

Characterization and Expression of a Phytochelatin Synthase Gene in Birch-leaf Pear (*Pyrus betulaeifolia* Bunge)

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Abstract Phytochelatin synthase (PCS) plays a major role in metal detoxification in plants. However, the molecular mechanisms of heavy metal detoxification remain unknown, and birch-leaf pear genes related to metal detoxification have not yet been identified. Here, we report the isolation of cDNA and genomic DNA sequences encoding a phytochelatin synthase (PCS) from birch-leaf pear (*Pyrus betulaeifolia* Bunge). The *PbPCS1* cDNA sequence contained 1,965 nucleotides of a 1,494 bp open reading frame (ORF), which encodes a 497-amino-acid protein with a molecular weight of 55.00 kDa. Its corresponding genomic DNA sequence consists of 3,820 nucleotides and eight exons separated by seven introns. The deduced amino acid sequence of *PbPCS1* contains five Cys residues (56, 90, 91, 109 and 113 amino acids) that are highly conserved in the plant PCS1 family, and 12 cysteine residues putatively involved in heavy metal binding sites, which included three adjacent Cys–Cys components (331–332, 351–352 and 369–370 amino acids) in the C-terminal variable domain. Homology analysis of the deduced *PbPCS1*

amino acid sequence revealed that it shares a high sequence similarity amongst N-terminal amino acids and low similarity with C-terminal amino acids with plant PCS1 proteins deposited with NCBI. *PbPCS1* was most related to *PCS1* from legume plants *Lotus japonicus* (GenBank accession no. AAT80342) and soybean (*Glycine max* L.; AAL78384) as they were clustered into the same clade in a neighbor-joining phylogenetic tree. Quantitative real-time PCR (qPCR) expression analysis revealed that *PbPCS1* had a very low basal expression level in untreated whole seedlings, and levels were higher in roots than in leaves and stems. After 24 h of exposure to 20 μ M CdSO₄, CuSO₄ or ZnSO₄, *PbPCS1* expression increased significantly in different organs. In addition, L-buthionine-sulfoximine (BSO) can inhibit *PbPCS1* expression in roots, stems and leaves, while L-glutathione-reduced (GSH) stimulates *PbPCS1* expression in different organs of birch-leaf pear.

Keywords Isolation · Phytochelatin synthase gene · Expression characteristic · *Pyrus betulaeifolia* Bunge

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Introduction

Plants are sessile organisms that cannot evade unfavorable habitats. Therefore, they have developed effective means to overcome stress conditions, which can include high levels of metal ions (Blum et al. 2010). Following the long-term use of improper agricultural practices, such as excessive use of phosphatic fertilizers, sludge application, and sewage irrigation, many agricultural soils in the world now suffer contamination with different heavy metals, usually Cd, Cu, Zn, Ni, Co, Cr, Pb, and As. To avoid metal ion toxicity, plants have evolved mechanisms to efficiently regulate the distribution of metal ions (Tennstedt et al. 2009). Phytochelatin

(PCs)—heavy metal binding thiolate peptides synthesized enzymatically from glutathione—play a major role in providing protection against heavy metals (Chaurasia et al. 2008). The general structure of PCs is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n varies from 2 to 11 (Ramos et al. 2008). After synthesis, PCs bind heavy metal ions and facilitate their transportation as complexes into the vacuole (Clemens 2006), where they eventually form high-molecular-weight (HMW) complexes, which is the homeostatic response mechanism to heavy metal poisoning among various plants (DalCorso et al. 2008). PCs can combine Cu^{2+} and Zn^{2+} —metals that are essential or vital processes—and transport them to the vacuole or target enzymes (Tennstedt et al. 2009). PCs also have the ability to chelate nonessential metal ions such as Cd^{2+} , thus preventing toxic metals coming into contact with enzymes or other active substances, thereby reducing heavy metal poisoning (Alberich et al. 2008).

Genes involved in the synthesis of PCs are phytochelatin synthases (PCS, EC 2.3.2.15), e.g., γ -glutamylcysteine transpeptidase (Ramos et al. 2008). PCS catalyze the net synthesis of PCs from glutathione. Induction of PCS activity by different heavy metal ions varies. Cd^{2+} is the best metal activator of the enzyme (Grill et al. 1989). Since 1999, many plant PCS genes have been isolated from different species, such as *Arabidopsis thaliana* (Ha et al. 1999; Vatamaniuk et al. 1999), *Triticum aestivum* (Clemens et al. 1999), *Brassica juncea* (Heiss et al. 2003), *Lactuca sativa* (He et al. 2005), and *Lotus japonicus* (Ramos et al. 2008). These genes have been proved to be involve in heavy metal detoxification processes.

Most heavy metals in plants stem from the soil, through root absorption, accumulation and transportation. Grafting of elite cultivars (scions) onto rootstocks is an efficient approach to improving fruit production. The heavy metal content of the scion is usually determined by the ability of the rootstock to absorb, accumulate and transport heavy metals. So the study of heavy metal detoxification mechanisms of rootstocks is major significance for fruit production. Birch-leaf pear is native to China, and is used widely as a rootstock for pear cultivation (Okubo and Sakuratani 2000). This rootstock has desirable traits such as tolerance to high humidity and drought (Kaneyoshi et al. 2001), flooding (Tamura et al. 1996) and salt stress (Okubo and Sakuratani 2000; Okubo et al. 2000; Matsumoto et al. 2006, 2007). However, information about the mechanism of heavy metal detoxification of birch-leaf pear remains limited. To determine whether PCs mediate heavy metal detoxification, PCS gene structure and expression should be investigated in birch-leaf pear. Therefore, this study was undertaken to isolate a PCS gene homologue from birch-leaf pear and investigate its expression patterns in different organs following heavy metal stress.

Materials and Methods

Plant Materials and Treatments

Mature seeds of birch-leaf pear were collected from Shankou town, Taian city, Shandong Province, China, in November 2009. The seeds were rinsed thoroughly with distilled water and laminated by sand under 4°C for 60 days. After sterilizing with NaClO at 50% (v/v) (5% active Cl_2) for 10 min, seeds were planted in pots with quartz sand as the matrix. One-quarter strength MS solution (pH 5.8), which contained 2.5 mM NH_4NO_3 , 4.7 mM KNO_3 , 0.75 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.375 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 mM KH_2PO_4 , 100 μM $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 30 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 100 μM H_3BO_3 , 5 μM KI, 1 μM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 μM CoCl_2 and 100 μM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, was adding to the pots every 3 days during seedling growth. When the fourth true leaf fully expanded, uniform plantlets were transferred to deionized water containing 20 μM cadmium sulfate (CdSO_4 , Sigma-Aldrich, St. Louis, MO), 20 μM zinc sulfate (ZnSO_4 , Sigma-Aldrich), 20 μM copper sulfate (CuSO_4 , Sigma-Aldrich), 200 μM BSO (Sigma-Aldrich), 200 μM GSH (Sigma-Aldrich), 20 μM CdSO_4 and 200 μM BSO, 20 μM CdSO_4 and 200 μM GSH, 20 μM CuSO_4 and 200 μM BSO, 20 μM CuSO_4 and 200 μM GSH, 20 μM ZnSO_4 and 200 μM BSO, or 20 μM ZnSO_4 and 200 μM GSH for 24 h. Plantlets grown in deionized water for 24 h were used as a control. The roots, stems and leaves were then collected for gene cloning and gene expression analysis, respectively. Seedlings were grown on solid or in liquid media with a 16 h day (25°C, light intensity 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$)/8 h night (20°C).

RNA Extraction, cDNA Synthesis and Gene Fragment Acquisition

Total RNA was extracted from leaf, stem and root samples using RNA plant plus Reagent (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. The template cDNA for cloning was prepared via PrimeScript® 1st Strand cDNA Synthesis Kit (TaKaRa, Kyoto, Japan). Degenerate PCR primers for the PCS genes were designed based on conserved amino acid sequences in the corresponding gene products of other plant species. The sequences of these oligonucleotides were 5'-GAAAGGGCCTTGGAGRTGG-3' (PbPCS-S1, forward primer, encoding TrpLysGlyProTrpArgTrp) and 5'-GATATDAGCACRAACCCYCT-3' (PbPCS-F1, reverse primer, encoding ArgGlyPheValLeulleSer). The reaction mixture (50 μl) contained 2 μl cDNA, 0.2 mM dNTPs, 1.5 mM MgCl_2 , 1 μM of each primer, and 2.5 U of Ex Taq® DNA polymerase (TaKaRa). Samples were heated to 94°C for 5 min and then subjected to 30 cycles (94°C

for 30 sec, 50.5°C for 30 sec and 72°C for 1 min) with a final extension at 72°C for 5 min.

Isolation of Full-length cDNAs and DNA Sequences

The nucleotide sequence of the full-length cDNA was determined by 3' rapid amplification of cDNA ends (RACE) as 3'-Full RACE Core Set Ver.2.0 instruction manual (TaKaRa) (Zhao et al. 2011) and 5' region amplification using a conserved nucleotide primer. The gene-specific primer PbPCS-3-1 for 3' RACE was 5'-TTCATCTACTGGACAACGCAGAGGGT-3'. The sequences of primers for the 5' region were 5'-ATGGCKATGGCGRGKTT RTATC-3' (PbPCS-5'-S1, forward primer) and 5'-GATCTCCAAAGGCTCGCAGCAG-3' (PbPCS-5'-F1, reverse primer). Finally, a pair of primers was designed to amplify the full-length cDNA: 5'-ATGGCGATGGCGGGTTG-3' (PbPCS-QC-S1, forward primer) and 5'-TCGCTCACATA TCACCACTATA-3' (PbPCS-QC-F1, reverse primer). PCR was performed as described above, except for the use of 0.4 μM of each primer and appropriate annealing temperatures.

Total DNA was extracted from birch-leaf pear leaves using the Sodium Pyrosulfite Method [extractive solution contains 2 M NaCl, 2% (w/v) polyvinylpyrrolidone, 200 mM Tris-HCl, 70 mM ethylenediaminetetraacetic acid, 20 mM sodium pyrosulfite]. After DNA was sedimentation, 20 μl ddH₂O and 2 μl Ribonuclease A (10 mg ml⁻¹, TaKaRa) were added at 37°C for 30 min to remove residual RNA. Gene-specific primers for DNA amplification were PbPCS-QC-S1 and PbPCS-QC-F1. Their sequences were described above. The reaction mixture (50 μl) contained 100 ng DNA, 0.2 mM dNTPs, 2.0 mM MgCl₂, 2 μM of each primer, and 2.5 U *Taq*TM DNA polymerase (TaKaRa). Samples were heated to 94°C for 5 min and then subjected to 30 cycles (94°C for 1 min, 55°C for 1 min and 72°C for 3 min) with a final extension at 72°C for 10 min.

Cloning and Sequencing of PCR Products

PCR products were separated by agarose gel electrophoresis, and the incised gels were purified using the TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa). All PCR products were cloned using the pGEM[®]-T Easy vector system (Promega, Madison, WI). The nucleotide sequence of each clone was determined using the dideoxy chain termination method with ABI3730 at Invitrogen Biotechnology Service Company (Shanghai, China).

Bioinformatics Analysis

Identification of ORFs in the nucleotide sequence and translation of the deduced amino acid sequences were completed

using BioXM 2.6. Genomic structure organization was investigated using Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn>). Homology searches of the NCBI (National Center for Biotechnology Information) databases were performed using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) with default parameters. Basic components of amino acids and typical motifs of PCS that are conserved among birch-leaf pear and other plants were analyzed via the ProtParam tool (<http://www.expasy.ch/tools/protparam.html>) and Pfam (<http://pfam.janelia.org/search>), respectively. Multiple alignments of deduced amino acid sequences were carried out DNAMAN software using default parameters (Guan et al. 2012). A phylogenetic relationship tree was then constructed using the neighbor-joining algorithm (Liu et al. 2010), implemented in Molecular Evolutionary Genetics Analysis (MEGA) 4.0 software (Tamura et al. 2007), after bootstrap re-sampling analysis with 1,000 replicates to assess branch support.

Quantitative Real-time PCR

Single-strand cDNA was prepared from 0.5 μg total RNA from each sample by reverse transcription using PrimeScript[®] RT reagent Kit (Perfect Real Time, TaKaRa). cDNA was diluted ten-fold for qPCR analysis. The *PbPCS1* primers for qPCR were 5'-AACGGACATCAAGCCAAAAAAA-3' (qPbPCS-S1, forward primer) and 5'-CAGTTAGCAGCAATTCAGCCA-3' (qPbPCS-F1, reverse primer). The birch-leaf pear actin gene was used simultaneously as a housekeeping gene as a control, with forward primer 5'-CTCCCAGGGCTGTGTTTCCTA-3' (qPbActin-S1) and reverse primer 5'-CTCCATGTCATCCAGTTGCT-3' (qPbActin-F1). The PCR mixture (25 μl total volume) comprised 12.5 μl 2×SYBR[®] Premix Ex *Taq*TM (TaKaRa), 0.5 μl of each primer (10 μM), 2 μl cDNA, and 9.5 μl dH₂O. PCR was performed on a Thermal Cycler Dice[®] Real Time System (TaKaRa). Samples were heated to 95°C for 30 sec and then subjected to 40 cycles (95°C for 5 s and 60°C for 30 s) with a melting curve analysis program. No-template blank controls were included in every reaction batch. For confirmation of amplicon presence and purity, the qPCR product was run on a 2% agarose gel, stained with ethidium bromide, and photographed. Expression levels are given as the ratio relative to the root sample without treatment, which was set as 1.

Results

Gene Isolation and Genomic Structure

To characterize the *PCS1* gene from birch-leaf pear, we first retrieved all plant *PCS1* gene sequences from NCBI

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atggcgatggcggggtgtatcgacgaatccttccctcctccggcagtcgaatttgcctccttacagggaaggtaacaaccttcagatgcagctccccaccccttactactttt 120
M A M A G L Y R R I L P S P P A U E F A S L Q G K
cttcaaaatcttcatTTTTTtaattcccccaatatttttggcgatgcttaaaatttagcattaaagcttaaaactttaattttctgtagatacccttctataatacaactgaaat 25
atgtcatcttccccaaatTTTTTgTTTgaggttaattgttctgtaagaattttgagttcagtgatgtatgatccacaattgTTTggctgccggtTTTTTgggaatgtagcaggtttgtca 360
gtaagccaagtcataattgtttgttctgtgtttttctagggagtgatgatTTTctgctgtgtgatgcttctgttctgaaccatttttctgggtactaaaatttgatggttttcgaa 480
ttcgaggagctTTTTTtgaagcattcagaggggaactatggagaactctataggttgatttcttattccagacacaatccgagcccgttattgggattggcgagcctgtccatg 600
E L F L E A I Q R G T M E N F Y R L I S Y F Q T Q S E P A Y C G R L A S L S M
gttttgatgcccctgtcattgatccggaaaggaaatggaaaggtacggtttaagttttgctcagctaataatcattgaaggtatcattctgttgaaccagctagtgatggcattttaa 720
U L N A L A I D P G R K W K G
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P W R W F D E S M L
agactgctgcgagccttggagaccgtcaaggaaaaaggcactcatttgggaagctgtgctgctggctcaactgtgctggagcaaaagtgaagcttccgcacaaatcatgcaacaat 1320
D C C E P L E T U K E K G I S F G K L U C L A H C A G A K U E A F R T N H S T I
tgatgattttcgtaaaatgatgattgagatgttctactctctgatgattgtcatgtgatcctatcagatagatcagttctcaaacagggttattactattctgctctacattttcttt 1440
D D F R K K Y U L R C T S T S D D C H U I S S Y D R S U L K Q
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tatgaatcaaatTTTctgacagacaggtactgtgctattttcccaaatgtgtgctatcatgctgaagagacatggcgttattttggatggtgcagctttaaagatcctccccatt 1680
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U P L K I L W D A M N N U D S S T G Q R R G
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F U L I
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S R P H S E P G Q L Y T L
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S C K H E S W U G U A R Y L M D D U P L L L K S R E U G L I Q E U L S
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K
tttaactcaataccaagtttttacttgcaggagggtactgagacaagtcaggacactggcctttttaaaccgctggcagaattattatcttctcagcgcattctgctgcggaact 2760
E E U L R Q U Q D T G L F K H U A E L L S S A H S C C G N L
tatattctggctatgaagaaaacttggccaacattgctgcgagcgtttgtgccaaggcgcaaaaatttggctgggaactctgcttctcgggggtgtagtctgctcaggaacatgctg 2880
Y S G H E E N L P N I A A S U C C Q G G A Q I L S G N S A F S G U Y C C Q E T C U
tgaattgcttcaaggcaaatggcgacaagcaggttggagttggagttggagttgaaatgggtggaaccgaggaagatggacatgcttcttcaaaaaaacaaatcaggat 3000
K C F K A N G D K P U T U U S G T U U N G G T E E K M D M L U P S S K T N S G C
gttctgtgcaattgggattcactcgtagcaatgagcttcaaacgacttctactggccttgcaccaggttacatggctgggctgaaagacgagaagcttcaaaagaaatatact 3120
S C A I G I H P A S N D U L T A L L L A L P P G T W S G I K D E K L S K E I S N
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L U S T A N L P T L L Q E E
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U L H L R R Q L H L L K K C Q E D R U D D D L G S
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P L A *
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Fig. 1 Nucleotide sequence of the *PbPCSI* gene (JN592477) and the deduced amino acid sequence. The amino acid sequence is shown in the single letter code below each nucleotide codon. Nucleotide and

amino acid numbers are indicated on the right. The asterisk below the nucleotide sequence indicates a stop codon

GenBank (<http://www.ncbi.nlm.nih.gov>). Sequence comparison revealed that most of the sequences are highly conserved among different plant species at the amino acid level. Primers were designed based on the conserved amino acids, and used to amplify cDNA from birch-leaf pear RNA. PCR products were sequenced, and a gene-specific primer for 3' RACE was designed according to the sequenced region. For 5' region amplification, a degenerate primer containing the start codon "ATG" and a gene-specific primer were designed according to the conserved amino acids and

the region sequenced above, respectively. After assembling fragments of the 5' and 3' regions using CAP3, we obtained a full-length *PCSI* gene, which contained 1,965 nucleotides of a 1,494 bp ORF, and 447 nucleotides of 3'-untranslated region (3' UTR) between the "TAG" stop codon and the putative terminal poly-A tail. A pair of primers (PbPCS-QC-S1 and PbPCS-QC-F1) based on the cDNA sequence was then designed for genomic DNA amplification. The full-length *PbPCSI* DNA and translated amino acid sequences are shown in Fig. 1. The DNA sequence consists of 3,820

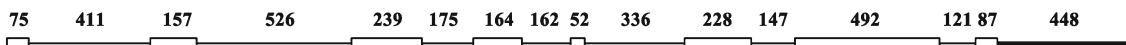


Fig. 2 Genomic organization of the *PbPCSI* gene (JN592477). White boxes exons, lines introns, thick black line untranslated region (UTR). Numbers nucleotide number

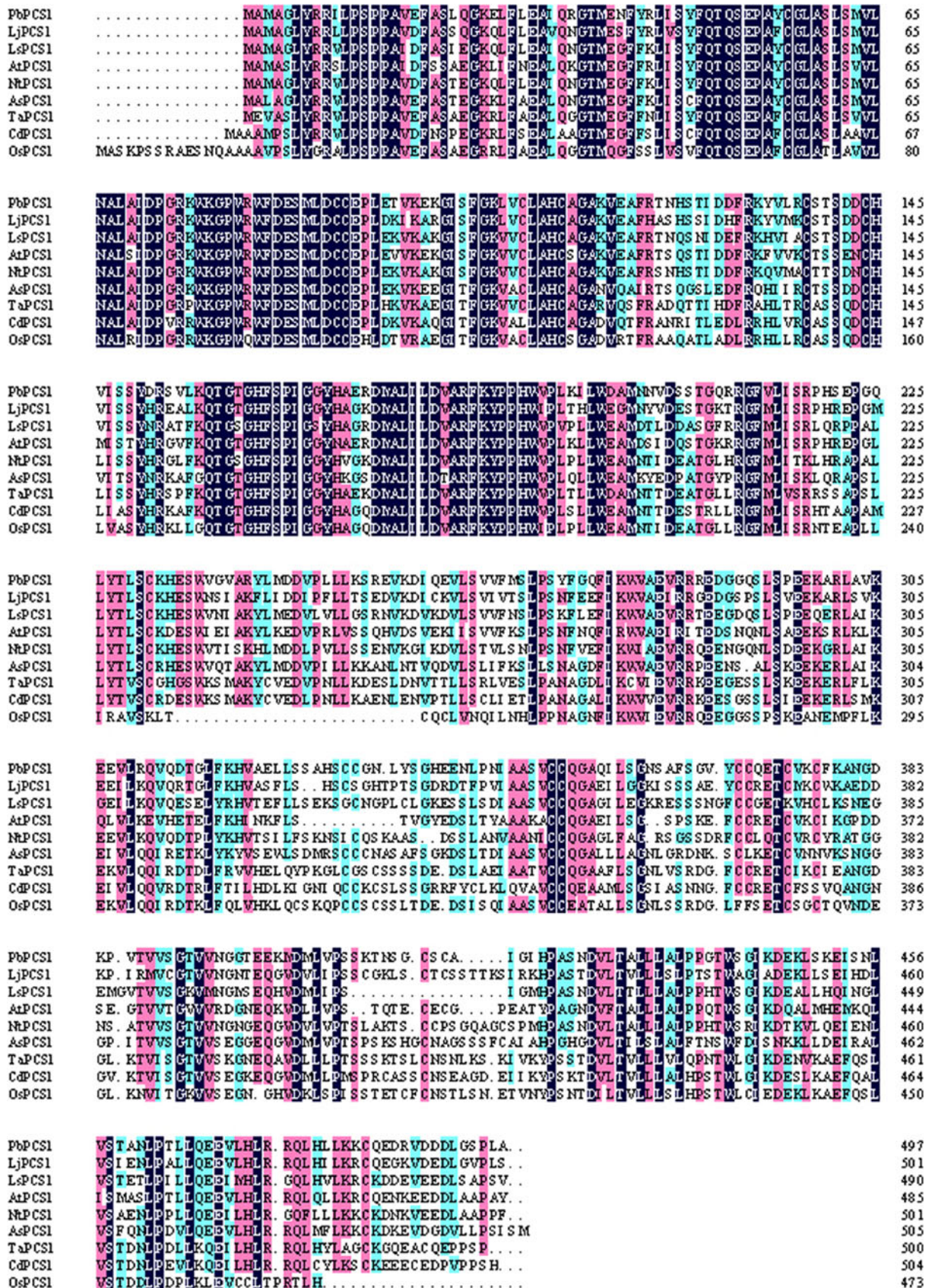


Fig. 3 Multiple alignment of amino acid sequences of the deduced protein of *PbPCS1* with PCS1 proteins from other organisms. Shaded regions identical amino acids

nucleotides, eight exons (1–75; 487–643; 1,170–1,408; 1,584–1,747; 1,910–1,961; 2,298–2,525; 2,673–3,164; 3,286–3,372 bp), and 7 introns (76–486; 644–1,169; 1,409–1,583; 1,748–1,909; 1,962–2,297; 2,526–2,672; 3,165–3,285 bp) as summarized in Fig. 2.

Deduced Protein Sequence Features

The putative translated PbPCS1 protein sequence contains 497 amino acids, has a predicted relative molecular weight of 55.00 kDa and theoretical isoelectric point of 9.36. PbPCS1 also harbors five amino acid residues that are highly conserved in plant PCS1, namely the cysteine residues at positions 56, 90, 91, 109 and 113. At the same time, 12 cysteine residues putatively involved in heavy metal binding sites, including three adjacent Cys-Cys components (331–332, 351–352 and 369–370 amino acids) are present at the C-terminal variable domain of the PbPCS1 protein.

The search for conserved domains (NCBI Conserved Domain Database and Pfam) in PbPCS1 protein clearly revealed a phytochelatin domain from amino acids 2 to 217 with an E-value of 3.17×10^{-88} . Three predicted active sites were found in the PC domain, which included the cysteine residue at position of 56, the histidine residue at 162 and the aspartic acid residue at 180. Additionally, phytochelatin C (a member of the functionally uncharacterized domains at the C-terminus of plant PCS family) was found from amino acids 220 to 477 with an E-value of 1.5×10^{-113} .

Sequence Alignment and Phylogenetic Analysis

Multiple sequence alignments with 16 plant PCS1 proteins revealed that they shared very high similarity amongst N-terminal amino acids and low similarity of C-terminal amino acids (Fig. 3). Sequence analysis by the BLASTX program in the NCBI database revealed that the putative amino acid sequence of *PbPCS1* shared a high similarity with known PCS1 from other plant species, such as *Lotus japonicus* (GenBank accession no. AAT80342, 73.5%), *Lactuca sativa* (AAU93349, 71.3%), *Arabidopsis thaliana* (AAD41794, 68.6%), *Nicotiana tabacum* (AAO74500, 68.6%), *Allium sativum* (AAO13809, 63.3%), *Triticum aestivum* (AAD50592, 61.1%), *Cynodon dactylon* (AAO13810, 57.1%), and *Oryza sativa* (AAO13349, 53.0%).

To analyze the phylogenetic relationship between PbPCS1 and PCS1 proteins from 15 other representative plants, full-length amino acid sequences were aligned by CLUSTALW and imported into the MEGA package version 4.0 (Tamura et al. 2007). Phylogenetic analyses were conducted using the neighbor-joining (NJ) method implemented in MEGA, with the pairwise deletion option for handling alignment gaps, and with the Poisson correction model for distance computation. Bootstrap tests were conducted using 1,000 replicates. Branch

lengths (drawn in the horizontal dimension only) are proportional to phylogenetic distances. The phylogenetic tree showed that PbPCS1 was most related to PCS1 from legume plants *Lotus japonicus* (GenBank accession no. AAT80342) and *Glycine max* (AAL78384) as they were clustered into the same clade, while it was most distant to AyPCS1 from *Athyrium yokoscense* (BAB64932) (Fig. 4).

Expression Analysis

The expression patterns of *PbPCS1* gene were analyzed by quantitative real-time PCR (Fig. 5). In untreated seedlings, expression of *PbPCS1* was barely detectable. However, after 24 h of exposure to 20 μM CdSO₄, CuSO₄ or ZnSO₄ treatment, expression of *PbPCS1* was enhanced, and was highest in roots compared to leaves and stems. The increases in roots were in order of 4.34-fold, 0.26-fold or 2.82-fold after exposure to CdSO₄, CuSO₄ or ZnSO₄, respectively.

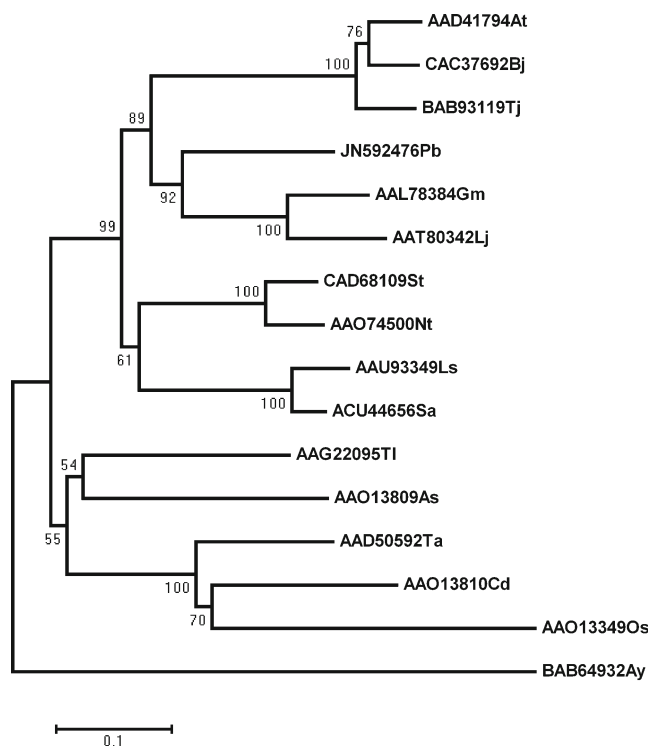


Fig. 4 Phylogenetic relationship of the deduced amino acid sequence of *PbPCS1* with the other plant phytochelatin synthases (PCS). A phylogenetic tree was constructed by the neighbor-joining algorithm with a total of 1,000 bootstrap replicates. AAO13809As from *Allium sativum* (GenBank accession No. AAO13809), AAD41794At from *Arabidopsis thaliana*, BAB64932Ay from *Athyrium yokoscense*, CAC37692Bj from *Brassica juncea*, AAO13810Cd from *Cynodon dactylon*, AAL78384Gm from *Glycine max*, AAT80342Lj from *Lotus japonicus*, AAU93349Ls from *Lactuca sativa*, AAO74500Nt from *Nicotiana tabacum*, AAO13349Os from *Oryza sativa*, JN592476Pb from *Pyrus betulifolia*, ACU44656Sa from *Sonchus arvensis*, CAD68109St from *Solanum tuberosum*, AAD50592Ta from *Triticum aestivum*, BAB93119Tj from *Thlaspi japonicum*, AAG22095TI from *Typha latifolia*

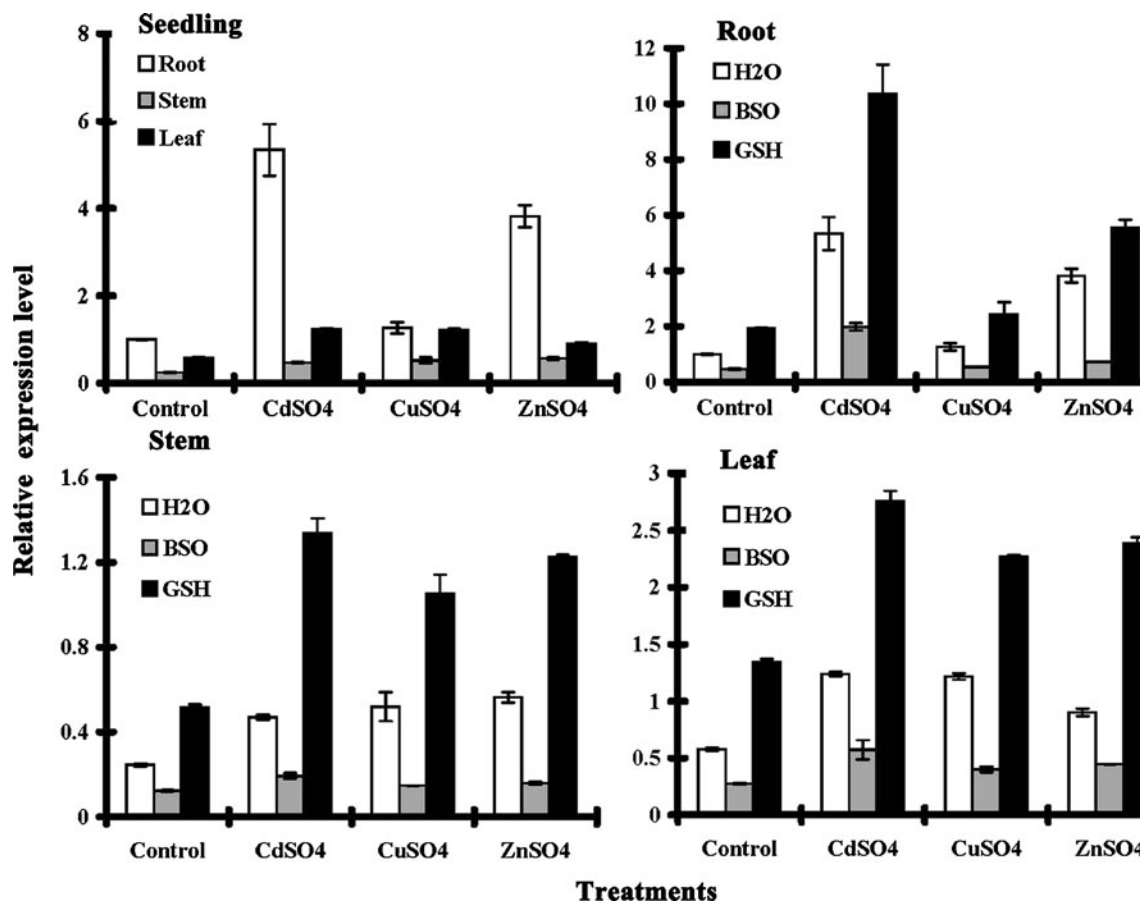


Fig. 5 Quantitative real-time PCR (qPCR) analysis of *PbPCS1* transcripts in birch-leaf pear. Mean values and standard errors (*bars*) were obtained from three independent biological experiments

The inducing abilities of these three heavy metal ions were different, with 20 μM CdSO₄ having the strongest effect among them. At the same time, *PbPCS1* mRNA accumulation in roots was highest, regardless of CdSO₄, CuSO₄ or ZnSO₄ treatment. Furthermore, 200 μM BSO or GSH was added to different heavy metal treatments. The results showed that the percentage decrease in *PbPCS1* expression levels ranged from 50.35% to 81.12% after exposed to single heavy metal combined with BSO. In contrast, elevated expression levels of *PbPCS1* were detected after adding GSH with a single heavy metal, with the fold changes ranging from 1.45 to 2.85. These findings imply that BSO would inhibit *PbPCS1* expression whether in roots, stems or leaves, but that GSH could stimulate *PbPCS1* expression in different organs of birch-leaf pear.

Discussion

Comparison of the genes structures of *PbPCS1*, *AtPCS1* and *LjPCS1* revealed that *PbPCS1* contains eight exons, *AtPCS1* consists of nine exons and *LjPCS1* has eight exons.

Interestingly, exons 1 to 6 of all three genes have identical sizes, i.e., 75, 157, 239, 164, 52 and 228 bp (Fig. 2). Also, the sequences from exons 1 to 6 of *PbPCS1* and *AtPCS1*, or *LjPCS1* show high sequence identity (72.2–80.5%). In contrast, intron sizes and sequences differed greatly among *PbPCS1*, *AtPCS1* and *LjPCS1*. Furthermore, comparisons of amino acid sequences from plant PCS1 proteins found that high similarity amongst N-terminal amino acids, but similarity was low for C-terminal amino acids (Fig. 3). Based on above results, the hypothesis could be proposed that the PCS enzyme from birch-leaf pear is functionally organized into an N-terminal (conserved) domain involved in catalysis and a C-terminal (variable) domain conferring metal sensing ability and enzyme stability as in *AtPCS1* or *LjPCS1* (Cobbett and Goldsbrough 2002; Ruotolo et al. 2004).

The position and arrangement of Cys residues, particularly in the N-terminal domain, are important for PCS activity and Cd tolerance (Cobbett and Goldsbrough 2002; Ruotolo et al. 2004; Rea 2006). Residue Cys⁵⁶ which is identical in all known PCS was subsequently proven to be indispensable for PCs synthesis activity and to represent the first site of acylation (Clemens 2006). Moreover, the

catalytic triad consisting of Cys⁵⁶, His¹⁶², and Asp¹⁸⁰ is essential for PCS activity (Ramos et al. 2007), and the conserved Cys⁵⁶, Cys⁹⁰, Cys¹⁰⁹ and Cys¹¹³ residues are reported to play important roles in heavy metal induced PCs catalysis (Maier et al. 2003). PbPCS1 also contains these specific amino acid sites. It should be pointed out that the Cys³⁵⁸Cys³⁵⁹XXXCys³⁶³XXCys³⁶⁶ (X represents any amino acid) motif of the PCS serves as a sensor for heavy metal ions (Vestergaard et al. 2008). This motif in PbPCS1 protein was shown to be Cys³⁶⁹Cys³⁷⁰GlnGluThrCys³⁷⁴VallLysCys³⁷⁷. After sensing the presence of a heavy metal ion, the C-terminal region transfers heavy metal ions to the N-terminal catalytic domain, which may function as a metal-chaperone to transfer metal ions (Vestergaard et al. 2008). In addition, *PbPCS1* expression levels in different organs after CdSO₄ treatment were higher than after CuSO₄ or ZnSO₄ treatment (Fig. 5), which indicates that the C-terminal region of PCS maybe bind Cd ions at high capacity, but has a much lower capacity for other metal ions such as Cu²⁺ and Zn²⁺ (Vestergaard et al. 2008).

The expression level of some *PCS1* genes (such as *AtPCS1*, *BjPCS1* and *LsPCS1*) is known to increase significantly in roots in the presence of heavy metals (Ha et al. 1999; Heiss et al. 2003; He et al. 2005; Zhang et al. 2005). *PbPCS1* also follows this rule, probably because the root is a major absorption organ. Once different heavy metals began to accumulate, the *PbPCS1* transcription level was enhanced quickly in roots. In plants, there is limited data concerning the regulation of expression of *PCS* in response to metal ions. After experimental treatments, *PbPCS1* showed a significant increase in expression levels at CdSO₄, CuSO₄ or ZnSO₄ concentrations of 20 μM. These increases were 4.34-fold, 0.26-fold or 2.82-fold, respectively, in roots after 24 h exposure (Fig. 5). The results suggest that regulation of the gene encoding PCS occurs at the transcriptional level in birch-leaf pear, which is in agreement with the hypothesis that PCS is controlled at the transcriptional level (Zhang et al. 2005).

The exposure of either cell cultures or intact plants to an inhibitor of GSH biosynthesis, BSO, led to inhibition of PC biosynthesis (Cobbett 2000). The same phenomenon was observed in birch-leaf pear during heavy metal treatment. *PbPCS1* transcript levels were obviously inhibited after BSO treatment (Fig. 5). Furthermore, numerous physiological, biochemical, and genetic studies have confirmed that GSH is the substrate for PC biosynthesis (Cobbett 2000). Exogenous GSH helped to recover *PbPCS1* expression abundance after single heavy metal treatment (Fig. 5). In conclusion, the PC biosynthesis pathway in birch-leaf pear may be similar to that in fission yeast (*Schizosaccharomyces pombe*) and Arabidopsis, which is catalyzed successively by γ-glutamylcysteine synthetase, glutathione synthetase and PCS (Clemens 2006).

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References

- Alberich A, Diaz-Cruz JM, Arino C, Esteban M (2008) Combined use of the potential shift correction and the simultaneous treatment of spectroscopic and electrochemical data by multivariate curve resolution: analysis of a Pb(II)-phytochelatin system. *Analyst* 133:470–477
- Blum R, Meyer KC, Wünschmann J, Lenzian KJ, Grill E (2010) Cytosolic action of phytochelatin synthase. *Plant Physiol* 153:159–169
- Chaurasia N, Mishra Y, Rai LC (2008) Cloning expression and analysis of phytochelatin synthase (*pcs*) gene from *Anabaena* sp. PCC 7120 offering multiple stress tolerance in *Escherichia coli*. *Biochem Biophys Res Commun* 376:225–230
- Clemens S (2006) Evolution and function of phytochelatin synthases. *J Plant Physiol* 163:319–332
- Clemens S, Kim EJ, Neumann D, Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *EMBO J* 18:3325–3333
- Cobbett CS (2000) Phytochelatin and their roles in heavy metal detoxification. *Plant Physiol* 123:825–832
- Cobbett C, Goldsbrough P (2002) Phytochelatin and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu Rev Plant Biol* 53:159–182
- DalCorso G, Farinati S, Maistri S, Furini A (2008) How plants cope with cadmium: staking all on metabolism and gene expression. *J Integr Plant Biol* 50:1268–1280
- Grill E, Löffler S, Winnacker EL, Zenk MH (1989) Phytochelatin, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific gamma-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). *Proc Natl Acad Sci USA* 86:6838–6842
- Guan LL, Wang YB, Shen H, Hou K, Xu YW, Wu W (2012) Molecular cloning and expression analysis of genes encoding two microsomal oleate desaturases (FAD2) from safflower (*Carthamus tinctorius* L.). *Plant Mol Biol Rep* 30:139–148
- Ha S, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB, Cobbett CS (1999) Phytochelatin synthase genes from Arabidopsis and the yeast *Schizosaccharomyces pombe*. *Plant Cell* 11:1153–1163
- He Z, Li J, Zhang H, Ma M (2005) Different effects of calcium and lanthanum on the expression of phytochelatin synthase gene and cadmium absorption in *Lactuca sativa*. *Plant Sci* 168:309–318
- Heiss S, Wachter A, Bogs J, Cobbett C, Rausch T (2003) Phytochelatin synthase (PCS) protein is induced in *Brassica juncea* leaves after prolonged Cd exposure. *J Exp Bot* 54:1833–1839
- Kaneyoshi J, Wabiko H, Kobayashi S, Tsuchiya T (2001) Agrobacterium tumefaciens AKE10-mediated transformation of an Asian pea pear, *Pyrus betulaefolia* Bunge: host specificity of bacterial strains. *Plant Cell Rep* 20:622–628
- Liu XM, Anderson JM, Pijut PM (2010) Cloning and characterization of *Prunus serotina* AGAMOUS, a putative flower homeotic gene. *Plant Mol Biol Rep* 28:193–203
- Maier T, Yu C, Küllertz G, Clemens S (2003) Localization and functional characterization of metal-binding sites in phytochelatin synthases. *Planta* 218:300–308
- Matsumoto K, Chun J, Tamura F, Kamamoto Y, Tanabe K (2006) Salt tolerance in *Pyrus* species is linked to levels of Na and Cl translocation from roots to leaves. *J Japan Soc Hort Sci* 75:385–391

- Matsumoto K, Tamura F, Chun J, Ikeda T, Imanishi K, Tanabe K (2007) Enhancement in salt tolerance of Japanese pear by using *Pyrus betulaefolia* rootstock. *Hortic Res (Japan)* 6:47–52
- Okubo M, Sakuratani T (2000) Effects of sodium chloride on survival and stem elongation of two Asian pear rootstock seedlings. *Sci Hortic* 85:85–90
- Okubo M, Furukawa Y, Sakuratani T (2000) Growth, flowering and leaf properties of pear cultivars grafted on two Asian pear rootstock seedlings under NaCl irrigation. *Sci Hortic* 85:91–101
- Ramos J, Clemente MR, Naya L, Loscos J, Perez-Rontome C, Sato S, Tabata S, Becana M (2007) Phytochelatin synthases of the model legume *Lotus japonicus*. a small multigene family with differential response to cadmium and alternatively spliced variants. *Plant Physiol* 143:1110–1118
- Ramos J, Naya L, Gay M, Abian J, Becana M (2008) Functional characterization of an unusual phytochelatin synthase, LjPCS3, of *Lotus japonicus*. *Plant Physiol* 148:536–545
- Rea PA (2006) Phytochelatin synthase, papain's cousin, in stereo. *Proc Natl Acad Sci USA* 103:507–508
- Ruotolo R, Peracchi A, Bolchi A, Infusini G, Amoresano A, Ottonello S (2004) Domain organization of phytochelatin synthase. *J Biol Chem* 279:14686–14693
- Tamura F, Tanabe K, Katayama M, Itai A (1996) Effect of flooding on ethanol and ethylene production by pear rootstocks. *J Japan Soc Hortic Sci* 65:261–266
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599
- Tennstedt P, Peisker D, Bottcher C, Trampeczynska A, Clemens S (2009) Phytochelatin synthesis is essential for the detoxification of excess Zn and contributes significantly to the accumulation of Zn. *Plant Physiol* 149:938–948
- Vatamaniuk OK, Mari S, Lu YP, Rea PA (1999) *AtPCS1*, a phytochelatin synthase from *Arabidopsis*: isolation and in vitro reconstitution. *Proc Natl Acad Sci USA* 96:7110–7115
- Vestergaard M, Matsumoto S, Nishikori S, Shiraki K, Hirata K, Takagi M (2008) Chelation of cadmium ions by phytochelatin synthase: role of the cysteine-rich C-terminal. *Anal Sci* 24:277–281
- Zhang H, Xu W, Guo J, He Z, Ma M (2005) Coordinated responses of phytochelatin and metallothioneins to heavy metals in garlic seedlings. *Plant Sci* 169:1059–1065
- Zhao D, Zhou C, Sheng Y, Liang G, Tao J (2011) Molecular cloning and expression of phytoene synthase, lycopene beta-cyclase, and beta-carotene hydroxylase genes in persimmon (*Diospyros kaki* L.) fruits. *Plant Mol Biol Rep* 29:345–351