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Plant Science 169 (2005) 1059–1065
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Coordinated responses of phytochelatins and metallothioneins to heavy metals in garlic seedlings

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Received 26 February 2005; received in revised form 22 June 2005; accepted 11 July 2005
Available online 8 August 2005

Abstract

To evaluate the roles of phytochelatins (PCs) and metallothioneins (MTs) in heavy metal tolerance in garlic (*Allium sativum* L.), the cDNAs encoding a phytochelatin synthase and a type 2 MT were cloned from the seedlings of garlic using RACE method and designated *AsPCS1* and *AsMT2a*. Semi-quantitative reverse transcriptase-PCR and PCs contents showed transcript levels of *AsPCS1* in roots exposed to cadmium increased significantly within 1 h concomitant with a sharp increase of PCs. However, the RNA expression of *AsMT2a* in roots was slightly increased within the preliminary phases of cadmium treatment and an obvious increase did not occurred until 10 h of Cd exposure. Different effects on RNA expression of *AsPCS1* and *AsMT2a* appeared under varied stresses. Treatment with cadmium, arsenic and heat shock resulted in a strong increase of *AsPCS1* transcripts and PCs contents in roots. Theses results showed that *AsPCS1* and *AsMT2a* displayed mutual coordination under the stresses. The implications of these results with respect to differential regulation of *AsPCS1* and *AsMT2a* during heavy metal exposure are discussed.

Keywords: Phytochelatin; Metallothionein; Heavy metal tolerance; Cadmium; Transcriptional regulation; *Allium sativum*

1. Introduction

Cells must be able to regulate the intracellular concentration of metal ions, since excess concentration of both essential and non-essential metal ions can cause growth inhibition or even death of the organism. Plants, like all living organisms, have evolved a variety of mechanisms to control and respond to uptake and accumulation of heavy metals. One of the major defense mechanisms involves the formation of proteinaceous complexes that chelate and sequestrate heavy metals by particular ligands. The two best-characterized heavy metal-binding ligands in plant cells are phytochelatins (PCs) and metallothioneins (MTs) [1]. PCs are cysteine (Cys)-rich peptides that chelate heavy metals, including Cd, Cu, Zn, on the included thiol moieties, and enzymatically synthesized from glutathione (GSH) by phytochelatin synthase (PC synthase). PCs have a general structure (γ-GluCys)*n*-Gly ($n = 2$–11) and are rapidly induced in plants by heavy metal treatment [1,2]. The acid–labile sulfide stabilized Cd–PC complexes and improves the efficiency of Cd sequestering [3]. Previous studies indicated that PC synthase was expressed constitutively and that the levels of this enzyme in plants were generally unaffected by exposure to Cd [4]. However, RT-PCR analysis of *TaPCS1* expression on wheat roots suggested that the mRNA of *TaPCS1* was induced on exposure to Cd [5]. MTs also are Cys-rich polypeptides, however, in contrast to PCs, they are products of mRNA translation. MTs typically contain two metal-binding and cysteine-rich domains that give these metalloproteins a

Abbreviations: PCs, phytochelatins; MTs, metallothioneins; Cys, cysteine; GSH, glutathione; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; AOS, active oxygen species; γ-EC, γ-glutamylcysteine

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doi:10.1016/j.plantsci.2005.07.010

dumb-bell conformation. Most of these MT proteins were classified into two types according to the deduced locations of the Cys residues as suggested by Robinson [6]. Heavy metals such as Cu and Cd treatment greatly induced the activities of *MT* gene in *Festuca rubra* and *Arabidopsis* [7,8]. Recently, Merrifield et al. [9] firstly reported that trivalent arsenic bound to MT from the seaweed *Fucus vesiculosus* and provided comparatively direct evidence to heavy metal detoxification of MT proteins.

Both MTs and PCs were found in plants [10–12], animals [13] and yeast [14]. Yu et al. [14] have assessed the relationship of yeast MTs and PCs in heavy metal ion buffering, and found that sequestration of metal ions by MTs appears to be the dominant metal detoxification pathway if expression of *MT* occurs, and that PCs function only under the conditions of the absence of MTs or inability to express *MT* genes. However, little is yet known on the relationship of PCs and MTs in plants in metal ion detoxification. Recently we found that garlic has strong tolerance to Cd stress and most Cd has been shown to accumulate in roots of garlic, where Cd might be bound and sequestrated by one or more ligands such as MTs and PCs. Thus, it was of our primary interest to analyze the expression of MTs and PCs formation in garlic seedlings in response to Cd and other multiple stresses. In this study, the cDNAs encoding a PC synthase (*AsPCS1*) and a type 2 MT (*AsMT2a*) were cloned from the seedlings of garlic. The transcript levels of *AsPCS1* and *AsMT2a* and the PCs contents were determined in roots and leaves of garlic seedlings after exposure to Cd and other stresses.

2. Materials and methods

2.1. Plant material

Seedlings were developed from cloves of garlic (*Allium sativum* L.) on a holder in polyethylene pots with 2000 ml aerated deionized water. The experiment was carried out under greenhouse conditions. For metal exposure and other stresses, 200 μM $CdCl_2$, 200 μM Na_3AsO_4, 50 μM $CuSO_4$ and 50 mM NaCl were added from concentrated stock solutions. For heat shock, some of plants were exposed to 40 °C for 45 min after the eighth day of culture. Seedlings were harvested at each sample date for analysis. After the different incubation times tissue samples for isolation of total RNA were shock-frozen in liquid nitrogen, and stored at −70 °C.

2.2. Determination of GSH and PCs contents

Plant material frozen in liquid nitrogen was extracted in 5% sulfosalicylic acid, 6 mM diethylenetriamine-pentaacetic acid as described in Ref. [15]. The homogenate was centrifuged at 10,000 × *g*, 4 °C for 10 min to remove cellular debris and precipitated proteins. Total non-protein thiols in the supernatant were then quantitated spectrophotometrically with Ellman's reagent at 412 nm by the method of De Knecht [15]. GSH determination was by HPLC on a reversed-phase column. An aliquot of the supernatant was loaded onto C_{18} reversed-phase HPLC column equilibrated with 0.06% TFA (v/v) at 37 °C and GSH was eluted over 20 min with a linear gradient of 0–5% acetonitrile in 0.06% TFA and then over 20 min 5% acetonitrile in 0.06% TFA. Standards were used for the identification of GSH. Total PCs concentration expressed as GSH equivalents was estimated from the difference between total non-protein thiols and GSH.

2.3. Cloning of the cDNA of AsMT2a

Total RNA from roots of Cd-stressed garlic seedlings was isolated using the Trizol reagent protocol (Invitrogen, Karlsruhe, Germany). Polyadenylated mRNAs were obtained using Oligotex mRNA Spin-Column (Clontech). The first-strand cDNA was prepared using Smart™ RACE cDNA amplification kit (Clontech) from 1 μg of RNA. *MT* genes are very conservative in the C- and N-terminal of all kinds of organisms. One primer was designed according to the C-terminal conservative domains of the *MT* cDNA from the monocotyledons published in GenBank. This primer 5′-ATGTCTTGCAGCTGCGGATCA-3′ and RACE 3′ CDS primer were used for amplifying the 3′ fragment. The product of PCR was cloned into pGEM-T Easy Vector (Promega) and sequenced. To isolate the full-length cDNA of *AsMT2a*, a gene specific primer 5′-CATAATGAAAT-GAAACTA-3 was synthesized according to the 3′ fragment and used for the 5′-RACE reactions together with the RACE Smart II primer. The sequence analysis and blast result showed that cloned cDNA has high homology with the type 2 MT genes from plants. Thus, it was designated *AsMT2a* (GenBank accession no. AY821676).

2.4. Cloning of the cDNA of AsPCS1

The cDNA of PC synthase of garlic has cloned previously in our laboratory. The details can be seen in Ref. [16].

2.5. RT-PCR analysis

RT-PCR analysis was performed according to the method described by Okamoto et al. [17]. Total RNA was extracted using the Trizol reagent protocol. The cDNA was synthesized from 2 μg of total RNA that was treated by Dnase I (Promega) before reverse transcription with the AMV reverse transcriptase (TaKaRa). For RT-PCR analysis, 5 μl single-stranded cDNA from each sample was added to PCR buffers, deoxynucleoside triphosphates and enzymes. The mixture was then split into three equal aliquots. The sequence-specific primers for *AsPCS1* (5′-ATGGCGCTTGCGGGACTTTATC-3′ and 5′-CAGTTCCAGTCTGACCGAAAGC-3′), *AsMT2a* (5′-CGGGGACAGATCATCA-3′ and 5′-CATAATGAAAT-GAAACTA-3′) or actin (5′-GCTCCAAGGGCAGTGTTTC-3′ and 5′-GATAGGTCCGACACGAAAGG-3′) designed

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                    *         20          *         40
AsMT2a  :  MSCCGGNCGCGSSCKCGNGCGGCKMYGDVG-EERSSMTTTSI  :  41
OsMT2   :  MSCCGGNCGCGSGCQCGSGCGGCKMYPEMA-EEVTTT-QTVI  :  40
TaMT2   :  MSCCGGNCGCGSGCKCGNGCGGCKMYPEMD-EGVNTTSQTLI  :  41
AtMT2a  :  MSCCGGSCGCGSACKCGNGCGGCKRYPDL----ENTATETLV  :  38
AtMT2b  :  MSCCGGSCGCGSACKCGNGCGGCKRYPDL----ENTATETLV  :  38
BjMT2   :  MSCCGGNCGCGSGCKCVG-CGGCKMYPDLSFSGETTTTETLV  :  41
NtMT2   :  MSCCGGSCGCGSGCKCGSGCGGCGMYPDL----ENTTTFTII  :  38
PsMT2   :  MSCCGGNCGCGTSCKCGSGCGGCKMYADLS-YTEATSSETLI  :  41
           MSCCGG CGCG3 C C   CGGC  Y  6      3   T 6

                    *         60          *         80
AsMT2a  :  LGVAPQR---SFEEFENEMIAEGGNACKCGSNCTCNPCTCK-  :  79
OsMT2   :  MGVAPSKGHAEGLEAGAAAGAGAENGCKCGDNCTCNPCNCGK  :  82
TaMT2   :  MGVAPSK-----GTP-SFEAAAENGGCKCGPNCSCNPCTCK-  :  76
AtMT2a  :  LGVAPAMNS--QYEASGETFVAENDACKCGSDCKCNPCTCK-  :  77
AtMT2b  :  LGVAPAMNS--QYEASGETFVAENDACKCGSDCKCNPCTCK-  :  77
BjMT2   :  LGVAPAMNS--QFEASGETFVAENDACKCGSDCKCNPCTCK-  :  80
NtMT2   :  EGVAPMKN----YEGSAEKATEGGNGCKCGANCKCDPCNC--  :  74
PsMT2   :  MGVGSEK-----TQFESAEMGAENDGCKCGANCTCNPCTCK-  :  77
            GV                      CKCG 1C C1PC C
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Fig. 1. Allignment of the deduced *AsMT2a* amino acids with other plant MT protein sequences. The protein sequences are deduced from the cDNA sequences of *Allium sativum* (As), *Oryza sativa* (Os), *Triticum aestivum* (Ta), *Arabidopsis thaliana* (At), *Brassica juncea* (Bj), *Nicotiana tabacum* (Nt) and *Pisum sativum* (Ps).

according to the published cDNA (*AsPCS1*, GenBank accession no. AF384110), the cloned full-length cDNA (*AsMT2a*) and the partial cDNA (*actin*) (GenBank accession no. AY821677) were added; and all three tubes were subjected to the same thermal cycler program. RT-PCR products were quantified by densitometric analysis of ethidium bromide-stained bands (Alphaimager). Three replicates of each sample were quantitated.

3. Results

3.1. Protein sequence deduced from AsMT2a cDNA

For the analysis of differential responses of *AsPCS1* and *AsMT2a* of garlic seedlings to Cd and other stresses, the full-length cDNA of a type 2 MT, named *AsMT2a*, was cloned from garlic seedlings and its nucleotide sequence was determined. The deduced protein sequence indicated *AsMT2a* presented the characteristic structure of most plants MTs, i.e. two Cys-rich domains separated by a long, Cys-free spacer domain (Fig. 1). *AsMT2a* cDNA contained 240 bp ORF encoding 79 amino acids with predicted molecular mass of 8020 Da. The deduced amino acids of *AsMT2a* showed 60–61% homology to the type-2 MT sequences from *Triticum aestivum* (TaMT2), *Arabidopsis thaliana* (AtMT2a, AtMT2b), *Nicotiana tabacum* (NtMT2), *Pisum sativum* (PsMT2) and 57% to that from *Brassica juncea* (BjMT2) and *Oryza sativa* (OsMT2). The number and positions of the Cys residues in the N- and C-terminal domains were completely conserved with those in other plant type-2 MT proteins (Fig. 1).

3.2. Changes in levels of phytochelatins, GSH and transcriptional expressions of AsPCS1 and AsMT2a in Cd-stressed garlic seedlings

To compare the temporal sequence of PCs formation in Cd-stressed garlic seedlings, garlic seedlings were exposed to 200 μM Cd and harvested at 1, 3, 10, 24 and 48 h of Cd

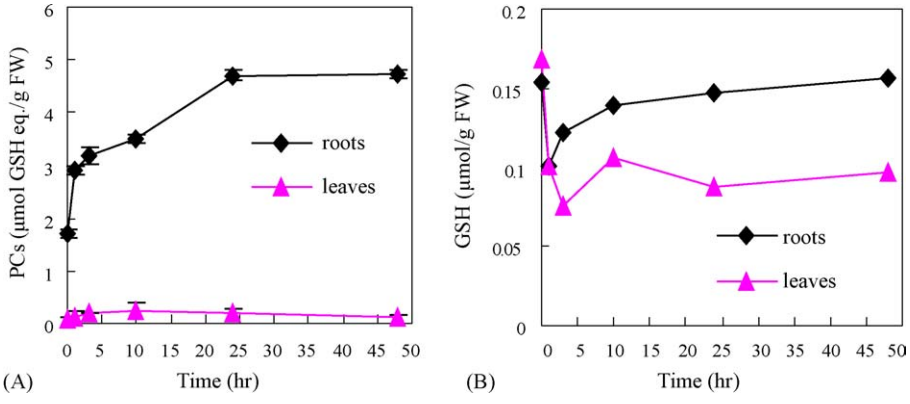

Fig. 2. PCs (A) and GSH (B) levels in roots and leaves of garlic seedlings in response to Cd exposure. Eight-day-old garlic plants were treated with 200 μM $CdCl_2$ for 1, 3, 10, 24 and 48 h. Each value of PCs represents the average of three experiments.

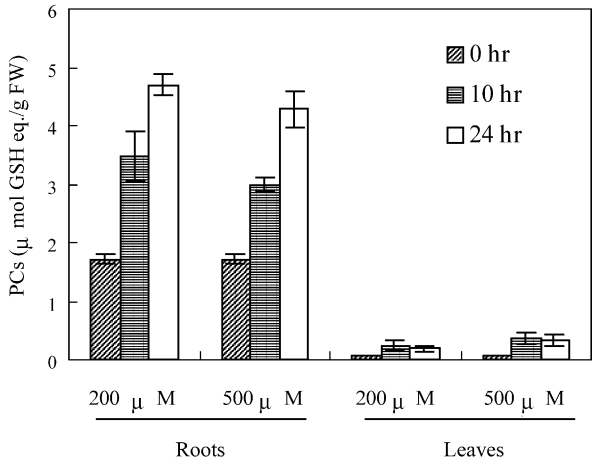

Fig. 3. PCs levels in roots and leaves of garlic seedlings in response to different concentrations of Cd. Eight-day-old garlic plants were treated for 10 or 24 h with 200 and 500 μM $CdCl_2$. Each value of PCs represented the average of three experiments (±S.D.).

treatment. PCs contents of roots were much higher than those of leaves under both normal conditions and 200 μM Cd stresses (Fig. 2). Upon exposure to Cd, PCs contents were increased with the increasing of incubation time in roots, while the strongest increase was detected within 1 h and the maximal yield of PCs was observed at 24 h. PCs contents plateaued between 24 and 48 h. PCs contents in leaves almost had no change, compared with roots. GSH contents were initially dramatically suppressed within 1–3 h of Cd exposure in roots and in leaves respectively, after that time, GSH contents gradually recovered to 57% of the control (in leaves) or even to the extent of the control (in roots) at the end of the experiment. Dose response of garlic seedlings to Cd are shown in Fig. 3. Higher concentrations (500 μM) of Cd failed to cause significant accumulations of PCs in roots and only slightly increased PCs contents in leaves.

To understand the molecular mechanisms of garlic seedlings to conditions of heavy metal stress, first of all, we compared the effects of Cd on the expression levels of *AsPCS1* and *AsMT2a* in roots and leaves. Fig. 4 shows the kinetics and dose response of the transcript levels of these two genes in response to Cd stress. A maximum increase of threefold of *AsPCS1* expression levels in roots was observed after 1 h exposure to Cd, subsequently its expression levels dropped but were still much higher than that of untreated roots and maintained at a steady-state level. However, although an obvious decrease of *AsPCS1* expression at transcriptional level in leaves was detected within 3 h of Cd stress, gradual increase of transcript levels was found after that time and 72% increase occurred at the end of the experiment compared with the control. In response to Cd, *AsMT2a* exhibited an expression pattern distinct from *AsPCS1*. Its transcript levels in roots were increased very slightly within 10 h of Cd treatment and evident increase occurred after that time. *AsMT2a* expression in leaves also presented the similar tendency as in roots with the increasing of incubation time. Dose–response analysis, in

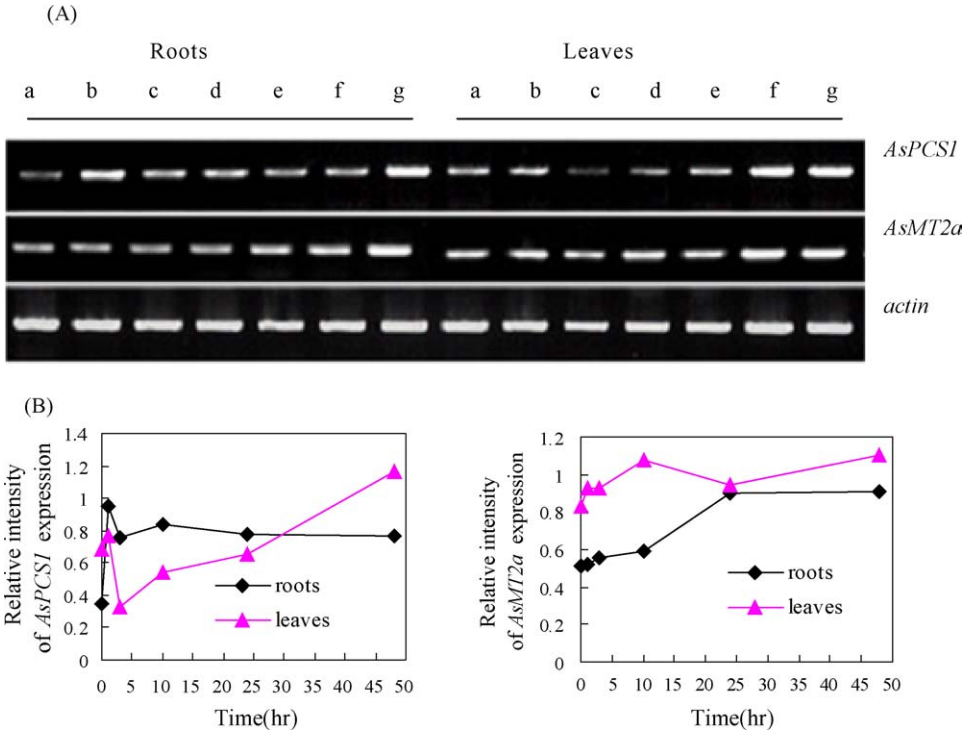

Fig. 4. Comparative RT-PCR analysis of *AsPCS1* and *AsMT2a* expression in roots and leaves of garlic seedlings in response to Cd exposure. (A) Transcript levels of *AsPCS1* and *AsMT2a* in roots and leaves from hydroponically grown plants treated for 0 h (a), 1 h (b), 3 h (c), 10 h (d), 24 h (e) and 48 h (f) with 200 μM $CdCl_2$ and 24 h (g) with 500 μM $CdCl_2$. (B) Relative amount of the normalized expression of *AsPCS1* (left) and *AsMT2a* (right) in roots and leaves of garlic seedlings in response to Cd exposure. The expression level of *actin* was used as a positive internal control.

which the cultures were exposed to 200 and 500 μM Cd for 24 h, revealed a trend that the increases in the expression levels of these two genes in both roots and leaves were dependent upon the Cd concentrations, with higher Cd concentrations causing larger increases in the expression levels of *AsPCS1* and *AsMT2a*.

3.3. Changes in levels of phytochelatins and transcriptional expressions of AsPCS1 and AsMT2a in heavy metal-, salt- and heat-stressed garlic seedlings

To determine whether the variation in PCs formation and transcriptional expression for these two genes was a general stress response or specific for stress elements, we challenged garlic seedlings with a range of different heavy metals (Cd, As and Cu) and other stresses (salt and heat shock) and subsequently analyzed PCs contents and transcript levels for these two genes. As shown in Fig. 5, both Cd and As had the effects on PCs induction in roots, whereas the former, the most stimulating ion, presented five-fold inductive activity over the latter. However, Cu stress and salt stress failed to induce the accumulation of PCs in roots. All the stresses elicited PCs formation in leaves but the PCs contents in leaves was far lower than that in roots. It is notable that heat shock led to significant induction of PCs in both roots and leaves.

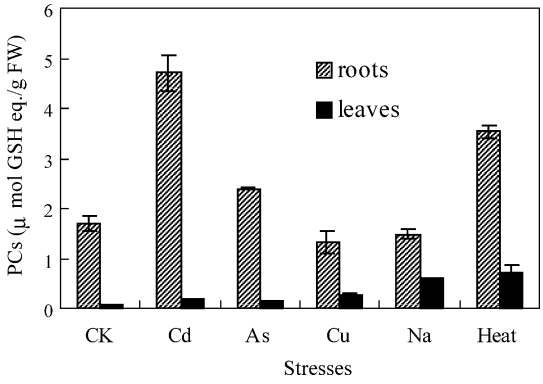

Fig. 5. PCs levels in roots and leaves of garlic seedlings in response to multiple stresses. Eight-day-old garlic plants were treated for 24 h with 200 μM $CdCl_2$, 200 μM Na_3AsO_4, 50 μM $CuSO_4$ and 50 mM NaCl. For heat shock, plants were exposed to 40 °C for 45 min. Each value of PCs represented the average of three experiments (±S.D.).

The responses of *AsPCS1* and *AsMT2a* genes to various stresses were shown in Fig. 6. The transcript levels of these two genes in roots were significantly increased only by Cd, As and heat shock, but not Cu and salt stress. Copper induced the obvious accumulation of *AsPCS1* mRNA in leaves, however, the elevated expression levels of *AsMT2a* in leaves were observed under all stresses.

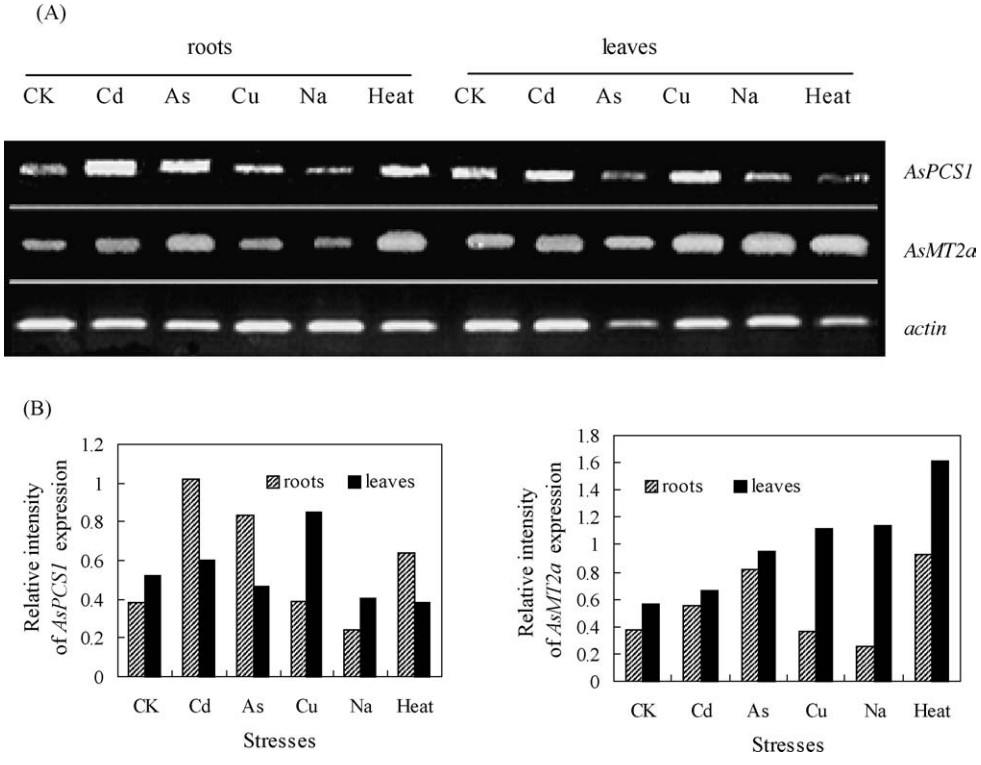

Fig. 6. Comparative RT-PCR analysis of *AsPCS1* and *AsMT2a* expression in roots and leaves of garlic seedlings in response to multiple stresses. Eight-day-old garlic plants were treated for 24 h with 200 μM $CdCl_2$, 200 μM Na_3AsO_4, 50 μM $CuSO_4$ and 50 mM NaCl. For heat shock, plants were exposed to 40 °C for 45 min. (A) Transcript levels of *AsPCS1* and *AsMT2a* in roots and leaves from hydroponically grown plants. (B) Relative amount of the normalized expression of *AsPCS1* (left) and *AsMT2a* (right) in roots and leaves of garlic seedlings in response to Cd exposure. The expression levels of *actin* was used as a positive internal control.

4. Discussion

A number of plant species have been shown to respond to heavy metals by activating PC synthase and thus increase PCs contents. Although PC synthase activity was increased in the presence of Cd, its expression was thought to be constitutive in plants [4]. But in another study, the wheat PC synthase gene *TaPCS1* was regulated by Cd at the transcriptional level [5]. Lee and Korban [18] reported that this transcriptional regulation of *Arabidopsis* PC synthase gene only occurred during the early stage of plant development, and as plants grow older this regulation disappeared. The results in this study were in agreement with the conclusion that PC synthase was regulated by heavy metals at the transcriptional level (Fig. 4). In addition, the temporal sequence of the transcriptional levels of *AsPCS1* indicated that *AsPCS1* might play a pivotal role in immediate detoxification of Cd (Fig. 4). Approximately two-fold induction of PCs contents in Cd-stressed roots within 1 h is consistent with its role in the instant effects in Cd detoxification. It suggested that PCs were predominant chelators of Cd in the early stages of Cd treatment (Fig. 2A). Slower accumulation of PCs in roots after 1 h of Cd exposure may result from self-regulation of *AsPCS1* activity by Cd and even maximal yield of PCs were obtained at 24 h of Cd treatment. *AsMT2a* also exhibited transcriptional regulation by heavy metals (Fig. 4) as reported earlier [19], however its expressional levels were obviously increased at the later stage (after 10 h of Cd treatment) in roots. It may be suggested that *AsMT2a* and *AsPCS1* could coordinately function in different phases of Cd treatment. *AsMT2a* might confer the long and persistent Cd tolerance on garlic seedlings, i.e. once an excess of Cd accumulation in roots over the chelating capacity of PCs happens, MTs synthesis is largely increased. This finding is inconsistent with the results from yeast [14]. Constitutive expression of the *Saccharomyces cerevisiae MT* (*CUP1*) gene inhibited the accumulation of metal-phytochelatin complexes in both *Candida glabrata* and *S. pomb* and PC complexes appear to function in metal detoxification in cells when *MT* genes are not present or well expressed [14].

MTs have been implicated to play a role in Cu tolerance in plants. For example, *MT* gene expression levels were correlated with Cu tolerance in 10 ecotypes of *Arabidopsis* [10]. Overexpression of pea *MT* gene in *Arabidopsis* enhanced the leve1 of copper accumulation [20]. Surprisingly, in garlic seedlings exposure to Cu could not lead to obvious induction of *AsMT2a* mRNA in roots (Fig. 6). Significant decrease of *MT2* mRNA was also observed in shoot and root tissues of *Brassica juncea* seedlings after treatment with 200 μM $CuSO_4$ [21]. At the same time, no distinct increase of *AsPCS1* mRNA and PCs contents in roots was also observed in response to Cu stress (Figs. 5 and 6). These results suggested that *AsMT2a* and *AsPCS1* genes and PCs did not make contributions to Cu tolerance in roots of garlic seedling and there might exist some other mechanisms to coordinate the uptake and accumulation of Cu ions in roots. For example, other *MT* genes can work well in this respect as *mcMT1* (type 1 *MT*) found in *Festuca rubra* cv. Merlin, which was induced significantly at the transcriptional level in roots exposed to 315 μM Cu (a four-fold increase) [7]. In addition, organic acids and amino acids are thought to be the effective chelator of heavy metals [22]. Otherwise, it was clear that the expression levels of *AsMT2a* and *AsPCS1* in Cu-stressed leaves, besides the elevated contents of PCs, were far higher than that in untreated ones (Figs. 5 and 6). It was deduced that PCs and MTs accumulated in leaves upon exposure to Cu stress may not be used to detoxify excess Cu since much higher concentration of Cu accumulated in roots than in leaves (data not shown). Therefore, these two peptides may play other important roles in leaves including essential heavy metal homeostasis, sulphur metabolism or as anti-oxidants. Recently it has been documented that both PCs and MTs peptides are potent scavengers for reactive oxygen species such as hydroxyl radicals in wild watermelon and a marine alga, *Dunalliela tertiolecta* [23,24]. Actually, the phytotoxicity of heavy metals is considered to increase the amount of active oxygen species (AOS) in plant tissues [25], leading to development of secondary oxidative stress. Thus PCs and MTs accumulated in leaves can protect photosynthesis metabolism from oxidative stress.

One (in root) and 3 h (in leaf) after Cd exposure, a pronounced reduction of GSH initially was observed (Fig. 2B). This is a common response to Cd caused by an increased consumption of GSH for PCs production [26]. With the extending of incubation time of Cd, elevated GSH contents in roots and leaves at later stages implicated that when plants were challenged to Cd, the homeostasis of GSH was perturbed and this temporary depletion was compensated by triggering a demand-driven synthesis of GSH either through alleviating the feedback inhibition of γ-EC (γ-glutamylcysteine) synthetase by GSH or through increasing sulfur uptake [27]. An alternative explanation is that the increase in GSH synthesis may be required for countering a Cd-induced oxidative stress at later stages and protecting plant cells from oxidative damage. It has been shown that GSH synthesis is regulated by oxidative stress. May and Leaver [28] have demonstrated that endogenously produced H_2O_2 increase GSH concentrations. Xiang and Oliver [27] also observed a twofold increase in cellular GSH level in samples treated with 5 mM H_2O_2 in liquid culture system.

Earlier studies showed that Cd was a most potent stimulator of PCs production, whereas some conditions such as oxidative stress, salt stress, jasmonic acid or salicylic acid treatment could not significantly induce PC synthase gene expression in *Arabidopsis thaliana* [11]. Recently it has been reported that gamma radiation increased the formation of PCs in roots of barley [29]. In our study, heat shock also resulted in obvious accumulation of PCs in both roots and leaves. These data indicated that PCs might have some other important roles outside of heavy metal detoxification as the

above-mentioned. However, the real mechanism of heat shock-induced PCs synthesis is yet unclear.

Acknowledgments

This work was supported by the National Science Foundation Council of PRC (Grant no. 30170086, 30370127), and the Special Project of Transgenic Research (JY03A2001). The authors thank Dr. Zhigang Ren (University of Newcastle, Australia) and Mr. Badis Bensid (University of Paris, Paris VI, France) for correcting the first draft of the manuscript.

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