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Proteomic analysis of *Alternaria alternata* (Fr.) Keissler responds to COS fumigation

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1. ABSTRACT

Carbonyl sulfide (COS) is a new fumigant which has been a potential alternative to methyl bromide and phosphine in many applications. In this study, we investigated the fungitoxicity of COS towards the pathogen of pear black spot disease *Alternaria alternata* (Fr.) Keissler (*A. alternata*). Moreover, proteomic analysis and RT-PCR was performed and our results showed that during the fumigation, the regulation of 21 proteins in protein expression and mRNA accumulation levels is involved, which respond to growth inhibition caused by COS. These results provide new clues for the mechanism of the fungitoxicity of COS.

2. INTRODUCTION

Fumigation is worldwide used as a disinfestation or quarantine measure against pests and fungal pathogens. Because methyl bromide, which is the most widespread used fumigant, is scheduled to be phased out due to its ozone depletion properties in the second decade of this century, peoples pay more attention on some new fumigants that are reported as alternatives to methyl bromide for certain applications (1). Carbonyl sulfide (COS) is one of those candidates which is present in nature (2). It was patented in 1992 by CSIRO Australia (3). Laboratory and field studies have shown that COS is effective on a wide range of pests at all life stages, such as

Sitophilus oryzae, *Rhyzopertha dominica*, *Tribolium castaneum* and *Trogoderma variabile* (4-8), without any adverse effects on grains and fresh products (1, 9-11).

Although there is a long history of investigation of its toxicity, little is known about the molecular mechanisms of COS against pests and fungal pathogens. In recent years, proteomics is becoming popular because a comprehensive result can be given. By proteomic methods including 2D-electrophoresis and MS analyses, the most of proteins regulated by drugs or elicitors can be identified in one experiment. Especially, when few proteins or genes regulated by the drugs is known, the proteomic results can give an initial illustration, and the differently expressed proteins identified by proteomic research can be investigated further by traditional methods.

In this study, we investigated the effect of COS as a fumigant on *A. alternate* which was the pathogen of pear black spot disease occurred in China, Japan and Korea (12,13). Further more, we made use of a proteomic approach to identify changes in the expression profiles of proteins caused by the fumigation of COS. Our findings show that several important proteins involved in general metabolism, RNA transcription, processing and translation, growth and division, signaling pathway, anti-oxidation and defense. The identification of the candidate genes provides some important clues on the molecular mechanisms of fungal pathogens responds to COS treatment.

3. MATERIALS AND METHODS

3.1. Preparation of inoculum of *A. alternata*

A. Alternata was obtained from Yali pears with black spot disease in several orchards of Hebei province, China. The fungus was cultured on the potato carrot agar (PCA) medium (2% potato, 2% carrot, 1.5% agar) and liquid PCA medium (150 rpm) at 25□ in the dark for 5 days, respectively.

A. Alternata on the plates were suspended in 1ml sterile water and coated on the surface of a new PCA plate. Discs 7 mm in diameter were removed from the new PCA plate, and 10 discs were placed on each strip of cellophane. The strips with fungal discs were placed inside of each 250ml narrow mouth bottle (Sigma Z263036-1PAK) for the determination of the fungitoxicity of COS.

The fungus in the PCA liquids was washed 3 times with sterile water, dried with filter paper and placed in the 250ml narrow mouth bottle for COS treatments.

3.2. Fumigation of *A. alternata*

COS was purchased as a compressed gas with 99% purity from Yanglilai company (Beijing, China). The gas was released into a 1L Tedlar sample bag (Delin, Dalian, China) and stored at 25C before fumigation. After the temperature was equilibrated, the bottles were sealed with a valve (Sigma 33304), and after removing a small amount of air, different amounts of COS gas were introduced by injectors(14). A stopcock on the lid was opened to bring the bottle back to normal pressure, then closed. The bottles were moved in a constant temperature incubator (Binder KBF720) to start the fumigation.

A. alternata was exposed to COS for 2, 4, 6, 8 and 10 hours at the dose of 50 g/m^3 respectively, in order to investigate the fungitoxicity of COS. The fumigated discs were transferred to PCA plates, and the diameters of the colonies were measured after 3 days. Inhibition rate = the average diameter of the fumigated fungus/the average diameter of the untreated fungus×100.

3.3. 2-DE

A. alternata prepared from PCA liquids was fumigated at the dose of 50 g/m^3 for 5 hours. And the untreated fungus was collected as control. Both of the fungus was plunged into liquid nitrogen and the grinded samples were suspended in 10% TCA. After precipitation, proteins were centrifuged at 12,000g for 10min, and washed with 3 volumes of ice cold acetone at -20□ for 2 h. The dried protein extracts were treated with the lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM DTT, and 2% v/v pharmalyte pH 4-7, from GE Healthcare) at room temperature for 4 h with rotation, followed by centrifugation at 100,000 g at 4°C for 1 h. The protein concentrations were determined using a 2-D Quant Kit (GE Healthcare).

Immobiline™ IPG strips (pH 4-7, 24 cm, GE Healthcare) were rehydrated with 450μl extracted protein in rehydration buffer (8 M urea, 2% CHAPS, 0.5% v/v IPG buffer pH 4-7, 7 μl DeStreak™ Reagent, GE Healthcare) for 20 h at room temperature. Using an Ettan IPGphorII system (GE healthcare), the IEF of proteins was carried out as following program: 100 V for 1 h, 300 V for 1 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 12 h (> a total of 90 kVh).

The focused strips were equilibrated twice with DTT and iodoacetamide in equilibration buffer (6 M urea, 30% w/v glycerol, 2% w/v SDS, and 50 mM Tris-HCl, pH 8.8) for 15 min, according to the manufacturer's protocol. Then the proteins were separated with 12.5% gels using Ettan Daltsix Electrophoresis System (GE Healthcare) according to the user manual. Subsequently, the gels were stained with PhastGel™ Blue R (CBB R-350) and scanned with ImageScanner using LabScan™ Version 5.0 software (all from GE Healthcare). The spots were identified and analyzed using ImageMaster™ 2D software (GE Healthcare, Version 5.0). In each group, at least three gels were analyzed. Statistical analysis by two tailed *t*-test was performed and $p < 0.05$ was considered significant.

3.4. Protein identification

Spots corresponding to differently expressed proteins were excised from the CBB-stained gel, washed, and digested in-gel with trypsin (sequencing grade, Promega) at 4°C for 30 min and at 37°C overnight. The digested peptides were extracted with 0.5% trifluoroacetic acid (TFA) by shaking at 37°C for 1 h, and mixed with one volume of 10 mg/ml R-cyano-4-hydroxycinnamic (CHCA) acid in 0.1%TFA/50% ACN for analysis using a Bruker Reflex III MALDITOF MS (Bruker-Franzen, Bremen,Germany) in positive ion mode at an accelerating voltage of 20 kV. The obtained PMFs were identified by searching NCBI nrdatabase of Mascot

Table 1. Proteomic analysis of differentially expressed proteins induced by COS in *A.alternata*

Spot No.	Gene Name	Genbank No.	Protein Name	pI/MW (kDa)	Coverage (%)	Peptide Matched	Exp. value	Mascot Score	Change folder [a]
Classification: Metabolism									
6	BCAT	gi|189189676	branched-chain-amino-acid aminotransferase	7.1/47	29	16	0.0023	83	0.45
10	ATPB	gi|189195394	ATP synthase subunit beta	5.4/54	61	24	4.9e-16	210	6.74
14	FABG	gi|189196532	3-oxoacyl-(acyl-carrier-protein) reductase	6.5/32	28	9	9.6e-05	97	0.36
15	β-CA	gi|189188506	carbonic anhydrase	6.1/27	24	10	0.043	71	0.57
17	SHMT	gi|119492035	serine hydroxymethyltransferase, putative	7.6/52	22	9	0.0049	80	0.60
18	ILVH	gi|189196802	acetolactate synthase small subunit	5.6/36	29	8	0.0025	83	0.38
20	AHCY-A	gi|189189746	adenosylhomocysteinase A	5.8/50	47	23	2.5e-13	183	0.39
21	KARI	gi|189190856	ketol-acid reductoisomerase	5.9/41	28	12	1.2e-05	106	0.51
28	HAL2	gi|189192783	3'-phosphoadenosine 5'-phosphatase isoform A	4.9/34	22	8	0.0006	89	0.34
29	PCBC	gi|121704379	oxidoreductase, putative	6.1/40	25	7	0.051	70	0.37
RNA transcription, processing and translation									
4	SRP1	gi|218722347	pre-mRNA splicing factor (Srp1)	9.1/25	30	11	0.00013	96	2.96
27	EIF3	gi|85109510	eukaryotic translation initiation factor 3	5.1/99	27	17	0.0036	81	3.87
32	VIP1	gi|189204968	RNA-binding protein Vip1	6.0/28	44	12	0.00043	91	3.94
Growth and division									
3	Rho_GDI	gi|213407484	rho GDP-dissociation inhibitor	5.5/22	41	6	0.054	70	0.29
25	EF1G	gi|189208786	elongation factor 1-gamma	5.3/44	25	14	9.8e-06	107	0.37
Signal transduction									
9	PP2A	gi|189188084	protein phosphatase 2A regulatory subunit A	4.7/69	47	32	3.9e-19	241	9.71
16	YWHAE	gi|189196909	14-3-3 protein epsilon	4.7/29	38	12	1.2e-05	106	4.10
Anti-oxidation									
1	PRDX6	gi|189202520	peroxiredoxin-6	5.9/25	27	9	0.0018	84	10.5
19	CCP	gi|189196316	Cytochrome c peroxidase	8.5/42	31	18	2.5e-05	103	3.89
Defense									
8	HSP70	gi|189197865	heat shock protein 70	5.3/81	30	30	4.9e-12	170	2.79
22	HSP90	gi|189202774	heat shock protein 90	4.9/80	27	16	0.014	76	2.96

[a]Average folder between COS-treated fungus and untreated fungus calculated for at least tri-replica gels (change folder > 1 means the up-regulation after COS treatment; change folder < 1 means the down-regulation; Student's *t*-test, $p < 0.05$)

(http://www.matrixscience.com), with peptide mass tolerance of ±100 ppm and one missed cleavage site. The Mascot score, expectation value, peptide query match, and coverage data were shown.

3.5. RT-PCR for transcription analysis

The total RNA was extracted by TRIzol RNA extraction kit (Tiangen Co., Beijing, China). The cDNA was synthesized using M-MLV Reverse Transcpritase (Promega) and Oligo-dT primer. The genes of interest were amplified using the primers in Table 1 and the following PCR program: 5min at 94°C, 30 cycles with 30s at 94°C, 30s at 60°C, and 40s at 72°C. Because the genome of A.alternata was unknown, all of the primers were designed according to their homologous genes in Pyrenophora tritici-repentis.

4. RESULTS

4.1. Inhibition of COS fumigation on the growth of *A.alternata*

The growth of *A.alternata* was significantly inhibited when exposed to COS (Figure 1). When treated with 50 g/m^3 COS, the inhibition rate was about 50% after 5 h, and this dose and treatment period were chosen for the proteomic analyses.

4.2. The differential proteomic profiles of *A.alternata* responds to COS treatment

The differential protein expression profile, potentially relating to COS fungitoxic mechanism, showed that the levels of 10 proteins increased, whereas 11 proteins decreased (Figure 2). The identification of these proteins by

Table 2. Primers used for RT-PCR analysis

Genes	Forward primers	Reverse primers
BCAT	5'-AGCCGCCAGACGAGTTCA-3'	5'-TGCCGCTTGACGACTTCC-3'
ATPB	5'-CGGTGGTTTCTCCGTCTT-3'	5'-CCCTTGGTGGTAGTGGTAAT-3'
FABG	5'-TAACCTCGGTGGCTCTGG-3'	5'-TCGCACGTTGATGTTGTATT-3'
β-CA	5'-ACCTCGGCATCCGCATTA-3'	5'-TGGCTTCTTCGCCTTCAT-3'
ILVH	5'-GAACTTCTTGCCCACCAC-3'	5'-CATCTCGCCTGGATTGTT-3'
AHCY-A	5'-TCTCCAGGCTGCCGTTTC-3'	5'-TGATGTGGCGACCGTTGT-3'
KARI	5'-AGCCCGACTACCGTGAGA-3'	5'-CAAATCCGTAATCGTAACAAAG-3'
HAL2	5'-CTGCCCGTAGACGACCAA-3'	5'-GCATCTGTGACCTCACCACC-3'
VIP1	5'-TCCAGTCCATCAGCGTAAA-3'	5'-CGACGACCTGTGCCTTTT-3'
EF1G	5'-AGCCTGCCAAGGTGTCTC-3'	5'-ACGCCTCGGTGATGAACT-3'
PP2A	5'-TCAGACGACCAGGATAGTGTA-3'	5'-CATGGGTAGAAGATGTGAAATG-3'
YWHAE	5'-GAGCGTTATGAGGAGATGGT-3'	5'-GGAGAAGTTCAGGGCAAGT-3'
PRDX6	5'-TTCACCATTCGCTCTGTC-3'	5'-TCTACTTGGGCGGTCATA-3'
CCP	5'-CCCTCACCAACGACTACTT-3'	5'-TTGCTCCCTTCAACAATCT-3'
HSP70	5'-AACTACCAGGAAAGATGAACCC-3'	5'-CGGCTGACAACGGCAAAT-3'
HSP90	5'-ATAGCGGTATCGGTATGAC-3'	5'-CACCAGCACTTGACTCCC-3'
ACTIN	5'-GAGTGGGTCTACTGGCAAACG-3'	5' –TGGTAACAAGCACCTCCTTCA-3'

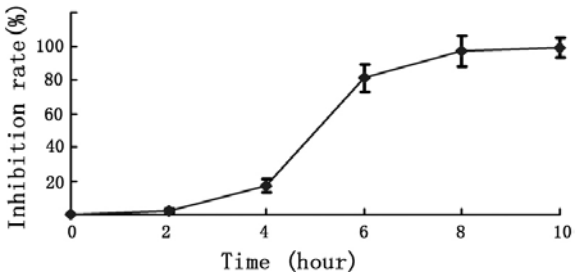

Figure 1. The inhibition of COS on growth of *A. alternate*. Fungus was treated with 50 g/m^3 COS and the inhibition rate was calculated. Data were averaged from ten replicates.

MALDI-TOF MS was listed in Table 2. The proteins were divided into six groups according to their molecular function. 10 proteins were assigned to the functional group of "metabolism" (48%), 3 proteins for "RNA transcription, processing and translation" (14%), 2 proteins for "growth and division" (10%), 2 proteins for "signal transduction" (10%) 2 proteins for "anti-oxidation" (10%) and 2 proteins for "defense" (10%).

4.3. Transcriptional analysis of COS regulated proteins

The transcription of 16 proteins by RT-PCR was illustrated and it was indicated that all of these genes' transcription showed an identical change with the protein level, whereas the internal control genes of *β–Actin* showed equal contents (Figure 3), indicating the regulation of these genes occurred at the transcription level.

5. DISCUSSION

In this study, 2DE and MALDI-TOF MS analysis were employed to provide the differential protein expression profiles of *A. alternate* following COS treatment. We showed that COS significantly inhibited the growth of *A. alternate*, and 21 proteins were found related to this process.

Our results showed exposure to COS affected many metabolic processes in *A. alternate*. Several proteins that participate in amino acid biosynthesis, protein biosynthesis and the fatty acid and lipid metabolism were down-regulate, such as branched-chain amino acid aminotransferase (BCAT) that catalyzes the final metabolic step in the production of the hydrophobic amino acids leucine, isoleucine and valine (15); 3-oxoacyl-(acyl-carrier-protein) reductase that belongs to the family of oxidoreductases and participates in fatty acid biosynthesis and polyunsaturated fatty acid biosynthesis (16); serine hydroxymethyltransferase (SHMT) that simultaneous conversions of L-serine to glycine and 5,6,7,8-tetrahydrofolate to 5,10-methylenetetrahydrofolate and plays an important role in cellular one-carbon pathways which provides the largest part of the one-carbon units available to the cell (17,18); acetolactate synthase that catalyses the first step in branched-chain amino acid biosynthesis (19) and ketol-acid reductoisomerase that participates in valine, leucine and isoleucine biosynthesis and pantothenate and coa biosynthesis (20). These results indicate that similar to the reports of other stress, COS fumigation inhibits the general metabolism of *A. alternate* (21,22). Moreover, rho GDP-dissociation inhibitor and elongation factor 1-gamma that related to cell growth and division are down-regulated either (23-26), and these should be the molecular basis of growth inhibition.

Two proteins participate in anti-oxidation are up-regulated. Peroxiredoxin 6 (Prdx6) is an enzyme with both GSH peroxidase and phospholipase A2 (PLA2) activities. The over-expression of Prdx6 strongly reduced the number of apoptotic cells after UVA or UVB irradiation, indicating it's a potent cytoprotective enzyme that detoxifies hydrogen peroxide and various organic peroxides (27). Cytochrome c peroxidase (CCP) is another key enzyme of defense systems against oxidative stress that takes reducing equivalents from cytochrome c and reduces hydrogen peroxide to water (28,29). These results indicate that oxidative stress must occur in treated fungus. Similar occurrence of antioxidant defense has been also reported in the studies of phosphine which is the most widely used fumigant throughout the world for insect control beside methyl bromide (2). And it is reported that, together with glutathione, peroxiredoxin and CCP are both thought to be key agents against phosphine-induced oxidative damage which is one of the most important mechanism of the toxicity of phosphine (30,31).

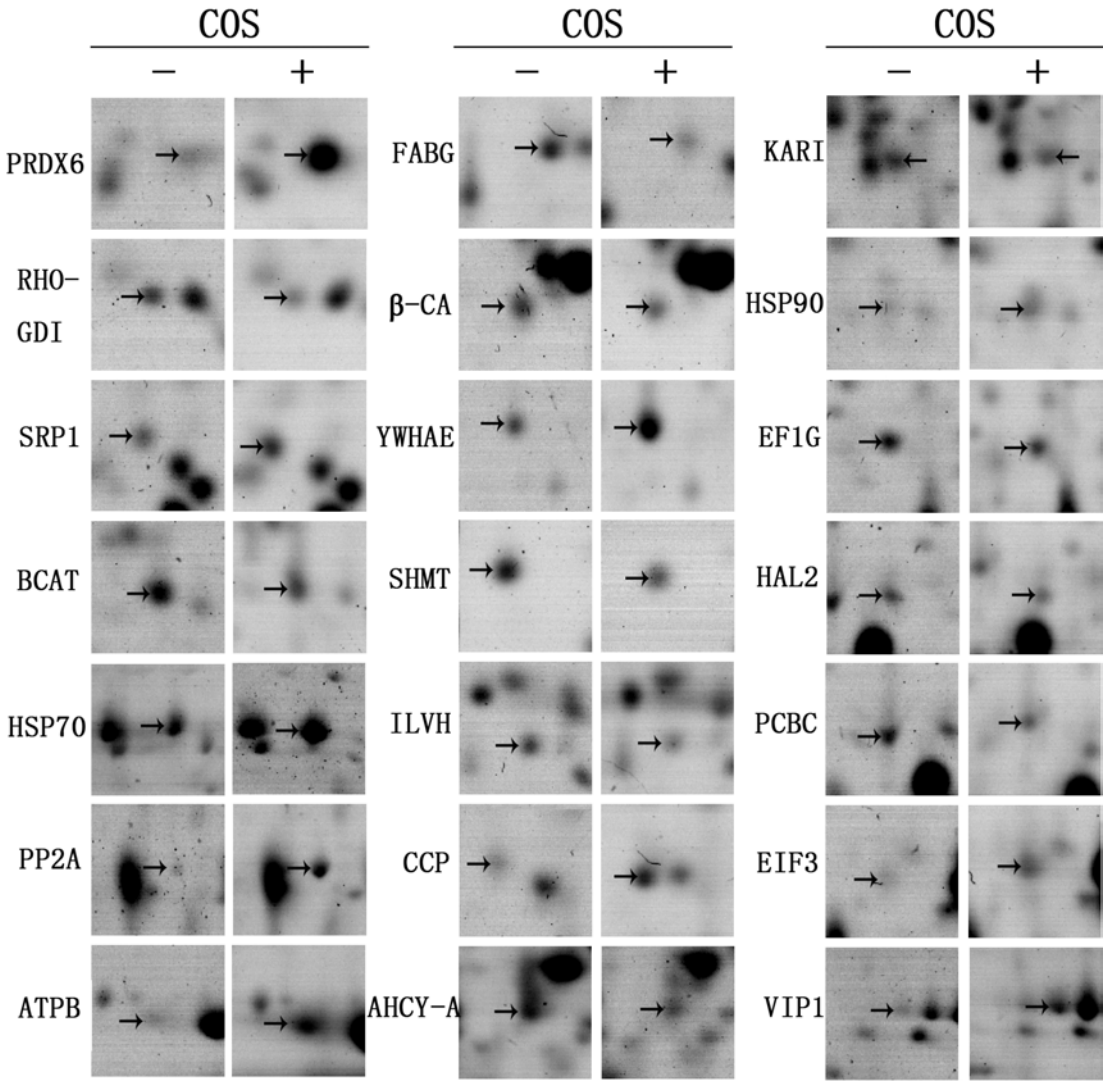

Figure 2. Spots of differentially expressed proteins identified in 2-DE gels. COS(-) means untreated fungus and COS(+) means fungus treated by 50 g/m^3 COS for 5 h..

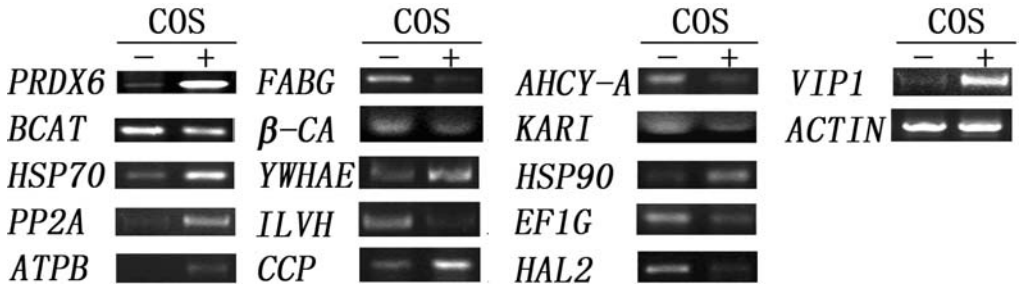

Figure 3. Differential transcription of genes corresponding to the differentially expressed proteins identified in 2-DE. The RNA transcription was detected by RT-PCR. COS(-) means untreated fungus and COS(+) means fungus treated by 50 g/m^3 COS for 5 h..

There are also two heat shock proteins (HSP) HSP70, HSP90 up-regulated. HSPs are a class of functionally related proteins whose expression is increased when cells are exposed to elevated temperatures or other stress (32). The up-regulation of anti-oxidative enzymes and some other defensive proteins such as HSP70 and HSP90 reveal the defense mechanism of *A. alternate* under COS treatment.

Finally, there are also two proteins involving in signal transductions up-regulated. Protein phosphatase 2A (PP2A) is a class of conserved Serine/Threonine phosphatase with broad substrate specificity and diverse cellular functions. PP2A act as a tumor suppressor by interacting with Raf, MEK, and Akt in mammals (33) and their roles in regulation of metabolism, cell cycle and development, as well as in light, stress and hormonal signaling in plants are also highlighted by Farkas *et al* (34). The 14-3-3 proteins are a family of acidic regulatory molecules found in all eukaryotes and involved in many processes through interacting with their partners, including cell cycle regulation, metabolism control, apoptosis, and control of gene transcription (35). Considering pre-mRNA splicing factor (Srp1), eukaryotic translation initiation factor 3 and RNA-binding protein Vip1 that are involved in RNA transcription, processing and translation are also up-regulated (36,37), we can suppose that there are complex transcriptional regulation underlying COS fumigation.

In conclusion, we identified differentially expressed proteins in *A. alternate* treated by COS in this study. Although further functional research should be preformed to investigate the differentially expressed proteins, our results indicate that COS fumigation affects many events in *A. alternate* and oxidative stress is one of the important mechanisms of the inhibition.

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Key Words: Carbonyl Sulfide; *A. alternata*; Proteomics, RT-PCR, Differential protein expression profiles

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