SclR, a Basic Helix-Loop-Helix Transcription Factor, Regulates Hyphal Morphology and Promotes Sclerotial Formation in *Aspergillus oryzae*†

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Most known basic-region helix-loop-helix (bHLH) proteins belong to a superfamily of transcription factors that have been well characterized, especially in the mammalian system (1, 16, 19). The bHLH domain contains approximately 60 amino acids and is comprised of two functionally distinctive regions: the basic region and the HLH region. The basic region is located at the N terminus of the domain and functions as a DNA-binding motif. It consists of approximately 15 amino acids, which typically include six basic residues (2). The HLH region, which is located at the C terminus of the domain, functions as a dimerization domain and is constituted mainly of hydrophobic residues that form two amphipathic α helices, along with a linking loop of variable length. The amphipathic α-helices of two bHLH proteins (or bHLH and HLH proteins) can interact, allowing the formation of homodimers or heterodimers (8, 28, 29). Some bHLH proteins have been shown to bind to sequences containing a consensus core element called the E box (5'–CANNTG–3'), with the G box (5'–CACGTG–3') being the most common form. In addition, the nucleotides flanking the core element may also have a role in binding specificity (2, 22, 24, 31).

In the animal system, bHLH proteins have been classified into six main groups (designated group A to F [1]) according to their phylogenetic relationships, DNA-binding motifs, and functional properties. The HLH family transcription factors play an important role in the regulation of neurogenesis, myogenesis, cell proliferation and differentiation, cell lineage determination, sex determination, and other essential processes in organisms ranging from yeasts to mammals (18, 26, 27, 32, 35). A phylogenetic classification of 242 bHLH proteins has been proposed (1, 3).

Compared to animals, only a small number of fungal bHLH proteins have been functionally characterized. In *Aspergillus nidulans*, three bHLH transcription factors (AnBH1, DevR, and StuA) were previously described. The AnBH1 is involved in the regulation of a penicillin biosynthesis gene and functions as a promoter of penicillin biosynthesis (5). DevR was shown to be a part of the *tcsA* signal transduction network and is required for both asexual and sexual development of *A. nidulans* (36). Another *A. nidulans* protein, StuA, conserved an APSES domain that was demonstrated to be a bHLH-like structure (7). The StuA regulates development and cell cycle progression. StuA has also been shown to act as a transcriptional repressor in *A. nidulans* (7).

The filamentous fungus *A. oryzae*, which is known for its capacity to secrete large amounts of hydrolytic enzymes, is widely utilized in the traditional food fermentative industry. On the basis of the available *A. oryzae* genomic sequence information (15, 21), 13 HLH proteins were predicted. However, none of the *A. oryzae* HLH transcription factors have been characterized to date.

In our previous study, we identified a novel bHLH protein-encoding gene (*sclR*; AO090011000215) by systemati-
cally deleting large chromosomal segments and further deletion analysis to screen a phenotype of dense conidia (14). A. oryzae SclR shares high similarity with those of putative proteins from genome sequencing projects of A. niger, A. fumigatus, and A. nidulans (14). The gene-disrupted strain was found to produce dense conidia, but sparse sclerotia, relative to the parent strain, suggesting that it possibly plays an important role in morphology and growth. Here, we further report the characterization of the sclR gene, along with analysis of its function. Overexpression of the sclR gene led to abnormal hyphal morphology and sclerotial formation. The loss of function of the sclR gene also led to rapid degradation of protein in liquid medium.

**MATERIALS AND METHODS**

**Strains and media.** The A. oryzae wild-type strain, RIB40, was used as a DNA donor, strain RkuAFN, a niaD derivative from strain RkuFp2-1ΔAF/F (Table 1), was used for the sclR overexpression in A. oryzae. To construct the RkuAFN strain, two arms were amplified by PCR. The L-arm with the primer pair 215F(SpeI) and 215R(SmaI). After digestion with SmaI and then inserted into the SmaI site of the plasmid pg5

**TABLE 1. Strains used in this study**

| Strain         | Genotype              | Source or reference |
|----------------|-----------------------|---------------------|
| RkuFp2-1ΔAF    | ∆ntrA ∆ΔF ∆ntrG       | 34                   |
| RkuFp2-1ΔAF/F  | ∆ntrA ∆ΔF/*         | 30                   |
| RkuAFN         | ∆ntrA ∆ΔF/*         | This study           |
| Control-1      | ∆ntrA ∆ΔF/*pAPT[∆ntrD] | This study           |
| OE-sclR        | ∆ntrA ∆ΔF/*pamy215[∆ntrD] | This study           |
| ∆sclR         | niaD::pGHHD[rc]PpLTLn[∆ntrD] | 14 |
| HHD[rc]Pp      | niaD::s'::pGHHD[rc]  | This study           |
| Control-2      | niaD::s'::pGHHD[rc](PpLTLn[∆ntrD]) | This study           |
| SclR-EGFP      | niaD::s'::pGHHD[rc](PpLTLn[∆ntrD]) | This study           |

**TABLE 2. Primers used in this study**

| Primer         | Sequence (5′−3′)                |
|----------------|--------------------------------|
| AoniaDD1       | GCAATGCAGCATCTAATAAAAC          |
| AoniaDD2       | TTGATTCATTGCTGCTTCTAACT         |
| AoniaDD3       | GAAACCTCAGATAGACCGGAAGAGCTAAAC |
| AoniaDD4       | CGCGAGGTGCTTCTACAGAAATGTC       |
| H2B5           | AATCCGGGTCGATTTGCAATGTTGCA      |
| H2BP-2         | TAGGACAGTGTAGTACACTGAGTG        |
| 215F(SpeI)     | CCAGGGGTAATACACCACTTGCTCC       |
| 215R(SmaI)     | TACATGCTGCTCTCTTCTCTTTG         |
| TMwetA-R       | AAACGACATGTATAACTCCGCCT        |
| TMwetA-F        | ATGGGAGGCTGTGTGTTCCA           |
| abaA-TR        | AGGCATTGGGTGAGTTGGGACC         |
| VeA2-TR         | GATTCAATCCACCACTTTTCA          |
| VeA2-F          | CATGTGACCCTTGGAAACCAT           |
| abaA-TR        | CCATCTAGCTGAGTATTGCTC          |
| VeA2-TR         | CTGTTGACCCTTGGAAACCAT           |
| abaA-TR        | GCCCTTGTTTGAGTTGAGGGGACC       |
| VeA2-TR         | TATCCGGGCAATCTTTCCTTGCG        |
| abaA-TR        | CATGTGACCCTTGGAAACCAT           |
| VeA2-TR         | CTGTTGACCCTTGGAAACCAT           |
| abaA-TR        | GCCCTTGTTTGAGTTGAGGGGACC       |
| VeA2-TR         | TATCCGGGCAATCTTTCCTTGCG        |

**Constructing plasmid for sclR overexpression.** To construct the plasmid for sclR overexpression, the sclR ORF was amplified by PCR using the C. oryzae RIB40 genomic DNA and the primer pair 215F(SpeI) and 215R(SmaI). After digestion with SpeI and SmaI, the fragment was ligated into the SpeI-SmaI site of the multicloning site of the pAPTLN vector (25), which contains the ampyB promoter and the niaD gene as a selectable marker. The generated plasmid pamy215 (Fig. 2a) was then used for the transformation.

**Southern blot analysis.** For Southern blot analysis, the overexpressing strains were identified by PCR analysis and Southern blot analysis. Genomic DNA of A. oryzae strains was extracted as previously described (33). After electrophoresis, the digested genomic DNA was transferred onto a Hybond-N+ membrane (Amersham Biosciences, Amersham, United Kingdom). Southern hybridization was performed as described previously (33). A digoxigenin (DIG)-labeled probe was constructed by using a PCR DIG labeling kit (Roche Diagnostics, Mannheim, Germany) with the primer pair 215F(SpeI) and 215R(SmaI). Hybridization and detection of signals with the DIG labeling kit (Roche Diagnostics, Mannheim, Germany) was performed according to the manufacturer's instructions. (Roche Diagnostics).

**RNA isolation and real-time PCR analysis.** Total RNA was isolated from the strains grown under different culture conditions by using biogen (Wako, Osaka, Japan) and a RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturers' protocols. The total RNA was treated with DNase I (TaKaRa, Japan) to remove the chromosomal DNA. Reverse transcription-PCR (RT-PCR) was performed with 1 μg of total RNA and a SuperScript VILO cDNA synthesis kit (Invitrogen) in accordance with the manufacturer's instructions. Semiquantitative real-time PCR was carried out with cDNA as a template. The primer pair OR215-F(SpeI) and OR215-R(SmaI) was used to amplify sclR, and the primer pair H2B-2F(20) and H2B-R(270) was used to amplify the histone H2B as a positive control. PCR was carried out for 25 cycles using a program of 94°C for 5 s and 60°C for 30 s. PCR products were collected and electrophoresed on a 2% agarose gel and stained with GelRed (Wako). Quantitative real-time PCR was performed for sclR using the same primer pair mentioned above, and TEF1, as a reference gene, was amplified by using the primer pair Tmef1F and Tmef1R.
The expression levels of brlA, wetA, abaA, veA, and sspA were tested by using the primer pairs TMbrlA-FW/TMbrlA-RV, TMwetA-FW/TMwetA-RV, abaA-TF/abaA-TR, veA2-TF/veA2-TR, and qRTsspA-F(191)/qRTsspA-R(291), respectively. Gene expression was analyzed with 300 nM primers using the Brilliant II Fast SYBR green QPCR master mix (Stratagene) and the Mx3000P instrument (Stratagene). Cycle conditions were 2 min at 95°C and 40 cycles of 5 s at 95°C and 20 s at 60°C. A standard curve method was used here for the quantification of gene expression. The relative level of expression was determined by the ratio of the target gene to the reference gene after amplification. All reactions were performed in duplicate, and the mixture included a negative no-RT control.

Conidial count and sclerotial count. Each strain was cultured onto a malt agar plate (diameter, 85 mm) at 30°C for 5 to 10 days. Conidia were suspended in 10 ml of 0.01% Tween 80 and filtered through a 70-μm-pore-size cell strainer (BD Falcon) to remove the mycelial matter. Separated conidia were further diluted and counted with a hemacytometer. Simultaneously, the mycelial matter containing the sclerotia after filtration was resuspended in 20 ml of distilled water and vortexed 10 times. Then, 1 ml of resuspension was extracted, and the black or dark brown sclerotial formations were counted.

Measurement of biomass dry weight. A total of 10⁶ conidia of each strain were cultivated in 40 ml of DYP liquid medium for 1, 3, and 6 days. Mycelia were collected by filtration through a Miracloth (Calbiochem) and then rapidly frozen in liquid nitrogen for 5 min. The frozen samples were lyophilized for 18 h. The weights of the freeze-dried samples were measured.

Construction of the strain for the production of the SclR-EGFP fusion protein. The DNA sequence containing the 1.0-kb sclR promoter region and full-length ORF was amplified by PCR using the A. oryzae RIB40 genomic DNA and the primer pair 215p-F(990IF) and 215R(IF). The PCR product was then inserted into a pAPTLN vector that was linearized by PCR with the primers V-F and V-R using an In-Fusion PCR cloning kit (Takara) according to the manufacturer’s protocols, because the designed PCR primers of the inserts have 16 bases with homology to the terminal sequences of the linearized pAPTLN vector. Next, the generated plasmid p215 was linearized by PCR with the primer pair amyl-F(IF) and 215R(IF). Using the plasmid pNH2BG harboring the h2b-egfp fusion gene (23) as a template, egfp was inserted into the terminus of the amyB promoter. The full-length sclR ORF is inserted into the terminus of the amyB promoter. The expected DNA sizes are shown by the arrow. Lane 1, parent strain; lanes 2 to 5, transformants with pamy215. (c) Confirmation of sclR overexpression by semiquantitative RT-PCR analysis. Total RNA was isolated from the strains cultured on CD or starch-containing CD media for 2 days. Negative-control PCR analysis using each mRNA as a template showed no amplified fragments (data not shown).
amplified by PCR with the primer pair GFPc-F(IF) and GFPc-R(IF) and then was further inserted into the C-terminal of the \textit{sclR} ORF of linearized p215 by use of an In-Fusion cloning kit (Takara). Finally, the plasmid (p215GFP) was generated and introduced into the HHDR strain to obtain the \textit{sclR}-EGFP fusion protein-expressing strain.

SDS-PAGE, CBB staining, and Western blot analysis. Conidia were cultured into 40 ml of DPY liquid medium at 30°C for 3 days. Proteins were extracted from the mycelia with radioimmunoprecipitation assay buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris-HCl [pH 7.4], and a 1% proteinase inhibitor [Wako]). A BCA protein assay kit (Pierce Chemicals) was used to quantitate the protein concentration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 10 to 20% polyacrylamide gel (Wako). Portions of 10 \mu l/well (24 \mu g) of each concentrated sample were loaded. The polyacrylamide gel was stained with a quick-CBB staining kit (Wako) for 30 min. For Western blot analysis, samples separated by electrophoresis on SDS-polyacrylamide gels were transferred onto a polyvinylidene difluoride membrane (GE Healthcare, Amersham, United Kingdom). The membrane was probed with primary antibody to green fluorescent protein (GFP; 1:5,000 dilution, ab290; Abcam, Cambridge, United Kingdom), and horseradish peroxidase-conjugated anti-rabbit antibody (1:10,000; GE Healthcare) was used as the secondary antibody. The peroxidase reaction products were detected using the ECL Plus Western blotting detection reagent (GE Healthcare).

SEM. Conidia were cultivated on malt agar medium at 30°C for 2 to 5 days. Samples were cut and fixed with 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for 3 h and postfixed with 1% osmium tetroxide in the same buffer for 1.5 h. They were rapidly frozen in liquid nitrogen for 3 to 5 min, and then lyophilization was performed. The specimens were examined by scanning electron microscopy (SEM; TM-1000 Miniscope; Hitachi High Technologies, Tokyo, Japan).

Microscopy and image analysis. Confocal microscopy was performed with an IX71 inverted microscope (Olympus, Tokyo, Japan) equipped with \times100 and \times40 Neofluor objective lenses (1.30 numerical aperture), 488-nm (Furukawa Electric, Tokyo, Japan) and 561-nm (Melles Griot, California) semiconductor lasers, GFP, DsRed and DualView filters (Nippon Roper, Chiba, Japan), a CSU22 confocal scanning system (Yokogawa Electronics, Tokyo, Japan), and an Andor iXon cooled digital charge-coupled device camera (Andor Technology, PLC, Belfast, United Kingdom). Images were analyzed using Andor iQ software (Andor Technology).

FIG. 3. Growth phenotype of the \textit{sclR}-overexpressing strain and the \textit{sclR}-disrupted strain. (a) Comparison of growth phenotypes. The Control-1 strain, \textit{sclR}-disrupted strain (\textit{ΔsclR}), and \textit{sclR}-overexpressing strain (OE-\textit{sclR}) were cultivated on malt agar medium at 30°C using spot and streak culture. (b) \textit{sclR} overexpression promotes sclerotial production. The OE-\textit{sclR} strain and the Control-1 strain were cultured on malt agar medium at 30°C for 9 days. The magnification of the panels on the right hand site is the same. SC, sclerotium.
RESULTS

Pattern of sclR gene expression. In our previous study, we identified a bHLH protein-encoding gene, sclR, the disruption of which led to dense conidia and sparse sclerotia. SclR is predicted to encode an unknown protein of 302 amino acids containing a putative bHLH motif (14).

To learn more about the mechanism of sclR gene, the timing of sclR gene expression were examined using quantitative RT-PCR. The wild-type strain A. oryzae RIB40 was grown under different culture conditions, the total RNA was extracted, and a target segment of the sclR gene was amplified from the purified RNA. The relative amounts of sclR message were determined from the ratio of the target to the reference gene after amplification (Qt/Qr). Quantitative RT-PCR (qRT-PCR) data analysis indicated that the sclR gene was expressed at the early stage (on the first day) when cultivated on both malt and CD agar media, and the expression level was found to gradually decrease from 2 days onward (Fig. 1). In addition, it is obvious that the expression level of sclR is higher in a liquid shaking incubator than on the agar medium, and the expression level showed an increasing tendency during 2-day cultivation. sclR also showed higher expression level in skim milk-containing CD liquid medium (Fig. 1).

Construction of A. oryzae sclR overexpression strain. To construct the sclR-overexpressing strain, RkuAFN was transformed with the generated plasmid, pamy215, as depicted in Fig. 2a. Genomic DNA extracted from transformants was isolated, digested with EcoRI, and then analyzed by Southern blot analysis using the sclR ORF as a probe. A single band was detected in the wild-type strain, while two individual bands were detected in that of the sclR-overexpressing strains. The size of the second band confirmed that the plasmid pamy215 has been inserted into the genomic niaD locus (Fig. 2b). To further verify whether the sclR gene was overexpressed in sclR-overexpressing strains, the transcription level of sclR was examined by semiquantitative RT-PCR analysis. For this analysis, total RNA was isolated from the strain grown under noninducing and inducing conditions. Starch-containing medium was used as an A. oryzae amyB promoter induction medium, since the promoter can be strongly induced by starch. The results of semiquantitative RT-PCR analysis show that sclR was more effectively transcribed in the sclR-overexpressing strain (OE-sclR) than in the wild-type strain when grown under the inducing culture condition. At the same time, no amplified fragment was detected in the sclR-disrupted strain (ΔsclR) (Fig. 2c).

sclR overexpression promotes sclerotium initiation. As shown in our previous research, the ΔsclR strain exhibited dense conidiation and sparse sclerotia relative to the control strain (14). Conversely, in the OE-sclR strain, hyphal aggregation was highly promoted, at the same time numerous sclerotial formations were observed (Fig. 3). When grown on the malt agar medium, the OE-sclR strain initially displayed abnormal polar growth and formed cottony and fluffy hyphae (Fig. 3a). Then, the abnormal hyphae gradually aggregated and a number of sclerotium-like structures were observed 7 to 9 days after inoculation (Fig. 3b). Measurements of the diameter of the colonies showed that the OE-sclR strain had a slower growth rate than the control strain, which was the opposite of what we observed with the ΔsclR strain (Fig. 4a). To verify whether the sclerotia of OE-sclR strain actually exceeded that of the control strain, the sclerotial number was counted. The result indicated that the sclerotial number of the OE-sclR strain had increased >5.5 times compared to the control strain when spot cultured on the malt agar medium (Fig. 4b). Conversely, the ΔsclR strain did not produce sclerotia or produced only a negligible number. At the same time, the axenic development-conidiation of the OE-sclR strain was notably reduced (Fig. 4c).

Electron microscopy of hyphal morphology. To further observe hyphal morphology, these strains were streak cultured on malt agar medium for 2 days. The marginal regions of the colonies were cut, fixed, and lyophilized. SEM indicated that a large number of the aerial hyphae of the OE-sclR strain were extremely branched. Instead of having the erect structure of conidial head, these branched aerial hyphae looked like a hand trying to grasp the neighboring hyphae and were highly intertwined with each other (Fig. 5a and b). The aberrant hyphal morphogenesis is a possible reason for the large number of sclerotial formation. In contrast, the sclR-disrupted strain pro-
duced almost no abnormal aerial hyphae such as those in the OE-sclR strain. The sclR-overexpressing strain was cultured continuously for 5 days, and many white sclerotia were further observed using SEM. Although the control strain was also seen hyphae connections and therefore produced a small number of the sclerotia, the OE-sclR strain displayed many more and tighter hyphal connections and fusions than did the control strain (Fig. 5c).

sclR gene-disruption leads to dramatic cell-lysis and protein degradation in DPY liquid medium. The OE-sclR strain, ΔsclR strain, and control strain were cultivated in DPY liquid medium at 30°C for 1, 3, and 6 days. Their phenotypes were observed and compared. We found that the ΔsclR strain produced an abnormal pellet that was comprised of a hollow structure surrounded by soft and fluffy hyphae (Fig. 6a). The time course of the change in biomass indicated that sclR gene disruption resulted in an evident decrease in biomass dry weight from the first day after inoculation, and the biomass was reduced to ca. 38% of the control strain on day 6 after inoculation. Conversely, the biomass of the OE-sclR strain increased slightly (Fig. 6b). The protein production pattern was further observed. The protein was extracted from the mycelia, and SDS-PAGE was performed. The protein products of the ΔsclR strain were severely decreased after 3 days (Fig. 6c). The results confirmed that the loss of sclR gene function led to a rapid degradation of the cellular proteins in the sclR-disrupted strain grown under DPY liquid medium conditions, and the sclR-encoding protein may play an important role in retaining normal cell function, at least in a liquid medium.

Localization of SclR-EGFP fusion protein. Construction of the strain for the expression of SclR-EGFP fusion protein was described in Materials and Methods. An ~1.0-kb upstream fragment of sclR was used as promoter, and egfp was fused to the C terminus of the full-length sclR ORF (see Fig. S1a in the supplemental material). The generated plasmid was used to transform the HHDR strain, the nuclei of which can be visualized by use of DsRed fluorescent protein fused to a nuclear localized protein, histone H2B (23). Genomic DNA of transformants was digested with EcoRI, and Southern blot hybridization was performed with the sclR probe. The results showed that the strains having the SclR-EGFP fusion gene had been obtained (see Fig. S1b in the supplemental material). Expression of the SclR-EGFP fusion protein was further confirmed by Western blot analysis (see Fig. S1c in the supplemental material). To verify whether the GFP fusion protein is functional, a complementation experiment was performed. The sclR-egfp expression plasmid, in which the niaD marker was replaced by pyrG marker, was used to transform the pyrG-deficient sclR-
disrupted strain. The phenotype of sclR-disrupted strain was restored by introducing sclR-egfp expression plasmid (data not shown).

The SclR-EGFP strain was cultured in DPY liquid medium at 30°C. GFP fluorescence was observed under light and fluorescence microscopy fitted with a GFP filter. In some mycelia, the GFP fluorescence was localized in the nucleus, which is in accord with the result observed with DsRed fluorescence, while in others the GFP fluorescence observed in the whole cytoplasm of mycelia on day 3 after the inoculation (Fig. 7b).
and c). On the fourth day, in the majority of mycelia, especially in the mycelia that existed in the central region of the pellet, the GFP fluorescence was easily observed to localize mainly in the nuclei (Fig. 7d).

qRT-PCR analysis. Since the sclR-overexpressing strain displayed significant morphological change, such as a large number of sclerotial formations and decreased conidiation, we assume that the expression of numerous genes involved in sexual/ asexual development should have been changed in this strain. A transcription factor cascade (BrlA→AbaA→WetA) that regulates asexual development has been well characterized in A. nidulans and A. oryzae (30). The changes in expression level of these genes in the OE-sclR and ΔsclR strains were tested by qRT-PCR. These strains were cultured onto the malt agar medium for 48 h, and the result indicated that the expression of brlA and abaA, which are associated with asexual development-conidiation, were reduced severely in the OE-sclR strain relative to the control strain (Fig. 8). In contrast to the OE-sclR strain, expression of these genes did not cause significant change in the ΔsclR strain. In addition, sclR overexpression did not result in important change in the expression of veA, which is involved in sexual development in A. nidulans. Besides the above-mentioned findings, the expression of a gene homologous to spp1, which was previously described as the major protein present in mature sclerotia of Sclerotinia sclerotiorum (17), was drastically increased in the OE-sclR strain (Fig. 8).

DISCUSSION

We report here the characteristics of a newly identified bHLH transcription factor, SclR (AO090011000215), in A. oryzae. The overexpression of sclR stimulates sclerotial production. A sclerotium is a compact and hardened mass of fungal mycelium. One important role of sclerotia is to endure extreme environments. In the OE-sclR strain, in addition to the numerous sclerotial formations, the conidia were severely decreased. In other word, instead of the conidia, the sclerotia formed by the sclR-overexpressing strain became the primary survival structure in extreme environments.

VeA is known as a global transcription factor with a role in activating sexual development and inhibiting asexual development in A. nidulans. In A. parasiticus and A. flavus, it has been reported that VeA has an effect on sclerotial formation (4, 6). Sclerotial bodies are similar to cleistothecia but have lost the capacity to produce spores. Therefore, the sclerotium is hypothesized to be a degenerate sexual structure and may represent a vestige of cleistothecium production (10). Deletion of veA results in a complete blockage of sclerotial production in both of the above-mentioned fungi (4, 6). However, in A. oryzae, the expression of veA was not increased, according to the qRT-PCR analysis (Fig. 8), even though the sclerotial formation increased in the sclR-overexpressing strain. Similarly, in the ΔsclR mutant, although the expression level of veA was unchanged, the sclerotial production was still largely blocked (Fig. 4b). We conclude from these results that the promotion of sclerotial formation by sclR overexpression seems to be not mediated via veA overexpression.

To further explore how the sclerotial formation initiates, we examined the hyphal morphology by using SEM. Sclerotial development could be divided into three distinguishable stages: (i) initiation, (ii) white sclerotium (development), and (iii) melanized sclerotium (maturatation). In the sclR-overexpressing strain, the three stages were readily observed. At the initial stage of sclerotial development, a large number of branched hyphae were observed (Fig. 5). These aberrant hyphae more easily aggregated, connected, and fused with neighboring hyphae and furthermore formed compact structures. The structures became progressively tighter and gradually developed into the mature sclerotia. Conversely, almost all of the sclR-disrupted strain produced normal hyphae on which conidiophores were formed (Fig. 5a). Phenotypic analysis further confirmed that SclR is a negative regulator of conidiation (asexual development) and, simultaneously, a positive regulator of sclerotial formation.

To further characterize sclR gene function, the phenotypes...
of the sclR-disrupted strain and sclR-overexpressing strain were examined when the strains were cultivated in DPY liquid medium. The ΔsclR strain produces an abnormal pellet and displays a soft and fluffy hyphal morphology (Fig. 6a). When the time in culture was sufficiently long, extreme cell lysis was observed in the ΔsclR strain. Rapid protein degradation (Fig. 6c) is the most likely reason why abnormal cell lysis happened in the sclR-disrupted strain. It is obvious that the sclR-encoding protein plays an important role in retaining both normal pellet structure and cell function in a liquid medium. The result shown in Fig. 1, where sclR expression shows a marked increase in liquid medium, also indicates that the SclR may serve a more important function in liquid medium, for example, by possibly promoting the connection and fusion of mycelia that is potentially useful for nutrition transportation. The sclR expression pattern observed by qRT-PCR also indicates that the sclR expression was strongly induced when glucose was used as the sole source of carbon or when skim milk was used as the source of nitrogen instead of NaNO₃ in liquid medium (Fig. 1).

As a DNA-binding transcription factor, SclR is expected to be localized in the nucleus. Although there is no obvious nuclear localization signal motif in the N-terminal region, the ScIR is still predicted to localize to the nucleus with the high probability of 73.9% as determined by the PSORT II prediction method. Interestingly, in the ScIR-EGFP strain, GFP fluorescence was observed to localize in the entire cytoplasm in the majority of mycelia during the early stages. After 3 days, two GFP fluorescence distribution states were observed. One was that the GFP fluorescence was localized in the entire cytoplasm, but the fluorescence in the nuclei was a little stronger. The other was that the GFP fluorescence was found to be abundant in the nuclei. We also found that the nuclear localization was readily observed in the mycelia that exist in the central region of the pellet. Considering this result together with the phenotype of the sclR-disrupted strain, which cannot form normal pellets and lysed mycelia in DPY liquid medium, SclR presumably plays an important role in regulating entwinement with neighboring mycelia and nutrition transportation to the central region of pellets for normal pellet formation in DPY liquid medium. This speculation is also in accord with another result, in which overexpression of the sclR gene promoted hyphal aggregation and sclerotial initiation on malt agar medium. Based on these observations, it is possible that, in a manner resembling the light-dependent nuclear localization of VeA, ScIR localizes in the entire cytoplasm under normal conditions, but a specific environ-
mental stimulus allows the SclR to enter the nucleus and function as a transcriptional regulator. Further research will be needed to fully understand this phenomenon.

SclR belongs to the family of bHLH transcription factors. Therefore, it seems very likely that the protein is able to form homodimers or heterodimers with as-yet-unknown partner proteins, which may include other HLH family members. The bHLH proteins bind DNA as dimers and usually recognize a short palindromic sequence, CANNTG, called the E-box element (19). SclR is a member of group B of the six main groups (A to F) of the bHLH family, since SclR contains the amino acids histidine (H), isoleucine (I), and arginine (R) at amino acid positions 5, 8, and 13 of the bHLH motif, respectively. Members of this subclass are suggested to bind the consensus sequence, most commonly either CACGTG or CAGCTG.

In the promoter region of sclR, there are three putative E-boxes in front of the transcriptional start site: E-box 1 (CAACTG) at -295, E-box 2 (CACCCG) at -228, and E-box 3 (CACTCTG) at -319. It is thus conceivable that SclR regulates its own expression. In addition, we investigated the 1.0-kb upstream region of the brlA and sspA ORFs since they both displayed significant changes in expression in the OE-sclR strain (Fig. 8). Within the promoter regions of brlA and sspA, five E-box sites and 11 E-box sites, respectively, were identified. It is likely that the SclR transcription factor also regulates the expression of the two genes through direct binding to the E-box elements. SclR also putatively contains 3 glycosylation sites and 18 phosphorylation sites. SclR may serve as a substrate for phosphorylation by cyclic AMP-dependent protein kinase, protein kinase C, and casein kinase II, based on ppscore (prosite search for protein motifs). It is consistent with the previous report that many HLH proteins are targets for phosphorylation, and it has been shown to be essential for functional activity as a transcription factor (11–13).

Vea is known to be involved in regulating conidiation and cleistothecial/sclerotial formation, as mentioned above. However, except for vea, no other gene has been previously identified for sclerotial development in Aspergillus. Thus, although the precise role of sclR remains to be determined, it is clearly required for sclerotial formation. These studies on SclR provide certain important clues to the formation and dynamics of sclerotia in A. oryzae.

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