The basal transcription factor II H subunit Tfb5 is required for stress response and pathogenicity in the tangerine pathotype of Alternaria alternata

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Abstract
The basal transcription factor II H (TFIIH) is a multicomponent complex. In the present study, we characterized a TFIIH subunit Tfb5 by analysing loss- and gain-of-function mutants to gain a better understanding of the molecular mechanisms underlying stress resistance and pathogenicity in the citrus fungal pathogen Alternaria alternata. Tfb5 deficiency mutants (ΔAatfb5) decreased sporulation and pigmentation, and were impaired in the maintenance of colony surface hydrophobicity and cell wall integrity. ΔAatfb5 increased sensitivity to ultraviolet light, DNA-damaging agents, and oxidants. The expression of Aatfb5 was up-regulated in the wild type upon infection in citrus leaves, implicating the requirement of Aatfb5 in fungal pathogenesis. Biochemical and virulence assays revealed that ΔAatfb5 was defective in toxin production and cell wall-degrading enzymes, and failed to induce necrotic lesions on detached citrus leaves. Aatfb5 fused with green fluorescent protein (GFP) was localized in the cytoplasm and nucleus and physically interacted with another subunit, Tfb2, based on yeast two-hybrid and co-immunoprecipitation analyses. Transcriptome and Antibiotics & Secondary Metabolite Analysis Shell (antiSMASH) analyses revealed the positive and negative roles of Aatfb5 in the production of various secondary metabolites and in the regulation of many metabolic and biosynthetic processes in A. alternata. Aatfb5 may play a negative role in oxidative phosphorylation and a positive role in peroxisome biosynthesis. Two cutinase-coding genes (AaCut2 and AaCut15) required for full virulence were down-regulated in ΔAatfb5. Overall, this study expands our understanding of how A. alternata uses the basal transcription factor to deal with stress and achieve successful infection in the plant host.

KEYWORDS
ACT toxin, Alternaria alternata, cutinase, DNA damage, oxidative stress, TFIIH, virulence
INTRODUCTION

The basal transcription factor II H (TFIHI) is a protein complex commonly found in different kingdoms, including animals, yeasts, plants, and protists (Compe and Egly, 2016; Rimel and Taatjes, 2018). The TFIHI complex consists of 10 subunits, which are grouped into two subcomplexes: a core TFIHI with an assembly of seven subunits including SsI2, Rad3, Tfb1, Tfb2, SsI1, Tfb4, and Tfb5, and a cyclin-dependent kinase-activating kinase (CAK) subcomplex comprising Tfb3, CcI1, and Kin28 (Kainov et al., 2010). The TFIHI complex regulates a wide array of cellular processes, including nucleotide excision repair (NER), cell cycle regulation, E3 ubiquitin ligase activity, cullin neddylation, and chromosome segregation (Compe and Egly, 2016).

In humans, mutations in TFIHI subunits decrease the ability of NER, causing severe developmental defects such as Cockayne syndrome (CS), xeroderma pigmentosum (XP), and trichothiodystrophy (TTD) (Lehmann, 2003). The TFIHI machinery stabilizes the genome by removing DNA damage induced by ultraviolet (UV) light or chemicals (Hoeijmakers, 2001). There are two types of NER: global genome NER and transcription-coupled NER (TC-NER) (Ding et al., 2007). The TC-NER is activated when RNA polymerase encounters DNA damage, and TFIHI is one of the important components of the RNA polymerase II complex (Ding et al., 2007; Rimel and Taatjes, 2018).

The tfb5 gene, originally identified in yeasts, encodes a small peptide that is highly conserved throughout evolution (Ranish et al., 2004). Tfb5 interacts with other subunits to stabilize and ensure the proper function of the entire TFIHI complex (Kainov et al., 2010). In yeasts, Tfb5 forms a heterodimer with Tfb2, both of which share similar structures (α/β split with C-terminal helix) (Theil et al., 2014; Compe and Egly, 2016). Interaction between Tfb5 and Tfb2 has been confirmed in yeasts and human cells (Zhou et al., 2007; Nonnekens et al., 2013). Studies on the function of Tfb5 in Drosophila and mouse systems have revealed similar roles in cell development and DNA damage responses (Aguilar-Fuentes et al., 2008; Theil et al., 2013). In yeasts, Tfb5 is required for growth and UV irradiation repair and maintenance of the stability of the TFIHI complex (Zhou et al., 2007). In the rice blast fungus Magnaporthe oryzae, tfb5 is required for conidiation and redox resistance, and its expression is down-regulated in a circadian-Twilight (twl) mutant (Deng et al., 2015). Despite the pioneering work in other species, the function of Tfb5 in Alternaria alternata remains unclear.

The necrotrophic fungal pathogen A. alternata is capable of infecting many economically important crops and resulting in yield losses. Some strains of A. alternata produce mycotoxins causing food contamination. Several pathotypes of A. alternata produce the host-selective toxin (HST) that acts as a pathogenicity factor to kill host cells before colonization (Tsuge et al., 2013; Meena et al., 2017). Alternaria brown spot of citrus, caused by the tangerine pathotype of A. alternata, is one of the important diseases on citrus worldwide. This disease mainly affects tangerines, grapefruit, and their hybrids (Akimitsu et al., 2003). The pathogen initiates its infection by secreting an ACT (A. alternata f. sp. citri tangerine) toxin, which shares a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid with that of AK (A. alternata f. sp. kikuchana) and AF (A. alternata f. sp. fragariae) toxin produced by the Japanese pear and strawberry pathotypes of A. alternata, respectively (Kohmoto, 1993; Imazaki et al., 2010). Genes encoding polypeptides involved in the biosynthesis of ACT toxin are clustered and located in a small, conditionally dispensable chromosome (Akamatsu et al., 1999; Ajiro et al., 2010; Tsuge et al., 2013).

In addition to ACT toxin, the ability to produce cell wall-degrading enzymes (CWDEs) is important for successful penetration and colonization of A. alternata in citrus. CWDEs play important roles in the virulence of some plant pathogenic fungi as they rely on CWDEs to degrade plant cell walls (Carvalho et al., 1999). Cutinase has recently been demonstrated to be required for full virulence in the tangerine pathotype of A. alternata (Ma et al., 2019). A fungal strain deleted at both cutinase-encoding genes (Aacut3 and Aacut7) has decreased cutinase activity and reduced virulence on unwounded citrus leaves. Moreover, A. alternata has to cope with toxic reactive oxygen species (ROS) in order to gain successful colonization in citrus leaves. Several proteins, including the NADPH oxidase (Yang and Chung, 2013), the redox-responsive transcription factor Yap1 (Lin et al., 2009), the mitogen-activated protein (MAP) kinase Hog1 (Lin and Chung, 2010), the stress response regulator Skn7 (Chen et al., 2012), and proteins in the thioredoxin and glutaredoxin systems (Ma et al., 2018), have been demonstrated to be required for resistance to ROS and full virulence of A. alternata on citrus. It has been hypothesized that H$_2$O$_2$ generated by the NADPH oxidase complex acts as a secondary messenger to activate the expression of Yap1 and Hog1, which activate both thioredoxin and glutaredoxin systems for ROS detoxification (Ma et al., 2018).

The filamentous phytopathogenic fungus A. alternata has a different lifestyle, gene regulation complexity, and metabolic diversity from yeasts. In this study, we explore the function of a Tfb5 ortholog in A. alternata. Our results demonstrate that A. alternata tfb5 (Aatfb5) is required for resistance to DNA-damaging and oxidative stress, maintenance of surface hydrophobicity, and cell wall integrity, as well as full virulence.

RESULTS

2.1 Identification and deletion of Aatfb5

The sequence of the tfb5 gene (accession number MT184174) in the tangerine pathotype of A. alternata Z7 (Aatfb5) was obtained from its genome data (GCA 001572055.1). The Aatfb5 gene encodes a polypeptide sharing 37% amino acid identity with that of the Saccharomyces cerevisiae tfb5 gene (Scfb5; KZV12316.1). Phylogenetic and domain analyses confirmed the orthology of Aatfb5 with other tfb5 genes (Figure S1a). Sequence alignment between Aatfb5 and Scfb5 indicated that both proteins have characteristic stretches of hydrophobic residues (Figure S1b). The Aatfb5 gene was found to contain a 326-bp open reading frame (ORF) interrupted by two small introns of 47 and 60 bp, which can be translated into a protein of 72 amino acids.
To explore the function of Aatfb5, we generated deletion mutants using the homology recombination strategy (Figure S1c). Candidate mutants were identified by PCR and successful disruption of Aatfb5 in two mutants (designated ΔAatfb5-1 and ΔAatfb5-4) was further confirmed by Southern blotting (Figure S1d,e). Because the two disrupted mutants were similar in terms of radial growth, sporulation, and pathogenicity, ΔAatfb5-1 was used in the study. A complementation strain designated Aatfb5-c was generated by transferring a functional copy of Aatfb5 into protoplasts prepared from ΔAatfb5. The complementation strain was also examined by PCR and the presence of the full-length Aatfb5 gene in the Aatfb5-c mutant was confirmed by Southern blotting (Figure S1d,e).

2.2 | Aatfb5 is involved in fungal development

Deletion of Aatfb5 reduced fungal radial growth by 23% on minimal medium (MM) but maintained wild-type growth on potato dextrose agar (PDA; Figure 1a). When grown on V8 juice medium, conidial production was reduced in ΔAatfb5 by c.50% compared with the wild type at 8 days of incubation. Sporulation was restored in the Aatfb5-c strain (Figure 1b). Germination rates of spores from the wild type, ΔAatfb5, and Aatfb5-c strains were similar (Figure 1c).

2.3 | Aatfb5 is involved in melanin synthesis and surface hydrophobicity

ΔAatfb5 reduced pigmentation dramatically when cultured on PDA or in potato dextrose broth (PDB; Figure 1d). Quantification of melanin revealed that ΔAatfb5 produced only c.33% melanin compared to the wild type and the Aatfb5-c strain (Figure 1e). Colony surface hydrophobicity assays showed that water drops could remain on the surface of mycelium of the wild type for more than 12 hr (Figure 1f). A solution containing sodium dodecyl sulphate (SDS) and ethylenediaminetetraacetic acid (EDTA) remained on the surface of the wild-type colony for about 30 min before soaking into the mycelium. In contrast, ΔAatfb5 mycelium failed to retain water as effectively as the wild type, producing wet spots on the colony surface, particularly in the presence of SDS and EDTA. Aatfb5-c displayed a similar ability to retain water drops on the colony surface to the wild type.

2.4 | Aatfb5 is required for resistance to DNA-damaging, oxidizing, and cell wall-interfering agents

As mentioned above, spores of ΔAatfb5 germinated as effectively as those of the wild type. On exposure to UV light (254 nm, 100 J/m²) for 15 s, spores produced by ΔAatfb5 germinated at rates slower than those of the wild type, reducing by 10%. Aatfb5-c spores germinated at rates comparable to those of wild type (Figure 2a). Chemical sensitivity assays on MM revealed that ΔAatfb5 increased sensitivity to DNA-damaging agents methanesulphonate (MMS, 0.02%), hydroxyurea (HU, 20 mM), and cisplatin (CDDP, 0.1 mM). Aatfb5-c displayed wild-type sensitivity to DNA-damaging agents (Figures 2b and S2a).

Compared with the wild type, ΔAatfb5 increased sensitivity to hydrogen peroxide (H₂O₂, 10 mM), diethyl maleate (DEM, 0.05%), cumyl hydroperoxide (CHP, 0.01%), menadione (VK3, 2 mM), and tert-butyl-hydroxyperoxide (T-BHP, 0.05%) (Figures 2c and S2b). Aatfb5-c showed wild-type resistance to those compounds.

As assayed on MM, ΔAatfb5 significantly increased sensitivity to sodium dodecyl sulphate (SDS, 100 µg/ml), Congo red (CR, 100 µg/ml), and calcofluor white (CFW, 200 µg/ml) compared to the wild type. The complementation mutant Aatfb5-c displayed wild-type sensitivity to cell wall-interfering agents (Figures 2d and S2c), indicating that Aatfb5 is involved in the maintenance of cell wall integrity.

2.5 | Aatfb5 is required for full virulence

Quantitative RT-PCR (RT-qPCR) analysis revealed that the expression level of Aatfb5 in the wild type increased by as much as 20-fold 3 hr postinoculation (hpi) and by 30-fold 6 hpi, then gradually decreased 9 hpi (Figure 3a), implying the involvement of Aatfb5 in pathogenesis. Pathogenicity tests using point inoculation by placing 10 µl spore suspensions (10⁴ spores/ml) on the surface of citrus leaves revealed that ΔAatfb5 failed to induce necrotic lesions 2 days postinoculation (dpi) on unwounded Hongjiv leaves, while both the wild type and Aatfb5-c induced necrotic lesions (Figure 3b). When citrus leaves were wounded before inoculation, ΔAatfb5-induced necrotic lesions, but the sizes of lesions were much smaller than those induced by the wild type (Figure 3c). Pathogenicity tests using spray inoculation of spore suspension also revealed that ΔAatfb5 failed to induce lesions on detached citrus leaves (Figure 3d).

2.6 | Aatfb5 is required for ACT toxin biosynthesis

Fungal strains (wild type and ΔAatfb5) were cultured in Richard's medium for 24 days for ACT toxin production. Cell-free culture filtrates were collected by passing through cheesecloth and a 0.45 µm filter, and used for bioassays on detached Hongjiv leaves that were wounded before inoculation. The results reveal that culture filtrates collected from ΔAatfb5 resulted in smaller lesions compared to those induced by culture filtrates of the wild type (Figure 3e). HPLC analysis of culture filtrates confirmed further that ΔAatfb5 produced a smaller quantity of ACT toxin than the wild type (Figures 3f and S3).

2.7 | Deletion of Aatfb5 affects cutinase activity

To test if Aatfb5 is involved in cell wall-degrading enzyme activities, fungal strains (27, ΔAatfb5, and Aatfb5-c) were cultured in modified Czapek's medium amended with inducers for 24 hr. Culture filtrates were mixed with p-nitrobenzoic acid (for cutinase activities)
or dinitrosalicylic acid (for cellulases) containing reagents and measured using a spectrophotometer. The results indicated that \( \Delta Aatfb5 \) produced significantly lower amounts of cutinase than the wild type and \( Aatfb5-c \) (Figure 4a). \( \Delta Aatfb5 \) produced slightly lower amounts of cellulase than the wild type and \( Aatfb5-c \) (Figure 4b).

### 2.8 Subcellular localization and interaction pattern of Aatfb5

A functional Aatfb5 was fused with green fluorescent protein (GFP) and expressed under control of its endogenous promoter in \( \Delta Aatfb5 \),
revealing a wide distribution of green fluorescence in the cytoplasm and nucleus except vacuoles (Figure 5a).

Given that tfb5 has been found to interact with the TFIIH complex subunit tfb2 in *S. cerevisiae* (Zhou et al., 2007), we conducted yeast two-hybrid (Y2H) and co-immunoprecipitation (Co-IP) assays to examine whether or not Aatfb5 interacts with Aatfb2 (accession number XM 018525809) in *A. alternata*. Y2H assays revealed that Aatfb5 interacted with Aatfb2 (Figures 5b and S4). The interaction of Aatfb5 with Aatfb2 was further confirmed by Co-IP assays. Both Aatfb2-FLAG and Aatfb5-GFP plasmids were cotransformed into ΔAatfb5, and the resulting strain was used for Co-IP assay. The results indicated that Aatfb5 proteins, after being incubated with the anti-FLAG agarose and eluted, could...
FIGURE 3  Tfb5 is required for Alternaria alternata pathogenesis to citrus leaves. (a) Expression of Aatfb5 in the wild-type strain inoculated to detached citrus Hongjv leaves. RNA was purified every 3 hr postinoculation (hpi), and used for cDNA synthesis and quantitative reverse transcription PCR analysis. (b) Fungal pathogenicity was assessed on detached Hongjv leaves (unwounded) inoculated by placing 10 µl of conidial suspensions (10^6 conidia/ml) prepared from wild type (Z7), ΔAatfb5, and Aatfb5-c on each spot. Spots treated with water were used as mock controls. (c) Fungal pathogenicity was assessed on detached Hongjv leaves that were wounded with a fine needle before inoculation. Leaves were inoculated with 10 µl of conidial suspensions (10^6 conidia/ml) prepared from Z7, ΔAatfb5, and Aatfb5-c on each spot. (d) Fungal pathogenicity was assessed by uniformly spraying conidial suspensions onto detached Hongjv leaves. Inoculated leaves were kept in a plastic box for 3 days for lesion development. (e) A leaf necrosis assay for the toxicity of ACT toxin by placing cell-free culture filtrates from the wild type or ΔAatfb5 on Hongjv leaves that were wounded with a fine needle before treatment. (f) HPLC analysis of ACT toxin purified from culture filtrates of Z7 and ΔAatfb5. ACT toxin is indicated by an arrow.
Transcriptomic analysis was performed to compare the whole-genome expression profiles between ΔAatfb5 and the wild type. Illumina libraries were constructed from ΔAatfb5 and the wild type and sequenced using the HiSeq platform. In total, 5,473 differentially expressed genes (DEGs) consisting of 2,673 up-regulated and 2,800 down-regulated genes were identified in ΔAatfb5 compared to the wild type (Figure S5a). Expression of the genes associated with a conditionally dispensable chromosome was decreased in ΔAatfb5 (Figure S5b). Gene ontology (GO) and KEGG pathway enrichment analyses revealed that Aatfb5 is probably involved in the regulation of many metabolic and biosynthetic processes, and may play a role in oxidative phosphorylation, the tricarboxylic acid (TCA) cycle, and the pentose phosphate pathway (Figure S5c,d). In addition, Aatfb5 may also regulate the thioredoxin and glutaredoxin system because expression of the genes AaTrr1 (AAL_g7769), AaTsa1 (AAL_g1812), AaGlr1 (AALT_g2809), and AaGpx3 (AALT_g41) was down-regulated in ΔAatfb5 (Figure 6b).

Biosynthesis gene clusters predicted by antiSMASH 4.0 revealed that Aatfb5 regulates a broad range of gene clusters involved in the biosynthesis of secondary metabolites (Figure 6a and Table S2). Significantly, several genes in cluster 6 were up-regulated and many genes in clusters 2 (alternariol) and 28 were down-regulated. Some genes in cluster 3 (melanin) and cluster 29 (ACT toxin) were also down-regulated in ΔAatfb5.

The expression profiles of some genes observed in transcriptome analysis were validated by RT-qPCR. The expression levels of five genes associated with oxidative phosphorylation (AAL_g5498, AAL_g4417, AAL_g1835, AAL_g4688, and AAL_g4321) were up-regulated and four genes associated with peroxisome including three catalases (AAL_g1664, AAL_g1666, and AAL_g1523) and a peroxin 24 (Aapex24) were down-regulated in ΔAatfb5 consistent with RNA-Seq (Figure 6b). RT-qPCR analysis also confirmed the expression profiles of genes associated with secondary metabolites. RT-qPCR analysis revealed the down-regulation of the AapksA coding genes involved in the biosynthesis of melanin in ΔAatfb5 (Figure 6b). Two genes (ACTTS3 and a metalloprotease-coding gene) located in the biosynthesis of ACT toxin cluster were down-regulated in ΔAatfb5 (Figure 6b).

Transcriptome analysis revealed that the expression of two cutinase-coding genes, Aacut2 (AAL_g5432) and Aacut15 (AAL_g7939), was significantly decreased in ΔAatfb5 (Figure 6b). To determine if Aacut2 and Aacut15 are involved in virulence in A. alternata, the genes were independently or simultaneously deleted in the genome of Z7 (Figure S6a,b). Fungal strains defective in Aacut2 or Aacut15 (single gene mutation) or both Aacut2 and Aacut15 (double mutation) displayed wild-type growth, conidiation, and ACT toxin production (Figures 7a,c, S3, and S6c). Assays for cutinase activity indicated that the double mutation strain produced much lower cutinase activity than the wild-type strain, while deletion of Aacut2 or Aacut15 alone had little or no effect on cutinase (Figure 7b). Pathogenicity tests revealed that single gene mutation strains ΔAacut2 and ΔAacut15 induced necrotic lesions at sizes slightly but significantly smaller than those induced by Z7 as assayed on unwounded Hongjv leaves. Necrotic lesions induced by the double mutation strain ΔAacut2ΔAacut15 were significantly smaller than those induced by Z7 at 2 dpi (Figure 7d).

3 | DISCUSSION

The NER machinery recognizes and removes damage in the DNA double helix in all cells (Hoeijmakers, 2001). Tfb5 is a highly conserved protein involved in cell processes, transcription, and DNA repair in eukaryotes. While Tfb5 is not essential in A. alternata, tfb5 deficiency
leads to embryonic lethality in mouse models (Theil et al., 2013). In this study, we characterized a yeast tfb5 homologue in the tangerine pathotype of A. alternata. Gain- and loss-of-function studies revealed that Aatfb5 is required for growth, conidiation, pathogenicity, maintenance of hyphal surface hydrophobicity, biosynthesis of secondary metabolites, cutinase production, and stress resistance in A. alternata. ΔAatfb5 reduces growth on MM and shows wild-type growth in PDB, suggesting that Aatfb5 is also involved in micronutrient metabolism.

FIGURE 5 Localization and interaction of Aatfb5 in Alternaria alternata. (a) Aatfb5-GFP is localized in the nucleus and cytoplasm but excluded from vacuoles. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). Fungal vacuoles were stained with 7-amino-4-chloromethylcoumarin (CMAC). Scale bars, 5 µm. (b) Yeast two-hybrid analysis reveals the interaction between Aatfb5 and Aatfb2. Serial dilutions of yeast cells (cells/ml) transferred with the bait and prey constructs indicated in the figure were assayed for growth on SD/-Ade/-His/-Leu/-Trp plates. The pGBK7-T7-53 and pGADT7 pair was used as a positive control and the pGBK7-LAM and pGADT7 pair was used as a negative control. (c) Co-immunoprecipitation analysis of Aatfb5-GFP and Aatfb2-FLAG in A. alternata.
FIGURE 6  Transcriptome analysis unravels the global regulatory role of Aatfb5. (a) Differential expression of gene clusters associated with the biosynthesis of secondary metabolites in the ΔAatfb5 mutant. pks, polyketide synthase; npr, non-ribosomal peptide synthetase; t1, type 1; t3, type 3; terpene, terpene synthetase. (b) Relative fold change of 31 genes involved in the oxidative phosphorylation, catalase, peroxin 24, biosynthesis of secondary metabolites, cutinase, and thiorredoxin and glutaredoxin systems in ΔAatfb5. The relative transcript levels of target genes were analysed by RNA-Seq (p < .05, log₂FoldChange > 1). The β-actin coding gene was used as the reference gene. The relative expression level of a gene in ΔAatfb5 was determined using a comparative Cₜ method in relation to that of the wild type.
FIGURE 7  Characteristics of Aacut2 and Aacut15 deficiency mutants.  
(a) Colonies of Alternaria alternata strains grown on potato dextrose agar.  
(b) Quantification of cutinase activity in wild type (Z7), the Cut2 mutant strain (ΔAacut2), the Cut15 mutant strain (ΔAacut15), and the Cut2Cut15 double mutant strain (ΔAacut2ΔAacut15). Means indicated by the same letter are not significantly different from one another, $p < .05$.  
(c) HPLC analysis of ACT toxin purified from culture filtrates of Z7, ΔAacut2, ΔAacut15, and ΔAacut2ΔAacut15. ACT toxin is indicated by an arrow.  
(d) Virulence assays on citrus. Hongjiv leaves were inoculated by placing 10 µl of conidial suspensions ($10^6$ conidia/ml) prepared from Z7 and the ΔAacut2, ΔAacut15, and ΔAacut2ΔAacut15.
Deletion of Aatfb5 apparently affects the formation of conidia in A. alternata. This observation is consistent with a previous report in the rice blast fungus M. oryzae (Deng et al., 2015). In M. oryzae, a circadian-regulated Twilight (TBL) gene is required for proper conidiation and pathogenesis. The expression of Motfb5 is significantly down-regulated in the Δtwl mutant, while overexpression of the Motfb5 gene restores a sporulation defect in the Δtwl mutant and increases sporulation in the wild-type strain. Furthermore, Tfb5-GFP fails to translocate to the nucleus in the Δtwl, indicating that Motfb5 is directly regulated by MoTBL (Deng et al., 2015). Conidia play an important role in the disease cycle of A. alternata. The formation of conidia is a complex process. In A. alternata, studies have identified several proteins that are involved in conidiation. These include the mitogen-activated protein kinases FUS3 (Lin et al., 2010) and SLT2 (Yago et al., 2011), the G-protein (Wang et al., 2010), the cAMP-dependent protein kinase (Tsai et al., 2013), the calcium-mediated signalling pathways (Tsai and Chung, 2014), the NADPH oxidase complex (Yang and Chung, 2013), and the COP9 signalosome (Wang et al., 2018). However, transcriptome analysis revealed that none of these genes showed significant changes at the transcriptional level in ΔAatfb5 (Table S3). Moreover, expression of several genes (G protein-mediated signalling pathways genes, FluG-mediated genes, BriA, AbaA and WetA central regulatory genes, and stuA-coding genes) required for conidiation in other filamentous fungi (Park and Chung, 2010) were not significantly different between ΔAatfb5 and the wild-type (Table S3). The results indicate that Aatfb5 may regulate conidiation via a novel yet unidentified pathway in A. alternata. Alternatively, Aatfb5 may not regulate conidiation via transcriptional activation.

TFIIH is a pivotal part in NER, a DNA-repair mechanism that removes DNA lesions caused by various environmental factors (Aguilar-Fuentes et al., 2008). S. cerevisiae tfb5 mutant cells are moderately sensitive to UV light (Zhou et al., 2007). Mouse embryonic cells lacking Tfb5 are highly sensitive to UV light (Theil et al., 2013). Sensitivity assays revealed that impairment of Aatfb5 increased sensitivity to UV light in A. alternata, consistent with findings in yeasts and mouse cells (Theil et al., 2013, 2014). In addition, the Aatfb5 deficiency strains showed an increased sensitivity to methyl methanesulphonate, hydroxyurea, and cisplatin. Re-expression of a copy of functional Aatfb5 in ΔAatfb5 restored its sensitivity to wild-type level, indicating that Aatfb5 plays a role in protection from DNA damage in the plant pathogenic fungus A. alternata.

Melanin has many functions in fungi (Toledo et al., 2017). In M. oryzae and Colletotrichum spp., melanin is essential for the formation of turgor pressure in appressoria, which are absolutely required for penetration of plant cuticles (Babitskaya et al., 2000). In A. alternata, melanin has been shown to be required for protection against UV light, but it is not required for pathogenicity (Kawamura et al., 1999; Ramona Fetzner et al., 2014). Several genes, including pksA, brm2, and CmrA, have been identified to be required for the biosynthesis of DHN melanin in A. alternata (Kimura and Tsuge, 1993; Tseng et al., 2011; Ramona Fetzner et al., 2014). The brm2 deletion mutant of the Japanese pear pathotype of A. alternata is unable to produce melanin, is hypersensitive to UV light, but displays wild-type virulence (Kawamura et al., 1999). In the current study, we found that deletion of Aatfb5 resulted in a significant decrease of melanin in ΔAatfb5, and conidia produced by ΔAatfb5 were hypersensitive to UV light. RNA-Seq and RT-qPCR analyses also revealed that the expression of pksA (AALT_g6058), a polyketide synthase-coding gene that is required for pigment synthesis, was down-regulated in ΔAatfb5. The results confirm further the involvement of Aatfb5 in the production of melanin.

In addition to resistance to DNA-damaging agents, Aatfb5 was required for resistance to several ROS-generating compounds such as hydrogen peroxide (H₂O₂), diethyl maleate (DEM), cumyl hydroperoxide (CHP), menadione (VK3), and tert-butyl-hydroperoxide (TBP) in A. alternata. Similar results have been reported in the t fb5 orthologues in mouse (Theil et al., 2013) and M. oryzae (Deng et al., 2015). The mechanisms underlying ROS resistance have been studied in A. alternata. Previous research has suggested that the NADPH oxidase complex generates low-level H₂O₂ that acts as a secondary signal to promote the nuclear translocation of Yap1 and Hog1, and to activate the expression of the genes encoding Yap1, Skn7, and Hog1, which in turn activate the thioredoxin (Aatsa1 and Aatrr1) and glutaredoxin (AaGpx3 and Aaglr1) systems in response to oxidative damage (Ma et al., 2018). In the present study, RT-qPCR revealed that the expression of Aatr1, Aagpx3, and Aaglr1 was significantly down-regulated in ΔAatfb5. These results indicate that the involvement of Aatfb5 in resistance to toxic oxidants is probably mediated via the regulation of the thioredoxin and glutaredoxin systems in A. alternata.

Pathogenicity assays performed on Hongjv leaves revealed that ΔAatfb5 failed to induce necrotic lesions on unwounded citrus leaves, indicating that Aatfb5 plays a profound role in A. alternata pathogenesis. This impairment may be largely due to the fact that ΔAatfb5 accumulates less ACT toxin than the wild type. The present study indicates that Aatfb5 regulates the biosynthesis of secondary metabolites. The ability to produce ACT toxin has been demonstrated to be crucial for pathogenicity in the tangerine pathotype of A. alternata (Musakana et al., 2005). The involvement of Aatfb5 in the biosynthesis of ACT is confirmed further based on the reduced expression of the genes (metalloprotease-encoding gene and ACTTS3) involved in the biosynthesis of ACT toxin in ΔAatfb5.

Because ΔAatfb5 incites necrotic lesions significantly smaller than the wild type on detached leaves that are wounded before inoculation, it appears that ΔAatfb5 is impaired for penetration. This could be due to a decreased activity of cell wall-degrading enzymes. Enzymatic assays revealed that Aatfb5 is required for cutinase production. Cutinase is a hydrolytic enzyme that has been shown to play an important role in virulence in many plant pathogenic fungi (Carvalho et al., 1999), such as Curvularia lunata (Liu et al., 2016), Fusarium solani (Rogers et al., 1994), and Pyrenopeziza brassicae (K. A. Davies, 2000). Disruption of CITUT7 in C. lunata leads to a significant decrease in virulence only on unwounded maize leaves (Liu et al., 2016). In the present study, RNA-Seq and RT-qPCR revealed that the expression of two cutinase-coding genes, Aacut2
A. alternata cutinase is required for full virulence in the tangerine pathotype of A. alternata. These results demonstrate that Aatfb5 is required for the expression of the cutinase-coding genes and virulence in A. alternata. These data verify our recent conclusion that cutinase is required for full virulence in the tangerine pathotype of A. alternata (Ma et al., 2019).

In the budding yeast, tfb5 interacts with tfb2 to stabilize the architecture of TFIIH and the interaction is important for the function of NER (Zhou et al., 2007). In living cells, the split-GFP system has shown that tfb5 interacts with tfb2, both of which are then incorporated into TFIIH (Nonneken et al., 2013). In the current study, we have verified the interaction of Aatfb5 with Aatfb2 by Y2H and Co-IP assays in A. alternata, indicating that a physical interaction between tfb5 and tfb2 plays an important role in the function of TFIIH. After multiple attempts, we were unable to obtain Aatfb2-deficient mutants, suggesting that Aatfb2 is essential for cell viability in A. alternata.

Peroxisomes carry out different functions in fungi, including metabolic activities, antioxidant defences, and pathogenicity (Pieuchoot and Jedd, 2012). Proxins encoded by the Pex genes are required for the biogenesis of peroxisomes. The expression of Aapex24 (AALT_g8342), a yeast Pex24 homologue, is down-regulated in ΔAatfb5. Pex24 is a peroxisome membrane protein, which is essential for peroxisome assembly in yeasts (Tam et al., 2002). Peroxisomes contain abundant catalasates, which are involved in scavenging ROS (Taheri and Kakooee, 2017). In the present study, the transcription levels of the genes encoding catalasates (AALT_g1664, AALT_g1666, and AALT_g1523) were significantly down-regulated in ΔAatfb5. Whether or not Aatfb5 is required for the formation of peroxisomes and catalase activity in A. alternata warrants further investigation.

In conclusion, we have demonstrated that the basal transcription factor II H subunit-coding gene tfb5 is involved in conidiation, pathogenicity, and resistance to DNA-damaging and oxidative stress in A. alternata. In addition, Aatfb5 affects the biosynthesis of secondary metabolites and cutinase by regulating the expression of the biosynthetic genes. Our research has established, for the first time, the biological functions of tfb5 in plant pathogenic fungi.

4 | EXPERIMENTAL PROCEDURES

4.1 | Fungal strain and culture conditions

The wild-type strain Z7 (CGMCC3.18907) of A. alternata, originally isolated from a diseased citrus leaf in Zhejiang, China (Huang et al., 2015; Wang et al., 2016), was used as a parent strain for mutagenesis. Unless otherwise indicated, the fungus was grown on PDA or PDB on a shaker set at 160 rpm at 26°C. Conidia were collected from fungal cultures incubated under fluorescent light for 7 days. All fungal strains used in this study were preserved in 15% glycerol solutions stored at −80°C.

4.2 | Targeted gene disruption and complementation

The deletion mutants were created using previously described protocols (Wang et al., 2018). Briefly, a double joint PCR was used to generate three hybrid DNA fragments (Figure S1). An upstream fragment (540 bp) and a downstream fragment (484 bp) of the Aatfb5 gene were independently amplified from the Z7 genome and fused with a bacterial phosphotransferase B gene (HPH). The fused fragments were transformed into protoplasts prepared from Z7 with polyethylene glycol and CaCl2 (Lin and Chung, 2010). Putative transformants were picked from PDA containing 100 μg/ml hygromycin, examined by PCR with specific primers, and confirmed further by Southern blotting assay. For genetic complementation, a full-length Aatfb5 gene including its promoter sequences (1,085 bp) and 3′-UTR sequences (323 bp) was amplified and cloned into the p1300-NEO plasmid (Wang et al., 2015). The resultant plasmid was then introduced into the protoplasts prepared from a ΔAatfb5 mutant. Transformants were picked from PDA containing 100 μg/ml G418, verified by PCR, and confirmed further by Southern blotting. The upstream (957 bp) and downstream (519 bp) fragments of the Aacet2 gene were amplified and fused with a neomycin resistance gene (NEO). The upstream (723 bp) and downstream fragments (616 bp) of the Aacut15 gene were amplified and fused with HPH. The fused fragments were independently transformed into protoplasts prepared from Z7 and putative transformants were picked from PDA containing 100 μg/ml G418 or hygromycin. For double deletion mutants, the Aacet2 fused fragments were transformed into protoplasts prepared from ΔAacet15. Putative transformants were picked from PDA containing 100 μg/ml hygromycin and G418, and examined by PCR with specific primers. All primers used in this study are listed in Table S1.

4.3 | Phenotypic analysis

Sporulation was assessed by culturing fungal strains on V8 juice agar for 7 days. The spore germination assay was performed by spreading 100 μl spore suspensions (10^5 spores/ml) on water agar (WA) plate, incubated at 26°C for 12 hr, and examined under a microscope (n = 300). Stress tolerance was assayed by transferring mycelium plugs onto minimal medium (MM) (Chung, 2003) amended with DNA-damaging agents, oxidants, cell wall-interfering agents, or other indicated chemicals at 26°C for 4 days. The growth inhibition rate was calculated by dividing the colony diameter of each strain grown on medium amended with or without a chemical treatment.

4.4 | Melanin extraction and measurement

Fungal mycelia cultured in PDB for 7 days were collected after filtration through a filter paper. Pigments were extracted from the filtrated mycelia (0.5 g) after being treated with 10 ml 1 M NaOH.
and boiled at 100°C for 5 hr. The solution containing boiled mycelia was centrifuged at 5,000 × g for 5 min. The pigment extracts were acidified with 5 M HCl to pH 2.0 and centrifuged at room temperature (6,000 × g, 15 min). The pellet was collected, washed three times with ddH₂O, and redissolved in 1 M NaOH. The solution was determined spectrophotometrically for absorbance at 459 nm and 1 M NaOH was used as a control (Babitskaya et al., 2000).

4.5 | Cutinase and cellulase assays

Freshly harvested mycelia were added to modified Czapek's medium (1 g glucose, 0.6 g NaNO₃, 0.6 g K₂HPO₄, 0.2 g MgSO₄, 0.2 g KCI, and 0.1 g FeSO₄·7H₂O per litre) amended with 0.1% tomato skins (preboiled in 4 g/L oxalate, 16 g/L ammonium oxalate, pH 3.8) (for cutinase activity) or 1.76% sodium carboxymethyl cellulose (for cellulases) and incubated at 26°C on a shaker for 24 hr (Rocha et al., 2008). Culture filtrates were centrifuged at 5,000 × g for 30 min. The supernatants were collected and tested for cutinase activity using p-nitrobenzoic acid (PNB)-mediated hydrolysis and measured for absorbance (A) at 405 nm with a spectrophotometer as described (Rocha et al., 2008). One unit of cutinase was defined by producing 1 μg p-nitrophenol per minute. Cellulase activity was determined using the dinitrosalicylic acid (DNS) method to determine the reduced glucose released from 1% citrus pectin and measured for A₅₄₀ nm with a spectrophotometer as described (Kapat et al., 1998; Ma et al., 2019). One unit of cellulase activity was defined to liberate 1 μmol glucose from 1% citrus pectin per minute.

4.6 | ACT toxin assays

ACT toxin was extracted from fungal culture filtrates as described (Kohmoto, 1993). Fungal strains were grown in a 300-ml Richards' solution at 26°C for 24 days. Culture filtrates after passing through four layers of cheesecloth were mixed with 10 ml Amberlite XAD-2 resins and incubated for 2 hr. Amberlite XAD-2 resins were collected by passing through a filter paper and ACT toxin eluted with 40 ml methanol. ACT toxin was analysed by HPLC as previously described (Ma et al., 2019). ACT toxin was separated in an XbridgeTMC18.5 column (4.6 × 250 mm) attached to a Waters 880-PU HPLC system using methanol/0.1% acetic acid as a mobile phase at a flow rate of 1 ml/min. ACT toxin was detected by a UV detector with absorbance measured at 290 nm. A peak of retention time at 36.9-37.9 min was collected and tested for toxicity on citrus leaves. The toxicity was assessed on detached citrus leaves (Citrus reticulata ‘Hongjiv’) by placing 10 μl of serially diluted filtrates on the wounded leaf surface. The treated leaves were incubated in a plastic box at 26°C for 3 days. Development of visible lesions 3 days after treatment was indicative of the presence of ACT toxin.

4.7 | Virulence assays

Virulence assay was conducted on detached Hongjiv leaves as described (Yago et al., 2011). Spore suspensions (10⁶ spores/ml) were sprayed or point-inoculated on detached Hongjiv leaves, which were or were not wounded by pricking prior to inoculation. The inoculated leaves were placed in a plastic box at 26°C for 3 days for lesion development. Each strain was tested on 12 leaves and the experiments were conducted at least twice.

4.8 | Transcriptome analysis

Mycelia of tested strains were cultured in PDB for 2 days, harvested, and ground in liquid nitrogen for RNA isolation. Total RNA was extracted using the AXxygen RNA purification kit (Capital Scientific). RNA-Seq was conducted using three biological replicates of each sample. Sequencing libraries were generated using Ultra RNA Library Prep kit (NEB) and were sequenced using an Illumina HiSeq2000 sequencer platform (Illumina Inc.) to generate 150 bp paired-end reads. Clean reads were obtained by removing reads containing adapters and low-quality reads from raw data using Trimmomatic v. 0.36 (Bolger et al., 2014). The resulting sequences were aligned to the A. alternata Z7 genome using Hisat2 v. 2.0.5 (Kim et al., 2015) and the number of reads mapped to each gene was counted by FeatureCounts v. 1.5.0-p3 (Liao et al., 2014). Differential expression between wild type and ΔAtf65 was analysed using the DESeq2 R package (Anders and Huber, 2012). Genes with an adjusted p value <.05 and an absolute value of log₂ fold change (log₂FC) greater than 1 by DESeq2 were assigned as differentially expressed. ClusterProfiler R package was used to test the statistical enrichment of differential expression genes in KEGG pathways and Gene Ontology (GO) (Yu et al., 2012). Gene clusters associated with secondary metabolites were predicted using antiSMASH 4.0 (Blin et al., 2017). The DEGs and SM gene clusters were plotted using Circos (Krzywinski et al., 2009).

4.9 | RT-qPCR analysis

Total RNA was extracted and reverse-transcribed to cDNA using a Prime Script RT reagent kit (Vazyme) (Wang et al., 2018). The relative expression of a gene was determined by quantitative real-time PCR in a CFX96 real-time system (Biorad). The actin coding gene (accession number KP341672) was used as a reference and each experiment was repeated three times using a comparative Cₘ method as previously described (Sun et al., 2011).

4.10 | Phylogenetic analysis

Multiple protein sequences were aligned by ClustalW program available in MEGA 5.0. The aligned sequences were performed
4.11 Yeast two-hybrid and co-immunoprecipitation

For Y2H assays, cDNA of each of the test genes was cloned into the yeast GAL4-binding domain vector pGBK7T and the GAL4-activation domain vector pGAD7T (Clontech). The pGBK7T-LAM and pGAD7T pair was used as a negative control and the pGBK7T-S3 and pGAD7T pair was used as a positive control. The pairs of Y2H plasmids were cotransformed into the yeast strain AH109. Transformants were grown on synthetic medium (SD) lacking Leu and Trp medium for 4 days, and then transferred to SD/–Ade–Leu–Trp–His medium and grown for 4 days at 30°C. At least three independent experiments were performed to confirm Y2H assay results (Liu et al., 2015).

A full-length gene including its promoter sequence was amplified and cloned into the p1532-GFP plasmid and pHZ-FLAG plasmid and verified by DNA sequencing. The resultant plasmids were introduced into the protoplasts prepared from a ΔAatfβ5 mutant. Transformants expressing fusion constructs were confirmed by western blotting with anti-FLAG (Abcam) and anti-GFP antibodies (Sigma). For Co-IP assays, total proteins were extracted and incubated with the anti-FLAG agarose (Sigma). Protein eluted from agarose was analysed by western blotting with anti-GFP antibodies. The protein samples were also detected with monoclonal anti-actin antibody (ABclonal Technology) as a control (Liu et al., 2015).

4.12 Statistical analysis

Data analyses and plotting were performed using SPSS statistics 19 (IBM) and Prism 5 (GraphPad). The significance of treatments was determined by analysis of variance and treatment means separated by Duncan's test (p ≤ .05).

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DATA AVAILABILITY STATEMENT

The raw sequence reads can be accessed at the NCBI SRA database at https://www.ncbi.nlm.nih.gov/sra with the accession numbers SRR11922316, SRR11922315, SRR11922314, SRR11922313, SRR11922312, and SRR11922311.

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**Supporting Information**

Additional supporting information may be found online in the Supporting Information section.

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