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ORIGINAL PAPER

Transcriptome analysis reveals downregulation of virulence-associated genes expression in a low virulence *Verticillium dahliae* strain

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Abstract

Verticillium dahliae causes wilt diseases and early senescence in numerous plants, including agricultural crops such as cotton. In this study, we studied two closely related *V. dahliae* strains, and found that V991w showed significantly reduced virulence on cotton than V991b. Comprehensive transcriptome analysis revealed various differentially expressed genes between the two strains, with more genes repressed in V991w. The downregulated genes in V991w were involved in production of hydrophobins, melanin, predicted aflatoxin, and membrane proteins, most of which are related to pathogenesis and multidrug resistance. Consistently, melanin production in V991w in vitro was compromised. We next obtained genomic variations between the two strains, demonstrating that transcription factor genes containing fungi specific transcription factor domain and fungal Zn2-Cys6 binuclear cluster domain were enriched in V991w, which might be related to pathogenicity-related genes downregulation. Thus, this study supports a model in which some virulence factors involved in *V. dahliae* pathogenicity were pre-expressed during in vitro growth before host interaction.

Keywords *Verticillium dahliae* · RNA-seq · Virulence · In vitro growth

Introduction

Verticillium spp. cause vascular wilt in a broad range of plant hosts, including economically important crops such as cotton, soybean, and tomato (Bhat and Subbarao 1999). Although verticillium wilt (VW) has been documented as the predominant disease of cotton, no effective chemical pesticides are available owing to its soil-borne nature (Xiong et al. 2014). The highly resistant resting structures, microsclerotia, of *Verticillium* spp. remain viable in the soil for

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more than a decade even under inhospitable conditions or in the absence of a susceptible plant (Green 1969; Fradin and Thomma 2006; Klosterman et al. 2009). Under inducible conditions, microsclerotia germinate, and the germinated hyphae penetrate the roots of susceptible plants and colonize the cortex. Subsequently, the hyphae cross the endodermis and enter the xylem vessels where conidia are produced. Vascular colonization occurs as conidia migrate to the aerial parts of the plant along with water, interrupting water movement from the roots to the leaves and resulting in characteristic wilt symptoms such as leaf chlorosis, vascular discoloration, and death of aerial tissues (Fradin and Thomma 2006; Klosterman et al. 2009).

Control of VW by breeding crop cultivars resistant to *Verticillium* infection has received worldwide attention (Cai et al. 2009). For developing anti-*Verticillium* strategies, identification of the pathogenicity factors and understanding of the mechanism of their interactions with hosts are apparently important. Since the 1950s–1960s, phytotoxins secreted by *Verticillium* spp. had been extensively studied (Jiang et al. 2005; Shi and Li 2008; Mansoori and Smith 2010). High molecular weight protein-lipopolysaccharide (PLP) complexes, hydrophobins, and cell wall degrading enzymes have been found in the crude *Verticillium* extracts and noted to be involved in the virulence of *Verticillium dahliae* (Meyer and Dubery 1993; Zhen and Li 2004; Klimes and Dobinson 2006; Klimes et al. 2008). Many studies have demonstrated that genes mediating pathogen–plant communications in signaling pathways are critical for fungal pathogenicity (Lengeler et al. 2000; Neves et al. 2002; Nishimura et al. 2003; Doehlemann et al. 2006; Casadevall 2007; Wouw and Howlett 2011; Kim et al. 2011; Tzima et al. 2012). Transcription factors (TFs) are required for the development and pathogenicity of some fungi, and nearly 5% of the genes in the fungal genome encode TFs, making them the largest family in *V. dahliae* (Lu et al. 2014; Xiong et al. 2015a). Transcriptome-wide analysis has revealed the elevated expression of certain TFs during microsclerotia formation (Xiong et al. 2014), and dysregulation of TFs has been reported to contribute to pathogenicity loss in *V. dahliae* (Santhanam and Thomma 2013; El Hadrami et al. 2015; Xiong et al. 2015b). However, the correlation between natural mutations in the TFs and fungal pathogenicity has not yet been investigated.

Pathogen–plant communications are complicated processes and essential for stimulating plant immune responses and inducing expression of virulence factors by fungal pathogens (Dodds and Rathjen 2010; Reniere et al. 2015). However, some virulence factors may be expressed before the fungus infects its host. For example, melanin is a well-known virulence factor of the animal fungal pathogen *Cryptococcus neoformans* (Casadevall et al. 2000). In addition, melanin has also been found to be a virulence factor of human pathogenic fungi (Nosanchuk et al. 2015), and has been recently reported to promote the pathogenicity of plant pathogen *Aspergillus* spp. (Akoumianaki et al. 2016). Although melanin production in *V. dahliae* is not required for pathogenesis or increased virulence (Bell et al. 1976; Wang et al. 2018), it has been observed to be clearly correlated with increased virulence in some genetic mutants, also affecting microsclerotia production (Fan et al. 2017). These pre-expressed virulence factors could play important roles in mediating pathogen-host communications. Thus, it is increasingly apparent that a more comprehensive understanding of the mechanisms underlying the biology and pathogenicity of *Verticillium* spp. is necessary for developing practical strategies for VW control.

Recently, we studied the pre-mRNA alternative splicing profile of two *V. dahliae* strains with different virulence, namely, V991w (low virulence) and V991b (high virulence), both naturally derived from the high-virulence strain V991 by serial subcultivation, and found no significant difference in their profiles under in vitro condition (Jin et al. 2017). In the present study, these two *V. dahliae* strains were demonstrated to significantly differ in their virulence during cotton infection. Furthermore, the differentially expressed genes (DEGs) between these two strains were analyzed by comparing their transcriptomes from in vitro culture. In V991w, more genes were downregulated. Using protein family (Pfam) and Gene Ontology (GO) annotation strategies to survey the functions of DEGs, we found that the downregulated genes in V991w encode hydrophobins and some other virulence factors, as well as in pathogenicity-related pathways. Moreover, upregulation of several genes potentially encoding aflatoxin was noted in the high-virulence *V. dahliae* strain. A comprehensive list of potential pathogenic genes in *V. dahliae* was obtained, and genomic sequence comparison between the two strains showed enriched variations in several types of TFs in V991w, which might contribute to downregulation of pathogenicity-related genes.

Materials and methods

Isolation and pathogenicity assessment of *V. dahliae* strains

The highly pathogenic *V. dahliae* V991 strain isolated from the wilt cotton plant was kindly provided by Prof. Xianlong Zhang (Huazhong Agricultural University, China). We obtained V991b and V991w strains, the natural variants of V991, by serial subcultivation (Jin et al. 2011). Prior to infection, the frozen fungal cells were activated on potato dextrose agar (PDA) plate for 3–4 days, and inoculated into Czapek broth and incubated on a shaker at 150 rpm and

25 °C for 10–14 days. The inoculum concentration was adjusted to 5×10^6 conidiospores/ml.

Three cotton cultivars with varying resistance to *V. dahliae* (Lu-Mian 28, Yu-Mian 2067, and Ji-Mian 11 representing the most resistant, medium resistant, and most susceptible cultivars, respectively) were used in this study, with each cultivar group comprising 50–70 seedlings. Before inoculation, the cotton seeds were germinated and grown in commercial sterilized soil (volume ratio of commercial matrix to sand was 5:5) at 24 °C/20 °C day/night temperatures for 2–3 weeks. Then, the cotton seedlings were gently removed from the soil (root cutting was reduced as much as possible) and immersed in *V. dahliae* spores suspension for 10 min. Finally, all the inoculated plants were planted into soil. The control cotton seedlings received no pathogen inoculation and were treated similarly with distilled water. Supplementary Figure S1 shows the representative cotton plants after inoculation with the two *V. dahliae* strains and control.

Pathogenicity assessment was conducted from day 20 after inoculation, and the evaluation was repeated on days 25 and 30. The disease index was measured according to the following grading standards: class 0, no incidence; class 1, cotyledons incidence; class 2, one euphylla (true leaf of the seedling and not the seed leaf) incidence; class 3, two or more euphylla incidence; and class 4, plant death.

Reads mapping and primary analysis

The two biological replicate RNA-seq data were obtained from a previous study by Jin et al. (Jin et al. 2017). The genome sequence and annotation files of *V. dahliae* strain VdLs.17 were downloaded from Broad Institute (Klosterman et al. 2011). Sequenced reads were quality-filtered (reads containing < 2 Ns) and adapter from ends were removed, and then the reads were aligned to the genome using TopHat software (Trapnell et al. 2009) with a maximum of two mismatches. The reads aligned to the annotated gene regions were subjected to statistical analysis. The expression level for each gene was normalized by the number of reads per kilo base per million aligned reads (RPKM).

DEG analysis

Differentially expressed genes between the strains V991b and V991w were analyzed using edgeR package (Robinson et al. 2010) in R. A p value of ≤ 0.01 and $|\log_2$ Fold Change$| \geq 1$ were set as thresholds.

Functional enrichment analysis

Two methods were used for functional enrichment analysis of DEGs, namely, the online Broad Institute platform (http://www.broadinstitute.org/annotation/genome/verticillium_dahliae) based on Pfam database (Punta et al. 2012) and the Blast2GO software with GO classification database (Conesa et al. 2005; Punta et al. 2012). Both the methods employed Fisher exact test with robust false discovery rate (FDR) correction to obtain an adjusted p value of certain tested gene groups with the total gene annotation as background (Conesa et al. 2005).

Validation of DEGs

Reverse transcription quantitative PCR (RT-qPCR) was applied to validate the randomly selected DEGs. The total RNA was prepared from the first batch of *V. dahliae* cultures, which represented a preparation different from that for transcriptome sequencing. The obtained total RNA (2 µg) was treated with RQ1 DNase (Promega) to remove genomic DNA and used for reverse-transcription. The first-strand cDNA was prepared using M-MLV reverse transcriptase (Invitrogen) and random hexonucleotides. RT-qPCR was performed with 7300 Real Time PCR System (ABI) using SYBR-Green Master Mix (Takara). Each experiment was performed in triplicate and two biological replicates were used to minimize variation. The amplification efficiencies of each of the primer sets were checked before experiment. The expression level of each gene was normalized to the 5S rRNA level (Xiao et al. 2012) to calculate the ΔC_t, and the altered gene expression between the samples was further compared to calculate the $\Delta\Delta C_t$. The fold change of the gene expression was visualized by calculating $2^{-\Delta\Delta C_t}$. The primers used in this study are listed in Supplementary Table S1.

DNA re-sequencing

The genomic DNA was extracted from the late-log phase culture of each strain using DNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. The purified DNA was sonicated into 100–500 bp fragments and 1 µg of the fragmented DNA was used for library preparation. Following end repair and A tailing, the DNA was ligated to the double-stranded Truseq DNA adaptors (Illumina) and PCR-amplified for 12 cycles. Then, fragments comprising 300–500 bp were purified with Ampure xp beads (Beckman), and the purified libraries were quantified and stored at − 80 °C until further sequencing. For high-throughput sequencing, the libraries were prepared following the manufacturer's instructions and subjected to illumina Next-Seq 500 system for 300-nt single-end sequencing by ABlife, Inc (Wuhan, China). Raw reads containing more than 2-N bases were discarded, and the remaining reads were processed by clipping adaptor and removing low-quality bases. Very short reads (< 20 nt) were also removed. FASTX-Toolkit (version 0.0.13) was used to obtain clean reads. The

clean reads were aligned to the reference genome by BWA (Li and Durbin 2010).

Single nucleotide polymorphisms/insertions or deletions calling and annotation

Samtools mpileup method (Li et al. 2009) was used for single nucleotide polymorphisms (SNPs)/insertions or deletions (Indels) calling. The SNPs/Indels were maintained with a quality of ≥ 20 and depth of ≥ 10 (Rosenblum et al. 2013). After filtering, the SnpEff software (Cingolani et al. 2012) was employed to annotate the SNPs/Indels. In addition to Indels, genome-wide analysis was performed for missense mutations causing alteration of amino acid code. Furthermore, variants that caused exon loss, frameshift, rare amino acid variant, splice acceptor and donor variant, stop loss, start loss, and stop gain were identified as high-impact variants.

Availability of data

All data generated or analyzed during this study are presented in the supplementary information files. The RNA-Seq datasets generated and/or analyzed in this study are available in the GEO repository under project GSE45936. The genome re-sequencing data have been deposited at the NCBI Sequence Read Archive under the accession number SRP074527.

Statistical analysis

Gene and SNP overlap analysis was performed using in-house Perl script, and Venn figures were obtained by R software. Fisher's exact test was employed for significance analysis by R software. Phylogenetic analysis was performed as described previously (Inderbitzin et al. 2011).

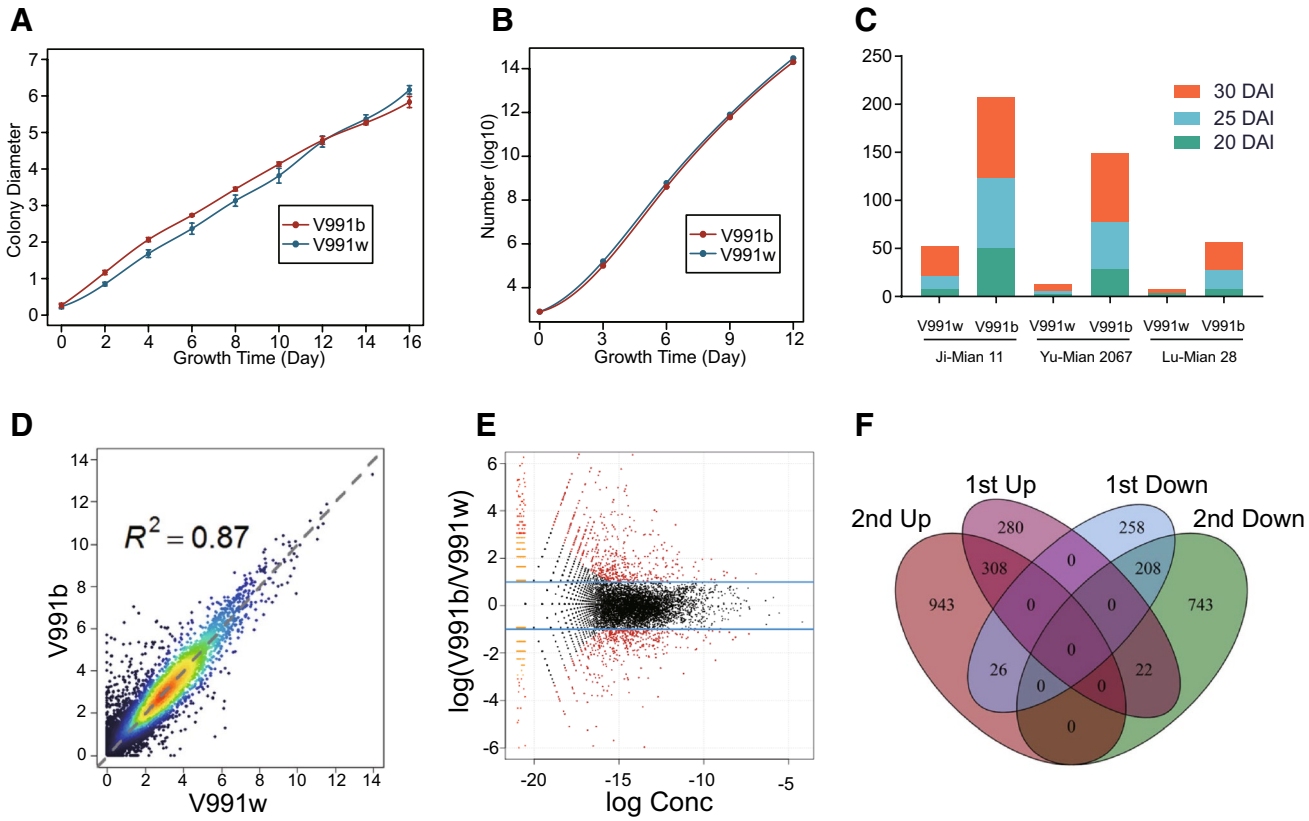

Fig. 1 Growth similarity and virulence difference between V991b and V991w. **a** Growth of the strains on PDA plates. The diameters of three colonies of each strain were measured on the indicated days. **b** Growth of the strains in Czapek broth. About 800 spores from each strain were inoculated and the growth was prolonged until 12 days. The number of spores were counted on the indicated days and plotted. **c** Statistics of *V. dahliae* disease index in three different cotton cultivars Ji-Mian 11, Yu-Mian 2067, and Lu-Mian 28 on 20, 25, and 30 day after infection (DAI). **d** Correlation of the expressed genes between V991b and V991w, 1st dataset. **e** Identification of the DEGs, 1st dataset. logConc represents the overall concentration of genes for the two strains. The smear of points on the left side signifies genes being observed in only one strain. **f** Venn diagram for the DEGs identified from the two independent RNA-seq datasets

Results

Growth and virulence of the two *V. dahliae* strains

The in vitro growth of V991b and V991w was essentially the same both on PDA and in the Czapek broth (Fig. 1a, b). However, upon inoculation at the root of cotton seedling, V991w showed substantially lower virulence during the entire course of cotton plant growth. The reduced virulence of V991w was observed in all the cotton cultivars with different pathogen susceptibility (Fig. 1c). With regard to the most susceptible cotton cultivar (Ji-Mian 11), after 20-day inoculation, more than 92% of plants were infected with V991b, presenting all levels of infection symptoms, including death. In contrast, only 31% plants inoculated with V991w showed cotyledons incidence, the least level of infection symptom. On day 30 of infection, 28 plants infected with V991b died, whereas only two plants inoculated with V991w died (Fig. 1c; Supplementary Figure S1 and Table S2). Similarly, the other two cotton cultivars also presented higher resistance to V991w than V991b (Fig. 1c; Supplementary Table S2). Therefore, it can be concluded that the pathogenicity of V991w was stably compromised.

Expression of potential virulence genes in the two *V. dahliae* strains

We hypothesized that the expression of some important pathogenicity-related genes may be downregulated before V991w infects the host (cotton plant). Therefore, two biological replicate RNA-seq data (1st and 2nd datasets) generated from the two *V. dahliae* strains in our previous work (Jin et al. 2017) were reanalyzed in this study. Consistent with similar in vitro growth phenotype of the two strains, the gene expression level in their transcriptomes was well-correlated (Fig. 1d; Supplementary Figure S2A). DEG analysis showed that most of the genes were expressed at similar level in the two strains, while some were differentially expressed (Fig. 1e). A total of 1102 and 2250 protein coding genes were identified as DEGs in the 1st and 2nd datasets, respectively. DEGs identified in the 1st and 2nd datasets were significantly overlapped (p value = 2.62e−120, hypergeometric test, Fig. 1f). The 1st dataset revealed 610 upregulated and 492 downregulated genes in the V991b strain (Supplementary Tables S3 and S4). The confidence of the DEGs identified in the 1st dataset of transcriptome sequencing was further validated by plotting the quantitative expression level (RPKM) of all the DEGs in both the datasets by Heatmap, which showed a highly consistent expression pattern of the putative virulence genes (Supplementary Figure S2B).

The upregulated genes in V991b might possibly contain a repository of virulence genes, whose downregulation might be correlated with the reduced pathogenicity of V991w. Consistent with this hypothesis, all of the five hydrophobin genes annotated in *V. dahliae* were found to be upregulated in V991b in both the datasets, when compared with those in V991w (Table 1). Moreover, the expression of a set of genes that were annotated to encode enzymes for the synthesis of aflatoxin was higher in V991b (Table 2), indicating that *V. dahliae* possibly synthesizes aflatoxin or its secondary metabolism products as a class of virulence factors. A total of 15 DEGs were selected for validation by RT-qPCR using total RNA prepared from a different batch of *V. dahliae* culture. Among them, 14 DEGs were validated, including five CFEM-domain-containing genes, eight major facilitator superfamily (MFS_1) genes, and one sugar transporter gene. In addition, most of the differential expressions detected by RT-qPCR were quantitatively correlated with the transcriptome sequencing data, strongly suggesting that the transcriptome reads well-reflected the expression levels of genes in the two genetically stable *V. dahliae* strains during their in vitro growth (Fig. 2). Besides, 11 of these 14 DEGs were also quantitatively validated by 2nd dataset of transcriptome sequencing, for which a different batch of *V. dahliae* culture and different cDNA libraries preparation method were employed (Fig. 2), thus clearly supporting the confidence of DEGs identified in this study.

Functional enrichment analysis of the DEGs in the two *V. dahliae* strains

To systematically study the possible pathogenicity functions of genes that were downregulated in V991w, the DEGs from the 1st dataset were subjected to functional enrichment analysis using a platform based on Pfam annotation. Consistent with our hypothesis, the downregulated genes in V991w were involved in several known and potential fungal pathogenicity-related functions (Table 3). These genes mostly encoded hydrophobins, CFEM-domain fungal membrane proteins, cytochrome P450 superfamily (CYP), MFS1, and sugar transporters. In particular, all the hydrophobins-coding genes were upregulated in V991b, when compared with those in V991w, with the expression of three genes presenting statistically significant differences (Table 1). Moreover, five out of the 17 genes encoding CFEM domain proteins were upregulated in V991b (Table 3; Fig. 2). Besides, nine and 12 of the 45 CYP genes were significantly upregulated in V991b in the 1st and 2nd datasets, respectively, suggesting that CYP genes played critical roles in *V. dahliae* pathogenesis (Table 3). The Pfam annotation result also showed that more than 30 MFS genes were significantly upregulated, whereas 16 MFS genes were downregulated in V991b. In contrast, transcription of the ABC transporter genes, another large family of membrane transporter, was not significantly upregulated in V991b. To confirm the globally

Table 1 Up-regulation of all five hydrophobin genes express in the high-virulence *V. dahliae* strain

Gene ID	Data set	V991b[a]	V991w[a]	Ratio (b/w)	Gene_ length (bp)	PFAM name	PFAM description	Nr. best-hit description	GO terms
VDAG_01586	1st	5.32	1.7	3.13	390	Hydrophobin 5	Hydrophobin_2, fungal hydrophobin	Hydrophobin	C: cell wall; hyphal cell wall; extracellular region; P: cell communication; hyphal growth; capsule organization
	2nd	2.68	1.39	1.93					
VDAG_07838	1st	1.96	0	–	353	Cerato-ulmin		Hydrophobin precursor	C: extracellular region; hyphal cell wall; P: interaction with host via protein secreted by type II secretion system; cell communication; hyphal growth; capsule organization
	2nd	37.46	6.46	5.80					
VDAG_07851	1st	95.55	14.51	6.59	869	Predicted protein		Hydrophobin-like protein	–
	2nd	103.02	31.96	3.22					
VDAG_08956	1st	39.37	4.58	8.60	580	Hypothetical protein		Hydrophobin	C: extracellular region; cell wall; P: cell communication
	2nd	7.66	1.30	5.89					
VDAG_02273	1st	33.43	0.97	34.46	683	Hypothetical protein		Cerato-ulmin	C: cell wall; extracellular region; P: cell communication; pathogenesis
	2nd	17.09	2.04	8.37					

RPKM mapped Reads Per Kilobase of mRNA per Million reads

[a]The expression level was indicated by RPKM value

altered expression of MFS_1 family genes between V991b and V991w, five upregulated and three downregulated MSF genes were chosen for RT-qPCR validation, and the altered expression of all the eight genes was validated (Fig. 2). Interestingly, four sugar transporter genes were identified to be upregulated in V991b (Supplementary Tables S3 and S4), highlighting the potential contribution of sugar transporters in the virulence of *V. dahliae*. Among these four sugar transporter genes, two were sugar transporter STL1 belonging to MFS_1 family and two were sugar transporter family protein classified as Sugar_tr Pfam family. Subsequently, three of these genes were selected for RT-qPCR validation (Fig. 2).

GO analysis of DEGs in the two *V. dahliae* strains

As shown in Fig. 3a, a large fraction of *V. dahliae* genes did not have Pfam annotation. To improve annotation and better understand the molecular mechanism associated with *V. dahliae* virulence genes, we annotated 10,535 known *V. dahliae* genes using Blast2GO software (Gotz et al. 2008; Gene Ontology 2012). A total of 8031 genes (76.2%) acquired at least one GO term, and about one-third of them were not annotated using Pfam strategy (Fig. 3a, left; Supplementary Table S5). This approach allowed us to annotate 572 (51.9%) DEGs, including 137 DEGs lacking Pfam annotation (Fig. 3a, right; Supplementary Tables S6 and S7).

Using the total GO terms of all the *V. dahliae* genes as background and a p value cutoff of 0.01, up-DEGs and down-DEGs were noted to be enriched in 22 and nine functional terms, respectively (Supplementary Table S8). A total of 21 terms ($p \leq 0.005$) showed several distinct biological pathways that could be associated with *V. dahliae* virulence, and most of them were related to the Pfam enriched terms (Fig. 3b). Among the 610 genes upregulated in V991b, 62 and 71 genes were involved in oxidation–reduction process and oxidoreductase activity, respectively (Fig. 3b). This result indicated that the V991b strain possessed a more robust oxidation–reduction capability than the V991w strain when grown in vitro, which is supported by the enriched short-chain dehydrogenases/reductases family and glucose-methanol-choline oxidoreductase family in Pfam description (Table 3). Transmembrane transport term was also enriched over the whole genome background (Fig. 3b), providing further evidence for the importance of multiple classes of membrane proteins in the pathogenicity of *V. dahliae*. A part of the globally increased oxidoreductase activity of V991b might probably be associated with the enforced CYP

Table 2 Genes encoding enzymes for aflatoxin synthesis is coordinately up-regulated in V991b strain

Gene ID (Chr)	Dataset	V991b	V991w	Ratio	Gene_length (bp)	Protein name	PFAM name	PFAM description
VDAG_02572 (Supercontig_1.4)	1st	43.13	37.82	1.14	1123	Aflatoxin B1 aldehyde reductase member 3	Aldo_ket_red	Aldo/keto reductase family
	2nd	112.56	48.10	2.34				
VDAG_03485 (Supercontig_1.5)	1st	597.73	330.48	1.81	771	Aflatoxin biosynthesis ketoreductase nor-1	adh_short	Short-chain dehydrogenase
	2nd	597.85	142.60	4.19				
VDAG_04535 (Supercontig_1.7)	1st	62.64	33.21	1.89	939	Aflatoxin biosynthesis ketoreductase nor-1	adh_short	Short-chain dehydrogenase
	2nd	118.07	36.19	3.26				
VDAG_09534 (Supercontig_1.26)	1st	10.36	0.21	49.33	6415	–	Aflatoxin biosynthesis polyketide synthase	Acyl_transf_1 (acyl transferase domain)
	2nd	4.41	0.08	55.13				

pathway because the high virulence associated oxidoreductase activity included 12 monooxygenases involved in the CYP pathway. Besides, the upregulation of heme/tetrapyrrole-binding genes in V991b also supported an enforced CYP pathway (Fig. 3b).

Melanin is a natural pigment found in most organisms, and its production in numerous pathogenic fungi is associated with pathogenicity (Jacobson 2000; Plonka and Grabacka 2006). Although melanin production in *V. dahliae* is not required for pathogenesis or increased virulence (Bell et al. 1976; Wang et al. 2018), an obvious correlation has been noted between melanin synthesis and increased virulence in some *V. dahliae* mutants, which also affected microsclerotia production (Fan et al. 2017). In the present study, GO annotation and functional clustering analysis revealed that four genes involved in melanin biosynthesis were coordinately upregulated in V991b, when compared with those in V991w (Fig. 3b). These genes were observed to encode essential enzymes catalyzing melanin synthesis, including polyketide synthase, versicolorin reductase, scytalone dehydratase, and tetrahydroxynaphthalene reductase. Consistently, only V991b was found to produce melanin when grown in vitro (Fig. 3c). In conclusion, these transcriptional evidences indicated pre-activation of phytotoxins, membrane proteins associated with cell signaling and transporter, as well as CYP pathways in *V. dahliae* for successful infection and pathogenicity (Fig. 4).

Genomic comparison between the two *V. dahliae* strains

The genomic variations between V991w and V991b, which might explain the difference in virulence as well as the differential expression of some virulence-related genes, were explored. First, phylogenetic analysis of the two *V. dahliae* strains was performed based on a geographically broad collection of *V. dahliae* strains (Supplementary Figure S3A). The selected genes showed slight differences, and the two *V. dahliae* strains were observed to be close to other *V. dahliae* strains (Supplementary Figure S3A). Subsequently, whole genome re-sequencing was accomplished for the two strains. In total, more than 3G raw reads were generated for each strain, which represented at least 90× average genome coverage, and about 84–89% of the reads were uniquely aligned to the reference genome.

Then, the potential distribution of copy number variations (CNVs) for the two *V. dahliae* strains was analyzed. A total of 446 and 419 CNVs were determined for V991b and V991w, respectively, most of which were common between these two strains, representing the genomic variation between V991 and VdLs.17 strain. Only 21 and 49 CNVs were specific for V991w and V991b, respectively. Furthermore, 3 out of 21 CNVs in V991w were overlapped with DEGs, including VDAG_00001 without a functional annotation, VDAG_05957 encoding a tetratricopeptide repeat protein, and VDAG_09411 amidohydrolase family protein (Supplementary Table S9).

Subsequently, the characteristics of SNPs and smaller Indels were analyzed. A total of 195,931 and 215,349 SNPs/Indels were found in V991b and V991w, respectively. As expected, most of the sequence variations were noted to be located within the intergenic or coding sequence (CDS) regions (Supplementary Table S10). While 26,421 SNPs/Indels were specific to V991w, only 7097 SNPs/Indels were exclusively found in V991b (Fig. 5a). Meanwhile, 2851 and 624 genes had specific SNPs/Indels in V991w and V991b, respectively, supporting that the low virulence strain might have undergone more genomic variations, resulting in virulence loss.

To validate the genetic variation detected by DNA re-sequencing, the RNA-seq reads were subjected to SNPs/Indels calling. Most of the exonic SNPs could be detected using RNA-seq SNPs, when genes were expressed in adequate levels (Supplementary Figure S3B). About 20% DNA SNPs/Indels were validated by RNA-seq, and

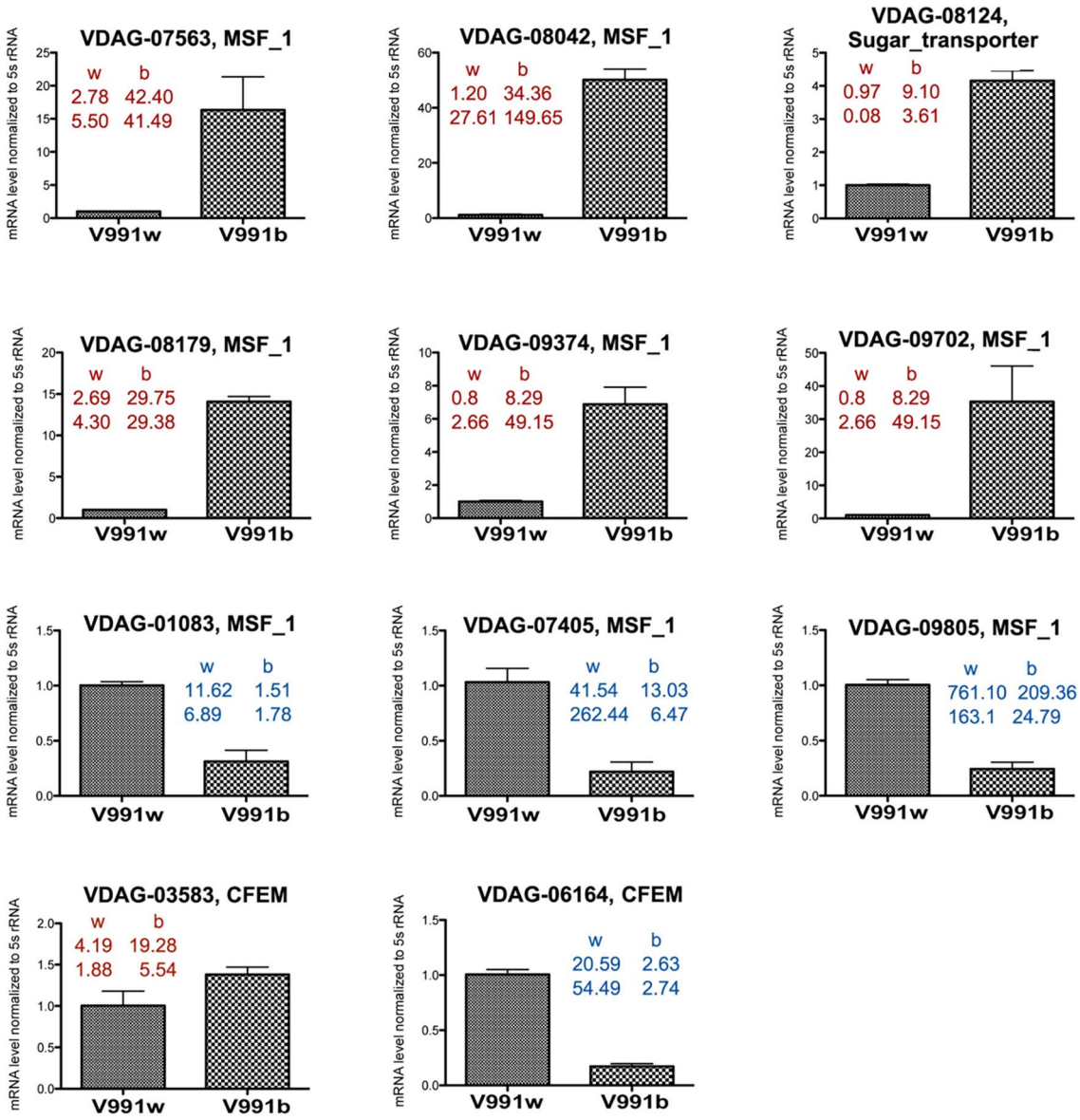

Fig. 2 RT-qPCR validation of DEGs in V991w and V991b strains. The expression level obtained from RNA-seq is indicated by RPKM for V991w/V991b. Only the validated genes supported by two independent RNA-seq data are shown. The values in each panel are the RPKM values in the two RNA-seq datasets

almost all genes (90.39%) containing RNA-seq SNPs/Indels were also included in DNA-seq (Supplementary Figure S3A). Some of the SNP/Indels were validated by PCR and Sanger sequencing (Supplementary Figure S3C, D). Overall, the SNPs/Indels detected by DNA re-sequencing were well-supported by RNA-seq and Sanger sequencing.

High impact SNPs/Indels variations in different classes of TFs in the two *V. dahliae* strains

The potential impact of missense SNPs in the protein-coding regions on the virulence of the two *V. dahliae* strains was examined. Among the missense SNPs, 1789 and 3297 SNPs were specific to V991b and V991w, respectively (Fig. 5b). While missense SNPs can alter the amino-acid identity, the possibility and level of influence on protein function could hardly be predicted. Subsequently, the high impact genetic variations causing a change in the start/stop codon or splice sites, or resulting in frameshift, which would have profound

Table 3 Functional clustering of genes significantly up-regulated in the high-virulence *V. dahliae* strain by Pfam annotation

Enriched terms	UP/total expressed genes		*p* value	Pfam description
	1st dataset	2nd dataset		
CFEM	5/17	8/17	0	CFEM domain
p450	9/45	12/45	0	Cytochrome P450
MFS_1	30/241	37/190	0	Major facilitator superfamily MFS1
DJ-1_PfpI	3/4	2/3	0	DJ-1/PfpI family protein
adh_short	13/70	27/75	0	The short-chain dehydrogenases/reductases family
PP-binding	3/5	1/2	0	Phosphopantetheine attachment site
GMC_oxred	6/22	9/22	0	Glucose-methanol-choline oxidoreductase family
ketoacyl-synt	2/3	3/6	0	Beta-ketoacyl synthase, N-terminal domain
Hydrophobin_2	3/5	4/5	0.001	Fungal hydrophobin
SGL	2/2	2/2	0.002	SMP-30/gluconolaconase/LRE-like region
Methyltransf_2	1/1	2/2	0.002	*O*-methyltransferase
AA_permease	4/16	0/16	0.003	Amino acid permease
Acyl_transf_1	1/4	0/1	0.004	Acyl transferase domain
PBP	2/3	1/3	0.005	Phosphatidylethanolamine-binding protein
Aa_trans	3/14	6/14	0.016	Transmembrane amino acid transporter protein
Fungal_trans	9/64	14/96	0.034	Fungal-specific transcription factor domain

impact on the gene function and phenotype outcome, were also analyzed. As shown in Fig. 5c, 939 and 315 specific high impact mutations, which corresponded to 623 and 202 genes, were detected in V991w and V991b, respectively. These data further supported the hypothesis that V991w would have undergone more genomic variation. Pfam annotation enrichment results showed that genes containing specific high impact mutations, including multiple types of TF families, in both the *V. dahliae* strains were involved in several functions (Fig. 5c, d). The V991w-specific SNP/Indels were enriched in MFS_1 genes that contributed to pathogen virulence.

Analysis of variations in TFs in the two *V. dahliae* strains

Through the Fungal TF Database search, 587 TFs were predicted in the *V. dahliae* genome. Overlapping analysis between genes with specific high impact variations and predicted TFs in the two *V. dahliae* strains showed 31 and 12 TFs in V991w and V991b, respectively (Fig. 6a; Supplementary Tables S11 and S12). Functional clustering of these TFs by Pfam revealed that two classes of TFs, fungal-specific TF domain and fungal Zn2-Cys6 binuclear cluster domain, were enriched in V991w, and one class of TFs, Zinc finger C2H2 type, was enriched in V991b (Fig. 6b). These data suggested that functional dysregulation of different types of TFs could have contributed to virulence loss in V991w.

It was observed that the TFs with high impact variation in V991w containing the Zn2Cys6-Zinc finger were GAL4-like TFs. GAL4 is an activator of transcription of various galactose-inducible genes, and the target site has highly conserved palindromic CGG sequences at its ends separated by 11-bp random bases, which allows GAL4-dependent transcriptional activation in vivo both in yeasts cells and mammalian tissue culture cells (Marmorstein et al. 1992; Liang et al. 1996). In the present study, to uncover the relationship between increased variation in TFs and DEGs in V991w, the target genes of the GAL4-like TFs were predicted. The consensus recognition site (5′-CGGN11CCG-3′) of GAL4 (Liang et al. 1996) was applied to search 0.5-kb upstream of DEGs. A total of 112 GAL4-like Zn2Cys6-Zinc finger target genes were predicted in the DEGs (Supplementary Table S13), representing about 10% of the total DEGs. The MFS_1 and Sugar transporters family, which showed significant expression change in V991w, were enriched in Zn2Cys6-Zinc finger TFs targets (Fig. 6c; Supplementary Table S14). Moreover, VDAG_03393, which was differentially expressed in the two *V. dahliae* strains and encodes scytalone dehydratase essential in the melanin biosynthesis process (Fig. 3b), was predicted to be the target gene of Zn2Cys6-Zinc finger TFs (Supplementary Table S14). Taken together, these data supported the hypothesis that the virulence loss in V991w could be owing to the accumulated variations in the TFs in the whole genome.

Discussion

Pre-expression of virulence pathways prior to *V. dahliae*–host interaction

We hypothesized that elevated expression of some virulence genes prior to plant infection might be important for

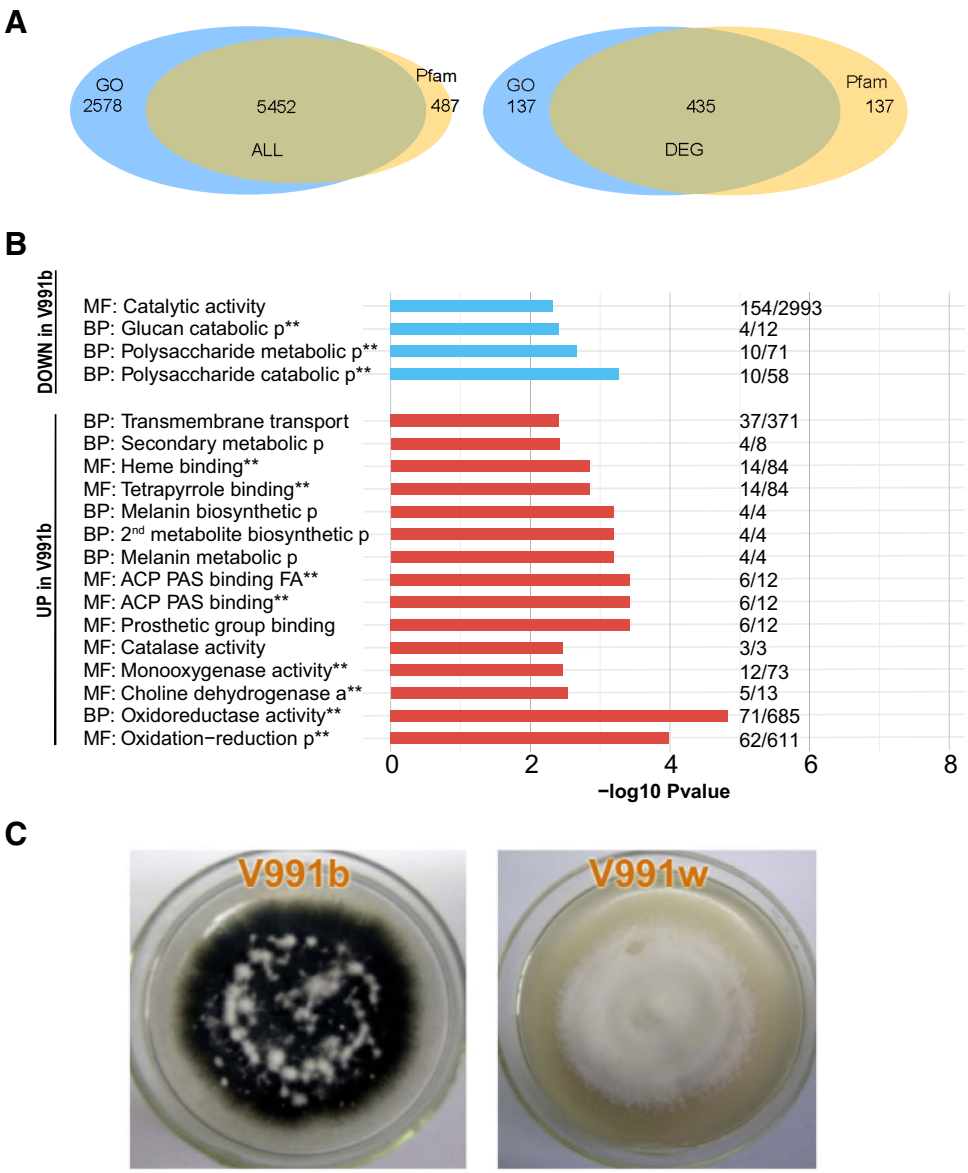

Fig. 3 Functional clustering of DEGs using Pfam and GO annotations. **a** Venn diagram showing the annotation of all *V. dahliae* genes (left) and DEGs (right) based on Pfam and GO approaches. **b** Bar plot showing the enriched GO terms ($p < 0.005$) of DEGs. **c** Melanin synthesis was compromised during the in vitro growth of V991w. The figure shows the colony formation of V991b and V991w on PDA plates

mediating pathogen–plant communication, thus contributing to pathogen virulence. In this study, hundreds of *V. dahliae* genes were found to be specifically upregulated in the high virulence *V. dahliae* strain, when compared with those in the low virulence *V. dahliae* strain, under in vitro growth condition. Using both Pfam and GO annotation approaches, several genes with potential virulence were identified, which had been previously reported as either virulence factors or being involved in multiple mechanisms of fungal pathogenicity.

Hydrophobins are unique to the fungal kingdom and described as pathogenicity factors in rice blast fungus *Magnaporthe grisea* (Talbot et al. 1993; Bayry et al. 2012). In the present study, all five hydrophobin-producing genes were upregulated in V991b, suggesting the potential importance of this kind of proteins in initiating a successful *V. dahliae* infection. Aflatoxin is a mycotoxin and its phytotoxic activity has been reported in *Aspergillus flavus* and *Aspergillus parasiticus* (McLean et al. 1995; Ehrlich et al. 2005; Baldwin et al. 2011; Kelley et al. 2012). Based on the Pfam annotation from Broad Institute, we predicted four homologous reductases in the aflatoxin pathway, all of which were overexpressed in V991b (Table 2), indicating that aflatoxin homologous proteins or their secondary metabolism pathway may also contribute to the infection and pathogenicity of *V. dahliae*. Besides, V991b also produced more melanin than V991w, consistent with the finding of higher expression of enzymes involved in melanin biosynthesis in V991b (Fig. 4b). Nevertheless, some studies have also shown that melanin production per se in *V. dahliae* is not required for pathogenesis or virulence (Bell et al. 1976; Wang et al. 2018).

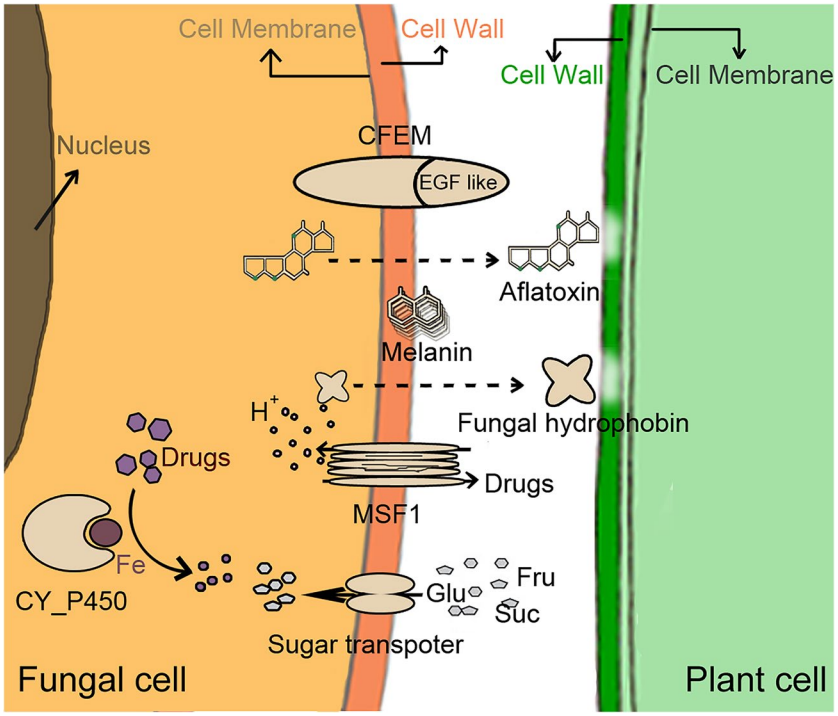

Fig. 4 Model diagram showing pre-activation of predicted pathogenicity-related pathways in *V. dahliae* prior to its infection on plant cell. The pre-activated synthesis of melanin and predicted aflatoxin (Butler et al. 2001; Kelley et al. 2012), as well as expression of hydrophobins (Bayry et al. 2012), CFEM, MSF proteins and sugar transporter (Kulkarni et al. 2003; Coleman and Mylonakis 2009; Klosterman et al. 2009) are depicted in the model. Drugs in this model indicate antifungal drugs in CYP pathway and drugs involved in pathogenesis produced by *V. dahliae* in MSF pathway

Melanin production is controlled by multiple regulators, such as VdCmr1 (Wang et al. 2018) and Vayg1 (Fan et al. 2017), and deletion of either of the two proteins can lead to repression of melanin production; however, deletion of only Vayg1 can result in reduced microsclerotia production and virulence, implying that lack of microsclerotia formation and associated changes in metabolism in the Vayg1 mutant influences fungal pathogenicity (Fan et al. 2017). The results of the present study suggested that melanin or its products may be associated with fungal virulence. In *V. dahliae*, genes controlling melanin production may also play important roles in pathogenesis on cotton by triggering the expression of other virulence-related genes (Fan et al. 2017).

The plant wilting symptom caused by *V. dahliae* requires a robust transporter system enabling the fungal cells to not only secrete signal molecules to compromise the host defense, but also to successfully compete with host cells under low concentrations of sugars, amino acids, and inorganic ions (Klosterman et al. 2009). We hypothesized that overexpression of a set of transporters could efficiently increase both the infection and survival capability of the highly aggressive *V. dahliae* strain and thus enhance the pathogenesis of *V. dahliae* on cotton. Membrane proteins of CFEM and MFS_1 with known functions in fungal pathogenesis and multi-drug resistance (Kulkarni et al. 2003; Coleman and Mylonakis 2009), respectively, were preferentially expressed in V991b. In addition, expression of oxidoreductase genes was systematically upregulated with the fungal pathogenicity, including the monooxygenases in CYP pathway and reductases in melanin synthesis. In summary, our results suggested pre-activation of several virulence-related pathways in *V. dahliae* for successful infection and pathogenicity (Fig. 4).

Correlation between reduced *V. dahliae* virulence and accumulated genomic variations

In this study, we sequenced the genomic DNA to obtain the CNVs and SNP/Indels profiles of V991b and V991w strains. As these strains were derived from the same high-virulence parent, the SNPs specific to either of the strains were good candidates accounting for *V. dahliae* virulence. We found that the low virulence strain presented more SNP/Indels than the high virulence strain, whereas the opposite trend was noted with regard to the CNVs. The strain-specific SNP/Indels were significantly enriched in multiple classes of TFs, and the enriched TF classes differed between the two strains. The SNP/Indels specific to V991w were more enriched in the MFS_1 genes that are important contributors to pathogen virulence. Given that a number of studies have reported that TFs are correlated with several fungal pathogenicity pathways, the results of the present study supported the model that reduced virulence of V991w may arise from the accumulated genomic variations.

Increasing amount of TFs have been found to encode the pathogenic proteins of *V. dahliae*, such as VdVta2, VdCrz1,

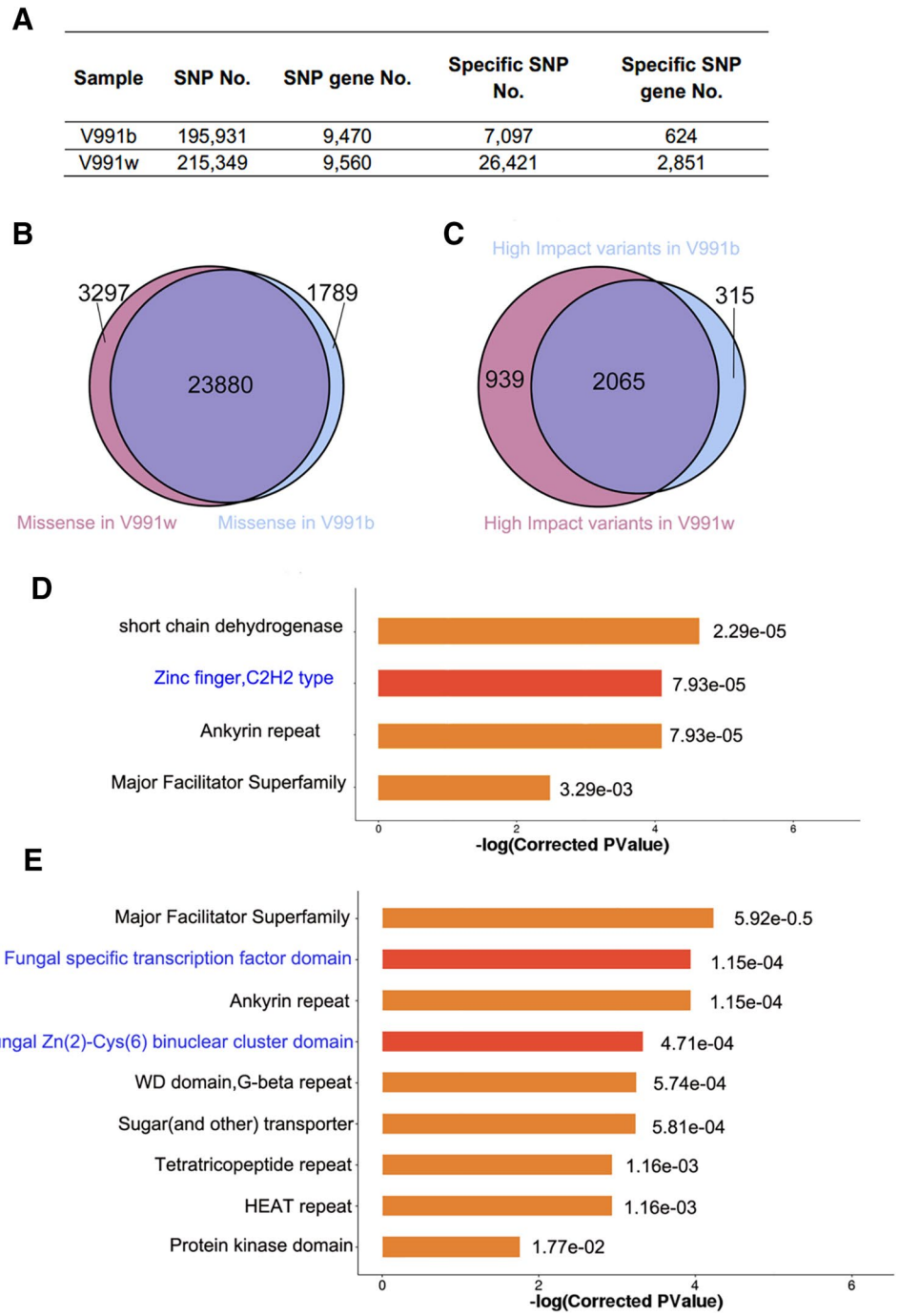

Fig. 5 Variations in V991w. **a** Statistical results of the SNP/Indels detection in the two *V. dahliae* strains. **b** Venn diagram of the missense variations identified from DNA re-sequencing data for the two *V. dahliae* strains. **c** Venn plot of the high impact variations identified from DNA re-sequencing data for the two *V. dahliae* strains. **d** Genes with high impact SNPs/Indels specific to V991b were annotated by Pfam, and the enrichment of functional clusters are presented. **e** Genes with high impact SNPs/Indels specific to V991w were annotated by Pfam, and the enrichment of functional clusters are indicated

VdSge1, VdCPC1, etc. (Santhanam and Thomma 2013; Timpner et al. 2013; Tran et al. 2014; Xiong et al. 2015b). In the present study, by searching the TF-binding sites, a significant number of DEGs were found to be the targets of TFs containing fungal Zn2-Cys6 binuclear cluster domain, and this class of TFs harbored more SNP/Indels in V991w. These data suggested that the enrichment mutations in TFs might contribute to the altered gene expression between V991b and V991w in vitro. Besides, the accumulated SNPs/Indels in *V. dahliae* TF genes might also contribute to the low virulence of V991w. Differences in gene regulation have been recognized as major contributors to phenotypic diversity, especially between closely related species (Romero et al. 2012; Villar et al. 2014). In the present study, TF variations were noted to be enriched under natural selection, indicating that TFs may be a hotspot for phenotypic diversity during fungal development in nature.

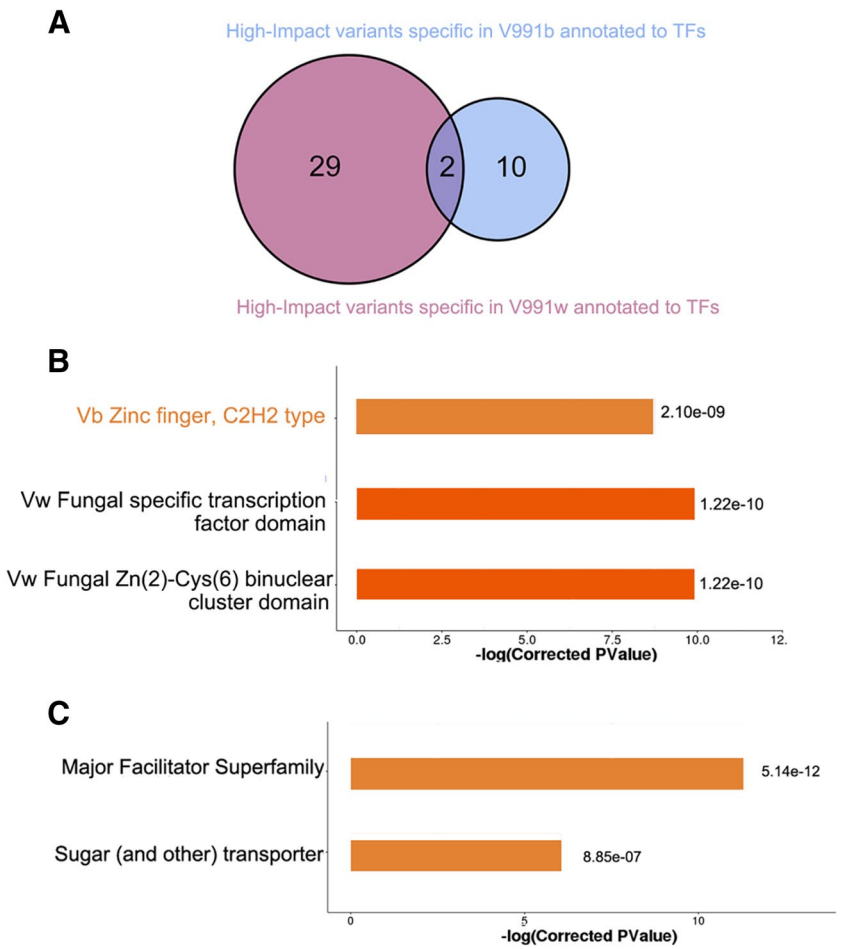

Fig. 6 TFs variations in the low-virulence strain V991w. **a** High-impact variants specific to V991b and V991w identified from DNA re-sequencing data were associated with the corresponding TFs in the two *V. dahliae* strains. These TFs were applied to develop Venn plot. **b** Different types of TFs were enriched for high impact variations specific to V991b or V991w. Functional clustering of the enriched TFs is presented. **c** Target genes of TFs with fungal Zn2-Cys6 binuclear cluster domain in the 0.5-kb DEGs promoter were predicted by motif search. Functional clustering of the target genes is presented

Taken together, by combining transcriptomic and genomic sequencing approaches, this study revealed that expression of a larger number of pathogenicity-related genes, including those involved in melanin synthesis, was reduced in low virulence *V. dahliae* strain under in vitro condition. This reduced expression could partially be explained by the accumulated SNP/Indels in TFs with Zn2-Cys6 binuclear cluster domain. These results supported the hypothesis that a fraction of the pathogenicity-related genes were pre-expressed at adequately high levels. Nevertheless, further analyses are needed to validate these findings obtained under in vitro condition. For example, overexpression of some of the identified potential virulence factors in V991w could be helpful to determine whether the virulence could be restored. Besides, the transcriptome profiles of the low and high virulence *V. dahliae* strains after coming into contact with the plant host should also be examined, and the function of the pre-activated expression of virulence factors and other pathogenicity-related genes in mediating plant–host communication should be investigated.

Conclusion

From RNA-seq data, we detected virulence-related genes and pathways that were upregulated during the vegetative growth stage of V991b. Then, we used comparative genomics data to investigate the mechanisms of the upregulated genes. The results revealed differences in the two *V. dahliae* strains, with the variations contributing to the expression of DEGs. The accumulated genetic variations in TFs were also associated with altered virulence of the fungal pathogens, which must be further studied with respect to pathogenicity regulation and anti-fungal applications.

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Author contributions Yi Z, DY, PW conceived the study, LJ cultured the two *V. dahliae* strains, performed the infection and disease assessment experiments. FY performed the transcriptome and DNA re-sequencing experimental work under the supervision of QW. DC performed all the transcriptome analysis, Yu Z performed the genomic data analysis. Yi Z, DC and SL interpret the data and wrote

the manuscript. All authors have read and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest D.C., S.L., F.Y., Yu Z., Q.W. and Yi Z. are employed by ABLife Inc. PW, LJ and DY are employed by Hubei Academy of Agricultural Sciences. The authors declare that they have no competing interests.

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