Colletotrichum orbiculare WHI2, a Yeast Stress-Response Regulator Homolog, Controls the Biotrophic Stage of Hemibiotrophic Infection Through TOR Signaling

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The hemibiotrophic fungus *Colletotrichum orbiculare* first establishes a biotrophic infection stage in cucumber (*Cucumis sativus*) epidermal cells and subsequently transitions to a necrotrophic stage. Here, we found that *C. orbiculare* established hemibiotrophic infection via *C. orbiculare* WHI2, a yeast stress regulator homolog, and TOR (target of rapamycin) signaling. Plant defense responses such as callose deposition, H₂O₂, and antimicrobial proteins were strongly induced by the *C. orbiculare whi2Δ* mutant, resulting in defective pathogenesis. Expression analysis of biotrophy-specific genes evaluated by the promoter VENUS fusion gene indicated weaker VENUS signal intensity in the *whi2Δ* mutant, thereby suggesting that *C. orbiculare* WHI2 plays a key role in regulating biotrophic infection of *C. orbiculare*. The involvement of *CoWHI2* in biotrophic infection was further explored with a DNA microarray. In the *Cowhi2Δ* mutant, TOR-dependent ribosomal protein-related genes were strikingly upregulated compared with the wild type. Moreover, callose deposition in the host plant after inoculation with the *Cowhi2Δ* mutant treated with rapamycin, which inhibits TOR activity, was reduced, and the mutant remained biotrophic in contrast to the untreated mutant. Thus, regulation of TOR by Whi2 is apparently crucial to the biotrophic stage of hemibiotrophic infection in *C. orbiculare*.

Colletotrichum orbiculare, the causal agent of anthracnose disease on cucurbitaceous plants, establishes infection by developing a series of specialized infection structures (Kubo and Takano 2013). Conidia adhering to the cuticle of host cells germinate and form a melanized dome-like structure called the appressorium that is necessary for successful infection of host plants (Kubo and Furusawa 1991). Recently, we revealed that proper cell cycle regulation during appressorium formation is essential for plant infection (Fukada and Kubo 2015). The infection hyphae emerging from the appressoria of *Colletotrichum* species initially form bulbous biotrophic hyphae, and the fungus later switches to a necrotrophic phase accompanied by the formation of narrower infection hyphae (Irieda et al. 2014; Perfect et al. 1998).

The biotrophic stage of hemibiotrophic fungi is a crucial step to obtain nutrients without killing the host cells. Against hemibiotrophic fungal infection, the host plant recognizes fungal-specific molecules such as β -glucans and chitin, called pathogen-associated molecular patterns (PAMPs), which immediately triggers its defense responses (Fujikawa et al. 2012; Latgé 2010). Therefore, hemibiotrophic fungi must suppress this pattern-triggered immunity caused by recognition of the PAMPs, via pattern recognition receptors to establish the biotrophic stage (Miya et al. 2007; Monaghan and Zipfel 2012).

To circumvent activation of host defense responses, hemibiotrophic fungi secrete effector proteins during the biotrophic stage (Kleemann et al. 2012; Zhang and Xu 2014). In *Magnaporthe oryzae*, effector protein Slp1 functions to suppress chitin-induced plant immunity (Mentlak et al. 2012). In *C. orbiculare*, effector proteins Nis1, Dn3, and Mc69 are predominantly expressed and secreted during the biotrophic stage (Saitoh et al. 2012; Yoshino et al. 2012). In addition, many secondary metabolite backbone synthesis genes are upregulated during the biotrophic stage compared with vegetative hyphae, and some genes are specifically expressed during the biotrophic stage in *C. orbiculare* and *C. higginsianum* (Gan et al. 2013; O'Connell et al. 2012). Thus, appropriate gene expression during the biotrophic stage is envisaged as a key for hemibiotrophic fungi to infect their host plants. However, the regulatory mechanisms involved in hemibiotrophic infection of hemibiotrophic fungi are still not clear.

The target of rapamycin (TOR) is widely conserved among eukaryotes from mammals to yeasts (Brown et al. 1994; Heitman et al. 1991; Kunz et al. 1993; Sabatini et al. 1994). TOR is known as a key factor for pleiotrophic intracellular events such as autophagy, reactive oxygen species, protein synthesis, ribosomal biosynthesis, and metabolism (Alers et al. 2012; Bonawitz et al. 2007; Martin et al. 2006). In plant-pathogenic fungi, TOR is also required for pathogenesis and infection-related morphogenesis. In the hemibiotrophic fungus *Fusarium graminearum*, the TOR signaling pathway is required for pathogenesis and vegetative hyphae, and serine/threonine kinase SCH9, which functions downstream of the TOR signaling pathway, is involved in conidium size, stress response, and pathogenesis (Chen et al. 2014; Yu et al. 2014). Furthermore, in *M. oryzae*, TOR activation in response to intracellular glutamine levels inhibits appressorium development and TOR activation is required for biotrophic growth inside epidermal cells of rice leaf (Fernandez et al. 2014; Marroquin-Guzman and Wilson 2015). However, how intracellular factors regulate the TOR signaling pathway activity in hemibiotrophic fungi is far less understood.

Nucleotide sequence data were deposited in the Gene Expression Omnibus (GEO) database under accession number GSE73096.

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In previous reports, *C. orbiculare* small GTP-binding protein CoRas2 positively regulates activation of the cAMP- protein kinase A (PKA) and the mitogen-activated protein kinase (MAPK) CoMekk1-Cmk1 signaling pathways involved in infection-related morphogenesis and undergo dynamic cellular localization during infection (Harata and Kubo 2014). Therefore, we assumed that the spatiotemporal regulation of CoRas2 has a crucial role in inducing infection-related morphogenesis. In *Saccharomyces cerevisiae*, the stress response regulator *WHI2* contributes to stabilizing F-actin structure in response to nutritional sensing, and F-actin requires the proper localization of the Ras protein (Leadsham et al. 2009). Therefore, to elucidate the relationship between the spatiotemporal regulation of CoRas2 and infection-related morphogenesis, we functionally analyzed *C. orbiculare* *CoWHI2*, the homolog of *WHI2* of *S. cerevisiae*.

In phytopathogens, the TOR signaling pathway is known to have pleiotropic roles during the infection process, but the regulatory mechanism for this pathway is far less understood in comparison with the cAMP-PKA and the MAPK signaling pathways required for successful infection. Activity of the transcription factor Msn2p is regulated by *S. cerevisiae* Whi2p and the TOR signaling pathway, but the relation between Whi2p and the TOR signaling pathway is not clearly defined (Sadeh et al. 2011). Therefore, in addition to analyzing the spatiotemporal regulation of CoRas2 in *CoWhi2* function, we also examined the relation between *CoWhi2* and the TOR signaling pathway.

In this study, we present evidence for the novel insight that *Colletotrichum orbiculare* Whi2 controls the highly regulated biotrophic stage of hemibiotrophic infection by *C. orbiculare* via TOR.

RESULTS

Targeted replacement of *CoWHI2* in *C. orbiculare*.

Using *C. orbiculare* genomic information, we searched for a homolog of *S. cerevisiae* *WHI2* that could potentially be involved in subcellular localization of the signaling protein Ras, in response to environmental sensing, and found the gene ENH78490, which putatively encodes a protein with 293 amino acids (Gan et al. 2013). The predicted amino acid sequence from ENH78490 showed significant similarity to that of Whi2p in *S. cerevisiae* (Supplementary Fig. S1). Therefore, we named this gene *Colletotrichum orbiculare* *CoWHI2*.

To analyze the function of *CoWHI2*, we constructed a *Cowhi2Δ* disruption vector designed to replace the wild-type *CoWHI2* gene with a hygromycin phosphotransferase gene via double crossover homologous recombination (Supplementary Fig. S2A and B). Using *Agrobacterium tumefaciens*-mediated transformation (ATMT), we obtained hygromycin-resistant transformants. Among these transformants, we selected bialaphos-sensitive transformants for exclusion of ectopic insertions and obtained three transformants having the phenotype with hygromycin resistance and bialaphos sensitivity. Successful replacement of the targeted *CoWHI2* gene in these transformants was confirmed by Southern blot analysis.

CoWHI2 is not involved in cell-wall integrity.

In *S. cerevisiae*, Whi2p is a regulator for transcription factor Msn2p, which regulates expression of stress responsive genes in stress conditions (Sadeh et al. 2011). In the *M. oryzae* mutant, *Momsn2Δ*, a homolog of *S. cerevisiae* Msn2p, showed increased tolerance to calcofluor white (CFW) that disturbs fungal cell-wall integrity due to binding to polysaccharides (Zhang et al. 2014). Thus, the regulator of Msn2 might be involved in cell-wall integrity in response to stress conditions. Therefore, to elucidate whether *CoWHI2* is involved in cell-wall integrity, we examined

the hyphal growth of the *Cowhi2Δ* mutant on potato dextrose agar (PDA) amended with different wall-perturbing agents, i.e., CFW, Congo red (CR) or sodium dodecyl sulfate (SDS). Hyphal growth of the *Cowhi2Δ* mutant on PDA was slightly slower than that of the wild type. On SDS, hyphal growth of the *Cowhi2Δ* mutant was inhibited nearly the same as the wild type, but the *Cowhi2Δ* mutant was more tolerant to CFW and CR than the wild type was (Supplementary Fig. S3).

CoWHI2 is not involved in regulating the cellular localization of CoRas2 during appressorium formation.

In our previous report, we showed the dynamic cellular localization of small GTP-binding protein CoRas2 during appressorium formation in *C. orbiculare*, suggesting that the regulation of the cellular localization of CoRas2 is important for infection-related morphogenesis and pathogenesis (Harata and Kubo 2014). *WHI2* is known to contribute to the appropriate localization of Ras via actin dynamics in response to nutritional sensing in *S. cerevisiae* (Leadsham et al. 2009). Therefore, to elucidate whether *CoWHI2* is involved in the regulation of the dynamic cellular localization of CoRas2 in *C. orbiculare*, we examined CoRas2 localization in the *Cowhi2Δ* mutant during appressorium formation. Cellular localization of CoRas2 in the *Cowhi2Δ* mutant was similar to that of the wild type, indicating that *CoWHI2* does not contribute to the cellular regulation of CoRas2, differing from the case in *S. cerevisiae* (Supplementary Fig. S4).

Host defense response with callose deposition is strongly induced by the *Cowhi2Δ* mutant in the initial infection stage.

To elucidate whether *CoWHI2* is involved in fungal pathogenesis, detached cucumber cotyledons were inoculated with the *Cowhi2Δ* mutant. The frequency of lesions larger than 0.4 mm in diameter caused by the *Cowhi2Δ* mutant was lower than that caused by the wild type and by transformants with *CoWHI2* reintroduced. The frequency of lesions smaller than

Fig. 1. Pathogenicity assay of *Colletotrichum orbiculare* *whi2Δ* mutants on cucumber cotyledons. **A**, Conidial suspensions of each strain were placed on detached cotyledons of cucumber and were incubated at 24°C for 5 days. WT = wild-type 104-Tn DWH2-1 and DWH2-2 = *Cowhi2Δ* mutants CWH2-1 = *CoWHI2*-complemented transformant of DWH2-1n CWH2-2 = *CoWHI2*-complemented transformant of DWH2-2n and DSD1 = *Cossd1Δ* mutant. **B**, Virulence of the wild type and the *Cowhi2Δ* mutant was scored at 5 days postinoculation. The diameter of the lesion caused by each strain was measured and was scored as shown. The total number of inoculation sites is indicated above each column.

0.29 mm in diameter caused by the *Cowhi2Δ* mutant was higher than that caused by the wild type and transformants with *CoWHI2* reintroduced, indicating that *C. orbiculare* *CoWHI2* is required for full virulence (Fig. 1). In *C. orbiculare*, we reported that the *Cossd1Δ* mutant that is defective in the constitution of its cell wall was less virulent to its host, due to an increased host defense response accompanied by rapidly

induced callose deposition at sites where appressoria tried to penetrate (Tanaka et al. 2009).

To elucidate whether this type of host defense response accompanied by callose deposition is induced by attack of the *Cowhi2Δ* mutant, we monitored callose deposition on cucumber epidermal cells beneath appressoria of the *Cowhi2Δ* mutant at 24 h postinoculation (hpi). By 24 hpi, large deposits of callose had accumulated at high frequency beneath these appressoria compared with the wild type (Fig. 2). Interestingly, the callose induced by the *Cowhi2Δ* mutant accumulated at around 24 hpi, while the callose induced by the *Cossd1Δ* mutant accumulated after 48 hpi. These data indicated that the deposition

Fig. 2. Callose deposition in epidermal cells on the abaxial surface of cucumber cotyledons beneath appressoria of the *Cowhi2Δ* mutant. Conidial suspensions of the strains were placed on the abaxial surface of cucumber cotyledons; after incubation at 24°C for 24 h, cotyledons were stained with aniline blue and were examined for callose deposition, using fluorescence microscopy. **A**, Variation in size of callose deposition induced by *Colletotrichum orbiculare*. LC indicates callose deposits larger than the boundary of the appressorium; SC, callose deposits smaller than the boundary of the appressorium; N, none detected. **B**, Callose deposition beneath appressoria formed by the wild type and the *Cowhi2Δ* mutant. **C**, Mean percentage of the sizes of callose deposition beneath appressoria formed by each strain. Approximately 200 appressoria were examined per inoculation site, three replicates were examined for each of three independent experiments; standard errors are indicated. WT = wild-type 104-Tn DWH2 = *Cowhi2Δ* mutant CWH2 = *CoWHI2*-complemented transformant of DWH2n and DSD1 = *Cossd1Δ* mutant.

Fig. 3. Expression of callose synthase genes in cucumber cotyledons inoculated with the *Cowhi2Δ* mutant. Conidial suspensions of each strain were placed on detached cotyledons of cucumber, which were incubated for 6, 12, or 24 h. Total RNA was extracted from the incubated leaves, and expression of callose synthase genes was analyzed by quantitative reverse transcription-polymerase chain reaction. Three independent experiments were conducted; standard errors are indicated. Asterisks represent significant differences between cucumber leaves inoculated with the wild type and each mutant (Student's *t* test: one asterisk (*) indicates $P < 0.05$ and two (**) $P < 0.01$). WT = wild-type 104-T, DWH2 = *Cowhi2Δ* mutant, and DSD1 = *Cossd1Δ* mutant.

of callose induced by the *Cowhi2Δ* mutant temporally differed from that induced by the *Cosd1Δ* mutant.

To test this hypothesis, we analyzed expression patterns of callose synthase genes of *Cucumis sativus* that represent homologs of *Arabidopsis thaliana* *GSL* genes involved in callose biosynthesis (Richmond 2000; Verma and Hong 2001) (Supplementary Fig. S5). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis showed that, in cucumber inoculated with the *Cowhi2Δ* mutant, the expression of callose synthases 8, 10, and 12 (*CS8*, *CS10*, *CS12*), at 6 hpi, was 2.2-, 1.6-, and 1.9-fold higher, respectively, than after inoculation with the wild type (Fig. 3). At 12 hpi, the expression of callose synthase genes was not significantly induced by the *Cowhi2Δ* mutant or the *Cosd1Δ* mutant compared with those inoculated with the wild type. At 24 hpi, the expression of *CS8*, *CS10*, and *CS12* in cucumber leaves inoculated with the *Cosd1Δ* mutant was 2.2-, 1.7-, and 1.7-fold higher, respectively, than in cucumber leaves inoculated with the wild type. Therefore, callose synthase genes *CS8*, *CS10*, and *CS12* are rapidly induced in the host cucumber leaves at the initial infection stage of *Cowhi2Δ* mutant.

Pleiotropic host defense responses induced by the *Cowhi2Δ* mutant.

H₂O₂ production by the host plant after attack by phytopathogenic fungi is one of the key factors for activating host defense responses. To elucidate whether H₂O₂ production in epidermal cells is induced by the *Cowhi2Δ* mutant, we evaluated H₂O₂ accumulation in epidermal cells beneath appressoria of the *Cowhi2Δ* mutant by treating with 3,3'-diaminobenzidine (DAB) to detect hydrogen peroxide. The epidermal cells beneath appressoria of the *Cowhi2Δ* mutant stained strongly with

DAB compared with the wild type, indicating that the *Cowhi2Δ* mutant does induce a host defense response accompanied by H₂O₂ accumulation (Fig. 4A and B). Interestingly, the epidermal cells at attempted penetration sites of the *Cowhi2Δ* mutant emitted significantly more autofluorescence compared with the wild type (Fig. 4C and D).

Plant NADPH oxidase is accepted as the key regulator of H₂O₂ generation associated with the plant defense response against attack by phytopathogenic fungi (Torres et al. 2005; Yoshioka et al. 2003). The *Cowhi2Δ* mutant induced H₂O₂ accumulation in cucumber epidermal cells. Therefore, to elucidate whether the plant defense response induced by the *Cowhi2Δ* mutant is triggered by the activity of NADPH oxidases, we examined callose deposition and H₂O₂ accumulation on cucumber epidermal cells beneath appressoria of the *Cowhi2Δ* mutant treated with 0.5 μM diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase activity. The frequency of callose deposition and H₂O₂ accumulation in the *Cowhi2Δ* mutant treated with DPI was lower than those in the untreated mutant (Supplementary Fig. S6).

The accumulation of phenolic compounds after pathogen attack has been previously reported (Chi et al. 2009; Nicholson and Hammerschmidt 1992), and our data suggested that the defense reaction of the host plant may be affected by infection with the *Cowhi2Δ* mutant. Therefore, to analyze whether plant defense gene expression was affected by infection with the mutant, we analyzed levels of the pathogenesis-related protein 1 (*PR-1*) gene and defense-related *DEFENSIN19* gene of *Cucumis sativus*, homologs of *PR1* and *PDF1.2*, that are induced by salicylic acid and jasmonic acid in *A. thaliana*, respectively (Penninckx et al. 1998; Tanaka et al. 2007). At 48 hpi, the expression of *DEFENSIN19* in cucumber leaves

Fig. 4. The *Cowhi2Δ* mutant induced activation of host defense responses. **A**, H₂O₂ accumulation beneath appressoria of each strain. Conidial suspensions of each strain were placed on the abaxial surface of cucumber cotyledons. After incubation at 24°C for 24 h, cotyledons were stained with 3,3'-diaminobenzidine and were then observed with microscopy for dark-brown staining to indicate H₂O₂ accumulation. WT = wild-type 104-T, DWH2 = *Cowhi2Δ* mutant, CWH2 = *CoWHI2*-complemented transformant of DWH2, DSD1 = *Cosd1Δ* mutant. **B**, Mean frequency of H₂O₂ accumulation beneath appressoria of each strain. Approximately 100 appressoria of each strain were observed per inoculation site, and three replicates were examined for each of three independent experiments; standard errors are indicated. Asterisks represent significant differences between cucumber leaves inoculated with the wild type and each strain (Student's *t* test: *P* < 0.01). **C**, Fluorescence in cells beneath appressoria formed by the *Cowhi2Δ* mutant. Conidial suspensions of each strain were placed on the abaxial surface of cucumber cotyledons; after incubation at 24°C for 48 h, host cells beneath appressoria were examined with fluorescence microscopy. **D**, Mean frequency of cells with fluorescence beneath appressoria formed by each strain. Approximately 100 appressoria of each strain were observed per inoculated site; three replicates were examined. Three independent experiments were conducted; standard errors are indicated. Asterisks represent significant differences between cucumber leaves inoculated with the wild type and *Cowhi2Δ* (Student's *t* test: *P* < 0.01).

inoculated with the *Cowhi2Δ* mutant was slightly higher than in the wild type, while the expression of *PR-1* in cucumber leaves inoculated with the *Cowhi2Δ* mutant was lower than in the wild type (Fig. 5). At 72 hpi, the expression of *DEFENSIN19* in cucumber leaves inoculated with the *Cowhi2Δ* mutant was significantly higher than in the wild type, while the expression of *PR-1* in cucumber leaves inoculated with the *Cowhi2Δ* mutant was lower than in the wild type (Fig. 5). Interestingly, the *Cosd1Δ* mutant strongly induced expression of *PR-1* compared with the wild type. Taken together, these results indicated that the initial host defense responses of host cucumber *Cucumis sativus* against the attack by the *Cowhi2Δ* mutant were much different than in the wild type.

The *Cowhi2Δ* mutation affects *C. orbiculare*–*Cucumis sativus* interactions in the biotrophic phase.

The *Cosd1Δ* mutant is unable to form infection hyphae on the host cells, due to callose deposition at attempted penetration sites below the appressoria, and has attenuated virulence (Tanaka et al. 2009). To determine whether the *Cowhi2Δ* mutant can form infection hyphae on cucumber leaves, we observed the infection process of the *Cowhi2Δ* mutant. The frequency of infection hyphae in the *Cowhi2Δ* mutant was similar to the wild type, and *CoWHI2* reintroduced transformants (Fig. 6A and B). Next, to evaluate whether the autofluorescence in epidermal cells induced by the attack of *Cowhi2Δ* mutant was associated with blocked appressorium-mediated penetration by the *Cowhi2Δ* mutant, we

Fig. 5. Expression of *DEFENSIN19* and *PR-1* in cucumber cotyledons after inoculation with each strain. Conidial suspensions of each strain were placed on the abaxial surface of cucumber cotyledons; at 48 and 72 h postinoculation (hpi), total RNA was extracted from cotyledons and expression of *DEFENSIN19* and *PR-1* was analyzed by quantitative reverse transcription-polymerase chain reaction. Three independent experiments were conducted and standard errors are indicated. WT = wild-type 104-T; DWH2 = *Cowhi2Δ* mutant; DSD1 = *Cosd1Δ* mutant. Asterisks represent significant differences between cucumber leaves inoculated with the wild type and each mutant (Student's *t* test: one asterisk (*) = $P < 0.05$, two (**) = $P < 0.01$).

examined the development of infection hyphae of the mutant at the autofluorescent cells. Interestingly, the autofluorescent epidermal cells were not penetrated and the *Cowhi2Δ* mutant did not grow in the host cell (Fig. 6C and D), but nonfluorescent adjacent cells around the autofluorescent cells did not prominently block penetration and infectious growth. These data indicated that the alteration causing or accompanying the autofluorescence in epidermal cells is involved in inhibiting the appressorium-mediated penetration and infectious growth of the *Cowhi2Δ* mutant.

Although infection hyphae growth with biotrophy in hemibiotrophic fungi is inhibited under the activation of plant defense responses, infection hyphae growth of the *Cowhi2Δ* mutant was not inhibited at 72 hpi when the *C. orbiculare* wild type forms a biotrophic infection hyphae. To elucidate whether the *Cowhi2Δ* mutant retains the ability to establish this biotrophic phase, we generated wild type and *Cowhi2Δ* transformants that expressed the fluorescent gene *VENUS* under the control of a biotrophy-specific promoter and the fluorescent gene *mCherry* under the control of the constitutively expressed *TRANSLATION ELONGATION FACTOR* (*TEF*) promoter derived from *Aureobasidium pullulans* and, then, assessed biotrophic fluorescence intensity normalized by *mCherry* signal intensity during the biotrophic stage. First, we selected two biotrophy-specific expression genes, polysaccharide deacetylase family protein (*CoPSD1*) and 4-aminobutyrate aminotransferase (*CoATF1*), that are prominently expressed at higher levels during the biotrophic stage than during appressorium development and the necrotrophic stage, and approximately 2.0 kb of their 5' upstream regions and *TEF* promoter was fused with the *VENUS* and *mCherry* genes, respectively (Gan et al. 2013). Then, we successfully generated transformants carrying the biotrophy-specific expression *VENUS* gene and the constitutively expressed *mCherry* gene. WT/*TEF*_{pro}:*mCherry*/*CoPSD*_{pro}:*VENUS* is the wild type and DWH2/*TEF*_{pro}:*mCherry*/*CoPSD*_{pro}:*VENUS* is the *Cowhi2Δ* mutant transformant, expressing the *VENUS* gene with the *CoPSD1* promoter and the *mCherry* gene with the *TEF* promoter. WT/*TEF*_{pro}:*mCherry*/*CoATF*_{pro}:*VENUS* is the wild type and DWH2/*TEF*_{pro}:*mCherry*/*CoATF*_{pro}:*VENUS* is the *Cowhi2Δ* mutant transformant, expressing the *VENUS* gene with the *CoATF1* promoter and the *mCherry* gene with the *TEF* promoter. At 72 hpi, the frequency and intensity of the *CoPSD*_{pro}:*VENUS* signal in infection hyphae of *Cowhi2Δ* was lower and weaker than in those in the wild type (Fig. 7A, C, and D). Similarly, the frequency and intensity of the *CoATF*_{pro}:*VENUS* signal in infection hyphae of *Cowhi2Δ* was lower and weaker than those in the wild type (Fig. 7B to D).

CoWhi2 directly interacts with *CoPsr1* and the *Copsr1Δ* mutant shows a similar phenotype to the *Cowhi2Δ* mutant.

In *S. cerevisiae*, *Whi2p* directly interacts with the phosphatase *Psr1p* and the complex dephosphorylates the transcription factor *Msn2p*. Therefore, to analyze whether *CoWhi2* interacts with *CoPsr1*, the homolog of *S. cerevisiae* *Psr1p*, we performed yeast two-hybrid assays. First, based on *C. orbiculare* genomic information, we searched for a homolog of *S. cerevisiae* *PSR1* in *C. orbiculare* and found gene ENH79657, which putatively encodes a 513-aa protein (Gan et al. 2013). The predicted amino acid sequence encoded by *CoPSR1* is significantly similar to that of *Psr1p* in *S. cerevisiae* (Supplementary Fig. S7). Next, we constructed the bait *CoWHI2* vector (BD-*CoWhi2*) and prey *CoPSR1* vector (AD-*CoPsr1*) and these vectors were used to transform the Gold and Y187 yeast strains, respectively. In the yeast two-hybrid assays, the mating type carrying BD-*CoWhi2* and AD-*CoPsr1* grew on selective medium, while growth of the negative control was inhibited on selective medium, indicating that *CoWhi2* directly interacts with *CoPsr1* in *C. orbiculare* as in *S. cerevisiae* *Whi2p*-*Psr1p* (Fig. 8A). To elucidate whether the function of *CoPSR1* is similar to that of *CoWHI2* during

infection by *C. orbiculare*, we conducted a functional analysis of *CoPSR1* with the *Copsr1Δ* mutant. In a virulence test, the *Copsr1Δ* mutant caused smaller lesions than the wild type did on cucumber cotyledons (Fig. 8B). Next, to elucidate whether the *Copsr1Δ* mutant induces a host defense response similar to that induced by the *Cowhi2Δ* mutant, we monitored callose deposition on cucumber cotyledons beneath appressoria of the *Copsr1Δ* mutant. The frequency of large callose depositions beneath appressoria of the *Copsr1Δ* mutant was higher than in the wild type at 24 hpi (Fig. 8C). Taken together, the similarity of the phenotype between the *Cowhi2Δ* mutant and the *Copsr1Δ* mutant supported the possibility that CoWhi2 and CoPsr1 work by forming a functional complex as in *S. cerevisiae*.

CoWHI2 is involved in TOR-dependent upregulation of ribosomal gene expression.

The transcription factor Msn2p is known to be dephosphorylated by the Whi2p-Psr1p complex and to bind to the core consensus 5'-WAGGGG-3' of stress response element (STRE) genes and to regulate STRE gene expression in *S. cerevisiae* (Görner et al. 1998; Martínez-Pastor et al. 1996). Therefore, to explore which genes are regulated by CoWhi2, we comprehensively analyzed gene expression in the *Cowhi2Δ* mutant at 4 hpi with a microarray, since expression of *CS8*, *CS10*, and *CS12* in the cucumber inoculated with the *Cowhi2Δ* mutant significantly increased at 6 hpi. This analysis revealed that 58 genes were upregulated and 21 genes were downregulated (>10-fold change) in the *Cowhi2Δ* mutant compared with the wild type (Table 1; Supplementary Table S1). Interestingly, 44 genes among the upregulated genes (>10-fold change) in the *Cowhi2Δ* mutant were ribosomal protein-related genes (Table 1).

Ribosome biogenesis in response to environmental conditions is crucial in regulating cell growth and cellular metabolism

(Martin et al. 2004). In mammals and *S. cerevisiae*, TOR is known to be a regulatory factor of ribosomal gene expression in response to environmental conditions (Crespo and Hall 2002; Martin et al. 2006). Therefore, we hypothesized that expression of ribosomal genes in the *Cowhi2Δ* mutant are upregulated by the activation of TOR. To verify this hypothesis, we performed a microarray focusing on ribosomal gene expression of the *Cowhi2Δ* mutant at 4 hpi treated with 100 nM rapamycin under conditions that decrease TOR activity. Surprisingly, ribosomal gene expression in the *Cowhi2Δ* mutant treated with rapamycin was significantly downregulated compared with the untreated mutant (Fig. 9). In *M. oryzae*, expression of *MoRS2* and *MoRS3* genes encoding ribosomal proteins is upregulated when TOR is activated but downregulated when TOR is inactivated (Fernandez et al. 2014; Marroquin-Guzman and Wilson 2015). We analyzed expression of *CoRS2* and *CoRS3* in the *Cowhi2Δ* mutant, homologs of *MoRS2* and *MoRS3*, by qRT-PCR with or without 100 nM rapamycin, at 4 hpi (Supplementary Fig. S8). The expression of *CoRS2* and *CoRS3* in the *Cowhi2Δ* mutant was upregulated compared with the wild type, while expression of *CoRS2* and *CoRS3* in the *Cowhi2Δ* mutant treated with rapamycin was downregulated compared with the untreated mutant (Fig. 10A and B). Taken together, these data suggested that expression of ribosomal genes in the *Cowhi2Δ* mutant is upregulated by activation of TOR.

Because the activation of the TOR pathway promotes biosynthesis of ribosomal proteins and translational initiation, resulting in the control of cell growth and cellular metabolism, we considered that the phenotype of the *Cowhi2Δ* mutant and the *Copsr1Δ* mutant was caused by a disturbance of the wild-type hemibiotrophic parasitic mode through the activation of TOR. To examine this hypothesis, we monitored callose

Fig. 6. Infection hyphae formed by the *Cowhi2Δ* mutant on cucumber cotyledons. **A**, Infection hyphae development of wild type (WT) and *Cowhi2Δ* mutant at 72 h postinoculation. Cotyledons were inoculated with a conidial suspension of the strain and were incubated at 24°C. WT = wild-type 104-T and DWH2 = *Cowhi2Δ* mutant. Scale bar, 20 μm. **B**, Frequency of infection hyphae of the *Cowhi2* mutant in epidermal cells of the abaxial surface of cotyledons. Approximately 100 appressoria of each strain were observed per inoculated site. Three independent experiments were conducted and standard errors are indicated. WT = wild-type 104-T, DWH2-1 = *Cowhi2Δ* mutant, CWH2 = *CoWHI2*-complemented transformant of DWH2, and DSD1 = *Cosd1Δ* mutant. Asterisks represent significant differences between the wild type and each mutant (Student's *t* test: *P* < 0.01). **C**, Infection hyphae formed by the *Cowhi2Δ* mutant in autofluorescent cells of autofluorescent cells. IH = infection hyphae and AC = autofluorescent cell. **D**, Frequency of infection hyphae formed by the *Cowhi2Δ* mutant in autofluorescent cells and the adjacent cells beneath approximately 100 appressoria of each strain. Three replicates were examined for each strain in three independent experiments; standard errors are indicated. Asterisks represent significant differences between the wild type and *Cowhi2Δ* mutant (Student's *t* test: *P* < 0.01).

deposition in cucumber epidermal cells beneath appressoria formed by the *Cowhi2Δ* mutant and the *Copsr1Δ* mutant treated with 100 nM rapamycin. The frequency of callose deposition beneath appressoria formed by the wild type treated with rapamycin was similar to the wild type without rapamycin treatment, while the frequency of the large callose deposits beneath appressoria of the *Cowhi2Δ* mutant and the *Copsr1Δ* mutant with rapamycin was lower than in the untreated mutant (Fig. 10C to E). Moreover, when we examined the biotrophic stage of the *Cowhi2Δ* mutant treated with 100 nM rapamycin, the intensity of the CoPSD1_{pro}:VENUS signal and

the CoATF1_{pro}:VENUS signal in the *Cowhi2Δ* mutant treated with rapamycin were stronger than in those without rapamycin treatment (Fig. 10F). These data indicated that *CoWHI2* and *CoPSR1* controls hemibiotrophic infection through TOR during infection of host epidermal cells.

In the *S. cerevisiae* cell cycle, G1 arrest is essential to change from a growth phase to a quiescence phase in response to nutrient starvation and *WHI2* is required for this G1 arrest (Radcliffe et al. 1997). In *S. cerevisiae*, TOR inactivation by rapamycin treatment or *TOR1* and *TOR2* double deletion can arrest G1 progression in the cell cycle (Loewith and Hall 2011). On the basis of the data

Fig. 7. Fluorescence visualization of expression of biotrophic-specific expression genes in the infection hyphae of the *Cowhi2Δ* mutant on cucumber cotyledons at 72 h postinoculation. **A**, Biotrophic-specific fluorescent signal detection in the infection hyphae of the *C. orbiculare whi2Δ* mutant carrying *TEF_{pro}:mCherry* and *CoPSD1_{pro}:VENUS* or **B**, *TEF_{pro}:mCherry* and *CoATF1_{pro}:VENUS* in cucumber cotyledons. Conidial suspensions of each strain that carried the fluorescence gene with the biotrophic-specific expression promoter were placed on the abaxial surface of cucumber cotyledons and were incubated at 24°C for 72 h. VENUS and mCherry fluorescence signals were observed using fluorescence microscopy. **C**, Frequency of the VENUS signal in the infection hyphae of the *Cowhi2Δ* mutant. Approximately 50 infection hyphae were observed per inoculated site and three replicates were examined. Three independent experiments were conducted; standard errors are indicated. Asterisks represent significant differences between cucumber leaves inoculated with the wild type and *Cowhi2Δ* mutant (Student's *t* test: two asterisks (**) = *P* < 0.01, one (*) = *P* < 0.05). **D**, Quantification of fluorescence intensity of biotrophy-specific expression of VENUS in the *Cowhi2Δ* mutant. Conidial suspensions of each strain, carrying *TEF_{pro}:mCherry* and *CoPSD1_{pro}:VENUS* or *TEF_{pro}:mCherry* and *CoATF1_{pro}:VENUS*, were placed on the abaxial surface of cucumber cotyledons and were incubated for 72 h. At least 50 VENUS fluorescence signals from infection hyphae were quantified for each strain. The mean biotrophic fluorescence intensity value was calculated by dividing the VENUS fluorescence intensity by the mCherry fluorescence intensity in infection hyphae of each strain. Asterisks represent significant differences between the wild type and *Cowhi2Δ* mutant (Student's *t* test: *P* < 0.01).

from these reports, suppression of the *Cowhi2Δ* mutant phenotype by rapamycin treatment could be due to control of G1 progression accelerated by *CoWHI2* deletion through TOR inactivation by rapamycin treatment. Therefore, to elucidate whether *CoWHI2* is involved in cell-cycle regulation, we generated a *Cowhi2Δ* mutant transformant expressing the histone H1-green fluorescent protein (GFP) fusion protein, which mainly localizes to the nucleus and, then, examined the cells for histone H1-GFP fluorescence signals (Fukada and Kubo 2015). During appressorium formation, the time course of nuclear division of the *Cowhi2Δ* mutant did not differ significantly from that of the wild type, while in the *Cobub2Δ* mutant, G1/S progression was accelerated during appressorium formation (Fig. 11).

DISCUSSION

C. orbiculare regulates the biotrophic stage of hemibiotrophic infection via *CoWhi2*.

It is generally accepted that plant-pathogenic fungi transmit a variety of extracellular signals such as physical signals and plant signals via an intracellular signaling pathway, resulting in the induction of infection-related morphogenesis and metabolism

(Kubo and Takano 2013; Wilson and Talbot 2009). In *Ustilago maydis*, plant surface-sensing factors Sho1 and Msb2 that act upstream of MAPK Kpp2 and Kpp6 are involved in appressorium formation (Lanver et al. 2010). In *M. oryzae*, Sho1 recognizes rice leaf wax as a signal for appressorium formation, while Msb2

Table 1. Upregulated genes (10-fold change) in the *Cowhi2Δ* mutant compared with the wild type (WT) at 4 h postinoculation

Target ID	Description	Whi2Δ/WT fold change
Cob_07269	NAD-dependent epimerase/dehydratase family protein	26.41
Cob_03272	60S acidic ribosomal protein P0	18.51
Cob_00779	40S ribosomal protein S26	18.37
Cob_10175	40S ribosomal protein S18	18.36
Cob_12426	60S ribosomal protein L28	17.37
Cob_02165	60S acidic ribosomal protein	16.89
Cob_00778	Ubiquitin	16.55
Cob_12837	60S ribosomal protein L37a	16.27
Cob_13019	60S ribosomal protein L24	16.22
Cob_09495	60S ribosomal protein L22	16.17
Cob_07358	60S ribosomal protein L15	15.90
Cob_13262	hsp70 chaperone	15.55
Cob_05002	40S ribosomal protein S4	15.52
Cob_07318	40S ribosomal protein S23	14.98
Cob_04116	60S ribosomal protein L3	14.22
Cob_00457	Alternative oxidase	14.00
Cob_03396	60S ribosomal protein L6	13.93
Cob_05469	UDP-glucose dehydrogenase UDP-ManNAc	13.91
Cob_10298	60S ribosomal protein L17	13.88
Cob_13037	Ribosomal protein L14	13.84
Cob_03403	60S ribosomal protein L35	13.82
Cob_01703	60S ribosomal protein L9	12.95
Cob_10141	60S ribosomal protein L10a	12.95
Cob_08099	60S acidic ribosomal protein P2	12.88
Cob_08240	40S ribosomal protein S17	12.83
Cob_11718	40S ribosomal protein S5	12.56
Cob_01807	40S ribosomal protein S20	12.45
Cob_02278	Amino acid transporter	12.43
Cob_11700	40S ribosomal protein S6	12.43
Cob_02253	Hypothetical protein	12.33
Cob_04000	40S ribosomal protein S10b	12.25
Cob_11705	40S ribosomal protein S8-b	11.91
Cob_12849	40S ribosomal protein S12	11.82
Cob_03549	60S ribosomal protein L11	11.78
Cob_02224	Fumarylacetoacetate hydrolase family protein	11.73
Cob_05690	60S ribosomal protein L8	11.70
Cob_07988	G-protein beta subunit	11.66
Cob_13407	HSCARG protein	11.64
Cob_03452	60S ribosomal protein L33	11.59
Cob_11185	60S ribosomal protein L20	11.52
Cob_07152	60S ribosomal protein L23	11.36
Cob_10127	60S ribosomal protein L39	11.21
Cob_12054	60S ribosomal protein L7	11.17
Cob_05317	60S ribosomal protein L34	10.97
Cob_03476	Ribosomal protein S28e	10.97
Cob_08116	40S ribosomal protein S15	10.95
Cob_13450	Partial/pseudo	10.82
Cob_10080	60S ribosomal protein L2	10.79
Cob_05721	40S ribosomal protein S27	10.68
Cob_06504	Translationally controlled tumor protein	10.61
Cob_09771	60S ribosomal protein L44	10.41
Cob_01044	Hypothetical protein	10.40
Cob_10929	60S ribosomal protein L31	10.28
Cob_13099	60S ribosomal protein L21-a	10.27
Cob_06269	GMP synthase	10.23
Cob_08573	Ribosomal protein S5	10.21
Cob_13036	Nascent polypeptide-associated complex subunit alpha	10.18
Cob_13030	60S ribosomal protein L37	10.02

Fig. 8. CoPsr1 directly interacted with CoWhi2, and the phenotype of the *Copsr1Δ* mutant was similar to that of the *Cowhi2Δ* mutant. **A**, Colony growth of each strain incubated at 28°C for 4 days on the selective medium. Negative control: pGBKT-LamxpGADT7-T. **B**, Conical suspensions of each strain were placed on detached cotyledons of cucumber and, then, were incubated at 24°C for 7 days. Shown are leaves inoculated with wild-type 104-T (WT), DPS2-1 and DPS2-2, the *Copsr1Δ* mutant, and EPS1, the ectopic strain. **C**, Mean frequency of each callose deposition type beneath appressoria of each strain at 24 h postinoculation. Approximately 200 appressoria of each strain were observed per incubated site; three replicates were examined per strain in each of three independent experiments. Standard errors are indicated.

Fig. 9. The upregulated ribosomal gene expression in the *Cowhi2Δ* mutant is regulated by TOR activity. Expression of ribosomal protein-related genes in the *Cowhi2Δ* mutant treated with rapamycin or without rapamycin. The heat map of genes depicts the top 150 most highly expressed genes in the *Cowhi2Δ* mutant compared with the wild type (WT) at 4 h post-inoculation. WT DW, the WT without rapamycin treatment; DWH2 DW1 and DW2 = the *Cowhi2Δ* mutant without rapamycin, DWH2 RAP = *Cowhi2Δ* mutant treated with 100 nM rapamycin. Red text is ribosomal protein-related gene.

recognizes surface hydrophobicity and cutin monomers as a signal for appressorium formation (Liu et al. 2011).

As mentioned earlier, hemibiotrophic fungi have two distinctive nutritional stages involved in infection; they initially establish primary bulbous-shaped infection hyphae inside the living host cell (biotrophy) and, subsequently, develop secondary infection hyphae that are narrower and grow rapidly, eventually leading to necrotic lesions at the infected site (necrotrophy). Transcriptome analyses of hemibiotrophic fungi such as *C. orbiculare* and *C. higginsianum* have revealed that gene expression significantly shifts during the switch from biotrophy to necrotrophy (Gan et al. 2013; O'Connell et al. 2012), but knowledge about the intracellular signaling pathway involved in this hemibiotrophic regulation has been lacking (Wilson and Talbot 2009).

In this study, we first found marked plant responses, rapid callose deposition and H_2O_2 production, induced at the initial stage of infection by the *Cowhi2Δ* mutant, similar to the earliest host-defense response against necrotrophic fungi such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Rossi et al. 2011; Williams et al. 2011). In addition, we showed that, at 72 hpi, the expression of *DEFENSIN19* in cucumber cotyledons inoculated with the *Cowhi2Δ* mutant was rapidly induced while the expression of *PR-1* was lower than in the wild type. *A. thaliana* *PDF1.2*, a homolog of *DEFENSIN19*, is also induced after attack by necrotrophic fungi such as *B. cinerea* and *Alternaria brassicicola* (Rossi et al. 2011; Su'udi et al. 2011). Moreover, the defense response of *A. thaliana* against *Alternaria brassicicola* activates jasmonic acid-dependent *PDF1.2* but suppresses salicylic acid-dependent *PR-1* (Spoel et al. 2007). Therefore, we considered that the host plant response triggered by the *Cowhi2Δ* mutant was similar to that induced by necrotrophic fungi. It is generally considered that the plant defense response against biotrophic pathogens is mainly mediated by salicylic acid-dependent signaling pathways (Glazebrook 2005). Based on data obtained in the present study, *C. orbiculare* first establishes a biotrophic stage in cucumber cotyledons between 48 and 72 hpi. Therefore, it is conceivable that the host defense response, from 48 to 72 hpi, against infection by wild-type *C. orbiculare* is a biotrophic-related plant defense response, in contrast to a necrotrophic infection-related host defense response against infection of the *Cowhi2Δ* mutant from 48 to 72 hpi. Therefore, the necrotrophic stage of the *Cowhi2Δ* mutant might occur earlier than the necrotrophic stage in the wild-type *C. orbiculare*.

Moreover, microscopic observations at 72 hpi revealed that the biotrophy-specific fluorescent signal in infection hyphae of the *Cowhi2Δ* mutant carrying the *VENUS* gene with the promoter of a biotrophic-specific gene was less frequent and weaker than in those of the wild type carrying the *VENUS* gene with the promoter of the biotrophic-specific gene (Fig. 7). Therefore, we assumed that *Cowhi2Δ* is a key factor in the regulation of the biotrophic stage in the *C. orbiculare*–*Cucumis sativus* interaction.

CoWhi2 regulates the TOR signal pathway and has crucial roles in the biotrophic stage of hemibiotrophic infection in *C. orbiculare*.

The biotrophic, bulbous-shaped infection hyphae in *C. orbiculare* develop in multiple host cells and, subsequently, switch to necrotrophy, but the mechanism underlying this switch in *C. orbiculare* has been unclear (Kubo and Takano 2013). In the hemibiotrophic fungus *M. oryzae*, TOR signaling is involved in several biological steps in the infection process (Fernandez et al. 2014; Marroquin-Guzman and Wilson 2015). For example, TOR signaling is involved in a checkpoint that controls primary hyphal growth in response to ATP produced via *MoTKL1* encoding transketolase and regulates appressorium formation

by blocking the cAMP-PKA signaling pathway (Fernandez et al. 2014; Marroquin-Guzman and Wilson 2015). Thus, these reports indicate that the role of TOR signaling seems to be involved mainly in processes such as the formation of the appressorium and primary hyphae in hemibiotrophic fungi. In *S. cerevisiae*, Msn2 activity is regulated by Whi2 and the TOR pathway, but the role of Whi2 in the TOR signaling pathway remains unclear (Sadeh et al. 2011). Although TOR activity regulates the bZIP protein MeaB and global nitrogen regulator AreA in response to environmental signals through Gln and NH₄ and controls virulence functions in *Fusarium graminearum* (López-Berges et al. 2010), the factors that are involved in transmitting environmental signals to the TOR signaling pathway in hemibiotrophic fungi are far less understood. Based on our analysis of plant responses against the *Cowhi2Δ* mutant and the biotrophic stage in the *Cowhi2Δ* mutant treated

with rapamycin, *CoWHI2* seems to be involved in regulating the biotrophic stage of hemibiotrophic infection through the TOR signaling pathway. Hemibiotrophic fungi undergo dynamic variations in gene expression in accordance with the individual infection process, such as appressorium formation, biotrophic infection growth and necrotrophic infection growth (Gan et al. 2013; O'Connell et al. 2012). Therefore, the regulatory system to control the expression of various genes is important for the highly regulated hemibiotrophic infection. In our study, microarray data revealed that many TOR-regulated ribosomal protein genes in the *Cowhi2Δ* mutant were notably upregulated compared with the wild type. We speculated that CoWhi2 has a central role through TOR in the regulation of the biotrophic stage of hemibiotrophic infection. In *Magnaporthe oryzae*, TOR activation inhibits appressorium formation (Marroquin-Guzman and Wilson 2015). In this study, we

Fig. 10. TOR signaling regulated by CoWhi2 is required for hemibiotrophic infection. **A**, Expression of *C. orbiculare* *RS2* and *RS3* in the *Cowhi2Δ* mutant at 6 h postinoculation (hpi). Conidial suspensions of each strain were placed on detached cotyledons of cucumber and incubated at 24°C. Total RNA was extracted from the cotyledons, and expression of *CoRS2* and *CoRS3* was analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Three independent experiments were conducted. Standard errors are indicated. WT = wild-type 104-T and DWH2 = *Cowhi2Δ* mutant. Asterisks represent significant differences between cucumber leaves inoculated with the WT and *Cowhi2Δ* mutant (Student's *t* test $P < 0.01$). **B**, Expression of *CoRS2* and *CoRS3* in the *Cowhi2Δ* mutant treated with rapamycin using qRT-PCR. RAP = 100 nM rapamycin and DW = distilled water. **C** to **E**, Mean frequency of the callose deposition types beneath appressoria of each strain treated with rapamycin. Approximately 200 appressoria were observed per inoculated site, and three replicates were examined for each of three independent experiments; standard errors are indicated. 100 nM RAP = 100 nM rapamycin, WT = wild-type 104-T, DWH2 = *Cowhi2Δ* mutant, DPS1 = *Copsr1Δ* mutant. **F**, Quantification of fluorescence intensity of biotrophy-specific expression of VENU in the *Cowhi2Δ* mutant at 80 hpi after treatment with rapamycin. At least 50 VENU fluorescence signals from infection hyphae were measured for each strain. The mean biotrophic fluorescence intensity value was calculated by dividing VENU fluorescence intensity by mCherry fluorescence intensity in infection hyphae of each strain. Asterisks represent significant differences between *Cowhi2Δ* mutant treated with rapamycin and untreated mutant (Student's *t* test: $P < 0.01$). RAP = 100 nM rapamycin, DW = distilled water.

revealed that the activity of the TOR signaling pathway is regulated by CoWhi2 at 4 hpi, when the conidial germ tube begins to swell to form the appressorium, leading to the regulation of hemibiotrophic infection necessary for host infection. Therefore, we considered that, in the hemibiotrophs *M. oryzae* and *C. orbiculare*, the highly regulated TOR activity during appressorium formation is essential for successful infection of the host plant. In *M. oryzae*, TOR activation is required for biotrophic growth (Fernandez et al. 2014). Our present analysis did not deal with the TOR signaling pathway during the biotrophic and necrotrophic stages after penetration; therefore, determining the relevance between CoWhi2 and the TOR signaling pathway during these two stages will provide a deeper understanding of the molecular mechanisms of hemibiotrophic infection. Interestingly, the microarray revealed that the top 150 upregulated genes, aside from the many ribosomal protein genes, in the *Cowhi2Δ* mutant treated with rapamycin were not downregulated compared with the *Cowhi2Δ* mutant without rapamycin treatment, suggesting that CoWhi2 also negatively regulates TOR-independent signal pathways. However, we consider that the biotrophic stage in hemibiotrophic infection in *C. orbiculare* is largely dependent on TOR rather than being a TOR-independent pathway, based on the plant responses against the *Cowhi2Δ* mutant and the biotrophic stage in the *Cowhi2Δ* mutant treated with rapamycin. In this study, we thus implicate a Whi2-mediated network in the regulation of hemibiotrophy in *C. orbiculare* (Fig. 12).

Environmental signal transduction through CoWhi2 and the downstream regulatory cascade that controls the biotrophic stage of hemibiotrophic infection.

What factor does CoWhi2 interact with during initial appressorium development? In *S. cerevisiae*, Whi2p physically interacts with phosphatase Psr1p, and this complex dephosphorylates the transcription factor Msn2p, which has a pivotal role in general stress responses by mediating the transcription of hundreds of genes (Boy-Marcotte et al. 1998; Kaida et al. 2002). In this study, we found that CoWhi2 physically interacts with CoPsr1 and that the phenotype of the *Copsr1Δ* mutant is similar to that of the *Cowhi2Δ* mutant. Therefore, we assumed that a CoWhi2–CoPsr1 signaling pathway may contribute to the biotrophic stage of hemibiotrophic infection. In *S. cerevisiae*, Whi2p–Psr1p has a role as a positive regulator of Msn2p, because Msn2p activity for Msn2p-mediated gene expression under stress conditions is lower in the *whi2Δ* and *psr1Δ* mutant (Kaida et al. 2002; Sadeh et al. 2011). On the other hand, in the TOR pathway, Tor1p and Tor2p act as negative regulators of Msn2p in *S. cerevisiae*, because Msn2p activity is elevated in *tor1Δ* and *tor2Δ* mutants (Sadeh et al. 2011). In addition, the activity of the TOR pathway is suppressed in response to nutrient starvation but activated in response to nutrients (Rohde et al. 2008). Thus, Msn2p activity, activated by Whi2–Psr1 signaling transduction and suppression of TOR activity in response to nutrient starvation, significantly induces Msn2p-mediated gene expression. Hemibiotrophic *M. oryzae* utilizes nutrients produced

Fig. 11. Nuclear division in the *Cowhi2Δ* mutant during appressorium formation. **A**, Time-course series of micrographs to show nuclei in conidia and infection structures of the wild type, the *Cowhi2Δ* mutant, and the *Cobub2Δ* mutant during appressorium development. The HISTONE H1-GFP gene fusion vectors were introduced into the three strains. Nuclei (green) were observed during appressorium development. Scale bar = 5 μ m. **B** to **D**, Mean percentage ($\pm$ standard error; $n = 3$) of cells with various patterns of nuclear distribution. Scoring as shown in top diagram: one nucleus retained in the conidium (black), two nuclei retained in the conidium (red), one nucleus in the conidium and the other in the appressorium through mitosis (blue), multiple nuclei in the conidium and appressorium through two or three rounds of mitosis (yellow), and one nucleus in the appressorium and degradation of the remaining nucleus in the conidium through autophagy (green). At least 200 conidia were scored at each timepoint. WT = wild type, DWH2 = *Cowhi2Δ* mutant, and DBU2 = *Cobub2Δ* mutant.

by autophagy in response to the lack of nutrients on the host plant surface until its infection hyphae can obtain nutrients from host tissues (Deng et al. 2012). In our study, TOR-dependent ribosomal gene expression in the *Cowhi2Δ* mutant is significantly upregulated at 4 hpi, suggesting that CoWhi2 negatively regulates TOR activity when nutrients are unavailable. In *M. oryzae*, *MoMSN2* encodes the C₂H₂ zinc-finger DNA-binding domain, the homolog of *MSN2* in *S. cerevisiae*, and is involved in pleiotropic functions, including conidiogenesis, appressorium formation, pathogenicity, and invasive hyphal growth, through Msn2-mediated gene expression (Zhang et al. 2014). Based on *C. orbiculare* genome information, we confirmed that the predicted amino acid sequence of gene ENH88318 in *C. orbiculare* conserves high similarity to *S. cerevisiae* Msn2p and *M. oryzae* MoMsn2, particularly in its zinc-finger domain. Moreover, we showed that the *Cowhi2Δ* mutant has increased resistance to CFW on PDA, similar to the case of the *M. oryzae* *Momsn2Δ* mutant, indicating that CoWhi2 may likely respond to a stress by regulating CoMsn2-mediated stress-responsive genes (Zhang et al. 2014). Therefore, we speculated that this gene in *C. orbiculare* acts as an *MSN2*, which putatively functions in the regulation of various genes necessary for infection. On the basis of our data and previous reports, we assumed that CoWhi2-CoPsr1 signal transduction and the inactivation of TOR by CoWhi2 increase activity of *C. orbiculare* CoMsn2, a homolog of Msn2p, which potentially may result in CoMsn2-mediated

gene expression necessary for the infection stage during nutrient deprivation.

An important question is the role of *CoWHI2* in hemibiotrophic infection by *C. orbiculare*. The TOR pathway in *S. cerevisiae* is activated in response to nutrient homeostasis, leading to synthesis of ribosomal proteins (Rohde et al. 2008). In our study, deletion of *CoWHI2* led to upregulation of ribosomal gene expression regulated by TOR activity. Therefore, we assumed that the loss of CoWhi2 function in response to nutrient homeostasis inside host tissues activates TOR. On the basis of our data and previous reports, we hypothesize that the downregulation of CoMsn2 resulting from the loss of the CoWhi2-CoPsr1 signal pathway and from the activation of TOR resulting from the loss of CoWhi2 function inside the nutrient-rich host tissues is a primary contributor as hemibiotrophic infection of *C. orbiculare* (Deng et al. 2012; Sadeh et al. 2011).

In *C. higginsianum*, the biotrophic, bulbous infection hyphae are confined to the initially infected epidermal cell, and development switches to narrower, necrotrophic infection hyphae, which then ramify throughout the host cells (O'Connell et al. 2012). In *Colletotrichum graminicola*, the biotrophic infection hyphae grow not only in the initially infected cell but also into cells adjacent to the first infected cell; it then switches to necrotrophic infection hyphae, which ramify throughout the host cells (Vargas et al. 2012). Therefore, we assumed that the switch from biotrophy to necrotrophy in *Colletotrichum* spp. could be

Fig. 12. Model for the *Colletotrichum orbiculare* Whi2 signal network involved in the biotrophic stage of hemibiotrophic infection of *C. orbiculare*. Environmental signals affect several intracellular events associated with infection-related morphogenesis in *C. orbiculare*. In the *C. orbiculare* wild type, CoWhi2 negatively regulates TOR activity at the time of appressorium development, resulting in establishment of the highly regulated biotrophic stage of hemibiotrophic infection. On the other hand, in the *Cowhi2Δ* mutant, TOR is activated at the time of appressorium development, resulting in the disturbance of the establishment of the biotrophic stage of hemibiotrophic infection.

achieved by sensing a particular condition in the host plant. Our future work will focus on revealing extracellular environmental signals and fungal-sensing mechanisms to control the transition from biotrophic to necrotrophic in *C. orbiculare* for a deeper understanding of hemibiotrophic fungi-host plant interactions.

MATERIALS AND METHODS

Fungal and bacterial strains, culture conditions, transformation, and genomic DNA blot analysis.

Strain 104-T (MAFF240422) of *Colletotrichum orbiculare* (Berk. & Mont.) Arx [syn. *C. lagenarium* (Pass.); Ellis & Halst.] was used as the wild type. All *C. orbiculare* strains were cultured on PDA (3.9% [wt/vol] PDA) (Difco Laboratories, Detroit) at 24°C. For observing vegetative growth under stress conditions, the fungal strains were cultured on PDA containing 0.1 mg of CFW per milliliter, 0.2 mg of Congo red (CR) per milliliter, and 0.005% SDS. *Escherichia coli* DH5 α -competent cells cultured in Luria-Bertani medium (Sambrook et al. 1989) at 37°C were used as the host for gene manipulation. When required, kanamycin was added to the medium at 50 μ g/ml. *Agrobacterium tumefaciens* C58C1 cultured in Luria-Bertani medium at 28°C was used to transform *C. orbiculare* based on a previous protocol (Harata and Kubo 2014). Hygromycin-resistant transformants were selected on PDA containing 50 μ g of hygromycin B per milliliter (Wako Chemicals, Osaka, Japan) and 25 μ g of Meropen per milliliter (Sumitomo Dainippon Pharma, Osaka, Japan). Bialaphos-resistant transformants of *C. orbiculare* were selected on SD medium containing 1.25 μ g of bialaphos per milliliter (Meiji Seika Pharma, Tokyo) and 25 μ g of Meropen per milliliter. Neomycin-resistant transformants were selected on PDA (Merck KGaA, Darmstadt, Germany) containing 50 μ g of G418 per milliliter (Nacalai Tesque, Kyoto, Japan) and 25 μ g of Meropen per milliliter. Sulfonyleurea-resistant transformants were selected on SD medium containing 4 μ g of chlorimuron-ethyl per milliliter (Chem Service, West Chester, PA, U.S.A.) and 25 μ g of Meropen per milliliter. Total DNA extraction from mycelia of *C. orbiculare* and DNA blot analysis were done as previously described (Harata and Kubo 2014).

Pathogenicity tests.

A pathogenicity assay on cucumber cotyledons (*Cucumis sativus* L. 'Suyo') was performed as described by Tsuji et al. (1997), using conidia of *C. orbiculare* from 7-day-old cultures. Drops of a conidial suspension (10 μ l of 5×10^5 conidia per milliliter) were placed at different locations on the surface of detached cucumber cotyledons. After inoculation, the cotyledons were incubated in a humid box at 24°C for 5 or 7 days.

Light microscopy.

For localization analysis of CoRas2 and histone H1-GFP during appressorium formation, conidia were harvested from 7-day-old PDA cultures and were suspended in distilled water. The conidial suspension, adjusted to 5×10^5 conidia per milliliter, was placed in an eight-well multitest slide (ICN Bio-medicals, Aurora, OH, U.S.A.) and was incubated in humid boxes at 24°C. Red fluorescent protein (RFP)-CoRas2 and histone H1-GFP signals were observed using a Carl ZEISS Imager M2 microscope (Zeiss, Göttingen, Germany), with an excitation/barrier filter set of 470 nm/509 nm for GFP and 595 nm/620 nm for RFP. For observing infectious hyphae in cucumber leaves, the abaxial surface of cucumber cotyledons was inoculated with drops of a conidial suspension and was incubated at 24°C for 3 days. Then, the epidermal cells at the inoculation site were excised with forceps and were stained with 0.1% (wt/vol) lactophenol-aniline blue. The stained

tissues were observed with a Nikon ECLIPSE E600 microscope (Nikon, Tokyo). For detecting callose deposition in epidermal cells, the abaxial surface of cucumber cotyledons was inoculated with a conidial suspension in distilled water or in water with 100 nM rapamycin or 0.5 μ M DPI and was incubated at 24°C for 24 h. The epidermal cells at the inoculation site were stained with 0.01% aniline blue in 0.15 M K₂HPO₄ and, then, were observed with a Nikon ECLIPSE E600 microscope. For detecting H₂O₂ cytochemically, inoculated cucumber cotyledons were stained with a 1 mg/ml DAB solution, were incubated for 24 h at room temperature in the dark, and were observed with the Nikon ECLIPSE E600 microscope. For observing VENUS signals under the control of the biotrophy-specific promoter and mCherry signals under the control of the *TEF* promoter of *C. orbiculare*, the abaxial surface of cucumber cotyledons was inoculated with a conidial suspension with or without 100 nM rapamycin and was incubated at 24°C for 72 or 80 h. Then, the epidermal cells at the inoculation site were excised with forceps and were observed with a Carl ZEISS Imager M2 microscope and an excitation barrier set of 470/509 nm for GFP and 595/620 nm for RFP. To quantify the biotrophic fluorescence intensity in the infection hyphae of each transformant, the images were analyzed using the software ImageJ (National Institutes of Health, Bethesda, MD, U.S.A.).

Vector construction.

All primers used in this study are listed in Supplementary Table S2. We constructed a *CoWHI2* complementation vector pBIG4MRBrev-*CoWHI2* as follows. The *CoWHI2* gene fragment, including 0.7 kb upstream of the translation start site of *CoWHI2* and 1.3 kb downstream of the translation termination site of *CoWHI2*, and the pBIG4MRBrev vector fragment carrying the bialaphos-resistance gene were amplified by PCR, using primer pairs CoWHI2F1A and CoWHI2R1B and pBICoWHI2F1A and pBICoWHI2R1B for the pBIG4MRBrev vector fragment, respectively. Then the pBIG4MRBrev-*CoWHI2* vector was constructed using the In-Fusion HD cloning kit (Takara Bio, Otsu, Japan) with the amplified product.

Next, we constructed the gene-replacement vector pBIG4MRBrev-cowhi2 as follows. The hygromycin-resistance gene and the pBIG4MRBrev-*CoWHI2* vector fragment, not including the region from the translation start site of *CoWHI2* to the translation termination site of *CoWHI2*, were amplified by PCR using primer pairs CoWHI2hphF1A and CoWHI2hphR1B and pBICoWHI2F2A and pBICoWHI2R2B, respectively. Then the pBIG4MRBrev-cowhi2 vector was constructed using the In-Fusion HD cloning kit with the amplified product.

We constructed the *CoPSR1* gene-replacement vector pBIG4MRSrev-copsr1 as follows. The *CoPSR1* gene fragment, including 1.3 kb upstream of the translation start site of *CoPSR1* and 1.1 kb downstream of the translation termination site of *CoPSR1*, and the pBluescriptSK vector fragment were amplified by PCR using primer pairs CoPSR1F1A and CoPSR1R1B and pBSCoPSR1F1A and pBSCoPSR1R1B, respectively. Then the pBluescriptSK-*CoPSR1* vector was constructed using the In-Fusion HD cloning kit with the amplified product.

Next, we amplified the neomycin-resistance gene and linearized the pBluescriptSK-*CoPSR1* vector fragment, not including the region from the translation start site of *CoPSR1* to the translation termination site of *CoPSR1*, by PCR, using primer pairs CoPSR1neoF1A and CoPSR1neoR1B and pBSCoPSR1F2A and pBSCoPSR1R2B, respectively. Then the pBluescriptSK-copsr1 vector was constructed using the In-Fusion HD cloning kit with the amplified product.

Finally, we amplified the neomycin-resistance fragment, including the upstream region of the translation start site of *CoPSR1* and downstream region of the translation termination

site of *CoPSR1*, and the pBIG4MRSrev vector fragment carrying the sulfonyleurea-resistance gene by PCR, using primer pairs CoPSR1F2A and CoPSR1R2B and pBIG4MRBSrevF1A and pBIG4MRBSrevR1B, respectively. Then the pBIG4MRSrev-copsr1 vector was constructed using the In-Fusion HD cloning kit with the amplified product.

To assess behavior of biotrophic-specific expression genes in the *Cowhi2* mutant, we constructed the pBICoPSD1_{pro}:VENUS vector carrying the biotrophy-specific expression *VENUS* gene by the following methods. The *CoPSD1* gene fragment, including 2.4 kb upstream of the translation start site of *CoPSD1* and 0.8 kb downstream of the translation termination site of *CoPSD1*, and the pBIG4MRBrev vector fragment were amplified by PCR using primer pairs CoPSD1F1A and CoPSD1R1B and pBIG4MRBSrevF1A and pBIG4MRBSrevR1B, respectively. Then, the pBIG4MRBrev-CoPSD1 vector was constructed, using the In-Fusion HD cloning kit with the amplified product. Next, we amplified the *VENUS* gene and pBIG4MRBrev-CoPSD1 vector fragment, not including the region from the translation start site of *CoPSD1* to the translation termination site of *CoPSD1*, by PCR using primer pairs VENUSF1A and VENUSR1B and pBICoPSD1F1A and pBICoPSD1R1B, respectively. Then, the pBICoPSD1_{pro}:VENUS vector was constructed, using the In-Fusion HD cloning kit with the amplified product.

To analyze the behavior of biotrophic-specific expression genes in the *Cowhi2Δ* mutant, we constructed the pBICoATF1_{pro}:VENUS vector carrying the biotrophic-specific expression gene *VENUS* as follows. The *CoATF1* gene fragment, including 2.1 kb upstream of the translation start site of *CoATF1* and 1.5 kb downstream of the translation termination site of *CoATF1*, and the pBIG4MRBrev vector fragment were amplified by PCR, using primer pairs CoATF1F1A and CoATF1R1B and pBIG4MRBSrevF1A and pBIG4MRBSrevR1B, respectively. Then the pBIG4MRBrev-CoATF1 vector was constructed using the In-Fusion HD cloning kit with the amplified product.

Next, we amplified the *VENUS* gene and pBIG4MRBrev-CoATF1 vector fragment, not including the region from the translation start site of *CoATF1* to the translation termination site of *CoATF1*, by PCR using primer pairs VENUSF1A and VENUSR1B and pBICoATF1F1A and pBICoATF1R1B, respectively. Then the pBICoATF1_{pro}:VENUS vector was constructed using the In-Fusion HD cloning kit with the amplified product.

To analyze the biotrophic fluorescence intensity from infection hyphae of the *Cowhi2Δ* mutant, we constructed the pBITEFpro:mCherry vector carrying the constitutive and highly expressed *mCherry* gene as follows. The *TEF* promoter fragment and the pBIG4MRSrev:mCherry vector fragment carrying the sulfonyleurea-resistance gene were amplified by PCR, using primer pairs TEFF1A and TEFR1B and pBIG4MRBSrevF1A and mCherryR1B, respectively. Then the pBITEFpro:mCherry vector was constructed, using the In-Fusion HD cloning kit with the amplified product. To visualize nuclear division, histone H1-GFP fusion gene cloned in pBICoHIS1-GFP was introduced into the wild type and *Cowhi2Δ* mutant by ATMT (Fukada and Kubo 2015).

qRT-PCR analysis.

A conidial suspension (10 µl of 1.0×10^6 conidia per milliliter) in distilled water or 100 nM rapamycin was placed on the surface of a detached cucumber cotyledon at different locations and was incubated in a humid box at 24°C. At 6, 12, 24, 48, and 72 hpi, total RNA was extracted from the cotyledons inoculated with the respective strains, using a Qiagen RNeasy plant mini kit (Qiagen) and was treated with RNase-free DNase kit (Qiagen). qRT-PCR was performed using the primers listed in Supplementary Table S3. Expression of *actin* genes in *Cucumber*

sativus and *C. orbiculare* was used as a control (Tanaka et al. 2007) for normalizing the level of mRNA. The Power SYBR Green RNA-to CT 1-step kit was used for all qRT-PCR tests (Life Technologies, Carlsbad, CA, U.S.A.). Quantitative analysis of each mRNA was performed using the Applied Biosystems StepOne System (Life Technologies).

Yeast two-hybrid interaction assays.

The yeast two-hybrid screen was performed following the instructions of the Matchmaker Gold yeast two-hybrid system (Takara Bio). Full-length cDNAs of putative interaction partners were generated from 3-day-old *C. orbiculare* mycelia. The genes encoding the proteins tested for interaction were cloned into pGBKT7 or pGADT7 vectors (Takara Bio), to express fusion proteins with the yeast GAL4 binding (BD) and activation domain (AD), respectively. All BD or AD constructs were transformed into the Gold or Y187 yeast strain (Takara Bio). After mating, diploid yeasts were plated on double dropout synthetic selective medium lacking Trp and Leu (DDO) for mating control, on stringent medium supplemented with 20 µg of X-α-Gal and 100 ng of aureobasidin A per milliliter (DDO/X/AbA) and four-dropout synthetic selective medium lacking Trp, Leu, His, and Ade (QDO) and were incubated at 28°C for 5 days. Growth of all diploid yeasts carrying both pGADT7-AD- and pGBKT7-BD-transformed vectors on DDO was examined. Protein interactions were assessed by the growth of diploid yeasts on DDO, DDO/X/AbA, and QDO media, compared with diploid yeasts carrying pGBKT7-Lam and pGADT7-T, used as a negative control.

Microarray analysis.

Microarray analyses were performed using the *Colletotrichum orbiculare* (8 × 60,000, 13,352 independent probes, Design ID: 060762) oligo microarray, according to the Agilent 60-mer Oligo Microarray Processing Protocol (Agilent Technologies). Total RNA samples (200 ng) were used to prepare Cy3-labeled cRNA, using the Low RNA Input fluorescent linear amplification kit (Agilent Technologies). Fluorescence-labeled cRNAs were purified using an RNeasy RNA purification kit (Qiagen, Hilden, Germany). Three independent RNA samples were used to confirm the reproducibility of the microarray analyses. The images were analyzed using Feature Extraction Software (ver. 10.7.3.1) and GeneSpring GX 12.1 software (Agilent Technologies). Normalization was performed as follows: i) intensity-dependent Lowess normalization; ii) data transformation, with measurements set to ≤0.01; iii) per chip: 75th percentile normalization of each array; and iv) per gene: Data were normalized to control samples (wild type). The top 150 upregulated genes in *Cowhi2Δ* compared with wild type were selected and were used for the hierarchical clustering (heat map) analysis. The raw and processed data were deposited in the Gene Expression Omnibus (GEO) database (accession number GSE73096).

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