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Expression of sulfur uptake assimilation-related genes in response to cadmium, bensulfuron-methyl and their co-contamination in rice roots

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ABSTRACT

The responses of sulfur (S) uptake assimilation-related genes' expression in roots of two rice cultivars to cadmium (Cd), bensulfuron-methyl (BSM) and their co-contamination (Cd+BSM) were investigated by gene-chip microarray analysis and quantitative real-time PCR (QRT-PCR) technology. Treatments of Cd and Cd+BSM induced expression of sulfate transporter and permease genes, and promoted sulfate uptake in rice roots. Cd+BSM could alleviate Cd toxicity to cv. Fengmeizhan seedlings, probably due to Cd+BSM promoting greater S absorption by seedlings. Cd and Cd+BSM induced expression of sulfate assimilation-related genes, and thus activated the sulfur assimilation pathway. Cd and Cd+BSM induced expression of phytochelatin synthase and metallothionein genes, and induced expression of glutathione S-transferases (GSTs), glutathione synthase (GS) and S-containing antioxidation enzyme genes, which detoxified Cd^{2+}. It is suggested that (to cope with the toxicity of Cd, BSM and their co-contamination) the S uptake and assimilation pathway was activated in rice roots by increased expression of related genes, thus enhancing the supply of organic S for synthesis of Cd or BSM resistance-related substances.

Introduction

Environmental pollution tends to occur in the form of combined pollution, which mainly involves organic and inorganic pollutants. For instance, co-contamination with sulfonylurea herbicides and heavy metals in paddy fields has become an environmental concern and a research focus (Wu et al., 2007).

Among heavy metals, cadmium (Cd) is one of the most toxic to plants; and in recent years, the role of sulfur (S) in decreasing Cd toxicity has interested researchers (Hassan et al., 2005; Fan et al., 2010). Cd stress activates the S assimilation pathway (Harada et al., 2002), and promotes S uptake in plants. S supply alleviates Cd toxicity to plants (Hassan et al., 2005), and changes Cd content and distribution in different organs of plants (Fan et al., 2010); however, S deficiency aggravates Cd toxicity (Sun et al., 2003). The major mechanism of S alleviation of Cd toxicity involves the synthesis of several Cd resistance-related substances, e.g. glutathione (GSH), phytochelatins (PCs) and metallothioneins (MTs) (Hassan et al., 2005; De la Rosa et al., 2005; Fotakis and Timbrell, 2006; Zhang and Ge, 2008).

Bensulfuron-methyl (BSM) is a sulfonylurea herbicide that is widely used in paddy fields. It is generally considered to be a new type of effective and safe herbicide; however, its residues have become one of the main organic pollutants in paddy soil (Yang et al., 2006). BSM remaining in the soil can be toxic to sensitive crops, and high concentrations of BSM absorbed by rice roots and leaves

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can inhibit cell division (Omokawa et al., 1996), decrease chlorophyll content and inhibit seedling growth (Huang et al., 2006; Wu et al., 2007).

Although researchers have begun investigating the co-contamination of BSM with Cd (Huang and Xiong, 2009), little is known about their combined toxicity in rice – especially their influence on the pathway of S uptake and assimilation.

The present work focused on the responses of S uptake- and assimilation-related gene expression in rice roots to Cd, BSM and their co-contamination using Affymetrix gene-chip expression analysis and Quantitative real-time PCR technology.

1 Materials and methods

1.1 Plant material

Two rice (*Oryza sativa* L.) cultivars that are widely used in China, Yuexiangzhan and Fengmeizhan (both indica rice), were employed as the test materials. Previous research (Wu et al., 2007) results have suggested that Yuexiangzhan seedlings have greater tolerance to Cd stress than Fengmeizhan, but Cd and BSM co-contamination alleviates Cd toxic effects on Fengmeizhan seedlings.

1.2 Rice seedling culture and treatments

Seeds of the two rice cultivars were sterilized in 10% H_2O_2 (V/V) solution for 30 min, followed by thorough washing with deionized water. The seeds were germinated on stainless steel wire-mesh submerged in water at 25°C. Then the rice seedlings were cultured using the complete nutrient solution of the International Rice Research Institute (pH 5.1), and grown in a growth chamber under controlled conditions (14-hr photoperiod, 75% relative humidity, and 30/25°C day/night regime). When the third leaves of the seedlings had completely emerged, $CdCl_2$ or BSM was added to the culture solution for the treatments of Cd (45 μmol/L Cd^{2+}), BSM (0.25 μmol/L BSM) or Cd+BSM (45 μmol/L Cd^{2+}+ 0.25 μmol/L BSM), and an equal volume of water was added to serve as the control (CK). Selection of the Cd and BSM treatment concentrations was based on previous research (Wu et al., 2007), and were the critical concentrations for significant inhibition of rice seedling growth. The solution was changed and the pH adjusted to about 5.1 daily. Rice seedlings were sampled after treatment for 3, 5 and 20 days.

1.3 Toxic effects of Cd, BSM and Cd+BSM on rice seedlings

Root length, root number, shoot height and root and shoot biomass were measured after 20 days of treatment, to determine the effect of Cd, BSM and Cd+BSM treatments on the growth of rice seedlings. The data was analyzed by the Duncan multiple range test.

1.4 Sulfur content determination in rice seedlings

Total S content in rice seedlings was determined after 5 days of treatment as described by Li et al. (2000). Data was analyzed by the Duncan multiple range test.

1.5 Microarray analysis of root gene expression

Affymetrix gene-chip expression analysis was employed to reveal the differentially expressed genes in rice roots under Cd, BSM and Cd+BSM treatments, and was performed by the Bioassay Laboratory of CapitalBio Corporation (Beijing). The brief steps are as follows.

Total RNA isolation: TRIzol Reagent (Invitrogen Life Technologies) was used to isolate total RNA from rice roots after 3 days of Cd, BSM and Cd+BSM treatment. RNA concentration and purity was quantified by spectrophotometric analysis at 260 and 280 nm.

Target preparation: Eukaryotic poly-A RNA control kit, one-cycle cDNA synthesis kit, IVT labeling kit and sample cleanup module (all from Affymetrix) were used to synthesize the first-strand and second-strand cDNA with one-cycle cDNA synthesis protocol, after poly-A RNA controls, RNA/T7-oligo (dT) primer mix, first-strand master mix, and second-strand master mix being prepared. Then, the sample cleanup module was used to purify the double-stranded cDNA in the cDNA cleanup spin column. Third, biotin-labeled cRNA was synthesized using the GeneChip IVT labeling kit (from Affymetrix), after the IVT reaction set-up and IVT reaction were prepared. Lastly, the cRNA fragmentation was performed using a fragmentation buffer (from Affymetrix) to break down full-length cRNA to 35 to 200 base fragments by metal-induced hydrolysis.

Target hybridization: GeneChip hybridization was performed in hybridization oven 640 (from Affymetrix) by use of the eukaryotic hybridization control kit and hybridization, wash and stain kit (all from Affymetrix).

Probe array washing, staining, and scanning: The hybridization, wash and stain kit was used for washing and staining of probe arrays in fluidics station 450 (from Affymetrix), and scanning of probe arrays in scanner 3000 7G (from Affymetrix).

Selection of differentially expressed genes: The call value in single chip results was processed by GCOS1.4 (Affymetrix® GeneChip® Operating Software1.4), and the output results were analyzed by dChip2008 software. Here, call in the B-channel being P and fold change $\geqslant 2$ (or call in the A-channel being P and fold change $\leqslant 0.5$) was taken as the criteria for selecting up-regulation (or down-regulation) genes under the treatment conditions. All differentially expressed genes were screened in an Excel document according to the criteria.

Functional analysis of differentially expressed genes:

Statistical analysis of the pathway and gene ontology (GO) involved in differentially expressed genes was performed with the CapitalBio® molecule annotation system (http://bioinfo.capitalbio.com/mas3/). Statistical significance of the pathway or GO was characterized by the p value and q value. The p value is probability value of error rejecting the null hypothesis (H_0: $p_0 = p_1$). It reflects the importance of the pathway or GO in the experimental results. The q value represents the false discovery rate (FDR) of the p value being selected as the threshold.

The molecular function, biological process and cellular component of differentially expressed genes was displayed by GO classification, and the Kyoto Encyclopedia of Genes and Genomes (KEGG), BioCarta and GenMAP was displayed by Pathway database analysis.

Hierarchical cluster of differentially expressed genes: Gene-chip data was clustered using hierarchical clustering based on Pearson correlation coefficients with Mev software (Mev 4.3.02 software, http://www.tm4.org/mev/).

1.6 QRT-PCR analysis of S uptake and assimilation gene expression

Primer design and synthesis: The differentially expressed S uptake- and assimilation-related genes detected by microarray were selected. cDNA sequences (CDS) of these genes were searched in the NCBI database. The primers (**Table 1**) were designed by Primer 5.0 software according to the CDS, and synthesized by Invitrogen Co. Ltd., USA.

Total RNA isolation: After the rice seedlings had been treated by Cd, BSM and Cd+BSM for 3 days, roots were harvested to extract total RNA using the RNAprep pure plant kit (Tiangen, Beijing), according to the manufacturer's manual.

First-strand cDNA synthesis: First-strand cDNA was synthesized by reverse transcribing 5 μL of total RNA in a final reaction volume of 20 μL using TIANScript RT kit (Tiangen, Beijing) according to the manufacturer's instructions. The cDNA concentration was determined by an Eppendorf Biophotometer. According to the cDNA concentration, the volume of reverse transcribing products were regulated to ensure each treatment had the same cDNA concentration.

Quantitative real-time PCR detection: Quantitative real-time PCR analysis was carried out using an ABI PRISM 7500 real-time PCR system (Applied Biosystems, USA). The Ubiquitin 5 (UBQ 5) gene was used as reference gene (Jain et al., 2006), and was amplified in parallel with the target gene allowing gene expression normalization and providing quantification. Detection of real-time RT-PCR products was done by using SYBR® Premix Ex Taq™II (Perfect real time) (TaKaRa, Japan) following the manufacturer's recommendations. The PCR mixture was adjusted to contain 10 μL SYBR® Premix Ex Taq™II (2×), 0.8 μL forward primer (10 μmol/L), 0.8 μL reverse primer(10 μmol/L), 0.4 μL ROX Reference Dye II (50×), 2 μL cDNA template, 6 μL ddH_2O. PCR cycling conditions comprised an initial cycle at 50°C for 1 min, one cycle at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and at 60–64°C (based on different primers) for 34 sec. For each sample, reactions were set up in triplicate

Table 1 Primer sequences of sulfate uptake- and assimilation-related genes

Gene	Forward primer	Reverse primer
Os03g0195500	5′-CTGGTGGATTTCCTGTCGC-3′	5′-ACGTCGGTGCTGTTGGTG-3′
Os01g0719300	5′-CCTGAGAAAGAAACTGCCAAAG-3′	5′-CGAAGATGCCTCCGATGAC-3′
Os09g0240500	5′-ATCGGGCATCATAGACAT-3′	5′-TTGGAGTGGTAACGGAGT-3′
Os03g0195800	5′-ATTGCACCACTCACTTCG-3′	5′-GGATTGATGCCTTTCTTTAT-3′
Os12g0608600	5′-TGGAAAGGGACTATTTAGCG-3′	5′-AACATCATACCCAGAAGCAC-3′
Os02g0102300	5′-CGAGCGACCTTATCACCT-3′	5′-CCTCCTCCCATGTTCTTC-3′
Os05g0386800	5′-GACTCCTGATGGCTATGTT-3′	5′-CTCAGTGGTCTGTGCTCC-3′
Os12g0263000	5′-GGGACAATCTGCGTGAAA-3′	5′-GAGTGATGCTGGTGGAAA-3′
Os01g0149800	5′-GGAGATGGCTGAGGAGGT-3′	5′-TTGCAGGTGCAGTTGT-3′
Os01g0974200	5′-GAACCCACGACCACGACCAC-3′	5′-TGCAGTTGCAGCAGGAGC-3′
Os05g0503300	5′-CTTGGCTATGGATACGCT-3′	5′-CAGGCACCTAATGATGAAC-3′
Os07g0509800	5′-CTTCCGCTACCGCTTCCATCT-3′	5′-TGCTGCTTCACGGCTCCAAT-3′
Os09g0115500	5′-TGGACTGACTGGCACAAAC-3′	5′-ACCGCAAGAGGCGAATAAG-3′
Os04g0111200	5′-CTGATGATGTCCCGTTGC-3′	5′-GTTAATCCGTGCCTTTGC-3′
Os01g0978100	5′-TCGGGTCCTCCTGCTTCT-3′	5′-TGGTCAGTGTAGGGTTGC-3′
Os10g0527800	5′-GCCCGTCCTCATCCACAA-3′	5′-CAGCCCGACCTCCATCTT-3′
Os10g0525500	5′-CTACCTGGACATCGCTCTTG-3′	5′-GTTTGGAACCTTCGCCTC-3′
Os05g0129000	5′-TACGCATTAGATGTCCCG-3′	5′-CATACAACAGCCCAACCC-3′
Os03g0133900	5′-AGAGGGCGTTGAGTTTCG-3′	5′-CTGAGCATCAAGGCGAAT-3′
AF023617.1	5′-GTAAAGAGGCGTGGTTGG-3′	5′-GAAGTATCGTCGGGAAGC-3′

to ensure the reproducibility of the results. At the end of the PCR run, a melting curve was generated and analyzed to verify the specificity of the PCR products.

Statistical analysis: The threshold cycle (CT) values of the triplicate PCRs were averaged and used for quantification of transcripts. The quantification of the relative transcript levels was performed using the comparative CT method (Livak and Schmittgen, 2001). The transcript levels of the target genes were normalized against the UBQ5 gene transcript levels as described in the ABI PRISM 7500 Real-Time PCR System. The relative quantitation (RQ) was calculated by the $2^{-\Delta\Delta CT}$ method:

$$\Delta\Delta CT = (CT_{Target} - CT_{UBQ5})_{treatment} - (CT_{Target} - CT_{UBQ5})_{control}$$

where ΔΔCT is the change in the CT value of the target gene of treatment compared with the control; CT_{Target} is the CT value of the target gene; CT_{UBQ5} is the CT value of UBQ5 gene; $(CT_{Target}–CT_{UBQ5})_{treatment}$ is the change in the CT value of treatment; $(CT_{Target}–CT_{UBQ5})_{control}$ is the change in the CT value of control.

Statistical analysis was conducted using procedures in Excel Software and SigmaPlot 2000.

2 Results

2.1 Toxic effects of Cd, BSM and Cd+BSM on rice seedlings

Root and shoot biomass after exposure of rice roots to Cd, BSM and Cd+BSM for 20 days are shown in **Table 2**.

Compared with control, the Cd, BSM and Cd+BSM treatments highly significantly inhibited root length, shoot height, shoot dry weight and root dry weight of Fengmeizhan seedlings (**Table 2**), indicating inhibited growth of the seedlings. Moreover, the root length when treated with BSM, the shoot height and dry weight treated with Cd, and root dry weight treated with Cd or BSM were all significantly or highly significantly lower than for treatment with Cd+BSM. The results suggested that the Cd+BSM combined treatment alleviated the toxic effects of Cd or BSM single treatments on Fengmeizhan seedlings.

Similarly, compared with control, the Cd, BSM and Cd+BSM treatments significantly or very significantly inhibited the root length, shoot height, shoot dry weight and root dry weight of Yuexiangzhan seedlings (**Table 2**), indicating inhibited growth of the seedlings. Of the two rice cultivars, the degree of inhibition of the Cd treatment on Yuexiangzhan root length, shoot height, and root and shoot dry weight were less than that for Fengmeizhan, suggesting the greater tolerance of Yuexiangzhan to Cd stress compared to Fengmeizhan. The differences in root length, shoot height, shoot dry weight and root dry weight between Cd+BSM and Cd (or BSM) treatments were not significant, suggesting that Cd+BSM treatment did not alleviate the toxic effects of Cd or BSM single treatments on Yuexiangzhan seedlings.

2.2 Differentially expressed genes detected by microarray analysis

For gene-chip microarray analysis, a total number of 57,381 probes were employed to reveal the expression signal. Compared with control, 4490 differentially expressed genes were revealed in the two variety roots under Cd, BSM and Cd+BSM treatments (**Fig. 1**). Among these, 2144 genes were up-regulated (964 genes in Fengmeizhan, 1599 genes in Yuexiangzhan), and 2346 genes were down-regulated (917 genes in Fengmeizhan, 1776 genes in Yuexiangzhan). Nearly twice as many differentially expressed genes were present in Yuexiangzhan roots as in Fengmeizhan roots under the three treatments, which confirmed previous speculation that Fengmeizhan and Yuexiangzhan roots exhibited different gene responses to

Table 2 Root and shoot biomass after exposure of Fengmeizhan and Yuexiangzhan roots to Cd, BSM and Cd+BSM for 20 days

Treatment	Root length (cm)	shoot height (cm)	Root number	Leaf number	Shoot dry weight (g)	Root dry weight (g)
Fengmeizhan						
CK	9.323	16.242	9.808	3.846	0.0609	0.0183
Cd	6.719**a	13.708**b	9.462b	3.846a	0.0452**b	0.00970**b
BSM	5.435**c	15.658**a	11.000*a	3.692b	0.0529**a	0.00900**b
Cd+BSM	6.427**a	15.031**a	12.500**a	4.192*a	0.0497**a	0.0116*a
Yuexiangzhan						
CK	8.381	13.965	11.615	3.923	0.051	0.0115
Cd	7.212**a	12.546**a	11.346a	3.692b	0.038**a	0.00830**a
BSM	7.327**a	13.085*a	12.385a	3.808a	0.047*b	0.00800**a
Cd+BSM	7.477*a	12.454**a	12.615a	4.077a	0.036**a	0.00760**a

* and ** represent significant ($p < 0.05$) and highly significant ($p < 0.01$) differences compared with CK, respectively in Fengmeizhan and Yuexiangzhan. a, b and c represent non-significant, significant ($p < 0.05$) and highly significant ($p < 0.01$) differences compared with Cd+BSM treatment, respectively in Fengmeizhan and Yuexiangzhan.

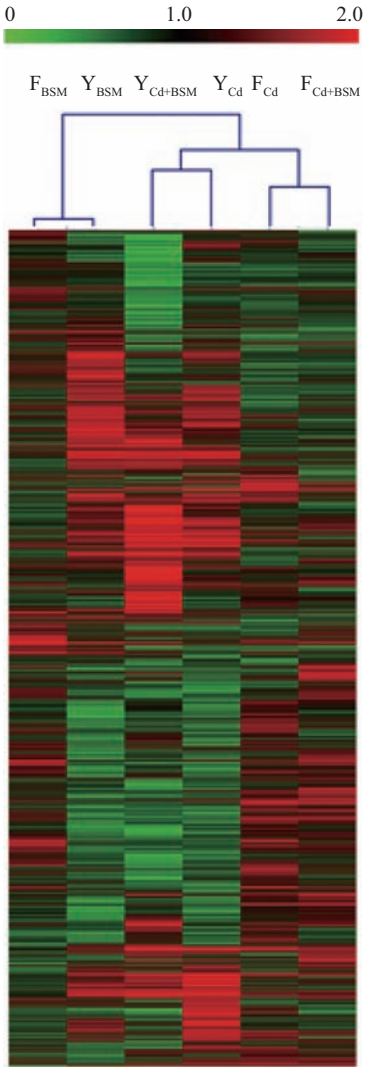

Fig. 1 Hierarchical clustering of differentially expressed genes in Fengmeizhan and Yuexiangzhan roots treated by bensulfuron-methyl (BSM), cadmium (Cd) and their co-contamination (Cd+BSM). Clustering was carried out using the TMeV software package. The relative transcript levels are indicated by the color scale (shown at the top of the figure, the colors red, green and black represent transcriptional up-, down-regulation and unchanged genes respectively). F_{Cd}, F_{BSM}, F_{Cd+BSM} represent Cd, BSM, Cd+BSM treatments for Fengmeizhan, Y_{Cd}, Y_{BSM}, Y_{Cd+BSM} represent Cd, BSM, Cd+BSM treatments for Yuexiangzhan.

Cd, BSM and their co-contamination (Wu et al., 2007).

Figure 1 displays hierarchical clustering and relative transcript levels of all differentially expressed genes. The differentially expressed genes of F_{Cd} (Fengmeizhan with Cd treatment) and Y_{Cd} (Yuexiangzhan with Cd treatment), F_{Cd+BSM} (Fengmeizhan with Cd+BSM treatment) and Y_{Cd+BSM} (Yuexiangzhan with Cd+BSM treatment) did not cluster, suggesting Fengmeizhan and Yuexiangzhan roots exhibited different gene responses to Cd, Cd+BSM co-contamination treatment. However, F_{Cd} and F_{Cd+BSM}, Y_{Cd} and Y_{Cd+BSM} were clustered, which may indicate that Cd plays the major role in co-contamination-treated roots. Meanwhile, F_{BSM} (Fengmeizhan with BSM treatment) and Y_{BSM} (Yuexiangzhan with BSM treatment) were clustered, and thus, the gene responses of both rice variety roots to BSM treatment may be similar.

The statistically significant metabolic pathways indexed by pathway-kegg related to transcriptional up-regulation genes in both cultivar roots under Cd treatment are shown in **Table 3**. Apparently, the metabolic pathways of Cd-inducible genes in both cultivar roots were significantly involved in sulfur metabolism (p-value = 3.38×10^{-6}, q-value = 2.25×10^{-6}), cysteine metabolism (p-value = 2.72×10^{-5}, q-value = 1.70×10^{-5}), and ascorbate metabolism (p-value = 5.48×10^{-5}, q-value = 1.83×10^{-5}), which indicated that Cd treatment might promote sulfate absorption and metabolism in both rice seedlings.

2.3 Influences of Cd, BSM and Cd+BSM treatments on S content in rice seedlings

Due to metabolic pathways of Cd inducible genes involvement with sulfate absorption and metabolism, the influences of Cd, BSM and Cd+BSM treatments on S content in rice seedlings were measured and are shown in **Fig. 2**.

Compared with control, the Cd and Cd+BSM treatments significantly increased the S content in seedlings of both cultivars, whereas BSM also significantly increased the S content in Yuexiangzhan seedlings. This suggested that Cd and Cd+BSM treatments promoted sulfate absorption in both cultivars, and that BSM also promoted sulfate uptake

Table 3 Metabolic pathways indexed by pathway-kegg related to transcriptional up-regulation genes in both cultivar roots under Cd treatment

Pathway	p-Value	q-Value
Phenylpropanoid biosynthesis	7.55E-07	1.51E-06
Sulfur metabolism	3.38E-06	2.25E-06
Fluorene degradation	2.49E-05	1.70E-05
Glycine, serine and threonine metabolism	2.72E-05	1.70E-05
γ-Hexachlorocyclohexane degradation	3.98E-05	1.70E-05
Limonene and pinene degradation	4.26E-05	1.70E-05
Ascorbate and aldarate metabolism	5.48E-05	1.83E-05
Lysine biosynthesis	0.00295	8.42E-04
Methionine metabolism	0.00376	9.41E-04
Alanine and aspartate metabolism	0.00784	0.00150
Phenylalanine, tyrosine and tryptophan biosynthesis	0.00817	0.00150
Methane metabolism	0.00882	0.00150
Arginine and proline metabolism	0.00914	0.00150
Phenylalanine metabolism	0.0101	0.00150
Nicotinate and nicotinamide metabolism	0.0122	0.00150

*p-Value is probability value of error rejecting the null hypothesis (H_0: $p_0 = p_1$). It reflects the importance of the pathway or GO in the experimental results. q-Value represents false discovery rate (FDR) of the p-value being selected as the threshold.

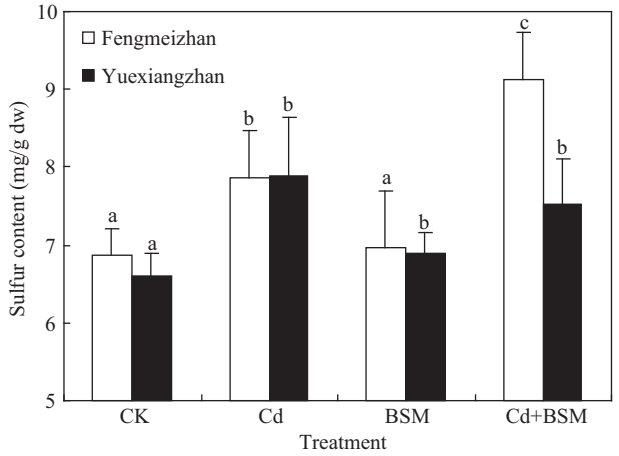

Fig. 2 Effects of Cd, BSM and Cd+BSM treatments on sulfur content in rice plants. a, b and c represent non-significant, significant ($p < 0.05$) and highly significant ($p < 0.01$) differences compared with control (CK), respectively.

in Yuexiangzhan. Moreover, the S content in Fengmeizhan seedlings treated with Cd+BSM was significantly higher than that for seedlings treated by Cd alone, which indicated that Cd+BSM promoted greater sulfate absorption by Fengmeizhan seedlings. This may be one of the mechanisms by which Cd+BSM treatment alleviated Cd toxicity in Fengmeizhan seedlings.

2.4 Differential expression of sulfate uptake- and assimilation-related genes detected by microarray

To reveal the mechanism by which Cd, BSM and Cd+BSM treatments promote sulfate absorption and metabolism, the genes whose functions are related to sulfate uptake and assimilation were further selected among differentially expressed genes detected by microarray, and hierarchical clustering was performed and is shown in **Fig. 3**.

Figure 3 shows the differential transcriptional profiling of sulfur uptake- and assimilation-related genes. As shown in **Fig. 3**, F_{Cd} and F_{Cd+BSM}, Y_{Cd} and Y_{Cd+BSM}, F_{BSM} and Y_{BSM} clustered into three different groups separately, which indicates that the expression responses of genes involved in sulfur uptake and assimilation to Cd and BSM treatments were distinct, and Cd played a predominant role in the Cd+BSM treatment.

In Fengmeizhan roots, the transcription of most Cd-inducible sulfur uptake- and assimilation-related genes was also induced by Cd+BSM treatment, but fewer were induced by BSM treatment. The transcription levels of several sulfur absorption-related genes, such as high affinity sulfate transporter (HAST), sulfate permease (SP), sulfate transporter (ST) 3.1 and ST 4.1 genes, were up-regulated under Cd and Cd+BSM treatments; only the ST 3.1 gene was up-regulated under BSM treatment, which indicates that Cd and Cd+BSM treatments promote greater sulfur absorption than BSM treatment. Similarly, the transcription of sulfur assimilation-related genes, such as rhodanese-like protein (RD-like), ATP sulfurylase (ATPS), cysteine synthase (CS), cystathionine-β-synthase (CBS) and PAPS reductase-like protein (PR), were induced by Cd and Cd+BSM treatments; only a CBS gene was up-regulated under BSM treatment. These results indicate that Cd and Cd+BSM promote sulfate absorption and assimilation by Fengmeizhan roots, which may be one of the mechanisms by which Cd and BSM co-contamination reduces the toxicity of BSM to Fengmeizhan seedlings.

In Yuexiangzhan roots, the transcription of most Cd-inducible sulfur uptake- and assimilation-related genes was also induced by BSM treatment, but the number of up-regulated genes decreased in Cd+BSM compared with Cd treatment. These results indicate that Cd, BSM and Cd+BSM promote sulfate absorption and assimilation in Yuexiangzhan roots, but unlike Fengmeizhan, Cd+BSM treatment did not induce the transcription of sulfur uptake- and assimilation-related genes to a greater degree than BSM or Cd treatments, which might explain why the Cd+BSM treatment did not alleviate the toxic effects of Cd or BSM single treatments in Yuexiangzhan seedlings.

Both in Fengmeizhan and Yuexiangzhan roots, the transcription of sulfate transporter ST1 and sulfite reductase (SiR) genes was only induced by Cd treatment, which further suggest that Cd might play a predominant role in the transcriptional induction of sulfur uptake- and assimilation-related genes in rice roots.

After inorganic sulfate is absorbed and assimilated, sulfide can be used as substrate for synthesis of various S-containing compounds associated with detoxification. As shown in **Fig. 3**, the transcription of phytochelatin synthetase-like protein (PCS-like) and phytochelatin synthetase-like protein 2 (PCS2) genes in both rice cultivar roots was induced by Cd and Cd+BSM treatments; and PCS-like gene in Yuexiangzhan roots was also induced by BSM treatment (**Fig. 2**), which suggests that Cd and Cd+BSM treatments promote the synthesis of phytochelatin (PC) in rice roots. Meanwhile, Cd and Cd+BSM treatments induced transcription of the plant metallothionein family 15 protein (MT15) and metallothionein-like protein (RicMT) genes in both rice cultivar roots; and induced transcription of metallothionein-like protein type 2 (MT-like2) gene in Yuexiangzhan roots. Thus, Cd and Cd+BSM treatments promoted synthesis of metallothionein (MT) in rice roots. Both PC and MT can chelate Cd to protect the cytosol from free Cd ions (Cobbett, 2000).

Glutathione S-transferases (GSTs) are typical phase II detoxification enzymes, which catalyze conjugation of thiol groups to endogenous or exogenous toxins (Hayes et al., 2005). As shown in **Fig. 3**, the transcription of different GST homologous genes in both rice cultivar roots was induced by Cd and Cd+BSM treatments. Moreover, more GST homologous genes were also induced by BSM treatment in Yuexiangzhan roots, whereas only a GST 31 gene was induced by BSM treatment in Fengmeizhan

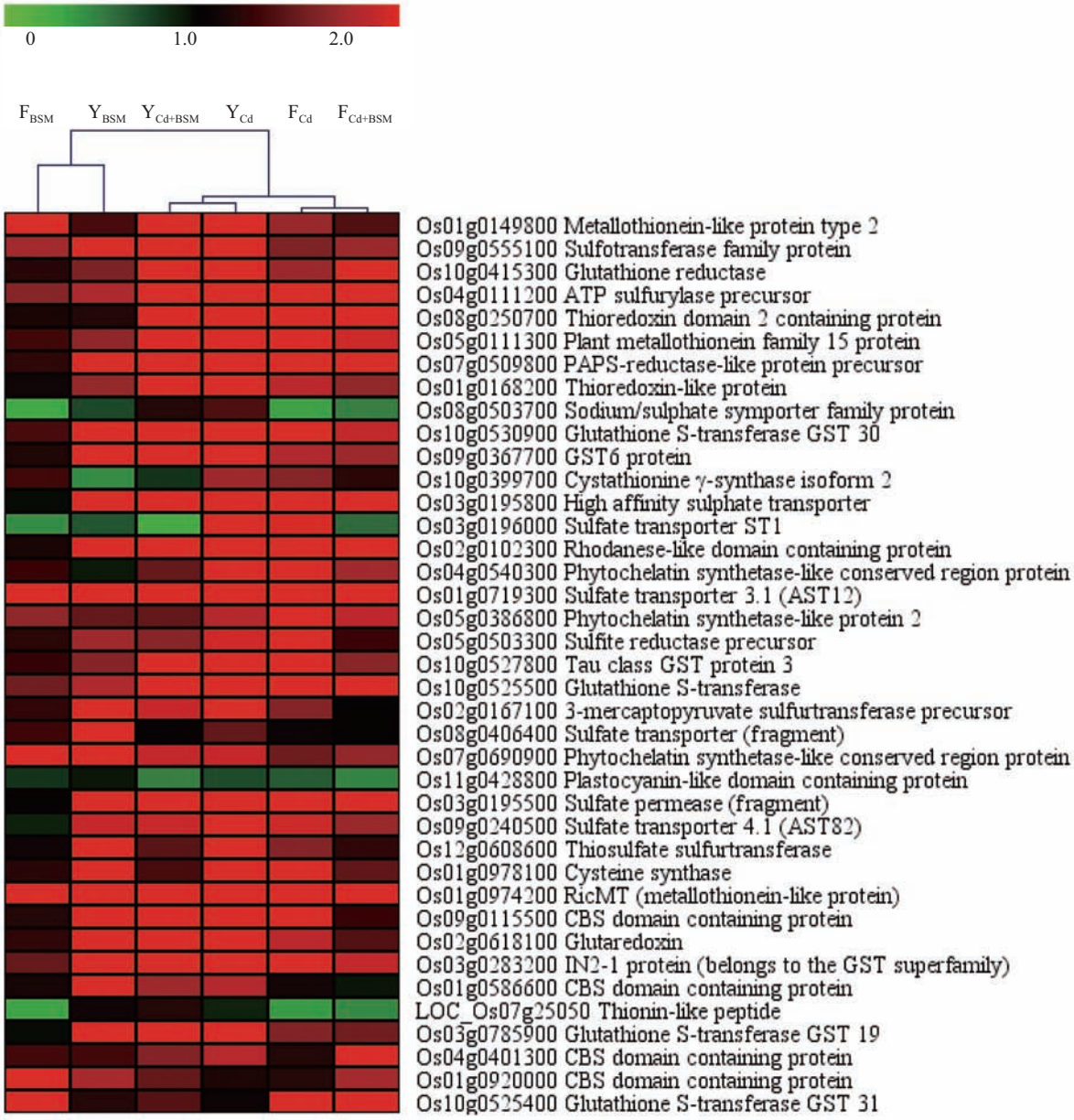

Fig. 3 Hierarchical clustering of sulfate uptake- and assimilation-related differentially expressed genes in Fengmeizhan and Yuexiangzhan roots treated by cadmium (Cd), bensulfuron-methyl (BSM) and their co-contamination (Cd+BSM). Clustering was carried out using the TMeV software package. The relative transcript levels are indicated by the color scale (shown at the top of the figure, the colors red, green and black represent transcriptional up-and down-regulation and unchanged genes respectively).

roots. These results suggest that greater amounts of sulfur absorbed by rice roots under Cd and Cd+BSM or BSM stresses can be used for detoxification.

In response to peroxide stress caused by Cd, BSM and Cd+BSM treatments, it is necessary to activate the antioxidant system in the treated seedlings. In addition to the transcriptional induction of different antioxidant enzyme genes, such as catalase isozymes and peroxidases, which were detected by microarray analysis (data not shown here), **Fig. 3** shows that the transcription of S-containing antioxidation-related enzyme genes, such as glutaredoxin, glutathione reductase, and thioredoxins was induced by Cd, Cd+BSM treatments in both rice cultivar roots, and glutaredoxin was also significantly induced by BSM treatment in Yuexiangzhan roots. The results suggest that the greater amounts of sulfur absorbed by rice roots under Cd and Cd+BSM stresses can be used for synthesis of antioxidant substances.

2.5 Expression levels of sulfate uptake- and assimilation-related genes verified by QRT-PCR

To verify the results of microarray analysis, quantitative real-time PCR was employed to further analyze the tran-

scriptional levels of important sulfate uptake- (**Table 4**) and assimilation-related (**Table 5**) genes in both cultivar roots under Cd, BSM and Cd+BSM treatments, as compared to control.

As shown by the results of QRT-PCR determination, the transcriptional levels of most selected genes agreed with those detected by microarray. **Table 4** shows that, compared with control, the transcriptions of sulfate uptake-related genes (including HAST, ST3.1, ST4.1 and SP) were significantly up-regulated in roots of both rice cultivars under Cd and Cd+BSM treatments. Furthermore, the transcriptions of HAST, ST3.1, ST4.1 were more strongly induced by Cd+BSM than by Cd treatment in Fengmeizhan roots, which might be the reason that Cd+BSM promoted greater sulfate absorption, and might be one of the mechanisms by which Cd+BSM co-contamination reduces the toxicity of Cd alone to Fengmeizhan seedlings. Additionally, the transcription of SP was also significantly induced by BSM treatment in Yuexiangzhan roots, which might lead to an increase of S content in BSM-treated Yuexiangzhan seedlings.

Table 5 shows that, compared with control, Cd treatment significantly induced transcriptions of all selected sulfate assimilation-related genes (including CBS, CS, RD-like, SiR, TSST, ATPS, PR) in roots of both rice cultivars. Except for CS and TSST genes, Cd+BSM treatment

Table 4 Relative expression of sulfate uptake-related gene in Fengmeizhan and Yuexiangzhan roots verified by quantitative real-time PCR

Gene	Protein	QRT-PCR analysis results			
		CK	Cd	BSM	Cd+BSM
Fengmeizhan					
Os03g0195800	HAST	1.00	50.377 ± 4.946	2.408 ± 1.786	62.932 ± 6.096
Os01g0719300	ST 3.1	1.00	2.782 ± 0.216	0.879 ± 0.049	3.147 ± 0.119
Os09g0240500	ST 4.1	1.00	1.918 ± 0.242	0.840 ± 0.178	2.444 ± 0.356
Os03g0195500	SP	1.00	3.069 ± 0.189	0.784 ± 0.053	1.683 ± 0.177
Yuexiangzhan					
Os03g0195800	HAST	1.00	5.561 ± 0.031	0.526 ± 0.049	3.434± 0.691
Os01g0719300	ST 3.1	1.00	1.828 ± 0.392	0.143 ± 0.037	0.043 ± 0.0023
Os09g0240500	ST 4.1	1.00	1.909 ± 0.126	0.474 ± 0.085	1.575 ± 0.155
Os03g0195500	SP	1.00	5.069 ± 0.189	3.190 ± 0.023	6.467 ± 0.227

HAST: high affinity sulfate transporter, SP: sulfate permease, ST: sulfate transporter.

Table 5 Relative expression of sulfate assimilation-related genes in Fengmeizhan and Yuexiangzhan roots verified by quantitative real-time PCR

Gene	Protein	Fengmeizhan			Yuexiangzhan		
		Cd	BSM	Cd+BSM	Cd	BSM	Cd+BSM
Os09g0115500	CBS	4.461 ± 0.074	1.133 ± 0.089	3.738 ± 0.636	2.893 ± 0.289	1.132 ± 0.078	1.636 ± 0.018
Os01g0978100	CS	1.760 ± 0.185	1.285 ± 0.090	0.770 ± 0.012	2.056 ± 0.248	1.105 ± 0.206	0.945 ± 0.452
Os02g0102300	RD-like	2.947 ± 0.046	0.898 ± 0.316	2.925 ± 0.778	3.245 ± 0.542	0.773 ± 0.105	2.291 ± 0.214
Os05g0503300	SiR	1.740 ± 0.145	0.985 ± 0.026	2.043 ± 0.374	5.206 ± 0.155	1.668 ± 0.189	1.574 ± 0.126
Os12g0608600	TSST	1.820 ± 0.233	1.213 ± 0.369	1.188 ± 0.371	1.572 ± 0.037	0.720 ± 0.115	0.946 ± 0.108
Os04g0111200	ATPS	5.217 ± 0.367	1.030 ± 0.257	3.149 ± 0.326	5.509 ± 0.074	0.377 ± 0.002	1.921 ± 0.384
Os07g0509800	PR	6.986 ± 0.211	2.280 ± 0.380	4.026 ± 0.481	25.328 ± 1.117	1.504 ± 0.684	6.332 ± 0.354
AF023617.1	APSR	4.233 ± 0.250	2.669 ± 0.077	5.367 ± 0.304	1.283 ± 0.002	0.617 ± 0.004	3.88 ± 0.154
Os03g0133900	SAT	4.730 ± 0.031	2.408 ± 0.025	6.054 ± 0.014	–	–	–
Os05g0129000	γ-GCS	5.087 ± 0.000	3.221 ± 0.002	6.419 ± 0.002	1.066 ± 0.102	0.584 ± 0.053	0.738 ± 0.055
Os12g0263000	GS	1.305 ± 0.006	0.787 ± 0.110	1.225 ± 0.102	1.352 ± 0.096	0.362 ± 0.070	2.717 ± 0.236
Os05g0386800	PCS-like	2.352 ± 0.219	0.700 ± 0.0172	2.592 ± 0.320	3.037 ± 0.151	0.246 ± 0.004	2.020 ± 0.287
Os01g0149800	MT-like 2	13.967 ± 0.253	3.469 ± 0.314	5.741 ± 0.175	6.407 ± 0.333	3.140 ± 0.133	2.440 ± 0.101
Os01g0974200	RicMT	1.307 ± 0.114	0.921 ± 0.126	2.404 ± 0.019	1.197 ± 0.033	0.607 ± 0.044	1.352 ± 0.024
Os10g0527800	T-GST 3	63.106 ± 1.278	0.782 ± 0.0638	12.164 ± 2.518	46.310 ± 1.612	6.384 ± 0.979	34.873 ± 0.568
Os10g0525500	GST	3.143 ± 0.892	0.367 ± 0.0754	1.909 ± 0.584	3.775 ± 0.422	0.950 ± 0.194	1.843 ± 0.163

CBS: cystathione-β-synthase domain protein; CS: cysteine synthase; RD-like: rhodanese-like domain protein; SiR: sulfite reductase; TSST: thiosulfate sulfurtransferase; ATPS: ATP sulfurylase; PR: PAPS-reductase-like protein; APSR: Adenosine-5′-phosphosulfate reductase; SAT: serine acetyltranserase; γ-GCS: γ-glutamylcysteine synthetase; GS: glutathione synthetase; PCS-like: phytochelatin synthetase-like; MT-like 2: metallothionein-like protein type 2; RicMT: Ric metallothionein; T-GST 3: Tau class GST protein 3; GST: glutathione S-transferases.

also induced transcriptions of the sulfate assimilation-related genes in both rice cultivar roots. Furthermore, Cd, Cd+BSM and BSM significantly induced transcriptions of γ-GCS, SAT and APSR genes in Yuexiangzhan, and Cd+BSM treatment also induced transcriptions of the APSR gene in Yuexiangzhan roots. Taken together, QRT-PCR determination confirmed that Cd and Cd+BSM treatments dramatically activated the expression of sulfate assimilation-related genes in rice roots.

Table 5 also shows that, compared with control, Cd and Cd+BSM treatments significantly induced transcriptions of PCS-like, MT-like 2, RicMT, T-GST 3 and GST in roots of both rice cultivars. BSM treatment also significantly induced transcriptions of MT-like 2 and T-GST 3 in Fengmeizhan roots and MT-like 2 in Yuexiangzhan roots. Moreover, it was found that transcription of glutathione synthase (GS) was slightly or significantly induced by Cd and Cd+BSM treatments in roots of both rice cultivars, which indicated that Cd and Cd+BSM treatments enhance synthesis of glutathione. Clearly, Cd and Cd+BSM treatment activation of the synthesis of detoxification-related S-containing compounds can be confirmed by QRT-PCR determination.

3 Discussion

Cd uptake in rice leads to various toxicity symptoms, growth reduction and cell death (He et al., 2008; Hu et al., 2011). Cd disturbs plant photosynthesis, carbohydrate metabolism, sulfate assimilation and activities of several enzymes even at low concentrations (Iqbal et al., 2010; Pokora and Tukaj, 2010; Devi et al., 2007; Lee and Leustek, 1999). Data in the present study showed that the growth of both cultivars of rice seedlings was significantly inhibited by Cd, BSM, and Cd+BSM treatments. The tolerance of Yuexiangzhan to Cd was higher than that of Fengmeizhan. Moreover, Cd+BSM seemed to relieve Cd toxicity in Fengmeizhan, but not Yuexiangzhan seedlings (**Table 2**).

Cadmium stress induced production of thiol compounds and activated the S uptake and assimilation pathway by increasing transcription of related genes (Harada et al., 2002; Alvarez-Legorreta et al., 2008), but S-deficiency results in severe injury in oilseed rape leaves (Sun et al., 2003). Excessive S supply decreases Cd accumulation in brown rice, but increases Cd accumulation in roots (Fan et al., 2010). In the present study, the S-content determinations suggested that Cd and Cd+BSM promoted sulfate uptake in rice seedlings. Moreover, S content in Fengmeizhan seedlings with Cd+BSM treatment was significantly higher than for the Cd treatment, which may be one of the main mechanisms by which Cd+BSM alleviated Cd toxicity in Fengmeizhan seedlings.

Plants' sulfate acquisition from the soil and transport through numerous cell membranes is mediated by a suite of sulfate transporters. Both the *Arabidopsis* and rice genomes contain 12 homologues of sulfate transporters classified into four distinct groups (Buchner et al., 2004). The transcript level of sulfate transporter genes have been shown to be regulated by the S status of the plant (Rouached et al., 2009; Koralewskaa et al., 2009), and by various abiotic and biotic stresses. With heavy metal stress, some genes involved in the S acquisition and assimilation pathway are known to be transcriptionally activated (Mendoza-Cózatl et al., 2005; Lee and Leustek, 1999). The present study suggested that Cd and Cd+BSM (or BSM) treatments enhanced sulfate absorption in both rice cultivar seedlings by transcriptional induction of a series of sulfate transporter and permease genes, such as HAST, ST3.1, ST4.1 and SP.

After being absorbed via a transport system, inorganic sulfate is reduced and assimilated as sulfide as an essential metabolic process for the synthesis of various S-containing compounds, e.g. sulfolipids, coenzymes and amino acids cysteine (Cys) and methionine (Met). Sulfate is first activated with ATP by ATP sulfurylase (ATPS) to form adenosine 5′-phosphosulfate (APS) (Phartiyal et al., 2006). In the next step, APS kinase phosphorylates APS to phosphoadenylyl sulfate (PAPS) (Gay et al., 2009), and PAPS-reductase (PR) catalyzes the first reductive step leading to the formation of sulfite (Savage et al., 1997). APS can also be reduced by glutathione dependent adenosine-5′-phosphosulfate reductase (APSR) to form sulfite and GSSG. Then sulfite reductase (SiR) catalyzes further reduction of sulfite to sulfide (Nakayama et al., 2000). Serine acetyltransferase (SAT) catalyzes the CoA-dependent acetylation of L-serine to form O-acetylserine. Sulfide from H_2S is incorporated into O-acetylserine catalyzed by O-acetylserine(thiol)-lyase to form cysteine (Saito, 2004). In another pathway of cysteine formation, cystathionine-β-synthase (CBS) catalyzes the first step of the trans-sulfuration pathway, from homocysteine to cystathionine, then cystathione γ-lyase converts cystathionine to cysteine (Banerjee et al., 2003). After cysteine is formed, the synthesis of glutathione and its derivatives are catalyzed by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthase (GS). In the present study, the expression of ATPS, PR, SiR, CBS, CS, TSST, γ-GCS, SAT and APSR genes in Fengmeizhan and/or Yuexiangzhan roots were all induced by Cd or Cd+BSM, which indicated that Cd and Cd+BSM promoted sulfate reduction and assimilation in both rice cultivar roots.

Furthermore, the expression of a RD-like gene was strongly induced by Cd and Cd+BSM in both roots of both rice cultivars. The role of RD (thiosulfate: cyanide sulfurtransferase) in plants is poorly understood. However, it is believed to be involved in cyanide detoxification in animals (Billaut-Laden et al., 2006). The expression induction of RD-like gene might be involved in the production of S

in sulfane form (Giuliani et al., 2010), which is suggested to be the relevant biologically active S-species. Certainly, further research is necessary to determine the role of RD in Cd detoxification.

A strategy for cells to detoxify heavy metal ions is the synthesis of high-affinity binding sites to suppress binding to physiologically important functional groups. The molecules carrying these functional groups have been described as metal chelators. The best-known and presumably most effective chelators for Cd^{2+} are small Cys-rich proteins, MTs and PCs. In plants, accumulation of PCs is triggered by exposure to various physiological and non-physiological metal ions (Scheidegger et al., 2011), and the dominant Cd^{2+} detoxification pathway is mainly mediated by PCs (Cobbett, 2000; Mendoza-Cózatl and Moreno-Sánchez, 2006). Glutathione molecules are the substrate for the biosynthesis of PCs, catalyzed by plant phytochelatin synthases (Ogawa et al., 2011; Jaeckel et al., 2005), which suggests the importance of the availability of reduced S as an essential factor for PC synthesis. In the present experiments, the over-expression of GS gene indicates that Cd and Cd+BSM treatments enhance synthesis of glutathione. Moreover, the expression of PCS-like and PCS2 gene in roots of both rice cultivars was induced by Cd and Cd+BSM, suggesting that Cd and Cd+BSM promoted the synthesis of PCs in rice roots. Additionally, it has been reported that MTs confer Cd^{2+} tolerance in mammals and fungi (Jaeckel et al., 2005; Sutherland and Stillman, 2008), but their contribution in plants is not clear. The expression of MT-like 2, RicMT and MT15 genes in Fengmeizhan and/or Yuexiangzhan roots was induced by Cd and Cd+BSM treatments, indicating that they promoted the synthesis of MTs in rice roots. Therefore, further research is needed to investigate whether MTs also have important detoxifying Cd functions in these two rice cultivars.

The heavy metal Cd and herbicide BSM are exogenous chemicals toxic to rice seedlings. Furthermore a variety of endogenous toxic substances, such as cytotoxic aldehydes, can be induced by Cd stress. The present results demonstrated that the expression of several groups of detoxification-related genes, including GSTs, cytochrome P450s, UDP glycosyltransferases, ABC transporters, were significantly induced in the roots of both rice cultivars. Among them, GSTs are S-transfer-related detoxification enzymes. The detoxification reactions catalyzed by GSTs involve the conjugation of glutathione (GSH) with substances containing electrophilic groups (including cytotoxic aldehydes). The over-expression of different GST homologous genes in both rice cultivar roots suggests that enhanced sulfur absorption by rice roots under Cd and Cd+BSM or BSM stresses is favorable for both the detoxification of substances containing electrophilic groups and for the elimination of cytotoxic aldehydes.

One major mechanism of the toxicity of Cd and BSM is that the stresses promote the excessive production of reactive oxygen species (ROS) and cause oxidative stress and lipid peroxidation (**Table 5**) (Wang et al., 2012). To cope with oxidative stress, up-regulation of antioxidation-related genes in plants has been widely reported (Zeng et al., 2011). The transcriptional induction of glutaredoxin, glutathione reductase, thioredoxin and GS genes found in the present experiments indicates that S uptake and assimilation promoted by Cd and Cd+BSM treatments is favorable for synthesis of antioxidant substances.

4 Conclusions

The present study demonstrated that Cd, BSM and Cd+BSM all inhibited rice seedling growth. Cd and Cd+BSM promoted sulfate uptake by inducing expression of sulfate transporter and permease genes in rice roots. Cd+BSM could alleviate the Cd toxicity in Fengmeizhan seedlings probably due to Cd+BSM promoting more S absorption by seedlings. Cd and Cd+BSM induced the expression of a series of sulfate assimilation-related genes, and thus activated the sulfate assimilation pathway. Cd and Cd+BSM induced expression of PCS, MT, GSTs and antioxidation-related genes, which performed the function of Cd^{2+} or BSM detoxification.

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