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ORIGINAL ARTICLE

VdERG2 was involved in ergosterol biosynthesis, nutritional differentiation and virulence of *Verticillium dahliae*

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Abstract

The ergosterol biosynthesis pathway plays an important role in model pathogenic bacteria *Saccharomyces cerevisiae*, but little is known about the biosynthesis of ergosterol in the pathogenic fungus *Verticillium dahliae*. In this study, we identified the *VdERG2* gene encoding sterol C-8 isomerase from *V. dahliae* and investigated its function in virulence by generating gene deletion mutants (ΔVdERG2) and complemented mutants (C-ΔVdERG2). Knockout of *VdERG2* reduced ergosterol content. The conidial germination rate and conidial yield of ΔVdERG2 significantly decreased and abnormal conidia were produced. In spite of *VdERG2* did not affect the utilization of carbon sources by *V. dahliae*, but the melanin production of ΔVdERG2 was decreased in cellulose and pectin were used as the sole carbon sources. Furthermore, the ΔVdERG2 mutants produced less microsclerotia and melanin with a significant decrease in the expression of microsclerotia and melanin-related genes *VaflM*, *Vayg1, VDH1, VdLAC*, *VdSCD* and *VT4HR*. In addition, mutants ΔVdERG2 were very sensitive to congo red (CR), sodium dodecyl sulfate (SDS) and hydrogen peroxide (H_2O_2) stresses, indicating that *VdERG2* was involved in the cell wall and oxidative stress response. The absence of *VdERG2* weakened the penetration ability of mycelium on cellophane and affected the growth of mycelium. Although ΔVdERG2 could infect cotton, its pathogenicity was significantly impaired. These phenotypic defects in ΔVdERG2 could be complemented by the reintroduction of a full-length *VdERG2* gene. In summary, as a single conservative secretory protein, *VdERG2* played a crucial role in ergosterol biosynthesis, nutritional differentiation and virulence in *V. dahliae*.

Keywords *Verticillium dahliae* · Ergosterol biosynthesis · Nutritional differentiation · Cell wall stress · Oxidative stress · Virulence

Introduction

As a soil-borne fungus, *V. dahliae* caused Verticillium wilt disease in more than 200 host plants, including some important economic crops(Johnson and Dung 2010; Song et al. 2020). The characteristic of Verticillium wilt is that fungal hyphae filled the vascular tissues of plants, resulting in plant leaves wilting and dying (Prieto, et al. 2009; Subbarao 2009). At present, cotton planting area in many parts of the world is seriously threatened by this disease. Once plants were infected, there was no efficient fungicide to cure them. The pathogen has strong variability and co-evolution ability with the host, and its pathogenic mechanism is very complex, besides, the interaction mechanism between the pathogen and host is still unclear (Depotter et al. 2018; Zhang et al. 2022b). The interaction mechanism between pathogens and hosts is still unclear. Therefore, the illumination of the molecular pathogenicity

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mechanism of *V. dahliae* will help to control the Verticillium wilt of cotton.

Ergosterol is the main sterol component in the fungal plasma membrane and is a fungal-specific sterol (Jordá and Puig 2020; Munn et al. 1999; Zinser et al. 1993). Which can maintain the integrity and fluidity of the cell membrane, regulate cell membrane permeability and membrane binding protein activity (Abe and Hiraki 2009; Wang et al. 2020). In fungi, ergosterol is synthesized by acetyl coenzyme A through a complex process (Hu et al. 2017; Zhang et al. 2020). The C-8 isomerase encoded by ERG2 catalyzed the biosynthesis of ergosterol, resulting in the formation of episterol by C-7 unsaturated in the sterol B ring (Lees et al. 2020). High expression of *ERG2* gene in *S. cerevisiae* inhibited the defect of ergosterol synthesis and indicated that *ERG2* played a role in ergosterol synthesis (Bhattacharya et al. 2018; Johnston et al. 2020; Sanglard et al. 2003). Studies have shown that the direct binding of the Hap1 transcription factor to ERG2 inhibited the transcription of *ERG2* and affected the synthesis of ergosterol (Jordá et al. 2022). Deletion of the ERG2 gene of *S. cerevisiae* caused disruption of ergosterol synthesis and accumulation of abnormal ergosterol, which led to sensitivity to stress. The sterol isomerase encoded by ERG2 in *S. cerevisiae* did not play a crucial role in the viability of fungi (Bhattacharya et al. 2018). Ergosterol has been proven to be an immunoactive fungal molecule (Rodrigues 2018). Deletion of *FgERG4* gene in *Fusarium graminearum* could block ergosterol synthesis and weaken the virulence of the strain (Liu et al. 2013). In addition, the inactivation of sterol D5,6-desaturase (Erg3) attenuated the virulence of *Candida albicans* in mice (Chau et al. 2005). Furthermore, the sterol C-14 reductase encoded by *FgERG24B* enhanced the intrinsic resistance of *F. graminearum* to amine fungicides (Liu et al. 2011). *ERG24* and *ERG2* were the main targets of amine fungicides (Hernández et al. 2016; Liu et al. 2011; Vlainić et al. 2021).

So far, there is little information about the ergosterol pathway in *V. dahliae*, and the role of *ERG2* in sterol biosynthesis and pathogenicity in *V. dahliae* is unclear. Therefore, the main purpose of this study is to investigate the role of *VdERG2* in the physiology and virulence of *V. dahliae* in vitro and in vivo. The results showed that as a single conservative secretory protein, *VdERG2* played a crucial role in ergosterol biosynthesis, nutri0tional differentiation and virulence in *V. dahliae*. Which will help us deeply understand the molecular pathogenicity mechanism of *V. dahliae*.

Materials and methods

Growth conditions of strains and plants

Vd080, a virulent strain of *V. dahliae* preserved in our laboratory, grew in darkness on Potato Dextrose Agar (PDA) at 25 °C. Susceptible variety Jimian 11 was preserved in our laboratory for the pathogen infection experiment. Cotton grew at 28 °C in a 16 h light/8 h dark cycle greenhouse. YTK12 was a sucrose enzyme deficient *S. cerevisiae* strain, which was widely used to analyze and identify the signal peptide of secreted protein.

Vector and bioinformatics analysis

The gene knockout vector B303-Hyg, YTK12 yeast strain and the pSUC2 vector were provided by researcher Fuguang Li's team from the Institute of Cotton Research, Chinese Academy of Agricultural Sciences. The gene complement vector pCAMBIA1302-neo was provided by researcher Xiaofeng Su from the Institute of Biotechnology, Chinese Academy of Agricultural Sciences. DNAMAN was used for the multi-sequence alignment of each protein. Potential signal peptide was predicted with SignalP V5.0 (http://www.cbs.dtu.dk/services/SignalP/index.php) (Almagro Armenteros et al. 2019).

Yeast signal peptide capture test

The function of signal peptides (SP) was verified by the yeast secretion system. The SP sequence of VdERG2 was cloned into pSUC2 vector with specific primers (pSUC2-VdERG2-F/R), and the obtained plasmid was transformed into YTK12 yeast strain. According to the previous method, positive colonies were screened on CMD-W medium (0.67% YNB, 2% sucrose, 0.1% glucose, 2% agar and 0.075% DO supplement-trp), and the utilization of raffinose by yeast was observed on YPRAA medium (1% yeast extract, 2% peptone, 2% raffinose, 2% agar and 2ug/ml antimycin A) to verify the function of signal peptide (Liu et al. 2021b). The activity of sucrose transferase was detected using a 2% TTC solution to verify the function of the signal peptide (Meng et al. 2022). The primers used in this assay were listed in Table S1.

Knockout and complemented of *VdERG2* gene

Both *VdERG2* knockout mutants and complemented mutants were obtained according to previous methods through *Agrobacterium*-mediated transformation (ATMT) (Li et al. 2012; Paz et al. 2011; Wang et al. 2016a). The 0.9 Kb sequence of upstream and downstream of *VdERG2* gene were selected. The target fragment was amplified from wild-type genomic DNA by specific primers (B303-VdERG2-Up-F/R, B303-VdERG2-Down-F/R). A hygromycin-resistant fragment (*HPH*) was amplified from B303 vector by specific primers (VdERG2-HPH-F/R). Three fragments were ligated with linearized B303 vector by homologous recombination ligase (ClonExpress Ultra

One Step Cloning Kit, Vazyme, Nanjing, China). After the obtained plasmid (B303-Up-HPH-Down) was transformed into the susceptible state of *Agrobacterium tumefaciens* AGL1, the ATMT method was used to screen the positive transformants on PDA medium containing hygromycin resistance, and the specific primers (VdERG2-F/R, HPH-F/R) were used for PCR verification. Using the same method, the 1.2 Kb upstream fragment of *VdERG2* gene, *VdERG2* fragment and linearized pCAM-BIA1302 vector were connected and transformed to obtain the recombinant plasmid (pCAM-BIA1302-Up-VdERG2). Based on the knock-out mutant strain, the positive transformants were screened according to the vector resistance, and the specific primers (VdERG2-F/R, HPH-F/R) were used for PCR verification. The primers used in this assay were listed in Table S1.

Southern blotting

According to the previous method, DIGHigh prime DNA marker and detection kit II (Roche, Germany) were used for southern blotting (Li et al. 1999; Okoli et al. 1993). The fragment of *HPH* was amplified with specific primers (HPH-F/R) as a probe. Genomic DNA was extracted from knockout mutant and wild type by the CTAB method. In the experiment, *HindIII* was used for enzyme digestion of genomic DNA (Glenn and Andreou 2013). The primers used in this assay were listed in Table S1.

Determination of ergosterol biosynthesis

To extract ergosterol, 5 mL conidial suspension (1×10^7 CFU/mL) of each strain was inoculated in 50 mL Potato Dextrose Broth (PDB) medium, and incubated at 25 °C for 3 days. Mycelia were harvested by filtration and washed three times with sterile water, then mycelia were dried at 60 °C for 3 h, and the dried mycelia were ground into a powder.

Specific extraction of ergosterol referred to a previously published method for extraction (Liu et al. 2013). The instrument used in this test was Waters 2695 high-performance liquid chromatograph equipped with Waters 2996 ultraviolet detector (Ping and Rong 2006). Ergosterol was separated at 30 °C on a Waters C18 column (5 μm, 4.6 mm × 250 mm) analytical column using 100% methanol (chromatography pure) as the mobile phase. The detection wavelength was 282 ŋm, and the standard was purchased from Shanghai yuan ye Bio-Technology Co, Ltd. The experiment was repeated three times (Chiocchio and Matković 2011; Gessner 2020; Nahar et al. 2020).

Oxidative stress

Oxidative stress was detected using the method of previous studies (Rehman et al. 2018; Tang et al. 2020). To test oxidative stresses, 100 μL conidial suspension (1×10^7 CFU/mL) of each strain was spread onto PDA plates, and filter paper discs containing 5 μL of 7.5%, 15% and 30% hydrogen peroxide (H_2O_2) were placed onto the centre of each plate, respectively. The zone of growth inhibition was measured after 3 days.

Expression analysis of microsclerotia and melanin-related genes

To study the impact of *VdERG2* knockout on the regulation of other genes related to microsclerotia and melanin formation were as follows: class II hydrophobin gene (*VDH1*) (Klimes et al. 2008; Klimes and Dobinson 2006), pigment biosynthesis protein Ayg1 (*Vayg1*) (Fan et al. 2017), scytalone dehydratase (*VdSCD*) (Duressa et al. 2013), laccase (*VdLAC*) (Li et al. 2020), tetrahydroxynaphthalene reductase (*VT4HR*) (Wang et al. 2018), and versicolorin reductase (*VaflM*) (Wang et al. 2016b).

1 μL conidial suspension (1×10^7 CFU/mL) of wild type, ΔVdERG2, and C-ΔVdERG2 strains was inoculated in 200 mL of PDB and incubated on the shaker (180 rpm) at 25 °C. After 5 days, the culture was filtered through four layers of clean gauze to collect hyphae. Total RNA was extracted from the respective hyphae using the RNA Extraction Kit (YPHBio, Tianjin, China). First-strand cDNA was synthesized with HiScript II QRT SuperMix for qPCR (+ g DNA wiper) (Vazyme) according to the instruction. qRT-PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme). The primers used in this assay were listed in Table S1.

Stress response and carbon utilization detection

For the determination of abiotic stress, 1 mol/L KCl, 1 mol/L NaCl, 1 mol/L Sorbitol, 0.004% SDS and 0.02% CR were added to PDA medium (Liu et al. 2021a; Zhang et al. 2022a). Normal PDA medium was used as the control. The conidial suspension was cultured on various types of PDA and kept 25 °C. In order to study the role of VdERG2 in the growth of V. *dahliae* during the absorption and utilization of specific carbon sources, sucrose (30 g/L), cellolose (5 g/L), skim milk (18 g/L), pectin (10 g/L) and starch (17 g/L) were added to Czapek-Dox medium without sucrose, respectively (Wang et al. 2021). The colony diameter of all strains was measured after 14 days of culture (Guo et al. 2022; Liu et al. 2021c; Su

et al. 2018; Zhang et al. 2022a, 2015). All experiments were repeated three times.

Mycelia penetration test

Spore suspension was obtained according to previous methods (Fraczek et al. 2019). 5 µL conidial suspension (1×10^7 CFU/mL) of wild type, ΔVdERG2, and C-ΔVdERG2 strains were added to the PDA plate with a layer of cellophane. After 3 days, the growth was observed and photographed, in the ultra-clean worktable, PDA plates were removed from the cellophane with mycelium, reclosed the petri dish, after 3 days of culture, the colony morphology was photographed (camera model: EOS 6D).

The mycelia above the removed cellophane were made into frozen sections, and the growth of mycelia was observed under the scanning electron microscope. The back of the cellophane was placed on the metal table and sprayed with gold under vacuum. The spores and mycelia were observed under a scanning electron microscope.

Pathogenicity test

The spore suspensions of wild type, ΔVdERG2 and C-ΔVdERG2 strains were prepared and the concentration was adjusted to 1×10^7 CFU/mL. The four-week-old cotton seedlings were gently pulled out of vermiculite. The roots were washed with water, and the seedlings without damage to the roots were selected. The cotton seedlings were soaked in the spore suspension for 10 min by dipping the roots. Subsequently, they were retransplanted into nutrient soil (PINDSTRUP), and placed in the cotton greenhouse for cultivation.

The disease index (DI) was investigated at 14, 18 and 21 days after inoculation. The DI was based on the previous method (Gong et al. 2017). The infected plants were classified into five grades (grade 0, 1, 2, 3, and 4) according to the symptoms on the cotyledons and true leaves (Wang et al. 2004; Zhang et al. 2012).

The stems of cotton plants 21 days after inoculation were selected for a fungal recovery test, which was performed as previously described (Zhang et al. 2016, 2019).

Moreover, the stems of cotton were longitudinally cut with a scalpel, and the browning degree of the stem was observed under the stereomicroscope (Leica, M165 FC, Germany), and photographed. At 21 days after inoculation, the total DNA of the cotton plant was extracted using the plant genome extraction kit (Vazyme), each sample of 100 ηg of DNA was employed for qRT-PCR reaction, and calculated by $2^{-\Delta\Delta CT}$ method. *Vdβt* was the target gene of *V. dahliae* biomass detection, and *GhUBQ7* was the reference gene.

Results

Identification of *VdERG2* in *V. dahliae*

Using *S. cerevisiae* sterol C-8 isomerase Erg2 as a query, we identified *VdERG2* (VDAG_01363) from the *V. dahliae* strain VdLs. 17 (https://www.ncbi.nlm.nih.gov/genome/832). The nucleotide sequence of *VdERG2* was 678 bp, encoding 225 amino acids. The protein homology analysis using DNAMAN showed that the amino acid sequence of VdERG2 was relatively conservative at the C-terminal with that of *S. cerevisiae* and other filamentous fungi, but mutated greatly at the N-terminal, the homology was 73.82% (Fig. 1A). SignalP V5.0 predicted that VdERG2 may contain a signal peptide at the N-terminal. YTK12-pSUC2-VdERG2sp was normally grown on CMD-W and YPRAA mediums according to the yeast signal peptide trap recovery test (Fig. 1B). TTC staining showed that yeast strain YTK12 carrying pSUC2-VdERG2sp secreted sucrase to hydrolyze sucrose into monosaccharides, which reacted with TTC to produce triphenyltetrazole chloride red insoluble in water (Fig. 1B). The above results indicated that VdERG2 was a highly conserved secretory protein in *V. dahliae*.

The knockout and complemented mutants of *VdERG2* in *V. dahliae*

In order to analyze the function of VdERG2 in detail, this study used the method of homologous recombination (Fig. 2A). Two mutants ΔVdERG2-1 and ΔVdERG2-2 were determined by PCR and southern blotting (Fig. 2C and D). In the genome of *V. dahliae*, the 1.2 Kb fragment of the upstream promoter region of the target gene *VdERG2* and the target gene *VdERG2* fragment were selected, and the complemented vector pCAMBIA1302-Neo-VdERG2 was constructed by homologous recombination further genetic transformation to knockout mutants. Two complemented mutants C-ΔVdERG2-1 and C-ΔVdERG2-2 were identified by PCR (Fig. 2B).

Decrease of ergosterol biosynthesis in *VdERG2* knockout mutant

High-performance liquid chromatography (HPLC) analysis of ergosterol extracted from mycelia of wild type, ΔVdERG2-1, ΔVdERG2-2, C-ΔVdERG2-1 and C-ΔVdERG2-2 revealed that there was an ergosterol-specific absorption peak at the retention time of 16.5 min in all strains (Fig. 3A). However, the ergosterol content of

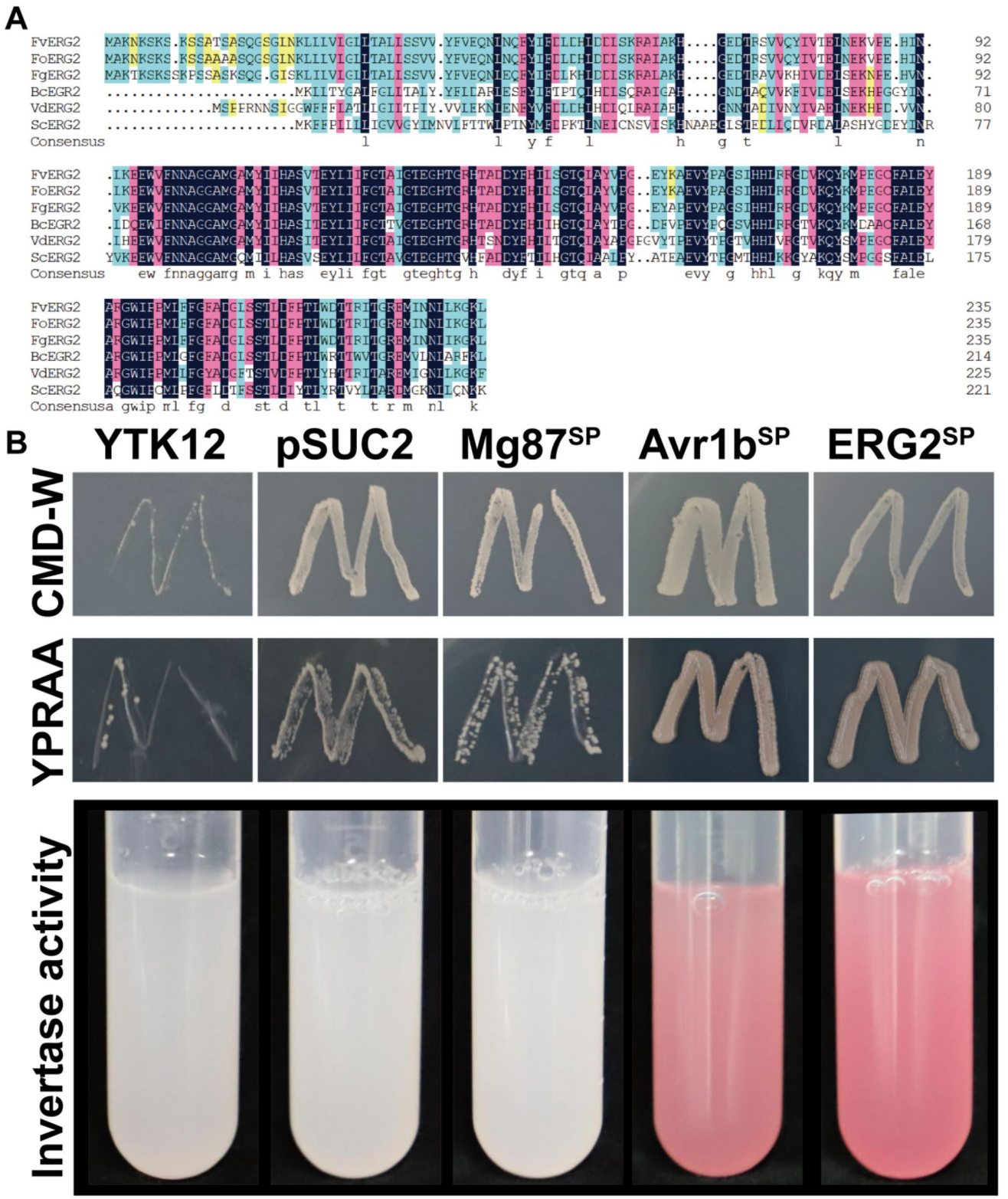

Fig. 1 VdERG2 was a highly conserved secretory protein in *V. dahliae*. **A** Homology analysis of VdERG2 with ERG2 in Saccharomyces cerevisiae and other filamentous fungi. Protein sequences from top to bottom were derived from *Fusarium verticillioide*, *Fusarium oxysporum*, *Fusarium graminearume*, *Botrytis cinerea*, *V. dahliae* and *S. cerevisiae*. **B** Signal peptide capture test to verify the signal peptide functions of VdERG2

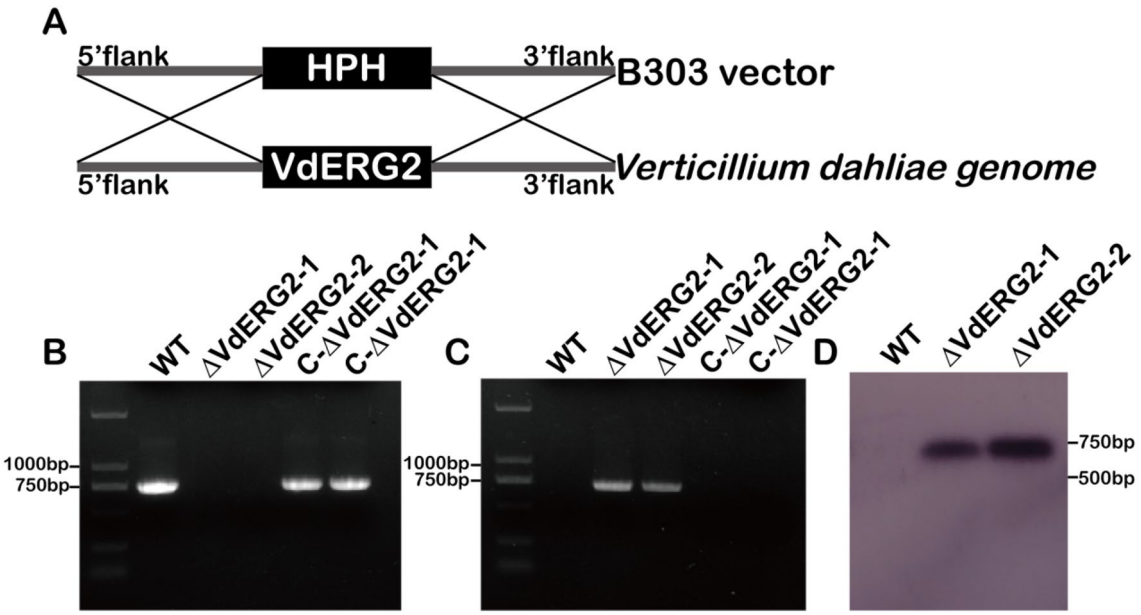

Fig. 2 The knockout and complemented mutants of VdERG2 in *V. dahliae*. **A** The homologous recombination of *VdERG2* in *V. dahliae*. **B** Identification of *VdERG2* gene knockout transformations by PCR. **C** Identification of *VdERG2* gene complementary transformations by PCR. **D** Identification of *VdERG2* gene knockout transformations by southern blotting

the ΔVdERG2-1 and ΔVdERG2-2 mutants were significantly lower than that of the wild type and complemented mutants (Fig. 3B). These results showed that *VdERG2* played an important role in ergosterol synthesis pathway in *V. dahliae*.

VdERG2 was involved in the regulation of conidia in *V. dahliae*

After the strains were cultured in a liquid medium for 5 days, the number of spores were observed under the fluorescence microscope and counted through the blood cell counting plate. The results showed that the conidia yield in ΔVdERG2-1 and ΔVdERG2-2 were significantly less than those in the wild type and the complemented mutants (Figure S1 and Fig. 4A). The same concentrations of spores of each strain were coated on PDA for 6 h, and the spore germination was observed. It was found that the germination rates of conidia in ΔVdERG2-1 and ΔVdERG2-2 were lower than those in the wild type and the complemented mutant (Figure S2 and Fig. 4B). The conidia were observed under a scanning electron microscope, and was found that the morphology of conidia was abnormal in ΔVdERG2-1 and ΔVdERG2-2 (Fig. 4C). These results showed indicated that the deletion of *VdERG2* could lead to a decrease of conidia yield and conidia germination rate, with influencing on the normal morphology of conidia.

VdERG2 did not affect the utilization of carbon source but reduced microsclerotia formation and melanin production

To further explore whether VdERG2 was involved in the utilization of carbon sources, sucrose, cellulose, skim milk, pectin and starch were selected as carbon sources. The results showed that *VdERG2* did not affect the utilization of carbon sources when sucrose, cellulose, skim milk, pectin and starch were used as carbon sources, respectively. However, when cellulose and pectin were used as carbon sources, the melanin and microsclerotia of the knockout mutants showed a decreasing trend (Figure S3). To determine whether *VdERG2* was associated with the production of melanin and microsclerotia, we examined the production of melanin and microsclerotia on basal modified medium (BMM, 0.2 g/L $NaNO_3$, 0.52 g/L KCl, 0.52 g/L $MgSO4{\cdot}7H_2O$, 1.52 g/L KH_2PO_4, 3 µmol/L thiamine, 0.1 µmol/L biotin, 5 g/L glucose, 15 g/L agar, pH = 11.5) after 30 days. The results showed that ΔVdERG2-1 and ΔVdERG2-2 significantly reduced the production of melanin and microsclerotia compared with wild-type and complement mutant strains (Fig. 5A). Furthermore, we similarly analyzed genes related to melanin and microsclerotia formation such as *VT4HR* (VDAG_03665), *VaflM* (VDAG_00183), *Vayg1* (VDAG_04954), *VdLAC* (VDAG_00189), *VdSCD* (VDAG_03393) and *VDH1* (VDAG_02273). As expected,

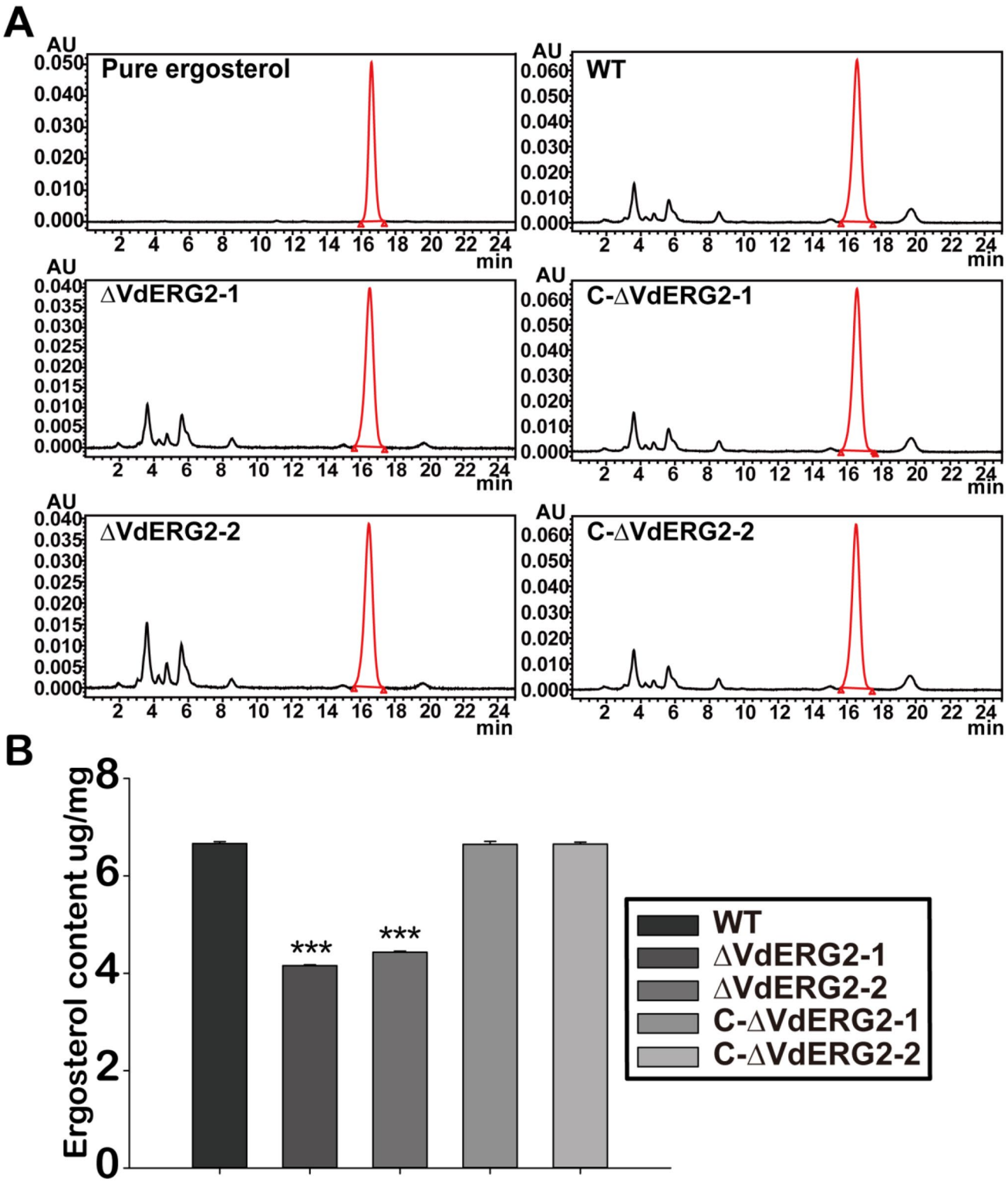

Fig. 3 Decrease of ergosterol biosynthesis in the *VdERG2* knockout mutant. **A** The ergosterol content in wild type, ΔVdERG2 and C-ΔVdERG2 strains were determined by high-performance liquid chromatography (HPLC). Commercial standard of ergosterol was used as the control. **B** The ergosterol content of the wild type, ΔVdERG2 and C-ΔVdERG2 strains. Values represent means ± standard deviation of three replicates. The asterisks represent statistical differences performed by a *t* test in comparison with the wild-type strains (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

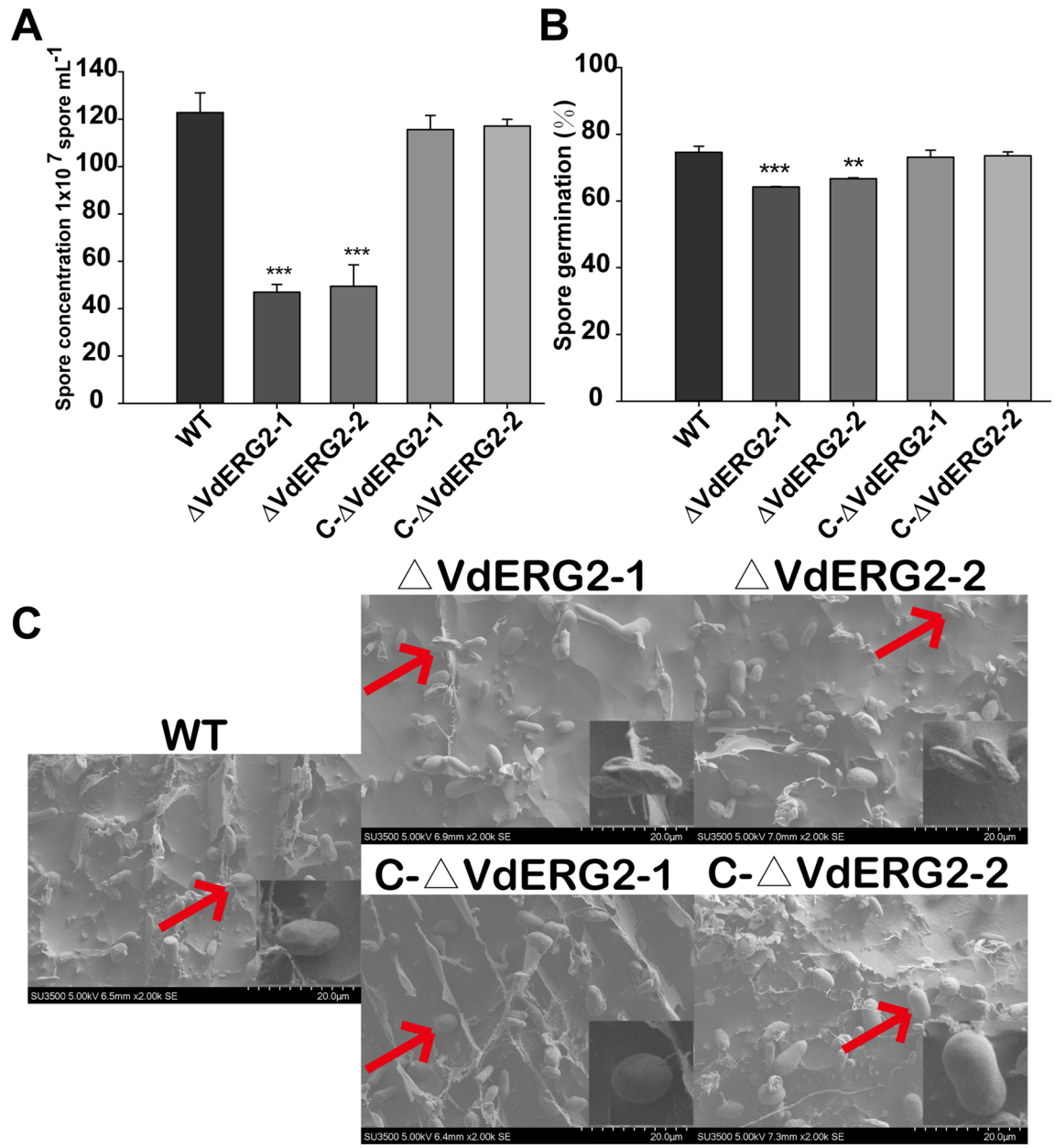

Fig. 4 VdERG2 involved in the regulation of conidia in *V. dahliae*. **A** The spore concentration of the wild type, ΔVdERG2 and C-ΔVdERG2 strains grown in liquid Czapek Dox medium for 5 days. **B** Spore germination rate of wild type, ΔVdERG2 and C-ΔVdERG2 strains cultured in solid PDA medium for 6 h. **C** The morphology of conidia was observed by scanning electron microscope. Scale bar = 20 μm. Values represent means ± standard deviation of three replicates. The asterisks represent statistical differences performed by a t-test in comparison with the wild type strains (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

the expression levels of these melanin-related genes in ΔVdERG2 were significantly lower than those in wild type and C-ΔVdERG2 (Fig. 5B). Thus, *VdERG2* was apparently involved in the production of melanin and microsclerotia in *V. dahliae*.

VdERG2 responded to CR, SDS and H_2O_2 stresses

The growth of *V. dahliae* was affected by various stresses in the soil. In this study, 1 mol/L KCl, 1 mol/L NaCl, 1 mol/L Sorbitol, 0.004% (mass/mass) SDS and 0.02% (mass/mass)

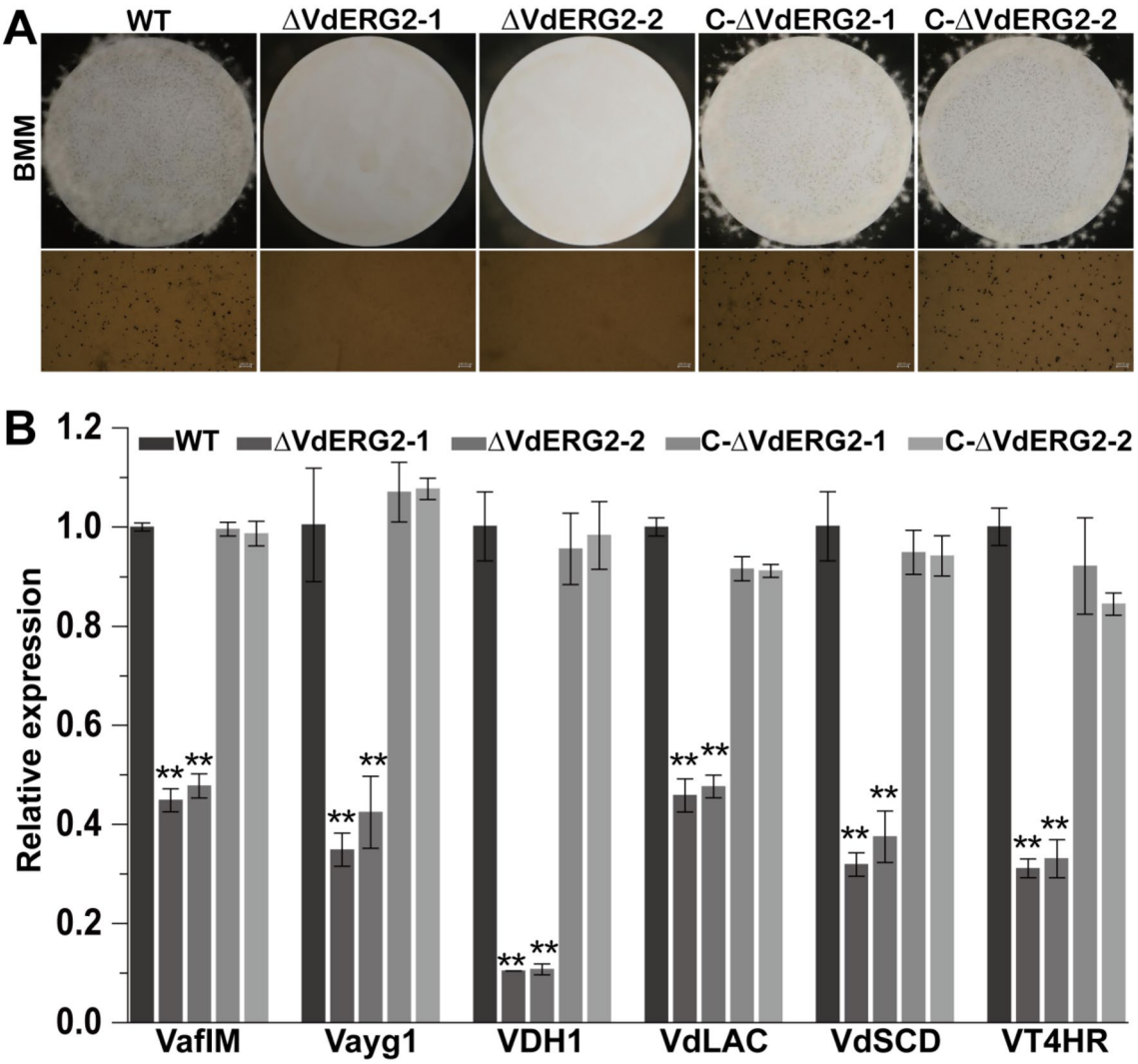

Fig. 5 *VdERG2* inhibited the production of microsclerotia. **A** Growth of microsclerotia of wild type, ΔVdERG2 and C-ΔVdERG2 strains cultured on BMM solid medium containing nitrocellulose membrane for 30 days. **B** Analysis using quantitative RT-qPCR to detect the expression of melanin-related genes *VaflM*, *Vayg1, VDH1, VdLAC*, *VdSCD* and *VT4HR*. Values represent means ± standard deviation of three replicates. The asterisks represent statistical differences performed by a t-test in comparison with the wild type strains (*$p < 0.05$, **$p < 0.01$)

CR were added to PDA medium to simulate abiotic stress, and the Plain PDA was used as the control. The results of 14 days of incubation showed that the growth of ΔVdERG2 in KCl, NaCl, Sorbitol and normal PDA medium were not different from those of wild type and complemented mutant (Figure S4). Under CR stress, the colony diameters of ΔVdERG2-1 and ΔVdERG2-2 were 37.39 mm and 38.33 mm, respectively, which were lower than those in normal PDA (63.5 mm and 63.75 mm). After gene complemented, the colony diameter increased, which was consistent with the wild type (Fig. 6A). Under SDS stress, the colony diameters of ΔVdERG2-1 and ΔVdERG2-2 were 31.61 mm and 32.03 mm, respectively, which were lower than those in normal PDA (63.5 mm and 63.75 mm). After the gene complemented, the colony diameter increased, which was consistent with that of the wild type (Fig. 6A). The above results showed that *VdERG2* was involved in the response of *V. dahliae* to CR and SDS-induced cell wall stress, the cell wall of mutant ΔVdERG2 was defective.

And then, we examined the growth status of these strains under H_2O_2 stress. The sensitivity of each strain to H_2O_2 was examined by measuring the diameter of the inhibition zone. Three gradients (7.5%, 15% and 30%) of H_2O_2 concentration were designed in the experiment. The results showed that the inhibition zones of ΔVdERG2-1 and ΔVdERG2-2 were larger than those of the wild type and complemented strains, and the phenotypes became more and more obvious with the increase of H_2O_2 concentration (Fig. 6B).

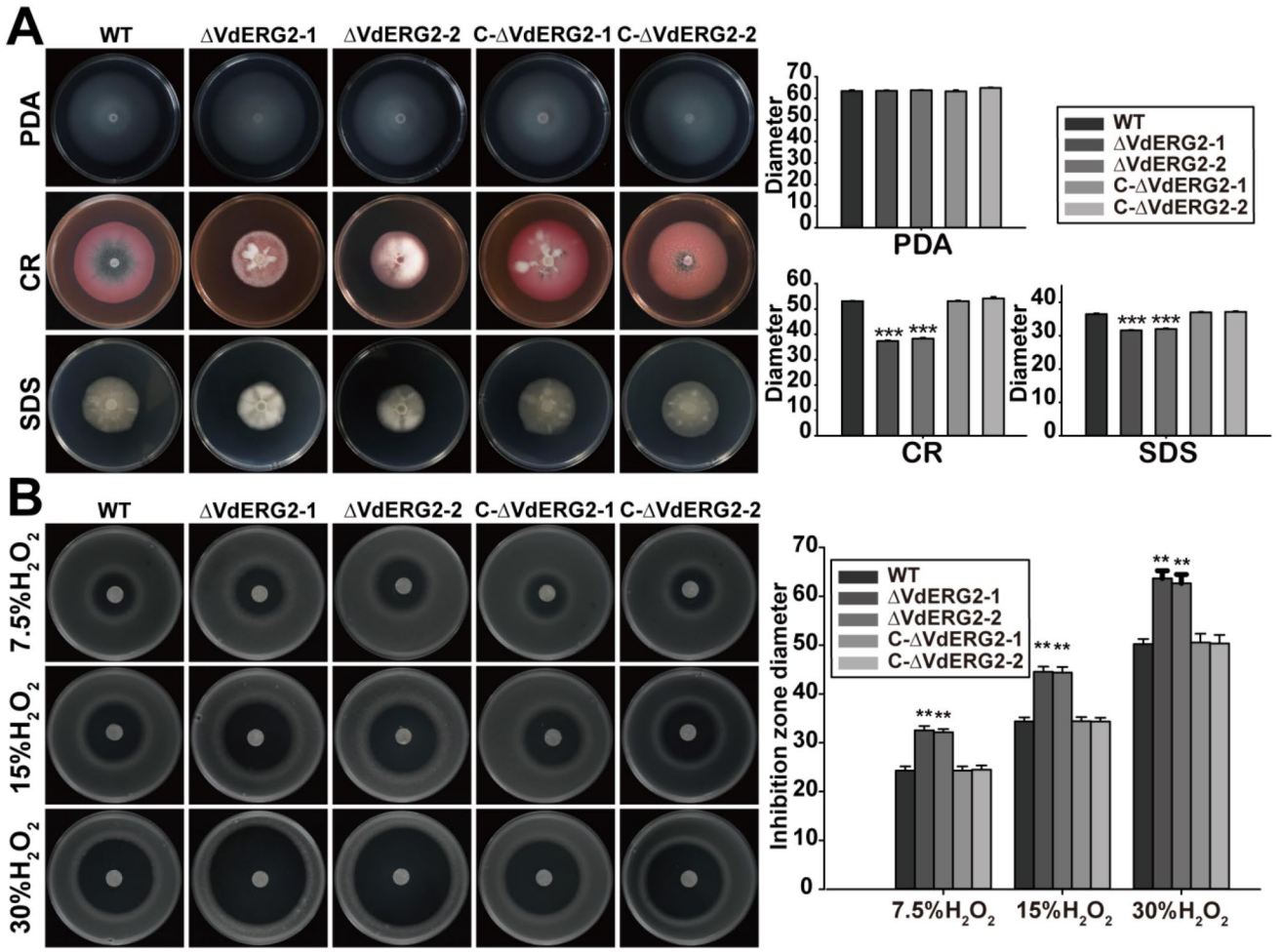

Fig. 6 *VdERG2* responded to CR, SDS and H_2O_2 stresses in *V. dahliae*. **A** Phenotype analysis and colony diameter determination of the wild type, ΔVdERG2 and C-ΔVdERG2 strains grown on PDA or PDA supplemented with 0.004% SDS and 0.02% CR for 14 days. **B** Similar numbers of spores (10^8 CFU/mL) of the wild type, ΔVdERG2 and C-ΔVdERG2 strains of *V. dahliae* were added to PDA plates. Five millimetre diameter sterile filter paper disks were placed in the centre of the plates, to which 5 µl of H_2O_2 (7.5%, 15% and 30%) was added. The plates were incubated at 25 °C for 2 days and the inhibition zones were recorded in millimetres. The suppression zone of the above plates after 2 days' incubation. Values represent means ± standard deviation of three replicates. The asterisks represent statistical differences performed by a *t* test in comparison with the wild-type strains (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

VdERG2 weakened mycelium penetration and affected mycelium growth on cellophane

To detect the penetration ability of cellophane, conidia of each strain were placed on PDA medium containing glass paper for 3 days at 25 °C. The results showed that all strains could grow normally on cellophane (Fig. 7A). By observing the growth of each strain on the cellophane, it was found that the mycelium growth of the knockout mutants was sparse and disordered, while the mycelium growth of the wild type and complemented strains were uniform and normal (Fig. 7C). By observing the back of the cellophane, it was found that all strains had conidia (Fig. 7B). When removing the cellophane, it was observed that the colony diameters of ΔVdERG2-1 and ΔVdERG2-2 were significantly smaller than those of the wild type and the complemented mutant (Fig. 7A). The above results showed that *VdERG2* could weaken the penetration of mycelium and affect the growth of mycelium on cellophane.

VdERG2 positively regulated the virulence of *V. dahliae*

To further study the function of *VdERG2* in the virulence of *V. dahliae*, the pathogenicity of wild type Vd080, ΔVdERG2-1, ΔVdERG2-2, C-ΔVdERG2-1 and C-ΔVdERG2-2 strains were determined using cotton root dipping method. The results showed that compared with the control inoculated with water, at 21 days, the leaves inoculated with the wild type strain showed severe yellowing

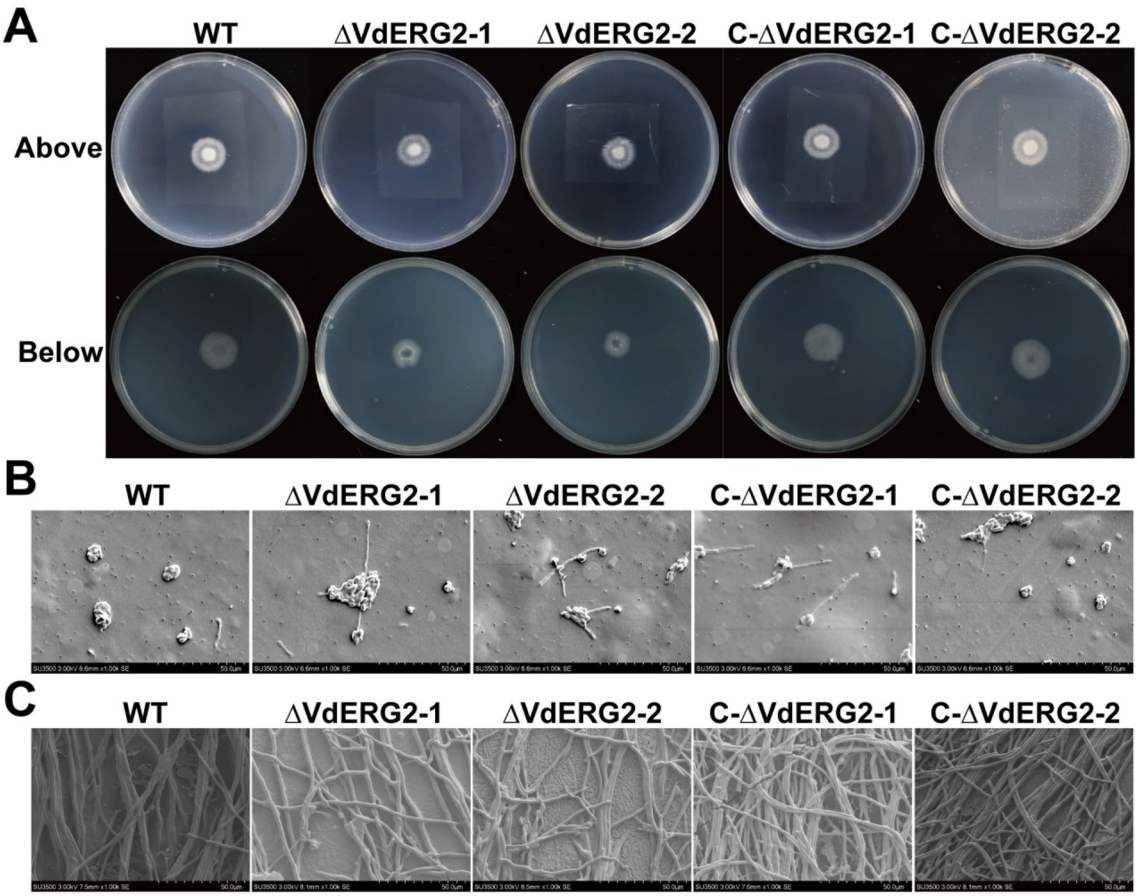

Fig. 7 *VdERG2* weakened hyphal penetration and affected hyphal growth on the cellophane of *V. dahliae*. **A** The cellophane membrane penetration assay. Penetration symptoms of the wild type, ΔVdERG2 and C-ΔVdERG2 strains grown on PDA medium overlaid with a cellophane layer (above) for 3 days and removal of the cellophane membrane for 5 days. **B** The existence of spores on the back of the cellophane membrane were observed by scanning electron microscope. Scale bar = 50 μm. **C** The morphology of hyphae on the cellophane membrane was observed by scanning electron microscope. Scale bar = 50 μm

wilting, necrosis, and typical symptoms of cotton verticillium wilt. After inoculation of the knockout mutant, the leaves also appeared yellowing and wilting, but it was significantly reduced compared with the inoculation of the wild type, and the leaf necrosis was rare. Verticillium wilt and leaf necrosis were aggravated after inoculation of the complemented mutants, and the incidence was not different from that of the wild type, indicating that the pathogenicity of *V. dahliae* to cotton was weakened after knockout of *VdERG2* (Fig. 8A). Observation of vascular bundle browning showed that the browning of stems inoculated with knockout mutant was significantly milder than that of stems inoculated with wild type and complemented mutants (Fig. 8B). Fungal recovery assay showed that the colonies of diseased cotton stems inoculated with knockout mutants were less than those of the wild type and complemented mutants (Fig. 8C). At 14, 18 and 21 days after inoculation, the disease index of cotton was investigated. The results showed that the disease index increased over time, at 21 days post inoculation, the disease index of cotton plants infected by deletion mutants (ΔVdERG2-1 and ΔVdERG2-2) were 34.56 and 34.22, respectively. While that of cotton plants infected by wild type, C-ΔVdERG2-1 and C-ΔVdERG2-2 were 71.11, 72.07 and 71.94, respectively (Fig. 8D). The fungal biomass in roots was evaluated by qRT-PCR, compared with the wild type strain, the fungal biomass of C-ΔVdERG2-1 and C-ΔVdERG2-2 strains did not differ significantly, whereas the fungal biomass of ΔVdERG2-1 and ΔVdERG2-2 strains decreased by 4.73 times and 4.54 times, respectively (Fig. 8E). These results showed that *VdERG2* positively regulated the pathogenicity of *V. dahliae*.

Discussion

Ergosterol is the major sterol component in the fungal plasma membrane (Liu et al. 2011, 2013). The biosynthetic pathway of ergosterol has been deeply studied in

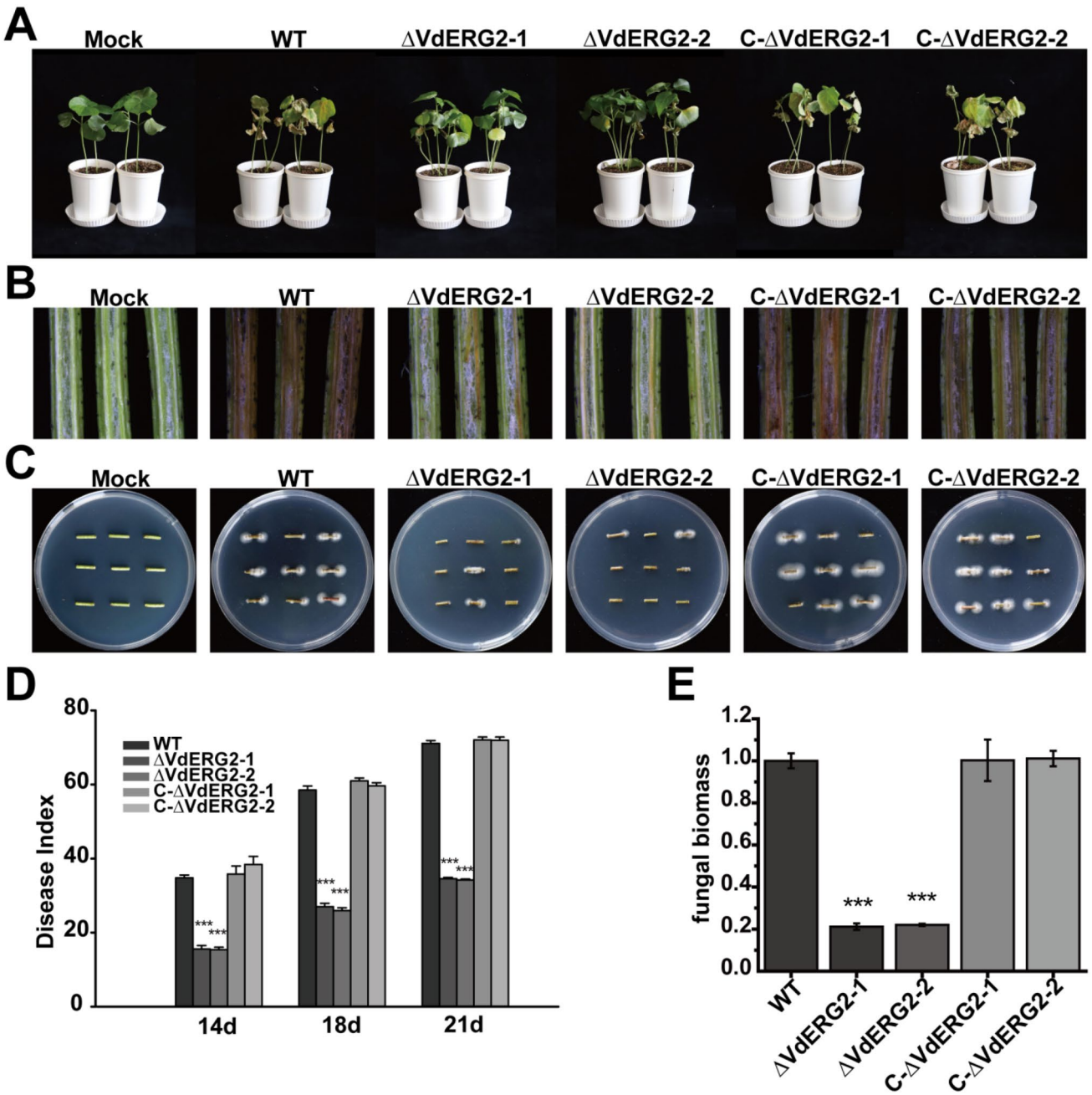

Fig. 8 *VdERG2* positively regulated the pathogenicity of *V. dahliae* in cotton **A** Disease symptoms of cotton after the wild type, ΔVdERG2 and C-ΔVdERG2 strains infection. Photographs were taken 21 days after fungal inoculation. **B** Vascular discoloration of the cotton stem tissue. **C** Reisolation of *V. dahliae* strains from the stem of inoculated cotton plants at 25 °C for 5 days. **D** Disease index of cotton plants at 14, 18, and 21 days after the wild type, ΔVdERG2 and C-ΔVdERG2 strains infection. **E** Fungal biomass in stems of cotton after the wild type, ΔVdERG2 and C-ΔVdERG2 strains infection at 21 days. *Vdβt* was used as the detection gene, and *GhUBQ7* of upland cotton was used as the endogenous control gene. Values represent means ± standard deviation of three replicates. The asterisks represent statistical differences performed by a *t* test in comparison with the wild type strains (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

S. cerevisiae. It has been found that the overexpression of *ERG2* gene promoted the biosynthesis of ergosterol, but the effect of *ERG2* gene knockout has not been demonstrated (Hu et al. 2017). In this study, we used *ERG2* in *S. cerevisiae* as the query and identified *VdERG2* in the genome of *V. dahliae*. VdERG2 was identified as a single conserved secretory protein in *V. dahliae* (Fig. 1). Knockout of *VdERG2* in *V. dahliae* reduced ergosterol production, suggesting that *VdERG2* played an important role in ergosterol biosynthesis in *V. dahliae* (Fig. 3). However, it also indicated that the

biosynthesis pathway of ergosterol in *V. dahliae* might be slightly different from that reported in *S. cerevisiae* due to the possibility of gene redundancy exists.

Verticillium wilt caused by *V. dahliae* is called cancer (Cai et al. 2009; Gong et al. 2017). Once the plant was infected, it was difficult for fungicides to effectively treat it, which may cause serious economic losses. Mycelium and conidia played an important role in the infection process of *V. dahliae* (Luo et al. 2016; Tang et al. 2020). The normal growth of *V. dahliae* was regulated by many genes. Previous studies have shown that the knockout of *FgERG4* in *F. oxysporum* resulted in decreasing conidial yield and conidial abnormalities (Liu et al. 2013). In *V. dahliae*, the knockout of *VdOGDH* resulted in abnormal conidia morphology and the absence of typical whorl branches, and the knockout of *VdHP1* also led to changes in conidia morphology (Li et al. 2020; Zhang et al. 2022a). In this study, it was found that the knockout of *VdERG2* led to a decrease in conidial yield and conidial germination, and abnormal conidial morphology (Fig. 4).

As a soil-borne fungus, environmental conditions in the soil caused various abiotic stresses and changes in nutritional conditions, thus affecting the normal growth of *V. dahliae*. Ergosterol production and cell membrane perturbations in *S. cerevisiae* directly affected the macromolecular structure and composition of the cell wall (Lesage and Bussey 2006). The stability of the cell wall was analyzed by CR treatment and it was determined that the strain overexpressing *ERG2* showed obvious growth defects on the medium containing CR (Bhattacharya et al. 2018). At the same time, we found that ΔVdERG2 was highly sensitive to CR and SDS stress, and confirmed the response of VdERG2 to cell wall stress. Oxidative stress and salt treatment inhibited the transcription of *ERG2* in *S.cerevisiae*, so overexpression of *ERG2* showed significant sensitivity to salt and oxidative stress (Jordá and Puig 2020). However, in our study, we found that ΔVdERG2 was highly sensitive to oxidative stress but did not respond to osmotic stress in *V. dahliae* (Fig. 6). The molecular mechanism of *V.dahliae* in response to osmotic stress may be different from that of *S. cerevisiae*. When *VdOGDH* was knocked out to supplement different carbon sources, the vegetative growth of *V. dahliae* was significantly inhibited (Li et al. 2020). After knocking out of *VdSNF1*, the growth of the mutant was significantly inhibited only when pectin or galactose was used as a carbon source (Tzima et al. 2011). In this study, when sucrose, cellolose, skim milk, pectin and starch as carbon sources, the growth of ΔVdERG2 was similar to those of the wild type and the complemented mutant. Interestingly, the melanin and microsclerotia production of the knockout mutant was weakened when cellulose or pectin was used as carbon sources (Figure S3). Previous studies have shown that melanin and microsclerotia were essential for survival in *V. dahliae* life cycle (Tzima et al. 2011). The virulence of *V. dahliae* seemed to be increasingly related to the development of microsclerotia. The ΔVdSsk2 strain showed severely delayed microsclerotia formation and melanization. Although the microsclerotia development of ΔVdste11 was similar to that observed in the wild type, it was obviously lack of melanin biosynthesis, indicating that both *VdSsk2* and *VdSte11* were involved in the microsclerotia or melanin biosynthesis (Yu et al. 2019). In this study, it was found that ΔVdERG2 mutant inhibited the formation of micronucleus and melanin, thereby reducing virulence (Fig. 5). Therefore, *VdERG2* not only participated in the response to CR, SDS and H_2O_2 abiotic stresses but involved in the formation of microsclerotia, thereby affecting virulence.

Infection results of pathogenic fungi showed that the knoukout of *VdERG2* reduced the penetration ability of *V. dahliae* mycelia, leading to disordered mycelial growth (Fig. 7). After inoculation with a conidia suspension of ΔVdERG2 strain, the cotton cotyledons turned slightly yellow with few deciduous leaves, compared with wild type and complemented mutant strains, the disease index of cotton inoculation with ΔVdERG2 significantly decreased (Fig. 8). The markedly reduced virulence of the knockout mutants might be caused by defects in multiple regulatory functions. First, fungal lipids, which were plasma membrane components exclusively, and demonstrated to be surface and extracellular components with key roles in fungal virulence (Rella et al. 2016). It was found that the knockout of *FgERG4* blocked the synthesis of ergosterol in *F. oxysporum*. *FgERG4* knockout mutants could successfully invade wheat and tomato, but the virulence of the mutants significantly decreased (Liu et al. 2013). Second, H_2O_2, one of the earliest cellular responses to infection, was produced when plant host cells challenged by biological and abiotic stresses (Fassler and West 2011). The sensitivity of ΔVdERG2 knockout mutants to H_2O_2 increased, which may explain the decreased virulence of ΔVdERG2 knockout mutants. In *V.dahliae*, *VdSkn7*, *VdSOD3* and *VdSOD5* were found to respond to oxidative stress, and the pathogenicity of knockout mutants was also significantly reduced (Fassler and West 2011; Li et al. 2021; Tang et al. 2020; Tian et al. 2020). Third, the cell wall of ΔVdERG2 knockout mutant was defective. In the presence of CR, knockout of *VdSsk1* or *VdSsk2* caused high expression of genes related to cell wall biosynthesis, and knockout of *VdCrz1* resulted in high sensitivity to SDS stress. Absence of these genes led to reduced pathogenicity of *V. dahliae* (Xiong et al. 2015; Yu et al. 2019; Zheng et al. 2019).In conclusion, sterol C-8 isomerase *VdERG2* of *V. dahliae* was a single conservative secretory protein and involved in the ergosterol biosynthesis, the regulation of conidia and the formation of melanin and microsclerotia. Furthermore, ΔVdERG2 was more sensitive to abiotic stresses (CR, SDS and H_2O_2), and weakened

the penetration ability of mycelium, thereby reducing the pathogenicity to cotton. This study showed that sterol C-8 isomerase was of great significance for the development, adaptability and pathogenicity of *V. dahliae*, which may provide a new perspective to further understand the molecular mechanism of ergosterol biosynthesis pathway in the virulence of *V. dahliae*.

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Data availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interests The authors declare no conflict of interest.

Informed consent Not applicable.

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