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Article

# The *Sclerotinia sclerotiorum* ADP-Ribosylation Factor 6 Plays an Essential Role in Abiotic Stress Response and Fungal Virulence to Host Plants

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**Abstract:** The ADP-ribosylation factor 6 (Arf6), as the only member of the Arf family III protein, has been extensively studied for its diverse biological functions in animals. Previously, the Arf6 protein in *Magnaporthe oryzae* was found to be crucial for endocytosis and polarity establishment during asexual development. However, its role remains unclear in *Sclerotinia sclerotiorum*. Here, we identified and characterized *SsArf6* in *S. sclerotiorum* using a reverse genetic approach. Deletion of *SsArf6* impaired hyphal growth and development, and produced more branches. Interestingly, knockout of *SsArf6* enhanced the tolerance of *S. sclerotiorum* to oxidative stress but the sensitivity to osmotic stress, and increased melanin accumulation, indicative of different roles of *SsArf6* in various stress responses. Moreover, the appressorium formation was severely impaired, and fungal virulence to host plants was significantly reduced. Overall, our findings demonstrate the essential role of *SsArf6* in hyphal development, stress responses, appressorium formation, and fungal virulence to host plants.

**Keywords:** *SsArf6*; hyphal growth; appressorium formation; stress response; virulence; melanin

## 1. Introduction

*Sclerotinia sclerotiorum*, as a notorious soilborne plant pathogenic fungus, which has an extremely broad host range, is capable of infecting economically significant crops such as rapeseed, sunflower, and soybeans, resulting in substantial agricultural losses [1–5]. In China, one of the main disease of oilseed rape is *Sclerotinia* stem rot (SSR) caused by *S. sclerotiorum*, leading to losses of approximate 8.4 billion RMB annually [6,7]. *S. sclerotiorum* produces sclerotia that can survive in the field for multiple years [8,9]. In addition, it employs different infection ways, including infection of host plant tissues through the hypha by germination of sclerotia. When conditions are favorable, sclerotia can also germinate to form apothecia, releasing many ascospores to infect the hosts [1,10].

Previous studies indicate that the interaction mechanisms between *S. sclerotiorum* and its host are intricate. To establish successful colonization, *S. sclerotiorum* undergoes specific morphological changes in its hyphal tips, leading to the formation of appressoria [11–13]. Appressoria play a crucial role in adhering to the host surface and penetrating the cuticle [14]. In the past few decades, some proteins related to the development and formation of appressoria have been identified and characterized in the *S. sclerotiorum*, including mitogen-activated protein kinase (MAPK) signaling pathway proteins *SsSte12* [15] and *SsFkh1* [16], autophagy-related proteins *SsATG8*, *SsNBR1* [17], *SsFoxE3* [18], and *SsAtg1* [19], as well as TOR signaling pathway protein *SsTOR* [20]. Additionally, secreted protein *SsRhs1* [21], *Ssams2* [22], *SsNsd1* [23] and *Magnaporthe* appressoria-specific (MAS) protein *Sscnd1* [24] has also been confirmed to participate in appressoria formation.

Growing evidences suggest that the infection of the host by *S. sclerotiorum* happens in stages [14,25]. During the early stages of infection, besides directly invading host tissues through appressoria, *S. sclerotiorum* also secretes virulent factors, for instance oxalic acid [26,27], cell wall-

degrading enzymes [28,29], and effectors [30,31] to impede mechanisms for recognizing and defending against pathogens [32]. Once colonization is achieved, *S. sclerotiorum* enters into the necrotrophic phase, characterized by the production of substantial amounts of reactive oxygen species (ROS) and virulent factors, which induce rapid cell death and the progression of necrotic symptoms [3,14,25,32,33]. Currently, due to the lack of resistant varieties, chemical method is mainly used to control SSR [34]. However, *S. sclerotiorum* has been reported to develop resistance to fungicides [35,36]. This has raised significant attention towards the control and prevention of *S. sclerotiorum*.

ADP-ribosylation factor 6 (Arf6) is a member of the ADP-ribosylation factors (Arf) family, which can regulate the endomembrane recycling and actin cytoskeleton remodeling at the cell surface [37,38]. In mammals, Arf6 plays a crucial role in regulating neutrophil energy metabolism [39], cancer cell invasion, metastasis and proliferation [40], as well as membrane lipid homeostasis [41]. Additionally, a homolog of Arf6 in *Aspergillus nidulans*, named ArfB, functions in endocytosis to play important roles in polarity establishment during isotropic growth and polarity maintenance during hyphal extension [42]. The Arf6's homolog in *Magnaporthe oryzae* also has similar functions [38]. However, the biological function of Arf6 in *S. sclerotiorum* remains unclear.

Through reverse genetic approaches, we characterized the roles of *SsArf6* in *S. sclerotiorum*, and found that *SsArf6* is highly conserved among various plant pathogenic fungi. Knockout of *SsArf6* led to hindered mycelium growth and development, producing more branches, aerial hyphae and fewer sclerotia. Surprisingly, *SsArf6* negatively regulates melanin accumulation and hydrogen peroxide resistance. In addition, deletion of *SsArf6* significantly increased the sensitivity of *S. sclerotiorum* to osmotic stress. Most importantly, the appressorium formation and virulence to host plants exhibited severe impairments in knockout mutants. Together, our results suggesting that *SsArf6* plays a significant role in the formation of appressorium, hyphal development, resistance to abiotic stress in *S. sclerotiorum* and fungal virulence to host plants.

## 2. Materials and Methods

### 2.1. Fungal Strains and Culture Conditions

Cultured in a constant temperature incubator at 20°C, the wild-type strain (WT), knockout mutant, and complemented strains were grown on potato dextrose agar (PDA) plates. The PDA plates were supplemented with 200 µg/mL hygromycin B (Roche) for the knockout mutants and PDA plates supplemented with 100 µg/mL G418 Sulfate (Geneticin) (Yeasen) for the complemented strain.

### 2.2. Plant Materials and Growth Conditions

*Arabidopsis thaliana*, oilseed rape (*Brassica napus*) and *Nicotiana benthamiana* seedlings were cultivated in a growth room at a temperature of 22°C, with a photoperiod of 16 hours of light followed by 8 hours of darkness. Except for *Brassica napus*, which were cultivated for 5 weeks, all other plants were cultivated for 4 weeks, were selected for the virulence assays with *S. sclerotiorum*.

### 2.3. Phylogenetic Tree Construction and Sequence Analysis

Homologous sequences of Arf6 in *Zyoseptoria tritici*, *S. sclerotiorum*, *Ustilago maydis*, *Botrytis cinerea*, *Magnaporthe oryzae*, *Fusarium oxysporum* and *Fusarium graminearum* were obtained from the NCBI database (<https://www.ncbi.nlm.nih.gov/>). The Arf6 gff3 files were got from the ensemblFungi database (<https://fungi.ensembl.org/>). The phylogenetic tree was constructed using MEGA11 programs with 1,000 bootstrap replicates and the neighbor joining method [43]. The gene feature was visualized by GSDS2.0 [44], and the alignment of Arf6 proteins was performed using Clustal Omega [45] and results viewed with Jalview [46].

#### 2.4. Knockout and Complementation of *SsArf6*

The split-marker method was employed to knockout the *SsArf6* (sscle\_03g022330) gene in *S. sclerotiorum*. As previously described [47], two rounds of PCR were used to construct the replacement fragments *SsArf6*-UP-HY and *SsArf6*-Down-YG, which were used to replace the target gene *SsArf6*. The PCR products were co-transformed into wild-type (WT) strain protoplasts. *SsArf6* knockout transformants were selected on PDA plates containing 200 µg/mL hygromycin B, and at least three rounds of hyphae tip transfer and protoplast purification were performed to obtain knockout mutant homozygotes.

In genetic complementation, a 1746-bp fragment consisting of upstream and full-length genomic DNA of *SsArf6* was amplified specifically through PCR. The resulting fragment was individually fused with NEO fragments, which were amplified from the *pCH-EF-1* plasmid (provided by D. Jiang from Huazhong Agricultural University). The resulting PCR products were co-transformed into protoplasts of the knockout mutant strain. Validation was achieved through PCR using six sets of primers (Table S1), and the transformed knockout and complementation strains were selected for further experimentation.

#### 2.5. DNA and RNA Manipulation

The mycelium of *S. sclerotiorum* was cultivated on PDA medium containing cellophane for 48 hours, and then ground into fine powder in liquid nitrogen. Genomic DNA was extracted from both WT and mutant strains using the cetyltrimethylammonium bromide (CTAB) method [48]. The extracted WT genomic DNA was used as a template for amplifying the full-length sequence and the flanking sequence of *SsArf6*. The extracted genomic DNA from the mutants was used to confirm the knockout and complementation of *SsArf6*. To evaluate the expression of *SsArf6* in the knockout and complementation strains at the transcriptional level, the mycelium was collected after 48 hours of cultivation on PDA medium containing cellophane, and total RNA was extracted using the SteadyPure Plant RNA Extraction Kit (Accurate Biology). The first-strand cDNA was synthesized from the total RNA using the Evo M-MLV RT-PCR Kit (Accurate Biology) as the template. Semi-quantitative RT-PCR was performed with cDNA as the template for 28 PCR cycles. All primers used in this study were listed in Table S1.

#### 2.6. Colony Morphology Observation

The growth lengths of the WT, knockout mutant ( $\Delta Ssarf6$ ) and complemented strain (*SsArf6*-C) on potato dextrose agar (PDA) medium were measured every 24 hours. The colony morphologies of the WT,  $\Delta Ssarf6$ , and *SsArf6*-C strains on PDA medium were documented using a digital camera at 24 hours, 48 hours, and 8 days of cultivation respectively.

#### 2.7. Stress Treatment

The WT,  $\Delta Ssarf6$ , and *SsArf6*-C strains were treated with 5mM, 10mM and 15mM of H<sub>2</sub>O<sub>2</sub>; cell wall inhibitor Congo Red (CR), 0.02% of Sodium Dodecyl Sulfate (SDS); osmotic stressors 0.5M NaCl, KCl and 1M sorbitol and glucose. After 48 hours, the colony morphology was recorded using a digital camera and the inhibition rate was calculated. The inhibition rate (%) was calculated as  $100 \times (\text{the colony radius of the strain on pure PDA} - \text{the colony radius of the strain under different stressors}) / \text{the colony radius of the strain on pure PDA}$ .

#### 2.8. Analysis of Compound Appressoria, OA and Virulence to Host Plants

The WT,  $\Delta Ssarf6$ , and *SsArf6*-C strains were inoculated onto a PDA medium containing 0.005% (W/V) Bromophenol Blue. After 48 hours, the color change was recorded using a digital camera. For appressorium formation observation, the WT,  $\Delta Ssarf6$ , and *SsArf6*-C strain blocks were inoculated onto a slide, and after 24 hours of cultivation, the morphology of the compound appressoria was observed and recorded under an optical microscope (Axio Imager 2, ZEISS, Oberkochen, Germany).

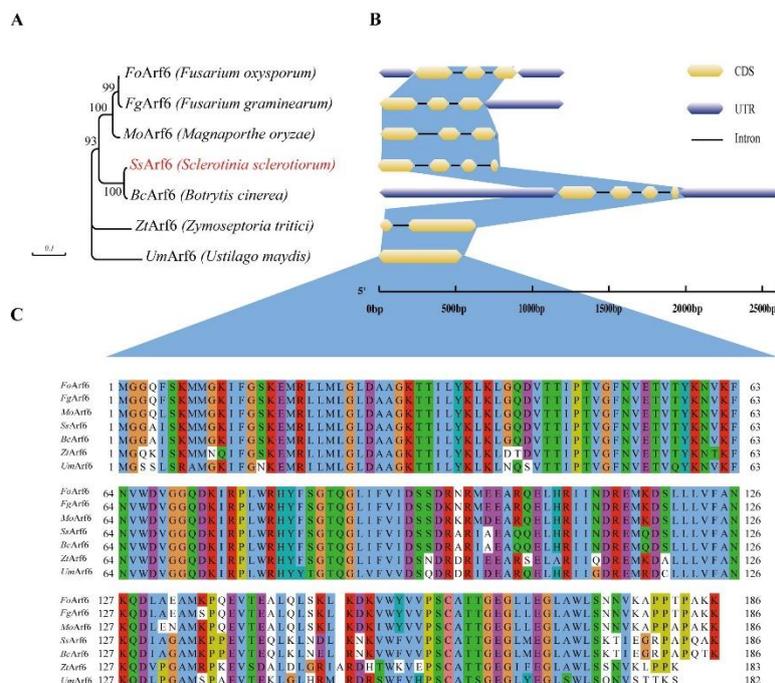
After a cultivation period of 36 hours, the appressorium of WT,  $\Delta Ssarf6$ , and  $SsArf6-C$  strain was recorded using a digital camera.

To observe the morphology of the compound appressoria formed on onion epidermal cells, the *S. sclerotiorum* strains were inoculated onto the onion epidermis cultured for 16 hours as previously described [49]. After that, the onion epidermis was soaked in a 0.5% Trypan Blue solution for 30 minutes. Next, a bleaching solution was prepared in a ratio of ethanol: acetic acid: glycerol of 3:1:1 and used to decolorize the samples. The morphology of the composite attached cells was then observed and recorded under an optical microscope. For the virulence analysis, the WT,  $\Delta Ssarf6$ , and  $SsArf6-C$  strains were inoculated onto detached leaves of *A. thaliana*, *B. napus* and *N. benthamiana*. After 36 hours of infection, the infection morphology was recorded using a digital camera, and the lesion areas are analyzed using Image J software [50].

### 3. Results

#### 3.1. Identification of the *Arf6* homolog in *S. sclerotiorum*

Previously, when using a forward genetic approach to screen hypo-virulent mutant of *S. sclerotiorum*, we got one mutant with a candidate gene which was speculated to be a guanine exchange factor for *Arf6* (*SsEFA6*, *sscle\_12g090120*), related to the pathogenic capacity of *S. sclerotiorum* (unpublished data). To determine whether *Arf6* is also associated with pathogenicity, we then performed a genomic blasting and identified *sscle\_03g022330* as the homologous gene of *MoArf6* in *S. sclerotiorum*, and named as *SsArf6*, which comprised four exons and three introns, spanning a length of 794 base pairs and encoding 186 amino acids. As shown in Figure 1A, *SsArf6* homologous proteins were prevalent among plant pathogenic fungi. When conducting gene structure analysis of these homologues, we discovered that *UmArf6* contained only one CDS region, while *SsArf6* and *BcArf6* had up to four CDSs (Figure 1B). In addition, protein sequence alignment revealed evolutionary conservation of *SsArf6*, which had a high similarity with *Arf6* homologs in *F. oxysporum*, *F. graminearum*, *B. cinerea*, *Z. tritici*, and *U. maydis*, with 86.02%, 84.95%, 85.48%, 98.39%, 76.92%, and 76.84% amino acid sequence identity respectively (Figure 1C).

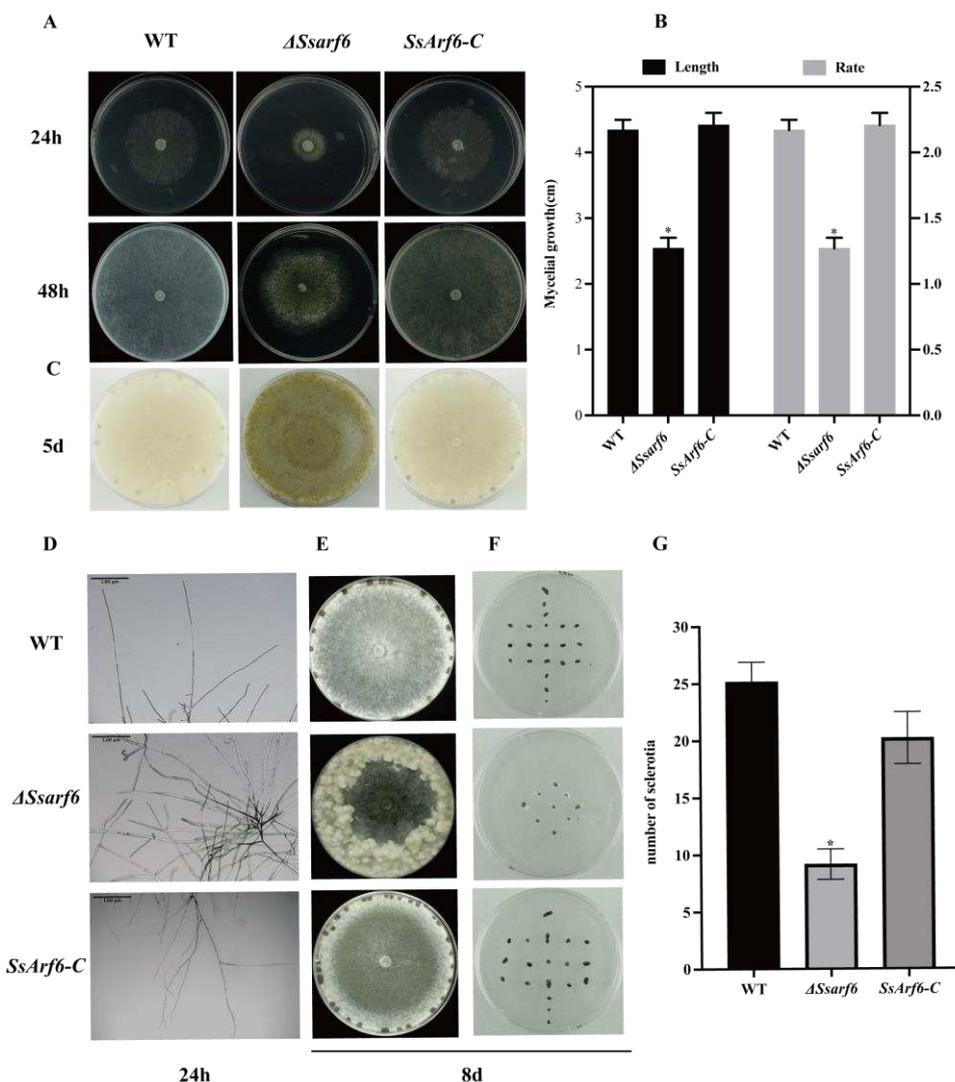


**Figure 1.** Phylogenetic and sequence analysis of *SsArf6*. **(A)** Phylogenetic analysis of *Arf6* between different species. **(B)** The *Arf6* gene features in *F. oxysporum*, *F. graminearum*, *M. oryzae*, *S. sclerotiorum*,

*B. cinerea*, *Z. tritici*, and *U. maydis* illustrated schematically by GSDS2.0. (C) Multiple sequence alignment of *FoArf6*, *FgArf6*, *PoArf6*, *SsArf6*, *BcArf6*, *ZtArf6*, and *UmArf6*. The alignment results were visualized using Jalview clustal Colour Scheme.

### 3.2. Knockout of *SsArf6* leads to aberrant mycelium growth, increased melanin accumulation and decreased sclerotium production

In order to investigate the biological function of *SsArf6* in *S. sclerotiorum*, we generated the *SsArf6* deletion mutant,  $\Delta Ssarf6$  (Figure S1A), using a split-marker method based on homologous recombination. The results indicated that *SsArf6* was completely replaced by *hph* (Figure S1B, Figure S3). The growth of  $\Delta Ssarf6$  strain mycelium was hindered, with an average growth rate of 1.27cm/24h, significantly lower than that of the WT strain (Figure 2A, B). In addition, the  $\Delta Ssarf6$  strains produced more branches and melanin compared to WT strains (Figure 2C, D). After incubation on PDA medium for 8 days, there were noticeable differences in colony morphology among WT and  $\Delta Ssarf6$  strains. Specifically, the  $\Delta Ssarf6$  strain formed many aerial hyphae (Figure 2E) but fewer sclerotia (Figure 2F, G) compared to WT. Furthermore, the phenotype of *SsArf6-C*, which was generated through *in situ* complementation in the  $\Delta Ssarf6$  background using a knock-in method (Figure S2A, B, Figure S3), was similar to WT (Figure 2). These findings strongly indicate that *SsArf6* plays an important role in hyphal growth, melanin accumulation and sclerotium production.

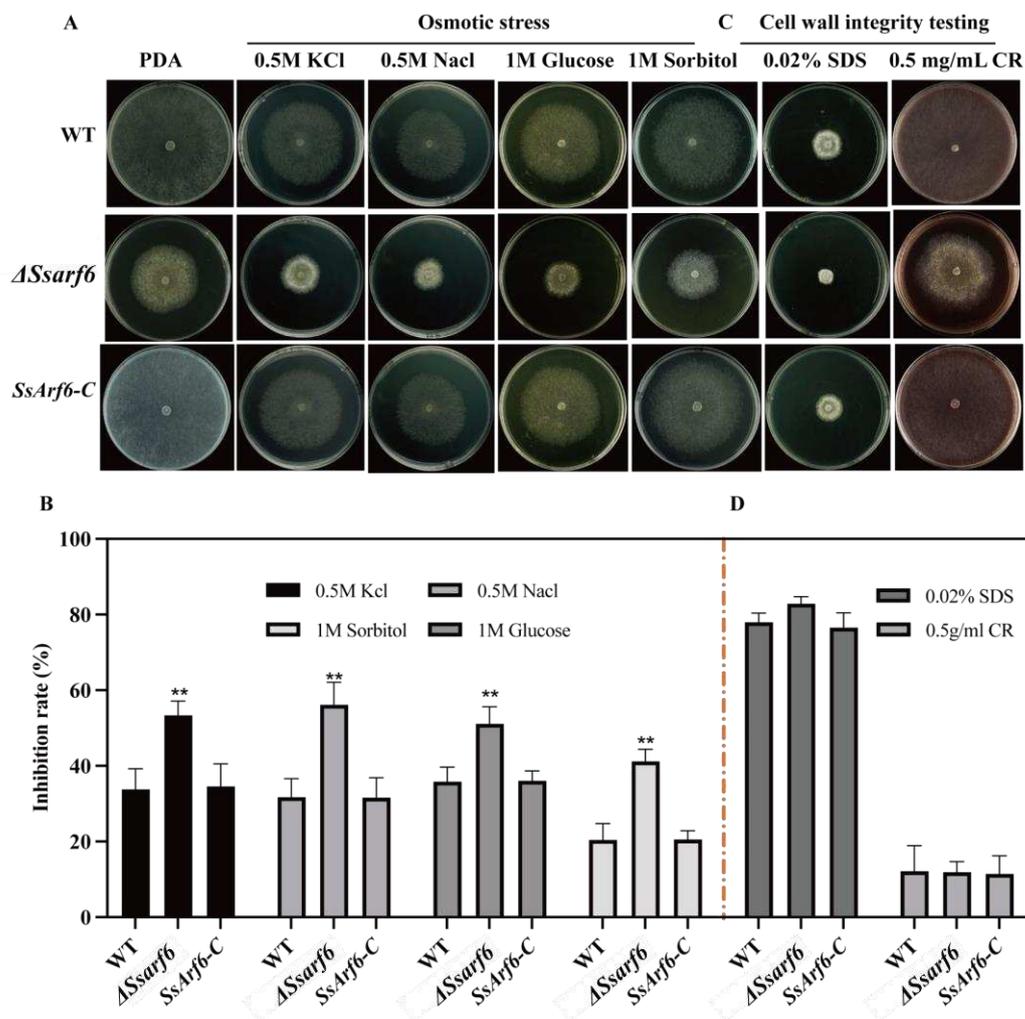


**Figure 2.** *SsArf6* contributes to mycelial development, melanin accumulation and sclerotia production in *S. sclerotiorum*. (A) The colony morphology of WT,  $\Delta Ssarf6$ , and *SsArf6-C* strains grown on PDA culture medium for 24h and 48h. (B) The mean length and growth rate of mycelia of WT,  $\Delta Ssarf6$ ,

and *SsArf6-C* strains 48 hours later. (C) Melanin accumulation of WT,  $\Delta Ssarf6$ , and *SsArf6-C* strains grown on PDA culture medium for 5d. (D) Branching patterns of mycelia of WT,  $\Delta Ssarf6$ , and *SsArf6-C* strains. (E) The colony morphology of WT,  $\Delta Ssarf6$ , and *SsArf6-C* strains grown on PDA medium for 8 days. (F, G) The number of sclerotia per plate. WT refers to the wild-type strain;  $\Delta Ssarf6$ , the knockout strain; and *SsArf6-C*, the complemented strain. The experiment was repeated three times with similar results. Error bars represent the standard deviation (SD). The statistical significance between WT and knockout mutant or complemented strains was analyzed using the student's t-test (\*  $p < 0.05$ ).

### 3.3. *SsArf6* is implicated in abiotic stress response in *S. sclerotiorum*

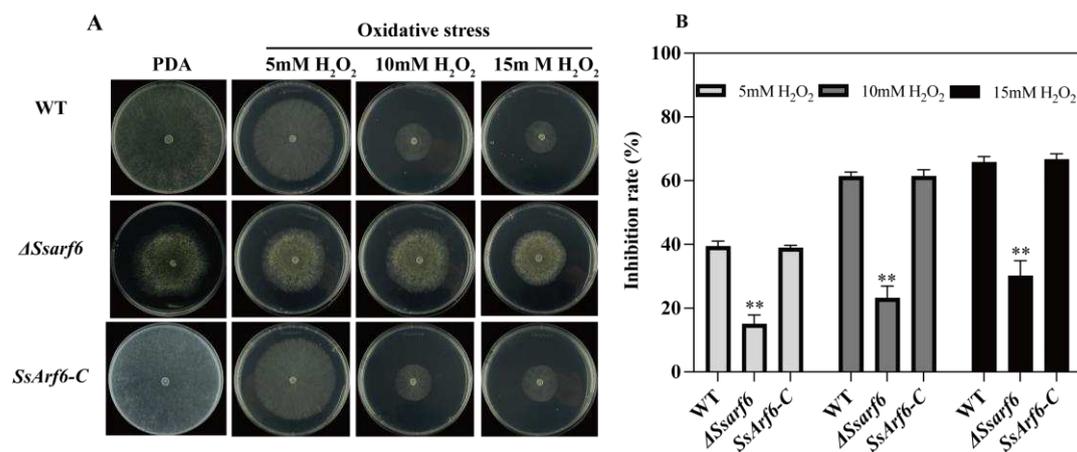
To explore the response of *SsArf6* to different stresses, *S. sclerotiorum* was treated with various cell wall inhibitors and osmotic stresses. When exogenous 0.5M NaCl, 0.5M KCl, 1M glucose and 1M sorbitol were applied, the colony radii of  $\Delta Ssarf6$  were significantly reduced, and the inhibition rate were significantly higher than that of WT and *SsArf6-C* strains (Figure 3A, B). However, in the medium supplemented with cell wall inhibitors Congo red (CR) and Sodium Dodecyl Sulfate (SDS),  $\Delta Ssarf6$  demonstrated a stress response that was comparable to both the WT and *SsArf6-C* strains (Figure 3C, D).



**Figure 3.** *SsArf6* contributes to the responses to osmotic stress in *S. sclerotiorum*. (A, B) The colony morphology and inhibition rate of WT,  $\Delta Ssarf6$ , and *SsArf6-C* strains grown on PDA medium containing different osmotic stressors, 0.5M NaCl, 0.5M KCl, 1M glucose and 1M sorbitol, for 48h. (C, D) The colony morphology and inhibition rate of WT,  $\Delta Ssarf6$ , and *SsArf6-C* strains grown on PDA medium containing different cell wall inhibitors, 0.5mg/mL Congo Red (CR) and 0.02% Sodium

Dodecyl Sulfate (SDS) for 48h.  $\Delta Ssarf6$  represents the knockout strain;  $SsArf6-C$ , the complemented strain. The experiment was conducted three times with similar results. Error bars represent the standard deviation (SD). The statistical significance between the WT and knockout mutant or complemented strains was analyzed using the student's t-test (\*\*  $p < 0.01$ ).

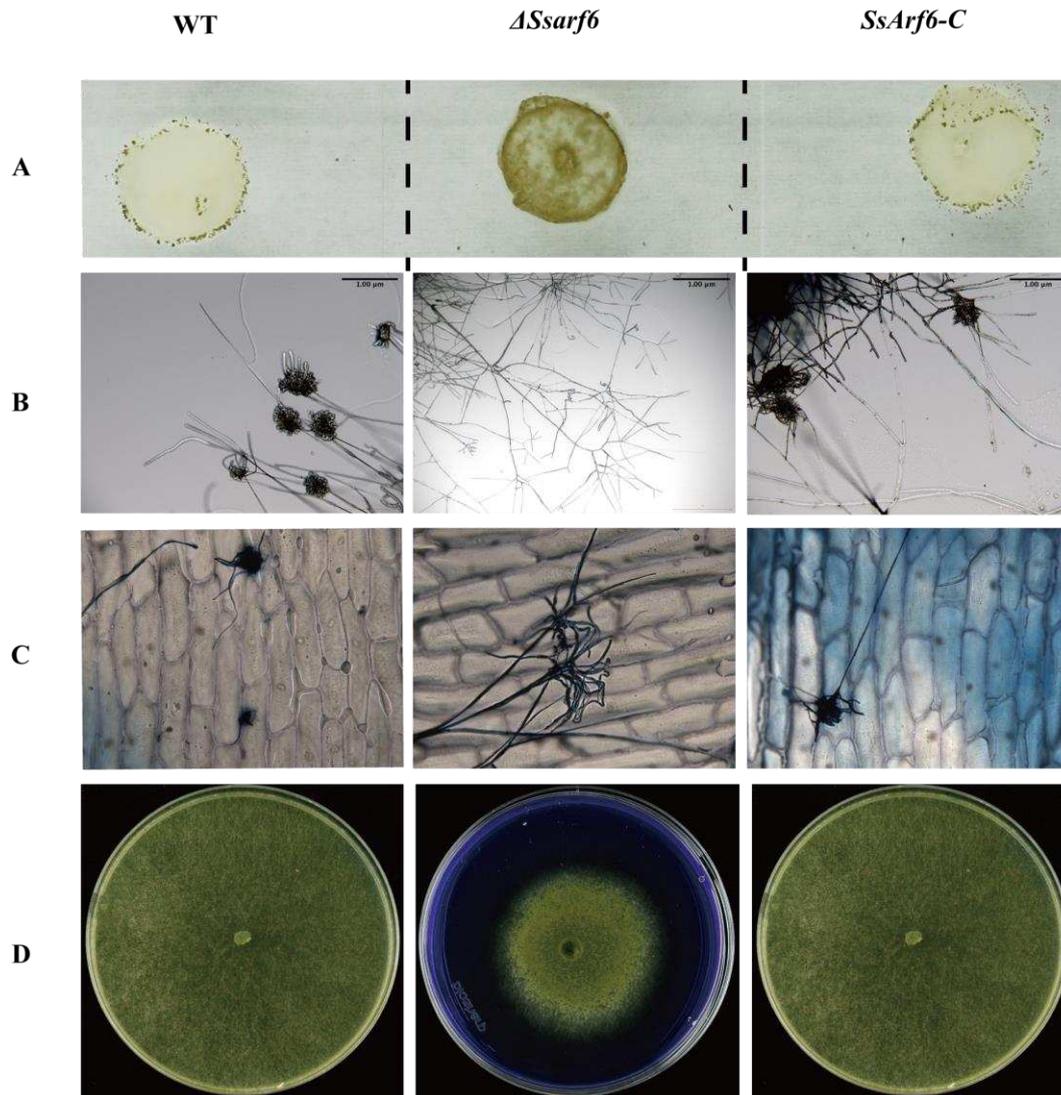
Former studies suggested that fungal melanin is frequently located intracellularly and has antioxidant properties, as it can neutralize reactive oxygen species and other free radicals [51]. Therefore, we assessed the antioxidant capacity of the  $\Delta Ssarf6$  mutant, and found that they exhibited significant tolerance to hydrogen peroxide compared to the WT and  $SsArf6-C$  strains (Figure 4A, B), indicative of the essential role of  $SsArf6$  in regulating the abiotic stress responses in *S. sclerotiorum*.



**Figure 4.**  $SsArf6$  negatively regulates the resistance of *S. sclerotiorum* to hydrogen peroxide. (A, B) The mycelium morphology and inhibition rate of WT,  $\Delta Ssarf6$ , and  $SsArf6-C$  strains grown on PDA medium containing 5mM, 10mM, and 15mM H<sub>2</sub>O<sub>2</sub> for 48h. The experiment was conducted three times with similar results. Error bars represent the standard deviation (SD). The statistical significance between the WT and knockout mutant or complemented strains was analyzed using the student's t-test (\*\*  $p < 0.01$ ).

#### 3.4. $SsArf6$ is involved in compound appressoria development

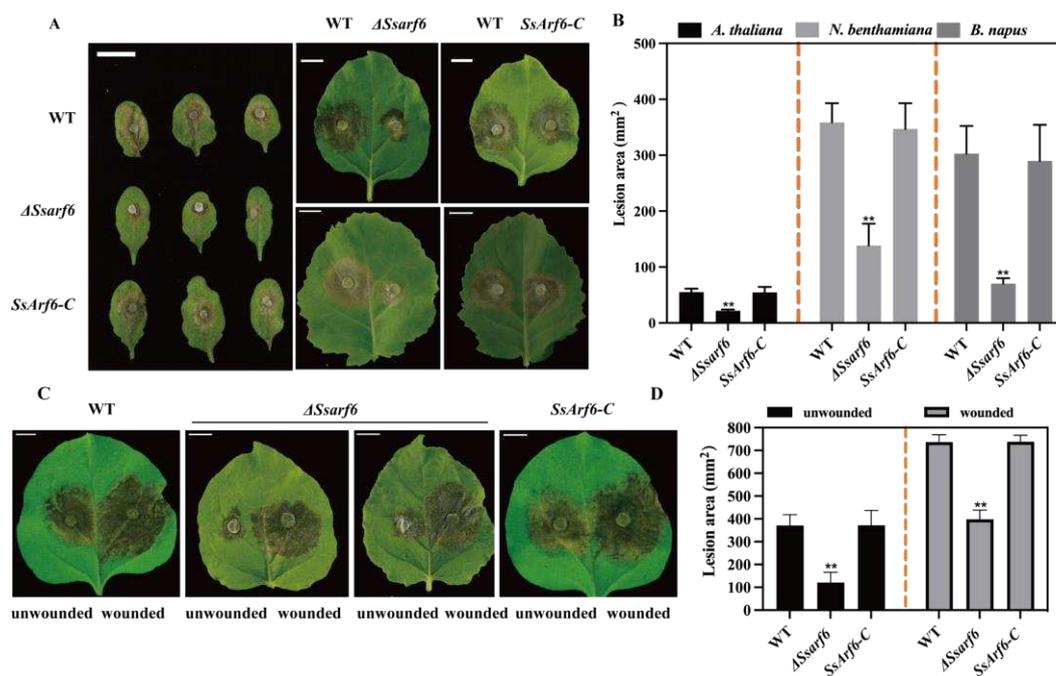
To successfully colonize, *S. sclerotiorum* undergoes specific morphological changes in its hyphal tips to form appressoria to break down the host cuticle [14]. Therefore, we analyzed the formation of compound appressoria in  $\Delta Ssarf6$  mutants, and found that the production of compound appressoria by  $\Delta Ssarf6$  mutants was significantly abnormal compared to the WT and  $SsArf6-C$  strain. Both WT and  $SsArf6-C$  strain formed compound appressoria on the slide surface as well as onion epidermal cells, while  $\Delta Ssarf6$  mutants did not (Figure 5A, B, C). Furthermore, we also assessed the secretion capacity of  $\Delta Ssarf6$  mutants for oxalic acid and revealed that the secretion of oxalic acid was normal in the  $\Delta Ssarf6$  strain, with no significant differences compared to the WT and  $SsArf6-C$  strains (Figure 5D). These results suggest that  $SsArf6$  is essential for the formation of complex appressoria and might be associated with the pathogenic ability of *S. sclerotium*.



**Figure 5.** *SsArf6* is involved in compound appressoria formation. (A) The Appressorium morphology after 48 hours on glass slides by WT,  $\Delta Ssarf6$ , and *SsArf6-C* strains (B) Appressorium formed after 24 hours on glass slides by WT,  $\Delta Ssarf6$ , and *SsArf6-C* strains observed under an optical microscope. Bar = 1  $\mu\text{m}$ . (C) Invasion assay of WT,  $\Delta Ssarf6$ , and *SsArf6-C* on onion epidermis. Invasion mycelial were stained by trypan blue. (D) Analysis of oxalic acid secretion ability of WT,  $\Delta Ssarf6$ , and *SsArf6-C* strains, with colony morphology after 48h cultivation on PDA medium containing bromophenol blue. When the pH of Bromophenol Blue is greater than or equal to 3.0 and less than 4.6, it appears yellow, and when the pH is greater than or equal to 4.6, it appears blue. WT represents the wild-type strain;  $\Delta Ssarf6$ , the knockout strain; *SsArf6-C*, the complemented strain.

### 3.5. *SsArf6* is essential for virulence to host plants

To confirm whether *SsArf6* is related to the pathogenicity of *S. sclerotiorum*, we inoculated WT,  $\Delta Ssarf6$ , and *SsArf6-C* strain on detached leaves of *A. thaliana*, *B. napus* and *N. Benthamiana*. After 36 hours of infection, the  $\Delta Ssarf6$ , WT and *SsArf6-C* strain caused water-soaked pale brown lesions on the leaves. However, the  $\Delta Ssarf6$  mutants showed a significant decrease in lesion area compared to the WT and *SsArf6-C* strains on leaf blades of different hosts (Figure 6A, B). In addition, when inoculated onto the wounded *N. Benthamiana* detached leaves, we observed a significant increase in the infection area of  $\Delta Ssarf6$  after 36 hours, compared to the intact detached leaves, under the same infection conditions, but the infection area of  $\Delta Ssarf6$  was still significantly less than that of WT and *SsArf6-C* strains (Figure 6C, D). These findings strongly indicate that *SsArf6* plays an essential role in the fungal virulence to hosts, except for its function in appressoria formation of *S. sclerotiorum*.



**Figure 6.** *SsArf6* contributes to the virulence of *S. sclerotiorum*. **(A)** Inoculated lesions of WT,  $\Delta Ssarf6$  and *Ssarf6-C* of detached leaves of *A. thaliana*, *B. napus* and *N. benthamiana*. **(B)** Lesion areas of WT,  $\Delta Ssarf6$  and *Ssarf6-C* on leaves of *A. thaliana*, *B. napus* and *N. benthamiana*. **(C)** Inoculated lesions of  $\Delta Ssarf6$  detached unwounded/wounded leaves of *N. benthamiana*. **(D)** Lesion areas of  $\Delta Ssarf6$  on wounded/unwounded leaves of *N. benthamiana*. Data were recorded at 36 h post inoculation. Bar = 10 mm. Image J was used to analyze the lesion area. The experiment was repeated three times with similar results. Error bars represent SD. The statistical significance between WT and knockout mutant strain or complemented strains was analyzed using Student's t-test (\*\* $p < 0.01$ ). WT represents the wild-type strain;  $\Delta Ssarf6$ , the knockout strain; *Ssarf6-C*, the complemented strain.

#### 4. Discussion

Arf6 is a conserved protein that plays a vital role in the development of fungi, deletion of *Arf6* in *M. oryzae* and *A. nidulans* results in slower mycelium growth, increased number of mycelial branches [38,42]. In this study, we identified a homologous protein *SsArf6* in *S. sclerotiorum*, which shares a high degree of similarity with *Arf6* proteins in other plant pathogenic fungi. Knockout of *SsArf6* led to impaired hyphal development, increased branching and melanin accumulation, excessive growth of aerial hyphae as well as negatively impacted sclerotia yield in *S. sclerotiorum*.

Fungal melanin, as a potent antioxidant, protects cells by scavenging hydrogen peroxide, hydroxyl radicals, and superoxide anions [52]. To investigate whether or not *SsArf6* mutant be tolerant to oxidative stress due to its melanin accumulation, we simulated oxidative stress by adding different concentrations of hydrogen peroxide exogenously to evaluate the antioxidant capacity of the *SsArf6* deletion mutant. As expected,  $\Delta Ssarf6$  mutants indeed exhibited significant tolerance to oxidative stress caused by hydrogen peroxide, compared to WT and *Ssarf6-C*. Additionally, we observed that  $\Delta Ssarf6$  mutants were more sensitive to hyperosmotic stress but unaffected by cell wall inhibitory agents, indicating that *SsArf6* plays an important but different roles in responding to different abiotic stress.

Appressoria and oxalic acid are essential for the interaction between *S. sclerotiorum* and its host. The formation of appressoria helps to break down the physical barriers of the host, such as the cell wall and cuticle [53], and oxalic acid is a key virulence factor in the invasion process, as its secretion enhances the activity of hydrolytic enzymes [26], induces programmed cell death in plants [54], and inhibits host defenses [27]. The absence of *SsArf6* resulted in abnormal appressoria development while it did not affect oxalic acid secretion in *S. sclerotiorum*, suggests that *SsArf6* is involved in the interaction between *S. sclerotiorum* and its host other than oxalic acid pathway.

In *M. oryzae*, *Arf6* was reported to be not essential for its pathogenicity [38]. When fungal virulence of the *SsArf6* deletion mutant was assessed, we observed significantly decreases compared to WT and *SsArf6-C* strain. Furthermore, when infection assays were performed on wounded leaves of *N. benthamiana*, the area of infection by the  $\Delta Ssarf6$  mutant significantly increased compared to unwounded leaves 36 hours post inoculation, although it still remained to be significantly lower than that of the WT and *SsArf6-C* strains under the same infection conditions. These evidences indicated an essential role of *SsArf6* in fungal virulence to hosts, except for its function in appressoria formation of *S. sclerotiorum*. Further characterization is needed regarding the specific mechanisms by which *SsArf6* regulates the formation of appressoria and pathogenicity.

Taken together, *SsArf6* is involved in mycelial growth, appressorium development, and stress response in *S. sclerotiorum*, and contributes to the infection process and fungal virulence to host plants. Our study provides evidence for understanding of the role of *Arf6* in the interaction between *S. sclerotiorum* and its hosts.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Knockout of *SsArf6* in *S. sclerotiorum*. (A) The schematic diagram of the strategy used for knockout of *SsArf6*. The *SsArf6*, *Hygromycin phosphotransferase (hph)*, and G418 resistance gene *NEO* were denoted by yellow, red, and green rectangles, respectively. The labeled primers in the diagram were used for amplifying flanking sequences of *SsArf6* or facilitating mutant screening. (B) The verification of knockout of *SsArf6* by PCR. Genomic DNA obtained from WT, knockout mutant  $\Delta Ssarf6$  were utilized as templates for PCR. A total of six primer pairs were employed to detect the insertion of *hph* and its upstream and down-stream fragments, as well as to confirm the knockout of *SsArf6*. The sizes of the amplified bands were indicated within brackets. The lanes labeled as M represent the DNA marker. Figure S2: Complementation of *SsArf6* in  $\Delta Ssarf6$  mutants of *S. sclerotiorum*. (A) The schematic diagram of the strategy used for the complementation of  $\Delta Ssarf6$ . The *SsArf6*, *Hygromycin phosphotransferase (hph)*, and G418 resistance gene *NEO* were denoted by yellow, red, and green rectangles, respectively. The labeled primers in the diagram were used for amplifying full-length genomic DNA of *SsArf6* or facilitating transformant screening. (B) The verification of complementation of *SsArf6* by PCR. Genomic DNA obtained from the deletion mutant  $\Delta Ssarf6$ , and complemented strain *SsArf6-C* were utilized as templates for PCR. A total of six primer pairs were employed to confirm the complementation of *SsArf6*. The sizes of the amplified bands were indicated within brackets. The lanes labeled as M represent the DNA marker. Figure S3: The semi-quantitative RT-PCR analysis of the mutant strains  $\Delta Ssarf6$  and *SsArf6-C*. Table S1: Primers in this study.

**Author Contributions:** K.W. and S.X. planned and designed the research. K.W. carried out most of the experiments. K.W., S.W. and S.X. wrote the original draft of the manuscript. S.W. and Q. X. contributed to the mutagenized population of *S. sclerotiorum*. T.W. analyzed and validated the data. All authors contributed to and approved the submitted version. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The GenBank accession numbers (species names) for organisms used in this study are as follows: *Zymoseptoria tritici* (*ZtArf6*, XP\_003857013.1), *Sclerotinia sclerotiorum* (*SsArf6*, APA07463.1), *Ustilago maydis* (*UmArf6*, XP\_011391884.1), *Botrytis cinerea* (*BcArf6*, XP\_001547581.1), *Magnaporthe oryzae* (*MoArf6*, XP\_003715902.1), *Fusarium oxysporum* (*FoArf6*, XP\_018253040.1), *Fusarium graminearum* (*FgArf6*, XP\_011321112.1), were obtained from the NCBI database (<https://www.ncbi.nlm.nih.gov/>). The *Arf6* gff3 files were got from the ensemblFungi database (<https://fungi.ensembl.org/>).

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**Conflicts of Interest:** All authors declare that there is no conflict of interest.

## References

1. Xia, S.; Xu, Y.; Hoy, R.; Zhang, J.; Qin, L.; Li, X. The notorious soilborne pathogenic fungus *Sclerotinia sclerotiorum*: An update on genes studied with mutant analysis. *Pathogens* **2019**, *9*, 27, doi:10.3390/pathogens9010027.
2. Boland, G.J.; Hall, R. Index of plant hosts of *Sclerotinia sclerotiorum*. *Can. J. Plant Pathol.* **1994**, *16*, 93-108, doi:10.1080/07060669409500766.

3. Ding, L.N.; Li, T.; Guo, X.J.; Li, M.; Liu, X.Y.; Cao, J.; Tan, X.L. Sclerotinia stem rot resistance in rapeseed: Recent progress and future prospects. *J. Agric. Food Chem.* **2021**, *69*, 2965-2978, doi:10.1021/acs.jafc.0c07351.
4. Fass, M.I.; Rivarola, M.; Ehrenbolger, G.F.; Maringolo, C.A.; Montecchia, J.F.; Quiroz, F.; García-García, F.; Blázquez, J.D.; Hopp, H.E.; Heinz, R.A.; et al. Exploring sunflower responses to *Sclerotinia* head rot at early stages of infection using RNA-seq analysis. *Sci. Rep.* **2020**, *10*, 13347, doi:10.1038/s41598-020-70315-4.
5. Hoffman, D.D.; Hartman, G.L.; Mueller, D.S.; Leitz, R.A.; Nickell, C.D.; Pedersen, W.L. Yield and seed quality of soybean cultivars infected with *Sclerotinia sclerotiorum*. *Plant Dis.* **1998**, *82*, 826-829, doi:10.1094/PDIS.1998.82.7.826.
6. Hu, Q.; Hua, W.; Yin, Y.; Zhang, X.; Liu, L.; Shi, J.; Zhao, Y.; Qin, L.; Chen, C.; Wang, H. Rapeseed research and production in China. *CROP. J* **2017**, *5*, 127-135, doi:https://doi.org/10.1016/j.cj.2016.06.005.
7. Zhang, X.; Cheng, J.; Lin, Y.; Fu, Y.; Xie, J.; Li, B.; Bian, X.; Feng, Y.; Liang, W.; Tang, Q.; et al. Editing homologous copies of an essential gene affords crop resistance against two cosmopolitan necrotrophic pathogens. *Plant Biotechnol. J.* **2021**, *19*, 2349-2361, doi:10.1111/pbi.13667.
8. Yang, G.; Tang, L.; Gong, Y.; Xie, J.; Fu, Y.; Jiang, D.; Li, G.; Collinge, D.B.; Chen, W.; Cheng, J. A ceratoplatanin protein SsCP1 targets plant PR1 and contributes to virulence of *Sclerotinia sclerotiorum*. *New Phytol.* **2018**, *217*, 739-755, doi:10.1111/nph.14842.
9. Harper, G.E.; Frampton, C.M.; Stewart, A. Factors influencing survival of sclerotia of *Sclerotium cepivorum* in New Zealand soils. *N. Z. J. Crop Hort. Sci* **2002**, *30*, 29-35, doi:10.1080/01140671.2002.9514196.
10. Willetts, H.J.; Wong, J.A.L. The biology of *Sclerotinia sclerotiorum*, *S. trifoliorum*, and *S. minor* with emphasis on specific nomenclature. *Bot. Rev.* **1980**, *46*, 101-165, doi:10.1007/BF02860868.
11. Zhang, J.; Xiao, K.; Li, M.; Hu, H.; Zhang, X.; Liu, J.; Pan, H.; Zhang, Y. SsAGM1-mediated uridine diphosphate-N-acetylglucosamine synthesis is essential for development, stress response, and pathogenicity of *Sclerotinia sclerotiorum*. *Front. Microbiol.* **2022**, *13*, 938784, doi:10.3389/fmicb.2022.938784.
12. Huang, L.; Buchenauer, H.; Han, Q.; Zhang, X.; Kang, Z. Ultrastructural and cytochemical studies on the infection process of *Sclerotinia sclerotiorum* in oilseed rape. *J. Plant Dis. Prot.* **2008**, *115*, 9-16, doi:10.1007/BF03356233.
13. Uloth, M.B.; Clode, P.L.; You, M.P.; Barbetti, M.J. Attack modes and defence reactions in pathosystems involving *Sclerotinia sclerotiorum*, *Brassica carinata*, *B. juncea* and *B. napus*. *Ann. Bot.* **2015**, *117*, 79-95, doi:10.1093/aob/mcv150.
14. Liang, X.; Rollins, J.A. Mechanisms of broad host range necrotrophic pathogenesis in *Sclerotinia sclerotiorum*. *Phytopathology.* **2018**, *108*, 1128-1140, doi:10.1094/PHYTO-06-18-0197-RVW.
15. Xu, T.; Li, J.; Yu, B.; Liu, L.; Zhang, X.; Liu, J.; Pan, H.; Zhang, Y. Transcription factor SsSte12 was involved in mycelium growth and development in *Sclerotinia sclerotiorum*. *Front. Microbiol.* **2018**, *9*, 2476, doi:10.3389/fmicb.2018.02476.
16. Cong, J.; Xiao, K.; Jiao, W.; Zhang, C.; Zhang, X.; Liu, J.; Zhang, Y.; Pan, H. The coupling between cell wall integrity mediated by MAPK kinases and SsFkh1 is involved in sclerotia formation and pathogenicity of *Sclerotinia sclerotiorum*. *Front. Microbiol.* **2022**, *13*, 816091, doi:10.3389/fmicb.2022.816091.
17. Zhang, H.; Li, Y.; Lai, W.; Huang, K.; Li, Y.; Wang, Z.; Chen, X.; Wang, A. SsATG8 and SsNBR1 mediated-autophagy is required for fungal development, proteasomal stress response and virulence in *Sclerotinia sclerotiorum*. *Fungal Genet. Biol.* **2021**, *157*, 103632, doi:10.1016/j.fgb.2021.103632.
18. Jiao, W.; Yu, H.; Cong, J.; Xiao, K.; Zhang, X.; Liu, J.; Zhang, Y.; Pan, H. Transcription factor SsFoxE3 activating SsAtg8 is critical for sclerotia, compound appressoria formation, and pathogenicity in *Sclerotinia sclerotiorum*. *Mol. Plant Pathol.* **2022**, *23*, 204-217, doi:10.1111/mpp.13154.
19. Jiao, W.; Yu, H.; Chen, X.; Xiao, K.; Jia, D.; Wang, F.; Zhang, Y.; Pan, H. The SsAtg1 activating autophagy is required for sclerotia formation and pathogenicity in *Sclerotinia sclerotiorum*. *J. Fungi* **2022**, *8*, 1314, doi.org/10.3390/jof8121314
20. Jiao, W.; Ding, W.; Rollins, J.A.; Liu, J.; Zhang, Y.; Zhang, X.; Pan, H. Cross-talk and multiple control of Target of Rapamycin (TOR) in *Sclerotinia sclerotiorum*. *Microbiol. Spectrum* **2023**, *11*, e0001323, doi:10.1128/spectrum.00013-23.
21. Yu, Y.; Xiao, J.; Zhu, W.; Yang, Y.; Mei, J.; Bi, C.; Qian, W.; Qing, L.; Tan, W. Ss-Rhs1, a secretory Rhs repeat-containing protein, is required for the virulence of *Sclerotinia sclerotiorum*. *Mol. Plant Pathol.* **2017**, *18*, 1052-1061, doi:10.1111/mpp.12459.

22. Liu, L.; Wang, Q.; Zhang, X.; Liu, J.; Zhang, Y.; Pan, H. *Ssams2*, a gene encoding GATA transcription factor, is required for appressoria formation and chromosome segregation in *Sclerotinia sclerotiorum*. *Front. Microbiol.* **2018**, *9*, 3031, doi:10.3389/fmicb.2018.03031.
23. Li, J.; Zhang, X.; Li, L.; Liu, J.; Zhang, Y.; Pan, H. Proteomics analysis of SsNsd1-mediated compound appressoria formation in *Sclerotinia sclerotiorum*. *Int. J. Mol. Sci.* **2018**, *19*, 2946., doi:10.3390/ijms19102946.
24. Ding, Y.; Chen, Y.; Yan, B.; Liao, H.; Dong, M.; Meng, X.; Wan, H.; Qian, W. Host-induced gene silencing of a multifunction gene *Sscnd1* enhances plant resistance against *Sclerotinia sclerotiorum*. *Front. Microbiol.* **2021**, *12*, 693334, doi:10.3389/fmicb.2021.693334.
25. Kabbage, M.; Yarden, O.; Dickman, M.B. Pathogenic attributes of *Sclerotinia sclerotiorum*: switching from a biotrophic to necrotrophic lifestyle. *Plant Sci.* **2015**, *233*, 53-60, doi:10.1016/j.plantsci.2014.12.018.
26. Monazzah, M.; Rabiei, Z.; Enferadi, S.T. The effect of oxalic acid, the pathogenicity factor of *Sclerotinia Sclerotiorum* on the two susceptible and moderately resistant lines of sunflower. *Iran. J. Biotechnol.* **2018**, *16*, e1832, doi:10.21859/ijb.1832.
27. Williams, B.; Kabbage, M.; Kim, H.J.; Britt, R.; Dickman, M.B. Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. *PLoS Pathog.* **2011**, *7*, e1002107, doi:10.1371/journal.ppat.1002107.
28. Wei, W.; Pierre-Pierre, N.; Peng, H.; Ellur, V.; Vandemark, G.J.; Chen, W. The D-galacturonic acid catabolic pathway genes differentially regulate virulence and salinity response in *Sclerotinia sclerotiorum*. *Fungal Genet. Biol.* **2020**, *145*, 103482, doi:10.1016/j.fgb.2020.103482.
29. Yang, B.; Yajima, W.; Das, D.; Suresh, M.R.; Kav, N.N. Isolation, expression and characterization of two single-chain variable fragment antibodies against an endo-polygalacturonase secreted by *Sclerotinia sclerotiorum*. *Protein Expr Purif.* **2009**, *64*, 237-243, doi:10.1016/j.pep.2008.12.007.
30. Fan, H.; Yang, W.; Nie, J.; Zhang, W.; Wu, J.; Wu, D.; Wang, Y. A novel effector protein SsERP1 inhibits plant ethylene signaling to promote *Sclerotinia sclerotiorum* infection. *J. Fungi* **2021**, *7*, doi:10.3390/jof7100825.
31. Seifbarghi, S.; Borhan, M.H.; Wei, Y.; Ma, L.; Coutu, C.; Bekkaoui, D.; Hegedus, D.D. Receptor-like kinases BAK1 and SOBIR1 are required for necrotizing activity of a novel group of *Sclerotinia sclerotiorum* necrosis-inducing effectors. *Front. Plant Sci.* **2020**, *11*, 1021, doi:10.3389/fpls.2020.01021.
32. Hossain, M.M.; Sultana, F.; Li, W.; Tran, L.P.; Mostofa, M.G. *Sclerotinia sclerotiorum* (Lib.) de Bary: Insights into the pathogenomic features of a global pathogen. *Cells* **2023**, *12*, doi:10.3390/cells12071063.
33. Seifbarghi, S.; Borhan, M.H.; Wei, Y.; Coutu, C.; Robinson, S.J.; Hegedus, D.D. Changes in the *Sclerotinia sclerotiorum* transcriptome during infection of *Brassica napus*. *BMC Genomics* **2017**, *18*, 266, doi:10.1186/s12864-017-3642-5.
34. Yang, G.; Tang, L.; Gong, Y.; Xie, J.; Fu, Y.; Jiang, D.; Li, G.; Collinge, D.B.; Chen, W.; Cheng, J. A ceratoplatanin protein SsCP1 targets plant PR1 and contributes to virulence of *Sclerotinia sclerotiorum*. *New Phytol.* **2018**, *217*, 739-755, doi:10.1111/nph.14842.
35. Zhou, F.; Zhang, X.L.; Li, J.L.; Zhu, F.X. Dimethachlon Resistance in *Sclerotinia sclerotiorum* in China. *Plant Dis.* **2014**, *98*, 1221-1226, doi:10.1094/pdis-10-13-1072-re.
36. Wang, Y.; Hou, Y.-P.; Chen, C.-J.; Zhou, M.-G. Detection of resistance in *Sclerotinia sclerotiorum* to carbendazim and dimethachlon in Jiangsu Province of China. *Australas. Plant Pathol.* **2014**, *43*, 307-312, doi:10.1007/s13313-014-0271-1.
37. Donaldson, J.G. Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane. *J. Biol. Chem.* **2003**, *278*, 41573-41576, doi:10.1074/jbc.R300026200.
38. Zhu, X.; Zhou, T.; Chen, L.; Zheng, S.; Chen, S.; Zhang, D.; Li, G.; Wang, Z. Arf6 controls endocytosis and polarity during asexual development of *Magnaporthe oryzae*. *FEMS Microbiol. Lett.* **2016**, *363*, doi:10.1093/femsle/fnw248.
39. Gamara, J.; Davis, L.; Leong, A.Z.; Page, N.; Rollet-Labelle, E.; Zhao, C.; Hongu, T.; Funakoshi, Y.; Kanaho, Y.; Aoudji, F.; et al. Arf6 regulates energy metabolism in neutrophils. *Free Radical Biol. Med.* **2021**, *172*, 550-561, doi:10.1016/j.freeradbiomed.2021.07.001.
40. Li, R.; Peng, C.; Zhang, X.; Wu, Y.; Pan, S.; Xiao, Y. Roles of Arf6 in cancer cell invasion, metastasis and proliferation. *Life Sci* **2017**, *182*, 80-84, doi:10.1016/j.lfs.2017.06.008.
41. Schweitzer, J.K.; Sedgwick, A.E.; D'Souza-Schorey, C. ARF6-mediated endocytic recycling impacts cell movement, cell division and lipid homeostasis. *Semin. Cell Dev. Biol.* **2011**, *22*, 39-47, doi:10.1016/j.semcdb.2010.09.002.

42. Lee, S.C.; Schmidtke, S.N.; Dangott, L.J.; Shaw, B.D. *Aspergillus nidulans* ArfB plays a role in endocytosis and polarized growth. *Eukaryot Cell*. **2008**, *7*, 1278-1288, doi:10.1128/EC.00039-08.
43. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022-3027, doi:10.1093/molbev/msab120.
44. Hu, B.; Jin, J.; Guo, A.Y.; Zhang, H.; Luo, J.; Gao, G. GSDS 2.0: an upgraded gene feature visualization server. *Bioinformatics* **2015**, *31*, 1296-1297, doi:10.1093/bioinformatics/btu817.
45. Madeira, F.; Pearce, M.; Tivey, A.R.N.; Basutkar, P.; Lee, J.; Edbali, O.; Madhusoodanan, N.; Kolesnikov, A.; Lopez, R. Search and sequence analysis tools services from EMBL-EBI in 2022. *Nucleic Acids Res.* **2022**, *50*, W276-W279, doi:10.1093/nar/gkac240.
46. Waterhouse, A.M.; Procter, J.B.; Martin, D.M.; Clamp, M.; Barton, G.J. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **2009**, *25*, 1189-1191, doi:10.1093/bioinformatics/btp033.
47. Goswami, R.S. Targeted gene replacement in fungi using a split-marker approach. *Methods Mol. Biol.* **2012**, *835*, 255-269, doi:10.1007/978-1-61779-501-5\_16.
48. Xu, Y.; Ao, K.; Tian, L.; Qiu, Y.; Huang, X.; Liu, X.; Hoy, R.; Zhang, Y.; Rashid, K.Y.; Xia, S.; et al. A forward genetic screen in *Sclerotinia sclerotiorum* revealed the transcriptional regulation of its sclerotial melanization pathway. *Mol. Plant-Microbe Interact.* **2022**, *35*, 244-256, doi:10.1094/MPMI-10-21-0254-R.
49. Yang, C.; Tang, L.; Qin, L.; Zhong, W.; Tang, X.; Gong, X.; Xie, W.; Li, Y.; Xia, S. mRNA turnover protein 4 is vital for fungal pathogenicity and response to oxidative stress in *Sclerotinia sclerotiorum*. *Pathogens* **2023**, *12*, 281, doi:10.3390/pathogens12020281.
50. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. **2012**, *9*, 671-675, doi:10.1038/nmeth.2089.
51. Smith, D.F.Q.; Casadevall, A. The role of melanin in fungal pathogenesis for animal hosts. *Curr. Top. Microbiol. Immunol.* **2019**, *422*, 1-30, doi:10.1007/82\_2019\_173.
52. Liu, R.; Meng, X.; Mo, C.; Wei, X.; Ma, A. Melanin of fungi: from classification to application. *World J. Microbiol. Biotechnol.* **2022**, *38*, 228, doi:10.1007/s11274-022-03415-0.
53. Gong, Y.; Fu, Y.; Xie, J.; Li, B.; Chen, T.; Lin, Y.; Chen, W.; Jiang, D.; Cheng, J. *Sclerotinia sclerotiorum* SsCut1 modulates virulence and cutinase activity. *J Fungi* **2022**, *8*, 526, doi:10.3390/jof8050526.
54. Kim, K.S.; Min, J.Y.; Dickman, M.B. Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Mol. Plant-Microbe Interact.* **2008**, *21*, 605-612, doi:10.1094/mpmi-21-5-0605.

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