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ACT-Toxin, the Key Effector for the Virulence of *Alternaria alternata* Tangerine Pathotype to Specific Citrus Species

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Abstract: Alternaria brown spot disease is caused by the *Alternaria alternata* tangerine pathotype, which relies on ACT-toxin for infection. At present, all identified ACT-toxin biosynthesis-related genes are multi-copy genes. In this study, we summarized the advances in important host-specific toxins (HSTs), and listed key genes required for the pathogenicity of the *A. alternata* tangerine pathotype. Toxin virulence test results revealed that different citrus species displayed distinctly different tolerances to ACT-toxin. The extraction method of ACT-toxin crude extract was described in schematic form to make the method easier to understand. In addition, target gene disruption of two copies of *ACTT5* (Δ*ACTT5*) displayed significantly reduced virulence, indicating that *ACTT5* is essential for the pathogenicity of the *A. alternata* tangerine pathotype.

Keywords: ACT-toxin; *ACTT5*; Virulence; *A. alternata*; HSTs

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1. Introduction

Host-specific toxins (HSTs) are essential for the pathogenicity of the corresponding phytopathogens [1]. Currently, numerous pathogens have been documented to produce HSTs, such as ACT toxin (*A. alternata*) [2], ABR toxin (*Alternaria brassicae*) [3], PC toxin (*Periconia circinata*) [4], HMT toxin (*Helminthosporium maydis*) [5], etc. HSTs range from low-molecular-weight metabolites to proteins, which are essential for the virulence of corresponding pathogens. However, the action mode of most HSTs remains unknown. Most HST-producing pathogens are necrotrophic or hemibiotrophic fungi. *Alternaria* species, which cause pathogenic disease on various economically important crops, are well-known producers of HSTs [6–10].

At present, more than 70 toxins with different chemical structures are known to be biosynthesized by *Alternaria* species [11], including *Alternaria alternata*, *Alternaria arborescens*, *Alternaria brassicae*, *Alternaria brassicola*, *Alternaria infectoria*, and *Alternaria radicina* [1,12,13]. The corresponding HSTs are toxic and could induce cell death, specifically at 10^{-9} to 10^{-8} M when applied to susceptible species [14]. The targets of these toxins include the plasma membrane, mitochondria, chloroplast, Golgi bodies, nucleus, etc. *Alternaria* species cause disease in about 400 plant species, of which *A. alternata* infects nearly 100 plant species [1]. Currently, 13 HSTs have been identified in *Alternaria* species, and most of them are produced by *A. alternata* [1]. Different pathotypes of *A. alternata* are determined by the toxins they produce. Chemical structures of at least six *A. alternata* HSTs have been determined [15].

For example, the *A. alternata* tangerine pathotype produces ACT-toxin [2], the Japanese pear pathotype produces AK-toxin [16], the strawberry pathotype produces AF-toxin [17], the tomato pathotype produces AAL-toxin [18], rough lemon produces ACR-toxin [19], etc. HSTs of tangerine, strawberry, and Japanese pear pathotypes were found to be structurally analogous metabolites that are esters of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (EDA). The primary site of ACT-toxin, which contains three components, EDA, polyketide, and valine, is the plasma membrane [20]. Most of the genes required for HSTs biosynthesis in *A. alternata* pathotypes are multi-copy genes, and disruption of any of these genes results in the loss of virulence [21–25].

Alternaria brown spot (ABS), which is a severe fungal disease resulting in defoliation and fruit drop of citrus, is caused by the fungus *A. alternata* tangerine pathotype [26]. This fungus is strictly pathogenic as a result of its capability to produce the HST, ACT-toxin [27]. All the mutants that fail to produce ACT-toxin are nonpathogenic [24,28]. To date, *ACTT2* [25], *ACTTS2* [22], *ACTT3* [27], *ACTTS3* [27], *ACTT5* [24], *ACTT6* [24], and *ACTTR* [28] encoding genes have been revealed to be required for ACT-toxin biosynthesis, and are located in the ACT-toxin gene cluster of the conditionally dispensable chromosome (CDC) [29]. Genetic inactivation of any ACTT gene will block ACT-toxin biosynthesis and lead to the complete loss of virulence [22,27]. Simultaneous treatment with HSTs and non-pathogenic *A. alternata* strains resulted in successful infection [30,31].

In addition to ACT-toxin-biosynthesis-related genes, other genes involved in ROS detoxification or cell wall degradation are also required for the virulence of the *A. alternata* tangerine pathotype. For example, nicotinamide adenine dinucleotide phosphate oxidase genes are required for the accumulation of cellular hydrogen peroxide (H_2O_2), reactive oxygen species (ROS) detoxification, and pathogenicity [32,33]. In addition, other genes (*AP1*, *Hog1*, *Skn7*, *Tsa1*, etc.) involved in ROS detoxification are also required for the virulence of *A. alternata* [34–37]. On the other hand, the capability to break through the citrus cuticle layer via cell-wall-degrading enzymes is also required for the full virulence of *A. alternata* [38,39].

In this study, to demonstrate whether the susceptibility of citrus to *A. alternata* is determined by the tolerance level to ACT-toxin, citrus species, which are resistant, tolerant, and susceptible to ABS, were analyzed. In addition, we performed pathogenicity analysis of mutants with one (*ACTT5*) ACT-toxin-biosynthesis-related gene disrupted on leaves of different citrus species, as all these genes are essential for ACT-toxin biosynthesis and are not involved in vegetative growth and conidiation according to previous study [22,24,25,27,28].

2. Materials and Methods

2.1. Strains and Plants

The wild-type strain of *A. alternata* Z7 (CGMCC3.18907) was isolated from infected tangerine 'Ougan' [26,29,40]. All strains were grown on potato dextrose agar medium (PDA), potato dextrose broth medium (PDB), regeneration medium (RMM), or V8 medium at 26 °C. Citrus leaves were collected from Dancy (*Citrus reticulata*), Clementina (*Citrus clementina*), Minneola, sweet orange (*C. sinensis*), *Citrus medica*, Carrizo citrange, and Sugar belle. All the citrus plants provided by the National Citrus Engineering Research Center or Fred G. Gmitter, Jr were grown in a greenhouse.

2.2. Medium or Solution

PDA medium: 20 g glucose, 200 g potato, 20 g agar, add ddH_2O to 1 L.

PDB medium: 20 g glucose, 200 g potato, add ddH_2O to 1 L.

Trace elements solution: 5 g citric acid, 5 g $ZnSO_4$, 1 g $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$, add ddH_2O to 100 mL.

V8 medium: 200 mL V8 broth, 3 g $CaCO_3$, 20 g agar, add ddH_2O to 1 L.

Richards' medium: 25 g glucose, 10 g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4$, 0.02 g $FeCl_3$, and 0.005 g $ZnSO_4$, add ddH_2O to 1 L.

2.3. Conidiation

Z7 and corresponding mutants were incubated on V8 medium at 26 °C for 8 days. Then, the conidia were harvested from colonies grown on V8 medium by immersion, scraped with sterile water, and passed through three layers of sterile cheesecloth.

2.4. ACTT5 Knockout

Previous studies have revealed that *ACTT5* contains at least three copies [24]. Two copies of *ACTT5* (*ACTT5-1* and *ACTT5-2*) were disrupted in Z7 strain using a homologous recombination method in this study. First, for *ACTT5-1* disruption in Z7 to obtain Δ*ACTT5*, a fragment containing *HPH* (phosphotransferase) encoding gene, 5′*ACTT5-1* fragment (1250 bp length, 2021 bp before the start codon of *ACTT5-1*), and 3′*ACTT5-1* fragment (1032 bp length, 1787 bp after the stop codon of *ACTT5-1*) was constructed. The fragment was transformed into protoplasts prepared from Z7 strain using $CaCl_2$ and polyethylene glycol, as described by Chung et al. [41]. Fungal transformants were recovered in a regeneration medium amended with 200 μg/mL hygromycin (Roche Applied Science, Indianapolis, IN, USA). Then, for *ACTT5-2* disruption in Δ*ACTT5* to obtain ΔΔ*ACTT5*, a fragment containing *Neo* encoding gene, 5′*ACTT5-2* fragment (914 bp length, 210 bp before the start codon of *ACTT5-2*), and 3′*ACTT5-2* fragment (1096 bp length, 381 bp after the stop codon of *ACTT5-2*) was constructed. The fragment was transformed into protoplasts prepared from Z7 strain using $CaCl_2$ and polyethylene glycol. Fungal transformants were recovered in a regeneration medium amended with 100 μg/mL G418. The corresponding gene knockout mutants were verified based on the phenotype according to a previous study [24].

2.5. Determination of Pathogenicity

Pathogenicity assay of corresponding strains was performed on detached leaves of Dancy, Clementina, Minneola, and Sugar belle with conidial suspension (1×10^4 conidia/mL). Conidial suspension was sprayed on tested leaves and the inoculated leaves were incubated in a mist chamber for 5 to 8 days for lesion development. Each fungal strain was tested on at least 10 leaves, and experiments were repeated twice.

2.6. ACT-Toxin Extraction and Virulence Assay

Z7 strain was grown in 200 mL of modified Richards' medium [2,42,43] at 26 °C for 25–35 days, and the culture filtrate was collected through three layers of gauze. The culture filtrate was adjusted to pH 5.5 with 10% NaH_2PO_4 and was stirred with 1 L of Amberlite XAD-2 resin (Aldrich Chemical Co., Inc., Milwaukee, WI, USA) for 2 h to absorb toxins. The XAD-2 was packed in a funnel and eluted with methanol. The eluate was concentrated using a rotary evaporator. The virulence assay of ACT-toxin crude extract was performed by using resistant (*C. clementina*, *C. medica*, *C. sinensis*, and Carrizo citrange) and susceptible (*C. reticulata*) leaves. The petioles of tested citrus leaves were inserted in PCR tubes containing ACT-toxin crude extract at 26 °C for 1–2 days. The phenotype appearing around petioles was recorded after incubation.

2.7. Phenotypic Analysis

Stress tolerance was assayed by placing mycelia plugs (5 mm × 5 mm) onto PDA or MM medium amended with oxidants, salts, cell wall-interfering agents, DNA-damaging agents, or other indicated chemicals at 26 °C for 3–5 days. The percentage of growth reduction was determined by comparing a cumulative percentage of the growth of Z7 and mutants grown on same medium. All tests were repeated at least twice with three replicates of each treatment. Tested compounds included sorbitol (1 mM), NaCl (1 M), H_2O_2 (10 mM), sodium dodecyl sulfate (SDS, 0.01%), and Congo red (CR, 0.2 mg/mL).

2.8. Statistical Analysis

The diameter of growth is presented as the mean ± standard deviation (SD) ($n = 6$). Significance was determined using Student's *t*-test ($p < 0.05$) for differences between the wild-type and Δ*ACTT5*.

3. Results

3.1. ACT-Toxin Biosynthesis and ROS Detoxification-Related Genes Are Involved in the Pathogenicity of *A. alternata*

HSTs produced by fungal plant pathogens are key factors responsible for pathogenicity during certain plant-pathogen interactions. Table 1 shows some important HSTs produced by phytopathogenic fungi. Nine different pathotypes of *A. alternata* known to produce HSTs are listed in Table 2. ACT-toxin, which is produced by the *A. alternata* tangerine pathotype, is essential for the pathogenesis of this pathogen. ACT-toxin biosynthesis is regulated by multiple genes in the ACT-toxin gene cluster, and the knockout of any one of these genes results in the complete loss of pathogenicity. Therefore, we listed relevant information (including accession number, copy number, and corresponding references) of these genes in Table 3. In addition to ACT-toxin, genes involved in ROS detoxification are also required for the virulence of *A. alternata*. Table 4 shows the studied genes required for ROS detoxification and pathogenicity in *A. alternata*.

Table 1. Examples of HSTs.

No	Pathogen	Toxin	Host	Reference
1	*A. alternata* Japanese pear pathotype	AK	Pear	[44,45]
2	*Helminthosporium victoriae*	HV	Oat	[4]
3	*Periconia circinata*	PC	Sorghum	[46]
4	*Helminthosporium turcicum*	HT	Maize	[47]
5	*A. alternata* apple pathotype	AM	Apple	[48,49]
6	*A. alternata* tangerine pathotype	ACT	Citrus	[2,49]
7	*Helminthosporium maydis*	HMT	Maize	[5]
8	*Helminthosporium sacchari*	HS	Sugarcane	[50]
9	*A. alternata* tomato pathotype	AAL	Tomato	[18]
10	*A. alternata* rough lemon pathotype	ACR	Rough lemon	[42,51]
11	*A. alternata* strawberry pathotype	AF	Strawberry	[52]
12	*A. alternata* tobacco pathotype	AT	Tobacco	[53]
13	*Helminthosporium carbonum*	HC	Maize	[54]
14	*Pyrenophora tritici-repentis*	PTR	Wheat	[55]
15	*A. alternata* sunflower pathotype	AS-I	Sunflower	[56]
16	*A. alternata* spotted knapweed pathotype	Maculosin	Knapweed	[57]
17	*A. brassicae*	ABR	*Brassica* spp.	[3]

Table 2. HSTs produced by *A. alternata*.

No	Pathotype	HSTs	Disease	Chemical Characteristics	Reference
1	Tomato pathotype	AAL	Alternaria stem canker of tomato	Aminopentol esters	[1,18,58]
2	Tangerine pathotype	ACT	Citrus brown spot	Epoxy-decatrienoic esters	[2]
3	Rough lemon pathotype	ACR	Leaf spot of rough lemon	Terpenoid	[19]
4	Strawberry pathotype	AF	Black spot of strawberry	Epoxy-decatrienoic esters	[17]
5	Japanese pear pathotype	AK	Black spot of Japanese pear	Epoxy-decatrienoic esters	[16]
6	Apple pathotype	AM	Alternaria blotch of apple	Cyclic peptide	[59]
7	Sunflower pathotype	AS-I	Leaf spot of sunflower	Tetrapeptide	[56]
8	Tobacco pathotype	AT	Brown spot of tobacco	—	[60]
9	Spotted knapweed pathotype	Maculosin	Black leaf blight of knapweed	Tetrapeptide	[61]

Table 3. Key genes essential for ACT biosynthesis.

No	Gene	Name	Copy Number	Accession Number	Reference
1	ACTT2	Hydrolase	≥2	AALT_g11743	[20]
2	ACTTS2	Enoyl-reductase	≥2	AALT_g12031	[22]
3	*ACTT3*	HMG-CoA hydrolase	≥2	AALT_g11755	[25]
4	*ACTTS3*	Polyketide synthase	≥3	AALT_g11750	[27]
5	*ACTT5*	Acyl-CoA synthetase	≥3	AALT_g11751	[24]
6	*ACTT6*	Enoyl-CoA hydratase	2	AALT_g12047	[24]
7	*ACTTR*	Zn(II)2Cys6 transcription factor	≥2	AALT_g11754	[28]

Table 4. ROS detoxification-related genes in *A. alternata* tangerine pathotype.

No	Gene	Vegetative Growth	Conidiation	Pathogenicity	Accession Number	Reference
1	*Hog1*	Required	Required	Required	GQ414509	[35]
2	*Skn7*	Required	Required	Required	JQ716919	[36]
3	*Ap1*	Required	—	Required	FJ376607	[62]
4	*Gpx3*	Required	Required	Required	ACY73852	[63]
5	*Tsa1*	Not required	Not required	Required	MG593564	[37]
6	*Trr1*	Required	Required	Required	MG593563	[37]
7	*Glr1*	Required	Required	Required	MG593559	[37]
8	*NoxA*	Required	Required	Required	JN900389	[32]
9	*NoxB*	Required	Required	Required	JX136700	[32]
10	*NoxR*	Required	Required	Required	JX207117	[32]
11	*MetR*	Required	Required	Required	Aa03030	[64]
12	*SSK1*	Required	Required	Required	KU170060	[65]
13	*Tbf1*	Required	Required	Required	MT184174	[39]
14	*Atg8*	Required	Required	Required	OK617334	[66]
15	*SreA*	Required	Required	Required	OWY49902.1	[67]

3.2. Different Citrus Species Display Distinct Sensitivity to ACT-Toxin

Until now, no schematic diagram of the process for obtaining ACT-toxin crude extract has been available, although ACT-toxin is crucial for the analysis of the pathogenicity mechanism in *A. alternata*. Therefore, we made a schematic diagram of ACT-toxin extraction. Briefly, the extraction protocol comprised five steps: toxin-producing cultivation, culture filtrate obtaining, toxin adsorption using XAD-2, toxin dissolution, and toxin concentration (Figure 1A). Subsequently, we obtained ACT-toxin crude extract from Z7 strain using this protocol. ACT-toxin virulence assay was performed on detached leaves of *C. reticulata*, *C. clementina*, *C. medica*, *C. sinensis*, and Carrizo citrange. Necrotic lesions quickly developed on the leaves of *C. reticulata*. On the contrary, *C. clementina*, *C. medica*, *C. sinensis*, and Carrizo citrange, which are citrus species resistant to ABS, displayed highly tolerance to ACT-toxin (Figure 1B). These results revealed that the ABS resistance capability of citrus was derived from the tolerance capability to ACT-toxin.

3.3. ACTT5 Is Required for the Pathogenicity of A. alternata

To verify that the sensitivity of citrus to ACT-toxin is the major factor in determining its resistance or susceptibility capability to ABS, we knocked out two copies of *ACTT5* using the developed multi-copy gene disruption strategy (Figure 2A, Table S1). Δ*ACTT5* means mutant with one copy of *ACTT5* knocked out. ΔΔ*ACTT5* means mutant with two copies of *ACTT5* knocked out. Both Δ*ACTT5* and ΔΔ*ACTT5* displayed wild-type radial growth on PDA medium (Figure 2B), indicating *ACTT5* was not involved in the vegetative growth of *A. alternata*. Pathogenicity analysis revealed that the wild-type Z7 strain could induce significantly enlarged necrotic lesions on leaves of ABS-susceptible citrus species (Dancy and Minneola), and tiny necrotic lesions on ABS-tolerant species (Sugar belle). No obvious necrotic lesions were observed on ABS-resistant accession (*C. clementina*)

(Figure 2C). ΔΔ*ACTT5* did not produce any visible lesions on ABS-resistant and -tolerant species (*C. clementina* and Sugar belle), and induced almost undetectable tiny lesions on susceptible citrus species (Dancy and Minneola) (Figure 2C).

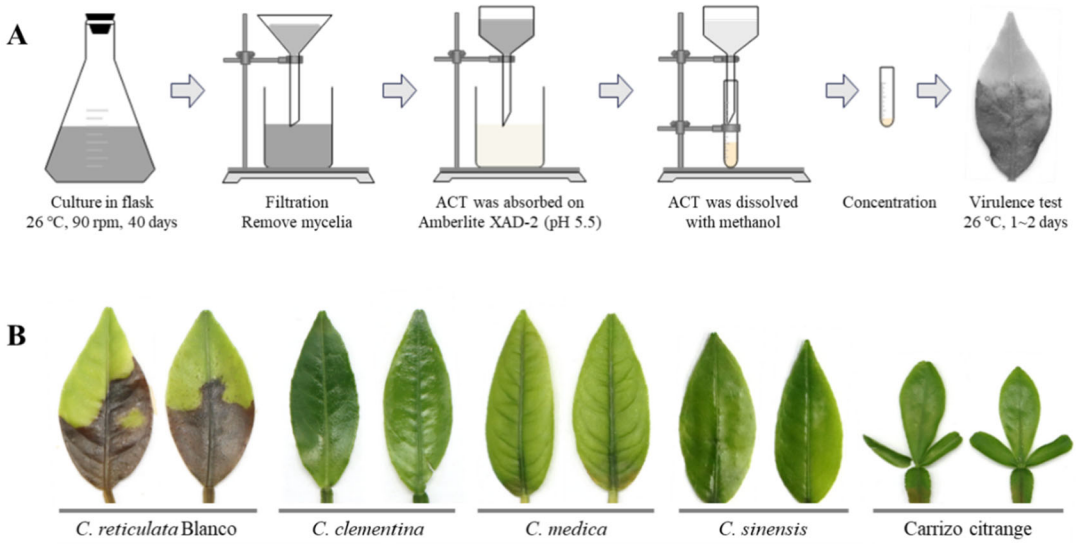

Figure 1. Different citrus accessions display distinct sensitivity to ACT-toxin. (**A**) Isolation procedure to obtain ACT crude extract. (**B**) Virulence test of ACT crude extract on leaves of different citrus accessions.

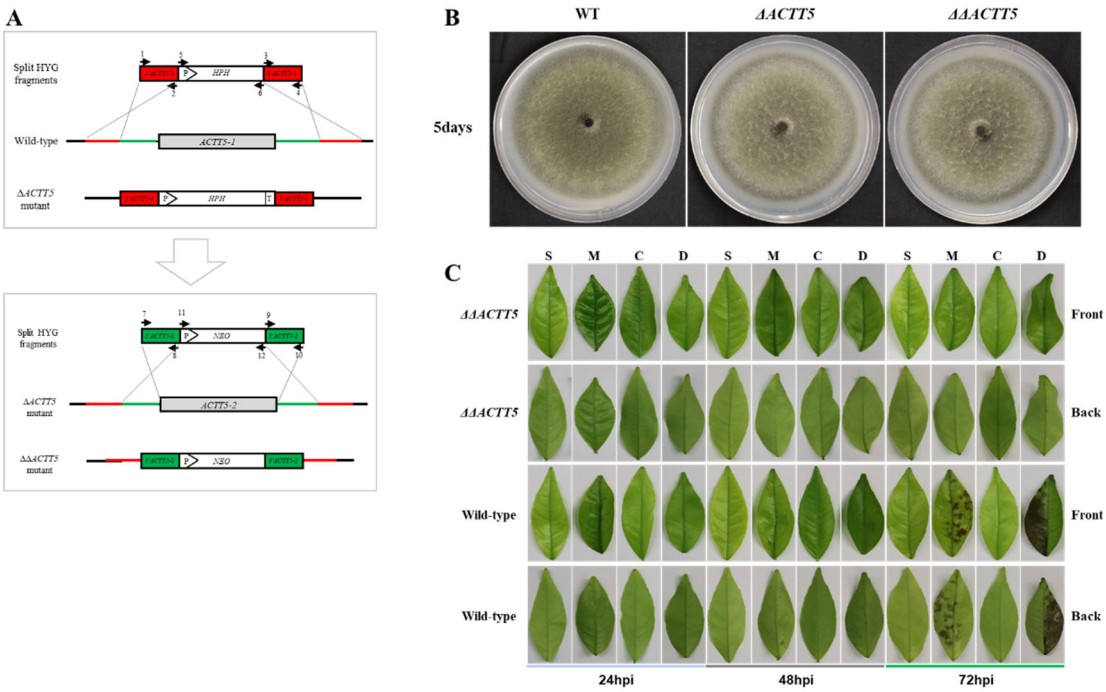

Figure 2. *ACTT5* knockout and virulence assay. (**A**) Schematic diagram for *ACTT5* knockout. (**B**) *ACTT5* was not involved in vegetative growth. (**C**) *ACTT5* was required for virulence. "S" means "Sugar belle", "M" means "Minneola", "C" means "Clementina", "D" means "Dancy".

3.4. ACTT5 Is Not Involved in Conidiation, Vegetative Growth, and Multi-Stress Resistance

The ΔΔ*ACTT5* produced regular mycelium and ovoid conidia with dark pigmentation, similar to those produced by the wild-type, indicating *ACTT5* is not involved in cell development (Figure 3A,B). ΔΔ*ACTT5* displayed wild-type sensitivity to sorbitol and

NaCl, indicating that *ACTT5* is not required for the resistance to osmotic stress. In addition, ΔΔ*ACTT5* also displayed wild-type sensitivity to H_2O_2, SDS, and Congo red (CR), indicating *ACTT5* is not involved in ROS detoxification and cell wall integrity (Figure 3C).

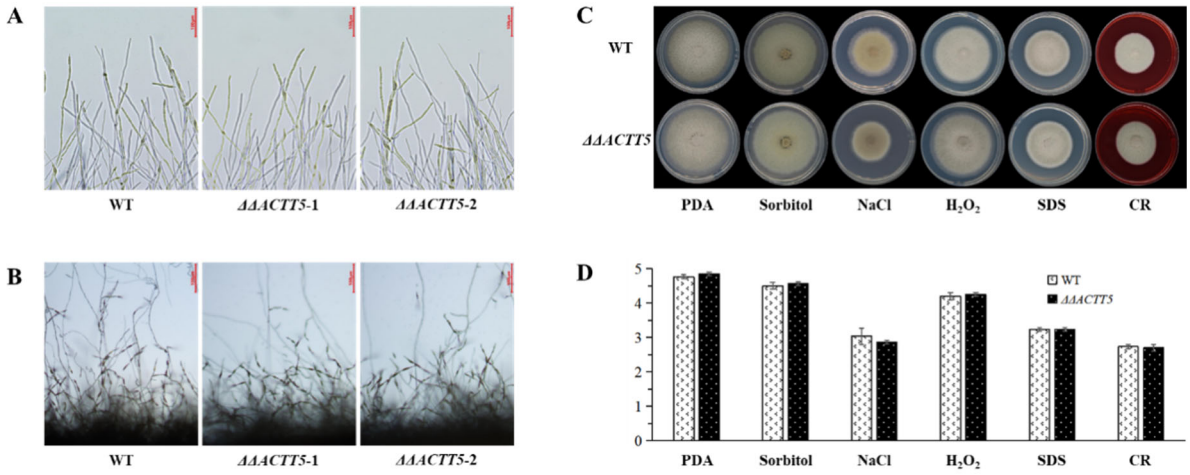

Figure 3. *ACTT5* is not involved in conidiation, vegetative growth, and multi-stress resistance. (**A**) Microscopic observation of mycelium of wild-type (WT) and ΔΔ*ACTT5*. (**B**) Conidiation of wild-type and ΔΔ*ACTT5*. (**C**) Multi-stress analysis of wild-type and ΔΔ*ACTT5*. (**D**) The percentage of growth reduction determined by comparing a cumulative percentage of the growth of Z7 and ΔΔ*ACTT5* grown on same medium is also shown.

4. Discussion

HSTs are a group of structurally complex and chemically diverse metabolites produced by specific phytopathogenic fungi and function as essential determinants of virulence. HSTs-producing species mainly include *Alternaria*, *Helminthosporium*, *Colletotrichum*, *Fusarium*, *Periconia*, *Phyllosticta*, *Corynespora*, etc. *Alternaria* species cause pathogenic disease on numerous economic crops. *A. alternata* consists of at least seven pathotypes, each of which can produce a specific HST toxic in the corresponding host plant [15]. Citrus is the world's largest type of fruit. The *A. alternata* tangerine pathotype, which produces ACT-toxin, causes ABS in tangerines (*C. reticulata*), grapefruits, (*C. paradisi* Macfad), hybrids of tangerine and sweet orange, and hybrids of tangerine and grapefruit [40,68]. Previous studies have revealed that ACT-toxin can cause necrotic lesions on citrus leaves at a concentration of 2×10^{-8} M [2,69]. Therefore, the study of the interaction mechanism of ACT-toxin and citrus is of great significance for the breeding of ABS-resistant citrus varieties. However, research on the action mechanism of ACT-toxin is still at the stage of microscopic observation. In this study, we summarized 17 HSTs produced by different pathogens, provided the details of 7 HSTs produced by *A. alternata*, and listed studied genes essential for the biosynthesis of ACT-toxin.

Previous studies have revealed that ACT-toxin biosynthesis and ROS detoxification capability is essential for the pathogenicity of *A. alternata*. ROS is generated from host cells during the infection of *A. alternata* [70]. *A. alternata* must be able to perform ROS detoxification for successful colonization. Therefore, many key genes essential for ROS detoxification were also revealed to be needed for the pathogenicity, such as Ap1 [62], Hog1 [35], Skn7 [36], etc. To date, the identified genes responsible for ACT-toxin biosynthesis include *ACTT1*, *ACTT2*, *ACTTS2*, *ACTT3*, *ACTTS3*, *ACTT5*, *ACTT6*, *ACTTR*, etc. [18,22,24,25,27,32]. These genes, which are mainly clustered and located in the conditionally dispensable chromosome, are multi-copy genes [29]. Among them, *ACTT1* and *ACTT2* are considered as homologous genes of *AKT1* and *AKT2* of the *A. alternata* Japanese pear pathotype [24]. *ACTTS2* (enoyl reductase) and *ACTTS3* (peptide synthetase) are also involved in ACT-toxin biosynthesis [22,27]. In addition, the corresponding homologous genes of *ACTT5* (acetyl-CoA synthetase) and *ACTT6* (enoyl-CoA hydratase) also exist in the Japanese pear

pathotype. *ACTTR* encoding Zn2Cys6 transcription factor has been proven to be involved in ACT-toxin biosynthesis and pathogenicity in the *A. alternata* tangerine pathotype [28]. In this study, we knocked out two copies of *ACTT5*. We did not conduct an RNA silencing experiment for *ACTT5* because the phenotype of Δ*ACTT5* met our experimental requirements. The virulence of Δ*ACTT5* significantly decreased. Moreover, Δ*ACTT5* displayed a wild-type phenotype of vegetative growth, conidiation, and multi-stress resistance. All these results revealed that ACT-toxin is a key factor for the virulence of the *A. alternata* tangerine pathotype.

Generally, the plasma membrane, mitochondria, chloroplast, and some important enzymes are the inhibitory sites for the action of HSTs of *A. alternata* [1,15]. Based on the chemical structures of HSTs produced by *A. alternata* pathotypes, HSTs can be classified into seven classes. The structure of ACT-toxin is related to AF- and AK-toxins, each of which share a common EDA moiety [2,17,44]. The structure of the other HSTs is as follows: AAL-toxin (sphinganine analogue), ACR-toxin (pyranones), AM-toxin (cyclic peptide), AS-I toxin (tetrapeptide), and maculosin (diketopiperazine) [71]. The target of ACT-, AK-, and AF-toxins is the plasma membrane [69]. ACR-toxin first targets the mitochondria and then other cell organelles [69]. Mitochondria and endoplasmic reticulum are the primary targets for the action of AAL-toxin. Chloroplasts are the primary target of AM-toxin and maculosin. The *A. alternata* tangerine pathotype produces ACT-toxins I and II, with ACT-toxin I being more toxic to citrus cells [2]. The ABS susceptibility of citrus species has been studied by numerous researchers [72–75]. In this study, we provided a schematic diagram for obtaining ACT-toxin crude extract. ACT-toxin inoculation results revealed that ABS-resistant citrus accession (*C. clementina*) was tolerant to ACT-toxin, and the ABS-susceptible citrus accession (*C. reticulata*) was highly sensitive to ACT-toxin. The disruption of the essential gene (*ACTT5*) involved in ACT-toxin biosynthesis resulted in the loss of virulence for *A. alternata*. All these results revealed that the ABS resistance capability of citrus is dependent on the ACT-toxin tolerance capability.

5. Conclusions

In this study, we reviewed HSTs produced by phytopathogenic fungi and summarized the HSTs of different *A. alternata* pathotypes. Our results further proved that ACT-toxin tolerance capability is an important basis on which to analyze whether the tested citrus species are resistant to ABS.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12123181/s1, Table S1: Primers used in this study.

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Conflicts of Interest: The authors declare no conflict of interest.

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