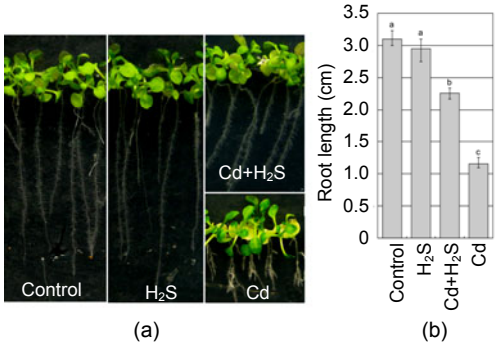

Fig. 6 Alleviation of Cd-triggered toxicity in *atcsr-2* by H_2S fumigation

atcsr-2 seeds were scattered on 1/2 MS for 12 d with or without 30 μmol/L $CdCl_2$, and then fumigated or not with 30 μmol/L H_2S for 4 d. (a) Representative images of *atcsr-2* seedlings with H_2S fumigation alone and with both Cd-stress and H_2S fumigation as well as with Cd-stress alone. The *atcsr-2* was cultivated with neither $CdCl_2$ nor H_2S fumigation as control group. (b) Root length corresponded to the seedlings of Fig. 6a. Values are expressed as mean±SE (n=10). Values with different letters indicate significant differences (P<0.05) among them

4 Discussion

PC can chelate heavy metals and then PC-metal complexes are transported across the tonoplast into vacuoles (Cobbett, 2000). PC can be synthesized from reduced GSH by the enzyme PC synthase through a transpeptidation reaction (Rea *et al*., 2004). GSH plays a vital role in protecting plants against Cd-triggered toxicity by providing a sequestration system (Sanità di Toppi and Gabbrielli, 1999). *GSH1* reportedly acts as a major control point under conditions of increasing demand for GSH (Yadav, 2010). In the present study, Cd^{2+} treatment did not trigger expression of *GSH1* in *atcsr-2*, in contrast to the wild type. The expression of *GSH2* was not induced by Cd-stress in the wild type and *atcsr-2* (Fig. 4b). Therefore, the synthesis of GSH was significantly restricted in *atcsr-2* (Fig. 3a). We propose that one reason for the Cd-sensitive phenotype of *atcsr-2* may be the decreased GSH content. We further studied the expression levels of *MRP3*, *CAX1*, *CAX2*, and *CAX4*, which are involved in Cd^{2+} transportation. All were sharply elevated in the wild type with Cd-stress compared to *atcsr-2* (Fig. 4c). This indicated that the increased expression levels of *MRP3*, *CAX1*, *CAX2*, and *CAX4* in the wild type were probably due to demand for plant cells to transport Cd^{2+} into the vacuole, thus protecting plants from Cd^{2+} toxicity. However, inducible expression of these genes by Cd-stress as for the wild type was not observed in *atcsr-2*. We hypothesized that dysfunctional Cd^{2+} transportation to the vacuole may be another aspect of the Cd-sensitive phenotype of *atcsr-2*. Additionally, *IRT* is one Fe transporter that could transfer environmental Cd^{2+} into plant cells. *IRT1* expression was induced by Cd-stress in *atcsr-2* (Fig. 4c). Consequently, *atcsr-2* transported more Cd^{2+} into the cells, accumulating superfluous Cd^{2+} in cytoplasm. This may be the third reason for the Cd-sensitive phenotype of *atcsr-2*. All of the discussed possibilities for the Cd-sensitive phenotype of *atcsr-2* could result in mounting accumulation of dissociated Cd^{2+} in cytoplasm, which could lead to increased MDA and H_2O_2, and inhibition of antioxidative enzymes' activities (Gonçalves *et al*., 2009), as demonstrated in *atcsr-2* (Figs. 3c–3e).

The expression pattern of most H_2S-release related genes (including *LCD* and *DCD*) was very consistent with that of Cd-chelation and Cd-transportation related genes both in the wild type and *atcsr-2* (Figs. 4c and 4d). This suggests that genes involved in H_2S release may participate in the process of Cd-stress response in *Arabidopsis*. The expression of *LCD* and *DCD* induced by Cd-stress was not found in *atcsr-2* (Fig. 4d). Similarly, the expression of genes related to Cd detoxification and transportation was not detected in *atcsr-2* (Fig. 4c). Other results indicated that H_2S functions in regulating the expression of genes associated with Cd^{2+} chelation and transportation (Fig. 5), and alleviates the Cd-triggered toxicity (Fig. 6). Together the results suggest that *AtCSR* expression affects the emission of H_2S, which as a gas-transmitter could regulate expression of a host of genes involved in Cd^{2+} chelation and transportation. The possible reason of Cd-sensitive phenotype of *atcsr-2* was due to down-regulation of *AtCSR* expression, which could suppress the expression of H_2S-emission related genes and, as a result, H_2S production decreased. Finally, the decreased H_2S emission possibly led to increased assimilation of Cd^{2+} from the environment, and weakened Cd^{2+} chelation and transportation. Thus the normal functions of Cd^{2+} chelation and transportation in *atcsr-2* were all destroyed. At the posttranscriptional level, the underlying mechanism of how the PPIase impacts the emission rate of H_2S remains unclear. We propose that PPIase could energetically accelerate isomerization of protein related to H_2S emission, promoting H_2S-release protein folding accurately, and as a result, H_2S gas-transmitters could be triggered rapidly.

References

Besson-Bard, A., Gravot, A., Richaud, P., Auroy, P., Duc, C., Gaymard, F., Taconnat, L., Renou, J.P., Pugin, A., Wendehenne, D., 2009. NO contributes to cadmium toxicity in arabidopsis by promoting cadmium accumulation in roots and by up-regulating genes related to iron uptake. *Plant Physiol*., **149**(3):1302-1315. [doi:10.1104/pp.108.133348]

Bloem, E., Riemenschneider, A., Volker, J., Papenbrock, J., Schmidt, A., Salac, I., Haneklaus, S., Schnug, E., 2004. Sulphur supply and infection with *Pyrenopeziza brassicae* influence L-cysteine desulphydrase activity in *Brassica* napus L. *J. Exp. Bot*., **55**(406):2305-2312. [doi:10.1093/jxb/erh236]

Bovet, L., Eggmann, T., Meylan-Bettex, M., Polier, J., Kammer, P., Marin, E., Feller, U., Martinoia, E., 2003. Transcript levels of *AtMRPs* after cadmium treatment:

induction of *AtMRP3*. *Plant Cell Environ*., **26**(3):371-381. [doi:10.1046/j.1365-3040.2003.00968.x]

Byczkowski, J.Z., Sorenson, J.R., 1984. Effects of metal compounds on mitochondrial function: a review. *Sci*. *Total Environ*., **37**(2-3):133-162. [doi:10.1016/0048-9697(84)90091-3]

Chance, B., Maehly, A.C., 1955. Assay of catalase and peroxidases. *Methods Enzymol*., **1**(2):764-775. [doi:10.1016/S0076-6879(55)02300-8]

Cherkasov, A.A., Overton, R.A.Jr., Sokolov, E.P., Sokolova, I.M., 2007. Temperature-dependent effects of cadmium and purine nucleotides on mitochondrial aconitase from a marine ectotherm, *Crassostrea virginica*: a role of temperature in oxidative stress and allosteric enzyme regulation. *J. Exp*. *Biol*., **210**(1):46-55. [doi:10.1242/jeb.02589]

Cho, U., Seo, N., 2005. Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. *Plant Sci*., **168**(1):113-120. [doi:10.1016/j.plantsci.2004.07.021]

Clemens, S., 2001. Molecular mechanisms of plant metal tolerance and homeostasis. *Planta*, **212**(4):475-486. [doi:10.1007/s004250000458]

Cobbett, C.S., 2000. Phytochelatins and their roles in heavy metal detoxification. *Plant Physiol*., **123**(3):825-832. [doi:10.1104/pp.123.3.825]

Connolly, E.L., Fett, J.P., Guerinot, M.L., 2002. Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. *Plant Cell*, **14**(6):1347-1357. [doi:10.1105/tpc.001263]

Dorta, D.J., Leite, S., Demarco, K.C., Prado, I.M., Rodrigues, T., Mingatto, F.E., Uyemura, S.A., Santos, A.C., Curti, C., 2003. A proposed sequence of events for cadmium-induced mitochondrial impairment. *J. Inorg*. *Biochem*., **97**(3):251-257. [doi:10.1016/S0162-0134(03)00314-3]

Gonçalves, J.F., Tabaldi, L.A., Cargnelutti, D., Pereira, L.B., Maldaner, J., Becker, A.G., Rossato, L.V., Rauber, R., Bagatini, M.D., Bisognin, D.A., *et al*., 2009. Cadmium-induced oxidative stress in two potato cultivars. *Biometals*, **22**(5):779-792. [doi:10.1007/s10534-009-9225-4]

Guo, J.B., Dai, X., Xu, W.Z., Ma, M., 2008. Over-expressing *GSH1* and *AsPCS1* simultaneously increases the tolerance and accumulation of cadmium and arsenic in *Arabidopsis thaliana*. *Chemosphere*, **72**(7):1020-1026. [doi:10.1016/j.chemosphere.2008.04.018]

Gupta, A.S., Webb, R.P., Holaday, A.S., Allen, R.D., 1993. Over-expression of superoxide dismutase protects plants from oxidative stress (induction of ascorbate peroxidase in superoxide dismutase-over-expressing plants). *Plant Physiol*., **103**(4):1067-1073. [doi:10.1104/pp.103.4.1067]

Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplast: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch*. *Biochem*. *Biophys*., **125**(1):189-198. [doi:10.1016/0003-9861(68)90654-1]

Hunter, T., 1998. Prolyl isomerases and nuclear function. *Cell*, **92**(2):141-143. [doi:10.1016/S0092-8674(00)80906-X]

Jimenez, A., Hernandez, J.A., Del Rio, L.A., Sevilla, F., 1997. Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiol*., **114**(1):275-284. [doi:10.1104/pp.114.1.275]

Jin, Z., Shen, J., Qiao, Z., Yang, G., Wang, R., Pei, Y., 2011. Hydrogen sulfide improves drought resistance in *Arabidopsis thaliana*. *Biochem*. *Biophys*. *Res*. *Commun*., **414**(3):481-486. [doi:10.1016/j.bbrc.2011.09.090]

Kiefhaber, T., Quaas, R., Hahn, U., Schmid, F.X., 1990. Folding of ribonuclease $T_{1.2}$. Kinetic models for the folding and unfolding reactions. *Biochemistry*, **29**(12):3061-3070. [doi:10.1021/bi00464a024]

Koren′kov, V., Park, S., Cheng, N.H., Sreevidya, C., Lachmansingh, J., Morris, J., Hirschi, K., Wagner, G.J., 2007. Enhanced Cd^{2+}-selective root-tonoplast-transport in tobaccos expressing Arabidopsis cation exchangers. *Planta*, **225**(2):403-411. [doi:10.1007/s00425-006-0352-7]

Li, Z.S., Lu, Y.P., Zhen, R.G., Szczypka, M., Thiele, D.J., Rea, P.A., 1997. A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis (glutathionato) cadmium. *PNAS*, **94**(1):42-47. [doi:10.1073/pnas.94.1.42]

Lu, K.P., Finn, G., Lee, T.H., Nicholson, L.K., 2007. Prolyl cis-trans isomerization as a molecular timer. *Nat*. *Chem*. *Biol*., **3**(10):619-629. [doi:10.1038/nchembio.2007.35]

Marie, V., Gonzalez, P., Baudrimont, M., Boutet, I., Moraga, D., Bourdineaud, J.P., Boudou, A., 2006. Metallothionein gene expression and protein levels in triploid and diploid oysters *Crassostrea gigas* after exposure to cadmium and zinc. *Environ*. *Toxicol*. *Chem*., **25**(2):412-418. [doi:10.1897/05-114R.1]

Rea, P.A., Vatamaniuk, O.K., Rigden, D.J., 2004. Weeds, worms, and more: papain's long-lost cousin, phytochelatin synthase. *Plant Physiol*., **136**(1):2463-2474. [doi:10.1104/pp.104.048579]

Riemenschneider, A., Wegele, R., Schmidt, A., Papenbrock, J., 2005. Isolation and characterization of a D-cysteine desulfhydrase protein from *Arabidopsis thaliana*. *FEBS J*., **272**(5):1291-1304. [doi:10.1111/j.1742-4658.2005.04567.x]

Romero-Puertas, M.C., Rodriguez-Serrano, M., Corpas, F.J., Gómez, M., del Río, L.A., Sandalio, L.M., 2004. Cadmium-induced subcellular accumulation of $O_2^{\cdot-}$ and H_2O_2 in pea leaves. *Plant Cell Environ*., **27**(9):1122-1134. [doi:10.1111/j.1365-3040.2004.01217.x]

Ruegsegger, A., Schmutz, D., Brunold, C., 1990. Regulation of glutathione synthesis by cadmium in *Pisum sativum* L. *Plant Physiol*., **93**(4):1579-1584. [doi:10.1104/pp.93.4.1579]

Salt, D.E., Prince, R.C., Pickering, I.J., Raskin, I., 1995. Mechanisms of cadmium mobility and accumulation in Indian mustard. *Plant Physiol*., **109**(4):1427-1433. [doi:10.1104/pp.109.4.1427]

Sandalio, L.M., Dalurzo, H.C., Gómez, M., Romero-Puertas, M.C., del Río, L.A., 2001. Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J. Exp*. *Bot*., **52**(364):2115-2126. [doi:10.1093/jexbot/52.364.2115]

Sanità di Toppi, L., Gabbrielli, R., 1999. Response to cadmium

in higher plants. *Environ. Exp. Bot.*, **41**(2):105-130. [doi:10.1016/S0098-8472(98)00058-6]

Sanni, B., Williams, K., Sokolov, E.P., Sokolova, I.M., 2008. Effects of acclimation temperature and cadmium exposure on mitochondrial aconitase and LON protease from a model marine ectotherm. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, **147**(1):101-112. [doi:10.1016/j.cbpc.2007.08.005]

Sokolova, I.M., 2004. Cadmium effects on mitochondrial function are enhanced by elevated temperatures in a marine poikilotherm, *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). *J. Exp. Biol.*, **207**(15):2639-2648. [doi:10.1242/jeb.01054]

Steffens, J.C., 1990. The heavy-metal binding peptides of plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **41**(1):553-575. [doi:10.1146/annurev.pp.41.060190.003005]

Thangavel, P., Long, S., Minocha, R., 2007. Changes in phytochelatins and their biosynthetic intermediates in red spruce (*Picea rubens* Sarg.) cell suspension cultures under cadmium and zinc stress. *Plant Cell Tiss. Org. Cult.*, **88**(2):201-216. [doi:10.1007/s11240-006-9192-1]

Thordal-Christensen, H., Zhang, Z., Wei, Y., Collinge, D.B., 1997. Subcellular localization of H_2O_2 in plants, H_2O_2 accumulation in papillae and hypersensitive response during barley-powdery mildew interaction. *Plant J.*, **11**(6):1187-1194. [doi:10.1046/j.1365-313X.1997.11061187.x]

Valko, M., Morris, H., Cronin, M.T.D., 2005. Metals, toxicity and oxidative stress. *Curr. Med. Chem.*, **12**(10):1161-1208. [doi:10.2174/0929867053764635]

Wang, Y., Qian, Y., Hu, H., Xu, Y., Zhang, H., 2011. Comparative proteomic analysis of Cd-responsive proteins in wheat roots. *Acta Physiol. Plant*, **33**(2):349-357. [doi:10.1007/s11738-010-0554-2]

Yadav, S.K., 2010. Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *South Afr. J. Bot.*, **76**(2):167-179. [doi:10.1016/j.sajb.2009.10.007]

Zientara, K., Wawrzyńska, A., Lukomska, J., López-Moya, J.R., Liszewska, F., Assunção, A.G., Aarts, M.G., Sirko, A., 2009. Activity of the *AtMRP3* promoter in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* plants is increased by cadmium, nickel, arsenic, cobalt and lead but not by zinc and iron. *J. Biotechnol.*, **139**(3):258-263. [doi:10.1016/j.jbiotec.2008.12.001]