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# Disruption of heat shock factor 1 reduces the formation of conidia and thermotolerance in the mycoparasitic fungus *Coniothyrium minitans*

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ABSTRACT

*Coniothyrium minitans* is a bio-control agent of *Sclerotinia* spp., and has the ability to produce abundant conidia to infect the host fungi. Mediation of heat shock factors (HSFs) is required to adapt to the acute temperatures, and to regulate the expression of heat shock proteins (HSPs) to function as molecular chaperones to assist in development, protein folding and stability. A heat shock factor 1 (*HSF1*) gene was identified from a T-DNA insertion mutant that lost the ability to form conidia in liquid culture as well as on solid media. Null mutants lacking *CmHSF1* were constructed by gene disruption strategy. Mutants lacking *CmHSF1* had reduced in conidial production and displayed decreased tolerance to heat and other abiotic stresses as compared to the wild type parent. Over-expression strains could recover faster from heat and abiotic stresses such as, ethanol, oxidative or osmotic stresses with or without heat shock. In over-expression strains, conidial germination was increased, and parasitic ability on sclerotia of *Sclerotinia sclerotiorum* was enhanced by 0.42–5.92% compared to the wild type strain. Increased expression levels in wild strain ZS-1 were observed when the fungus was grown at 37 °C or 45 °C with other abiotic stresses. *CmHSF1* plays an important role in conidial production, conidial germination, and tolerance against heat and other abiotic stresses.



## 1. Introduction

*Coniothyrium minitans* is a bio-control agent of *Sclerotinia sclerotiorum*, a pathogen that infect a large range of plant hosts worldwide. *C. minitans* can parasitize the sclerotia and mycelia of *S. sclerotiorum* (Merriman et al., 1979; Trutmann et al., 1982). Efficient production of conidia is important in *C. minitans* for commercial use. *C. minitans* has the potential to infect sclerotia of *S. sclerotiorum*, *S. minor*, *S. cepivorum* and *S. trifoliorum* (Whipps and Gerlagh, 1992; Van Toor et al., 2005). Conidial production in *C. minitans* may be affected by light, temperature, nutrition, air and secondary metabolites (Calvo et al., 2002; Roncal et al., 2002; Flaherty and Dunkle, 2005). Media requirements and environmental factors for optimum growth of *C. minitans* have been studied (McQuilken et al., 1997; Cheng et al., 2003). The conidial development of *C. minitans* is divided into five stages, from the hyphal growth stage at 48 hpi to the pycnidial maturation stage at 96 hpi (Li et al., 2010). *C. minitans* in soil can survive for long periods in the presence of *S. sclerotiorum*, by subsisting on exudates such as oxalate from mycelia or sclerotia of *Sclerotinia* (Bennett et al., 2006; Ren et al., 2010). The recognition and attack on the sclerotial host may be related to the conidia of *C. minitans* and the properties of the host surface and some other factors such as, hydrophobicity, surface charge, and lectin binding have been found (Smith et al., 1998, 1999). The commercial production and use of this bio-control agent is increasing against *sclerotial* diseases in some countries, and two commercial products, Contans (Prophyta, Germany) and Koni (Biovéd, Hungary) have been registered.

Heat shock proteins occur as conserved families and are regulated by the activation of heat shock factor proteins (HSFs). HSF acquires DNA binding activity to heat shock elements (HSEs) for survival and development in response to activators such as temperature, heavy metals, osmotic stress, oxidants, and pathogen infection, and then mediates the transcription of heat shock genes (Morimoto et al., 1992; Lindquist and Craig, 1988). HSF (i.e. HSF1) is responsible for the activation and expression of HSP 70 chaperone during stress conditions, and also stress dependent expression of SSA1 and SSA4 in yeast (Voellmy, 1994). HSF enhances the expression of chaperones during stress conditions by increasing the level of partially unfolded proteins, and these unfolded proteins could induce heat shock response in the cell in the absence of temperature shifts (Ananthan et al., 1986; Mifflin and Cohen, 1994). Cells shows heat shock response (HSR) by the preferential synthesis of heat shock proteins under elevated temperature or other stresses (Parsell and Lindquist, 1993; Feder and Hofmann, 1999), and it has been observed that organisms from thermally dif-

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ferent habitats vary in their heat shock response (Tomanek, 2008). In *Candida albicans*, HSF is required for the regulation of heat shock element containing genes in response to elevated temperature and also works for the expression of chaperones machinery (i.e. Hsp104, Hsp90, and Hsp70) even in the absence of stress conditions (Nicholls et al., 2009). All HSF contain DNA-binding domain and oligomerization domain that are located at the N-terminal, and these are conserved throughout family of HSF proteins. Hydrophobic heptad repeats and nuclear localization signals are located at the C-terminal of HSF genes (Mager and De Krujiff, 1995; Wu, 1995). In yeast *Saccharomyces cerevisiae*, HSF is required for the regulation of basal HSP gene expression in response to stress conditions (Smith and Yaffe, 1991), and also necessary for the cellular processes and development in the absence of stress conditions (Sorger and Pelham, 1988; Gallo et al., 1993; Andrew et al., 1995). HSF's knock out strain analysis showed that hsf1 is essential in the mycelial growth, and hsf2 is required for the asexual development in *Neurospora crassa* (Thompson et al., 2008).

In this research, a T-DNA insertion mutant ZS-1T16231 was found to have lost the ability to form conidia on agar or liquid cultures. In this mutant, a gene encoding a HSF-type DNA-binding domain was obtained by TAIL-PCR and inverse PCR. Knock out of this gene, *CmHSF*1 reduced conidial formation, sclerotial parasitism, and tolerance to heat or other abiotic stresses. Over-expression increased the production of conidia, and tolerance to heat or other abiotic stresses. The results indicate that *CmHSF*1 plays an important role in conidial production, conidial germination, and tolerance against heat and other abiotic stresses.

## 2. Materials and methods

### 2.1. Strains and culture conditions

*C. minitans* wild type strain ZS-1 (CCAM 041057) was used in all studies. It can produce abundant conidia on potato dextrose agar (PDA) plates and potato dextrose broth (PDB) (Cheng et al., 2003). *S. sclerotiorum* strain (Ep-1PNA367) was isolated by a single sporing (Xie et al., 2006). All strains were cultured at 20 °C and maintained on PDA slants at 4 °C. *Escherichia coli* strain JM109 and *Agrobacterium tumefaciens* strain EHA105 were prepared and stored at −80 °C and used for cloning and propagation of plasmids.

### 2.2. Construction of disruption and over-expression vectors and transformation

To construct the knock out vector of *CmHSF*1, a deletion cassette was made with the *hph* gene that confers resistance to hygromycin flanked by a 5′ fragment of 905 bp with restriction sites *Xho*I and *Pst*I and a 3′ fragment of 701 bp with restriction sites of *Xba*I and *Sac*I. Both fragments were amplified by PCR with primers HSF1XhoFP, HSF1PstRP for the 5′ fragment (position 449–1339), and HSF1XbaFP, HSF1SacRP for the 3′ fragment (position 3808–4509). This deletion cassette was constructed in pBluescript II SK(+) vector and digested with *Xho*I and *Sac*I enzymes and ligated into pCAMBIA3300 neo vector to obtain the final hsfRPLp3300 plasmid.

To construct the over-expression vector, an over-expression cassette was developed by using the promoter (PtrpC) and terminator (TtrpC) from *Aspergillus nidulans*. A 1.8 kb fragment with primer pair OVXHSF1fp and OVXHSF1rp of *CmHSF*1 gene ORF was amplified from genomic DNA of the wild type strain ZS-1 and ligated between the PtrpC and TtrpC. Another cassette was constructed by using a 1.4 kb fragment of hygromycin (*hph*) resistant gene and ligated into PtrpC and TtrpC by using the primary cloning vector pSKH. The *CmHSF*1 gene cassette was first ligated into the backbone of pCAMBIA3300 binary vector by digesting it with *XcmI* and later it was digested with *Xba*I to ligate the *hph* resistance gene cassette. To confirm the orientation of ligated *CmHSF*1 fragment, a forward primer PtrpFP from the PtrpC and a gene specific reverse primer OVXHSF1rp were used and about 1.9 kb fragment was amplified. The *hph* gene cassette ligation was confirmed by amplifying 890 bp fragment by a pair of primer hph-FP and hph-RP. All primers are listed in Table S1.

*A. tumefaciens* mediated transformation (ATMT) was carried out by using 10 days old conidia of the wild type strain ZS-1 as describe by Li et al. (2006), and candidates were screened against hygromycin and neomycin resistance.

### 2.3. DNA manipulation and southern blot analysis

The wild type strain and all screened transformants were cultured on the PDA plates covered with cellophane membranes, and mycelia were harvested after 4 days of incubation at 20 °C. Genomic DNA was extracted by using the CTAB method as described by Sambrook and Russell (2001).

Southern blot analysis was performed according to Sambrook et al. (1989). A gene-specific fragment of 843 bp (P 1) from the disrupted region and *hph* gene (P 2) were labeled with $\alpha$-$^{32}$P as probes. A gene-specific primer pair HSF1koFP and HSF1koRP (P 1) from the disrupted region of the gene and primer pair 'hph-FP' and 'hph-RP' from the hygromycin gene (P 2) were used for PCR based confirmation.

### 2.4. Expression analysis of CmHSF1 in C. minitans

Mycelia were collected from 4-day-old colonies of the various strains and stored at −80 °C. RNA extraction was carried out using the TRIzol plus RNA purification kit (Invitrogen), and total RNA was treated with DNase I (RNase free) (TaKaRa) following the kit protocol. First strand cDNA synthesis was done using Revert AidTM First strand cDNA synthesis kit (MBI, Fermentas) following manufacturer instructions.

Expression analysis of *CmHSF1* was carried out by RT-PCR and SYBR Green Real Time RT-PCR on a CFX96TM Real time System (Bio-Rad). A gene-specific primer pair, OVXRTfp and OVXRTrp, was use for targetting a 161 bp region in *CmHSF1*. The PCR conditions were 2 min at 95 °C, and then 40 cycles of 20 s at 95 °C, 15 s at 56 °C and 20 s at 72 °C. The *CmACTIN* gene amplified with primer pair, CmACT289 and CmACT419, was used as an internal control for all reactions.

### 2.5. Conidiation and pathogenicity assays

Conidial production was determined by collecting three 1 cm$^2$ mycelial agar plugs from 14-day-old colonies of each strain, vortexing in 1 ml dd$H_2O$ for 1 min, and calculating the number of conidia by using a haemocytometer (Li et al., 2010). Conidial germination rates from all strains were determined using different media ($H_2O$, PDB, *S. sclerotiorum* mycelial extract and plant leaf extract) for 24 h at 20 °C.

The ability of the various *C. minitans* strains to parasitize the sclerotia of *S. sclerotiorum* was assessed. Surface sterilized sclerotia were dipped in 4-day-old mycelial suspension (100 mg/ml) of *C. minitans* for 30 min, and transferred to moist sterilized soil for 30 days. Rot index was calculated according to Cheng et al. (2003). Experiments were repeated twice with three replicates.

### 2.6. Heat and abiotic stress conditions

All strains were observed for tolerance by measuring the hyphal growth rates when incubated under different stress conditions.