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The cAMP-dependent protein kinase A pathway perturbs autophagy and plays important roles in development and virulence of *Sclerotinia sclerotiorum*

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ABSTRACT

Previous research has demonstrated that sclerotia production is suppressed by exogenous cyclic AMP (cAMP) in *Sclerotinia sclerotiorum* and enhanced upon deletion of the adenylate cyclase gene. This study focuses on further functionally characterizing the cAMP-dependent protein kinase A (PKA) signaling pathway in *S. sclerotiorum*. Here, we demonstrate functions for two components of cAMP signaling: the catalytic, SsPKA, and the regulatory, SsPKAR, subunits of cAMP-dependent PKA. Growth and virulence were greatly reduced by disruption of either *Sspka2* or *SspkaR* in addition to deficiencies in appressorium development. Surprisingly, disruption of both *Sspka2* (*dSspka2*) and *SspkaR* (*dSspkaR*) display an up-regulation of autophagy without nutrient starvation suggesting that properly regulated PKA activity is required for control of autophagy. SsPKAR is demonstrated to be required for carbohydrate metabolism and mobilization, which are required for appressorium development and sclerotium initiation. A closer examination of *dSspkaR* during *Nicotiana benthamiana* infection revealed that an oxalic acid (OA)-independent necrosis protein(s) or metabolite(s) may be involved in the lesion development in *dSspkaR-N. benthamiana* interaction. In summary, these data demonstrate that the cAMP-dependent PKA signaling is essential for multiple forms of *S. sclerotiorum* development as well as virulence which rely on optimal regulation of autophagy.

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a devastating soil-borne necrotrophic pathogen in the fungal phylum Ascomycota. It causes diseases on a broad range of hosts from over 60 plant families including economically important crops such as vegetables, legumes and oilseeds (Boland and Hall, 1994). *S. sclerotiorum* lacks the ability to produce asexual spores; therefore, the sclerotium, a mass of compact melanized hyphae, plays an important role as the provider of primary inoculum and long-term survival (Purdy, 1979). Initiation of sclerotium development can be triggered by physical and chemical factors including contact with physical barriers or nutrient deprivation which disrupt hyphal polar elongation (Willetts and Wong, 1980). Chemical screening has revealed that supplying exogenous cAMP or pharmacologically increasing cellular cAMP levels inhibit the initiation of sclerotial development and stimulate oxalic acid (OA) accumulation significantly (Rollins and Dickman, 1998).

S. sclerotiorum mostly penetrates its host directly through the cuticle layer rather than through natural openings. This direct penetration is dependent on the compound appressorium, a multicellular, cushion-shaped, branched structure arising from vegetative hyphae that functions in adhesion and penetration of the host cuticle (Li et al., 2012). Appressorium complexity is correlated with the nature of the host tissue such as physical resistance to penetration (Tariq and Jeffries, 1984). Fungal mutants that fail to differentiate compound appressoria lose the ability to penetrate the host cuticle layer (Bashi et al., 2016; Jurick II and Rollins, 2007; Li et al., 2012; Liang et al., 2015b; Xiao et al., 2014; Yu et al., 2017)

Development depends on the ability to perceive and respond to external and internal environments and eukaryotes employ various signaling pathways to coordinate these stimuli. cAMP signaling, and mitogen-activated protein (MAP) kinases pathways are the two of most studied signaling transduction pathways in fungi. In the yeast, *Saccharomyces cerevisiae*, the cAMP-dependent protein

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kinase A (PKA) pathway is one of the major signaling pathways responding to the availability of glucose. Glucose sensing and signaling pathways of filamentous fungi consist of G-protein coupled receptors and heterotrimeric G proteins. The Gα subunit is required for glucose sensing, cAMP cascade, and conidia germination (Doehlemann et al., 2006; Lafon et al., 2005; Schumacher et al., 2008). cAMP production is stimulated in response to glucose, and the enzyme adenylate cyclase (AC) is the key membrane bound enzyme catalyzing the conversion of adenosine triphosphate (ATP) to cAMP (Tamaki, 2007; Mitts et al., 1990). PKA is the main intracellular target of cAMP and, in its inactive form, exists as a tetramer consisting of two catalytic and two regulatory subunits. Binding of cAMP by the regulatory subunits reduces their inhibitory activity through the release of the catalytic subunits (Kim et al., 2005).

Although the cAMP-dependent PKA signaling is well conserved among yeast and filamentous fungi, it regulates species-specific downstream processes (D'Souza and Heitman, 2001). These include roles in vegetative growth, morphological transition, mating, virulence, pathogenicity, appressorial development, conidiation, sexual development, toxin production, pigmentation, and stress responses (Adachi and Hamer, 1998; Caza and Kronstad, 2019; Choi and Xu, 2010; Durrenberger et al., 1998; Gold et al., 1994, 1997; Hu et al., 2014; H.-S. Kim et al., 2011; C. Li et al., 2018; Mitchell and Dean, 1995; Park et al., 2016; Schumacher et al., 2008; Takano et al., 2001; Tsai et al., 2013; Tzima et al., 2010; Xu et al., 2011, 1997; Yamauchi et al., 2004; Yang and Dickman, 1999; Zhu et al., 2017). In *S. sclerotiorum*, components of cAMP-dependent PKA signaling have been studied for roles in fungal growth and development as well as pathogenesis. Deletion of *sac1*, encoding AC in *S. sclerotiorum*, revealed that cAMP is required for hyphal growth, sclerotial development, compound appressorium formation, and pathogenicity (Jurick and Rollins, 2007). Two paralogs of the PKA catalytic subunit, SsPKA1 and SsPKA2, are encoded by *S. sclerotiorum* akin to many other filamentous ascomycete fungi. Deletion of *Sspka1* did not affect PKA enzyme activity, growth, development, or pathogenicity (Jurick et al., 2004). Additionally, phylogenetic analysis of PKA catalytic subunit coding genes from other plant pathogenic ascomycetes revealed that SsPKA1 clusters phylogenetically with catalytic subunits that exhibit minor roles in PKA-dependent processes, while SsPKA2 clusters with catalytic subunits that have major roles in regulating PKA-dependent activities (Jurick et al., 2004; Schumacher et al., 2008). This evidence suggests that SsPKA2 might be the primary regulator of cAMP-dependent PKA activities in *S. sclerotiorum*.

Autophagy in *S. cerevisiae* is control by both cAMP-dependent PKA signaling and the target of rapamycin (Tor) pathway cooperatively (Stephan et al., 2009, 2010). Autophagy is a ubiquitous cellular process for recycling amino acids and other macromolecular precursors within eukaryotic cells. Autophagy has three forms: starvation-induced macroautophagy, cytoplasm to vacuole targeting (Cvt) autophagy, and microautophagy (Liu et al., 2016; Tanida, 2011; Thorburn, 2008). In the presence of glucose-activated PKA inhibits the association of pre-autophagosomal structure and the ATG1 complex, which leads to inhibition of autophagy (Stephan et al., 2009). Defects in autophagy regulation have been observed with a null mutant of the regulatory subunit of PKA (*PKR*) in *Fusarium graminearum*. Deletion of *PKR* caused over-induction of autophagy in the macroconidia resulting in a low germination rate (C. Li et al., 2018).

Although pharmacological studies demonstrated that cAMP signaling regulates sclerotial development and OA accumulation in *S. sclerotiorum*, the functional roles of PKA have not been studied completely. This study aims to further characterize the cAMP-dependent PKA signaling pathway in *S. sclerotiorum*. To study the functions of the catalytic subunit and regulatory subunits of PKA in vegetative growth, sexual development, and pathogenicity, we disrupted the genes encoding these proteins, *Sspka2* and *SspkaR*, respectively and tested the hypothesis that the cAMP-dependent PKA signaling pathway controls carbon and nitrogen starvation-induced autophagy.

2. Material and methods

2.1. Fungal strains, media, and culture conditions

The wild-type *S. sclerotiorum* isolate UF1, isolated from infected petunia in Florida, USA, was used to generate mutants in this study. All strains were routinely cultured in PDA (Difco) at room temperature. To induce in vitro compound appressoria development, fungal strains were inoculated on PDA media overlaid with cellophane (Bio-Rad, Hercules, CA, USA). Liquid culture conditions were modified from the procedure described previously (Rollins and Dickman, 2001). Two hundred and fifty ml of YPSu media (4 g/l of yeast extract (Difco), 15 g/l of sucrose, 1 g/l of K_2HPO_4, and 0.5 g/l of $MgSO_4$, pH 6.5) in a 500-ml flask was inoculated with 10–20 50mm^3 agar-mycelium plugs collected from the leading edge of fungal colonies on PDA. The liquid culture was incubated at room temperature, on an orbital shaker at 150 rpm for 3 days. Fungal hyphae were collected on filter paper by vacuum filtration and washed twice with sterile deionized water (dH_2O). Five hundred grams of hyphae were suspended in 50 ml buffered YPGlu (4 g/l of yeast extract (Difco), 15 g/l of glucose (Fisher Scientific, Waltham, MA, U.S.A), 1 g/l of K_2HPO_4, and 0.5 g/l of $MgSO_4$, and 0.5 M 3-(N-morpholino) propanesulfonic acid (MOPS) buffer, pH 7.0) in a 125-ml flask and then incubated for an additional 24 h while shaking at 150 rpm. Total hyphae were collected, washed with sterile dH_2O, vacuum dried and then stored at −80 °C until required for protein extraction.

2.2. Targeted gene disruption and genetic complementation

All DNA oligomers used in this study are listed in Table 1. Two genes, *Sspka2* and *SspkaR* (GenBank: XP_001585338.1 and XM_001588040.1, respectively), were disrupted with the modified vector, pCRISPR-Cas-U6-Hyg carrying the hygromycin B resistance gene, derived from CRISPR-Cas9-TrpC-Hyg (J. Li et al., 2018). The *Sspka2* single guide RNA (sgRNA) sequence consisted of the 20 nucleotides (5′- AAACGGACGAAGGAGTCTCC-3′) directly upstream of the protospacer adjacent motif (PAM: GGG). The *SspkaR* sgRNA sequence, 5′- GTAGCGACGGAAGTGGCATG-3′, is directly upstream of the PAM site: GGG. Since the transcription initiation of the U6 promoter prefers purine, the sequences of these sgRNA sequences start with A or G (Ma et al., 2014). For restriction enzyme cloning, compatible overhang sequences, GATT and AAAC, were added to the 5′ end of sgRNA sense strand and antisense strand, respectively. To construct the CRISPR-Cas9 vector, annealed nucleotides were inserted into *Aar*I-digested and dephosphorylated pCRISPR-Cas-U6-Hyg as previously described (Arazoe et al., 2015). Constructs were sequenced and verified using the forward sense primer specific to the appropriate sgRNA and the U6_test_FP2 primer.

For complementation of the *Sspka2*-disruption mutant, a modified *Sspka2* sequence consisting of the endogenous promoter obtained from the 1 kb sequence directly upstream of the *Sspka2* translation start site, a modified coding region with silent mutations at the sgRNA target site, and a 0.3-kb terminator obtained from the nucleotide sequences directly downstream of the *Sspka2* translation stop site. This modified *SsPka2* sequence was synthesized and inserted into the pUC57-Mini vector by GeneScript Biotech (Piscataway, NJ, U.S.A.) resulting in vector pUC-Mini-*Sspka2*

Table 1
Primers used in this study.

Primer name	Sequence	Description
Sspka2_sgRNA_F2	GATTGTGTGGGGGTTATCGGACTTG	Sense strand of sgRNA targets *Sspka2*
Sspka2_sgRNA_R2	AAACCAAGTCCGATAACCCCCACA	Antisense strand of sgRNA targets *Sspka2*
SspkaR_sgRNA_F1	GATTGTAGCGACGGAAGTGGCATG	Sense strand of sgRNA targets *SspkaR*
SsspkaR_sgRNA_R1	AAACCATGCCACTTCCGTCGCTAC	Antisense strand of sgRNA targets *SspkaR*
U6_test_FP2	CGCCCACAAGAGCAGAAACACCTACC	Reverse primer for CRISPR-Cas9 expression vector verification
Sspka2_F1	TCCTACCTTCTCTTCCGGGTA	Forward primer for verifying CRISPR-Cas9 expression vector integration event
Sspka2_R2	TGGGACCTTCTTGGCGAATC	Reverse primer for verifying CRISPR-Cas9 expression vector integration event
SspkaR_F1	GCAGGGTTCCTGACATCTCG	Forward primer for verifying CRISPR-Cas9 expression vector integration event
SspkaR_R1	CCGATATTACCGTGGCTGCT	Reverse primer for verifying CRISPR-Cas9 expression vector integration event
PKA2-7-seq-R1	CGAAAAGATGGGCGTAGGTT	Sequencing insertion event of *dSspka2-7* mutant
PKA2-7-seq-F2	ACCATCGGCGCAGCTATTTA	Sequencing insertion event of *dSspka2-7* mutant
PKA2-7-F2	ATTGAAGAAGGCTCAGGTGC	Sequencing insertion event of *dSspka2-7* mutant
PKA2-7-Chr1-F1	GGCAATCGCTACCACATCCA	Sequencing insertion event of *dSspka2-7* mutant
PKA2-7-Cas9-R1	GAAGTCCTCCTGTCGACGGA	Sequencing insertion event of *dSspka2-7* mutant
Hyg_ORF_5′	ATGCAGCTCTCGGAGGGCGA	Forward primer for amplifying coding sequence of hygromycin resistance gene (HygR)
Hyg_3_1	CTATTCCTTTGCCCTCGGACGAGTGCT	Reverse primer for amplifying coding sequence of hygromycin resistance gene (HygR)

(Fig. S3). The modified *Sspka2* sequence was excised from the pUC57-Mini-*Sspka2* with restriction enzymes *Sal*I and *Sac*I. The gene silencing vector pSilent-Dual1 (Nguyen et al., 2008) was digested by *Sal*I and *Sac*I to remove the dual promoters. The insert and backbone vector were gel purified through E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek Inc, Norcross, GA, U.S.A.), and then the modified *Sspka2* sequence was cloned into the linearized pSilent-Dual1 plasmid harboring the Neomycin/G418 selection marker. Plasmid DNA was purified using Wizard® *Plus* Minipreps DNA Purification System (Promega, Madison, WI, U.S.A).

For fungal transformation, 6 μg of plasmid DNA was transformed into protoplasts prepared from the wild type UF1 isolate (WT) or *dSspka2-7* mutant through PEG-mediated protoplast transformation as described previously, and the transformants were selected with 100 ppm hygromycin or G418 (geneticin) according to standard procedures (Rollins, 2003).

2.3. Evaluation of transforming DNA integration events

Fungal genomic DNA was purified as previously described (Saghai-Maroof et al., 1984) and used as a template for PCR. LongAmp® Taq DNA Polymerase (New England Biolabs, lpswich, MA, U.S.A.) was used for standard PCR reactions with the following program: 94 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 65 °C for 8 min. To verify the integration events at the *Sspka2 and SspkaR* loci, Sspka2_F1 and Sspka2_R2 primers were used to amplify across the *Sspka2* sgRNA target site. For the *SspkaR* locus, SspkaR_F1 and SspkaR_R1 were used. The insertion at *dSspka2-7* was sequenced by the primer walking method. Primers are listed in Table 1.

2.4. Protein extraction and quantification

To extract total protein, 0.1 g of lyophilized, ground fungal mycelia was suspended in 1-ml protein extraction buffer (10 mM Tris−HCl (Fisher Scientific), 1 mM ethylenediaminetetraacetic acid (EDTA, Fisher Scientific), 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Fisher Scientific), pH 7.5), and incubated on ice for 30 min. Samples were centrifuged for 15 min at 12000 rmp, 4 °C, and the supernatant was transferred to a 1.5 ml eppendorf tube. Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, USA) was used for measuring protein concentration according to the user guide.

2.5. Apothecia development assay

Apothecia induction was conducted as described previously with some modifications (Li and Rollins, 2010). Mature sclerotia were collected from the fungal culture grown on PDA at room temperature. Sclerotia were surface sterilized with 0.5% bleach for 5 min followed by a rinse with sterile deionized water and the process was repeated three times. Sterilized sclerotia were then washed in 90% ethanol for 5 min, and then dried on sterile paper towels in a laminar flow hood overnight. Fifty sclerotia were placed in a 10 cm diameter glass petri dish containing sterile, water-saturated vermiculite. The plate was incubated in a growth chamber at 15 °C with constant fluorescent cool white lighting.

2.6. Pathogenicity assays

Fava bean (*Vicia faba* cv. Windsor), soybean (*Glycine max* DP 2330 RR), and tomato (*Solanum lycopersicum* cv. Better Boy) were grown in the greenhouse. *Nicotiana benthamiana* plants were grown in a growth chamber at 20 °C with a 12 h day and 12 h night cycle. Mature and fully expanded leaves were collected from the plants and inoculated with 5-mm mycelia-agar plugs. For wounded inoculations, each detached leaf was cut with a scalpel to make a 5-mm cut through the leaf, and the mycelial plug was placed directly on the wound. Infected leaves were incubated in a moist chamber, and lesion sizes were measured at 48 or 72 h post inoculation. Inoculation assays were repeated independently three times.

2.7. In planta oxalate accumulation assay

To quantify OA concentration within lesions of infected *N. benthamiana* leaves, samples were collected with a 5 mm diameter cork borer, lyophilized, homogenized, and resuspended in 50 or 100 μl sample dilution buffer. Mixtures were centrifuged at 13200 rpm for 1 min. OA in the supernatants were quantified via an enzymatic reaction using a commercial oxalate kit (Trinity Biotech, Wicklow, Ireland) according to the manufacturer's instructions. The absorbance of the mixture at 590 nm was read by iMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, CA, U.S.A.). To determine the detection limit of the OA assay, an OA stock solution (624.8 μM) was diluted by 2-fold serial dilutions and used to generate a standard curve. The concentration of OA was normalized with the lesion area collected from leaves inoculated with different fungal strains at 5 days post inoculation. To measure OA level beneath the cellophane treated with 20 mM potassium oxalate or inoculated with different fungal strains, a 4 cm^2 area of leaf tissue

was excised at 24-h post inoculation. The tissues were processed as described before. All the experiments were repeated three times independently.

2.8. Oxalic acid treatment of plants

Mature and fully expanded *N. benthamiana* leaves were used for OA treatment. A 4 cm^2 cellophane sheet was place on the detached leaves and overlaid with 20 μl of 20 mM potassium oxalate (Fisher Scientific) adjusted to pH 3, 5, or 7 with HCl. Tap water with the same pH values were used as controls. For fungal challenge, 5-mm mycelial plugs collected from the leading edge of colonies were inoculated directly onto 4 cm^2 cellophane sheets overlaid on top of detached leaves. Observations were made 2 days post inoculation and recorded by digital photography.

2.9. Staining and microscopic observation

Infected *N. benthamiana* leaves were used for the visualization of compound appressorium development by Trypan Blue staining. A nylon woven mesh with a pore size of 60 μm (Thomas Scientific, Swedesboro, NJ, U.S.A) was kept between the inoculum plug and the leaf surface to assist separation of the plug from plant tissue. Inoculated leaves were sampled at the point of inoculation 24 h (wild-type (WT) and complemented (CP) strains) or 72 h (*dSspak2* and *dSspkaR* mutants) post inoculation. Samples were incubated in a 0.5% Trypan Blue solution (0.5 g of Trypan Blue in 100 ml H_2O) for 5 min, and samples washed with H_2O to remove excess dye. The stained samples were examined by bright field light microscopy with a Leica DMR compound light microscope (Wetzlar, Germany).

For monitoring autophagic activity, fungal hyphae were stained with monodansylcadaverine (MDC, Sigma–Aldrich, St. Louis, MO, U.S.A.). Nutrient starvation media contain solid minimal media (MM, 1 g/l K_2HPO_4, 0.5 g/l $MgSO_4$, 0.5 g/l KCl, 0.01 g/l $FeSO_4$, 15 g/l agar). Carbon starvation media (MM + N) contains MM supplemented with 2 g/l $NaNO_3$. Nitrogen starvation media (MM + C) contains MM supplemented with 30 g/l sucrose. PDA was used as a rich medium control. To avoid transferring nutrients from the PDA media, cultures were incubated on MM + N for two days prior to treatment. Mycelial plugs collected from the leading edge of colonies were inoculated on cellophane-overlaid media. Five 1 cm^2 squares of hypha growing on the surface of the cellophane were cut from the edges of 3 independent fungal colonies of each strain grown in agar media and incubated with 5 mM MDC (5 ml of 10 mM in dimethylsulfoxide (DMSO) in 5 ml phosphate buffered saline, pH 7.0) at 37 °C for 30 min in the dark. The MDC-stained hyphae were observed with an epifluorescence microscope (Leica DMR) with a narrow UV filter (excitation wavelength: 370 nm, emission wavelength: 509 nm). A minimum of five fields of view from three independent colonies of each strain were examined and the experiment was repeated five times.

2.10. Glycerol assays

The glycerol content of hyphae was determined from 3 or 6 mg of total protein using the Glycerol Assay Kit according to manufacturer specifications (Sigma–Aldrich #MAK117). In short, glycerol standards (0–1 mM) were used to generate a colorimetric standard curve. The reaction mixture contains 100 μl of master mix (100 μl assay buffer, 2 μl enzyme mix, 1 μl ATP, and 1 μl dye reagent) and 10 μl of the standard or protein sample. The mixtures were incubated at room temperature for 20 min in the dark and absorbance was measured at 570 nm. This glycerol assay was repeated three times independently using total protein extracted from three fungal cultures for each strain.

2.11. Statistical analysis

All statistical analyses were performed through R (version 3.5.1), and multiple comparisons of the means were conducted through one-way analysis of variance (ANOVA) followed by a post-hoc test Duncan's new multiple range test.

3. Results

3.1. Disruption of the Sspka2 and SspkaR loci

The *Sspka2* and *SspkaR* coding sequences were annotated in the *S. sclerotiorum* isolate 1980 reference genome (Amselem et al., 2011) as locus IDs Sscle14g098350 and Sscle09g071910, respectively (GenBank: XP_001585338.1 and XM_001588040.1). To test if SsPKA2 and SsPKAR are required for functional cAMP-dependent PKA signaling in *S. sclerotiorum*, gene-disruption mutants were generated through CRISPR-Cas9-mediated insertional mutagenesis. Mutants were verified via PCR screening and mutation sites characterized by DNA sequencing of the target sites and intervening sequences. Mutants with disruptions in SsPKA2 and SsPKAR coding sequences were designated *dSspka2*-7, *dSspka2*-9, *dSspkaR*-13, and *dSspkaR*-39 (Fig. S1A). An 8-kb amplicon was amplified from the *dSspka2*-7 insertion site (Fig. S1B). Twenty eight nucleotides upstream of the clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9 (Cas9) cut site (3–4 bp upstream of the PAM: TGG) were deleted in the coding region of *Sspka2*, and the intervening sequence contains plasmid vector sequences originating from the Cas9 expression vector including a partial Cas9 coding sequence, the SV40 nuclear localization signal (NLS), 3xFLAG peptide, TEF promoter, U6 promoter, gRNA scaffold, sgRNA, TrpC promoter, and the hygromycin B resistance gene (HygR) truncated by 36 bp at the 3' end (Fig. S2A). No full-length copy of the HygR coding sequence was detected by PCR from d*Sspka2*-7, indicating that the Cas9 expression vector sequences were not ectopically integrated outside of the CRISPR target site (Fig. S2B). For d*Sspka2*-9, utilizing LongAmp PCR, no amplicon was produced at the target site indicating a large (>10 kb) insertion in the *Sspka2* coding sequence. The d*SspkaR*-13 and -39 mutants were also determined to have large sequence inserts at the Cas9 target site as no amplicon was produced from either transformant following LongAmp PCR (Fig. S1B). CRISPR-Cas9-mediated vector sequence integration events have been reported previously in *S. sclerotiorum*, and serve as an efficient means of producing insertion mutants (J. Li et al., 2018; Wang and Rollins, 2021).

A wild-type copy of the *Sspka2* sequence in which the Cas9 target site sequence was modified utilizing synonymous nucleotide substitutions, coupled with the endogenous promoter and terminator was used for genetic complementation of the d*Sspka*2-7 strain (Fig. S3). The complemented strain (CP) was verified via PCR screening and sequencing (Fig. S1B). The number and quality of protoplasts generated from d*SspkaR* mutants were extremely low and repeated attempts to generate and, subsequently, genetically transform the d*SspkaR* mutants were unsuccessful.

3.2. Mutation of Sspka2 and SspkaR produce morphological aberrations

Two days after transfer to fresh PDA growth media, the radial growth of *dSspka2* and *dSspkaR* mutants was reduced 85% and 70% when compared with WT, respectively (Fig. 1A). A 70% growth reduction was also seen in *dSspkaR* mutants when grown in liquid shake cultures of YPGlu. Biomass accumulation of the *dSspka2* mutants in YPGlu shake culture however was the same as WT.

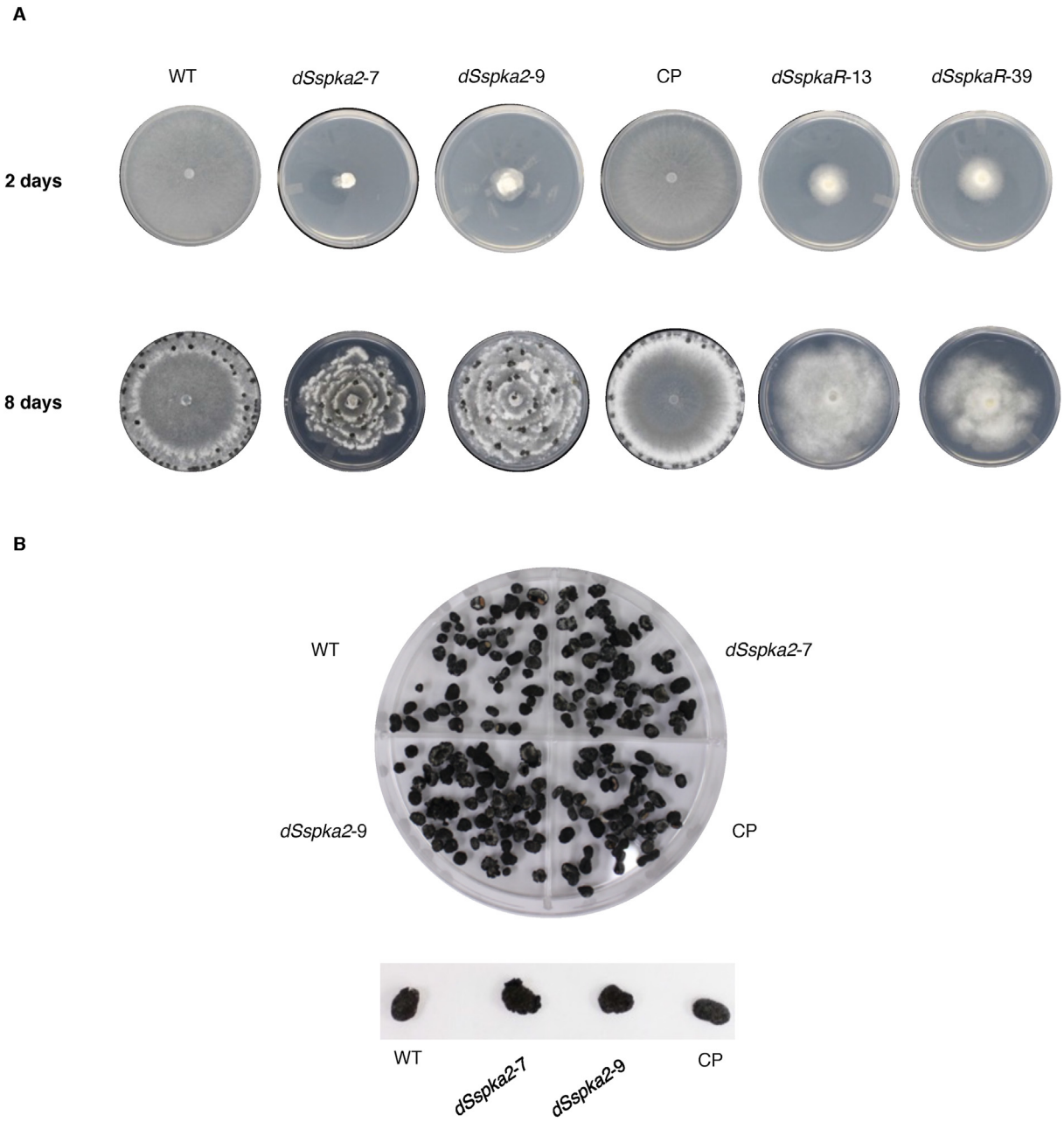

Fig. 1. Morphology of wild type (WT), *Sspka2*-disruption mutants (d*Sspka2*-7 and d*Sspka2*-9), *SspkaR*-disruption mutants (d*SspkaR*-13, d*SspkaR*-39), and d*Sspka2*-7 complementation strain (CP). (A) Fungal growth and sclerotial differentiation of fungal strains grown on PDA. (B) Characteristics of sclerotia derived from WT, d*Sspka2*-7, d*Sspka2*-9, and CP.

Colony morphology and the pattern of sclerotia development in *dSspka2 mutants* are distinct from the WT. *dSspka2* mutants formed irregular and compact colonies (Fig. 1A). By 8 days of growth, the WT had formed sclerotia along the wall of the petri dish while *dSspka2* mutants developed sclerotia in multiple concentric rings as the colony expanded. The rind surface of WT sclerotia were smooth while the surface of sclerotia derived from *dSspka2* mutants tend to be irregular and bumpy. Sclerotia formed by the *dSspka2* mutants sometimes merged to produce large sclerotia (Fig. 1B). Carpogenic germination of WT sclerotia began 4–5 weeks after incubation, but it took an additional two weeks for *dSspka2* mutants. By 8–10 weeks, the germination rate of WT sclerotia reached 75%, while 25 and 36% of the sclerotia collected from *dSspka2*-7 and *dSspka2*-19, respectively, germinated in this same period.

The colony growth of *dSspkaR* mutants consisted of thinly dispersed mycelia that fail to produce sclerotia (Fig. 1A). The *dSspkaR* mutants also failed to survive after prolonged incubation at room temperature (about a month) and could not be recovered from storage on dried, colonized filter paper stored at −20 °C, a standard method for long-term storage of the WT. The *dSspkaR* mutants could be preserved for extended periods of time (greater than a year) as 3-day old actively growing fungal culture on PDA at 4 °C.

3.3. Sspka2-*and* SspkaR-*disruption mutants exhibit attenuated virulence*

Pathogenicity of *dSspka2* and *dSspkaR* mutants was evaluated. *Sspka2* and *SspkaR* disruption mutants failed to cause symptoms on intact host tissues in most inoculations (Fig. 2A). *dSspkaR* mutants were able to develop symptoms on *N. benthamiana* without wounding, and yet the virulence was greatly reduced (Figs. 2A and

S4). To bypass the host cuticle penetration, wounded host tissues were also evaluated. Wounding did not restore the full virulence of *dSspka2* or *dSspkaR*. *dSspka2* mutants were generally nonpathogenic but occasionally caused small areas of necrosis surrounding the inoculum plug (Figs. 2B and S4). *dSspkaR* mutants however generally produced limited lesions and failed to fully colonize inoculated leaves even during prolonged incubation (Figs. 2B and S4).

3.4. dSspka2 *and* dSspkaR *mutants exhibit varying defects in compound appressorium differentiation*

Microscopic examination of fungal cultures revealed that the WT developed mature compound appressoria after 24 h of incubation on cellophane-overlaid PDA (Fig. 3B). These compound appressoria were macroscopically visible in the WT and CP at 48 h post incubation (Fig. 3A). Only after 72 h incubation were the

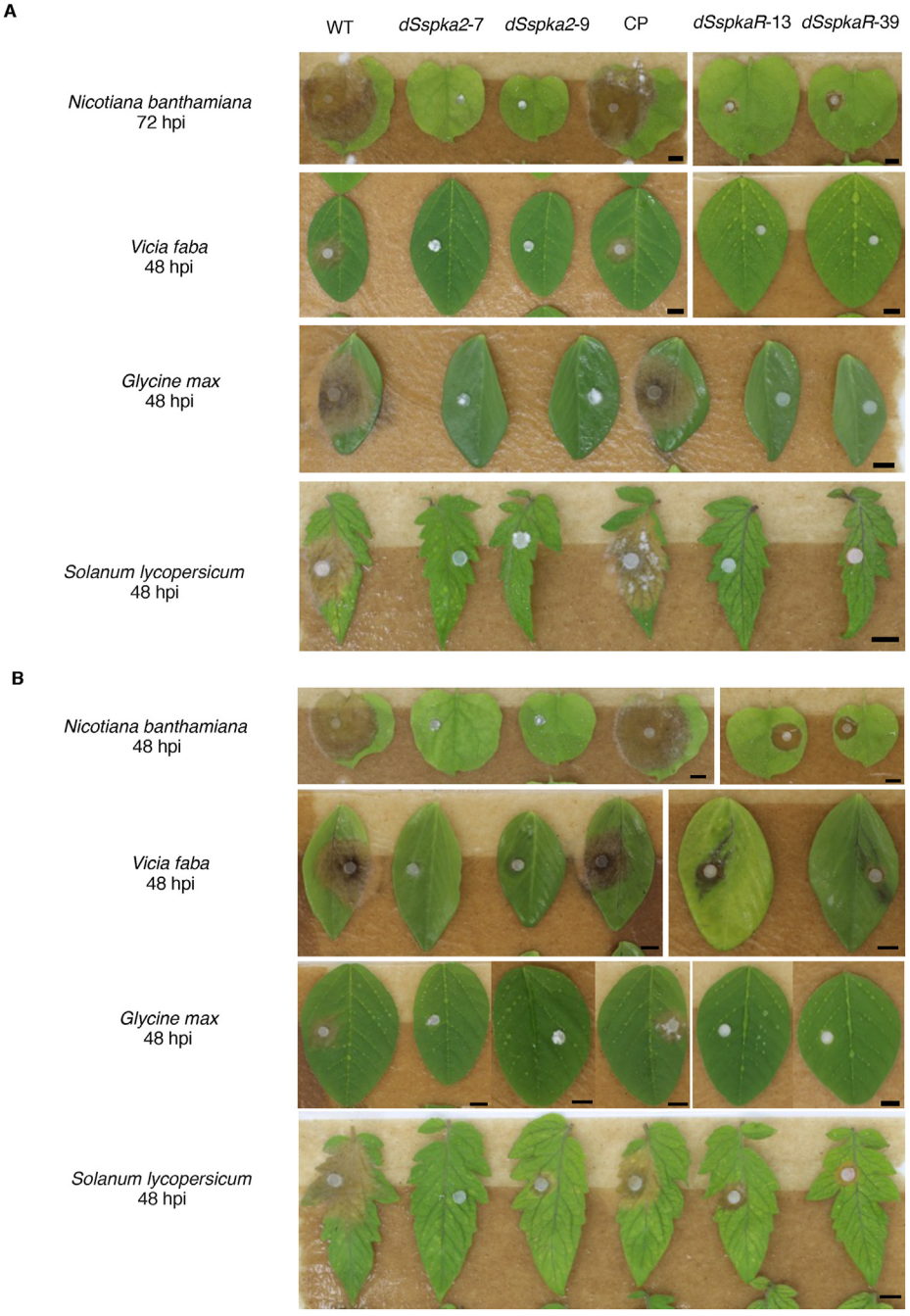

Fig. 2. Pathogenicity and virulence assay on detached leaves. (A) Detached leaves were inoculated with mycelial agar plugs directly. (B) The detached leaves were wounded before inoculation. Lesions were photographed at 48 or 72 h post inoculation. Scale bar = 1 cm. Wild type (WT), *Sspka2*-disruption mutants (d*Sspka2*-7 and d*Sspka2*-9), *SspkaR*-disruption mutants (d*Sspka*R-13, d*SspkaR*-39), and d*Sspka2*-7 complementation strain (CP).

dSspka2 mutants observed to develop mature compound appressoria with numbers and complexity similar to WT appressoria at 24 h of incubation. In contrast, *dSspkaR* mutants were unable to form compound appressoria on all artificial surfaces tested: plastic coverslips (data not shown), parafilm (data not shown), and cellophane (Fig. 3B).

Since the *dSspkaR* mutants were capable of infecting unwounded host tissues (Fig. 2A), we hypothesized that *dSspkaR* mutants were able to produce compound appressoria on plant tissue. At 24 h post inoculation, compound appressoria produced by the WT and CP in different development stages were observed on *N. benthamiana* leaves (Fig. 4B). After 72 h of incubation, fewer and less complex compound appressoria were derived from *Sspka2* and *SspkaR* disruption mutants on the host tissue compared with WT. *dSspkaR* mutants were also observed to frequently penetrate through stomata, which is uncommon in the WT (Fig. 4B). Defective compound appressorium formation in *dSspka2* and *dSspkaR* mutants, along with the slow growth rate both contribute to low virulence.

3.5. An unknown plant necrosis-inducing fungal factor(s) contributes to lesion production in dSspkaR

The size of lesions produced following inoculation with *dSspkaR* mutant was inconsistent with the number of appressoria and hyphae developed from the inoculum plug (Fig. 4A). This observation suggested that the *dSspkaR* mutant might overproduce a known virulence factor or produce a novel factor(s) that causes necrosis in *N. benthamiana*. To further characterize the infection event of *dSspkaR* mutants, a 5-mm mycelia-agar plug was inoculated onto a 4 cm^2 cellophane sheet overlaid on the leaf surface. *S. sclerotiorum* could not degrade or penetrate the cellophane, so the hyphae and appressoria remain physically separated from the plant tissue (Fig. 5A). Without direct contact with the host tissues, only *dSspkaR* mutants produced necrotic lesions beneath the cellophane sheet (Fig. 5B).

As OA has been demonstrated to be an important necrotizing virulence factor in *S. sclerotiorum* (Kim et al., 2008; Liang et al., 2015a) and necrotic lesions developed on *N. benthamiana* leaves infiltrated with potassium oxalate at pH 3, 5, and 7 (Fig. S5), we

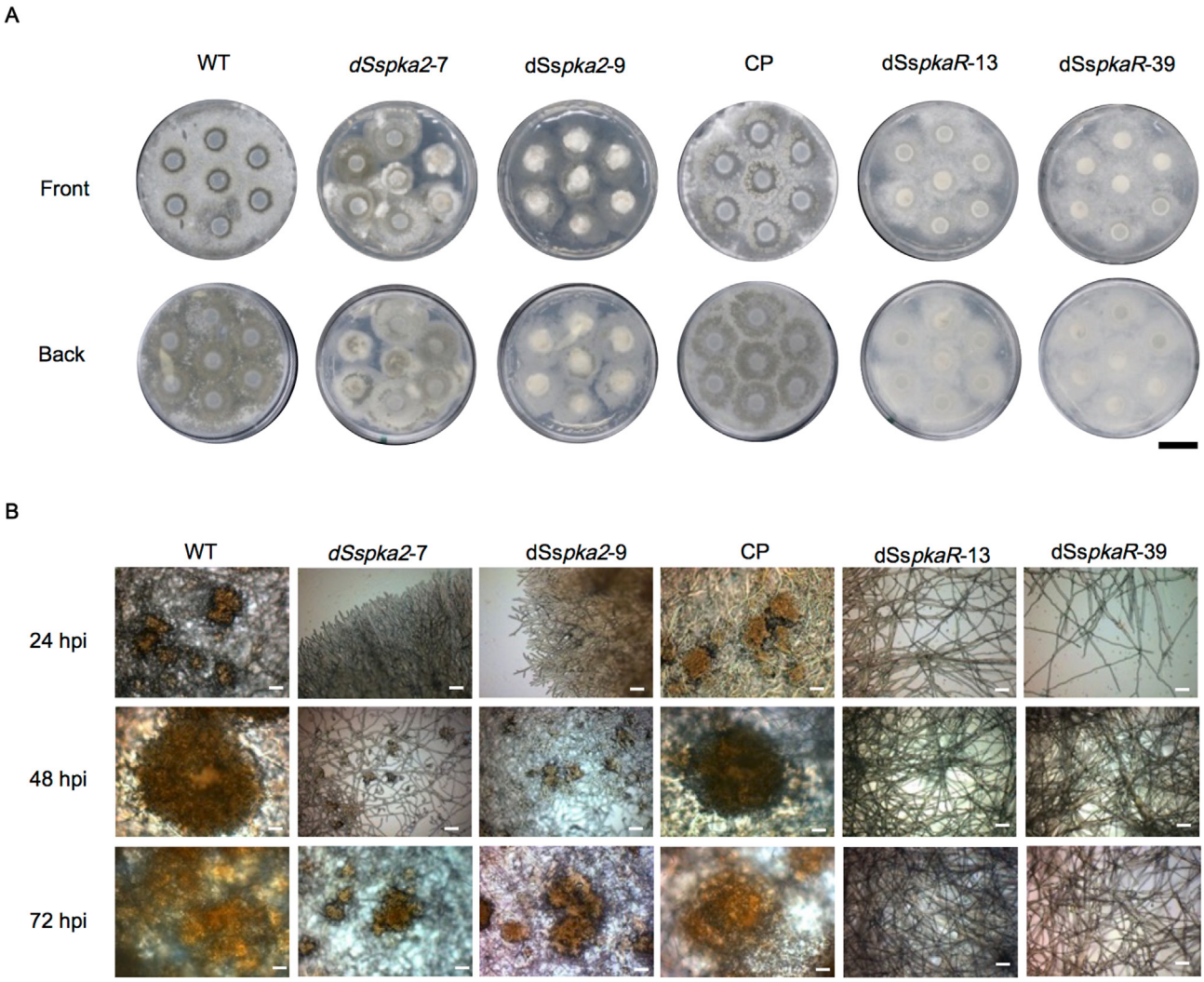

Fig. 3. Formation of compound appressoria on cellophane overlaid on PDA growth medium. (A) Macroscopic examination of compound appressoria after 48 h of growth. Scale bar = 1 cm (B) Microscopic observations of compound appressoria formation at 24, 48 and 72 h post inoculation (hpi). Scale bar = 100 μm. Wild type (WT), *Sspka2*-disruption mutants (d*Sspka2*-7 and d*Sspka2*-9), *SspkaR*-disruption mutants (d*SspkaR*-13, d*SspkaR*-39), and d*Sspka2*-7 complementation strain (CP).

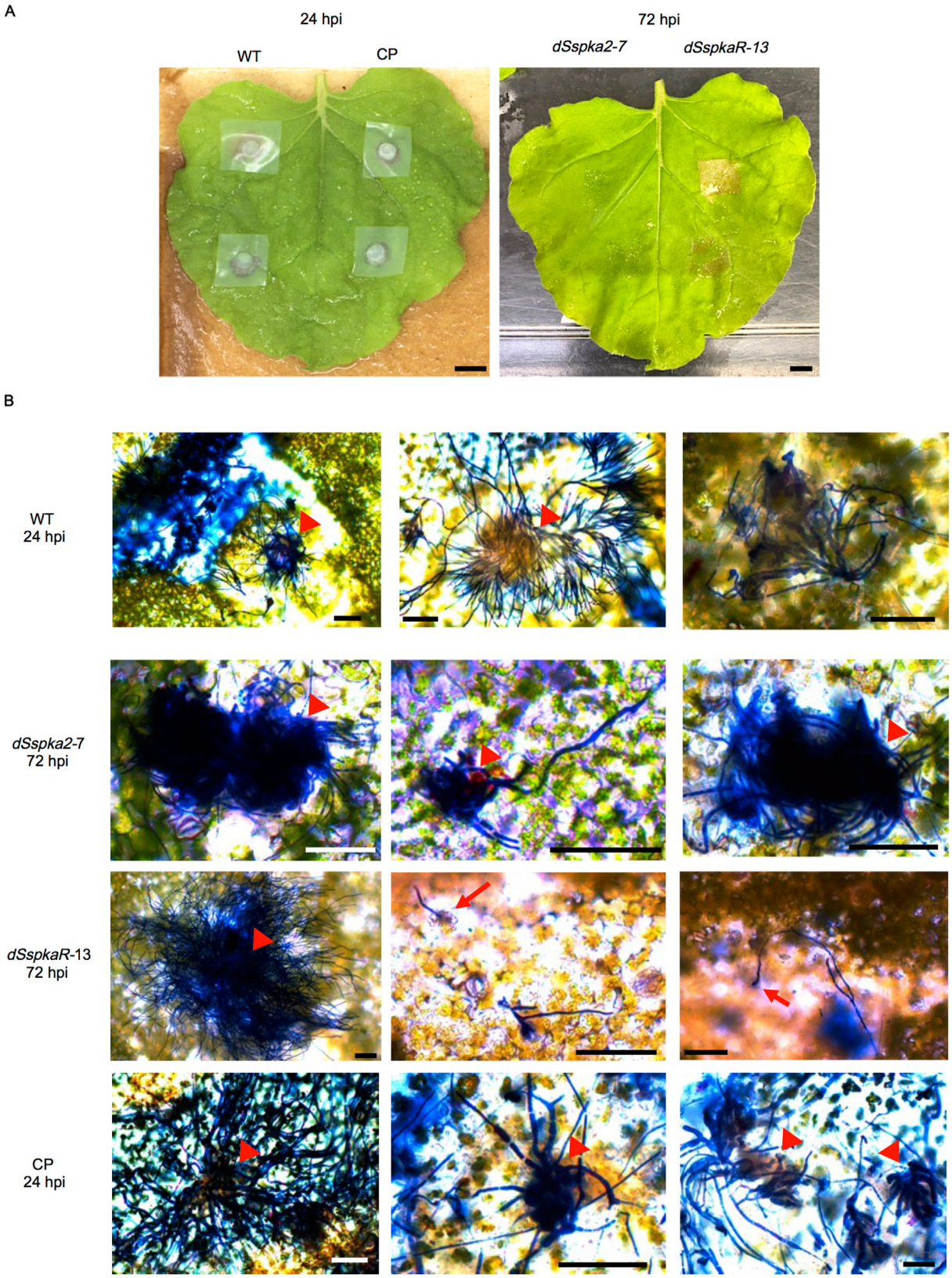

Fig. 4. Compound appressorium and lesion development on *Nicotiana benthamiana*. (A) Lesion development was photographed at 24 h post inoculation (hpi) or 72 hpi. (B) Hyphae and appressoria were visualized on the leaf surface by bright field light microscopy following Trypan Blue staining. Arrows indicate stomata. Arrowheads indicate compound appressoria. Scale bars in (A) and (B) represent 1 cm and 100 μm, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

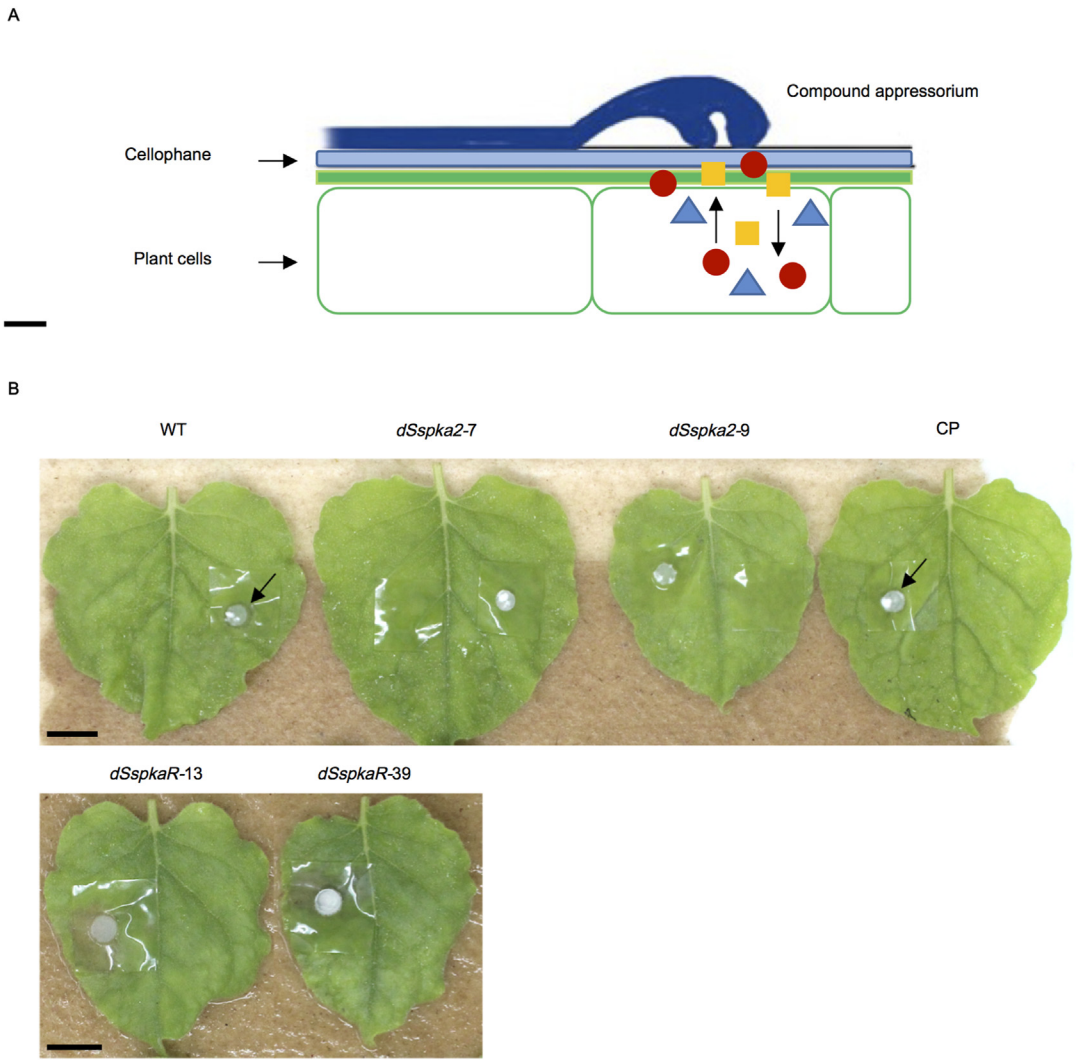

Fig. 5. *SspkaR* disruption mutants induced necrosis without physical contact with leaves. (A) Schematic demonstrating how diffusible molecules were exchanged between plant cells and fungal cells through cellophane membrane without direct contact. (B) Necrosis appearing at 24 h post inoculation on *SspkaR* disruption mutants-inoculated onto cellophane overlaying the leaves. Arrows indicate formation of compound appressoria. WT: wild-type; *dSspka2*-7 and *dSspka2*-9: *Sspka2* disruption mutants; CP: complementation strain of *dSspka2*-7; *dSspkaR*-13 and *dSspkaR*-39: *SspkaR* disruption mutants. Scale bar = 1 cm.

tested OA for the ability to produce necrotic lesions under the experimental conditions utilized for infection studies. In contrast to the infiltration result, direct application of potassium oxalate on the cellophane placed on leaves did not trigger necrotic lesion development. Based on this finding, the plant tissue under the cellophane inoculated with WT, *dSspkaR* or *dSspka2* mutants was collected for OA quantification at 24 h post inoculation. OA levels for all treatments was below the detection range of the oxalate quantification assay (<78 μM). To further explore the possibility that OA concentrations are higher during infection in the *dSspkaR* mutant than in the WT or *dSspka2* mutant, OA concentration from lesions was quantified at 5 days post inoculation. Analysis of the concentrations with a one-way ANOVA did not identify differences among the WT, the *dSspka2* mutant, the CP, and the *dSspkaR* mutant (Fig. 6).

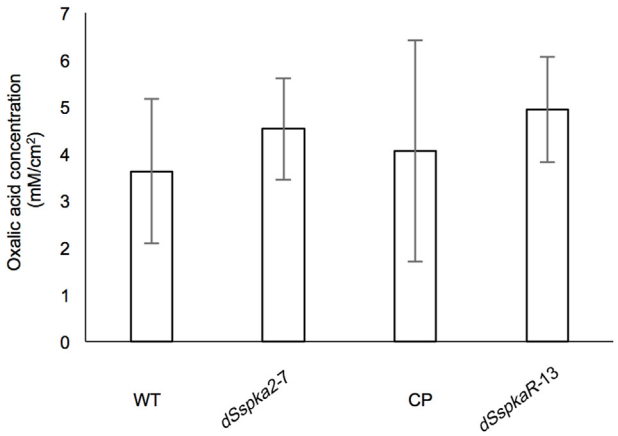

Fig. 6. Oxalic acid concentration within lesions collected from infected *Nicotiana benthamiana* leaves. Concentration of oxalic acid was normalized with area of lesion collected from the leaves inoculated with different fungal strains at 5 days post inoculation. Six detached leaves (n = 6) were inoculated for each strain. Scale bars represent standard deviation.

3.6. cAMP-dependent PKA signaling is required for proper regulation of autophagy

To study the role of SsPKA2 and SsPKAR in regulation of autophagy, we examined autophagy induction in *dSspka2* and *dSspkaR*

mutants by monitoring monodansylcadaverine (MDC) staining in vegetative hyphae. MDC is a selective autofluorescent compound, which conjugates to cadaverine accumulating in acidic autophagic vacuoles and interacts with autophagosome lipid membrane (Biederbick et al., 1995; Niemann et al., 2000). MDC staining has been used as autophagy indicator in other fungal pathogens including the *Pyricularia oryzae* (Marroquin-Guzman et al., 2017; Veneault-Fourrey, 2006) and *Fusarium* spp. (Josefsen et al., 2012; Khalid et al., 2019). While uniform, background staining was observed in WT and CP hyphae under the nutrient-rich condition, there is a clear increase in discrete fluorescent points under starvation conditions (Fig. 7). These MDC-labeled autophagosomes were distributed within the cytoplasm of hyphae of the *dSspka2 and dSspkaR* mutant visible as distinct fluorescent points under starvation as well as non-starvation conditions (Fig. 7). The result suggested that disruption of *Sspka2* or *SspkaR* causes induction of

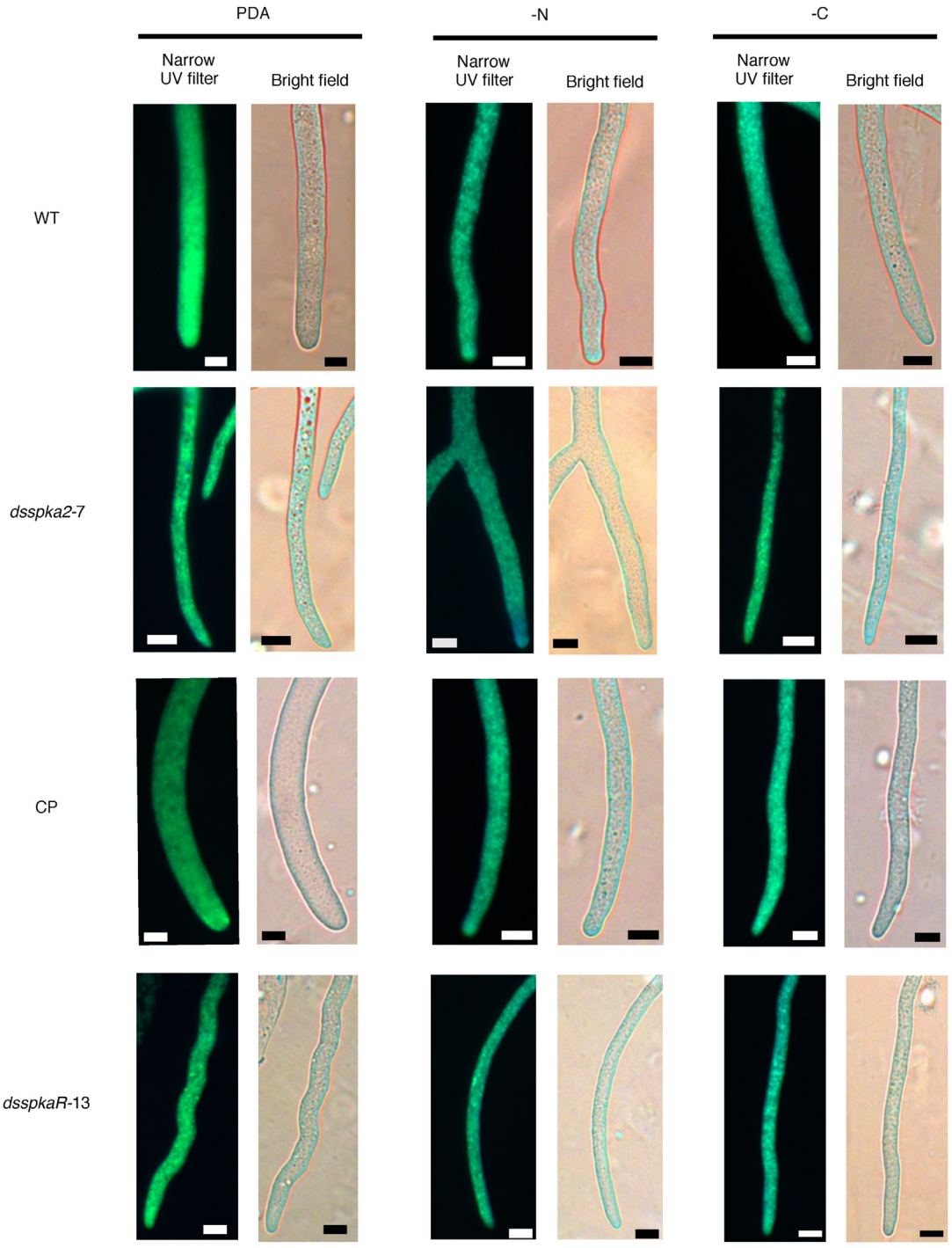

Fig. 7. Visualizing induction of autophagy through the monodansylcadaverine staining in hyphae collected from PDA, minimal media with carbon source (-N) or nitrogen source (-C). Scale bar = 10 μm. WT: wild-type; *dSspka2*-7 and *dSspka2*-9: Sspka2 disruption mutants; CP: complementation strain of *dSspka2*-7; *dSspkaR*-13 and *dSspkaR*-39: *SspkaR* disruption mutants.

autophagy during hyphal growth under nutrient non-limiting conditions whereas the WT experiences autophagy only under starvation conditions.

3.7. Disruption of SspkaR results in hyper-accumulation of glycerol in vegetative hyphae

cAMP-dependent PKA signaling has been demonstrated to be required for glycerol accumulation in appressoria of *Pyricularia oryzae* through PKA catalytic subunit-regulated tracylglycerol lipase activity (Thines et al., 2000). To test if the SsPKA2 and SsPKAR are involved in glycerol biosynthesis in *S. sclerotiorum*, the glycerol content of hyphae was measured. Glycerol dramatically accumulates to higher concentrations in *dSspkaR*-13, while *dSspka2*-7 maintains a level similar to WT (Fig. 8).

4. Discussion

The cAMP-dependent PKA signaling pathway controls a myriad of processes in fungi. In filamentous ascomycetes, regulation of morphological transitions and pathogenicity are the most studied (Adachi and Hamer, 1998; Mitchell and Dean, 1995; Schumacher et al., 2008; Tsai et al., 2013; Xu et al., 1997). The current study adds to our current knowledge of this signaling pathway in *S. sclerotiorum* by demonstrating that SsPKA2 and SsPKAR are required for optimal vegetative growth, regulation of autophagy, compound appressorium and sclerotium development, and pathogenicity.

4.1. Fungal colony morphology

Previous phylogenetic analysis and experimental evidence demonstrated that *Sspka1* encoded only minor or redundant PKA activity (Jurick et al., 2004). These results and those published for other fungi suggested that the paralog characterized here, *Sspka2*, is primarily responsible for the primary PKA activity in *S. sclerotiorum* (Jurick et al., 2004; Schumacher et al., 2008). Supporting this hypothesis, the *dSspka2* disruption mutant described here, developed sclerotia in concentric rings similar to those described for the adenylate cyclase (*sac1*) null mutant (Jurick and Rollins, 2007). Likewise, the elimination of sclerotia development in the *dSspkaR* mutant is consistent with cAMP being a negative regulator of sclerotia development (Rollins and Dickman, 1998). Our results indicate that a lack of PKA activity over-stimulates sclerotial development while excess PKA activity inhibits the initiation of sclerotial development. Perhaps the slowed growth rate, coupled with defects in metabolic flux as a consequence of eliminating SsPKA activity, underlie the hyper-sclerotium initiation phenotype. Sexual development-related phenotypes are associated with the mutation of the PKA regulatory subunit in *F. graminearum* and *Ustilago maydis* as well. In *U. maydis*, deletion of the regulatory subunit encoding gene *ubc1* caused *U. maydis* to lose the ability to develop galls in maize as well as sexual development/teliosporogenesis (Gold et al., 1997). In *F. graminearum*, the regulatory subunit of PKA is essential for developing perithecia (C. Li et al., 2018). Although sexual fruiting body development was not grossly affected in the *SsPka2* mutants, their delayed carpogenic germination relative to WT is indicative of a metabolic defect rather than a direct role in regulating morphology.

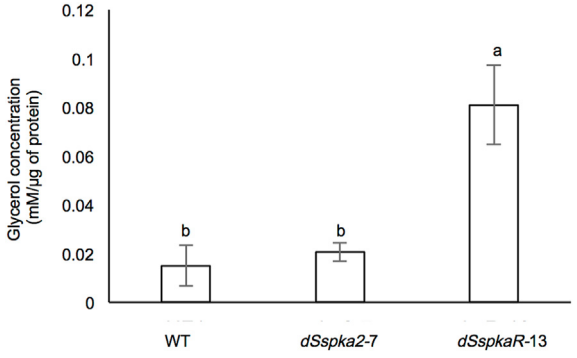

Fig. 8. Glycerol concentration within hyphae of WT: wild type, *dSspka2*-7: Sspka2 disruption mutant, and *dSspkaR*-13: *SspkaR* disruption mutant. Different letters are statistically different ($p < 0.01$) as determined by one-way ANOVA followed by a post-hoc test Duncan's new multiple range test. Scale bars represent standard deviation. One representative replicate from three independent experiments is shown. Fungal mycelia for total protein extraction were collected from three independent cultures ($n = 3$) separately from each strain.

4.2. Appressorium development

Many phytopathogenic fungi produce appressoria, specialized unicellular or multicellular structures that function in attachment and penetration of the host cuticle. *S. sclerotiorum* produces a multicellular infection structure known variously as a compound appressorium or an infection cushion (Choquer et al., 2021; Lumsden and Dow, 1973). *S. sclerotiorum* hyphae can differentiate compound appressoria on hydrophobic and hydrophilic surfaces. Disruption of *SMK3*, *ggt1*, *caf1*, *sac1*, or *odc2* compromises the develop of compound appressoria, which leads to loss of pathogenicity on unwounded plants (Bashi et al., 2016; Jurick II and Rollins, 2007; Li et al., 2012; Liang et al., 2015b; Xiao et al., 2014), yet the signaling pathways that are involved in perception of surface or chemical signals for initiating compound appressoria formation remain uncharacterized. The *sac1* null mutant does not produce compound appressoria (Jurick and Rollins, 2007); however, mutation of *Sspak2* did not fully block appressorium formation but rather delayed appressorium development on both hydrophobic and hydrophilic surfaces. The *SspkaR*-disruption mutant did not form compound appressoria on artificial surfaces but was able to develop compound appressoria of limited complexity on the plant surfaces. These findings indicate that cAMP-dependent PKA signaling regulation is involved in surface sensing during the initiation of appressorium development similar to what has been reported for *P. oryzae* (Xu and Hamer, 1996).

Unlike *S. sclerotiorum*, *P. oryzae* germlings only differentiate appressoria on hydrophobic surfaces, but appressorium formation can be induced on hydrophilic surface by supplying exogenous cAMP (Lee and Dean, 1993). Genetic analyses of cAMP-dependent PKA signaling in *P. oryzae* further support that cAMP is involved in appressorium development. Null mutants of the AC coding gene, *mac*1, do not form appressoria, but the defect could be restored by cAMP treatment or deletion of the PKA regulatory subunit—coding gene *sum1*. Deletion of *sum1* also led to appressorium formation on hydrophilic surface or hydrophobic surface with the addition of nutrients (Adachi and Hamer, 1998; Mitchell and Dean, 1995; Xu et al., 1997). The deletion of the *P. oryzae CPKA*, orthologous to *Sspka2*, caused a delay in appressorium development similar to what was observed here with *S. sclerotiorum* (Xu et al., 1997). The conservation of cAMP-dependent regulation of appressorium development between species that produce single-celled versus multi-cellular appressoria suggests a common regulation and perhaps evolutionary origin of these highly specialized structures despite their lack of morphological similarity.

4.3. Crosstalk between cAMP signaling and MAP kinase cascade involved in glycerol accumulation

The defects in multicellular development of compound appressoria and sclerotia observed with the *Sspka2* and *SspkaR* mutants suggest an underlying metabolic defect. In the *dSspkaR* mutant, this is manifest as a hyperaccumulation of glycerol in vegetative hyphae. Studies of *P. oryzae* demonstrate that glycerol rapidly accumulates to a high concentration within the appressorium and is utilized to build turgor within this structure (De Jong et al., 1997; Foster et al., 2017; Thines et al., 2000). Lipid and glycogen metabolism are required for this glycerol accumulation, and are cross-regulated by the cAMP-dependent PKA and *PMK1* MAPK signaling pathways (Thines et al., 2000; Xu and Hamer, 1996). Rapid degradation of glycogen and lipid translocation dependent on *PMK1* occurs during appressorium maturation. These processes are greatly delayed in the loss of function PKA catalytic subunit gene, *ΔcpkA*. Defects in glycogen and lipid metabolism in *P. oryzae ΔcpkA* suggests that loss of pathogenicity in the mutant is due to an impairment in the ability to generate turgor pressure (Thines et al., 2000). Similarly, a defect in lipid degradation is observed in the PKA catalytic subunit (CPK1) deletion mutant of *Colletotrichum lagenarium* where lipid metabolism homeostasis is required for appressorium penetration (Yamauchi et al., 2004). However, evidence of a requirement for turgor generation and signaling pathways that regulate solute accumulation in compound appressoria is lacking in *S. sclerotiorum.* Disruption of melanin biosynthetic gene *Sspks13* of *S. sclerotiorum* produces albino appressoria but does not impair their function (J. Li et al., 2018). This suggests that melanin is dispensable for *S. sclerotiorum* pathogenicity and that extreme turgor generation within compound appressoria is not a requirement for penetration. Still, distribution and mobilization of nutrients is required for supporting multicellular development transitions. The hyperaccumulation of glycerol in the hyphae of the *dSspkaR* mutant suggests a defect in metabolic flux.

Both lipid metabolism and glycogen biosynthesis are potential sources of glycerol biosynthesis (Talbot, 2003) and are controlled by autophagy in plant pathogenic fungi (Josefsen et al., 2012; Liu et al., 2007, 2017; Lu et al., 2009; Nguyen et al., 2011; Ren et al., 2018). In *P. oryzae*, *Botrytis cinerea*, and *F. graminearum*, deletion of autophagy-related genes, ATGs, caused defects in lipid metabolism affecting conidiation, conidium germination, appressorium development and function, and sexual fruiting body formation (Liu et al., 2007, 2017; Lu et al., 2009; Nguyen et al., 2011; Ren et al., 2017). In *P. oryzae*. Furthermore, glycerol accumulates to high levels in the hyphae of the *ΔBcatg1 B. cinerea* mutant (Ren et al., 2017).

In *S. cerevisiae*, cAMP-dependent PKA signaling negatively regulates autophagy by inhibiting the induction process of autophagy in the presence of glucose (Stephan et al., 2009). Genetic evidence supporting a direct connection between cAMP-dependent PKA signaling and autophagy has not been extensively established for filamentous fungi with the exception of *Aspergillus* species where cAMP-dependent PKA signaling also negatively regulates autophagy (Y., Y. Kim et al., 2011; Shao et al., 2017). In *P. oryzae*, TOR and cAMP signaling also regulate autophagy cooperatively, which is required for appressorium function. Differing from *S. cerevisiae* and *Aspergillus* spp., cAMP signaling was demonstrated through pharmacological studies to positively regulate autophagy in *P. oryzae* (Sun et al., 2018). Similarly, deleting the PKA regulatory subunit-coding gene in *F. graminearum* causes over-induction of autophagy in hyphae and conidia (C. Li et al., 2018). Interestingly, no antagonistic effect between disruption of *Ssapk2* and *SspkaR* were observed in terms of autophagy regulation here in *S. sclerotiorum.* Disruption of either *Sspka2* or *SspkaR* induced autophagy in the hyphae without nutrient starvation treatment. In contrast to the *dSspkaR* mutant, disruption of *Sspka2* does not change the intracellular glycerol concentration. During sclerotium development, the mobilization of carbohydrate from hyphae to the sclerotium initials has been reported (Cooke, 1971). Potentially, the *dSspka2* mutant is unable to store carbohydrates within hyphae for later mobilization whereas the *dSsPkaR* mutant is unable to metabolize glycerol to support sclerotial development. This hypothesis would explain the hyper-initiation of sclerotia phenotype of the *dSspka2* mutant and the inability to initiate sclerotia in the *dSspkaR* mutant. Regardless of the specific metabolic consequences, as discussed above, mis-regulation of autophagy impacts development and pathogenicity. It appears that balanced PKA activity is required for proper regulation of autophagy in *S. sclerotiorum*, which contributes to the fungal differentiation and pathogenicity-related development.

4.4. A necrosis-inducing factor(s) is over-secreted in dSspkaR mutants

dSspkaR mutants exhibited reduced virulence on unwounded and wounded host plants. This attenuated virulence likely stems from slow hyphal growth and impaired appressorium development and function. Although *dSspkaR* mutants are unable to form compound appressoria in vitro, they do produce some, although delayed, low complexity compound appressoria on host tissues. The reduced complexity and high frequency at which these compound appressoria were observed to penetrate through stomata indicates that they were impaired in direct host penetration. The incongruence between the lesion size and number of appressoria formed by the *dSspkaR* mutant indicated that necrosis may be initiated independent of penetration. Investigations using cellophane as a barrier between fungal and plant tissue, demonstrated that whereas the WT and *dSspka2* mutants failed to infect the host and no symptoms were observed, the *SspkaR* null mutant was able to induce necrosis in the host without infecting. Although cellophane provides a physical barrier between hyphae and plant tissues, the cellophane membrane allows exchange of diffusible factors up to 90 kDa (Kullnig et al., 2000). The results support the possibility that a diffusible necrosis-inducing factor(s) might be overproduced by the *dSspkaR* mutant.

Fungal toxin production is controlled by the cAMP-dependent PKA signaling pathway in some filamentous fungi. In *Aspergillus* spp., aflatoxin or sterigmatocystin biosynthesis are regulated through PKA which post-transcriptionally regulates AflR, a $Zn(II)_2$-Cys_6 transcription factor (Roze et al., 2004; Shimizu et al., 2003). In phytopathogenic fungi, mycotoxins and phytotoxin biosynthesis are also regulated through cAMP-dependent PKA signaling (Hu et al., 2014; C. Li et al., 2018; Park et al., 2016; Xu et al., 2011). As OA is the important virulence factor in *S. sclerotiorum* that, in addition to being strongly regulated by ambient pH, has also been suggested to be regulated by cAMP (Rollins and Dickman, 1998). OA's role in the observed necrosis was investigated. The *dSspkaR* mutant did not overproduce OA in vitro (data not shown) or in planta suggesting that OA was not the factor responsible for the observed d*SspkaR* necrosis. This evidence strengthens the idea that a necrosis-inducing factor(s) other than OA might be over-secreted in the *dSspkaR* mutant. In *S. sclerotiorum*, protein elicitors of cell death as well as necrosis and ethylene-inducing peptides have been isolated and heterologously expressed to induce necrotic hypersensitive-like lesions (Dallal Bashi et al., 2010; Seifbarghi et al., 2020; Zhang et al., 2014). These as well *S. sclerotiorum* produced metabolites such as botcinic acid are candidates for this necrosis-inducing activity. Characterization of this activity in future studies will provide a better understanding of regulation of

necrosis-inducing factors through cAMP-PKA signaling in *S. sclerotiorum*.

5. Conclusions

In summary, this study demonstrates that *dSspka2*-and *dSspkaR* mutants have pleiotropic defects in hyphal growth, appressorium development, sclerotium initiation, and virulence of *S. sclerotiorum*. Our results imply that autophagy is regulated via cAMP-dependent PKA signaling in *S. sclerotiorum* and both constitutive or lack of PKA activity appear to induce autophagy in hyphae. However, only *dSspkaR* mutants exhibited hyperaccumulation of glycerol. Defects in this sugar alcohol metabolism lead to an inability to form multicellular structures including appressoria and sclerotia which require its translocation from undifferentiated hyphae to support their development. Mis-regulation of glycerol synthesis also suggests a defect in lipid and glycogen metabolism, which are required for appressorium maturation in other pathogenic fungi. The ability to colonize host tissue has been demonstrated to require cooperation between hyphae at the center and at the margins of the expanding lesion (Peyraud et al., 2019). Thus, many processes important for *S. sclerotiorum* colony formation in culture are equally likely to be important for lesion development in planta.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2021.09.004.

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