Functional analysis of CfSnf1 in the development and pathogenicity of anthracnose fungus *Colletotrichum fructicola* on tea-oil tree

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**Abstract**

**Background:** Tea-oil tree (*Camellia oleifera*) is a unique edible-oil tree in China, and anthracnose occurs in wherever it is cultivated, causing great economic losses each year. We have previously identified the Ascomycete fungus *Colletotrichum fructicola* as the major pathogen of anthracnose in *Ca. oleifera*. The purpose of this study was to characterize the biological function of Snf1 protein, a key component of the AMPK (AMP-activated protein kinase) pathway, for the molecular pathogenic-mechanisms of *C. fructicola*.

**Results:** We characterized CfSnf1 as the homolog of *Saccharomyces cerevisiae* Snf1. Targeted *CfSNF1* gene deletion revealed that CfSnf1 is involved in the utilization of specific carbon sources, conidiation, and stress responses. We further found that the ΔCfSnf1 mutant was not pathogenic to *Ca. oleifera*, resulting from its defect in appressorium formation. In addition, we provided evidence showing crosstalk between the AMPK and the cAMP/PKA pathways for the first time in filamentous fungi.

**Conclusion:** This study indicate that CfSnf1 is a critical factor in the development and pathogenicity of *C. fructicola* and, therefore, a potential fungicide target for anthracnose control.

**Keywords:** Conidiation, Appressorium formation, Pathogenicity, *C. fructicola*

**Background**

Tea-oil tree (*Camellia oleifera*) is a commercial shrub native to China and has been widely cultivated throughout southern China for more than 2000 years with abundant edible oil in its seeds [1]. Owing to its lower cholesterol concentrations and the ability to decrease lipid concentration and prevent hypertension and arteriosclerosis, tea oil is considered as an excellent source of oil for human health, similar to that of olive oil [2, 3]. In China, Tea-oil tree covers more than 30,000 km² and produces 250,000 tons of edible oil each year, however, it is still unable to meet the consumer demand for tea oil. One of the major limiting factors is the serious diseases occurred in Tea-oil tree.

Anthracnose is the most devastating disease in Tea-oil tree and happens in wherever it is cultivated [4]. The buds, fruits, and leaves of Tea-oil tree all are susceptible to the disease, causing the wilt or even fall of the plant tissues. Anthracnose typically results in 10%~30% reduction of tea oil each year, and the severely affected areas often experience more than 50% of tea oil losses. Our previous studies demonstrated that there are at least five pathogens of anthracnose in Tea-oil tree, namely: *Colletotrichum fructicola*, *Colletotrichum siamense*, *Colletotrichum gloeosporioides*, *Colletotrichum camelliae*, and *Colletotrichum karstii*. Among them, *C. fructicola* showed the widest distribution and highest pathogenicity, acting as the major pathogen [4, 5].
**Results**

**Identification and phylogenetic analysis of CfSnf1 in C. fructicola**

Using *S. cerevisiae* Snf1 sequence as the trace, we acquired its single homolog in *C. fructicola* genome database by a BLAST_P search. Then, we submitted its sequence to NCBI database ([https://www.ncbi.nlm.nih.gov/](https://www.ncbi.nlm.nih.gov/)) (GenBank accession number MN094751) and named it CfSnf1. CfSnf1 was predicted to encode 741 amino acids and phylogenetic analysis revealed that CfSnf1 shows sequence conservation among other fungi Snf1 proteins; CfSnf1 shows higher amino acid sequence homology with that of *C. gloeosporioides* (96% identify and 96% similarity) and lesser homology with *S. cerevisiae* Snf1 (still 43% identify and 56% similarity) (Fig. 1a). This result indicates that Snf1 proteins are well conserved in fungi.

The domain prediction using the SMART website ([http://smart.embl-heidelberg.de/](http://smart.embl-heidelberg.de/)) suggested that CfSnf1 contains a S_TKc (Serine/Threonine protein kinases, catalytic) domain, a UBA (ubiquitin-associated) domain, an Adenylate Sensor domain, and three low complexity regions (Fig. 1b).

**Targeted deletion of CFSNF1 gene in C. fructicola**

To characterize the functions of CfSnf1, a CFSNF1 gene-deletion construct was generated according to the homologous recombination principle by replacing the coding region with a hygromycin-resistance cassette (HPH) (Additional file 1: Figure S1a). Putative transformants were screened on hygromycin media, then verified by PCR amplification, and we thus obtained two deletion mutants ΔCfSnf1#3 and ΔCfSnf1#5 (Additional file 1: Figure S1b). Since these two mutants showed the same biological phenotypes, we selected ΔCfSnf1#3 for further analysis. To complement the mutant strain, the genomic DNA sequence of CFSNF1 containing a 1.5-kb promoter was retransformed to the ΔCfSnf1#3 mutant and restored all of the mutant defects.

**CfSnf1 is involved in the utilization of specific carbon sources and aerial hyphae growth**

Snf1 proteins have been demonstrated to be important for the utilization of specific carbon sources in *V. dahliae* and *M. oryzae* [26, 27]. To investigate the functions of CfSnf1 in utilization of carbon sources, Wide-Type (WT), ΔCfSnf1 mutant, and complemented strain
ΔCfSnf1/CfSNF1 were cultured in the plates of PDA (Potato Dextrose Agar), MM (Minimal Medium), and MM supplemented with 1% Glycerol or 1% Pectin as sole carbon source for 4 days. All the strains showed similar growth levels on PDA and MM, while ΔCfSnf1 mutant showed significantly reduced growth rates to WT and ΔCfSnf1/CfSNF1 on MM supplemented with Glycerol or Pectin (Fig. 2a and b). Meanwhile, we also found that ΔCfSnf1 mutant exhibit a flat colony compared with the fluffy colony of WT and ΔCfSnf1/CfSNF1, owing to its reduced aerial hyphal growth (Fig. 2a and c).

CfSnf1 is important in asexual development
Like most fungal pathogens, asexual conidia are important for the disease cycle and infection in Colletotrichum [29, 30]. To examine the role of CfSnf1 in conidiation, WT, ΔCfSnf1 mutant, and ΔCfSnf1/CfSNF1 were cultured in liquid shaking PDB for 4 days. WT and ΔCfSnf1/CfSNF1 showed more than 220 × 10⁴ conidia per milliliter, comparing with the less than 5 × 10⁴ conidia per milliliter for ΔCfSnf1 mutant (Fig. 3). This result demonstrates that CfSnf1 is important in conidiation.

CfSnf1 is essential for pathogenicity
As a plant-pathogenic fungus, we concentrated our interests on the role of pathogenicity for CfSnf1 in C. fructicola. Mycelial plugs of the WT, ΔCfSnf1 mutant, and ΔCfSnf1/CfSNF1 were inoculated onto the intact Ca. oleifera leaves. After 4 days incubation, ΔCfSnf1 mutant caused no lesions, in contrast to the large and typical lesions produced by the WT and ΔCfSnf1/CfSNF1 (Fig. 4a left panel). To examine whether CfSnf1 plays roles in infectious growth, we further carried out the pathogenicity assay on wounded leaves. The result showed that ΔCfSnf1 mutant still causes no lesions, compared with the typical lesions of WT and ΔCfSnf1/CfSNF1 (Fig. 4a right panel). This result indicates that CfSnf1 is essential for pathogenicity.

To further analyze the pathogenicity defect of ΔCfSnf1 mutant, we performed the cellophane penetration assay. Colonies of the strains were first cultured on top of cellophane membranes placed on PDA plates for 3 days incubation, then the cellophane membranes with fungal dishes were removed. After the further incubation for 3 days, we found ΔCfSnf1 mutant could not penetrate the cellophane, compared with the mycelial growth of WT and ΔCfSnf1/CfSNF1 on the plates (Fig. 4b). This result suggests that CfSnf1 is required for penetration.

CfSnf1 is localized to cytoplasm
To detect the subcellular localization of CfSnf1, we fused a green fluorescent protein (GFP) tag to the C-terminus of CfSnf1 and introduced it into the ΔCfSnf1 mutant. Strong GFP signals were uniformly distributed throughout the cytoplasm, but not in vacuoles, both in hyphae and conidia (Fig. 5). This result suggests the expression and cytoplasm-localized pattern of CfSnf1 in hyphae and conidia.

CfSnf1 is involved in response to osmotic stress
For normal growth and infection, fungi must undergo many types of stresses in nature, such as osmotic stress, oxidative...
stress and cell wall integrity stress etc. Studies in yeast concluded that ScSnf1 plays critical roles in response to various environmental stresses [20, 31, 32]. Here we investigated the roles of CfSnf1 in the response to environmental stresses. We first cultured the WT, ΔCfSnf1 mutant, and ΔCfSnf1/CfSNF1 on PDA plates supplemented with osmotic stress (1 M NaCl, 1 M KCl, and 1 M sorbitol) for 4 days. We found that ΔCfSnf1 mutant showed significant higher inhibition rates than that of WT and ΔCfSnf1/CfSNF1 in all three osmotic stresses (Fig. 6a and b). These results indicate that CfSnf1 is involved in response to osmotic stress. Then, we examined the sensitivity of the strains to two cell wall integrity inhibitors, Congo red (CR) and sodium dodecyl sulfate (SDS), and oxidative stress H2O2. Consistently, all the strains showed similar inhibition rates on CR, SDS and H2O2 plates (Additional file 2:Fig. S2).

CfSnf1 is required for appressorium formation

Based on the fact that ΔCfSnf1 mutant caused no lesions on both unwounded and wounded tea-oil tree leaves and its non-penetration on cellophane membranes, we wondered whether the mutant could not produce any functional appressorium. To further elucidate the mechanisms of the abolished pathogenicity in ΔCfSnf1 mutant, the conidia of WT, ΔCfSnf1 and ΔCfSnf1/CfSNF1 were inoculated onto hydrophobic artificial surfaces to induce the appressorium formation. At 4 h post inoculation (hpi), all the strains exhibited more than 80% conidial germination rate and showed no significant difference among them. However, the germ tubes of ΔCfSnf1 were elongated abnormally and no appressoria were developed at 24 hpi, in contrast with the normal germ tubes and more than 80% appressorium formation rate in WT and ΔCfSnf1/CfSNF1 (Fig. 7a and b). This result indicates that CfSnf1 is required for appressorium formation.

**Fig. 2** CfSnf1 is involved in the utilization of specific carbon sources and aerial hyphae growth. (a) Growth of WT, ΔCfSnf1 mutant, and complemented strain ΔCfSnf1/CfSNF1 on the PDA, MM and MM plates supplemented with 1% Glycerol or 1% Pectin as sole carbon source for 4 days at 28 °C. (b) Colony diameters were measured and statistically analyzed by Duncan analysis from three replicates. Asterisks indicate significant differences (p < 0.01). (c) Aerial hyphae growth is reduced in ΔCfSnf1 mutant. Strains were cultured in PDA for 4 days and colony side views are shown from three replicates.

**Fig. 3** CfSnf1 is important in asexual development. The conidia produced by WT, ΔCfSnf1 and ΔCfSnf1/CfSNF1 cultured in PDB medium for 4 days were collected and quantified, then analyzed by Duncan analysis (p < 0.01) from three replicates. Three independent experiments yielded similar results.
cAMP treatment partially restores the appressorium formation defect of ΔCfSnf1 mutant

Studies in *M. oryzae*, which acts as the model for studying the mechanisms of fungal pathogenesis and host-microbe interactions, have revealed that the loss of cAMP/PKA signaling pathway abolishes appressorium formation and appressorium formation can be rescued in strains carrying deletions upstream of cAMP/PKA by cAMP treatment [31, 33, 34]. Therefore, we performed cAMP treatment for ΔCfSnf1, when inducing appressorium formation. The result showed that 88% of ΔCfSnf1 conidia germinated but did not formed appressoria and the rest conidia were not germinated, however, about 20% of ΔCfSnf1 conidia formed appressoria when cAMP was added (Fig. 8). This result reveals that cAMP treatment could partially restore the appressorium formation defect of ΔCfSnf1 mutant.

Discussion

In the present study, we characterized CfSnf1 as the homolog of *S. cerevisiae* Snf1 in *C. fructicola*. We found that CfSnf1 plays critical roles in the utilization of specific carbon sources, conidiation, appressorium formation, stress responses and pathogenicity in *C. fructicola*.

Targeted CfSnf1 gene deletion mutant ΔCfSnf1 showed similar mycelial growth rate as the WT in PDA and MM plates, but showed decreased growth rate compared to WT on MM with Glycerol or Pectin as sole carbon source. Considering the fact that PDA and MM medium use glucose for carbon source and the yeast Snf1 is inactivated in high glucose concentrations and activated in low glucose [21], we speculate that CfSnf1 also mainly functions in low glucose condition in *C. fructicola*. Moreover, the homologs of Snf1 in *V. dahliae* and *C. carbonum* are also required for the utilization of specific carbon sources [26, 27], which is consistent with our study. These findings indicate that Snf1 proteins shared conserved mechanisms during carbon utilization.

Asexual conidia play critical roles in the disease cycle and infection of *Colletotrichum* [29, 30]. The ΔCfSnf1 mutant produced dramatically decreased conidiation, which is consistent with the studies in *M. oryzae* [28, 35]. However, the ΔCcSnf1 mutant showed normal conidiation in *C. carbonum* and the ΔVdSnf1 mutant even produced more conidia in *V. dahliae* [26, 27]. These differences in conidiation by SNF1
mutants of the fungal pathogens reflect distinct functions of Snf1 proteins during asexual development in different fungal species.

The studies in yeast revealed the roles of Snf1 in response to various environmental stresses through the activation of downstream proteins [20, 36]. Our study demonstrated that CfSnf1 is involved in response to osmotic stress but plays no role in the response to cell wall integrity stress and oxidative stress in C. fructicola. Such differences probably contribute to the specificity of CfSnf1 in stress responses. Furthermore, since fungi must undergo many types of stresses in nature for normal infection, the function of CfSnf1 in osmotic stress response might foretell its role in pathogenicity.

The ΔCfSnf1 mutant was non-pathogenic on intact Tea-oil leaves, indicating the penetrating defect of the mutant and its non-penetration on cellophane membranes confirmed this. Furthermore, the lack of pathogenicity on osmotic stress but plays no role in the response to cell wall integrity stress and oxidative stress in C. fructicola. Such differences probably contribute to the specificity of CfSnf1 in stress responses. Furthermore, since fungi must undergo many types of stresses in nature for normal infection, the function of CfSnf1 in osmotic stress response might foretell its role in pathogenicity.

The ΔCfSnf1 mutant was non-pathogenic on intact Tea-oil leaves, indicating the penetrating defect of the mutant and its non-penetration on cellophane membranes confirmed this. Furthermore, the lack of pathogenicity on
wounded Tea-oil leaves also revealed the host-colonizing defect of ΔCfSnf1 mutant. We reasoned that the abolished pathogenicity of ΔCfSnf1 mutant was directly due to the defect in appressorium formation, which is essential for the penetration and host-colonization of Colletotrichum [29, 30]. In addition, the defect of appressorium formation in ΔCfSnf1 mutant was partially restored by cAMP, which is a positive determinant in cAMP/PKA pathway [31]. Thus, we predict a relationship between Snf1 and cAMP/PKA pathway in C. fructicola, which has also been reported but not clearly clarified in yeast and mammalian cells [21, 37]. Though the related mechanism is unknown and needs to be further investigated, at least, we establish the first crosstalk between Snf1 and cAMP/PKA pathway in filamentous fungi.

Conclusion
This study concluded that CfSnf1 is involved in utilization of specific carbon sources, conidiation, and stress responses in C. fructicola. CfSnf1 also functions as a key regulator for appressorium formation that are crucial for the pathogenicity of the fungus.

Methods
Strains and culture conditions
C. fructicola CFLH16 was used as the wild-type strain. All strains were cultured on PDA (200 g peeled potato, 20 g dextrose, and 15 g agar in 1 L ddH2O) plates at 28 °C in the darkness, unless indicated otherwise. The strains were cultured in liquid PDA medium in darkness shaking at 28 °C for 2 days and collected for the extraction of genomic DNA.

Phylogenetic tree construction and domain prediction
The Snf1 proteins of C. fructicola, C. gloeosporioides, V. dahliae, Neurospora crassa, M. oryzae, C. carbonum, and S. cerevisiae were acquired from the NCBI database (https://www.ncbi.nlm.nih.gov/). The phylogenetic tree was constructed by MEGA 5.05 programs using neighbor-joining method with 1000 bootstrap replicates. The domain of CfSnf1 was predicted by the SMART website (http://smart.embl-heidelberg.de/).

Gene deletion and complementation assays
CfSNF1 targeted gene deletion was performed by one-step replacement strategy [38]. Two about 1.0-kb DNA fragments flanking the CfSNF1 gene and 1.4-kb HPH gene were amplified using primes (Additional file 3: Table. S1), then the two flanking sequences were ligated to the flanks of HPH, respectively, by overlap PCR. The PCR products were further cloned into the pMD19-T vector. After sequencing, the 3.4-kb fragments, which contain the flanking sequences and hygromycin cassette, were amplified and transformed into the protoplasts of wild-type strain. The transformants were selected by hygromycin and screened by PCR. For complementation assays, approximately 1.5-kb native promoter and the full-length of CfSNF1 were amplified and ligated to the pYF11 vector (bleomycin resistance). After sequencing, the fused-pYF11 plasmids were transformed into the protoplasts of ΔCfSnf1 mutant for complementation.

Growth assays on different carbon sources
The strains were cultured on PDA, MM (6 g NaNO3, 0.52 g KCl, 0.152 g MgSO4·7H2O, 1.52 g KH2PO4, 0.01 g VB1, 1 ml 1000 × trace elements, 10 g Glucose, and 15 g agar in 1 L ddH2O), and MM with the Glucose was substituted by 1% Glycerol or 1% Pectin. After 4 days incubation, the colony diameters were measured and statistically analyzed.

Stress response assays
The strains were cultured on PDA and PDA added with different stresses, including osmotic stress (1 M NaCl, 1 M KCl, and 1 M Sorbitol), cell wall integrity stress (0.01% SDS and 400 μg/ml CR), and oxidative stress (10 mM H2O2). After 4 days incubation, the colony diameters were measured and the inhibition rates were statistically analyzed.

Conidiation and appressorium formation assays
For conidiation, the strains were cultured in liquid PDB (Potato Dextrose Broth) for 4 days, then filtered with three layers of lens paper and the conidia were collected and statistically analyzed. For appressorium formation, the collected conidia were resuspended to a concentration of 1 × 10⁵ spores/mL and inoculated onto hydrophobic artificial surfaces for germination and appressorium formation. For cAMP treatment, the conidial suspensions of ΔCfSnf1 mutant were added with 10 mM cAMP and then were induced for appressorium formation.

Pathogenicity and penetration assays
For pathogenicity assay, the mycelial plugs of the strains were inoculated onto the leaf margin of the detached Tea-oil tree leaves. The inoculated leaves were kept in a humidity plate with a 12 h light and 12 h dark cycle for 4 days and then photographed. The penetration assay on cellophane membranes was performed as previously described [39, 40] with minor modifications, colonies of the strains were first cultured on top of cellophane membranes placed on PDA plates. After 3 days incubation, the cellophane membranes with fungal dishes were removed, and the plates were further cultured for 3 days.
Localization observation
The green fluorescent protein (GFP) tag was fused to the C-terminus of CfSnf1 and transformed into the ΔCfSnf1 mutant. The fluorescence of the hyphae and conidia was observed under microscope.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12863-019-0796-y.

Additional files 1: Figure S1. Generation of the CSNFI gene deletion mutants in C. fructicola. (A) Strategy for the construction of gene replacement fragment. ~ 1.0-kb left fragment (LF) and right fragment (RF) flanking the CSNFI gene and 1.4-kb HPH gene from pCXY62 vector were amplified, then the LF and RF were ligated to the flanks of HPH, respectively, by overlap PCR. The PCR products were further cloned into the pMD19-T vector and obtained the vector of pMD-CSNFI-KO. After sequencing, the 3.4-kb fragments, which contain the LF, RF and HPH, were amplified for gene replacement. (B) Schematic illustration for deletion strategy of CSNFI gene. (C) Validation of the gene deletion mutants by PCR amplified with primers 1 (NBF/NBR) and primers 2 (BWF/HPHR). M: marker; +: positive control; -: negative control; #3 and #5: mutants.

Additional files 2: Figure S2. CSNFI is not involved in the tolerance of cell wall stress and oxidative stress. (A) The WT, ΔCSNFI and ΔΔCSNFI/CSNFI strains were cultured onto PDA plates with different cell wall stresses (CR and SDS) and oxidative stress (H2O2) at 28 °C for 4 days and photographed. (B) Statistical analysis of inhibition rates of the strains to cell wall stresses and oxidative stress. Error bars represent SD of three replicates.

Additional files 3: Table S1. Primers used in this study.

Abbreviations
CR: Congo red; PCR: Polymerase chain reaction; SDS: Sodium dodecyl sulfate

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Authors’ contributions
SZ, YG, and SL carried out the experiment and drafted the manuscript. GZ, JL, and HL participated in the design and coordination. JX help with the English writing. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary additional files.

Ethics approval and consent to participate
Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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