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The methionine biosynthesis regulator AaMetR contributes to oxidative stress tolerance and virulence in *Alternaria alternata*

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ABSTRACT

The tangerine pathotype of *A. alternata,* which produces a unique host-selective ACT toxin causes brown spots on citrus leaves and fruits. In this study, we report a methionine biosynthesis regulator (MetR), which belong to bZIP transcription factor, is required for methionine metabolism, oxidative stress tolerance and pathogenicity. We generated two Δ*AaMetR* mutants in the tangerine pathotype of *Alternaria alternata* and investigated the resulting mutant phenotypes. The *ΔAaMetR* disruption mutant grew poorly in the absence of methionine and unable to produce conidia. Furthermore, pathogenicity tests have shown that *ΔAaMetR* mutant on their tangerine host can neither penetrate nor cause disease. These *ΔAaMetR* mutants exhibit an increased sensitivity to exogenous H_2O_2 and many ROS generating oxidants. To elucidate the transcription network of AaMetR, we performed RNA-Seq experiments on wild-type and *ΔAaMetR* mutant and identified genes that were differentially expressed between the two genotypes. Transcriptome data demonstrated that AaMetR contributes in many other biological processes including ROS detoxification, sulfur transfer, and amino acid metabolism. Comparative transcriptome analysis indicated that the *ΔAaMetR* mutant up-regulated several genes involved in cysteine and methionine metabolism. In conclusion, our results highlight the global regulatory role of AaMetR in cysteine and methionine metabolism and provide new insights into the crucial role of ROS detoxification, sporulation and pathogenicity in the tangerine pathotype of *A. alternata*.

1. Introduction

To successfully cause disease, pathogenic microorganisms must overwhelm the host's immune defenses, spread throughout the entire tissue and acquire nutrients from the cells. When pathogens infect plant, inducers in the plant cells bind to receptors on cell membranes, activate G protein, and then Ca^{2+} enters the cell to activate the protein kinase, which acts on the membrane of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to produce toxic reactive oxygen species (ROS) (Bowler and Fluhr, 2000; Gilroy et al., 2014). ROS are reactive chemical species containing oxygen, such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH·) and superoxide (O_2^-) (Schopfer et al., 2001; Giorgio et al., 2007). Under normal condition, host cells typically maintain ROS at relatively low, sub-toxic levels. However, ROS levels can increase dramatically after the host recognizes pathogens (Wojtaszek, 1997). Excessive production of ROS can cause oxidative damage to membrane lipids, proteins, other cellular molecules, and ultimately kill pathogens and host cells, which is known as hypersensitive response (HR) (Bandyopadhyay et al., 1999). Therefore, the pathogen must be able to withstand the toxic ROS after penetrating the host cell.

Pathogens have evolved strategies to overcome plant defense responses. For example, the filamentous fungus *Alternaria alternata* (Fr.) Keissler produces a unique host-selective toxin (HST) that kills host cells prior to invasion and nutrient acquisition (Akimitsu et al., 2014; Tsuge et al., 2016). *A. alternata* consists of at least seven pathotypes, each of which can cause diseases in a variety of important crops, including Japanese pear, tangerine, apple, strawberry, rough lemon, tomato, and tobacco (Akimitsu et al., 2014). Two distinct pathotypes of *A. alternata* have been found in citrus. One is the rough lemon pathotype

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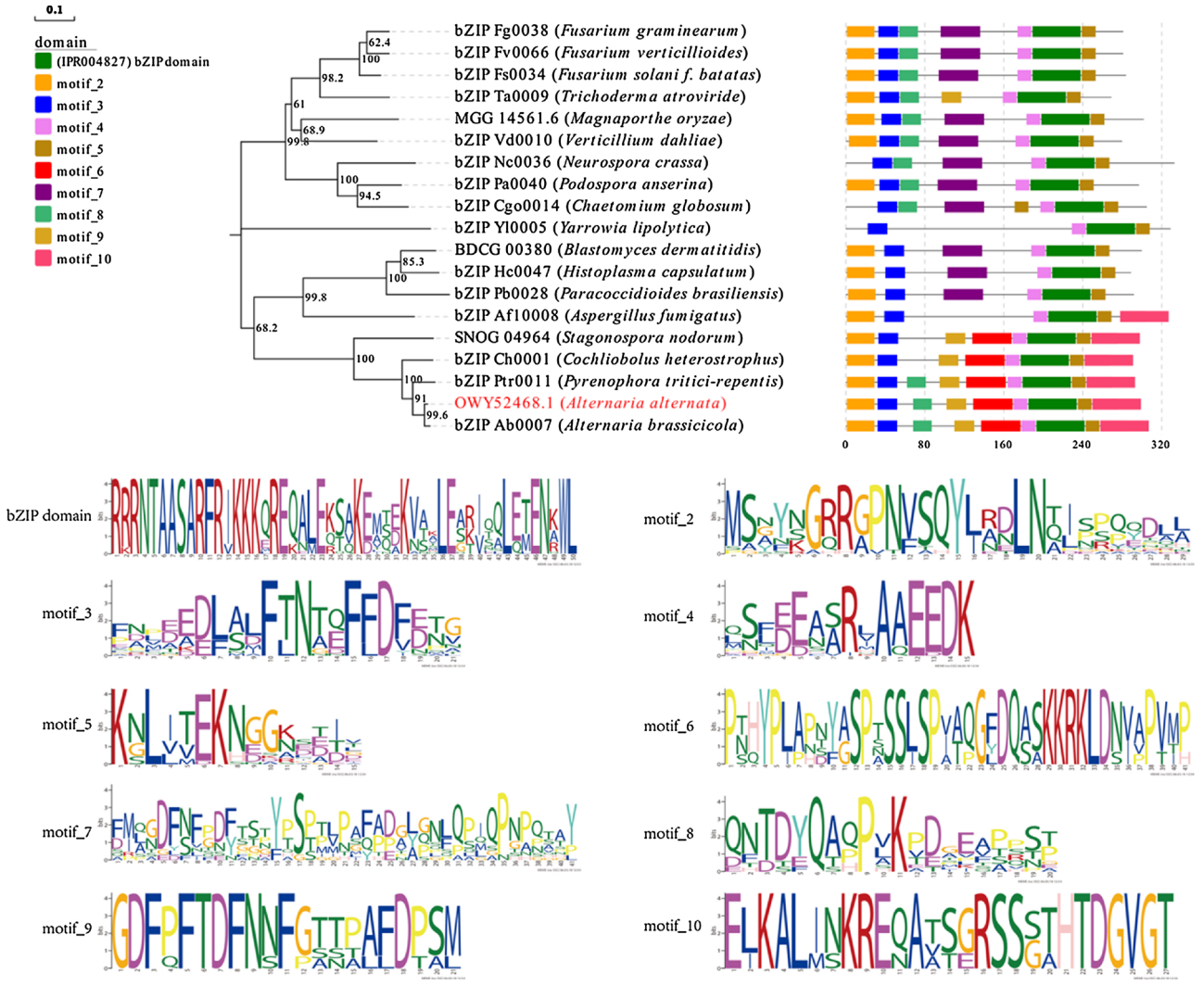

Fig. 1. Evolutionary analysis of MetR proteins from nineteen species and conserved motifs of according to the evolutionary relationship. The phylogenetic tree was constructed based on the amino acid sequence alignments of 19 MetR transcription factor (TFs) in nineteen fungal species. These sequences were aligned with ClustalW, and tree reconstruction was performed using the neighbor joining method by MEGA7.0. The numbers representing the percentage of the occurrence in 1000 bootstrap replicates. The conserved motifs in the MetR proteins were identified by MEME software. The scale bar shows the number of motifs. Grey lines represent the non-conserved sequences, and each motif is indicated by a colored box at the left. The length of motifs in each protein was exhibited proportionally.

of *A. alternata,* which produces host-selective ACRL toxin that is pathogenic to rough lemon (*Citrus jambhiri* Lush) and Pangpur lime (*Citrus limonia* Osbech), causing leaf spot (Akimitsu et al., 1989; Peever et al., 1999, 2004). The other is the tangerine pathotype of *A. alternata,* which produces a unique host-selective toxin called ACT toxin that causes brown spots on citrus leaves and fruits (Kohmoto et al., 1991; Akimitsu et al., 2003; Huang et al., 2015). The tangerine pathotype of *A. alternata* is pathogenic to tangerines (*Citrus reticulata* Blanco), grapefruit (*Citrus paradisi* Merced.), their hybrids, as well as hybrids from tangerine and sweet orange (*Citrus sinensis* (L.) Osbeck), but not toxic in rough lemon (Ma et al., 2018). Previous studies have shown that at least seven *ACTT* genes are present in multiple copies of gene clusters on less than 2.0 Mb of the conditionally dispensable chromosome (CDC), which is essential for the biosynthesis of ACT toxin (Tsuge et al., 2016; Wang et al., 2017). RNA silencing of any *ACTT* gene leads to no production of ACT toxin and loss of pathogenicity (Miyamoto et al., 2008; Wang et al., 2017).

Sulfur is an essential mineral element that plays an important role in many biological processes (Kellogg et al., 1972). Sulfur is a crucial constituent of some amino acids, such as methionine and cysteine (Brosnan and Brosnan, 2006). Methionine plays a critical role in various cellular metabolic pathways in all organisms (Shoveller et al., 2005). For example, under the catalysis of methionine adenosyltransferase, methionine can bind to adenosine triphosphate (ATP) to synthesize S-adenosyl methionine (SAM), which is a key source of activated methyl groups in all organisms. It can also be served as a source of activated C3 aminopropyl units for polyamine biosynthesis in the cell cycle (Wang et al., 2005). S-adenosyl methionine is the major methyl donor in biomethylation and involves in methyl transfer, transsulfurization and aminopropylation, which are required for cell growth and regeneration (Cantoni, 1975; Lu, 2000). Microorganisms, plants, and animals are different in terms of obtaining methionine. Plants and microorganisms can acquire mineral sulfur from the environment through the aspartate

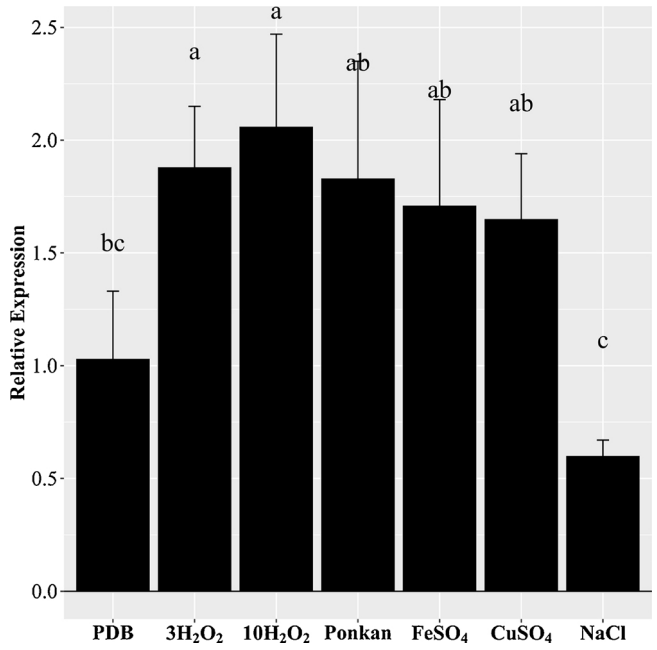

Fig. 2. Expression of *AaMetR* gene in *A. alternata* wide-type strain under different conditions. Mycelia of *A. alternata* wide-type strain Z7 are inoculated on PDB media for 48 h and treated with different chemical or injured Ponkan leaves to obtain a final concentration of PDB only, 3 mM H2O2, 10 mM H2O2, Ponkan (*Citrus reticulata* Blanco var. *Ponkan*), 10 mM FeSO4, 1 mM CuSO4, and 1 M NaCl, respectively.

biosynthetic pathway and *de novo* synthesize methionine (Ravanel et al., 2004). In contrast, animals cannot synthesize methionine and must acquire methionine from their diet (Ball et al., 2006). As a result, the methionine biosynthetic pathway is a common target for antifungal agents because of the presence of methionine biosynthetic pathway in microorganisms and absence in the animal kingdom. Ebelactone A is a beta-lactonease inhibitor that inhibits N-formylmethionine aminopeptidases on cell surface and stimulates host defense (Paterson and Hulme, 1995). Another compound strongly affecting methionine biosynthesis is the antibiotic azoxybacillin, which is shown to induce five sulfate assimilation enzymes, especially ATP sulfurylase and homoserine transacetylase (Jastrzębowska and Gabriel, 2015).

In the present study, a homolog of the bZIP transcription factor MetR in the tangerine pathotype of *A. alternata* was identified and functionally characterized to further understand the mechanism of methionine biosynthesis. Comparative transcriptome analysis of wild-type and *AaMetR* disruption mutant *ΔAaMetR* was performed to provide a novel data for AaMetR-mediated regulation of gene expression in the tangerine pathotype of *A. alternata*. The *ΔAaMetR* mutation perturb the expression of a large number of genes, including those associated with the cysteine and methionine metabolism pathway. Most of the differentially expressed genes are up-regulated in the cysteine and methionine metabolism pathways in the *ΔAaMetR* mutant, suggesting that AaMetR plays a negative regulatory role in cysteine and methionine metabolism.

2. Methods

2.1. Fungal strains and culture conditions

The reference *A. alternata* strain Z7, which was previously isolated from infected citrus in Zhejiang Province, China, was used as a wild-type strain in this work (Huang et al., 2015). The strain Z7 and its derivative mutants were cultured on potato dextrose agar (PDA) medium (potato 200 g, glucose 20 g, and agar 20 g, per liter of purified water), V8 medium (V8 broth 200 mL, $CaCO_3$ 3 g and agar 20 g, per liter of purified water), minimal medium (MM: KCl 0.5 g, $NaNO_3$ 2 g, KH_2PO_4 1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $FeSO_4$ 0.01 g, Sucrose 10 g, Trace elements 200 µL and agar 20 g, per liter of purified water) and complete medium (CM: MM medium added with yeast extract 1 g, casein hydrolysate 1 g, and peptone 2 g, per liter of MM medium) at 25 °C to evaluate their growth and colony characteristics (Hopwood, 1967). The trace elements solution consists of 5 g of $ZnSO_4$, 5 g of citric acid, 0.25 g of $CuSO_4{\cdot}5H_2O$, and 1 g of $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ per 100 mL of purified water. For conidia collection, mycelia of 2-weeks-old culture of each strain on V8 medium was rubbed in 5 mL of sterile water. The conidia suspension was filtered with a layer of filter paper and calculated by hemocytometer under an optical microscope.

2.2. Identification and phylogenetic analysis of MetR in A. alternata

To identify all bZIP transcription factors in *A. alternata*, the annotated genome sequence of *A. alternata* strain Z7 was downloaded from NCBI and used as the reference genome for data analysis. The Hidden Markov Model (HMM) profiles of the bZIP domains (PF00170) downloaded from Protein family (Pfam) (http://pfam.xfam.org) is employed as a query sequences against the genome of Z7 using HMMER 3.0 with default E-value (Finn et al., 2009). Search results below the threshold e-value ($< 10^{-4}$) were selected and their corresponding protein sequences were obtained from the Z7 genome. The homologous sequences of *MetR* from different fungi for phylogenetic analysis were downloaded from GenBank Sequence Database and the Fungal Transcription Factor Database (FTFD) (Park et al., 2008). MEME SUITE program were used to identify protein domains (Bailey et al., 2015). Multiple protein sequence alignment were carried out using ClustalW programs (Chenna et al., 2003). Phylogenetic trees were constructed using the neighbor-joining (NJ) method with 1000 bootstrap replicates by MEGA7 software (Kumar et al., 2016).

2.3. Quantitative RT-PCR of AaMetR gene

To quantify the expression of *AaMetR* gene under different stress conditions, the quantitative reverse transcription PCR (qRT-PCR) was performed using Bio-Rad CFX96 Real-Time PCR Detection Systems (Bio-Rad, USA). The fungus *A. alternaria* strain Z7 was cultivated in 150-mL Erlenmeyer flasks containing 50 mL of liquid PDB medium (PDA without agar) and incubated at 25 °C on a rotary shaker at 180 rpm in the dark for two days. The two-days-old Z7 cultures were supplemented with different chemical or injured Ponkan leaves to obtain a final concentration of PDB only, 3 mM H_2O_2, 10 mM H_2O_2, the injured tangerine (*Citrus reticulata* Blanco var. *Ponkan*) Leaves, 10 mM FeSO4, 1 mM CuSO4, and 1 M NaCl, respectively. After incubation for 30 min, the treated mycelia of Z7 with different chemicals were immediately filtered through four layers of cheesecloth, freeze-dried and ground to fine powder in liquid nitrogen for RNA extraction.

Fungal RNA was extracted from each sample using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen Biotechnology, Hangzhou, China) according to the manufacturer's instructions. 5 µg RNA was reverse transcribed into cDNA using a HiScript II Q RT SuperMix Kit (Vazyme Biotech Co., Ltd, Nanjing, China). Quantitative real-time PCR was performed using the ChamQ SYBR qPCR Master Mix Kit (Vazyme Biotech Co., Ltd, Nanjing, China). The Bio-Rad CFX96 Real-Time PCR Detection Systems was used for qRT-PCR with initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at

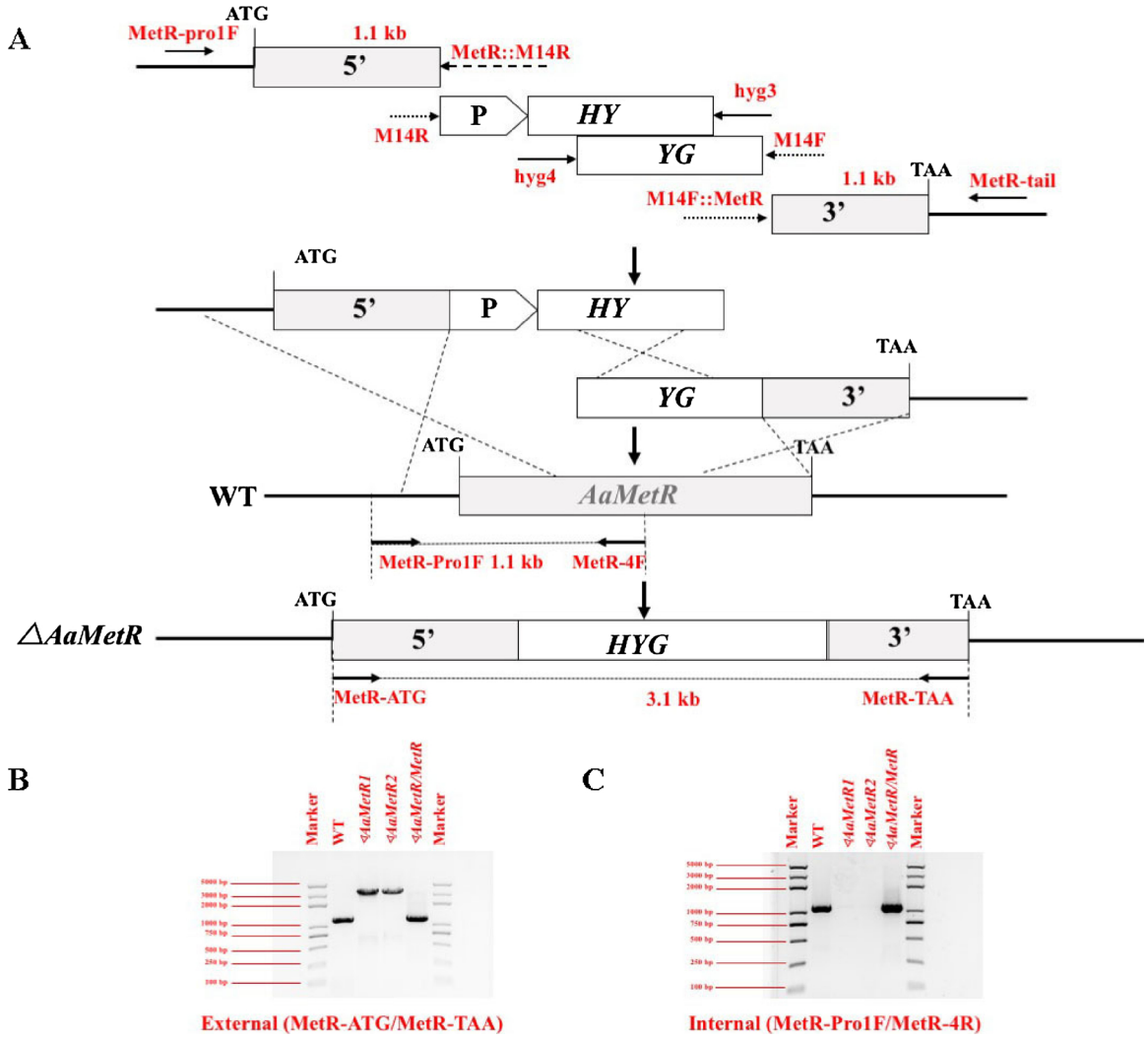

Fig. 3. Construction and confirmation of *AaMetR* disrupted and complemented mutants. (A) Schematic depiction of generation of truncated but overlapping hygromycin phosphotransferase gene (HYG) under control by the *Aspergillus nidulans* trpC promoter (P) and terminator (T) within AaMetR. Oligonucleotide primers used to amplify each fragment are indicated. A 162-bp fragment of AaMetR gene was replaced by a 2,144-bp fragments including hygromycin phosphotransferase gene (HYG) gene and the *Aspergillus nidulans* trpC promoter. (B) PCR verification from genomic DNA of wild type (Z7), two transformants (*AaMetR1* and *AaMetR2*) and the complementation strain *ΔAaMetR/MetR* with the primers MetR-ATG and MetR-TAA. Two *AaMetR* specific primers (MetR-ATG/MetR-TAA) amplified an expected 1,182-bp fragment from genomic DNA of wild-type Z7 and the complementation strain *ΔAaMetR/MetR* but amplified an expected 3,164-bp fragment from genomic DNA of two transformants (*AaMetR1* and *AaMetR2*). (C) PCR verification from genomic DNA of wild type (Z7), two transformants (*AaMetR1* and *AaMetR2*) and the complementation strain *ΔAaMetR/MetR* with the primers MetR-Pro1F and MetR-4R. Two *AaMetR* specific primers (MetR-Pro1F/MetR-4R) amplified an expected 1,068-bp fragment from genomic DNA of wild-type Z7 and the complementation strain *ΔAaMetR/MetR* but failed to amplify any fragments from two transformants (*AaMetR1* and *AaMetR2*) carrying a defective *AaMetR*.

95 °C for 10 s, annealing and extension at 60 °C for 30 s. For each sample, all treatments were performed in triplicate. *A. alternata* actin gene was used as an endogenous control in this study.

2.4. Mutation and complementation of AaMetR

AaMetR disruption mutants were generated by homologous recombination following protoplast transformation as previously described (Yang and Chung, 2013). Briefly, two DNA fragments (5′*MetR*::5′*HYG* and 3′*MetR*::3′*HYG*) overlapped with the hygromycin phosophotransferase gene (HYG) cassette were constructed separately by fusion PCR. Two purified fragments were transformed into protoplasts of *A. alternaria* strain Z7 using polyethylene glycol (PEG) and $CaCl_2$ (Yang and Chung, 2013). The transformants were cultured on PDA medium amended with 100 ug/mL hygromycin. To identify transformants carrying the mutation, the resistant transformants were screened by PCR using the inner and flanking primers of the *HYG* and *MetR* genes. For *AaMetR* gene complementation, a 3.6 kb DNA fragment containing *AaMetR* promoter and *AaMetR* gene was PCR amplified and cloned to the linearized pA1300-NEO vector by recombination method using ClonExpress II (Vazyme, Nanjing, China). After confirming the integrity of the recombinant plasmid by sequencing, the plasmid containing *AaMetR* gene were transformed into the protoplasts of *ΔAaMetR*. Transformants were selected on the MM medium amended with 3 mM H_2O_2 and the H_2O_2 resistant transformants were examined by PCR. MM medium amended with H_2O_2 was used as a selective medium, because

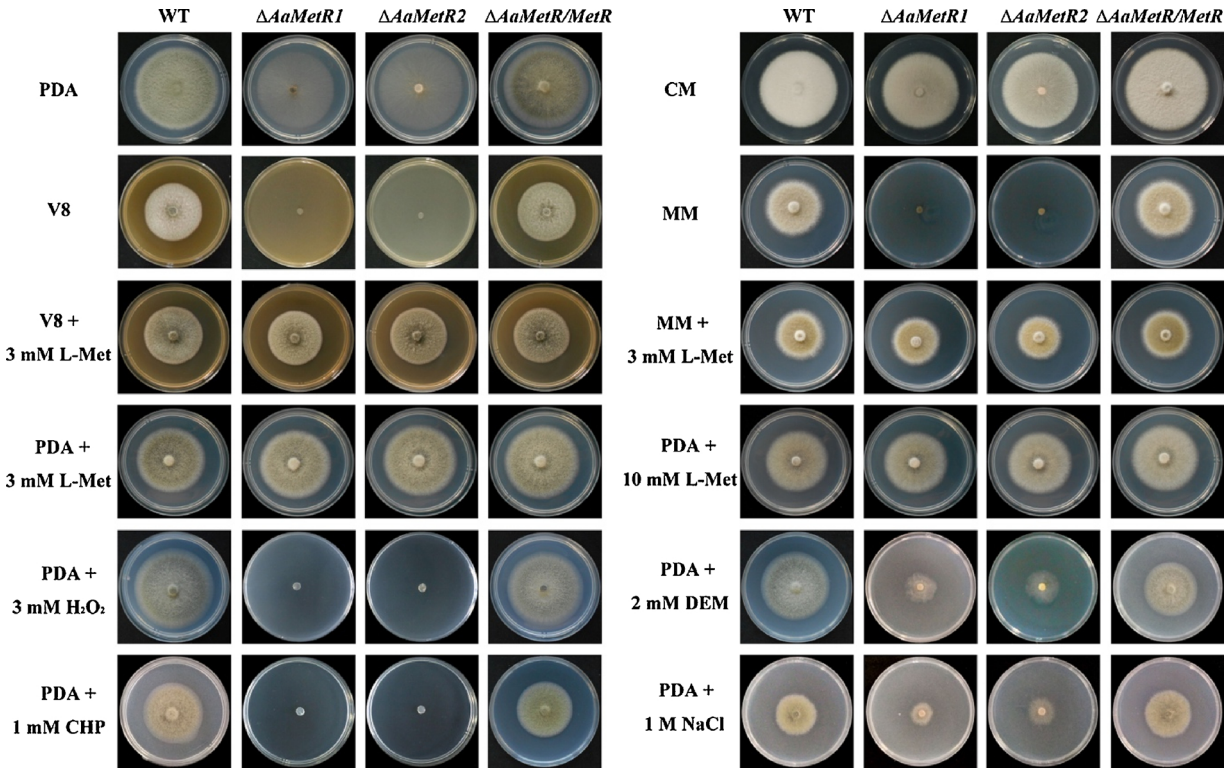

Fig. 4. Morphological characteristics of wild type strain (Z7), *ΔAaMetR* mutant (*AaMetR1*, *ΔAaMetR2*), and complemented strain *ΔAaMetR/MetR* on different media. Each strain was inoculated on PDA, V8, complete medium (CM), minimal medium (MM), PDA, V8, MM with 3 mM L-methionine, PDA with 10 mM L-methionine, PDA with 3 mM H2O2, 1 mM Cumyl hydroperoxide (CHP), 2 mM Diethyl maleate (DEM), 1 M NaCl, and cultured at 25℃ in the dark for 5 days and photographed.

further studies indicated that the *ΔAaMetR* mutant was hypersensitive to H_2O_2 and could not grow on MM medium. All the primers used in this study are listed in the supplementary table S1.

2.5. Characterization of the ΔAaMetR

To test the methionine auxotrophic phenotype, *A. alternaria* wild-type strain Z7, *ΔAaMetR* mutant *AaMetR1*, *AaMetR2* and complementary strain *ΔAaMetR/MetR* were inoculated on PDA, V8 and MM media supplemented with 3.0 mM and 10.0 mM L-methionine supplementation and cultured at 25 ˚C in the dark for 5 days. To examine the vegetative growth under oxidative stress, wild-type strain Z7, *ΔAaMetR* mutant *AaMetR1*, *AaMetR2* and complementary strain *ΔAaMetR/MetR* were inoculated on PDA plates containing 3 mM H_2O_2, 1 mM Cumyl hydroperoxide (CHP) and 2 mM Diethyl maleate (DEM), respectively. The diameter of fungal colonies was measured after culturing at 25 ˚C for 5 days. All growth assays were repeated twice, with three replicates per treatment. To investigate whether AaMetR is required for conidia formation, 2-weeks-old cultures of each strain on PDA medium were flooded with sterile water and scraped with glass rod under sterile condition. Since *ΔAaMetR* mutant is unable to grow on V8 and MM medium, we examined the 2-weeks-old culture on PDA medium.

2.6. Pathogenicity assay

Fungal virulence was evaluated by placing 5 mm mycelial plug from PDA medium on detached leaves of tangerine (*Citrus reticulata* Blanco var. *Ponkan*) and Hongjv (*Citrus tangerina* Hort.Ex *Tanaka*). After inoculation, leaves were placed in a humidified plastic box and incubated at 25 ˚C for 3 days to observe the development of disease symptoms. We tested at least 30 leaves per strain, and the entire experiment was repeated twice. To assess whether *ΔAaMetR* regained its virulence after supplementation with exogenous methionine, *ΔAaMetR* was grown on PDA medium amended with 3.0 mM methionine, then inoculated on tangerine leaves, and wild-type Z7 and *ΔAaMetR* grown on methionine-free PDA medium were used as control.

2.7. RNA-seq

For transcriptome analysis, ten mycelial plugs (5 mm in diameter) excised the margins of 4-day-old colonies of *ΔAaMetR* as well as wild-type strain Z7 were transferred into 150 mL Erlenmeyer flasks containing 50 mL PDB medium and shaken at 180 rpm on a rotary shaker in the dark at 25 ˚C for 2 days. The mycelium produced in PDB was filtered, freeze-dried, frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle for RNA extraction. Total RNA was extracted using AxyPrepTM Multi-Source Total RNA Miniprepkit (Axygen, USA). RNA degradation was monitored on 1% agarose gel. RNA purity was checked using the NanoPhotometer spectrophotometer (IMPLEN, CA, USA) and its concentration measured using Qubit RNA Assay Kit on Qubit 2.0 Flurometer (Life Technologies, CA, USA). RNA-seq libraries were performed using the Illumina TruSeq RNA sample preparation kit (Illumina Inc., USA) according to the manufacturer's protocol. Transcriptome sequencing was performed using an Illumina HiSeq2000 sequencer (Illumina Inc., USA). RNA-Seq was performed for three biological replicates of each sample.

The genome of *A. alternata* Z7 downloaded from GenBank was employed as the reference genome. Poor-quality reads and adapter were trimmed using Trimmomatic (Bolger et al., 2014) and the cleaned reads were subsequently mapped to the reference genome using Bowtie

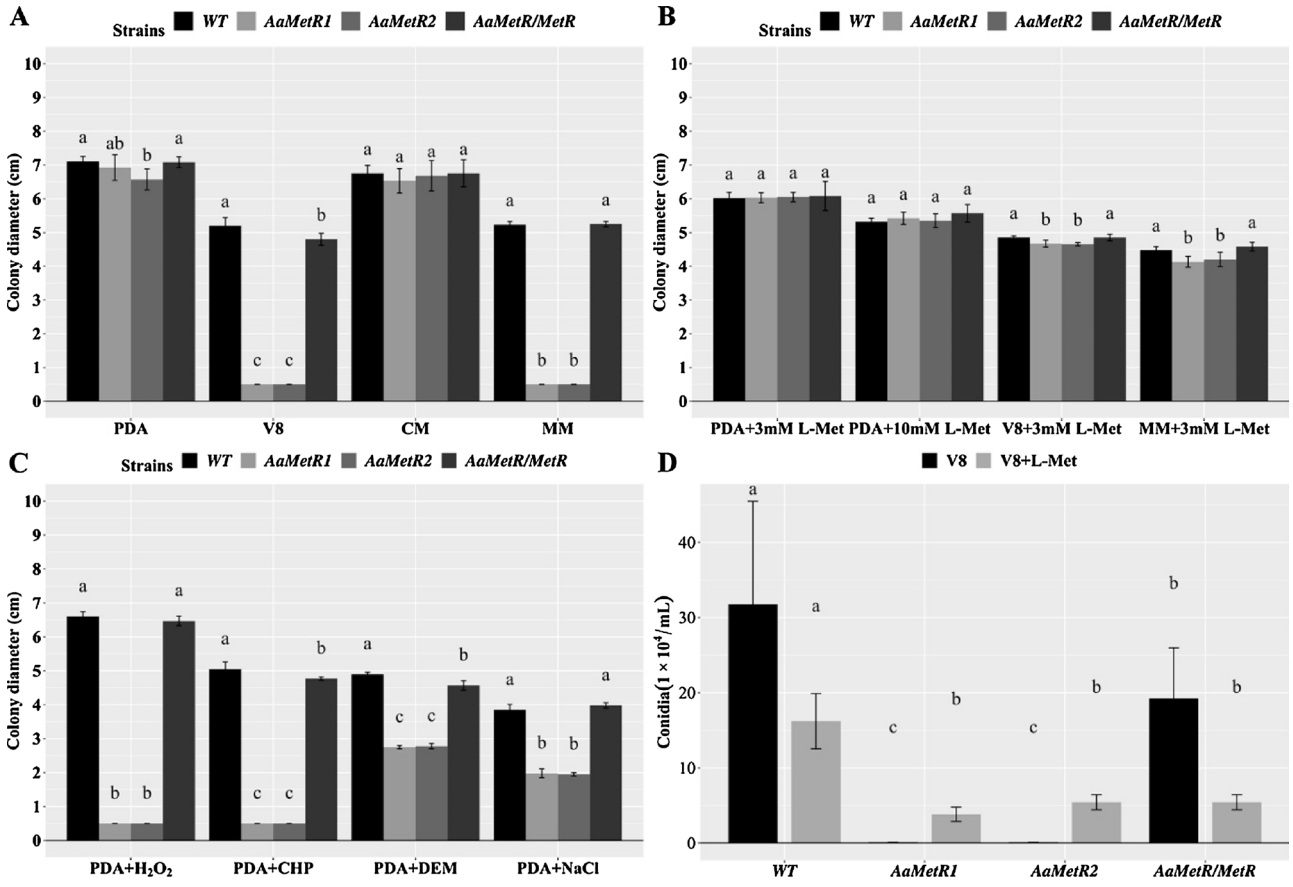

Fig. 5. The bar chart of wild type strain (Z7), *ΔAaMetR* mutant (*AaMetR1*, *ΔAaMetR2*), and complemented strain *ΔAaMetR/MetR* on different media. (A) Each strain was inoculated on PDA, V8, complete medium (CM), minimal medium (MM); (B) Each strain was inoculated on PDA, V8, MM with 3 mM L-methionine, and PDA with 10 mM L-methionine at 25℃ in the dark for 5 days; (C) Each strain was inoculated on PDA with 3 mM H2O2, 1 mM Cumyl hydroperoxide (CHP), 2 mM Diethyl maleate (DEM), and 1 M NaCl at 25℃ in the dark for 5 days; (D) Each strain was inoculated on V8 and V8 with 3.0 mM L-methionine at 25℃ in the dark for 14 days and count the conidial production. The Duncan's multiple comparison was used for the comparison of group mean differences. Statistical significance was set as $P < 0.05$. Same letter means same level.

(Langmead and Salzberg, 2012) and TopHat (Trapnell et al., 2009). Mapped reads were transformed into a count matrix with rows of gene and columns of sample using htseq-count to quantify gene expression levels (Anders et al., 2015). DESeq2 R package was used to identify differentially expressed (DE) genes under two conditions with a preset p-value lower than a threshold 0.001 as well as $\log_2$ fold change higher than a threshold 1.5 (Love et al., 2014). Gene Ontology (GO) annotations and functional term mapping were performed using Blast2GO software (Conesa et al., 2005). The GO annotation results were classified to categories using Web Gene Ontology Annotation Plot (WEGO) (Ye et al., 2006). Secondary metabolite (SM) biosynthesis gene clusters was identified in *A. alternata* Z7 genome by antiSMASH 4.0 (Blin et al., 2017). The differentially expressed genes (DEGs) and SM gene clusters were plotted using Circos (Krzywinski et al., 2009). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed for mapping enzymes onto known metabolic pathways (Kanehisa and Goto, 2000).

2.8. Quantitative RT-PCR

The RNAs of wide-type strain Z7 and *ΔAaMetR* were used for quantitative RT-PCR to verify the transcriptome data obtained by RNA-Seq. A total of 20 randomly selected *A. alternata* genes were tested on Bio-Rad CFX96 Real-Time PCR Detection Systems (Bio-Rad, USA). 5 μg of total RNA were reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara Biotechnology, Dalian, China). Quantitative real-time PCR was performed using Vazyme ChamQ SYBR Green qPCR master Mix Kit (Vazyme Biotech Co., Ltd, Nanjing, China). The qRT-PCR reaction with a volume of 20 μL was composed of template cDNA 20 ng, 0.8 μM of primers, and 10 μL of ChamQ SYBR Green qPCR master Mix. The reaction procedure was initial denaturation at 95 ˚C for 30 s, followed by 40 cycles of denaturation at 95 ˚C for 10 s, annealing and extension at 60 ˚C for 30 s. For each sample, all treatments were performed in triplicate. *A. alternata* actin gene was used as an endogenous control in this study.

3. Results

3.1. Identification and characterization of MetR in A. alternata

To identify MetR homologs in *A. alternata,* we used HMMER3 with the Hidden Markov Model (HMM) profile of the bZIP domain (PF00170) to query the whole genome of *A. alternata* Z7. The sequence of *Mocys-3* (MGG 14561, XM_003709705.1) and its protein sequence (XP_003709753.1) in *Magnaporthe oryzae* were used for comparison. We identified a single gene Aa03030 in *A. alternata* Z7 with a predicted

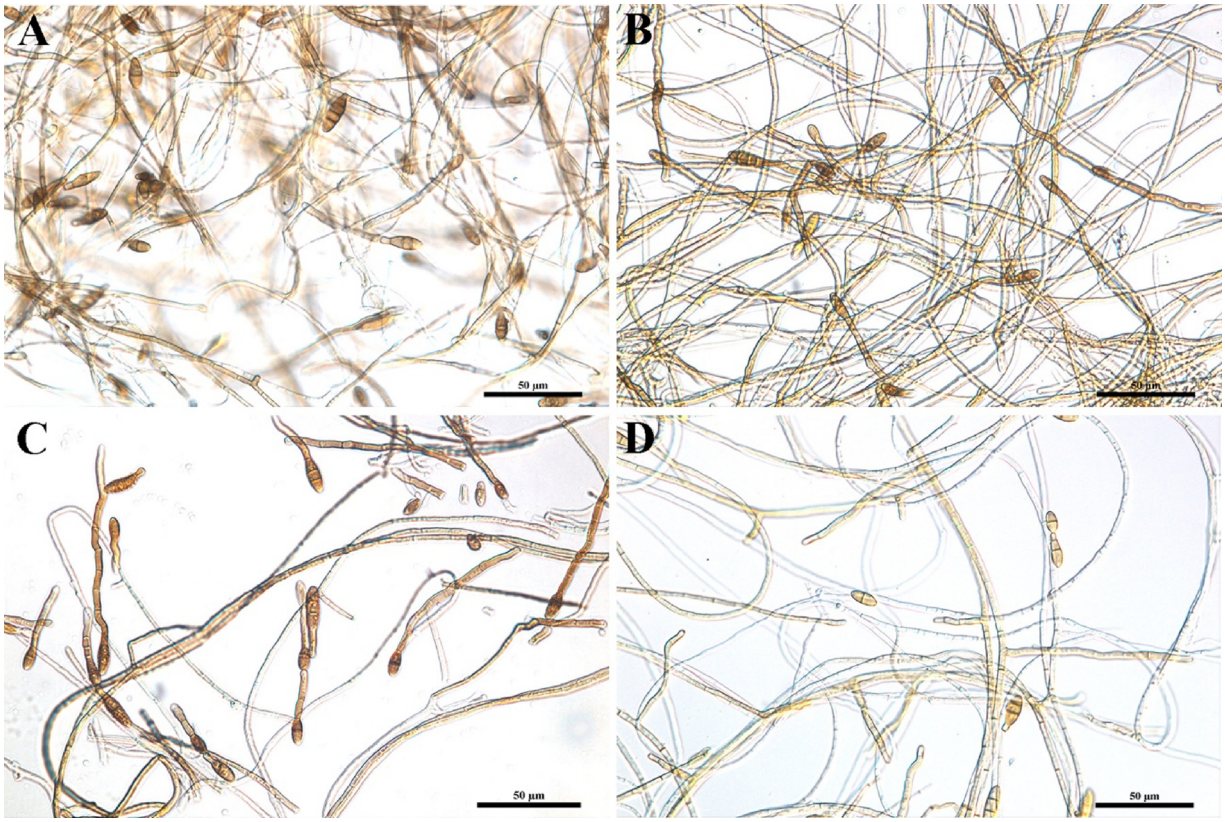

Fig. 6. Exogenous methionine compensate for the conidial formation. (A) *A. alternata* wild type strain Z7; (B) *ΔAaMetR mutant AaMetR1*; (C)*AaMetR2*; (D) complemented strain *ΔAaMetR/MetR*. All strains were inoculated on V8 media with 3.0 mM L-methionine and cultured at 25℃ in the dark for 14 days and photographed.

bZIP domain, and its product shared 52% amino acid identity with MoCys-3 homolog of *M. oryzae* (Fig. S1). The Aa03030 gene contains an 855 bp open reading frame interrupted by a single 333 bp intron, encoding a protein containing 284 amino acids. Sequence alignment and phylogenetic analysis of MetR homologs from different fungi confirmed that they shared a highly conserved bZIP domain, and Aa03030 was most similar to bZIP_Ab0007 of *A. brassicicola* (Fig. 1). We tentatively designated this gene *AaMetR*.

3.2. Oxidative and abiotic stress modifies AaMetR expression in A. alternata

The expression level of *AaMetR* gene in the *A. alternata* wild-type strain Z7 is different under different environmental stresses. In brief, the expression of *AaMetR* was up-regulated under the conditions of 3 mM H_2O_2, 10 mM H_2O_2, injured tangerine leaves, FeSO4, CuSO4, but down-regulated under the stress of NaCl. Among all conditions, *A. alternata* strain Z7 had the highest *AaMetR* expression in 10 mM H_2O_2. Oxidative stress and metal ion stress induce *AaMetR* to be up-regulated significantly. Similar to ROS stress, the expression of *AaMetR* gene in *A. alternata* was up-regulated under the stimulation of injured tangerine leaves (Fig. 2).

3.3. Targeted disruption of AaMetR in A. alternata

Reverse genetics strategy was implemented to investigate the role of *AaMetR* gene in abiotic stress tolerance. The *AaMetR* gene was disrupted with a split marker protocol using two DNA fragments overlapping in the hygromycin selection marker (5′*AaMetR*::5′*HYG* and 3′*AaMetR*::3′*HYG*) (Fig. 3A). This protocol showed up to 100% gene disruption efficiency in the tangerine pathotype of *A. alternata* (Lin et al., 2009; Yang and Chung, 2013). The transformation of wild-type Z7 protoplasts with two overlapping DNA fragments resulted in more than 40 hygromycin resistant transformants. The disruption of *AaMetR* gene was confirmed by PCR using outside primers pairs (Fig. 3B) and inside primers flanking the insertion site and included an annealing site upstream of the 5′ recombination target (Fig. 3C). Using an upstream primer with one internal to the deleted *AaMetR* fragment did not amplified a PCR product in either of the putative mutants, but amplified a PCR product from wild-type DNA consistent with gene disruption (Fig. 3C).

Cmplementary analysis was conducted to confirm that the observed phenotype was due to the disruption of *AaMetR* gene. A recombinant plasmid pA1300-NEO vector containing *AaMetR* gene and the promoter was introduced into protoplasts of the mutant *AaMetR1*. Presumptive complementary transformants were screened on the MM medium amended with 3 mM H_2O_2 and verified by PCR amplification (Fig. 3B and C). The complementary strain was named *ΔAaMetR/MetR*.

3.4. AaMetR is involved in vegetative growth

The growth of *ΔAaMetR* mutant were significantly different from wild-type on PDA, V8 and MM medium. *A. alternata* wild-type Z7 grew well on all four media, while *AaMetR1* and *AaMetR2* were only able to grow on PDA and CM. The *ΔAaMetR* mutant could not grow on V8 and MM media (Figs. 4 and 5A). Furthermore, the colony morphology of *ΔAaMetR* mutant were distinct from the wild-type strain on PDA. On PDA medium, the colony of wild-type strain Z7 was initially light

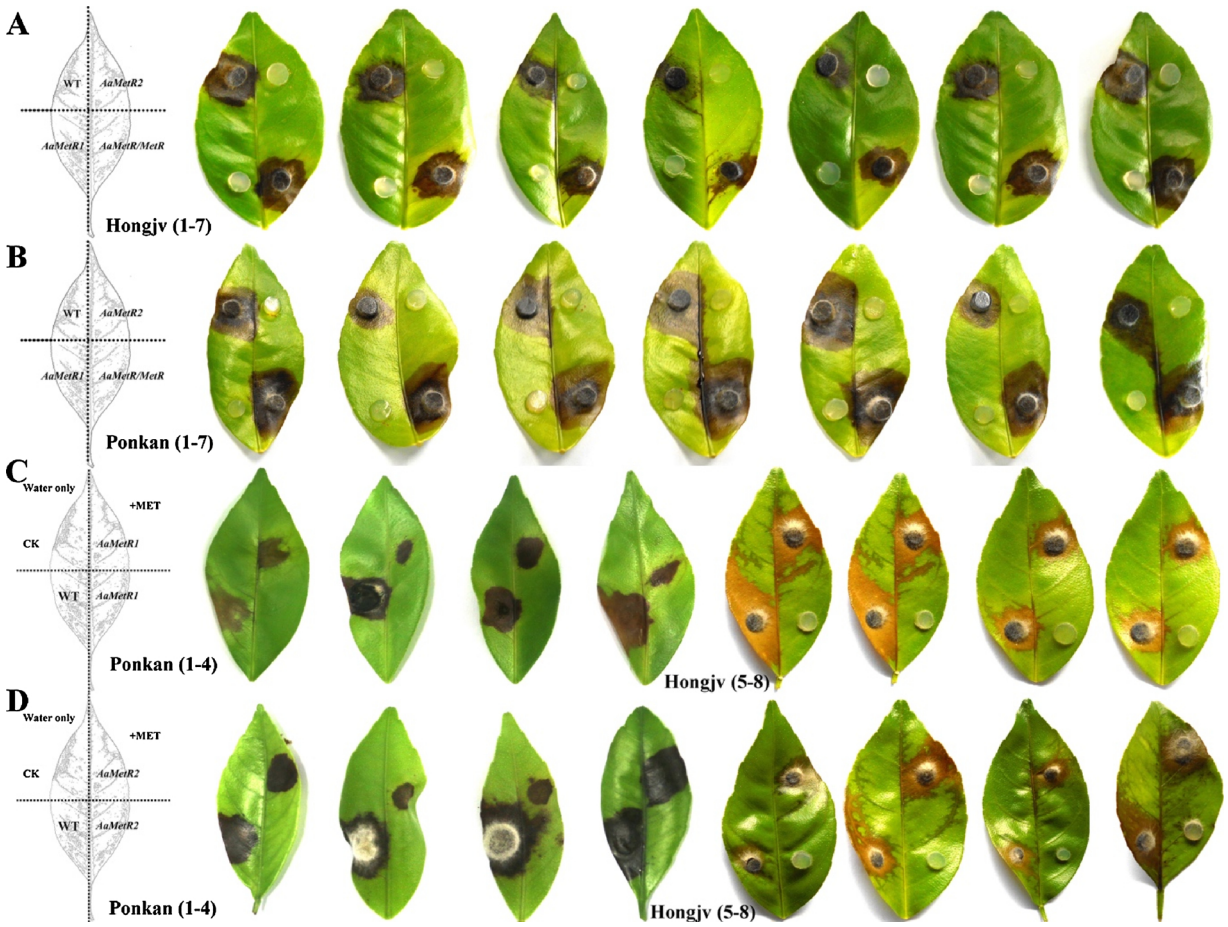

Fig. 7. Pathogenicity test of *A. alternata* with wild type strain Z7, *ΔAaMetR mutant* and complemented strain *ΔAaMetR/MetR* on detached leaves. Exogenous L-methionine compensate for the pathogenicity test of AaMetR mutant. (A) The detached leaves of Hongjv (*Citrus tangerina* Hort.Ex *Tanaka*) were inoculated with *A. alternata* wild type, *ΔAaMetR mutant* and complemented strain *ΔAaMetR/MetR*. (B) The detached leaves of Ponkan (*Citrus reticulata* Blanco var. *Ponkan*) were inoculated with *A. alternata* wild type, *ΔAaMetR mutant* and complemented strain *ΔAaMetR/MetR*. (C) The detached leaves of Ponkan (the 1st to 4th leaves) and Hongjv (the 5th to 8th leaves) were inoculated with *A. alternata* wild type, *AaMetR1* and *AaMetR1 with* L-methionine. (D) The detached leaves of Ponkan (the 1st to 4th leaves) and Hongjv (the 5th to 8th leaves) were inoculated with *A. alternata* wild type, *AaMetR2* and *AaMetR2 with* L-methionine for 3 days and photographed.

brown, then turned brown to dark brown within 3–5 days, producing abundant aerial hyphae. In contrast, the *ΔAaMetR* mutant consistently produced white to pale colonies without aerial hyphae (Fig. S2). After introducing the *AaMetR* gene, the complementary strain *ΔAaMetR/MetR* was able to grow on all four media and exhibited colony morphology similar to wild-type, indicating that the aberrant morphological phenotypes were caused by *AaMetR* disruption (Figs. 4 and 5A).

3.5. AaMetR contributes to conidia formation

To assess conidia formation, conidia were harvested from two-weeks-old cultures of each strain on V8 medium with 3 mL of water. Under optical microscope, wild-type strain Z7 produces conidia in long chains, characterized by pale to light brown in color, with a beak, and muriform septation (Fig. 6). The conidia yield of wild-type was approximately $3.2 \pm 1.4 \times 10^5$ conidia/mL (Fig. 5D). In contrast, *ΔAaMetR* mutant could not produce any conidia on V8 and PDA medium. After reintroduction of *AaMetR* gene, the complemented strain *ΔAaMetR/MetR* regained the ability to produce conidia, yielding $2.0 \pm 0.7 \times 10^5$ conidia/mL on PDA with two-week-old colonies (Fig. 5D). These results indicate that *AaMetR* is required for mycelial growth conidial production.

To determine whether the mutant phenotype can be complemented by exogenous methionine, *ΔAaMetR* mutant were inoculated on PDA, V8 and MM plates supplemented with 3 mM methionine. Both *AaMetR1* and *AaMetR2* were able to grow on V8 and MM media supplemented with 3 mM and 10 mM L-methionine. The colony morphology was similar to that of wild-type strain Z7 (Figs. 4 and 5B). A large number of conidia of *ΔAaMetR* mutant were observed on PDA and V8 media supplemented with 3 mM L-methionine, indicating that AaMetR is essential for methionine biosysthesis (Figs. 5D and 6).

3.6. AaMetR is required for fungal virulence

To determine whether *AaMetR* is involved in the pathogenicity of *A. alternata* on tangerine, thirty detached tangerine leaves were inoculated with 5-days-old mycelial plugs of the wild-type strain Z7, *AaMetR1*, *AaMetR2* and the complemented strain *ΔAaMetR/MetR*. Three days after inoculation, symptoms with circular to irregular brown necrosis were fully developed on detached citrus leaves inoculated with the wild-type strain Z7, but no lesion were observed on the leaves inoculated with *ΔAaMetR* mutant (Fig. 7A and B). The loss of pathogenicity was complemented in *ΔAaMetR/MetR*. To further investigate the loss-of-pathogenicity due to the inability of the *ΔAaMetR* mutant to

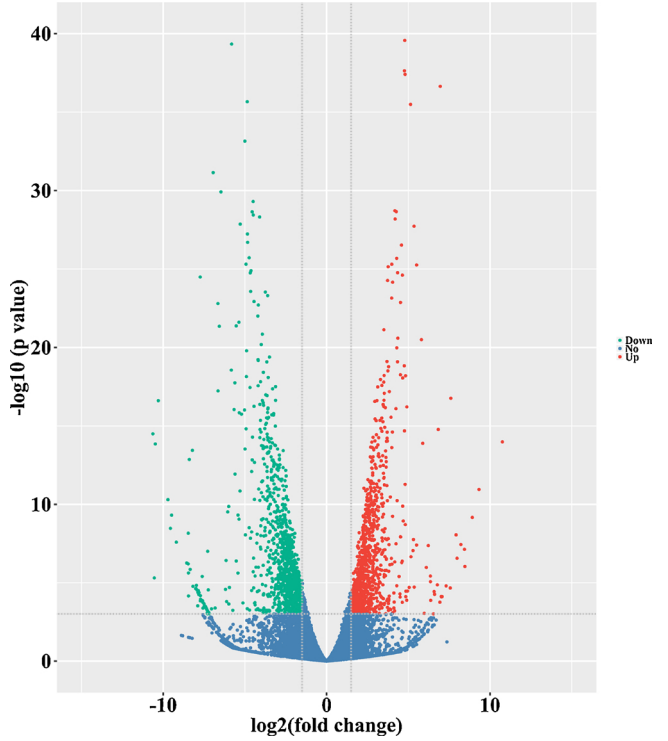

Fig. 8. Volcano plot of *ΔAaMetR* mutant gene expression pattern. In the volcano plot, the y-axis corresponds to the mean expression value of log10 (p-value), and the x-axis displays the log2 fold change value. The red dots represent the significantly differentially expressed transcripts ($p < 0.05$, false discovery rate (FDR) $q < 0.05$) which have upregulated in *ΔAaMetR* mutant than wild type; the gray dots represent the transcripts whose expression levels did not reach statistical significance ($p > 0.05$, FDR $q > 0.05$) between the high and low groups.

penetrate the host cells, wound inoculation experiments were conducted on detached tangerine leaves. Three days after inoculation, symptoms with brown spot were observed on the wound tangerine leaves inoculated with wild-type strain Z7. However, no visible symptoms were observed on wounded leaves inoculated with *AaMetR* mutant. Therefore, our results demonstrated that the disruption of *AaMetR* gene leaded to the complete loss of virulence of *A. alternata*.

To further investigate the loss of pathogenicity of *ΔAaMetR* mutant due to the observed methionine auxotrophy, we inoculated detached tangerine leaves with 5-days-old mycelial plugs of *ΔAaMetR* mutant grown on PDA amended with 3 mM L-methionine. We used wild-type and *ΔAaMetR* mutant without methionine supplementation as control. Three days after inoculation, typical symptoms of brown spot were observed on detached leaves inoculated with wild-type strain Z7 as well as the *ΔAaMetR* mutant grown with methionine supplementation. The detached leaves inoculated with *ΔAaMetR* mutant without methionine supplementation were asymptomatic. Although the lesions induced by the *ΔAaMetR* mutant with methionine supplementation were less than that caused by the wild-type strain Z7 (Fig. 7C and D). This experiment suggests that the loss of pathogenicity in the *ΔAaMetR* mutant is caused by a defect in methionine biosysthesis. The *ΔAaMetR* mutant partially restored their virulence after supplementation with extraneous methionine.

3.7. AaMetR is crucial for resistance to oxidative stress

To investigate whether *MetR* is participated in the oxidative stress resistance and salt stress, *A. alternata* wild-type strain Z7, *ΔAaMetR* mutant *AaMetR1*, *AaMetR2*, and *ΔAaMetR/MetR* were inoculated onto PDA plates supplemented with 3 mM H_2O_2, 1 mM Cumyl hydroperoxide (CHP), 2 mM Diethyl maleate (DEM), and 1 M NaCl. The mycelial growth of the *ΔAaMetR* mutant was significantly affected by oxidizing agents H_2O_2, CHP, DEM, and NaCl. H_2O_2 and CHP completely inhibited fungal growth of the *ΔAaMetR* mutant on PDA. In contrast, growth of the wild-type fungus did not appear to be impacted by the supplements (Figs. 4 and 5C). Complementation of *AaMetR* mutation restored the ability of the *ΔAaMetR* mutant to withstand the induced oxidative stress confirming that loss of *AaMetR* function confers oxidative stress sensitivity.

3.8. Transcription profiling of AaMetR by RNA sequencing

RNA-seq was implemented to identify genes differentially expressed in *AaMetR* mutant, which may be the downstream of the putative transcription factor. Illumina libraries were constructed from *AaMetR1* and wild-type Z7 and sequenced them on the HiSeq platform. In total, 122,477,108 and 125,603,640 reads were obtained from the RNA-Seq libraries of *AaMetR1* and wild-type Z7. Of these, 107,023,771 and 110,948,646 reads were uniquely mapped to the reference genome of Z7, accounting for 87.38% and 88.33% mapping rate for *AaMetR1* and wild-type Z7, respectively. The absolute value of $\log_2$ratio ≥ 1.5 and FDR ≤ 0.001 was used as the threshold. In total, more than 2042 genes met these criteria and were considered to be differentially expressed genes (DEGs). Among the 2042 DEGs, 956 were up-regulated and 1086 were down-regulated in the mutant *AaMetR1* compared to the wild-type (Figs. 8 and 9). Quantitative RT-PCR experiment was performed to verify the results of RNA-Seq. Twenty genes were randomly selected and validated. Although there is a slightly difference in folding changes, each gene expression pattern was consistent with the gene expression pattern in the RNA-Seq data (Fig. 10).

In total, 127 SM gene clusters were identified in the genome of *A. alternata* Z7 using antiSMASH 4.0. A custom Perl script was used to screen the gene cluster which at least 3 genes are upregulated or downregulated with a log2FC $\geq$ 2.0 and the average value of log2FC $\geq$ 1.0 (Sum of log2FC /Number of genes $\geq$ 1.0). Overall, 21 gene clusters showed considerable upregulated or downregulated in *ΔAaMetR* mutant. The transcript level of cluster4 was significantly downregulated in *ΔAaMetR* mutant, indicating there may be positive regulation cechanism between AaMetR and cluster4. Among those genes, Aa00442, Aa00443, and Aa00444 belong to Cytochrome P-450 family, which is involved in the toxication of toxic substances and steroids and fatty acids. Aa00444 encodes a FAD/FMN-dependent oxygenase/oxidase. Aa00446 encodes PksC, which is responsble for polyketide synthase. Therefore, AaMetR showed extensive regulation of the secondary metabolite (SM) biosynthesis gene clusters (Fig. 11).

Of all DEGs, 841 up-regulated genes and 821 down-regulated genes were categorized into 49 GO groups. The genes were enriched significantly within 29 GO term, including organic substance metabolic process (772 genes), primary metabolic process (739), cellular metabolic process (704), nitrogen compound metabolic process (665), organic cyclic compound binding (521), heterocyclic compound binding (520), ion binding (509), biosynthetic process (477), hydrolase activity (377), small molecule binding (303), establishment of localization (300), oxidation-reduction process (291), oxidoreductase activity (289), transferase activity (282), regulation of biological process (226), regulation of cellular process (217), small molecule metabolic process (211), carbohydrate derivative binding (197), cellular component organization (194), drug binding (181), cofactor binding (164), cellular component biogenesis (163), regulation of metabolic process (158), cellular response to stimulus (139), catalytic activity, acting on a protein (129), transmembrane transporter activity (110), protein binding

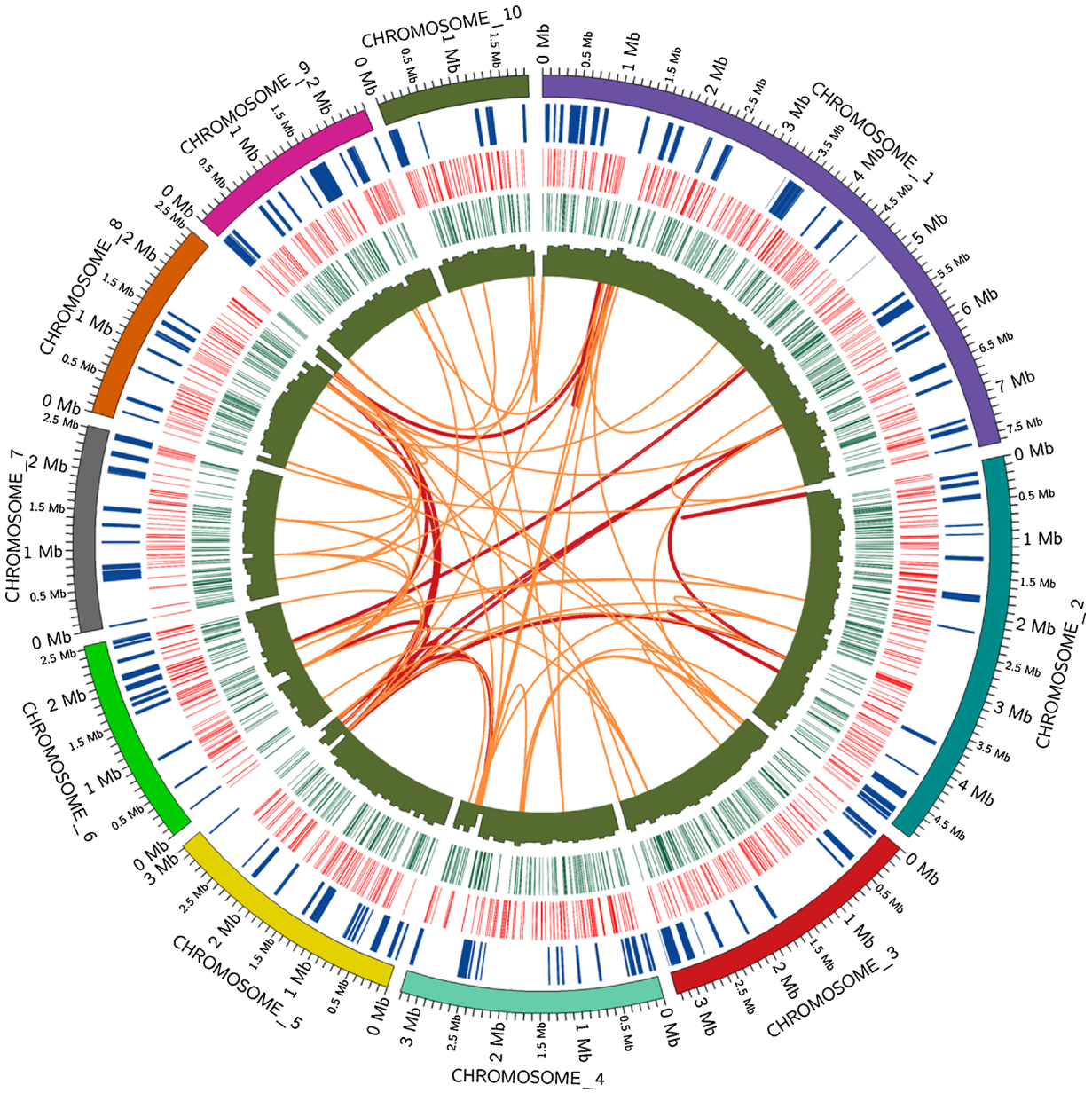

Fig. 9. Circos plot displaying the differences in gene expression, and miRNA expression in *ΔAaMetR* mutant compared to the wild-type Z7. Each circle from the periphery to the core represents the following: chromosomal location, secondary metabolite gene clusters, differentially expressed genes (DEGs), up-regulation in red, down-regulation in green, and GC content. Gene duplications are shown in the center. The conditionally dispensable chromosome (CDC) are not including in this figure.

(108), structural constituent of ribosome (104), response to stress (89) (Fig. 12).

A total of 798 genes were mapped to the KEGG pathways, and the top 20 pathways in enrichment degree are listed (Fig. 13). The pathways with significant enrichment including, Aminoacyl-tRNA biosynthesis (18 genes), Cysteine and methionine metabolism (13), Proteasome (11), Valine, leucine and isoleucine biosynthesis (10), Alanine, aspartate and glutamate metabolism (10), Phenylalanine, tyrosine and tryptophan biosynthesis (10), Glutathione metabolism (9), Arginine biosynthesis (9), Nitrogen metabolism (8), Lysine biosynthesis (8), Butanoate metabolism (8), Pantothenate and CoA biosynthesis (7), Quorum sensing (6), Vitamin B6 metabolism (5), Antifolate resistance (5), Selenocompound metabolism (5), Carbon fixation pathways in prokaryotes (5), Sulfur relay system (4), Synthesis and degradation of ketone bodies (4), C5-Branched dibasic acid metabolism (3).

3.9. AaMetR affect several critical genes in cysteine and methionine metabolism pathway

As shown in the KEGG pathway enrichment scatter plot, AaMetR significantly affects cysteine and methionine metabolism (ko00270), which contains the greatest number of differentially expressed genes in all DEGs-enriched pathways. Twelve DEGs were mapped to the cysteine and methionine metabolism pathway by $\log_2$ratio ≥ 1.5, 9 of which

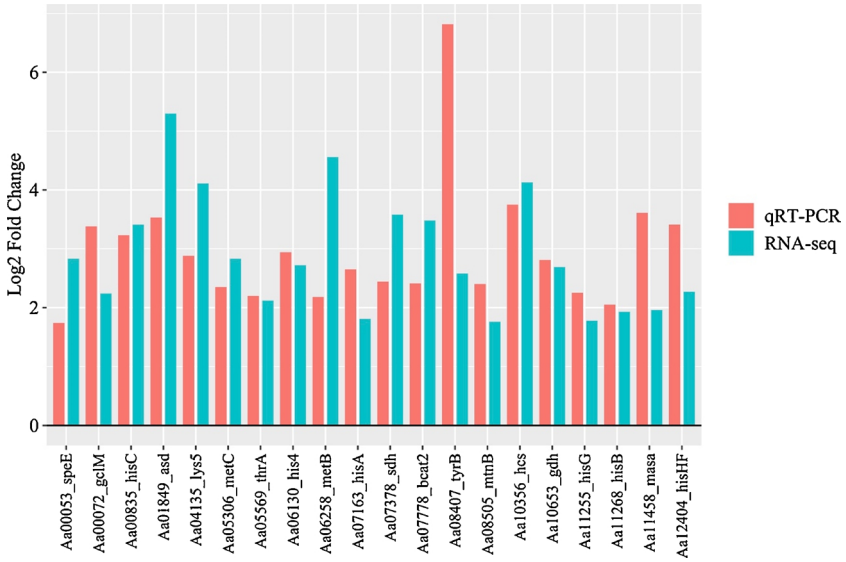

Fig. 10. Validation of the RNA-seq data using qRT-PCR. Fold change expression levels were normalized to actin expression levels in each sample and data is presented relative to wildtype expression levels using $2^{-\Delta\Delta Ct}$ method.

were differentially expressed by a $\log_2$ratio ≥ 2.0 (Fig. 14). *AaMetR* significantly affects Aa10494 (*msrC*, K08968), Aa01849 (*asd*, K00133), Aa07778 (K00826), Aa01614 (*MetX*, K00641), Aa06258 (*MetB*, K01739), *Aa05306* (*MetC*, K01760), Aa00053 (*speE*, K00797), Aa05948 (*MetE*, K00549), Aa10458 (K00641), Aa05569 (K00003) and Aa08024 (K17989). Among these genes, Aa05306 is a *MetC* ortholog (cystathionine beta-lyase), which converts L-cystathionine to homocysteine. Aa05948 is a *MetE* ortholog, which is involved in catalyzing homocysteine to methionine. Aa10494 is an ortholog of *msrC* (methionine sulfoxide reductase C) that is involved in oxidative stress response and is specific for repairing free methionine sulfoxide. After disrupting *AaMetR* gene in *A. alternata*, the $\log_2$ratio value of *msrC* was 5.28-folds compare with wild-type. Another important gene that *MetR* affected is *MetB*, which is cystathionine y-synthase, the second enzyme of methionine biosynthetic pathway.

Taken together, these results indicate that *AaMetR* perturb the expression of a large number of genes, including several crucial genes involved in the cysteine and methionine metabolism. Most of the genes are up-regulated in the cysteine and methionine metabolism pathway in the *ΔAaMetR* mutant, indicating that AaMetR plays a crucial role in cysteine and methionine metabolism.

4. Discussion

The present study identified a homolog of the methionine biosynthesis regulator MetR, designated AaMetR, and further investigated the key role of this bZIP transcription factor in regulating methionine metabolism. Similar to other homologs of the bZIP transcription factor family, AaMetR contains a conserved leucine zipper (bZIP) domain and several conserved motifs that are critical for binding to the promoter region of the gene and regulating their expression. By analyzing the loss of function and complementation of *AaMetR* gene, we found that *AaMetR* gene is required for methionine metabolism, vegetative growth, conidial formation, pathogenicity and anti-oxidative stress. This work further demonstrated that the citrus pathogen *A. alternata* utilizes a complex pathway that modulates methionine biosynthesis through transcriptome analysis of the *ΔAaMetR* mutant and the wide-type strain Z7. Quantitative real-time PCR was performed to quantify *AaMetR* expression under various stress condition, including H_2O_2, the injured tangerine leaves, FeSO4, CuSO4 and NaCl. The injured tangerine leaves are used to stimulate *A. alternata*, because after the plant is infected by microorganisms, NADPH oxidase can utilizes molecular electrons provided by NADPH to catalyze molecular oxygen (O_2) to superoxide anion (O^{2-}), and then converted to hydrogen peroxide (H_2O_2). Therefore, the injured tangerine leaves can be used to stimulate *A. alternata* and to determine gene expression during the pathogen infecting the host plant. Compared to *A. alternata* wild-type Z7 in PDB, the expression of *AaMetR* gene was up-regulated at different levels of oxidative stress, metal ionic stress and injured tangerine leaves, implicating that the bZIP transcription factor AaMetR may be involved in the regulation of genes in responding to the exogenous oxidative stress.

The ability of detoxified host-generating ROS is critical in the infection and colonization of pathogenic microorganisms. After disrupting *AaMetR* gene in *A. alternata*, the mutant became hypersensitivity to hydrogen peroxide and various ROS-generating agents. The *ΔAaMetR mutant* was unable to grow on PDA medium containing 3 mM H_2O_2 and other ROS-generating oxidants. ROS such as hydrogen peroxide (H_2O_2) are by-products of aerobic metabolism, which are mainly generated by mitochondria and NADPH oxidases. Previous studies have demonstrated that NADPH oxidase components, including NoxA, NoxB and NoxR, are involved in the resistance to oxidative stress (Yang and CHUNG, 2012,2013). *A. allternata* lacking *NoxA*, *NoxB* and *NoxR* genes displayed a slightly increased sensitivity to oxidants. Only double disrupting mutants lacking *NoxA/NoxB* or *NoxA/NoxR* genes exhibited significant sensitivity to hydrogen peroxide (H_2O_2)[41]. The ROS sensitivity phenotype of *ΔAaMetR mutant* is similar to that of the *GPx3*, *Yap1* and *hog1* mutants, but more severe than any of these mutants (Lin et al., 2009; Lin and Chung, 2010). Deletion of *ap1*, *hog1*, or *GPx3* in *A. allternata* caused a considerable decrease in mycelial growth under oxidative stress. However, the *ap1* and *hog1* mutants were still able to grow on the PDA media containing H_2O_2 and other ROS-generating oxidants, albeit much slower than wide-type. In contrast, *A. alternata* lacking *AaMetR* was unable to survive on PDA medium containing 3 mM H_2O_2. It appears that AaMetR significantly affects the ability to resist oxidative stress in *A. alternata*.

Our study demonstrated that AaMetR is required for the

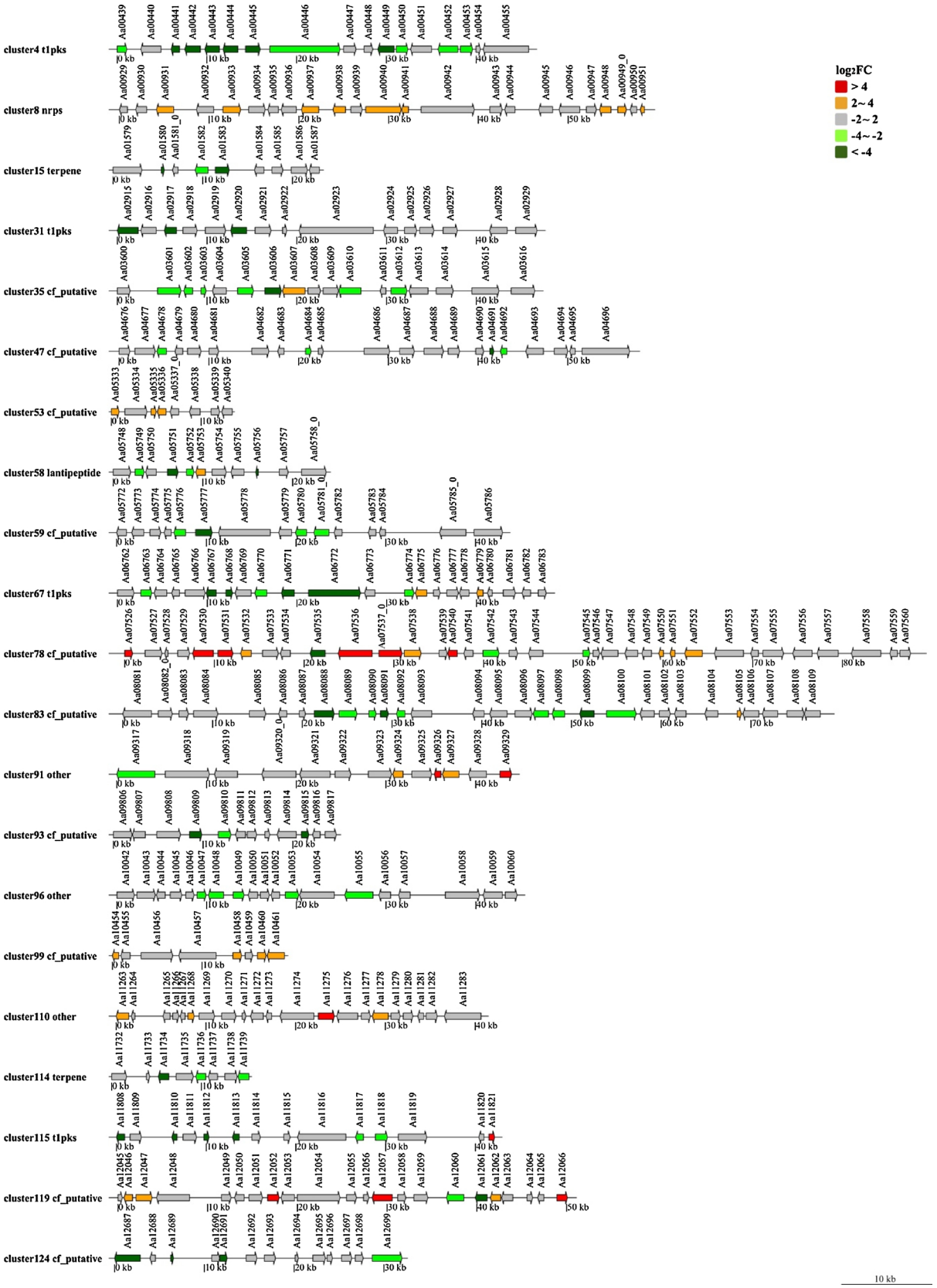

Fig. 11. The differential gene expression of transcriptome data between the *ΔAaMetR* mutant and the wild-type Z7 were mapped against the secondary metabolite gene clusters. The different colors of genes represent differential gene expression in the *ΔAaMetR* mutant compared to the wild-type. pks, polyketide synthase; nrps, nonribosomal peptides synthetase; t1, Type1; cf_putative, putative biosynthetic types (unknown types); terpene, terpene synthetase.

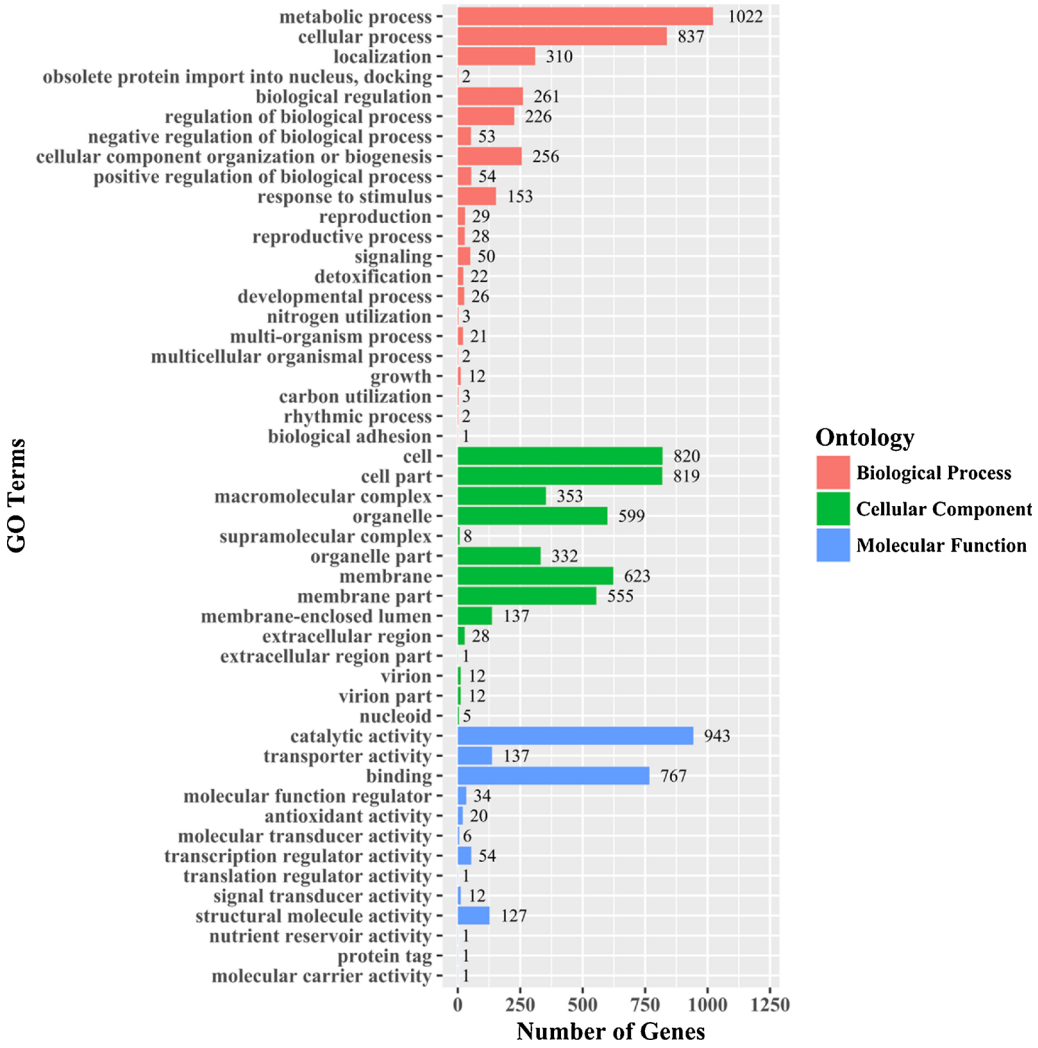

Fig. 12. Gene Ontology (GO) enrichment analysis of the differential expressed genes (DEGs) between the transcriptome of *ΔAaMetR1* mutant and wide-type strain Z7. The results are summarized in three main GO categories (cellular component, molecular function and biological process). The x-axis indicates the number of DEGs in a category. The y-axis indicates the GO term.

pathogenicity of the tangerine pathotype of *A. alternata.* When pathogens invade a host plant, the level of ROS in the host cell increase dramatically, which kills the invading pathogen. However, microorganisms have evolved mechanisms to detoxify the ROS generated by host plants (Apel and Hirt, 2004). *AaMetR* expression in wild-type *A. alternata* was significantly up-regulated in response to citrus leaves tangerine stimulation, which suggests that *AaMetR* may be involved in ROS response and host-pathogen interaction. Virulence assays demonstrate that the *ΔAaMetR mutant* is unable to induce necrotic lesions on detached citrus leaves, further confirming that *AaMetR* is required for the pathogenicity. Previous studies have demonstrated that *MoMetR,* the *M. oryzae* ortholog of *AaMetR* was essential for virulence of *M. oryzae* on rice [43]. However, the pathogenesis of *M. oryzae* and *A. alternata* is different. Many factors can affect the virulence of pathogenic fungi including the environment, host genotype, and the pathogen itself. In *A. alternata*, ROS detoxification and ACT toxin synthesis have been reported to be two critical factors that influence pathogenicity. Dysfunction of any *ACTT* genes of CDC could prevent ACT toxin secretion leading to loss-of-pathogenicity in *A. alternata. AaMetR* null mutants failed to induce necrotic lesions on both detached wounded and unwounded citrus leaves. The loss of pathogenicity by *AaMetR* null mutants may be due to their inability to resist or otherwise detoxify the ROS produced by host plant. *ΔAaMetR mutant* regain pathogenicity after complementation of *AaMetR* gene or after receiving methionine supplementation from media.

The results of present study demonstrate that *MetR* plays a crucial role in vegetative growth and conidiogenesis. *ΔAaMetR mutant* could not grow on either V8 or MM media, and grew poorly on PDA when compared with wild-type strain Z7. It would probably be worth noting somewhere in the manuscript the differences between the different media types. The colorless mycelia suggest that *ΔAaMetR mutant* produce less of the black pigment melanin than wild-type fungus. Intriguingly, the rice pathogen *Magnaporthe grisea* requires melanization of its appressoria development and host penetration (Howard and Ferrari, 1989). Unlike *M. grisea*, the appressoria of *A. alternata* is colorless and melanin is not required for appressoria development or host penetration (Kawamura et al., 1997). Conidiogenesis is an important mechanism for the dispersal of *A. alternata*. The conidia of *A. alternata* in the field can be spread widely by wind and rain. Our study demonstrates that *MetR* is required for conidia formation. While wild-type Z7 produces typical *Alternaria* brown conidia, the *ΔAaMetR mutant* could not produce conidia on either PDA or CM media. The *AaMetR mutant*,

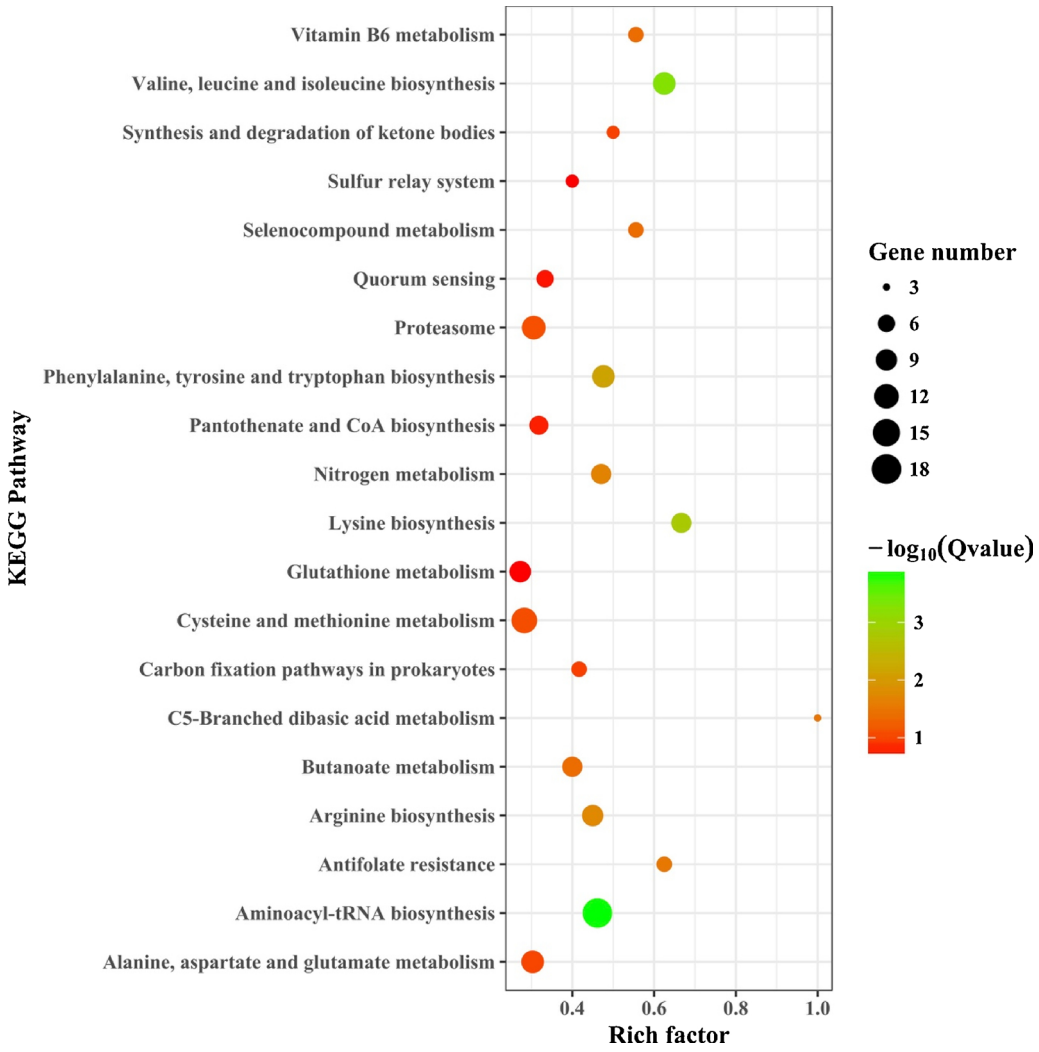

Fig. 13. Scatter plot of KEGG pathway enrichment statistics. different expressed genes in the *ΔAaMetR* disruption mutant. Rich Factor represents the ratio of numbers of differentially expressed genes annotated in the pathway term to the numbers of all genes annotated in the same pathway. Q-value is corrected P-value, with a lower value means greater intensiveness. Top 20 pathway terms enriched are displayed in the figure.

however, produced wild-type levels of conidia the media supplemented with methionine indicating that methionine is required for conidiogenesis.

In order to candidate methionine metabolism and oxidative stress resistance genes that are downstream of *AaMetR* transcription factor, we identified the differentially expressed genes in *AaMetR* mutant. In total, we identified 2042 genes with transcription levels $\log_2$ FC ≥ 1.5 different from wild-type. Several of these genes, including *MsrC*, *MetB*, *MetC*, *MetE* and *MetX* were up-regulated, suggesting the genes in the cysteine and methionine metabolism pathway were regulated by *MetR*. Previous studies have shown *MsrC* plays an important role in the oxidative stress response of *Salmonella Typhimurium* (Denkel et al., 2011, 2013). *ΔmsrC* mutant of *S. Typhimurium* could not survive when exposed to H_2O_2, and were unable to grow in either macrophages or in mice. Another important gene in this pathway *MetR* have affected is *MetB*, which is cystathionine y-synthase, the second enzyme of methionine biosynthetic pathway.

5. Conclusions

In summary, our work functionally characterized the *A. alternata* AaMetR as a global regulator in methionine biosynthesis, which also contributes to oxidative stress tolerance. This work highlights the role of *AaMetR* in regulation of conidia formation, ROS detoxification, mycelial growth and fungal pathogenicity in the tangerine pathotype of *A. alternata*. Transcriptomics analysis was used to identify candidate genes that AaMetR might regulate. Our data demonstrate that AaMetR is essential for the fungal pathogen to infect its host. This study contributes to our understanding of the relationship of methionine biosynthesis and oxidative stress response.

Author contributions

Yunpeng Gai, Mingshuang Wang and Hongye Li contributed to the conception of the study. Yunpeng Gai, Bing Liu, Haijie Ma and Lei Li performed the experiments. Yunpeng Gai contributed to analysis and

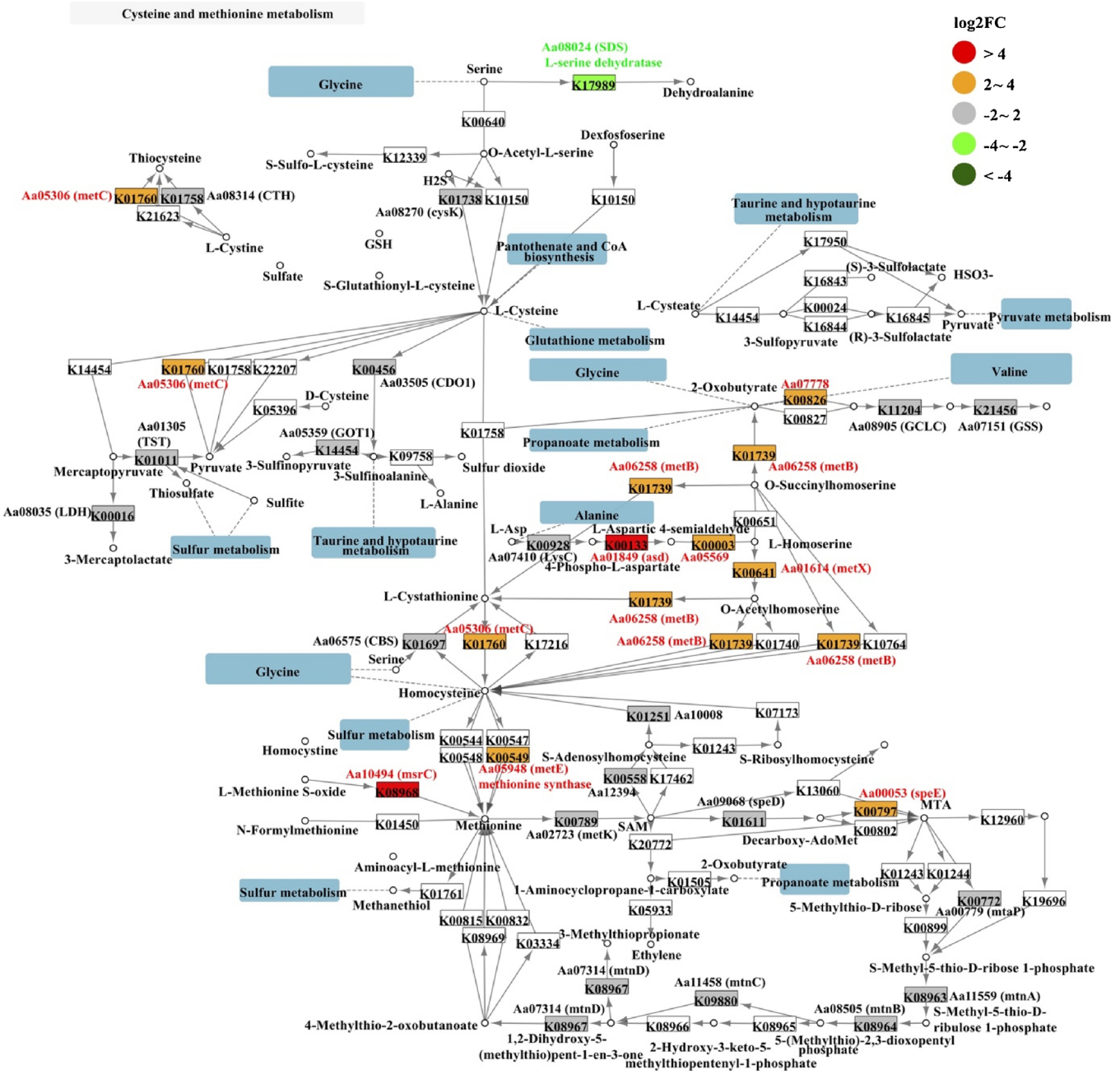

Fig. 14. The KEGG pathway figure shows differential expression of genes in the cysteine and methionine metabolic pathway of *ΔAaMetR* mutant. The differentially expressed genes of transcriptome data between the *ΔAaMetR* mutant and the wildtype were mapped against the cysteine and methionine metabolic pathway. The different colors of the squares represent differential gene expression in the *ΔAaMetR* mutant compared to the wildtype. The square without color indicates the gene that is not present in *Alternaria alternata*.

manuscript preparation. Yunpeng Gai, Xinglong Chen, Susan Moenga, Brendan Riely and Amna Fayyaz performed the data analyses and wrote the manuscript. Susan Moenga, Brendan Riely, Amna Fayyaz, Mingshuang Wang, Hongye Li helped perform the analysis with constructive discussions.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.11.007.

References

Akimitsu, K., Kohmoto, K., Otani, H., Nishimura, S., 1989. Host-specific effects of toxin from the rough lemon pathotype of *Alternaria alternata* on mitochondria. Plant. Physiol. 89 (3), 925–931.

Akimitsu, K., Peever, T.L., Timmer, L., 2003. Molecular, ecological and evolutionary approaches to understanding Alternaria diseases of citrus. Mol. Plant. Pathol. 4 (6), 435–446.

Akimitsu, K., Tsuge, T., Kodama, M., Yamamoto, M., Otani, H., 2014. Alternaria host-selective toxins: determinant factors of plant disease. J. Gen. Plant. Pathol. 80 (2), 109–122.

Anders, S., Pyl, P.T., Huber, W., 2015. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31 (2), 166–169.

Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55 (x), 373–399.

Bailey, T.L., Johnson, J., Grant, C.E., Noble, W.S., 2015. The MEME suite. Nucleic Acids Res. 43 (W1), W39–W49.

Ball, R.O., Courtney-Martin, G., Pencharz, P.B., 2006. The in vivo sparing of methionine by cysteine in sulfur amino acid requirements in animal models and adult humans. J. Nutr. 136 (6), 1682S–1693S.

Bandyopadhyay, U., Das, D., Banerjee, R.K., 1999. Reactive oxygen species: oxidative damage and pathogenesis. Curr. Sci. India 658–666.

Blin, K., Wolf, T., Chevrette, M.G., Lu, X., Schwalen, C.J., Kautsar, S.A., Suarez Duran, H.G., De Los Santos, E.L., Kim, H.U., Nave, M., 2017. antiSMASH 4.0—improvements in chemistry prediction and gene cluster boundary identification. Nucleic Acids Res. 45 (W1), W36–W41.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30 (15), 2114–2120.

Bowler, C., Fluhr, R., 2000. The role of calcium and activated oxygens as signals for controlling cross-tolerance. Trends Plant. Sci. 5 (6), 241–246.

Brosnan, J.T., Brosnan, M.E., 2006. The sulfur-containing amino acids: an overview. J. Nutr. 136 (6), 1636S–1640S.

Cantoni, G.L., 1975. Biological methylation: selected aspects. Annu. Rev. Biochem. 44 (1), 435–451.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G., Thompson, J.D., 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 31 (13), 3497–3500.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21 (18), 3674–3676.

Denkel, L.A., Horst, S.A., Rouf, S.F., Kitowski, V., Böhm, O.M., Rhen, M., Jäger, T., Bange, F.-C., 2011. Methionine sulfoxide reductases are essential for virulence of Salmonella typhimurium. PloS One 6 (11) e26974.

Denkel, L.A., Rhen, M., Bange, F.-C., 2013. Biotin sulfoxide reductase contributes to oxidative stress tolerance and virulence in Salmonella enterica serovar Typhimurium. Microbiology 159 (7), 1447–1458.

Finn, R.D., Mistry, J., Tate, J., Coggill, P., Heger, A., Pollington, J.E., Gavin, O.L., Gunasekaran, P., Ceric, G., Forslund, K., 2009. The Pfam protein families database. Nucleic Acids Res. 38 (suppl_1), D211–D222.

Gilroy, S., Suzuki, N., Miller, G., Choi, W.-G., Toyota, M., Devireddy, A.R., Mittler, R., 2014. A tidal wave of signals: calcium and ROS at the forefront of rapid systemic signaling. Trends Plant. Sci. 19 (10), 623–630.

Giorgio, M., Trinei, M., Migliaccio, E., Pelicci, P.G., 2007. Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? Nat. Rev. Mol. Cell. Biol. 8 (9), 722.

Hopwood, D.A., 1967. Genetic analysis and genome structure in Streptomyces coelicolor. Bacteriol. Rev. 31 (4), 373.

Howard, R.J., Ferrari, M.A., 1989. Role of melanin in appressorium function. Exp. Mycol. 13 (4), 403–418.

Huang, F., Fu, Y., Nie, D., Stewart, J.E., Peever, T.L., Li, H., 2015. Identification of a novel phylogenetic lineage of *Alternaria alternata* causing citrus brown spot in China. Fungal Biol. 119 (5), 320–330.

Jastrzębowska, K., Gabriel, I., 2015. Inhibitors of amino acids biosynthesis as antifungal agents. Amino Acids 47 (2), 227–249.

Kanehisa, M., Goto, S., 2000. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28 (1), 27–30.

Kawamura, C., Moriwaki, J., Kimura, N., Fujita, Y., Fuji, S.-i., Hirano, T., Koizumi, S., Tsuge, T., 1997. The melanin biosynthesis genes of *Alternaria alternata* can restore pathogenicity of the melanin-deficient mutants of Magnaporthe grisea. Mol. Plant. Microbe In 10 (4), 446–453.

Kellogg, W.W., Cadle, R.D., Allen, E.R., Lazrus, A.L., Martell, E.A., 1972. The sulfur cycle. Science 175 (4022), 587–596.

Kohmoto, K., Akimitsu, K., Otani, H., 1991. Correlation of resistance and susceptibility of citrus to *Alternaria alternata* with sensitivity to host-specific toxins. Phytopathology 81 (7), 719–722.

Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., Jones, S.J., Marra, M.A., 2009. Circos: an information aesthetic for comparative genomics. Genome Res. 19 (9), 1639–1645.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33 (7), 1870–1874.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9 (4), 357.

Lin, C.-H., Chung, K.-R., 2010. Specialized and shared functions of the histidine kinase- and HOG1 MAP kinase-mediated signaling pathways in *Alternaria alternata*, a filamentous fungal pathogen of citrus. Fungal. Genet. Biol. 47 (10), 818–827.

Lin, C.-H., Yang, S.L., Chung, K.-R., 2009. The YAP1 homolog–mediated oxidative stress tolerance is crucial for pathogenicity of the necrotrophic fungus *Alternaria alternata* in citrus. Mol. Plant. Microbe In 22 (8), 942–952.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15 (12), 550.

Lu, S.C., 2000. S-adenosylmethionine. Int. J. Biochem. Cell. B 32 (4), 391–395.

Ma, H., Wang, M., Gai, Y., Fu, H., Zhang, B., Ruan, R., Chung, K.-R., Li, H., 2018. Thioredoxin and glutaredoxin systems required for oxidative stress resistance, fungicide sensitivity and virulence of Alternaria alternata. Appl. Environ. Microb. AEM 00086-18.

Miyamoto, Y., Masunaka, A., Tsuge, T., Yamamoto, M., Ohtani, K., Fukumoto, T., Gomi, K., Peever, T., Akimitsu, K., 2008. Functional analysis of a multicopy host-selective ACT-toxin biosynthesis gene in the tangerine pathotype of *Alternaria alternata* using RNA silencing. Mol. Plant. Microbe In 21 (12), 1591–1599.

Park, J., Park, J., Jang, S., Kim, S., Kong, S., Choi, J., Ahn, K., Kim, J., Lee, S., Kim, S., 2008. FTFD: an informatics pipeline supporting phylogenomic analysis of fungal transcription factors. Bioinformatics 24 (7), 1024–1025.

Paterson, I., Hulme, A.N., 1995. Total synthesis of (-)-ebelactone A and B. J. Org. Chem. 60 (11), 3288–3300.

Peever, T., Canihos, Y., Olsen, L., Ibanez, A., Liu, Y.-C., Timmer, L., 1999. Population genetic structure and host specificity of *Alternaria* spp. causing brown spot of Minneola tangelo and rough lemon in Florida. Phytopathology. 89 (10), 851–860.

Peever, T., Su, G., Carpenter-Boggs, L., Timmer, L., 2004. Molecular systematics of citrus-associated *Alternaria* species. Mycologia 96 (1), 119–134.

Ravanel, S., Block, M.A., Rippert, P., Jabrin, S., Curien, G., Rébeillé, F., Douce, R., 2004. Methionine metabolism in plants chloroplasts are autonomous for de novo methionine synthesis and can import s-adenosylmethionine from the cytosol. J. Biol. Chem. 279 (21), 22548–22557.

Schopfer, P., Plachy, C., Frahry, G., 2001. Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. Plant. Physiol. 125 (4), 1591–1602.

Shoveller, A.K., Stoll, B., Ball, R.O., Burrin, D.G., 2005. Nutritional and functional importance of intestinal sulfur amino acid metabolism. J. Nutr. 135 (7), 1609–1612.

Trapnell, C., Pachter, L., Salzberg, S.L., 2009. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25 (9), 1105–1111.

Tsuge, T., Harimoto, Y., Hanada, K., Akagi, Y., Kodama, M., Akimitsu, K., Yamamoto, M., 2016. Evolution of pathogenicity controlled by small, dispensable chromosomes in *Alternaria alternata* pathogens. Physiol. Mol. Plant. Pathol. 95, 27–31.

Wang, H., Pang, H., Bartlam, M., Rao, Z., 2005. Crystal structure of human E1 enzyme and its complex with a substrate analog reveals the mechanism of its phosphatase/enolase activity. J. Mol. Biol. 348 (4), 917–926.

Wang, M., Fu, H., Shen, X.-X., Ruan, R., Pun, N., Xu, J., Li, H., Rokas, A., 2017. Genomic features and evolution of the conditionally dispensable chromosome in the tangerine pathotype of Alternaria alternata. bioRxiv 207746.

Wojtaszek, P., 1997. Oxidative burst: an early plant response to pathogen infection. Biochem. J. 322 (Pt 3), 681.

Yang, S.L., Chung, K.R., 2013. Similar and distinct roles of NADPH oxidase components in the tangerine pathotype of Alternaria alternata. Mol. Plant. Pathol. 14 (6), 543–556.

Yang, S.L., CHUNG, K.R., 2012. The NADPH oxidase-mediated production of hydrogen peroxide (H2O2) and resistance to oxidative stress in the necrotrophic pathogen *Alternaria alternata* of citrus. Mol. Plant. Pathol. 13 (8), 900–914.

Ye, J., Fang, L., Zheng, H., Zhang, Y., Chen, J., Zhang, Z., Wang, J., Li, S., Li, R., Bolund, L., 2006. WEGO: a web tool for plotting GO annotations. Nucleic Acids Res. 34, W293–W297 Web Server issue.