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Stress responsive glycosylphosphatidylinositol-anchored protein SsGSP1 contributes to *Sclerotinia sclerotiorum* virulence

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ABSTRACT

Fungal cell wall acts as a defense barrier, shielding the cell from varying environmental stresses. Cell wall proteins, such as glycosylphosphatidylinositol (GPI)-anchored proteins, are involved in swift and appropriate responses to minor environmental changes in fungi. However, the roles of these proteins in the pathogenic *Sclerotinia sclerotiorum* remain largely unexplored. Here, we identified a novel GPI-anchored protein in *S. sclerotiorum*, SsGSP1, comprising a Kre9_KNH domain. *SsGSP1* was upregulated during infection, and the loss-of-function mutants of *SsGSP1* exhibited the compromised cell wall integrity and reduced β-glucan content. During inoculation on *Arabidopsis thaliana*, *Nicotiana benthamiana*, and *Brassica napus*, the *SsGSP1*-deletion strains demonstrated the decreased virulence. The transgenic *A. thaliana* line carrying the sRNA targeting *SsGSP1* enhanced resistance to *S. sclerotiorum* via Host-Induced Gene Silencing (HIGS). The *SsGSP1*-deficient strains displayed the heightened sensitivity to various stresses, including osmotic pressure, oxidative stress, and heat shock. The yeast two-hybrid and BiFC assays confirmed that SsGSP1 interacted with the key stress-related proteins catalase SsCat2, heat shock protein Sshsp60, and ABC transporter SsBMR1. Accordingly, transcriptome analysis revealed that the disruption of *SsGSP1* downregulated the expression of genes involved in oxidative stress response, heat shock response, and chemical agent resistance. These results collectively delineate the intricate role of GPI-anchored protein SsGSP1 in β-glucan, cell wall integrity, and virulence and may act as a potential surface sensor to elicit signal transduction in response to environmental stresses in *S. sclerotiorum*.

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Introduction

Sclerotinia sclerotiorum, a necrotrophic phytopathogenic fungus, poses a significant threat by infecting more than 600 plant species, including rapeseed, peanut, soybean, and dry pea, leading to substantial yield and quality losses [1–4]. The extensive damage inflicted by *S. sclerotiorum* is exacerbated by the absence of high-level host resistance and the intricate nature of its pathogenic mechanisms.

Currently, the extensive studies of pathogenic factors in *S. sclerotiorum* have revealed a complex pathogenic mechanism for *S. sclerotiorum*. *S. sclerotiorum* acidifies its ambient environment by producing oxalic acid [5]. The cell wall degrading enzymes secreted by *S. sclerotiorum* induce the degradation of plant cell walls [6–8]. The NADPH oxidases such as SsNox1 and thioredoxin SsTrx1 affect the virulence of *S. sclerotiorum* by regulating reactive oxygen species (ROS) [9,10]. Additionally, effectors such as SsCP1, SSITL, SsSSVP1, and SsPINE1 are involved in the plant-*S. sclerotiorum* interaction during infection [11–14]. However, there are 14,522 genes in the genome of *S. sclerotiorum* [15], and the function of numerous genes still remains unknown.

Fungal cell wall is a dynamic structure that plays essential roles in morphology, development, virulence, and pathogen–host interactions and is mainly comprised chitin, β-glucan, and cell wall proteins [16,17]. KRE9 and KNH1 proteins with the Kre9_KNH domain (pfam: PF10342) at the N-terminal are involved in encoding secreted or cell surface O-glycoproteins, required for cell wall β-1,6-glucan synthesis in yeast [18–20]. Glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) form a class of cell wall proteins that are essential for cell adhesion, enzyme catalytic activity, or cell wall integrity [16]. A few GPI-APs

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have been identified in fungi. *Candida albicans* GPI-AP Hwp1 and *Aspergillus fumigatus* GPI-AP Afu3g00880 are both involved in fungal adhesion and invasion [21–23]. In addition, *C. albicans* GPI-APs CRH, and ECM33 are involved in cell wall integrity and virulence [24,25]. A GPI-anchored superoxide dismutase (SOD) in *C. albicans* is related to the regulation of fungal redox status [26]. *Cryphonectria parasitica* GPI-AP CpGap1 exists antioxidizing properties and is involved in the response to ROS [27]. Moreover, GPI-APs in *Magnaporthe oryzae* and *Ustilaginoidea virens* are reported to evade host defenses or elicit plant immunity [28,29].

In this study, we identified a novel GPI-AP, SsGSP1, with a Kre9_KNH domain in *S. sclerotiorum*, and characterized its roles in β-glucan, cell wall integrity, stress response and pathogenicity. Furthermore, we demonstrated that host plant expressing hairpin (hp) RNAs targeting *SsGSP1* enhanced Sclerotinia resistance. Our research contributes to an understanding of GPI-APs with a Kre9_KNH domain as virulence factors in the pathogenic mechanism of *S. sclerotiorum* and provides a potential target for the safety control of Sclerotinia stem rot.

Materials and methods

Fungal strains and plants

The *S. sclerotiorum* wild-type strain 1980 [15], provided by Dr. Yang Yu (Southwest University), was cultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit). Transformants were cultured on PDA supplemented with 300 μg/mL hygromycin B (Yeasen, Shanghai, China) or 100 μg/mL G418 (Coolaber, Beijin, China). The wild-type *Arabidopsis thaliana* (Columbia ecotype, Col-0) and transgenic lines were grown in a chamber with 16 h/22°C at day with 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and 8 h/16°C at night.

Bioinformatic analysis

The *SsGSP1* gene was characterized from the genomic sequence database of *S. sclerotiorum* (http://www.ncbi.nlm.nih.gov/bioproject/15530). Protein domains were predicted by Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The signal peptide sequence, transmembrane domain, and subcellular localization were predicted using SignalP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP/), TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), and WoLF PSORT (https://www.genscript.com/wolf-psort.html), respectively. The identification of GPI-anchor signals was predicted by GPI-SOM (http://gpi.unibe.ch/) and PredGPI (http://gpcr.biocomp.unibo.it/predgpi/pred.htm).

RNA extraction and quantitative real-time PCR

Total RNA of fungi and plants was extracted with the Ultrapure RNA Kit (Cwbio, Jiangsu, China), and the first-strand cDNA was synthesized using MonScript™ RTIII All-in-One Mix with dsDNase (Monad, Wuhan, China). qRT-PCR was carried out using the Bio-Rad CFX96 Real-Time System (America) and SYBR Green I technology according to the manufacturer's instructions. *Sstub1* (*SS1G_04652*) was used as the internal control. The transcript level of the genes of interest was calculated from the threshold cycle the $2^{-\Delta\Delta CT}$ method [30] with three independent replicates. The primers are listed in Table S1.

Signal peptide of SsGSP1

The feature of the signal peptide of SsGSP1 was validated using the YTK12 yeast invertase secretion assay as described previously [8]. Briefly, the signal peptide sequence of SsGSP1 was predicted and ligated into the pSUC2 vector with the restriction sites *Eco*R I and *Xho* I. The vector pSUC2-SsGSP1 was transferred into the yeast strain YTK12. Transformants were grown on YPDA medium (1% yeast extract, 2% peptone, 2% glucose, 0.003% adenine hemisulfate, 2% agar powder), CMD-W medium (0.67% yeast nitrogen base without amino acids, 0.075% -Trp DO supplement, 2% sucrose, 0.1% glucose, 2% agar powder), and YPRAA medium (1% yeast extract, 2% peptone, 2% raffinose, 2% agar powder, and 2 μg/mL antimycin A) to evaluate the secretory function. Invertase secretion was detected by examining yeast cultures grown in YPDA liquid medium using the 2,3,5-triphenyltetrazolium chloride (TTC) assay. The YTK12 strain transformed with pSUC2-Avr1b was used as a positive control, while pSUC2-Mg87 and the untransformed YTK12 strain were used as negative controls.

Construction of gene-knockout vector and transformation in S. sclerotiorum

The *SsGSP1* in *S. sclerotiorum* was knocked out using the split-marker method as described by Yang et al. [11]. The upstream fragment *SsGSP1*-5' and the downstream fragment *SsGSP1*-3' of the gene were amplified from the genome of *S. sclerotiorum* with primers P1/P2 (contain *Sac* I/*Not* I site) and T1/T2 (contain *Spe* I/*Apa* I site), respectively. The amplicons were cloned into the

vector pSKH [31] by homologous recombination. Primers P1/hy and yg/T2 were used to amplify two sequences, *SsGSP1*-5′-hy and yg-*SsGSP1*-3′, with truncated hygromycin-resistant gene fragments, respectively. The purified *SsGSP1*-5′-hy and yg-*SsGSP1*-3′ were mixed in equal molar quantities and used for protoplast transformation of *S. sclerotiorum* via the PEG-mediated method [32]. The transformants were screened on PDA medium supplemented with hygromycin B at 300 μg/mL and verified by evaluating the expression level of *SsGSP1* with semi-quantitative PCR and qRT-PCR.

Subcellular localization

To verify the anchoring position of *SsGSP1*, the *BamH* I/*EcoR* I-digested *SsGSP1* fragment was ligated into the pCB vector to generate pCB-*SsGSP1*-GFP. The *npt*II gene cassette was cloned into pCB-*SsGSP1*-GFP, which was then transferred into the *SsGSP1*-deletion strains via the protoplast method and screened on 100 μg/mL G418 medium. The GFP fluorescence of transformants was assessed under a laser scanning confocal microscope (LSM800).

Cell wall integrity and sensitivity assays

The mycelium-colonized agar plugs (0.6 cm in diameter) were cultured on PDA medium supplemented with 2.5 mg/mL Congo red to evaluate the cell wall stress of *SsGSP1*-deletion strains and cultured under hypertonic environments (1 M glucose and 1 M sorbitol), high salt (0.5 M NaCl and 0.5 M KCl), hyperoxia stress (10 mM H_2O_2), and heat shock (28°C) to evaluate stress response. Inhibition rates were calculated by measuring the diameter of hyphal growth at 36 h post-inoculation (hpi). All of the experiments were independently repeated three times, with three plates in each replicate.

Congo red staining was performed to further evaluate the cell wall integrity of *SsGSP1*-deletion strains. The deletion strains and wild-type strain were stained with 1% Congo red for 30 min, washed with 1X Phosphate Buffered Saline (PBS) for 1 min, and then observed fluorescence under a laser scanning confocal microscope (LSM800).

Cell wall β-glucan content assay

An aniline blue assay was performed to assess the content of cell wall β-glucan as described by Fortwendel et al. [33]. Briefly, 50 μL of each sample treated with 1 M NaOH was mixed with 185 μL of aniline blue solution (0.067% aniline blue, 0.35 M HCl, 0.98 M glycine-NaOH, pH 9.5), and the fluorescence was measured at Ex/Em = 405/460 nm. To calculate β-glucan content (μg/mg dry biomass), the β-1,3-glucan analog curdlan (Sigma) was used as a standard.

Compound appressoria assay

Compound appressoria formation of *SsGSP1*-deletion strains and wild-type strain was observed as described by Yu et al. [34]. The mycelium-colonized agar plugs (0.6 cm in diameter) were inoculated onto rapeseed leaves, and the plugs were removed at 9 hpi. The inoculated rapeseed leaves were cleared with ethanol/acetic acid (3:1 v/v) solution for 12 h and then stained with 5% trypan blue for 12 h. The appressoria were observed under a microscope, and the experiment was set up with three independent replicates.

Pathogenicity assays

To assay the pathogenicity, the mycelium-colonized agar plugs obtained from the actively growing colony edges were inoculated onto the leaves of *A. thaliana*, *Nicotiana benthamiana*, and *Brassica napus* (Zhongshuang 11) at 90% relative humidity at 22°C. The lesion size was measured at 24 hpi for *A. thaliana*, 36 hpi for *N. benthamiana*, and 48 hpi for *B. napus* leaves. Each strain was inoculated on three leaves, with at least three repeats.

A 352-bp specific *SsGSP1* fragment was ligated into the pLabc vector to form a "sense-intron-antisense" palindromic sequence, which was used to create an RNAi-based HIGS construct for *A. thaliana* plant transformation according to the previous study [35]. The 35-day-old HIGS-*SsGSP1* transgenic *A. thaliana* lines were inoculated with 0.2 cm mycelium-colonized agar plugs of the *S. sclerotiorum* wild-type strain 1980 from the actively growing colony edges. Lesion size was measured at 24 hpi for in *vitro* inoculation and 48 hpi for in *vivo* inoculation. The experiments were performed at least three times with five leaves or plants for every line in each replicate.

Transcriptome sequencing and data analysis

The mycelia of the wild-type strain 1980 and the *SsGSP1*-deletion strain on cellophane over PDA were harvested at 48 hpi for transcriptome sequencing at Shanghai Meiji Biomedical Technology Co. Ltd. (Shanghai, China) following the manufacturers' procedure. The high-quality sequence data (clean reads) after

filtering was aligned to the reference genomes of *S. sclerotiorum*. The raw counts were normalized by TPM (Transcripts Per Million reads), and the differentially expressed genes (DEGs) between wild-type strain 1980 and the *SsGSP1*-deletion strain were identified by DEGseq (*p*-adjust <0.001 & $|\log_2 FC| \geq 1$) [36]. Functional enrichment of DEGs was performed on FunCat (https://elbe.hki-jena.de/fungifun/).

Yeast two-hybrid

The GAL4-based Matchmaker Gold Yeast Two-Hybrid System (Clontech, USA) was used to screen and verify SsGSP1-interacting proteins as described by Yang et al. [11]. *SsGSP1* (without signal peptide, SP) was cloned into pGBKT7 as bait, while putative interaction proteins were cloned into pGADT7 as prey. The bait and prey plasmids were co-transformed into the yeast strain Y2H Gold according to the manufacturer's instructions. The yeast transformant growth was analyzed on synthetic dropout (SD)/-Trp-Leu plates and SD/-Trp-Leu-His-Ade plates containing X-*a*-galactosidase (X-*a*-gal).

Bimolecular fluorescence complementation

Bimolecular fluorescence complementation (BiFC) assay was performed as described by You et al. [37]. The full-length sequences of *SsCat2*, *Sshap60*, and *SsBMR1* were inserted into the nYFP vector, respectively, while *SsGSP1* (without signal peptide, SP) was inserted into the cYFP vector. The recombination plasmids were transiently expressed in *N. benthamiana* leaves by *Agrobacterium*-mediated transfection. After 48 h of expression, the fluorescent signals were visualized using a laser scanning confocal microscope (LSM800).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8 software (California, San Diego, CA) with Student's *t*-test, one-way ANOVA, and Holm–Sidak test.

Results

SsGSP1 is a GPI-anchored protein on the cell surface of S. sclerotiorum

In the previous study, three DEGs encoding proteins with a Kre9_KNH domain in *S. sclerotiorum* during the inoculation of *Brassica oleracea* stems were identified by RNA-seq [38]. Among the three DEGs, the *S. sclerotiorum* gene *SS1G_03230* had the highest expression level (Figure S1). The SS1G_03230 belongs to the GPI-anchored serine-threonine rich family protein and encodes a 212-amino-acid polypeptide (NCBI Reference Sequence: XP_001595142.1), of which the 17 amino acids at the N-terminal form a signal peptide predicted by SignalP 5.0 Server, followed by a Kre9_KNH (pfam: PF10342) domain at residues 22–113 (Figure 1a). The GPI binding site G-ω was predicted at C-31/30 (residues 182/183) by GPI-SOM and PredGPI (Figure 1a). SS1G_03230 was predicted to be located extracellularly without the transmembrane domain by TMHMM and WoLF PSORT, suggesting that it may be a secreted protein. Therefore, we speculated that *SS1G_03230* encodes a GPI-anchored secreted protein and designated it as SsGSP1.

To verify the function of secretory in SsGSP1, the fusion expression vector carrying the signal peptide of SsGSP1 (pSUC2-SsGSP1) was constructed and transferred into the YTK12 yeast strain. The YTK12 strains carrying the signal peptides of Avr1b and Mg87 were used as positive and negative controls, respectively. All the yeast strains could grow on YPDA, but only the strains transformed with pSUC2-SsGSP1 and the positive control pSUC2-Avr1b could grow on both media of CMD-W and YPRAA, and convert TTC to insoluble red-colored triphenylformazan (Figure 1b). It indicated that SsGSP1 is a probable secretory protein.

To determine the subcellular localization of SsGSP1, a binary vector containing GFP-labeled *SsGSP1* was constructed and transformed into the *SsGSP1*-deletion strains. The fluorescence was filled in the whole mycelia of the control (GFP strain: wild-type strain 1980 carrying the GFP binary vector), while the fluorescence in the SsGSP1-GFP strain was concentrated along the surface of the mycelia using a laser scanning confocal microscope, indicating that SsGSP1 is anchored on the cell surface (Figure 1c).

SsGSP1 is associated with cell wall integrity of S. sclerotiorum

To assay the role of *SsGSP1* in cell wall integrity of *S. sclerotiorum*, two *SsGSP1*-deletion strains (Δ*SsGSP1*-2, Δ*SsGSP1*-25) were developed with extremely low transcript detected by semi-quantitative PCR and qRT-PCR analysis (Figure S2). The inhibition growth rates of Δ*SsGSP1*-2 and Δ*SsGSP1*-25 were 1.53-fold and 1.58-fold higher than those of the wild-type strain at 36 hpi (Figure 2a) on PDA medium supplemented with 2.5 mg/mL Congo red (CR), which specifically binds to β-glucan in the cell wall and inhibits the growth of fungi [39]. Moreover, Congo red staining of *SsGSP1*-deletion

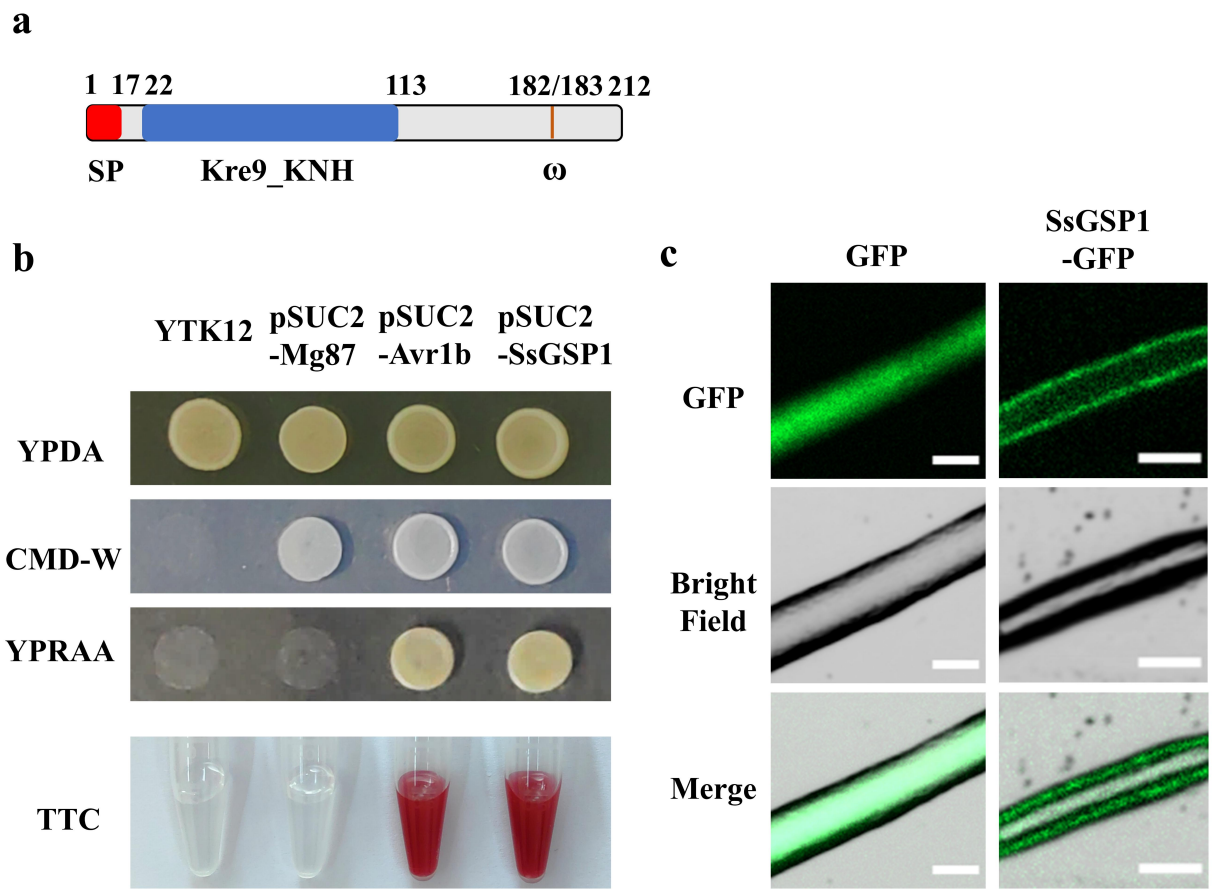

Figure 1. SsGSP1 is a GPI-anchored protein. (a) The structure schematic of SsGSP1 with the signal peptide (residues 1–17), the Kre9_KNH domain (residues 22–113), and the GPI binding site G-ω at C-31/30 (residues 182/183). (b) The secreted characteristic of SsGSP1 using the yeast invertase secretion assay. The pSUC2-Avr1b was used as a positive control, while pSUC2-Mg87 and YTK12 were used as negative controls. Secreted invertase activity was detected by 2,3,5-triphenyltetrazolium chloride (TTC). The red color confirms the occurrence of invertase activity. (c) Subcellular localization of the SsGSP1-GFP protein under a laser scanning confocal microscope (LSM800). Scale bars, 20 μm.

strains revealed impaired cell wall integrity compared with the wild-type strain (Figure 2b). Aniline blue assay was used to measure the content of cell wall β-glucan, and the results showed that deletion of *SsGSP1* led to a significant reduction in β-glucan level (Δ*SsGSP1*-2: 25.67%, Δ*SsGSP1*-25: 28.74%), but the complemented strain had no differences compared with the wild-type strain (Figure 2c). These findings demonstrated that the *SsGSP1* gene impacts the β-glucan content of the cell wall and is related to the cell wall integrity of *S. sclerotiorum*.

SsGSP1 is involved in the virulence of S. sclerotiorum

The qRT-PCR analysis showed that the expression level of *SsGSP1* was gradually up-regulated during the early inoculation of *B. napus* leaves and up to the top of expression at 48 hpi, which was 9-fold higher than that at 0 hpi (Figure 3a). To further analyze whether the *SsGSP1* gene is involved in the virulence of *S. sclerotiorum*, we explored whether it is related to the compound appressoria formation during the infection stage. However, it was found that the compound appressoria of the *SsGSP1*-deletion strains was similar to those of the wild-type strain at 9 h after inoculation of rapeseed leaves (Figure S3), suggesting that the *SsGSP1* gene is not involved in the formation of compound appressoria in *S. sclerotiorum*. Subsequently, we inoculated *A. thaliana* leaves in *vivo* with *SsGSP1*-deletion strains, and found that the virulence of *SsGSP1*-deletion strains was reduced with a smaller lesion size (0.48 ± 0.18 cm^2) in comparison with the wild-type strain (0.96 ± 0.12 cm^2) (Figure 3b). In *vitro* assays by inoculating the detached leaves of *A. thaliana*, *N. benthamiana*, and *B. napus* with wild-type strain, *SsGSP1*-deletion strains, and complemented strain, the *SsGSP1*-deletion strains produced smaller lesions (*A. thaliana*: 0.48 ± 0.12 cm^2, *N. benthamiana*: 5.44 ± 0.61 cm^2, and *B. napus*: 8.82 ±

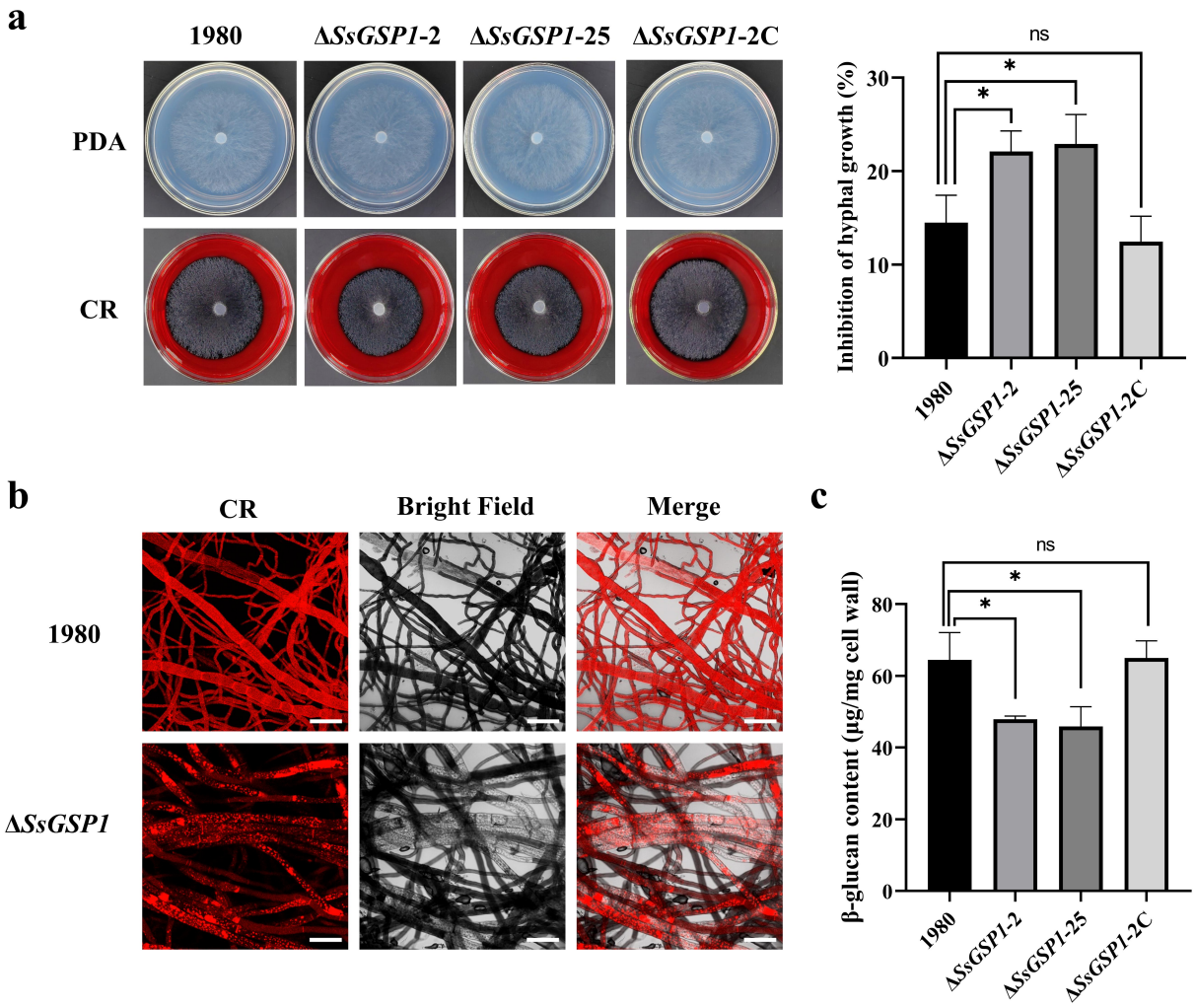

Figure 2. *SsGSP1* is associated with cell wall integrity of *S. sclerotiorum*. (a) Growth inhibition of *SsGSP1*-deletion strains inoculated on PDA supplemented with 2.5 mg/mL Congo red (CR) at 36 h post-inoculation (hpi). (b) Congo red staining of *SsGSP1*-deletion strains. The fluorescence was observed under a laser scanning confocal microscope (LSM800). Scale bars, 50 μm. (c) The β-glucan content in cell wall was determined using an aniline blue assay. Asterisks indicate statistical significance when compared with wild-type strain 1980. *$p < 0.05$; ns, not significant; one-way ANOVA. Error bars represent SD.

0.81 cm^2) than those of the wild-type strain (*A. thaliana*: 0.95 ± 0.16 cm^2, *N. benthamiana*: 8.34 ± 0.95 cm^2, and *B. napus*: 12.29 ± 1.52 cm^2), but the complemented strain (Δ*SsGSP1*-2C) restored virulence to the level of the wild-type strain (*A. thaliana*: 0.94 ± 0.16 cm^2, *N. benthamiana*: 8.19 ± 0.74 cm^2, and *B. napus*: 11.53 ± 1.51 cm^2) (Figure 3c).

To further investigate the role of *SsGSP1* in virulence, the HIGS-*SsGSP1* vector was constructed and transformed into the wild-type *A. thaliana*. The amplified sequence of *SsGSP1* showed no homologous or similar sequences in *A. thaliana* when queried against the BLAST database of NCBI. Two homozygous transgenic HIGS-*SsGSP1* lines (HIGS-*SsGSP1*-3, HIGS-*SsGSP1*-14), along with wild-type *A. thaliana* (Col-0) and empty vector (EV) as controls, were inoculated with wild-type *S. sclerotiorum*. The expression of *SsGSP1* of *S. sclerotiorum* inoculated HIGS-*SsGSP1* transgenic lines was significantly lower than that of the controls at 12 hpi, with an average reduction of 46.24 ~ 50.63% (Figure 4a), indicating that the expression of the *SsGSP1* gene in *S. sclerotiorum* was silenced during inoculation in HIGS-*SsGSP1* transgenic lines. The lesion size on the leaves of HIGS-*SsGSP1*-3 and HIGS-*SsGSP1*-14 varied from 0.22 to 0.36 cm^2, which was significantly smaller than that of Col-0 (0.77 cm^2) and EV (0.71 cm^2) at 24 hpi in *vitro* assay (Figure 4b). It is in accordance with the assay in *vivo*, where the incidence of Sclerotinia disease in HIGS-*SsGSP1* transgenic lines was less than that in Col-0 and EV at 48 hpi (Figure 4c). Taken together, these findings suggest that the *SsGSP1* gene is involved in the pathogenesis of *S. sclerotiorum*.

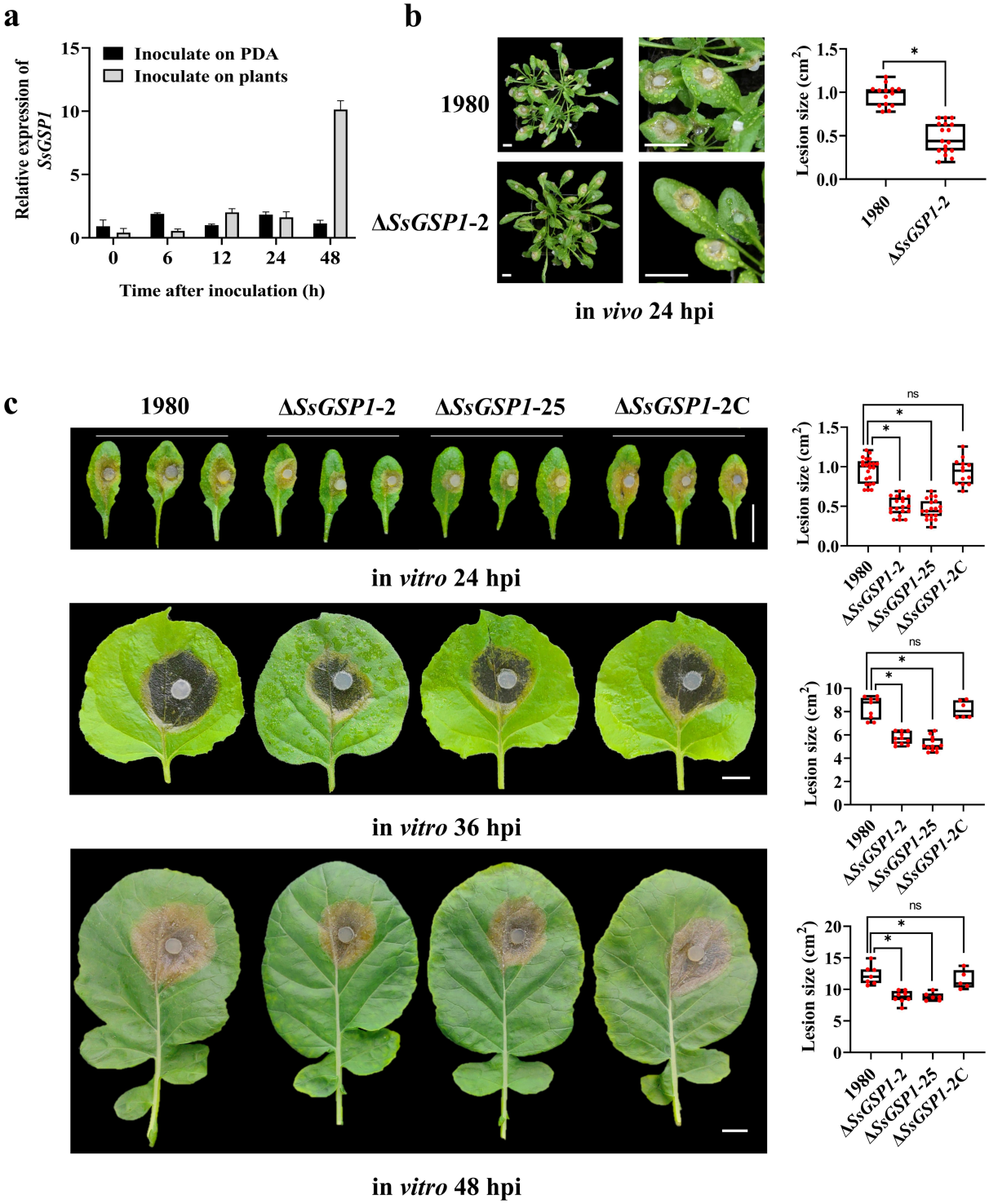

Figure 3. *SsGSP1* is involved in the virulence of *S. sclerotiorum*. (a) Relative expression of *SsGSP1* gene in *S. sclerotiorum* during the infection of rapeseed leaves and growth on PDA medium was determined by qRT-PCR. Disease phenotypes and lesion size when strains were inoculated on *A. thaliana* leaves in *vivo* (at 24 hpi) (b), and in *vitro* on *A. thaliana* leaves (at 24 hpi), *N. benthamiana* leaves (at 36 hpi), and rapeseed leaves (at 48 hpi) (c). Scale bars, 1 cm. Lesion size is shown as box plots, with boxes displaying the 25th–75th percentiles, the center line indicating the median with all individual data points overlaid and whiskers extending to the minimum and maximum values. Asterisks indicate statistical significance when compared with wild-type strain 1980. *$p < 0.05$; ns, not significant; Student's *t*-test (b) and one-way ANOVA (c).

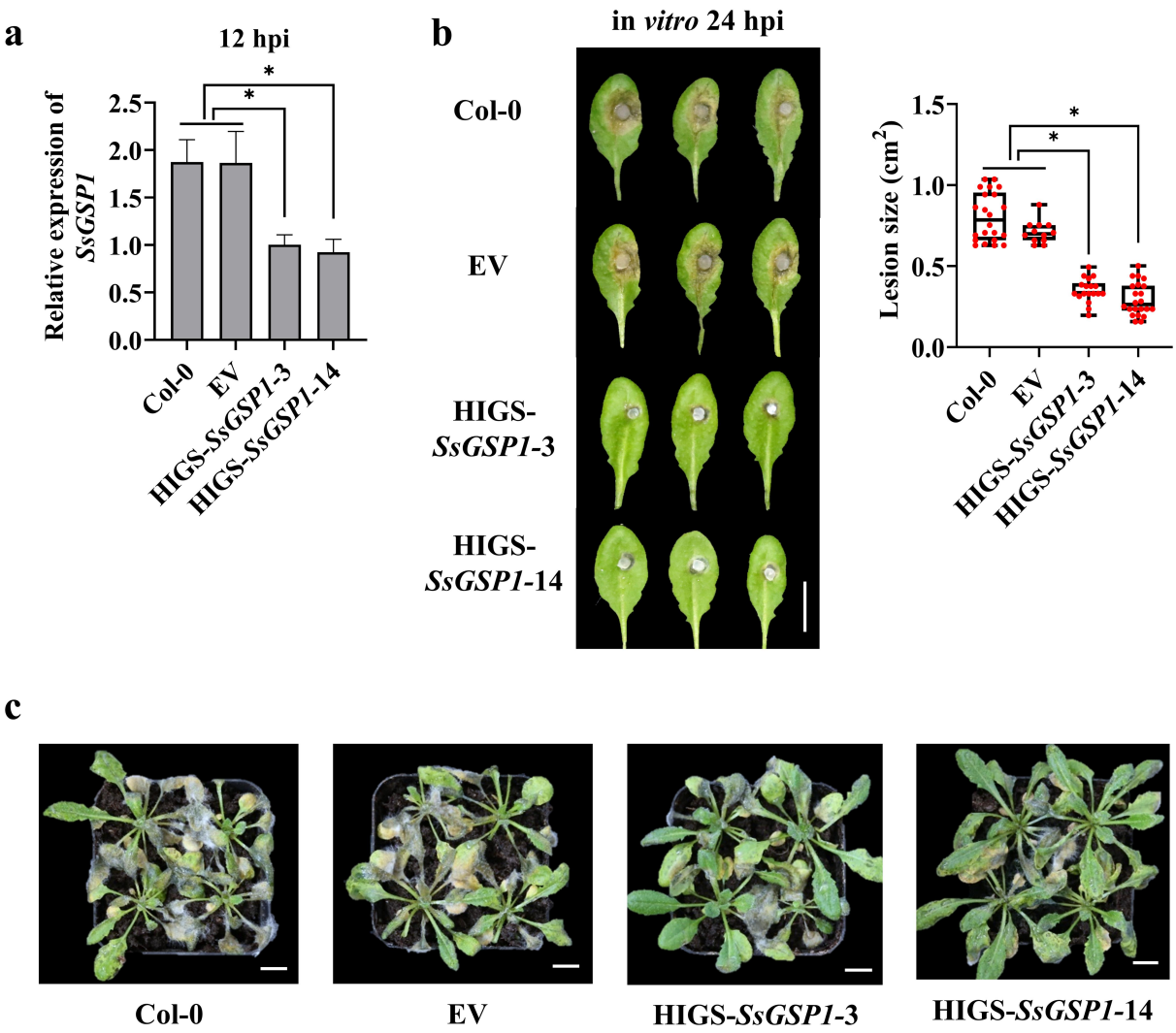

Figure 4. HIGS-*SsGSP1* transgenic *A. thaliana* enhances resistance to *S. sclerotiorum*. (a) The relative expression levels of *SsGSP1* among the HIGS-*SsGSP1* transgenic *A. thaliana* lines (HIGS-*SsGSP1*-3, HIGS-*SsGSP1*-14), wild-type *A. thaliana* (Col-0), and empty vector (EV) plants at 12 hpi. (b) Disease phenotypes and lesion size when Col-0, EV, and HIGS-*SsGSP1* transgenic *A. thaliana* lines were inoculated with *S. sclerotiorum* wild-type strain 1980 in *vitro* (at 24 hpi). Scale bars, 1 cm. Lesion size is shown as box plots, with boxes displaying the 25th–75th percentiles, the center line indicating the median with all individual data points overlaid and whiskers extending to the minimum and maximum values. Asterisks indicate statistical significance when compared with Col-0 or EV. *$p < 0.05$; one-way ANOVA. (c) Disease phenotypes of Col-0, EV, and HIGS-*SsGSP1* transgenic *A. thaliana* lines after inoculation with *S. sclerotiorum* wild-type strain 1980 in *vivo* (at 48 hpi). Scale bars, 1 cm.

SsGSP1 responds to stress tolerance in S. sclerotiorum

The mycelia of the wild-type strain 1980 and the *SsGSP1*-deletion strain cultured on PDA medium for 2 days were collected for RNA-seq analysis, and 1192 DEGs were obtained in the *SsGSP1*-deletion strain in comparison with the wild-type strain. Of which, 518 downregulated DEGs were mainly enriched in oxidative stress response, heat shock response, and chemical agent resistance in cell rescue, defense and virulence pathways by FunCat functional enrichment analysis (Figure 5a). Subsequently, qRT-PCR was used to detect the expression level of nine stress marker genes (*SS1G_00547*, *SS1G_05899*, *SS1G_12928*, *SS1G_02087*, *SS1G_00134*, *SS1G_12558*, *SS1G_04483*, *SS1G_02042*, and *SS1G_13659*) on the PDA medium. Consistent with the transcriptome data, these stress genes were downregulated in the *SsGSP1*-deletion strain compared to the wild-type strain. Additionally, following treatment with stresses (oxidative stress (10 mM H_2O_2), heat stress (28°C), and osmotic pressure (0.5 M KCl)), these genes were up-regulated in all strains, but the degree of

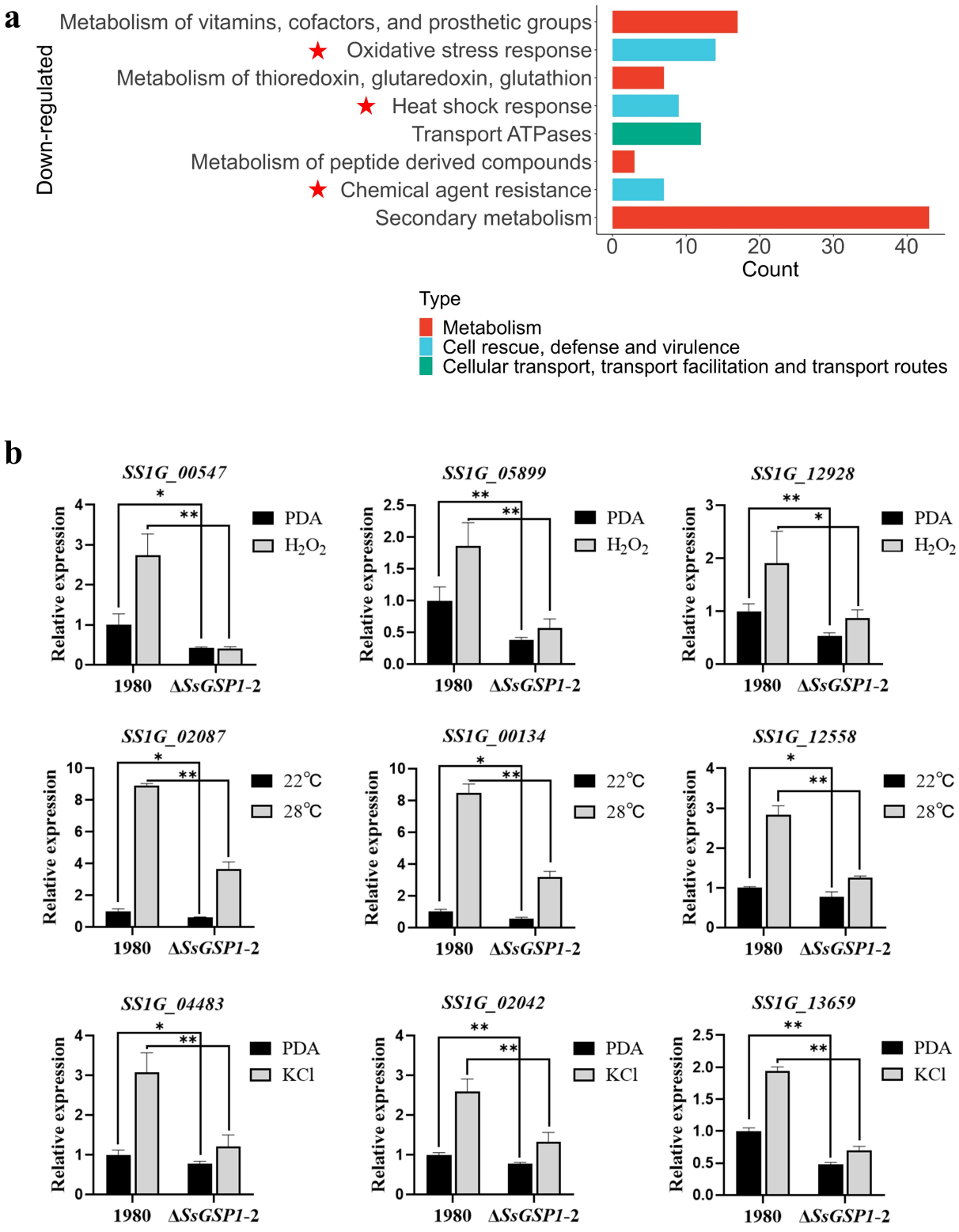

Figure 5. Key stress genes are downregulated in *SsGSP1*-deletion strains. (a) Functional enrichment analysis (FunCat) for the downregulated differentially expressed genes (DEGs). Specifically, the terms of oxidative stress response, heat shock response, and chemical agent resistance were marked with red stars. (b) The expression level of important stress genes under oxygen stress (10 mM H_2O_2), heat stress (28°C), and salt stress (0.5 M KCl) was determined by qRT-PCR. Error bars represent SD. Asterisks indicate statistical significance when compared with wild-type strain 1980. *$p < 0.05$; **$p < 0.01$.

upregulation in the deletion strain was lower than that of the wild-type strain (Figure 5b). Those findings indicate that *SsGSP1* may respond to environmental stress in *S. sclerotiorum*.

Tolerance to several environmental stresses in *SsGSP1*-deletion strains was then assessed. The hyphal growth was not significantly different among the wild-type strain and two *SsGSP1*-deletion strains on the PDA medium (Figure S4), but the inhibition of hyphal growth in *SsGSP1*-deletion strains was 1.11–1.69-fold higher than that of the wild-type strain on the medium added with 1 M glucose, 1 M sorbitol, 0.5 M NaCl, 0.5 M KCl, and 10 mM H_2O_2, and under heat shock (28°C) (Figure 6a,b). It indicated that *SsGSP1*-deletion strains are more sensitive to hypertonicity, high oxygen, and heat shock.

Furthermore, we detected 36 proteins that interacted with SsGSP1 by screening the yeast two-hybrid library (Table S2), including three stress response proteins: SS1G_00547 (SsCat2, catalase),

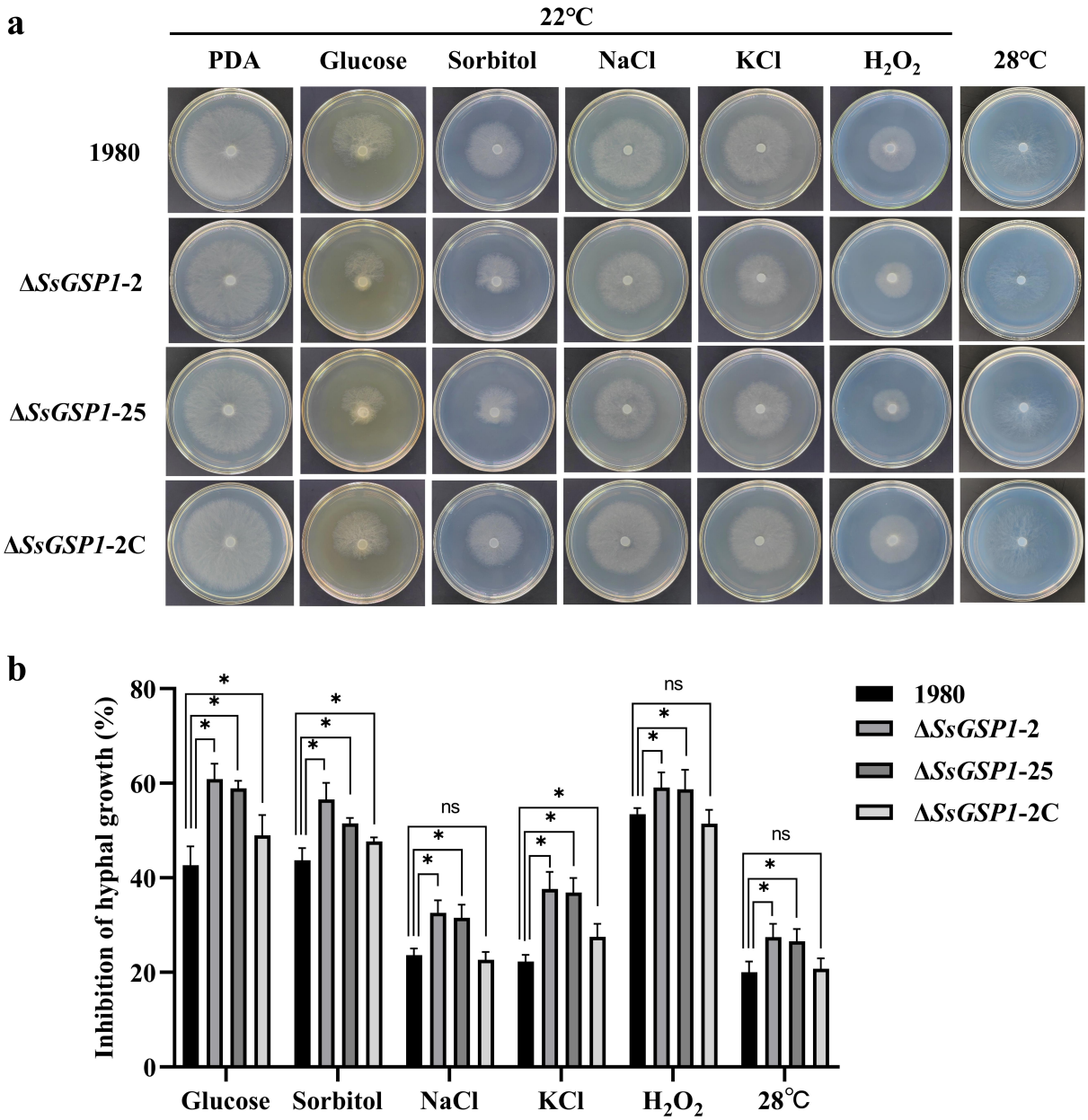

Figure 6. *SsGSP1* responds to stress tolerance in *S. sclerotiorum*. (a) Sensitivity of *SsGSP1*-deletion strains to various stress factors. Phenotype photographs were taken at 36 hpi. (b) The growth inhibition of *SsGSP1*-deletion strains. Asterisks indicate statistical significance when compared with wild-type strain 1980. * $p < 0.05$; ns, not significant; Holm–Sidak test. Error bars represent SD.

SS1G_02087 (Sshsp60, heat shock protein), and SS1G_04483 (SsBMR1, the pleiotropic drug resistance protein of the ABC transporter) (Figure 7a). Moreover, the BiFC assay further confirmed their interaction in *vivo*. The yellow fluorescence signals were detected when cYFP-SsGSP1$^{\Delta SP}$ and nYFP-SsCat2, cYFP-SsGSP1$^{\Delta SP}$ and nYFP-Sshap60, cYFP-SsGSP1$^{\Delta SP}$ and nYFP-SsBMR1 were transiently coexpressed in *N. benthamiana* leaves, but no fluorescence was detected when cYFP and nYFP-SsCat2, cYFP and nYFP-Sshap60, cYFP and nYFP-SsBMR1 were coexpressed (Figure 7b). Hence, these collective results indicate that *SsGSP1* is involved in the tolerance response to environmental stress in *S. sclerotiorum*.

Discussion

S. sclerotiorum, a necrotrophic phytopathogenic fungus with a broad host range infecting over 600 plant species, faces diverse stress challenges during infection processes. In this investigation, we identified that the deletion of a specific GPI-AP with a Kre9_KNH domain, SsGSP1, resulted in the reduced β-glucan content, the compromised cell wall integrity, the diminished virulence, and the impaired tolerance to osmotic pressure, oxidative stress, and heat shock in *S. sclerotiorum*. Notably, we observed interactions between SsGSP1 and key stress factors, including SsCat2, Sshsp60, and the ABC transporter SsBMR1. This study represents the first instance of elucidating

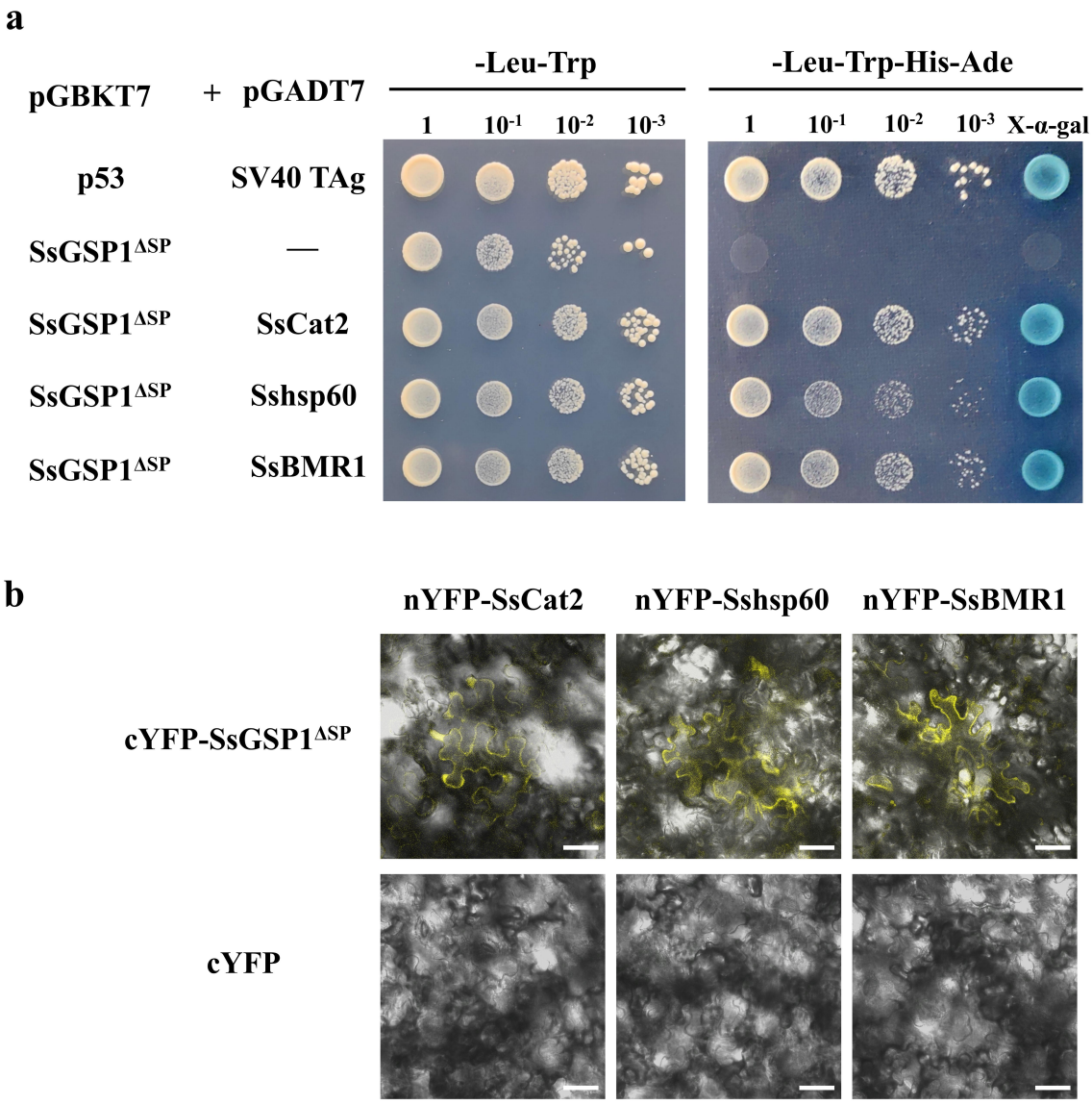

Figure 7. SsGSP1 interacts with SsCat2, Sshsp60 and SsBMR1 in *S. sclerotiorum*. (a) Yeast-two hybrid assay showed that SsGSP1 interacted with SsCat2, Sshsp60, and SsBMR1. pGBKT7-p53 together with pGADT7-SV40 Tag was used as a positive control. pGBKT7-SsGSP1$^{\Delta SP}$ was used as a negative control. (b) Bimolecular fluorescence complementation (BiFC) confirmed the interaction of SsGSP1 with SsCat2, Sshsp60, and SsBMR1. Scale bars, 50 μm.

the role of a GPI-AP as a potential cell surface sensor involved in responding to environmental stresses in *S. sclerotiorum*.

A substantial proportion of GPI-APs is located on the cell wall of fungi [40]. Liu and Free [17] identified 24 GPI-anchored cell wall proteins in *S. sclerotiorum* via cell wall proteome analysis. Among them, a GPI-anchored cell wall protein SsGsr1 was characterized in cell wall integrity and virulence of *S. sclerotiorum* [41]. In addition to these reported 24 GPI-anchored cell wall proteins, we identified a novel GPI-AP, SsGSP1 in *S. sclerotiorum* via transcriptome sequencing during inoculation on *B. oleracea*, which comprises a Kre9_KNH domain, and found the reduced β-glucan content of cell wall in the *SsGSP1*-deletion strains. In yeast, disruption of Kre9 led to severe growth defects and a decrease in β-1,6-glucan content of cell wall [20]. Kre9/KNH proteins may play roles in anchoring or cross-linking the newly synthesized β-1,6-glucan in the wall or serving as extracellular components of a β-1,6-glucan synthase [42].

Most pathogenic fungi have an inner cell wall layer of chitin and β-glucan, which provides cellular integrity, while the outer layer proteins of cell wall can also maintain cell wall integrity and have diverse functions [16]. The β-1,3-glucanosyltransferase GEL family proteins, which are members of GPI-APs, have been reported to be related to cell wall integrity [42]. In *M. oryzae*, the Δgel2 and the Δgel1Δgel2 mutants altered cell wall composition and reduced virulence [43]. And gel proteins affected the structural modification of the fungal cell wall during appressorium-mediated plant infection [44]. Our investigation revealed that *SsGSP1*-deletion strains compromised cell wall integrity and diminished virulence when inoculated on wild-type *A. thaliana*, *N. benthamiana*, and *B. napus*. Accordingly, we speculate that the deletion of *SsGSP1* decreases the β-glucan content of cell wall, impairing cell wall integrity and virulence in *S. sclerotiorum*.

The fungal cell wall represents the first line of defense for protecting the cell from a wide range of environmental challenges, such as heat, osmotic stress, and other adverse conditions [17]. In this study, we detected that *SsGSP1*-deletion strains showed increased sensitivity to osmotic pressure, oxidative stress, and heat shock. Similarly, all mutant strains of GPI-APs *gel* 1–5 in *M. oryzae* exhibited heightened susceptibility to chemicals that perturb cell walls, osmotic pressure, and oxidative stress [28]. Additionally, the GPI-anchored aspartyl protease Cgyps1 mutant was found to be growth-attenuated in the presence of high concentrations of Na^+, Zn^{2+}, and Mn^{2+} metal ions in *Candida glabrata* [45], and deletion of Dfg5 in *Trichoderma atroviride* led to impaired osmotic stress resistance at 50 mM sorbitol or 1 M NaCl [46]. Furthermore, we identified three proteins (SsCat2, Sshsp60, and SsBMR1) associated with environmental stress responses that interacted with SsGSP1 by yeast two-hybrid and BiFC assays. The catalase genes were reported to be involved in the response to oxidative stress, including *SsCat2* in *S. sclerotiorum* [47]. Heat Shock Proteins (HSPs) can interact with cell surface receptors to initiate signaling cascades, particularly in response to stress [48,49]. Signal transduction systems and ABC transporters often work together to adapt cellular responses to environmental changes [50]. Several cell membrane sensors (Wsc family, Mid2, Mtl1, Sho1, and Sln1) detect specific cell wall perturbing agents and transduce the signal to elements in MAP kinase cascade pathway in order to regulate cell wall remodeling and cell integrity under adverse environmental conditions [16,51]. Collectively, our findings speculate that SsGSP1 may function as a cell surface sensor, initiating signal transduction, and responding to environmental stresses swiftly and appropriately. Future studies are essential to elucidate the specific mechanisms through which SsGSP1 engages in signal transduction.

Previous studies revealed that eliminating GPI-anchored cell wall proteins or disrupting the GPI-anchored synthetic pathway have been proposed as effective strategies to control fungal diseases [28]. In this study, the utilization of dsRNA to target the *S. sclerotiorum SsGSP1* gene through HIGS significantly bolstered resistance against Sclerotinia in *A. thaliana*. This underscores the potential application of HIGS as a valuable tool for controlling *S. sclerotiorum* in crop improvement. The HIGS technology has been successfully employed to enhance Sclerotinia host resistance by silencing various genes, including *SsPG1*, *SsCBH*, *SsOAH1*, *SsTrx1*, and *Sscnd1* [10,35,52–54].

Conclusion

Our study reveals the existence of a novel GPI-AP, SsGSP1 in *S. sclerotiorum*, which comprises a Kre9_KNH domain. This protein is implicated in β-glucan, maintaining fungal cell wall integrity, and influencing virulence, making it a potential target for the safety control of Sclerotinia stem rot via HIGS. Additionally, our findings suggest that SsGSP1 may serve as a potential cell surface sensor involved in responding to environmental stresses in *S. sclerotiorum*.

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Author contributions

Hongmei Liao and Yijuan Ding conceived and designed the experiments. Hongmei Liao, Yangui Chen, Yujia He, Minghong Zou, Lintao Zheng, and Jinghang Liao performed methodology, validation, investigation, data curation, and result analysis. Kusum Rana, Wei Qian, and Yijuan Ding performed the reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are openly available in figshare (10.6084/m9.figshare.27292140), and the transcriptome raw data are openly available in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/bioproject/1132500).

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