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The succinate dehydrogenase *PsSDHB* is involved in hyphal morphology, chemical stress response and pathogenicity of *Phytophthora sojae*

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ABSTRACT

Soybean blight caused by Phytophthora sojae is a serious disease that may occur during any developmental stage of soybean. Many proteins participate in the process of pathogenetic, like the existence of a set of preset perfect procedures that command these proteins to perform their duties at the appropriate time and space, such as necrosis-inducing proteins. In this study, we investigated the function of the *PsSDHB* gene by constructing the *PsSDHB*-silencing vector (pTOR-*PsSDHB*) and transforming this vector into *P. sojae* protoplasts using polyethylene glycol (PEG)-mediated protoplast transformation. After the transformation, two silenced mutants (B1 and B2) with good inhibitory effects were obtained from the screening. Further experiments indicated that both of the silenced mutants showed slower mycelial growth. The growth of *P. sojae* in medium containing NaCl, H_2O_2, and Congo Red (sodium salt of 3,3′-bis) showed that silenced mutants had a lower resistance than the wild-type (WT) strain to stress conditions. Moreover, pathogenicity assays showed that both of the silenced mutants showed significantly reduced pathogenicity compared with the WT strain. Our experimental results may help improve our understanding of the energy supply chain of *P. sojae* and provide a theoretical basis for the prevention of disease.

1. Introduction

Phytophthora sojae mainly invades the roots of soybeans (*Glycine max*) [1] and leads to root rot, a soil-borne disease first discovered in the northeastern region of China [2,3] and subsequently found in Hebei, Shandong, and Anhui. Root rot has become a major plant disease affecting soybean production both in China and around the world causing [4]. Moreover, *P. sojae* infection causes the sudden death of soybean seedlings and root rot in adult plants [5], thereby severely affecting the quality and yield of soybeans due to its high invasiveness and devastating effects on the yield and quality of soybeans.

Succinate dehydrogenase (SDH) is a key enzyme of the tricarboxylic acid (TCA) cycle and plays important roles as a link between oxidative phosphorylation and electron transport [6,7]. SDH is a protein complex that consists of several subunits; among these subunits, subunit A (SDHA) has a covalently attached flavin adenine dinucleotide (FAD), succinate-binding site, and coenzyme Q binding site, whereas subunit B (SDHB) has multiple iron-sulfur clusters involved in electron transport [8]. The enzymatic activity of SDH directly reflects mitochondrial activity, which in turn determines the function of the organism. Moreover, SDHB is involved in electron transport and signal transmission and affects the TCA cycle.

After invading soybeans, the hyphae of *P. sojae* differentiate into sporangiophores, which form sporangia at the tips when the temperature and humidity of the surrounding environment become favorable [9]. The sporangia form mononuclear zoospores and release them to the environment once matured [10]. The zoospores can swim over a short distance to the root of host plant and rapidly form aplanospores, which re-invade the host plant after germination. *P. sojae* often switches from asexual to sexual reproduction by producing oospores when the external environment becomes unfavorable. Oospores exhibit characteristics that render it resistant to extreme temperatures, microorganisms, and other adverse environmental factors. Hence, oospores can survive the winter in the soil

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or plant debris and germinate when the external environment becomes favorable, thereby becoming the primary source of infection. During infection, *P. sojae* disrupts the cell wall near the roots by secreting abundant amounts of toxic or destructive effectors; this process requires high levels of energy as well as signal transmission and electron transport mechanisms [3]. Therefore, the invasiveness of *P. sojae* can be inhibited by disrupting its functional links or the process of electron transport and signal transmission.

The vegetative body of *P. sojae* is a diploid with a low probability of homologous recombination; thus, molecular manipulation techniques effective for haploids, such as gene knockout [11]. With continuous advancements in elucidation of the whole genome sequence of *P. sojae*, RNA interference (RNAi) has been employed as an alternative to conventional gene knockout techniques for functional genomic analysis of *P. sojae* [12,13]. A complete whole-genome sequence of *P. sojae* has been reported, as have the sequences of other oomycetes, including *Phytophthora capsici*, *Phytophthora infestans*, *Phytophthora ramorum*, and *Hyaloperonospora parasitica* [14,15]. Sequencing of many other species is also currently underway to further improve the genome database.

Studies on SDH in animals have indicated that SDH is a tumor suppressor that serves as a link between oxidative phosphorylation and electron transport. Numerous medical studies have explored the relationship between SDH and cancer occurrence and development. For example, SDH inactivation leads to a massive accumulation of succinate, acting as an oncometabolite and which levels, assessed on surgically resected tissue are a highly specific biomarker of SDHx-mutated tumors [16]. Moreover, reduction of SDH activity leads to disruption of the TCA cycle, hampering the function of succinic acid in electron transport and thereby affecting mitochondrial function [17]. The SDH-deficient renal cell carcinoma (RCC) has only recently been well-characterized and it is included as a specific subtype of RCC in the new World Health Organization (WHO) classification published in 2016 [18].

In plants, structural analysis of SDH in *Arabidopsis thaliana* have indicated that this enzyme consists of three subunits (sdh2-1, sdh2-2, and sdh2-3) [19], of which sdh2-1 and sdh2-2 exhibit highly similar spatial structures. Functional studies on SDH in yeast have indicated that SDH plays a role in regulating the embedding of catalytic subunits in the phospholipid bilayer without relying on energy derived from the submembrane. Analysis of the role of SDH in the drug resistance of bacterial blight in rice showed that mutations in the iron-sulfur subunit of SDH (SDHB) at amino acid position 229 leads to significant changes in drug resistance in bacterial blight in rice [20].

Thus, compared with plant pathogens, SDH has been extensively studied in animals and humans, including *in vivo* metabolism and application studies. However, the studies on SDH in plant pathogens is just emerging.

In this study, we constructed an expression vector based on the principle of RNAi-mediated gene silencing by inserting the target gene in the reverse direction *in vitro*, and silencing of the endogenous target gene *SDHB* was induced via PEG-mediated protoplast transformation. PEG-mediated *P. sojae* protoplast transformation was conducted to screen for *PsSDHB*-silenced mutants, which were then subjected to some simple analyses, such as gene identification and growth rate under stress conditions. Our study provides a foundation for further studies of the main functions and pathogenicity of the *PsSDHB* gene.

2. Materials and methods

2.1. Gene identification and bioinformatics

The existing SDHB nucleic acid and protein sequences were downloaded from the *Saccharomyces* genome database, and a homologous sequence search of the protein sequence through the basic local alignment search tool (BLAST) [21] in the *P. sojae* genome database was conducted [22]. Conserved domain searches were performed using Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de) [11]. *P. sojae* was compared and contrasted with *Arabidopsis thaliana* (GenBank Accession No. NP_189374.1), *Azorhizobium caulinodans* (WP_012172269.1), *Blastocystis hominis* (XP_012898243.1), *Caenorhabditis elegans* (NP_495992.1), *Chlamydomonas reinhardtii* (XP_001696290.1), *Enterobacteriaceae* (WP_003858617.1), *Homo sapiens* (NP_002991.2), *Inquilinus limosus* (WP_034834809.1), *Magnaporthe oryzae* (XP_003718958.1), *Methylobacterium* (WP_047574474.1), *Microvirga lupini* (WP_036362637.1), *Nannochloropsis gaditana* (EWM29428.1), *Neurospora crassa* (XP_011392866.1), *Oryza sativa* (XP_015650211.1), *P. infestans* (XP_002901751.1), *Phytophthora parasitica* (XP_008891746.1), *Puccinia graminis* (XP_003321977.1), *Saccharomyces cerevisiae* (ONH74473.1), *Spizellomyces punctatus* (XP_016612031.1), *Zea mays* (XP_008677742.1) to create a phylogenetic tree by ClustalX1.83 [23]. MEGA3.1 [24].

2.2. P. sojae strains and culture conditions

P. sojae strain P6497 was used in this study, which is preserved at Anhui Agricultural University at 4 °C and is routinely grown on 10% V8 medium at 25 °C in the dark [25]. P6497 was used to generate all other strains in our study. We collected vegetative hyphae, immediately freezed them in liquid nitrogen and then pestled them for RNA extraction.

2.3. DNA and RNA manipulation of P. sojae

Genomic DNA (gDNA) of different strains were separated from hyphae grown in 10% V8 liquid medium, as described by Tyler [26]. Total RNA was isolated using the E. Z. N. A.™ Total RNA Kit I (Solarbio, Beijing, China) and was treated with RNAse-free DNAse I (TaKaRa Biotechnology, Dalian, China). According to the protocol specifications, 1.2 μg RNA measured by a Nano Drop 1000 Spectrophotometer (Thermo Scientific, USA) and tested by agarose gel electrophoresis was used to synthesize the first-strand cDNA.

To clone *PsSDHB*, cDNA of mycelia from *P. sojae* strain P6497 was used as a PCR template with the primers *PsSDHB*-F and *PsSDHB*-R (Table 1). PCR was performed with 32 cycles of 30 s at 94 °C, 30 s at 55 °C and 90 s at 72 °C. The PCR products were cloned into pMD19-T-simple vectors (TaKaRa) and sequenced.

2.4. Plasmid construction and P. sojae transformation

The sequence of *PsSDHB* was amplified using PrimeStar polymerase (TaKaRa) from SDHB-pMD19. The PCR fragments of *PsSDHB* were ligated into the pTOR-GFP vector, which carries the geneticin

Table 1
Oligonucleotide primer pairs and qRT-PCR detection primer.

Primer name	Primer sequence (5′-3′)
PsSDHB-F	ATGCTGGCGGCTGTGCGTTCCAT
PsSDHB-R	TTAGTGCAGGCTGGCAAGTCGG
*Ps*ACT RT-F	ACTGCACCTTCCAGACCATC
*Ps*ACT RT-R	CCACCACCTTGATCTTCATG
PsSDHB RT-F	GCCAAGCCCAAGAACATCAA
PsSDHB RT-R	GGTCCTGCTCGTTCTTGATC
pTOR Forward	AGGCTCATTCTCCTTTTCACTC
pTOR Reverse	ATCTCGAACTCGTGGCCGTTC