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# Differential Expression Profiles of *Alternaria alternate* Genes in Response to Carbonyl Sulfide Fumigation

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**Carbonyl sulfide (COS) is a new fumigant used in phytosanitary treatments. It was developed as a potential alternative to methyl bromide, which is being phased out because of its ozone-depletion properties. To understand the molecular and cellular mechanisms occurring in fungal pathogens in response to COS fumigation, we cloned 510 cDNA fragments of *Alternaria alternata* (Fr.) Keissler genes that are differentially expressed; these genes were cloned using suppression subtractive hybridization. Changes in the levels of transcripts of 79 fragments were confirmed by microarray analysis and qRT-PCR. Further homology search revealed that they are highly homologous to 41 genes of other fungi, which were related to general metabolism, growth and division, defense, cellular transport, and signal transduction. These results provide an overview of differential expression profiles of *A. alternata* genes following COS treatment and some new clues about the mechanism of COS fungitoxicity.**

***Keywords*:** carbonyl sulfide, *A. alternata*, suppression subtractive hybridization (SSH), expression profiles

Black spot disease is caused by *Alternaria alternata*, and it is one of the most harmful diseases of pears, particularly in China, Japan, and Korea. This disease has greatly reduced the export of Yali pear (*Pyrus bretschneideri* Rehd.) in recent years (Baudry *et al.*, 1993; Zhang *et al.*, 2003). Fumigation with carbonyl sulfide (COS), which is naturally present in the atmosphere (Fields and White, 2002), is a candidate postharvest control method for this disease. COS was patented in 1992 by CSIRO Australia and has been widely studied as an alternative to methyl bromide for many applications (Banks *et al.*, 1993). Laboratory and field studies have shown that COS is effective against a wide range of pests at all life stages (Desmarchelier, 1994; Zettler *et al.*, 1997; Obenland *et al.*, 1998; Weller, 1999; Xianchang *et al.*, 1999) and has no adverse effects on grains and fresh products when applied at reasonable concentrations (<80 g/m$^3$) for reasonable exposure times (1-24 h) (Chen and Paull, 1998; Weller *et al.*, 1998; Bell, 2000; Aung *et al.*, 2001). The toxicity of COS in mammals has also been reported. COS has low acute inhalational toxicity and no genotoxicity or developmental toxicity (Ruishu *et al.*, 1999; Herr *et al.*, 2007), but at certain doses, it can reversibly impair male fertility. COS presents neurotoxicity hazards similar to those of the structurally and toxicologically related compound, carbon disulfide (Ruishu *et al.*, 1999; Morgan *et al.*, 2004; Bartholomaeus and Haritos, 2005; Sills *et al.*, 2005).

Despite a long history of investigation of COS toxicity, little is known about its molecular and cellular mechanisms against pests and fungal pathogens. Suppression subtractive hybridization (SSH) is a powerful technique for cloning ESTs that are differentially expressed in two different populations. Combining suppression PCR with normalization and subtraction steps in a single reaction increases the possibility of identifying rarely expressed genes (Diatchenko *et al.*, 1999). Recently, SSH has been successfully used to identify abiotic stress-regulated genes (Nguyen *et al.*, 2009; Sahu and Shaw, 2009; Tian *et al.*, 2009).

In this study, we investigated the effect of COS fumigation on *A. alternata*. SSH was used to analyze gene expression profiles after COS fumigation. In addition, the induction specificity of isolated expressed sequence tags (ESTs) was assessed with a cDNA microarray. Identifying the candidate genes could provide important clues about the molecular mechanisms involved in the response of fungal pathogens to COS treatment.

## Materials and Methods

### Preparation of *A. alternata* inoculum

*A. alternata* L-3 was obtained from Yali pears with black spot disease; the pears were collected from several orchards in Hebei Province, China. The fungus was cultured on potato carrot agar (PCA) medium (2% potato, 2% carrot, and 1.5% agar) and liquid PCA medium (150 rpm) at 25°C in the dark for 5 days.

The spores and mycelium on the plate were suspended in 1 ml 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 a strip of cellophane with the fungus facing upward. The strips with the fungal discs were placed inside a 250-ml narrow mouth bottle (Sigma Z263036-1PAK, Germany) and stored at 25°C in order to determine the fungitoxicity of COS.

The fungus in PCA liquid was washed 3 times with sterile water, dried with filter paper, and placed in 250-ml narrow mouth bottles before COS treatment.

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### Pathogenicity analysis

The inoculation test was performed to determine the pathogenicity of the fungus. Ya pears (*P. pyrifolia*) were purchased from a local orchard without fungicide treatment 1 month before harvest. After storing at 25°C for 1 day, the surfaces of the fruit were disinfested with 70% ethanol for 30 sec. Several small wounds (2 mm in depth and 5 mm in diameter) were made on each pear using a sterile pin. The wounded pear was inoculated with *A. alternata* by covering the fruit with small pieces of sterile filter paper that have been dipped in the previously prepared fungi suspension. The pears were then stored at 25°C for 7 days.

### Fumigation of *A. alternata*

COS was purchased as a 99% pure compressed gas from Yanglilai Company (China). The gas was released into a 1-L Tedlar sample bag (Delin, China) and stored at 25°C before fumigation. After the temperature was equilibrated, the bottles were sealed with a valve (Sigma 33304, Germany), and after removing a little air, different amounts of COS gas were introduced using injectors (Ren *et al.*, 2007). The stopcock was opened to bring the pressure back to normal and then closed. The bottles were moved to a constant-temperature incubator (25°C, Binder KBF720 Germany) for fumigation.

COS fumigation was performed for 4 h at doses of 25, 50, 75, 100, 125, 150, and 200 g/m$^3$ to investigate COS fungitoxicity. The discs were transferred to PCA plates after fumigation, and the diameters of the colonies were measured after 3 days. To calculate inhibition rate, we used the following formula.

Inhibition rate = the average diameter of the fumigated fungus/the average diameter of the untreated fungus×100.

*A. alternata* cultivated with PCA liquid was fumigated with 80 g/m$^3$ COS for 0.5, 1, and 2 h and then plunged into liquid nitrogen to prepare the samples for RNA extraction.

### RNA extraction and mRNA purification

Total RNA was extracted using TRIzol (TIANGEN). DNase I (TaKaRa, Japan) was used to digest genomic DNA. mRNA was purified with a PolyATract mRNA Isolation System III (Promega, USA) used according to the manufacturer's instructions.

### Subtractive hybridization

SSH was performed with a PCR-Select™ cDNA Subtraction kit (Clontech, USA) according to the user's manual. In order to obtain both of upregulated and downregulated genes by COS, mRNA isolated after fungus exposure to COS for 0.5, 1, and 2 h were mixed and used for cDNA synthesis. Forward and reverse subtractions were both performed: cDNAs from the control and fumigated fungus served as either driver or tester populations. Products of the secondary PCR were directly cloned into a pGEM T-easy Vector (Promega) and transformed into *Escherichia coli* DH5α cells.

### Amplification of cDNA inserts

The cDNA inserts were amplified with PCR performed using primers T7 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-ATTTAGGT GACACTATAG-3′). The PCR procedure was as follows: initial denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min; and a final extension of 72°C for 5 min. The PCR products were separated with electrophoresis on a 1.5% agarose gel to determine the quality of amplification and the quantity of the products.

### cDNA microarray analysis

In order to identify the positive clones, the treated and untreated RNA labeled with Cy3 and Cy5 were used as probes, and the PCR products were arrayed onto 2 poly-L-lysine-coated microscope slides (Ma *et al.*, 2001, 2002). cDNA microarray analysis was then performed in the microarray laboratory at Beijing Genomics Institute (China) according to a standard procedure (Eisen and Brown, 1999). Each sample had at least 3 biological replications to minimize systematic errors, and actin was used as the internal control. Separate TIFF images of the Cy5 and Cy3 channels were obtained using a ScanArray Lite scanner (Perkin-Elmer), and spot intensities were quantified using the Axon GenePix Pro 5.1 image analysis software. The data were then normalized and processed as described by Wei *et al*. (2009), and the differential expression levels of the cDNA clones were defined between paired samples with a $P$ value of $<0.01$ ($Z$ test).

### Sequence analysis

Differentially expressed clones whose expression changed by more than 3-fold were sequenced using the T7 primer (Beijing Genomics Institute, China). After the adaptor and vector sequences were removed, all the ESTs were annotated on the basis of the existing annotation of nonredundant databases at NCBI by using BLASTX. Sequence similarity was represented by the BLASTX probability e-value. E-values of $<1e^{-5}$ indicated significant homology, and these sequences were categorized according to their functions.

### qRT-PCR analysis

qRT-PCR was performed to further confirm the transcriptional regulation after COS treatment. The genes of interest were amplified using the primers in Table 1 and the following PCR program: 5 min at 94°C, 30 cycles with 30 sec at 94°C, 30 sec at 60°C, and 40 sec at 72°C. *ACTIN* gene was used as the internal control.

## Results

### The pathogenicity of *A. alternata*

The pathogenicity of *A. alternata* was determined by the inoculation test. After 3 days storage, black spots could be clearly observed on the surface of Ya pears. The spots were about 3 cm in diameter after 7 days, indicating the prepared *A. alternata* inoculum was pathogen of black spot disease (Fig. 1).

### COS fumigation inhibits *A. alternata* growth

COS significantly inhibited the growth of *A. alternata* in a dose-dependent manner (Fig. 2), indicating the strong fungitoxicity of COS's. The LD50 was approximately 80 g/m$^3$ at 4 h, and this dose was used in further experiments.

### Identification of COS-responsive genes

Genes responsive to COS fumigation were identified by

**Fig. 1.** Pathogenicity analysis of *A. alternate* on Ya pears.

**Table 1.** Primers used for qRT-PCR analysis

| Genes | Molecular functions | Forward primers | Reverse primers |
|---|---|---|---|
| *GRX* | glutaredoxin domain containing protein | 5′-AGCGTGGTCGCGGCCGAGGTACGGT-3′ | 5′-GAAGGACGCAGTGAAGAAGT-3′ |
| *ADH* | alcohol dehydrogenase | 5′-CGCAGCGTGGACCTTTAC-3′ | 5′-ACCGTGGTAGTTGCTGTTT-3′ |
| *ATPT* | ATP transporter | 5′-GCAGGTACGTCTTCCACT-3′ | 5′-TGGGTATGTGCGGATTGT-3′ |
| PRK | flavohemoprotein | 5′-ACCTCGGCATCCGCATTA-3′ | 5′-GCACCCGTCTGTCTATGAAA-3′ |
| *CAX* | vacuolar calcium ion transporter /H(+) exchanger | 5′-TGGGAAGAGGAGTTATGCT-3′ | 5′-GTCTATGAAATCCGTCCGTG-3′ |
| *SIW14* | tyrosine-protein phosphatase SIW14 | 5′-CTGGTGTCAAGGCTCGCTTCC-3′ | 5′-ACCCAATGCCTACTGGTCCTA-3′ |
| *TRPD* | TPR domain protein | 5′-ATTCTGCGATGATGTCTGG-3′ | 5′-GCTAAGGAAACCGTATCAC-3′ |
| *PTRD* | FAD dependent oxidoreductase | 5′-CTGCCCGTAGACGACCAA-3′ | 5′-GCATCTGTGACCTCACCACC-3′ |
| *APSR* | APS reductase alpha subunit | 5′-TCCAGTCCATCAGCGTAAA-3′ | 5′-CGACGACCTGTGCCTTTT-3′ |
| *FTSJ* | cell division protein ftsj | 5′-AGCCTGCCAAGGTGTCTC-3′ | 5′-ACGCCTCGGTGATGAACT-3′ |
| *ASPF* | Asp f 13-like protein | 5′-TCAGACGACCAGGATAGTGTA-3′ | 5′-CATGGGTAGAAGATGTGAAATG-3′ |
| *ACTIN* | | 5′-GAGTGGGTCTACTGGCAAACG-3′ | 5′-TGGTAACAAGCACCTCCTTCA-3′ |

constructing a subtractive cDNA library from mRNA isolated from untreated fungus and mixed fungus exposed to 80 g/m$^3$ COS for 0.5, 1, and 2 h. A total of 635 vector-containing bacterial colonies were picked from the library and assayed by PCR to check for the presence for a cDNA insert. Of the 635 clones, 501 clones that yielded a single PCR product were investigated further by differential screening by using microarray analysis.

The PCR products amplified from the inserts were arrayed on a chip, and the chip was hybridized with cDNA probes of treated and untreated fungus labeled with Cy3 and Cy5. On the basis of the results of the microarray analysis, 13 downregulated clones (16%) and 66 upregulated clones (84%), whose expression levels changed more than 3-fold after fumigation, were identified for sequence analysis (Tables 1 and 2).

## Analysis of COS-regulated cDNA sequences

The cDNA sequences were compared against those in the NCBI database. Forty-one full-length cDNA clones were identified, and their definitive or putative functions were assigned (Tables 2 and 3). The cDNA clones were divided into 6 groups according to their molecular function. Nineteen clones were assigned to the functional group "general metabolism" (46%); 2 clones, to "growth and division" (5%); 6 clones, to "defense" (15%); 3 clones, to "cellular transport" (7%); 4 clones, to "signal transduction" (10%); and 7 clones, to "unclassified" (17%).

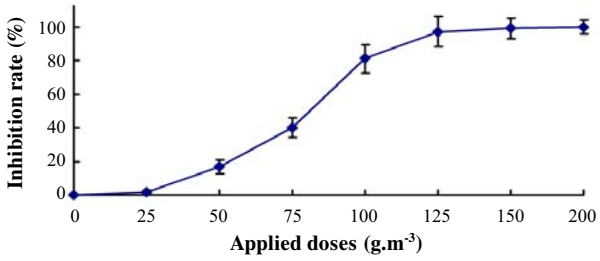


**Fig. 2.** Inhibition rates of *A. alternata* exposed to different doses of COS for 4 h. All data were averaged from 10 replicates. Error bars indicate SE.

There were 31 upregulated genes identified. 13 of them belonged to the group "general metabolism", 6 belonged to the group "defense", 3 belonged to to "cellular transport", 4 belonged to "signal transduction" and 5 belonged to "unclassified" (Table 2). Most of upregulated genes in the group "general metabolism" encoded oxidoreductases, indicating that oxidoreductases play an important role in the molecular and cellular mechanisms involved in the response to COS fumigation. All defense related genes were upregulated, indicating a stress response occurred after COS fumigation. All signal transduction related genes were upregulated, indicating COS may function as a signal molecular and regulate the downstream events through these signal transduction related genes.

There were 10 downregulated genes identified. 6 of them belonged to the group "general metabolism", 2 belonged to the group "growth and division" and 2 "unclassified" (Table 3). The downregulated genes in the group "general metabolism" included the nitroreductase family protein, glutamine amidotransferase subunit pdxT, and fructose dehydrogenase small subunit which contributed to nitrogen, sugar, and amino acid metabolism. The downregulation of these genes and the genes relevant to growth and division indicated that the vitality and energy of *A. alternata* were greatly inhibited after COS exposure. These results indicate that complex reactions occur in response to COS fumigation.

## Transcriptional analysis of COS regulated genes

The transcription of 11 genes by qRT-PCR was consistant with the microarray results, whereas the internal control genes of *Actin* showed equal contents (Fig. 3).

# Discussion

In this study, SSH and microarray analysis were used to identify the *A. alternata* genes that were differentially expressed after COS treatment in order to understand the molecular and cellular mechanisms occurring in fungal pathogens in

**Table 2.** BLAST-X search and microarray analysis of SSH fragments of genes upregulated by COS fumigation

| Molecular functions | GenBank no. | E value | Change fold[a] | P value | No. of ESTs |
|---|---|---|---|---|---|
| General metabolism | | | | | |
| *N*-Acetylglucosaminyltransferase | CAD90583 | 5e-07 | 4.03 | 3e-08 | 1 |
| Disulfide oxidoreductase | XP_001941921 | 6e-61 | 4.15 | 1e-08 | 1 |
| Glutaredoxin domain containing protein | XP_001930380 | 9e-26 | 5.16 | 5e-08 | 7 |
| Glutathione reductase | XP_001934501 | 1e-25 | 3.32 | 5e-06 | 4 |
| Alternative oxidase | XP_001941938 | 2e-47 | 15.23 | 2e-27 | 4 |
| ATP transporter | XP_001931582 | 3e-41 | 25.23 | 2e-38 | 1 |
| Alcohol dehydrogenase | XP_001939356 | 5e-28 | 3.35 | 2e-06 | 1 |
| Phosphopantothenate-cysteine ligase | XP_001937503 | 3e-55 | 4.34 | 5e-09 | 1 |
| Oxidoreductase | XP_002342004 | 1e-23 | 3.07 | 8e-06 | 2 |
| Glycerol-3-phosphate acyltransferase | XP_001599857 | 2e-26 | 25.54 | 2e-37 | 3 |
| Cysteine dioxygenase | XP_001930573 | 2e-24 | 10.51 | 9e-14 | 1 |
| Alpha-ketoglutarate-dependent sulfonate dioxygenase | XP_002376025 | 4e-22 | 4.78 | 4e-10 | 2 |
| Defense | | | | | |
| Superoxide dismutase | AJ496411 | 9e-09 | 4.87 | 1e-12 | 7 |
| Flavohemoprotein | XP_001931846 | 8e-12 | 3.42 | 9e-07 | 1 |
| DNAJ heat shock family protein | XP_001931291 | 2e-31 | 5.74 | 3e-12 | 1 |
| Metallo-beta-lactamase domain protein | XP_001262659 | 2e-45 | 19.65 | 3e-18 | 8 |
| Sulfide:quinone oxidoreductase | XP_001934772 | 1e-13 | 6.10 | 6e-13 | 2 |
| Putative puroindoline b protein | CAQ43070 | 6e-07 | 16.8 | 2e-29 | 1 |
| Cellular transport | | | | | |
| Vacuolar calcium ion transporter /H(+) exchanger | XP_001939162 | 4e-34 | 14.6 | 1e-09 | 1 |
| Archaerhodopsin-2 | XP_001937696. | 4e-16 | 3.44 | 9e-07 | 1 |
| Sodium bile acid symporter family protein | XP_001937222 | 3e-25 | 3.87 | 7e-07 | 1 |
| Signal transduction | | | | | |
| Tyrosine-protein phosphatase SIW14 | XP_001934454 | 2e-24 | 21.021 | 9e-16 | 1 |
| MADS FLC-like protein 3 | ACL54967 | 2e-09 | 30.25 | 7e-42 | 4 |
| MADS FLC-like protein 2 | ACL54966 | 1e-07 | 15.10 | 3e-31 | 3 |
| TPR domain protein | XP_001261011 | 7e-34 | 5.97 | 3e-11 | 1 |
| Unclassified | | | | | |
| Conserved hypothetical protein | XP_001937113 | 2e-37 | 3.31 | 2e-06 | 1 |
| Conserved hypothetical protein | XP_001941762 | 1e-06 | 4.86 | 3e-10 | 1 |
| Hypothetical protein PTRG_04401 | XP_001934734 | 2-41 | 4.54 | 1e-10 | 1 |
| Conserved hypothetical protein | XP_001805925 | 5e-30 | 9.98 | 7e-20 | 1 |
| Hypothetical protein PTRG_05096 | XP_001935429 | 5e-12 | 3.74 | 6e-07 | 1 |

[a] values were averaged from all ESTs.

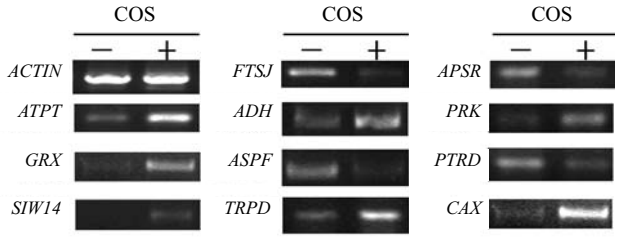


**Fig. 3.** Differential transcription of genes identified by microarray. The RNA transcription was detected by RT-PCR. COS(-) means untreated fungus and COS(+) means mixture of fungus treated by 80 g/m$^3$ COS for 0.5, 1, and 2 h.

response to COS fumigation.

Exposure to COS affects many metabolic processes in *A. alternata*, in particular sulfur redox systems. Sulfur acts as a redox "chameleon" and has around 10 oxidation states *in vivo* (Jacob and Anwar, 2008). The activation of enzymes such as disulfide oxidoreductase, glutaredoxin domain-containing protein, alternative oxidase, phosphopantothenate-cysteine ligase, and cysteine dioxygenase changes sulfur from its -2 state in COS to other chemotypes to help *A. alternata* resist the adverse effects of COS.

The redox balance is also disrupted. Glutathione reductase, which reduces glutathione disulfide (GSSG) to the sulfhydryl form of glutathione (GSH) (Meister, 1988), is upregulated, and APS reductase, an enzyme of the sulfur assimilation pathway that uses glutathione as an electron donor (Rouhier

**Table 3.** BLAST-X search and microarray analysis of SSH fragments of genes downregulated by COS fumigation

| Molecular functions | GenBank No. | E value | Change fold[a] | P value | No. of ESTs |
|---|---|---|---|---|---|
| General metabolism | | | | | |
| Nitroreductase family protein | XP_001930653 | 3e-06 | -3.06 | 8e-06 | 1 |
| Glutamine amidotransferase subunit pdxT | XP_001930736 | 2e-08 | -3.14 | 3e-07 | 1 |
| FAD dependent oxidoreductase | XP_001930724 | 3e-36 | -3.28 | 3e-06 | 1 |
| APS reductase alpha subunit | CAP03145 | 5e-16 | -3.33 | 2e-06 | 2 |
| Fructose dehydrogenase small subunit | BAH36945 | 8e-08 | -4.61 | 1e-09 | 3 |
| Trehalose phosphorylase | XP_001931619 | 2e-65 | -4.56 | 9e-09 | 1 |
| Growth and division | | | | | |
| Cell division protein ftsj | XM_001941993 | 5e-21 | -9.25 | 3e-19 | 1 |
| Von Willebrand factor | XP_001940802 | 1e-37 | -3.35 | 1e-06 | 1 |
| Unclassified | | | | | |
| Asp f 13-like protein | AAQ87930 | 2e-15 | -3.12 | 5e-06 | 1 |
| Conserved hypothetical protein | XP_001792216 | 3e-48 | -5.06 | 1e-10 | 1 |

[a] values were averaged from all ESTs

*et al.*, 2008), are downregulated, indicating that *A. alternata* needs GSH after COS fumigation. Glutathione, which is an antioxidant, is crucial for oxidative stress management in living cells (Pompella *et al.*, 2003). Other genes encoding enzymes relative to oxidative stress are also upregulated. These genes include those encoding superoxide dismutases (SODs), a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, which are important molecules for antioxidant defense in nearly all cells exposed to oxygen (Galecka *et al.*, 2008).

These results indicate that oxidative stress must occur in treated fungus. A similar antioxidant defense mechanism has also been reported in studies of phosphine, which is the fumigant most widely used for insect control worldwide (Fields and White, 2002). Glutathione and SOD are both believed to protect against phosphine-induced oxidative damage, which is one of the most important mechanisms of phosphine toxicity (Hsu *et al.*, 2002; Proudfoot, 2009).

Moreover, 3 genes related to cellular transport are also upregulated: Vacuolar calcium ion transporter/H(+) exchanger, which is a member of the cation/H(+) exchanger family, which helps maintains $Ca^{2+}$ homeostasis (Kamiya *et al.*, 2005); archaerhodopsin-2 (aR2), a retinal protein-carotenoid complex found in the claret membrane of *Halorubrum* sp. aus-2, which functions as a light-driven proton pump (Uegaki *et al.*, 1991); and sodium bile acid symporter family proteins, which are related to the human bile acid:sodium symporters. The symporters are transmembrane proteins that function in the liver and are involved in the uptake of bile acids from portal blood plasma, a process mediated by the co-transport of $Na^{+}$ (Hagenbuch *et al.*, 1991). All cells acquire the molecules and ions they need from their surrounding extracellular fluid, and the unceasing traffic of molecules and ions is not only related to the maintenance of normal cellular trafficking but also to signals required for gene regulation (Lang *et al.*, 2007; Rhodes and Sanderson, 2009). Further studies are needed to determine the actual functions of the 3 abovementioned genes in COS fumigation.

Finally, some genes involved in signal transductions are also upregulated. Protein tyrosine phosphatases, MADS FLC-like protein 2 and 3, and the TPR domain protein are signaling molecules that participate in many important events such as cell growth, cellular differentiation, and mitotic cycles (Goebl and Yanagida, 1991; Becker and Theissen, 2003; Mustelin *et al.*, 2005; den Hertog *et al.*, 2008). Genes encoding protein tyrosine phosphatases and MADS FLC-like proteins 2 and 3 are 21, 30, and 15 times upregulated, respectively. Therefore, we infer that there are complex signal transductions underlying the response to COS fumigation.

In conclusion, we identified *A. alternata* genes that are differentially expressed in response to COS treatment. Although further functional studies should be performed to investigate these genes, our results indicate that COS fumigation affects many events in *A. alternata* and that oxidative stress is an important inhibition mechanism.

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