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Effect of ochratoxin A and buthionine sulfoximine on proteome and ascorbate-glutathione cycle enzymes in *Arabidopsis thaliana*

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Abstract

In this study, proteome and activities of glutathione (GSH)-related enzymes were investigated in detached leaves of *Arabidopsis thaliana* treated with ochratoxin A (OTA) alone or supplemented with buthionine sulfoximine (BSO, a specific inhibitor of the first step in GSH biosynthesis). A comparative proteomic study using two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry (MALDI-TOF/TOF MS/MS) identified 12 differentially expressed proteins mainly involved in GSH metabolism, energy metabolism, sugar metabolism, and photosynthesis. The treatment with OTA significantly enhanced the activities of glutathione-S-transferase (GST) and glutathione reductase (GR) through up-regulating the corresponding genes (*GSTF7, GR1*), an the diminishing effect of BSO on them counteracted the results. However, both OTA and BSO decreased the activity of ascorbate peroxidase (APX), and OTA also decreased the monodehydroascorbate reductase (MDHAR) and glutathione peroxidase (GPX) activities. Briefly, the OTA-induced phytotoxicity to the *A. thaliana* detached leaves was increased slightly by addition of BSO, and the fluctuation in GSH synthesis, GSH metabolism and disorder of cellular metabolism happened.

Additional key words: ascorbate peroxidase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, monodehydroascorbate reductase.

Introduction

Tripeptide glutathione (γ-Glu-Cys-Gly, GSH), the most abundant source of low molecular mass thiols in plants and other organisms, is synthesized in two steps. The first step is the formation of γ-glutamylcysteine from glutamate and cysteine catalyzed by γ-glutamylcysteine synthetase (γ-GCS, EC 6.3.2.2.) and the second step is the conjugation of γ-glutamylcysteine with glycine catalyzed by glutathione synthase (GSH-S, EC 6.3.2.3.) (Meister 1995). GSH serves a broad range of biochemical functions (Penninckx 2000, Ogawa 2005, Kumar *et al*. 2011, Wójcik and Tukiendorf 2011), especially it is a major cellular antioxidant and determinant of redox state (Noctor *et al*. 2002, Meyer 2008, Szalai *et al*. 2009). GSH protects plants against oxidative stress by

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Abbreviations: APX - ascorbate peroxidases; ASH - reduced ascorbate; AsA - ascorbate; BSO - buthionine sulfoximine; DGE - digital gene expression; DHA - dehydroascorbic acid; DHAR - dehydroascorbate reductase; GSH - reduced glutathione; GPX - glutathione peroxidases; GR - glutathione reductase; GSH-S - glutathione synthase; GS2 - glutamine synthetase 2; GSSG - oxidized glutathione; GST - glutathione-S-transferase; HCF136 - high chlorophyll fluorescence 136; HR - hypersensitive response; MDHAR - monodehydroascorbate reductase; OTA - ochratoxin A; PCD - programmed cell death; ROS - reactive oxygen species; TIM - triosephosphate isomerase.
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scavenging reactive oxygen species (ROS) *via* oxidation of reduced GSH to its oxidized form (GSSG). Then, GSSG can be reduced by glutathione reductase (GR, EC 1.6.4.2). Besides, GSH acts as reducing agent to scavenge H_2O_2 not only in the ascorbate-glutathione (ASC-GSH) cycle, where dehydroascorbate reductase (DHAR, EC 1.8.5.1) catalyses dehydroascorbic acid (DHA) reduction to ascorbic acid (AsA) (Srivalli and Khanna-Chopra 2008), but also in a reaction catalyzed by glutathione peroxidase (GPX, EC 1.11.1.9) (Chang *et al*. 2009). Moreover, as the substrate of glutathione-S-transferases (GST, EC 2.5.1.18), GSH can remove lipid peroxides, heavy metals, and herbicides by the formation of conjugates (Sharma and Dietz 2006, Sappl *et al*. 2009, Szalai *et al*. 2009). GSH and GSSG, together with GSH S-conjugates, provide a control or signal for functional changes, such as ROS levels maintaining, signalling pathways mediation, *etc*. (Jones 2002, Srivalli and Khanna-Chopra 2008).

In view of the diverse functions of GSH, GSH-related metabolic processes have raised considerable concern in research on mycotoxin-induced phytotoxicity, and it was shown that GSH functions importantly in the defence responses of plants to mycotoxin through ROS elimination. For example, trichothecene causes programed cell death (PCD) and severe wilting in tomato plants due to increased ROS accumulation associated with shift of reduced AsA and GSH towards their oxidised forms (Paciolla *et al*. 2008, Arunachalam and Doohan 2013). In addition, in the previous study of barley-deoxynivalenol interaction, expressions of genes encoding GSTs and cysteine synthases were up-regulated and the nonenzymatic formation of deoxynivalenol-GSH adducts were detected *in vitro*, both indicating the important role of glutathione in deoxynivalenol detoxification (Gardiner *et al*. 2010).

Ochratoxin A (OTA), a mycotoxin produced by several species of *Penicillium* and *Aspergillus,* routinely contaminates cereals and agricultural products, which results in common exposure to OTA in human population (Khoury and Atoui 2010, Tittlemier *et al*. 2011). In the presence of OTA, root growth of *Allium cepa* (Lerdal *et al*. 2010) and *A. thaliana* (Peng *et al*. 2010, Wang *et al*. 2013) was inhibited, and anthocyanin accumulation in *A. thaliana* was observed (Wang *et al*. 2013). In addition, cell death induced by OTA was observed with features resembling the hypersensitive response (HR)-type lesions in excised *A. thaliana* leaves, where the occurrence of an oxidative burst was happened (Peng *et al*. 2010). Besides, the mechanism of OTA-induced phytotoxicity to *A. thaliana* has been discussed to be a complex process from both digital gene expression (DGE) transcriptomic and two-dimensional electrophoresis (2-DE) proteomic analyses (Wang *et al*. 2012, 2013). The results showed that OTA increases ROS production, activates the corresponding antioxidant system, induces changes in transcription factors, photosynthesis, photorespiration, and redox homeostasis.

Our previous transcriptional analysis of *A. thaliana* leaves showed that 29 GSH-related genes are OTA-responsive (Table 1 Suppl., Wang *et al*. 2012). Genes encoding GST, GR, GPX, DHAR, monodehydro-ascorbate reductase (MDHAR, EC 1.6.5.4), *etc*., all exhibit more than 2-fold modification, and most increase. Of these, 17 are GST-related genes. Besides, we have recently demonstrated that the accumulation of GSH in the detached leaves of *A. thaliana* is induced by an OTA treatment through changing related enzymes and genes, and the ROS, malondialdehyde, and GSH accumulations are significantly affected in mutants *gsh1*, *gr1*, and *gpx2* after the treatment with OTA (Wang *et al*. 2014). These results indicate the direct involvement of GSH in the response of *A. thaliana* to OTA. To further explore the role of GSH in the response of plants to OTA, buthionine sulfoximine (BSO) was used as modulator of GSH. Both *in vivo* and *in vitro*, BSO is a strong inhibitor of γ-GCS which catalyzes the rate-limiting step in the *de novo* synthesis of GSH in animals (Cuperus *et al*. 2011). Therefore, a reduced GSH content by a BSO treatment can influence the OTA-induced changes in stress response and cell death of *A. thaliana*. To examine these hypotheses, we employed BSO as GSH modulator to investigate the effects of OTA on *A. thaliana* from physiological, genetic, and proteomic aspects. The results could provide a further insight into the mechanism of OTA-induced phytotoxicity to *A. thaliana*.

Materials and methods

Plants and treatments: *Arabidopsis thaliana* L. ecotype Columbia-0 (Col-0) were cultivated as described (Peng *et al*. 2010). Briefly, seeds were germinated on a Murashige and Skoog medium (*Sigma-Aldrich*, St. Louis, MI, USA) containing 2 % (m/v) sucrose and 0.8 % (m/v) agar, pH 5.8. After 3-d vernalization at 4 °C, the seeds grew under the following conditions: a 16-h photoperiod, a photosynthetic photon flux density of 100 μmol m^{-2} s^{-1}, a temperature of 20 ± 2 °C, and a relative humidity of 40 - 60 %. Seven-day-old seedlings were planted into soil. Leaves of 4-week-old plants were used for experiments. OTA was extracted and purified as described previously (Gómez *et al*. 2006, Liang 2008, Peng *et al*. 2010). OTA-producing *Aspergillus ochraceus* strain 3.4412 grew on maize particle medium for 28 d and then 200 cm^3 of trichloromethane in 0.1 M phosphoric acid (10:1) solution was added. After shaking for 30 min, the mixture was filtered with gauze and OTA was extracted in trichloromethane through liquid-liquid extraction (trichloromethane and 0.1 M $NaHCO_3$, HCl

was used for adjusting pH). After rotary evaporation, OTA was dissolved in 3 cm^3 of methanol and then purified by thin layer chromatography, in which a mixture of methylbenzene, ethyl acetate and acetic acid (v/v/v 6:3:1) was used as developing solvent. The OTA-contained band was cut under UV radiation and then washed by a solution containing trichloromethane and methanol (v/v 2:1). After rotary evaporation, OTA was finally dissolved in 1 cm^3 of methanol and filtered through 0.45 nm microfilter. *Waters LCM1* chromatograph (*Waters Corporation*, Milford, MA, USA) with a fluorescence detector *Waters 2475* was used to determine the purity and concentration of OTA with the following parameters: a 250 × 4.6 mm C18 column, excitation wavelength of 330 nm, emission wavelength 460 nm, and the mobile phase (57 % acetonitrile, 41 % water, and 2 % acetic acid) at rate of 1 $cm^3\ min^{-1}$. The standard OTA and all chromatographically pure chemicals mentioned above were purchased from *Sigma-Aldrich*.

Excised leaves from four-week-old *A. thaliana* were pre-incubated with 2 mM BSO (*B2515, Sigma-Aldrich*) or water in Petri dishes for 6 h. Then, OTA (150 μM) or the same volume of solvent (methanol) were added. Samples were classified as control (water + methanol), BSO (BSO + methanol), OTA (water + OTA), and BSO+OTA and collected 6 and 24 h after the addition of OTA.

Protein extraction and two-dimensional electrophoresis: The extraction of total proteins was performed, and 2-DE was done as described before (Wang *et al.* 2012). The leaves (1 g) were washed with water and ground in liquid nitrogen before they were homogenized in 1 cm^3 of homogenization buffer (20 mM Tris-HCl, pH 7.5, 250 mM sucrose,10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiotreitol (DTT), 1 % (v/v) *Triton X-100*, 1 % (m/v) polyvinylpolypyrrolidone (PVPP), 2 % (m/v) nonidet P40 (NP-40) and 0.5 % (v/v) protease inhibitor cocktail) (*Sigma-Aldrich*). The homogenate was centrifuged at 12 000 *g* and 4 °C for 10 min and the supernatant were precipitated with 10 % (m/v) trichloroacetic acid (TCA) on ice for 60 min, and then centrifuged at 20 000 *g* and 4 °C for 20min. After washing three times with acetone the pellet was solubilized in 0.5 cm^3 of lysis buffer containing 7 M urea, 2 M thiourea, 4 % (m/v) 3-[(3-cholamido-propyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1 % (m/v) DTT, 1 % (v/v) nonlinear (NL) IPG buffer (pH 3 - 10), and 0.5 % (v/v) protease inhibitor cocktail (*GE Healthcare*, Waukesha, WI, USA. The extracted protein was quantified using a 2-DE *Quant* kit *NJ08855* (*GE Healthcare*) with bovine serum albumin as standard. The total protein (1 mg) was loaded onto 24-cm *pH 3-10 NL* IPG strips (*GE Healthcare*) for isoelectic focusing. Then, the IPG strips were equilibrated with DTT and iodoacetamide prior to electrophoresis on 12.5 % (m/v) sodium dodecylsulphate (SDS)-polyacrylamide gels in the second dimension. The equilibrated strips were run at 20 mA per gel for 1 h, then adjusted the electric current to 50 mA per gel and run until the dye front reached the bottom end of the gel. Proteins were visualized with *Coomassie Brilliant Blue R-250* after a 45 min protein fixation in solution containing 50 % ethanol, 10 % acetic acid and 40 % water. After destaining in solution containing 30 % ethanol, 8 % acetic acid, and 62 % water for 2h, the gels were washed with water. Then, image digitization was carried out with an *Image Scanner III* (*GE Healthcare*). Protein amounts in 2-DE gel images were compared using the *Image Master 2-DE Platinum 7.0* software (*GE Healthcare*). At least three gels were analyzed for each treatment and according to previous studies (Zhang *et al*. 2006, Wang *et al*. 2012, 2013) protein spots with significant changes (at least 1.5-fold) were carefully cut out and then subjected to in-gel trypsin digestion and matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry (MALDI-TOF/TOF MS/MS) experiments. The *GPS Explorer* software, *v. 3.6* (*Applied Biosystems,* Foster City, CA, USA) was used to create and search files with the *MASCOT* search program for peptide and protein identification. The *NCBI Greenplant* database restricted to tryptic peptides was used for the search. All proteins identified had protein scores ≥ 58 with expected *P*-values < 0.05. All of the MS/MS spectra were further validated manually. The function of the identified proteins was confirmed by searching the *UniProt* database (http://www.ebi.uniprot.org) and *TAIR* database (http://www.arabidopsis.org/).

Determination of enzyme activities: Enzymes were extracted from samples of all variants 6 and 24 h after the treatment according to Ma and Cheng (2004) with minor modifications. Briefly, 1 g of leaf samples was homogenized with a pre-cooled mortar and pestle in 10 cm^3 of an ice-cold extraction buffer containing 50 mM K_2HPO_4-KH_2PO_4 (pH 7.4), 0.1 mM ethylenediaminetetraacetic acid, and 4 % (m/v) insoluble polyvinylpolypyrrolidone. Afterwards, the extract was centrifuged at 12 000 g and 4 °C for 30 min and the supernatant was used for enzyme activity measurements.

The specific activities of ascorbate peroxidase (APX, EC 1.11.1.11), MDHAR, DHAR, and GR were determined according to the methods of Nakano and Asada (1981), Knöraer (1996), Hossain and Asada (1984), and Ma and Cheng (2004), respectively. GPX and GST specific activities were assayed with GPX and GST detection kits (*A005 and A004, Nanjing Jiancheng Bioengineering Institute,* Nanjing, China). Protein content was determined as described Bradford (1976) using bovine serum albumin as standard.

RNA extraction and real-time quantitative PCR analysis: According to the manufacturer's instruction, total RNAs from samples of all variants 6 h after the

treatment were prepared using a *RNAprep* pure plant kit (*Tiangen,* Beijing, China) and purified using a RNeasy RNA purification kit (*Qiagen,* Dusseldorf, Germany) with on-column DNase digestion. Total RNA was reverse-transcribed into cDNA using a first-strand cDNA *Quantscript RT* kit (*Tiangen*). Real-time PCR experiments were performed in an *ABI 7500* real-time PCR apparatus (*Applied Biosystems*) from 35 to 40 cycles with primers listed in Table 2 Suppl. for *GPX6*, *GSTF7*, *SAPX*, *DHAR1*, *MDHAR1*, *GR1*, and *Actin2* at respective annealing temperatures.

Statistical analysis: Each experiment was performed independently for at least three times. Statistical calculations were performed with SPSS Statistics v. 18. Results were considered statistically significant when $P \leq 0.05$.

Results

Detached leaves of *A. thaliana* pretreated with or without 2 mM BSO were exposed to 150 μM OTA for 24 h. BSO alone showed no visible phytotoxic effect on leaves, however, the OTA exposure induced visible macroscopic lesions on the leaves. Both the amount and the area of the lesions slightly increased when the leaves were pretreated with BSO (Fig. 1 Suppl.).

Quantitative changes (at least 1.5-fold) in 18 proteins were revealed among the experimental variants (Fig. 1, Table 1). Differentially expressed protein spots were analyzed by MALDI-TOF/TOF MS/MS and *MASCOT* searching. Though 6 protein spots (SPs 1, 2, 5, 6, 7, and 18) showed relatively low total ion scores, 12 other proteins were identified unambiguously. The identified proteins were mainly involved in the following processes: the synthesis and metabolism of GSH, energy metabolism, sugar metabolism, and photosynthesis.

It is noteworthy that 3 protein spots (SPs 3, 4, and 15) were identified as GSTs of which 2 were AtGSTU19 and AtGSTF2. Besides, 2 protein spots (SPs 12 and 13) were identified as glutamine synthetase 2 (GS2, EC 6.3.1.2). For the spots identified as the same protein AtGSTU19 or GS2, neither the pI nor the molecular mass was the same indicating that there might be different post-translational modifications which could regulate activities or localizations of the protein. The accumulation of AtGSTU19 was shown in both the groups treated with BSO or OTA alone, and OTA improved AtGSTU19

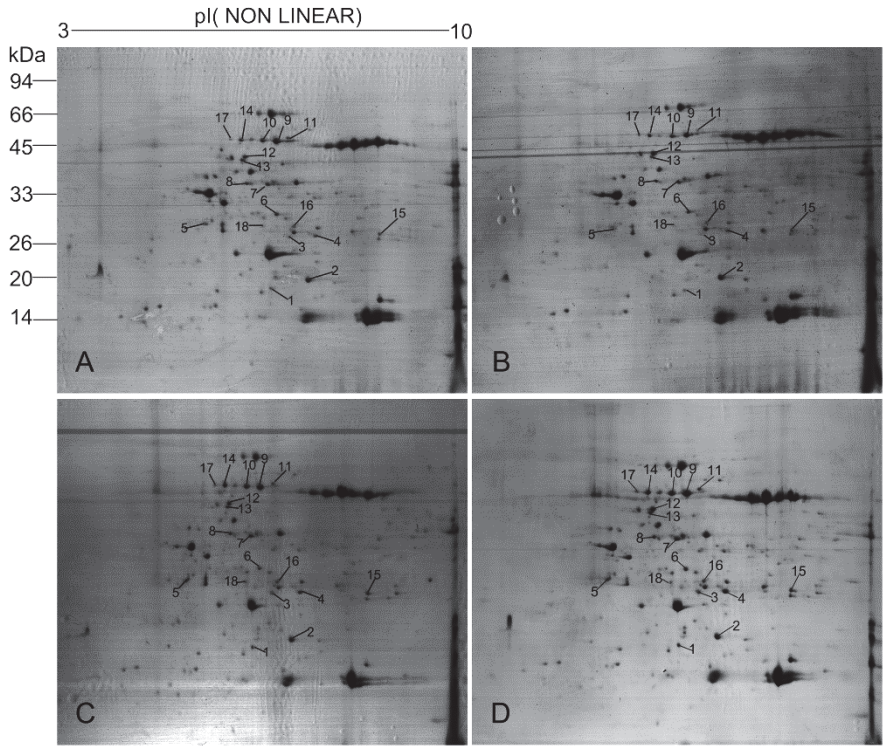

Fig. 1. Images of 2-DE gels of total proteins of *A. thaliana* leaves after treatments: *A* - control, *B* - BSO, *C* - OTA, *D* - BSO+OTA. *Arrows* indicate proteins that are differentially expressed. Protein spots are numbered corresponding to the numbers in Table 1.

Table 1. Identification of *A. thaliana* intracellular proteins showing differential expressions under BSO, OTA, and BSO+OTA treatments compared with the control using the MS/MS analysis. Spot numbers correspond to those in Fig. 1. Protein score - the score obtained from *MASCOT* for all matches. Fold change - the average change in abundance expressed as the mean from three independent experiments.

Spot	NCBI acc. No.	Protein name	Mr [kDa] theor/exp	pI theor/exp	Protein score	[%]	Fold change BSO	OTA	BSO +OTA
3	gi|18411929	AtGSTU19 (glutathione-S-transferase U19; glutathione-S-transferase 8)	25.63/35.1	5.8/5.52	62	88.36	-1.19	2.30	3.12
4	gi|18411929	AtGSTU19 (glutathione-S-transferase U19; glutathione-S-transferase 8)	25.63/35.6	5.8/5.74	123	100	1.62	2.93	5.45
8	gi|15237225	HCF136 (high chlorophyll fluorescence 136)	44.07/43.4	6.79/5.16	68	96.876	-1.74	-1.16	1.12
9	gi|7525040	ATP synthase CF1 beta subunit	53.9/51	5.38/5.39	72	98.836	-2.20	1.10	1.22
10	gi|7525040	ATP synthase CF2 beta subunit	53.9/51	5.38/5.29	102	99.999	-1.35	1.01	1.88
11	gi|18415911	ATP synthase beta chain 2, mitochondrial	59.63/53	6.18/5.52	62	87.527	-1.89	1.34	1.39
12	gi|15238559	GS2 (glutamine synthetase, chloroplastic/mitochondrial); glutamate-ammonia ligase	47.38/47.6	6.43/5.14	58	70.081	1.42	1.21	2.43
13	gi|15238559	GS2 (glutamine synthetase, chloroplastic/mitochondrial); glutamate-ammonia ligase	47.38/46.3	6.43/5.13	105	100	-1.68	-1.75	1.06
14	gi|7525018	ATP synthase CF1 alpha subunit	55.29/52	5.19/5.13	142	100	-1.06	1.79	2.90
15	gi|15235401	AtGSTF2 (glutathione-S-transferase class phi 2)	24.113/35.5	5.92/6.2	165	100	1.00	3.64	3.08
16	gi|15233272	TIM (triosephosphate isomerase, cytosolic)	27.152/36.5	5.39/5.54	109	100	1.04	1.09	1.61
17	gi|7525018	ATP synthase CF1 alpha subunit	55.294/52	5.19/5.07	152	100	1.19	2.61	3.84

stronger. The most significant increase was exhibited in the BSO+OTA-treated group (3.12- and 5.45-fold changes). BSO showed no effect on AtGSTF2, but OTA induced the accumulation of stress-responsive AtGSTF2 and AtGSTU19. Therefore, a significant increase of all the three spot expressions in the BSO+OTA group indicated that the metabolism of GSH was influenced, mainly by OTA. In addition, 5 protein spots (SPs 9, 10, 11, 14, and 17) were ATP synthase subunits, which were reduced by BSO but promoted by both the OTA and BSO+OTA treatments, suggesting the ATP synthesis was OTA/BSO-responsive. Furthermore, cytosolic triosephosphate isomerase (TIM) (SPs 16) involved in sugar metabolism, and the high chlorophyll fluorescence 136 (HCF136) (SPs 8), which participated in the assembly of *A. thaliana* photosystem (PS) II reaction center complexes, were also identified.

Considering the alteration in morphology of leaves treated with BSO+OTA and the changes in proteins involved in GSH-related processes, specific activities of GST, GPX, and enzymes involved in the ASC-GSH cycle (APX, DHAR, MDHAR, and GR) were measured after 6 and 24 h of the treatment. There were no obvious differences in the specific activities of GPX between the leaves exposed to OTA alone and its combination with BSO (Fig. 2*A*). A diminished GPX specific activity after 24 h in the samples treated with OTA and BSO+OTA compared with the control and the BSO-treatment alone was due to the toxic effect of OTA. At 6 h, the GST specific activity was enhanced obviously in the samples treated with OTA alone, but the decreasing effect of BSO led to the parallel GST specific activity in the BSO+OTA group with control (Fig. 2*B*). In addition, a similar effect was observed in the measurement of the DHAR (Fig. 2*D*) and GR (Fig. 2*F*) specific activities and BSO showed a more significant effect on the latter one (the BSO treatment alone decreased the GR specific activity by 33.6 % at 24 h). However, both OTA and BSO inhibited the APX specific activity in comparison with the control and an aggravated decrease (by about 40 %) was detected in the BSO+OTA group (Fig. 2*C*). There was no apparent fluctuation in the specific activity of MDHAR until 24 h when OTA showed a decreasing effect (Fig. 2*E*).

The changes in transcription of GSH-associated genes (*GPX6*, *GSTF7*, *SAPX*, *DHAR1*, *MDHAR1*, and *GR1*) in leaves treated for 6 h (Fig. 3) further verified the effect of BSO coupled with OTA on the GSH-related system. Compared with the control, increases in expressions of *SAPX* (by about 80 %), *DHAR1* (20 %), and *GR1* (60 %) were induced by BSO alone, and increases in expressions of *GPX6* (by about 100 %), *GSTF7* (140 %), *SAPX* (150 %), *DHAR1* (150 %), and *GR1* (140 %) were induced by OTA alone. However, when BSO+OTA were applied, increases in expresions were lower (*GSTF7* by

about 30 % and *SAPX* by about 20 %) compared with OTA alone. Interestingly, both BSO and OTA inhibited the expression of *MDHAR1* by 34 % and 22 %, respectively, but the combination of them showed an increase compared with the OTA-treated group (Fig. 3).

Discussion

OTA is toxic to both animals and plants with induced oxidative stress and other symptoms. GSH serves to a broad range of biochemical functions involving oxidative stress elimination and detoxification of xenobiotics. Moreover, the promotion of GSH-related genes was one of the remarkable effects of OTA to *A. thaliana* (Wang

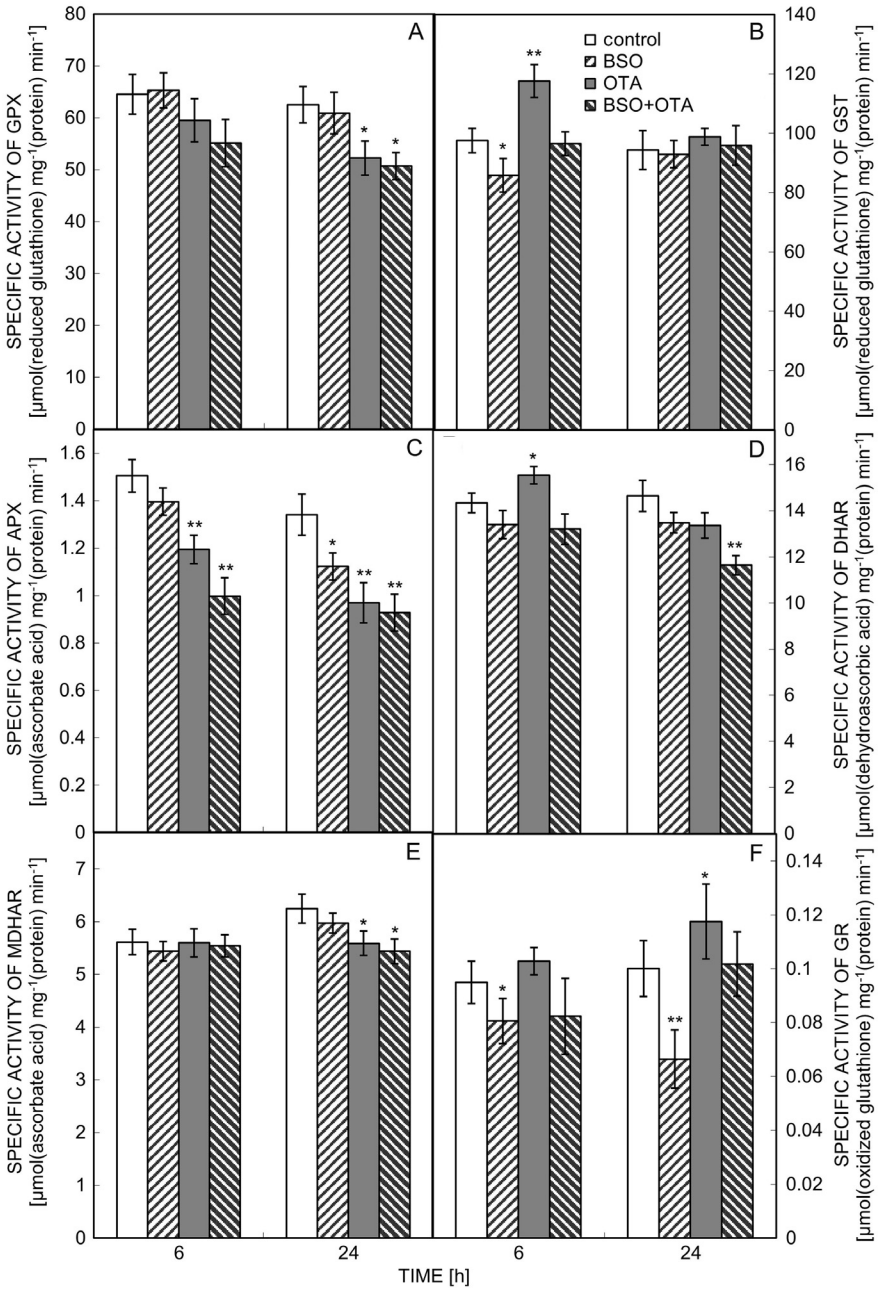

Fig. 2. Specific activities of selected enzymes: *A* - GPX, *B* - GST, *C* - APX, *D* - DHAR, *E* - MDHAR, *F* - GR. Four-week-old *A. thaliana* leaves were treated with methanol (control), BSO, OTA, and BSO+OTA, and harvested after 6 or 24 h. Means ± SD, $n = 3$. *, ** indicate values that differ significantly at $P < 0.05$ and 0.01, respectively, from controls.

et al. 2012, 2014). In this study, BSO, which could reduce about 90 % of GSH content in *A. thaliana* proved by our recent study (Wang *et al*. 2014), was used as modulator of effects of OTA. According to the morphology observation, the BSO+OTA treatment just slightly aggravated the damage of detached *A. thaliana* leaves caused by the OTA stress under the conditions investigated. Therefore, other changes must happen to make up the effect of BSO-induced low GSH. After the treatment with BSO+OTA for 6 h, the GR specific activity showed little difference in the BSO+OTA group compared with the BSO group, but the up-regulated transcription of *GR1* was observed, and after 24 h, the GR specific activity increased in the BSO+OTA group. The results indicate that although BSO inhibited the GSH synthesis, GSH regeneration by GR was enhanced by OTA. In addition, the total AsA content showed little changes in response to OTA and the increased AaA pool induced by BSO might compensate for the loss of glutathione (Wang *et al*. 2014), which was also in line with the decreased APX, DHAR, and MDHAR activities caused by BSO+OTA in our study.

GPXs, as important ROS scavengers, can catalyze H_2O_2 and lipid hydroperoxide conversions to H_2O and alcohol, respectively (Ursini *et al*. 1995, Fu *et al*. 2002, Chang *et al*. 2009). The elevated content of ROS and lipid hydroperoxides in leaves of *A. thaliana* promoted by OTA (Peng *et al*. 2010) might account for the up-regulation of *GPX6.* In addition, from the proteomic perspective, we observed that, when GSH was inhibited, the metabolism of GSH by GSTs was up-regulated in response mainly to the stress by OTA, and the energy metabolism, sugar metabolism, and photosynthesis fluctuated in the leaves. Previous studies have shown that GSTs function as GSH transferases, GSH-dependent peroxidases, isomerases, oxido-reductases, as well as non-enzymatic carrier proteins (Edwards and Dixon 2005, Sappl *et al*. 2009). In *A. thaliana*, *tau* and *phi* GSTs are most abundant and involved mainly in response to abiotic stresses including xenobiotics (Liu *et al*. 2013). In this study, the increased GSTs indicate the important role of them in *A. thaliana* defence against the OTA stress. In

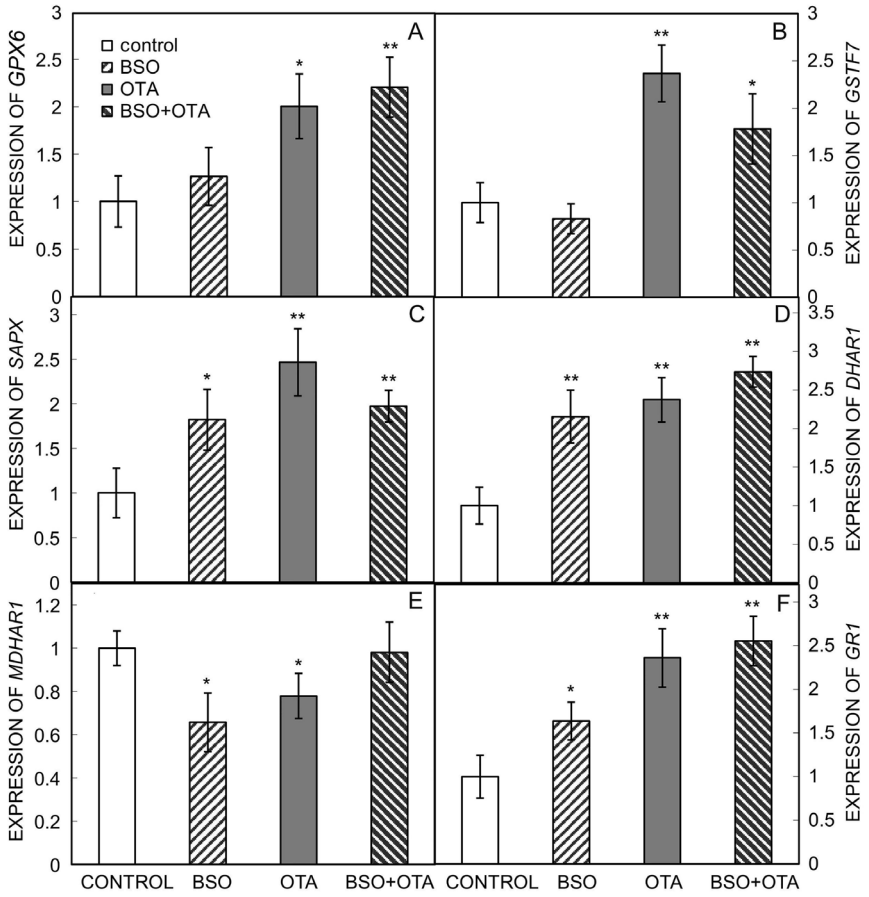

Fig. 3. Real-time qPCR analyses of the selected genes encoding glutathione peroxidases, *GPX6* (*A*); glutathione-S-transferase, *GSTF7* (*B*); ascorbate peroxidase, *SAPX* (*C*); dehydroascorbate reductase, *DHAR1* (*D*); monodehydroascorbate reductase, *MDHAR1* (*E*); glutathione reductase, *GR1* (*F*). Four-week-old *A. thaliana* leaves were treated with methanol, BSO, OTA, and BSO+OTA, and samples were harvested 6 h after the addition of OTA. Means ± SD, $n = 3$. *, ** indicate values that differ significantly at $P < 0.05$ and 0.01, respectively, from controls.

the BSO-supplemented leaves treated with OTA for 24 h, 3 spots of differently expressed proteins were identified as tau or phi GSTs, and two of them were identified as AtGSTU19. Considering that the gene expression of *GSTU19* is induced in *A. thaliana* when exposed to salicylic acid, H_2O_2, hypoxia, and OTA (Sappl *et al*. 2009, Wang *et al*. 2012), AtGSTU19 is stress sensitive. When GSH was inhibited by BSO, the up-regulated AtGSTU19 was shown in the BSO+OTA group compared with BSO treated alone, which provided evidence that OTA was the main reason for the sharpened toxicity. More remarkable, *A. thaliana phi* class glutathione transferase AtGSTF2, often highly stress-inducible, was 3.08 fold higher in the OTA+BSO group than in the control or BSO group. In addition, although BSO showed the inhibition effect on the activity of GST and the gene expression of *GSTF7*, a rising tendency induced by OTA still existed in the BSO+OTA group. In view of lipid peroxidation caused by OTA (Peng *et al*. 2010), the increased GSTs might play roles in assisting GSH in lipid peroxidation elimination when suffering from OTA exposure. Moreover, a previous study showed that a recombinant AtGSTU19 protein can conjugate GSH and benzyl ITC (Dixon *et al*. 2009, Hara *et al*. 2010). The binding and regulating function of AtGSTF2 by biologically active heterocyclic compounds suggests an allosteric enhancement in GSH-conjugating activity (Dixon *et al*. 2011). Therefore, the formation of conjugates was one of the possible mechanisms for GSTs functions in response to OTA in *A. thaliana*, but a further study should be taken to find the conjugates.

Increased ATP synthase subunits induced by OTA of the BSO-pre-treated leaves indicated the accelerated degradation of ATP synthase, in line with reports that ATP synthase α and β chains were degraded under chilling stress in rice (Yan *et al*. 2006). The degradation unavoidably resulted in a decreased ATP production and even photosynthetic activity of leaves under the OTA stress, in line with the study that ATP hydrolysis and the ATP-driven proton translocation activity of CF1CFo were both reduced under the impact of singlet oxygen dramatically in *Spinacia oleracea* (Buchert and Forreiter 2010, Gill and Tuteja 2010). Therefore, OTA-induced ROS might be responsible for the decrease in ATP synthase activity. Furthermore, decreased GSH might aggravate the impact caused by ROS and finally resulted in a decreased photosynthetic activity in response to OTA. Interestingly, HCF136, as a thylakoid membrane associated protein and PS II assembly/stability factor, increased in the proteome to suggest a possible improvement of plant photosynthesis ability, but in view of the fact that the photosynthesis is inhibited by OTA (Wang *et al*. 2012), a more reasonable explanation is that the up-regulation of HCF136 was to repair or avoid more serious damage to the photosystems. Similar effects has been seen in recent studies of the response to Pb stress in *Catharanthus roseus* (Kumar *et al*. 2011) and salt stress in rice (Li *et al*. 2011). All above were consistent with the idea that PS II is a target for both biotic and abiotic stresses (Pérez-Bueno *et al*. 2004, Shinya *et al*. 2010). Glutamine synthetase (GS) functions as a major assimilatory enzyme for ammonia and plays a key role in maintaining the balance of nitrogen forms. GS2, the major GS isoform at the vegetative stage of *A. thaliana* (Maaroufi-Dguimi *et al*. 2011), is responsible for the reassimilation of ammonia generated by photorespiration (Taira *et al*. 2004). So, the accumulation of GS2 identified by the proteome in the BSO+OTA group provided to some extent a possibility of the imbalance of nitrogen metabolism. In addition, TIM exists in glycolysis and the improved expression of it suggests that the sugar metabolism was also influenced. All these changes might in turn influence the effect of OTA+BSO on *A. thaliana*.

Taken together, the inhibition of GSH by BSO just slightly potentiated the OTA-induced cell damage in detached leaves of *A. thaliana*. The analyses on the proteome, gene expression and enzyme activities of the BSO-pretreated detached leaves under the OTA stress implied that OTA increased the metabolism of GSH by increased GSTs, promoted the degradation of ATP synthase, and accelerated the disorder of cellular metabolism. The present work provides a detail of the effect of OTA coupled with BSO on *A. thaliana* and a further insight into the phytotoxicity of OTA.

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