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Transcription factor SsFoxE3 activating *SsAtg8* is critical for sclerotia, compound appressoria formation, and pathogenicity in *Sclerotinia sclerotiorum*

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[Correction added on 9 November 2021, after first online publication: the article title has been updated in this version.]

Abstract

Sclerotinia sclerotiorum, the notorious necrotrophic phytopathogenic fungus with wide distribution, is responsible for sclerotium disease in more than 600 plant species, including many economic crops such as soybean, oilseed rape, and sunflower. The compound appressorium is a crucial multicellular infection structure that is a prerequisite for infecting healthy tissues. Previously, the Forkhead-box family transcription factors (FOX TFs) SsFoxE2 and SsFKH1 were shown to play a key regulatory role in the hyphae growth, sexual reproduction, and pathogenicity of *S. sclerotiorum*. However, little is known about the roles of SsFoxE3 regulating growth and development and pathogenicity. Here, we report *SsFoxE3* contributes to sclerotium formation and deletion of *SsFoxE3* leads to reduced formation of compound appressoria and developmental delays. Transcripts of *SsFoxE3* were greatly increased during the initial stage of infection and *SsFoxE3* deficiency reduced virulence on the host, while stabbing inoculation could partially restore pathogenicity. The *SsFoxE3* mutant showed sensitivity to H_2O_2, and the expression of reactive oxygen species detoxification and autophagy-related genes were reduced. Moreover, expression of *SsAtg8* was also decreased during the infection process of the *SsFoxE3* mutant. Yeast 1-hybrid tests suggested that SsFoxE3 interacted with the promoter of *SsAtg8*. Disruption of *SsAtg8* resulted in a phenotype similar to that of the *SsFoxE3* mutant. Comparative analysis of the level of autophagy in the wild type and *SsFoxE3* mutant showed that N starvation-induced autophagy was reduced in the *SsFoxE3* mutant. Taken together, our findings indicate that SsFoxE3 plays an important role in compound appressorium formation and is involved in transcriptional activation of *SsAtg8* during infection by *S. sclerotiorum*.

KEYWORDS
autophagy, compound appressoria, pathogenicity, *Sclerotinia sclerotiorum*, SsFoxE3

1 | INTRODUCTION

Sclerotinia sclerotiorum, a pathogen with a complex of pathogenicity factors (cell wall-degrading enzymes, transcription factors [TFs], effectors, and oxalic acid), has two different lifestyles: biotrophic and necrotrophic (Bashi et al., 2016; Hegedus & Rimmer, 2005; Kabbage et al., 2015; Liang & Rollins, 2018; Lyu et al., 2016; Sang et al., 2019; Veluchamy et al., 2012). During infection, *S. sclerotiorum* saprotrophic hyphae differentiate to form a special infection structure named the multicellular appressorium (infection cushion) that penetrates the cuticle and cell walls of healthy plant issue (Li et al., 2012; Lumsden, 1973; Tariq & Jeffries, 1984). Several phytopathogenic fungi, such as *Rhizoctonia solani*, *Fusarium graminearum*, and *Botrytis cinerea*, use complex appressoria similar to infection cushions (Armentrout & Downer, 1987; Choquer et al., 2021; Jeffries, 1986; Mentges et al., 2020; Xu et al., 2018). *SsGgt1*, which encodes γ-glutamyl transpeptidase involved in redox homeostasis, is known to functions in the regulation of infection cushion development (Li et al., 2012). In addition, transcriptomic studies of infection cushions in *F. graminearum* and *B. cinerea* have shown that infection cushions are arsenals: virulence-associated factors including cell wall-degrading enzymes, effector proteins and secondary metabolites are synthesized in infection cushions (Choquer et al., 2021; Mentges et al., 2020). Therefore, the formation and development of compound appressoria in *S. sclerotiorum* should be studied systematically.

Members of the Forkhead-box (FOX) TF family contain a wing-like helix structure in the DNA-binding region, also called the winged helix TF (Postnikoff et al., 2012). FOX TFs show great functionality in growth, development, and cell division and participate in the control of the cell cycle process, growth, differentiation, and other biological processes (Bulmer et al., 2004; Postnikoff et al., 2012; Shimada et al., 2008). FOX TFs are conserved in animal and fungal genomes, but are not found in plants. *Saccharomyces cerevisiae* contains four FOX TFs: FKH1, FKH2, HCM1, and FHL1 (Arsenault et al., 2015; Pataki et al., 2017; Postnikoff et al., 2012). Fhl1 of *Schizosaccharomyces pombe* regulates the nitrogen starvation response and acts in the target of rapamycin signalling pathway (Pataki et al., 2017). CaFKH2, the FOX TF of *Candida albicans*, controls the morphogenesis of fungal hyphae and the toxicity of pseudohyphae (Bensen et al., 2002). In *Magnaporthe oryzae*, the absence of *MoFKH1* affects mycelial growth, conidial germination, and its pathogenicity; the absence of *MoHCM1* leads to defects in mycelial growth and conidial germination (Park et al., 2014). Fox1 is exclusively expressed during the biotrophic stage of *Ustilago maydis* infection; deficiency of *fox1* can lead to reduced virulence, impaired tumour development, and induces the accumulation of H_2O_2 in and around infected cells (Zahiri et al., 2010). In *S. sclerotiorum*, there are four FOX TF members: SsFkh1, SsFoxE2, SsFoxE3, SsFox1 Silencing *SsFkh1* resluts in significantly reduced pathogenicity and no formation of sclerotia (Fan et al., 2017). SsFoxE2 is required for apothecial development (Wang et al., 2016). However, the functions of SsFoxE3 and SsFox1 are still unknown.

Autophagy is a programmed cell degradation mechanism that is ubiquitous in eukaryotic cells. Organisms can remove and degrade excess biological macromolecules and self-damaged cells that are produced in biological processes through autophagy and use the degradation products to provide energy, which is considered to be a strategy for obtaining nutrients by recycling their own cell components under starvation conditions, and is essential for maintaining cell homeostasis and the operation of life activities. Autophagy in filamentous fungi is not only related to nutrition balance but also to developmental processes such as cell differentiation, secondary metabolism, and pathogenicity, and is often a prerequisite for pathogenicity (Liu et al., 2012; Zhu et al., 2019). *MoAtg8* is necessary for the differentiation of the *M. oryzae* infection structure (Liu et al., 2010). Deletion of *ATG8* causes *U. maydis* autophagosomes to accumulate in vacuoles and reduces virulence (He et al., 2018). *SmAtg8* is required for *Sordaria macrospora* fruiting body development and ascospore germination (Voigt et al., 2013). In *Colletotrichum orbiculare*, deficiency of *coatg8* causes it to fail to form normal appressoria in the early steps of morphogenesis (Asakura et al., 2009). *FgAtg8* is involved in the formation of fruiting bodies and the production of conidia of *F. graminearum* (Josefsen et al., 2012), which means that the autophagy process has a variety of functions in different species. However, the transcriptional regulation of autophagy-related genes in *S. sclerotiorum* is not yet known, and the role of autophagy is still unclear.

In this study, we identified SsFoxE3 as a FOX TF and showed it is involved in polarized growth, pathogenicity, and the stress response in *S. sclerotiorum*. Moreover, we reveal that *SsAtg8* may be the downstream target of SsFoxE3, which is also required for pathogenicity.

2 | RESULTS

2.1 | Characterization of SsFoxE3 in *S. sclerotiorum*

The FOX family TFs are important regulators in controlling the cell cycle, primary metabolism, and morphogenesis. Four putative FOX TFs exist in *S. sclerotiorum* (SsFoxE2, SsFoxE3, SsFkh1, SsFox1). SsFoxE2 deletion mutants are deficient in apothecial development (Wang et al., 2016), SsFkh1 is involved in sclerotial formation and pathogenicity (Fan et al., 2017), suggesting that FOX TFs are transcriptional regulators that are shared by multiple pathways. We therefore analysed the function of SsFoxE3 in *S. sclerotiorum*. To distinguish between the TFs, we constructed a phylogenetic tree of the conserved domain (Forkhead domain IPR001766) of FOX TFs from the pathogenic fungi *F. oxysporum*, *U. maydis*, and *M. oryzae*, which revealed that SsFoxE3, Umfox1, and AfFox are in the same branch (Figure 1a,b). Alignment of Forkhead domains showed the most conserved residues to be Y707, L724, Y728, W746, R751, H752, N753, and L754 in SsFoxE3 (Figure 1c). The SsFoxE3 protein sequence contains 1232 amino acids including the forkhead-associated (FHA) domain (IPR000253), which is a phosphopeptide recognition domain, suggesting that the protein has diverse functions (Figure 1b).
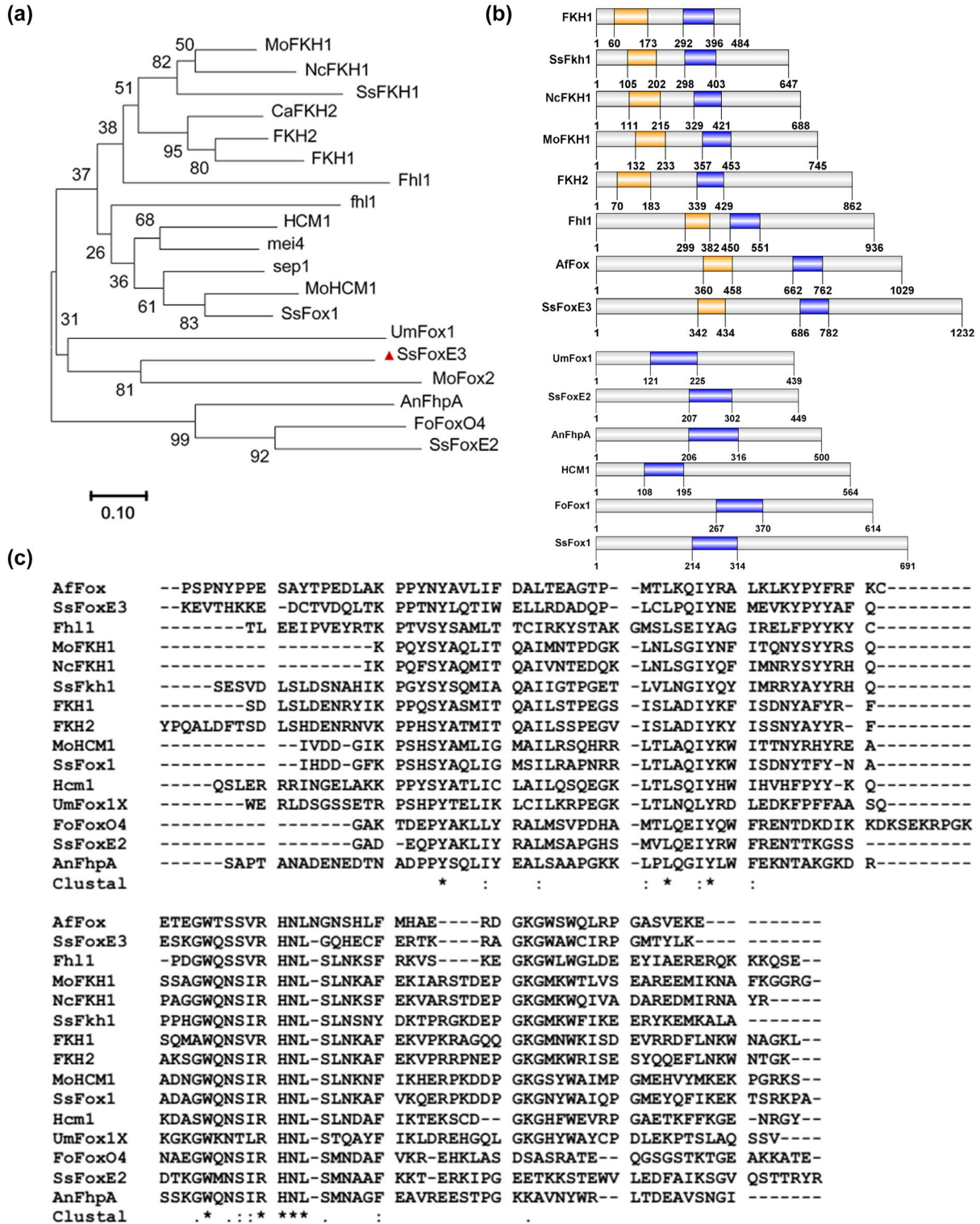
(a)

(b)

(c)

AfFox	--PSPNYPPE	SAYTPEDLAK	PPYNYAVLIF	DALTEAGTP-	-MTLKQIYRA	LKLKYPYFRF	KC--------
SsFoxE3	--KEVTHKKE	-DCTVDQLTK	PPTNYLQTIW	ELLRDADQP-	-LCLPQIYNE	MEVKYPYYAF	Q---------
Fhl1	--------TL	EEIPVEYRTK	PTVSYSAMLT	TCIRKYSTAK	GMSLSEIYAG	IRELFPYYKY	C---------
MoFKH1	----------	--------K	PQYSYAQLIT	QAIMNTPDGK	-LNLSGIYNF	ITQNYSYYRS	Q---------
NcFKH1	----------	-------IK	PQFSYAQMIT	QAIVNTEDQK	-LNLSGIYQF	IMNRYSYYRH	Q---------
SsFkh1	-----SESVD	LSLDSNAHIK	PGYSYSQMIA	QAIIGTPGET	-LVLNGIYQY	IMRRYAYYRH	Q---------
FKH1	--------SD	LSLDENRYIK	PPQSYASMIT	QAILSTPEGS	-ISLADIYKF	ISDNYAFYR-	F---------
FKH2	YPQALDFTSD	LSHDENRNVK	PPHSYATMIT	QAILSSPEGV	-ISLADIYKY	ISSNYAYYR-	F---------
MoHCM1	----------	--IVDD-GIK	PSHSYAMLIG	MAILRSQHRR	-LTLAQIYKW	ITTNYRHYRE	A---------
SsFox1	----------	--IHDD-GFK	PSHSYAQLIG	MSILRAPNRR	-LTLAQIYKW	ISDNYTFY-N	A---------
Hcm1	-----QSLER	RRINGELAKK	PPYSYATLIC	LAILQSQEGK	-LTLSQIYHW	IHVHFPYY-K	Q---------
UmFox1X	--------WE	RLDSGGSETR	PSHPYTELIK	LCILKRPEGK	-LTLNQLYRD	LEDKFPFFAA	SQ--------
FoFoxO4	----------	-------GAK	TDEPYAKLLY	RALMSVPDHA	-MTLQEIYQW	FRENTDKDIK	KDKSEKRPGK
SsFoxE2	----------	------GAD	-EQPYAKLIY	RALMSAPGHS	-MVLQEIYRW	FRENTTKGSS	----------
AnFhpA	------SAPT	ANADENEDTN	ADPPYSQLIY	EALSAAPGKK	-LPLQGIYLW	FEKNTAKGKD	R---------
Clustal			*	:	:	: * :*	:

AfFox	ETEGWTSSVR	HNLNGNSHLF	MHAE----RD	GKGWSWQLRP	GASVEKE---	-------
SsFoxE3	ESKGWQSSVR	HNL-GQHECF	ERTK----RA	GKGWAWCIRP	GMTYLK----	-------
Fhl1	-PDGWQSSVR	HNL-SLNKSF	RKVS----KE	GKGWLWGLDE	EYIAERERQK	KKQSE--
MoFKH1	SSAGWQNSIR	HNL-SLNKAF	EKIARSTDEP	GKGMKWTLVS	EAREEMIKNA	FKGGRG-
NcFKH1	PAGGWQNSIR	HNL-SLNKSF	EKVARSTDEP	GKGMKWQIVA	DAREDMIRNA	YR-----
SsFkh1	PPHGWQNSIR	HNL-SLNSNY	DKTPRGKDEP	GKGMKWFIKE	ERYKEMKALA	-------
FKH1	SQMAWQNSVR	HNL-SLNKAF	EKVPKRAGQQ	GKGMNWKISD	EVRRDFLNKW	NAGKL--
FKH2	AKSGWQNSIR	HNL-SLNKAF	EKVPRRPNEP	GKGMKWRISE	SYQQEFLNKW	NTGK---
MoHCM1	ADNGWQNSIR	HNL-SLNKNF	IKHERPKDDP	GKGSYWAIMP	GMEHVYMKEK	PGRKS--
SsFox1	ADAGWQNSIR	HNL-SLNKAF	VKQERPKDDP	GKGNYWAIQP	GMEYQFIKEK	TSRKPA-
Hcm1	KDASWQNSIR	HNL-SLNDAF	IKTEKSCD--	GKGHFWEVRP	GAETKFFKGE	-NRGY--
UmFox1X	KGKGWKNTLR	HNL-STQAYF	IKLDREHGQL	GKGHYWAYCP	DLEKPTSLAQ	SSV----
FoFoxO4	NAEGWQNSIR	HNL-SMNDAF	VKR-EHKLAS	DSASRATE--	QGSGSTKTGE	AKKATE-
SsFoxE2	DTKGWMNSIR	HNL-SMNAAF	KKT-ERKIPG	EETKKSTEWV	LEDFAIKSGV	QSTTRYR
AnFhpA	SSKGWQNSIR	HNL-SMNAGF	EAVREESTPG	KKAVNYWR--	LTDEAVSNGI	-------
Clustal	.* .::* ***		. :		.	

FIGURE 1 Comparison of Forkhead (FH) domains and the phylogenetic tree of fungal Forkhead-box family (FOX) transcription factors (TFs). (a) Dendrogram of FOX TFs. The phylogenetic tree was constructed by MEGA 7. GenBank accession numbers and sequence information are in Table S1. (b) Distribution map of Forkhead-associated (FHA) and FH domains in FOX TFs. the information of the FHA (orange) and FH (purple) domains were analysed by Interpro (http://www.ebi.ac.uk/interpro) and drawn by GPS 2.0. (c) Alignments of the conserved FH domains of FOX TFs by ClustalW. Identical amino acids residues are marked as *

2.2 | *SsFoxE3* contributes to sclerotia formation

To investigate the role of *SsFoxE3* in *S. sclerotiorum*, we constructed an SsFoxE3 mutant strain ΔSsFoxE3 by disruption with the hygromycin gene (Figure S1). First, we analysed the colony morphology of strain ΔSsFoxE3 by evaluating ΔSsFoxE3 and wild-type UF-1 strain growth on potato dextrose agar (PDA), minimal medium (MM), and complete medium (CM) for 48 h at 25°C. There was no

significant difference in colony diameter between ΔSsFoxE3 and wild-type UF-1 (Figure 2a,b). To investigate whether SsFoxE3 interferes with cell wall synthesis related to hyphal development, the cell wall synthesis inhibitors Congo red, calcofluor white (CFW), and sodium dodecyl sulphate (SDS) were added to the medium. In each case, growth of ΔSsFoxE3 was significantly inhibited compared to the wild type (Figure 2a,b). When we used CFW to stain the cell wall, obvious blue fluorescence was seen at the growing tip of the wild-type hyphae, while fluorescence in the mutant was diffuse, indicating that SsFoxE3 deficiency affected the normal development of the cell wall (Figure 2c,d). SsFoxE3 disruption also led to a decrease in the number of sclerotia (Figure 2e,f). The results indicate that SsFoxE3 may have an effect on the cell wall and affects the normal development of sclerotia.

2.3 | SsFoxE3 is required for compound appressoria formation and pathogenicity

Compound appressoria, play a vital role during the disease cycle of S. sclerotiorum (Fan et al., 2017; Kabbage et al., 2015). Compound appressoria formation of ΔSsFoxE3, the wild-type UF-1, and complemented strain SsFoxE3-C was determined on a hydrophobic interface after incubation for 48 h. The formation of brown compound appresoria by the SsFoxE3 mutant was significantly reduced, and the number of compound appressoria was reduced almost 7-fold (Figure 3a,b). The deficiency of SsFoxE3 also significantly reduced pathogenicity (Figure 3c). We ruled out the influence of oxalate (Figure S2). The pathogenicity of ΔSsFoxE3 onsoybean leaves was restored to a certain extent by wounding compared with that on unwounded leaves (Figure 3d,e). These results suggest that SsFoxE3 affects the development of the compound appressoria and the virulence of S. sclerotiorum.

There was no obvious difference in morphology between the wild type and ΔSsFoxE3 compound appressoria on the glass slides (Figure S3); however, we found that SsFoxE3 disruption led to developmental retardation of compound appressoria (Figure 4a). To further clarify the influence of SsFoxE3 deletion on compound appressoria development during the infection process, we compared the ability to penetrate onion cells by ΔSsFoxE3 and the wild-type strain. By staining with lactophenol blue 12 h after inoculation, it was found that the wild type formed a large group of compound appressoria (invasive hyphae) in onion cells but ΔSsFoxE3 did not (Figure 4b). These results indicate that the effect of SsFoxE3 deletion on the loss of pathogenicity is due to defects in the formation of compound appressoria.

2.4 | SsFoxE3 disruption caused hypersensitivity to reactive oxygen species

For plant pathogens to successfully colonize and further develop in host plants, they need a tolerance mechanism to overcome plant defence mechanisms (Chi et al., 2009). Therefore, to explore whether SsFoxE3 plays an active role in tolerance to osmotic stress and exogenous H_2O_2, strains were inoculated on 5, 10, 15, 20, and 25 mM H_2O_2-containing PDA. The hyphal growth of ΔSsFoxE3 was affected, the growth inhibition rate was higher than the wild type (Figure 5a,b). There was no significant difference in response to osmotic stress (Figure S4a). Subsequently, we used 3,3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining to measure the changes in H_2O_2 and O^{2-} accumulation in the host after inoculation, respectively. The DAB staining of the leaf tissue of the mutant after inoculation was higher than that of the wild type, and the NBT staining was similar to that of wild type (Figure S4b). Because of this difference, we measured the expression of reactive oxygen metabolism-related genes in the wild type and mutant, and found that SsSOD1 expression did not change significantly but Scat1 was significantly different. There were also significant differences in the expression of autophagy-related genes in the mutant (Figure S4c). These results indicate that the weakened pathogenicity of the mutant may be because it lacks the mechanism to detoxify active oxygen.

2.5 | SsFoxE3 transcriptional activation of autophagy-related genes during invasion

To investigate the function of SsFoxE3 in S. sclerotiorum pathogenicity, we inspected its expression during the infection stage of S. sclerotiorum by reverse transcription quantitative PCR (RT-qPCR). The expression of SsFoxE3 transcripts in invasive hyphae increased 2-fold by 5 h postinoculation (hpi) on soybean leaves (Figure 6a). The results suggest that SsFoxE3 is highly activated during infection.

SsFoxE3 is a transcriptional activator that can self-activate and make medium supplemented with X-α-gal appear blue (Figure S5). To clarify the effect of SsFoxE3 deletion on the expression of autophagy-related genes (SsAtg1, SsAtg4, SsAtg6, SsAtg8, and SsAtg9), we analysed their expression levels in the wild type and ΔSsFoxE3 during the infection process (Figure S4). The expression of SsAtg8 increased in the early stages of wild-type infection, but its expression in the ΔSsFoxE3 mutant was lower than that of the wild type, which indicates that SsFoxE3 deletion affected the normal expression of SsAtg8 (Figure 6b). Furthermore, yeast 1-hybrid (Y1H) analysis showed that SsFoxE3 interacted with the promoter of SsAtg8 (Figure 6c). These results indicate that SsAtg8 may be down-regulated in ΔSsFoxE3 during the infection process, which means SsFoxE3 could transcriptionally activate the expression of SsAtg8, enhancing autophagy to provide energy to form infectious hyphae.

2.6 | SsAtg8 is involved in the formation of compound appressoria, sclerotia, and pathogenicity of S. sclerotiorum

To clarify the role of autophagy in the growth and development of S. sclerotiorum, SsAtg8 was knock out. Compared with the wild type,
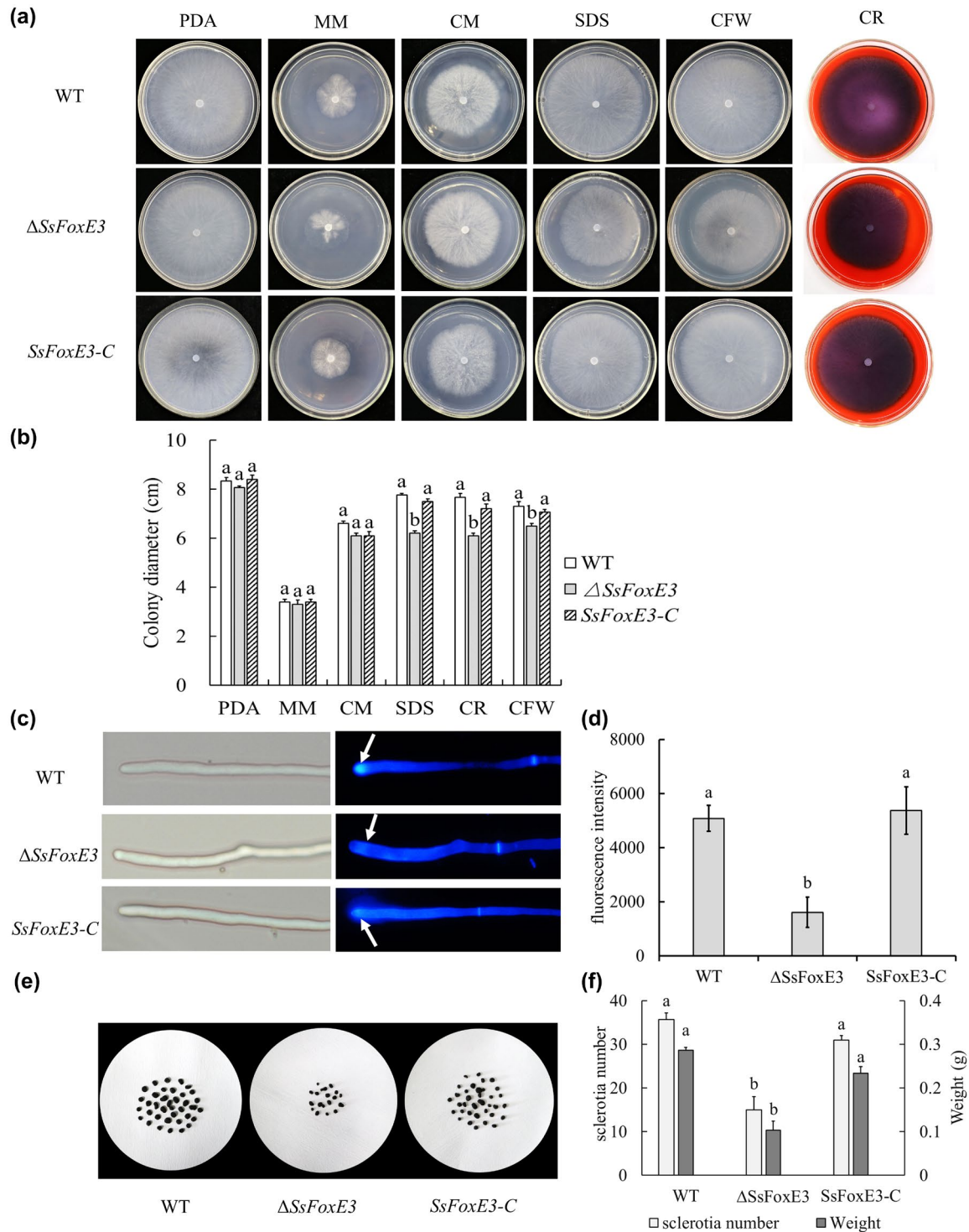
FIGURE 2 *SsFoxE3* contributes to cell wall and sclerotia formation. (a) Mycelial growth is not affected by SsFoxE3. Strains were inoculated on potato dextrose agar (PDA), minimal medium (MM), and complete medium (CM), and cultured for 48 h at 25°C. (b) Δ*SsFoxE3* is sensitive to cell wall synthesis inhibitors. Colony diameters of strains on medium amended with Congo red (CR), calcofluor white (CFW), or 0.001% sodium dodecyl sulphate (SDS) were measured by the cross method after 48 h. Error bars are the standard deviation, significant difference in data using SPSS software ($p < 0.05$). Each set of experiments was repeated three times. (c) *SsFoxE3* is involved in the formation of the cell wall. The tips of hyphae were stained with CFW and observed under the fluorescence microscope. Arrows show the chitin content at the top of the mycelial growth. (d) The fluorescence intensity assay of strains. ImageJ was used to analyse the fluorescence intensity after staining by CFW. Different letters represent statistically significant differences (Student's *t* test, $p < 0.05$). (e) Sclerotial formation was reduced in Δ*SsFoxE3*. Strains were grown on PDA and cultured for 2 weeks at 25°C. (f) The number and the weight of sclerotia were measured and subjected to statistical analysis. Three repeats were performed and error bars represent the standard deviations. Different letters represent statistically significant differences (Student's *t* test, $p < 0.05$)
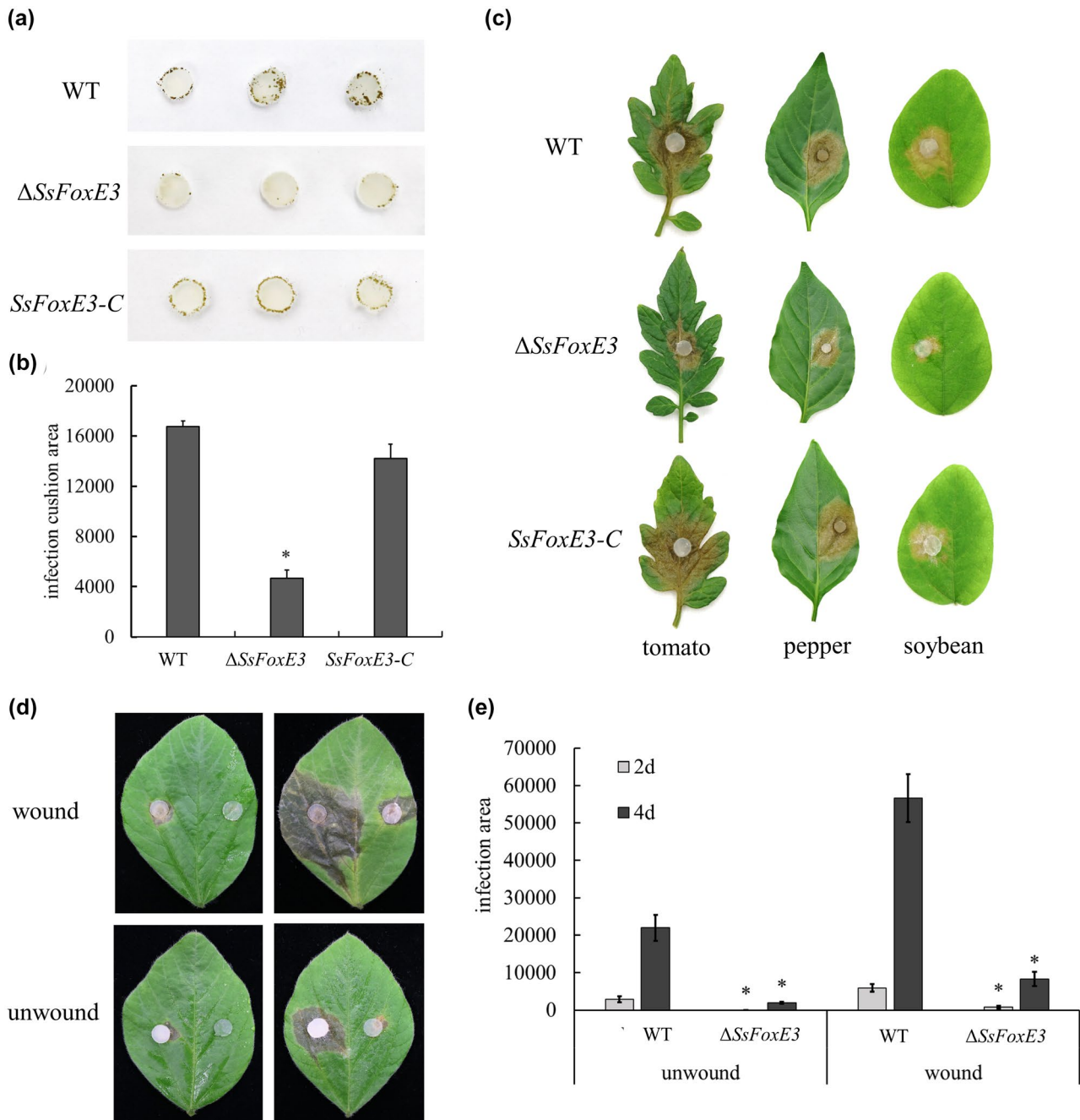
FIGURE 3 *SsFoxE3* contributes to compound appressoria formation and pathogenicity. (a) Development of compound appressoria on a hydrophobic interface. Strains were grown on potato dextrose agar (PDA) for 48 h, then an agar disk containing mycelium (0.5 mm diameter) was inoculated on a glass slide, moisturized, then cultured for 48 h. (b) Statistical analysis of compound appressoria formation. The compound appressoria produced by strains were measured by ImageJ. Error bars represent standard deviations. *Significant differences from compound appressoria of wild type at α = 0.05 (Student's *t* test). (c) The *SsFoxE3* deletion mutant showed reduced virulence on host leaves. Agar disks containing mycelium (0.5 mm diameter) of strains were inoculated on tomato, pepper, and soybean leaves. Leaves were harvested 48 h after inoculation. (d, e) Pathogenicity of Δ*SsFoxE3* on unwounded and wounded soybean leaves. These experiments were performed three times with 10 pieces of soybean leaves. *Significant differences from wild type at α = 0.05 (Student's *t* test)

SsAtg8 deficiency reduced the formation of sclerotia (Figure S6). In Δ*SsAtg8* and in Δ*SsFoxE3*, the number and the weight of sclerotia was significantly reduced on minimal medium, indicating that both of them were necessary for the development of sclerotia when growing under conditions of nutritional deficiency. The formation of compound appressoria decreased in Δ*SsAtg8* compared to the wild type (Figure 7a–c). After inoculation of leaves, the pathogenicity of *SsAtg8* mutant was significantly decreased, and was recovered by the complemented strain *SsAtg8*-C (Figure 7d,e). The phenotype of the *SsAtg8* mutant indicated that the effect of *SsFoxE3* on sclerotia formation and infection may be related to the autophagy pathway.
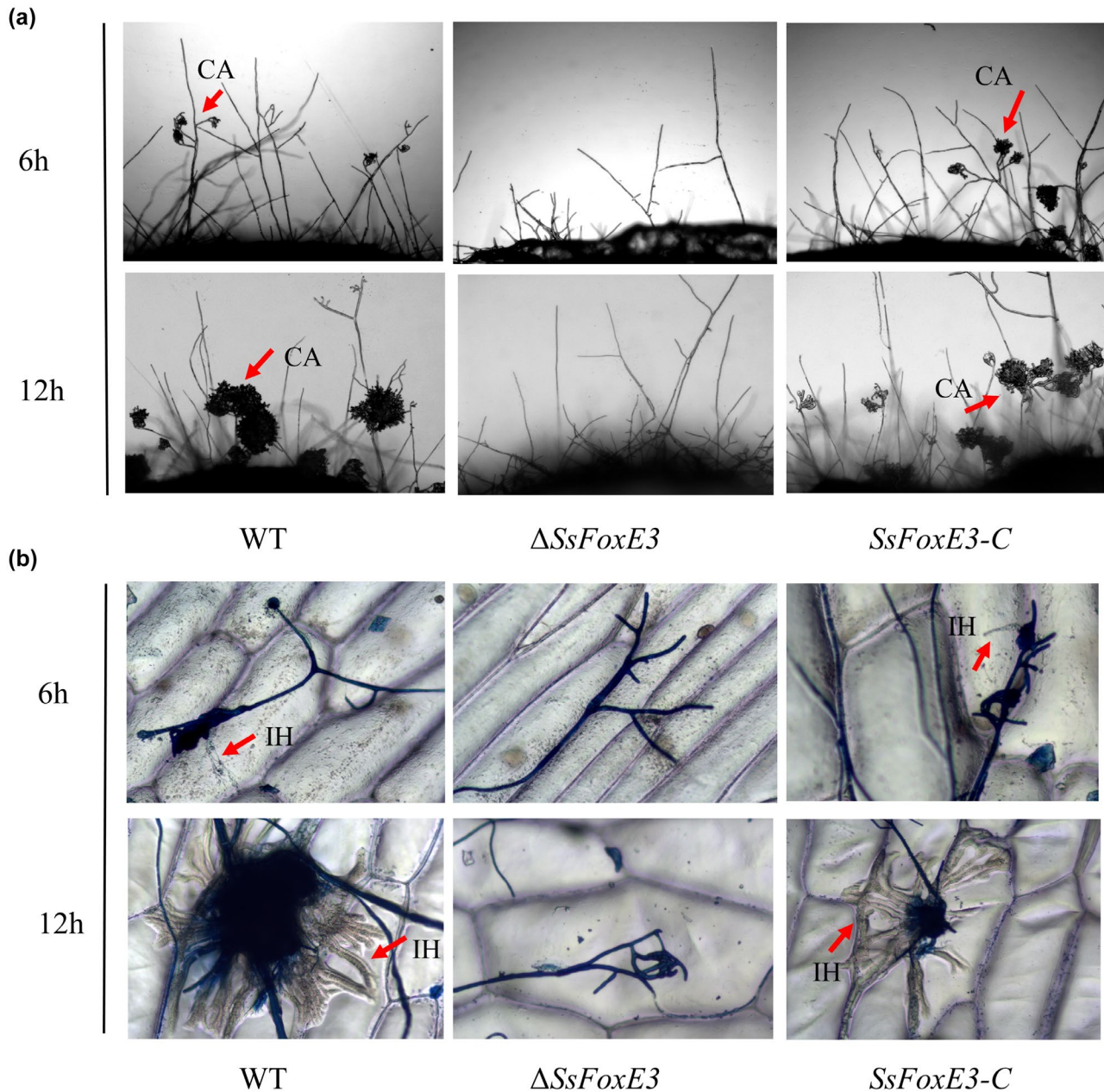
(a)

(b)

FIGURE 4 *SsFoxE3* affects the formation of invasion hyphae. (a) Development of compound appressoria on a glass slide. The compound appressoria were induced by placing mycelial blocks on a glass slide for 6 and 12 h. (b) Penetration assay of the *SsFoxE3* mutant on onion epidermis cell. The invasion hypha was stained with lactophenol blue and observed using differential interference contrast microscopy. Arrows indicate compound appressoria (CA) or invasive hyphae (IH) inside cells

2.7 | *SsFoxE3* response to N starvation-activated autophagy

The generation and modulation of reactive oxygen species is essential for *S. sclerotiorum* during pathogenic development, successful colonization, and fungal development (Ding et al., 2020; Huang et al., 2021; Liang & Rollins, 2018). To examine whether *SsAtg8* is involved in H_2O_2 tolerance, the wild type was treated with different H_2O_2 concentrations then changes in the expression level of SsAtg8 were measured. The growth of the Δ*SsAtg8* mutant was significantly inhibited by 10 mM H_2O_2 (Figure S7). In addition to H_2O_2 induction,

N starvation is the main condition for autophagy induction. Thus, we used minimal medium without nitrogen source (MM−N) medium for Δ*SsAtg8* and Δ*SsFoxE3* culture and found that the growth of Δ*SsAtg8* and Δ*SsFoxE3* was restricted (Figure 8a,b), indicating that H_2O_2 and nitrogen starvation could affect the autophagy of *S. sclerotiorum*.

The autophagy process was observed using green fluorecent protein (GFP)-tagged SsAtg8. Autophagosome formation of the wild type and Δ*SsFoxE3* after N starvation-induced autophagy was observed under the microscope. It was found that autophagosomes in Δ*SsFoxE3* were decreased, and after induction on MM−N the number of autophagosomes in Δ*SsFoxE3* were significantly lower
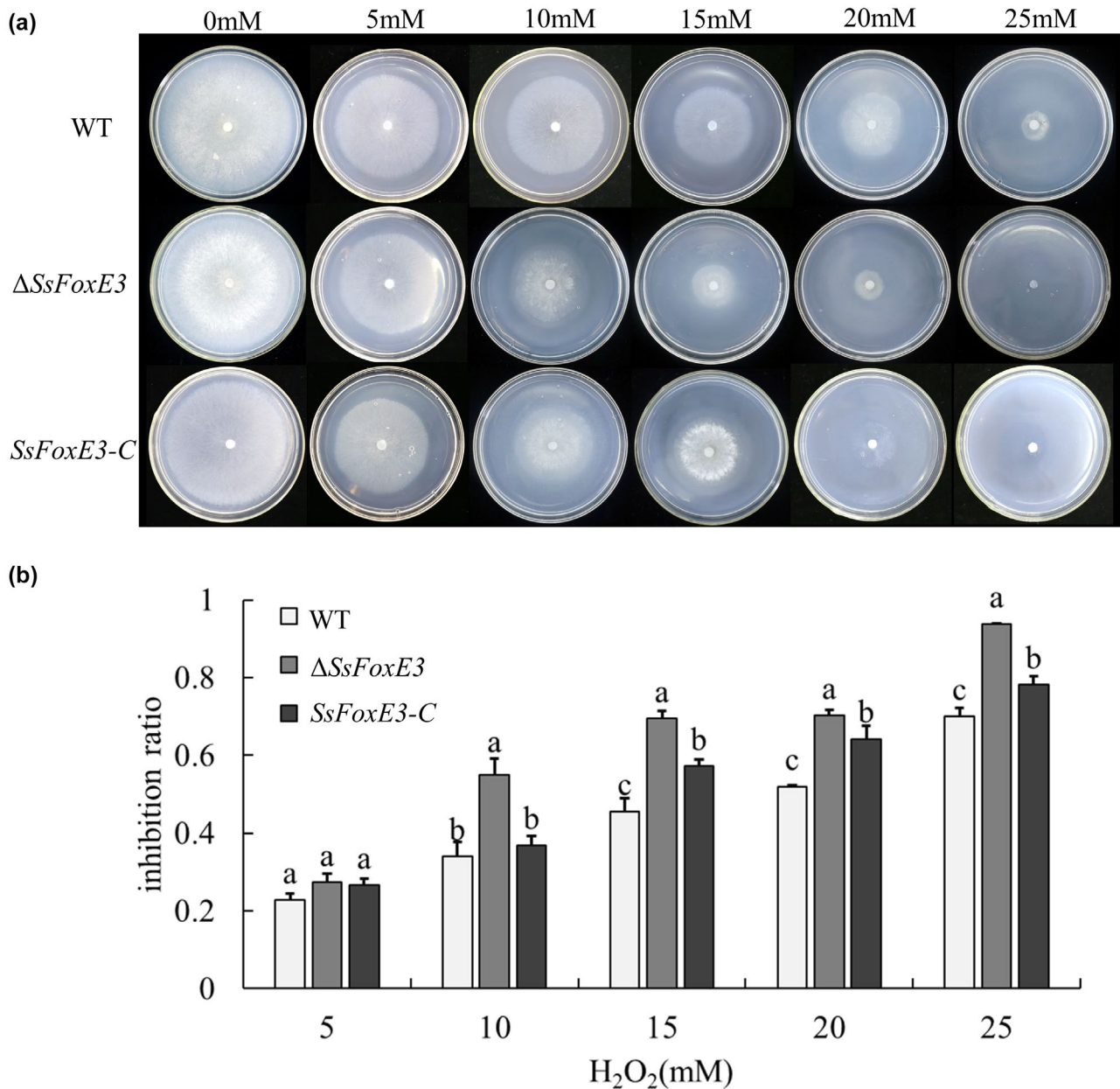
(a)

(b)

FIGURE 5 The *SsFoxE3* deletion mutant is hypersensitive to oxidative stress. (a) Colonial morphology and mycelial growth of Δ*SsFoxE3* under different concentrations of H_2O_2. (b) Growth inhibition rate of Δ*SsFoxE3*. Inhibition rate (%) = 100 × (colonial diameter of strain without H_2O_2 – colonial diameter of strain with H_2O_2)/(colonial diameter of strain without H_2O_2). Error bars are standard deviation, different letters represent statistically significant differences (Student's *t* test, $p < 0.05$)

than that of WT (Figure 8c–e). The results suggest that SsFoxE3 is involved in the autophagy pathway, and that the autophagy pathway is involved in the process of sclerotia formation and infection.

3 | DISCUSSION

TFs are often the downstream effectors of signalling cascades and initiate the transcription and expression of specific genes, finally regulating responses to internal and external signals through the effect of gene products and playing an important regulatory role in

environmental responses such as growth and development, morphogenesis, and biological and abiotic stress (Cho et al., 2012; van der Does et al., 2016; John et al., 2021). TFs have been widely used as novel antifungal drug targets (Bahn, 2015). FOX TF SsFkh1 is critical for sclerotia and pathogenicity in *S. sclerotiorum* (Fan et al., 2017). SsFoxE2 is involved in sexual development and affects the development of ascomycetes (Wang et al., 2016). In *M. oryzae*, deficiency of *MoFKH1* affects mycelial growth, conidial germination, and its pathogenicity, while deficiency of *MoHCM1* results in mycelial growth and conidial germination (Park et al., 2014). FKH1, FKH2, HCM1, and FHL1 play different roles in *Saccharomyces cerevisiae* (Arsenault

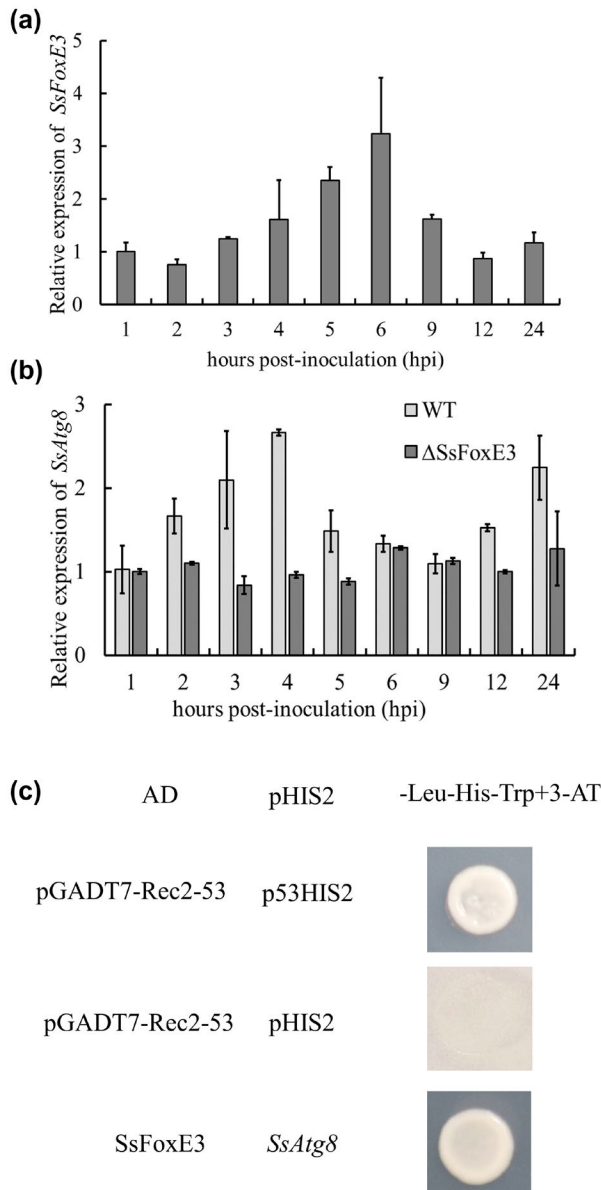**(a)**

(b)

(c)

FIGURE 6 SsFoxE3 transcriptional activation of an autophagy-related gene, *SsAtg8*, during invasion. (a) Expression of *SsFoxE3* during invasion. (b) *SsAtg8* gene expression analysis in the Δ*SsFoxE3* mutant and wild type strain (WT) during invasion. (c) SsFoxE3 transcriptional activation of *SsAtg8*. Yeast 1-hybrid analysis showed SsFoxE3 interacted with the promoter of *SsAtg8*. 3-amino-1,2,4-triazole (3-AT) was added to inhibit SsFoxE3 self-activation

et al., 2015; Martin et al., 2004; Pataki et al., 2017; Postnikoff et al., 2012). Our study of the function of SsFoxE3 is expected to further clarify the division and cooperation of the FOX TFs in *S. sclerotiorum*. First of all, we analysed the domain sequence of FOX TFs in *S. sclerotiorum* and other plant-pathogenic fungi, and found that SsFoxE3 had the same domain distribution as SsFkh1 and MoFKH1 of *M. oryzae*, as well as FKH1 and FKH2 in yeast (Figure 1b). In addition, we conducted a *cis*-acting regulatory element analysis on the promoter

of SsFoxE3 and found that the promoter region contains light responsive (ATCT-motif, Bo x 4, Box II, G-Box, TCT-motif, GT1-motif), defence and stress responsive (TC-rich repeats), methyl jasmonate (MeJA) responsive (TGACG-motif and CGTCA-motif), and GCN4 motifs, which provides ideas for the subsequent clarification of the expression response of SsFoxE3.

Further study on the function of SsFoxE3 showed that the number and weight of sclerotia of the mutant were significantly reduced, the formation of compound appressoria was abnormal, and the pathogenicity of the mutant was lower than that of the wild type.

The pathogenicity of the Δ*SsFoxE3* mutant was reduced. Inoculation and staining revealed that the reduction of pathogenicity was related to the development of the compound appressoria and the formation of infectious hyphae, but was not related to the production of oxalic acid (Figure S2). Subsequently, the expression levels of *SsFoxE3* and pathogenicity-related genes were measured at different inoculation times, and it was found that the expression of *SsAtg8* was reduced during the Δ*SsFoxE3* infection process (Figure 6b). As an autophagy-related protein, Atg8 is usually used as a marker protein to monitor the occurrence of autophagy (Liu et al., 2012, 2016). SsFoxE3 localizes in the nucleus (Figure S5a) and has transcriptional activation activity (Figure S5b). In RT-qPCR and Y1H assays SsFoxE3 activated the transcription of *SsAtg8* in response to starvation signals (Figures 6c and 8c). In addition, the *SsAtg1*, *SsAtg8*, and *Scat1* genes in the *SsFoxE3* mutant were still expressed, indicating that there are other TFs involved besides SsFoxE3 (Figure S4c).

Autophagy processes, which are highly conserved cellular processes, degrade cytoplasmic constituents in vacuoles and play important roles in filamentous fungal pathogenicity (Zhu et al., 2019). Subsequently, we analysed the biological characteristics of Δ*SsAtg8*. After deletion of *SsAtg8*, the number of sclerotia was reduced (Figure S6), the compound appressoria were defective, and the pathogenicity was reduced (Figure 7). The phenotype was similar to that of the Δ*SsFoxE3* mutant. To further examine the relationship between *SsFoxE3* and autophagy, we used a GFP-SsAtg8 fusion protein as a marker. Under N starvation, the number of autophagosomes in the wild type was significantly higher than in Δ*SsFoxE3*, further indicating that *SsFoxE3* is involved in the autophagy pathway (Figure 8c).

In the infection cycle of *S. sclerotiorum*, compound appressoria are important as an infection structure. They directly determine the parasitic relationship and the degree of disease. Autophagy of filamentous fungi is important not only to their nutritional balance, but also to developmental processes such as cell differentiation, secondary metabolism, and pathogenicity. Understanding the role of the SsFoxE3 TF in the regulation of *S. sclerotiorum* compound appressoria and autophagy is not only helpful for exploring the classic autophagy model, but also for the development of *S. sclerotiorum* compound appressoria and provides new clues for pathogenicity. However, the molecular mechanism of autophagy affecting the pathogenicity of *S. sclerotiorum* still needs further study, and the developmental mechanism of its sclerotia requires further analysis.
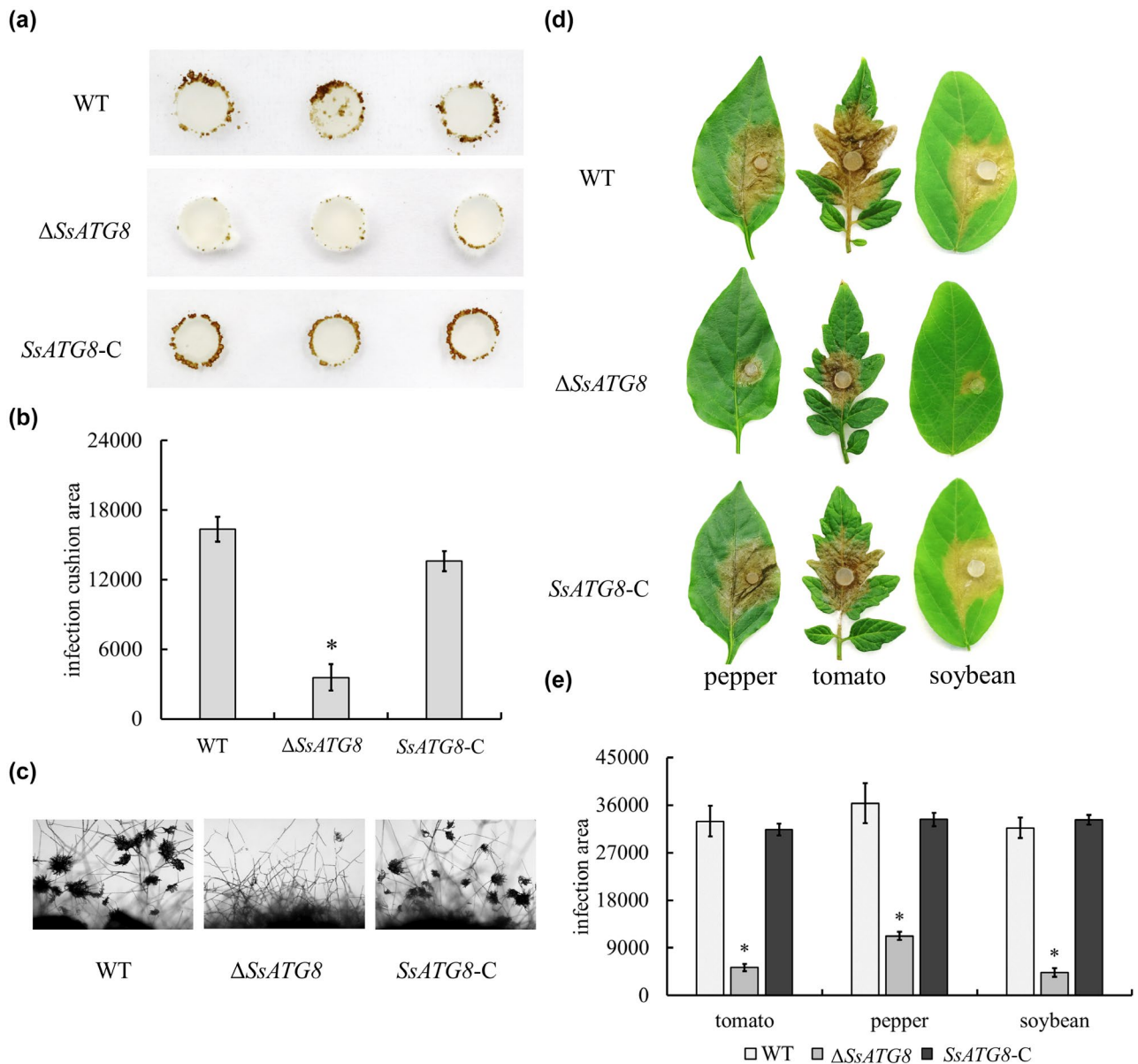
FIGURE 7 *SsAtg8* contributes to compound appressoria formation and pathogenicity. (a) Development of compound appressoria on a hydrophobic interface. Strains were grown on potato dextrose agar for 48 h, then an agar disk containing mycelium (0.5 mm diameter) was inoculated on a sterilized glass slide, moisturized, and cultured for 48 h. (b) Statistical analysis of compound appressoria formation was measured by ImageJ. *Significant differences compared to compound appressoria of wild type (WT) at α = 0.05 (Student's *t* test). (c) Microscopic observation of compound appressoria formation. (d) Pathogenicity test of Δ*SsAtg8* on pepper, tomato, and soybean leaves. (e) Statistical analysis of the lesion area. The experiments were performed three times with 10 pieces of host leaves for each strain. *Significant differences from the wild-type strain at α = 0.05 (Student's *t* test)

4 | EXPERIMENTAL PROCEDURES

4.1 | Strains and culture condition

S. sclerotiorum UF-1 was used as the wild-type strain in this study. Δ*SsFoxE3* and *SsFoxE3*-C (complemented strain), Δ*SsAtg8* and Δ*SsAtg8*-C, WT::GFP-SsAtg8, and Δ*SsFoxE3*::GFP-SsAtg8 were initially grown on PDA (200 g potato, 20 g glucose, 15 g agar per litre) at 25°C.

4.2 | Identification and sequence analysis of *SsFoxE3*

The phylogenetic tree was inferred using the neighbour-joining method. The associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches and the evolutionary analyses were conducted in MEGA 7. The domains contained in the sequence were analysed by Interpro (http://www.ebi.ac.uk/interpro) and visualized by GPS.

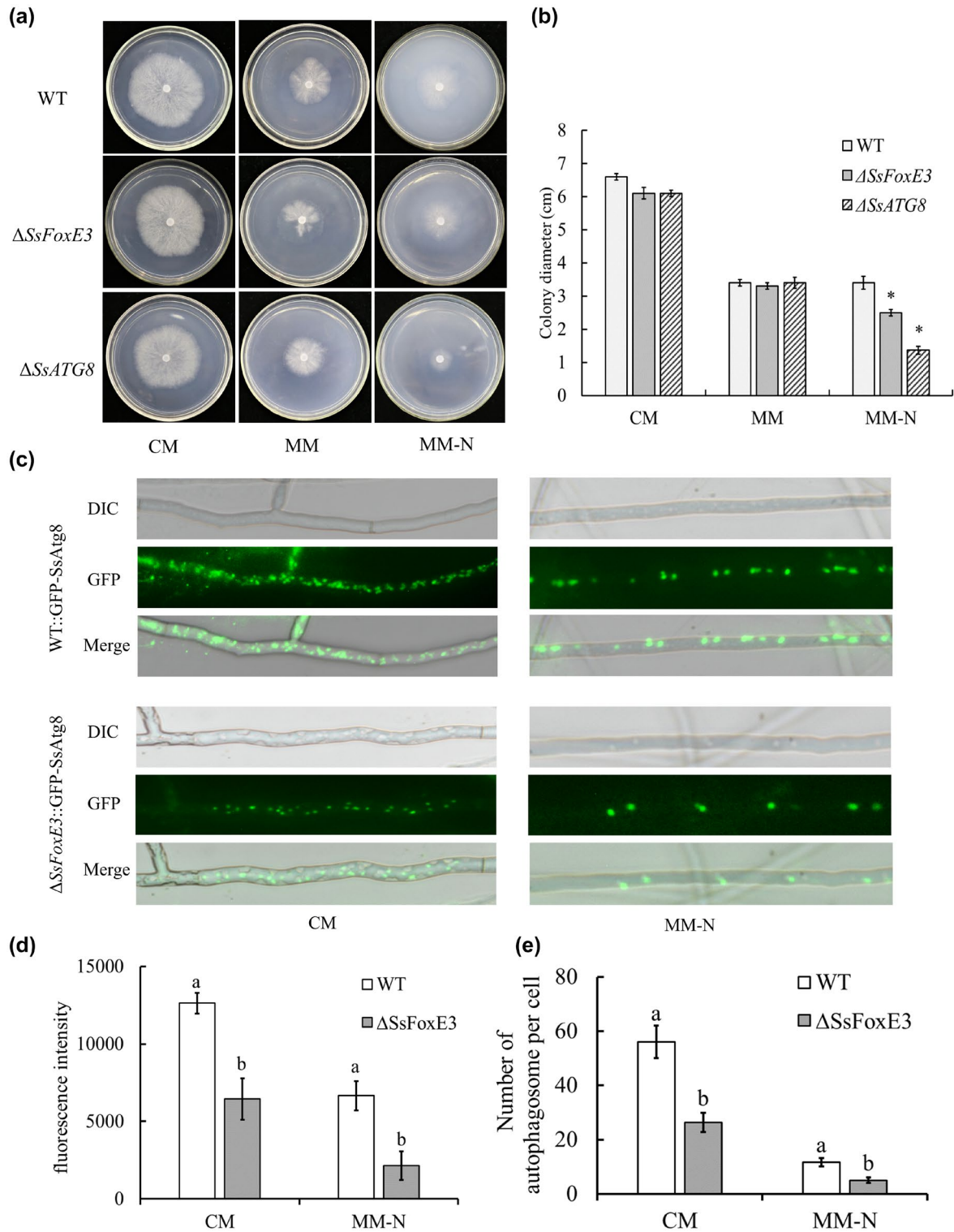**FIGURE 8** SsFoxE3 in response to N starvation and activated autophagy. (a) Colony morphology of ΔSsFoxE3 and Δ SsAtg8 incubated on complete medium (CM), minimal medium (MM), and minimal medium without nitrogen source (MM−N at 25°C for 48 h. (b) Colony diameters of ΔSsFoxE3 and Δ SsAtg8 incubated on CM, MM, and MM-N for 48 h. Error bars are standard deviation and * indicates significant differences from the wild-type strain at α = 0.05 (Student's t test). (c) Observation of the formation of autophagosomes in WT::GFP-SsAtg8 and ΔSsFoxE3::GFP-SsAtg8. WT::GFP-SsAtg8 and ΔSsFoxE3::GFP-SsAtg8 were first grown in liquid CM at 25°C for 24 hr, then transferred to liquid MM−N for 6 hr. (d) Fluorescence intensity assay of WT::GFP-SsAtg8 and ΔSsFoxE3::GFP-SsAtg8. ImageJ was used to analyse the fluorescence intensity by split channels. (e) The number of autophagosomes per cell. The experiments were performed three times with 20 cells of each strain. Different letters represent statistically significant differences (Student's t test, $p < 0.05$)

4.3 | Cell integrity and stress treatment

Cell integrity of strains were determined by culture on PDA with Congo red (CR, 300 mg/ml), CFW (30 μg/ml), 0.001% SDS, 1 M glucose, 1 M sorbitol, 1 M KCl, and 1 M NaCl. For the analysis of oxidative stress, strains were inoculated on PDA with different concentrations of H_2O_2 (5, 10, 15, 20, 25 mM) (Huang et al., 2021). For testing nutrient uptake, strains were grown on CM (0.2 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$, 0.15 g NaCl, 1 g $Ca(NO_3)_2.4H_2O$, 10 g glucose, 1 g yeast extract, 1 g casein hydrolysate, 15 g agar) and MM (without 1 g yeast extract and 1 g casein hydrolysate) and MM−N (i.e., MM without $Ca(NO_3)_2.4H_2O$). To observe oxalic acid content, strains were grown on PDA with 100 μg/ml bromophenol blue (Li et al., 2018; Liu et al., 2018). The colony diameters were measured after 48 h and the inhibition of hyphal growth was calculated. Each experiment was repeated three times.

4.4 | Analysis of virulence and the compound appressoria

To evaluate the virulence, mycelia-colonized plugs (0.5 mm diameter) from wild type, ΔSsFoxE3, SsFoxE3-C, ΔSsAtg8, and SsAtg8-C were harvested from colony margins of 48 h-old PDA cultures and then inoculated on tomato, pepper, and soybean leaves. The diameter of the lesions were measured after 48 h and each experiment was repeated three times. Compound appressoria of strains were observed on hydrophobic (glass slide) and onion surfaces, stained with lactophenol cotton blue (Sigma). The formation of compound appressoria was observed at 6 and 12 hpi under a microscope. The experiments were conducted three times.

4.5 | Plasmid constructs and transformation

Plasmid pXEH-SsFoxE3, which was used to generate the SsFoxE3 knock-out transformants, was constructed as follows: plasmid pXEH, containing the HYG marker for hygromycin resistance, was used as the vector backbone. Oligonucleotides SsFoxE3F1EcoRI/ SsFoxE3R1XhoI (5′-GAATTCTGTATTGGATAGAGATGTTG-3′/5′ -CTCGAGTCGTCCACCTTCTATTGT-3′) and SsFoxE3F2 BamHI/ SsFoxE3R2PstI (5′-GGATCCCATTTCGTGGGAAGTAAG-3′/5′-CT GCAGGGATCCTATGGGCTGCGATGTGAT-3′) were used to amplify the 5′ and 3′ fragments of SsFoxE3, which was digested with EcoRI/XhoI and PstI/BamHI, respectively, and cloned into pXEH to construct pXEH-SsFoxE3. pXEH-SsAtg8 was constructed as before with SsAtg8F1EcoRI/SsAtg8R1KpnI (5′-GAATTCTCGGTCCT GGGTAACTAT-3′/5′-GGTACCGGGAAAGGGTATGGGAGT-3′) and SsAtg8F1BamHI/SsAtg8R1PstI (5′-GGATCCGAGGCGGATGATGGA TAG-3′/5′-CTGCAGAGTGGAATGAGCGGAACA-3′). These plasmids were used to transform S. sclerotiorum UF-1 using the polyethylene glycol (PEG)-mediated transformation method (Rollins, 2003) and

protoplasts of S. sclerotiorum were prepared as described previously (Qu et al., 2014). Plasmids pYF11::RP27::eGFP::SsAtg8 and pYF11::RP27::eGFP::SsFoxE3 were used for the overexpression of mutants. Transformants were purified by using hygromycin (100 μg/ml) and G418 (100 μg/ml) selection of hyphal tips at least five times, respectively.

4.6 | RT-qPCR analysis

Gene expression during infection was observed in inoculated soybean leaves at hourly intervals. The RNA of test samples was extracted using a TransZol Up Plus RNA Kit and cDNA synthesis using EasyScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) (TransGen Biotech). Quantitative expression assays were performed using TransStart Green qPCR SuperMix (TransGen Biotech). Primers used for qPCR are shown in Table S2 and actin was used as internal reference.

4.7 | Yeast 1-hybrid assays

The yeast 1-hybrid (Y1H) assay is a method to analyse the interaction between DNA and proteins in vitro. The bait construct was generated by cloning the cDNA sequence of SsFoxE3 into pGADT7, the prey constructs were generated by cloning ProSsATG8 into pHIS2, and the pairs were cotransformed into yeast strain Y187 and grown on SD−Leu−His medium, then transferred to SD−Leu−His− Trp + 3-amino-1,2,4-triazole. The positive and negative control strains were obtained from the BD library construction and screening kit.

4.8 | Autophagy assay

To visualize the autophagic process in S. sclerotiorum, GFP-SsAtg8 fusion protein plasmid pYF11::RP27:: eGFP::SsAtg8 was constructed and transformed into the wild-type UF-1 and ΔSsFoxE3. The autophagosomes were observed in the mycelia of WT::GFP-SsAtg8 and ΔSsFoxE3::GFP-SsAtg8, which were cultured in liquid CM at 25°C for 24 h then transferred to liquid MM−N for 6 h and viewed under a fluorescence microscope.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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