Disruption and Overexpression of the Gene Encoding ACC (1-Aminocyclopropane-1-Carboxylic Acid) Deaminase in Soil-Borne Fungal Pathogen *Verticillium dahliae* Revealed the Role of ACC as a Potential Regulator of Virulence and Plant Defense

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Accepted 3 December 2018.

It has been suggested that some microorganisms, including plant growth-promoting rhizobacteria, manipulate the level of ethylene in plants by degrading 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor, into α -ketobutyrate and ammonia, using ACC deaminase (ACCD). Here, we investigated whether ACCd of *Verticillium dahliae*, a soil-borne fungal pathogen of many important crops, is involved in causing vascular wilt disease. Overexpression of the *V. dahliae* gene encoding this enzyme, labeled as *ACCd*, significantly increased virulence in both tomato and eggplant, while disruption of *ACCd* reduced virulence. Both types of mutant produced more ethylene than a wild-type (70V-WT) strain, although they significantly differed in ACC content. Overexpression strains lowered ACC levels in the roots of infected plants, while the amount of ACC in the roots of plants infected with deletion mutants increased. To test the hypothesis that ACC acts as a signal for controlling defense, roots of WT and *Never-ripe* (*Nr*) tomato plants were treated with ACC before *V. dahliae* inoculation. Plants pretreated with ACC displayed less severe symptoms than untreated controls. Collectively, our results suggest a novel role of ACC as a regulator of both plant defense and pathogen virulence.

Verticillium dahliae is a soil-borne fungal pathogen that causes Verticillium wilt disease in a wide variety of economically important annual herbaceous, perennial, and woody plants

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Funding: This work was supported by the Cyprus University of Technology Dimitris Tsaltas Startup Grant EX064 2011–2014, Research Activities Fund, and Researcher's Fund and by funds from the State Scholarships Foundation of Greece (grant 2012-IIIE2-3339) via the Operational Program "Education and Lifelong Learning" of the European Social Fund (ESF) within the National Strategic Reference Framework (2007–2013) with a Ph.D. scholarship awarded to Maria-Dimitra Tsolakidou.

*The e-Xtra logo stands for "electronic extra" and indicates that six supplementary figures are published online.

(Bhat and Subbarao 1999; Fradin and Thomma 2006; Khasabab et al. 2017). Due to the broad host range, the ability to survive many years as microsclerotia in the soil and very limited control options, *V. dahliae* continuously causes severe crop losses in many temperate and subtropical regions around the world (Fradin and Thomma 2006; Robison et al. 2001; Tjamos 1989). *V. dahliae* evolved various mechanisms to overcome plant defense systems and facilitate in planta proliferation (Klosterman et al. 2009; Maruthachalam et al. 2011). Secretion of a variety of molecules, including cell wall-degrading enzymes, phytotoxins, and effectors, is one such mechanism (Luo et al. 2014; Tzima et al. 2011). Some regulatory proteins that control signal transduction pathways, such as GTP-binding proteins, mitogen-activated protein kinases, and protein kinase A, also play critical roles in virulence (Tzima et al. 2010, 2012).

Ethylene is a gaseous plant hormone that affects developmental processes such as flowering, senescence, fruit ripening, and leaf abscission and also participates in regulating responses to biotic and abiotic stresses (Abeles et al. 1992; Johnson and Ecker 1998). Certain bacteria and fungi also produce ethylene, presumably to manipulate plant ethylene-mediated regulation of these processes in ways that facilitate their colonization of plants. Potential roles of pathogen-produced ethylene in virulence and disease development have been studied in *V. dahliae* and a few other pathogens (Al-Masri et al. 2006; Cristescu et al. 2002; Tzeng and Devay 1985; Tzima et al. 2010, 2012; Zhu et al. 2012, 2017). Ethylene may be involved in causing Verticillium wilt symptoms such as epinasty, stunting, premature senescence, and leaf abscission (Cronshaw and Pegg 1976; Wiese and Devay 1970). Ethylene perception is required for spore germination and appressorium formation in *Colletotrichum gloeosporioides* (Kim et al. 2000). In *Botrytis cinerea*, ethylene affected transcriptional reprogramming of the genes that may be involved in plant interaction (Chagué et al. 2006). However, its role in plant infection remains unclear (Chagué et al. 2002).

In plants, ethylene is synthesized from methionine through two intermediates, S-adenosyl-methionine (S-AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang 1979). In microorganisms, ethylene biosynthesis can occur via three pathways, one of which is the pathway

employed by plants (Jia et al. 1999). Another pathway involves a multifunctional enzyme called ethylene-forming enzyme (EFE), which converts 2-oxoglutarate (OXO) to ethylene (Chagué 2010; Fukuda et al. 1986; Hottiger and Boller 1991). Ethylene can also be produced through oxidation of α -keto- γ -methylthiobutyric acid (KMBA), a substrate synthesized through deamination of methionine (Chagué et al. 2002; Cristescu et al. 2002; Primrose 1977). Tzeng and De Vay (1984) showed that two *V. dahliae* isolates from cotton could produce ethylene via the KMBA pathway and that addition of ACC to culture medium inhibited ethylene production. The genome of *V. dahliae* contains genes encoding ACC synthase (ACS), ACC deaminase (ACCd), and EFE. However, their role in pathogenicity and ethylene biosynthesis has not been elucidated. Many plant growth-promoting rhizobacteria (PGPR) that produce ACCd, which cleaves ACC into α -ketobutyrate and ammonia, promote plant growth, presumably by lowering plant ethylene levels (Glick 2014; Honma and Shimomura 1978). Vanderstraeten and Van Der Straeten (2017) stated that the secretion of ACC from plant roots into the rhizosphere to attract and interact with PGPR suggests an ethylene-independent function for ACC. Several other studies also supported a model that ACC functions as a signal molecule (Tsang et al. 2011; Tsuchisaka et al. 2009; Van de Poel and Van Der Straeten 2014; Xu et al. 2008; Yoon and Kieber 2013).

Here, we investigated if and how ACCd affects the pathogenicity of *V. dahliae* and its growth, development, and ethylene production, by disrupting the gene encoding this enzyme, entitled *ACCd*, and also by overexpressing it. We also investigated the role of ACC in disease development and whether

plant response to ACC could be ethylene-independent by evaluating how WT and *Nr* (ethylene-insensitive) tomato plants pretreated with ACC respond to infection by *V. dahliae*.

RESULTS

Overexpression and disruption of the *ACCd* gene.

The *V. dahliae* *ACCd* gene consists of an open reading frame (ORF) of 1,038 bp, which is interrupted by one intron of 90 bp located at position 186, and encodes a protein of 345 amino acids. Its ORF fused to the *Magnaporthe oryzae* ribosomal protein promoter, a strong constitutive promoter, was introduced into strain 70V-WT, a race 1 isolate from tomato (Pantelides et al. 2010), to increase its expression (Supplementary Fig. S1). Based on the amount of *ACCd* transcripts in 12 transformants, three transformants (*Vd6ADM1*, *Vd6ADM2*, and *Vd6ADM10*), which produced up to 12 times more transcripts than 70V-WT, were chosen for subsequent experiments. The gene was also deleted (Supplementary Fig. S2). *ACCd* transcripts were absent in deletion mutants *70ΔAD1.4* and *70ΔAD3.4*. A functional copy of *ACCd* was introduced into *70ΔAD1.4* for complementation, resulting in *70CAD1.2.2*. The amount of *ACCd* transcripts in *70CAD1.2.2* was comparable to that in 70V-WT.

Effect of both mutations on growth, microsclerotia formation, and conidial production and germination.

70V-WT and its mutants were cultured on minimal medium to investigate the role of *ACCd* in growth and development. Their colony sizes were similar (Fig. 1A), however, compared

Fig. 1. Colony growth, microsclerotia formation and spore production of 70V-WT and 1-aminocyclopropane-1-carboxylic acid deaminase (*ACCd*) mutants. **A**, Colony growth of 70V-WT, *ACCd* deletion mutants (*70ΔAD1.4* and *70ΔAD3.4*), overexpression strains (*Vd6ADM1* and *Vd6ADM2*), and complemented deletion mutant *70CAD1.2.2* over time and **B**, their spore production on potato dextrose agar. Vertical bars indicate the standard errors of five and three replicates, respectively. Different letters in B note statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$. **C** and **D**, Colony appearances from, respectively, the top and bottom of representative plates 30 days after inoculation.

with 70V-WT, which formed abundant darkly pigmented microsclerotia, both types of mutants produced fewer microsclerotia but more aerial hyphae (Fig. 1C and D). When cultured on potato dextrose agar (PDA), deletion mutants *70ΔAD1.4* and *70ΔAD3.4* produced significantly more conidia than 70V-WT (Fig. 1B), but no significant difference was noted in liquid sucrose sodium nitrate (SSN) medium (Supplementary Fig. S3). However, on PDA amended with 10 or 100 μ M ACC, significantly reduced conidial production was observed in *70ΔAD3.4* (Fig. 2B). Conidial production by overexpression strains *Vd6ADM1* and *Vd6ADM2* and complemented deletion mutant *70CAD1.2.2* was comparable to 70V-WT when cultured on PDA (Fig. 1B), in SSN, and on PDA supplemented with ACC (Fig. 2B). ACC at 500 μ M inhibited colony growth of 70V-WT, *Vd6ADM1*, and *70CAD1.2.2* (Fig. 2A). Spore germination was similar among all strains.

Involvement of *ACCd* in pathogenesis.

Virulence of deletion mutants and overexpression strains in tomato and eggplant was evaluated. Infection of tomato plants was performed in both a growth room and greenhouse. Compared with 70V-WT, two deletion mutants caused significantly less disease, whereas plants infected with two overexpression strains displayed more severe wilting symptoms (Fig. 3A). Tomato plants inoculated with 70V-WT and overexpression strains *Vd6ADM1* and *Vd6ADM2* exhibited the first wilting symptoms at 8 days postinoculation (dpi). At 14 dpi, the disease severity caused by *Vd6ADM1* and *Vd6ADM2* reached 50 and 42%, respectively, while that caused by 70V-WT reached 25%. Plants inoculated with deletion mutants *70ΔAD1.4* and *70ΔAD3.4* displayed only mild chlorosis in older leaves at 14 dpi, with disease severity being 12 and 12.5%, respectively (Fig. 3B). Consistent with the onset and severity of symptom development, relative area under the disease progress curve (AUDPC) values showed that *70ΔAD1.4* and *70ΔAD3.4* (7%) caused significantly less disease than 70V-WT (13.9%), complemented deletion mutant *70CAD1.2.2* (10.3%), and overexpression strains *Vd6ADM1* and *Vd6ADM2* (31.3 and 26.4%, respectively) (Fig. 3C). Compared with plants inoculated with 70V-WT, average heights and fresh weights of plants inoculated with *70ΔAD1.4* and *70ΔAD3.4* were higher, while those of plants inoculated with *Vd6ADM1* and *Vd6ADM2* were significantly lower (Figs. 3D and E). Although the difference between 70V-WT and two overexpression strains was less pronounced in greenhouse evaluations, similar patterns were observed (Supplementary Fig. S4). Relative AUDPC values after infection by *Vd6ADM1* and *Vd6ADM2* were 17 and 20%, respectively, while those caused by 70V-WT, *70ΔAD1.4*, and *70ΔAD3.4* were 13.6, 7.6, and 9.8%, respectively.

Fungal biomass in the vascular tissues of infected plants was assessed, using quantitative polymerase chain reaction (qPCR), to determine whether it correlates with disease severity. At 8 dpi, fungal DNA was detectable in all treatments. However, the amount of fungal DNA in plants infected with *Vd6ADM1* and *Vd6ADM2* was significantly higher than that in plants infected with 70V-WT (Fig. 4A). The amount of DNA for deletion mutants *70ΔAD1.4* and *70ΔAD3.4* was similar to that of 70V-WT (Fig. 4A). At 21 dpi, the amount of DNA for *Vd6ADM1* and *Vd6ADM2* was 20- and 11-fold higher than that of 70V-WT. However, the difference between 70V-WT and deletion mutants was not significant (Fig. 4B).

In eggplant inoculated with three overexpression strains, disease progression was more rapid (Fig. 5A). At 14 dpi, about 60% of the leaves of plants infected with *Vd6ADM1*, *Vd6ADM2*, and *Vd6ADM10* displayed wilt symptoms, while approximately 50% of the leaves showed wilt symptoms in plants infected with 70V-WT (Fig. 5B). Relative AUDPC

values were consistent with higher virulence of *Vd6ADM1*, *Vd6ADM2*, and *Vd6ADM10* (35, 33, and 37%, respectively) than that of 70V-WT (23%) (Fig. 5C). Similar to tomato, eggplant infected with deletion mutants showed less chlorosis and wilting symptoms (Fig. 6A) with disease severity at 13 dpi being 40 and 33%, respectively compared with 60% caused by 70V-WT (Fig. 6B). Relative AUDPC values after infection by *70ΔAD1.4* and *70ΔAD3.4* were 23 and 19%, respectively, while that of 70V-WT was 33% (Fig. 6C).

Both mutations did not affect conidial adhesion to tomato roots and root surface colonization.

To facilitate monitoring of conidial attachment to roots and early stages of root colonization, deletion mutants *70ΔAD1.4* and *70ΔAD3.4*, overexpression strain *Vd6ADM1*, and 70V-WT were transformed to constitutively express a red fluorescent protein (AsRed), resulting in *70ΔAD1.4R*, *70ΔAD3.4R*, *Vd6ADM1R*, and 70V-WTR. At 1 dpi, conidia of all strains attached to the root surface similarly (Fig. 7A to D). At 5 dpi, no significant differences in spore attachment and root colonization were observed (Fig. 7E to H). Subsequently, disease severity in plants infected with these AsRed transformants was calculated. At 17 dpi, plants inoculated with *Vd6ADM1R* showed symptoms of wilting, necrosis, and leaf abscission, with disease severity of 18.5% (Fig. 8B). In contrast, 70V-WTR, *70ΔAD1.4R*, and *70ΔAD3.4R* caused the disease severity of 11.5, 4.3, and 5.3%, respectively (Fig. 8B). Relative

Fig. 2. A, Colony diameter and **B**, spore production of 70V-WT and 1-aminocyclopropane-1-carboxylic acid deaminase (*ACCd*) mutants in the presence of ACC. 70V-WT, deletion mutant *70ΔAD3.4*, overexpression strain *Vd6ADM1*, and complemented deletion mutant *70CAD1.2.2* were cultured on potato dextrose agar supplemented with 0, 10, 100, or 500 μ M ACC. Vertical bars indicate the calculated standard errors of five (A) and three (B) replicates. All values were subjected to analysis of variance. For data that showed a significance level of $P \leq 0.05$, Tukey's post hoc analysis was also performed.

AUDPC values confirmed the lower virulence of *70ΔAD1.4R* and *70ΔAD3.4R* (0.7 and 0.9%) and the higher virulence of *Vd6ADM1R* (6.1%) compared with *70V-WTR* (3.5%) (Fig. 8C).

Both mutations affected ethylene production.

Since ACC is the precursor for ethylene in the methionine-ACC biosynthetic pathway, we hypothesized that *ACCd* deletion and overexpression would affect ethylene production.

Fig. 3. Disease severity of tomato plants infected with *70V-WT* and 1-aminocyclopropane-1-carboxylic acid deaminase (*ACCd*) mutants. Tomato plants were inoculated with *70V-WT*, deletion mutants (*70ΔAD1.4* and *70ΔAD3.4*), overexpression strains (*Vd6ADM1* and *Vd6ADM2*), and complemented deletion mutant *70CAD1.2.2*. **A**, Representative tomato plants at 19 days after inoculation. **B**, Disease progress over time, **C**, amount of disease expressed as relative area under the disease progress curve, **D**, height, and **E**, fresh weight of plants. Vertical bars indicate the standard errors based on 24 replicates. All values were subjected to analysis of variance. Different letters in **C**, **D**, and **E** note statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$.

Ethylene production by 70V-WT and its mutants fluctuated during 14 days of cultivation (Fig. 9A). The amount of ethylene produced by 70 Δ AD3.4 was higher than that of 70V-WT at multiple timepoints, with the maximum difference being observed at day 12. Interestingly, the amount of ethylene produced by *Vd6ADM1* was also higher than that by 70V-WT at days 4, 8, and 12, but at days 6 and 14, no significant difference was observed (Fig. 9A).

Expression patterns of *ACS* and *EFE* were determined to investigate whether both mutations affect the expression of other genes involved in ethylene production. The amount of *ACS* transcripts in 70 Δ AD3.4 and *Vd6ADM1* appeared lower than that in 70V-WT at day 4, but at days 6, 8, and 12, they looked similar (Fig. 9B). The amount of *EFE* transcripts was highest at day 4 in all three strains and subsequently decreased. However, no significant differences among the strains were observed at all timepoints (Fig. 9C). We also checked the production of KMBA, another substrate for ethylene, in 70V-WT and mutants. All strains produced KMBA as evidenced by the formation of a yellow precipitate, an indicator for KMBA presence (Supplementary Fig. S5) (Chagué et al. 2002; Primrose 1977).

ACCd manipulations altered fungal and plant ACC content.

Consistent with the role of ACCd in cleaving ACC, after 7 days of culture, ACC concentration in overexpression strains *Vd6ADM1* and *Vd6ADM2* was significantly lower than that in 70V-WT, and ACC levels in deletion mutants 70 Δ AD1.4 and 70 Δ AD3.4 were higher than that in 70V-WT (Fig. 10A).

To investigate if *ACCd* manipulations in *V. dahliae* could affect ACC production in tomato plants, the ACC content in the roots and shoots of infected plants was analyzed at 2 and 4 dpi. The ACC content in roots of plants infected with *Vd6ADM1* and *Vd6ADM2* were significantly lower than those in plants infected with 70V-WT and the mock-inoculated controls at both timepoints (Fig. 10B). At 2 dpi, the ACC content in roots of plants infected with 70 Δ AD1.4 and 70 Δ AD3.4 deletion mutants was not significantly different from those observed in 70V-WT and mock-inoculated plants (Fig. 10B). However, at 4 dpi, root ACC levels in 70V-WT and mock-inoculated controls were reduced, while those in 70 Δ AD1.4 and 70 Δ AD3.4 infected plants remained unchanged (Fig. 10B). The ACC content in the shoots of infected plants was also measured at 2 and 4 dpi and showed no significant differences among all treatments (Fig. 10C). ACC was also measured in plants root-drenched with ACC and the amount of ACC in both the roots and shoots of ACC-treated plants was found to be significantly greater compared with the mock-inoculated controls (Supplementary Fig. S6).

Exogenous application of ACC before infection reduced disease symptoms in tomato plants.

To determine whether ACC affects disease development, roots of WT and *Nr*, a mutant insensitive to ethylene, tomato plants were treated with ACC prior to infection with 70V-WT. After ACC treatment, both plants displayed reduced symptoms and slower disease progression compared with untreated plants (Fig. 11A). At 16 dpi, about 23% of the leaves in untreated plants displayed wilt symptoms, while only 10 and 8%, respectively, of the leaves in ACC treated WT and *Nr* plants showed symptoms (Fig. 11B). The relative AUDPC analysis showed similar trends (Fig. 11C).

DISCUSSION

Ethylene performs diverse regulatory functions in plants (Abeles et al. 1992; Johnson and Ecker 1998). Besides its role

in regulating plant development and plant defense responses to abiotic or biotic stresses, ethylene may be employed as a virulence factor by plant pathogens (Abeles et al. 1992; van Loon et al. 2006). Effect of exogenously applied ethylene on virulence and disease development by diverse pathogens compelled inquiries into whether ethylene produced by microbes affect their interaction with plants (Chagué et al. 2006; Cristescu et al. 2002; Cronshaw and Pegg 1976; Marco and Levy 1979; Tzeng and DeVay 1985; Weingart et al. 2001). Tzeng and DeVay (1984) reported that two *V. dahliae* isolates from cotton produced ethylene via the KMBA pathway. Nondefoliating isolate SS4 produced more ethylene than T9, a defoliating isolate, in vitro, but cotton plants inoculated with T9 produced more ethylene than those inoculated with SS4 (Tzeng and DeVay 1984; Wiese and DeVay 1970). Since the source of ethylene in infected plants was unknown, it was not clear about whether ethylene produced by *V. dahliae* contributed to virulence. Here, we manipulated the *ACCd* gene of *V. dahliae* in two ways to investigate its role in growth, development, pathogenesis, and ethylene production. Both mutations

Fig. 4. Quantification of fungal biomass in infected plants. **A**, The amount of fungal DNA in the stem of tomato plants was quantified at 8 and **B**, 21 days after inoculation with 70V-WT, deletion mutants (70 Δ AD1.4 and 70 Δ AD3.4), overexpression strains (*Vd6ADM1* and *Vd6ADM2*), and complemented deletion mutant 70 Δ AD1.2.2. Vertical bars indicate the calculated standard errors of three replicates (pool of three plants per replicate per treatment). Columns with different letters were statistically significantly different according to Tukey's multiple range test at $P \leq 0.05$.

did not significantly affect colony growth, spore production (cultured in SSN), and spore germination (Fig. 1) but suggested the involvement of the gene and ACC in determining disease severity.

The ACC-dependent pathway may only play a minor role in ethylene synthesis in *V. dahliae*.

In phytopathogenic fungi such as *Penicillium digitatum* and *Fusarium oxysporum*, more than one ethylene biosynthetic pathway exists (Billington et al. 1979; Chagué 2010; Fukuda et al. 1986; Hottiger and Boller 1991; Jia et al. 1999). The production of KMBA and the presence of genes encoding enzymes involved in the ACC and 2-OXO dependent ethylene biosynthesis pathways in *V. dahliae* suggested the participation of multiple pathways in synthesizing ethylene. Since ACCd cleaves ACC into α -ketobutyrate and ammonia (Honma and Shimomura 1978), we hypothesized that if the ACC-dependent pathway mainly produces ethylene, overexpression of *ACCd* would lead to decreased ethylene production while its deletion would increase production. Although the amount of ethylene produced by deletion mutants was higher than that by overexpression strains at most timepoints, both mutants produced more ethylene than did 70V-WT (Fig. 9). This result does not support the hypothesis and, instead, suggests that ACC,

α -ketobutyrate, ammonia, or combinations of these metabolites might affect the production of ethylene via other pathways. Transcript levels of the *ACS* and *EFE* genes in 70V-WT, overexpression strain *Vd6ADM1*, and deletion mutant *70 Δ AD3.4* were not significantly different, with the exception of the *ACS* gene at one timepoint (Fig. 9), suggesting that the effect does not occur at the gene-expression level.

Manipulation of the *ACCd* gene affects the virulence of *V. dahliae*.

ACCd has been shown to affect plant health in different ways, depending on where it is expressed. Glick et al. (1998) suggested that some ACC produced by plants is released through roots and is metabolized by ACCd of root colonizing bacteria into α -ketobutyrate and ammonia, potentially lowering the amount of ethylene produced by plants. Several studies have shown that plants inoculated with PGPR strains expressing this enzyme formed longer roots and shoots and became more resistant to a variety of abiotic stresses, including flooding, drought, contamination by heavy metals, and salinity (Cheng et al. 2007; Glick 2014; Grichko et al. 2000; Mayak et al. 2004; Penrose and Glick 2003; Saleem et al. 2007), and exhibited increased resistance against pathogenic nematodes, bacteria, fungi, and oomycetes (Husen et al. 2011; Nascimento

Fig. 5. Disease symptoms in eggplant infected with 70V-WT and 1-aminocyclopropane-1-carboxylic acid deaminase (*ACCd*) overexpression strains. Eggplant was infected with 70V-WT and three overexpression strains. **A**, Symptoms at 14 days after inoculation, **B**, disease progression over time, and **C**, relative area under the disease progress curve. Vertical bars indicate the standard errors based on 20 replicates. All values were subjected to analysis of variance. Different letters in **C** note statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$.

et al. 2013; Toklikishvili et al. 2010). For example, *Pseudomonas fluorescens* CHA0 transformed with *ACCd* of *Pseudomonas putida* UW4 caused enhanced protection of cucumber from *Pythium* spp. and potato from *Pectobacterium carotovorum* subsp. *carotovorum* but failed to protect tomato from *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Wang et al. 2000).

The *ACCd* gene is also present in some plant-pathogenic bacteria, but its role is not clearly understood (Nascimento et al. 2018). *Pseudomonas brassicacearum* expressing *ACCd* caused a wide range of effects on inoculated tomato plants, including growth promotion, neutral or increased disease severity depending on the number of bacterial cells inoculated and environmental conditions (Belimov et al. 2007). Introduction of a bacterial *ACCd* gene into *Agrobacterium tumefaciens* enhanced its virulence, increasing transformation frequency of several plant species (Nonaka and Ezura 2014). Exogenous application of ethylene and ACC caused reduced T-DNA transfer and transformation of melon and tomato (Davis et al. 1992; Ezura et al. 1999), suggesting the role of ACC as a negative regulator of virulence. However, whether *ACCd* or ACC affects virulence (*vir*) gene expression was not determined. Similarly to *A. tumefaciens*, overexpression of the endogenous *ACCd* gene in *V. dahliae* enhanced virulence in tomato and eggplant (Figs. 3 and 5). Overexpression strains, which contained lower levels of ACC than 70V-WT (Fig. 10A), colonized the vascular

system of infected plants to a greater extent than 70V-WT (Fig. 4). In contrast, plants inoculated with deletion mutants *70ΔAD1.4* and *70ΔAD3.4*, which produced higher levels of ACC than 70V-WT (Fig. 10A), showed significantly reduced symptoms than those infected with 70V-WT and overexpression strains (Figs. 3 and 6).

Does ACC act as a potential regulator of virulence and plant defense?

ACC seems to act as a potential negative regulator of virulence in *V. dahliae* but functions as a positive regulator of defense, which was suggested by three lines of evidence: i) *ACCd* deletion mutants that had a higher ACC content than the wild-type strain (Fig. 10A) caused significantly less disease in tomato and eggplant (Figs. 3 and 6), ii) ACC content in roots of plants infected with *ACCd* deletion mutants was higher than that in mock-inoculated and 70V-WT infected plants (Fig. 10B), and iii) ACC treatment of plants significantly reduced disease in both WT and *Nr* tomato plants (Fig. 11). It is also possible that the enhanced virulence of overexpression strains was caused by the increased degradation of the amount of plant ACC. Application of ACC to *Arabidopsis thaliana* plants resulted in enhanced resistance against *Pseudomonas syringae* pv. *tomato* (Pieterse et al. 1998, 2000; van Wees et al. 1999), also supporting the role of ACC as a positive regulator of defense. Other studies also suggested that ACC could be more

Fig. 6. Disease symptoms in eggplant infected with 70V-WT and 1-aminocyclopropane-1-carboxylic acid deaminase (*ACCd*) deletion mutants. Eggplant was infected with 70V-WT, two deletion mutants (*70ΔAD1.4* and *70ΔAD3.4*), and complemented deletion mutant *70CAD1.2.2*. **A**, Symptoms at 13 days after inoculation, **B**, disease progression over time, and **C**, relative area under the disease progress curve. Vertical bars indicate the standard errors based on 20 replicates. All values were subjected to analysis of variance. Different letters in C note statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$.

than just a precursor for ethylene but acts as a signaling molecule (Tsang et al. 2011; Tsuchisaka et al. 2009; Van de Poel and Van Der Straeten 2014; Xu et al. 2008; Yoon and Kieber 2013). Root cell elongation in *Arabidopsis thaliana* appears to be controlled by ACC, which is ethylene-independent (Tsang et al. 2011; Xu et al. 2008). ACC also plays a possible role in phytohormone crosstalk (Nascimento et al. 2018; Van de Poel and Van Der Straeten 2014).

The findings of the present study provide evidence for the role of the *ACCd* gene in the pathogenicity of soil-borne fungus *V. dahliae*. Contrary to the plant growth-promoting function of *ACCd* conferred by PGPR, overexpression of the *ACCd* gene in *V. dahliae* resulted in enhanced virulence, demonstrating a different role of this gene in phytopathogenic fungi. Given the fact that even subtle changes in the production of plant hormones can affect plant-microbe interactions differently, further investigation is needed into how ACC is produced on both the fungal and plant sides and how its changes influence the outcome of infection.

MATERIALS AND METHODS

Fungal isolates and culture conditions.

For long-term storage, 70V-WT and its transformants were preserved as a conidial suspension (4×10^7 conidia per milliliter) in 25% glycerol at -80°C . They were activated by culturing on PDA (Oxoid) for 5 days at 25°C . Transformants were cultured on a medium supplemented with 50 μg of geneticin (Life Technologies) or hygromycin B (Duchefa Biochemies) per milliliter.

Minimal medium (Puhalla and Mayfield 1974) was used for measuring colony growth. Conidial production at 25°C was assessed under two culture conditions: i) culturing on PDA for 20 days and ii) shaking (140 rpm) in liquid SSN medium (Sinha and Wood 1968). Spores produced in SSN medium were used to observe spore germination and infect plants. Ethylene production was measured after culturing in liquid Czapek-Dox medium (Oxoid) supplemented with 10 mM methionine (Sigma-Aldrich) by shaking (140 rpm) at 25°C .

Nucleic acid manipulation.

Fungal genomic DNA was extracted as previously reported (Cary et al. 2009) with a few modifications. After culturing on PDA for 7 days at 25°C , mycelia were scraped off and were placed in a 1.5-ml tube. The mycelia were macerated, using a mini plastic pestle after adding 700 μl of LETS buffer (0.1 M LiCl, 20 mM EDTA, 10 mM Tris-HCl), were mixed by inversion, and were incubated at room temperature for 5 min. After adding 700 μl of phenol/chloroform/isoamyl alcohol (25:24:1), they were mixed by inversion and were centrifuged at $16,000 \times g$ at 4°C for 15 min. The supernatant was transferred to a new tube, and 1 ml of 95% ethanol was added. Nucleic acids were precipitated by centrifugation at $16,000 \times g$ for 10 min at room temperature, were washed with 500 μl of 70% ethanol, were dried at room temperature for 5 min, and were resuspended in 30 μl of distilled water. For quantification of fungal biomass in tomato stems, total genomic DNA was isolated as described by Dellaporta et al. (1983).

DNA amplification for gene manipulation was performed using Kapa HiFi DNA polymerase (Kapa Biosystems). PCR

Fig. 7. Microscopic observation of tomato root colonization by 70V-WTR, *Vd6ADM1R*, *70ΔAD1.4R*, and *70ΔAD3.4R*. **A to D**, Root surface was imaged at 1 and **E to H**, 5 days postinoculation. Magnification used for **A to E** was 200 $\times$, whereas for **F, G**, and **H**, it was 400 $\times$.

conditions included i) one cycle of 95°C for 3 min, ii) 35 cycles of 98°C for 20 s, 72°C for 15 s, and 72°C for 30 s, and iii) one cycle of 72°C for 1 min. Transformants were screened by PCR with Kapa polymerase (Kapa Biosystems), using the following conditions: i) one cycle of 95°C for 3 min, ii) 35 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 3 min, and iii) one cycle of 72°C for 1 min. *Escherichia coli* DH5a was used for plasmid propagation. Plasmid DNA isolation, digestion with restriction enzymes, ligation, and *E. coli* transformation were performed as described by Sambrook et al. (1989).

Total RNA was extracted from mycelia ground in the presence of liquid nitrogen, using RNAiso plus reagent (Takara). RNA samples were treated with DNase (HT Biotechnology) to eliminate DNA contamination. First-strand complementary (c)DNAs were synthesized using the Prime-Script RT kit (Takara).

Vector construction and fungal transformation.

A construct for overexpressing *ACCd* was prepared. Primers ACD_OX_F and ACD_OS_R (Table 1) were used to amplify the ORF of *ACCd* (1,038 bp). ACD_OX_F and ACD_OS_R contained the *Xba*I and *Sph*I restriction sites, respectively, at the 5' end to facilitate cloning of the ORF between the *Magnaporthe oryzae* ribosomal protein promoter and the *Neurospora crassa*

β -tubulin terminator in vector pSK1226. The resulting construct (1,835 bp) was cloned between the *Eco*RI and *Hind*III sites of binary vector pSK879, which carries the geneticin resistance cassette, to produce p1226VdAD. This plasmid was introduced into *A. tumefaciens* AGL1 for fungal transformation. Transformation of 70V-WT was performed as described by Mullins et al. (2001) with the following modifications. i) Cocultivation of spores and *A. tumefaciens* cells was performed on a positively charged nylon membrane (Porablot NY Plus, Macherey-Nagel), and ii) PDA amended with 50 μ g of geneticin or hygromycin B per milliliter, 100 μ g of moxalactam per milliliter, and 200 μ M cefotaxime was used to select transformants. Transformants were purified via single-spore isolation.

Construction of a mutant allele for disrupting *ACCd* was done. Primer pairs ACD_EX-F1/ACD_K-R1 and ACD_X-F2/ACD_H-R2 (Table 1) were used to amplify the promoter (827 bp) and terminator (864 bp) regions of the gene, respectively. The amplified regions were cloned into the 5' and 3' ends of the geneticin resistance gene in vector pSK666 (Tzima et al. 2010). The resulting mutant allele (3,171 bp) was amplified with primers ACD-P_F1 and ACD_H-R2 and was cloned between the *Pst*I and *Hind*III sites of binary vector pGKO2 (Khang et al. 2005), which carries the herpes simplex virus thymidine kinase gene as a negative selection marker

Fig. 8. Disease severity of tomato plants infected with 70V-WT and 1-aminocyclopropane-1-carboxylic acid deaminase (*ACCd*) mutants in a hydroponic system. **A**, Tomato plants at 17 days after inoculation with 70V-WTR, *Vd6ADM1R*, and *70ΔAD3.4R*. **B**, Disease progression at 14 and 17 days postinoculation and **C**, the amount of disease expressed as relative area under the disease progress curve. Vertical bars indicate the calculated standard errors based on six replicates. All values were subjected to analysis of variance. Different letters in **B** and **C** indicate statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$.

against ectopic transformants. The resulting plasmid pGVdAD in *A. tumefaciens* AGL1 was used for disrupting *ACCd*. Transformants were selected as described above except that 50 μ M 5-fluoro-2-deoxyuridine (Sigma-Aldrich) was used to select against ectopic transformants (Khang et al. 2005).

For complementation, a 2,728-bp fragment that covers the *ACCd* gene, including the 5' (1,000 bp) and 3' (600 bp) regions flanking the ORF, was amplified from 70V-WT, using primers ACD_X-C-F1 and ACD_H-R2 (Table 1). The resulting PCR product was cloned between the *Xba*I and *Hind*III sites of pSK561 (Tzima et al. 2010), a binary vector that carries a hygromycin resistance gene cassette.

Molecular characterization of resulting mutants.

Deletion and complemented mutants were identified by PCR, using primers ACD_EX-F1 and ACD_H-R2 (Table 1). Transcripts from *ACCd* in deletion mutants were determined via reverse transcription (RT)-PCR, using ACD_OX_F and ACD_OS_R primers on cDNAs. *ACCd* transcripts in overexpression

strains and transcripts from other genes involved in ethylene biosynthesis were measured by real-time qRT-PCR analysis. The *V. dahliae* β -tubulin gene was used as an internal standard. Primers used are listed in Table 1. Averaged threshold cycle values derived from three independent biological samples were calculated for each gene (Pfaffl 2001).

Analysis of growth and developmental characteristics.

For each strain, a 20- μ l conidial suspension (1×10^6 conidia per milliliter) was spot-inoculated on minimal medium (five plates per strain) and was incubated at 25°C. Colony diameter and morphology were examined at 8, 10, 12, 14, 16, and 21 dpi. For conidial production, individual strains were cultured on PDA at 25°C for 20 days (three plates per strain). After adding 4 ml of sterilized water to each plate, the surface was rubbed using a sterile swab and plates were shaken gently to release conidia. A hemocytometer was used to count the number of conidia in the 20- μ l conidial suspension, using a light microscope (Axiostar plus; Carl Zeiss International). Results were presented as the

Fig. 9. Ethylene production and expression of two genes involved in ethylene biosynthesis. **A**, Ethylene production over time by 70V-WT, overexpression strain *Vd6ADM1*, and deletion mutant *70 Δ AD3.4*. **B**, Transcript levels of the ACC synthase and **C**, ethylene-forming enzyme genes over time. Vertical bars indicate the calculated standard errors of three replicates. All values were subjected to analysis of variance. For data that showed a significance level of $P \leq 0.05$, Tukey's post hoc analysis was also performed.

number of conidia produced per square centimeter of individual colonies.

Conidial production in liquid SSN medium was quantified by inoculating a 300- μ l spore suspension (1×10^6 conidia per milliliter) into 100 ml of medium in a 250-ml Erlenmeyer flask (three flasks per strain) and shaking (150 rpm) at 25°C. After 7 days, the number of spores produced was counted as described above. The spore germination rate was determined by placing a 10- μ l spore suspension (1×10^6 conidia per milliliter) on sterile glass slides (three slides per strain) and, subsequently, observing approximately 100 conidia per slide after 12, 16, and 20 h of incubation at 25°C under 100% humidity. Individual strains were cultured on PDA supplemented with 0, 10, 100, and 500 μ M ACC to evaluate the effect of ACC on colony growth and spore production.

Conditions for plant growth and infection.

Seeds of two tomato (*Solanum lycopersicum*) lines, including Nr (obtained from the Tomato Genetics Resource Center at the University of California, Davis) and cultivar Ailsa Craig, and eggplant (*Solanum melongena* cv. Black Beauty) were stored at 4°C. For infection assays in a growth room, seedlings grown in plastic trays were transplanted to 10.5 cm-diameter pots (Teku VCG), with each pot containing approximately 450 ml of soil (Plantaflor Potting Soil). These pots were placed in a growth room set at 65 to 70% relative humidity, 25°C, and a 16-h photoperiod, with a photon flux density of $200 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were watered every 2 days to maintain approximately 70% soil humidity. For infection assays in a greenhouse, seeds were sown directly in the same pots and plants were watered as needed.

Five-day-old fungal culture in SSN medium was passed through cheesecloth to remove mycelia, and the conidial concentration was adjusted to 1×10^7 conidia per milliliter. Each seedling was inoculated with a 10-ml conidial suspension via root drenching when they reached the four-leaf (tomato) or two-leaf stage (eggplant) stage.

Disease severity was measured, based on the number of wilted leaves, as a percentage of the total number of leaves for each plant. Symptoms were recorded for up to 19 days and were plotted over time to generate the AUDPC, using the trapezoidal integration method (Campbell and Madden 1990). Disease was expressed as a percentage of the maximum possible area for the whole period of each experiment, referred to as relative AUDPC. Plant height and fresh weight were also measured. The experiments were repeated two times with 12 (growth room) and five (greenhouse) plants per experiment for tomato and 10 seedlings per experiment for eggplant.

The effect of ACC on disease development was evaluated by applying a solution of 100 μ M ACC by root drenching (10 ml per plant) two-week-old plants 12 h prior to fungal inoculation. Pathogen inoculation was performed as described above. Six plants per treatment were used, and the experiment was repeated twice. Symptoms were recorded for up to 20 days.

Hydroponically grown plants were also infected to observe root attachment and colonization by individual *V. dahliae* strains. Tomato seedlings grown in plastic trays were transplanted to 1-liter containers (one plant per container), containing complete nutrient solution, at the first-leaf stage. Containers were covered with aluminum foil (Fig. 8) to prevent algae growth and temperature fluctuation. Plants were grown for 2 weeks to allow recovery from transplanting stress. Complete nutrient solution consisted of the following minerals: $\text{NO}_3^- = 14.29$, $\text{K} = 8.43$, $\text{PO}_4^{3-} = 1.07$, $\text{Ca} = 6.96$, $\text{Mg} = 2.93$, $\text{SO}_4^{2-} = 1.56$, $\text{Na} = 1.91$ mmol per liter, respectively, and $\text{B} = 25.00$, $\text{Fe} = 20.85$, $\text{Mn} = 10.00$, $\text{Cu} = 0.77$, $\text{Zn} = 3.85$, and $\text{Mo} =$

0.52 μmol per liter, respectively (Marinou et al. 2013). At the end of the second week, six plants were inoculated with conidia at the final concentration of 1×10^6 conidia per milliliter. Additional nutrient solution was added weekly. The pH and

Fig. 10. Effect of 1-aminocyclopropane-1-carboxylic acid deaminase (*ACCd*) mutations on the amount of ACC in *V. dahliae* and infected tomato. **A**, The amounts of ACC in 70V-WT, *ACCd* deletion mutants (70 Δ AD1.4 and 70 Δ AD3.4), overexpression strains (Vd6ADM1 and Vd6ADM2), and complemented deletion mutant 70CAD1.2.2, after 7 days of culture. **B**, The amount of ACC in roots and **C**, shoots of mock-inoculated tomato plants and plants infected with 70V-WT, Vd6ADM1, Vd6ADM2, 70 Δ AD1.4, and 70 Δ AD3.4 mutants. Vertical bars indicate the calculated standard errors of three replicates. All values were subjected to analysis of variance. For data that showed a significance level of $P \leq 0.05$, Tukey's post hoc analysis was also performed.

electric conductivity of the solution were maintained at 5.8 and 2.1 mS cm⁻¹, respectively. Due to the alkalinity of water used, the pH was measured every third day and was adjusted if needed, using H₂SO₄ (5% vol/vol). Symptoms were recorded at 14 and 17dpi.

Quantification of fungal biomass in planta.

Fungal biomass in infected plants was quantified by qPCR. For each biological sample (pool of three plants per treatment), the above-ground parts of inoculated plants were collected and were ground to a fine powder, using an autoclaved mortar and

Fig. 11. Effect of 1-aminocyclopropane-1-carboxylic acid deaminase (ACC) treatment on disease development in tomato plants infected with 70V-WT. Plants were treated with 100 μ M ACC or water by root drenching (10 ml per plant) 12 h before fungal inoculation. **A**, Symptoms at 18 days after inoculation, **B**, disease progress curves, and **C**, the amount of disease expressed as relative area under the disease progress curve. Vertical bars indicate the calculated standard errors of 12 replicates. All values were subjected to analysis of variance. Different letters in C note statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$.

pestle, in the presence of liquid nitrogen. DNA was extracted as described above, was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific), and was adjusted to 100 ng μL^{-1} . qPCR reactions were performed using the IQ5 thermocycler (Bio-Rad) and the Kapa SYBR FAST qPCR kit (Kapa Biosystems). Primers ITS1-F and ST-VE1-R were used for quantifying *V. dahliae*, and the *Solanum lycopersicum* β -tubulin gene was used to normalize the amount of fungal DNA in different samples (Table 1).

Microscopic observation of root infection.

To compare root attachment by conidia and colonization during early stages of infection between 70V-WT and its mutants, hydroponically grown tomato plants were infected with spores of 70V-WTR, *Vd6ADM1R*, *70 Δ AD1.4R*, and *70 Δ AD3.4R*, transformants of 70V-WT, *Vd6ADM1*, *70 Δ AD1.4*, and *70 Δ AD3.4* with the *AsRed* gene under the control of the *Fusarium verticillioides* elongation factor 1 α promoter (Kim et al. 2011). At 1 and 5 dpi, plants were removed, were washed with distilled water, and the root surface was imaged using a Zeiss Axio Imager D1 epifluorescence microscope equipped with a Zeiss AXIO Cam MRc5 camera (Carl Zeiss International). Filter sets used for observing *AsRed* were 576 nm (excitation) and 592 nm (emission). Collected images were stored as jpeg files and were processed using Adobe Photoshop CS software (Adobe Systems Inc.).

Ethylene production.

Individual strains were cultured in a 50-ml Erlenmeyer flask containing 20 ml of Czapek Dox broth supplemented with 10 mM methionine (all strains) and 50 μg of geneticin or hygromycin (only for transformants) per milliliter. Flasks inoculated with 10^6 conidia were placed in an orbital shaker (150 rpm) at 25°C. Before ethylene measurement, each culture flask was flushed with air to eliminate any accumulated ethylene. After sealing the flask with a rubber stopper for 1 h, a syringe was used to collect headspace air. The amount of ethylene in a 1-ml air sample was measured using a 7890A Agilent gas chromatographer (Agilent Technologies) equipped with a flame ionization detector and an Agilent stainless steel column (2 mm id $\times$ 2 m) packed with Porapak Q 80/100 mesh, as described by Petrou et al. (2013), with minor modifications.

Helium was used as a carrier gas under a constant inlet pressure of 20 psi. Inlet and oven temperatures were maintained at 80°C, and the detector temperature was set at 200°C. The total run time was 3 min, with ethylene being detected at 1.8 min. Calibration was performed using ethylene at 1 ppm. After collecting headspace air, mycelia and conidia in each flask were harvested by centrifugation and were lyophilized to determine dry weight. Ethylene production rate was calculated as nanoliters of ethylene produced per hour per gram. The experiment was repeated twice, and the data shown correspond to the averages of three independent biological samples.

ACC measurement in fungal and plant tissues.

For quantifying the amount of ACC in *V. dahliae* 70V-WT and its *ACCd* transformants, they were cultured in liquid SSN medium at 25°C by shaking (140 rpm) for 7 days, and harvested spores and mycelia were freeze-dried for 48 h. The amount of ACC was also determined in roots and shoots of tomato plants i) inoculated with 70V-WT and *ACCd* transformants as described above and ii) root-drenched with 10 ml of 100 μM ACC. Plants were uprooted 2 and 4 days after infection and 12 h after ACC treatment, respectively, were rinsed thoroughly with sterile, deionized water, and were lyophilized for 5 days. All samples were immediately frozen by immersing them in liquid nitrogen before freeze-drying. The amount of ACC was measured using the method described by Lizada and Yang (1979), in which the amount of ACC formed was measured via its conversion to ethylene. Ethylene was determined by gas chromatography as described above. Fungal ACC contents shown correspond to the averages of three independent biological samples. For plant ACC determination, three biological samples (pool of two plants per treatment) were used.

Statistical analysis.

Statistical analyses were performed using R package (R 2016, version 3.3.1). Data obtained in all experiments were subjected to analysis of variance, and significant differences between individual means were then determined using Tukey's post hoc pairwise comparison test at the 5% confidence level. Dunnett's multiple comparisons test was used for analyzing gene expression data.

Table 1. Primers used in this study

Name	Sequence ^a	Target ^b	Reference
ACD_EX-F1	5'-ATGAATTCATTCAGGCGGTATCCAGGTCAACAACA-3'	<i>ACCd</i> (VDAG_10392)	This study
ACD_P-F1	5'-ATCTGCAGAGGCGGTATCCAGGTCAACAACA-3'		This study
ACD_K-R1	5'-AA ^{GGT} ACCGGGGGTTCATCCATGCCTATCACT-3'		This study
ACD_X-F2	5'-AA ^{TCT} AGCGGAGGGCGACATCACA-3'		This study
ACD_H-R2	5'-TTTAA ^{GCTT} CATCCTCCACCTGGGCTCTTTC-3'		This study
ACD_X-C-F1	5'-ATTCTAGAAGGCGGTATCCAGGTCAACAACA-3'		This study
ACD_OX_F	5'-CCACGATCTAGACCACACCACCACCATC-3'		This study
ACD_OS_R	5'-CCATT ^{TGC} ATGCATTGACCCTCGCCCTCTC-3'		This study
ACD_Rt1_F	5'-GCCAAACGCGAGGACTGC-3'		This study
ACD_Rt1_R	5'-CACGCCGCCAATGCTGAC-3'		This study
ACS_Rt1_F	5'-AACTCGGGCGCCACAACATT-3'	<i>ACS</i> (VDAG_05021.1)	This study
ACS_Rt1_R	5'-ACAGGGCGCGGAGCAAGGAT-3'		This study
EFE_RT1_F	5'-ACGACGTCGGTGCCCTGTTTGTTC-3'	<i>Ethylene/succinate forming enzyme</i> (VDAG_09492.1)	This study
EFE_RT1_R	5'-TGCGCTGGGGGTAGTTCTCTGATGA-3'		This study
ITS1-F	5'-AAAGTTTAAATGGTTCGCTAAGA-3'	<i>ITS1</i>	Gardes and Bruns 1993
ST-VE1-R	5'-CTTGGTCATTTAGAGGAAGTAA-3'	18S rDNA	Lievens et al. 2006
VdBt-F	5'-TTCCCCCGTCTCCACTTCTTCATG-3'	β -tubulin (VDAG_10074.1)	Tzima et al. 2012
VdBt-R	5'-GACAAGATCGTTTCATGTTGGACTC-3'		
LeTUB-F	5'-GATTTGCCCACTAACCTCTCGT-3'	β -tubulin gene (DQ205342)	Pantelides et al. 2010
LeTUB-R	5'-ACCTCCTTTGTGCTCATCTTACCC-3'		

^a Underlined sequences correspond to restriction enzyme sites introduced to facilitate subsequent cloning.

^b With the exception of LeTUB-F and LeTUB-R, all primers correspond to *Verticillium dahliae* genes.

ACKNOWLEDGMENTS

We acknowledge N. Tzortzakakis and A. Chrysargyris for helping with the hydroponic infection assays and A. Triantafyllopoulou for supporting the greenhouse work.

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