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Full paper

An exocyst component, Sec5, is essential for ascospore formation in *Bipolaris maydis*

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ABSTRACT

In this study, we identified Sec5 in *Bipolaris maydis*, a homologue of Sec5 in *Saccharomyces cerevisiae* and a possible exocyst component of the fungus. To examine how Sec5 affects the life cycle of *B. maydis*, we generated null mutant strains of the gene (Δ*sec5*). The Δ*sec5* strains showed a strong reduction in hyphal growth and a slight reduction in pathogenicity. In sexual reproduction, they possessed the ability to develop pseudothecia. However, all ascospores were aborted in any of the asci obtained from crosses between Δ*sec5* and the wild-type. Our cytological study revealed that the abortion was caused by impairments of the post-meiotic stages in ascospore development, where ascospore delimitation and young spore elongation occur.

Keywords: *Cochliobolus heterostrophus*, exocytosis, membrane traffic, sexual reproduction

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1. Introduction

The exocyst, which is highly conserved in eukaryotic systems, is thought to regulate the transport of the secretory vesicle and to tether the secretory vesicle to the plasma membrane before vesicle fusion (Chen, Ebbole, & Wang, 2015). The exocyst was first identified in *Saccharomyces cerevisiae* and consists of eight components: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (TerBush, Maurice, Roth, & Novick, 1996). These exocyst components, which encoded a single gene in each fungus, were confirmed by co-purification and co-immunoprecipitation experiments to also form an octameric complex in the filamentous fungi *Magnaporthe oryzae* and *Neurospora crassa* (Riquelme et al., 2014; Gupta et al., 2015). Subsequently, these exocyst components were characterized in several fungi, indicating the possibility that exocyst components are involved in morphogenesis and pathogenicity in fungi (e.g., Finger & Novick, 1997; Guan et al., 2019). However, most of exocyst components are essential for viability, with some exceptions, and their importance is dependent on the fungal species. This suggests that the exocyst components should be investigated in many fungal species to gain further insight into the roles of exocysts in their life cycles.

Among the exocyst components, Sec5 plays unique and important roles in the construction of the exocyst complex in *S. cerevisiae*. The structure of the yeast exocyst complex was made up of two subcomplexes: subcomplex I, consisting of Sec3, Sec5, Sec6, and Sec8; and subcomplex II, consisting of Sec10, Sec15, Exo70, and Exo84. Sec5 mediates the interaction of these subcomplexes (Mei et al., 2018; Mei & Guo, 2018). In *N. crassa*, only the *Sec5* gene is dispensable with regard to viability, but required for proper vegetative growth (Riquelme et al., 2014). In *M. oryzae*, the disruption of Sec5 significantly reduces pathogenicity (Giraldo et al., 2013). However, little has been reported on the functional role of Sec5 in development and morphogenesis in filamentous fungi, especially in sexual reproduction.

The filamentous ascomycete *Bipolaris maydis* (syn.: *Cochliobolus heterostrophus*) is a necrotrophic pathogen that causes southern corn leaf blight (Rossman, Manamgoda, & Hyde, 2013). The fungus uses fusiform asexual spores, called conidia, as a primary inoculum. In addition, *B. maydis* is a heterothallic fungus with bipolar mating types (*MAT1-1* and *MAT1-2*) and easily mates between strains with different mating types in laboratory conditions. In the mating process, the fungus develops ascocarps, called pseudothecia. As a result of mating, filiform ascospores within pseudothecia can be observed readily in the laboratory. Therefore, *B. maydis* has often been used as a model organism to investigate fungal asexual and sexual development (Turgeon, 1998; Raju, 2008; Sumita, Izumitsu, & Tanaka, 2017; Kitade, Sumita, Izumitsu, & Tanaka, 2019).

In the present study, an orthologue of *S. cerevisiae Sec5* was identified in *B. maydis*. Then, we performed gene disruption and obtained null mutant strains of the gene. Here, we characterize the roles of Sec5 in the life cycle of *B. maydis* by comparing it with the wild-type and mutants.

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2. Materials and methods

2.1 Strains and growth conditions

All strains used in this study are shown in Table 1. To express gene symbols in this paper, a proposed genetic nomenclature by Yoder, Valent, and Chumley (1986) was generally followed, and a two-lettered acronym of a fungal scientific name (species prefix) was placed in front of a gene symbol, if necessary. *Bipolaris maydis* HITO7711 (*SEC5*, *ALB3*, *MAT1-2*) was used as a wild-type strain throughout this study. The compatible albino strain, M3alb3 (*SEC5*, *alb3*, *MAT1-1*; Tanaka, Kubo, & Tsuda, 1991), was used in crossing experiments. Pseudothecia of the *alb3* strain were light tawny colored; thus, they were easily distinguishable from black pseudothecia developed by strains that carry a wild-type allele (*ALB3*) at the *Alb3* locus, e.g., HITO7711, etc. Disruptants of the *Sec5* gene derived from the wild-type strain HITO7711 were generated by homologous recombination. All strains were maintained on a complete medium agar (CMA; Tanaka et al., 1991) or V8 agar (V8A; Ribeiro, 1978) at 25 °C.

2.2 Identification of the Sec5 gene in B. maydis

The amino acid sequence of Sec5 in *S. cerevisiae* (Accession No. KZV12402) was obtained from the DDBJ/EMBL/GenBank. To identify its orthologue in *B. maydis*, we used it as a query in PSI-BLAST searches of the NCBI protein database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sec5 orthologues in other fungal species were also acquired from the NCBI protein database. Amino acid sequence alignments were carried out using CLUSTAL X (Larkin et al., 2007).

2.3 Disruption of the Sec5 gene in B. maydis

All primers used in this study are shown in Table 2. Molecular experiments were performed according to Sambrook, Fritsch, and Maniatis (1989). The entire coding regions and introns of the wild-type allele (*SEC5*) were deleted using the targeted replacement method in homologous recombination. A fungal genomic DNA was obtained using the method of Izumitsu et al. (2012). A DNA fragment for gene disruption was constructed using the PCR fusion method (Szewczyk et al., 2006) with a Hyg^R cassette (*Aspergillus nidulans TrpC* promoter (P_{TrpC}) fused to a hygromycin B phosphotransferase gene (*HPH*)) obtained from the plasmid pCB1004 (Carroll, Sweigard, & Valent, 1994). In the first-round PCR, 5′- and 3′-noncoding regions of the *SEC5* gene were amplified from a genomic DNA of the wild-type HITO7711 using an ExTaq polymerase (Takara Bio, Otsu, Japan) and primer set (BmSec5-f1/BmSec5-r1 for the 5′-noncoding region; BmSec5-f2/BmSec5-r2 for the 3′-noncoding region; Table 2). The Hyg^R cassette was also amplified from the plasmid pCB1004 using ExTaq polymerase and the primers pCB1004-f1 and pCB1004-r1 (Table 2). These DNA fragments were used as substrates for the second-round PCR in order to fuse the three fragments into one deletion cassette using primers BmSec5-f1 and BmSec5-r2 (Table 2). The resulting major PCR product was purified by ethanol precipitation and resuspended at half of the original volume in a TE buffer for fungal transformation. Transformation experiments were performed using the method described by Izumitsu et al. (2009). Integration of the Hyg^R cassette into the targeted region was confirmed by PCR using primers BmSec5-f3 and BmSec5-r3, which annealed to a flanking region outside the deletion cassette (Table 2; Fig. 1A).

Table 1. Wild-type and mutant strains used in this study

Strain	Genotype	Reference
HITO7711	*SEC5*, *ALB3*, *MAT1-2*	Tanaka et al. (1991)
M3alb3	*SEC5*, *alb3*, *MAT1-1*	Tanaka et al. (1991)
DSE5-1	Δ*sec5*, *ALB3*, *MAT1-2*	This study
DSE5-2	Δ*sec5*, *ALB3*, *MAT1-2*	This study
CSE5	Δ*sec5*:: *SEC5*, *ALB3*, *MAT1-2*	This study

Table 2. Sequences of primers used in this study

Name	Nucleotide sequence
BmSec5-f1	5′-CTGGCGCTTGGTGCCGCGAC-3′
BmSec5-r1	5′-CCTCAGGCATTTGAGAAGCAC CATGCAGAGAGACGGGCGAAATTC-3′
BmSec5-f2	5′-CTCATCATTGGAAAACGTTCTTCG GACGTGAGCAGGAAAGGGGGTTG-3′
BmSec5-r2	5′-CAAAGTCTCGCTTTCCCCAGCATC-3′
BmSec5-f3	5′-CACTCACAAGCTGTCGTCTTTGC-3′
BmSec5-r3	5′-CAAACCCAACACAACGGACCTTCTC-3′
BmSec5-r4	5′-CCAATCACTTCACCGACATATCTACATG-3′
BmSec5-r5	5′-CTCCTTGGACCCCTCTCATGCAG-3′
BmSec5-f4	5′-CAATCCGGCATTTTCTCCCCAACC-3′
BmSec5-f5	5′-CTGCCAAGCACTACGCGTGTCTG-3′
BmSec5-r6	5′-CAATCATGCCCGATGAGAGGTTTGC-3′
pCB1004-f1	5′-GTGCTTCTCAAATGCCTGAG-3′
pCB1004-r1	5′-CGAAGAACGTTTTCCAATG-3′
Nat-chk-f1	5′-ATGAGCATGCCCTGCCCCTG-3′

The underlines show a reverse complement sequence of the Hyg^R cassette.

2.4 Reconstitution of the SEC5 gene to the Δsec5 strain

For functional complementation of the null mutant, we amplified the *SEC5* gene including 5′- and 3′-noncoding regions from genomic DNA of the wild-type HITO7711 with a primer set BmSec5-f1/BmSec5-r4 using PrimeStar GXL DNA polymerase (Takara Bio; Table 2). The amplified fragment was ligated into the EcoRV site of pZNAT1 (Izumitsu et al., 2009) carrying a nourseothricin-resistance gene (*NAT*) under the control of the *TrpC* promoter. The plasmid in which *SEC5* was oriented in a tail-to-tail configuration with *NAT* was selected by PCR with BmSec5-f4 and NAT-chk-f1. Reconstitution of the *SEC5* gene in the Δ*sec5* strain was made by a double crossing over event of homologous recombination within 5′-noncoding regions of the *Sec5* genes and the *TrpC* promoter regions of resistance gene cassettes. The constructed vector was linearized by *Spe*I digestion, and the vector's transformation into a Δ*sec5* strain was performed using a method described previously (Izumitsu et al., 2009). Strains showing resistance to nourseothricin (300 μg/mL) were isolated individually, and the reintroduction of the gene was confirmed by PCR using two primer sets: BmSec5-f3/BmSec5-r5 and BmSec5-f5/BmSec5-r6 (Table 2).

2.5 Penetration assay and pathogenicity assay

A penetration assay was performed on onion epidermises. These epidermises were killed by heat in an oven (65 °C, 1 h). Conidial suspensions (100 μL, 1×10^4 conidia/mL) of each strain were prepared and inoculated on these epidermises. These samples were incubated for 24 h at 25 °C in the dark. After staining with cotton blue (0.05% (w/v) in lactophenol), the penetration sites were observed microscopically.

For pathogenicity assays, maize plants (*Zea mays* cv. Takanester; Sato, Sawano, Shigemori, Mejima, & Miki, 2008) were grown in a greenhouse. Leaves were harvested from 3- to 4-wk-old plants and placed in a transparent polystyrene box lined with paper moistened with sterile water. Ten microliters of the conidial suspensions (5×10^4 conidia/mL) of each strain was inoculated onto the leaves. The

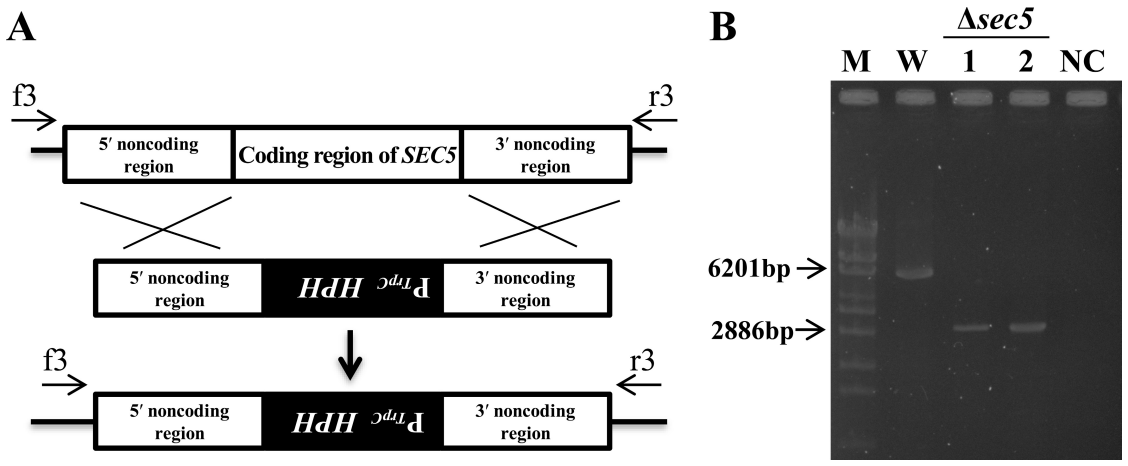

Fig. 1.—Disruption of the *Sec5* gene in *Bipolaris maydis*. A: Schematic illustration of the disruption of the *SEC5* allele in the wild-type strain and the location of the primers. The primer BmSec5-f3 is derived from the sequence of 5′-noncoding regions of *SEC5* outside the disruption fragment. The primer BmSec5-r3 is derived from the sequence of 3′-noncoding regions of *SEC5* outside the disruption fragment. B: PCR results for the confirmation of *Sec5* gene disruption. M, *λ/Sty*I digest; W, wild-type HITO7711; Δ*sec5*, null mutants of *Sec5*; N, negative control (TE buffer)

conidial suspensions were prepared from a 2-wk-old culture on V8A. The inoculated leaves were incubated for 3 d under dark conditions at room temperature. Pathogenicity assays were repeated three times using three leaves per assay.

2.6 Crossing experiments

Crossing was performed on a sterilized corn leaf on Sachs's agar medium according to the method described by Tanaka et al. (1991). The wild-type HITO7711 and Δ*sec5* strains were crossed with their compatible albino strain, M3alb3. The inoculated cultures were incubated at 25 °C for 4 wk, and the observation of pseudothecia on the corn leaf was made with a stereomicroscope. To inspect the ascus and ascospore development, mature pseudothecia were harvested and crushed in sterilized water on glass slides. Subsequently, these asci and ascospores were observed microscopically.

2.7 Cytology for ascospore development

For ascus cytology, nuclei in an ascus were stained with fluorochrome bisbenzimide H33258 trihydrochloride (Hoechst; Nacalai Tesque, Kyoto, Japan). Developing pseudothecia on the corn leaf from 10 to 30 d after inoculation were harvested and dissected with tweezers on a glass slide. They were fixed with 5% (v/v) glutaraldehyde at 4 °C for 1 d and washed with phosphate buffer. These samples were then stained with Hoechst solution (10 μg/mL) for 20 min in the dark and washed with phosphate buffer. After the addition of 5% (v/v) glycerol, a cover slip was applied, and the stained material was carefully spread by pressure exerted with a toothpick. The samples were inspected using DIC-epifluorescent microscopy (Leica DML with A cube (BP 340–380 nm excitation filter, 400 nm dichromatic mirror, LP 425 nm suppression filter); Leica Microsystems, Wetzler, FRG). Over 200 pseudothecia were dissected, and thousands of developing asci at various stages of nuclear division and ascospore developments were examined.

3. Results

3.1 Sec5 in B. maydis *was identified.*

In order to identify a Sec5 orthologue in *B. maydis*, an *S. cerevisiae* Sec5 protein sequence was subjected to PSI-BLAST searches with default parameters. The resulting hit was only one protein (ID: 1193032); thus, that protein was designated as Sec5 in this study. A gene encoding the Sec5 protein in *B. maydis* is composed of four exons interrupted by three introns; it also encodes a protein of 1065 amino acids. It showed 20% identity with *S. cerevisiae* Sec5. On the other hand, the sequence of BmSec5 of *B. maydis* showed higher identity with Sec5 orthologues in other filamentous fungi: 45% identity with AnSec5 in *A. niger* (Accession No. XP_001392674), 47% identity with NcSec5 in *N. crassa* (Accession No. XP_962284), 47% identity with MoSec5 in *M. oryzae* (Accession No. XP_003715348), and 47% identity with VdSec5 in *Verticillium dahliae* (Accession No. RBQ89220). Previous studies have reported that orthologues of Sec5 in *V. dahliae* and *N. crassa* contain a Vps51/Vps67 domain (Yang et al., 2013; Riquelme et al., 2014). The region predicted to be the Vps51/Vps67 domain was highly conserved among some filamentous fungi, including *B. maydis* (Supplementary Fig. S1). These results suggested that Bm*Sec5* was an orthologue of the *S. cerevisiae Sec5*.

3.2 Disruption of the Sec5 *gene affected hyphal growth.*

To investigate the role of Sec5 in *B. maydis*, we generated null mutants of the gene. We obtained 19 transformants that showed resistance to hygromycin B. Subsequently, all hygromycin B-resistant transformants were screened using the PCR method described above, and four strains were characterized as *Sec5* null-mutant (Δ*sec5*) strains. Two null mutants were arbitrarily chosen for subsequent experiments (Fig. 1B). To confirm the effects of *Sec5* gene disruption, we also attempted to generate a reconstituted strain by reintroducing the wild-type allele (*SEC5*) into the Δ*sec5* strain. The reconstituted strain was generated by a double-crossover event in which the wild-type gene (*SEC5*) was inserted into a null allele (Δ*sec5*). The transformant—named CSE5—was confirmed by PCR and characterized as reconstituted strain (data not shown).

To understand the role of Sec5 in colonial development and conidiation, all strains were grown on CMA or V8A at 25 °C. Δ*sec5* strains developed a melanized colony, as did the wild-type. However, Δ*sec5* strains showed a drastic reduction in radial growth as compared with the wild-type and reconstituted strains (Fig. 2; Table 3). On the other hand, the conidial morphology of Δ*sec5* strains

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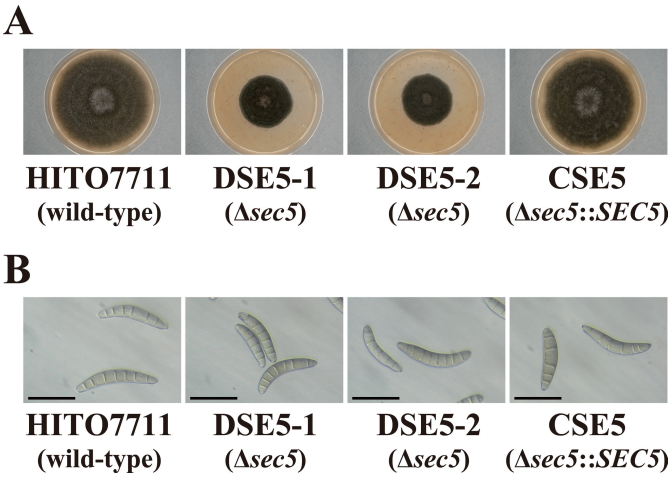

Fig. 2.—Phenotypic effects of *Sec5* disruption on colonial growth (A) and conidial morphology (B). The label of each picture indicates a strain name and its genotype in parentheses. All strains were grown on V8A, and pictures were taken 7 d after inoculation. *Bars*: 50 μm

Table 3. Vegetative growth and conidiation of the wild-type and Δ*sec5* mutants

Strain	Colony diam. (mm)	No. of conidia/cm^2 (× 10^5)
HITO7711	63.8±0.3^a	2.2±0.1^a
DSE5-1	23.8±1.1^b	3.1±0.3^{ab}
DSE5-2	30.5±0.1^c	3.8±0.1^b
CSE5	64.5±1.4^a	2.7±0.2^a

Values represent the mean±standard errors ($n = 5$). Different letters indicate a significant difference between strains ($P < 0.05$) according to the Tukey's multiple comparison test of mean separation.

was similar to that of the wild-type strain. The conidial density (number of conidia per cm^2) of Δ*sec5* strains was slightly increased as compared to those of the wild-type and reconstituted strains (Table 3); however, there is no statistic significance among categories of the wild-type, Δ*sec5*, and reconstituted strains (statistic results from Tukey's multiple comparison test are not shown).

3.3 Sec5 is required for full virulence.

We investigated whether Sec5 is important for penetration and pathogenicity. Conidia of the wild-type and reconstituted strains developed numerous appressoria on the heat-killed onion epidermis. Infection hyphae from these appressoria were observed in onion epidermises (Fig. 3A). Δ*sec5* strains also showed normal appressoria and infection hypha (Fig. 3A). The infection rate was similar to those of the wild-type and reconstituted strains (Fig. 3B).

For a pathogenicity test, we inoculated conidial suspensions of the wild-type, Δ*sec5*, and reconstituted strains on host corn leaves. The wild-type and reconstituted strains produced well-developed lesions. On the other hand, the lesion size formed by Δ*sec5* strains was slightly reduced, as compared to those of wild-type and reconstituted strains (Fig. 4A, B). These observations suggested that Sec5 is required for full virulence after infection.

3.4 Sec5 is essential for ascospore formation.

To assess the role of the Sec5 in sexual reproduction, we crossed the Δ*sec5* strains (*MAT1-2*) with the compatible albino strain, M3alb3. In crossing the wild-type strain with the albino strain, the former formed black pseudothecia, and the latter developed light tawny-colored ones along the interface of the two strains (Fig. 5A). In the crosses of Δ*sec5* strains with the albino strain, black and light tawny pseudothecia were formed along the interface of the strains, as in the cross of the wild-type and the albino strains. These results suggested that Sec5 is not required for pseudothecial development. In order to assess the involvement of Sec5 in ascus and ascospore development, pseudothecia obtained by crossing of the Δ*sec5* strains with the albino strain were inspected microscopically. In results of crosses of the wild-type strain with the albino strain, seven or eight mature ascospores per ascus were typically formed in both black and light tawny pseudothecia (Fig. 5B). In asci from a cross between the reconstituted and the albino strains, ascospores developed. However, in the crosses of Δ*sec5* strains with the albino strain, any asci in either black or light tawny pseudothecia had no ascospores (>1000 asci; Fig. 5B). These results implied that Sec5 is not essential for female organ development but is essential for female and/or male functions in the sexual reproduction process of this fungus. Further attempts to clarify the defective step affected by Δ*sec5* in sexual reproduction were carried out by cytological study. Black pseudothecia from 10 to 30 d old after inoculation were dissected, and each ascus was stained with Hoechst dye. In pseudothecia from crosses between wild-type and albino strains,

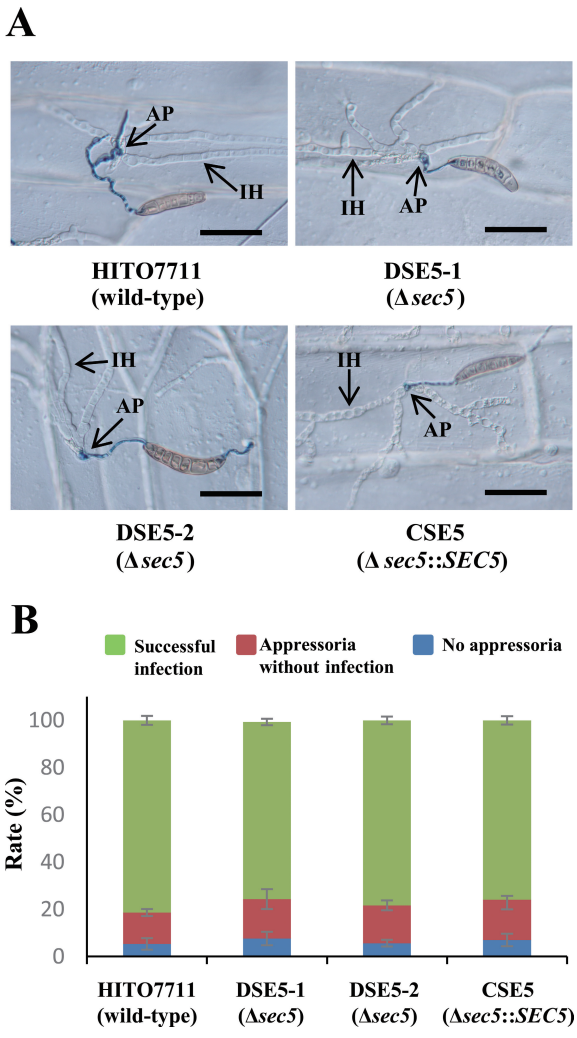

Fig. 3.—Penetration assay of Δ*sec5* mutants. A: Infection assay on onion epidermises. Ap, appressorium; IH, infected hypha. *Bars*: 50 μm. B: The rate of appressorial development and successful infection into onion epidermis cells in each strain. In all strains, 100 conidia on onion epidermis cells were observed microscopically and counted per each conidium. Error bars represent the standard error of the mean calculated from three replicates.

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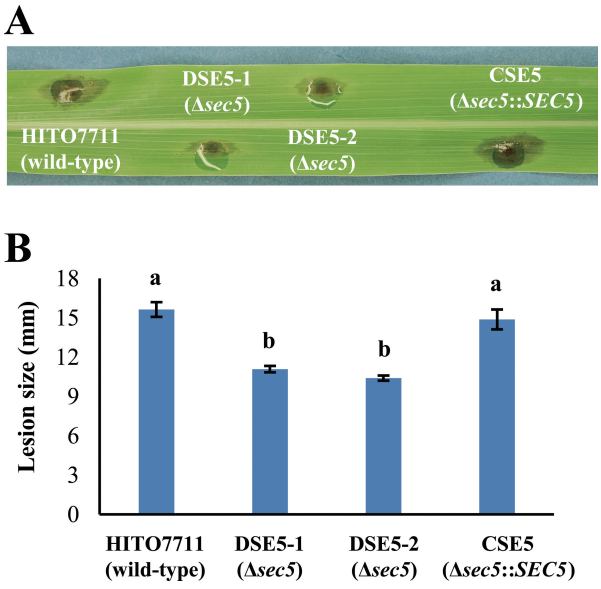

Fig. 4.—Pathogenicity of Δ*sec5* mutants. A: Pathogenic assay on corn leaves. Photos were taken 3 d after inoculation. B: Lesion size of each strain on corn leaves 3 d after inoculation. Error bars represent the standard error of the mean ($n = 5$). Different letters indicate a significant difference between strains ($P < 0.05$) according to Tukey's multiple comparison test of mean separation.

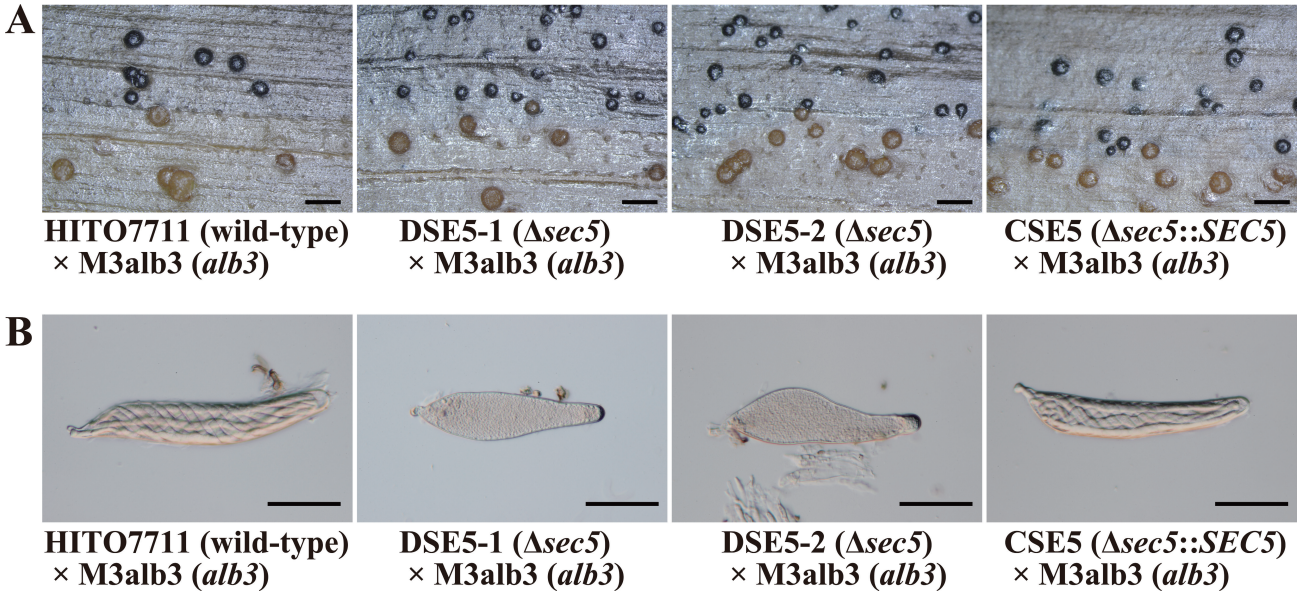

Fig. 5.—Sexual reproductivity of Δ*sec5* mutants. A: Pseudothecial developments. The tested strains (the wild type, Δ*sec5* mutants, and the reconstituted strain) were crossed with the albino strain, M3alb3. The former strains developed black pseudothecia, and the latter strain developed light tawny ones. *Bars*: 1 mm. B: Ascospore developments. Ascospore did not develop in any asci obtained from either black or light tawny pseudothecia from the crosses of Δ*sec5* mutants with the albino strain. *Bars*: 50 μm

we could observe all stages of asci with a single nucleus, as well as 2-nucleus, 4-nucleus, 8-nucleus, 16-nucleus, and multinucleate ascospores (Fig. 6A–F), as Raju (2008) reported previously. In crosses of Δ*sec5* strains with the albino strain, meiotic stages of asci were found (Fig. 6G–I). Asci with 8 and 16 nuclei were also observed, which implied that the post-meiotic first and second mitoses progressed (Fig. 6J–N). However, few asci with 8 nuclei were delimited (Fig. 6K). In most of the asci with 16 nuclei, the nuclei were not enclosed in ascospores (Fig. 6L), and they migrated to lower part of the ascus later (Fig. 6M). Delimited binucleate ascospores might be unable to elongate, meeting the same fate as unenclosed nuclei (Fig. 6N). No further stages for multinucleating ascospores were found in the crosses of Δ*sec5* strains. Thus, they seemed to be delimitation failures or impaired in the elongation of delimited young ascospores, and further processes including the third post-meiotic mitosis were aborted. Our observations implied that a defection of Sec5 did not affect the mating process prior to the meiosis of the fungus but was critical for ascospore development posterior to meiosis.

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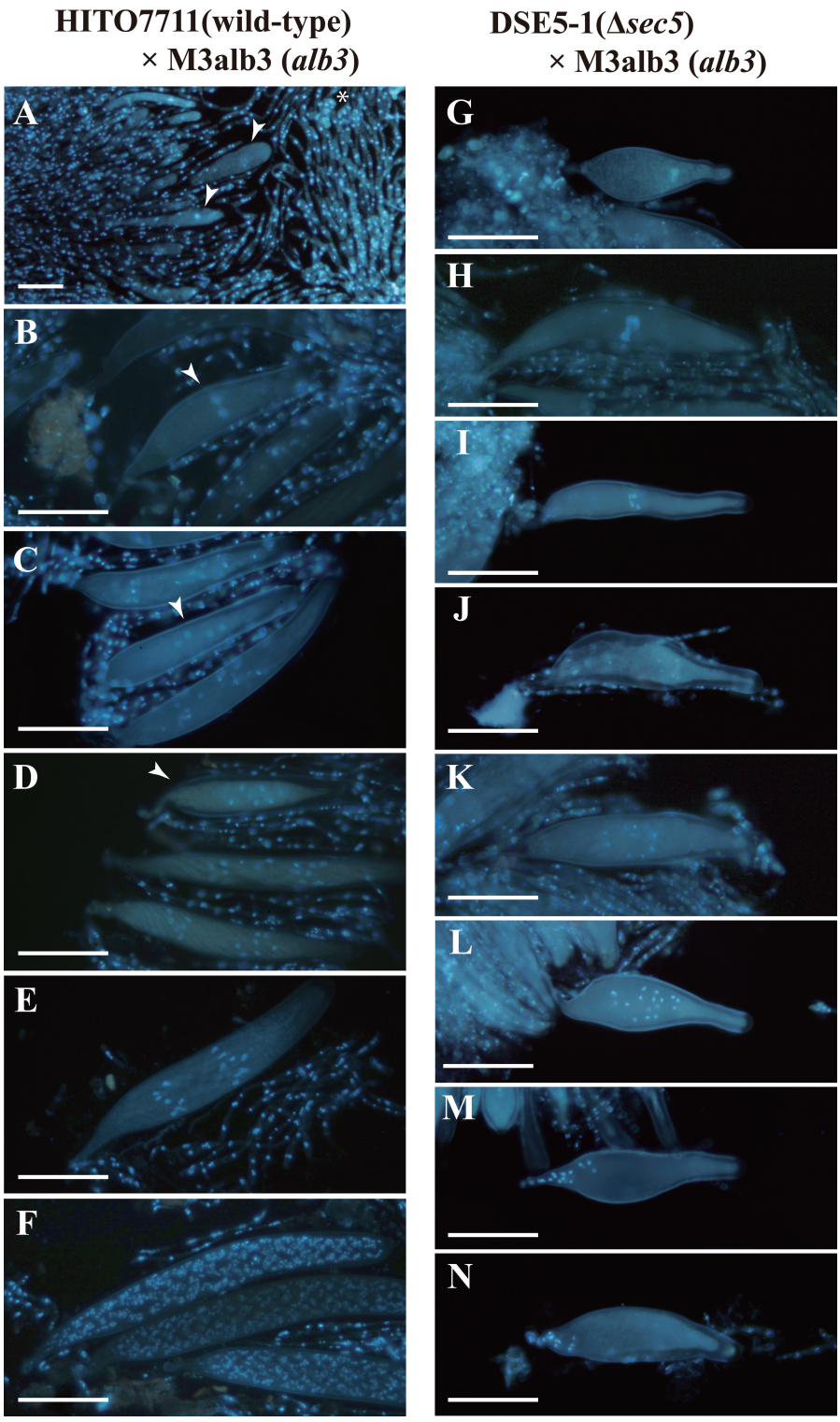

Fig. 6.—Nuclear behavior and ascospore development. Photographs (A–F) in the left column were taken of samples from crosses of the wild-type strain with the albino strain, both of which carried wild-type *SEC5* alleles. Pictures in the right column (G–N) were taken of samples from the crosses of the Δ*sec5* strain with the albino strain, in which crosses the conjugated diploid nuclei were heterozygous to Δ*sec5*/*SEC5* alleles. The bottom-to-top direction of the ascus is arranged in a picture horizontally (left to right). A: Partial contents of a young pseudothecium, showing a developing ascus with a single diploid nucleus (arrowhead) and a crozier (asterisk). B: 2-Nucleus stage of an ascus (arrow-head), showing interphase I of the nuclei in meiosis. C: 4-Nucleus stage of an ascus (arrowhead), showing interphase II of the nuclei after meiosis. D: 8-Nucleus stage of an ascus posterior to the first post-meiotic mitosis and prior to ascospore delimitation (arrowhead). Unmarked asci were in 16 nuclei or in a further stage, in which ascospores had been delimited and their filiform shape was recognized. E: 16-Nucleus stage of an ascus, showing extended filiform binucleate ascospores after the second post-meiotic mitosis. F: Mature asci showing multinucleate, multi-segmented, helically coiled ascospores. G: Single nucleus ascus. H: Ascus showing the first meiotic division. I: 4-Nucleus stage of an ascus, showing interface II of the nuclei after meiosis. J: 8-Nucleus stage of an ascus without delimitation. K: 8-Nucleus stage of an ascus with delimitation. L: 16-Nucleus stage of an ascus without delimitation. M: Ascus containing 16 nuclei without delimitation, where the nuclei are located only in the lower part of the ascus. N: Another sample of an ascus containing 16 nuclei. *Bars*: 50 μm

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4. Discussion

The composition of the exocyst complex is evolutionally conserved among eukaryotic cells. In fungi, each of the eight exocyst components presents a single gene, and most of these genes are essential for viability, with the exceptions of *Sec3* in *S. cerevisiae*, *Sec5* in *N. crassa*, and *Sec5* and *Exo70* in *M. oryzae* (Finger & Novick, 1997; Giraldo et al., 2013; Riquelme et al., 2014). This suggests the possibility that the same exocyst components may not have a common function in different fungi. Thus, these homologues were needed to investigate functions in different fungi. Moreover, to our knowledge, only a few studies have focused on the broad function of exocyst components in the fungal life cycle. In this study, we identified the *Sec5* orthologue of *B. maydis* and investigated its broad functions in the fungal life cycle, using null mutants.

Our results revealed that Bm*Sec5* is required for hyphal growth (Fig. 2; Table 3). Similar results were reported for the *Sec5* mutant of *N. crassa* (Riquelme et al., 2014). These results show that an exocyst complex or Sec5 is important for normal hyphal growth. In addition, the study indicated that Bm*Sec5* is not important for conidiation (Table 3). However, few studies have focused on the function of Sec5 in conidiation of other fungi.

In infectious development of *B. maydis*, Δ*sec5* strains formed numerous appressoria on the onion epidermis and infectious hypha in the onion epidermis, which was similar to the wild-type strain (Fig. 3A, B). The results indicated that BmSec5 is not required for infectious development. In *M. oryzae*, lack of the exocyst component MoSec5 or MoExo70 shows significant pathogenicity to host plants (Giraldo et al., 2013; Gupta et al., 2015). These exocyst components are thought to be required for the secretion of cytoplasmic effectors after the infection of host plants (Giraldo et al., 2013). These results suggest that the exocyst complex or several of its components play important roles in the secretion of virulence factors. In *B. maydis*, here, Δ*sec5* strains showed a slight reduction in lesion size. Unlike the case of *M. oryzae*, BmSec5 is not critical for pathogenicity in the postinfectious stage in *B. maydis* (Fig. 4A, B). The result implies that the role of Sec5 in pathogenicity can be different between two species.

In this study, we found that Bm*Sec5* is required for ascospore formation, but not for the development of the female organ, the pseudothecium. Ascospore formation processes in *Bipolaris* (syn.: *Cochliobolus*) spp. has been studied by Hrushovetz (1956); Guzman, Garber, and Yoder (1982); and Raju (2008). Meiosis occurs in a common cytoplasm of the ascus, which has arisen from an ascogenous hypha. Meiosis is followed by the first post-meiotic mitosis, which generates eight daughter nuclei in the ascus. Typically, delimitation happens after the first post-meiotic mitosis to enclose a single nucleus in each ascospore. These delimited young ascospores elongate to filiform shape and are parallel to one another along the long axis of the ascus. The following second mitosis synchronously or asynchronously occurs in each uninucleate ascospore, producing binucleate ascospores, which occupy about 75–80% of the ascus length. The ascospores elongate further with the third and additional mitoses, and septa appear at random in the spores, resulting in helically coiled mature ascospores. Hrushovetz (1956) has described his observation of such asci that failed to delimit ascospores after the first post-meiotic mitosis, noting the possibility that they never do delimit and form normal spores in *B. sorokiniana*. In the crosses of Δ*sec5*, we did not find any filiform young ascospores or further stages of ascus resulting from the second post-meiotic mitosis.

The functions of exocysts in mating and ascospore formation have been studied in yeast (Sharifmoghadam, de Leon, Hoya, Curto, & Valdivieso, 2010). In fission yeast *S. pombe*, exocyst components were reported to be involved in mating and ascospore development. Exocyst component mutants in *S. pombe* reduce mating efficiency as compared with a wild-type. These mutants also exhibit abnormal ascospores or no ascospore formation (Sharifmoghadam et al., 2010). Exocyst components are known to be important for forespore membrane (FSM) development (Sharifmoghadam et al., 2010; Imada & Nakamura, 2016). An FSM is a double membrane within the cytoplasm of the diploid mother cell, later becoming an ascospore membrane. These results may show that BmSec5 has similar functions in the delimitation of ascospores or in the elongation of delimited young ascospores for a filiform shape to that of exocysts in ascospore formation of the yeast, although there are some differences in mating behavior and ascospore development between the yeast and *B. maydis*.

Disclosures

The authors declare that they have no conflict of interest. All of the experiments undertaken in this study comply with the current laws of Japan, where the research was performed.

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Reference

Carroll, A. M., Sweigard, J. A., & Valent, B. (1994). Improved vectors for selecting resistance to hygromycin. *Fungal Genetics Reports, 41,* Article 5. https://doi.org/10.4148/1941-4765.1367

Chen, X., Ebbole D.J., & Wang, Z. (2015). The exocyst complex: delivery hub for morphogenesis and pathogenesis in filamentous fungi. *Current Opinion in Plant Biology, 28,* 48–54. https://doi.org/10.1016/j.pbi.2015.09.003

Finger, F.P., & Novick, P. (1997). Sec3p is involved in secretion and morphogenesis in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell, 8,* 647–662. https://doi.org/10.1091/mbc.8.4.647

Giraldo, M. C., Dagdas, Y. F., Gupta, Y. K., Mentlak, T. A., Yi, M., Martinez-Rocha, A. L., Saitoh, H., Terauchi, R., Talbot,N. J., & Valent, B. (2013). Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*. *Nature Communication, 4,* 1996. https://doi.org/10.1038/ncomms2996

Guan, W., Feng, J., Wang, R., Ma, Z., Wang, W., Wang, K., & Zhu, T. (2019). Functional analysis of the exocyst subunit BcExo70 in *Botrytis cinerea*. *Current Genetics*. https://doi.org/10.1007/s00294-019-01002-9

Gupta, Y. K., Dagdas, Y. F., Martinez-Rocha, A. L., Kershaw, M. J., Littlejohn, G. R., Ryder, L. S., Sklenar, J., Menke, F., & Talbot, N. (2015). Septin-dependent assembly of the exocyst is essential for plant infection. *The Plant Cell, 27,* 3277–3289. https://doi.org/10.1105/tpc.15.00552

Guzman, D., Garber, R. C., & Yoder, O. C. (1982). Cytology of meiosis I and chromosome number of *Cochliobolus heterostrophus* (Ascomycetes). *Canadian Journal of Botany*, *60*, 1138–1141. https://doi.org/10.1139/b82-143

Hrushovetz, S. B. (1956). Cytological studies of ascus development in *Cochliobolus sativus*. *Canadian Journal of Botany*, *34*, 641–651. https://doi.org/10.1139/b56-047

Imada, K., & Nakamura, T. (2016). The exocytic Rabs Ypt3 and Ypt2 regulate the early step of biogenesis of the spore plasma membrane in fission yeast. *Molecular Biology of the Cell, 27,* 3317–3328. https://doi.org/10.1091/mbc.E16-03-0162

Izumitsu, K., Yoshimi, A., Kubo, D., Morita, A., Saitoh, Y., & Tanaka, C. (2009). The MAPKK kinase ChSte11 regulates sexual/asexual development, melanization, pathogenicity, and adaption to oxidative stress in *Cochliobolus heterostrophus*. *Current Gentics, 55,* 439–448. https://doi.org/10.1007/s00294-009-0257-7

Izumitsu, K., Hatoh, K., Sumita, T., Kitade, Y., Morita, A., Gafur, A., Ohta, A., Kawai, M., Yamanaka, T., Neda, H., Ota, Y., & Tanaka, C. (2012). Rapid and simple preparation of mushroom DNA directly from colonies and fruiting bodies for

PCR. *Mycoscience, 53,* 396–401. https://doi.org/10.1007/S10267-012-0182-3

Kitade, Y., Sumita, T., Izumitsu, K., & Tanaka, C. (2019). Cla4 PAK-like kinase is required for pathogenesis, asexual/sexual development and polarized growth in *Bipolaris maydis*. *Current Genetics, 65,* 1229–1242. https://doi.org/10.1007/s00294-019-00977-9

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., & Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics, 23,* 2947–2948. https://doi.org/10.1093/bioinformatics/btm404

Mei, K., Li, Y., Wang, S., Shao, G., Wang, J., Ding, Y., Luo, G., Yue, P., Liu, J. J., Wang, X., Dong, M. Q., Wang, H. W., &Guo, W. (2018). Cryo-EM structure of the exocyst complex. *Nature Structural & Molecular Biology, 25,* 139–146. https://doi.org/10.1038/s41594-017-0016-2

Mei, K., & Guo, W. (2018). The exocyst complex. *Current Biology, 28,* R922–R925. https://doi.org/10.1016/j.cub.2018.06.042

Raju, N. B. (2008). Meiosis and ascospore development in *Cochliobolus heterostrophus*. *Fungal Genetics and Biology, 45,* 554–564. https://doi.org/10.1016/j.fgb.2007.08.007

Ribeiro, O. K. (1978). *A source book of the genus* Phytophthora. J. Cramer.

Riquelme, M., Bredeweg, E. L., Callejas-Negrete, O., Roberson, R. W., Ludwig, S., Beltran-Aguilar, A., Seiler, S., Novick, P., & Freitag, M. (2014). The *Neurospora crassa* exocyst complex tethers Spitzenkörper vesicles to the apical plasma membrane during polarized growth. *Molecular Biology of the Cell, 25,* 1312–1326. https://doi.org/10.1091/mbc.E13-06-0299

Rossman, A. Y., Manamgoda, D. S., & Hyde, K. D. (2013). Proposal to conserve the name *Helminthosporium maydis* Y. Nisik. & C. Miyake (*Bipolaris maydis*) again *H. maydis* Brond. and *Ophiobolus heterostrophus* (Ascomycota: Pleosporales: Pleosporaceae). *Taxon, 62,* 1332–1333. https://doi.org/10.12705/626.22

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (2nd ed.) Cold Spring Harbor Laboratory Press.

Sato, H., Sawano, T., Shigemori, I., Mejima, H., & Miki, M. (2008). Breeding of a silage maize hybrid cultivar "Takanestar". *Bulliten of the Nagano Chushin Agricultural Experiment Station, 18,* 11–24 [In Japnaese].

Sharifmoghadam, M. R., de Leon, N., Hoya, M., Curto, M. A., & Valdivieso, M. H. (2010). Different steps of sexual development are differentially regulated by the Sec8p and Exo70p exocyst subunits. *FEMS Microbiology Letters, 305,* 71–80. https://doi.org/10.1111/j.1574-6968.2010.01915.x

Sumita, T., Izumitsu, K., & Tanka, C. (2017). Characterization of the autophagy-related gene *BmATG8* in *Bipolaris maydis*. *Fungal Biology, 121,* 785–797. https://doi.org/ 10.1016/j.funbio.2017.05.008

Szewczyk, E., Nayak, T., Oakley, C. E., Edgerton, H., Xiong, Y., Taheri-Talesh, N., Osmani, S. A., & Oakley, B. R. (2006). Fusion PCR and gene targeting in *Aspergillus nidulans*. *Nature Protocols, 1,* 3111–3120. https://doi.org/10.1038/nprot.2006.405

Tanaka, C., Kubo, Y., & Tsuda, M. (1991). Genetic analysis and characterization of *Cochlioborus heterostrophus* colour mutants. *Mycological Research, 95,* 49–56. https://doi.org/10.1016/S0953-7562(09)81360-9.

TerBush, D. R., Maurice, T., Roth, D., & Novick, P. (1996). The exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. *The EMBO Journal, 15,* 6483–6494. https://doi.org/10.1002/j.1460-2075.1996.tb01039.x

Turgeon, B. G. (1998). Application of mating type gene technology to problems in fungal biology. *Annual Review of Phytopathology, 36,* 115–137. https://doi.org/10.1146/annurev.phyto.36.1.115

Yang, X., Ben, S., Sun, Y., Fan, X., Tian, C., & Wang, Y. (2013). Genome-wide identification, phylogeny and expression profile of vesicle fusion components in *Verticillium dahliae*. *PLoS ONE, 8,* e68681. https://doi.org/10.1371/journal.pone.0068681

Yoder, O. C., Valent, B., & Chumley, F. (1986). Genetic nomenclature and practice for plant pathogenic fungi. *Phytopathology*, *76*, 383–385. https://doi.org/10.1094/Phyto-76-383