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The cyclase-associated protein UvCap1 is required for mycelial growth and pathogenicity in the rice false smut fungus

Hui-Juan Cao[1†], Jin-Jin Zhang[2†], Ming-Li Yong[1], Mi-Na Yu[1], Tian-Qiao Song[1], Jun-Jie Yu[1], Xia-Yan Pan[1] and Yong-Feng Liu[1,2*]

Abstract

Rice false smut caused by *Ustilaginoidea virens* is one of the widespread rice diseases across the globe in recent years, however, we know little about its molecular mechanism of infection. The cAMP signaling pathway functions directly in the development and formation of infectious structures to regulate the infection process in many pathogenic fungi. In order to investigate the role of the cAMP signaling pathway in *U. virens*, UvCap1, a cyclase-associated-protein homologous to *Saccharomyces cerevisiae* Srv2 was identified. Three targeted deletion mutants of the *UvCAP1* gene were obtained with gene replacement strategy assisted with CRISPR-Cas9 system. The *UvCAP1* deletion mutants showed defects in mycelial growth and conidial production. Inoculation experiments demonstrated that Δ*Uvcap1* exhibited defects in pathogenicity. Compared with the wild-type strain, Δ*Uvcap1* showed decreased tolerance to sorbitol and H_2O_2, and increased tolerance to NaCl, CFW and SDS, and the intracellular cAMP level was significantly reduced in Δ*Uvcap1*. Yeast two-hybrid assay identified the interactions of UvCap1 with UvAc1 (adenylase cyclase), two Ras proteins (UvRas1 and UvRas2) and UvSte50. Taken together, as a component of cAMP signaling pathway, UvCap1 plays important roles in the development and pathogenicity of *U. virens*.

Keywords: Rice fungal disease, *Ustilaginoidea virens*, Cyclase-associated protein UvCap1, Mycelial growth, Pathogenicity

Background

Rice false smut has become one of the most prevalent fungal diseases of rice in recent years, and is currently seriously threatening food security (Zhou et al. 2008; Sun et al. 2020). This disease is caused by the ascomycete fungus *Ustilaginoidea virens* (Cooke) Takahashi (teleomorph: *Villosiclava virens*), which initially infects the stamen filaments and then extends to all floral organs of rice to produce false smut balls in spikelets (Tang et al. 2013; Song et al. 2016). The occurrence of rice false smut not only seriously affects the yield and quality of rice, but also produces a large number of mycotoxins that are harmful to humans and animals (Tsukui et al. 2015). Therefore, there is an ongoing and urgent need for research into prevention of the disease. As a nonobligate biotrophic pathogenic fungus, the infection process of *U. virens* is significantly different from some other pathogenic fungi such as *Magnaporthe oryzae*. *M. oryzae* forms appressorium, a specialized infection structure that accumulates enormous turgor pressure to penetrate the host tissue (Talbot 2003). *U. virens*, in contrast, does not form specialized infection structures: after the mycelium penetrates the host epidermis, it cannot penetrate the plant cell wall and enter the host cell, but only grows in the intercellular space (Tang et al. 2013). Elucidating the unique infection mechanism of *U. virens* at the molecular level is of great theoretical significance for understanding the infection process of this fungus.

* Correspondence: liuyf@jaas.ac.cn
†Hui-Juan Cao and Jin-Jin Zhang contributed equally to this work.
[1]Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China
[2]College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China

The cAMP signaling pathway is essential for normal development of many eukaryotes. In plant pathogenic fungi, the conserved cAMP-PKA cascade regulates various developmental and infection processes (Adachi and Hamer 1998; Kang et al. 1999). Cyclase-associated proteins (CAPs), as important components of the cAMP signaling pathway, are well conserved in eukaryotes from yeast to humans (Hubberstey and Mottillo 2002). CAPs have been identified in *Saccharomyces cerevisiae, Candida albicans*, *Cryptococus neoformans*, *Ustilago maydis* and *M. oryzae* (Kawamukai et al. 1992; Rocha et al. 2001; Bahn et al. 2004; Zhou et al. 2012). In *S. cerevisiae*, a CAP gene named as *SRV2* was identified in the Ras-responsive adenylyl cyclase complex (Field et al. 1990; Mintzer and Field 1994; Yu et al. 1999). The N-terminus of Srv2 interacts with Ras2 and Cyr1 (adenylyl cyclase), and the C-terminus of Srv2 interacts with actin monomers (Quintero-Monzon et al. 2009). In *C. albicans*, Cap1 also interacts with adenylyl cyclase and Ras proteins to influence the intracellular cAMP level, and the *CAP1* mutant is defective in bud-hypha transitions under specific environmental conditions (Bahn and Sundstrom 2001; Zou et al. 2010). In *C. neoformans*, the CAPs homologous protein Aca1 interacts with the C-terminus of adenylyl cyclase to regulate cell fusion, capsule formation and pathogenesis (Bahn et al. 2004). In *U. maydis*, Cap1 is an important component of the cAMP signaling pathway that interacts with adenylate cyclase Uac1 and regulates morphogenesis and pathogenesis (Takach and Gold 2010). In *M. oryzae*, *CAP1* functions directly in the activation of adenylate cyclase, and regulates appressorium formation and plant infection. *CAP1* is also involved in the feedback inhibition of the Ras2 signaling pathway in *M. oryzae* (Zhou et al. 2012). Although the cAMP signaling pathway is conserved and has essential functions in numerous plant pathogenic fungi, there is still no advancing research on *CAP* genes in *U. virens*.

In this study, we identified and characterized a CAP-coding gene, *UvCAP1*, in *U. virens*. UvCap1 was found to interact with UvAc1, UvRas1, UvRas2 and UvSte50 in a yeast two-hybrid assay. Δ*Uvcap1*, the deletion mutant of *UvCAP1* was defective in mycelial growth, conidial production, some stress responses and pathogenicity. Collectively, our results demonstrate that UvCap1 plays important roles in the development and pathogenicity of the rice false smut fungus.

Results

Identification of CAP ortholog in *U. virens*

Srv2 is a CAP in the Ras-responsive adenylyl cyclase complex in the budding yeast *S. cerevisiae* (Yu et al. 1999). In this study, we identified *UvCAP1* (KDB15885.1, UV8b_3197), a gene homologous to the yeast *SRV2* gene in the *U. virens* genome by searching the National Center for Biotechnology Information (NCBI) database (accession number: GCA_000687475.1). UvCap1 was the only CAP ortholog we found in *U. virens*, and pfam analysis indicated that this protein contains two domains, CAP_N (11–377 aa) and CAP_C (399–557 aa) (Fig. 1a). A phylogenetic analysis of protein sequences showed that UvCap1 shares the highest similarity with the adenylate CAP from the filamentous fungi *Claviceps purpurea* (Fig. 1b).

UvCap1 is required for mycelial growth and conidiation in *U. virens*

To investigate the function of *UvCAP1* in *U. virens*, we generated the *UvCAP1* deletion mutant in the wild-type strain P-1 by replacing the open reading frame (ORF) of *UvCAP1* with the hygromycin resistance gene (*HYG*) through gene replacement strategy assisted with CRISPR-Cas9 system (Liang et al. 2018). The replacement plasmid was created by homologous recombination (Additional file 1: Figure S1a) (Zheng et al. 2016), and the Cas9-gRNA vector was constructed as described previously (Liang et al. 2018). The resulting vectors were co-transformed into the protoplasts of strain P-1. *HYG*-resistant transformants were first screened by PCR and further confirmed by sequencing. The results showed that the *HYG* gene replaced the ORF of *UvCAP1* in Δ*Uvcap1* (*#11*, *#14* and *#29*) (Additional file 1: Figure S1b). The copy number of *HYG* in Δ*Uvcap1* (*#11*, *#14* and *#29*) was close to 1.0, demonstrating that the *HYG* gene was inserted as a single copy. These three mutants showed similar phenotypic characteristics, so Δ*Uvcap1* (*#11*) was selected for subsequent study. The complemented transformants *Uvcap1-c* (*#1* and #2) were obtained by complementing the Δ*Uvcap1* (*#11*) mutant with the *UvCAP1* gene driven by its native promoter, and were confirmed by RT-PCR and phenotypic analysis (Additional file 1: Figure S1c).

To explore if UvCap1 is involved in mycelial growth and colony morphology in *U. virens*, the wild-type strain P-1, the mutant strains Δ*Uvcap1* (*#11*, *#14*) and the complemented strain *Uvcap1-c* (*#1*) were cultured on PSA and TB3 medium plates for 20 days. Δ*Uvcap1* (*#11*, *#14*) had a reduced mycelial growth compared with those of P-1 and *Uvcap1-c* (*#1*) (Fig. 2a, b). On PSA plate, the average colony diameter for P-1 was 47.6 mm, while those for Δ*Uvcap1* (*#11*) and Δ*Uvcap1* (*#14*) were 23.4 mm and 24.0 mm, respectively. The colony sizes of Δ*Uvcap1* (*#11*, *#14*) in PSB were also significantly smaller than those of P-1 and *Uvcap1-c* (*#1*) (Fig. 2a, b). There was no significant difference in conidial morphology between Δ*Uvcap1* (*#11*, *#14*) and P-1, however, Δ*Uvcap1* (*#11*, *#14*) produced significantly fewer conidia than P-1, with Δ*Uvcap1* (*#11*, *#14*) having only 28–35% of the number of conidia produced by P-1 (Fig. 2c). In

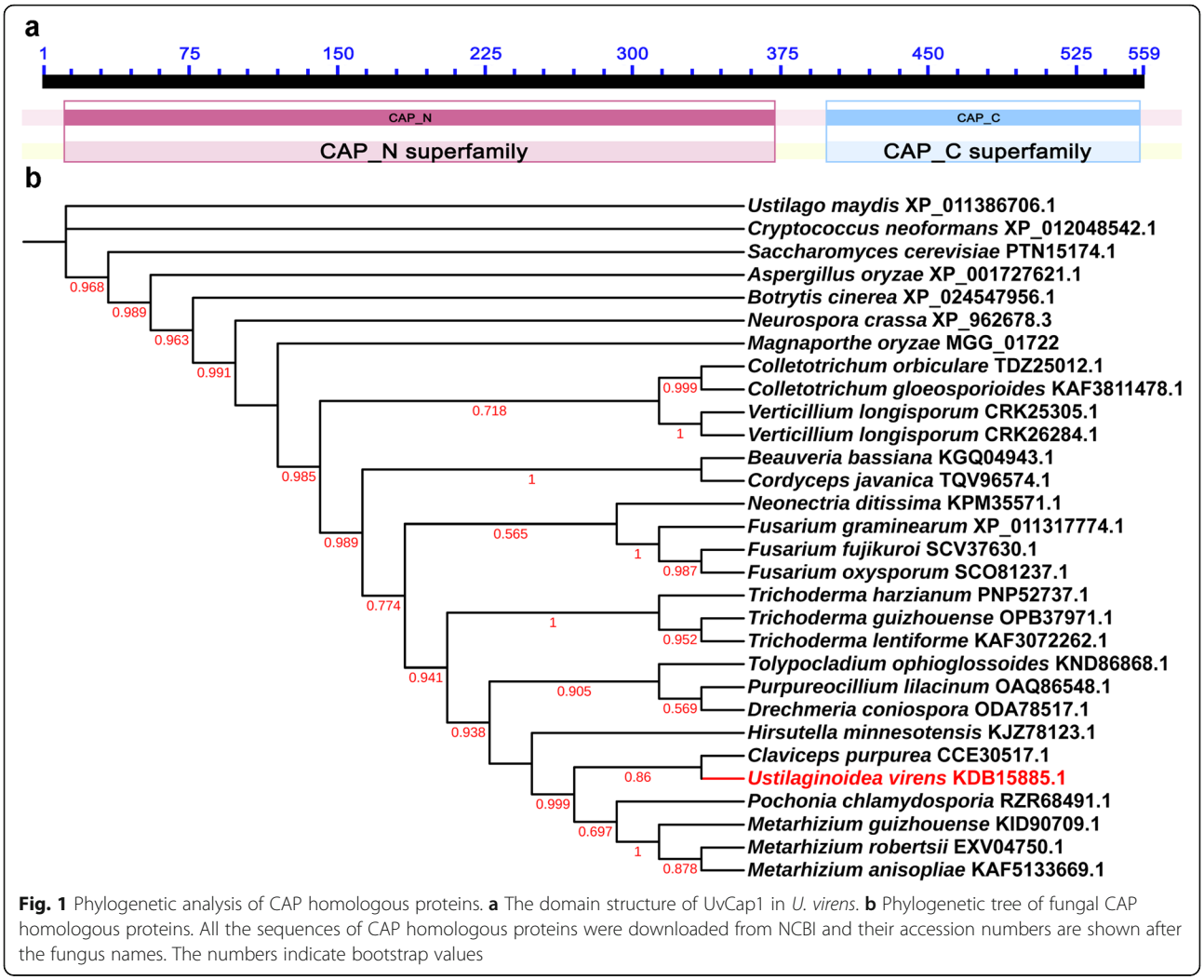

Fig. 1 Phylogenetic analysis of CAP homologous proteins. **a** The domain structure of UvCap1 in *U. virens*. **b** Phylogenetic tree of fungal CAP homologous proteins. All the sequences of CAP homologous proteins were downloaded from NCBI and their accession numbers are shown after the fungus names. The numbers indicate bootstrap values

Uvcap1-c (*#1*), the defects in growth and conidiation were restored to the level of the wild-type strain (Fig. 2). These results indicate that UvCap1 is required for mycelial growth and conidial production in *U. virens*.

UvCap1 is required for full virulence of *U. virens*

We next investigated the role of UvCap1 in regulating the virulence of *U. virens*. Suspensions of shattered hyphae and conidia from P-1, Δ*Uvcap1* (*#11*) and *Uvcap1-c* (*#1*) were inoculated into Liangyoupeijiu panicles at booting stage (5–7 days before heading). At 30 days post inoculation (dpi), the false smut balls produced on rice panicles were counted to evaluate the virulence of the inoculated strains. The average number of false smut balls per panicle for P-1, *Uvcap1-c* (*#1*) and Δ*Uvcap1* (*#11*) were 26.5, 27.3 and 19.0, respectively (Fig. 3a, b). Additionally, mycelial expansion of these strains inside the spikelets of rice was observed at 10, 14 and 18 dpi, and the results demonstrated that mycelial expansion was slower in spikelets inoculated with Δ*Uvcap1* (*#11*) than with P-1 and *Uvcap1-c* (*#1*) (Fig. 3c). To further observe the difference in the infection process, P-1, Δ*Uvcap1* (*#11*) and *Uvcap1-c1* (*#1*) were tagged with GFP, and used to inoculate rice plants. Results of fluorescence microscopic examination showed that there were fewer GFP-tagged hyphae inside the spikelets infected by Δ*Uvcap1* (*#11*) than by the wild-type and complemented strains (Fig. 3d). These results indicate that UvCap1 affects the growth of infection hyphae to regulate the pathogenicity of *U. virens*.

UvCap1 regulates stress responses in *U. virens*

The cAMP signaling pathway is essential in sensing and response to changes of extracellular environments. Δ*Uvac1* shows increased tolerance to sodium chloride (NaCl) and decreased tolerance to Congo red (CR) (Guo et al. 2019). Here we investigated if UvCap1 is involved in responses of *U. virens* to osmotic, oxidative and cell wall stresses. The wild-type strain P-1, the Δ*Uvcap1* (*#11*) mutant and its complemented strain *Uvcap1-c*

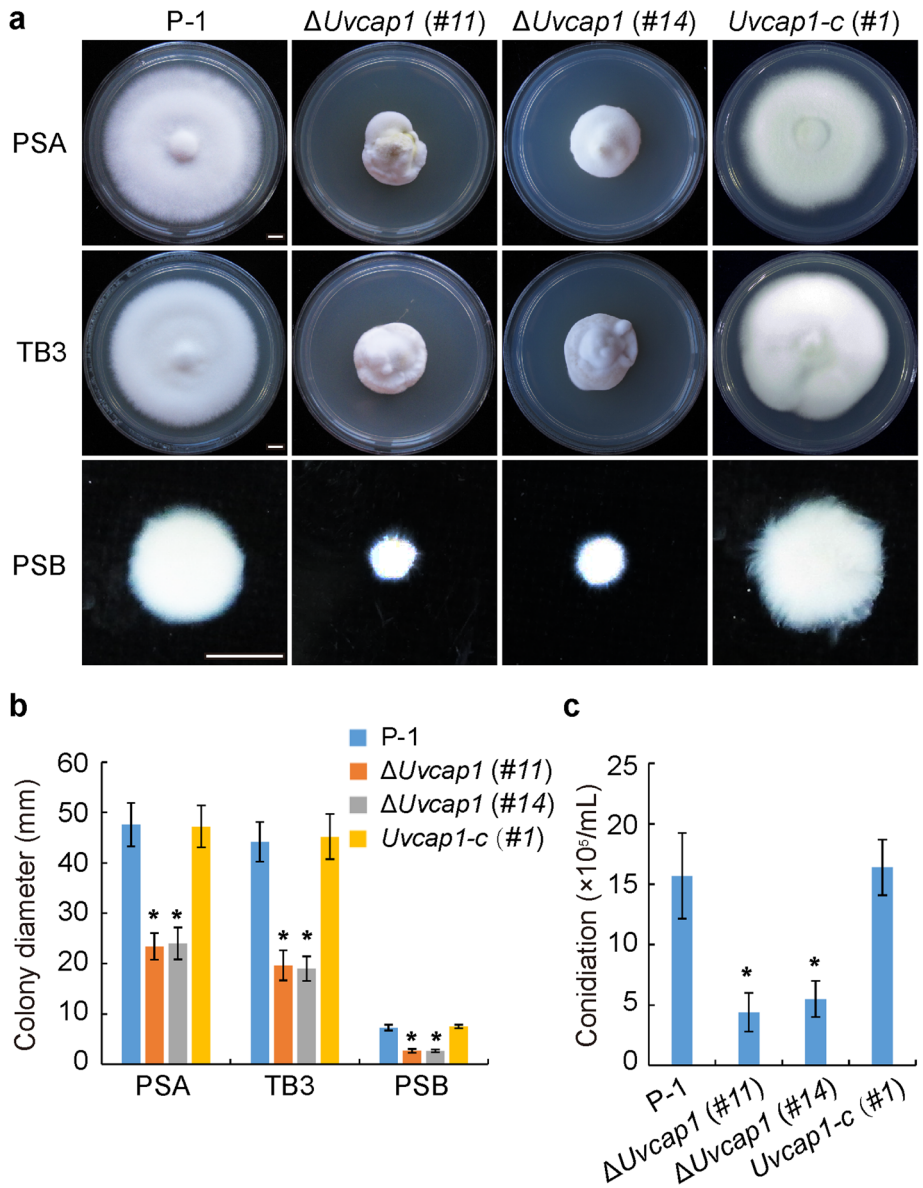

Fig. 2 UvCap1 is required for mycelial growth and conidiation in *U. virens*. **a** Colony morphology of the wild-type strain P-1, the Δ*Uvcap1* (*#11*, *#14*) mutant strains and the complemented strain *Uvcap1-c* (*#1*). The tested strains were incubated on PSA and TB3 plates for 20 days or cultured in PSB with shaking for 7 days. Bar, 5 mm. **b** Column diagram showing colony diameters of strains cultured on different media in a. **c** Conidial production of P-1, Δ*Uvcap1* (*#11*, *#14*) and *Uvcap1-c* (*#1*) in PSB incubated for 7 days at 28 °C, 150 rpm. Error bars represent SD. * indicates significant differences between the mutant and the wild-type/or complemented strains as estimated by Duncan's new multiple range test ($P < 0.05$)

(*#1*) were cultured on PSA medium plate supplemented with different stress agents. Compared with P-1 and *Uvcap1-c* (*#1*), the growth inhibition rate of Δ*Uvcap1* (*#11*) was significantly different. Under 0.6 M sorbitol and 0.05% H_2O_2 treatment, Δ*Uvcap1* (*#11*) showed decreased tolerance (Fig. 4a, b). Δ*Uvcap1* (*#11*) exhibited increased tolerance to 0.5 M NaCl, 500 μg/mL CFW and 0.05% SDS. On PSA medium plate with 100 μg/mL Congo red (CR), the growth inhibition rate of Δ*Uvcap1* (*#11*) showed no difference from P-1 and *Uvcap1-c* (*#1*) (Fig. 4a, b). These results suggest that UvCap1 is required for regulating the responses of *U. virens* to osmotic, oxidative and cell wall stresses.

UvCAP1 was highly expressed during the early infection stage of *U. virens*

According to previous studies on the infection process of *U. virens*, conidia germinate to form hyphae to extend to the interior of spikelets through the gap between lemma and palea at 1–2 dpi. The hyphae then infect

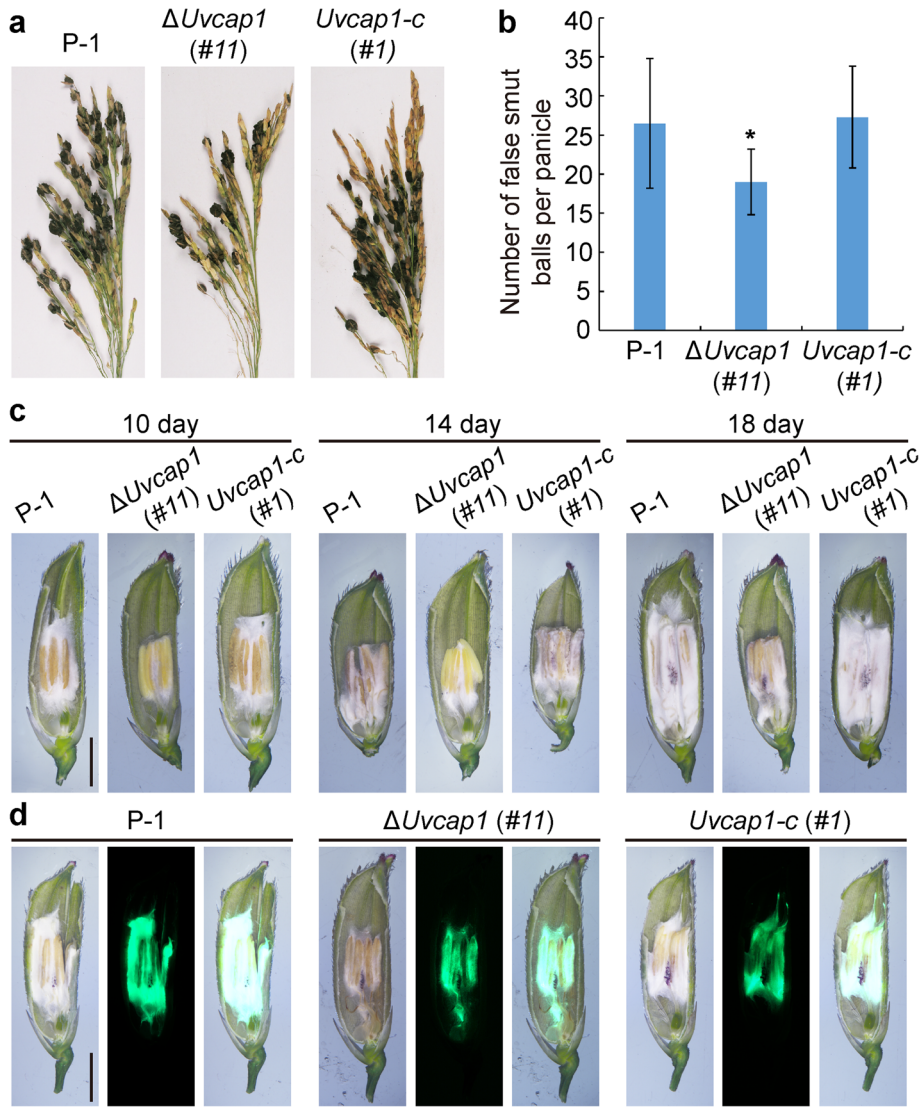

Fig. 3 UvCap1 is required for full virulence of *U. virens*. **a** The rice false smut balls generated by the wild-type strain P-1, the Δ*Uvcap1* (*#11*) mutant and its complemented strain *Uvcap1-c* (*#1*). Pictures were taken at 30 dpi. **b** Column diagram showing the average numbers of false smut balls in an infected panicle in a. Error bars represent SD. * indicates significant differences between the mutant and the wild-type/or complemented strains as estimated by Duncan's new multiple range test ($P < 0.05$). **c** Mycelial extension of P-1, Δ*Uvcap1* (*#11*) and *Uvcap1-c* (*#1*) inside the spikelets at 10, 14 and 18 dpi. Bar, 10 μm. **d** Mycelial extension of GFP-tagged P-1, Δ*Uvcap1* (*#11*) and *Uvcap1-c* (*#1*) inside the spikelets at 10 dpi. Bar, 10 μm

flower organs including filaments, anthers and stigma at 3–7 dpi, and fungal mycelia develop quickly and enclose the entire flower organs, filling the whole spikelets by 8–15 dpi (Song et al. 2016). In order to gain a deeper understanding of the function of *UvCAP1* in the pathogenicity of *U. virens*, RT-qPCR was used to detect the relative expression level of *UvCAP1* during the infection process of *U. virens*. The inoculated rice spikelets at 0, 1, 2, 3, 5, 7 and 14 dpi were collected for RT-qPCR assay. The results showed that the expression level of *UvCAP1* had an upward trend at the initial stage of inoculation, reaching the highest value at 3 dpi, which was 5.14 times the expression level at the time of initial inoculation (Fig. 5). After that, the expression level of the gene showed a downward trend (Fig. 5).

UvCap1 interacts with UvAc1, Ras proteins and UvSte50

Previous studies found that the CAPs function as a component of the cAMP signaling pathway, interacting with adenylate cyclase and regulating morphogenesis and pathogenesis (Bahn et al. 2004; Quintero-Monzon et al. 2009; Takach and Gold 2010; Zou et al. 2010; Zhou et al. 2012). In *S. cerevisiae* and *C. albicans*, Cap1 interacts with Ras and adenylyl cyclase proteins (Quintero-Monzon et al. 2009; Zou et al. 2010). In *U. maydis* and *M. oryzae*, Cap1 interacts with adenylate cyclase to positively regulate

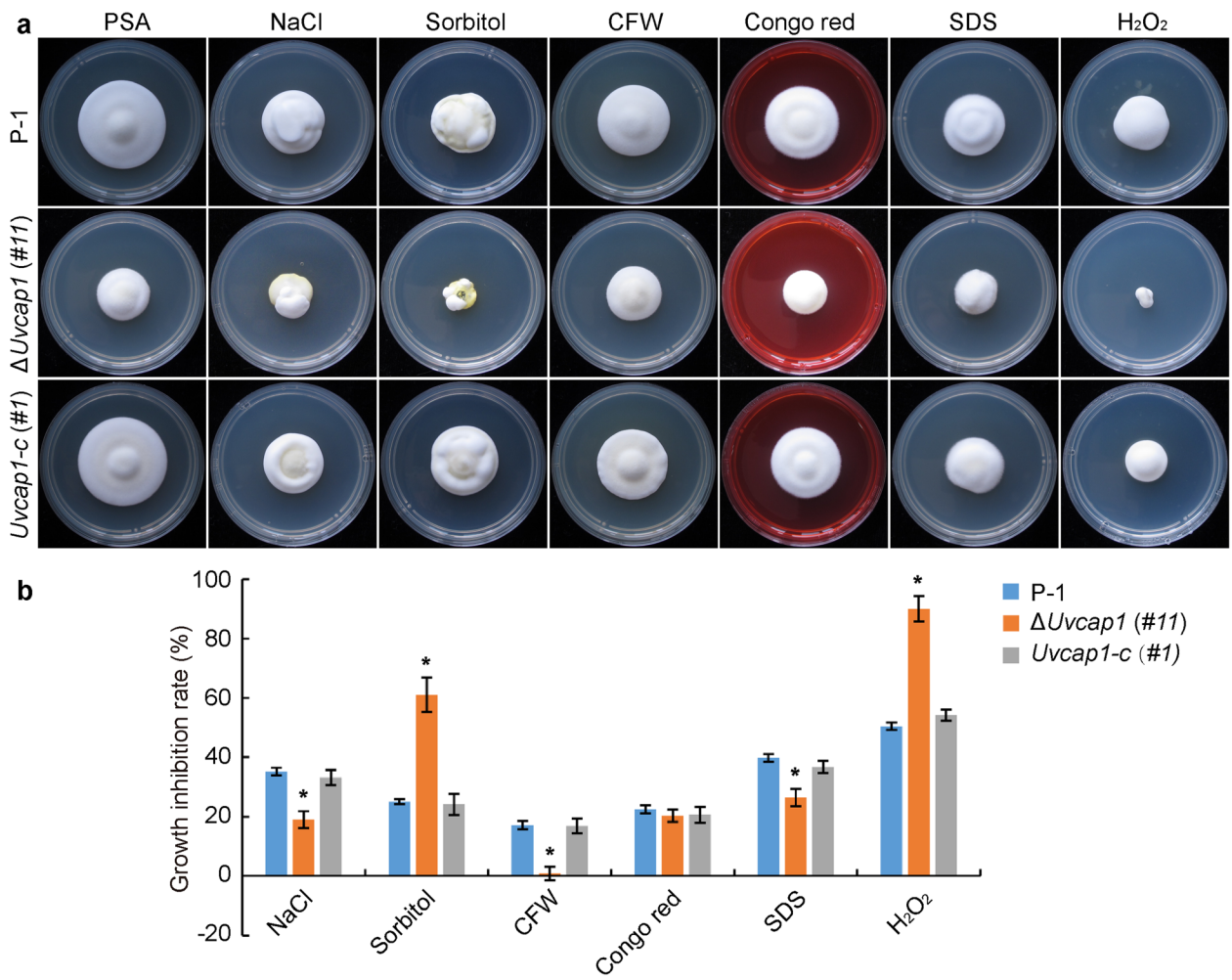

Fig. 4 UvCap1 regulates stress responses in *U. virens*. **a** Mycelial growth of the wild-type strain P-1, the Δ*Uvcap1* (*#11*) mutant and its complemented strain *Uvcap1-c* (*#1*) on PSA plate or PSA with different concentrations of stress agents, including 0.5 M NaCl, 0.6 M sorbitol, 500 μg/mL CFW, 100 μg/mL Congo red, 0.05% SDS and 0.05% H_2O_2. **b** Column diagram showing the growth inhibition rate of strains cultured under different stress conditions in a. Error bars represent SD. * indicates significant differences between the mutant and the wild-type/or complemented strains as estimated by Duncan's new multiple range test ($P < 0.05$)

adenylyl cyclase (Takach and Gold 2010; Zhou et al. 2012). Here, we used yeast two-hybrid assays to identify the interactions of UvCap1 with UvAc1, Ras proteins (*U. virens* has two Ras proteins in the genome database) and UvSte50. The results showed that UvCap1 interacts with UvAc1 (the C-terminal region), UvRas1, UvRas2 and UvSte50 (Fig. 6).

UvCap1 affects the intracellular cAMP level in *U. virens*

cAMP is a ubiquitous second messenger that plays a critical role in the activation of downstream pathways in both eukaryotic and prokaryotic cells. cAMP is produced from ATP by adenylate cyclase and the intracellular cAMP level is significantly decreased (17.0-fold) in the Δ*Uvac1* mutant (Guo et al. 2019). Here we assayed the cAMP level in the wild-type strain P-1, the Δ*Uvcap1* (*#11*) mutant and its complemented strain *Uvcap1-c* (*#1*). Compared with that in P-1, the intracellular cAMP content in Δ*Uvcap1* (*#11*) was significantly reduced (2.0-fold) (Fig. 7). These results indicate that just like UvAc1, UvCap1 plays an important role in maintaining the intracellular cAMP level in *U. virens*.

UvCap1 was distributed as spots in the cytoplasm of hyphae and conidia

In order to explain the possible role of UvCap1 in *U. virens*, a subcellular localization assay was carried out. The UvCap1:GFP transformant was observed using a confocal laser scanning microscope (CLSM). UvCap1 showed strong fluorescence signals as spots in the cytoplasm of hyphae (especially in the apical regions of hyphae) and conidia (Fig. 8a, b). The distribution of

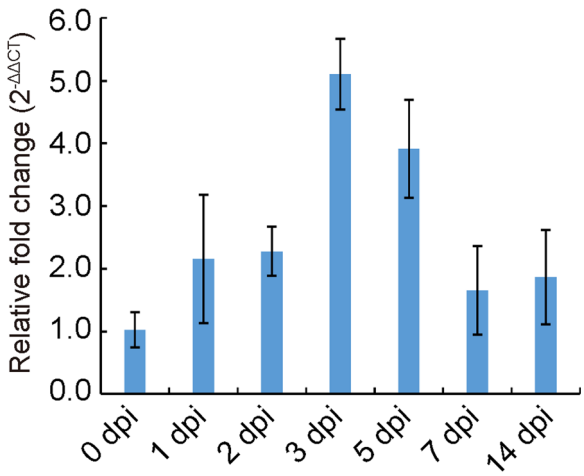

Fig. 5 Expression pattern of *UvCAP1* during the infection process of *U. virens*. The inoculated panicle at 0, 1, 2, 3, 5, 7 and 14 dpi were collected to detect the expression level of *UvCAP1*. RT-qPCR was performed with five biological replicates; *β-TUBULIN* gene was used as the reference gene. Error bars represent SD

UvCap1 in hypha of *U. virens* was similar to that of Cap1 in *M. oryzae* (Zhou et al. 2012). Western blot analysis was performed to detect UvCap1 expression in the UvCap1:GFP transformant. UvCap1:GFP showed a 86-kDa band against the anti-GFP antibody, which is consistent with the protein size of UvCap1 fused with GFP (Fig. 8c). The results suggest that UvCap1 is constitutively expressed in the cytoplasm.

Discussion

In this study, the PEG-mediated transformation and CRISPR/Cas9-based targeted gene replacement method was used to obtain the *UvCAP1* gene deletion mutants. The CRISPR/Cas9 system significantly promoted gene replacement frequency in *U. virens* as previously reported by Liang et al. (2018).

CAPs are involved in regulating the development and pathogenesis of many pathogenic fungi. In human pathogens, the mutant of *ACA1* (homologous to CAPs) in *Cryptococcus neoformans* and the *CAP1* mutant in *Candida albicans* are non-pathogenic (Bahn and Sundstrom 2001; Bahn et al. 2004). In *M. oryzae*, the Δ*cap1* mutant has defects in invasive growth in host cells, so lesions

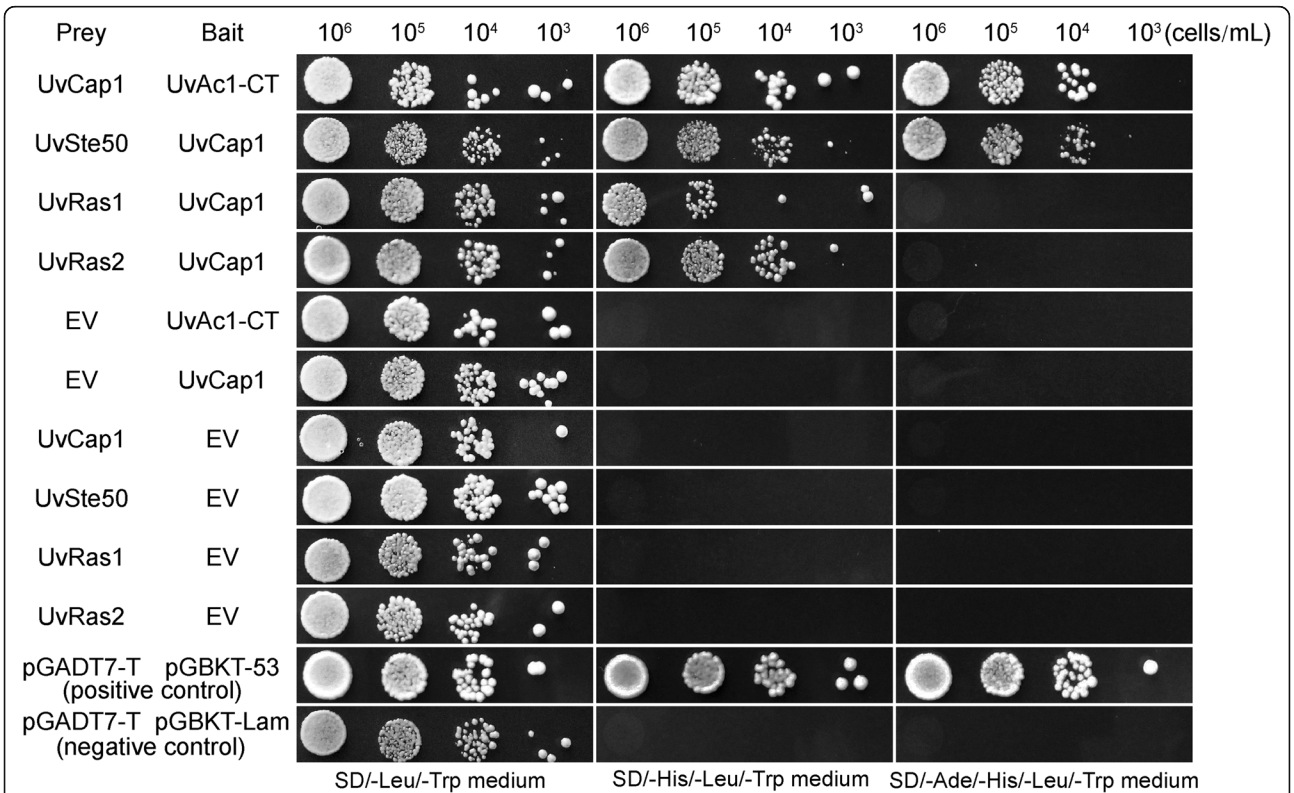

Fig. 6 Assays for interactions between UvCap1 and other proteins in *U. virens*. A yeast two-hybrid assay was used to examine the interaction between UvCap1 and the C-terminal end of UvAc1, Ras proteins (Ras1 and Ras2) and Uvste50. Yeast cells (10^3–10^6 cells/mL) of transformants containing prey and bait vectors were assayed for growth on SD/-Leu/-Trp, SD/-His/-Leu/-Trp and SD/-Ade/-His/-Leu/-Trp. EV, empty vector

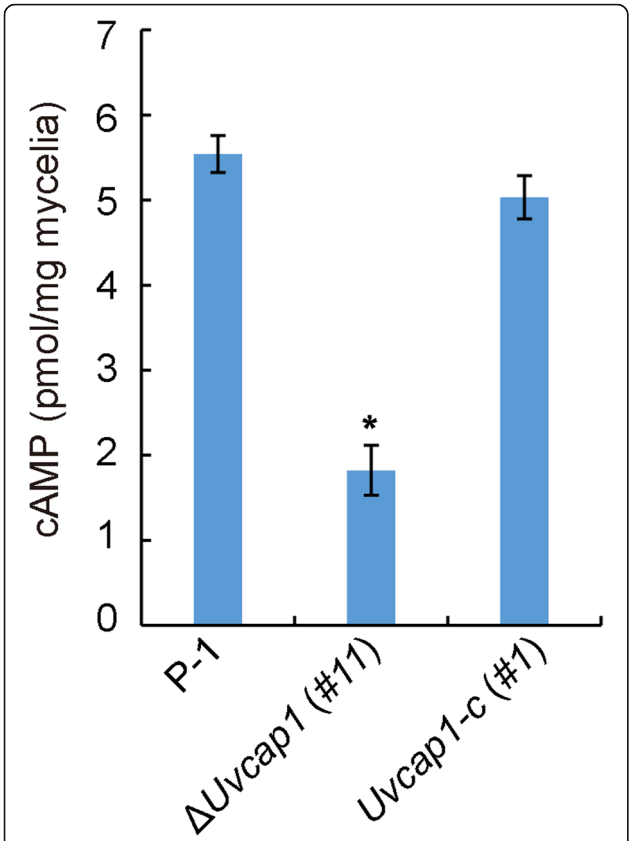

Fig. 7 UvCap1 affects the intracellular cAMP content in *U. virens*. The intracellular cAMP level in wild-type strain P-1, the Δ*Uvcap1* (*#11*) mutant and its complemented strain *Uvcap1-c* (*#1*) was detected. Error bars represent SD. * indicates significant differences between the mutant and the wild-type/or complemented strains as estimated by Duncan's new multiple range test ($P < 0.05$)

caused by this mutant are significantly reduced compared with those by the wild-type strain (Zhou et al. 2012). Adenylate cyclase UvAc1 functions directly in the infection process of *U. virens*: the Δ*Uvac1* mutant cannot form false smut balls on rice panicles (Guo et al. 2019). Our results showed that UvCap1 is required for full virulence of *U. virens* (Fig. 3). Compared with Δ*Uvac1*, Δ*Uvcap1* had a much milder effect on the virulence of *U. virens* to rice. In the early stage of infection, the growth of infection hyphae of Δ*Uvcap1* in the diseased spikelets was slower than that of the wild-type strain, and the number of false smut balls produced by Δ*Uvcap1* was reduced (Fig. 3). Δ*Uvcap1* showed serious defects in mycelial growth and conidiation (Fig. 2). UvCap1 is also required for regulating the responses of *U. virens* to multiple stresses; Δ*Uvcap1* showed increased tolerance to NaCl, CFW and SDS, and decreased tolerance to H_2O_2 and sorbitol (Fig. 4). UvCap1 thus has regulatory roles in the development and infection process of *U. virens*.

The localization of CAPs varies slightly in different organisms at different developmental stages. In yeast, CAP/Srv2p is located in cortical actin patches to regulate the cytoskeleton (Yu et al. 1999). In *Dictyostelium discoideum*, CAP is distributed throughout the cytoplasm and shows enrichment at plasma membrane regions (Gottwald et al. 1996; Noegel et al. 1999). In mouse, Cap1 is widely present in nonmuscle cells, while Cap2 is mainly present in developing striated muscles. Cap1 colocalizes with cofilin-1 to dynamic regions of the cortical actin cytoskeleton in cultured NIH3T3 and B16F1 cells (Bertling et al. 2004). Cap1, as an actin shuttle, provides a link from actin cytoskeleton to mitochondria, and in cells induced for apoptosis, Cap1 is rapidly translocated to the mitochondria (Wang et al. 2008). In *M. oryzae*, Cap1 also has an actin-like localization pattern in hyphae and germ tubes (Zhou et al. 2012). In *U. virens*, the strain UvCap1:GFP showed strong GFP signals as spots in the cytoplasm of the hyphae (especially in the apical regions of hyphae) and conidia (Fig. 8a, b). Whether these spots are actin-like positioning patterns remains to be determined.

CAPs are well conserved in eukaryotes from yeast to mammals (Hubberstey and Mottillo 2002). The Cap1 protein in *U. virens* has typical structural characteristics

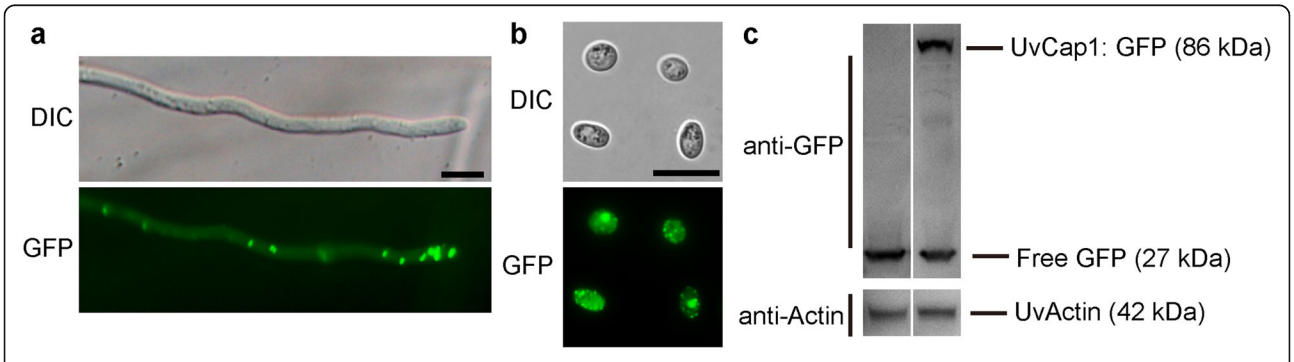

Fig. 8 Subcellular localization of UvCap1 in *U. virens*. **a** Location pattern of UvCap1 in the hyphae of *U. virens*. Bar, 10 μm. **b** Location pattern of UvCap1 in the conidia of *U. virens*. Bar, 10 μm. **c** Verification of UvCap1:GFP transformant by western blot using anti-GFP antibody

of CAPs (Fig. 1a). In *S. cerevisiae*, the conserved N-terminal and C-terminal ends of CAPs interact with adenylate cyclase and agonist protein, respectively (Gerst et al. 1991; Freeman et al. 1995; Paavilainen et al. 2004). Our results also showed that the C-terminal region of UvAc1 interacted with UvCap1 in *U. virens* (Fig. 6). Adenylyl cyclase is a key component of the cAMP signaling pathway, and its function is to maintain the intracellular cAMP level and to participate in the proper infection morphogenesis in many pathogens (Choi and Dean 1997). The localization of adenylate cyclase is slightly different in mammals and yeast. In mammalian cells, adenylate cyclase is a transmembrane protein (Taussig and Gilman 1995), while in budding yeast, it is a peripheral membrane protein (Mitts et al. 1990; Huang et al. 1997).

The binding of CAPs to adenylate cyclase is important in regulating the post-translational modification of Ras2, which in turn is critical for the Ras-dependent activation of adenylate cyclase (Shima et al. 1997). Ras proteins are small GTP-binding proteins that recruit effectors to the cytoplasm membrane, and the activation of Ras requires Ras GTP-binding protein (RasGAP) (Shima et al. 2000; Wennerberg et al. 2005). In yeast, Ira1/2 (Ras GTPase activating protein) induces the GTPase activity of Ras1/2, leading to the activation of adenylate cyclase, meanwhile promoting the synthesis of cAMP (Tanaka et al. 1991). The disruption of the *IRA1* gene leads to the accumulation of a large amount of adenylate cyclase in the cytoplasm (Mitts et al. 1991). In *U. virens*, there is no interaction between UvCap1 and UvGap1 (unpublished data), but UvCap1 interacted with UvRas1 and UvRas2 (Fig. 6). The interaction of UvCap1 with two Ras proteins and UvAc1 may also play an important role in the activation of UvAc1.

The adaptor protein Ste50 interacts with multiple upstream components to activate the MAP kinase cascade in *M. oryzae* (Park et al. 2006). In *S. cerevisiae*, Ste50 interacts with Ste11 and is involved in the cell wall integrity in vegetative cells (Jansen et al. 2001; Ramezani-Rad 2003; Kwan et al. 2006). In *Schizosaccharomyces pombe* and *U. maydis*, Ste4 and Ubc2 (homologous to Ste50 in *S. cerevisiae*) act directly upstream of the MAP kinase cascade and are involved in mating and other developmental processes (Barr et al. 1996; Mayorga and Gold 2001). Our study showed that UvCap1 also interacted with Uvste50 in *U. virens* (Fig. 6). UvCap1 may has an important relationship with MAPK signaling pathways to affect the development and infection process of *U. virens*.

Rice false smut has become one of the most important panicle diseases of rice in recent years. *M. oryzae* is also a filamentous ascus fungus that infects rice. The Cap1 proteins showed many similar functions in these two pathomycetes. Mycelial growth and conidiation were both reduced in the *CAP1* mutants of *U. virens* (Fig. 2) and *M. oryzae* (Zhou et al. 2012). *CAP1* regulates the appressoria formation and invasive growth in *M. oryzae* (Zhou et al. 2012). Although *U. virens* does not form a special appressorium, the expansion of infection hyphae was influenced by UvCap1 in this fungus (Fig. 3). As is the case in *M. oryzae*, the intracellular cAMP level in the *CAP1* deletion mutant of *U. virens* was reduced compared with that in the wild-type strain (Fig. 7). Previous study demonstrated that deletion of *CAP1* suppresses the effects of $RAS2^{DA}$ on appressoria formation in *M. oryzae* (Zhou et al. 2012). In this study, we found that UvCap1 interacted with UvRas2 in *U. virens* (Fig. 6). The subcelluar localization of UvCap1 in hyphae was similar to that of Cap1 in *M. oryzae*, and GFP-tagged UvCap1 was mainly localized in the apical regions of hyphae (Fig. 8).

In summary, we analyzed the function of *UvCAP1* in the development and pathogenicity of *U. virens*. Further characterization of the functional relationships among UvCap1, UvAc1, Ras proteins and UvSte50 will provide valuable information to better understand the role of UvCap1 during the infection process of the rice false smut fungus.

Conclusions

We identified a cyclase-associated protein UvCap1, which showed a high expression level during the early infection process stage of *U. virens*. The deletion of *UvCAP1* led to defects in mycelial growth, conidial production and pathogenicity. Δ*Uvcap1* exhibited more sensitive to sorbitol and H_2O_2 stresses. The intracellular cAMP level was significantly reduced in Δ*Uvcap1* compared with the wild-type strain. Yeast two-hybrid assay showed UvCap1 interacted with UvAc1, UvRas1, UvRas2 and UvSte50. Taken together, UvCap1 functions as a component of the cAMP signaling pathway, interacting with UvAc1, UvRas1, UvRas2 and UvSte50 to regulate the develpoment and pathogenicity of *U. virens*. Further work should focus on the role of UvCap1 in the cAMP signaling pathway and the relationship of UvCap1 with the PMKA signaling pathway during the infection process of the rice false smut fungus.

Methods

Strains and culture conditions

The wild-type strain P-1 of *U. virens* was used as the starting strain for subsequent strain construction; P-1 and the derivative strains were stored in 20–30% glycerol solution at -70 °C. The strains were cultured on potato sucrose agar (PSA) medium (200 g/L potato, 20 g/L sucrose, 15 g/L agar) or TB3 medium (3 g/L yeast extract, 3 g/L acid hydrolyzed casein, 20 g/L sucrose) at

28 °C in the dark for 10–20 days (Tsukui et al. 2015). The rice cultivar Liangyoupeijiu, which is susceptible to strain P-1, was used in the inoculation experiments (Yu et al. 2019). Yeast strain Y2HGold was cultured on YPDA medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 0.3 g/L adenine hemisulfate and 20 g/L agar) at 30 °C.

Phylogenetic analysis of CAP homologous proteins

All the homologous protein sequences of CAP in different fungi were obtained from the NCBI database and their accession numbers are shown as below: *Ustilago maydis* (XP_011386706.1), *Cryptococcus neoformans* (XP_012048542.1), *Saccharomyces cerevisiae* (PTN15174.1), *Aspergillus oryzae* (XP_001727621.1), *Botrytis cinerea* (XP_024547956.1), *Neurospora crassa* (XP_962678.3), *Magnaporthe oryzae* (MGG_01722), *Colletotrichum orbiculare* (TDZ25012.1), *Colletotrichum gloeosporioides* (KAF3811478.1), *Verticillium longisporum* (CRK25305.1), *Verticillium longisporum* (CRK26284.1), *Beauveria bassiana* (KGQ04943.1), *Cordyceps javanica* (TQV96574.1), *Neonectria ditissima* (KPM35571.1), *Fusarium graminearum* (XP_011317774.1), *Fusarium fujikuroi* (SCV37630.1), *Fusarium oxysporum* (SCO81237.1), *Trichoderma harzianum* (PNP52737.1), *Trichoderma guizhouense* (OPB37971.1), *Trichoderma lentiforme* (KAF3072262.1), *Tolypocladium ophioglossoides* (KND86868.1), *Purpureocillium lilacinum* (OAQ86548.1), *Drechmeria coniospora* (ODA78517.1), *Hirsutella minnesotensis* (KJZ78123.1), *Claviceps purpurea* (CCE30517.1), *Ustilaginoidea virens* (KDB15885.1), *Pochonia chlamydosporia* (RZR68491.1), *Metarhizium guizhouense* (KID90709.1), *Metarhizium robertsii* (EXV04750.1), *Metarhizium anisopliae* (KAF5133669.1). A neighbor-joining tree based on the above sequences was built using the Phylogeny.fr platform as described previously (Dereeper et al. 2008) with 1000 bootstrap repeats for distance estimation.

Construction of the *UvCAP1* deletion mutant and its complemented strain

Targeted deletion mutants of the *UvCAP1* gene were obtained with gene replacement strategy assisted with CRISPR-Cas9 system. First, we created a homologous recombination construct pMD19-*UvCAP1* by inserting the *HYG* cassette between the two flanking sequences of the *UvCAP1* gene. The 1025-bp upstream and 1104-bp downstream flanking sequences of *UvCAP1* were amplified with primer pairs 1F/2R and 3F/4R, respectively (Additional file 2: Table S1). The up- and downstream flanking sequences and *HYG* cassette were cloned to T-Vector pMD19 (simple) (TaKaRa, Japan) using a ClonExpress MultiS One Step Cloning Kit (Vazyme, Nanjing).

Then the gRNA designer program was used to design *UvCAP1*-gRNA spacers, and the Cas9 off program was used to screen the high-score spacer to minimize potential off-target effects in the genome (Doench et al. 2014; Guo et al. 2014). The sense and antisense strands of *UvCAP1*-gRNA spacer (Additional file 2: Table S1) were synthesized and annealed to generate double-stranded gRNA spacers (Arazoe et al. 2015; Liang et al. 2018). Then the double-stranded gRNA spacers were cloned to the two *Bsm*BI sites of pmCas9:tRp-gRNA (Liang et al. 2018) using a ClonExpress II One Step Cloning Kit (Vazyme, Nanjing). The resulting construct was confirmed by sequencing.

The vectors pMD19-*UvCAP1* and *UvCAP1*-gRNA were co-transformed into protoplasts of the wild-type strain P-1 using the PEG-mediated method as described previously (Liang et al. 2018). Hygromycin-resistant transformants were screened for deletion of *UvCAP1* by PCR with primers 5F/6R (Additional file 2: Table S1). Overlapping PCR with primers 7F/8R and 9F/10R was used to detect whether *UvCAP1* was replaced by the *HYG* cassette. The *ΔUvcap1* mutants were verified by sequencing analysis (Additional file 2: Table S1).

For complementation assays, the *UvCAP1* gene, along with 1025-bp upstream and 365-bp downstream sequences was amplified with primers *UvCAP1*-comF/comR (Additional file 2: Table S1) and inserted into the pKO1-NEO (G-418 resistance) vector. Then the construct was transformed into Δ*Uvcap1* (*#11*) using the *Agrobacterium*-mediated transformation (ATMT) method (Lv et al. 2016). The resulting transformants were first screened by recovery of growth defects, followed by RT-PCR amplification, and verified by fully restored phenotype characterization.

Subcellular location of UvCap1

To construct the subcellular location strain UvCap1:GFP, we amplified 1955 bp coding sequence of *UvCAP1* with primers *UvCAP1*-GFPF/GFPR (Additional file 2: Table S1). The resulting fragment was cloned into the *Bam*HI and *Sma*I sites of vector pKD2-GFP and then transformed into the wild-type strain P-1 by ATMT as described previously (Lv et al. 2016). G-418-resistant transformants were screened for further fluorescence microscopic observation.

Phenotypic analysis of *U. virens* strains

In the assays for mycelial growth of *U. virens* strains, the mycelia of the wild-type strain P-1, the Δ*Uvcap1* (*#11*, *#14*) mutant and the complemented *Uvcap1-c* (*#1*) were ground with a tissue blender (Waring Commercial Blender 8011S, USA) and diluted with potato sucrose broth (PSB), then individually spread evenly on PSA medium plates, and cultured at 28 °C. A mycelial block (5 mm in diameter) cut from the margin of 5-day-old colony was inoculated on the center of a PSA or TB3 medium plate at 28 °C for 20 days with 5 replicates for each group (Yu et al. 2019; Yong et al. 2020). Colony

diameters were measured using the cross-measurement method.

In the conidiation assays, the same method as above-mentioned was used to obtain mycelial blocks. Three mycelial blocks were transferred into 50 mL of YT (1 g/L yeast extract, 1 g/L tryptone and 1 g/L glucose) liquid medium and incubated with shaking (150 rpm) at 28 °C for 6 days. The number of conidia was recorded under a microscope using a hemocytometer. All the experiments were performed three times with three replicates.

Plant infection assays of *U. virens* strains

To detect if UvCap1 is involved in the infection process of *U. virens*, the susceptible rice variety Liangyoupeijiu was inoculated as described previously (Tang et al. 2013; Yu et al. 2015). The wild-type strain P-1, the Δ*Uvcap1* (*#11*) mutant and its complemented *Uvcap1-c* (*#1*) were cultured in PSB with shaking at 150 rpm, 28 °C for 7 days. A tissue blender (Waring Commercial Blender 8011S, USA) was used for the preparation of a mixture of hyphae and conidia, and the conidia were diluted to a concentration of 1×10^6 conidia/mL with PSB. About 5–7 days before heading, 1–2 mL of hyphae and conidia suspensions were injected into the panicles of rice plants using sterilized syringes. Inoculated rice plants were cultured in a humid environment with a water spray system and the number of rice false smut balls per panicle were counted at 30 dpi (Hu et al. 2014; Yong et al. 2020). The expansion of infection hyphae inside the spikelets of the wild-type strain P-1, the Δ*Uvcap1* (#11) mutant and its complemented *Uvcap1-c* (#1) were observed at 10, 14 and 18 dpi. GFP-tagged strains of *U. virens* were also constructed to further ascertain if UvCap1 is involved in the infection process. The binary vector pKD2-GFP was transformed into P-1, Δ*Uvcap1* (*#11*) and *Uvcap1-c* (*#1*) by ATMT as described previously (Lv et al. 2016). The expansion of infection hyphae inside the spikelets of these GFP-tagged strains was observed at 10 dpi under the stereo fluorescence microscope (Olympus IX71, Japan). The test was repeated three times in total.

Stress response assays of *U. virens* strains

In the stress response assays, the same method as above-mentioned was used to obtain mycelial blocks. A mycelial block (5 mm in diameter) cut from the margin of 5-day-old colony was inoculated on the center of a PSA medium plate or PSA supplemented with different concentrations of stress agents, including 0.5 M NaCl, 0.6 M sorbitol, 500 μg/mL CFW, 100 μg/mL Congo red, 0.05% SDS and 0.05% H_2O_2. The plates were incubated in 28 °C for 20 days, with five replicates for each group (Yu et al. 2019; Yong et al. 2020). Colony diameters were measured using the cross-measurement method. Inhibition rates were calculated as described previously (Xie et al. 2019; Yong et al. 2020).

RT-qPCR analysis of the expression level of *UvCAP1*

Rice spikelets were collected at 0, 1, 2, 3, 5, 7 and 14 dpi. RNA was extracted using an RNA extraction kit (BioTeKe, China) and cDNA reverse transcription was performed with the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan) as previously described (Yong et al. 2020). qPCR was performed in an ABI Q6 Real-Time System with primers *UvCAP1*-qF/qR (Additional file 2: Table S1). TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Japan) was used to evaluate the relative abundance of target gene transcripts, and the average threshold cycle (Ct) was normalized to that of the *TUBULIN* gene (*TUBULIN*-qF/-qR) (Additional file 2: Table S1).

Yeast two-hybrid assay

Protein-protein interactions were performed using the yeast two-hybrid system (Clontech, USA). The ORF regions of *UvCAP1* was amplified from the cDNA of the wild-type strain P-1 with primers *UvCAP1*-BDF/BDR (Additional file 2: Table S1) and cloned into pGBKT7 vector using the ClonExpress II One Step Cloning Kit (Vazyme, China). The ORF regions of *UvRAS1*, *UvRAS2* and *UvSTE50* were individually cloned into the pGADT7 vector to obtain prey constructs. Then the resulting bait and prey constructs were co-transformed into yeast strain Y2HGold. The transformants were grown on SD/-Leu/-Trp, SD/-His/-Leu/-Trp and SD/-Ade/-His/-Leu/-Trp medium for 3–5 days at 30 °C.

Abbreviations

ATMT: *Agrobacterium*-mediated transformation; CAPs: Cyclase-associated proteins; CFW: Calcofluor white; CLSM: Confocal laser scanning microscope; CR: Congo red; dpi: Days post inoculation; *HYG*: Hygromycin resistance gene; GFP: Green fluorescent protein; NCBI: National Center for Biotechnology Information; ORF: Open reading frame; PSA: Potato sucrose agar; PSB: Potato sucrose broth; RT-qPCR: Reverse transcription quantitative PCR

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-021-00083-0.

Additional file 1: Figure S1. Construction of the *UvCAP1* deletion mutant and its complemented strain. **a** Schematic diagram of the construction of replacement plasmid. **b** Hygromycin-resistant transformants were screened for deletion of *UvCAP1* by PCR. I, bands for the *UvCAP1* gene with primers 5F/6R; II and III, bands for overlapping PCR with primers 7F/8R and 9F/10R to detect whether *UvCAP1* was replaced by the *HYG* cassette. IV, bands for *β-TUBULIN* (as a control). **c** G-418-resistant transformants were screened for complementation of Δ*Uvcap1*(*#11*) by RT-PCR. I, bands for the *UvCAP1* gene with primers 5F/6R; II, bands for *β-TUBULIN* (as a control).

Additional file 2 : Table S1. Primers used in this study.

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Authors' contributions
HJC conceived the study and wrote the manuscript. JJZ and HJC carried out the experiments. MLY, MNY, TQS, JJY and XYP contributed to the materials and analysis procedure. YFL revised the manuscript critically. All authors have read and approved the final manuscript.

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The authors declare that they have no competing interests.

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