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VdCrz1 is involved in microsclerotia formation and required for full virulence in *Verticillium dahliae*

Dianguang Xiong, Yonglin Wang *, Chen Tang, Yulin Fang, Jingyi Zou, Chengming Tian *

The Key Laboratory for Silviculture and Conservation of Ministry of Education, College of Forestry, Beijing Forestry University, Beijing, China

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ABSTRACT

Calcium signaling plays crucial roles in ion stress tolerance, sporulation and pathogenicity in fungi. Although the signaling pathway mediated by calcineurin and the calcineurin-responsive zinc finger transcription factor *Crz1* is well characterized in other fungi, this pathway is not well characterized in the phytopathogenic fungus, *Verticillium dahliae*. To better understand the role of this calcineurin-dependent transcription factor in *V. dahliae*, an ortholog of *CRZ1*, *VdCrz1*, was identified and characterized functionally. Transcriptional analysis of *VdCrz1* and GFP expression driven by the *VdCrz1* promoter indicated that *VdCrz1* was involved in microsclerotia development. After targeted deletion of *VdCrz1*, microsclerotia formation and melanin accumulation were impaired. Furthermore, the Δ*VdCrz1* mutants were hypersensitive to high concentrations of Ca^{2+} and cell wall-perturbing agents, such as sodium dodecyl sulfate. The addition of Mg^{2+} to the medium restores the microsclerotia formation in Δ*VdCrz1* mutants. The Δ*VdCrz1* mutants exhibited delayed Verticillium wilt symptoms on smoke tree. These results suggest that *VdCrz1* plays important roles in Ca^{2+} signaling, cell wall integrity, microsclerotia development and full virulence in *V. dahliae*.

1. Introduction

The phytopathogenic fungus *Verticillium dahliae* causes destructive plant vascular wilt diseases on over 200 species throughout the world (Klosterman et al., 2009). The symptoms of smoke tree (*Cotinus coggygria*) vascular wilt, caused by *V. dahliae*, include stunted growth in stems, early senescence in leaves, and the disease may result in mortality. Consequently, Verticillium wilt has had a detrimental effects on the red-leaf scenery in Beijing, which is largely composed of stands of smoke trees (Wang et al., 2013).

Verticillium wilt disease is difficult to control, largely because in the late stages of the disease cycle, *V. dahliae* forms melanized resting structures, also known as microsclerotia, which can survive for years in the soil, and are resistant to environmental extremes. When suitable hosts are available, microsclerotia can germinate and infect the plant root as primary infectious propagules (Klosterman et al., 2009). Thus, generation of microsclerotia represents a critical event in the disease and life cycles of *V. dahliae*. Insight into the molecular machinery and signaling pathways that regulate this lifecycle event may prove useful in designing novel strategies to control Verticillium wilt.

Cellular responses to external stimuli are governed primarily by signal transduction pathways, which transduce external input into appropriate cellular responses. The calcium (Ca^{2+}) signaling transduction pathway has been studied in many organisms, including the fungi, revealing involvement of this pathway in cell growth and development (Berridge et al., 2003). Ca^{2+} binds to and activates the protein calmodulin, which in turn activates calmodulin-dependent enzymes, such as calcineurin (Chin and Means, 2000). Calcineurin possesses a catalytic A subunit (CNA) and a regulatory B subunit (CNB) (Guerini, 1997). Calcineurin plays important roles in fungal growth and development, as well as in the production of conidia, pathogenicity, and stress tolerance (Harel et al., 2006; Kothe and Free, 1998; Rasmussen et al., 1994; Steinbach et al., 2007; Viaud et al., 2003, 2002). For example, the CNA and CNB silenced mutants in *Magnaporthe oryzae* (Nguyen et al., 2008) and CNA deleted mutants in *Aspergillus fumigatus* (Steinbach et al., 2006) exhibit defects in fungal growth, sporulation and virulence, suggesting diverse functions of calcineurin in fungi.

The transcription factor *Crz1* (calcineurin-responsive zinc finger) is an important downstream regulator of Ca^{2+} signaling in fungi (Stathopoulos and Cyert, 1997). In response to Ca^{2+} stimuli, wild-type calcineurin regulates activation of *Crz1* by dephosphorylation causing its translocation from the cytosol to the nucleus. *Crz1* contains two typical C_2H_2 zinc fingers and acts as a major mediator of Ca^{2+} signaling, which regulates calcineurin-dependent gene

* Corresponding authors.
E-mail addresses: ylwang@bjfu.edu.cn (Y. Wang), chengmt@bjfu.edu.cn (C. Tian).

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expression (Schumacher et al., 2008; Stathopoulos-Gerontides et al., 1999; Yoshimoto et al., 2002). *Crz1* also possesses an atypical C_2H_2 zinc finger with an unusual spacing of the conserved CCHC sequence, which is similarly present in various fungi (Spielvogel et al., 2008). In *Saccharomyces cerevisiae*, *Crz1* is required for tolerance of high concentrations of ions such as Ca^{2+}, as it induces regulated genes such as *PMC1* which is a Ca^{2+} transporting ATPase (Matheos et al., 1997). *CRZ1* also confers tolerance to chitosan and alkaline stress, and regulates mating in *S. cerevisiae* (Zakrzewska et al., 2005).

The function of *CRZ1* homologs are also well characterized in many pathogenic fungi (Hagiwara et al., 2008; Hernandez-Ortiz and Espeso, 2013; Lev et al., 2012). For example, *CRZ1* in *M. oryzae* is essential for tolerance to ion stress, maintaining cell wall integrity, sporulation, appressorium-mediated penetration, and virulence (Choi et al., 2009; Zhang et al., 2009). In *Botrytis cinerea*, the *CRZ1* homolog is required for sclerotia formation and pathogenicity (Schumacher et al., 2008). However, *CRZ1* deletion mutants in *Candida albicans* exhibited only a slight defect in virulence (Karababa et al., 2006). Genes regulated by *Crz1* have been identified in some fungi, such as in *A. fumigatus* (Karababa et al., 2006; Kim et al., 2010; Soriani et al., 2008, 2010; Yoshimoto et al., 2002). Surprisingly, only two of the genes regulated by *Crz1* are shared among *M. oryzae*, *S. cerevisiae*, and *A. fumigatus*, which might indicate the divergent evolution of the genes regulated by *Crz1* in response to the unique conditions faced by each species (Kim et al., 2010).

Since the release of genomic resources for *V. dahliae* (Klosterman et al., 2011), the molecular and functional genomics tools available for *V. dahliae* have significantly improved, leading to functional characterizations of diverse genes. Among these findings, genes involved in signal transduction cascades have been characterized that are essential for pathogenicity or asexual development. For example, disruption of the G protein β subunit in *V. dahliae* causes defects in virulence, as well as increased microsclerotia production and conidiation (Tzima et al., 2012). The cAMP-dependent protein kinase A catalytic subunit *VdPKAC1* regulates virulence and fungal development (Tzima et al., 2010, 2011), and the Fus3 orthologous gene *VMK1* affects microsclerotia production and pathogenicity (Rauyaree et al., 2005). The MAPK kinase Msb2 (Tian et al., 2014) and MAPK Hog1 (Wang et al., unpublished data) are important for microsclerotia formation and virulence in *V. dahliae*. Furthermore, genes encoding transcriptional regulators with important roles in virulence and development have also been characterized in this fungus (Santhanam and Thomma, 2013; Tran et al., 2014).

In this work, we examined the function of a putative C_2H_2 zinc finger transcription factor, *VdCrz1*, which was previously identified as highly expressed during microsclerotia formation (Xiong et al., 2014). Deletion of *VdCrz1* in *V. dahliae* in this study resulted in hypersensitivity to high concentrations of Ca^{2+}, and the cell membrane-perturbing agent, sodium dodecyl sulfate. The *VdCrz1* deletion mutant exhibited reduced microsclerotia formation and reduced virulence on smoke trees. Our results indicate that *VdCrz1* is involved in microsclerotia formation and required for full virulence of *V. dahliae* on smoke tree.

2. Materials and methods

2.1. Fungal strain and culture conditions

V. dahliae strain XS11 was isolated from smoke tree, *C. coggygria* in Fragrant Hills, Beijing (Wang et al., 2013) and used as the wild-type strain for these experiments. All strains were cultured on PDA plates (200 g potato, 20 g glucose, 15 g agar) at room temperature. Cultures were incubated in liquid complete medium (1 l CM, 50 ml 20 × nitrate salts, 1 ml 1000× Trace, 10 g glucose, 2 g peptone, 1 g yeast extract, 1 g casamino acids, 1 ml vitamin solution) for the collection of 7-day-old mycelia and genomic DNA extraction. Conidia of *V. dahliae* were spread on basal medium (1 l BM, 10 g glucose, 0.2 g sodium nitrate, 0.52 g KCl, 0.52 g $MgSO_4{\cdot}7H_2O$, 1.52 g KH_2PO_4, 3 μM thiamine HCl, 0.1 μM biotin and 15 g agar) for analyses of gene expression during microsclerotia formation. For analyses of conidia germination, conidia were inoculated in liquid BM at room temperature for 24 h.

2.2. Bioinformatics analysis

The complete sequence of *Crz1* was downloaded from the *V. dahliae* sequence available at genome database (http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html). *Crz1* homologs in other fungal species were identified with the Blastp search of databases (Broad Institute and Joint Genome Institute). Multiple sequence alignments were conducted with ClustalX 2.0 program (Larkin et al., 2007) using full-length protein sequences. The phylogenetic tree was constructed by MEGA 6.0 with full length protein sequences and a neighbor-joining with 1000 bootstrap replications (Tamura et al., 2013).

2.3. Targeted disruption of VdCrz1 and mutant complementation

The split-marker method was used for targeted gene replacement of *VdCrz1* (Goswami, 2012). The two flanking sequences of *Crz1* (each approx. 1.2 kb) were amplified using primers Crz1-5Ffor and Crz1-5Frev for the 5′ flanking sequence and primers Crz1-3Ffor and Crz1-3Frev for the 3′ flanking sequence. The geneticin resistance cassette was amplified with primers Geneticinfor and Geneticinrev so that it contained approximately 20 bp overlaps with the 5′ and 3′ flanking sequences, respectively. Subsequently, the resulting 5′ and 3′ flanking sequences were fused to the geneticin resistance cassette through fusion PCR respectively. Finally, the overlapping DNA fragments were verified by sequencing and used directly for protoplast transformation. Transformants were selected with 50 μg/ml geneticin.

For complementation of the Δ*Crz1* strain, a fragment containing the coding sequence of wild type *VdCrz1* without the termination codon was amplified from genomic DNA with primers Crz1-Compfor, and Crz1-Comprev and inserted into a *Sma*I-digested pKD5-GFP plasmid (kindly provided by Prof. Jianping Lu, Zhejiang University). A fragment containing the H3 promoter (pKD5-GFP), the *VdCrz1* coding region (without a stop codon) and eGFP was PCR amplified with primers H3 + Crz1 + eGFPfor and H3 + Crz1 + eGFPrev. This fragment was co-transformed into protoplasts Δ*VdCrz1* with a hygromycin resistance cassette amplified from the pRF-HU vector. Transformants were selected on TB3 medium (3 g yeast extract, 3 g casamino acids, 20% sucrose, and 0.7% agar) supplemented with 50 μg/ml geneticin and 25 μg/ml hygromycin.

2.4. Construction of the Crz1 promoter::eGFP vector

To analyze *VdCrz1* expression during microsclerotia formation, the promoter sequence of *VdCrz1* (2410 bp) was amplified with primers Crz1-Pfor and Crz1-Prev and inserted into the *Sma*I-digested plasmid pKD5-GFP. The *VdCrz1*(p2.4)::eGFP fragment was amplified with primers Crz1-Pfor and Pro + eGFPrev and co-transformed into wild-type protoplasts with a geneticin resistance cassette. Positive transformants were selected on TB3 medium supplemented with 50 μg/ml geneticin and by the presence of a fluorescence signal. Single-spore purified transformants were used for observation.