Suppression Subtractive Hybridization (SSH)-Based Method for Estimating Cd-Induced Differences in Gene Expression at Cultivar Level and Identification of Genes Induced by Cd in Two Water Spinach Cultivars

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The abilities to accumulate cadmium (Cd) are different among cultivars (cv.) in many species. The characteristic of Cd concentration among cultivars is heritable and is probably controlled by genes, but rather limited information about the relevant genes in vegetable crops has been published. In the present study, a suppression subtractive hybridization (SSH) approach was used to identify genes induced by Cd in two water spinach (an important vegetable in southern China) cultivars that differ in Cd accumulation in their edible parts. The two cultivars were cv. Qiangkunqinggu (QK), a low Cd accumulative cultivar and cv. Taiwan 308 (TW), a high Cd accumulative cultivar. In the construction of QK and TW libraries, the plants without Cd treatment were taken as drivers and the plants exposed to 6 mg L^{-1} Cd for 24 h as testers. Four hundred clones were sequenced, and 164 nonrepeated sequences (112 from the QK library and 52 from the TW library) were assigned to being functional genes or proteins. A tremendous difference in Cd-induced gene expressions between the two libraries was observed. In the QK library, genes implicated in disease/defense comprised one of the largest sets (20.6%), whereas the proportion was only 8.8% in the TW library. An *MT3* gene (Q5), a wound inductive gene (Q22), an antioxidation relevant gene (Q34), a lectin gene (Q45), an f-box family protein gene (Q319), a 20S proteasome subunit gene (T17), a multidrug resistance associated protein gene (T156), and a cationic amino acid transporter gene (T218) were selected to compare semiquantitatively their expression between cv. QK and cv. TW using the RT-PCR method, and obvious differences were detected. The relationships between the identified differences in the expressions of the genes and the Cd accumulation of the two cultivars were discussed, and it was concluded that the SSH approach is useful for finding the difference in expression of Cd-induced gene even at the cultivar level and is applicable in the investigation of the mechanisms of low Cd accumulation.

KEYWORDS: Cadmium; cultivar; differential gene expression; water spinach (*Ipomoea aquatica* Forsk.); suppression subtractive hybridization

INTRODUCTION

Cadmium (Cd) is a nonessential as well as a toxic trace element. Increased heavy metal contamination resulting from natural processes or recent human activities such as mining, industrial, and agricultural practices has attracted great attention worldwide (*1*). Phytoremediation has become a vital method to remove heavy metals such as Ni, Cd, As, Cu, Co, and Pb from highly contaminated soil (*2*), but it is a time-consuming process that may take many years to completely clean up a site (*3*). Most hyperaccumulators grow slowly and have small biomass; many of them are not suitable for practical applications (*4*). The physical, chemical, and biological methods used to remediate heavy metal contaminated soil are often limited by effect, cost, and time (*5*, *6*). Furthermore, Cd contamination resulting from agricultural practices is usually light and not fit for phytoremediation. Selection for pollution-safe cultivars (PSC), based on the variations of Cd uptake among cultivars (cv.) within species that are heritable, is a feasible method to reduce Cd concentration in crops (*7*−*9*). This method makes it possible to produce safe food from lightly contaminated soil and avoid leaving the soil unused. It is useful for developing countries with great population pressures such as China.

The abilities of Cd accumulation are different among cultivars in many species, such as asparagus bean (*Vigna unguiculata* subsp. *Sesquipedalis* L.), barley (*Hordeum vulgare* L.), carrot (*Daucus carota* var. *sativa*), corn (*Zea mays* L.)., cucumber (*Cucumis sativus* L.), common wheat (*Triticum aestivum* L.),

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durum wheat (*Triticum turgidum* L. var. *durum*), linseed (*Linum usitatissimum* L.), lettuce (*Lactuca sativa* L.), oat (*Avena sativa* L.), pea (*Pisum sativum* L.), peanut (*Arachis hypogaea* L.), potato (*Solanum tuberosum* L.), rice (*Oryza sativa* L.), ryegrass (*Lolium perenne* L.), soybean (*Glycine max* (L.) Merr.), and willow (*Salix viminalis* L.) (*8*). More and more studies focus on selecting cultivars with a low Cd accumulation characteristic in to minimize Cd accumulation in the edible parts of crops (*9*, *10*). Some of the low-Cd cultivars have been commercialized successfully. Strongfield, a low-Cd durum wheat cultivar, is now sown on >25% of the durum area in Canada (*8*). Peanut cultivars such as Florunner and soybean cultivars such as Soy 791 have been recommended for use in Australia for their low Cd accumulation characteristic (*11*). All of these studies strongly proved that the characteristic of Cd concentration among cultivars is heritable. Using PSCs could provide an option for farmers to reduce the influx of pollutants to the human food chain (*7*).

The low Cd concentration characteristic is probably controlled by genes. Some studies focused on the genes related to low Cd concentration. Clarke et al. found that low Cd concentration is controlled by a single dominant gene in durum wheat (*12*). Ishikawa et al. identified the putative quantitative trait loci for Cd concentration in brown rice (*13*). Kilchevsky et al. reported the general type of heavy metal accumulation inheritance in tomato (*Lycopersicon esculentum*) fruits—overdominance toward reduction of pollutant value (*14*). Li et al. used RNA interference (RNAi) to suppress the expression of phytochelatin synthase (PCS) gene *OsPCS1* in rice. They found that Cd accumulation was reduced by about half in the seeds of RNAi rice and that no apparent difference of growth appeared between RNAi and wild-type plants (*15*). The purpose of the present study was to find the genes associated with the control of low Cd accumulation in water spinach (*Ipomoea aquatica* Forsk.) cultivar.

Suppression subtractive hybridization (SSH) based on suppression PCR is a good method to find genes differentially expressed in two groups (*16*). Some studies used SSH to find genes related to heavy metal tolerance and accumulation in plants. For example, Srivastava et al. isolated several differentially expressed cDNA clones, including a type 2 metallothionein (MT) gene, which was differentially regulated in lead-treated *Sesbania drummondii*, by the SSH method (*17*). By using the SSH method, Lewandowska et al. observed the phenomenon of 26S rRNA polyadenylation and degradation in shoots of *Nicotiana tabaccum* plants grown in the presence of Cd (*18*). Guo et al. compared differentially expressed genes in the roots of two wheat near-isogenic lines after aluminum stress through the SSH method (*19*).

Water spinach is a popular vegetable in southern China and is found to be easily polluted by soil Cd (*20*, *21*). In our previous study, two water spinach cultivars, cv. Taiwan 308 (TW) and cv. Qiangkunqinggu (QK), with different Cd accumulation abilities were selected. When grown in the contaminated soil with 0.593 mg kg^{-1} Cd, the shoot Cd concentrations of cv. QK and cv. TW were 0.082−0.084 and 0.253−0.282 mg kg^{-1} (fresh weight), respectively, and the differences between the two cultivars were 3.1−3.4-fold (*20*).

In the present study, the SSH method was used to find the differentially expressed genes in the two water spinach cultivars after Cd treatment. We hypothesize that different responses in gene expressed under Cd stress shall be experimentally visible if the Cd accumulation is genotype dependent, and thus the SSH approach will be useful for studying the difference in the induced gene expression at cultivar level. This is the first attempt to use the SSH approach to explain the mechanisms and to identify the genes governing the low Cd accumulation in leafy vegetable.

MATERIALS AND METHODS

Preparation of Plant Material. Two cultivars of water spinach, cv. TW and cv. QK, with different abilities to accumulate Cd were used in this study. After germination, three seedlings were grown in a beaker with 200 mL of Hoagland solution with the following composition: 5 mM Ca(NO$_3$)$_2$·2H$_2$O, 5 mM KNO$_3$, 2 mM MgSO$_4$·7H$_2$O, 1 mM KH$_2$PO$_4$, 0.1 mM EDTA−Fe, 47 μM H$_3$BO$_3$, 1 μM MnCl$_2$·4H$_2$O, 1 μM ZnSO$_4$·7H$_2$O, 0.01 μM H$_2$MoO$_4$, and 0.25 μM CuSO$_4$·5H$_2$O. Solutions were replaced every 3 days over the duration of the experiment. All of the used chemicals in this work were analytical reagents. Twelve beakers for each cultivar were arranged randomly in a growth chamber at a day temperature of 32 °C and a night temperature of 28 °C with a photoperiod of 16 h (12000 lx). Three weeks later, half of the plants were transplanted into the Hoagland solution containing 6 mg L^{-1} cadmium chloride for 24 h, and the other half were kept in the original Hoagland solution as control. After the 24 h treatment, all of the plants were sampled at the same time.

Some of the fresh shoots (for SSH), leaf, stem, and root (for RT-PCR) were frozen in liquid nitrogen before being stored at −75 °C for RNA isolation. The rest of the plant samples were separated into shoots (including leaves) and roots for analyzing Cd concentrations in the different plant issues. The root samples were rinsed with tap water and immersed in 20 mM Na$_2$EDTA for 15 min to remove trace elements adhering to the tissue. Roots and shoots were then washed thoroughly with deionized water. The samples were oven-dried at 70 °C to constant weight.

Analysis of Cd Concentrations in Roots and Shoots. Cd concentrations in the root and shoot samples were determined by an atomic absorption spectrophotometer (AAS, Hitachi Z-2300, Japan) after digestion with HNO$_3$−H$_2$O$_2$ (G.R.) in a microwave oven (microwave digester MDS-6, Shanghai Sineo Microwave Chemistry Technology Co., Ltd., China). A certified reference material (CRM) of the plant (GBW-07603, provided by the National Research Center for CRM, China) with a Cd concentration of 0.057 mg kg^{-1} was used to control the precision of the analytical procedures.

RNA Extraction. For RNA isolation, the prepared shoot samples were homogenized in liquid nitrogen and resuspended in a solution containing 500 μL of extraction buffer 1, 500 μL of extraction buffer 2, and 2% β-mercaptoethanol (*22*). Extraction buffer 1 was composed of 2% CTAB (hexadecyltrimethylammonium bromide), 2% PVP (polyvinylpyrrolidone K 30), 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 1.4 M NaCl. Extraction buffer 2 was composed of 2% CTAB, 2% PVPP (polyvinylpolypyrrolidone), 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 1.4 M NaCl. After incubation at room temperature for 10 min, the suspension was mixed violently with 800 μL of chloroform. After centrifugation at 12000g for 15 min at 4 °C, phenol/chloroform (1:1) and chloroform/isoamyl alcohol (24:1) extractions were performed. The supernatant was transferred to another PE tube, and then total RNA was precipitated by the addition of a one-third volume of 8 M LiCl; this mixture was incubated overnight at −20 °C with subsequent centrifugation at 12000g for 20 min at 4 °C. The total RNA was washed twice with 75% ethanol and then resuspended in a volume of 40 μL of H$_2$O$_{DEPC}$. The isolation of poly(A)$^+$ RNA was carried out with the Oligotex Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany).

Suppressive Subtractive Hybridization (SSH). SSH was carried out using the PCR-Selected Subtractive Hybridization Kit (Clontech) following the instructions of the manufacturer. The cDNA was made using 2 μg of mRNA from shoot with random primers and MMLV reverse transcriptase. Then the synthesized cDNA was completely digested with *Rsa*I. Two SSH libraries were constructed: a QK library, in which cDNA prepared from the 24 h Cd-treated seedlings of cv. QK was used as the "tester" and that from the control samples of cv. QK as the "driver"; a TW library, in which cDNA prepared from the 24 h Cd-treated samples of cv. TW was used as the "tester" and that from the control samples of cv. TW as the "driver". Thus, the subtracted cDNAs represented genes induced in the 24 h Cd treated samples of cv. QK and in the 24 h Cd treated samples of cv. TW, respectively. GAPDH was used to test the subtraction efficiency of the corresponding libraries before cloning, and the primers are shown in **Table 1**. The subtracted cDNAs were finally cloned into pGEM-T Easy (Promega) and transformed into DH5α *Escherichia coli* cells. Subtracted

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Table 1. Primer Sequences Used for Semiquantitative Reverse Transcription PCR

clone	sequence of forward (F) and reverse (R) primers	length of the amplified fragment (bp)
GAPDH	F 5′-CAGGAACCCTGAAGATATCCC R 5′-GCAGTTGGTACTCTGAAGGCC	478
actin	F 5′-CAGCCTTCAATCATCGGAATG R 5′-GGGCCGGATTCATCATACTCT	313
Q5	F 5′-ATGTCGGACAAGTGCGGAAACTGCG R 5′-TTAGTGGCCACAGGTGCGGTCGGTA	202
Q22 (T337)	F 5′-GCGAATAAGCAAGTTGATTGTGT R 5′-GAAGATGAGCACTCTGAGGGTTGT	215
Q34	F 5′-CCCAGAACGCTGCATAACGAG R 5′-GGCGGAGGGAATTGACT	330
Q45	F 5′-ATTCATTATGGCACTGGTAACAAC R 5′-CCGTACGCTTTCACATTGGTA	239
Q319 (T126, T24)	F 5′-GCGTATCGAACCTTGGACCTG R 5′-GTGTGAAAGCCTGCGAAAGTTG	272
T17	F 5′-GGCATTAAATACAGGGACGGT R 5′-TTGAACTTATTGCGCCGATTAT	275
T156	F 5′-GCGTGAAAACATACTATTCGGTTC R 5′-GCCTCTTAATTCTCCCCTGATAC	286
T218	F 5′-TGCAGCTTGTGTGGTATCCCTC R 5′-GGCAGCAATAATGGCAATAACT	199

expressed sequence tag (EST) clones were obtained and stored at −70 °C as glycerol stocks for PCR amplification of the cDNA inserts.

Sequencing and Sequence Analysis. Sequencing of cloned DNA was determined using SP6 and T7 promoter primers and by an ABI Prism 377-96 genetic analyzer (BigDye terminator v2.0, Applied Biosystems). The sequences were analyzed with the BLAST programs provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA of leaf, stem, and root was extracted according to the method mentioned above, treated with DNase, and subsequently converted to cDNA using ReverTra Ace (Toyobo). The cDNA (1 μL) was used directly for PCR. The PCR conditions were as follows: 26−30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed by 10 min of a final extension at 72 °C. The gene-specific primers used are shown in **Table 1**. The actin gene was used as control. The PCR products were analyzed on a 2% agarose gel with ethidium bromide at 0.5 μg mL^{-1} using a gel documentation and Gene Tools analysis software (GeneGenius; Syngene Co., Cambridge, U.K.). The level of the test gene was expressed in arbitrary units after normalization of the band intensity with that of the corresponding actin control.

RESULTS

Cd Concentration in Plant. After 24 h of Cd treatment, the Cd concentration in the shoots of cv. QK (25.1 ± 5.97 mg kg^{-1}, DW basis) was significantly ($p < 0.05$) lower than that of cv. TW (40.5 ± 6.23 mg kg^{-1}, DW basis), being consistent with that reported by Wang et al. (20). The Cd concentration in the roots of cv. QK (319 ± 6.77 mg kg^{-1}, DW basis) was also significantly ($p < 0.05$) lower than that of cv. TW (366 ± 15.8 mg kg^{-1}, DW basis).
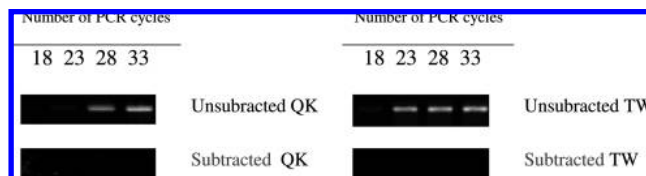

Figure 1. Analysis of subtraction using PCR. Tester cDNA was prepared from the poly (A)$^+$ RNA of plants with Cd treatment and driver cDNA from control plants. Aliquots of the samples were taken after 18, 23, 28, and 33 cycles of PCR amplification, and the products were analyzed on a 2% agarose gel. PCR was performed on the subtracted or unsubtracted secondary PCR product with GAPDH primers.

Analysis of Subtraction Efficiency. PCR amplification was used to estimate the efficiency of subtraction by comparing the abundance of a housekeeping gene GAPDH before and after subtraction. The GAPDH fragment was hardly detectable even after 33 cycles of amplification in the subtracted sample, whereas it is clearly detectable in the unsubtracted sample after 23 or 28 cycles (**Figure 1**). The result indicated that the subtraction had worked well.

Construction of cDNA Library. Two subtracted cDNA libraries were constructed in this study. They were a QK library and a TW library. With reference to the common practices of SSH (23), 200 colonies from each library were randomly picked and amplified for sequencing. Among the 400 cDNA sequences, 220 nonrepeat sequences (150 from the QK library and 70 from the TW library) were obtained. By comparison with the databases of BLASTN and BLASTX, 164 nonrepeat sequences (112 from the QK library and 52 from the TW library) were homologous with the published sequences in the databases (**Table 2**), and 56 nonrepeat sequences (38 from the QK library and 18 from the TW library) had no significant similarity with published sequences. Among the 164 known sequences, 136 (93 from the QK library and 43 from the TW library) of them could be assigned to functional proteins or genes, but 28 sequences (19 from the QK library and 9 from the TW library) were classified into gene or protein without any known function. cDNA fragments representing the same gene were counted as a single gene, and a total of 102 different genes (68 from the QK library and 34 from the TW library) with putative function was obtained.

Functional Classification. One hundred and two genes in the two libraries were assigned to putative function. The involved functions included metabolism, energy, cell growth/division, transcription, protein synthesis, protein destination and storage, transporters, cell structure, signal transduction, and disease/defense (**Figure 2**; **Table 2**), and the gene or protein function with intracellular traffic and secondary metabolism was not identified, according to the functional categories (FUN.CAT) described in Bevan et al. (24). In the QK library, one of the largest sets of genes (14 genes) was involved in disease/defense category (FUN. CAT = 11), and the proportion was as high as 20.6%. For the TW library, however, only 3 genes (8.8%) were relevant to the disease/defense among the 34 genes with known function, and the genes involving energy (FUN.CAT. = 2) occupied the dominant position (11 genes and 32.4% in proportion); in contrast, only 7 genes (10.3%) involving energy were identified in the QK library. These results implied the rather different responses to Cd stress between the two tested cultivars. Another major difference between the two cDNA libraries was that the genes involving protein synthesis occupied 11.8% in QK library and 5.9% in TW library. The proportion of genes involved in transcription was also higher in the QK library (7.4%) than in the TW library (2.6%). The proportions of genes involved in metabolism,

Table 2. Cd-Induced Water Spinach Genes

SSH clone[a]	base pairs	nearest homologue	score	accession no.	function catalog[b]
Q168	279	*Ipomoea nil* ln35 mRNA for hypothetical protein, complete cds	3e−120	AB267830.1	0
Q31	495	*Lycopersicon esculentum* clone 133171F, mRNA sequence	2e−81	BT014071.1	0
Q58	339	*Lycopersicon esculentum* clone 135057F, mRNA sequence	2e−22	BT013331.1	0
Q18	307	*Oryza rufipogon* (W1943) cDNA clone: ORW1943C104F01, full insert sequence	1e−42	CT841984.1	0
*Q133	249	plant synaptotagmin [*Populus trichocarpa*]	5e−35	XP_002312964.1	0
*Q200, Q201	332	predicted: hypothetical protein [*Vitis vinifera*]	2e−20	XP_002271692.1	0
Q77, Q105, Q393	424	*Solanum lycopersicum* cDNA, clone: FC18AB11, HTC in fruit	4e−57	AK246646.1	0
Q312	411	*Solanum lycopersicum* cDNA, clone: LEFL1004AF11, HTC in leaf	5e−38	AK319467.1	0
Q391	503	*Solanum lycopersicum* cDNA, clone: LEFL1004DE06, HTC in leaf	1e−59	AK320084.1	0
Q356	320	*Solanum lycopersicum* cDNA, clone: LEFL1012CG05, HTC in leaf	5e−49	AK320730.1	0
Q345	277	*Solanum lycopersicum* cDNA, clone: LEFL1012DC08, HTC in leaf	9e−20	AK320745.1	0
Q390	388	*Solanum lycopersicum* cDNA, clone: LEFL1023BC11, HTC in leaf	1e−25	AK319506.1	0
Q222	355	*Solanum lycopersicum* cDNA, clone: LEFL1073DB11, HTC in leaf	2e−23	AK324212.1	0
Q339	371	*Solanum lycopersicum* cDNA, clone: LEFL1096AH08, HTC in leaf	5e−50	AK325408.1	0
Q21	293	*Solanum lycopersicum* tomato chromosome 2, C02SLe0089P21, complete sequence	9e−146	AC215459.1	0
Q68	497	*Solanum tuberosum* Drm3-like protein mRNA, complete cds	7e−68	DQ191667.1	0
*Q254, Q185	520	SOUL heme-binding family protein [*Arabidopsis thaliana*]	2e−46	NP_173153.1	0
Q344	343	*Vitis vinifera*, whole genome shotgun sequence, contig VV78X057517.7, clone ENTAV 115	3e−20	AM438874.1	0
Q56	289	*Vitis vinifera*, whole genome shotgun sequence, contig VV78X266006.21, clone ENTAV 115	8e−20	AM461090.1	0
Q14	507	*Arabidopsis thaliana* bis(5′-adenosyl)-triphosphatase, putative (AT5G58240) mRNA, complete cds	8e−67	NM_203228.2	1
Q191	552	*Arabidopsis thaliana* TMS membrane family protein/tumor differentially expressed (TDE) family protein (AT1G16180) mRNA, complete cds	3e−61	NM_101485.2	1
*Q209	276	ATHDH (histidinol dehydrogenase); histidinol dehydrogenase [*Arabidopsis thaliana*]	2e−13	NP_851260.1	1
*Q116	183	ATPH1 (*Arabidopsis thaliana* Pleckstrin homologue 1); phosphoinositide binding [*Arabidopsis thaliana*]	8e−10	NP_565687.1	1
Q205	269	*Capsicum chinense* acyl-ACP thioesterase (FatA) mRNA, complete cds	3e−32	AF318288.1	1
*Q198	271	CNX2 (cofactor of nitrate reductase and xanthine dehydrogenase 2); catalytic [*Arabidopsis thaliana*]	1e−26	NP_850177.2	1
Q137	416	*Gossypium hirsutum* β-D-glucosidase mRNA, complete cds	7e−68	AY335818.1	1
Q218	345	*Lycopersicon esculentum* arginase 1 (ARG1) mRNA, complete cds	4e−83	AY656837.1	1
Q179	222	*Nicotiana tabacum* obtusifoliol-14-demethylase (NtCYP51-2) mRNA, complete cds	3e−49	AY065641.1	1
Q73	497	*Nicotiana tabacum* phosphoribosylaminoimidazole carboxylase (purEK) mRNA, partial cds	4e−128	AY429422.1	1
Q385	449	predicted: *Vitis vinifera* hypothetical protein LOC100254959 (LOC100254959), mRNA, carbohydrate metabolism	2e−132	XM_002276836.1	1
Q135	490	predicted: *Vitis vinifera* similar to hydrolase, α/β fold family protein (LOC100265524), mRNA	6e−51	XM_002265985.1	1

Table 2. Continued

SSH clone[a]	base pairs	nearest homologue	score	accession no.	function catalog[b]
Q208	418	*Solenostemon scutellarioides* CBS domain-containing protein mRNA, complete cds	3e−41	EF076754.1	1
Q316	533	*Zea mays* haloacid dehalogenase-like hydrolase domain-containing protein 1A (LOC100280513), mRNA	1e−76	NM_001153433.1	1
Q165, Q352, Q365, Q371	346	*Brassica rapa* mRNA for putative δ subunit of ATP synthase, partial cds	8e−41	D78493.1	2
Q273	311	*Capsicum annuum* mRNA for chloroplast ferredoxin-NADP+ oxidoreductase precursor (fnr gene)	5e−76	AJ250378.1	2
Q20	188	*Lycopersicon esculentum* 33 kDa precursor protein of oxygen-evolving complex (PsbO) mRNA, partial cds	1e−46	DQ539439.1	2
Q375	227	*N. tabacum* mRNA for chloroplast Rieske FeS precursor protein 1	2e−40	X66009.1	2
Q333	236	*Prunus persica* clone Mdh1 NAD-dependent malate dehydrogenase (mdh1) mRNA, complete cds	8e−20	AF367442.1	2
Q27, Q176	308	*S.tuberosum* cyc1 gene encoding cytochrome *c*1	2e−91	X62124.1	2
Q120, Q206, Q413	307	*Zea mays* clone 1695472 ferredoxin-1 mRNA, complete cds	5e−43	EU958479.1	2
Q3, Q114, Q175, Q188, Q271, Q304, Q323	276	*Zea mays* clone 1695472 ferredoxin-1 mRNA, complete cds	1e−44	EU958479.1	2
Q122	502	*Nicotiana tabacum* mRNA for histone H3, complete cds	3e−136	AB015760.1	3
Q217	443	*Nicotiana tabacum* mRNA for histone H3, complete cds	4e−71	AB015760.1	3
Q158, Q162	567	*Populus trichocarpa* precursor of protein cell division protease ftsh-like protein, mRNA	2e−159	XM_002301891.1	3
Q377	453	*Camellia sinensis* zinc finger protein mRNA, complete cds	1e−46	DQ869863.1	4
Q57	446	*L. esculentum* mRNA for homeobox protein	3e−72	X94947.1	4
Q322	822	*Nicotiana tabacum* poly(A)-binding protein (PABP) mRNA, complete cds	0	AF190655.1	4
Q406	470	*Populus trichocarpa* AP2 domain-containing transcription factor (RAP2), mRNA	3e−80	XM_002310679.1	4
*Q379	293	zinc finger (C3HC4-type RING finger) family protein [*Arabidopsis thaliana*]	6e−18	NP_001078548.1	4
Q359	294	*Arabidopsis thaliana* 60S ribosomal protein L10A (RPL10aA) (AT1G08360) mRNA, complete cds	1e−74	NM_100709.4	5
Q187	321	*Arabidopsis thaliana* putative hydroxyproline-rich glycoprotein (At3g22440) mRNA, complete cds	5e−56	AY096625.1	5
Q170	466	*Nicotiana tabacum* (clone: L24-2) chloroplast ribosomal protein L24 mRNA, complete cds	6e−82	M87839.1	5
Q308	633	*Solanum tuberosum* 60s acidic ribosomal protein-like protein mRNA, complete cds	1e−74	DQ191633.1	5
Q147	428	*Solanum tuberosum* clone 081G01 ribosome-associated protein p40-like mRNA, complete cds	1e−121	DQ207864.1	5
Q409	675	*Solanum tuberosum* clone 154B05 ripening regulated protein DDTFR10-like mRNA, complete cds	1e−162	DQ235167.1	5
Q128	472	*Solanum tuberosum* EF-1-α mRNA for elongation factor 1-α, complete cds	3e−156	AB061263.1	5
Q28, Q74	497	*Solanum tuberosum* ribosomal protein S27-like protein mRNA, complete cds	2e−87	DQ191664.1	5
Q157	248	*Ipomoea batatas* aspartic protease mRNA, complete cds	3e−113	DQ903691.1	6
Q123	547	*Ipomoea batatas* cathepsin B-like cysteine proteinase (CathB) mRNA, complete cds	0	AF101239.1	6
Q324, Q400	326	*Lycopersicon esculentum* leucine aminopeptidase preprotein (LapN) mRNA, complete cds	3e−58	AF510743.1	6
Q194	235	*Nicotiana tabacum* B38 mRNA for DnaJ homologue, complete cds	2e−51	AB032545.1	6

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Table 2. Continued

SSH clone[a]	base pairs	nearest homologue	score	accession no.	function catalog[b]
Q90	948	*Nicotiana tabacum* Ntubc1 mRNA for ubiquitin-conjugating enzyme (E2), complete cds	4e−106	AB026055.1	6
Q155	382	*Populus tomentosa* cultivar Hebeinica cysteine proteinase inhibitor (HBMYCPI) mRNA, complete cds	5e−29	DQ020096.1	6
Q319	485	*Populus trichocarpa* f-box family protein (FBL2), mRNA	1e−104	XM_002321300.1	6
*Q367	453	predicted protein [*Populus trichocarpa*] serine-type endopeptidase inhibitor activity	1e−11	XP_002314645.1	6
Q130, Q207, Q389	361	sweet potato sporamin B mRNA, complete cds	7e−29	M16883.1	6
Q24	167	*Tamarix androssowii* ubiquitin-conjugating enzyme family protein mRNA, complete cds	3e−41	AY587772.1	6
Q63	290	*Momordica charantia* auxin influx carrier-like protein 2 (AIC2) mRNA, complete cds	3e−25	AF522029.1	7
Q260	291	*Nicotiana tabacum* mRNA for sucrose transporter (sut1x gene), cultivar Samsun	3e−32	FM164640.1	7
*Q224	492	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein [*Arabidopsis thaliana*]	5e−14	NP_188456.1	7
Q239, Q403	367	*Pyrus communis* Py-PIP2-1 mRNA for plasma membrane intrinsic protein 2-1, complete cds	3e−65	AB058678.1	7
Q64	159	*Pyrus communis* Py-PIP2-1 mRNA for plasma membrane intrinsic protein 2-1, complete cds	1e−27	AB058678.1	7
Q418	470	*S. tuberosum* pPOM36I mRNA for 36 kDA porin I	2e−81	X80388.1	7
*Q417	349	glycoside hydrolase family 28 protein/polygalacturonase (pectinase) family protein [*Arabidopsis thaliana*].	4e−35	NP_194081.1	9
Q12	664	*Arabidopsis thaliana* protein kinase family protein (AT1G64630) mRNA, complete cds	4e−41	NM_105138.4	10
Q150, Q369	288	*Nicotiana benthamiana* ADP-ribosylation factor 1 (ARF1) mRNA, complete cds	2e−98	DQ531849.1	10
Q101	317	*Calystegia sepium* lectin mRNA, complete cds	1e−87	U56820.1	11
Q117	378	*Calystegia sepium* lectin mRNA, complete cds	1e−109	U56820.1	11
Q121	384	*Calystegia sepium* lectin mRNA, complete cds	8e−111	U56820.1	11
Q13, Q302	319	*Calystegia sepium* lectin mRNA, complete cds	2e−90	U56820.1	11
Q132, Q203, Q330	366	*Calystegia sepium* lectin mRNA, complete cds	1e−109	U56820.1	11
Q159	338	*Calystegia sepium* lectin mRNA, complete cds	1e−100	U56820.1	11
Q169	351	*Calystegia sepium* lectin mRNA, complete cds	6e−106	U56820.1	11
Q17	208	*Calystegia sepium* lectin mRNA, complete cds	2e−50	U56820.1	11
Q171	337	*Calystegia sepium* lectin mRNA, complete cds	2e−98	U56820.1	11
Q182	332	*Calystegia sepium* lectin mRNA, complete cds	1e−95	U56820.1	11
Q204	456	*Calystegia sepium* lectin mRNA, complete cds	1e−26	U56820.1	11
Q225	340	*Calystegia sepium* lectin mRNA, complete cds	6e−99	U56820.1	11
Q252	348	*Calystegia sepium* lectin mRNA, complete cds	2e−104	U56820.1	11
Q266	380	*Calystegia sepium* lectin mRNA, complete cds	4e−108	U56820.1	11
Q307	495	*Calystegia sepium* lectin mRNA, complete cds	1e−134	U56820.1	11
Q328	400	*Calystegia sepium* lectin mRNA, complete cds	3e−97	U56820.1	11
Q45	703	*Calystegia sepium* lectin mRNA, complete cds	0	U56820.1	11
Q85	110	*Calystegia sepium* lectin mRNA, complete cds	9e−26	U56820.1	11
Q96	306	*Calystegia sepium* lectin mRNA, complete cds	1e−80	U56820.1	11
Q227	403	*Ipomoea batatas* metallothionein-like protein (SPMT) mRNA, complete cds	3e−155	AF242374.1	11
Q5, Q301	414	*Ipomoea batatas* metallothionein-like protein (SPMT) mRNA, complete cds	4e−158	AF242374.1	11
Q373	198	*Ipomoea batatas* mRNA for ipomoelin, complete cds	1e−35	D89823.1	11
Q143	220	*Ipomoea batatas* mt2k mRNA for metallothionein-like type 2 protein, complete cds	6e−59	AB193157.1	11
Q202	279	*Ipomoea batatas* proteinase inhibitor (SPLTI-b) mRNA, complete cds	4e−75	AF404833.1	11
Q67	442	*Nicotiana tabacum* polyphenol oxidase gene, partial cds	3e−21	DQ356947.1	11
Q313	808	*Nicotiana tabacum* secretory peroxidase (PER) mRNA, complete cds	0	AF149251.1	11
Q22, Q29	358	*Nicotiana tabacum* wound inducive mRNA, complete cds	2e−79	AB009885.1	11

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Table 2. Continued

SSH clone[a]	base pairs	nearest homologue	score	accession no.	function catalog[b]
*Q34, Q263	500	polyphenol oxidase [*Populus trichocarpa*]	5e−42	XP_002331793.1	11
*Q415	480	polyphenol oxidase [*Populus trichocarpa*]	6e−39	XP_002331793.1	11
*Q192	612	polyphenol oxidase [*Populus trichocarpa*]	7e−49	XP_002331793.1	11
Q329	520	*Populus* EST from severe drought-stressed leaves	3e−35	CU227612.1	11
Q370	270	*Populus* EST from severe drought-stressed leaves	2e−21	CU227595.1	11
Q407	369	*Populus* EST from severe drought-stressed leaves	3e−21	CU227595.1	11
*Q219	335	protease inhibitor, putative [*Arabidopsis thaliana*]	2e−13	NP_030435.1	11
Q7, Q30, Q395	429	sweet potato mRNA for catalase (EC 1.11.1.6)	2e−180	X05549.1	11
*Q156	180	wound-responsive protein-related [*Arabidopsis thaliana*]	5e−21	NP_177671.1	11
*T14, T148	348	*Arabidopsis thaliana* unknown protein	1e−26	NP_564055	0
T183	789	*Lycopersicon esculentum* clone 113819F, mRNA sequence	0	BT012803.1	0
T290, T313	330	*Populus trichocarpa* predicted protein, mRNA	4e−31	XM_002330046.1	0
*T364	706	predicted: hypothetical protein [*Vitis vinifera*]	4e−16	XP_002277015.1	0
T378	389	predicted: *Vitis vinifera* hypothetical protein LOC100248166 (LOC100248166), mRNA	1e−46	XM_002263937.1	0
T265	350	*Solanum lycopersicum* cDNA, clone: LEFL1090AD06, HTC in leaf	2e−73	AK325009.1	0
T284	605	*Solanum lycopersicum* cDNA, clone: LEFL1097CH09, HTC in leaf	5e−59	AK325542.1	0
T253	362	*Sorghum bicolor* hypothetical protein, mRNA	1e−20	XM_002456021.1	0
T26	264	*Vitis vinifera,* whole genome shotgun sequence, contig VV78X145709.7, clone ENTAV 115	1e−28	AM480356.1	0
*T174	315	*Arabidopsis thaliana* GAE6 (UDP-D-glucuronate 4-epimerase 6), catalytic	4e−39	NP_189024	1
T244	304	*Gossypium hirsutum* trans-2-enoyl-CoA reductase (ECR2) mRNA, complete cds	2e−55	EU001743.1	1
T180, T298	404	*Ipomoea batatas* soluble acid invertase Ib2FRUCT3 (Ibbfruct3) mRNA, complete cds	2e−167	AY037938.2	1
*T143	186	long-chain-fatty-acid−CoA ligase family protein/ long-chain acyl-CoA synthetase family protein (LACS8) [*Arabidopsis thaliana*]	3e−16	NP_178516.1	1
*T151, T295	281	long-chain-fatty-acid−CoA ligase family protein/ long-chain acyl-CoA synthetase family protein (LACS8) [*Arabidopsis thaliana*]	1e−30	NP_178516.1	1
T120, T139, T142	317	*Lupinus luteus* mRNA for putative metallophosphatase (ppd2 gene)	6e−80	AJ421010.1	1
T124	382	*Nicotiana glutinosa* NGR2 mRNA for RNase NGR2, complete cds	4e−64	AB032256.1	1
T300	240	*Nicotiana tabacum* sterol-C5(6)-desaturase homologue mRNA, complete cds	1e−42	AF099969.1	1
T221, T311, T358, T412	333	*Solanum lycopersicum* ext mRNA for endoxyloglucan transferase, complete cds	3e−83	D16456.1	1
T175	332	*Betula pendula* mRNA for ribulose-1,5-bisphosphate carboxylase (rbcS gene)	8e−52	Y07779.1	2
T279, T287	188	*Betula pendula* mRNA for ribulose-1,5-bisphosphate carboxylase (rbcS gene)	5e−33	Y07779.1	2
T266	525	*Capsicum annuum* mRNA for chloroplast ferredoxin-NADP+ oxidoreductase precursor (fnr gene)	8e−172	AJ250378.1	2
T16	219	*Ipomoea aquatica* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast gene for chloroplast product	3e−107	AY100958.1	2
T243	461	*Ipomoea aquatica* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast gene for chloroplast product	0	AY100958.1	2
T105, T129, T133, T192, T219, T222, T269, T275, T283, T302, T327, T344, T361, T366, T385, T391, T414	249	*Ipomoea batatas* rubisco activase (rca) mRNA, complete cds	3e−107	EU287993.1	2
T211	605	*Ipomoea batatas* rubisco activase (rca) mRNA, complete cds	6e−108	EU287993.1	2
T281	467	*Ipomoea batatas* rubisco activase (rca) mRNA, complete cds	5e−108	EU287993.1	2

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Table 2. Continued

SSH clone[a]	base pairs	nearest homologue	score	accession no.	function catalog[b]
T179	364	*Ipomoea nil* CAB-like protein mRNA, complete cds	3e−90	AY547298.1	2
T262	312	*Ipomoea nil* mRNA for nonphotosynthetic ferredoxin, complete cds	1e−63	AB038037.1	2
T272	241	*Lycopersicon esculentum* ascorbate free radical reductase (AFRR), complete cds	2e−71	L41345.1	2
T146	316	*Musa acuminata* chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (rbcS-Ma5) gene, complete cds; nuclear gene for chloroplast product	8e−28	DQ088101.1	2
T291, T307, T326, T386	342	*Musa acuminata* chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (rbcS-Ma5) gene, complete cds; nuclear gene for chloroplast product	1e−45	DQ088101.1	2
T212	378	*Oryza sativa* Japonica group mRNA for cytochrome *c*, complete cds	1e−52	D12634.1	2
T215, T371	269	*Solanum tuberosum* clone 154D06 fructose-bisphosphate aldolase-like mRNA, complete cds	3e−63	DQ235169.1	2
T106, T110, T203, T278, T301, T336, T392	307	*Zea mays* clone 1695472 ferredoxin-1 mRNA, complete cds	5e−43	EU958479.1	2
T382	645	*Zea mays* clone 1695472 ferredoxin-1 mRNA, complete cds	1e−42	EU958479.1	2
T132, T334	305	*Pisum sativum* mRNA for Ftsh-like protease (ftsh11 gene)	5e−68	AJ786652.1	3
T108	294	Japanese morning glory high-mobility group protein mRNA sequence	2e−111	L12169.1	4
T185	501	*Solanum tuberosum* clone 145H06 putative 60S ribosomal protein L7-like protein mRNA, complete cds	6e−100	DQ294270.1	5
T188, T255	523	*Solanum tuberosum* EF-1-α mRNA for elongation factor 1-α, complete cds	0	AB061263.1	5
T317	440	*Arabidopsis thaliana* CSN complex subunit 7ii (CSN7) mRNA, complete cds, alternatively spliced	5e−89	AF395066.1	6
T114, T125, T138, T150, T163, T197, T214, T232, T274, T282, T304, T322, T328, T346, T357	228	*Lotus japonicus* ubiquitin mRNA, complete cds	2e−76	DQ249171.1	6
T17, T400	519	*Nicotiana tabacum* partial mRNA for putative *β*7 proteasome subunit (b7 gene)	8e−118	AJ291743.1	6
T126	758	*Populus trichocarpa* f-box family protein, mRNA	5e−48	XM_002320135.1	6
T24, T166, T207, T261, T370, T380, T393, T413	400	*Populus trichocarpa* f-box family protein, mRNA	3e−47	XM_002320135.1	6
T156, T303	394	*Capsicum chinense* mRNA for putative multidrug resistance-associated protein, complete cds	3e−116	AB372261.1	7
T218, T259	402	*Populus trichocarpa* cationic amino acid transporter (PtrCAT9), mRNA	3e−53	XM_002302454.1	7
T324	443	*Populus trichocarpa* predicted protein, mRNA (proton-transporting two-sector ATPase complex)	8e−99	XM_002319212.1	7
T22, T149, T167, T169, T194, T229, T236, T332	198	*Arabidopsis thaliana* *ρ*-GTPase-activating protein-related (AT4G35750) mRNA, complete cds	8e−24	NM_119741.2	10
T173	457	*Nicotiana tabacum* callus-expressing factor (CEF1) mRNA, complete cds	4e−26	AY286010.1	11
T337	290	*Nicotiana tabacum* wound inducive mRNA, complete cds	4e−44	AB009885.1	11
T102, T136, T160, T162, T190, T224, T225, T238, T247, T254, T268, T286, T330, T345, T351	381	*Solanum tuberosum* temperature-induced lipocalin (TIL) mRNA, complete cds	5e−93	DQ222995.1	11
T242	635	*Solanum tuberosum* temperature-induced lipocalin (TIL) mRNA, complete cds	2e−77	DQ222995.1	11

[a] Q, the 24 h Cd treatment library of cv. QK; T, the 24 h Cd treatment library of TW; *, from BLASTX, the E-value < 10e^{-10}. [b] Function catalog of plant genes: 0, unclassified; 1, metabolism; 2, energy; 3, cell growth/division; 4, transciption; 5, protein synthesis; 6, protein destination and storage; 7, transporters; 8, intracellular traffic; 9, cell structure; 10, signal transduction; 11, disease/defense; the E-value < 10e^{-20}.
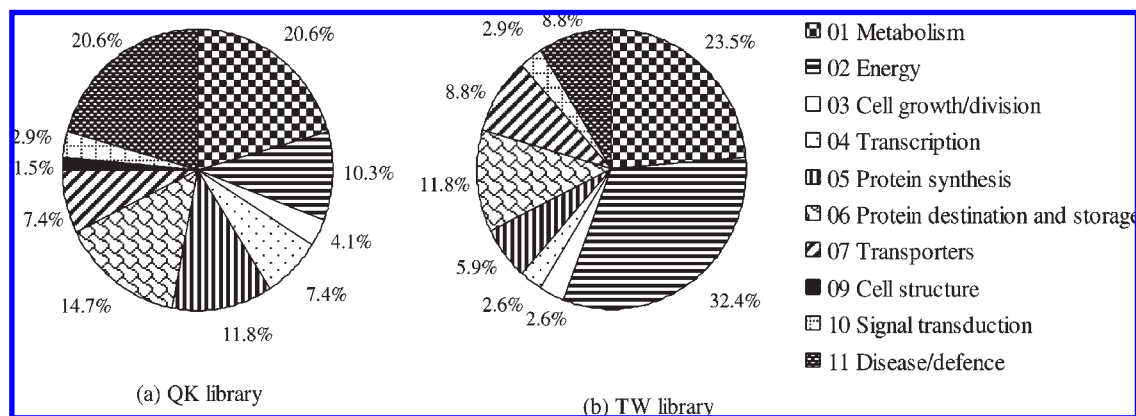
Figure 2. Proportions of the fraction of Cd-induced genes classified according to the functional categories described in Bevan et al. (*24*).

transporters, and protein destination and storage were rather similar between the two libraries. Therefore, the genes involved in disease/defense, energy, protein synthesis, and transcription groups are considered to be probably relevant to different mechanisms responding to the Cd stress.

Semiquantitative Reverse Transcription PCR (RT-PCR). Semi-quantitative RT-PCR was performed to analyze gene expression after 24 h of Cd treatment in the two tested cultivars (**Figure 3**). Eight genes including Q5 (an *MT3* gene), Q22 (as well as T337, a wound inductive gene), Q34 (an antioxidation relevant gene), Q45 (a lectin gene), Q319 (as well as T126 and T24, f-box genes), T17 (a 20S proteasome subunit gene), T156 (a multidrug resistance associated protein gene), and T218 (a cationic amino acid transporter gene) were selected because of their known functions in response to Cd or other abiotic stress. Q5 belongs to a metallothionein type 3 gene. It was highly expressed in leaf, and the expression was up-regulated in leaf of cv. TW and cv. QK, but was down-regulated in the root of both cultivars. Q22 (as well as T337) and Q319 (as well as T126 and T24) were two genes found in both libraries. Q22 was up-regulated in the leaf of both cultivars after Cd treatment, but the up-regulated level in leaf of cv. QK was higher than that of cv. TW. A similar response of Q319 was observed. The expression of Q34 remained unchanged in root and was up-regulated in the leaf and stem of cv. QK by the Cd treatment, whereas the expressions in the root and stem of cv. TW were down-regulated instead, as responding to the Cd treatment, especially in the root. Clone Q45 appeared to express at higher levels after Cd treatment in the leaf of cv. QK, whereas the expression in the leaf of cv. TW was irrelevant to the Cd treatment. In the stem and root, opposite responses of Q45 to the Cd treatment were observed between the two cultivars. The expression of clone T17 was much higher in cv. QK than in cv. TW in the absence of Cd stress, and it was up-regulated markedly in the leaf, stem, and root of cv. TW (especially in the leaf and root) but down-regulated markedly in the root of cv. QK under Cd treatment. The expression of T156 was up-regulated in the leaf, stem, and root of both cultivars after Cd treatment, and the up-regulated level in the leaf and stem of cv. TW was higher than that of cv. QK. T218, a cationic amino acid transporter gene, was up-regulated in the leaf of both cultivars, whereas in the root it was greatly up-regulated in cv. QK but slightly down-regulated in cv. TW.

DISCUSSION

The different Cd accumulations in shoots between cv. QK and cv. TW were verified again in the present study, and the result was

consistent with that obtained by Wang et al. (*20*). It enhanced the understanding that the Cd accumulation in water spinach is genotype dependent. However, the exuberant Cd compartment effect in the root of cv. QK observed by Wang et al. did not appear in the present study, probably attributable to the shorter Cd exposure time in the present study.

Although the Cd accumulation ability among cultivars of some vegetable species is proved to be different and inheritable (*9*, *11*), few studies have been conducted on the mechanisms. In the present study, SSH analysis was used to compare the Cd-induced gene expressions between cv. QK and cv. TW. Some differentially expressed genes were identified, which may be helpful to explain the mechanisms at the molecular level.

A tremendous difference in the Cd-induced gene expressions between the two libraries was observed. The speed of response to the Cd stress of cv. QK might be faster than that of cv. TW and may be one of the considered explanations. In other words, in the 24 h Cd (6 mg L^{-1}) treatment, the toxicity of Cd was probably not enough to make cv. TW begin biochemical behaviors as cv. QK did. However, in another study, similar Cd-induced gene expressions were found in both cultivars after gradient Cd treatment as long as 21 days (unpublished data).

Plant genes responding to Cd stress have been identified in some plants such as *Arabidopsis thaliana* (*25*), *Thlaspi caerules-cens* (*26*), *Datura innoxia* (*27*), and *Pisum sativum* (*28*). For the water spinach in the present study, the SSH method was used to estimate the difference in the expression of the Cd accumulation related genes at cultivar level. Among the 220 nonrepeated sequences from the Cd-induced cDNA libraries of cv. QK and cv. TW, 56 sequences (25.5%) did not have significant hits with the BLASTN and BLASTX databases. This indicated that the sequences were probably new or water spinach-specific genes, and further investigations about these clones are of interest. Increased expression of disease/defense genes seemed to be an important response of water spinach to Cd toxicity, especially for cv. QK, the Cd-PSC. Under Cd exposure, the genes involved in disease/defense in the QK library were 20.6% of the total genes, whereas the proportion was only 8.8% in the TW library. This suggested that cv. QK might suffer more seriously from Cd stress than cv. TW, so that cv. QK had to mobilize more resources to construct a defense system. The most characterized response to Cd stress in cv. TW was the obvious increase of the expression of genes or protein involved in energy. Growth of cv. TW under high Cd exposure was proved to be faster than that under low Cd exposure (*20*), which might be one of the reasons that cv. TW needed to mobilize actively energy relevant processes. It remains unknown how the genes involved in energy associated with the Cd detoxication, and further studies are needed. Probably the in-
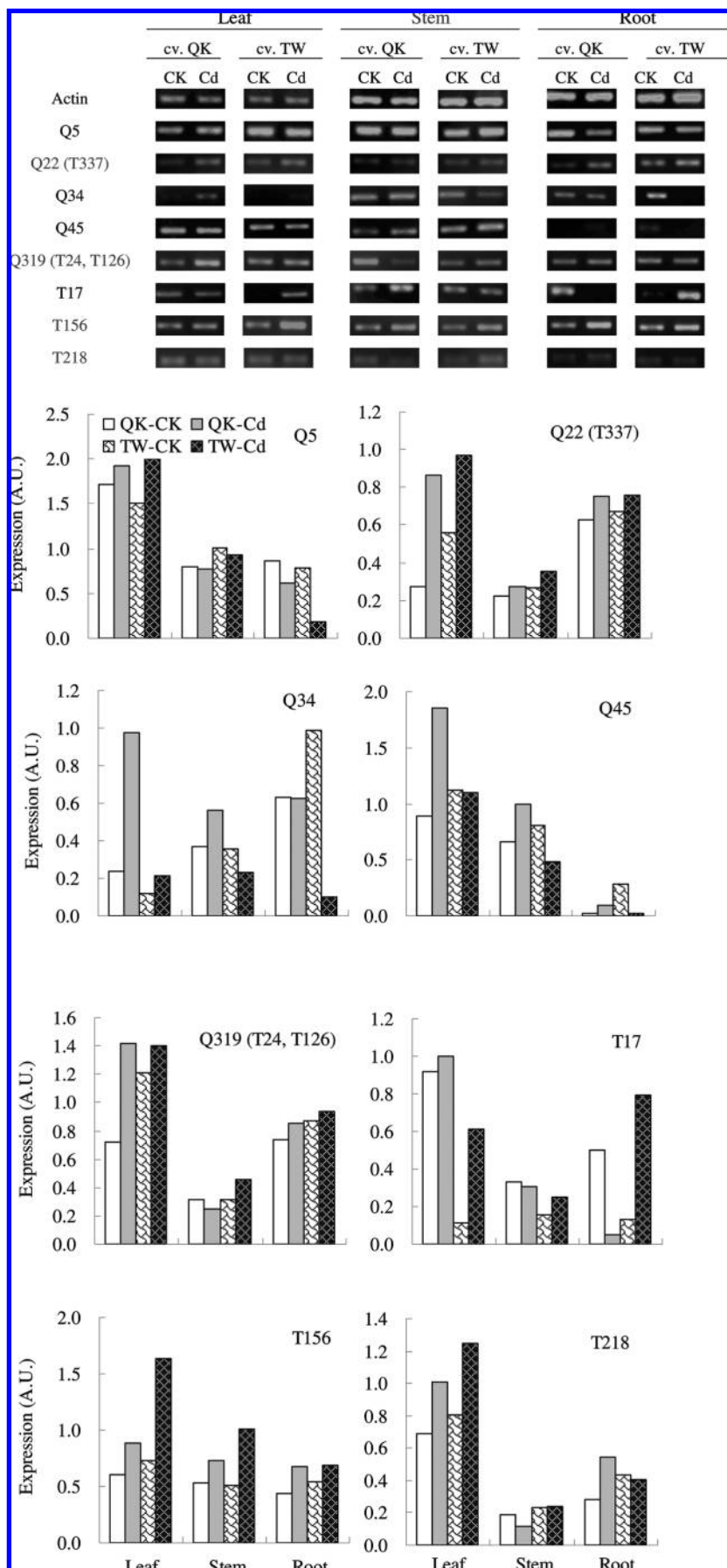
Figure 3. Gene expression determined by semiquantitative RT-PCR. Expression of genes in arbitrary units (AU) corresponds to the ratio of the target gene divided by the ratio of the constitutive control actin gene. QK-CK, cv. QK under Cd stress absent; QK-Cd, cv. QK under Cd exposure; TW-CK, cv. TW under Cd stress absent; TW-Cd, cv. TW under Cd exposure; Q5, Q34, Q45, Cd-induced genes in the QK library; T17, T156, T218, Cd-induced genes in the TW library; Q22 (T337), Q319 (T24, T126), Cd-induced genes in both libraries.
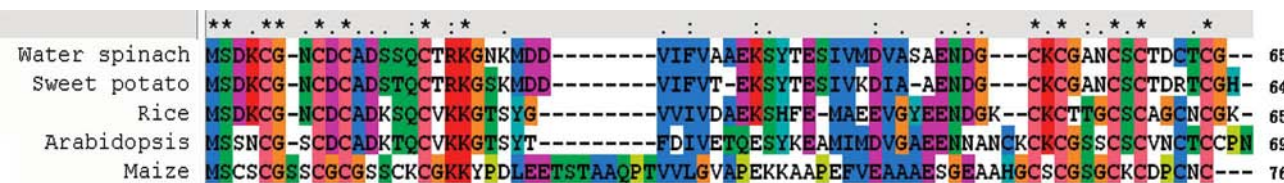
Figure 4. Amino acid sequence alignments of the putative MT from various species: water spinach, a sequences from water spinach (*Ipomoea aquatica* Forsk.) in the present study (Q5); sweet potato MT (AAK27970); rice MT (NP_001054885.1); *Arabidopsis*, MT (NP_566509.1); maize MT (NP_001105465.1). ∗ indicates positions that have a single, fully conserved residue.

cipient response of cv. TW to the Cd stress was to protect its photosystem to maintain faster growth as a priority, because 7 (rbcS gene, chloroplast ferredoxin-NADP+ oxidoreductase gene, rbcL gene, rubisco activase gene, CAB-like protein gene, rbcS-Ma5 gene and ferredoxin-1 gene) among the 11 expressed energy-relevant genes or protein in TW library were associated with photosynthesis.

Some genes related to metal chelate complex were found in the libraries. For example, a gene coded as Q5 in the present study was highly similar to the sweet potato *G14* gene, which encodes an MT-like protein, and was up-regulated in the 24 h Cd treated seedlings of cv. TW. Sequence analysis of the Q5 showed that it encodes a protein of 65 amino acids with two cysteine-rich domains separated by a central cysteine-free spacer (**Figure 4**). The range of amino acids encoded by Q5 has high similarity with similar MTs found in other plants, especially the positions of cysteine, indicating that Q5 is a similar MT, which has been proved to be effective to resist heavy metal toxicity in many plant species (*29, 30*). In the leaf of cv. TW, the up-regulated Q5 expression suggested that cv. TW needed more MT encoded by the Q5 to relieve the toxicity from the Cd transported into the leaf, which was more than that in cv. QK (*20*).

Cd is toxic to plants and can induce oxidative stress. Using diverse enzymes such as superoxide dismutase, ascorbate peroxidase, guiacol peroxidase, and catalase as well as low molecular weight antioxidants such as cysteine, nonprotein thiol, and ascorbic acid to scavenge different types of reactive oxygen species, plants are well equipped to minimize oxidative stress (*31*). It has been demonstrated that Cd amplified the activities of catalase, peroxidases, and superoxide dismutase in maize (*Zea mays* L.) (*32*). Metwally et al. also found that the activity of catalase significantly increased in all pea geno-types after treatment with Cd (*33*). In the present study, the expression of several genes on the subjects of catalase, poly-phenol oxidase, and peroxidase were enhanced within 24 h after Cd treatment. Q7, similar to the catalase gene, was found in the QK library. Q67, Q34, and Q415, similar to the poly-phenol oxidase gene, were also found in the QK library. Q313 is a gene similar to *Nicotiana tabacum* secretory peroxidase (PER) mRNA. The four genes are all grouped into the disease/defense category, which was expressed much more intensely in cv. QK than in cv. TW. Species of the Cd-induced disease/defense genes were particularly abundant (20.6%) in cv. QK and were with various functions. Except for the genes involved in the antioxidation system (Q7, Q34, and Q313), there were lectin genes (Q101 et al.), being relevant to salt tolerance and pathogen defense (*34*), wound-inducible genes (Q22 and Q156), and metallothionein-like protein genes (Q5, Q143, and Q227). These results exhibited that cv. QK may be more easily damaged by oxidation caused by Cd exposure than cv. TW and may, accordingly, more speedily and more actively start an antioxidation mechanism than cv. TW within the 24 h after Cd treatment. The expression of the two disease/

defense genes, Q34 and Q45, being up-regulated in the leaf and shoot of cv. QK is clear evidence proving that Cd exposure caused the quick response of the disease/defense genes in cv. QK.

Various types of transporters play important roles in heavy metal resistance and accumulation in plant. Some studies found transporters are associated with Cd transfer. For example, the ATP binding cassette (ABC) transporter AtPDR8 is a Cd extrusion pump conferring heavy metal resistance (*35*). Several cation exchangers isolated from *A. thaliana* encode tonoplast-localized transporters that appear to be major contributors to vacuolar accumulation/sequestration of Cd (*36*). *AtMRP6/AtABCC6*, an ATP-binding cassette transporter gene, was up-regulated by Cd in *A. thaliana* (*37*). In the present study, we also found some genes similar to transporter genes in the QK library, including Q63 and Q239, which were similar to *Momordica charantia* auxin influx carrier-like protein 2 (AIC2) mRNA; Q260, which was similar to *N. tabacum* mRNA for sucrose transporter; and Q418, which was similar to *S. tuberosum* pPOM36I mRNA for 36kDA porin I. There were also Cd-induced expressions of the transporter gene found in the TW library, such as T156, which was similar to *Capsicum chinense* mRNA for putative multidrug resistance-associated protein (MRP); T218, which was similar to *Populus trichocarpa* cationic amino acid transporter; and T324, which was similar to *P. trichocarpa* predicted protein mRNA (proton-transporting two-sector ATPase complex). Among these genes involved in transport, only MRP (T156) has been reported to relate with Cd transport. The Cd-induced expression increment of T156 in the leaf and stem of cv. TW was higher than that of cv. QK. Thus, T156 may associate with higher Cd accumulation in cv. TW.

T17 belongs to $\beta7$ proteasome, one of the 20S proteasome subunits. It has been reported that two types of 20S proteasome subunits in the cell of *A. thaliana* increased about 60% after Cd treatment (*38*). Djebali et al. found that the up-regulation of the proteasome subunit expression is part of the acclimation response of the tomato plant to oxidative stress (*39, 40*). In the present study, however, although cv. QK compartmentalized much more Cd in its root cells, and thus should suffer much more than cv. TW, gene T17 was down-regulated in cv. QK but up-regulated in cv. TW in response to the Cd stress. This did not coincide with the above-stated responses to Cd stress in the two cultivars and was left for further investigation.

In conclusion, the SSH approach is useful for finding the difference in expression of Cd-induced genes even at the cultivar level, and our hypothesis is thus acceptable. The Cd-induced genes in two water spinach cultivars differing in Cd accumulation were also successfully identified, which is helpful for explaining he mechanisms concerning the differences in Cd tolerance and Cd accumulation between the two tested cultivars. Further investigations for more identified genes will produce more information about the different genetic responses of water spinach at the cultivar level.

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