VdThit, a Thiamine Transport Protein, Is Required for Pathogenicity of the Vascular Pathogen *Verticillium dahliae*

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Verticillium dahliae causes a serious wilt disease of important crops and is difficult to control. Few plasma-membrane transport proteins for nutrient acquisition have been identified for this fungus, and their involvement in the disease process is unknown. Here, a plasma-membrane protein, the *V. dahliae* thiamine transporter protein VdThit, was characterized functionally by deletion of the *VdThit* gene in *V. dahliae*. Disruption strains were viable, but growth and conidial germination and production were reduced and virulence was impaired. Interestingly, by supplementing exogenous thiamine, growth, conidiation, and virulence of the *VdThit* mutants were partially restored. Stress-tolerance assays showed that the *VdThit* mutant strains were markedly more susceptible to oxidative stress and UV damage. High-pressure liquid chromatography-mass spectrometry (HPLC-MS) and gas chromatography-mass spectrometry (GC-MS) analyses showed low levels of pyruvate metabolism intermediates acetoin and acetyl coenzyme A (acetyl-CoA) in the *VdThit* mutant strains, suggesting that pyruvate metabolism was suppressed. Expression analysis of *VdThit* confirmed the importance of VdThit in vegetative growth, reproduction, and invasive hyphal growth. Furthermore, a green fluorescent protein (GFP)-labeled *VdThit* mutant (*VdThit-7-GFP*) was suppressed in initial infection and root colonization, as viewed with light microscopy. Together, these results showed that VdThit plays an indispensable role in the pathogenicity of *V. dahliae*.

Verticillium dahliae is a soil-borne, filamentous fungal pathogen that causes a vascular wilt in annual and perennial herbaceous plants and woody angiosperms (Hillocks 1992; Klosterman et al. 2009) and several billions of dollars of annual crop losses throughout the world (Cai et al. 2009; Fradin and Thomma 2006; Klosterman et al. 2009). As such, *V. dahliae* has been likened to a bottleneck in commercial crop productivity; its microsclerotia can survive for many years in soil and dead plant tissues, making the disease difficult to control with pesticides (Cai et al. 2009; Klosterman et al. 2011). Gaining insight into the signaling pathways and molecular machinery responsible for pathogenicity of the fungus should inform new strategies to

prevent infection by *V. dahliae* (Fradin and Thomma 2006; Klosterman et al. 2011). However, only a few studies have focused on pathogenicity-related molecular mechanisms in the fungus and the identification or functional characterization of resistance-related molecular mechanisms in the host (Luo et al. 2014). Thus, characterization of fungal development and pathogenicity-related molecular mechanisms is urgently needed for developing strategies to control the fungus.

The long-lived microsclerotia of *V. dahliae* are induced to germinate in the soil by plant root exudates and hyphae then colonize the root surface (Pegg and Brady 2002). Hyphae penetrate the epidermal cell layer without forming appressoria and grow intracellularly and intercellularly through the root cortex, entering the xylem vessels, changing the endogenous environment of the plant cells, and forming a parasitic environment (Klosterman et al. 2009; Pegg and Brady 2002; Reusche et al. 2012; Zhao et al. 2014). In this parasitic environment, the hyphae take up plant nutrients to colonize the host tissue further and eventually conidiate (Fradin and Thomma 2006). The conidia are transported within the xylem vessels in stems and leaves through the plant by its transpiration flow (Eynck et al. 2007). When the hyphae colonize the plant's entire vascular system, flow in small areas can be disrupted, resulting in the wilt phenotype—plant stunting and wilting, vascular browning, and foliar chlorosis and necrosis (Pegg and Brady 2002). Finally, microsclerotia form in the parenchyma of the dying stem and are released into the soil to complete the life cycle (Klosterman et al. 2009).

Our knowledge of the mechanism of pathogenesis of *V. dahliae*, however, has remained at a preliminary exploratory stage. Secreted pathogenic proteins are known to play important roles during the initial infection process, which includes degradation of the host cell walls, scavenging of reactive oxygen species (ROS) generated by the host, stress responses, and protein metabolism (Chu et al. 2015; Hogenhout et al. 2009). How nutrients from the host plant are absorbed and ultimately enhance virulence when *V. dahliae* colonizes host plants is still unknown. Therefore, exploring *V. dahliae* pathogenesis and finding the key protein factors involved in fungal metabolism during invasion and colonization are very important.

The soluble B vitamin (B1) thiamine is essential for all organisms as a key cofactor of enzymes with an important role in carbohydrate metabolism (Butterworth 2003; Rapala-Kozik 2011). Only bacteria, fungi, and plants can naturally synthesize thiamine; animals and humans must obtain their thiamine from food (Friedrich 1987). For fungi, thiamine is also essential for vegetative growth, development, sporulation, host invasion, and adaptation to environmental stress (Hohmann and Meacock 1998; Rapala-Kozik 2011).

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The thiamine molecule consists of a thiazole and a pyrimidine moiety, which are synthesized independently at different branches of the thiamine biosynthetic pathway (Hohmann and Meacock 1998; Young 1986). After their independent formation, 2-methyl-4-amino-5-hydroxymethylpyrimidine pyrophosphate (HMP-PP) and 4-methyl-5-(2-hydroxy-ethyl) thiazole monophosphate (TH-P) are condensed to form thiamine monophosphate, which is finally pyrophosphorylated to the active thiamine pyrophosphate (TPP) (Mojzita and Hohmann 2006). Fungal organisms either take up thiamine from the medium or synthesize it *de novo*, but they primarily acquire exogenous thiamine, using a thiamine transporter protein in their plasma membrane (Mojzita and Hohmann 2006). In *Saccharomyces cerevisiae*, the thiamine transporter protein is Thi7p (Thi10) (Enjo et al. 1997; Singleton 1997).

The thiamine transporter protein is a high-affinity transporter for thiamine, belonging to the reduced folate carrier transporter family, and transports extracellular thiamine into the cell (Dutta et al. 1999; Lagarde et al. 2004; Larkin et al. 2012). Targeted deletion of the *SLC19a2* gene (encoding a high-affinity thiamine transporter) in mice on a thiamine-free diet led to diabetes, sensorineural deafness, and megaloblastosis (Oishi et al. 2002). However, the thiamine transporter protein itself has not yet been reported in *V. dahliae* and other fungi, except for *S. cerevisiae*. Additionally, how a limited nutrient supply is taken up by plasma-membrane transporter proteins and how it contributes to pathogenicity are poorly understood in this

filamentous fungus. In an effort to better understand *V. dahliae* virulence and pathogenesis, a *V. dahliae* Δ *Thit* deletion mutant strain was generated. The growth and development, its infection and colonization capacity, the pyruvate metabolism intermediates, stress tolerance, and virulence of this strain were characterized. Characterization of the mutant suggested that the *V. dahliae* Thit (VdThit) protein plays an important role in the pathogenicity of *V. dahliae*.

RESULTS

Deletion and complementation of *VdThit* in *V. dahliae*.

The *VdThit* gene in wild-type strain 991 of *V. dahliae* was replaced with a neomycin phosphotransferase resistance gene (*neo*) cassette that conferred neomycin resistance. Twelve transformants of *V. dahliae* 991 with neomycin resistance were analyzed by polymerase chain reaction (PCR), and five showed that the *VdThit* gene was replaced through homologous recombination. Two independent *VdThit* deletion mutants (*VdThit-2* and *VdThit-7*) were used for further analysis (Fig. 1B and C). Additionally, to complement the *VdThit* gene, a functional copy of *VdThit* containing the promoter *trpC* and terminator *TtrpC* was successfully reintroduced in the two *VdThit* mutants (*VdThit-2* and *VdThit-7*) by PCR (Fig. 1D). *VdThit* transcription was restored in the resulting complementation strains (*VdThit-2-com* and *VdThit-7-com*).

Fig. 1. *VdThit* gene disruption in *Verticillium dahliae* and complementation of the *VdThit* mutant strains *VdThit-2* and *VdThit-7*. **A**, Disruption scheme used for *VdThit* in the wild-type strain. **B** and **C**, Confirmation of gene disruption by polymerase chain reaction (PCR). Genomic DNA from wild type (*Vd-wt*) and the *VdThit* mutant strains were used as templates for amplification with primer pairs Thit-J-F and Thit-J-R (**B**) and neo-J-F and neo-J-R (**C**). A 1.2-kb fragment was amplified from *Vd-wt* with primer pair Thit-J-F and Thit-J-R (**B**) and a 2.0-kb fragment was amplified using primer pair neo-J-F and neo-J-R (**C**) from the *VdThit* mutant strains. **D**, Confirmation of gene complementation of the *VdThit* mutant strains (*VdThit-2-com* and *VdThit-7-com*) by PCR with primer pair Thit-J-F and Thit-J-R; a 1.2-kb fragment was amplified.

Mycelial radial growth, conidiation, and conidial germination were significantly reduced in the *VdΔThit* mutants.

The role of *VdThit* in mycelial growth was studied, using two *VdΔThit* mutant strains, *VdΔThit-2* and *VdΔThit-7*, grown on agar minimal medium (MM) with different carbon sources (sucrose, pectin, starch, galactose, or xylose) and compared with that in the *V. dahliae* wild-type strain and complementation *VdΔThit* mutant strains *VdΔThit-2-com* and *VdΔThit-7-com*. Colony diameter of the *VdΔThit* mutants was less than

that of the parental strain on MM plates with different carbon sources. Colony diameter of the *VdΔThit* mutants on different carbon sources (sucrose, pectin, starch, galactose, and xylose) was reduced to 68, 62, 53, 48, and 50%, respectively, of the level of the wild type (Fig. 2A and B). Likewise, conidial production of the *VdΔThit* mutants was significantly reduced compared with the wild-type strain and complementation *VdΔThit* mutant strains cultured on Czapek–Dox agar plates (Fig. 3A). In addition, conidia of the *VdΔThit* mutant strains germinated more slowly than those of the wild-type strain and

Fig. 2. Mycelial growth of *VdΔThit*. **A**, Growth of *Verticillium dahliae* wild-type *VdΔThi* mutant strains (*VdΔThit-2* and *VdΔThit-7*) and complementation *VdΔThit* mutant (*VdΔThit-2-com* and *VdΔThit-7-com*) strains on minimal medium agar with different carbon sources (per liter, 10 g of xylose, galactose, pectin, or starch or 30 g of sucrose). Colony diameters were measured after 3, 6, 9, 12, and 15 days. **B**, Colony morphology was observed at 15 days. Values represent the mean $\pm$ standard deviation from three independent replicates.

complementation *VdΔThit* mutant strains; after 30 h of incubation in liquid complete medium (CM), only about 36% of the conidia of the *VdΔThit* mutant strains had germinated, compared with 90% of the wild-type conidia (Fig. 3B). These results suggested that growth and development in the *VdΔThit* mutant strains were impaired remarkably under these conditions.

Loss of VdThit resulted in reduced stress tolerance.

Thiamine plays an important role in tolerance to various abiotic stresses (Rapala-Kozik et al. 2012). To determine whether the *VdThit* gene was involved in abiotic stress tolerance, various types of abiotic stress (UV irradiation, oxidative stress, and osmotic stress) were tested for their effects on the relative growth inhibition of the *VdΔThit* mutant strains. To ascertain the contribution of *VdThit* to UV-irradiation resistance, the viability of spores was assessed with a vital strain. Significantly fewer colonies of the *VdΔThit* mutant strains survived

after UV exposure compared with those of the wild-type strain and complemented *VdΔThit* mutant strains ($P < 0.01$). Specifically, only 1.2% of the UV-treated *VdΔThit* mutant colonies survived UV irradiation at 50 J m^{-2} , compared with 11.5% of the wild-type colonies (Fig. 4A). Our results for *VdΔThit* mutant strains are similar to previous studies on *VdΔThi4* (involved in thiazole biosynthesis of the de novo thiamine synthesis pathway) mutant strains that cannot repair damage caused by UV (Hoppenau et al. 2014).

The contribution of *VdThit* to strain tolerance to various stresses was investigated by growing the *VdΔThit* mutant strains, wild-type strain, and complemented strains on Czapek-Dox agar plates with $10 \mu\text{g}$ of menadione per liter (oxidative stress) or 1 M sorbitol (osmotic stress) and comparing subsequent growth of the strains. The *VdΔThit* mutant strains grew much slower than the wild-type strain and the complemented *VdΔThit* mutant strains on menadione (Fig. 4B and C). However, when the *VdΔThit* mutant strains were compared with *V. dahlia* wild-type strains on sorbitol, the growth of all strains was inhibited to an equivalent degree (Supplementary Fig. S1). In short, our results showed that the *VdΔThit* mutant strains were more susceptible to oxidative stress than the other strains (Fig. 4).

VdThit was required for virulence.

We then assessed the role of *VdThit* in virulence of the *VdΔThit* mutant strains in *Nicotiana benthamiana* by dipping intact roots in a conidial suspension of the wild-type strain, the complementation *VdΔThit* mutant strains, or the *VdΔThit* mutant strains. Interestingly, after 8, 10, and 12 days postinoculation (dpi), the wilting phenotype was significantly reduced in plants inoculated with the *VdΔThit* mutant strains compared with the wild-type strain and complementation *VdΔThit* mutant strains. The plants inoculated with the wild-type strain or complemented *VdΔThit* mutant strains exhibited typical symptoms, including stunting, chlorosis, wilting, and death, at 12 dpi. In contrast, those inoculated with the *VdΔThit* mutant strains showed only a mild interveinal chlorosis of leaves, which never became necrotic (Fig. 5A and C). Fewer fungal colonies grew from excised stem tissue sections of plants inoculated with the *VdΔThit* mutant strains than with the wild-type strain or the complemented *VdΔThit* mutant strains (Fig. 5B). Furthermore, less fungal DNA was amplified from infected plants inoculated with the *VdΔThit* mutant strains than with the wild-type strain or the complemented *VdΔThit* mutant strains (Fig. 5D). These results suggested that the *VdThit* mutant strains were defective in virulence.

Pyruvate metabolism intermediates in the VdThit mutant strains decreased, as shown by mass spectrometry analyses.

Because the cofactor thiamine diphosphate TPP is required for the activity of pyruvate decarboxylase in the Krebs cycle and for pyruvate dehydrogenase during pyruvate metabolism (Lu et al. 2000; Moe et al. 1974; Mojzita and Hohmann 2006), we used gas chromatography-mass spectrometry (GC-MS) and high-pressure liquid chromatography-mass spectrometry (HPLC-MS) to ascertain whether the lowered virulence and the stress phenotypes of the *VdΔThit* mutant strains and the depletion of TPP were also associated with restricted pyruvate dehydrogenase and pyruvate decarboxylase by analyzing the Krebs cycle and pyruvate metabolism intermediates. These analyses indicated that acetoin in pyruvate metabolism (Fig. 6) and acetylcoenzyme A (acetyl-CoA) in the Krebs cycle were significantly reduced in the *VdΔThit* mutant strains compared with those in the *V. dahlia* wild type (Fig. 7). We thus concluded that the TPP depletion in the *VdΔThit* mutant strains was confined to the Krebs cycle and pyruvate metabolism, restricting their energy production and leading to the defective virulence and stress phenotypes.

Fig. 3. Deletion of *VdThit* affects conidial production and germination. **A**, Conidial production by the *Verticillium dahliae* wild-type strain, *VdΔThit* (*VdΔThit-2* and *VdΔThit-7*) mutant strains, and complementation *VdΔThit* mutant (*VdΔThit-2-com* and *VdΔThit-7-com*) strains was recorded 15 days after inoculation on Czapek-Dox agar plates. **B**, Percentages of germinated conidia of the *V. dahliae* wild type, *VdΔThit* (*VdΔThit-2* and *VdΔThit-7*) mutant strains, and complementation *VdΔThit* mutant (*VdΔThit-2-com* and *VdΔThit-7-com*) strains were calculated 5, 10, 15, 20, 25, and 30 h after inoculation in liquid complete medium. Values represent the mean $\pm$ standard deviation from three independent replicates. Asterisks (**) indicate significant difference in Student's *t* test at $P < 0.01$.

Exogenous thiamine partially restored vegetative growth, conidiation, and virulence of the *VdΔThit* mutants.

Our earlier experiments showed the *VdΔThit* mutant strains grew noticeably slower than the wild type on MM agar plates. When supplemented with 50 to 1,000 μg of exogenous thiamine per liter, growth of the *VdΔThit* mutant strains was partially restored to the level of the wild type (Fig. 8A and B). To determine whether exogenous thiamine could restore the defective conidiation of the *VdΔThit* mutants, the Czapek-Dox agar plates were supplemented with 50 to 1,000 μg of exogenous thiamine per liter. When cultured with exogenous thiamine, the *VdΔThit* mutant strains produced significantly more conidia than on the unsupplemented with exogenous thiamine Czapek-Dox agar plates but not at the level of the wild type (Fig. 8C).

To examine the virulence of the recovered conidia, we inoculated *N. benthamiana* roots with a suspension of conidia collected from the colonies grown on Czapek-Dox agar plates with exogenous thiamine (0, 1, and 50 $\mu\text{g}/\text{liter}$). As shown in Figure 8D, at 12 dpi, the virulence defect was partially overcome in *VdΔThit* mutant strains grown from spores from the medium with 50 μg of thiamine per liter (Fig. 8D). Similarly, in the *ΔMoi1v1* mutant of *Magnaporthe oryzae*, a disruptant of *Moi1V1*, vegetative growth was fully restored and conidiation and virulence were partially restored on MM supplemented with exogenous isoleucine (Du et al. 2014). These results strongly suggest that *VdThit* contributes to thiamine absorption and transport during vegetative growth, development, and infection.

Fig. 4. Effect of abiotic stresses on the growth of the *Verticillium dahliae* wild-type strain, *VdΔThit* (*VdΔThit-2* and *VdΔThit-7*) mutant strains, and complementation *VdΔThit* mutant (*VdΔThit-2-com* and *VdΔThit-7-com*) strains. **A**, The *VdΔThit* mutant strains were hypersensitive to UV-irradiation. Colony survival rates for the *V. dahliae* wild type and *VdΔThit* mutant strains and complementation *VdΔThit* mutant strains were calculated after UV exposure at 50, 100, 150, and 200 J m^{-2} . **B**, Colony morphology of the *V. dahliae* wild-type strain, *VdΔThit* (*VdΔThit-2* and *VdΔThit-7*) mutant strains, and complementation *VdΔThit* mutant (*VdΔThit-2-com* and *VdΔThit-7-com*) strains after 15 days of incubation on Czapek-Dox agar plates containing 10 μg of menadione per liter compared with no-stress controls at 25°C. **C**, Colony diameters at 15 days after inoculation on Czapek-Dox agar plates with 10 μg of menadione per liter compared with no-stress controls. Values represent the mean $\pm$ standard deviation from three independent replicates. Asterisks (**) indicate significant difference in Student's *t* test at $P < 0.01$.

Upregulation of de novo thiamine synthesis genes in the *VdΔThit* mutant.

To confirm that *VdThit* plays an important role in thiamine biosynthesis, transport, and usage, we first focused on regulation by profiling the transcripts of the *VdSti35* and *Vdthi11* genes from the de novo thiamine synthesis pathway, using quantitative reverse transcription (qRT)-PCR analyses of cDNA samples from 7-day-old cultures of the *VdΔThit* mutant strains, wild-type strain, and complemented *VdΔThit* mutant strains. In

the *VdΔThit* mutant strains, *VdSti35* and *Vdthi11* gene transcript levels were five- and sixfold higher, respectively, than in the wild-type strain and complemented *VdΔThit* mutant strains (Fig. 9A and C). In liquid CM supplemented with 50 to 1,000 μg of exogenous thiamine per liter, transcription of the *VdSti35* and *Vdthi11* genes in the *VdΔThit* mutant strains also increased compared with the wild-type strain and complemented *VdΔThit* mutant strains in the same medium and compared with the levels of the respective *VdΔThit* mutant strains in liquid CM without

Fig. 5. *VdThit* was required for virulence of *Verticillium dahliae* on *Nicotiana benthamiana*. **A**, Representative images of plants infected with the *V. dahliae* wild-type strain, *VdΔThit* (*VdΔThit-2* and *VdΔThit-7*) mutant strains, and complementation *VdΔThit* mutant (*VdΔThit-2-com* and *VdΔThit-7-com*) strains at 12 days postinoculation (dpi). **B**, Fungal regrowth on potato dextrose agar 7 days after incubation of plant stem tissue sections excised at 12 dpi. **C**, Disease level of plants after a 2-min root-dip in conidia of the *V. dahliae* wild-type strain, *VdΔThit* (*VdΔThit-2* and *VdΔThit-7*) mutant strains, and complementation *VdΔThit* mutant (*VdΔThit-2-com* and *VdΔThit-7-com*) strains at 8, 10, and 12 dpi. **D**, Determination of the *V. dahliae* DNA concentration in *N. benthamiana* hypocotyls after infection with *V. dahliae* wild-type strain, *VdΔThit* (*VdΔThit-2* and *VdΔThit-7*) mutant strains, and complementation *VdΔThit* mutant (*VdΔThit-2-com* and *VdΔThit-7-com*) strains at 12 dpi. Tap water-inoculated plants (mock) as a control did not yield any *V. dahliae* DNA. Values represent the mean ± standard deviation from three independent replicates. Asterisks (**) indicate significant difference in Student's *t* test at *P* < 0.01.

exogenous thiamine (Fig. 9A and C). In short, our results showed that de novo thiamine synthesis genes were upregulated in the *VdΔThit* mutants, suggesting that thiamine synthesis de novo compensated for the shortage of thiamine when the thiamine transport system was not functional and was also regulated by the addition of thiamine. Thus, we speculate that *VdThit* may play another important role in *V. dahlia*.

Root infection and fungal colonization by the *VdΔThit* mutant were impaired.

To analyze the defect in infection and colonization of plant root tissues of the *VdΔThit* mutant strains, we used *VdΔThit-7-GFP* and *Vd-wt-GFP* transformant strains that constitutively express enhanced green fluorescent protein (eGFP), to enable observation of roots after inoculation with the *VdΔThit-GFP* mutant strain or *Vd-wt-GFP* strain by laser scanning confocal microscopy (LSCM). First, there was no difference in growth, development, or virulence between the wild-type strain and *Vd-wt-GFP* strain or between the *VdΔThit* mutant strain and *VdΔThit-GFP* strain (Supplementary Fig. S2). Roots of *N. benthamiana* seedlings at the third leaf stage were dipped into a conidial suspension of *VdΔThit-7-GFP* strain or *Vd-wt-GFP* strain to monitor infection and fungal colonization. By 24 h postinoculation (hpi), germ tubes and hyphae from *Vd-wt-GFP* strain conidia had elongated extensively at random positions on the *N. benthamiana* root surface- and hyphae had elongated and surrounded the root (Fig. 10). In contrast, *VdΔThit-7-GFP* strain conidia formed germ tubes but they did not elongate, so no hyphae developed (Fig. 10). By 7 dpi, strains *VdΔThit-7-GFP* and *Vd-wt-GFP* had colonized the vascular system of the roots, but hyphae of the *Vd-wt-GFP* strain had proliferated within the vascular tissue in contrast to a small hyphal net in xylem vessels infected with

VdΔThit-7-GFP strain (Fig. 10). The *VdΔThit* mutant was thus defective in the infection and colonization of plant root tissues.

The *VdThit* gene of *V. dahliae* plays an important role in vegetative development and invasive hyphal growth as shown by qRT-PCR.

The thiamine transporter is expressed ubiquitously in various tissues of *Saccharomyces cerevisiae* (Enjo et al. 1997; Singleton 1997). For analyzing the expression pattern of *VdThit* during spore germination, hyphal growth, and conidiogenesis of *V. dahliae* on media and in infected roots of *N. benthamiana*, RNA was extracted from conidia or hyphae harvested after 0, 3, 6, 9, 12, 24, 72, 96, and 120 h of growth in liquid CM for qRT-PCR. *VdThit* expression was highest during late hyphal growth at 48 h and was also higher during conidiogenesis than during conidial germination (Fig. 11A). In infected *N. benthamiana*, *VdThit* expression was low during early stages of disease (0 to 3 days) with the lowest expression at 1 to 2 days, even lower than in harvested conidia (Fig. 11B). From 4 to 8 days, *VdThit* expression was higher than in harvested conidia and was highest at 5 to 6 days (Fig. 11B). This experiment suggested that the *VdThit* gene plays an important role in vegetative growth and reproductive development and in invasive hyphal growth.

DISCUSSION

In the present study, we analyzed the biological function of the *V. dahliae Thit* gene. Deletion of *VdThit* resulted in reduced vegetative growth, conidial production, stress-response capacity, and invasive growth and colonization of plant hosts. Interestingly, the defects in vegetative growth, conidiation, and stress responses of the *VdΔThit* mutant were accompanied by

Fig. 6. High-pressure liquid chromatography-mass spectrometry (MS) analyses of acetyl-CoA in the Krebs cycle of *VdΔThit* mutant strains and wild-type strain. **A**, Multiple-reaction monitoring (MRM) chromatograms of acetyl-CoA standard solution and **B**, MS spectrum (precursor ion scan of 810 m/z). **C**, MRM chromatograms of acetyl-CoA in extracts from mycelium of wild-type strain cultivated for 5 days and **D**, MS spectrum (precursor ion scan of 810 m/z) at retention time of 5.53 min. **E**, MRM chromatograms in extracts from mycelium of *VdΔThit* mutant strains cultivated for 5 days and **F**, MS spectrum (precursor ion scan of 810 m/z). cps = counts per second.

greatly impaired virulence. Our results provide evidence that *VdThit* is required for the pathogenicity of *V. dahliae*.

In previous research, *VdThi4* (*Vdsti35*, a gene of the de novo thiamine synthesis pathway) was found to be required for *V. dahliae* infection of tomato and might contribute to the oxidative-stress response but be unrelated to osmotic stress (Hoppenau et al. 2014). However, thiamine uptake and transport regulation as well as the contribution of the stress response to species pathogenicity was not examined. The *Fusarium oxysporum* ortholog of *Vdsti35*, *Fosti35*, did not influence virulence, and an ambient thiamine supply was suggested to be necessary for this fungus to grow (Ruiz-Roldán et al. 2008). Nevertheless, our study showed that growth rate, conidiation, and stress responses (oxidative stress and osmotic stress) of the *VdΔThit* mutants were significantly reduced compared with the *V. dahliae* wild type. We speculate that uptake of exogenous thiamine by the thiamine transporter protein is the main way that *V. dahliae* obtains thiamine. In *S. cerevisiae*, a thiamine transporter (*thi7*) serves as a high-capacity, primary uptake system for the bulk of the thiamine acquired (Mojzita and Hohmann 2006). In *S. cerevisiae*, *Yor71c* and *Yor192c*, which encode proteins that contribute to thiamine uptake, have a low capacity and low affinity for thiamine. And in the *V. dahliae* Ls.17 genome (Verticillium Comparative Genomics Project database) (Klosterman et al. 2011), there are no paralogs of *Yor71c* and *Yor192c*.

When exogenous thiamine is low or absent, the expression of several *THI* genes (de novo thiamine synthesis) is strongly upregulated to synthesize thiamine for organism use (Hohmann and Meacock 1998; Mojzita and Hohmann 2006). Similarly, deletion of *VdThit* resulted in the upregulation of *Vdsti35* and *VdThi11* expression for de novo thiamine synthesis. And when the *VdΔThit* mutant strains were supplemented with high exogenous thiamine, the de novo thiamine synthesis genes (*Vdsti35* and *VdThi11*) were more strongly upregulated when *VdThit* had been deleted (Mojzita and Hohmann 2006). In *Schizosaccharomyces pombe*, thiamine transport activity is regulated by extracellular thiamine (Iwashima et al. 1979) and the expression of *THI7*, which encodes a thiamine transporter protein, is induced when extracellular levels of thiamine increase to 1.2 μM per liter. Some exogenous thiamine is transferred into cells by a non-saturable or passive diffusion mechanism when the extracellular concentration of thiamine is high in *S. cerevisiae* (Rindi 1992; Singleton 1997), and de novo thiamine synthesis genes are then up-regulated to meet the needs of the fungus. In our study of the *VdΔThit* mutant, high supplemented levels of exogenous thiamine did not completely restore vegetative growth, conidiation, and virulence to the wild-type levels. We thus suggest that *VdThit* plays an irreplaceable role in the growth, development, and virulence of *V. dahliae*.

In fungi, various genes are up- or down-regulated to meet the requirements of different stages of growth and development

Fig. 7. Gas chromatography-mass spectrometry analyses of acetoin in pyruvate metabolism of *VdΔThit* mutant strains and wild-type strain. **A**, Gas chromatograms of acetoin standard solution (ethanol solvent) and **B**, mass spectrum (precursor ion scan of 87 m/z). **C**, Gas chromatograms of acetoin in extracts from mycelium of wild-type strain cultivated for 5 days and **D**, mass spectrum (precursor ion scan of 87 m/z) at retention time of 5.53 min. **E**, Gas chromatograms in extracts from mycelium of *VdΔThit* mutant strains cultivated for 5 days and **F**, mass spectrum. cps = counts per second.

(Gomes et al. 2016; Ikegami et al. 2000; Lee et al. 2008; Schumacher et al. 2016; Zeier et al. 2015). For instance, adapting to different hosts and stages of growth and development, *Fusarium graminearum* expressed distinct sets of genes

associated with transport proteins and secondary metabolism during infection of wheat, barley, and maize (Harris et al. 2016). Genes for transport proteins and secondary metabolism were also up- or down-regulated to meet the requirements of

Fig. 8. Exogenous thiamine could restore the growth, conidiation, and virulence in defective *VdΔThit* mutant strains on Czapek-Dox agar plates. **A**, Colony morphology of the *Verticillium dahliae* wild-type strain and *VdΔThit* (*VdΔThit-2* and *VdΔThit-7*) mutant strains after incubation on Czapek-Dox agar supplemented with exogenous thiamine (from 0 to 1,000 µg per liter) for 7 days at 25°C. **B**, Colony diameters and **C**, conidial production of the *V. dahliae* wild-type strain, *VdΔThit* (*VdΔThit-2* and *VdΔThit-7*) mutant strains, and complementation *VdΔThit* mutant (*VdΔThit-2-com* and *VdΔThit-7-com*) strains were measured 7 days after inoculation. **D**, Disease level of *Nicotiana benthamiana* plants at 8, 10, and 12 days postinoculation (dpi) after a 2-min root-dip in conidia of the *V. dahliae* wild-type strain, *VdΔThit* (*VdΔThit-2* and *VdΔThit-7*) mutant strains, and complementation *VdΔThit* mutant (*VdΔThit-2-com* and *VdΔThit-7-com*) strains grown on Czapek-Dox agar supplemented with exogenous thiamine (0, 1, or 50 µg per liter) for 7 days at 25°C. Values are means $\pm$ standard deviation from three replications. Asterisks (*) indicate significance in Student's *t* test at *P* < 0.05.

different stages of growth and development (Harris et al. 2016). In *Magnaporthe oryzae*, an impairment in the temporal expression of different chitin synthase genes during hyphal growth, conidiogenesis, appressorium development, and plant infection processes indicated that these genes were indispensable for growth, development, and plant infection (Kong et al. 2012). In our study of *VdThit* expression profiles at different developmental stages, expression of *VdThit* was elevated during vegetative growth and conidiogenesis on CM and during infection of *N. benthamiana*, suggesting that *VdThit* may play a role in growth and development and in plant infection. We speculate that the expression of *VdThit* may be regulated to help the fungus adapt to external conditions at different stages of growth and development and infection of its host (Callejas-Negrete et al. 2015). In addition, the continued expression of *VdThit* during various developmental and plant infection processes confirmed the necessity of *VdThit* for *V. dahliae* growth, development, and plant infection.

Just as thiamine (vitamin B1) is essential for humans (Sriram et al. 2012), thiamine and thiamine diphosphate, the active form of thiamine, are essential for cellular metabolism in fungi and green plants. Thiamine can relieve the effects of several environmental stresses in *Arabidopsis thaliana* by protecting the plant from oxidative and osmotic stress damage (Ahn et al. 2005; Rapala-Kozik et al. 2008, 2012). Thiamine-treated rice, *Arabidopsis thaliana*, and vegetable crop plants showed resistance to

infection by various fungal, bacterial, and viral pathogens, because the treatment induces the transient expression of pathogenesis-related genes in plant defense responses and improves resistance to abiotic stresses, such as salt and ROS (Goyer 2010). In fungi, thiamine also increases resistance to stress (Hoppenau et al. 2014; Ruiz-Roldán et al. 2008). Recently, research has confirmed that thiamine biosynthetic pathway genes (*THI4* and *THI6*) are necessary for responses to stress conditions in *S. cerevisiae* and *V. dahliae* (Hoppenau et al. 2014; Ruiz-Roldán et al. 2008). Consistent with this research, our results showed that deletion of *VdThit* increased susceptibility to oxidative stress, osmotic stress, and DNA damage, suggesting that *VdThit* helps the fungus adapt to specific environmental conditions. Research on *Beauveria bassiana* suggests that the virulence defect in the $\Delta BbMBF1$ mutant was likely due primarily to impaired hyphal morphogenesis and adaptation to specific environmental stresses (Ying et al. 2014). In *Phytophthora sojae*, the impaired ability of *Psim1* mutants to tolerate and remove ROS resulted in decreased virulence (Yang et al. 2015). The intrinsic osmotic conditions in plant tissues can also reduce mycelial growth; we speculate that susceptibility to abiotic stress conditions may be responsible for the virulence defect in the *Vd Δ Thit* mutant (Shao et al. 2015).

The thiamine diphosphate TPP, the active form of thiamine, as a key cofactor of pyruvate decarboxylase and pyruvate dehydrogenase, plays an important role in the metabolism of pyruvate (Lu et al. 2000; Moe et al. 1974; Mojzita and Hohmann

Fig. 9. Expression of de novo thiamine synthesis genes (*VdSti35* [VDAG_01137] and *Vdthi11* [VDAG_07211]) in the *Vd Δ Thit* (*Vd Δ Thit-2* and *Vd Δ Thit-7*) mutant strains was higher than in the *Verticillium dahliae* wild-type strain and complementation *Vd Δ Thit* mutant (*Vd Δ Thit-2-com* and *Vd Δ Thit-7-com*) strains. **A** and **B**, Quantitative real-time reverse-transcription polymerase chain reaction was used to measure *VdSti35* and **C** and **D**, *Vdthi11* expression levels. The constitutively expressed β -tubulin gene was used as an internal standard. Total RNA was extracted from the mycelia of 7-day-old *V. dahliae* wild-type strain, *Vd Δ Thit* (*Vd Δ Thit-2* and *Vd Δ Thit-7*) mutant strains, and complementation *Vd Δ Thit* mutant (*Vd Δ Thit-2-com* and *Vd Δ Thit-7-com*) strains grown in thiamine-free (**A** and **C**) and thiamine-supplemented (**B** and **D**; 1 to 1,000 μ g per liter) liquid complete medium. Values are means $\pm$ standard deviation of three independent experiments performed in duplicate. Asterisks (**) indicate significant difference in Student's *t* test at *P* < 0.01.

2006). Our HPLC-MS and GC-MS results demonstrated that the pyruvate metabolism intermediates of the *VdΔThit* mutant strains, acetoin and acetyl-CoA, were markedly reduced, affecting the metabolism of intermediates in the Calvin cycle, and may be the cause of the sensitivity to stress and of decreased virulence of *VdΔThit* mutant strains. Furthermore, acetoin and acetyl-CoA in the *VdΔThit* mutant strains were not detectable by HPLC-MS or GC-MS. We speculate that acetoin and acetyl-CoA may be related to the regulation of pyruvate metabolism, but further study is needed to explore how they contribute to regulating pyruvate metabolism in *V. dahliae*. The HPLC-MS and GC-MS results further confirmed that uptake of exogenous thiamine by the thiamine transporter protein is the main way that *V. dahliae* obtains thiamine; the thiamine de novo synthesis pathway is only an accessory pathway in *V. dahliae*.

Verticillium, a vascular pathogen, inhabits the vascular tissue of a host plant during colonization and infection because the xylem sap is the only source of accessible nutrients, including water, inorganic compounds, amino acids, and organic acids (Floerl et al. 2012; Iven et al. 2012; Kehr et al. 2005; Ratzinger et al. 2009; Satoh et al. 1998; Singh et al. 2010, 2012). Fungi acquire nutrients from their environment by their uptake systems (Ratzinger et al. 2009; Timpner et al. 2013), which include numerous ion channel systems in the plasma membrane for the uptake and transport of nutrient elements. In *Verticillium longisporum*, to absorb and use the amino acids present in the plant xylem tissue, cross-pathway control is required for acquiring amino acids from the imbalanced supply in the xylem so that it can further colonize the host (Singh et al. 2010; Timpner et al. 2013). In this study, we described the first construction of a *V. dahliae* mutant strain for the plasma-membrane protein transporting extracellular thiamine into the cell. The GFP-tagged strain *VdΔThit-GFP* was deficient in early infection processes and few strains could successfully colonize and penetrate the root

epidermis of *N. benthamiana*. Furthermore, at later stages of infection, only a few hyphae and conidia were observed in the xylem vessels of the roots. Growth of the GFP-tagged strain *VdΔThit-GFP* was restricted enough within the vascular system to impair the ability of the fungus to colonize the vascular system. These results indicate that *VdThit* in *V. dahliae* plays an important role in the penetration, infection, and colonization of its host. In short, deletion of *Thit* in *V. dahliae* markedly decreased virulence of the fungus in *N. benthamiana*.

The adaptation of *V. dahliae* to life inside vascular tissues of its plant hosts requires a large number of channel proteins to control the absorption of nutrients across the plasma membrane. In this study, the thiamine transporter protein was functionally characterized and, for the first time, the function of this protein and its contribution to pathogenicity of *Verticillium* spp. were evaluated, providing novel insights into the molecular pathogenic mechanisms of *V. dahliae*.

MATERIALS AND METHODS

Fungal strains, plant material, plasmids, and media.

V. dahliae 991 was provided by G. Jian from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences in Beijing, China. *Nicotiana benthamiana* used in this study was preserved in our laboratory. *V. dahliae* 991 and its variants were cultivated on potato dextrose agar, CM (6 g of yeast extract, 6 g of casein acid hydrolysate, and 10 g of sucrose per liter), or Czapek-Dox agar (per liter, 3.0 g of NaNO₃, 0.5 g of MgSO₄·7H₂O, 0.5 g of KCl, 0.01 g of FeSO₄·7H₂O, 1.0 g of K₂HPO₄, and 15 g of agar). *N. benthamiana* plants were grown in a growth chamber at 23 to 25°C, with 16 h of light and 8 h of dark, and 60 to 70% relative humidity. *Agrobacterium tumefaciens* AGL1 and plasmids pGKO2, pCAM-neo, pCM-Hyg, and pCAMgfp were provided by X. Dai from the Institute of Agro-products Processing Science

Fig. 10. Infection and fungal colonization of roots by the *VdΔThit-7-GFP* mutant strains and *Vd-wt-GFP* strain, 24 h (left) or 7 days (right) after inoculation.

Plasmid construction and fungal transformation.

The *VdThit* knockout cassette was constructed using In-Fusion cloning as described previously (Frandsen et al. 2012; Qi et al. 2015). Briefly, the 2.6-kb *neo* cassette was amplified from pCAM-*neo* using primer pair *neo-F* and *neo-R* (Table 1). A 1,071-bp 5' flanking region and a 1,012-bp 3' flanking region of *VdThit* were amplified from genomic DNA with primer pairs *VdThit-5F* and *VdThit-5R* and *VdThit-3F* and *VdThit-3R*, respectively (Table 1). Vector pGKO2 (Khang et al. 2005), carrying the HSVtk (herpes simplex virus thymidine kinase) gene as a negative selection marker against ectopic transformants, was digested with *EcoRI* or *HindIII*. For introducing compatible ends for cloning, primer pair *VdThit-5F* and *VdThit-3R* contained 15-bp-long 5' overhangs identical to the corresponding pGKO2 sequence digested with *EcoRI* or *HindIII*. Similarly, primer pair *VdThit-5R* and *VdThit-3F* contained 15-bp 5' overhangs identical to those in the *neo-F* and *neo-R* sequences. Next, all three amplicons (the 5' flanking region, the *neo* cassette, and the 3' flanking region) and the digested pGKO2 were fused together with an in-fusion enzyme (Clontech, Mountain View, CA, U.S.A.) to create the pGn-*VdThit* plasmid (pGKO2 [*EcoRI*]:*VdThit-5'*:*neo*:*VdThit-3'*:pGKO2 [*HindIII*]) was fused according to the instructions for the In-Fusion dry-down PCR cloning kit [Clontech]; each gene fragment contained 15-bp 5' overhangs [Fig. 1 A]). Similarly, for generating a *VdThit* complementation vector, the *TrpC*-promoter sequence (amplified using the primers *C-TrpC-F* and *C-TrpC-R*, [Table 1]), the full-length *VdThit* cDNA sequence (amplified with primers *C-VdThit-F* and *C-VdThit-R* [Table 1]), the *Nos*-terminator fragment (with primers *C-Nos-F* and *C-Nos-R* [Table 1]), and the digested pCM-Hyg (carrying the hygromycin B resistance

gene [*hph*] cassette, digested with *KpnI* and *XbaI*) were fused together with an In-Fusion enzyme to construct the pCM-Hyg-*VdThit* plasmid.

Agrobacterium tumefaciens-mediated transformation (ATMT).

The plasmids pGn-*VdThit* (carrying the neomycin-resistance gene) and pCM-Hyg-*VdThit* were introduced into *Agrobacterium tumefaciens* AGL1 via electroporation. ATMT of *V. dahliae* Vd991 with pGn-*VdThit* and the *VdΔThit* mutant strains with pCM-Hyg-*VdThit* was done as described previously (Hooykaas et al. 1979; Mullins et al. 2001). Similarly, plasmid pCAMgfp (with the eGFP gene and *hph*) was introduced into the wild-type strain and the *VdΔThit* mutant strains via ATMT, to observe the infection process in plants.

Confirmation of *VdThit* gene disruption or complementation.

Transformants were initially screened for neomycin phosphotransferase resistance to obtain the desired mutant strain, using PCR with primers *VdThit-J-F* and *VdThit-J-R* (Table 1). Replacement of *VdThit* with hygromycin B resistance was further analyzed by PCR, using primers *neo-J-F* and *neo-J-R* (Table 1). The selected complementation transformants were PCR-amplified, using primers *VdThit-J-F* and *VdThit-J-R* (Table 1).

Growth, conidiogenesis, and germination assays.

VdΔThit mutant strains were characterized by measuring colony diameter every 2 to 3 days and recording colony morphology on minimal medium (MM) (per liter, 6 g of NaNO₃, 0.52 g of KCl, 0.152 g of MgSO₄, 1.52 g of KH₂PO₄, 0.01 g of thiamine, 10 ml of trace elements, and 15 g of agar) amended with different carbon sources (per liter, 10 g of xylose, galactose, pectin, or starch or

Fig. 11. Expression pattern analysis of *VdThit* gene during the development of *Verticillium dahliae* on media and assays of plant infection. **A**, Analysis of *VdThit* gene expression during conidial germination (at 0, 3, 6, and 9 h), hyphal growth (at 12, 24, and 48 h), and conidiogenesis (at 72, 96, and 120 h) in liquid complete medium. **B**, Total RNA was extracted individually from hypocotyls of plants of *Nicotiana benthamiana* at 1, 2, 3, 4, 5, 6, 7, 8, and 9 days infected with *V. dahliae* to estimate the *VdThit* gene expression by quantitative reverse transcription-polymerase chain reaction.

30 g of sucrose [Puhalla and Mayfield 1974; Tzima et al. 2011]). Conidial production by wild-type strain 991 of *V. dahliae*, complementation *VdΔThit* mutant strains, and *VdΔThit* mutant strains cultured on Czapek-Dox agar were assessed as described previously (Qi et al. 2015). For germination tests, 10^4 spores per milliliter of the *V. dahliae* wild-type strain, complementation *VdΔThit* mutant strains, and *VdΔThit* mutant strains were added to liquid CM and, then, were shaken for 30 h at 140 rpm at 25°C. At 5-h intervals, 100 conidia were examined for germination, and percent germination was calculated (Tzima et al. 2012). Each assay was done three times.

Phenotypic analysis under stress conditions.

Fresh conidia, 1,000 each from the *V. dahliae* wild-type strain, complementation *VdΔThit* mutant strains, and *VdΔThit* mutant strains were plated onto Czapek-Dox agar plates and were incubated at 25°C for 1 h. For determining conidial survival of these strains in response to UV, conidia were exposed to UV radiation at 50, 100, 150, and 200 J m⁻², using a UV Crosslinker (Spectrolinker XL-1000A, Spectronics Corp., Westbury, NY, U.S.A.), and then, were incubated for 3 days at 25°C before CFU were counted on each plate. The UV-irradiation assay was repeated three times independently.

Next, 2 μl of conidial suspensions (5×10^6 spores per milliliter) of the *V. dahliae* wild-type strain, complementation *VdΔThit* mutant strains, and *VdΔThit* mutant strains were dropped onto the center of Czapek-Dox agar plates supplemented with 10 μg of menadione per liter (oxidative stress) or 1 M sorbitol (osmotic stress) or without menadione or sorbitol. The plates were incubated at 25°C for 7 days, and colony phenotypes were then observed. The assay was repeated three times independently.

qRT-PCR reaction.

Transcript levels of the genes *VdSti35* (VDAG_01137) and *VdThi11* (VDAG_07211) for de novo thiamine synthesis were examined in the *VdΔThit* mutant strains, wild-type strain, and complementation *VdΔThit* mutant strains grown in liquid CM. Total RNA was extracted from cells, using an RNA miniprep kit (Axygen, Union City, CA, U.S.A.) according to the manufacturer's instructions. cDNA was synthesized from purified RNA using a Toyobo RT kit (Osaka, Japan). The qRT-PCR reaction was performed with the SYBR Fast qPCR kit (Kapa Biosystems, Boston). Transcript levels of *VdSti35* and *VdThi11* were quantified relative to the constitutively expressed β-tubulin gene (DQ266153), which was amplified with primers VdBt-F and VdBt-dn, using qRT-PCR in an ABI7500 PCR thermocycler (Applied Biosystems, Foster City, CA, U.S.A.). The qRT-PCR analysis of the *VdΔThit* mutant strains and wild-type strain was done three times.

RNA was extracted from conidia or hyphae of *V. dahliae* harvested after 0, 3, 6, 9, 12, 24, 72, 96, and 120 h of growth in liquid CM, for identifying distinct stages of fungal development, and *VdThit* gene expression was analyzed by qRT-PCR. Total RNA was extracted from individual hypocotyls of diseased plants daily from day 1 to day 9 after inoculation to estimate *VdThit* gene expression, by qRT-PCR, using the SYBR Fast qPCR kit. The qRT-PCR analysis of *VdThit* gene expression in the wild-type strain was done three times.

Infection assays and quantification

of *VdΔThit* fungal biomass in plant tissue, using qRT-PCR.

Infection assays and quantification of the *VdΔThit* fungal biomass in plant tissue, using qRT-PCR, were performed as described previously (Qi et al. 2015). *N. benthamiana* seedlings with 6 to 7 true leaves were removed from the soil and roots were dipped into a conidial suspension (5×10^6 spores per milliliter) of *V. dahliae* wild-type strain, complementation *VdΔThit* mutant strains, or *VdΔThit* mutant strains (Qi et al. 2015). Disease level was recorded and calculated at 8, 10, and 12 dpi. Total DNA was

extracted individually from the hypocotyls of diseased plants at 12 days after inoculation, to estimate fungal biomass by qRT-PCR, using the SYBR Fast qPCR kit (Kapa Biosystems). To normalize any differences in DNA template amounts, the *N. benthamiana* actin gene was amplified with primer pair Nb-actin-F and Nb-actin-R (Table 1) and was used as an internal standard. Primer pair Vd-F and Vd-R (Table 1), designed based on the ITS1 and ITS2 regions of the ribosomal RNA genes (Z29511) of *V. dahliae*, were used to quantify fungal DNA in each sample. Fungal outgrowth of the infected plant material was measured from harvested stems as described previously (Santhanam and Thomma 2013). The infection assay and qRT-PCR analysis of *N. benthamiana* tissue were done three times (with 10 plants per strain).

HPLC-MS and GC-MS analysis of the *VdΔThit* mutant strains.

For chemical assays of pyruvate metabolism intermediates in cell extracts, mycelial samples grown for 6 days in liquid CM were harvested and were powdered in liquid nitrogen. A highly sensitive HPLC-MS was performed, as described previously, to analyze acetyl-CoA in the Krebs cycle of *VdΔThit* mutant and wild-type strains (Hayashi et al. 2006). A previously published GC-MS method (Güler-Akin et al. 2009), with slight modifications, was used to analyze acetoin in pyruvate metabolism in the *VdΔThit* mutant strain and wild-type strain. The extracts from *V. dahliae* wild-type strain or *VdΔThit* mutant strain were analyzed using a Shimadzu GCMS-QP2010 Plus system (Shimadzu, Sao Paulo, Brazil) and a Chromolith Performance DB-WAX column (30 m × 0.25 mm × 0.25 μm) and hydrogen carrier gas (flow rate 1 ml min⁻¹) with injector temperature of 250°C and ion-source temperature of 200°C. The oven temperature was programmed from 40°C (isothermal for 2 min), with an increase of 5°C min⁻¹ to 120°C, and then, 10°C min⁻¹ to 200°C, ending with a 5-min isothermal at 200°C. Ions were detected in full-scan monitoring mode from 35 to 500 m/z. The

Table 1. Primers used in this study

Primer name	Sequences (5' → 3')
Thit-5F	GTACCCAAATTCGAATTCTTAGTGCCTGGACGTT TTAGTGA
Thit-5R	CAAGACAGCCCGCAAACCCGGGGTGGTGAGGATG
Thit-3F	CCCGAATGACAGGTACCTTCCCCGACGTG TTTTCA
Thit-3R	GACGGTATCGATAAGCTTTAGCCGGACGCGTTG TAGGT
C-TrpC-F	TTGAAGGAGCATTTTTGGGC
C-TrpC-R	ATCGATGCTTGGGTAGAATAGGT
C-VdThit-F	ATGTGGAAGAACATTGTGCGAGAAG
C-VdThit-R	TCACTCCCTCAAGTACCGTATCTTC
C-Nos-F	AGATGCCGACCGGGATCCACTT
C-Nos-R	TTATCTTTGCGAACCCAGGG
neo-F	GTTTGCGGGCTGCTTGACG
neo-R	TACCTGTGCATTCTGGGTAA
Thit-J-F	GCTATGATCAGCAGCTGGAGCTC
Thit-J-R	ACCCCTTGTTCTCTCGCCTGAT
noe-J-F	GCGGTTCAAGACACCTCGA
noe-J-R	TATCTTTGCGAACCCAGG
VdBt-F	TTCCCCCGTCTCCACTTCTCATG
VdBt-R	GACGAGATCGT TCATGTTGAACCTC
Vdsti35-F	GGCCCCAAGACCAACAC
Vdsti35-R	GAGCGAGTAGGCGGCGGAGAG
Vdthi11-F	TCATGGGCTCGGACCTCAACC
Vdthi11-R	GACGGCAGCGATCTCAATGG
Nb-actin-F	GGACCTTTATGGAACATTGTGCTCAGT
Nb-actin-R	CCAAGATAGAACCCTCAATCCAGACAC
Vd- F	CCGCCGGTCCATCAGTCTCTCTGTTTATAC
Vd- R	CGCTGCGGGACTCCGATGCGAGCTGTAAC

mass spectra of the products detected by GC-MS were then compared with those in the National Institute of Standards and Technology library (NIST011). Quantities were calculated using an external standard and area normalization method.

Growth, conidiation, and virulence of the *VdΔThit* mutant strains under different exogenous thiamine levels.

The *VdΔThit* mutant strains were characterized according to their colony diameter, conidia production, and virulence (infection assays) on Czapek-Dox agar plates supplemented with 1 to 1,000 µg of exogenous thiamine per liter or without thiamine, as described above. The experiment was repeated three times.

Microscopic observation of initial infection.

Roots of 30 *N. benthamiana* seedlings with three to four true leaves were inoculated with *Vd-wt-GFP* strain and *VdΔThi-7-GFP* mutant strain as described earlier, respectively. At 24 hpi and 7 dpi, *N. benthamiana* plants were uprooted and were gently washed in tap water. The roots of the *N. benthamiana* plants were cut vertically and were microscopically examined using LSM (LSM 700; Zeiss, Jena, Germany). The experiment was done three times.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Verticillium Comparative Genomics Project database:
http://www.broadinstitute.org/annotation/genome/verticillium_dahliae