Journal of Cotton Research

RESEARCH Open Access

Succinate dehydrogenase SDH1–1 positively regulates cotton resistance to *Verticillium dahliae* through a salicylic acid pathway

ZHANG Xiangyue[1†], FENG Zili[1†], ZHAO Lihong[1], LIU Shichao[1], WEI Feng[1,2], SHI Yongqiang[1], FENG Hongjie[1,2*] and ZHU Heqin[1,2*]

Abstract

Background: Verticillium wilt, caused by the soil-borne fungus of *Verticillium dahliae Kleb*., is one of the most devastating diseases of cotton. The complex mechanism underlying cotton resistance to Verticillium wilt remains uncharacterized. Identifying an endogenous resistance gene may be helpful to control this disease. Previous studies revealed that succinate dehydrogenase (SDH) is involved in reactive oxygen species (ROS)-induced stress signaling pathway that is likely to be triggered by salicylic acid (SA). Here, through the metabolomics and differential expression analyses in wilt-inoculated cotton (*Gossypium hirsutum)*, we noticed that *GhSDH1–1* gene in cotton may play an important role in the resistance to *V. dahlia*. Then we reported *GhSDH1–1* gene and its functional analysis in relation to the resistance of cotton to *V. dahliae*.

Results: The *GhSDH1–1* gene in cotton root was significantly up-regulated after *V. dahlia* inoculation, and its expression level peaked at 12 and 24 h post-infection. SA can also induce the up-regulation of *GhSDH1–1*. Additionally, the functional analysis showed that *GhSDH1–1*-silenced cotton was more susceptible to *V. dahliae* than the control because of the significant decrease in abundance of immune-related molecules and severe damage to the SA-signaling pathway. In *Arabidopsis thaliana*, high expression of *GhSDH1–1* conferred high resistance to *V. dahliae*. *Arabidopsis* that overexpressed *GhSDH1–1* had higher resistance to *V. dahliae* infection compared with the wild-type.

Conclusions: Our findings provide new insights into the role of *GhSDH1–1*; it positively regulates cotton resistance to Verticillium wilt. The regulatory mechanism of *GhSDH1–1* is closely related to SA-related signaling pathway.

Keywords: Cotton, Verticillium wilt, Resistance gene, *GhSDH1–1*, Salicylic acid

Background

Cotton is a globally important economic crop and its fiber is an important textile to economies and people's livelihoods (Chen et al. 2007; Egbuta et al. 2017). Unfortunately in China, more than 200 million hectares of cotton are infected by Verticillium wilt, causing serious economic losses (Erdogan et al. 2006; Malik et al. 2014). Verticillium wilt is caused by the soil-borne fungus *Verticillium dahliae* Kleb (Yan et al. 2016). As the most destructive soil-borne disease to cotton growth, *V. dahliae* infects individuals through the root and causes wilting, discoloration, necrosis, defoliation, and ultimately causes plants to death. Consequently, the fungus seriously threatens cotton yield and fiber quality (Gerik and Huisman 1998; Xu et al. 2011; Jiménez-Díaz et al. 2012).

* Correspondence: fenghongjie@caas.cn; heqinanyang@163.com
†Zhang XY and Feng ZL contributed equally to this work.
[1]State Key Laboratory of Cotton Biology, Institute of Cotton Research of Chinese Academy of Agricultural Sciences, Anyang 455000, Henan, China
Full list of author information is available at the end of the article

Verticillium dahliae has a wide range of hosts, and in its dormant form, micro-sclerotium, is strongly resistant to environmental stresses. It inhabits the soil all the year round and can invade and occur in various growth stages of cotton, and thus it is difficult to control (Shaban et al. 2018). In agricultural production, the prevention and control of Verticillium wilt of cotton faces severe challenges caused by difficulties in implementing crop rotation, which leads to continuous-cropping of cotton in fields and results in high numbers of the pathogen in soils, as well as the lack of resistant cotton varieties and effective fungicides (Cai et al. 2009; Malik et al. 2014). Long-term production practices have shown that the search for the key genes for disease resistance in cotton is essential for managing Verticillium wilt (Cai et al. 2009). Recent studies have identified some key genes for resistance of cotton Verticillium wilt, such as *GbVIP1* gene and *GbTRP1* gene, which can regulate cotton resistance through positive and negative regulation, respectively (Zhang et al. 2019; Miao et al. 2019).

Complex II [succinate dehydrogenase (succinate-ubiquinone oxidoreductase); EC 1.3.5.1] is the only enzyme shared by both the tricarboxylic acid (TCA) cycle and electron transport chain in mitochondria (Huang and Millar 2013). Succinate dehydrogenase (SDH) usually consists of four subunits, flavoprotein (SDH1), iron-sulfur (Fe-S) protein (SDH2) and two small integral membrane proteins (SDH3 and SDH4). These four classical SDH1–4 subunits form a natural complex, which could catalyzes the oxidation of succinate to fumarate in the mitochondria matrix and transfers electrons to ubiquinone (Tretter et al. 2016; Huang et al. 2019). As a highly conserved protein, SDH is involved in mitochondrial oxidative phosphorylation and is a major source of ATP production in aerobic eukaryotic cells. In both mammalian and plant systems, the mitochondrial reactive oxygen species (mtROS) was a significant production of SDH, and the specific SDH inhibitors suppressed the ROS production, then impairing plant growth (Gleason et al. 2011; Ralph et al. 2011; Quinlan et al. 2012; Chouchani et al. 2014; Jardim-Messeder et al. 2015; Huang et al. 2016). A study using *Arabidopsis* SDH1 mutants *dsr1* and *sdhaf2* revealed that mutating SDH1 can reduce SDH activity, demonstrated a higher susceptibility to specific bacterial and fungal pathogens by disrupting the ROS-induced stress salicylic acid (SA) signaling pathway (Gleason et al. 2011; Belt et al. 2017; Huang et al. 2019). Although these studies indicate that SDH has an important relationship with plant disease resistance, it is not clear whether SDH is involved in the cotton resistance mechanism and how SA may interact with SDH to produce mtROS in cotton. A more comprehensive characterization of cotton SDH function may contribute to the breeding of cotton varieties resistant to Verticillium wilt.

Plants evolves defense mechanisms to identify and resist the infection of pathogens, including constitutive defense systems and inducible defense systems (Daayf 2015; Shaban et al. 2018). In the process of plant interaction with pathogens, a series of signals are transmited through plant signal molecules, such as reactive oxygen species (ROS), SA, jasmonic acid (JA), nitric oxide (NO), etc. They play an important role in regulating defense signaling networks and stimulating plant defense systems, enabling plants to develop disease-resistant responses (Chen et al. 1993; Moreau et al. 2012; Shaban et al. 2018). SA is an important signaling molecule. Once the SA pathway is activated at the site of infection, it usually triggers a defense response in distal plant parts to protect undamaged tissues. This long-lasting broad-spectrum sensing resistor is called system-acquired resistance (SAR) (Norman et al. 2004; Häffner et al. 2014; Verma et al. 2016). In addition, elevated levels of SA in pathogen-exposed tissues trigger the NON-EXPRESSOR OF PR GENE 1 (NPR1), leading to the expression of PATHOGENESIS RELATED (PR) genes and subsequent defense responses (Grant and Lamb 2006; Fu et al. 2012). The activation of plant immune responses is also associated with increases in the production of reactive oxygen intermediates and NO levels (Nie et al. 2015; Caarls et al. 2015). These complex signaling molecules and signal transduction networks provide plants with powerful means of regulating immune responses (Fradin et al. 2009; Li et al. 2011; Cheng et al. 2016).

In this study, we identified 18 SDH genes in cotton and found that four *SDH1* genes (*GhSDH1–1*, *GhSDH1–2*, *GhSDH1–3*, *GhSDH1–4*) were up-regulated after infection. Especially, the expression levels of *SDH1–1* peaked at 6 and 12 h after infection. Silencing this gene compromised cotton resistance and weakened SA signaling pathway. In contrast, overexpression of *GhSDH1–1* conferred resistance to pathogen infection in *Arabidopsis* plants. Our results provide important evidence illustrating that SDH positively regulates cotton resistance to Verticillium wilt through the SA signaling pathway.

Results

Analysis of *GhSDH1–1* structure and expression patterns in cotton treated with *V. dahliae* or salicylic acid

Based on the results of an unpublished metabolomics analysis of disease responses in cotton, the metabolite succinic acid content decreased and the fumaric acid content increased after inoculation (Additional file 1: Figure S1). The two substances were regulated by SDH, which can convert succinic acid into fumaric acid. Succinate dehydrogenase consists of four classical subunits and several accessory subunits and assembly factors

(Huang and Millar 2013). We used the SDH protein sequence of *Arabidopsis* to compare with that of *G. hirsutum* plants and identified 18 genes (the homology analysis of these genes are shown in Additional file 1: Figure S2). To further investigate the function of these genes, RT-qPCR analysis was performed to detect the expression of the SDH gene in roots of inoculated cotton (Fig. 1). The results showed that the genes of SDH1 subunit (GhSDH1–1, GhSDH1–2, GhSDH1–3, GhSDH1–4) were up-regulated obviously after infection. Especially, the expression level of *GhSDH1–1* peaked at 6 and 12 h after infection. This may be play an important role in cotton defense responses to *V. dahliae* infection.

To identify the signaling pathway associated with *GhSDH1–1*, we examined the *GhSDH1–1* expression pattern in hormone (SA)-treated 'Zhongzhimian 2' plants. The expression of *GhSDH1–1* was rapidly induced and peaked at 6 h after treatment. In addition, a lower SA concentration induced a higher level of *GhSDH1–1* expression (Fig. 2a). These results suggested that *GhSDH1–1* enhanced the resistance of cotton to *V. dahliae* and affected the SA-mediated signaling pathway.

Silencing of *GhSDH1–1* in cotton suppressed the resistance to *V. dahliae*

To clarify the function of *GhSDH1–1* in cotton responses against *V. dahliae*, we generated *GhSDH1–1*-knockdown plants by TRV-based virus-induced gene silencing (VIGS). At approximately 2 weeks post-infiltration, the positive contrast TRV-PDS plants started to display the albino phenotype in the true leaves. Additionally, we used qRT-PCR to assess gene-silencing efficiency. The abundance of *GhSDH1–1* transcripts was significantly lower in TRV:*GhSDH1–1* plants than that in the controls (Fig. 2b, c), indicating that *GhSDH1–1* was effectively silenced in these plants. Furthermore, after *V. dahliae* inoculation, disease symptoms were not visually apparent on the tissues of control plants, while necrotic, yellowish, stunted, and wilting leaves were observed on

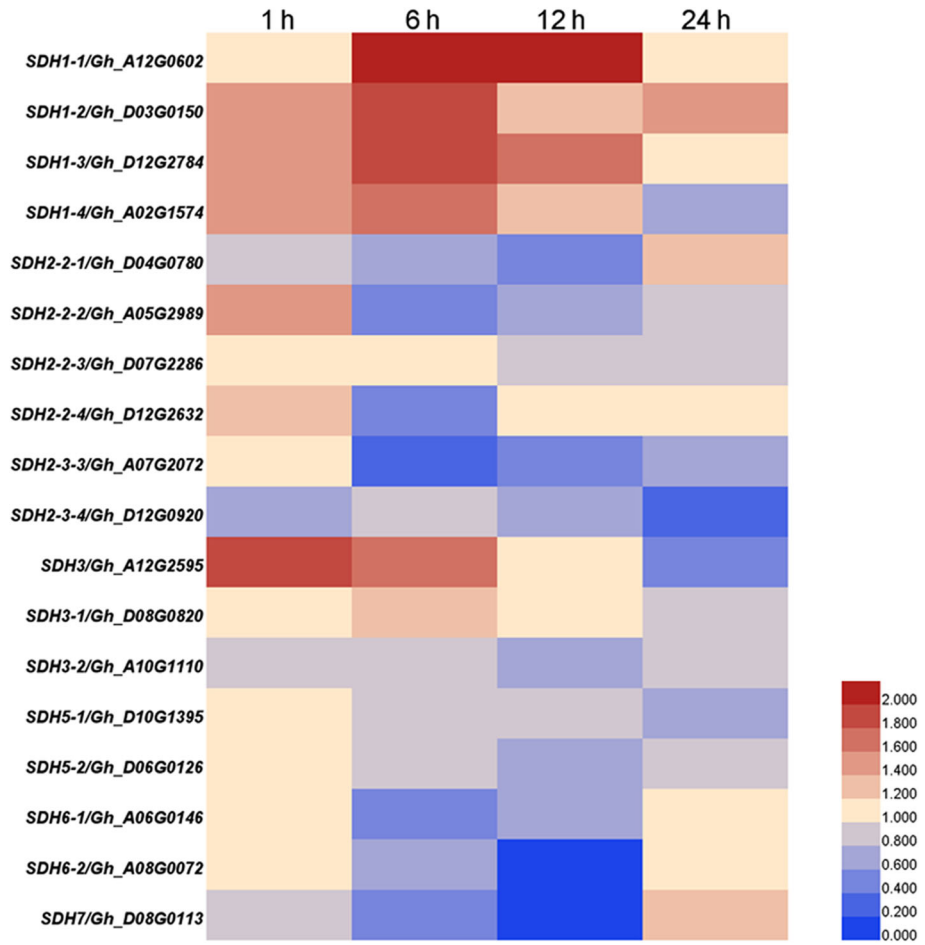

Fig. 1 Expression patterns of *GhSDH1–1* under *Verticillium dahliae* stress treatment in cotton. The cotton 'Zhongzhimian 2' were treated with *Verticillium dahliae* (Vd080) or an equal amount of sterile water, and the mock and treated roots were harvested at 0, 6, 12, 24 h post inoculation (hpi), respectively. The expression levels were determined by qRT-PCR, and the expression level of the mock control was normalized to '1'

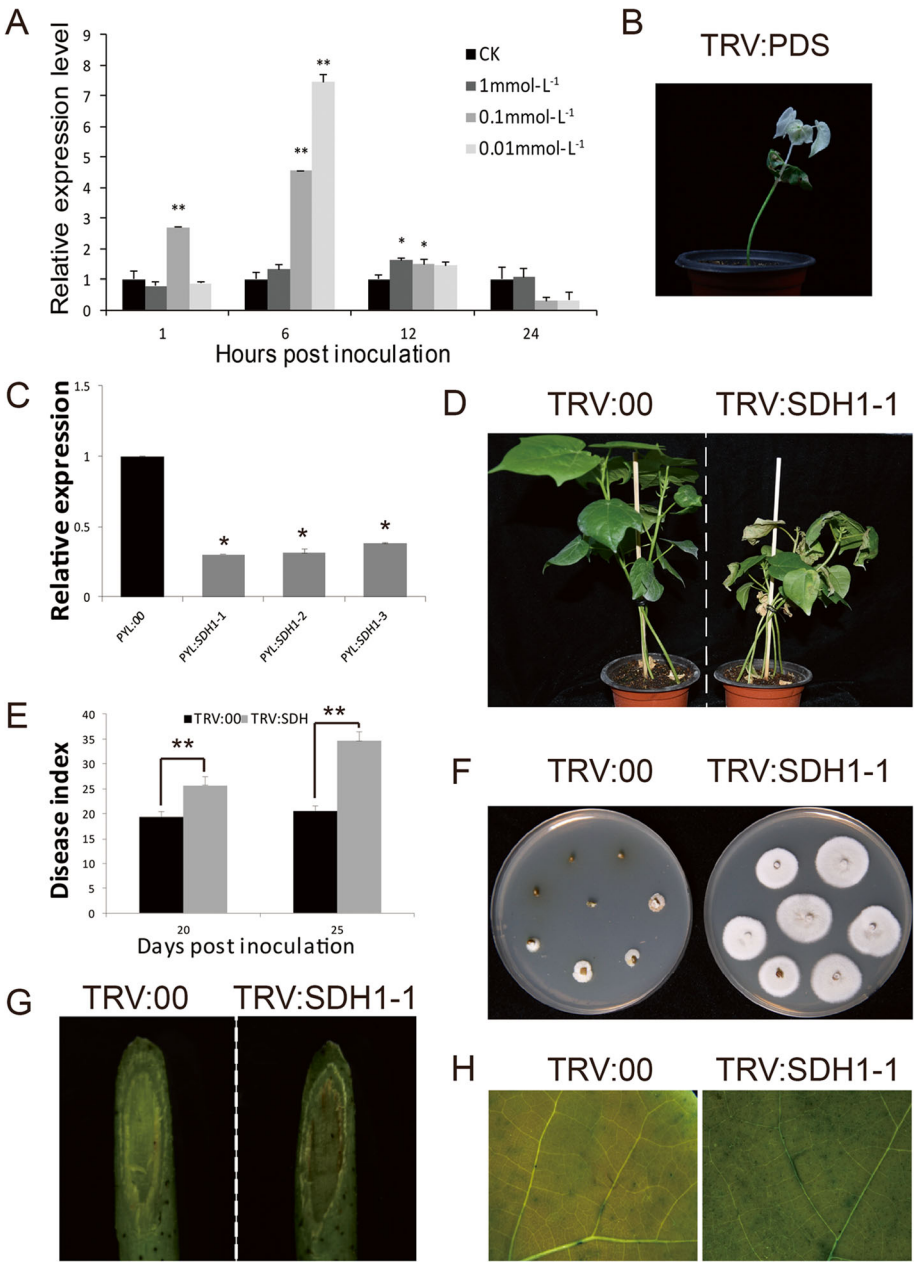

Fig. 2 **a** *GhSDH*1-1 expression pattern in hormone (SA)-treated 'Zhongzhimian 2' plants. **a**, Treatment with exogenous hormone SA (1 mmol·L^{-1}, 0.1 mmol·L^{-1}, 0.01 mmol·L^{-1}) or an equal amount of sterile water. Mimetics and treated roots were harvested at 1, 6, 12, 24 hpi, respectively, and expression levels were determined by qRT-PCR. The expression level of the simulated control was normalized to '1'. Silencing of *GhSDH1–1* seriously compromises Verticillium wilt resistance in cotton. **b**, TRV:PDS as the visual marker for silencing efficiency. Albino phenotype appeared on TRV:*SDH1–1* plants. **c**, Relative transcript levels in control and TRV:*SDH1–1* plants 15 d after hand-infiltration. **d**, Disease symptoms induced with *V. dahliae* on control and TRV:*SDH1–1* plants at 20 dpi and 25 dpi. **e**, Assessment of DI for TRV:*SDH1–1* plants at 20 dpi and 25 dpi. **f**, *Verticillium dahliae* recovery assay. **g**, Vascular browning found in xylem from infected plants at 25 dpi. **h**, Trypan blue staining. Error bars represent the standard deviation of three biological replicates. Asterisks indicate statistically significant differences as determined by Student's t test (*$P < 0.05$; **$P < 0.001$)

TRV:*GhSDH1–1* plants. The DI of TRV:*GhSDH1*–1 plants was significantly higher than that of the control at 20 and 25 days post-inoculation (Fig. 2d, e). The results confirmed that the resistance of cotton plants to *V. dahliae* was compromised because of the absence of *GhSDH1–1* expression.

In order to study the degree of colonization of *V. dahliae* in the stem of infected plants, the recovery assay

showed that the number of fungi on TRV:*GhSDH1–1* was significantly higher than that of control. In addition, the xylem of TRV:*GhSDH1–1* plants showed greater vascular browning than that of the controls (Fig. 2f, g). To visually compare the differences in plant leaves inoculated with *V. dahliae*, we stained dead cells with trypan blue. The trypan blue stained area was larger and the color was stronger in TRV:*GhSDH1–1* leaves than in control leaves (Fig. 2h).

Silencing of *GhSDH1–1* attenuated the expression of resistance-related genes

To further elucidate the effect of *GhSDH1–1* on Verticillium wilt resistance in cotton plants, we compared the expression levels of disease-related genes in silenced plants with control inoculated with *V. dahliae* and sampled at different times. The genes of the enzymes peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) are involved in lignin synthesis. When not vaccinated, the gene expression of TRV:*GhSDH1–1* plants was similar to that of the control or even higher than that of the control. However, at 1, 3, 6, 12, 24, and 48 h after inoculation, the responses of these genes to *V. dahliae* infection were significantly disrupted in the TRV:*GhSDH1–1* plants, and the difference was greatest at 24 h (Fig. 3). The expression level of *HIN* (HR maker gene) and *JAZ* (JA signaling pathway-related gene) were significantly decreased in the TRV:*GhSDH1–1* plants (Fig. 3).

Salicylic acid pathways are important signaling pathways in plant disease resistance. We examined the expression levels of SA signaling pathway-related genes, including those involved in SA biosynthesis, such as non-expressor of pathogenesisrelated genes 1 (*NPR1*), pathogenesis-related gene 1 (*PR1*), and pathogenesis-related gene 3 (*PR3*). After inoculation, the expression levels of *NPR1*, *PR1* and *PR3* in TRV:*GhSDH1–1* plants were significantly weakened. The evidence suggested that silencing of *GhSDH1–1* greatly attenuated the expression of resistance-related genes, such as the lignin synthesis-related genes. Consequently, significant effects occurred on the SA biosynthesis signal transduction pathway (Fig. 3).

Salicylic acid, nitric oxide and H_2O_2 decreased in *GhSDH1–1*-silenced cotton plants upon *V. dahliae* infection

To further evaluate the role of *GhSDH1–1* and its relationship with the SA pathway in cotton defense responses to *V. dahliae* infection, we analyzed the abundance of fumaric acid, SA and several other immune-responsive compounds (NO, H_2O_2) in silenced and control plants. We observed a decrease in fumaric acid content *GhSDH1–1*-silenced plants (Fig. 4a). The content of SA in TRV:*GhSDH1–1* plants was also significantly reduced compared with that of control plants (Fig. 4b). Reactive oxygen is considered an important indicator of plant disease resistance. The results showed that the content of H_2O_2 in silenced plants was lower than that of the control within the first hour after inoculation, while the content of NO in silenced plants increased first and then decreased compared with that of control plants (Fig. 4c, d). Furthermore, we observed the physical occurrence of reactive oxygen species by diaminobenzidine (DAB) staining in cotton leaves 24 h after inoculation. The DAB-stained area was large and the color was intense in the silenced plant leaves, while there was no obvious staining in control leaves (Fig. 4e). These results indicate that silencing *GhSDH1–1* in wilt-resistant cotton plants inhibits *V. dahliae*-induced production of SA, NO and H_2O_2, further confirming that *GhSDH1–1* is closely related to the SA signaling pathway.

Overexpression of *GhSDH1–1* in transgenic *Arabidopsis* plants enhanced *V. dahliae* resistance

To further detect whether *GhSDH1–1* confers resistance to *V. dahliae*, an overexpression strategy in *Arabidopsis* plants was used. The *GhSDH1–1* gene was inserted into the pCAMBIA2300 plant vector and infected into *A. thaliana* ecotype Col-0. Two methods were used to select homozygous *GhSDH1–1*-overexpressing transgenic lines, including a 0.1% Kanamycin-screening and qPCR (Additional file 1: Figure S3). Two lines (line2 and line3) with the highest expression level of *GhSDH1–1* were chosen for further analysis. Transgenic and WT plants were subjected to *V. dahliae* infection. Fourteen days after inoculation, WT plants showed more serious yellowing and wilting than transgenic plants (Fig. 5a, b). The disease indexes of line2 and line3 were 16.3 and 11.7, respectively, which were significant lower than that of control line (Fig. 5c). This further confirms that the transgenic *A. thaliana* plants overexpressing *GhSDH1–1* were more resistant to Verticillium wilt than the WT plants.

Subcellular localization of *GhSDH1–1*

To gain direct evidence for Gh SDH1–1 subcellular localization, the full length of SDH1–1 gene was cloned into the vector pBin-GFP, and constituted the transient expression vector SDH1–1-GFP. Mt-rk is a mitochondrial marker gene for mitochondria as a positive control. The fluorescence was observed by confocal laser-scanning microscopy. The results showed that a spotty distribution of the green fluorescence of SDH1–1-GFP in the cytoplasm, and the red fluorescence of the mitochondrial marker mt-rk was punctate and elongated (Fig. 6). In addition, agrobacterium cells containing

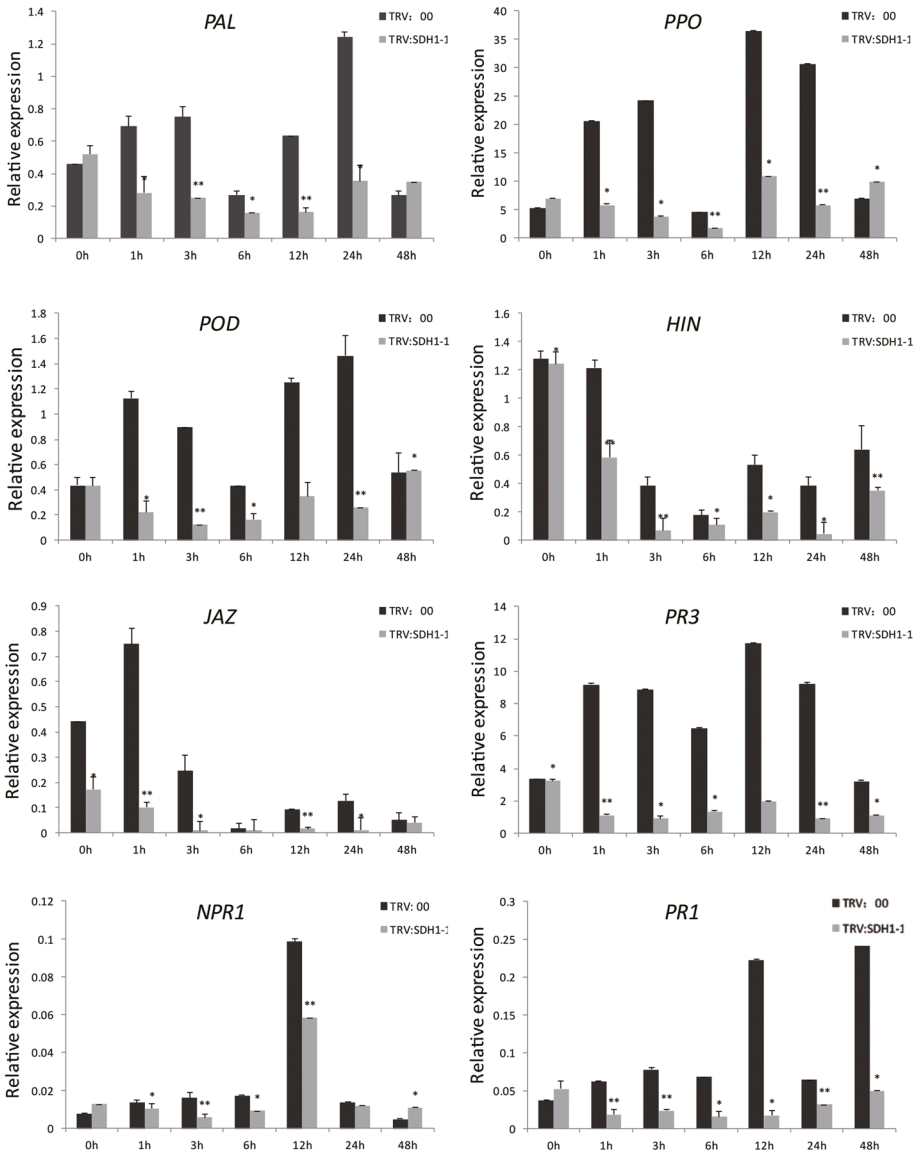

Fig. 3 Detection of expression levels of disease resistance-related genes in *GhSDH1–1*-silenced and control cotton plants. Total RNAs were extracted from *GhSDH1–1*-silenced and control cotton plants after treatment with *V. dahliae*, and roots were harvested at 0, 1, 3, 6, 12, 24, and 48 hpi, respectively. The expression levels of resistance-related genes were determined by qRT-PCR. Error bars represent the standard deviation of three biological replicates. Asterisks indicate statistically significant differences, as determined by Student's t-test (*$P < 0.05$; **$P < 0.001$)

SDH1–1-GFP and mt-rk were co-infiltrated into *N. benthamiana* leaves. Indeed, the SDH1–1 protein localizes to mitochondria.

Discussion

In recent years, in-depth studies of genomics, transcriptomics and proteomics have laid the foundation for the analysis of cotton candidate genes and regulatory mechanisms for Verticillium wilt and other diseases, with the ultimate aim of generating disease-resistant cultivars by molecular breeding. In recent years, cotton leaf curl disease (CLCuD) is also a severe disease of cotton. It caused destructive damage to cotton production in Pakistan and India (Sattar et al. 2013). Some genes involved in cotton defense responses have been identified. For example, the key role of the thioredoxin-encoding gene *GbNRX1* in *V. dahliae* infection is associated with homeostasis of apoplastic reactive oxygen species (Li et al. 2016; Zhou et al. 2018). The ribosomal protein GaRPL18 (Gong et al. 2017) regulates cotton resistance to Verticillium wilt through a SA signaling pathway. However, the development of these genes for treatment is still far from adequate. Recent research showed that the identification of cotton earlier resistance genes is

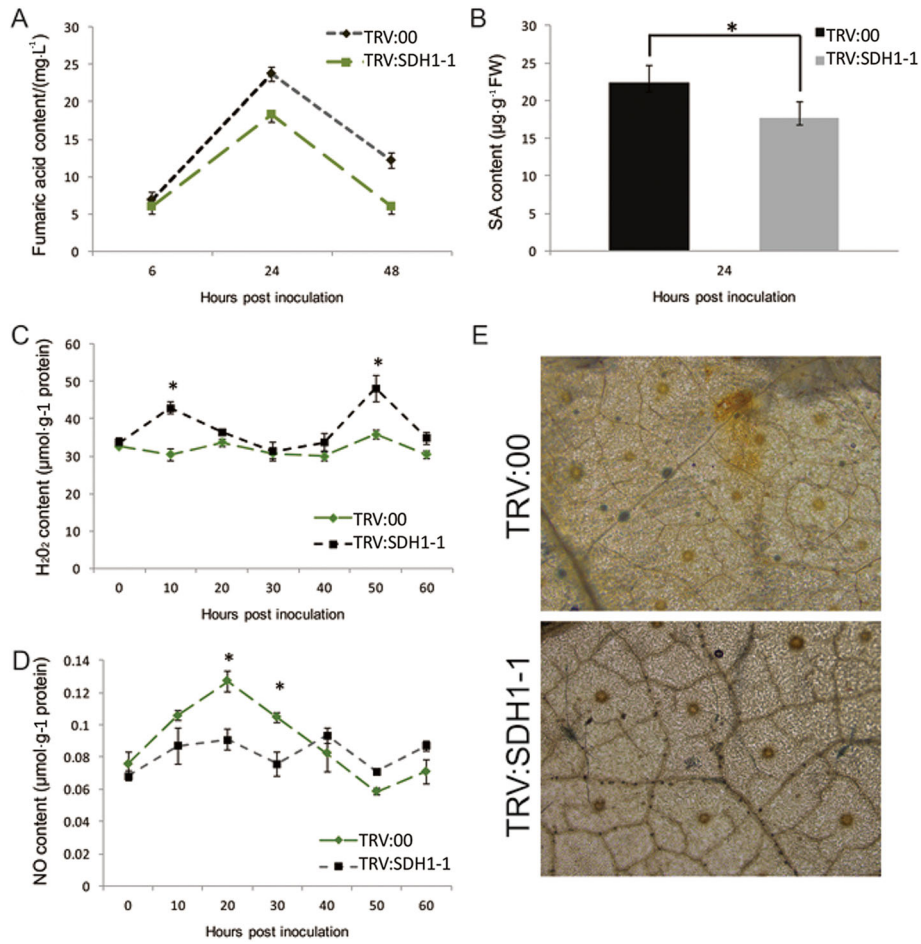

Fig. 4 The abundance of fumaric acid, SA and several other immune-responsive compounds (NO, H_2O_2) in *V. dahliae* treated silencing and control plants. **a**, The abundance of fumaric acid. **b**, The abundance of SA. **c**, **d**, The content of H_2O_2 and NO in *GhSDH1–1*-silenced and control cotton plants within 1 h after inoculation. **e**, Diaminobenzidine (DAB) staining to the plants at inoculation 24 hpi

one of the most effective ways to prevent the disease. When cotton is attacked by pathogens, it first generates hormone signals and then activates a series of resistance responses, such as the elicitation of PR genes, production of phytoalexins, and reinforcement of cell wall. The discovery of early resistance genes is critical to the development of new varieties of disease-resistant cotton. In the present study, we demonstrated that *GhSDH1–1* encoding SDH affects resistance to Verticillium wilt. And as an early resistance gene, it is likely to have an important resistance not only in cotton resistance to Verticillium wilt, but also in cotton resistance to leaf curl disease.

As a highly conserved protein, SDH has been thoroughly researched in mitochondrial oxidative phosphorylation and production of ATP in aerobic eukaryotic cells. Moreover, recent in-depth studies report that SDH also has important roles for mtROS production in both mammalian and plant systems (Yankovskaya et al. 2003; Quinlan et al. 2012). Complex II generates ROS in both the forward reaction, with electrons supplied by succinate, and the reverse reaction, with electrons supplied from the reduced ubiquinone pool (Quinlan et al. 2012). The *Arabidopsis* SDH1 mutants dsr1 and sdhaf2 showed that SDH is involved in a ROS-induced stress signaling pathway that is likely to be triggered by SA (Gleason et al. 2011; Belt et al. 2017). These evidences suggest that SDH plays an important role in plant disease resistance and may be associated with the SA signaling pathway. Our study results also indicate that the SDH gene plays a role in disease resistance. The data show that SDH can positively regulate cotton Verticillium wilt. We found that the genes of the SDH1 subunit in cotton roots were significantly up-regulated after inoculation, especially *GhSDH1–1*, which peaked at 12 and 24 h post-infection. The study of *dsr1* suggested that the mutation of SDH1 reduced the catalytic efficiency of SDH, and the *K*m value as a binary switch of improving plant stress sensitivity and tolerance was lower, interrupting the SA signal pathway and reduced disease resistance

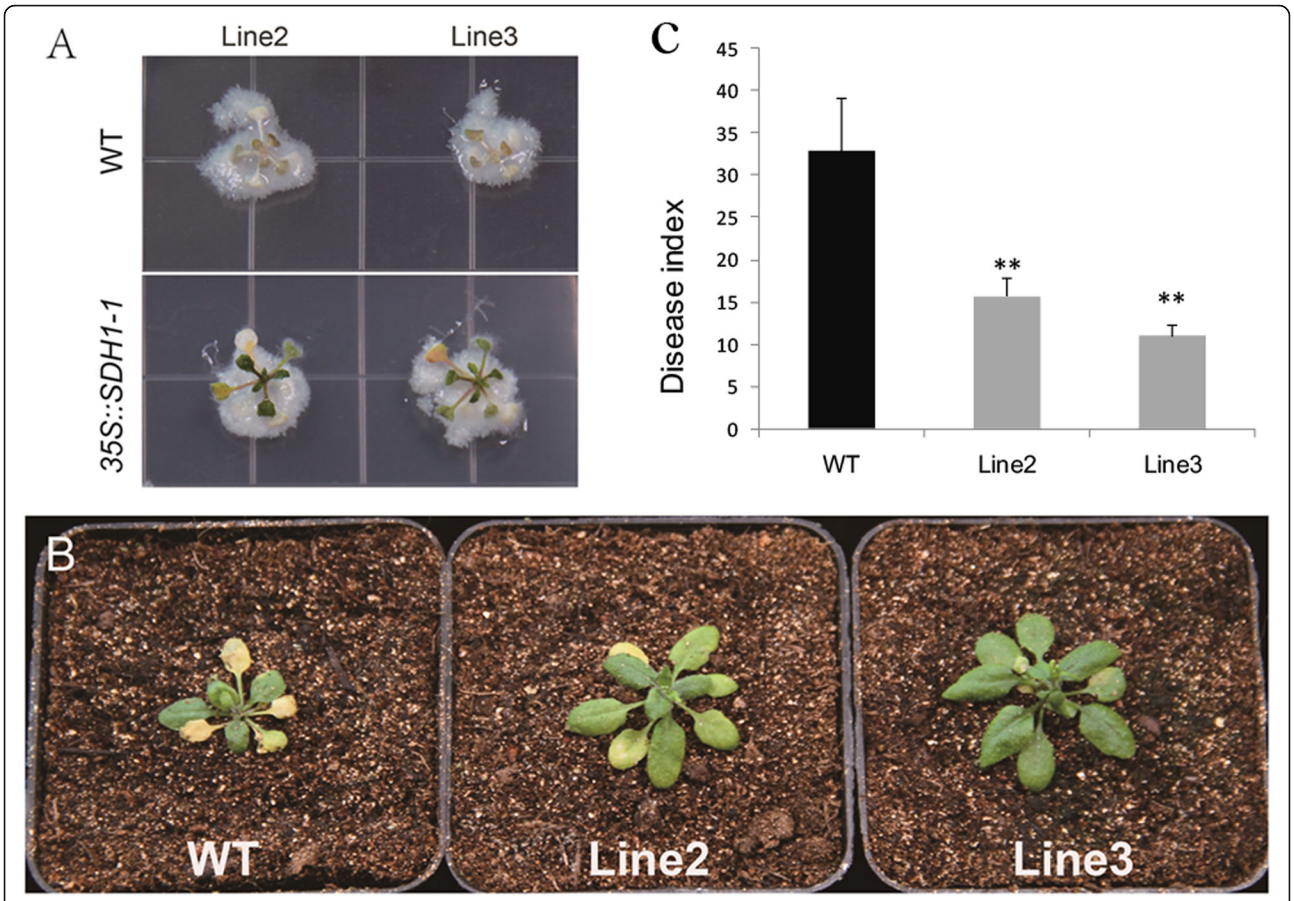

Fig. 5 Effects of overexpression of *GhSDH1–1* in defense response to *V. dahliae* in *A. thaliana*. Selecting Line2 and Line3 which have higher expression levels of *GhSDH1–1* for testing. Twenty dpi old seedings were inoculated with *V. dahliae*, the control plants were sprayed with water. Photos were taken at 15 dpi (**a**) and 20 dpi (**b**). **c**, Assessment of disease index (DI) at 15 dpi. Error bars represent the standard deviation of three biological replicates ($n \geq 30$). Asterisks indicate statistically significant differences when compared with control

(Gleason et al. 2011; Belt et al. 2017). Using VIGS technology, we found that the content of fumaric acid decreased as expected when the gene was silenced, which may be due to the reduction of SDH catalytic efficiency. In addition, DI values and fungal recovery assay data confirmed that silencing of *GhSDH1–1* significantly reduced the resistance of cotton to *V. dahliae*. When we tested the outbreak of reactive oxygen species in *GhSDH1–1*-silenced and control plants within 1 h after inoculation, the H_2O_2 content of silenced plants was lower than that of the control plants. An active oxygen burst is an important indicator of the initial stage of

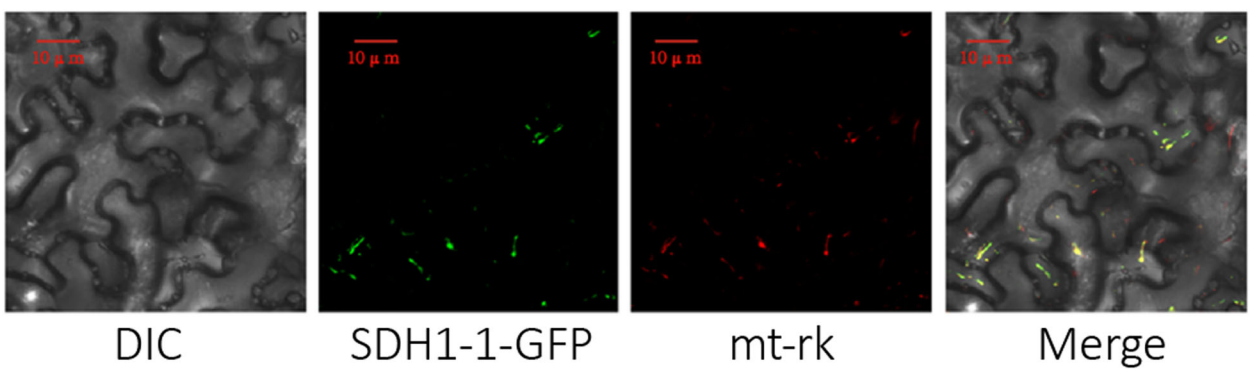

Fig. 6 Co-localization of SDH1–1-GFP with mitochondria markers

plant disease resistance. The in vitro experiments also confirmed that the control plants had more reactive oxygen bursts than the silenced plants. The content of NO in silenced cotton plants was slightly higher than that of control plants at the initial stage of infection, and then was lower than the control in the latter stage. The change in NO content may be due to the transient dynamic equilibrium between NO and H_2O_2 (Rao et al. 1997; Niu and Liao 2016). This reduction of ROS in *GhSDH1–1*-knockdown plants may be because the destruction of that, which the SA stimulation of SDH lead to reactions with oxygen to form ROS including superoxide (O_2^-) (Belt et al. 2017). In *GhSDH1–1*-knockdown plants, the expression levels of *PPO*, *POD*, and *PAL* related to lignin synthesis were also disrupted. The expression of disease-related genes showed that the expression levels of *PPO*, *POD*, and *PAL* related to lignin synthesis were lower in silent plants than in control plants. Lignin is one of the main components of the cell wall. Generally, the higher the lignin content of the plant, the stronger the resistance to pathogens, especially of the vascular bundles infected by *V. dahliae* (Sun et al. 2013). These results indicate that with the silencing of *GhSDH1–1*, H_2O_2, NO, lignin synthesis and other important disease-resistance processes are inhibited and significantly increase plant susceptibility to infection, thus supporting our conclusion of the important role of *GhSDH1–1* in cotton disease resistance. Furthermore, we analyzed disease resistance traits in transgenic *Arabidopsis* plants based on DI values. The results indicate that WT plants showed more severe yellowing, chlorosis, and dysplasia than the overexpressed lines after inoculation. In summary, we found that *GhSDH1–1* positively regulates cotton resistance to *V. dahliae*.

The use of different concentrations of SA to treat cotton and detection of expression of *GhSDH1–1* helped further elucidate the relationship of *GhSDH1–1* and the SA signaling pathway. The different concentrations of SA induced the expression of *GhSDH1–1*. The peak level occurred at 6 h after inoculation where the lowest concentration of SA induced the strongest expression of *GhSDH1–1* (Yalpani et al. 1991). This evidence suggests that *GhSDH1–1* may be associated with the SA signaling pathway. Moreover, it is demonstrated that the SA content was low in *GhSDH1–1*-silenced plants. SA is required for both local and systemic immunity against a wide range of the related genes of the pathogens (Fu et al. 2012; Kazan and Lyons 2014; Huang et al. 2016).

Three essential genes of the SA signaling pathway include *NPR1, PR1,* and *PR3* (Häffner et al. 2014). Our results show that the expression levels of these genes were significantly decreased in the silenced plants, indicating that silencing *GhSDH1–1* disrupted the response of the SA signaling pathway. Previous studies have demonstrated that *Arabidopsis* mutants of SDH1−1 have increased sensitivity to fungi and are more susceptible to disease because the SA-mediated downstream stress response was disrupted and mROS production reduced (Gleason et al. 2011; Edward et al. 2014; Huang et al. 2016; Belt et al. 2017). This is consistent with our findings, demonstrating that *GhSDH1–1* interacts with the SA signaling pathway and positively regulates cotton resistance to *Verticillium dahliae*. Therefore, *GhSDH1–1* is a promising candidate gene to improve the breeding of cotton varieties resistant to Verticillium wilt.

Materials and methods

Plant materials and growth conditions

Seeds of *G. hirsutum* Linn cv. 'Zhongzhimian 2' (a wilt-resistant cultivar) were obtained from the Institute of Cotton Research of Chinese Academy of Agricultural Sciences. For disease assays, plants were grown in greenhouse at a 16 h day/8 h night cycle and 25 °C. The *GhSDH1–1* overexpression vector (35S::*GhSDH1–1*) was inserted into wild-type (WT) *A. thaliana* Columbia ecotype (Col-0) plants. *Arabidopsis* seedlings were grown in a temperature- and light-controlled greenhouse between 22 °C and 28 °C with a 16 h/8 h light cycle.

Culturing of *V. dahliae* and inoculation of plants

Verticillium dahliae strain V080 was cultured on potato dextrose agar (PDA) medium at 25 °C for 5 days and then transferred to liquid Czapek's medium at 25 °C for 6 days. The final concentration of spore suspension was adjusted to 1×10^{10} conidia·L^{-1} with sterile water (Zhang et al. 2015).

The first euphylla was fully expanded, and cotton seedlings were applyed slight damage to roots. And then 10 mL of the V080 conidia suspension (1×10^{10} spores·L^{-1}) was injected into the soil. Control plants were inoculated with an equal volume of sterile distilled water. Similarly, after of growth in *Arabidopsis*, seedlings were grown in pots, and 10 mL of the V080 conidia suspension (1×10^{10} spores·L^{-1}) was injected into the soil at 20 days after germination. While, seedlings on the medium (1/2 MS), were inoculated with 2 μL of conidia suspension (1×10^{8} conidia·L^{-1}) after 2 weeks of germination (Gong et al. 2017).

Transcription level analysis of genes by real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from wilt-resistant cultivars (Zhongzhimian 2) using an RNAprep Pure Plant Kit (Tiangen, Beijing, China) and the instructions of its manual. Total RNA was reversely transcribed into cDNA using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Japan). Ubiquitin10 was used as an endogenous reference for normalization of cotton mRNA. All RT-

qPCR primers were designed by the Primer Premier 5.0 program, and listed in Additional file 1: Table S1. RT-qPCR was performed to determine the relative expression levels of several defense-related genes using SYBR Green PCR Master Mix (TIANGEN Biotech). The three-step method involved the following PCR conditions: 40 cycles of 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 30 s. A minimum of three technical replicates were performed for each sample with at least three biological replicates. RT-qPCR analysis was determined using the $2^{-\Delta\Delta Ct}$ methods (Hu et al. 2018). Statistical significances were evaluated by *t*-test using SigmaPlot version 12.0. And, the confidence level of all analyses was set at 95% and *P*-values < 0.05 were considered statistically significant.

Generation of the virus-induced gene-silencing construct and pathogen inoculation

The *GhSDH1–1* fragment was amplified by PCR using 'Zhongzhimian 2' cDNA with the V*GhSDH1–1*-F/V*GhSDH1–1*-R primers (Additional file 1: Table S1). The amplified fragments were subsequently cloned into the pTRV (TRV156) vector. The constructs (TRV:156, TRV:192, and TRV:*SDH1–1*) were then used to transform *Agrobacterium tumefaciens* strain GV3101 (Gao et al. 2011). An equivalent amount of TRV-156 vector was then injected into the cotyledons of 7-day-old cotton seedlings resistant to Verticillium wilt. After 24 h of incubation in the dark, cotton seedlings were grown in the greenhouse and inoculated with *V. dahliae* 10 days after infiltration with the vector.

Arabidopsis thaliana transformation

The full-length coding sequence of *GhSDH1–1* (1 902 bp) containing *XbaI* and *SacI* linkers was cloned using the primers *GhSDH1–1* (Table S1). For overexpression studies, the 35S::*GhSDH1–1* vector was constructed by digesting the *GhSDH1–1* coding sequence with *XbaI* and *SacI* (BioLabs, U.K). The digested sequence was then inserted into a modified pCAMBIA2300 (Kana, Japan) plant binary vector. *Arabidopsis* Col-0 was transformed by floral-dip method using *A. tumefaciens* strain GV3101 containing the *GhSDH1–1* overexpression vector (Hu et al. 2019). Transgenic seeds were then plated on Murashige and Skoog (MS) medium containing 0.1% Kanamycin to select positive transformants. The TransDirect Plant Tissue PCR Kit (TRAN, Beijing, China) and qPCR method were used to detect the T3 line with the transgene and the correct separation ratio. Only stable homozygous T3 lines that exhibited high levels of *GhSDH1–1* expression were selected for further functional analysis.

Salicylic acid treatments

Cotton seedlings were grown in pots incubated in a greenhouse. They were sprayed with 1 mmol·L^{-1}, 0.1 mmol·L^{-1}, or 0.01 mmol·L^{-1} SA at the foliar stage as described previously (Belt et al. 2017). Control plants were treated with sterile distilled water at the same pH and volume.

Measurements of free salicylic acid, nitric oxide, H_2O_2, and fumaric acid levels

The abundances of immune system-related molecules SA, H_2O_2, NO and fumaric acid were monitored using different methods. The cotton root samples were collected at the corresponding time point after virus-induced gene silencing (VIGS) treatment, and stored in the − 80 °C refrigerator after liquid nitrogen treatment. The NO and H_2O_2 from root tissue were measured using a Quantitative Assay Kit (Nanjing Jiancheng, Beijing, China) (Gong et al. 2017). In addition, the roots sample was grinded, then added 2 mL of mobile phase, ultrasonic extraction for 30 min, centrifuge at 5 000 r·min^{-1} for 5 min, and the supernatant was taken through a 0.22 μm filter. Finally, the free SA and fumaric acid contents were determined via the high performance liquid chromatography (HPLC) system (Agilent 1 260) as previously described (Hu et al. 2019).

Cell death assay

The cell death assay performed is described by Gong et al. (2017). The cotton euphylla was taken at 7 days after inoculation with *V. dahliae*. Leaves were soaked in trypan blue dye (1 g phenol, 1 mg Trypan blue, 1 mL lactic acid, and 1 mL glycerol dissolved in 1 mL sterile distilled water) and then boiling for 2 min. After cooling to room temperature overnight, samples were soaked with a chloral hydrate solution (1.25 kg·L^{-1}) for 3 days. Then observed the leaves with a stereo microscope and took photos.

Verticillium dahliae recovery assay

To confirm the effects of *V. dahliae* on cotton and *A. thaliana* plants, the stem fragments from the first stem node were analyzed. The stem samples collected from cotton and *Arabidopsis* plants were 5 cm and 3 cm long, respectively. The stem was cleaned with 75% ethaol, 3% sodium hypochlorite and sterile distilled water according to a previously described method (Zhang et al. 2011). Then cleaned stems were cut into seven sections. The stem fragment was placed on potato dextrose agar (PDA medium) in a plate and incubated at 25 °C. The number of stem segments that developed fungal growth indicated the degree of susceptibility of the plant to infection.

Analysis of disease index

The assessment of disease was conducted according to Yuan et al. (2017). For cotton plants, the disease index (DI) was calculated according to the following formula: DI = [(Σ disease grade × number of infected plants) / (total number of sampled plants × 4)] × 100. According to the symptoms observed from cotyledons and true leaves, the seedlings were divided into five grades (0, 1, 2, 3 or 4). The disease severity of *Arabidopsis* plants was graded on a scale of 0–4, and DI was calculated using the following formula (Gao et al. 2011): DI = [(Σdisease grade × number of infected plants) / (total number of sampled plants) × 4)] × 100.

Subcellular localization

The full-length coding sequence of GhSDH1–1(without the stop codon) was inserted into the binary plant vector pBin-GFP, to constitute the transient expression vector SDH1–1-GFP. Then we transformed it to *A. tumefaciens* strain GV3101. The 3-week-old *N. benthamiana* plants at the 4–5 leave stage were infiltrated with the GV3101 mixture of SDH1–1-GFP and mt-rk (mitochondrial marker) via a syringe. The fluorescence in leaves was observed 48 h later (Pečenková et al. 2017).

Conclusions

Characterizing the function of *GhSDH1–1* in cotton and *Arabidopsis* plants revealed that *GhSDH1–1* positively regulates cotton resistance to *Verticillium dahliae*. Silencing of *GhSDH1–1* significantly reduced the abundance of immune-related molecules and severely impaired the SA signaling pathway. Consequently, resistance of cotton to Verticillium wilt was significantly reduced. The overexpressed lines exhibited stronger resistance. These results provide new insights into the role of *GhSDH1–1*, confirming the important role of SDH in cotton against Verticillium wilt and its association with the SA signaling pathway.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s42397-020-00052-6.

Additional file 1 : **Table S1.** Primers used in this study. The bold and underlined parts are adaptor sequence; the primers for RT-qPCR are marked by 'RL'. **Figure S1.** Pathway mapping of the identified succinic and fumaric in metabolomics analysis of cotton toot after *V. dahliae* inoculation. Red colour represents significantly increased in cotton toot after *V. dahliae* inoculation. Blue colour represents significantly decreased in cotton toot after *V. dahliae* inoculation. **Figure S2.** Homology analysis of the 18 genes. Made by software DNAMAN. **Figure S3.** Selecting homozygous *GhSDH1–1*-overexpressing transgenic lines, including a 0.1% Kana screening (A) and RT-qPCR (B).

Acknowledgments

We thank Dr. XU, Xiangming at NIAB East malling research for his kind assistance with experiments and critical reading of the manuscript.

Authors' contributions

Feng HJ and Zhu HQ designed the experiments; Zhang XY and Zhao LH performed experiment and wrote the manuscript; Liu SC, Feng ZL, Wei F and Shi YQ assisted in some experiments and review the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (31701479).

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author details

[1]State Key Laboratory of Cotton Biology, Institute of Cotton Research of Chinese Academy of Agricultural Sciences, Anyang 455000, Henan, China. [2]Zhengzhou Research Base, State Key Laboratory of Cotton Biology, School of Agricultural Sciences, Zhengzhou University, Zhengzhou 450001, China.

Received: 2 December 2019 Accepted: 30 March 2020
Published online: 06 May 2020

References

Belt K, Huang S, Thatcher LF, et al. Salicylic acid-dependent plant stress signaling via mitochondrial succinate dehydrogenase. Plant Physiol. 2017;173:2029–40. https://doi.org/10.1104/pp.16.00060.

Caarls L, Pieterse CMJ, Van W. How salicylic acid takes transcriptional control over jasmonic acid signaling. Front Plant Sci. 2015;6:170. https://doi.org/10.3389/fpls.2015.00170.

Cai Y, Xiaohong H, Mo J, et al. Molecular research and genetic engineering of resistance to Verticillium wilt in cotton: a review. Afr J Biotechnol. 2009;8:1684–5315.

Grant M, Lamb C. Systemic immunity. Curr Opin Plant Biol. 2006;9:414–20.

Chen Z, Silva H, Klessig D. Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science. 1993;262:1883–6. https://doi.org/10.1126/science.8266079.

Chen ZJ, Scheffler BE, Dennis E, et al. Toward sequencing cotton (*Gossypium*) genomes. Plant Physiol. 2007;145:1303–10. https://doi.org/10.1104/pp.107.107672.

Cheng HQ, Han LB, Yang CL, et al. The cotton MYB108 forms a positive feedback regulation loop with CML11 and participates in the defense response against to *Verticillium dahliae* infection. J Exp Bot. 2016;67:1935–50. https://doi.org/10.1093/jxb/erw016.

Chouchani ET, Pell VR, Gaude E, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature. 2014;515:431–5. https://doi.org/10.1038/nature13909.

Daayf F. Verticillium wilts in crop plants: pathogen invasion and host defence responses. Can J Plant Pathol. 2015;37:8–20. https://doi.org/10.1080/07060661.2014.989908.

Erdogan O, Sezener V, Ozbek N, et al. The effects of verticillium wilt (*Verticilli1m dahliae* Kleb.) on cotton yield and fiber quality. Asian J Plant Sci. 2006;5:867–70. https://doi.org/10.3923/ajps.2006.867.870.

Fradin EF, Zhang Z, Juarez Ayala JC, et al. Genetic dissection of *Verticillium* wilt resistance mediated by tomato Ve1. Plant Physiol. 2009;150:320–32. https://doi.org/10.1104/pp.109.136762.

Fu ZQ, Yan S, Saleh A, et al. NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature. 2012;486:228-32. https://doi.org/10.1038/nature11162.

Gao X, Wheeler T, Li Z, et al. Silencing *GhNDR1* and *GhMKK2* compromises cotton resistance to Verticillium wilt. Plant J. 2011;66:293–305. https://doi.org/10.1111/j.1365-313X.2011.04491.x.

Gerik JS, Huisman OC. Study of field-grown cotton roots infected with *Verticillium dahliae* using an immunoenzymatic staining technique. Phytopathology. 1988;78:1174–8. https://doi.org/10.1094/phyto-78-1174.

Gleason C, Huang S, Thatcher LF, et al. Mitochondrial complex II has a key role in mitochondrial-derived reactive oxygen species influence on plant stress gene regulation and defense. Proc Natl Acad Sci U S A. 2011;108:10768–73. https://doi.org/10.1073/pnas.1016060108.

Gong Q, Yang Z, Wang X, et al. Salicylic acid-related cotton (*Gossypium arboreum*) ribosomal protein GaRPL18 contributes to resistance to *Verticillium dahliae*. BMC Plant Biol. 2017;17:59. https://doi.org/10.1186/s12870-017-1007-5.

Häffner E, Karlovsky P, Splivallo R, et al. ERECTA, salicylic acid, abscisic acid, and jasmonic acid modulate quantitative disease resistance of *Arabidopsis thaliana* to *Verticillium longisporum*. BMC Plant Biol. 2014;14:85. https://doi.org/10.1186/1471-2229-14-85.

Hu Q, Min L, Yang X, et al. Laccase *GhLac1* modulates broad-spectrum biotic stress tolerance via manipulating phenylpropanoid pathway and jasmonic acid synthesis. Plant Physiol. 2018;176:1808–23. https://doi.org/10.1104/pp.17.01628.

Hu Q, Zhu L, Zhang X, et al. *GhCPK33* negatively regulates defense against *Verticillium dahliae* by phosphorylating *GhOPR3*. Plant Physiol. 2019;178:876–89. https://doi.org/10.1104/pp.18.00737.

Huang S, Hans B, Ryan M, et al. Mitochondrial complex II of plants: subunit composition, assembly, and function in respiration and signaling. Plant J. 2019;98(3):405–17. https://doi.org/10.1111/tpj.14227.

Huang S, Millar AH. Succinate dehydrogenase: the complex roles of a simple enzyme. Curr Opin Plant Biol. 2013;16(3):344–9. https://doi.org/10.1016/j.pbi.2013.02.007.

Huang S, Van AO, Schwarzlder M, et al. Roles of mitochondrial reactive oxygen species in cellular signalling and stress response in plants. Plant Physiol. 2016;171(3):1551–9. https://doi.org/10.1104/pp.16.00166.

Jardim-Messeder D, Caverzan A, Rauber R, et al. Succinate dehydrogenase (mitochondrial complex II) is a source of reactive oxygen species in plants and regulates development and stress responses. New Phytol. 2015;208(3):776–89. https://doi.org/10.1111/nph.13515.

Jiménez-Díaz RM, Cirulli M, Bubici G, et al. Verticillium wilt, a major threat to olive production: current status and future prospects for its management. Plant Dis. 2012;96(3):304–29. https://doi.org/10.1094/PDIS-06-11-0496.

Kazan K, Lyons R. Intervention of phytohormone pathways by pathogen effectors. Plant Cell. 2014;26:2285–309. https://doi.org/10.1105/tpc.114.125419.

Li A, Zhang R, Pan L, et al. Transcriptome analysis of H_2O_2-treated wheat seedlings reveals a H_2O_2-responsive fatty acid desaturase gene participating in powdery mildew resistance. PLoS One. 2011;6(12):e28810. https://doi.org/10.1371/journal.pone.0028810.

Li YB, Han LB, Wang HY, et al. The thioredoxin gbnrx1 plays a crucial role in homeostasis of apoplastic reactive oxygen species in response to *Verticillium dahliae* infection in cotton. Plant Physiol. 2016;170(4):2392–406. https://doi.org/10.1104/pp.15.01930.

Malik W, Ashraf J, Iqbal MZ, et al. Molecular markers and cotton genetic improvement: current status and future prospects. Sci World J. 2014;2014:607091. https://doi.org/10.1155/2014/607091.

Egbuta MA, McIntosh S, Waters DL, et al. Biological importance of cotton by-products relative to chemical constituents of the cotton plant. Molecules. 2017;22:93. https://doi.org/10.3390/molecules22010093.

Miao Y, Zhu L, Zhang X. Down regulation of cotton *GbTRP1* leads to accumulation of anthranilates and confers resistance to *Verticillium dahliae*. J Cotton Res. 2019;2:19. https://doi.org/10.1186/s42397-019-0034-1.

Moreau M, Tian M, Klessig DF. Salicylic acid binds NPR3 and NPR4 to regulate NPR1-dependent defense responses. Cell Res. 2012;22:1631–3. https://doi.org/10.1038/cr.2012.100.

Nie S, Yue H, Zhou J, Xing D. Mitochondrial-derived reactive oxygen species play a vital role in the salicylic acid signaling pathway in *Arabidopsis thaliana*. PLoS One. 2015;10:e0119853. https://doi.org/10.1371/journal.pone.0119853.

Niu L, Liao W. Hydrogen peroxide signaling in plant development and abiotic responses: crosstalk with nitric oxide and calcium. Front Plant Sci. 2016;7:230. https://doi.org/10.3389/fpls.2016.00230.

Norman C, Howell KA, Millar AH, et al. Salicylic acid is an uncoupler and inhibitor of mitochondrial electron transport. Plant Physiol. 2004;134:492–501. https://doi.org/10.1104/pp.103.031039.

Pečenková T, Pleskot R, Žárský V. Subcellular localization of *Arabidopsis* pathogenesis-related 1 (PR1) protein. Int J Mol Sci. 2017;18:825. https://doi.org/10.3390/ijms18040825.

Quinlan CL, Orr AL, Perevoshchikova IV, et al. Mitochondrial complex II can generate reactive oxygen species at high rates in both the forward and reverse reactions. J Biol Chem. 2012;287(32):27255–64. https://doi.org/10.1074/jbc.M112.374629.

Ralph SJ, Moreno-Sánchez R, Neuzil J, Rodríguez-Enríquez S. Inhibitors of succinate: quinone reductase/complex II regulate production of mitochondrial reactive oxygen species and protect normal cells from ischemic damage but induce specific cancer cell death. Pharm Res. 2011;28:2695-730. https://doi.org/10.1007/s11095-011-0566-7.

Rao MV, Paliyath G, Ormrod DP, et al. Influence of salicylic acid on H_2O_2 production, oxidative stress, and H_2O_2-metabolizing enzymes, salicylic acid-mediated oxidative damage requires H_2O_2. Plant Physiol. 1997;115:137–49. https://doi.org/10.1104/pp.115.1.137.

Sattar MN, Kvarnheden A, Saeed M, Briddon RW. Cotton leaf curl disease-an emergingthreat to cotton production worldwide. J Gen Virol. 2013;94(4):695–710. https://doi.org/10.1099/vir.0.049627-0.

Shaban M, Miao Y, Ullah A, et al. Physiological and molecular mechanism of defense in cotton against *Verticillium dahliae*. Plant Physiol Biochem. 2018;125:193–204. https://doi.org/10.1016/j.plaphy.2018.02.011.

Sun Q, Jiang H, Zhu X, et al. Analysis of sea-island cotton and upland cotton in response to *Verticillium dahliae* infection by RNA sequencing. BMC Genomics. 2013;14:852. https://doi.org/10.1186/1471-2164-14-852.

Tretter L, Patocs A, Chinopoulos C. Succinate, an intermediate in metabolism, signal transduction, ros, hypoxia, and tumorigenesis. Biochimica et Biophysica Acta (BBA) – Bioenergetics. 2016;1857(8):1086–101. https://doi.org/10.1016/j.bbabio.2016.03.012.

Verma V, Ravindran P, Kumar P. Plant hormone-mediated regulation of stress responses. BMC Plant Biol. 2016;16:86. https://doi.org/10.1186/s12870-016-0771-y.

Xu L, Zhu L, Tu L, et al. Differential gene expression in cotton defence response to *Verticillium dahliae* by SSH. J Phytopathol. 2011;159:606–15. https://doi.org/10.1111/j.1439-0434.2011.01813.x.

Yalpani N, Silverman P, Wilson TM, et al. Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell. 1991;3(8):809–18. https://doi.org/10.1105/tpc.3.8.809.

Yan R, Liang C, Meng Z, et al. Progress in genome sequencing will accelerate molecular breeding in cotton (*gossypium* spp.). 3 Biotech. 2016;6:217. https://doi.org/10.1007/s13205-016-0534-3.

Yankovskaya V, Horsefield R, Törnroth S, et al. Architecture of succinate dehydrogenase and reactive oxygen species generation. Science. 2003;299(5607):700–4. https://doi.org/10.1126/science.1079605.

Yuan Y, Feng HJ, Wang LF, et al. Potential of endophytic fungi isolated from cotton roots for biological control against Verticillium wilt disease. PLoS One. 2017;12(1):e0170557. https://doi.org/10.1371/journal.pone.0170557.

Zhang K, Zhao P, Wang H, et al. Isolation and characterization of the *GbVIP1* gene and response to Verticillium wilt in cotton and tobacco. J Cotton Res. 2019;2:2. https://doi.org/10.1186/s42397-019-0019-0.

Zhang Y, Wang X, Yang S, et al. Cloning and characterization of a Verticillium wilt resistance gene from *Gossypium barbadense* and functional analysis in *Arabidopsis thaliana*. Plant Cell Rep. 2011;30:2085–96. https://doi.org/10.1007/s00299-011-1115-x.

Zhang YL, Li ZF, Feng ZL, et al. Isolation and functional analysis of the pathogenicity-related gene $VdPR_3$ from *Verticillium dahliae* on cotton. Curr Genet. 2015;61:555–66. https://doi.org/10.1007/s00294-015-0476-z.

Zhou Y, Sun L, Wassan GM, et al. Gbsobir1 confers *Verticillium* wilt resistance by phosphorylating the transcriptional factor GbbHLH171 in *Gossypium barbadense*. Plant Biotechnol J. 2018;17:152–63. https://doi.org/10.1111/pbi.12954.