LAC2 Encoding a Secreted Laccase Is Involved in Appressorial Melanization and Conidial Pigmentation in *Colletotrichum orbiculare*

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Both *Colletotrichum* and *Magnaporthe* spp. develop appressoria pigmented with melanin, which is essential for fungal pathogenicity. 1,8-Dihydroxynaphthalene (1,8-DHN) is believed to be polymerized to yield melanin around the appressorial cell wall through the oxidative activity of laccases. However, no 1,8-DHN laccase has yet been identified in either *Colletotrichum* or *Magnaporthe* spp. Here, we report a laccase gene, *LAC2*, that is involved in the appressorial melanization of *Colletotrichum orbiculare*, which causes cucumber anthracnose. *LAC2* encodes a protein with a signal peptide and has high homology to fungal laccases. The conidial color of *lac2* mutants is distinct from that of the *C. orbiculare* wild type, and the mutants are nonpathogenic. Notably, the mutant appressoria are defective in melanization, and a host invasion assay showed that the appressoria are nonfunctional. *LAC2* was induced during appressorial melanization. These results suggest that *LAC2* oxidizes 1,8-DHN in the appressoria. The *LAC2* homologues of other fungi located in the same phylogenetic clade as *LAC2* fully complemented the *lac2* mutants. Interestingly, a *LAC2* homologue, located in a different clade, complemented the conidial pigmentation but not appressorial melanization of the mutants, suggesting that the *LAC2* function in appressorial melanization might only be conserved in laccases of the *LAC2* clade.

Colletotrichum orbiculare is a hemibiotrophic fungus that causes anthracnose on cucurbits (Agrios 2005; Perfect et al. 1999), producing a specialized infection structure called the “appressorium”. After the conidia of *C. orbiculare* germinate, the tips of the germ tubes form swollen appressoria, which are then melanized to become mature appressoria. *Magnaporthe oryzae*, the causal agent of rice blast disease, also develops melanized appressoria. A series of studies of *C. orbiculare* and *M. oryzae* has shown that melanization is required for appressorial function and fungal pathogenicity (Chumley and Valent 1990; Kubo and Furusawa 1991; Kubo et al. 1982). The melanin layer formed in the appressorial cell wall is thought to determine the correct penetration site because melanin is excluded from the penetration site (Kubo and Furusawa 1991). Melanin also provides the rigidity and selective permeability of the appressorial cell wall, and is linked to generation of the immense

turgor pressure in the appressoria to penetrate the plant cuticle and cell wall (Bechinger et al. 1999; Howard and Ferrari 1989).

Colletotrichum and *Magnaporthe* spp. commonly produce dihydroxynaphthalene (DHN) melanin. The biosynthesis of DHN melanin starts with the synthesis of pentaketide, and proceeds to 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-T4HN). The subsequent reduction of 1,3,6,8-T4HN generates scytalone. From scytalone, there are two dehydration steps and one reduction step, i.e., the dehydration of scytalone to 1,3,8-trihydroxynaphthalene (1,3,8-T3HN), the reduction of 1,3,8-T3HN to vermeline, and the dehydration of vermeline to 1,8-DHN. 1,8-DHN is then oxidized and polymerized to yield melanin. Genes encoding the biosynthetic enzymes for DHN melanin have been isolated from several fungal pathogens, including *C. orbiculare*. In *C. orbiculare*, three genes, *PKS1*, *SCD1*, and *THR1*, have been identified and characterized (Kubo et al. 1996; Perpetua et al. 1996; Takano et al. 1995). *PKS1* encodes a polyketide synthase involved in the synthesis of 1,3,6,8-T4HN (Fujii et al. 2000; Takano et al. 1995). *SCD1* is required for i) the dehydration of scytalone to 1,3,8-T3HN and ii) the dehydration of vermeline to 1,8-DHN. *THR1* is required for the reduction of 1,3,8-T3HN to vermeline, and also functions in the reduction of 1,3,6,8-T4HN to scytalone, together with a 1,3,6,8-T4HN reductase. Although the 1,3,6,8-T4HN reductase was identified in *M. oryzae* (Thompson et al. 2000), its functional orthologue has not yet been identified in *C. orbiculare*. Studies of the transcriptional patterns of *PKS1*, *SCD1*, and *THR1* have also indicated that the expression of the three genes is strongly induced in conidia when they are forming appressoria, suggesting the coordinated regulation of these genes in appressorial melanization (Takano et al. 1997b).

In addition to the biosynthetic enzymes for melanin described above, several genes have been identified as encoding factors involved in melanin biosynthesis. *C. orbiculare* *CMR1* encodes a transcriptional factor essential for the expression of *SCD1* and *THR1* during mycelial melanization whereas, interestingly, *CMR1* is not required for the expression of these two genes during appressorial melanization (Tsuji et al. 2000). *CoPEX6* and *CoPEX13* have been reported to be essential for appressorial melanization and the pathogenicity of *C. orbiculare* (Fujihara et al. 2010; Kimura et al. 2001). Both *CoPEX6* and *CoPEX13* are required for peroxisome biogenesis, indicating that peroxisome function is important for melanin biosynthesis in the appressoria. The requirement for *PEX6* in appressorial melanization has also been reported in *M. oryzae* (Ramos-Pamplona and Naqvi 2006). In *C. lindemuthianum*, which is pathogenic on the common bean, *clap1*, encoding a copper-transporting ATPase, is involved in both appressorial and mycelial melani-

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zation (Parisot et al. 2002). Thus, the sets of genes involved in melanin synthesis have been characterized in several fungal pathogens. However, there is still a lack of information regarding the genes responsible for the last step in the biosynthesis of DHN melanin. 1,8-DHN is believed to be polymerized to yield melanin through the oxidative activity of laccases (Langfelder et al. 2003). However, no DHN laccase has yet been characterized from either *Colletotrichum* or *Magnaporthe* spp.

Laccase is a copper-containing enzyme that catalyzes the oxidation of a phenolic substrate by coupling it to the reduction of oxygen to water. Laccases have been detected in a variety of organisms, such as plants, insects, bacteria, and fungi. Fungal laccases are the most widely studied group and have been shown to play a variety of roles, such as in morphogenesis, pathogenesis, and lignin degradation (Baldrian 2006). For example, in *Botrytis cinerea*, the causal agent of gray mold, a polyphenol oxidase (laccase-like enzyme) can degrade several phytoalexins of grape vines and, thus, contributes to fungal pathogenicity (Sbaghi et al. 1996). Laccase is also thought to be involved in melanin synthesis in fungi. A mutant of *Cochliobolus heterostrophus* lacking putative laccase activity has a defect in mycelial melanization, which suggests a relationship between laccase and the conversion of 1,8-DHN to melanin (Tanaka et al. 1992). However, no laccase gene responsible for the biosynthesis of 1,8-DHN melanin has been identified. Moreover, although the *Colletotrichum orbiculare* *LAC1* gene, which encodes a putative secreted laccase, was previously isolated, a phenotypic analysis of *lac1* mutants indicated that *LAC1* is not essential for melanin biosynthesis in *C. orbiculare* (Tsuji et al. 2001).

Here, we identified a new laccase gene, designated *LAC2*, in *C. orbiculare*. A knockout analysis of *LAC2* revealed that it is essential for the pathogenicity of *C. orbiculare*. Notably, a phenotypic analysis of the *lac2* mutants indicated that *LAC2* is involved in appressorial melanization, suggesting that *LAC2* is a DHN laccase indispensable for appressorial melanization.

RESULTS

Identification of *LAC2* and generation of *lac2*-knockout mutants.

To identify the genes responsible for the pathogenicity of *C. orbiculare*, a random insertional genomic library of *C. orbicu-*

lare was generated with a transposon-arrayed gene knockout method (Hamer et al. 2001). We sequenced the transposon insertional sites in the genomic clones generated. The sequences obtained were screened to find genes potentially involved in fungal pathogenicity. A putative laccase gene was identified with this sequence-based screening. As mentioned above, melanization is one of the important factors required by *C. orbiculare* to infect its hosts, and no laccase for DHN melanin has yet been identified in fungal pathogens, including *C. orbiculare*. Therefore, we focused on the identified gene.

The gene with homology to a putative laccase gene was designated laccase 2 (*LAC2*) because another laccase gene (*LAC1*), distinct from *LAC2*, has already been isolated and character-

Fig. 1. *LAC2* is required for the pathogenicity of *Colletotrichum orbiculare*. **A**, Schematic drawing of the annotated domains within *LAC2* of *C. orbiculare*. *LAC2* has three multicopper oxidase domains: the multicopper oxidase type 3 domain (Cu-oxidase_3), the multicopper oxidase type 1 domain (Cu-oxidase), and the multicopper oxidase type 2 domain (Cu-oxidase_2). *LAC2* also has a signal peptide sequence (SP) at the N terminus. Numbers indicate the amino acid residues. **B**, Colony phenotype of the *lac2* mutants. Wild-type (WT) strain 104-T and a *lac2* strain (DLA2-1) were incubated on potato dextrose agar (PDA) for 7 days. Conidia of each strain were formed around the center of melanized mycelial colony. **C**, Color of conidia collected from colony on PDA. Conidia of each strain were removed from the colony on PDA by pouring water, and concentrated by centrifugation. Conidial color of both the WT strain and *pks1* strain (ALB1) is orange, whereas that of *lac2* strain is brown. **D**, Pathogenicity assay of the *lac2* mutants on host cucumber plants. On the left halves of the cucumber cotyledons, the WT strain 104-T was inoculated as the positive control. Test strains were inoculated onto the right halves. Inoculated leaves were incubated for 7 days. **E**, Assay for infectious growth of the *lac2* mutants inside the host plant tissues. Mycelial blocks of the test strains were inoculated onto the wound sites of cucumber cotyledons. On the left halves of the cotyledons, the WT strain 104-T was inoculated as the positive control. On the right halves, the *lac2* strain DLA2-1 or the *cmk1* strain DCM1 was inoculated. Inoculated plants were incubated for 7 days. We obtained similar results from three independent experiments.

ized in *C. orbiculare* (Tsuji et al. 2001). The *LAC2* gene contains an open reading frame (ORF) of 1,785 bp, with two introns, and encodes a 595-amino-acid protein (GenBank accession number AB713188). At the N terminus, a putative signal peptide consisting of 19 amino acid residues was identified (Fig. 1A; Supplementary Fig. S1). *LAC2* has three multicopper oxidase domains with copper-binding motifs (Fig. 1A). To investigate the function of *LAC2* in *C. orbiculare*, we performed a targeted gene disruption of *LAC2* in *C. orbiculare*. We constructed the disruption vector pCB1636*LAC2* for *LAC2* and introduced it into the wild-type (WT) strain of *C. orbiculare* (Supplementary Fig. S2). Genomic polymerase chain reaction (PCR) identified two independent *lac2* null mutants (DLA2-1 and DLA2-2). The *lac2* mutants exhibited normal growth on potato dextrose agar (PDA), as did the WT strain (Supplementary Table S1). The deletion of *LAC2* did not appear to affect melanization for mycelial colonies formed on PDA (Fig. 1B). The amount of conidia formed in a mycelial culture of the *lac2* mutants on PDA was similar to that in the WT strain. However, whereas the color of conidia formed on a mycelial colony of WT appeared light brown, the conidial color of the *lac2* mutants on the colony appeared dark brown (Fig. 1B). To verify conidial color without the effects of mycelial colony, we collected conidia from the colony of each strain. As a result, we found that the color of collected conidia is orange in the WT whereas it is brown in the *lac2* mutants (Fig. 1C). This suggests that *LAC2* is likely involved in the

pigmentation of the conidia of *C. orbiculare*. Also, the color of conidia collected from the *pks1* albino mutant is orange, which is identical to that of the WT strain (Fig. 1C).

***LAC2* is necessary for the pathogenicity of *C. orbiculare* on the host cucumber plant.**

To investigate whether *LAC2* is involved in the pathogenicity of *C. orbiculare*, conidial suspensions of the *lac2* mutants were drop inoculated onto detached cucumber cotyledons. The *lac2* mutants exhibited severely reduced pathogenicity on the cotyledons. The WT strain 104-T formed yellowish necrotic lesions at the inoculated sites. In contrast, the *lac2* mutants were unable to form lesions on the cucumber cotyledons (Fig. 1D). An inoculation assay through wounds was also performed to investigate whether the *lac2* mutants retained the ability to grow invasively inside the host plant tissues. *CMK1*, which encodes a mitogen-activated protein kinase, was previously reported to be essential for the infectious growth of *C. orbiculare* inside the host plant tissues (Takano et al. 2000). Therefore, we used the *cmk1* mutant as a negative control. The results of the assay indicated that the *lac2* mutants could form necrotic lesions through wounds on detached cucumber cotyledons, whereas the *cmk1* mutant could not form necrotic lesions when applied through wounds (Fig. 1E; Supplementary Fig. S3). This finding indicates that the *lac2* mutants can grow invasively in the host plant tissues if the appressorial penetration stage is bypassed, suggesting that the mutants are defective in the preinvasion stages of infection.

Appressorial melanization is impaired in the *lac2* mutants.

We investigated the preinvasion morphogenesis of the *lac2* mutants on a glass surface. The conidia of the *lac2* mutants germinated normally, as did the WT strain, and the germ tubes of the mutants developed swollen appressoria effectively, indicating that neither germination nor appressorial differentiation were affected in the mutants. However, the appressorial melanization of the *lac2* mutants was obviously impaired compared with that of the WT strain. The appressoria of the WT strain were melanized when incubated for 5 h. In contrast, the appressoria of the *lac2* mutants were rarely pigmented after incubation for 5 h. At 24 h, the appressoria of the WT strain were completely melanized and mature, whereas the appressoria of the *lac2* mutants exhibited only slight pigmentation (Fig. 2). This phenotypic difference in the appressoria of WT and the *lac2* mutants was also detected on the host plants (Fig. 2). These results indicate that appressorial melanization is impaired in the *lac2* mutants. To verify that the loss of pathogenicity in the *lac2* mutants is attributable to the defect in appressorial melanization, we investigated whether the appressoria of the *lac2* mutants were functional, using an appressorial penetration assay on the host cucumber. Over 30% of the appressoria of the WT strain formed invasive hyphae in the host plant tissue at 4 days postinoculation (dpi). However, the appressoria of the *lac2* mutants were unable to form invasive hyphae at 4 dpi (Fig. 3), indicating that the appressoria of the *lac2* mutants are nonfunctional. Because the melanin in appressoria is essential for the pathogenicity of *Colletotrichum* and *Magnaporthe* spp., these results suggest that *LAC2*-dependent appressorial melanization is required for the pathogenicity of *C. orbiculare*.

To investigate the expression period of *LAC2*, we generated a WT strain expressing green fluorescent protein (GFP) under the control of the putative native *LAC2* promoter (Fig. 4). Our observation of GFP fluorescence in the generated strains indicated that the expression of *LAC2* commenced at the initiation of appressorium formation (at 3 h), and its expression increased significantly when the appressoria were melanized (at

Fig. 2. Appressorial morphogenesis and melanization in the *lac2* mutants. Conidial suspensions of the test strains were inoculated onto either glass for 3, 5, or 24 h or cucumber cotyledons (plant) for 24 h; a, appressorium; c, conidium. Bar = 10 μ m.

6 h). The GFP signal was also detected in the conidia and appressoria after further incubation (for 16 h). These data suggest that LAC2 is preferentially expressed during the appressorial maturation steps, including melanization. Thus, these results suggest that LAC2 is a DHN laccase involved in appressorial melanization in *C. orbiculare*.

Secretion of LAC2 is crucial for its functionality.

We investigated the localization of LAC2 by the expression of LAC2 fused to GFP under the control of the LAC2 promoter (LAC2:GFP). GFP fused to full-length LAC2 restored the conidial pigmentation, appressorial melanization, and pathogenicity of the *lac2* mutants (Figs. 5A and 6), indicating that LAC2-GFP was functional. To determine the localization of LAC2 in the conidia and appressoria, the GFP fluorescence was monitored in the LAC2-GFP-complemented strains (Fig. 5B). However, no GFP fluorescence was observed in the strains, even when the appressoria were melanized (Fig. 5B). We also did not detect the GFP fluorescence in nonmelanized appressoria treated with a melanin inhibitor, carpropamid (Hattori et al. 1994) (data not shown). As mentioned above, a signal cleavage site was predicted in LAC2 (using the SignalP 4.0 server program) (Emanuelsson et al. 2007) (Fig. 1A). This raised the possibility that the majority of LAC2 is secreted ex-

tracellularly, and that the LAC2-GFP signals inside the cells and around the cell walls are below the level of detection. Thus, instead of the LAC2 promoter, we used a constitutive and strong promoter (*TEF* promoter) to express LAC2-GFP more intensely to confirm whether LAC2 is secreted extracellularly (Vanden Wymelenberg et al. 1997). When LAC2-GFP was expressed under the *TEF* promoter (TEFP:LAC2:GFP), GFP fluorescence accumulated close to the cell surface of the conidia (Fig. 5C), suggesting that LAC2 is secreted extracellularly.

A SignalP analysis suggested that 19 amino acids from the start methionine (Met) to the cleavage site constitute the signal peptide of LAC2 (Fig. 1A). To verify the importance of the correct secretion of LAC2, a GFP construct fused to LAC2 lacking the signal peptide (LAC2 Δ SP:GFP) was generated and introduced into the *lac2* mutants. The LAC2 Δ SP:GFP gene failed to restore the conidial color, pathogenicity, or appressorial melanization of the *lac2* mutants (Figs. 5A and 6), suggesting that the signal peptide of LAC2 is essential for its function. Furthermore, the GFP signal of LAC2 Δ SP:GFP was detected in the cytoplasm when it was expressed under the LAC2 promoter, whereas the signal of LAC2:GFP was not detected because it was secreted (Fig. 5B). These results suggest that LAC2 is secreted by the 19-amino-acid signal peptide and that the secretion of LAC2 is critical for its functionality. Because melanin is deposited around the cell walls of the appressoria (Kubo and Furusawa 1991; Takano et al. 1997a), the final biosynthetic step of melanin is assumed to occur in the extra-

Fig. 3. The *lac2* mutants are defective in appressorium-mediated host invasion. **A**, The *lac2* mutants failed to form hyphae that invaded the host plants. A conidial suspension of each strain was inoculated onto the lower surfaces of cucumber cotyledons and incubated for 4 days. Invasive hyphae were stained with lactophenol aniline blue; a, appressorium; ih, invasive hypha. Bars = 10 μ m. **B**, Quantitative assay of appressorium-mediated host invasion. Conidial suspensions of each strain were inoculated onto the lower surfaces of cucumber cotyledons, and the cotyledons were incubated for 4 days. The proportion of appressoria forming invasive hyphae was calculated. In each experiment, at least 100 appressoria were examined and counted to calculate the percentage of invasive hyphae. Means and standard deviations were calculated from three independent experiments.

Fig. 4. Green fluorescent protein (GFP)-based reporter assay of the transcriptional pattern of the LAC2 gene during prepenetration morphogenesis. Conidia of the wild-type line carrying the LAC2 promoter-GFP gene were incubated on a glass surface for 15 min or 3, 6, or 16 h; a, appressorium; c, conidium. Bar = 10 μ m.

Fig. 5. Functional analysis of the signal peptide of LAC2. **A**, Signal peptide (SP) is required for LAC2 function in conidial pigmentation. Full-length LAC2 fused to green fluorescent protein (GFP) (LAC2:GFP) or LAC2 lacking the SP sequence fused to GFP (LAC2ΔSP:GFP) was expressed under the LAC2 promoter in the *lac2* mutants. Expression of LAC2:GFP restored conidial pigmentation to the *lac2* mutants, whereas LAC2ΔSP:GFP did not. **B**, GFP fluorescence of LAC2:GFP or LAC2ΔSP:GFP in the conidia-forming appressoria of each transgenic strain. Each strain was incubated on a glass surface for 6 h. No GFP fluorescence was detected in the *lac2* strains carrying LAC2:GFP. In contrast, GFP fluorescence was detected inside the *lac2* strains carrying LAC2ΔSP:GFP. Bars = 10 μm. **C**, Expression of LAC2:GFP under a constitutive and strong promoter (*TEF* promoter). The LAC2:GFP construct with the *TEF* promoter (TEFP:LAC2:GFP) was introduced into the *lac2* mutant. Conidia of the generated strain were incubated on glass for 15 min. Bar = 10 μm.

cellular space. Therefore, the importance of the signal peptide in LAC2 further supports the proposition that (secreted) LAC2 oxidizes 1,8-DHN to yield melanin around the appressorial cell wall.

Evolutionary relationships of LAC2 in filamentous fungi.

To further understand the evolutionary position of LAC2 among the filamentous fungi, an evolutionary phylogenetic tree was constructed based on the laccase sequences of other fungi homologous to LAC2. To generate the phylogenetic tree, we selected seven putative laccases showing high similarity to LAC2: *C. graminicola* GLRG_06593 (GenBank accession number EFQ31449), *C. higginsianum* CH063_12092 (CCF41971), *M. oryzae* MGG_08523 (XP_36291), *C. graminicola* GLRG_08638 (EFQ33709), *Trichoderma reesei* TRIREDRAFT_54239 (EGR53027), *Gibberella zeae* FG10395.1 (XP_390571), *Fusarium oxysporum* lcc5 (ABS19941), and *C. orbiculare* LAC1 (BAB32575), which is structurally unrelated to LAC2 (Fig. 7A; Supplementary Fig. S4). The four LAC2 homologues (GLRG_06593, CH063_12092, MGG_08523, and GLRG_08638) were

Fig. 6. Secretion of LAC2 is required for its function in appressorial melanization and pathogenicity. **A**, Pathogenicity assay of the *lac2* mutants carrying LAC2:green fluorescent protein (GFP) or LAC2ΔSP:GFP on the host plant. Wild-type (WT) strain 104-T was inoculated onto the left halves of the cucumber cotyledons as the positive control. Test strains were inoculated onto the right halves. Inoculated leaves were incubated for 7 days. We obtained similar results from three independent experiments. **B**, Appressorium formation assay. Conidial suspensions of the test strains were incubated on a glass surface for 24 h. Bar = 10 μm.

from plant-pathogenic fungi that form melanized appressoria (*Colletotrichum* and *Magnaporthe* spp.). CH063_12092 of *C. higginsianum* showed the highest similarity (83%) to LAC2, and GLRG_06593 of *C. graminicola* showed the next highest similarity (80%) to LAC2. LAC2, CH063_12092, and GLRG_06593 occurred in the same clade, designated the “LAC2 clade” (Fig. 7A). Two laccases within *C. graminicola* shared homology with LAC2 but another LAC2 homologue from *C. graminicola* (GLRG_08638) had only 49% similarity to LAC2, and was located in a clade distinct from the LAC2 clade. MGG_08523 of *M. oryzae* occurred in the same clade as GLRG_08638 (Fig. 7A). *T. reesei* TRIREDRAFT_54239, *G. zeae* FG10395.1, and *F. oxysporum* lcc5 also formed a clade distinct from the LAC2 clade, although they still showed high homology to LAC2.

To determine whether the LAC2 homologues from other fungi could complement the function of LAC2, selected laccase genes (GLRG_06593, CH063_12092, MGG_08523, GLRG_

08638, and *LAC1*) were expressed under the *LAC2* promoter in the *lac2* mutants. Both GLRG_06593 of *C. graminicola* and CH063_12092 of *C. higginsianum* complemented the conidial pigmentation, appressorial melanization, and pathogenicity of the *lac2* mutants (Figs. 7B and 8). In contrast, *LAC1*, unrelated to *LAC2*, did not complement any aspect of the *lac2* mutant phenotype (Figs. 7B and 8). Nor was GLRG_08638 able to complement any aspect of the *lac2* mutant phenotype. These findings suggest that both GLRG_06593 and CH063_12092, located in the LAC2 clade, are the functional orthologs of LAC2 in *C. graminicola* and *C. higginsianum*, respectively.

Interestingly, the LAC2 homologue of *M. oryzae*, MGG_08523, complemented the conidial pigmentation of the *lac2* mutants but failed to restore their appressorial melanization (Figs. 7B and 8). This result suggests a possibility that the putative biosynthesis pathway for the conidial pigment is separate from the pathway of appressorial melanization. MGG_08523 also failed to complement the pathogenicity of the *lac2* mutants, indicating that LAC2-dependent appressorial melanization but not conidial pigmentation is required for the fungal pathogenicity of *C. orbiculare*.

DISCUSSION

In this study, we identified a novel laccase gene, *LAC2*, encoding an important factor in the appressorial melanization of *C. orbiculare*. We have shown that *LAC2* is essential for the pathogenicity of *C. orbiculare*. An inoculation assay through

Fig. 7. Complementation assay of the *lac2* mutants with *LAC2* homologues from other fungal pathogens. **A**, Phylogenetic relationships of the *LAC2*-related laccases from filamentous fungi. *LAC2*, *LAC2*-related laccases, and *LAC1* were aligned using ClustalW (Thompson et al. 1994). The following alignment parameters were used: for the pairwise alignment, gap opening = 10.0 and gap extension = 0.1; for the multiple alignment, gap opening = 10.0 and gap extension = 0.05. The resulting alignments were submitted to MEGA5 software to generate a neighbor-joining tree derived from 1,000 replicates (Saitou and Nei 1987; Tamura et al. 2011). Bootstrap values (%) are indicated on the node of each branch. **B**, Functionality of the *LAC2* homologues in the conidial pigmentation in *Colletotrichum orbiculare*. The *lac2* strains carrying the *LAC2* homologues of other fungal pathogens (GLRG_06593, CH063_12092, MGG_08523, and GLRG_08638) or *C. orbiculare* *LAC1* were incubated on potato dextrose agar for 7 days.

Fig. 8. *LAC2* homologues located in the *LAC2* clade restored the appressorial melanization and pathogenicity of the *lac2* mutants. **A**, Pathogenicity assay of the *lac2* mutants carrying the *LAC2* homologues of the other fungal pathogens (GLRG_06593, CH063_12092, MGG_08523, and GLRG_08638) or *Colletotrichum orbiculare* *LAC1*. On the left halves of the cucumber cotyledons, the wild-type (WT) strain 104-T was inoculated as the positive control. Test strains were inoculated onto the right halves. Inoculated leaves were incubated for 7 days. We obtained similar results from three independent experiments. **B**, Appressorium formation assay. Conidial suspensions of the test strains were incubated on a glass surface for 24 h. Bar = 10 µm.

wounds indicated that the *lac2* mutants have no defect in their postinvasive growth inside the host plant tissues, suggesting that their loss of pathogenicity occurs in a preinvasion stage. Analysis of the preinvasion stages demonstrated a defect in the appressorial melanization of the *lac2* mutants. An appressorial penetration assay further showed that the appressoria of the *lac2* mutants, which are defective in melanization, were unable to form invasive hyphae into the host plant tissue. These findings are consistent with the previous observation that melanization around the appressorial cell wall is essential for the function of the appressoria (Kubo and Furusawa 1991). Therefore, we concluded that LAC2-dependent appressorial melanization is essential for the pathogenicity of *C. orbiculare*. LAC2 is preferentially expressed during appressorial melanization. We also demonstrated that the signal peptide of LAC2 is essential for LAC2 function in appressorial melanization. Melanin accumulated around the appressorial cell wall, suggesting that the final biosynthesis step occurs in the extracellular space. Together with the data from previous reports that suggest the involvement of laccases in DHN melanin synthesis (although the corresponding genes are not yet identified) (Edens et al. 1999; Tanaka et al. 1992), our findings strongly suggest that LAC2 is responsible for the oxidation of 1,8-DHN to melanin in the appressorial cells of *C. orbiculare*. Appressoria of the *lac2* mutants are slightly pigmented (Fig. 2). If the gray-colored pigment is melanin, this suggests that laccases other than LAC2 also partially contribute a basal level of DHN polymerization in appressoria.

When the four LAC2-related laccases and LAC1 were individually expressed in the *lac2* mutants under the LAC2 promoter, only the two closest homologues of LAC2 restored the appressorial melanization of the *lac2* mutants (Figs. 7 and 8). This finding indicates that 1,8-DHN is not oxidized by a wide range of laccases but can be oxidized by a specific class of laccases, including LAC2. SCD1 catalyzes two dehydration steps: scytalone to 1,3,8-T3HN and vermeline to 1,8-DHN. A SignalP 4.0 analysis predicted that SCD1 has no signal peptide, suggesting that, unlike LAC2, SCD1 is not secreted. This suggests that 1,8-DHN generated by SCD1 is moved to the apoplastic space of the appressorial cells and is then oxidized by secreted LAC2, allowing the deposition of melanin around the appressorial cell walls. The *LAC2-GFP* gene under the control of the LAC2 promoter restored the appressorial melanization of the *lac2* mutants but the LAC2-GFP signal was not detected around the appressorial cell walls of the complemented strains, probably because the signal was below the limit of detection of the confocal microscope in our laboratory. Secreted LAC2 might only accumulate transiently around the cell wall, which might be sufficient to oxidize 1,8-DHN for appressorial melanization.

The fungus *Gaeumannomyces graminis*, a phytopathogenic ascomycete that causes “take-all”, is known to produce 1,8-DHN melanin and complete its infection with melanized ectotrophic hyphae. In this fungus, the melanization of the hyphae is crucial for its pathogenicity. It has been reported that a purified laccase of *G. graminis* can catalyze the polymerization of 1,8-DHN into melanin in vitro, although it remains to be clarified whether the purified laccase is involved in mycelial melanization (Edens et al. 1999). The *C. heterostrophus* mutant defective in mycelial melanization also lacks a laccase activity (Tanaka et al. 1992). These findings suggest the presence of laccases in fungi for mycelial melanization. *C. orbiculare* also forms darkly melanized mycelial colonies on nutrient medium but, interestingly, the melanization of mycelial colonies is unlikely affected in the *lac2* mutants (Fig. 1B), implying that a different laccase is involved in mycelial melanization in *C. orbiculare*.

We have previously reported that the three melanin biosynthesis genes *PKS1*, *SCD1*, and *THR1* are commonly induced during the process of appressorium formation (Takano et al. 1997b), which is similar to the case of LAC2. CMR1 is a known transcription activator, controlling the expression of *SCD1* and *THR1* during mycelial melanization in *C. orbiculare*. However, CMR1 is not essential for the expression of *SCD1* and *THR1* during appressorial melanization (Tsuji et al. 2000). This indicates the presence of an unidentified transcription activator that controls the expression of the melanin biosynthesis genes during appressorial melanization. Because appressorial melanization is affected in the *lac2* mutants, the expression of LAC2 probably might depend on a putative transcription activator that controls the other melanin biosynthesis genes during appressorial melanization.

An analysis of conidial pigmentation gene clusters in the filamentous fungus *Aspergillus fumigatus* revealed that *A. fumigatus* synthesizes its conidial pigment through a pathway similar to the 1,8-DHN melanin pathway. However, the final conidial pigment produced by *A. fumigatus* is bluish green, which differs from the color of DHN melanin (black). Therefore, although the melanin-like biosynthetic pathway for the conidial pigment in *A. fumigatus* is similar to the DHN melanin biosynthetic pathway, the pathway of *A. fumigatus* must have diverged at steps before the final melanin product is generated. Three additional genes—*abr1*, *abr2*, and *ayg1*—are involved in the *A. fumigatus* conidial pigmentation pathway, two encoding laccases (*Abr1* and *Abr2*), which might explain the divergence of the final products (Tsai et al. 1999). A comparative analysis of LAC2 and *Abr1* and *Abr2* might reveal the molecular background of the evolutionary divergence of 1,8-DHN melanin and the bluish-green pigment of *A. fumigatus*.

Interestingly, the deletion of LAC2 also affected the conidial pigmentation of *C. orbiculare*. The conidial color of *C. orbiculare* is orange, distinct from both melanin (black) and the conidial pigment of *A. fumigatus* (bluish green). Our finding that conidial color of the albino *pks1* mutant is identical to that of the WT strain (Fig. 1C) suggests that the conidial pigment of *C. orbiculare* is not related to the DHN melanin pathway. Consistently, a previous experiment with the carotenoid inhibitor diphenylamine suggested that the orange pigment in *C. graminicola* conidia is a carotenoid (Epstein et al. 1998). Thus, LAC2 might have roles for two distinct secondary metabolic pathways, although the details of the putative biosynthetic pathways for the conidial pigment of *C. orbiculare* are currently unclear. Further studies are required to understand the change in the conidial color of the *lac2* mutants.

The closest homologues of LAC2 in *C. higginsianum* and *C. graminicola* (CH063_12092 and GLRG_06593, respectively) restored the conidial pigmentation, appressorial melanization, and pathogenicity of the *lac2* mutants. Both homologues were located in the same phylogenetic clade as LAC2 (the LAC2 clade). The *M. oryzae* homologue MGG_08523 localized to a different clade from LAC2, and failed to complement the appressorial melanization or pathogenicity of the *lac2* mutants. However, MGG_08523 restored the conidial pigmentation of the *lac2* mutants. These findings suggest that i) the function of LAC2 in appressorial melanization is likely only conserved among the *Colletotrichum* laccases located in the LAC2 clade and ii) the possible function of LAC2 in conidial pigmentation might even be conserved in those LAC2 homologues that do not cluster in the LAC2 clade.

Because *M. oryzae* forms appressoria pigmented with DHN melanin, it is plausible that the pathogen has a laccase for synthesis of the melanin in appressoria. The restoration of conidial pigment change by MGG_08523 indicates that MGG_08523 is functional in the *lac2* mutant at this aspect. Nevertheless,

MGG_08523 failed to restore appressorial melanization in the *lac2* mutant. Thus, MGG_08523 might not be involved in appressorial melanization of *M. oryzae*; and an unidentified *lac*-case, with no strong homology to LAC2, might function in the appressorial melanization of *M. oryzae*. However, LAC2 and MGG_08523 might need a partner protein for synthesis of melanin in appressoria. If this is true, it is possible that MGG_08523 is involved in appressorial melanization of *M. oryzae* but is not functional in appressoria of the *lac2* mutant, because of the failure to work together with the partner protein of *C. orbiculare*.

The laccase proteins are reported to be involved in fungal morphogenesis, lignin degradation, and pathogenesis. However, there has been no report of the laccase proteins involved in the synthesis of DHN melanin in the filamentous fungi. This study is the first to identify a laccase gene that is involved in the biosynthesis of 1,8-DHN melanin in the appressorial cells of fungal pathogens. Because the functional orthologs of LAC2 are probably conserved among the *Colletotrichum* spp., this finding provides a new target for the development of novel melanin-inhibitory fungicides, especially against *Colletotrichum* spp.

MATERIALS AND METHODS

Fungal strains, culture conditions, and nucleic acid analysis.

C. orbiculare (syn. *C. lagenarium*) wild-type strain 104-T (MAFF240422) is a stock culture of the Laboratory of Plant Pathology, Kyoto University. For the study of the LAC2 homologues in other fungi, *C. higginsianum* strain MAFF305635, *M. oryzae* strain 70-15, and *C. graminicola* strain M1.001 (M2) were used. All fungal strains were maintained on PDA medium (3.9% [wt/vol] PDA) (Difco Laboratories, Detroit) at 24°C in the dark. Transposon-arrayed gene knockout was performed as reported previously (Hamer et al. 2001). The total DNA of *C. orbiculare* was isolated from mycelia with the DNeasy plant mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The total RNA of the fungal strains was isolated from mycelia grown in liquid medium with the RNeasy plant mini kit (Qiagen) and treated with DNase (Promega RQ1 RNase-free, Charbonnières, France) to remove any contaminating DNA. The cDNA of the fungal strains was synthesized with the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). Restriction enzyme digestion, cloning, plasmid isolation, and gel electrophoresis were performed according to the manufacturer's instructions and standard methods (Sambrook et al. 1989).

Plasmid constructs.

The primers used for plasmid construction are listed in Supplementary Table S2. To delete the LAC2 gene of *C. orbiculare*, a gene disruption vector, pCB1636LAC2, was constructed. A 3.3-kb fragment containing the 5'-flanking region of LAC2 was amplified by PCR with primers LAC2PROSN1 and LAC2PROASS1, which contain a *NotI* site and a *SpeI* site, respectively. The amplified fragment was digested with *NotI* and *SpeI* and introduced into the *NotI*–*SpeI* sites of pCB1636 (Sweigard et al. 1997), containing the *HPH* gene, to produce the plasmid pCB5LAC2. The 2-kb fragment containing the 3'-flanking region of LAC2 was amplified by PCR with primers LAC2TERSA1 and LAC2TERASK1, which contain an *ApaI* site and a *KpnI* site, respectively. The amplified fragment was digested with *ApaI* and *KpnI* and then introduced into the *ApaI*–*KpnI* sites of pCB5LAC2 to produce pCB1636LAC2. To construct the LAC2-complementary GFP expression vector for the *lac2* mutants, the entire promoter and ORF of LAC2 were amplified with primers LAC2PROSN1 and LAC2ORFASX1.

The amplified fragment was digested with *NotI* and *XbaI* and introduced into pBAFPGFP (Kojima et al. 2004), which contains a bialaphos resistance gene (*bar*), to produce pBAFPGFPLAC2. To construct the LAC2-promoter-driven GFP-expressing vector, the entire promoter of LAC2 was amplified with LAC2PROSN1 and LAC2PROASS1. The amplified fragment was digested with *NotI* and *XbaI* and introduced into pBAFPGFP to produce pBAFPGFPLAC2P. To clarify the importance of the signal peptide of LAC2, a plasmid carrying *GFP* fused to LAC2 with the LAC2 promoter but lacking the region encoding the signal peptide (LAC2ΔSP:GFP) was constructed. The LAC2 ORF lacking the signal peptide was amplified with primers LAC2DSPX1 and LAC2ORFASX1. The amplified fragment was digested with *XbaI* and introduced into pBAFPGFPLAC2P to produce pBAFPGFPLAC2DSP. To express LAC2-GFP under the *Aureobasidium pullulans* *TEF* promoter (Vanden Wymelenberg et al. 1997), the *TEF* promoter was isolated from pBATTEFPMR (Asakura et al. 2009) with *NotI* and *XbaI*, and was introduced into pBAFPGFP to produce pBAFPGFPTEFP. The entire ORF region of LAC2 was amplified with COLAC2ORFXI-s1 and LAC2ORFASX1, and the amplified fragment was digested with *XbaI* and introduced into pBAFPGFPTEFP to produce pBAFPGFPTEFLAC2. To construct the expression plasmids for the LAC2 homologues and LAC1, the LAC2 promoter was amplified from pBAFPGFPLAC2P and digested with *NotI* and *XbaI*. The LAC2 promoter fragment was then introduced into pBAT (Kimura et al. 2001) to form pBATLAC2P. The cDNAs of *C. orbiculare*, *C. graminicola*, *C. higginsianum*, and *M. oryzae* were used as the templates to amplify the LAC2 homologues or LAC1 of these strains with the following primers: COLAC2SXbaI and COLAC2ASSpeI for LAC2; CGLAC2SXbaI065 and CGLAC2ASSpeI065 for GLRG_06593; CGLAC2SXbaI086 and CGLAC2ASSpeI086 for GLRG_08638; CHLAC2SXbaI and CHLAC2ASSpeI for CH063_12092; MGLAC2SXbaI and MGLAC2ASSpeI for MGG_08523; and COLAC1SXbaI and COLAC1ASSpeI for LAC1. The amplified fragments were digested with *XbaI* and *SpeI* and introduced into pBATLAC2P to form pBATCoLAC2, pBATCg065, pBATCg086, pBATCh063, pBATMg085, and pBATCoLAC1, respectively.

Fungal transformation.

Protoplasts were prepared and *C. orbiculare* was transformed according to methods described previously (Takano et al. 2001). Hygromycin- and bialaphos-resistant transformants were selected on regeneration medium containing hygromycin B (Wako Pure Chemicals, Osaka, Japan) at 100 µg/ml and bialaphos (Wako Pure Chemicals) at 25 µg/ml (Sweigard et al. 1997), respectively.

Pathogenicity assays.

For the pathogenicity assays, conidial suspensions collected from 7-day-old colonies on PDA (5×10^5 conidia/ml) were drop inoculated onto detached cucumber cotyledons (*Cucumis sativus* L. 'Suyo'), with 10 µl in each drop. The cotyledons were picked from 7- to 10-day-old cucumber. For inoculation through wounded sites, the mycelial block from a 7-day-old PDA colony was placed onto a site that had been wounded with a needle. Each pathogenicity test was performed at least three times under the same inoculation and incubation conditions.

Light microscopy.

Conidia were collected from 7-day-old colonies on PDA medium. Conidial suspensions (20 µl of 5×10^5 conidia/ml in 0.1% yeast extract solution) were loaded onto glass slides (eight-well multitest slides; ICN Biomedicals, Aurora, OH) and incubated in a humid chamber at 24°C in the dark. After

incubation for 1 h, the 0.1% yeast extract solution was removed and replaced with distilled water (Takano et al. 2001). The incubated samples were observed under a FluoView FV500 confocal microscope (Olympus, Tokyo). To observe appressorial melanization on plants, the conidial suspensions (5×10^5 conidia/ml) were sprayed onto the lower surfaces of cucumber cotyledons. After incubation for 4 days in a humid chamber, the lower epidermal layers were peeled off and observed under a FluoView FV500 confocal microscope (Olympus). To investigate appressorium-mediated host invasion, conidial suspensions were sprayed onto the lower surfaces of cucumber cotyledons. After incubation for 4 days, the lower epidermal layers were peeled off and stained with lactophenol aniline blue. The invasive hyphae were observed with a bright-field Olympus BX53 fluorescence microscope (Olympus) equipped with an Olympus DP72 camera and Olympus cellSens software.

Fluorescence microscopy.

In all experiments involving the observation of fluorescence, the conidia collected from 7-day-old colonies on PDA were loaded onto glass slides and incubated with 0.1% yeast extract solution in a humid chamber at 24°C in the dark. After incubation for 1 h, the 0.1% yeast extract solution was removed and replaced with distilled water or carpropamid at 10 µg/ml, which inhibits melanin biosynthesis (Asakura et al. 2006). The fluorescence of GFP was observed under a FluoView FV500 confocal microscope (Olympus), as previously described (Asakura et al. 2006).

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