




Nuclear Ssr4 Is Required for the *In Vitro* and *In Vivo* Asexual Cycles and Global Gene Activity of *Beauveria bassiana*

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ABSTRACT Ssr4 serves as a cosubunit of chromatin-remodeling SWI/SNF and RSC complexes in yeasts but remains functionally uncharacterized due to its essentiality for yeast viability. Here, we report pleiotropic effects of the deletion of the *ssr4* ortholog nonessential for cell viability in *Beauveria bassiana*, an asexual insect mycopathogen. The deletion of *ssr4* resulted in severe growth defects on different carbon/nitrogen sources, increased hyphal hydrophilicity, blocked hyphal differentiation, and 98% reduced conidiation capacity compared to a wild-type standard. The limited Δ *ssr4* conidia featured an impaired coat with disordered or obscure hydrophobin rodlet bundles, decreased hydrophobicity, increased size, and lost insect pathogenicity via normal cuticle infection and 90% of virulence via intrahemocoel injection. The expression of genes required for hydrophobin biosynthesis and assembly of the rodlet layer was drastically repressed in more hydrophilic Δ *ssr4* cells. Transcriptomic analysis revealed 2,517 genes differentially expressed in the Δ *ssr4* mutant, including 1,505 downregulated genes and 1,012 upregulated genes. The proteins encoded by hundreds of repressed genes were involved in metabolism and/or transport of carbohydrates, amino acids, and lipids, inorganic ion transport and energy production or conversion, including dozens involved in DNA replication, transcription, translation, and posttranslational modifications. However, purified Ssr4 samples showed no DNA-binding activity, implying that the role of Ssr4 in genome-wide gene regulation could rely upon its acting as a cosubunit of the two complexes. These findings provide the first insight into an essential role of Ssr4 in the asexual cycle *in vitro* and *in vivo* of *B. bassiana* and highlights its importance for the filamentous fungal lifestyle.

IMPORTANCE Ssr4 is known to serve as a cosubunit of chromatin-remodeling SWI/SNF and RSC complexes in yeasts but has not been functionally characterized in fungi. This study unveils for the first time the pleiotropic effects caused by deletion of *ssr4* and its role in mediating global gene expression in a fungal insect pathogen. Our findings confirm an essential role of Ssr4 in hydrophobin biosynthesis and assembly required for growth, differentiation, and development of aerial hyphae for conidiation and conidial adhesion to insect surface and its essentiality for insect pathogenicity and virulence-related cellular events. Importantly, Ssr4 can regulate nearly one-fourth of all genes in the fungal genome in direct and indirect manners, including dozens involved in gene activity and hundreds involved in metabolism and/or transport of carbohydrates, amino acids, lipids, and/or inorganic ions. These findings highlight a significance of Ssr4 for filamentous fungal lifestyle.


KEYWORDS virulence, aerial conidiation, chromatin-remodeling SWI/SNF subunit, entomopathogenic fungi, gene expression and regulation, hyphal differentiation, hyphal hydrophobicity, insect pathogenicity

Citation Shao W, Cai Q, Tong S-M, Ying S-H, Feng M-G. 2020. Nuclear Ssr4 is required for the *in vitro* and *in vivo* asexual cycles and global gene activity of *Beauveria bassiana*. mSystems 5:e00677-19. <https://doi.org/10.1128/mSystems.00677-19>.

Editor Michelle Heck, USDA Agricultural Research Service, Boyce Thompson Institute, Cornell University

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 Nuclear Ssr4, a co-subunit of chromatin-remodeling SWI/SNF and RSC complexes, is nonessential for cell viability of *Beauveria bassiana* but required for the *in vitro* and *in vivo* asexual cycles and global gene activity of the asexual insect mycopathogen

Received 18 October 2019

Accepted 9 April 2020

Published 21 April 2020

Multisubunit SWI/SNF (switch/sucrose nonfermentable) and RSC (remodel the structure of chromatin) complexes can alter DNA-histone contacts in nucleosomes in an ATP-dependent manner and activate gene transcription and other chromatin-relating processes in model yeasts (1, 2). In *Saccharomyces cerevisiae*, the SWI/SNF complex serves as a chromatin-remodeling complex by binding to the binding sites of transcription factors, and its binding to both DNA and nucleosomes presents high affinity but little specificity (3, 4). Upon binding, the complex is recruited to specific genes for transcriptional activation through chromatin remodeling (5) and catalyzes formation of nucleosome dimers with mono- and dinucleosome templates assembled from histones and DNA of high-affinity nucleosome positioning sequence (6). The yeast RSC complex is required for cell viability (7, 8), mediates gene transcription by RNA polymerases (9–13), and plays a crucial role in cell cycle (14), kinetochore function (15), sister chromatid cohesion (16), and DNA repair (17, 18). These studies demonstrate important roles of both SWI/SNF and RSC complexes in most chromatin-related processes of *S. cerevisiae*.

A comprehensive study has unveiled that *Schizosaccharomyces pombe* SWI/SNF and RSC complexes consist of 12 and 13 components essential or nonessential for cell viability and are compositionally distinct from the *S. cerevisiae* counterparts comprising 12 and 17 components (1). The two complexes of *S. pombe* share four SSR (SWI/SNF and RSC) components (Ssr1 to Ssr4), which are all required for yeast viability. Also unveiled in the study, Ssr1/2 and Ssr3 are orthologous to Swi3 and Snf12, which act as nonessential SWI/SNF components, and to Rsc8 and Rsc6, which are essential RSC components in *S. cerevisiae*. In *Candida albicans*, some SWI/SNF or RSC components have also been characterized, such as Swi1 essential for formation of infective hyphae (19) and Snf6 required for differentiation of invasive hyphae in response to stress cues and growth on fermentable and nonfermentable carbon sources (20).

Despite intensive studies in model yeasts, either SWI/SNF or RSC components remain unexplored in filamentous fungal pathogens, leaving it unknown how they function in host-pathogen interactions. Revealed in our previous transcriptomic analysis, an orthologous Ssr4-coding gene was increasingly upregulated in the first 48-h infection course of *Beauveria bassiana* against the larvae of *Plutella xylostella*, a cosmopolitan lepidopteran pest (21). The transcript level of *ssr4* in the fungus-insect interaction increased 109-fold at 36 h versus 24 h postinfection and 2-fold at 48 h versus 36 h postinfection, while so striking transcript changes were not found in the coding genes of other SWI/SNF or RSC components (21). This implies that Ssr4 may serve as a critical player in the fungus-insect interaction. As a classic insect mycopathogen, *B. bassiana* has been widely applied for arthropod pest control. Its biological control potential depends mainly on an ability to infect the host through the normal route of cuticular penetration for entry into host hemocoel (22), where the penetrating hyphae turn into unicellular blastospores (also called hyphal bodies) to proliferate rapidly by yeast-like budding until host mummification to death (23–27), as well as an ability to overcome stress cues from host immunity defense and environment (28, 29). Our fungal genome survey revealed a wide existence of orthologous Ssr4 in filamentous fungi. This study seeks to elucidate a role of Ssr4 in the *in vitro* and *in vivo* asexual cycles of *B. bassiana* through multiphenotypic analyses of *ssr4* deletion/rescue mutants and transcriptomic analysis of differentially expressed genes (DEGs) in the Δ *ssr4* mutant versus a wild-type strain. In contrast to an essentiality of *ssr4* for the viability of *S. pombe* (1), our viable Δ *ssr4* mutant offers the first opportunity to gain in-depth insight into the regulatory role of Ssr4 in a fungus. As presented below, Ssr4 localizes in the nucleus and plays an essential role in the *in vitro* and *in vivo* asexual cycles of *B. bassiana*. Importantly, the expression of 2,517 genes, namely, one-fourth of the fungal genome, was significantly deregulated in the null mutant of *ssr4*.

RESULTS

Protein domain analysis, transcript levels, and subcellular localization of Ssr4 in *B. bassiana*. The nucleotide sequence of *ssr4* (locus tag BBA_01960) in the *B.*

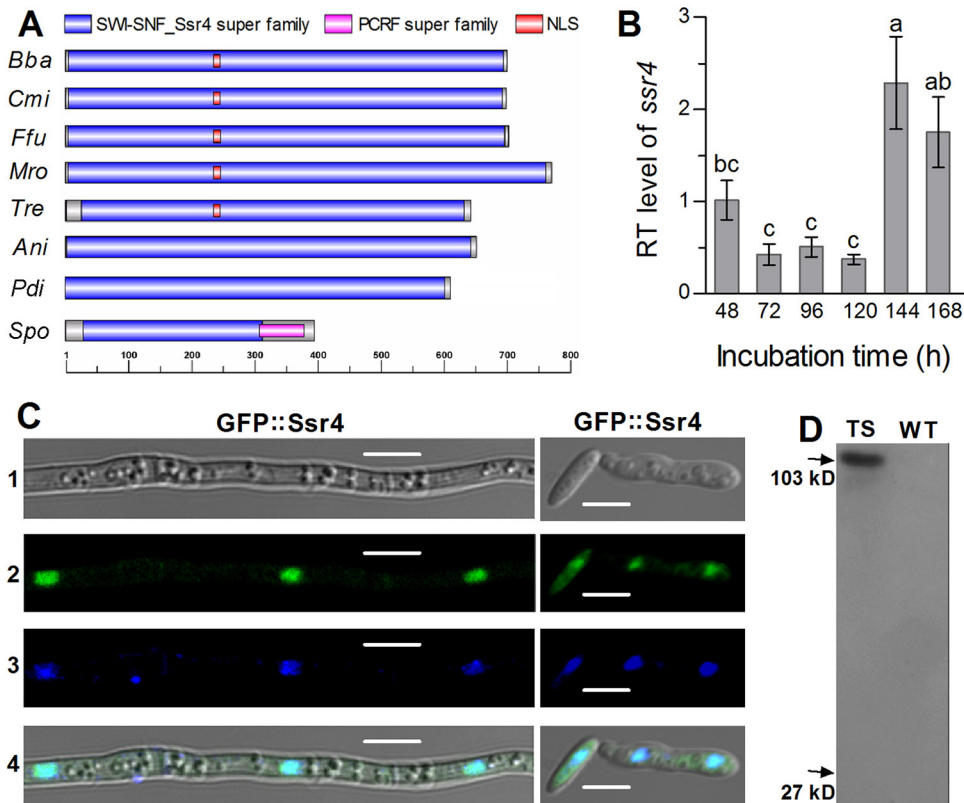


FIG 1 Properties of Ssr4 in *B. bassiana*. (A) Structural comparison of Ssr4 orthologs found in *B. bassiana* (*Bba*), *Cordyceps militaris* (*Cmi*), *Fusarium fujikuroi* (*Ffu*), *Metarhizium roberstii* (*Mro*), *Trichoderma reesei* (*Tre*), *Aspergillus nidulans* (*Ani*), *Penicillium digitatum* (*Pdi*), and *S. pombe* (*Spo*). The main domains and nuclear localization signal (NLS) were predicted at <http://smart.embl-heidelberg.de> and http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi, respectively. (B) Relative transcript (RT) level of *ssr4* in the SDAY culture of a WT strain during a 7-day incubation at the optimal regimen with respect to a standard at the end of 48 h. Values are means \pm standard deviations (SD) (error bars) from three cDNA samples. Different lowercase letters denote significant differences (Tukey's HSD test, $P < 0.05$). (C) LSCM images for subcellular localization of GFP::Ssr4 fusion protein in the hyphal cells collected from 3-day-old SDBY culture and stained with DAPI. Panels 1 to 4 show bright, expressed, stained, and merged views of the same field, respectively. Note that the expressed signal (green) overlapped well with the stained signal (blue) in nuclei. Bars, 5 μ m. (D) Western blots for GFP (27 kDa) and GFP::Ssr4 (102.9 kDa) in the protein extracts isolated from the 3-day-old SDBY cultures of transgenic strain (TS) versus WT (control) and probed with anti-GFP antibodies.

bassiana genome (30) is 2,231 bp long with two introns and encodes 701 amino acids (NCBI:protein accession no. or code [EJP68925](https://.ncbi.nlm.nih.gov/nucl/EJP68925)) with a molecular mass of 75.9 kDa and an isoelectric point of 4.74. The deduced protein sequence is more identical to the counterparts of filamentous fungi (34 to 84%) than to the *S. pombe* ortholog (26%) (see Fig. S1 in the supplemental material). In *B. bassiana*, Ssr4 features a large conserved SWI-SNF_Ssr4 domain (residues 6 to 695) and a nuclear localization signal (NLS) motif (residues 234 to 244) (Fig. 1A). Both the conserved domain and the NLS motif are also present in the orthologs of all examined filamentous fungi except *Aspergillus nidulans* and *Penicillium digitatum*. The latter two Eurotomyete species lack a predictable NLS motif. All of these orthologs are somewhat distinct from the *S. pombe* Ssr4, which has a peptide chain release factor (PCRf) domain overlapping the C-terminal end of the conserved domain and also lacks an NLS motif. Aside from Ssr4, most orthologs of the SWI/SNF or RSC components characterized in *S. pombe* (1) also exist in *B. bassiana* and four other filamentous fungi examined (see Table S1 in the supplemental material). Exceptionally, these filamentous fungi have one protein orthologous to both Ssr1 and Ssr2 and lack an ortholog of Snf30 in *S. pombe*, suggesting that only one of these components participates in filamentous fungal SWI/SNF complex.

The gene *ssr4* was consistently transcribed in the wild-type strain *B. bassiana* ARSEF 2860 (designated the wild type [WT]) during a 7-day incubation on Sabouraud dextrose

agar plus yeast extract (SDAY) at the optimal regimen of 25°C in a light/dark (L/D) cycle of 12 h/12 h (Fig. 1B). Its transcription was significantly downregulated during days 3 to 5 and upregulated to a peak on day 6 with respect to the standard level at the end of a 2-day incubation.

The presence of an NLS motif in the *B. bassiana* Ssr4 implicates its possible localization in the nucleus. This was verified by expression of green fluorescence protein (GFP)-tagged Ssr4 fusion protein in the WT strain. As shown in laser scanning confocal microscopic (LSCM) images, the expressed fusion protein aggregated mainly in the nuclei of 4',6'-diamidino-2'-phenylindole dihydrochloride (DAPI)-stained hyphal cells and merged well with the stained color (shown in blue) in the nuclei (Fig. 1C). However, the signal of the fusion protein in the cytoplasm was very weak. In a Western blot experiment to detect GFP alone and GFP::Ssr4 with anti-GFP antibody, only the fusion protein (92.9 kDa) was well detected in the protein extract isolated from the hyphal cells (Fig. 1D), indicating that the fusion protein was expressed as expected. The main localization of Ssr4 in the nuclei implies its involvement in nuclear events of *B. bassiana*.

Ssr4 is required for aerial conidiation and cell hydrophobicity. The gene *ssr4* was deleted from the WT strain and rescued in an identified Δ *ssr4* mutant as described in Materials and Methods. The expected recombinant events were confirmed through PCR and Southern blotting analyses (Fig. S2) with paired primers and amplified probe (Table S2). As a consequence, the Δ *ssr4* mutant suffered extremely severe defect in aerial conidiation during a 15-day incubation at the optimal regimen on SDAY plates, which were spread with 100- μ l aliquots of a 10^7 conidia/ml suspension for culture initiation. The control (WT and complemented) strains produced a mean yield of 2.6×10^7 conidia/cm² on day 4 and approached a peak of $\sim 5.5 \times 10^8$ conidia/cm² on day 8 (Fig. 2A). Compared to the control strains, the mutant showed a 2-day delay in conidiation and a drastic (98%) decrease in final conidial yield during a 15-day incubation. Revealed by scanning electron microscopy (SEM), plenty of conidia formed in the 5-day-old cultures of the control strains, contrasting to a scarcity of conidia in the Δ *ssr4* culture comprising abundant hyphae not differentiated yet (Fig. 2B). We also measured biomass levels of each strain during the period and found that fresh (wet) biomass level increased significantly by 12 to 17% (Tukey's honestly significance difference [HSD] test, $P < 0.05$) in the Δ *ssr4* cultures grown for 7 to 15 days in comparison to those measured from the cultures of the control strains (Fig. 2C). Intriguingly, dry biomass levels quantified from the 3- to 15-day-old Δ *ssr4* cultures were significantly (22 to 35%) lower than those of the control strains (Fig. 2D). Such measurements implicate a marked increase in hyphal hydrophilicity or water content of Δ *ssr4*. This implication was confirmed by dipping 10- μ l aliquots of sterile water containing 0.02% Tween 80 on the surfaces of 4-day-old SDAY cultures and monitoring the 72-h stability of dew-like water droplets. As a consequence, the water droplets remained consistently intact on the culture surfaces of the control strains but gradually sank into the Δ *ssr4* culture and disappeared at the end of 72 h (Fig. 2E). These data indicate that Ssr4 is required for aerial conidiation but nonessential for biomass accumulation of *B. bassiana* on the SDAY plates spread evenly with conidia for culture initiation. The increased water content in hyphal cells and the water droplets disappeared on the culture surface of Δ *ssr4* implicate higher hydrophilicity of its hyphae and hence an important role for Ssr4 in sustaining hyphal hydrophobicity and aerial growth, which are critical for normal differentiation of aerial hyphae for conidiation (31).

Aside from the severe conidiation defect, the *ssr4* deletion resulted in impaired conidial quality indicated by several features. The mutant conidia were compromised not only in viability due to a 3.7-h-longer time required for 50% germination (GT_{50}) at optimal 25°C but also in hydrophobicity due to a 45% reduction in comparison to those of the control strains (Fig. 2F). Shown in the SEM images of conidia (Fig. 2G), the control strains had a well-defined rodlet layer of conidial coat comprising hydrophobin-borne fascicles or bundles (32). In contrast, such fascicles or bundles became obscure on the

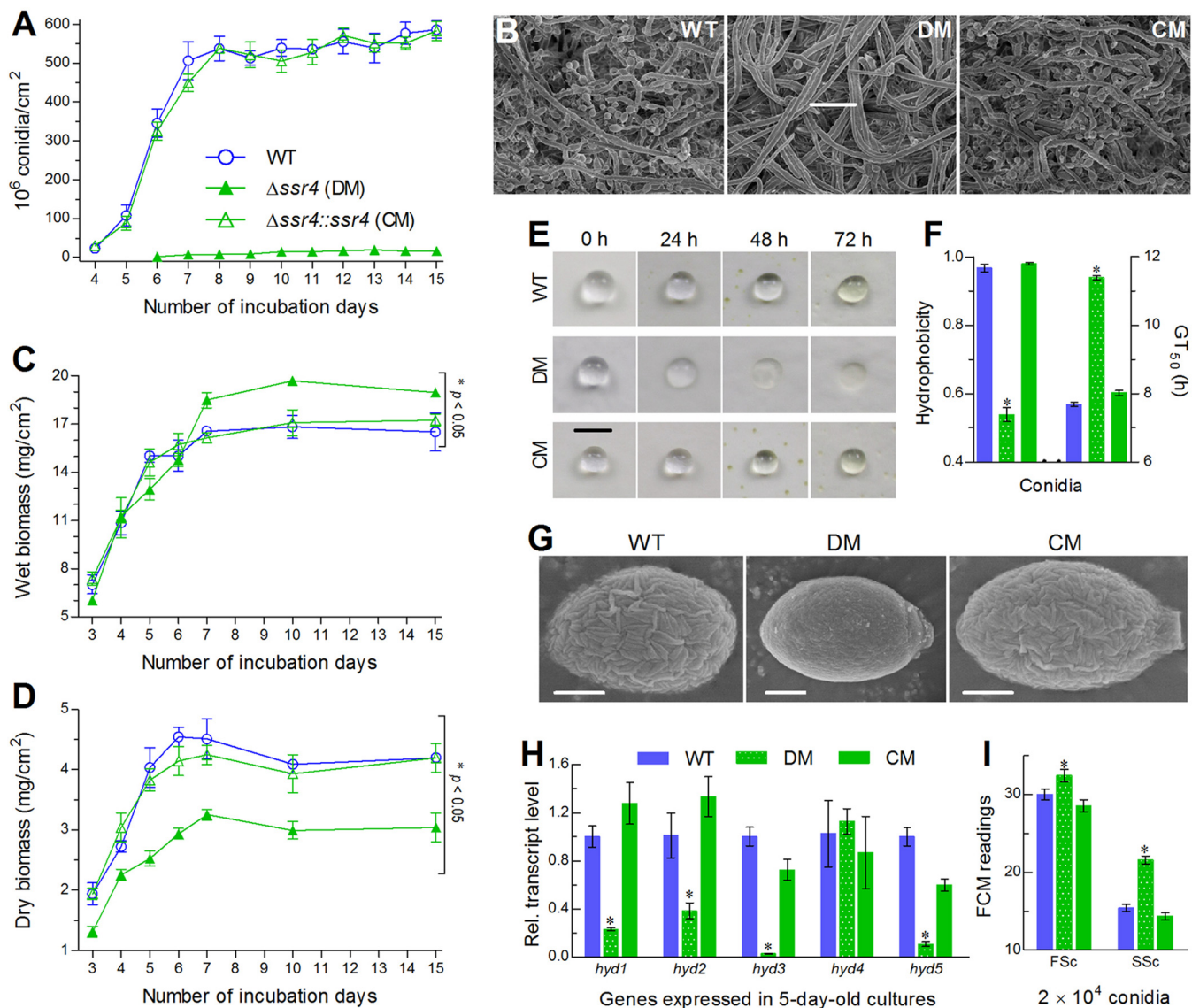


FIG 2 Ssr4 is required for aerial conidiation and cell hydrophobicity of *B. bassiana*. (A) Conidial yields quantified from the SDAY cultures during a 15-day incubation at the optimal regimen. The WT, deletion mutant (DM), and complemented mutant (CM) strains are shown. (B) SEM images for the status of conidiation in the 5-day-old SDAY cultures. Bar, 10 μ m. (C and D) Wet (fresh) and dry biomass levels from the SDAY cultures, respectively. (E) Visual images for the stability of dew-like water droplets on the surfaces of the SDAY cultures over 72 h. Note that the water droplet gradually sank and disappeared in the Δ *ssr4* culture. Bar, 3 mm. (F) Conidial hydrophobicity assessed in an aqueous-organic system and median germination time (GT_{50}) at 25°C. (G) SEM images for ultrastructural features of conidial coat. Bars, 10 μ m. (H) Relative transcript levels of five hydrophobin genes (*hyd1* to *hyd5*) in the 5-day-old SDAY cultures of *ssr4* mutants versus WT. (I) Conidial size and complexity (density) indicated by the forward scatter (FSc) and side scatter (SSc) readings from flow cytometry (FCM) of 2×10^4 conidia per sample. All SDAY cultures were initiated by spreading 100 μ l of a 10^7 conidia/ml suspension per plate (9-cm diameter). Values are means \pm SD (error bars) from three replicates. Values that are significantly different ($P < 0.05$) for the Δ *ssr4* mutant and its control strains by Tukey's HSD test are indicated by an asterisk.

surfaces of apparently larger Δ *ssr4* conidia. Obviously, the Δ *ssr4* conidia were coated with distorted or fewer hydrophobin-borne bundles, coinciding well with the marked decrease in hydrophobicity. This observation is also supported by transcript levels of five hydrophobin family genes, which were repressed by 62 to 97% in the 5-day-old SDAY cultures of the Δ *ssr4* mutant versus the WT strain except for unaffected expression of *hyd4* (Fig. 2H). In addition, conidial size and complexity (density) were significantly increased in the absence of *ssr4* (Fig. 2I). The measured mean size of the mutant conidia coincided with the conidial size shown in the SEM image. All of these changes were well restored by targeted gene complementation and hence indicated an essential role of Ssr4 in maintenance of conidial quality, transcription and translation of hydrophobin genes, and assembly of hydrophobins into the bundles.

Ssr4 is indispensable for fungal infection and virulence. The virulence of each strain against the fifth-instar larvae of greater wax moth (*Galleria mellonella*) was assayed by topical application (immersion) of a 10^7 conidia/ml suspension for normal infection through cuticular penetration and intrahemocoel injection of ~ 500 conidia ($5 \mu\text{l}$ of a 10^5 conidia/ml suspension) per larva for cuticle-bypassing infection. All treated larvae died from mummification by the control strains within 5 or 6 days postimmersion (Fig. 3A) and 4 or 5 days postinjection (Fig. 3B). In contrast, the Δssr4 mutant caused no substantial mortality through the normal infection and only 11% mortality by the end of 12 days postinjection.

Noticeably, the deletion mutant was unable to grow out of cadavers, which were heavily covered with outgrowths of the control strains 5 days after death from the injection (inset images in Fig. 3B). This implied that the Δssr4 cells inside the cadaver could not penetrate the cuticle for outgrowth. To verify this speculation, biomass levels and total activities of extracellular (proteolytic, chitinolytic, and lipolytic) enzymes (ECEs) and Pr1 proteases involved in cuticle degradation (22, 23, 27) were quantified from the 3-day-old submerged cultures of a 10^6 conidia/ml suspension in Czapek-Dox broth (CDB), which contained the sole nitrogen source of 0.3% bovine serum albumin (BSA) for enzyme induction. As a result, the Δssr4 mutant exhibited a 21% decrease in biomass level (Fig. 3C) and 50% and 11% reductions in total ECE and Pr1 activities (Fig. 3D) in comparison to the corresponding WT quantities. These data hint at a main link of reduced ESC and Pr1 activities to decreased biomass level in the submerged Δssr4 culture and some other more important cellular events that might be involved in the mutant inability to infect insects through cuticular penetration.

For insight into drastic attenuation of the mutant virulence via the cuticle-bypassing infection, we examined the presence/absence and abundance of hyphal bodies in the hemolymph samples taken from the larvae surviving the injection. Such hyphal bodies are usually produced from penetrating hyphae after entry into host hemocoel and can propagate rapidly by yeast-like budding for acceleration of host mummification to death (24–27). The control strains formed abundant hyphal bodies (i.e., blastospores) in the samples at the time of 84 h postinjection, whereas the Δssr4 mutant formed no hyphal body until 216 h postinjection (Fig. 3E). Blocking the dimorphic (hypha-blastospore) transition *in vivo* critical for fungal virulence was further examined in the following *in vitro* experiments, in which conidia were incubated 3 days in CDB and trehalose-peptone broth (TPB) mimicking host hemolymph. As a result, many more blastospores formed in the submerged cultures of the control strains than those of the Δssr4 mutant (Fig. 3F) although their biomass levels in CDB or TPB were similar or close to each other (Fig. 3G). Intriguingly, blastospore concentrations in the CDB and TPB cultures of the Δssr4 mutant decreased by 90% and 79%, respectively, in comparison to those estimated from the WT cultures (Fig. 3H). Thus, the dimorphic transition rate of the Δssr4 mutant on the basis of its biomass level was reduced by 86% in CDB and 79% in TPB (Fig. 3I), accompanied by $\sim 40\%$ decrease in both size and complexity of the blastospores produced in TPB (Fig. 3J). These results indicated a block of dimorphic transition *in vitro* or *in vivo* in the absence of *ssr4*.

Since the limited decreases of the ECE and Pr1 activities hardly accounted for the abolished host infection of the Δssr4 mutant via cuticular penetration, we evaluated radial growth rates of each strain on rich SDAY, minimal CDA (CDB plus agar) and 34 CDAs amended with different carbon (sugars or polyols) or nitrogen (inorganic or organic) sources by spotting $1 \mu\text{l}$ of a 10^6 conidia/ml suspension per plate for colony initiation. After a 7-day incubation at 25°C , the deletion mutant showed severe or extremely severe growth defects on all tested media. As illustrated in Fig. 4, the mean size of the Δssr4 colonies decreased by 69% on SDAY, 55% on CDA, and 54 to 95% on all amended CDAs in comparison to the corresponding colony sizes of the control strains. Growth of the mutant was suppressed more on the carbon sources of fructose and sodium acetate (95%) than on sucrose and glucose ($\sim 55\%$) and also on the nitrogen sources of most amino acids (77 to 94%) than on inorganic nitrogen sources

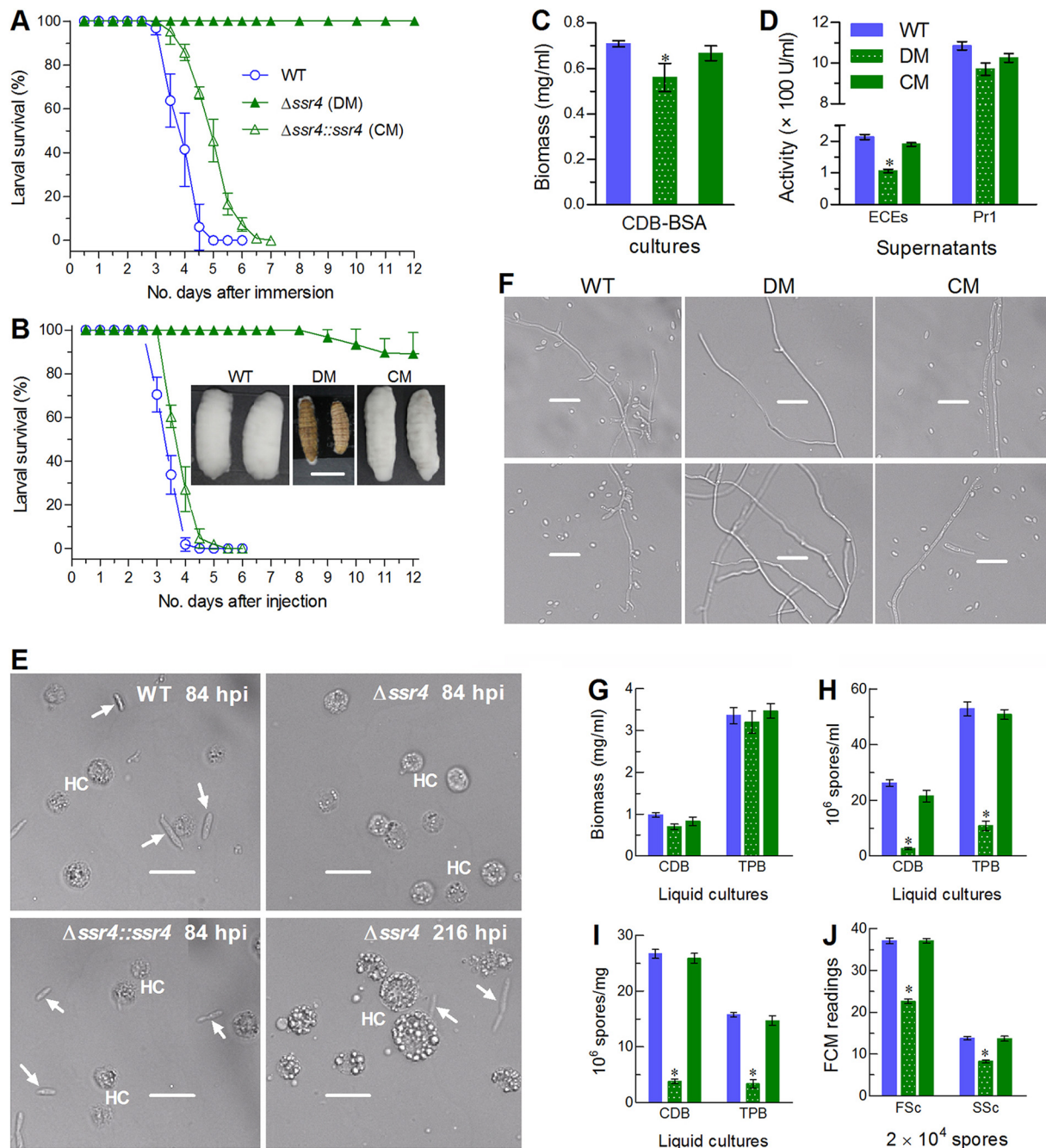


FIG 3 Ssr4 is indispensable for host infection and virulence of *B. bassiana*. (A and B) Survival trends of *G. mellonella* larvae after topical application (immersion) of a 10^7 conidia/ml suspension for normal cuticle infection and intrahemocoel injection of ~ 500 conidia per larva for cuticle-bypassing infection, respectively. The insets in panel B show fungal outgrowths on the surfaces of cadavers 5 days after death from injection. Bar, 10 mm. (C and D) Hyphal biomass levels and total activities of extracellular enzymes (ECEs) and Pr1 proteases quantified from the 3-day-old CDB-BSA cultures initiated with 10^6 conidia/ml. (E) Microscopic images showing the presence or absence of hyphal bodies (indicated by white arrows) in the hemolymph samples taken from surviving larvae after injection. hpi, hours postinfection; HC, host hemocytes. Bars, 20 μ m. (F to I) Microscopic images (scale: 20 μ m), biomass levels, blastospore concentrations, and dimorphic transition rates quantified from 3-day-old CDB and TPB cultures, respectively. (J) Blastospore size and complexity (density) indicated by the FSc and SSc readings from flow cytometry (FCM) of 2×10^4 blastospores per sample. *, $P < 0.05$ (Tukey's HSD) for marked differences between $\Delta ssr4$ and its control strains. Error bars show SD from three replicates.

tested (55 to 72%). These data demonstrate that Ssr4 is an essential sustainer of radial growth on nutrient-rich or scant substrata and hint at a main cause to interpret a complete loss of insect pathogenicity by the $\Delta ssr4$ mutant, which could be greatly suppressed in hyphal growth on oligotrophic insect integument as shown on the scant media and hence blocked in hyphal invasion into host.

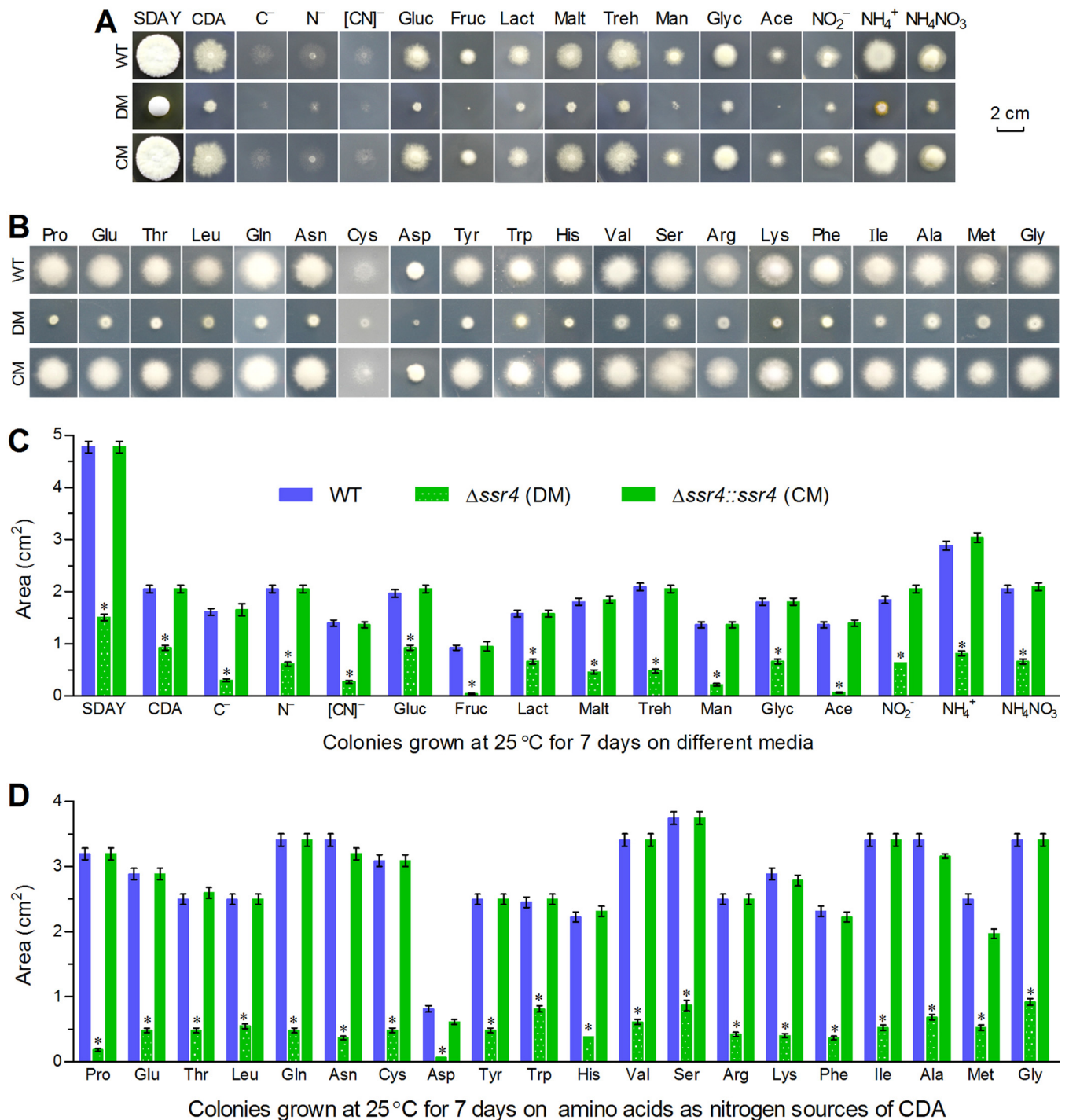


FIG 4 Essential role of *Ssr4* in radial growth of *B. bassiana*. (A to D) Images and areas of fungal colonies initiated by spotting 1- μ l aliquots of a suspension of 10^6 conidia/ml and incubated 7 days at 25°C on the plates of rich SDAY, minimal CDA, and modified CDAs, which contained different carbon sources (Gluc, glucose; Fruc, fructose; Lact, lactose; Malt, maltose; Treh, trehalose; Glyc, glycerol; Ace, acetate) (A) or inorganic/organic (amino acids) nitrogen sources (B) or were free of carbon (C⁻), nitrogen (N⁻), or both ([CN]⁻). *, $P < 0.05$ (Tukey's HSD) for marked differences between $\Delta ssr4$ and its control strains. Error bars show SD from three replicates.

***Ssr4* enables regulation of global gene expression.** Transcriptomes based on three 4-day-old SDAY cultures (replicates) of $\Delta ssr4$ and WT strains (Fig. 5A) were constructed and analyzed for in-depth insight into the essential role of *Ssr4* in the *in vitro* and *in vivo* asexual cycles of *B. bassiana*. Among 9,308 genes mapped to the *B. bassiana* genome, 2,517 were differentially expressed in the $\Delta ssr4$ mutant versus WT

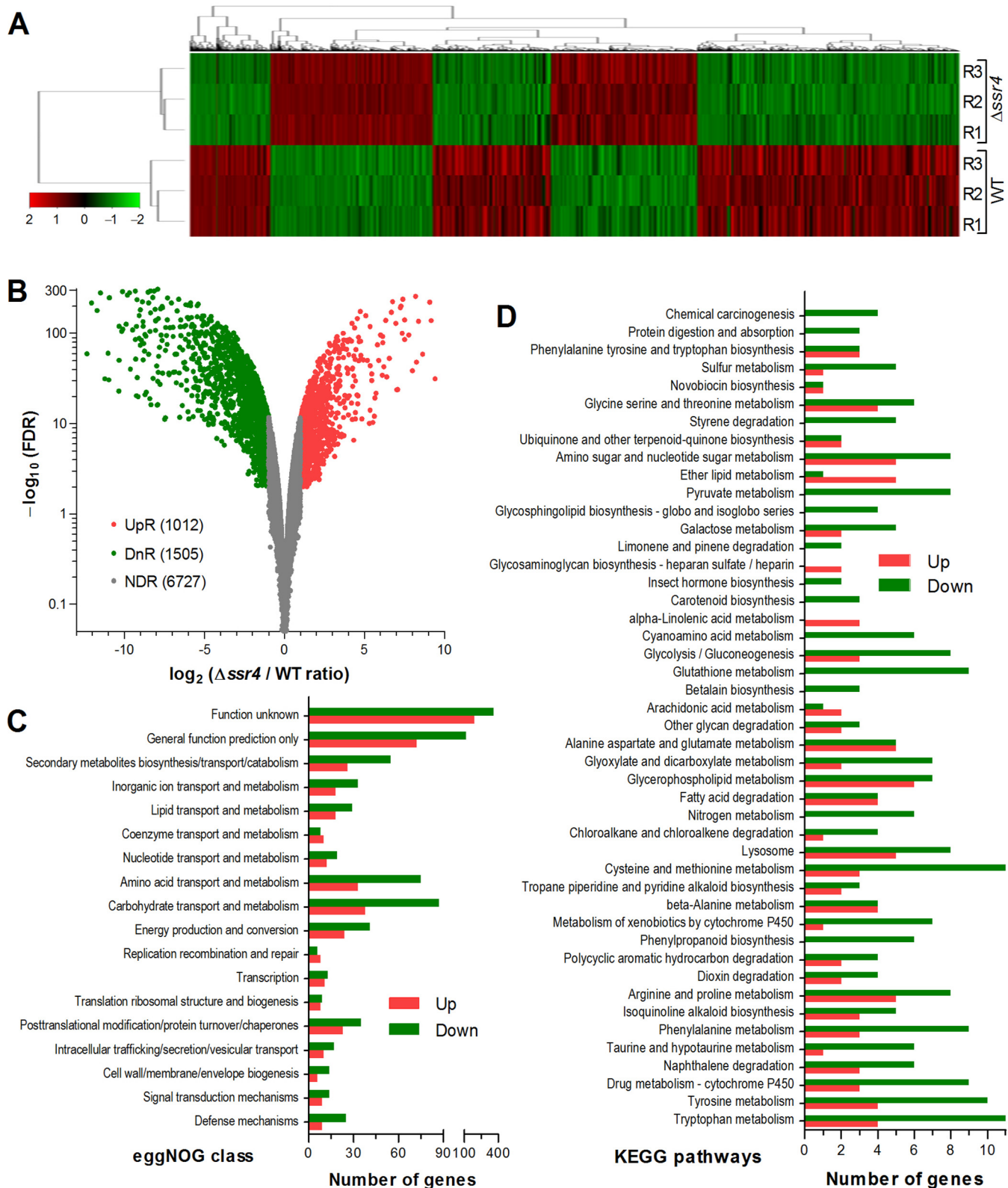


FIG 5 Genome-wide regulatory role of Ssr4 in *B. bassiana*. (A) Cluster analysis of differentially expressed genes (DEGs) in the transcriptomes generated from three 4-day-old SDAY cultures (three replicate cultures [R1, R2, and R3]) of $\Delta ssr4$ and WT strains grown at the optimal regimen of 25°C and 12-h/12-h L/D cycle. (B) Distributions of \log_2 ratios and FDR values for all genes significantly upregulated (UpR) (\log_2 ratio ≥ 1), downregulated (DnR) (\log_2 ratio ≤ -1), or not differentially regulated (NDR) ($-1 < \log_2$ ratio < 1). (C and D) Counts of DEGs significantly enriched in eggNOG function classes (C) and KEGG pathways (D).

strain at significant levels (Fig. 5B and Table S3), including 1,505 downregulated genes ($-12.35 \leq \log_2 \text{ ratio } [R] \leq -1.00$) and 1,012 upregulated genes ($1.00 \leq \log_2 R \leq 9.41$), and 433 recognized as new genes. All DEGs accounted for 24.3% of the genome of *B. bassiana* (30). Such a high proportion of DEGs in the absence of *ssr4* indicates a requirement of *Ssr4* for global gene activity. However, the developmental activator genes *brlA* (BBA_07544), *abaA* (BBA_00300), *wetA* (BBA_06126), and *vosA* (BBA_01023), which are evidently required for conidiation and conidial maturation of *B. bassiana* (33, 34), were markedly upregulated ($\log_2 R$, 6.67 for *brlA*, 3.38 for *abaA*, and 1.62 for *wetA*) or unaffected (*vosA*). None of the genes encoding signal transducers of the upstream developmental activation pathway required for activation of the critical *brlA* in *A. nidulans* (35–37), namely, *flbABCDE* (BBA_02968, BBA_06988, BBA_03181, BBA_07259, and BBA_01716, respectively) was differentially expressed although the upstream *fluG* (BBA_04942) was sharply repressed ($\log_2 R = -8.35$). The transcript changes of these development-required genes provided little insight into the severe conidiation defect of Δssr4 under normal culture conditions.

Nearly 60% DEGs (526 up- and 962 downregulated genes) were significantly enriched in 21 function classes by eggNOG function classification (Fig. 5B and Table S4), including one class containing a single DEG (data not shown) and two uncharacterized classes containing 191 (general function predicted only) and 554 (function unknown) DEGs, respectively. There were 14, 24, 17, and 58 DEGs involved in DNA replication, transcription, translation, and posttranslational modifications, respectively. Many more downregulated genes were enriched in the carbohydrate transport and metabolism class than those upregulated (87 versus 38). Many repressed genes encode important enzymes essential for utilization of carbon sources, such as ribose 5-phosphate isomerase (BBA_04969) acting in both the pentose phosphate pathway and the Calvin cycle (38), six hexose transporters (BBA_05766, BBA_03013, BBA_00397, BBA_09477, BBA8728, and BBA5674) critical for glucose uptake (39), and most of the glycosyl hydrolases (BBA_03946, BBA_05847, BBA_09379, and BBA_08796) involved in the hydrolysis of glycosidic bonds, the processing of N-linked glycoproteins and the degradation of carbohydrates (40, 41). Twenty-seven other repressed genes encode membrane transporter proteins (major facilitator superfamily [MFS]), including two sugar transporters (BBA_09393 and BBA_06639). These genes are known to function in the movement of small solutes across cell membranes (42, 43) or act as sugar transporters (40, 44, 45). Up to 75 repressed genes were involved in amino acid transport and metabolism, contrasting to only 33 upregulated in the class. The repressed genes encode five aminotransferases (BBA_08426, BBA_09446, BBA_03201, BBA_06530, and BBA_04988) involved in transamination reaction and the synthesis and breakdown of amino acids (41), five amino acid permeases essential for the transport of amino acids into cells (44, 46), five carboxypeptidases (BBA_02206, BBA_02865, BBA_00850, BBA_05763, and newGene_144) involved in the hydrolysis of peptide bond, the mediation of translocation across the endoplasmic reticulum and the targeting of molecules to the vacuole (47), three γ -glutamyl transpeptidases (BBA_05887, BBA_06332, and BBA_04223) involved in the γ -glutamyl cycle as a pathway of glutathione synthesis and degradation (48), and three dihydrodipicolinate synthases (BBA_06627, BBA_02585, and BBA_05664) essential for lysine biosynthesis (49). Also, more repressed genes than those upregulated were functionally enriched in energy production and/or conversion (41 versus 24), lipid transport and metabolism (29 versus 18), inorganic ion transport and metabolism (33 versus 18), and secondary metabolite biosynthesis, transport and catabolism (55 versus 26) classes. Apparently, differential expression and malfunction of so many important genes associated with nutritional utilization and energy production/conversion were causative of not only the severe growth defects of the Δssr4 mutant on all tested carbon and nitrogen sources but also both abolishment of its insect pathogenicity through cuticular penetration and great attenuation of its virulence through intrahemocoel injection.

Aside from the eggNOG classification, 334 DEGs (236 down- versus 98 upregulated) were significantly ($q < 0.05$) enriched in 46 Kyoto Encyclopedia of Genes and Genomes

(KEGG) pathways (Fig. 5D and Table S5). Many repressed genes were enriched in the metabolism pathways of carbohydrates (amino sugars and nucleotide sugars) and amino acids (tryptophan, tyrosine, phenylalanine, β -alanine, cysteine, methionine, arginine, and proline), providing further insights into the fungal phenotypes markedly impaired in the absence of *ssr4*.

Ssr4 has no DNA-binding activity. Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions (50) was conducted to examine whether purified Ssr4 extract can bind to amplified promoter DNA fragments of six selected genes, which were sharply repressed (\log_2 ratio < -8) in the absence of *ssr4* (Fig. S3A). The resultant gels showed no sign of any DNA fragment bound by 0.8 to 4.0 μ g of purified Ssr4 extract through agarose gel electrophoresis (Fig. S3B). The staining of each gel with Coomassie brilliant blue also revealed no activity of each protein sample bound to any DNA fragment. Obviously, Ssr4 had no DNA-binding activity, excluding a possibility of its acting as a transcription factor in *B. bassiana*.

DISCUSSION

As a cosubunit of SWI/SNF and RSC complexes, Ssr4 is required for yeast cell viability (1), leaving its functions unexplored yet. In this study, orthologous Ssr4 was proven to localize in the nucleus, act as a key player in genome-wide gene expression, and hence have pleiotropic effects on radial growth, cell hydrophobicity and differentiation, aerial conidiation, insect pathogenicity, and virulence-related cellular events in *B. bassiana*. These findings provide the first in-depth insight into an essential role for Ssr4 in the *in vitro* and *in vivo* asexual cycles of a filamentous fungal pathogen. Importantly, our transcriptomic analysis unveiled nearly one-fourth of all *B. bassiana* genes to be transcriptionally mediated by Ssr4 in direct and indirect manners, making it possible to understand its pleiotropic effects, as discussed below.

Asexual development is critical for the survival and dispersal of a filamentous fungus and genetically controlled by sequential activation of *brlA*, *abaA*, and *wetA* in the central developmental pathway and downstream of *vosA* (35–37). The four genes were proven to be required for aerial conidiation and conidial maturation of *B. bassiana* due to abolished conidiation in the absence of *brlA* or *abaA* (34) and greatly reduced (98% or 88%) conidial yield and impaired conidial quality in the absence of *wetA* or *vosA* (33). In this study, deletion of *ssr4* resulted in a 98% decrease in conidiation capacity, but none of the four genes was repressed at the transcriptional level, suggesting that some other factors were responsible for the severe conidiation defect. Aside from the developmental activators, multifunctional hydrophobins are well-known to take part in aerial hyphal growth and differentiation essential for conidiophore development and conidiation in filamentous fungi (31). In *B. bassiana*, conidial hydrophobicity was reported to be significantly lowered by the deletion of *hyd1* (class I hydrophobin gene) that resulted in bald conidial surface with altered rodlet fascicles/bundles or the deletion of *hyd2* (class II hydrophobin gene) that led to distorted conidial surface bundles with truncated/incomplete rodlets, and much more reduced by *hyd1-hyd2* double deletion (32). However, impacts of the single and double deletions on the fungal conidiation were not disclosed in the previous study. In this study, *hyd1*, *hyd2*, and two other hydrophobin-like genes (*hyd3* and *hyd5*) were markedly repressed in the absence of *ssr4*. In the Δ *ssr4* mutant, the reduced transcript levels of these genes correlated well with impaired hydrophobicity of hyphal culture, which featured an enhanced capability of water absorption and an increased water content. As a result of this correlation, hyphal differentiation and aerial conidiation were severely blocked, as shown in the SEM images. The limited conidia produced by the Δ *ssr4* mutant were also featured by an impaired coat with obscure, distorted, and fewer hydrophobin rodlet bundles and hence a marked decrease in hydrophobicity. Taken together, a block of aerial conidiation in the absence of *ssr4* is attributable to blocked differentiation of more hydrophilic hyphae, in which the *hyd* genes required for hydrophobin biosynthesis and assembly were largely downregulated at the transcription level, but not associated with any effect on the central pathway. In other words, Ssr4 mediates hyphal

differentiation and aerial conidiation of *B. bassiana* by its involvement in transcription of the *hyd* family genes.

Our Δ *ssr4* mutant was unable to cause any insect mortality through the cuticle infection and also lost ~90% of its lethal action against the model insect tested via cuticle-bypassing infection by intrahemocoel injection. This highlights the indispensability of *Ssr4* for fungal pathogenicity and virulence and that it plays a role as a supervirulence factor in *B. bassiana*. Fungal lethal action against insects depends on not only an ability to infect the host by hyphal penetration through insect cuticle but also intrahemocoel transition of penetrating hyphae to unicellular blastospores for rapid proliferation by yeast-like budding until host death from mummification. In general, hyphal penetration through insect cuticle relies upon the cuticle-degrading activities of secreted enzymes, including Pr1 family proteases (22, 51). In *B. bassiana*, pathogenicity abolished or greatly reduced via cuticle infection is often correlated with hindered secretion of such enzymes in the absence of one of some genes that function in different pathways, such as those encoding the Hsp40 family member *Mas5* (23), the Na^+/H^+ antiporter *Nhx1* (24), the eisosome proteins *Pil1A* and *Pil1B* (25), the blue-light receptor *VVD* (27), and the histone acetyltransferase *Gcn5* (26) or deacetylase *Rpd3* (52). In this study, however, complete abolishment of cuticle infection by the *ssr4* deletion is unlikely an outcome from a limited decrease in total activity of secreted enzymes involved in cuticle degradation. Instead, this abolished phenotype is likely due to the severe Δ *ssr4* defects in conidial adhesion, germination, and hyphal growth. Impaired coat, lowered hydrophobicity, and increased size of the mutant conidia could have reduced conidial adhesion to the surface of the insect. This inference is supported by a correlation of reduced conidial adhesion with blocked cuticle infection in the double deletion mutant of *hyd1* and *hyd2* (32), both of which were downregulated with *hyd3* and *hyd5* in the Δ *ssr4* mutant. Aside from the reduced adhesion, the Δ *ssr4* mutant could be slower in conidial germination and greatly suppressed in hyphal growth on oligotrophic insect integument, as indicated by its delayed germination *in vitro* and severe growth defects on nutritionally limited media. Such growth defects of the Δ *ssr4* mutant are close to those caused by single-gene deletions of some other SWI/SNF subunits in *C. albicans*, such as an inability for the Δ *swi1* mutant to form true hyphae essential for pathogenicity (19) and hyphal growth defects of the Δ *snf6* mutant on fermentable and nonfermentable carbon sources (20). On the other hand, the fungal virulence via cuticle-bypassing infection is also largely attenuated by inactivated genes as mentioned above due to repressed expression of developmental activator genes associated with blocked transition of hyphae to blastospores and vice versa. Such dimorphic transition has proved a developmental process governed by the key developmental activator gene *brlA* or *abaA* in *B. bassiana* (34). In this study, injection of the Δ *ssr4* conidia resulted in a very low (only 11%) insect mortality, accompanied by a failure to form hyphal bodies *in vivo* until 216 h postinjection and a drastic reduction in dimorphic transition *in vitro*. The delayed formation of hyphal bodies after injection may account for the low insect mortality, but it is not linked to either accumulation of hyphal biomass in the submerged cultures or repressed expression of any developmental activator gene. Our transcriptomic analysis revealed many repressed genes involved in different pathways of carbon/nitrogen metabolism and transport. These repressed genes could exert comprehensive effects on cell growth, differentiation, and development, helping us to understand severe growth defects of the Δ *ssr4* mutant on various carbon and nitrogen sources, a complete loss of its pathogenicity via the cuticle infection, and a 90% loss of its virulence via cuticle-bypassing infection.

The pleiotropic effects and genome-wide regulatory role of *Ssr4* help us to understand an increasingly upregulated expression of *ssr4* during the first 48-h period of *B. bassiana* infection comprising sequential steps of conidial germination, hyphal penetration through cuticle and hemocoel colonization (21). More than 24% of all genes regulated by *Ssr4* is far beyond those regulated by *Snf6* (~10%) in *C. albicans* (20) and *Rei1*-like protein (13.5%), a pre-60S subunit export factor that was also increasingly upregulated at the transcriptional level in the 48-h infection course and involved in

nutritional metabolism and transport required for the asexual cycle *in vitro* and *in vivo* of *B. bassiana* (53). Importantly, a large number of genes downregulated in the Δ *ssr4* mutant are involved in metabolism and/or transport of carbohydrates, amino acids, lipids and inorganic ions, energy production and conversion, and biosynthesis, transport, and catabolism of secondary metabolites. These genes positively regulated by Ssr4 could exert fundamental impacts on nutrition utilization, energy supply, and intracellular ion homeostasis. Intriguingly, our EMSA results excluded the possibility of Ssr4 acting alone as a transcription factor, hinting at the likelihood that the mediation of global gene expression by Ssr4 may rely on the role of its taking part in the chromatin remodeling of the SWI/SNF complex, which can bind to the binding sites of transcription factors in model yeast (3, 4). Notably, 113 genes differentially expressed in the Δ *ssr4* mutant are functionally involved in DNA replication, transcription, translation, and posttranslational modifications, thereby expanding the role of Ssr4 in genome-wide transcription regulation. Therefore, we infer that Ssr4 regulates global gene expression in *B. bassiana* by acting directly as a subunit of the chromatin-remodeling complex or indirectly through transcriptional regulation of those genes that enable mediation of gene activity.

Altogether, our findings uncover for the first time the pleiotropic effects of Ssr4 required for the asexual cycle *in vitro* and *in vivo* of *B. bassiana* and also its essential role in global gene expression and recall research attention to molecular mechanisms underlying the role of Ssr4 in the chromatin-remodeling SWI/SNF complex and interactions between Ssr4 and other subunits of the complex in filamentous fungal pathogens.

MATERIALS AND METHODS

Subcellular localization of Ssr4 in *B. bassiana*. The coding sequence of *ssr4* revealed by previous analysis of transcriptomes (21) was amplified from the cDNA of the WT strain with paired primers (see Table S2 in the supplemental material). The protein sequence deduced from the coding sequence and those of Ssr4 orthologs found in the genomic databases of *S. pombe* and several filamentous fungi via online BLAST analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) were aligned with the SMART program (<http://smart.embl-heidelberg.de>) for structural comparison, followed by analysis of their phylogenetic relationships with MEGA7 at <http://www.megasoftware.net>. A nuclear localization signal was predicted from each ortholog using cNLS Mapper software at <https://omictools.com/cnls-mapper-tool>.

The N terminus of the amplified *ssr4* cDNA was fused to the C terminus of *gfp* (GenBank accession no. or code U55763), inserted into linearized pAN52-bar, and transformed into the WT as described previously (53). A transgenic strain best expressing the fusion gene *gfp::ssr4* was chosen for incubation and full conidiation at 25°C on SDAY (4% glucose, 1% peptone, and 1.5% agar plus 1% yeast extract). The resultant conidia were suspended in SDBY (i.e., agar-free SDAY) and incubated 3 days on a shaking (150 rpm) bed at 25°C. Hyphal cells collected from the culture were stained 30 min with the nucleus-specific dye DAPI (4',6'-diamidino-2'-phenylindole dihydrochloride; Sigma) at ambient temperature and visualized through LSCM for subcellular localization of expressed fusion protein. The protein extracts isolated from the 3-day-old cultures of the WT and transgenic strains were probed with mouse monoclonal anti-GFP antibodies (Cell Signaling Technology, Boston, MA, USA) for Western blots of GFP alone and GFP::Ssr4 to verify whether the fusion protein was expressed as expected.

Generation of *ssr4* mutants. The gene *ssr4* was deleted by homogenous recombination of its 5' and 3' fragments separated by *bar* maker (p0380-5'*ssr4*-bar-3'*ssr4*) in the WT and rescued in an identified deletion mutant by ectopic integration of a cassette consisting of its full-length sequence and *sur* marker (p0380-*sur-ssr4*) through *Agrobacterium*-mediated transformation, as described previously (53). Briefly, the deletion plasmid was constructed by amplifying the 5' and 3' coding/flanking fragments (1,260 and 1,381 bp, respectively) of *ssr4* from the genomic DNA of the WT strain with paired primers (Table S2) under the action of LaTaq DNA polymerase from Promega (Madison, MI, USA) and inserting them into appropriate enzyme sites in our backbone plasmid p0380-bar. The complementing plasmid was constructed by amplifying a full-length coding/flanking sequence of *ssr4* (3,833 bp in total) from the WT DNA and inserting it into p0380-*sur*-gateway to take the place of the gateway fragment. After transformation, putative mutant colonies were screened by the *bar* resistance to phosphinothricin (200 μ g/ml) or the *sur* resistance to chlorimuron ethyl (10 μ g/ml), followed by PCR and Southern blot analyses with paired primers and amplified probe (Table S2) to verify the expected recombination events. Positive Δ *ssr4* and Δ *ssr4::ssr4* mutants were evaluated together with the WT strain in the following experiments of three independent cultures (replicates) or samples from the cultures.

Experiments for pleiotropic effects. The 1- μ l aliquots of a 10⁶ conidia/ml suspension were centrally spotted on the plates of SDAY, CDA (3% sucrose, 0.3% NaNO₃, 0.1% K₂HPO₄, 0.05% KCl, 0.05% MgSO₄ and 0.001% FeSO₄ plus 1.5% agar) and 34 CDA-derived media, which were prepared by deleting 3% sucrose or 0.3% NaNO₃ or both from the standard CDA, replacing the carbon source with 3% glucose, fructose, lactose, maltose, trehalose, mannitol, glycerol, or acetate (sodium acetate [NaAc]), and replacing the

nitrogen source with 0.3% NH_4Cl , NaNO_2 , NH_4NO_3 or one of 20 amino acids, respectively. After a 7-day incubation at 25°C, the mean diameter of each colony was estimated with two measurements taken perpendicular to each other across the colony center to calculate colony area as a radial growth index of each strain on each medium.

Conidiation capacity was quantified from the SDAY cultures initiated by spreading 100 μl of a 10^7 conidia/ml suspension per plate (9-cm diameter) and incubated 15 days at the optimal regimen of 25°C and 12-h/12-h light/dark (L/D) cycles. From day 4 onwards, three plugs (5-mm diameter) were taken daily from each plate culture using a cork borer. All conidia were released into 1 ml of 0.02% Tween 80 from each plug by supersonic vibration. The conidial concentration in the resultant suspension was assessed with a hemocytometer and converted to the number of conidia per unit area (squared centimeters) of plate culture. For assessment of biomass level, SDAY plates overlaid with cellophane were spread with 100- μl aliquots of the same conidial suspension for culture initiation, followed by a 15-day incubation at the same regimen. During the period of incubation, biomass levels were quantified from the fresh culture (wet biomass) of each plate and the same culture dried 2 h at 70°C (dry biomass), respectively. To reveal hydrophobic and hydrophilic features of hyphal cultures, 10- μl aliquots of sterile water containing 0.02% Tween 80 were dipped onto the surfaces of 4-day-old SDAY cultures initiated with the same method. The dew-like water droplets were monitored for their stability on the culture surfaces of different strains during a period of 72 h at 25°C. In addition, conidiation status of each strain in the 5-day-old SDAY cultures and ultrastructural changes on the surfaces of conidia were examined via scanning electron microscopy (SEM) after pretreatments as described elsewhere (27, 32).

Conidial virulence of each strain was assayed on *G. mellonella* larvae in two infection modes. Briefly, three batches of ~35 larvae per batch were separately immersed for 10 s in 30 ml of a 10^7 conidia/ml suspension for normal infection through cuticular penetration. Alternatively, 5 μl of a 10^5 conidia/ml suspension was injected into the hemocoel of each larva in each batch for cuticle-bypassing infection. All batches treated with each strain in either infection mode were maintained at 25°C for 12 days and monitored every 12 or 24 h for survival/mortality records. During the period of monitoring, hemolymph samples were taken from surviving larvae and examined for the presence/absence and abundance of hyphal bodies under a microscope. Cadavers 5 days after death were observed to show an ability for intrahemocoel cells of each strain to penetrate insect cuticle for outgrowth and conidiation on cadaver surfaces.

Infection- and virulence-related cellular events of all strains tested were examined. First, conidial hydrophobicity as an important index of conidial adhesion to insect integument was assessed in a diphasic (aqueous/organic) system as described previously (54, 55). Second, total extracellular enzyme (ECE) and Pr1 activities essential for cuticle degradation were quantified from the supernatants (crude extracts) of 3-day-old submerged cultures in CDB (i.e., agar-free CDA) by reading optical densities at 442 nm (OD_{442}) and 410 nm (OD_{410}), respectively, as described elsewhere (23, 26, 51). The submerged cultures were initiated with 50-ml aliquots of a 10^6 conidia/ml suspension in CDB containing the sole nitrogen source of 0.3% BSA for enzyme induction. One unit of enzyme activity was defined as the enzyme amount required for an increase of each OD value by 0.01 after 1-h reaction of each extract versus a control; total activity was expressed as units per milliliter of supernatant with respect to dry biomass level estimated from the culture. Third, 20-ml aliquots of a 10^6 conidia/ml suspension in CDB and TPB (amended CDB containing 3% trehalose [sole carbon source] and 0.5% peptone [sole nitrogen source]) were incubated by shaking (150 rpm) for 3 days at 25°C. Blastospore concentration and dry biomass level (in milligrams per milliliter) were quantified from each culture. The two quantities were used to compute dimorphic transition rate (number of blastospores per milligram of biomass) *in vitro*. Finally, standardized suspensions (10^7 cells/ml) were prepared with three samples of conidia from the SDAY cultures or samples of blastospores from the TPB cultures of each strain. The size and complexity of conidia or blastospores were assessed with the respective readings from forward scatter (FSc) and side scatter (SSc) detectors in flow cytometry of 2×10^4 conidia or blastospores per sample.

All data from the experiments of three replicates were subjected to one-way (strain) analysis of variance, followed by Tukey's honestly significant difference (HSD) test for phenotypic changes among the tested fungal strains.

Transcriptional profiling. Aliquots of 100 μl of the 10^7 conidia/ml suspension were spread onto cellophane-overlaid SDAY plates and incubated 5 or 7 days at the optimal regimen. Total RNAs were extracted daily from the WT cultures during the 7-day cultivation or from the 5-day-old cultures of the $\Delta\text{ssr}4$ mutant and control strains using an RNAiso Plus kit (TaKaRa, Dalian, China) and reverse transcribed into cDNAs using a PrimeScript^{RT} reagent kit (TaKaRa), respectively. Three samples of each cDNA were used as the templates to assess transcript levels of *ssr4* in the daily WT cultures and of five hydrophobin genes (*hyd1* to *hyd5*) in the 5-day-old cultures of the $\Delta\text{ssr}4$ mutant and control strains through real-time quantitative PCR (qPCR) with paired primers (Table S2) under the action of SYBR Premix Ex Taq (TaKaRa). The fungal 18S rRNA was used as an internal control. The threshold cycle ($2^{-\Delta\Delta\text{CT}}$) method was used to calculate the relative transcript level of *ssr4* over the incubation days of the WT strain with respect to a standard transcript at the end of 48-h incubation and of each *hyd* gene in the *ssr4* mutants with respect to the WT standard. Each qPCR was repeated three times using cDNA samples derived from independent cultures.

Transcriptomic analysis. Three 4-day-old cultures (replicates) of $\Delta\text{ssr}4$ and WT strains grown on cellophane-overlaid SDAY plates were prepared as mentioned above and sent to Microanaly Gene Co. (Shanghai, China) for transcriptome constructs and analysis. Extraction of total RNA from each culture, isolation of mRNA from the total RNA, fragmentation of mRNA, and syntheses of first- and second-strand cDNAs followed the same protocols of transcriptome construction as described previously (53). Each of

the double-stranded cDNAs was purified and end repaired, followed by adding a single adenine to the ends of the cDNA molecules. The final cDNA library was generated by adding proper adaptors to the cDNA and sequenced on an Illumina Novaseq 6000 platform.

All clean tags generated through filtration of raw reads from the cDNA sequencing were mapped to the *B. bassiana* genome (30). All data were normalized as fragments per kilobase of exon per million fragments mapped (FPKM). Differentially expressed genes (DEGs) were identified in terms of \log_2 ($\Delta\text{ssr4}/\text{WT}$ ratio) ≤ -1 (downregulated) or ≥ 1 (upregulated) at the significance level of a false-discovery rate (FDR) of less than 0.01. All identified DEGs were annotated with known or putative gene information in the nonredundant NCBI protein databases and subjected to eggNOG function classification with eggNOG v4.5 (<http://eggnoG.embl.de>). All function classes were enriched to four categories: cellular process and signaling, information storage and processing, metabolism, and uncharacterized. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (<http://www.genome.jp/kegg/>) was also performed to enrich DEGs to various KEGG pathways at the significance level of $q < 0.05$.

EMSA for binding activity of Ssr4 to promoter DNA fragments. Electrophoretic mobility shift assay (EMSA) was conducted as described previously (53). Briefly, Ssr4 expressed in competent *Escherichia coli* cells was detected in cell lysates through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by purification through affinity chromatography column, dialysis, and standardization to 0.4 mg/ml. The purified protein samples were used as the templates to detect the binding activity of Ssr4 to each of six promoter DNA fragments amplified from the genomic DNA of the WT with paired primers (Fig. S3A). The 4- μl aliquots of each DNA extract were mixed with 2 to 10 μl (0.8 to 4.0 μg) of the purified protein extract, respectively, in 10- μl aliquots of binding buffer comprising 25 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid [pH 7.4]), 50 mM KCl, 5 mM MgCl_2 , 0.5 mM EDTA, 1 mM dithiothreitol, and 5% glycerol. The combinations of 4 μl DNA extract with no protein extract and of 10 μl protein extract with no DNA extract were used as negative controls in each EMSA. All reactions lasted for 30 min at ambient temperature, followed by agarose gel electrophoresis for detection of Ssr4-bound DNA. The resultant gels were stained with Coomassie brilliant blue and rinsed repeatedly in washing buffer for visualization of the targeted protein.

Data availability. All data generated or analyzed during this study are included in the published paper and associated supplemental files. All transcriptomic data aside from those reported in supplemental files (Tables S3 to S5) of this paper are available at the NCBI's Gene Expression Omnibus under the accession no. [GSE132211](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132211).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, JPG file, 0.2 MB.

FIG S2, JPG file, 0.2 MB.

FIG S3, JPG file, 1.1 MB.

TABLE S1, PDF file, 0.4 MB.

TABLE S2, PDF file, 0.3 MB.

TABLE S3, XLSX file, 0.4 MB.

TABLE S4, XLSX file, 0.2 MB.

TABLE S5, XLSX file, 0.04 MB.

ACKNOWLEDGMENTS

She-Long Zhang (Core Facilities, College of Life Sciences, Zhejiang University [ZJU]) and Jun-Ying Li (Analysis Center of Agrobiological and Environmental Sciences, ZJU) are acknowledged for their assistance with LSCM, SEM, and flow cytometry.

Funding of this study was provided by the National Natural Science Foundation of China (31772218 and 31801795), Ministry of Science and Technology of the People's Republic of China (2017YFD0201202), and China Postdoctoral Science Foundation (2019M652068).

We declare that we have no conflicts of interest.

M.-G.F. and W.S. conceived and designed the study. W.S., Q.C., S.-M.T., and S.-H.Y. performed experiments. M.-G.F. and W.S. analyzed the data. M.-G.F. prepared figures and tables and wrote the manuscript with input from all authors. All authors read and approved the final manuscript.

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