Microbial Pathogenesis 108 (2017) 40–48

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Cloning and functional analysis of succinate dehydrogenase gene *PsSDHA* in *Phytophthora sojae*

Yuemin Pan [1], Tao Ye [1], Zhimou Gao*

Department of Plant Pathology, College of Plant Protection, Anhui Agricultural University, Hefei 230036, China

ARTICLE INFO

Article history:
Received 3 November 2016
Received in revised form
15 March 2017
Accepted 15 March 2017
Available online 21 April 2017

Keywords:
Phytophthora sojae
Succinate dehydrogenase
RNAi
Phenotypic analysis

ABSTRACT

Succinate dehydrogenase (SDH) is one of the key enzymes of the tricarboxylic acid cycle (TCA cycle) and a proven target of fungicides for true fungi. To explore the roles of the *SDHA* gene in *Phytophthora sojae*, we first cloned *PsSDHA* to construct the *PsSDHA* silenced expression vector pHAM34-*PsSDHA*, and then utilized PEG to mediate the *P. sojae* protoplast transformation experiment. Through transformation screening, we obtained the silenced mutants A1 and A3, which have significant suppressive effect. Further study showed that the hyphae of the silenced mutant strains were shorter and more bifurcated; the growth of the silenced mutants was clearly inhibited in 10% V8 agar medium containing sodium chloride (NaCl), hydrogen peroxide (H_2O_2) or Congo Red, respectively. The pathogenicity of the silenced mutants was significantly reduced compared with the wild-type strain and the mock. The results could help us better to understand the position and function of SDH in *P. sojae* and provide a proven target of fungicides for the oomycete.

1. Introduction

Phytophthora sojae belongs to the oomycota [15,21] and leads to severe reduction in soybean yields [42]. It furthermore affects the production of the oil crop and interferes with stable economic development [43]. The infection begins with *P. sojae* secreting a series of extracellular proteins to degrade the plant cell wall and effector molecules to suppress or inhibit the defense responses in different parts of the cells, changing the metabolic pathway of the host by secreting inhibitory proteins and infecting the host successfully [47]. The infected soybeans can respond changes in the external environment by regulating their gene expression levels. In the process, the soybeans synthesize and release defensive reactive oxygen species to promote thickening of the cell wall to prevent and postpone the invasion and diffusion of the pathogenic microbes [27,36].

Molecular-level research on *P. sojae* has been conducted in great depth, and particularly countless studies highlighting the role of effectors [6,19,44], while most of them tend to be fruitless due to the proposed size and redundancy of the effector repertoire [48]. As for chemical control, the control efficiency of the fungicides currently in common use is becoming unsatisfactory due to pathogen resistance [11]. The rapid and heritable variation of *P. sojae* isolates renders many traditional fungal control methods invalid for the prevention of *P. sojae* [12]. Therefore, it is particularly important to explore new thinking and methods for the prevention and treatment of *P. sojae*. Molecular-level research on the growth behavior and pathogenesis of *P. sojae* and specific potential drug targets will, to some extent, provide a theoretical basis for the prevention of *P. sojae*.

The diploid nature of oomycetes, with a low probability of homologous recombination, makes very slow progress in molecular genetic technology research, such as gene disruption. However, with the whole-genome sequencing of several species including *P. sojae* and *Phytophthora ramorum* [41], *Phytophthora capsici* [23], *Phytophthora infestans* [13] and *Hyaloperonospora parasitica* [35], *Pythium ultimum* [26] and *Plasmopara halstedii* [34] of oomycetes, the study of oomycetes is gradually developing new perspectives and achieving new heights. The wide applicability, high efficiency, strong specificity, short cycle, easy operation and high throughput of RNAi technology makes it more effective in functional genomics research than other technologies such as gene disruption [3,4,18,25,33]. Therefore, with genome-wide data on *P. sojae* completed, RNAi technology will be applied to the functional genomics research of *P. sojae* [16,40], taking the place of traditional

* Corresponding author.
E-mail address: gaozhimou@126.com (Z. Gao).
[1] Yuemin Pan and Tao Ye contributed equally to the paper.

http://dx.doi.org/10.1016/j.micpath.2017.03.012
0882-4010/

gene-disruption technology [10,30,39]. It is more advanced that [9] a CRISPR/Cas9 system had been established for rapid and efficient genome editing in *P. sojae* by Fang Y and Tyler BM.

The functional analysis of succinate dehydrogenase (SDH) has long been a scientific focus. SDH is composed of 4 subunits, including SDHA, SDHB, SDHC and SDHD. The FAD-containing flavoprotein (SDHA) and the iron–sulfur protein (SDHB), which contains 3 iron–sulfur complexes, [2Fe-2S], [4Fe-4S], [3Fe-4S], are two hydrophilic parts forming a peripheral domain and confer the succinate dehydrogenase activity. Two hydrophobic subunits SDHC and SDHD anchor the complex to the mitochondrial membrane [7]. SDH is a proven target for fungicides. Succinate dehydrogenase inhibitor (SDHI) fungicides, such as carboxin, and thifluzamide, play an important role in the control of plant diseases, which could been specifically interfered fungal respiration by blocking the ubiquinone-binding sites in the mitochondrial complex II [2,31]. However, a SDH inhibitor fungicide for an oomycete has not yet been developed, unlike the case for SDH inhibitors for true fungi.

RNAi technology was used to examine the functions of *PsSDHA* in *P. sojae* in this paper. An expression vector was constructed for inserting into the target genes in reverse, silencing the endogenous target gene *PsSDHA* through PEG/$CaCl_2$-mediated transformation of protoplasts. The silenced mutants of *PsSDHA* were screened and analyzed to identifiy the functions of *PsSDHA* in *P. sojae*. Thus, this paper will provide a basis for further study of the major functions and pathogenic mechanism of SDH, offering reference values for later research on other functional genes in the SDH family.

2. Materials and methods

2.1. Gene identification and bioinformatics

The existing SDHA 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) [1] in the *P. sojae* genome database was conducted [37]. Conserved domain searches were performed using Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de) [47]. *P. sojae* was compared and contrasted with *Arabidopsis thaliana* (GenBank Accession No. NP_201477.1), *Azorhizobium caulinodans* (WP_012172268.1), *Blastocystis hominis* (XP_012896852.1), *Caenorhabditis elegans* (NP_492798.1), *Chlamydomonas reinhardtii* (XP_001689842.1), *Escherichia coli* (WP_059342177.1), *Homo sapiens* (NP_004159.2), *Inquilinus limosus* (WP_026868485.1), *Magnaporthe oryzae* (XP_003718957.1), *Methylobacterium* (WP_055882807.1), *Microvirga lupini* (WP_036360904.1), *Nannochloropsis gaditana* (EWM29068.1), *Neurospora crassa* (XP_001728186.2), *Oryza sativa* (XP_015646992.1), *P. infestans* (XP_002997801.1), *Phytophthora parasitica* (ETM03646.1), *Pinus massoniana* (AIZ74343.1), *Puccinia graminis* (XP_003329108.2), *Saccharomyces cerevisiae* (NP_012774.1), *Spizellomyces punctatus* (XP_016606951.1), *Zea mays* (ACG43057.1) to create a phylogenetic tree by ClustalX1.83 [38]. MEGA3.1 [22].

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 [8]. 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 [50]. Total RNA was extracted 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 *PsSDHA*, cDNA of mycelia from *P. sojae* strain P6497 was used as a PCR template with the primers *PsSDHA*01-F and *PsSDHA*01-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 vectors (TaKaRa) and sequenced.

2.4. Plasmid construction and P. sojae transformation

The sequence of *PsSDHA* was amplified using PrimeStar polymerase (TaKaRa) from SDHA-pMD19. The PCR fragments of *PsSDHA* were ligated into the pHam34 vector digested with *Sm*a I the antisense orientation and sequenced for confirmation.

PEG-mediated DNA transformation was used to introduce a 3:1 mixture of the target plasmid pHam34 [17] and the selection plasmid pTH209 [14], which carries the geneticin (G418, Shanghai Sangon BS723)-resistance gene, into protoplasts of *P. sojae* strain P6497 by co-transformation [5,28]. The mock strain was the mutant which introduced the selection plasmid pTH209 only.

2.5. Quantitative real-time-PCR (qRT-PCR)

To evaluate the expression level of *PsSDHA* in the different transformants and wild type or distinct developmental stages of *P. sojae*, including the mycelium (MY), sporulating hyphae (SP), mixed zoospores (ZO) and cysts (CYST), germinating cysts (GC) and infected plant tissue, qRT-PCR was performed on an Applied Biosystems StepOne™ Real-Time PCR System Thermal Cycling Block (Applied Biosystems, USA) using the SYBR Green RT-PCR Kit (TaKaRa Biotechnology, Dalian, China). Primer pairs were designed to anneal specifically to the gene of interest. The primer pairs used in this study are listed in Table 1. The *Actin* from *P. sojae* was used as a constitutively expressed endogenous control, and the expression of each gene was determined relative to *Act*A using the ΔΔCt method [45].

2.6. Detection of survivability in stress media

To detect the survivability of hyphae cells, strains were cultured on 10% V8 medium with several separate stress factors, including 0.7 M NaCl, 200 μg/mL Congo Red or 3 mM H_2O_2. The strains inoculated in 10% V8 medium without any treatment (no NaCl, no Congo Red and no H_2O_2) for control. The inhibition of hyphal

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

Primer name	Primer sequence (5′-3′)
*PsSDHA*01-F	CTGACCAAGCCTGGTGATAAG
*PsSDHA*01-R	TTAGTAGACACGCGCGAAGG
*Ps*ACT RT -F	ACTGCACCTTCCAGACCATC
*Ps*ACT RT-R	CCACCACCTTGATCTTCATG
PsSDHA RT -F	TGGAGATGCAGAAGACGATG
PsSDHA RT-R	GTCAGGTCCGTGTTCCAGAT
pHAM34 Forward	TTCTCCTTTTCACTCTCACG
pHAM34 Reverse	AGACACAAAATCTGCAACTTC