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Research

Two homologous Zn_2Cys_6 transcription factors play crucial roles in host specificity of *Colletotrichum orbiculare* by controlling the expression of cucurbit-specific virulence effectors

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Summary

- Fungal plant pathogens preferentially express a set of effector genes at specific infection stages to successfully colonize the host. However, it remains unclear how effector gene expression is regulated during host infection.
- This study identified a Zn_2Cys_6 transcription factor, *TFV1* (*Transcription Factor for Virulence 1*), whose deletion weakened virulence of *Colletotrichum orbiculare* on its cucurbit hosts. The additional deletion of a *TFV1* paralog gene, *TVL1* (*TFV1-like 1*), resulted in a further reduction in virulence on the cucurbits. Notably, *TFV1 TVL1* double mutants retained wild-type virulence on the Solanaceae host *Nicotiana benthamiana*.
- Expression of putative effector genes, including four cucurbit host-specific virulence effectors (*effector protein for cucurbit infection, EPC1-4*), was commonly downregulated in the *TFV1* knockout mutants. Yeast one-hybrid assays suggested that TFV1 binds to the putative promoter regions of *EPC2*, *EPC3*, and *EPC4*, indicating the importance of TFV1 for the induced expression of key effector genes in cucurbit infection.
- Among the effector-like genes whose expression was affected by *TVL1* deletion, a novel LysM effector gene, *EPC5*, was identified as being specifically required for virulence on cucurbit hosts. Our study extends the knowledge of the regulatory mechanisms governing host- and stage-specific effectors in *C. orbiculare*.

Introduction

Plant fungal pathogens impose a huge burden of pressure on agricultural productivity and threaten global food security by causing destructive diseases on plants (Avery *et al.*, 2019), and elucidating their infection mechanisms at the molecular level is therefore essential for addressing this challenge. The ascomycete genus *Colletotrichum* contains > 190 accepted species and causes anthracnose disease in a wide variety of plants, including numerous economically important crops (Cannon *et al.*, 2012; Dean *et al.*, 2012; O'Connell *et al.*, 2012; Jayawardena *et al.*, 2021). In general, *Colletotrichum* species exhibit a hemibiotrophic lifestyle in host plant infection: pathogens initially invade but keep host cells alive (the biotrophic phase), and later kill them by developing necrotrophic hyphae as well as secreting toxins and lytic enzymes (the necrotrophic phase) (Münch *et al.*, 2008; Kleemann *et al.*, 2012; O'Connell *et al.*, 2012). Among *Colletotrichum* species, *C. orbiculare* infects multiple cucurbitaceous plants, such as cucumber, watermelon, and melon (Kubo & Takano, 2013; Matsuo *et al.*, 2022), and can also infect *Nicotiana benthamiana*, which is distantly related to cucurbitaceous plants (Takano *et al.*, 2006; Inoue *et al.*, 2023).

During invasion and colonization of host plants, plant pathogenic fungi secrete a suite of effectors (Kale & Tyler, 2011; Bozkurt *et al.*, 2012). Effectors are typically small, secreted cysteine-rich proteins that act by suppressing plant immunity or manipulating host environmental factors (Selin *et al.*, 2016). Genome sequence analyses of *C. orbiculare* revealed that numerous effector candidate genes are present in this pathogen (Gan *et al.*, 2013). So far, several virulence-related effectors have been identified in *C. orbiculare*. For example, an effector named NIS1 (necrosis-inducing secreted protein 1) that can induce cell death on *N. benthamiana* was identified by functional screening of *C. orbiculare* cDNAs (Yoshino *et al.*, 2012), whereas CoDN3 was described as an effector that suppresses NIS1-induced cell death. Subsequently, it was reported that NIS1 targets the plant immune kinases BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1) and BIK1 (BOTRYTIS-INDUCED KINASE 1) to suppress plant immune responses triggered by PAMPs (pathogen-associated molecular patterns) (Irieda *et al.*, 2019). Also, the effector CoMC69 was shown to be required for virulence of *C. orbiculare* on both cucumber and *N. benthamiana* (Saitoh *et al.*, 2012).

In addition, secreted protein genes of *C. orbiculare* exhibit different expression profiles during the infection, that is more

effector-like genes are upregulated during the establishment of biotrophy, whereas genes encoding degradative enzymes are upregulated in the necrotrophic phase (Gan *et al.*, 2013; Irieda *et al.*, 2016), suggesting the importance of effectors for the early infection steps. Consistent with this idea, we have recently reported the identification of four effector genes, named *EPC1* (*effector protein for cucurbit infection 1*) to *EPC4*, that are preferentially expressed during early stages of infection and are required for fungal virulence in the cucurbit hosts cucumber and melon (Inoue *et al.*, 2023). Several plant pathogenic fungi such as *Colletotrichum higginsianum* and *Magnaporthe oryzae* display expression profiles of secreted protein genes similar to those of *C. orbiculare* (O'Connell *et al.*, 2012; Gan *et al.*, 2013; Yan *et al.*, 2023). These findings suggest the importance of infection stage-specific expression of effectors for virulence of fungal pathogens; however, it remains to be elucidated how the expression of effector genes is regulated in these pathogens.

Transcription factors are sequence-specific DNA-binding proteins that are essential for regulating gene expression in various contexts such as development and environmental responses. Thus, it is plausible that plant pathogenic fungi deploy a suitable set of transcription factors for successful establishment of host infection, including those that regulate the expression of key effector genes. Until now, multiple transcription factors required for fungal pathogenicity and development have been identified and characterized in several fungal species including *C. orbiculare* (John *et al.*, 2021). Regarding transcription factors that regulate the expression of effector genes, it was reported that the transcription factor-encoding gene *AbPf2* is involved in controlling the expression of 13 genes encoding hydrolytic enzymes and eight encoding putative effector proteins in *Alternaria brassicicola* (Cho *et al.*, 2013). More recently, two fungal transcription factors (Moeitf1 and Moeitf2) were identified as required for full virulence of *M. oryzae* on rice, and Moeitf2 controls the expression of the secreted protein gene *T2REP*, which is involved in pathogen virulence (Cao *et al.*, 2022).

Currently, it is unknown how the infection-related expression of effector genes, including *EPC1–EPC4*, is regulated in *C. orbiculare*. Interestingly, the quadruple mutant of the four *EPC* genes displayed almost complete loss of virulence on the cucurbit hosts but still maintained full virulence on *N. benthamiana* (Inoue *et al.*, 2023), suggesting the specific involvement of the four EPC effectors in virulence on the cucurbit hosts. Importantly, further studies revealed that the *EPC* genes are preferentially expressed after inoculation on cucumber compared with inoculation on *N. benthamiana*, although the expression profiles of the majority of genes were common to both hosts (Inoue *et al.*, 2023).

In this study, based on RNA-Seq data of *C. orbiculare* at multiple stages, we selected transcription factor genes that are preferentially expressed in the early infection phase of *C. orbiculare* as candidates for transcription factors involved in virulence and effector regulation. We then performed knockout analysis of these candidate genes and identified one gene required for full virulence of *C. orbiculare* on the cucurbit hosts, which was named *TFV1* (*Transcription Factor for Virulence 1*). *TFV1* encodes a putative transcription factor containing a Zn_2Cys_6 binuclear cluster DNA-binding domain. Phenotypic analyses of the Δ*tfv1* mutant showed that *TFV1* is involved in appressorial host invasion into cucurbit hosts but that its deletion does not affect the development of appressoria. The additional deletion of *TVL1* (*TFV1-like 1*), which is the closest homolog of *TFV1* in *C. orbiculare*, in the Δ*tfv1* mutant further reduced lesion development on the cucurbit hosts. Importantly, the Δ*tfv1* Δ*tvl1* double mutants were as virulent on *N. benthamiana* as the wild-type (WT) strain, suggesting that *TFV1* and *TVL1* of *C. orbiculare* are specifically important for the infection of cucurbit hosts. Remarkably, RNA-Seq and reverse transcription quantitative polymerase chain reaction (RT-qPCR) analyses revealed that the expression of all four *EPC* genes was commonly downregulated in both the Δ*tfv1* and Δ*tfv1* Δ*tvl1* mutants. Furthermore, yeast one-hybrid assays showed that TFV1 exhibits binding activity to the putative promoter sequences of *EPC2*, *EPC3*, and *EPC4* genes. Therefore, the *TFV1*-encoded transcription factor is critical for the induced expression of key virulence effector genes in cucumber infection. Further analysis also successfully identified a novel virulence effector gene, named *EPC5*, containing the LysM domain, whose expression is significantly reduced in the Δ*tfv1* Δ*tvl1* mutant but not in the Δ*tfv1* mutant.

Materials and Methods

Fungal strains and transformation

Colletotrichum orbiculare (syn. *C. lagenarium*) WT strain 104-T (MAFF240422) is stored at the Laboratory of Plant Pathology, Kyoto University (Akai & Ishida, 1968). For disruption of selected candidate transcription factor genes in the initial screening, the *lig4* mutant strain of 104-T (Zhang *et al.*, 2021) was used as the parental strain. In the subsequent knockout analysis of *TFV1* and *TVL1*, we used 104-T as the parental strain. All *C. orbiculare* strains were maintained on 3.9% (w/v) potato dextrose agar (PDA; Nissui, Tokyo, Japan) at 24°C in the dark. The transformation of *C. orbiculare* is based on a method described previously (Takano *et al.*, 2001). Hygromycin-resistant transformants were selected on PDA plates containing 100 μg ml^{-1} hygromycin B. Bialaphos-resistant transformants were selected on minimal medium plates (0.16% yeast nitrogen base without amino acids, 0.2% asparagine, 0.1% NH_4NO_3, and 2% glucose) with 25 μg ml^{-1} bialaphos. Fungal transformants were evaluated by colony PCR using the primers shown in Supporting Information Table S1.

Plasmid construction

Total DNA of *C. orbiculare* was isolated from mycelia with the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. All plasmids used for gene disruption in *C. orbiculare* were constructed using the In-Fusion Cloning System (TaKaRa Bio, Kusatsu, Japan). Fragments encompassing *c.* 2.0 kb upstream and downstream of each target gene were amplified by PCR with the corresponding primers, while the *HPH* (for hygromycin resistance) fragment was amplified with the primers Hyg_F and Hyg_R. The fragments were then

introduced into the *Xho*I-*Hin*dIII site of pCB1636 (Sweigard *et al.*, 1997) to produce each gene disruption vector. The *Bar* (for bialaphos resistance) fragment amplified with the primers Bar_F and Bar_R, instead of *HPH*, was inserted into pCB1636 to construct the *TVL1* disruption vector to generate the Δ*tfv1* Δ*tvl1* double mutant. The primers used for plasmid construction are listed in Table S1.

Virulence assays of *C. orbiculare*

For the virulence assays of *C. orbiculare*, conidial suspensions (5×10^5 conidia ml^{-1}) from a 7-d-old culture of each strain were drop-inoculated on detached cucumber (*Cucumis sativus* L. cv Suyo) and melon (*Cucumis melo* L. cv Lennon) cotyledons from 10-d-old plants and detached *Nicotiana benthamiana* leaves from 28 to 35-d-old plants. Inoculated samples were incubated for 7 d (cucumber and *N. benthamiana*) or 5 d (melon) at 24°C. A total of 24 inoculated spots for each fungal strain were measured in virulence assays. The lesion size data on cucumber and melon were obtained from a total of six cotyledons in three independent experiments (two cotyledons per experiment). In the case of *N. benthamiana*, the lesion size data were obtained from a total of three leaves in three independent experiments (one leaf per experiment).

Evaluation of conidiation, appressorium development, and appressorium invasion

To investigate conidiation, each fungal strain of *C. orbiculare* was incubated at 24°C for 7 d on PDA. Conidia were then harvested from the resultant colonies by suspending in 1 ml of distilled water per plate. The number of conidia generated per cm^2 of colony was calculated using a Neubauer counting chamber. To investigate appressorium development, conidia of each strain suspended in 0.1% yeast extract solution were placed on 8-well glass slides (MP Biomedicals, Solon, OH, USA) and incubated for 1 h, after which the solution was replaced with distilled water and incubation continued (Takano *et al.*, 2000). After 24 h of incubation, the percentage of appressorium formation was measured under a microscope. To investigate the appressorium invasion ratio on host plants, a conidial suspension (1×10^6 conidia ml^{-1}) of each strain was inoculated on the abaxial surface of cucumber and melon cotyledons. The proportion of total appressoria forming invasive hyphae was calculated at 3-, 4-, 5-, and 6-d postinoculation (dpi) for cucumber and 2, 3, and 4 dpi for melon.

Molecular phylogenetic analyses

TFV1 homologs in *C. orbiculare* were identified from the NCBI's nonredundant protein sequences database. Four protein sequences (TFV1, TVL1, TVL2, TVL3) were aligned using the ClustalW program (Thompson *et al.*, 1994), and a phylogenetic tree of the four protein sequences was produced with the neighbor-joining method, with 100 bootstrap replications, by MegaX (Stecher *et al.*, 2020). Blastp searches against the NCBI nr database identified 26, 30, 28, and 55 homologs (percent identity ≥ 70%, *e* value = 0, query cover ≥ 90%) of TFV1, TVL1, TVL2, and TVL3, respectively, in different fungal species, and in total 143 protein sequences were aligned using ClustalW. A phylogenetic tree was constructed as described above.

RT-qPCR analysis

The abaxial surfaces of cucumber cotyledons were drop-inoculated with a conidial suspension (1×10^6 conidia ml^{-1}) of each *C. orbiculare* strain. After incubation for 0, 4, 8, 12, 24, 48, and 72 h, the inoculated epidermis strips were peeled off from three cotyledons for each sample and immediately frozen in liquid nitrogen. For inoculation on *N. benthamiana*, leaves were spray-inoculated with a conidial suspension (1×10^6 conidia ml^{-1}) of each *C. orbiculare* strain. Whole leaves, one leaf for each sample, were then sampled at each time point and frozen in liquid nitrogen. The frozen tissues were ground, and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). Three biological replicates were prepared for each time point. The primers used for RT-qPCR are listed in Table S1. TB Green Premix Ex Taq (TaKaRa Bio) was used with a Thermal Cycler Dice Real Time System TP800 (TaKaRa Bio) for RT-qPCR. Relative expression levels were normalized against the *C. orbiculare* actin gene (GenBank accession no. AB778553.1).

RNA-Seq analysis

To identify *C. orbiculare* genes regulated by the transcription factor TFV1 and/or TVL1, the gene expression profiles of the Δ*tfv1* mutant (Δ*tfv1*-1), the Δ*tfv1* Δ*tvl1* double mutant (Δ*tfv1*Δ*tvl1*-1), and the WT strain at 24-h postinoculation (hpi) of cucumber were investigated by RNA-seq. To prepare RNA samples for sequencing, 20 cucumber cotyledons were inoculated with a conidial suspension (1×10^6 conidia ml^{-1}) of each strain, as much as possible on the abaxial surface, as one biological replicate. After 24 h, inoculated epidermis containing fungal cells was peeled off from 20 cotyledons for each sample, and they were immediately frozen in liquid nitrogen to fix gene expression profiles. The frozen tissues were ground, and total RNA was extracted using the Agilent Plant RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA, USA). Three biological replicates were prepared for Δ*tfv1*-1, Δ*tfv1*Δ*tvl1*-1, and the WT strains. RNA-Seq was conducted using the Illumina Hiseq2000. RNA-Seq reads from different tissues/conditions (Table S2) were mapped to the published *C. orbiculare* genome assembly (Gan *et al.*, 2019) using Hisat2 (Kim *et al.*, 2019). After read mapping, read counts per gene were obtained by using an R package rsubread (v.1.24.2; Liao *et al.*, 2019). Then, read counts were transformed into RPKM (reads per kilobase per million mapped reads) values by using 'rpkm()' function in an R package edgeR (v.3.28.1; Robinson *et al.*, 2010).

Yeast one-hybrid assay

To determine whether TFV1 binds to the promoter regions of the four *EPC* genes, we performed yeast one-hybrid (Y1H) assays

(TaKaRa Bio). Upstream fragments of *EPC1* (1169 bp), *EPC2* (1004 bp, 501 bp), *EPC3* (1500 bp, 1500 bp), and *EPC4* (1005 bp, 501 bp) were ligated into pAbAi to generate the bait vectors. The full-length coding sequence of *TFV1* was amplified and cloned into pGADT7, resulting in the prey vector pGADT7-TFV1. The bait vectors were linearized and integrated into the Y1HGold yeast genome and selected on SD/−Ura agar plates at 30°C for 3 d. The prey vector pGADT7-TFV1 was then introduced into the Y1HGold bait strains, and the yeast strains were selected on SD/−Leu agar plates with 200 ng ml^{-1} Aureobasidin A (AbA). p53-AbAi and pGADT7-Rec-53 were introduced into the Y1HGold yeast strain as a positive control, while p53-AbAi and pGADT7-TFV1 were introduced as a negative control. All primers used are listed in Table S1.

Results

TFV1 encodes a fungal transcription factor required for full virulence of *C. orbiculare* on cucurbitaceous plants but not on *N. benthamiana*

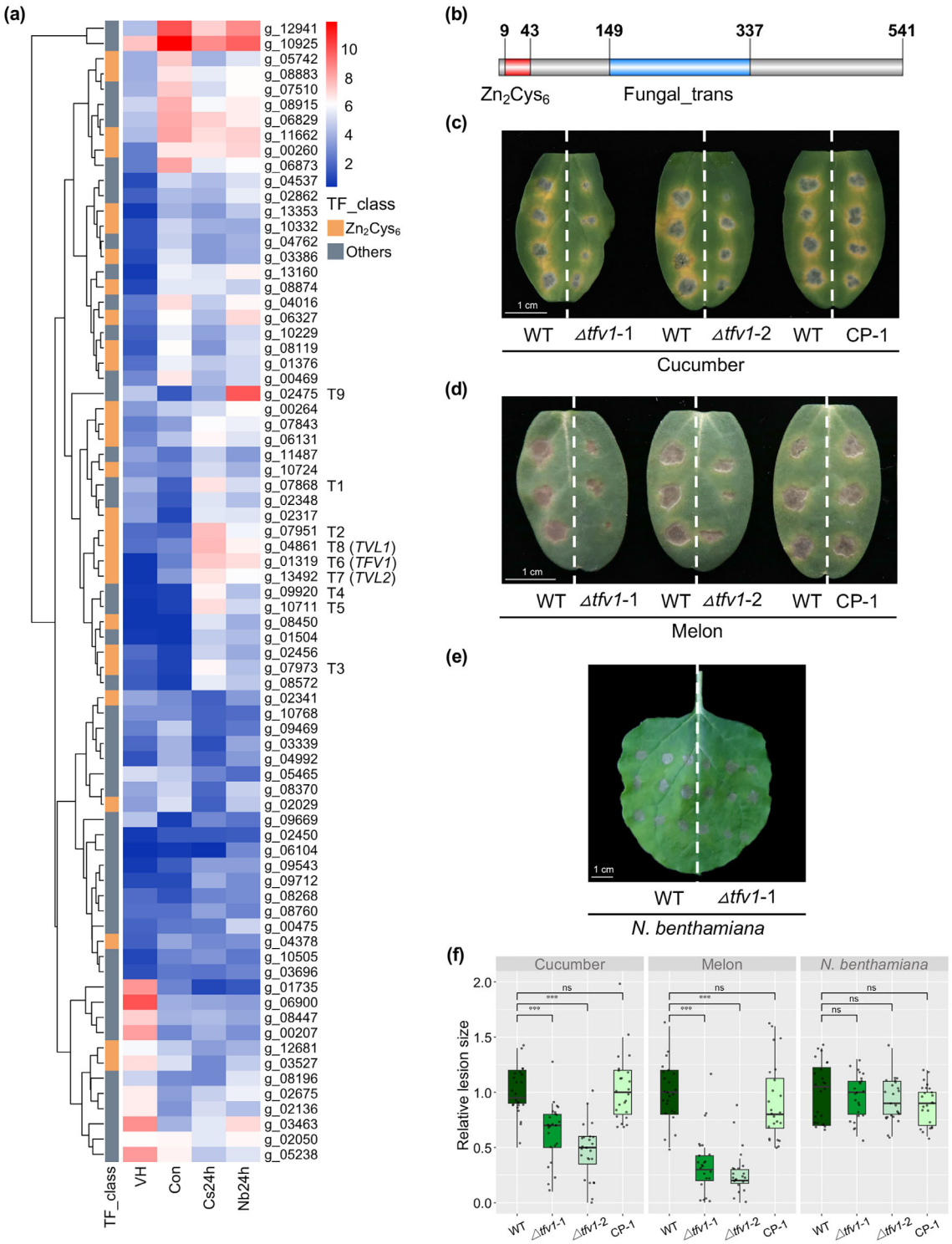
To identify key transcription factors that are involved in virulence and regulation of effector genes in *C. orbiculare*, we selected a set of genes encoding putative transcription factors that are preferentially expressed in the early infection phase (24 hpi) of *C. orbiculare* via RNA-Seq of the pathogen under multiple conditions and subsequent comparative analyses. We selected nine candidate genes (hereafter called T1–T9) that showed low expression (RPKM value < 20) in conidia before inoculation but high expression (RPKM value > 90) at 24 hpi on cucumber (T1–T8) or *N. benthamiana* (T9), when the pathogen has already developed melanized appressoria but has not yet developed invasive hyphae, regarded as the preinvasive stage (Fig. 1a). To investigate whether these candidate genes are involved in fungal virulence, we generated knockout mutants of each gene in the background of the *lig4* mutant of *C. orbiculare* strain 104-T for efficient homologous recombination (Zhang *et al.*, 2021) by targeted gene replacement with a hygromycin B resistance gene (*HPH*) cassette (Fig. S1).

The inoculation test with T1–T9 disruption mutants on cucumber cotyledons revealed that T6 is involved in the virulence of *C. orbiculare* on cucumber, because the T6 knockout mutant displayed a marked reduction in lesion development (Fig. S2). On the other hand, the knockout mutants of the other genes caused disease symptoms similar to the parental *lig4* mutant at 7 dpi (Fig. S2). In the case of T9, which is highly expressed on *N. benthamiana* at 24 hpi, we also investigated the lesion development ability of the T9 knockout mutant on *N. benthamiana* but observed no clear difference in lesion development between the T9 knockout and the parental strain (Fig. S2). We renamed T6 as *TFV1* (*Transcription Factor for Virulence 1*). *TFV1* encodes a 541 amino acid protein with two domains showing Pfam matches (Fig. 1b). The first domain is a fungal Zn_2Cys_6 binuclear cluster DNA-binding domain (residues 9–43) and the second is a fungal-specific transcription factor domain (residues 149–337). For further analyses of *TFV1*, we generated the Δ*tfv1* mutants Δ*tfv1*-1 and Δ*tfv1*-2 in the background of the WT strain 104-T, instead of the *lig4* mutant. We also generated a complementation strain (CP-1) by reintroducing *TFV1* into Δ*tfv1*-1 (Fig. S3). The generated fungal strains were then inoculated onto cucumber, melon, and *N. benthamiana*. Lesions caused by the two Δ*tfv1* mutants were commonly smaller on both cucumber and melon than those of the WT strain. By contrast, CP-1 was as virulent as the WT strain on cucumber and melon (Fig. 1c,d). Interestingly, the Δ*tfv1* mutants showed virulence on *N. benthamiana* at the same level as the WT strain (Fig. 1e). Quantitative analysis of the lesion area confirmed a significant reduction in the lesion development of the Δ*tfv1* mutants on cucumber and melon, but not on *N. benthamiana*, compared with the WT and CP-1 (Fig. 1f). Taken together, these results suggest that *TFV1* encodes a fungal transcription factor that is essential for full virulence on cucumber and melon, but not on *N. benthamiana*.

Deletion of *TFV1* affects appressorial invasion on both cucumber and melon

We next performed multiple phenotypic analyses on the two Δ*tfv1* mutants (Δ*tfv1*-1 and Δ*tfv1*-2) of *C. orbiculare*. The vegetative growth rate of the Δ*tfv1* mutants on PDA medium was found to be similar to that of CP-1 and the WT strain (Fig. 2a).

Fig. 1 *TFV1* encodes a Zn_2Cys_6 binuclear cluster transcription factor that is specifically required for virulence of *Colletotrichum orbiculare* on cucurbit hosts. (a) Selection of nine candidate genes (T1–T9) encoding putative transcription factors. Heat map showing the gene expression levels (log_2RPKM values) of each candidate gene in vegetative hyphae (VH), conidia (Con), inoculated cucumber (*Cucumis sativus* L. cv Suyo) cotyledons at 24-h postinoculation (Cs24h), and inoculated *Nicotiana benthamiana* leaves at 24-h postinoculation (Nb24h). The color key represents the log_2 ratio of RPKM, with red indicating higher expression levels and blue indicating lower expression levels. T1–T8 demonstrated high expression during the early stages of cucumber infection, while T9 was highly expressed in the early stages of *N. benthamiana* infection. Zn_2Cys_6-type transcription factors are shown in orange in the TF_class column. (b) Protein sequence organization of TFV1. The predicted coding region of *TFV1* encodes a 541 amino acid protein with two significant Pfam matches: a fungal Zn_2Cys_6 binuclear cluster domain (Zn_2Cys_6), located at amino acid resideus 9–43, and a fungal-specific transcription factor domain (Fungal_trans), located at amino acid residues 149–337. (c) *TFV1* knockout mutants displayed reduced virulence on cotyledons of cucumber. Symptom development of *C. orbiculare* wild-type 104-T (WT), two Δ*tfv1* strains (Δ*tfv1*-1 and Δ*tfv1*-2), and complementation strain Δ*tfv1*-1/*TFV1* (CP-1) on cucumber cotyledons at 7-d postinoculation (dpi). (d) The *TFV1* knockout mutants also showed reduced virulence on cotyledons of melon (*Cucumis melo* L. cv Lennon) at 5 dpi. (e) Symptom development of Δ*tfv1*-1 was maintained when compared with WT on *N. benthamiana* leaves at 7 dpi. (f) Quantified lesion size in spot-inoculated host plants. At 5 dpi (melon) or 7 dpi (cucumber and *N. benthamiana*), relative lesion size was calculated using ImageJ. The two *TFV1* disruption mutants displayed reduced lesion size on both cucumber and melon, but not on *N. benthamiana*, compared to lesions caused by WT. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to 1.5 times the interquartile range from the 25th and 75th percentiles. The statistical significance of the difference between the means was tested using the Welch's *t*-test (***, $P < 0.001$; ns, not significant, $P > 0.05$).



Conidia production by the Δ*tfv1*-1 mutant was also comparable to that by the WT strain (Fig. 2b). In the Δ*tfv1*-1 mutant, there was also no defect in appressorium development on a glass slide, that is 80–90% of conidia formed appressoria with normal morphology in the Δ*tfv1*-1 mutant at 24 hpi. The appressorium development ratio of the mutant was similar to that of the WT and CP-1 (Fig. 2c). These results indicated that the deletion of *TFV1* has no detectable effects on vegetative growth, conidia production, or appressorium development in *C. orbiculare*.

We then asked whether appressorial invasion was affected by the disruption of *TFV1*. Via microscopic analysis, we quantified the ratio of appressoria that formed invasive hyphae at three

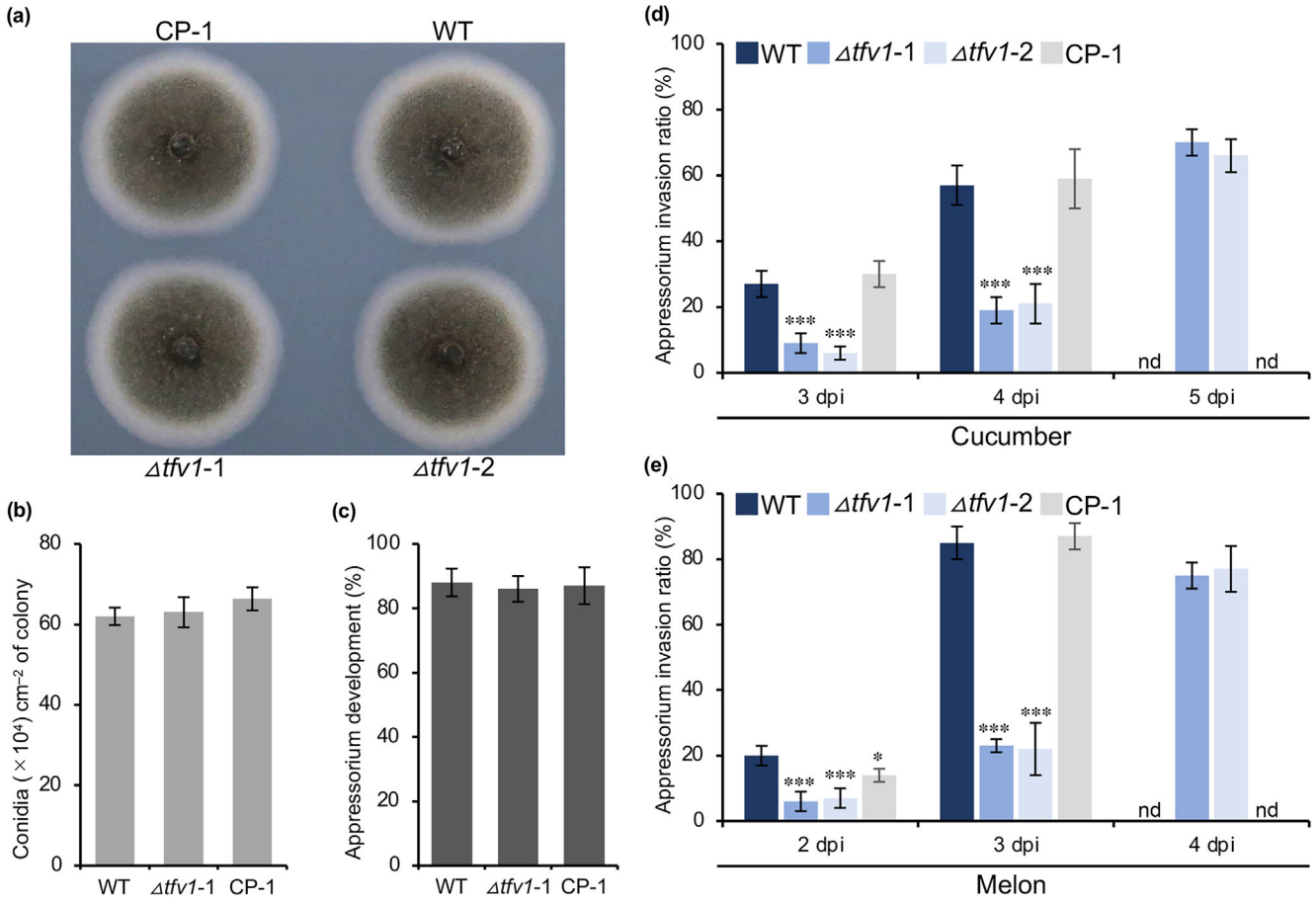


Fig. 2 Deletion of *TFV1* has no detectable effect on appressorium development but results in a significant delay of subsequent appressorium-mediated invasion into cucurbit hosts. (a) *TFV1* is not essential for vegetative growth. *Colletotrichum orbiculare* wild-type104-T (WT), two Δ*tfv1* strains (Δ*tfv1*-1 & Δ*tfv1*-2), and complementation strain (CP-1) were incubated for 7 d on a potato dextrose agar (PDA) plate. (b) *TFV1* is dispensable for conidia production on PDA plates. After incubation on PDA plates for 7 d, the conidia formed in mycelial colonies were counted for each strain. The indicated values are means of three biological replicates and SD is indicated by the error bars. There were no significant differences between group means as determined by one-way ANOVA. (c) Deletion of *TFV1* has no detectable effect on appressorium development of *C.orbiculare*. Conidial suspensions of each strain were incubated on slide glass for 24 h, and the percentage of appressorium formation was calculated. The indicated values are means of three biological replicates; error bars indicate SD. There were no significant differences between group means as determined by one-way ANOVA. (d, e) Appressorium-mediated invasion of the *TFV1* disruption mutants is delayed on cucumber (*Cucumis sativus* L. cv Suyo) (d) and melon (*Cucumis melo* L. cv Lennon).(e). A conidial suspension of each strain was inoculated onto the abaxial surface of cucumber cotyledons (or melon cotyledons), and then incubated at 24°C. The mean percentage of appressoria forming invasive hyphae at 3-, 4-, and 5-d postinoculation (dpi) on cucumber (at 2, 3, and 4 dpi for inoculation on melon) was calculated. nd indicates that the data could not be determined due to collapse of the tissue caused by severe invasive growth. Asterisks indicate a significant difference from WT in a Tukey's test (***, $P < 0.001$; *, $P < 0.05$). Error bars indicate the SD for five biological replicates. One hundred appressoria were scored per replication.

time points that were adjusted to different host plant preferences. Importantly, the Δ*tfv1* mutants showed significantly delayed appressorial invasion on both cucumber and melon when compared with the WT and CP-1. In detail, on cucumber, *c.* 30% appressoria of the WT and CP-1 developed invasive hyphae at 3 dpi, whereas the appressorial invasion ratio of the two Δ*tfv1* mutants was < 10% in both cases. At 4 dpi, more than half of appressoria developed invasive hyphae in the WT and CP-1, whereas the invasion ratio of the Δ*tfv1* mutants just reached 20%. At 5 dpi, the WT and CP-1 produced necrotrophic hyphae, which caused cell death on plant tissues; meanwhile, *c.* 60% of appressoria developed invasive hyphae in the Δ*tfv1* mutants (Fig. 2d). We also found a reduction in appressorial invasion of the Δ*tfv1* mutants on melon at 2 and 3 dpi compared to the WT and CP-1 (Fig. 2e). Collectively, these findings demonstrate that the deletion of *TFV1* in *C. orbiculare* results in reduced virulence on cucurbitaceous plants, which is at least partially due to inhibition of the appressorial invasion step.

Phylogenetic analysis of TFV1

TFV1 is highly conserved in *C. orbiculare* species complex (COSC) fungi, such as *Colletotrichum trifolii* and *Colletotrichum sidae* (Fig. S4). A BLASTP search in the NCBI database also revealed that three genes homologous to *TFV1* were present in

C. orbiculare, which we named *TVL1*, *TVL2*, and *TVL3* (*TFV1-like 1*, *2*, and *3* respectively) (Fig. S5). Actually, *TVL1* and *TVL2* correspond to T8 and T7, respectively, in the selected candidates of this study (Fig. 1a). The phylogenetic analysis indicated that TVL1 was most closely related to TFV1 (Fig. S6).

We then performed BLASTP searches against the NCBI protein database using TFV1, TVL1, TVL2, and TVL3 as the query sequences and identified 26 putative TFV1 orthologs, 30 putative TVL1 orthologs, 28 putative TVL2 orthologs, and 55 putative TVL3 orthologs in different fungal species; these sequences were subjected to phylogenetic analysis (Fig. 3a). This revealed that the orthologs of TFV1, TVL1, and TVL2 are highly conserved in *Colletotrichum* species but are not found in other fungal genera, implying that TFV1, TVL1, and TVL2 orthologs are restricted to *Colletotrichum* species (Fig. 3a). On the other hand, putative TVL3 orthologs were found in many fungal species belonging to the division Ascomycota, such as *Verticillium* and *Fusarium* spp. (Fig. 3a).

A heatmap analysis based on obtained RNA-Seq data suggested that *TFV1*, *TVL1*, and *TVL2* are highly expressed during the plant infection process compared with *TVL3* (Fig. S6). By contrast, *TVL3* was constantly expressed at all tested stages including vegetative mycelia and preinoculated conidia, which is distinct from the case of *TFV1*, *TVL1*, and *TVL2* (Fig. S6). We also found that the expression level of *TVL1* was higher than that of *TVL2* (Fig. S6). Based on these data, together with the finding that TVL1 is most closely related to TFV1, we decided to perform further studies on not only TFV1 but also TVL1. We monitored the expression of both *TFV1* and *TVL1* during infection of *C. orbiculare* on cucumber and *N. benthamiana* by RT-qPCR (Fig. 3b,c). On cucumber, *TFV1* expression was lowest in conidia, and it was highly induced at 4 hpi. The high-expression level of *TFV1* was maintained until 24 hpi and reduced at 48 hpi (Fig. 3b). By contrast, the expression of *TVL1* was low until 12 hpi and then highly induced at 24 hpi (Fig. 3c). These results suggest a distinct transcriptional regulation between *TFV1* and *TVL1* during cucumber infection. When inoculated on *N. benthamiana*, *TFV1* showed a similar expression pattern to that seen in cucumber, but the expression level was lower than in cucumber, in agreement with the finding that *TFV1* is required for virulence on cucumber but not *N. benthamiana* (Fig. 3b). We also found a more striking difference for *TVL1*: its expression was not clearly induced in the inoculation on *N. benthamiana*, in contrast with the case on cucumber (Fig. 3c).

Involvement of *TVL1* in the virulence of *C. orbiculare* on cucurbits

As mentioned above, the single knockout mutant of *TVL1* (also called T8 in the selected candidate list) showed no observed differences in virulence on cucumber compared to the parental strain (the *lig4* mutant) (Figs S2, S7). To explore the potential functional relationship between TFV1 and TVL1, we generated double-knockout mutants of *TFV1* and *TVL1* by replacing *TVL1* with a bialaphos resistance cassette in the background of the Δ*tfv1* mutant (Δ*tfv1*-1) (Fig. S8). Remarkably, the virulence assays revealed that two independent Δ*tfv1* Δ*tvl1* double mutants commonly decreased lesion development on both cucumber and melon compared with the Δ*tfv1* single mutants (Fig. 3d,e). Quantitative data from replicated experiments supported this finding (Fig. S9). We further performed a virulence assay of each mutant on *N. benthamiana*. Importantly, there was no significant difference between WT and the Δ*tfv1* Δ*tvl1* mutant in lesion development on *N. benthamiana* (Figs 3f, S10). These findings indicate that (1) the *TFV1*-homologous gene *TVL1* is involved in virulence of *C. orbiculare* on cucurbit hosts, at least in the absence of *TFV1*, and (2) both *TFV1* and *TVL1* are dispensable for virulence on *N. benthamiana*.

To investigate whether the Δ*tfv1* Δ*tvl1* double mutant has more severe defects in the host invasion step compared with the Δ*tfv1* single mutant, the appressorial invasion ratio on cucumber was investigated. The ratio for the Δ*tfv1* mutant was *c.* 60% at 5 dpi, whereas that for the Δ*tfv1* Δ*tvl1* mutant was below 40% (Fig. 3g), suggesting the involvement of *TVL1* in host invasion, at least in the background of the Δ*tfv1* mutant.

Plant-induced expression of key virulence effector genes *EPC1*–*EPC4* largely depends on the *TFV1*-encoded transcription factor

Phenotypic analysis of the Δ*tfv1* and Δ*tfv1* Δ*tvl1* knockout mutants revealed that *TFV1* and *TVL1* are specifically required for full virulence on cucurbit hosts but not on *N. benthamiana*. Also, both *TFV1* and *TVL1* encode putative transcription factors containing the Zn_2Cys_6 binuclear cluster domain. Consistent with this, nuclear localization signals were predicted in both TFV1 and TVL1 by cNLS Mapper (Kosugi *et al.*, 2009; Fig. S11a). Furthermore, yeast two-hybrid assays revealed that yeast cells co-transformed with pGBKT7-TFV1/pGADT7 or pGBKT7-TVL1/pGADT7 grew properly on SD/−Trp/−Leu/−His/−Ade medium (Fig. S11b), indicating that TFV1 and TVL1 were able to activate transcription of target genes when fused to the GAL4 DNA-binding domain. These results collectively indicated that both TFV1 and TVL1 function as transcription factors required for the cucurbit-specific virulence of *C. orbiculare*.

We next performed a comparative RNA-Seq analysis between the WT strain, the Δ*tfv1* mutant, and the Δ*tfv1* Δ*tvl1* mutant. Each strain was inoculated on cucumber cotyledons and the inoculated samples were collected at 24 hpi, regarded as the preinvasion stage. Total RNA was then extracted and subjected to RNA-Seq analysis using Illumina Hiseq 2000. Around 6–10% of the total reads were mapped to *C. orbiculare* genes. We calculated RPKM values for all *C. orbiculare* genes in each strain, and then focused on genes with RPKM values ≥ 100 in the WT strain. In both the Δ*tfv1* mutant and the Δ*tfv1* Δ*tvl1* mutant, 113 genes were concurrently downregulated with at least a twofold change in the expression level compared to the WT strain (Fig. 4a). Among these genes, about two-thirds (71) encode proteins having putative signal peptides, suggesting the involvement of TFV1 in the upregulation of putative effector genes (Fig. 4a).

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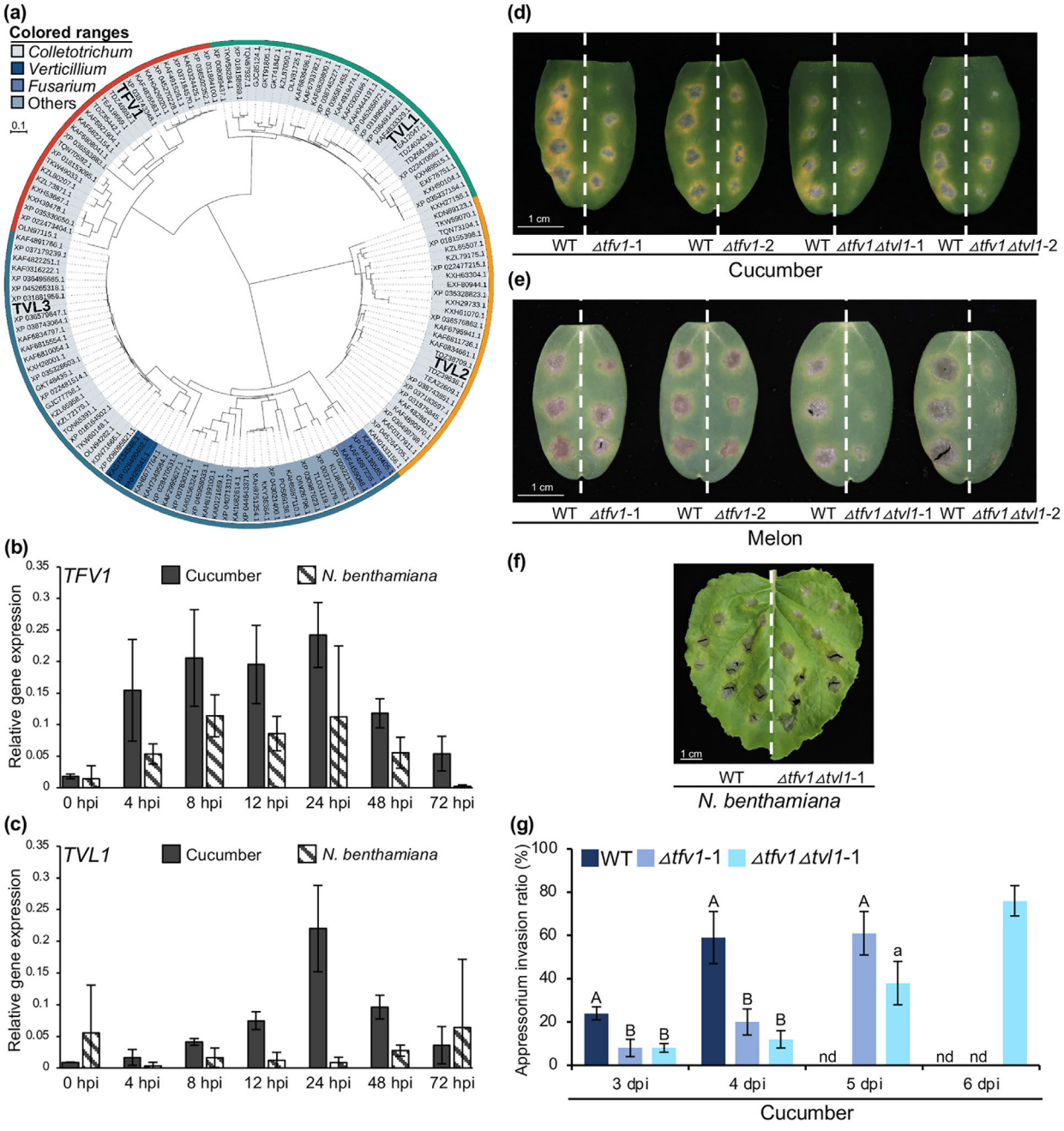


Fig. 3 Specific involvement of *TVL1* in the virulence of *Colletotrichum orbiculare* on cucurbits. (a) Phylogenetic analysis of TFV1, TVL1, TVL2, and TVL3 of *C. orbiculare* and their putative orthologs from different fungal species. The phylogenetic tree was constructed with 143 protein sequences in MEGAX using the neighbor-joining method. Different genera are marked with different colors. The bar indicates the evolutionary distance in amino acid substitutions per site. (b, c) Expression patterns of *C. orbiculare TFV1* and *TVL1* on cucumber (*Cucumis sativus* L. cv Suyo) and *Nicotiana benthamiana*. A conidial suspension of *C. orbiculare* was drop-inoculated onto the abaxial surface of detached cucumber cotyledons or spray-inoculated onto detached *N. benthamiana* leaves. Samples were collected at 0-, 4-, 8-, 12-, 24-, 48-, or 72-h postinoculation (hpi). Expression of *TFV1* (b) and *TVL1* (c) relative to the *C. orbiculare* reference gene Actin was assessed by quantitative RT-PCR. The indicated values are means of three biological replicates; error bars indicate SD. (d, e) *TFV1* and *TVL1* double-knockout mutants show reduced lesion development compared to the *TFV1* single mutants on both cucumber (at 7-d postinoculation) and melon (*Cucumis melo* L. cv Lennon) (at 5-d postinoculation). (f) Gene disruption of both *TFV1* and *TVL1* had no visible effect on the virulence of *C. orbiculare* on *N. benthamiana*. (g) Appressorial invasion on cucumber is delayed by the deletion of *TVL1* in the Δ*tfv1* single mutant. A conidial suspension of each strain was inoculated onto the abaxial surface of cucumber cotyledons and incubated at 24°C. The mean percentage of appressoria forming invasive hyphae was calculated at 3-, 4-, 5-, 6-d postinoculation (dpi). nd indicates that the data could not be determined because of the collapse of the tissues by severe invasive growth. At the same time point, different capital letters on the bars indicate significant differences between samples ($P < 0.01$) and the lowercase letter indicates that the difference between samples was statistically significant ($P < 0.05$) in a Tukey HSD test. Error bars indicate the SD for five biological replicates. One hundred appressoria were scored per replication.

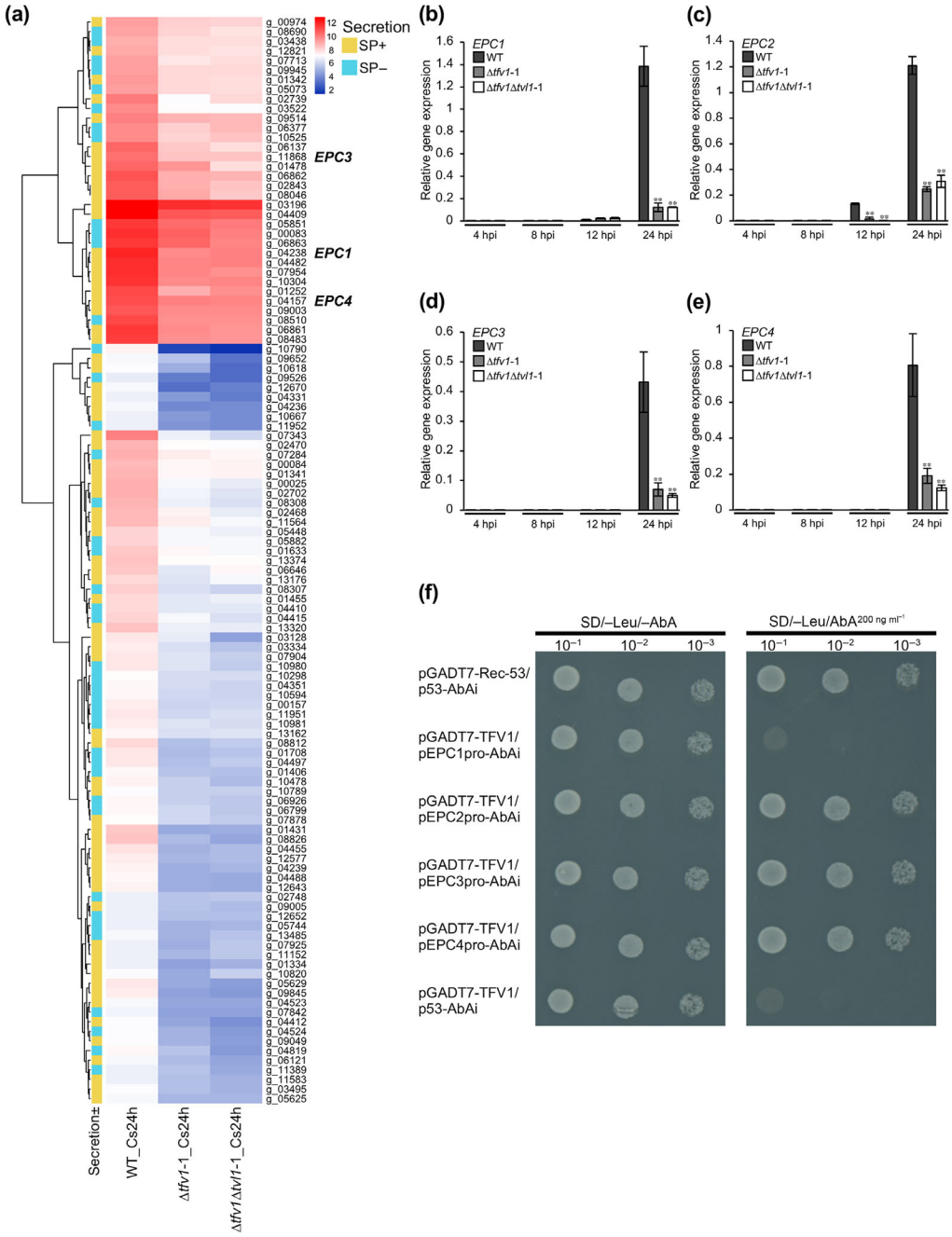


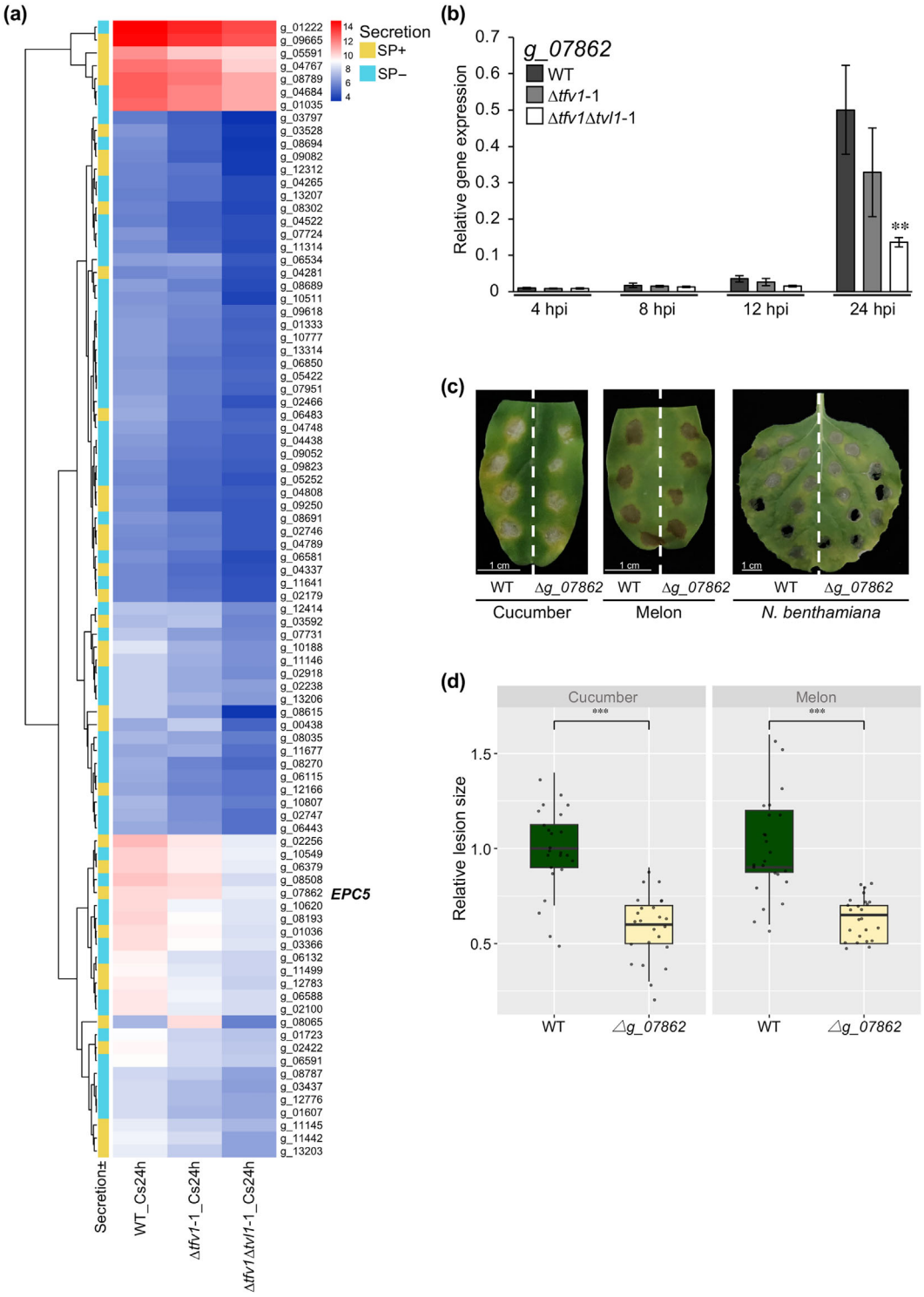
Fig. 4 TFV1 is essential for the induced expression of effector genes including *EPC1*–*EPC4* during cucumber (*Cucumis sativus* L. cv Suyo) infection. (a) Expression profiles of downregulated genes in the Δ*tfv1* mutant and the Δ*tfv1* Δ*tvl1* mutant. The set of 113 downregulated genes (fold change ≥ 2) at 24-h postinoculation (hpi) on cucumber in the Δ*tfv1* mutant (Δ*tfv1*-1_Cs24h) and the Δ*tfv1* Δ*tvl1* mutant (Δ*tfv1*Δ*tvl1*-1_Cs24h) compared to *Colletotrichum orbiculare* wild-type 104-T (WT) (WT_Cs24h) is shown in this heat map. The color key represents the $\log_2$ ratio of RPKM, with red indicating higher expression levels and blue indicating lower expression levels. The annotation of secreted type shown in yellow represents 71 genes encoding proteins with putative signal peptides (SP+). The virulence-related effector genes *EPC1*, *EPC3*, and *EPC4* are located in this group. (b–e) Verification of the gene expression of (b) *EPC1*, (c) *EPC2*, (d) *EPC3*, and (e) *EPC4* in the transcription factor knockout mutants. Conidial suspensions of *Colletotrichum orbiculare* WT strain, *C. orbiculare* Δ*tfv1* strain, and *C. orbiculare* Δ*tfv1* Δ*tvl1* strain were drop-inoculated onto the abaxial surface of detached cucumber cotyledons. Samples were collected at 4, 8, 12, and 24 hpi. The expression of each virulence effector gene relative to the *C. orbiculare* reference gene Actin was assessed by quantitative RT-PCR. The indicated values are means of three biological replicates; error bars indicate SD. Asterisks indicate a significant difference from WT in an independent samples *t*-test (**, $P < 0.01$). (f) Yeast one-hybrid assays were employed to assess the binding activity of TFV1 with the promoter region of *EPC*s. The yeast strains transformed with the plasmid combinations pGADT7-TFV1/pEPC2pro-AbAi, pGADT7-TFV1/pEPC3pro-AbAi, pGADT7-TFV1/pEPC4pro-AbAi, and the positive control (pGADT7-Rec-53/p53-AbAi) could grow on SD/−Leu medium with 200 ng ml^{-1} Aureobasidin A (AbA) in a 10-fold serial dilution (10^{-1}, 10^{-2}, 10^{-3}). By contrast, two transformants with pGADT7-TFV1/pEPC1pro-AbAi and the negative control (pGADT7-TFV1/p53-AbAi) were unable to grow on the same medium.

Recently, four host-specific virulence effector genes, named *EPC1–EPC4*, have been identified in *C. orbiculare* (Inoue *et al.*, 2023). Knockout mutant analyses revealed that the four *EPC* genes are specifically required for virulence on cucurbit hosts (cucumber and melon) but are dispensable for virulence on *N. benthamiana* (Inoue *et al.*, 2023). Remarkably, comparative RNA-Seq analysis identified *EPC1*, *EPC3*, and *EPC4* as commonly downregulated genes in both the Δ*tfv1* mutant and the Δ*tfv1* Δ*tvl1* mutant. To verify these results, we performed RT-qPCR analysis to investigate the expression level of *EPC*s in each strain at different time points in the cucumber inoculation (Fig. 4b–e). This revealed that the expression of all *EPC* genes was not induced by 12 hpi but was highly induced at 24 hpi, in agreement with the previous report (Inoue *et al.*, 2023). Consistent with the comparative RNA-Seq analysis, the expression of *EPC1*, *EPC3*, and *EPC4* was severely reduced in both the Δ*tfv1* mutant and the Δ*tfv1* Δ*tvl1* mutant (Fig. 4b,d,e). Although *EPC2* was not included in the downregulated gene list based on RNA-Seq data, the RT-qPCR analysis revealed that the expression of *EPC2* was significantly reduced in both Δ*tfv1* and Δ*tfv1* Δ*tvl1* mutants compared to the WT strain (Fig. 4c). Furthermore, there were no observed differences in the expression of *EPC1–EPC4* between the Δ*tfv1* mutant and the Δ*tfv1* Δ*tvl1* mutant, indicating that the *TFV1*-encoded transcription factor acts as a crucial positive regulator for the expression of the virulence effector genes *EPC1*, *EPC2*, *EPC3*, and *EPC4* (Fig. 4b–e). Next, we investigated whether TFV1 directly regulates the expression of the four *EPC* genes. For this purpose, a yeast one-hybrid assay was performed to test the physical binding activity of TFV1 in the putative promoter regions of the *EPC* genes. We found that yeast cells co-transformed with TFV1 (prey) and the *EPC2*, *EPC3*, or *EPC4* upstream regions (bait) commonly exhibited normal growth on SD/−Leu agar plates with 200 ng ml^{-1} AbA, indicating that TFV1 bound in the upstream regions of *EPC2*, *EPC3*, and *EPC4* (Fig. 4f). On the other hand, yeast cells transformed with TFV1 and the *EPC1* upstream region did not grow on the same medium (Fig. 4f). Original and extended transformation results are also presented in Fig. S12. These results suggested that TFV1 can directly bind to the promoter regions of *EPC2*, *EPC3*, and *EPC4* to regulate their expression; by contrast, TFV1 may control the expression of *EPC1* indirectly.

Comparative RNA-Seq analysis identifies a novel virulence effector whose expression depends on TVL1

The Δ*tfv1* Δ*tvl1* mutant exhibited a reduction in virulence on cucurbits in comparison with the Δ*tfv1* mutant (Fig. 3d,e), raising the possibility that TVL1 positively regulates the expression of unidentified virulence effector genes, at least in the absence of TFV1. To focus on this issue, we listed 88 genes that were specifically downregulated in the Δ*tfv1* Δ*tvl1* mutant, but not in the Δ*tfv1* mutant, compared to the WT strain, with at least a twofold change in expression level (Fig. 5a). More than a third of the genes (33) encode proteins with putative signal peptides, suggesting that TVL1 also positively regulates the expression of putative effector genes. Among these selected 33 protein genes, we decided to focus on *g_07862*, which encodes a putative effector having a LysM domain (Fig. 5a). Secreted LysM effectors of multiple phytopathogenic fungi are known to inhibit chitin-triggered plant immunity (Tariqjaveed *et al.*, 2021). RNA-Seq data at 24 hpi on cucumber showed that the expression of *g_07862* was the highest among the four putative LysM effector genes of *C. orbiculare* (Fig. S13). It is also noteworthy that *ChELP4*, a putative ortholog of *g_07862*, is not essential for the virulence of *C. higginsianum* (Fig. S13; Takahara *et al.*, 2016). We first verified the expression pattern of *g_07862* in WT, the Δ*tfv1* mutant, and the Δ*tfv1* Δ*tvl1* mutant upon cucumber inoculation by RT-qPCR. This analysis revealed that *g_07862* expression was highly induced at 24 hpi and significantly reduced in the Δ*tfv1* Δ*tvl1* mutant, but not in the Δ*tfv1* mutant, compared to the WT strain (Fig. 5b). We then generated a knockout strain of *g_07862* in the background of *C. orbiculare* WT strain. The resultant knockout strain showed a significant reduction in lesion development on both cucumber and melon, but not on *N. benthamiana*, indicating that the *g_07862*-encoded LysM effector is a novel effector that is specifically required for the virulence of *C. orbiculare* on cucurbitaceous plants (Fig. 5c,d). We therefore named *g_07862* as *EPC5* (*effector proteins for ucurbit infection 5*). In common with *EPC1–EPC4* genes, the RNA-Seq data suggested the preferential expression of *EPC5* (*g_07862*) on cucumber (RPKM value = 986.1) compared with that on *N. benthamiana* (RPKM value = 384.4). This finding was confirmed by an RT-qPCR experiment (Fig. S14). We also generated a knockout

Fig. 5 Comparative RNA-Seq analysis of wild-type and the mutants identifies a novel virulence effector whose expression depends on TVL1. (a) Expression profiles of downregulated genes in the Δ*tfv1* Δ*tvl1* mutant. The set of 88 downregulated genes (fold change ≥ 2) at 24-h postinoculation (hpi) on cucumber (*Cucumis sativus* L. Suyo) in the Δ*tfv1* Δ*tvl1* mutant (Δ*tfv1*Δ*tvl1*-1_Cs24h), but not in the Δ*tfv1* mutant (Δ*tfv1*-1_Cs24h), compared to *Colletotrichum orbiculare* wild-type 104-T (WT) (WT_Cs24h) is shown in this heat map. The color key represents the log_2 ratio of RPKM, with red indicating higher expression levels, and blue indicating lower expression levels. The annotation of secreted type shown in yellow represents 33 genes encoding proteins with putative signal peptides (SP+). A newly identified effector (*g_07862*; *EPC5*) has been included here. (b) Verification of *g_07862* gene expression in the transcription factor knockout mutants. The expression of *g_07862* relative to the *Colletotrichum orbiculare* reference gene Actin was assessed by quantitative RT-PCR. The indicated values are means of three biological replicates; error bars indicate SD. Asterisks indicate a significant difference from WT in an independent samples *t*-test (**, $P < 0.01$). (c) Pathogenicity test of the *g_07862* single knockout mutant on different plants. Compared to the WT strain, Δ*g_07862* showed reduced lesion development on cucumber and melon (*Cucumis melo* L. cv Lennon) cotyledons, but not on *Nicotiana benthamiana* leaves. (d) Quantification of lesion size in spot-inoculated plants. At 5-d postinoculation (melon) or 7-d postinoculation (cucumber), relative lesion size was calculated using ImageJ. There was a significant reduction in lesion development caused by the *g_07862* disruption mutant compared to WT on both cucumber and melon. Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to 1.5 times the interquartile range from the 25th and 75th percentiles. The statistical significance of the difference between the means was tested using the Welch's *t*-test (***, $P < 0.001$).



mutant of *g_01431* in *C. orbiculare*, because the LysM effector gene *ChELP2*, a putative ortholog of *g_01431*, was reported to be required for the virulence of *C. higginsianum* (Fig. S13; Takahara *et al.*, 2016). However, the knockout mutant of *g_01431* showed WT-level virulence on both cucumber and *N. benthamiana* (Fig. S15), which contrasted with the case of *EPC5* (*g_07862*).

Discussion

Plant pathogenic fungi deploy hundreds of secretory effector proteins to suppress plant defense responses and manipulate plant physiological conditions for their successful infection (Snelders *et al.*, 2018). *Colletotrichum* fungi including *C. orbiculare* express a different set of effector genes at each infection stage, suggesting

the presence of a regulatory machinery for stage-specific expression of effector genes (O'Connell *et al.*, 2012; Gan *et al.*, 2013). We also previously reported that inappropriate expression of the effector NLP1 (NECROSIS- AND ETHYLENE-INDUCING PEPTIDE1-LIKE PROTEIN1) completely abolished the virulence of *C. orbiculare* on multiple cucurbit hosts (Azmi *et al.*, 2018), indicating the importance of stage-specific effector expression for fungal virulence. Recently, we identified four effector genes named *EPC1–EPC4* that are crucial for the virulence of *C. orbiculare* on cucurbit hosts but not on *N. benthamiana* (Inoue *et al.*, 2023), suggesting that the *EPC* genes are specifically involved in virulence of *C. orbiculare* on cucurbits. As shown in the previous and current studies, the *EPC* genes are not induced at 12 hpi but are highly induced at 24 hpi on cucumber, suggesting the specific induction of the *EPC* genes at particular infection stages. Interestingly, the *EPC* genes are preferentially expressed in cucumber infection compared with *N. benthamiana* infection, although gene expression profiles of the majority of genes were similar in both hosts (Inoue *et al.*, 2023). These findings suggested the concept that the selective expression of virulence effector genes shapes fungal host specificity (Inoue *et al.*, 2023). However, it remained unclear how this host-specific and stage-specific expression of the critical effectors is regulated during the infection process of *C. orbiculare*.

In this study, we identified a Zn_2Cys_6 transcription factor, TFV1 that is required for full virulence of *C. orbiculare* on cucurbit hosts but not on *N. benthamiana*, which is consistent with the behavior of the EPC effectors. Deletion of *TFV1* did not interfere with vegetative growth, conidiation, germination, or appressorium formation but reduced appressorium-mediated invasion on both cucumber and melon, further supporting the idea that TFV1 is not involved in fungal morphogenesis but is involved in some aspect of the interaction with host plants, such as effector-mediated suppression of plant immunity. Targeted gene disruption of *TVL1*, the closest homolog of *TFV1*, in the Δ*tfv1* mutant resulted in enhanced reduction in virulence on cucurbits compared to the parental Δ*tfv1* mutant, whereas the Δ*tvl1* single mutant exhibited WT-level virulence.

As mentioned above, four virulence effector genes (*EPC1–EPC4*) are highly expressed at the preinvasion stage (24 hpi) of *C. orbiculare* infection on cucumber. In this study, comparative RNA-Seq and subsequent RT-qPCR analyses revealed that all *EPC* genes were commonly and strongly downregulated in both the Δ*tfv1* mutant and the Δ*tfv1* Δ*tvl1* mutant, revealing the essential role of TFV1 for the host-specific and stage-specific expression of all *EPC* genes. Furthermore, yeast one-hybrid assays demonstrated that TFV1 binds directly to the promoter regions of *EPC2*, *EPC3*, and *EPC4* (Figs 4f, S12). Consequently, these findings indicate that the *TFV1*-encoded transcription factor plays a pivotal role in the stage-specific expression of these key virulence effector genes. For further understanding on molecular function of TFV1, it is important to identify the putative binding sequence of TFV1 in the upstream regions of *EPC2*, *EPC3* and *EPC4*.

RT-qPCR analysis revealed that the expression of both *TFV1* and *TVL1* was higher during cucumber infection than *N. benthamiana* infection, which was concordant with the expression of the *EPC* genes, suggesting the idea that host-preferential effector expression can be explained by the host-preferential expression of upstream transcription factors. Recent studies have shown that the overall transcriptome profiles of *C. orbiculare* during infection are similar between cucumber and *N. benthamiana*, with only a small proportion of DEGs (differentially expressed genes) enriched for putative secreted protein genes (Inoue *et al.*, 2023). In this study, only one downregulated effector-like gene in the Δ*tfv1* mutant belonged to the *N. benthamiana*-specific DEGs, whereas 13 downregulated effector-like genes in this mutant belonged to the cucumber-specific DEGs (Table S3), further suggesting that *C. orbiculare* utilizes TFV1 to induce host-specific transcription of effector-like genes on cucurbitaceous plants.

The RNA-Seq data suggested that the expression of *TVL1* was not affected in the Δ*tfv1* mutant (RPKM value = 239.3) compared with WT (RPKM value = 186.7). Thus, we also tried to identify genes that were downregulated in the Δ*tfv1* Δ*tvl1* mutant but not in the Δ*tfv1* mutant compared with WT. Subsequent knockout analysis in the WT background of *C. orbiculare* successfully identified a novel effector gene that is required for virulence on cucurbits but not on *N. benthamiana*; this gene was designated *EPC5* and encodes an effector containing a LysM domain (Fig. 5c,d). Consistent with this, the comparative RNA-Seq and subsequent RT-qPCR analyses revealed preferential expression of *EPC5* on cucumber compared with that on *N. benthamiana* (Fig. S14). By contrast, *ChELP4*, an ortholog of *EPC5*, was shown to be nonessential for the virulence of *C. higginsianum* on *Arabidopsis thaliana* (Takahara *et al.*, 2016). On the other hand, *ChELP2*, encoding a distinct LysM effector, was shown to be involved in the virulence of *C. higginsianum* (Takahara *et al.*, 2016), and interestingly we also found that its ortholog *g_01431* is dispensable for the virulence of *C. orbiculare* (Figs S13, S15). These findings suggest functional diversification of LysM-type fungal effectors, with fungal pathogens likely deploying distinct LysM effectors to establish the infection of each host.

Our study reveals that TFV1 and TVL1 regulate the expression of virulence effector genes (*EPC1–EPC4* and *EPC5*) in *C. orbiculare* on cucurbit hosts. Interestingly, while the *EPC* genes show a common induction pattern at 24 hpi, TFV1 exhibits high-expression levels as early as 4 hpi, whereas *TVL1* is not expressed at 4 hpi and but is highly expressed at 24 hpi. This suggests the possibility that TFV1 activity is temporally regulated in infection-related morphogenesis. Considering the vast number of effector-like genes in phytopathogenic fungi, pinpointing those contributing to virulence is challenging (Tariqjaveed *et al.*, 2021). Our approach involves comparative RNA-Seq analysis on transcription factor mutants defective in fungal virulence, which has proven effective in narrowing down candidate virulence effectors. We also propose that TFV1 and TVL1's preferential expression on cucumber is important for establishing host specificity of *C. orbiculare*. Future research should focus on further understanding the molecular basis of stage-specific and host-specific effector expression by TFV1 and TVL1, as well as

the regulatory system that governs the induced expression of *TFV1* and *TVL1*.

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Competing interests

None declared.

Author contributions

RZ and YT designed the research. RZ, YI, SS-O and TO performed experiments and collected data. RZ and YT analyzed data. RZ, KM, AM and YT wrote the manuscript.

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Data availability

The data that support the findings of this study are available in Figures S1–S15 and Tables S1–S3 of this article.

References

Akai S, Ishida N. 1968. An electron microscopic observation on the germination of conidia of *Colletotrichum lagenarium*. *Mycopathologia* **34**: 337–345.

Avery SV, Singleton I, Magan N, Goldman GH. 2019. The fungal threat to global food security. *Fungal Biology* **123**: 555–557.

Azmi NSA, Singkaravanit-Ogawa S, Ikeda K, Kitakura S, Inoue Y, Narusaka Y, Shirasu K, Kaido M, Mise K, Takano Y. 2018. Inappropriate expression of an NLP effector in *Colletotrichum orbiculare* impairs infection on Cucurbitaceae cultivars via plant recognition of the C-terminal region. *Molecular Plant–Microbe Interactions* **31**: 101–111.

Bozkurt TO, Schornack S, Banfield MJ, Kamoun S. 2012. Oomycetes, effectors, and all that jazz. *Current Opinion in Plant Biology* **15**: 483–492.

Cannon PF, Damm U, Johnston PR, Weir BS. 2012. *Colletotrichum* – current status and future directions. *Studies in Mycology* **73**: 181–213.

Cao Y, Chen J, Xie X, Liu S, Jiang Y, Pei M, Wu Q, Qi P, Du L, Peng B *et al.* 2022. Characterization of two infection-induced transcription factors of *Magnaporthe oryzae* reveals their roles in regulating early infection and effector expression. *Molecular Plant Pathology* **23**: 1200–1213.

Cho Y, Ohm RA, Grigoriev IV, Srivastava A. 2013. Fungal-specific transcription factor *AbPf2* activates pathogenicity in *Alternaria brassicicola*. *The Plant Journal* **75**: 498–514.

Dean R, Van Kan JA, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J *et al.* 2012. The top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology* **13**: 414–430.

Gan P, Ikeda K, Irieda H, Narusaka M, O'Connell RJ, Narusaka Y, Takano Y, Kubo Y, Shirasu K. 2013. Comparative genomic and transcriptomic analyses reveal the hemibiotrophic stage shift of *Colletotrichum* fungi. *New Phytologist* **197**: 1236–1249.

Gan P, Tsushima A, Narusaka M, Narusaka Y, Takano Y, Kubo Y, Shirasu K. 2019. Genome sequence resources for four phytopathogenic fungi from the *Colletotrichum orbiculare* species complex. *Molecular Plant–Microbe Interactions* **32**: 1088–1090.

Inoue Y, Vy TTP, Singkaravanit-Ogawa S, Zhang R, Yamada K, Ogawa T, Ishizuka J, Narusaka Y, Takano Y. 2023. Selective deployment of virulence effectors correlates with host specificity in a fungal plant pathogen. *New Phytologist* **238**: 1578–1592.

Irieda H, Inoue Y, Mori M, Yamada K, Oshikawa Y, Saitoh H, Uemura A, Terauchi R, Kitakura S, Kosaka A *et al.* 2019. Conserved fungal effector suppresses PAMP-triggered immunity by targeting plant immune kinases. *Proceedings of the National Academy of Sciences, USA* **116**: 496–505.

Irieda H, Ogawa S, Takano Y. 2016. Focal effector accumulation in a biotrophic interface at the primary invasion sites of *Colletotrichum orbiculare* in multiple susceptible plants. *Plant Signaling & Behavior* **11**: e1137407.

Jayawardena RS, Hyde KD, de Farias ARG, Bhunjun CS, Ferdinandez HS, Manamgoda DS, Udayanga D, Herath IS, Thambugala KM, Manawasinghe IS *et al.* 2021. What is a species in fungal plant pathogens? *Fungal Diversity* **109**: 239–266.

John E, Singh KB, Oliver RP, Tan KC. 2021. Transcription factor control of virulence in phytopathogenic fungi. *Molecular Plant Pathology* **22**: 858–881.

Kale SD, Tyler BM. 2011. Entry of oomycete and fungal effectors into plant and animal host cells. *Cellular Microbiology* **13**: 1839–1848.

Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. 2019. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology* **37**: 907–915.

Kleemann J, Rincon-Rivera LJ, Takahara H, Neumann U, van Themaat EVL, van der Does HC, Hacquard S, Stuber K, Will I, Schmalenbach W *et al.* 2012. Sequential delivery of host-induced virulence effectors by appressoria and intracellular hyphae of the phytopathogen *Colletotrichum higginsianum*. *PLoS Pathogens* **8**: e1002643.

Kosugi S, Hasebe M, Matsumura N, Takashima H, Miyamoto-Sato E, Tomita M, Yanagawa H. 2009. Six classes of nuclear localization signals specific to different binding grooves of importin α. *Journal of Biological Chemistry* **284**: 478–485.

Kubo Y, Takano Y. 2013. Dynamics of infection-related morphogenesis and pathogenesis in *Colletotrichum orbiculare*. *Journal of General Plant Pathology* **79**: 233–242.

Liao Y, Smyth GK, Shi W. 2019. The R package RSUBREAD is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Research* **47**: e47.

Matsuo H, Ishiga Y, Kubo Y, Yoshioka Y. 2022. *Colletotrichum orbiculare* strains distributed in Japan: race identification and evaluation of virulence to cucurbits. *Breeding Science* **72**: 306–315.

Münch S, Lingner U, Floss DS, Ludwig N, Sauer N, Deising HB. 2008. The hemibiotrophic lifestyle of *Colletotrichum* species. *Journal of Plant Physiology* **165**: 41–51.

O'Connell RJ, Thon MR, Hacquard S, Amyotte SG, Kleemann J, Torres MF, Damm U, Buiate EA, Epstein L, Alkan N *et al.* 2012. Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. *Nature Genetics* **44**: 1060–1065.

Robinson MD, McCarthy DJ, Smyth GK. 2010. EDGER: a BIOCONDUCTOR package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**: 139–140.

Saitoh H, Fujisawa S, Mitsuoka C, Ito A, Hirabuchi A, Ikeda K, Yoshino K, Yoshida K, Matsumura H, Tosa Y *et al.* 2012. Large-scale gene disruption in *Magnaporthe oryzae* identifies MC69, a secreted protein required for infection by monocot and dicot fungal pathogens. *PLoS Pathogens* **8**: e1002711.

Selin C, De Kievit TR, Belmonte MF, Fernando WD. 2016. Elucidating the role of effectors in plant-fungal interactions: progress and challenges. *Frontiers in Microbiology* **7**: 600.

Snelders NC, Kettles GJ, Rudd JJ, Thomma BP. 2018. Plant pathogen effector proteins as manipulators of host microbiomes? *Molecular Plant Pathology* **19**: 257.

Stecher G, Tamura K, Kumar S. 2020. Molecular evolutionary genetics analysis (MEGA) for macOS. *Molecular Biology and Evolution* **37**: 1237–1239.

Sweigard JA, Chumley F, Carroll A, Farrall L, Valent B. 1997. A series of vectors for fungal transformation. *Fungal Genetics Reports* **44**: 19.

Takahara H, Hacquard S, Kombrink A, Hughes HB, Halder V, Robin GP, Hiruma K, Neumann U, Shinya T, Kombrink E *et al.* 2016. *Colletotrichum higginsianum* extracellular LysM proteins play dual roles in appressorial function and suppression of chitin-triggered plant immunity. *New Phytologist* **211**: 1323–1337.

Takano Y, Kikuchi T, Kubo Y, Hamer JE, Mise K, Furusawa I. 2000. The *Colletotrichum lagenarium* MAP kinase gene CMK1 regulates diverse aspects of fungal pathogenesis. *Molecular Plant–Microbe Interactions* **13**: 374–383.

Takano Y, Komeda K, Kojima K, Okuno T. 2001. Proper regulation of cyclic AMP-dependent protein kinase is required for growth, conidiation, and appressorium function in the anthracnose fungus *Colletotrichum lagenarium*. *Molecular Plant–Microbe Interactions* **14**: 1149–1157.

Takano Y, Takayanagi N, Hori H, Ikeuchi Y, Suzuki T, Kimura A, Okuno T. 2006. A gene involved in modifying transfer RNA is required for fungal pathogenicity and stress tolerance of *Colletotrichum lagenarium*. *Molecular Microbiology* **60**: 81–92.

Tariqjaveed M, Mateen A, Wang S, Qiu S, Zheng X, Zhang J, Bhadauria V, Sun W. 2021. Versatile effectors of phytopathogenic fungi target host immunity. *Journal of Integrative Plant Biology* **63**: 1856–1873.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

Yan X, Tang B, Ryder LS, MacLean D, Were VM, Eseola AB, Cruz-Mireles N, Ma W, Foster AJ, Osés-Ruiz M *et al.* 2023. The transcriptional landscape of plant infection by the rice blast fungus *Magnaporthe oryzae* reveals distinct families of temporally co-regulated and structurally conserved effectors. *Plant Cell* **35**: 1360–1385.

Yoshino K, Irieda H, Sugimoto F, Yoshioka H, Okuno T, Takano Y. 2012. Cell death of *Nicotiana benthamiana* is induced by secreted protein NIS1 of *Colletotrichum orbiculare* and is suppressed by a homologue of CgDN3. *Molecular Plant–Microbe Interactions* **25**: 625–636.

Zhang R, Isozumi N, Mori M, Okuta R, Singkaravanit-Ogawa S, Imamura T, Gan P, Shirasu K, Ohki S, Takano Y. 2021. Fungal effector SIB1 of *Colletotrichum orbiculare* has unique structural features and can suppress plant immunity in *Nicotiana benthamiana*. *Journal of Biological Chemistry* **297**: 101370.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Disruption of target genes.

Fig. S2 Virulence assay of knockout mutants on cucumber cotyledons or *Nicotiana benthamiana* leaves.

Fig. S3 Disruption of *TFV1* in *Colletotrichum orbiculare* and complementation of *TFV1* in this single knockout mutant.

Fig. S4 TFV1 is highly conserved in two fungi of the *Colletotrichum orbiculare* species complex.

Fig. S5 Alignment analysis of TFV1, TVL1, TVL2, and TVL3.

Fig. S6 Phylogenetic tree of *TFV1* and its homologs in *Colletotrichum orbiculare*.

Fig. S7 Virulence assay of *TVL1* single knockout mutant on different hosts.

Fig. S8 Disruption of *TVL1* in *TFV1* single knockout mutant.

Fig. S9 Quantified lesion size in virulence assays on host plants.

Fig. S10 Quantified lesion size of WT and Δ*tfv1* Δ*tvl1* mutant on *Nicotiana benthamiana*.

Fig. S11 Prediction of nuclear localization signals and detection of transcriptional activation activity of TFV1 and TVL1.

Fig. S12 Original and extended transformation results of the yeast one-hybrid assay.

Fig. S13 Characterization of LysM effector genes in two *Colletotrichum* species.

Fig. S14 Expression pattern of *EPC5* on cucumber and *Nicotiana benthamiana*.

Fig. S15 Pathogenicity test of the *g_01431* knockout mutant on cucumber and *Nicotiana benthamiana*.

Table S1 List of primers used in this study.

Table S2 Summary of RNA-Seq reads and mapping statistics.

Table S3 List of downregulated genes shown in this study.

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