

Article

The AMP-Activated Protein Kinase Homolog Snf1 Concerts Carbon Utilization, Conidia Production and the Biosynthesis of Secondary Metabolites in the Taxol-Producer *Pestalotiopsis microspora*

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Received: 10 November 2017; Accepted: 17 January 2018; Published: 24 January 2018

Abstract: Highly conserved, the Snf1/AMPK is a central regulator of carbon metabolism and energy production in the eukaryotes. However, its function in filamentous fungi has not been well established. In this study, we reported functional characterization of Snf1/AMPK in the growth, development and secondary metabolism in the filamentous fungus *Pestalotiopsis microspora*. By deletion of the yeast *SNF1* homolog, we found that it regulated the utilization of carbon sources, e.g., sucrose, demonstrating a conserved function of this kinase in filamentous fungus. Importantly, several novel functions of *SNF1* were unraveled. For instance, the deletion strain displayed remarkable retardation in vegetative growth and pigmentation and produced a diminished number of conidia, even in the presence of the primary carbon source glucose. Deletion of the gene caused damages in the cell wall as shown by its hypersensitivities to Calcofluor white and Congo red, suggesting a critical role of Snf1 in maintaining cell wall integrity. Furthermore, the mutant strain $\Delta snf1$ was hypersensitive to stress, e.g., osmotic pressure (1 M sorbitol), drug G418 and heat shock, though the mechanism remains to be illustrated. Significantly, disruption of the gene altered the production of secondary metabolites. By high-performance liquid chromatography (HPLC) profiling, we found that $\Delta snf1$ barely produced secondary metabolites, e.g., the known product pestalotiollide B. This study suggests that Snf1 is a key regulator in filamentous fungus *Pestalotiopsis microspora* concerting carbon metabolism and the filamentous growth, conidiation, cell wall integrity, stress tolerance and the biosynthesis of secondary metabolites.

Keywords: Snf1/AMPK; secondary metabolism; cell wall integrity; conidiation; *Pestalotiopsis microspora*

1. Introduction

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is widely found in eukaryotic cells, which plays diverse, yet critical regulatory roles in the metabolism of nutrients such as sugar, fat and protein and thus concerts the response of the cell to environmental stress [1,2]. Many fungi have the homologs of AMPK, which is one of the subunits in a heterotrimeric protein consisting of one catalytic subunit— α subunit, known as *SNF1* in yeast—and two regulatory subunits— β

and γ subunits. Generally, upstream kinases, e.g., Sak1, Tos3 or Elm1, can activate SNF1 protein kinase through phosphorylation of the kinase domains at Thr210 [3–7]. The protein phosphatase complex (Reg1/Glc7), which responds to nutrient signals (such as high concentration of glucose) dephosphorylates SNF1 to inactivated its function [8]. In the baker's yeast, SNF1 controls the transcription of a large set of genes that covers the cellular processes of carbon sources, gluconeogenesis, respiration, material transport and meiosis. Known as the gauge of energy, SNF1 is generally responsive to the in vivo ratio of adenosine triphosphate (ATP) to AMP to regulate carbon metabolism. *SNF1* mutated strain cannot accumulate glycogen and the intracellular glucose-6-phosphate concentration decreases significantly [9–11]. On the other hand, SNF1 complex is one of the key regulators in the responsive processes to stress, e.g., SNF1 activates the heat shock transcription factor (HSF), stress-response transcription factor Msn2 and GATA transcription factor Gln3 [12–17]. The general function of the AMPK kinase family is largely conserved over the eukaryotic domains [16,17]. However, its role in filamentous fungi remains largely uncharacterized.

As a part of our efforts to the understanding of the regulation of secondary metabolism in *Pestalotiopsis microspora*, we identified a homolog of *snf1* in the genome of *P. microspora* NK17. This strain was originally isolated as a producer of taxol-like molecules [18,19]. And an analog of a new class of cholesterol ester transfer protein inhibitor, pestalotiollide B (PB) was also produced by the fungus [20]. Through targeting disruption, we found that *P. microspora snf1* gene was involved in carbon source metabolism and controlled the mycelial growth and development.

It played crucial roles in cell wall biosynthesis, drug resistance and osmotic stress tolerance. Some of the functions are divergent from the baker's yeast. Our study opens an avenue to explore the function of AMPK in concerting the primary and the secondary metabolism in filamentous fungi.

2. Materials and Methods

2.1. Strains and Culture Conditions

P. microspora NK17, a taxol-producing strain was previously isolated by our laboratory [18]. Its uracil auxotrophic strain, Δ ura3-NK17, was used as the host for gene manipulation in this study. Unless otherwise specified, fungal strains were grown or maintained in PDA (20% potato, 1.0% dextrose and 1.5% agar) at 25–28 °C, or PDB (PDA without agar) with shaking at 180 rpm, 25–28 °C (MAXQ 6000, Thermo Scientific, Waltham, MA, USA).

2.2. Construction of *snf1* Mutant and Complemented Strains

The technique, *Agrobacterium tumefaciens*-mediated genetic transformation of NK17, was previously described by Hao et al. [19]. To achieve the deletion of *snf1* (Δ *snf1*), briefly, the deletion vector pOSCAR-URA-Snf1 was constructed through an OSCAR one-step protocol [21]. The selective marker was the gene *pm-ura3* that restores the prototrophic phenotype of uracil biosynthesis. The homologous fragments for targeting of *snf1* were obtained by polymerase chain reaction (PCR) amplification and gel-purified using the AxyPrep DNA Gel Extraction Kit (Axygen, Corning, NY, USA). Thereafter, pOSCAR-URA-Snf1 was delivered into *A. tumefaciens* LBA4404 by electroporation [22]. Then, *A. tumefaciens* carrying pOSCAR-URA-Snf1 was co-cultured with 10^7 conidia from Δ ura3-NK17 at 28 °C on IM plate (50 mgL⁻¹ uracil and 40 mgL⁻¹ acetosyringone) on a nitrocellulose filter. After 2-day incubation, the filter was transferred onto YNB plates supplied with 100 mgL⁻¹ cefotaxime. Finally, individual fungal transformants were obtained through single-spore purification. All primers used in this study are listed in Table 1.

Table 1. Primers used in this study.

Primer	Sequence (5'—3')
Snf1-up(F)	GGGGACAGCTTTCTTGTACAAAAGTGGAA TCATCAGGCTCGTTATTG
Snf1-up(R)	GGGGACTGCTTTTTTGTACAAACTTGT GTAGGTTCTCGGGTTTCA
Snf1-down(F)	GGGGACAACCTTTGTATAGAAAAGTTGTT AGGCGATTCTATTTCTGT
Snf1-down(R)	GGGGACAACCTTTGTATAATAAAGTTGT TAACTGCTCGTGTCTGG
Snf1(F)	TTACCGTCCATCCACAAGAT
Snf1(R)	AAGGGTCTGGGTGGTATGAT
Snf1-PRO(F)	GGGGACAGCTTTCTTGTACAAAAGTGGAAATCATACCACCCAGACCCTT
Snf1-TER(R)	GGGGACAACCTTTGTATAATAAAGTTGT ATCTTGTGGATGGACGGTAA
Ura3(F)	CGAGGTCGACATAACTTCGT
Ura3(R)	ACGAAGTTATTTCACTGGCA
Hyg(F)	GCCCTCCTCCCTTATT
Hyg(R)	TGTTGGCGACCTCGTATT

F: Forward; R: reverse.

For complementation, plasmid pOSCAR-Snf1C was constructed using the BP Clonase reaction and *Hph*, conferring hygromycin B resistance, was used as a selective marker. The complementation strain was created by introducing a 4.4-kb fragment containing the wild-type copy of *snf1* into $\Delta snf1$. Genomic DNA was used as a template in the amplification of *snf1* by the high-fidelity PCR amplification. Total DNA from each strain was extracted from mycelium grown in 200mL PLB (0.1% lactose to replace of dextrose in PDB) for 4 days, as described by Hao et al. [19]. PCR amplification and Southern blotting were performed to characterize the disruption of *snf1* in $\Delta snf1$ strain and in the complementation strain *snf1*-C [23].

2.3. Analysis of *snf1* Roles on Carbon Source Utilization

To determine the effects of alternative carbon source utilization on mycelium growth, equal numbers of conidia ($\sim 5 \times 10^4$) from NK17, $\Delta snf1$ and the complementation strain *snf1*-C were placed separately on a YNB (0.17% YNB, 0.5% $(\text{NH}_4)_2\text{SO}_4$) supplemented with 2% glucose, 2% sucrose, 2% glycerol, 2% xylose, 2% fructose, 2% maltose and 2% galactose, individually. The plates were then incubated at 28 °C. Three plates were set for each type of sugar.

2.4. Quantification of Conidia Production and Mycelia

Conidia were collected from cultures inoculated on PDA at 28 °C for 7 days. The plates were washed twice with sterile distilled water and the concentration of conidia suspension in sterile distilled water was determined by hemocytometer. Total mycelium was isolated from cultures inoculated in PDB, at 28 °C, for 2, 3, 4, 5, 6, 8 and 10 days, respectively. Fresh mycelium was isolated by vacuum filtration and dry mycelium was obtained by vacuum freeze-drying. The weight of the dry mycelium was determined. Triplicates were set in each treatment.

2.5. Heat Tolerance Assay

To test the effect of *snf1* on heat tolerance, fresh conidia and mycelium were harvested from cultures inoculated on PDA at 28 °C for six days, or in PDB at 28 °C for three days (*P. microspora* can undergo sporulation readily in liquid culture). Conidia and mycelium were washed with sterile ddH₂O (double distilled water). Fresh conidia and mycelium were then incubated at 45 °C in water bath for 2 h. Equal numbers of conidia ($\sim 5 \times 10^4$) or equal volume of mycelium was inoculated on PDA at 28 °C for 168 h. Conidia without heat-shocked served as control. Assays were conducted in triplicates.

2.6. Phenotype Observation and Secondary Metabolite Profiling for $\Delta snf1$

Equal numbers of conidia ($\sim 5 \times 10^4$) from NK17, $\Delta snf1$ and the complement strain *snf1-C* were inoculated into PDB, or onto PDA plates, respectively, for monitoring phenotypic variation. Each assay was set in triplicate.

To analyze the secondary metabolites, equal numbers of conidia ($\sim 5 \times 10^5$) from different strains were cultured in 200 mL PDB (in 500-mL flask), 28 °C, with shaking at 180 rpm for 168 h. Mycelium and the liquid phase were separated by centrifugation (Sigma 2-16 KL, Sigma Laborzentrifugen GmbH, Germany) followed by vacuum filtration onto 3M filter paper. The culture supernatant was extracted with an equal volume of ethyl acetate. The organic phase was transferred for evaporation to yield a concentrated residue, which was suspended in 1 mL of methanol. Samples in 20 μ L of methanol were injected for high performance liquid chromatography (HPLC) analysis. The device used was the CoM HPLC system (CoMetro Technology, South Plainfield, NJ, USA). It was conducted at 30 °C, using a C-18 ODS column (4.6 \times 250 mm) (Plainfield) and eluted with methanol/H₂O. The flow rate was 1 mL/min. The solvent gradient for HPLC was: Solvent A (95% methanol in H₂O, *v/v*) and solvent B (70% methanol in H₂O, *v/v*). The chromatographic data were collected and analyzed digitally using the Agilent EZChrom Elite software (Agilent Technologies, Santa Clara, CA, USA). Yield was determined three times in parallel and variations were calculated as a standard error. Cultured in flasks could reflect vegetative growth and conidiation in liquid culture.

2.7. Drug and Stress Sensitivity Assays

For drug and stress sensitivity assays, 5×10^4 conidia from NK17, $\Delta snf1$ and *snf1-C* were incubated on PDA plates supplemented separately with 0.02% Congo red, 0.8 M KCl, 1 M NaCl, 1 M sorbitol, 2 mM H₂O₂, 50 μ g/mL G418, 300 ng/mL caspofungin and 50 ng/mL calcofluor white. All cultures were incubated at 28 °C for seven days, in triplicate.

3. Results

3.1. Characterization of *snf1* Homolog in *Pestalotiopsis microspora*

The genome of *P. microspora* NK17 has been sequenced (unpublished data). When searching using the amino acid sequence of Snf1 from *Saccharomyces cerevisiae* as the query, only one homologous protein was found in *P. microspora* NK17 (38.27% identity and designated as *snf1*, GenBank accession no. MG462730). An open reading frame (ORF) of *snf1* in *P. microspora* NK17 was predicted to be 2544 bp in length, encoding a peptide of 745 amino acids (Figure 1a). Structural analysis of Snf1 suggested that this protein contains a catalytic kinase domain (KD) (residues 64-318) and a C-terminal domain (CTD) (563-745 residues). Amino acid sequence alignment of KD (74.12% identity) and CTD (25.13% identity) indicated that Snf1 shared high identity with Snf1 homologs in *S. cerevisiae* (Figure 1a). The high similarity among these sequences suggests a conserved function of Snf1 in *P. microspora*.

To investigate the role of *snf1* in *P. microspora* NK17, we made a targeted disruption mutant for *snf1* via homologous recombination. The disruption cassette was described in detail in the Materials and Methods section. The open reading frame (ORF) of Snf1 was replaced by the selection marker (*ura3* gene) (Figure 1b). Three transformants were confirmed as anticipated by PCR amplification and Southern blotting (Figure 1b,c). The transformant No.3 was used for further study. The complementation strain was obtained by way of a procedure described in the Materials and Methods section and verified by Southern blotting (Figure 1c).

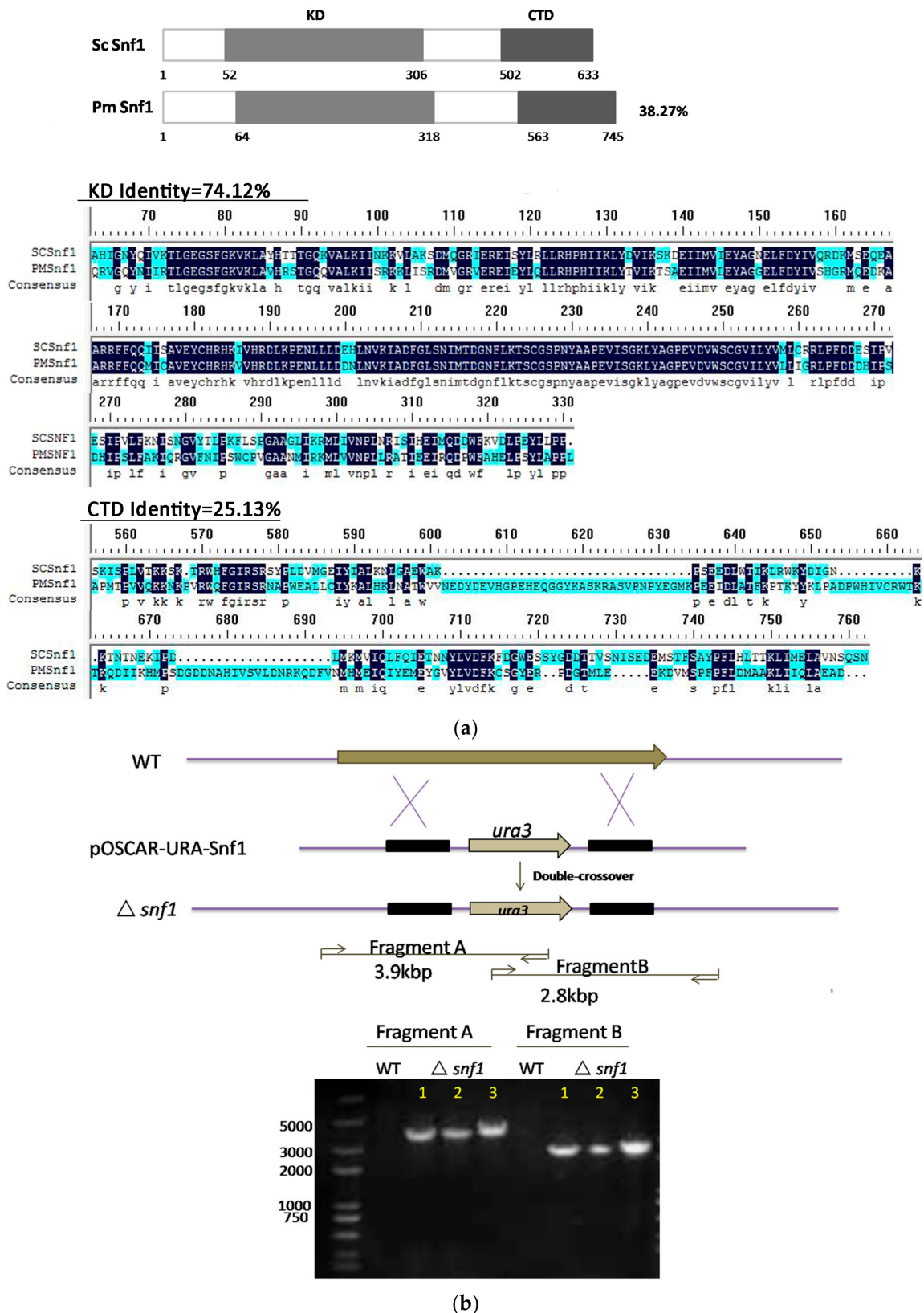


Figure 1. Cont.

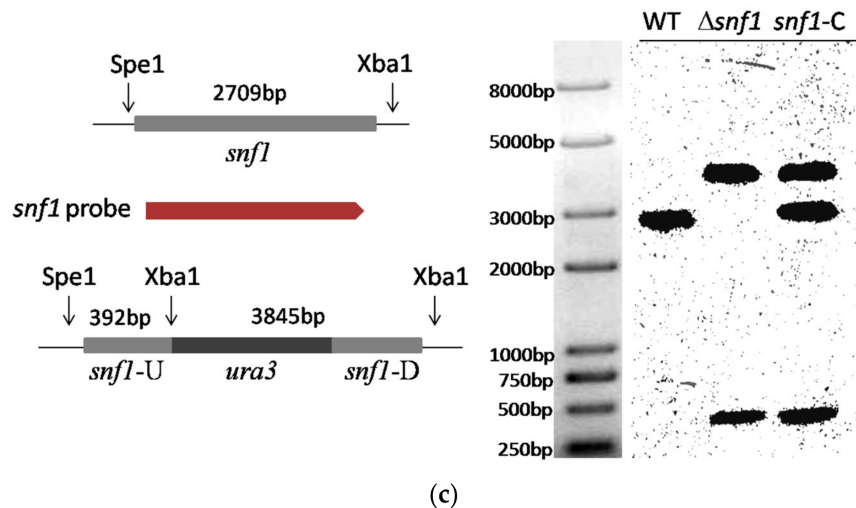


Figure 1. Identification and deletion of *snf1* in *Pestalotiopsis microspora* NK17. (a) Schematic diagram of the structure of Snf1 protein from *P. microspora* NK17 and amino-acid sequence (partial sequence) alignments of Snf1 homologs from *Saccharomyces cerevisiae*. Numbers indicate the length and the location of the conserved domain of the protein, including kinase domain (KD) and C-terminal domain (CTD). The overall percentage of amino acid sequence identity with ScSnf1 is shown on the right. The identical amino acids are starred and highlighted in blue and the less conserved amino acids are shown in turquoise; (b) The disruption cassette on the vector pOSCAR-URA-Snf1 carried the homologous fragments of *snf1*. Bottom panel showed the polymerase chain reaction (PCR) verification of the deletion in the mutants. Two fragments, 3.9 kb and 2.8 kb, were obtained by PCR amplification for $\Delta snf1$, while no band was seen for NK17; (c) The left panel, the wild-type copy that was reintroduced in the deleted strain $\Delta snf1$ was shown. And the probe used in Southern blotting (the right panel) was highlighted in red. Southern blot was shown on the right to confirm the deletion of *snf1*. Genomic DNAs from NK17, $\Delta snf1$ and the complementation strain *snf1-C* were digested with *Spe* I and *Xba* I. The probe used for Southern blotting was amplified by primers Snf1-up (F) and Snf1-down (R). Two bands on the membrane, at 0.4 and 3.8 kb, were obtained for $\Delta snf1$, while in wild-type (WT) NK17, one band of 2.7 kb was observed. In the complementation strain *snf1-C*, there were three bands (0.4, 2.7 and 3.8 kb).

3.2. *Snf1* Is Necessary for Conidiation, Colonial Pigmentation and Vegetative Growth

As yeast Snf1 did not show significant effect on vegetative growth, we first tested whether *P. microspora* NK17 *snf1* has a similar role. We cultivated the fungal strains NK17, $\Delta snf1$ and the complement *snf1-C* on PDA and in PDB. We found that the growth and conidiation of $\Delta snf1$ decreased remarkably (Figure 2a). The subsequent detection of conidiation and growth curve (based on dry weight of mycelia) also confirmed this phenomenon (Figure 2b,c).

Disruption of *snf1* led to dramatically decreased production of conidia on PDA (Figure 2b). The mutant strain $\Delta snf1$ produced an average of $0.87 \pm 0.15 \times 10^6$ ($p < 0.01$) conidia per plate, whereas NK17 produced about $5.74 \pm 0.35 \times 10^6$ ($p < 0.01$) conidia per plate and the complement strain *snf1-C* restored the number of conidia to $5.93 \pm 0.32 \times 10^6$ ($p < 0.01$) conidia per plate. Furthermore, compared to NK17, $\Delta snf1$ produced much less mycelium and exhibited lighter colonies (Figure 2a,c). Thus, *snf1* plays a critical role in conidiation, mycelial pigmentation and vegetative growth in *P. microspora* NK17.

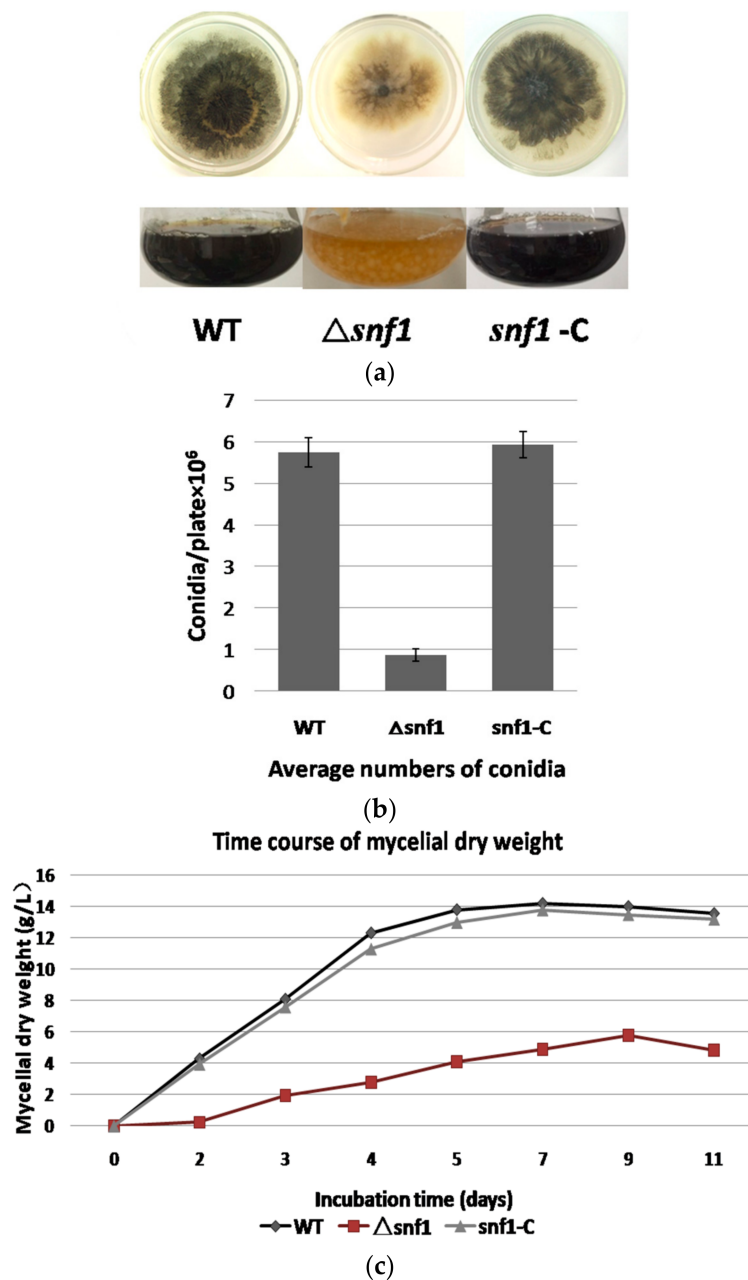


Figure 2. Phenotype of $\Delta snf1$. (a) Difference in pigmentation and growth rate between the wild type (WT) or the complement strain *snf1*-C and $\Delta snf1$. Strains were grown either on PDA or in PDB; (b) The number of conidia produced by NK17, $\Delta snf1$ and the complement strain *snf1*-C was determined as $5.74 \pm 0.35 \times 10^6$ ($p < 0.01$), $0.87 \pm 0.15 \times 10^6$ ($p < 0.01$) and $5.74 \pm 0.35 \times 10^6$ ($p < 0.01$), per plate, respectively. Each strain was incubated at 28 °C for 168 h. Error bars represent standard deviation; (c) A growth curve based on the quantity of dry-weight of mycelia for NK17, $\Delta snf1$ and the complementation strain *snf1*-C in PDB at the indicated time points. Dry weight was obtained as the mean value of three parallel cultures. Fungal strains were incubated at 28 °C.

3.3. Requirement of *snf1* for Utilization of Carbon Sources

Previous studies have established that yeast *snf1* gene is a key regulator in the utilization of secondary carbon sources under glucose-limiting condition. To examine whether *P. microspora* NK17 *snf1* played this canonical role, we tested the growth of $\Delta snf1$ on galactose, sucrose, glycerol, xylose, fructose and maltose as sole carbon source (Figure 3a). In contrast to the wild type, $\Delta snf1$ exhibited

apparently reduced growth rate on all above carbon sources, in particular, on xylose, fructose, sucrose and galactose. This phenomenon confirmed the classic role of *P. microspora* Snf1 has in the baker's yeast in sucrose utilization (it was originally named for Sucrose-Non-Fermenting). On the other hand, disruption of *snf1* led to dramatically decrease in conidia production on all the carbon sources (Figure 3b). As we have shown, unlike in the baker's yeast, *P. microspora* *snf1* was also needed for the utilization of glucose (Figure 2a), thus, we conclude that Snf1 plays a critical role in the regulation of general carbon metabolism in *P. microspora*.

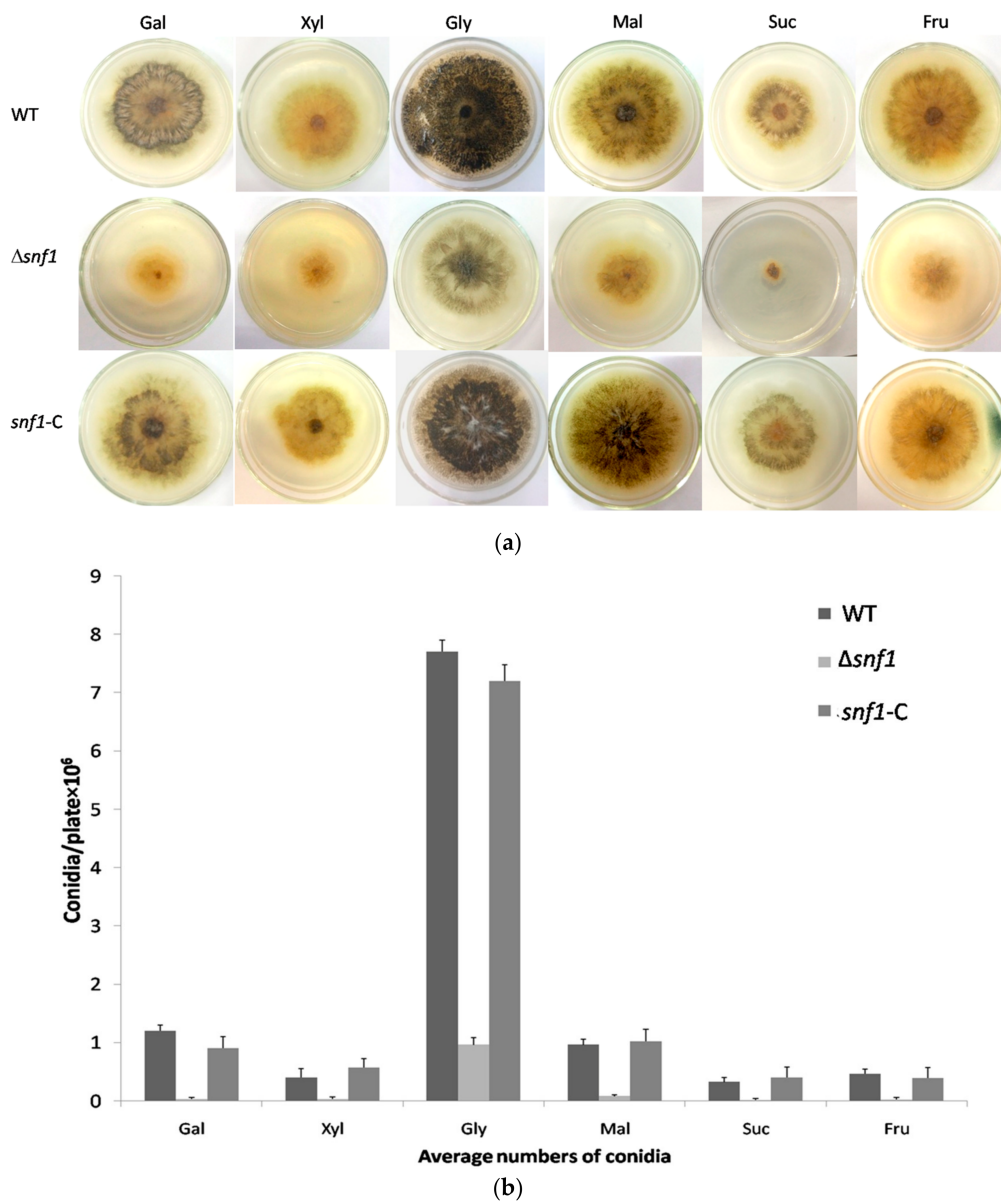


Figure 3. Effect of *snf1* on the use of alternative carbon sources. (a) Changes of conidiation/pigmentation and vegetative growth were observed in $\Delta snf1$ comparing to the wild-type (WT) and the complement strains. The conidiation (indicated by pigmentation) and vegetative growth of $\Delta snf1$ decreased obviously on sucrose, xylose, fructose, maltose and galactose, while the change was less dramatic on glycerol; (b) Numbers of conidia produced by NK17, $\Delta snf1$ and the complement strain *snf1*-C was determined in the carbon sources as indicated. Triplicate PDA plates for each strain were incubated at 28 °C for 168 h. Error bars represent standard deviations.

3.4. Roles of *Snf1* in Tolerance to External Stress

S. cerevisiae Snf1 plays an important role in stress resistance. To test the role of NK17 *snf1* in response to stress conditions, assays were performed in the presence of the following chemical reagents at appropriate concentrations: cell wall inhibitors Congo red, calcofluor white and caspofungin; osmotic reagents KCl and NaCl; oxidant H₂O₂; osmotic stabilizer sorbitol; and the antifungal drug G418. In order to verify whether the deletion of *snf1* affected the integrity of the cell wall, we respectively tested the sensitivity of the mutant strain to three cell wall inhibitors in the medium. As shown in Figure 4a, addition of 0.02% Congo red to PDA plates resulted in profound growth inhibition of $\Delta snf1$ compared to the controls. Likewise, 50 ng/mL calcofluor white and 300 ng/mL caspofungin also remarkably inhibited the growth of the mutant, while the wild-type and the complement strains underwent a normal growth (Figure 4a, bottom panels). This data clearly demonstrated that cell wall integrity was disrupted in the mutant strain $\Delta snf1$. Treatment with high concentrations of salts 0.8 M KCl, 1 M NaCl and 1 M sorbitol, could dramatically inhibit vegetative growth of the mutant strain (Figure 4b), comparing to the control strains (bottom plates in Figure 4b). This result suggests that the cellular responses in NK17 to osmotic pressure requires the function of *snf1*. On plates supplemented with 2 mM oxidant H₂O₂, $\Delta snf1$ obviously showed a slower growth, implying a role of *snf1* in tolerance to this stress condition (Figure 4b). Lastly, the mutant strain $\Delta snf1$ exhibited a hypersensitivity to 50 μ g/mL G418 (Figure 4b). Moreover, disruption of *snf1* also had a significant impact on conidiation in external stress (Figure 4c).

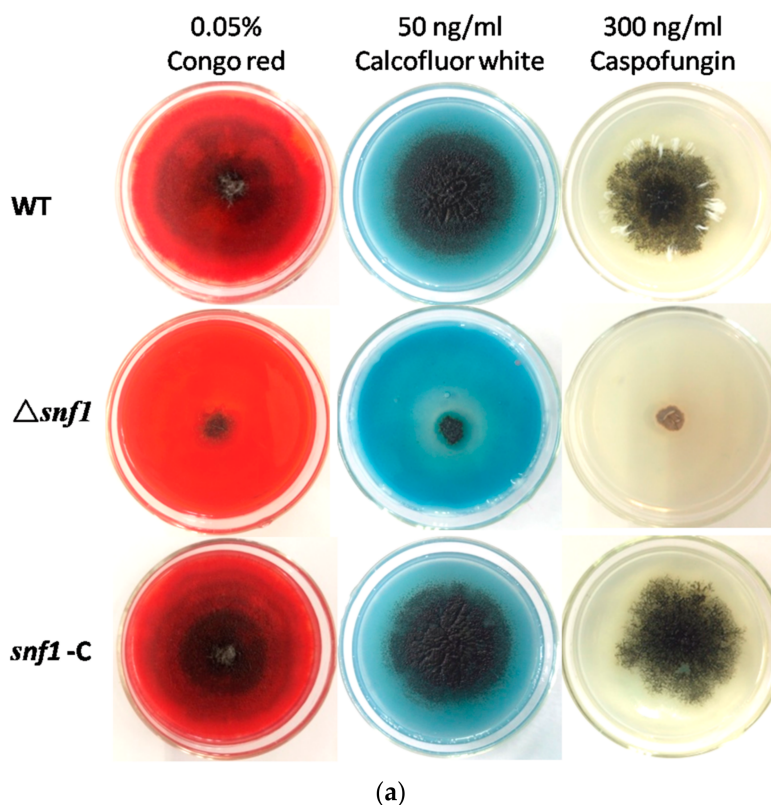
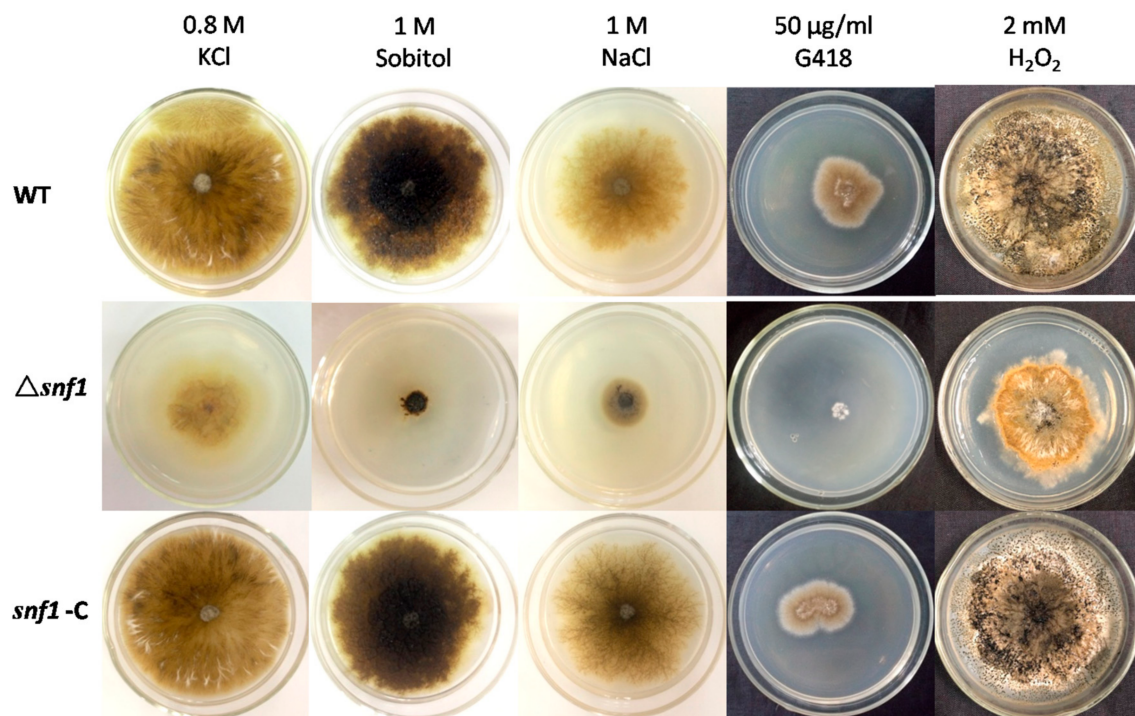
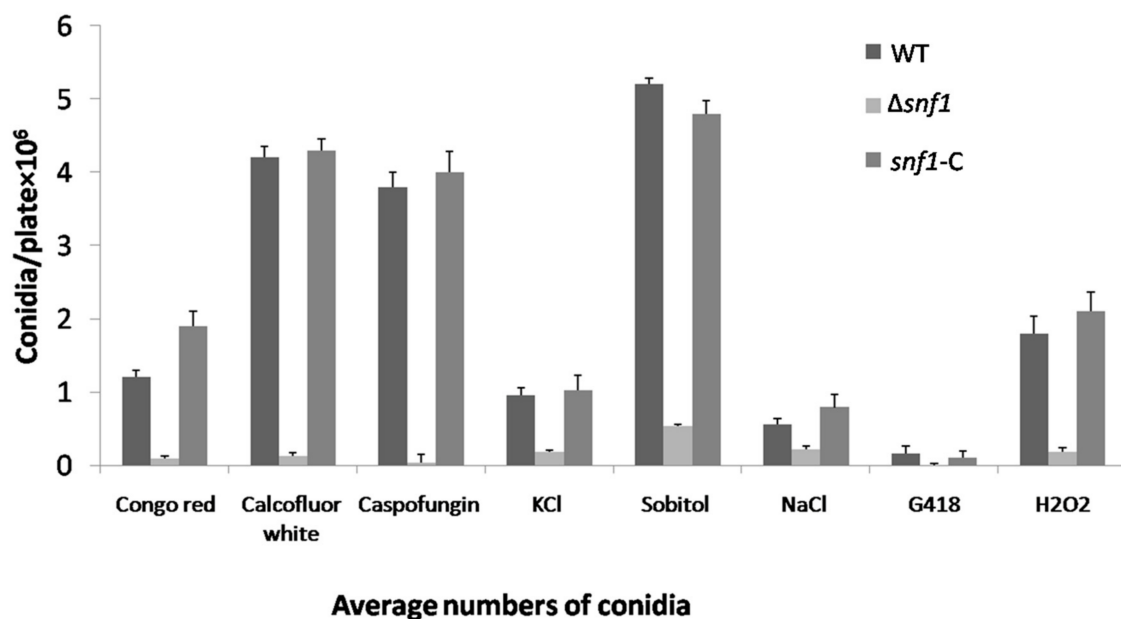


Figure 4. Cont.



(b)



(c)

Figure 4. Susceptibility tests for $\Delta snf1$ to adverse culture conditions. (a) Stress tolerance to cell wall inhibitors, Congo red, calcofluor white and caspofungin on PDA. PDA without the supplements served as the control. Conidiation and vegetative growth of $\Delta snf1$ was significantly affected; (b) Stress tolerance to osmotic pressure generated by high concentration of KCl, NaCl and sorbitol. Growth test under oxidative stress (2 mM H₂O₂), plus G418 Susceptibility tests for $\Delta snf1$; (c) The number of conidia produced by NK17, $\Delta snf1$ and the complementation strain *snf1-C* was determined in the indicated conditions and triplicate PDAs for each strain were incubated at 28 °C for 168 h. Error bars represent standard deviations.

3.5. Sensitivity of $\Delta snf1$ to Heat Shock

We tested the growth of $\Delta snf1$ under heat shock to see whether the gene played any role in this process. The result was shown in Figure 5. As shown, the mutant showed a profound growth defect comparing to the wild-type and the complemented strain (Figure 5a). Moreover, the mutant showed a dramatically decrease in conidiation (Figure 5b). It was indicated that *P. microspora* NK17 Snf1 played a crucial role in heat shock. Interestingly, the mutant spores were more sensitive than the mutant mycelium under incubation at 45 °C for 2 h.

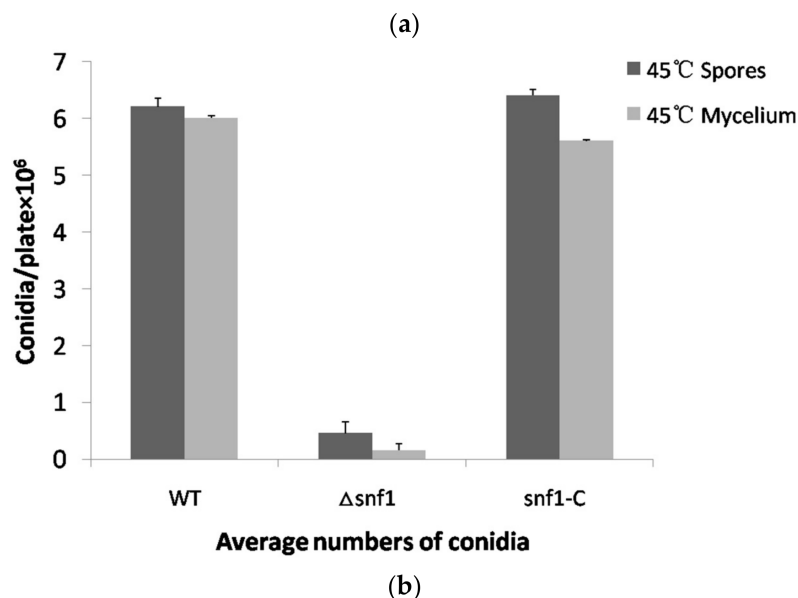
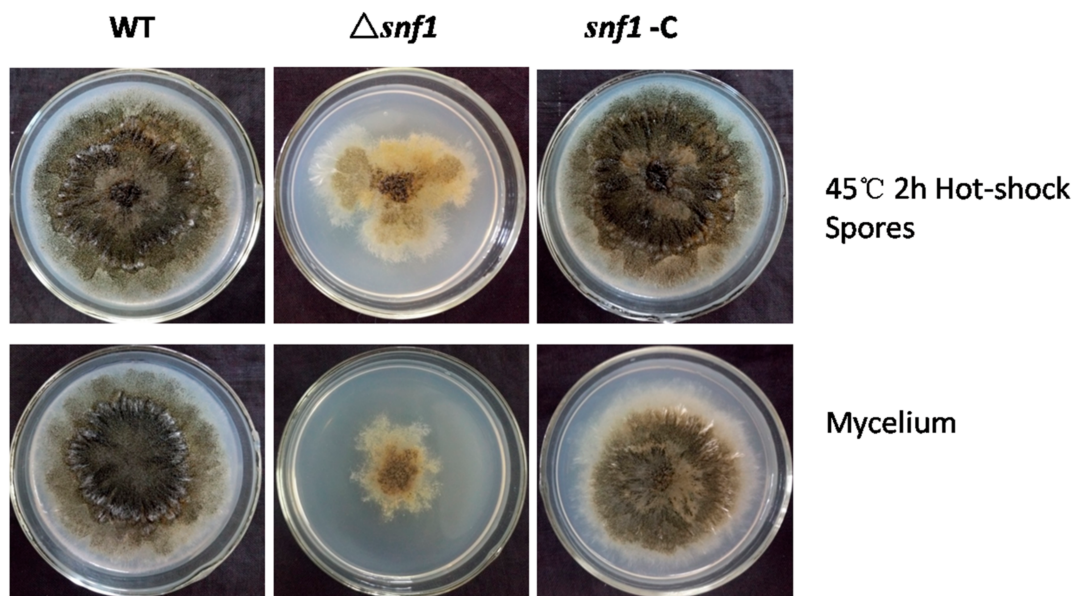


Figure 5. Sensitivity of $\Delta snf1$ to heat shock. (a) Fresh spores and mycelium were incubated in sterile ddH₂O at 45 °C for 2 h. Equal numbers of conidia ($\sim 5 \times 10^4$) or equal volume of mycelium from NK17, $\Delta snf1$ and the complementation strain *snf1*-C were inoculated on PDA at 28 °C for seven days. $\Delta snf1$ showed a high sensitivity to heat shock. Conidia without heat-shocked served as control; (b) The number of conidia produced by NK17, $\Delta snf1$ and the complementation strain *snf1*-C was determined in every medium and triplicate PDA plates for each strain were incubated at 28 °C for 168 h and used for quantification. Error bars represent standard deviations.

3.6. Roles of Snf1 in the Biosynthesis of Secondary Metabolites

Regulation of the biosynthesis of secondary metabolites is complex in filamentous fungi and is usually intimately coordinated with fungal development [24]. To examine the role of Snf1 in biosynthesis of secondary metabolites in *P. microspora* NK17, we examined the profile of secondary compounds in $\Delta snf1$ cultures by HPLC. We found that deletion of *snf1* obviously altered the profile of secondary metabolites (Figure 7a). Based on HPLC profiling, peaks of most secondary metabolites in $\Delta snf1$ were substantially lower than that of the wild type. Interestingly, the polyketide pestalotiollide B [24] that we described before was considerably reduced in the mutant strain $\Delta snf1$ (Figure 7a). Most significantly, when strains were cultured in galactose as sole carbon source, the general production of secondary metabolites in $\Delta snf1$, including pestalotiollide B, was almost inhibited (Figure 7b). The production of secondary metabolites was restored to nearly the wild-type level and pattern in the profile of the complement strain (Figure 7b, bottom panel). Above data showed that $\Delta snf1$ lead to a decrease in mycelium production (Figure 2c), whether it was the determinant of PB change. The ratio of peak area of PB peak relative to the dry weight of mycelium was analyzed (Figure 7c). The result suggested that *snf1* had effects on the production of PB. The above results demonstrate that *snf1* is a regulator of secondary metabolism and may be involved in either activation or silencing of certain gene clusters in NK17.

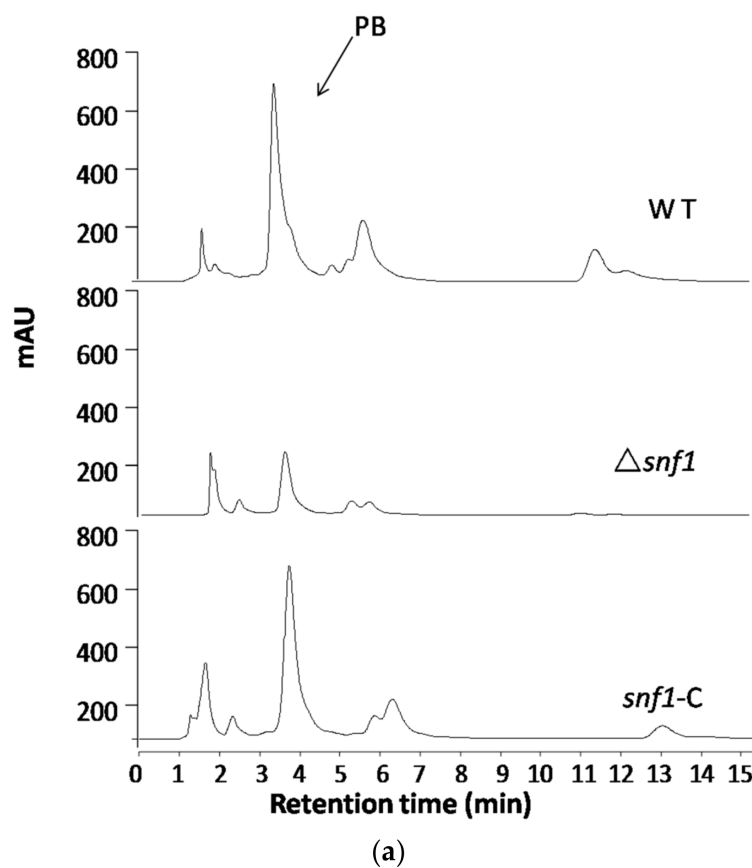


Figure 6. Cont.

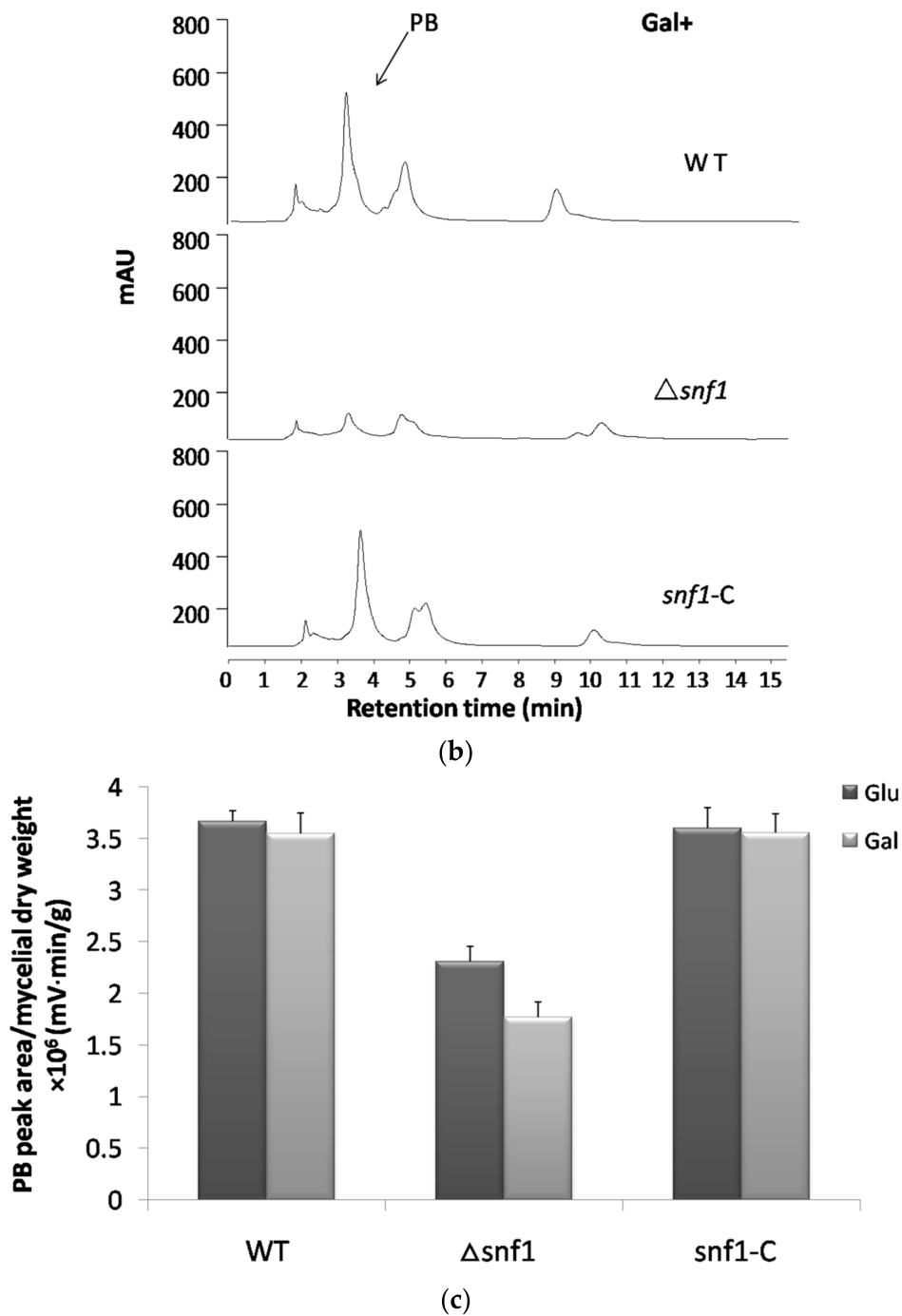


Figure 7. High performance liquid chromatography (HPLC) profiling for secondary metabolites in $\Delta snf1$. (a) HPLC profiling of the secondary metabolites in $\Delta snf1$ cultured in PDB. The peak patterns including pestalotiollide B peak of $\Delta snf1$ was distinct from that of the wild type; (b) HPLC profiling of the secondary metabolites in $\Delta snf1$ in galactose as sole carbon source. HPLC profiling analysis was repeated for three times; (c) The ratio of peak area of PB peak relative to the dry weight of mycelium was analyzed. The relative yield of PB in $\Delta snf1$ significantly decreased comparing with wild-type, especially in the medium of galactose. Error bars represent standard deviations.

4. Discussion

The Snf1/AMPK is highly conserved among eukaryotic organisms and is an important regulator in carbon metabolism and in energy homeostasis. Many studies have established that Snf1 concert

carbon metabolism by sensing glucose limitation. In *S. cerevisiae*, *SNF1* acts as an activator for more than 400 glucose-repressing genes and some of them are involved in alternative carbon source utilization [25]. Also, other regulatory functions of Snf1 involve in the response to various environmental stress. In this study, we firstly demonstrated Snf1 of *P. microspora* played a comparable role in the utilization of alternative carbon sources. For instance, it is required for the use of sucrose (Figures 2 and 3). However, distinct from the baker's yeast *snf1*, our data suggest that *P. microspora snf1* took part in the utilization of glucose, as disruption of *snf1* showed a significant delay in growth on PDA plate under permissive condition (Figure 2). This result suggests a complexity of carbon metabolism in fungus [26].

Nonetheless, our observation suggests that *snf1* in *P. microspora* shares similar roles in several other aspects, e.g., in stress response. We found that *P. microspora snf1* was involved in heat tolerance (Figure 5). This function of *snf1* homologs was previously reported in another yeast fungus *Cryptococcus neoformans* [27]. In our fungus, either the spores or the mycelium of the mutant $\Delta snf1$ was shown to be sensitive to 45 °C heat-shock (Figure 5a). Another important finding is that *snf1* is essential for the cell wall biosynthesis in this filamentous fungus. By challenging the mutant strain $\Delta snf1$ with the wall inhibitors, the dyes Calcofluor white and Congo, we demonstrated that the growth of the mutant strain was substantially inhibited (Figure 4a). A similar result was obtained for the drug caspofungin which is a chemotherapeutic chemical targeting fungal cell wall biosynthesis. The dyes Calcofluor white and Congo red, which target the biosynthesis of (1, 3) β -glucans and chitin during cell growth and protoplast wall generation, have been used in test of fungal wall integrity [28,29]. Taking into account of this result, we speculated that sensitivity of the mutant strain $\Delta snf1$ to heat shock might be resulted from the impairment of the cell wall integrity. In addition, previous studies in *S. cerevisiae* have shown that the heat shock transcription factor (HSF) is a target of Snf1 in response to glucose limitation but not to heat shock [30]. Whether HSF is the target of Snf1 is not clear in *P. microspora*.

Our data suggest that *snf1* is required for conidiation in the filamentous fungus *P. microspora* (Figures 2b and 3b). Disruption of *snf1* in NK17 led to dramatic decrease in conidia production. Although there has been considerable progress in the genetic regulation and light effect on conidiation [31,32], the molecular mechanisms regulating asexual sporulation are still not fully understood in filamentous fungi. Evidence from other study support this view. For example, disruption of *PdSNF1*, a *snf1* homology, also resulted in impaired conidiation and caused malformation of the conidiophore structures in *Penicillium digitatum* [33] and the germination and nucleation of both ascospores and conidia were delayed in *GzSNF1* mutants [34]. Taken together, these data suggest that *snf1* may have a role in concerting the carbon metabolism and conidia development in filamentous fungi, though the underlying mechanism remains to be identified.

Many filamentous fungi have the capacity to produce a myriad of secondary metabolites. It is still puzzling in many fungi how the synthesis of secondary metabolites is modulated with fungal differentiation and growth, especially with the formation of conidia [24,33]. Fungal secondary metabolism is reportedly regulated by mitogen-activated protein kinase (MAPK) pathway [35], G-protein/cAMP/PKA pathway [36] and velvet complex pathway [32,37]. In this study, we found a possible regulatory role of *snf1* in the production of secondary metabolites. Through HPLC profiling for $\Delta snf1$, it was revealed that *snf1* participates in the regulation of the biosynthesis of a polyketide metabolite pestalotiollide B (Figure 7a). Besides, the general pattern of the peaks for products declined sharply in the HPLC profile in the mutant strain $\Delta snf1$ compared to the wild type and the complement strain. Therefore, this result suggests that Snf1 likely plays a positive regulation in the biosynthesis of these metabolites including pestalotiollide B (PB) (Figure 7c). Notably, in the complement strain, production of PB seems to be restored to the wild-type level. However, this strain displayed a different secondary production profile from the wild type (Figure 7c). This is perhaps caused by the ectopic insertion of the reintroduced copy of *snf1*, which affect the way of its expression. Still more, when the strains were grown in galactose as the sole carbon source, deletion of *snf1* almost abolished the biosynthesis of secondary metabolites (Figure 7b). The above results clearly suggest that *snf1* can function as a critical global regulator controlling secondary metabolism.

5. Conclusions

Our work identified and characterized a SNF1/AMPK homolog in the taxol-producing fungus *Pestalotiopsis microspora*. Snf1 plays a critical role in the regulation of the stress response, alternative carbon source utilization and thermotolerance. Importantly, Snf1 is a key regulator in the growth, development and the biosynthesis of secondary metabolites of filamentous fungi.

Acknowledgments: This work was financially supported by the National Science Foundation of China (#31470251), the National High Technology Research and Development Program (“863” Program #2012AA022105), a grant from the Fundamental Research Funds for the Central Universities (2015NT10), and an Open Fund of the Key Laboratory of Cell Proliferation and Regulation Biology, Ministry of Education.

Author Contributions: X.Z. conceived and designed the experiments, and the writing and correction of the manuscript; Da.W. and Y.L. performed most of the experiments, repeated them side by side. H.W. conducted bioinformatics; Do.W. constructed the vectors for the laboratory. X.H. participated in analysis of the data and polished the first draft; B.X. contributed reagents and materials; O.A. and Y.L. conducted the HPLC profiling separately. Da.W. and X.H. prepared the first draft of the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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