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Synchronous Activation of Cell Division by Light or Temperature Stimuli in the Dimorphic Yeast *Schizosaccharomyces japonicus*

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Many fungi respond to light and regulate fungal development and behavior. A blue light-activated complex has been identified in *Neurospora crassa* as the product of the *wc-1* and *wc-2* genes. Orthologs of WC-1 and WC-2 have hitherto been found only in filamentous fungi and not in yeast, with the exception of the basidiomycete pathogenic yeast *Cryptococcus*. Here, we report that the fission yeast *Schizosaccharomyces japonicus* responds to blue light depending on Wcs1 and Wcs2, orthologs of components of the WC complex. Surprisingly, those of ascomycete *S. japonicus* are more closely related to those of the basidiomycete. *S. japonicus* reversibly changes from yeast to hyphae in response to environmental stresses. After incubation at 30°C, a colony of yeast was formed, and then hyphal cells extended from the periphery of the colony. When light cycles were applied, distinct dark- and bright-colored hyphal cell stripes were formed because the growing hyphal cells had synchronously activated cytokinesis. In addition, temperature cycles of 30°C for 12 h and 35°C for 12 h or of 25°C for 12 h and 30°C for 12 h during incubation in the dark induced a response in the hyphal cells similar to that of light. The stripe formation of the temperature cycles was independent of the *wcs* genes. Both light and temperature, which are daily external cues, have the same effect on growing hyphal cells. A dual sensing mechanism of external cues allows organisms to adapt to daily changes of environmental alteration.

Many organisms on Earth have adapted themselves to the alternating day/night environment, particularly with regard to light and temperature cycles. Responses to light have been thoroughly studied in unicellular organisms. Among eukaryotes, many fungi respond to light and regulate fungal development and behavior, and the wavelengths they respond to are mostly blue light (1). A blue light-activated photoreceptor has been identified in *Neurospora crassa* as the product of the *wc-1* gene (2). The *wc-2* gene is also essential for photoresponse. These gene products associate with each other and make a transcription factor activated by blue light via a flavin adenine dinucleotide (FAD) chromophore. WC-1 has an LOV (light, oxygen, or voltage) domain with a flavin binding site (3, 4) and two PAS (PER-ARNT-SIM) domains, and WC-2 has one PAS domain. The PAS domain is a protein structural motif that is often seen in a variety of signal sensor molecules and is involved in protein-protein interactions (5). Orthologs of WC-1 and WC-2 have hitherto been characterized only in filamentous fungi and not in yeast, with the exception of the basidiomycete pathogenic yeast *Cryptococcus* (6, 7).

N. crassa WC-1 and WC-2 are subject to light-dependent phosphorylation, and transient phosphorylation of WC-1 is crucial for desensitization of the photoreceptor (8). Importantly, the function of the WC complex is not only as a blue light sensor but also as a regulator of expression of FRQ (frequency) (3). FRQ is one of the components for the circadian clock oscillator (9). The circadian clock oscillator depends on regulatory interactions between the WC complex and FRQ (10). Thus, the WC complex is incorporated into the circadian feedback loop (10) and is a crucial component for the circadian rhythm that causes periodic formation of conidia in *N. crassa* (11). Once the circadian response of each cell of *N. crassa* is synchronized by the daily light/dark cycle (L/D), the daily development of conidiation is visible as a band pattern that is formed by altering zones of mycelium and sporulating mycelium. After synchronization by the light/dark cycle, this band pattern progresses during dark/dark cycles (D/D) or light/light cycles (L/L). In addition, the circadian rhythm is entrained by temperature changes (12).

It has recently been reported that a circadian rhythm of metabolic activity is shown in *Saccharomyces cerevisiae* after entrainment of temperature cycles, although the molecular basis of sensing temperature remains unknown (13). Here, we found orthologs of WC-1 and WC-2 in the genome of the fission yeast *Schizosaccharomyces japonicus*, which possesses characteristics common to filamentous fungi and whose cells change from yeast growth to hyphal growth in response to environmental stress, including nutrient loss or DNA damage (14, 15). If the DNA-damaging reagent camptothecin (CPT) is included in nutrient-rich medium, hyphal cells will develop. We discovered that hyphal cell growth is a response to light and temperature and that the former is dependent on the WC-1 and WC-2 orthologs.

MATERIALS AND METHODS

Media. *Schizosaccharomyces japonicus* was cultivated as previously described (16). YE medium (yeast extract, 5 g; glucose, 30 g/liter) was used as a rich medium. To induce growth of nutrient-dependent hyphae, malt agar (malt extract, 30 g), EMM2 (17), and morphology agar (Difco Yeast Morphology agar; Becton, Dickinson and Company, USA) were used. If necessary, a final concentration of 2% agar was added to make solid me-

Received 24 April 2013 Accepted 14 July 2013
Published ahead of print 19 July 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/EC.00109-13.

doi:10.1128/EC.00109-13

TABLE 1 Strains used in this study

Strain	Genotype	Reference or source
NIG2017	h$^+$ *mat-2017*	16
NIG2028	h$^-$ *mat-P2028*	16
NIG5097	h$^-$ *mat-P2028 hht3*-GFP::Kan	16
NIG3001	h$^+$ *mat-2017 wcs1*::*natMX6*	This study
NIG3002	h$^+$ *mat-2017 wcs2*::*kanMX6*	This study
NIG3003	h$^+$ *mat-2017 wcs1*::*natMX6 wcs2*::*kanMX6*	This study
NIG3004	h$^-$ *mat-P2028 wcs1*::*natMX6*	This study
NIG3005	h$^-$ *mat-P2028 wcs2*::*kanMX6*	This study
NIG3006	h$^-$ *mat-P2028 wcs1*::*natMX6 wcs2*::*kanMX6*	This study

dium. For marker selection in YE medium, 40 μg/ml of Geneticin was used. To induce hyphal cells on agar plates, 2 μl of overnight-cultured yeast cells was spotted at the center and incubated at 30°C for at least a week. Burkholder's synthetic medium (18) was modified in this study as follows: nicotinic acid (10.0 mg/liter), citric acid (1.0 mg/liter), potassium chloride (0.1 mg/liter), and sodium sulfate (40.0 mg/liter) were supplemented instead of riboflavin, pyridoxine, thiamine, and niacin. The optical density at 660 nm (OD_{660}) of cultures was automatically recorded every 15 min with a TVS062CA biophotorecorder (Advantec Co. Ltd., Japan).

Strains. Strains used in this study are summarized in Table 1. Transformation of plasmids into yeast cells was performed by electroporation (16). The *S. japonicus* orthologs of *wc-1* and *wc-2* genes were identified by searching the database available at the Broad Institute (http://www.broadinstitute.org//annotation/genome/schizosaccharomyces_group/MultiHome.html) (19). These *S. japonicus* genes were *wcs1*, SJAG_02860, and *wcs2*, SJAG_05242.

Photography. Colonies with hyphal zones were photographed with illuminating light coming through a plate from the bottom. Images were taken by a Nikon D3s camera with AF-S Micro Nikkor 60-mm f/2.8G ED or a Nikon D5200 camera with AF-S DX Micro Nikkor 85-mm f/3.5G ED VR. Interval images were carried out by shutter release mode under red light-emitting diode (LED) light (Flat Beam Aurora; GEX Corporation, Osaka, Japan). Images were processed by iPhoto imaging software (Apple Inc., USA). A fluorescence stereomicroscope (SMZ1500; Nikon, Japan) was used for hyphal cells with green fluorescent proteins (GFP). Distance between septa was measured by using MacSCOPE image-processing software (Mitani Co., Ltd., Japan).

Incubation under light and temperature cycles. Under light conditions, plates were placed in a stainless steel sterilizing box (240 mm by 240 mm by 200 mm) under an LED lamp. The intensity of the LED lamp was 50 lx on the surface of a plate. The temperature of the incubator was 30.6 ± 0.2°C when the LED lamp was on and 30.3 ± 0.2°C when the LED lamp was off. The time of lighting was controlled by a programmable timer. The hyphal response by temperature cycles was induced using incubators MIR-153 (Panasonic Corporation, Japan) and FMU-0541 and FMU-1331 (Fukushima Industries Corp., Japan).

Deletion of *wcs* genes. To create a *wcs1* deletion mutants (NIG3001), the 5′ untranslated region (UTR) and 3′ UTR of *wcs1* were amplified from NIG2017 genomic DNA using the following primers: 5′ UTR, AACAGG ATCCACGGTCATTTCACGCTTCC and AGTTTAATTAAACGGATGT TGGAGAATAGC, and 3′ UTR, ACAGTTTAAACTAAGTGGTCTGCCT GATGC and ATTGTGAATTCCGTGTGATGAGATGCGGAG. To create *wcs2* deletion mutants (NIG3001), the following primers were used: 5′ UTR, ACCCAGGATCCACTGTATCCCAAAG and GGCATATTAATTA ACCACACCTTGAGG, and 3′ UTR, CTGCGTTTAAACGTCGATC AGTG and GCAACAGAATTCTTAGCTGTTCTTAGC. The gene disruption mutants for each of these *S. japonicus* genes were constructed as described previously (14).

Cloning of *wcs* genes. DNA fragments containing the *wcs1* or *wcs2* gene with its putative promoter and terminator regions were amplified from *S. japonicus* genomic DNA (NIG2017) using the following primers: for *wcs1*, TAGGCTGCGGCCGCATTGCATGCAGTTCCATTTC and CG TAAAGCGGCCGCATACACTTCGTAGCATG, and for *wcs2*, CAGACA GCGGCCGCTCAAGTGATCTTACCC and GCTCTTGCGGCCGCAAA CTGTAATCTTGC. Both PCR DNA fragments were digested with NotI restriction enzyme. These DNA fragments were inserted into the NotI site of a vector plasmid, pSJK11 (20).

Phylogenetic tree and alignment of amino acid sequences. Homologues of *Neurospora crassa* WC-1 (accession no. X94300) or WC-2 (accession no. Y09119) were searched for in genomic databases of fungi by using the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Homologues of *N. crassa* WC-1 with E values lower than 3E−25 were further analyzed by the neighbor-joining algorithm, ClustalW (http://clustalw.ddbj.nig.ac.jp/index.php?lang=ja). A phylogenetic tree of the homologues of Wcs1 was drawn by using FigTree software (http://tree.bio.ed.ac.uk/software/figtree/). Bootstrap values in this analysis were performed based on 1,000 replications by using ClustalW. The homologues of Wcs1 were aligned by using clustalX (http://www.clustal.org/clustal2/).

RESULTS

Photoresponse of growing hyphae. When a colony of yeast cells is formed, hyphal cells extend from the periphery of the colony. Eventually, hyphae cover a large area of an agar plate called the "hyphal zone." We noticed that when culture dishes were kept in an incubator with a window, concentric rings were occasionally formed within the hyphal zone. We assumed that the elongating hyphae responded to daily alterations of light coming through the window. When regular cycles of light illumination (12-h light and 12-h dark [12-h L/12-h D]) were applied throughout the hyphal growth phase under experimentally controlled conditions, distinct dark- and bright-colored stripes formed on the agar plate (Fig. 1A). In contrast, those stripes were not formed in hyphal cells when the incubation was under constant light (LL) or darkness (DD). Thus, hyphal cells of the fission yeast made concentric circles of dark- and bright-colored stripes surrounding the colony according to the number of light cycles.

The growth pattern of hyphal cells varied with the nutrient conditions of the agar plates. Concentric circles were clearly made on morphology agar and malt agar plates by elongated hyphal cells, given the proper light cycle, although sections were made within the hyphal zone (see Fig. S1 in the supplemental material). In conclusion, hyphal cells of *S. japonicus* have the ability to respond to light regardless of nutrient conditions for hyphal induction.

Effects of light and dark on growing hyphae. Incubation of hyphal cells in continuous dark or light completely inhibited the formation of the hyphal stripes (Fig. 1A). This result suggests that the photoresponse is dependent either on periodic changes or on a single change of light or dark. We tested the effect of a single stimulus of light on hyphal growth. First, a yeast colony on an agar plate was incubated for several days in the dark to form a well-growing hyphal zone, and then light was applied. The plate was further incubated in the light for several days. A single transition from the darkness to the light induced a single dark-colored hyphal stripe band (Fig. 1B). Thus, a single stimulus of light after continuous darkness is sufficient for induction of the photoresponse of hyphal cells.

Next, we carried out an experiment with transition from continuous light to continuous darkness. Interestingly, darkness after continuous light also induced the hyphal photoresponse (Fig. 1B). In this case, two dark-colored bands appeared after the removal of

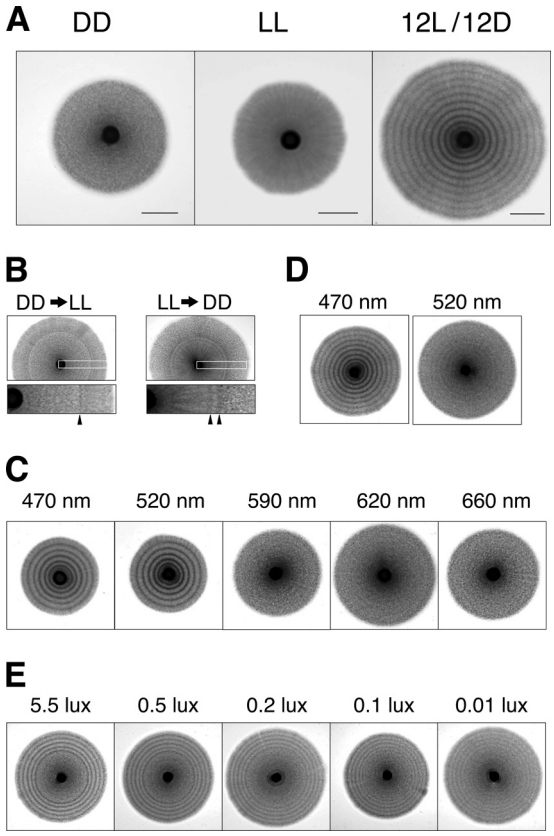


FIG 1 Photoresponse of hyphal cells. (A) Yeast cells (NIG2017) were spotted on the center of EMM2 agar plates and incubated at 30°C for longer than a week with constant dark (DD), constant light (LL), and the light cycle of 12-h light and 12-h dark (12L/12D). Bars, 1 cm. (B) An agar plate was incubated at 30°C until forming a hyphal zone during DD and then transferred into growth conditions at LL (DD→LL). Conversely, another was transferred from growth conditions under LL to DD (LL→DD). The portion of the bands shown in the white boxes is enlarged, and arrowheads indicate the dark-colored bands in each image. (C) Light from various LED lamps was tested for its ability to induce the photoresponse of hyphal cells. The power of the lamps was 50 lx at 10 cm of distance, and the light cycles were 12-h L/12-h D. (D) The power of the lamps was 2.5 lx at 10 cm. (E) Yeast cells were spotted on the center of EMM2 agar plates and incubated at 30°C for a week under constant dark and then under the light cycle of 12-h light and 12-h dark (12L/12D). The power of the LED lamp (470 nm) is indicated above each image.

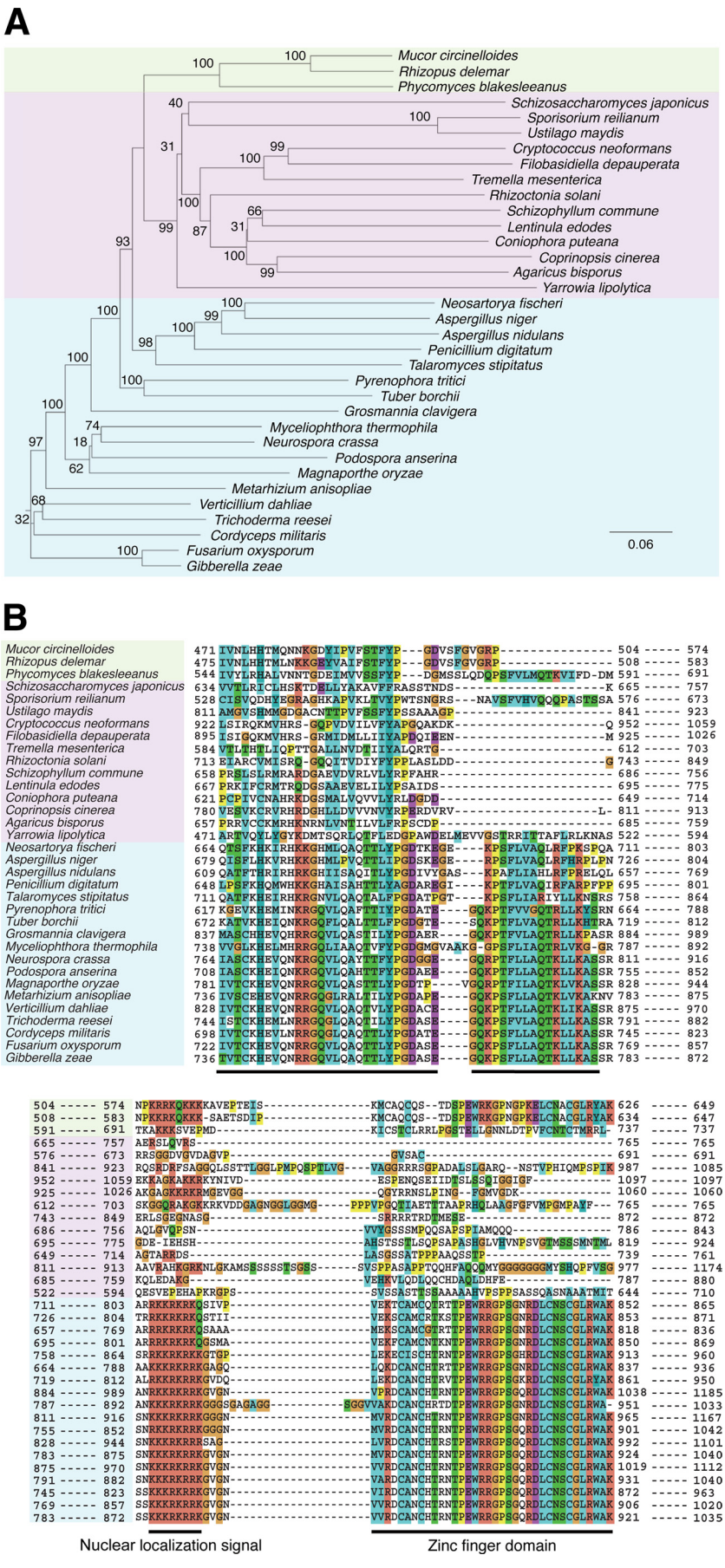
light. We determined how long it took until the dark-colored stripes began to form after the transition. Several agar plates were incubated in continuous light until the hyphal zone was well formed, and then they were incubated in the dark. The first band appeared 10 h later, and the second stripe appeared 20 h later (see Fig. S2 in the supplemental material). Interestingly, we found that both light and darkness induced the photoresponse. These results suggested that the alteration of light intensity triggers the photoresponse. Incubation for many hours in the light is necessary for the induction of two dark-colored bands by darkness (see below).

Hyphal photoresponse by blue light. It is known that many filamentous fungi have a photoresponse ability that is activated by a specific wavelength of light (1). We tested which wavelength activated the photoresponse of hyphal cells of *S. japonicus*. We used five different colors of light-emitting diode (LED) lamps, blue (470 nm), green (520 nm), orange (590 nm), red (620 nm), and deep red (660 nm), to illuminate hyphal cells. The blue and green lamps were highly effective in inducing the photoresponse, but the orange, red, and deep red lamps were not (Fig. 1C). Furthermore, the blue light was the most effective, as it could induce the formation of hyphal stripes at a lower power than could the green light (Fig. 1D). At least 0.1 lx of intensity of the blue lamp is sufficient for distinct formation of hyphal stripes (Fig. 1E). These results indicate that blue light contains the most effective wavelength to induce hyphal ring formation.

Hyphal photoresponse by blue light sensors. Our results suggested that *S. japonicus* hyphae might have a blue light receptor for the photoresponse. In filamentous fungi, the photoreceptor system for blue light sensing is widespread, and genes encoding orthologs of photoreceptor proteins, WC-1 and WC-2, are well conserved in their genome. In the genome of *S. japonicus*, we found orthologs of WC-1 and WC-2 with low similarity of amino acid sequences (Fig. 2; see also Fig. S3 and S4 in the supplemental material) and referred to them as Wcs1 and Wcs2, respectively. Orthologs of cryptochrome and of photolyase, which are related to photoreactivation, are not found in the genome of *S. japonicus*. To confirm the involvement of these orthologs in blue light sensing, deletion mutations in these genes were constructed. Both deletion mutants were unable to form any stripes in response to blue light (Fig. 3A). Furthermore, a single stimulus of light or dark also had no effect on the photoresponse to hyphal cells (Fig. 3B). Complementation tests with cloned *wcs1* and *wcs2* genes showed that formation of the stripes by the blue light is recovered in each deletion mutant (Fig. 3C). Thus, Wcs1 and Wcs2 were certainly responsible for blue light sensing in *S. japonicus*, suggesting that they form the blue light receptor.

To test whether a circadian rhythm was involved in the stripe formation of hyphal growth, a free-running experiment was carried out. The rhythm of the stripe formation was entrained to 12-h L/12-h D cycles for several days and then changed to DD or LL. The patterning of the dark- and bright-colored stripes ceased formation after the change (see Fig. S5 in the supplemental material). In fact, other genes related to the circadian rhythm of *N. crassa* (*frq*, *frh*, and *vvd*) are not found in the *S. japonicus* genome.

The growing front of the hyphae is photosensitive. Hyphal cells show apical growth, which means the filamentous cells elongate at only one of the cell tips. When hyphal cells radially spread from a yeast colony, distal daughter cells of the hyphae continue to divide after cell growth while proximal daughter cells tend to suspend cell growth. As a result, hyphal cells that are located at the front of the hyphal zone are always actively growing. This suggests that cells at the forefront of the hyphal zone are likely to respond to light and then form a dark-stripe zone. To test whether cells at the forefront of the hyphal zone become dark colored after light stimuli, we carried out time-lapse imaging of the photoresponse. Hyphal cells on a plate were grown in the dark and then irradiated by a blue LED lamp for 1 h, and subsequently the cells were incubated under a red LED lamp that does not trigger the photoresponse. We were able to take images because of the red LED lamp during further incubation. Distinct dark-colored stripes were visible in the hyphal cell zone 43 h later (Fig. 3D). We compared the photoimages of the hyphal zone 1 h after blue light exposure with that 43 h after exposure (Fig. 3D). The position of the dark-colored stripes that appeared on the plate 43 h after blue light exposure corresponded to the forefront of the hyphal zone on the plate 1 h after exposure where there were apical growing hyphal cells. We determined how long it took until the dark stripes began to form


A
Mucor circinelloides
Rhizopus delemar
Phycomyces blakesleeanus
Schizosaccharomyces japonicus
Sporisorium reilianum
Ustilago maydis
Cryptococcus neoformans
Filobasidiella depauperata
Tremella mesenterica
Rhizoctonia solani
Schizophyllum commune
Lentinula edodes
Coniophora puteana
Coprinopsis cinerea
Agaricus bisporus
Yarrowia lipolytica
Neosartorya fischeri
Aspergillus niger
Aspergillus nidulans
Penicillium digitatum
Talaromyces stipitatus
Pyrenophora tritici
Tuber borchii
Grosmannia clavigera
Myceliophthora thermophila
Neurospora crassa
Podospora anserina
Magnaporthe oryzae
Metarhizium anisopliae
Verticillium dahliae
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Cordyceps militaris
Fusarium oxysporum
Gibberella zeae
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Nuclear localization signal
Zinc finger domain

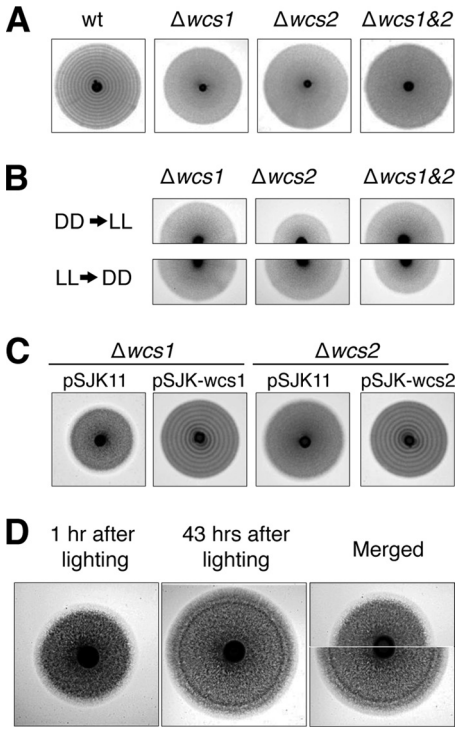

FIG 3 The effect of *wcs* genes on stripe formation. (A) Wild-type (wt) yeast cells (NIG2017) and deletion mutants for *wcs* genes (NIG3001, NIG3002, and NIG3003) were spotted on EMM2 agar plates and incubated at 30°C under 12-h L/12-h D light cycles. (B) EMM2 agar plates with deletion mutants of *wcs* genes (NIG3001, NIG3002, and NIG3003) were incubated at 30°C until forming a hyphal zone during DD and then were transferred into growth conditions at LL (DD→LL, top). Conversely, another was transferred from growth conditions under LL to DD (LL→DD, bottom). (C) The complementation tests of the deletion mutants of the *wcs* genes were performed by using the plasmid harboring *wcs1* (pSJK-wcs1) or *wcs2* (pSJK-wcs2). The deletion mutants (NIG3001 and NIG3002) harboring these plasmids or the vector plasmid pSJK11 were incubated at 30°C under 12-h L/12-h D light cycles. (D) An EMM2 agar plate with yeast strain NIG2017 was incubated at 30°C in the dark until forming a hyphal zone and then illuminated by a blue LED lamp for 1 h. It was further incubated under constant red light (620 nm). After stimulation by blue light, images were taken at 1-h intervals. Images shown are at 1 h and 43 h. Upper and lower halves of the images were combined.

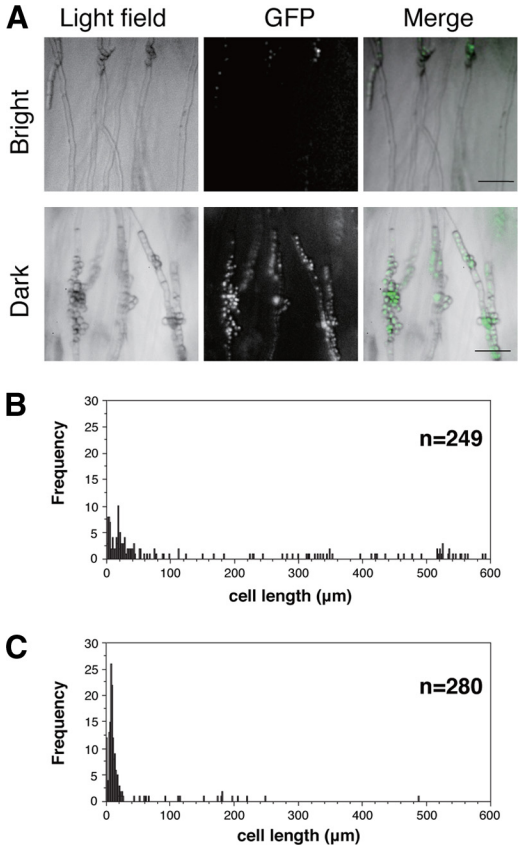

FIG 4 Hyphal cells in dark- and bright-colored stripes. (A) Images of hyphal cells taken by a fluorescence stereomicroscope. Hyphal cells are shown in a bright-colored stripe (upper row) and a dark-colored stripe (lower row). A yeast strain (NIG5097) with histone H3 proteins labeled by GFP was used to detect nuclei. Bar, 0.1 mm. (B and C) Distributions of cell length in the bright-colored stripe (B) and dark-colored zone (C). The average length of cells in the bright-colored segment is 142 ± 189 μm (mean ± standard deviation [SD]; $n = 249$), and the average length of cells in the dark-colored segment is 24 ± 51 μm (mean ± SD; $n = 280$).

after exposing the hyphal cells to blue light. A series of time-lapse images indicated that incubation for 20 h in light caused darkness at the edge of the hyphal zone (see Fig. S6 in the supplemental material). These results indicated that light stimulates only hyphal cells at the forefront, and although distal daughter cells continue to elongate, proximal daughter cells remain at their position and synchronously activate cell division 20 h after light stimulus.

Active cytokinesis in the dark zone of the stripes. What is the nature of the dark-colored stripes of the hyphae? We directly observed hyphal cells under a fluorescence stereomicroscope. A strain of *S. japonicus* that constitutively expressed histone H3 fused with green fluorescent protein (GFP) was used so that nuclei were detectable in hyphal cells. Zones of the brightly colored stripes contained long filamentous hyphae that were transparent tubular cells without septa (Fig. 4A). In contrast, septated hyphae, in which septation occurred at a higher frequency, were enriched in the dark zones. Because many septations by cytokinesis had diminished the transparency of the hyphae, the zones had become

FIG 2 Phylogenetic tree and alignment of amino acid sequences of representative members (34 species) in the Wcs1 protein family. A phylogenetic tree (A) is shown for three phyla of fungi: Ascomycota (blue), Basidiomycota (pink), and Zygomycota (green). Bootstrap values are indicated as percentages. The scale bar indicates an evolutionary distance of 0.06 amino acid substitutions per position. Phylogenetic trees of all members of WC families are shown as Fig. S3 and S4 in the supplemental material. The color scheme in partial alignment of amino acid sequences is used as the default scheme of ClustalX (B). The orange indicates G as glycine. The yellow indicates P as proline. The green indicates polar amino acid residues STQN when those residues were conserved more than 50% or hydrophobic residues ACFHILMVWYP when they were conserved more than 60%. The blue indicates hydrophobic residues ACFHILMVWYP when those residues were conserved more than 60%. The cyan indicates H as histidine and Y as tyrosine when each residue was conserved more than 85% or hydrophobic residues ACFILMVWP when they were conserved more than 60%. The magenta indicates negatively charged residues DE when those residues were conserved more than 50%. The blood orange color indicates positively charged residues KR when those residues were conserved more than 60%. Black bars indicate the amino acid sequences that are well conserved in Ascomycota.

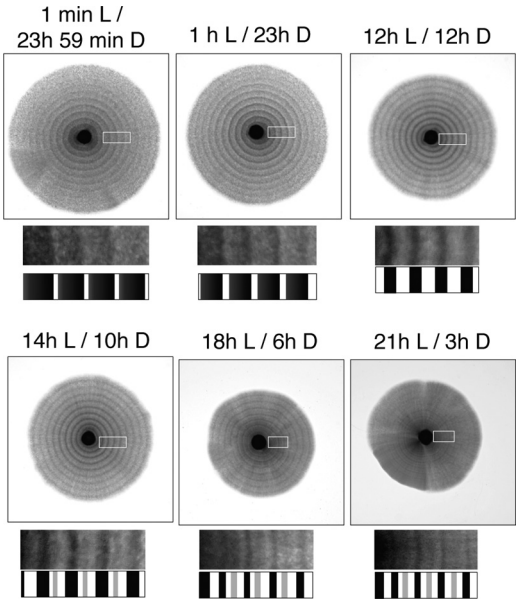

FIG 5 Photoresponse to various ratios of L/D. Yeast cells (NIG2017) were spotted on the center of agar plates and incubated at 30°C for 2 weeks under various lighting times and 24-h light cycles. Blue light was applied according to each light cycle as indicated above each image. A portion of the stripes shown in the white boxes is enlarged, and the patterns of the stripes are illustrated below each image.

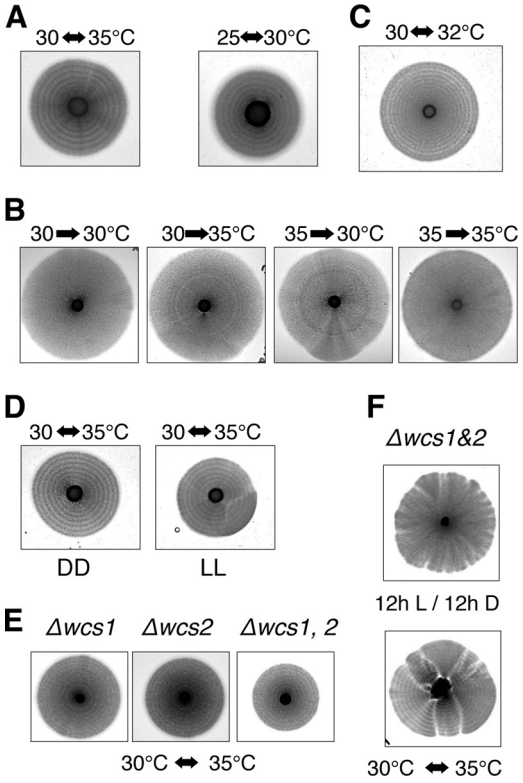

FIG 6 Effect of temperature shift on hyphal cells. (A) Yeast cells (NIG2017) were spotted on the center of EMM2 agar plates and incubated under DD for more than a week: temperature cycles were 12 h at 30°C and 12 h at 35°C or 12 h at 25°C and 12 h at 30°C. (B) EMM2 agar plates were incubated at 30°C until forming hyphal zones under DD and then were transferred into growth condition at 35°C. Conversely, others were transferred from growth conditions under 35°C to 30°C. (C) Yeast cells (NIG1056) were spotted on the center of YE agar plates and incubated under DD for more than a week: temperature cycles were 12 h at 30°C and 12 h at 32°C. (D) An EMM2 agar plate was incubated under constant blue light with temperature cycles of 12 h at 30°C and 12 h at 35°C. The power of the lamps was 50 lx at a 10-cm distance. (E) Yeast cells of deletion mutants of *wcs* genes were spotted on EMM2 agar plates and incubated at 30°C with temperature cycles of 12 h at 30°C and 12 h at 35°C. (F) The double deletion mutant of *wcs1* and *wcs2* genes was spotted on morphology agar plates and incubated at 30°C under a 12-h L/12-h D light cycle (top) with temperature cycles of 12 h at 30°C and 12 h at 35°C (bottom).

dark colored. In addition, we confirmed that hypha septation was also accompanied by nuclear division (Fig. 4A). Fluorescent nuclei were distributed in each septated hypha- and yeast-like cell. Statistical analyses of distance between septa in hyphal cells showed that the length of cells in the bright-colored segment is greater than that in the dark-colored segment, indicating that hyphal cells undergo high-frequency division in the dark-colored segment (Fig. 4B and C). These results indicated that growing hyphal cells are actively undergoing cytokinesis and nuclear division. We conclude that the cell cycle of hyphal cells is synchronously stimulated by light.

Patterning of hyphal stripes in response to various ratios of L/D. In the natural environment of the Earth, the light cycle is a constant 24 h of daily rhythms. However, the day-to-night ratio varies according to the seasons and latitude. It seemed that the photoresponse of *S. japonicus* adapts to the lighting cycles in its natural habitat. We tested the effect of varied ratios of light and dark (L/D) in a 24-h light cycle on the formation of dark- and bright-colored stripes. At least 1 min of blue light illumination was effective in causing the photoresponse in a 24-h light cycle (Fig. 5). Given a prolonged lighting time, the pattern of the dark- and bright-colored stripes changed. At first, the dark-colored area of the stripes was narrowed as the period of the dark was shortened. Simultaneously, the pattern of the stripes was clear and enhanced under 12-h L/12-h D. Interestingly, when 14-h L/10-h D was applied, an additional dark-colored stripe was induced between the usual dark stripes (Fig. 5). The additional dark-colored stripes were faint and thinner than the usual dark-colored stripes. They also appeared under 16-h L/8-h D, 18-h L/6-h D, and 21-h L/3-h D. Finally, the stripes of 21-h L/3-h D were weakened, and further prolonged illumination inhibited the formation of stripes in the hyphal zone. This result indicates that at least 3 h of darkness is necessary for the formation of stripes during the light cycle.

Hyphal photoresponse by temperature cycles. We tested whether other environmental cues could cause the formation of the hyphal stripes. Alternation in temperature can be a daily external cue. We incubated plates with hyphal cells in an incubator with temperature cycles of 30°C for 12 h and 35°C for 12 h. After incubation for more than 10 days, patterning with the dark- and bright-colored stripes was observed regardless of incubation in the dark (Fig. 6A). As seen in the photoresponse, hyphal cells in the dark-colored stripes were septated, and the cells in the bright-colored stripes were transparent and filamentous (see Fig. S7 in the supplemental material). It is obvious that alteration of temperature induced a similar response in the hyphal cells to light.

Temperature cycles of 25°C for 12 h and 30°C for 12 h also caused hyphal cells to form stripes (Fig. 6A). The stripes caused by alteration in temperature were induced by both a shift up, from 30°C to 35°C, and a shift down, from 35°C to 30°C (Fig. 6B). We previously isolated a spontaneous mutant that constitutively induces hyphal cells regardless of the nutrient conditions. We found

that hyphal cells of the mutant strain were able to form stripes with temperature cycles of 30°C for 12 h and 32°C for 12 h (Fig. 6C). Only a 2°C alteration in external temperature was required to affect the cell division cycle of hyphal cells, suggesting that this heat effect is different from heat shock or cold shock that causes structural changes in proteins and macromolecules. Thus, the response to alterations in temperature was quite sensitive in the hyphal cells.

The effect of temperature cycles was tested either in constant dark or in constant light. Constant blue light had no obvious effect on the temperature cycles (Fig. 6D). The stripes caused by the temperature cycles formed in all *wcs* gene mutants (Fig. 6E). Although the contrast between the bright- and dark-colored stripes was weakened on EMM2 agar plates, the response to alteration in temperature was retained in mutant cells as seen by the formation of temperature-dependent hyphal rings on morphology agar plates (Fig. 6F). These results suggest that each signaling pathway for light and temperature independently functions to induce a synchronous cell division cycle in hyphal cells.

Wcs proteins in yeast cells. It was previously reported that light enhances sexual flocculation in *S. japonicus* under conditions of vegetative growth (21). Sexual flocculation is made prior to sporulation when light is adequate (Fig. 7A). This sexual flocculation was inhibited in the dark (Fig. 7B). In addition, the sexual flocculation was completely inhibited in the *wcs1* or *wcs2* deletion mutant or double mutants, even though the culture was illuminated (Fig. 7C). As a result, zygotes could not develop in the mutants. On the other hand, when the nitrogen source was eliminated from the medium to induce sporulation, even in the mutants, they were able to induce sexual flocculation and succeed in sporulation regardless of the dark. Thus, although neither *wcs1* nor *wcs2* was essential for vegetative growth and sporulation *per se*, given the light-dependent physiology of this yeast, sporulation and vegetative growth were affected via the function of the *wcs* genes under certain nutrient conditions.

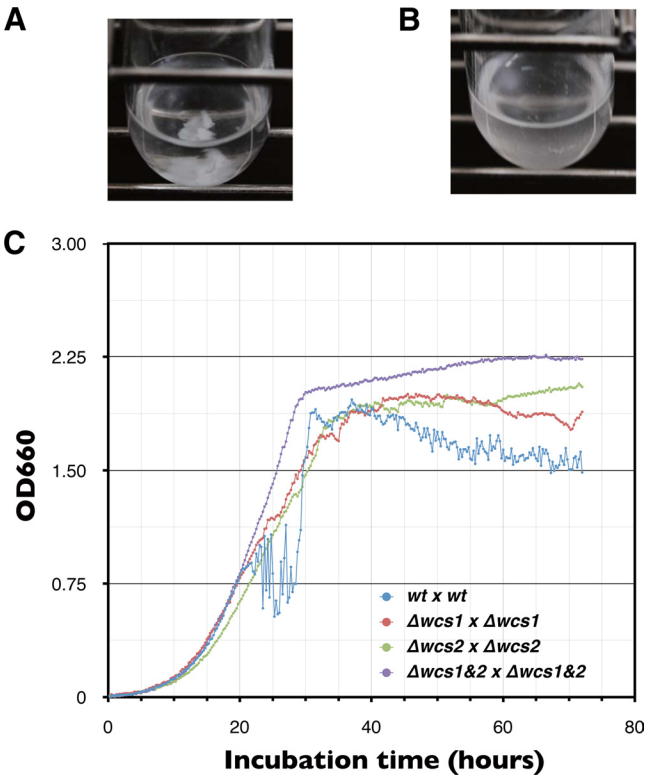

FIG 7 Effect of *wcs* genes on sexual flocculation. *S. japonicus* cells can sporulate in a nutrient-rich medium, Burkholder synthetic medium. (A) Sexual flocculation enhances precipitation of cells at the bottom of gently shaking test tubes in which wild type (wt) (h^+) and wt (h^-) are mixed. (B) Sexual flocculation is completely inhibited in test tubes incubated in the dark. Heterothallic strains of *S. japonicus* cells were independently cultivated in modified Burkholder synthetic medium overnight. Cells were washed once with fresh modified Burkholder synthetic medium and then mixed in test tubes according to each combination of mating types as follows: wt (h^+) × wt (h^-), Δ*wcs1* (h^+) × Δ*wcs1* (h^-), Δ*wcs2* (h^+) × Δ*wcs2* (h^-), Δ*wcs1&2* (h^+) × Δ*wcs1&2* (h^-). (C) The optical density of test tubes was automatically monitored during conjugation. Optical density of mating cultures was remarkably reduced because of precipitation of cells by sexual flocculation. After completion of spore formation, cells separated from one another and then optical density increased. The OD_{660} of mixing cultures was automatically recorded every 15 min.

DISCUSSION

Many organisms adjust the timing of cell division or differentiation to the night in order to escape photodamage. From our experiments applying various ratios of L/D, the light cycles tended to activate cytokinesis during the dark period. This might be beneficial to cells, as they can avoid light-induced damage during cell division. However, it seems that adjustment of the cell cycle to darkness is not perfect. In the case of *S. japonicus*, cellular responses to changes in either light or temperature lead the growing hyphal cells to the same consequence: synchronous activation of the cell division cycle. Both the temperature cycles and the light cycles might independently adjust the cellular activity to an ideal time of day in the natural environment. Given that aborting the light or temperature cycles immediately interrupted the hyphal stripe formation, each is a one-time-only response to daily alternating environmental stimuli, and circadian rhythmic responses are not involved. Without a circadian rhythm, this organism can still make periodic adaptations to environmental stresses by using a dual sensing mechanism for temperature and light. In other words, *S. japonicus* cells adapt their physiology and behavior directly to environmental circumstances rather than by anticipation of daily changes by a circadian clock.

S. japonicus has quite a high sensitivity to light and temperature, and it might be that even moonlight affects the hyphal cells, because the intensity of moonlight may reach 0.7 lx (22). Circadian clocks can sense even a 2°C difference in temperature (23–25). Curiously, the present sensing mechanism can respond to both negative and positive stimuli from light and temperature. In general, the WC complex, which includes FAD to catch photons, is activated by light. Loss of the *wcs1* and *wcs2* genes caused loss of sensitivity to light stimuli both from DD to LL and from LL to DD. The WC complex of *S. japonicus* is activated somehow not only by light but also by its absence.

By providing light for only 1 min after continuous dark, two stimuli are generated: exposure to light and its removal. In general, light is a major stimulus for organisms. An adequate lighting period is required for the effect of darkness, and this lighting period is longer than 14 h for *S. japonicus*. We inferred this requirement from the appearance of faint stripes due to stimulation by darkness after continuous illumination for longer than 14 h (Fig. 8). Dark-colored stripes caused by the effect of light emerged 20 h after illumination. Two dark-colored stripes emerged after re-

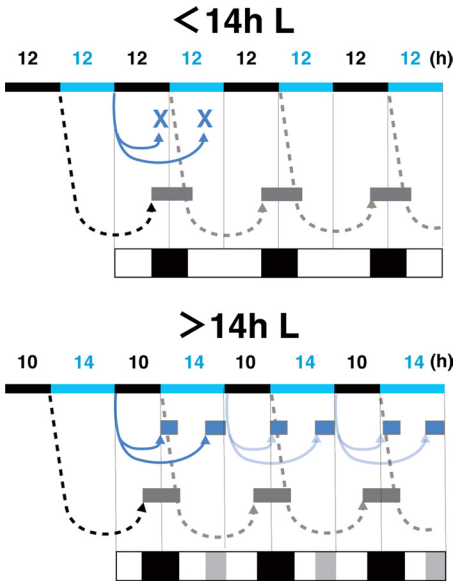

FIG 8 Diagrams of the photoresponse caused by light and darkness. The light cycles are indicated by the top bars (black, dark; blue, light). The effect of light (black dotted lines) induced synchronous cell division 20 h later (gray boxes). When the lighting time was longer than 14 h, the photoresponse by darkness (blue line) induced synchronous cell division 10 and 20 h later, respectively (blue boxes). The patterns of the dark- and bright-colored stripes are illustrated at the bottom, and additional dark-colored stripes are shown as gray.

moval of light: the first stripe emerged 10 h after darkness, and the second stripe emerged at 20 h. In the case of more than 14 h of light, the dark-colored stripes caused by light and the first stripes caused by darkness merged, and then the second stripes were seen as additional faint bands.

Activation of the cell division cycle in hyphal cells is synchronously induced with a constant time delay after stimulation with light or temperature and then synchronously reduced so that regular patterning of hyphal cells is formed. This temporal regulation suggests that the response of hyphal cells is controlled by a timer, like an hourglass. This means that once triggered, an event will be performed. An hourglass timer requires resetting to be repeated. In fact, a 22-h L/2-h D light cycle does not result in formation of hyphal stripes. More than 3 h of darkness for reactivation of the photoresponse might be necessary to reset the putative hourglass timer.

A relatively broad range of the ratio of light to dark affects the photoresponse of hyphal cells. When lighting is more than 14 h, an additional effect is observed when darkness is applied. The photoresponse of *S. japonicus* has probably been adapted to its particular natural environment. While the natural habitat of *S. pombe* is in the tropics, *S. japonicus* is endemic to temperate regions such as Michigan, USA, and Fukuoka, Japan, where periods of daylight are more than 14 h before and after the summer solstice (26, 27).

There is a homologue of WC-1 in the basidiomycete yeast *Cryptococcus neoformans* (6). In addition, we recently found homologues of *Neurospora crassa* WC-1 in two species of the Ascomycota yeasts, *S. japonicus* and *Yarrowia lipolytica*. For genome databases of fungi that have been analyzed, *S. japonicus* and *Y. lipolytica* are the only ascomycete yeasts that retain proteins of the WC family. The WC-1 orthologs of these two species lack the conserved zinc finger domains, the nuclear localization signal sequences, and an unknown functional domain found in Ascomycota (Fig. 2B). Interestingly, their WC orthologs are more closely related to those of the Basidiomycota rather than to those of the Ascomycota. In *S. japonicus*, not only do hyphal cells show a periodic response to daily alterations, but also yeast cells show light-dependent flocculation during sporulation. Further analyses of gene expression will reveal whether yeast respond to light and temperature during vegetative growth.

Except for the WC orthologs, genes involved in circadian rhythms are not found in the genome of *S. japonicus*. This observation is consistent with the loss of a free-running rhythm after disruption of the light or temperature cycle. During adaptation to its particular environment, this yeast had apparently lost those genes and only the photoactivated proteins remained. Alternatively, these clock components arose later in a small branch of the Ascomycota. In any case, the acquirement of photoactivated proteins was a sufficient adaptation for *S. japonicus*. Moreover, the temperature cycle-dependent response provides this fission yeast with an additional strategy for hyphal cells to adapt to environmental conditions.

ACKNOWLEDGMENTS

We thank N. Ishihara for technical assistance and H. Iwasaki for discussions of our results.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to H.N. and an NIG Postdoctoral Fellowship to S.O.

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