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The laccase gene (*LAC1*) is essential for *Colletotrichum gloeosporioides* development and virulence on mango leaves and fruits

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ABSTRACT

Colletotrichum gloeosporioides is the main causal agent of anthracnose of mango (*Mangifera indica*), which has great economic impact on mango industry worldwide. Laccase has versatile biological functions including: melanin biosynthesis and pathogenicity in several other pathogenic fungi. A laccase gene *LAC1*, homologous to *C. lagenarium* laccase, was cloned from *C. gloeosporioides*. To evaluate its role, *LAC1* was disrupted via gene replacement, and the mutant *Δlac1* was analyzed. Compared with the wild-type, the *Δlac1* mutant had less pigmentation and dramatically reduced aerial mycelial mass and radial growth rates, and rarely produces conidia in vitro. Microscopic observation showed that *Δlac1* hyphae were lighter in color, less branched and septated with obviously more condensed protoplasm than wild-type. The *Δlac1* mutant was obviously different in nutritional utilization compared with the wild-type. Virulence tests showed that *Δlac1* mycelia were weakly virulent on non-wounded mango leaves and fruits, and was significantly less virulent on wounded mango leaves and fruits. The *Δlac1* produced less extracellular cell wall-degrading enzymes (CWDE) and no detectable extracellular laccase activity in vitro. These results suggest that the *C. gloeosporioides* LAC1 laccase is involved not only in virulence, but also in mycelial growth and differentiation, conidiation, appressorium formation, pigmentation, melanin biosynthesis, secretion of extracellular hydrolytic enzymes, and utilization of exogenous nutrition.

1. Introduction

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. (teleomorph: *Glomerella cingulata*) is an economically important plant pathogenic fungus that attacks a wide variety of tropical and subtropical crops. Anthracnose caused mainly by *C. gloeosporioides*, is the most serious disease in all mango growing regions of the world [1–4]. The fungus attacks most organs of mango tree, resulting in anthracnose on leaves, twigs, flowers and young fruits [5,6]. It also appears as a storage disease of mango fruits following storage and transport postharvest. The symptoms of the disease are black, slightly sunken lesions of irregular shape, which gradually enlarge and cause blossom blight, leaf spotting, staining and fruit rot [7].

C. gloeosporioides produces conidia that germinate and penetrate into the host epidermis by formation of melanized appressoria at the end of a germ-tube. After penetration, the infection hyphae are restricted and remain quiescent until the mango fruits ripen. The resistance of unripe mango fruit to fungal attack during quiescence has been reported to depend on the presence of preformed antifungal compounds, mainly phenolic substances, and lack of secretion of fungal virulence factors, such as pectic lyase [8]. Guetsky et al. [9] reported that biotransformation of flavonoid epicatechin by *C. gloeosporioides* laccase in ripening avocado fruits declined the preformed antifungal diene compound which biodegradation is inhibited by epicatechin, resulting in the

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activation of quiescent infections. Isolates of *C. gloeosporioides* with reduced laccase activity and no capability to metabolize epicatechin showed reduced virulence on ripening fruits, and conversely, Mexican isolates with increased capability to metabolize epicatechin showed early symptoms of the disease in unripe fruits, indicating that laccase is related to the virulence of *C. gloeosporioide*.

Laccases are copper-containing phenol oxidases that belong to a small group of enzymes known as blue copper proteins or blue copper oxidases [10,11]. Laccases are widely distributed in higher plants and fungi, and have been found in insects and bacteria [12]. In higher plants, laccase are involved in lignin degradation, so recently, they are routinely used to degrade environmental pollutants [13]. Plant pathogenic fungi may produce a number of laccase isozymes encoded by multiple genes [14,15]. Thus far, about 10 laccase genes functions in plant pathogenic fungi have been identificated. In *Botrytis cinerea*, laccase *Bclcc2* can degrade several phytoalexins of grape vines (*Vitis rupestris*), and thus contributes to fungal virulence [16,17]. *Aspergillus nidulans*, *A. fumigatus*, *C. orbiculare* and *Setosphaeria turcica* laccases are required for pigment biosynthesis during conidial development, and for pathogenesis [18–20]. In *Fusarium oxysporum* f.sp. *lycopersici*, a vascular wilt pathogen, laccase genes *lcc*1 and *lcc*3 are involved in antioxidant capacity [14]. However, laccase *LAC1* mutants of *C. lagenarium* retained laccase activity and had no significant phenotypic differences in melanin production, which indicated *LAC1* was not involved in melanin biosynthesis and pathogenicity [21]. Two laccase genes *Mgg-00551.5* and *Mgg-02876.5* of *Magnaporthe oryzae* are also not essential for fungal differentiation, development, and pathogenicity [22]. These indicate laccases of fungal plant pathogens play a variety of biological roles and have diverse functions in melanin biosynthesis, osmotic regulation, antioxidant capacity, morphogenesis, pathogenesis, and lignin degradation [14,20,23].

The activity of exogenously applied laccase enhaced the virulence of *C. gloeosporioides* on mango leaves and fruits (unpublished data). However, antifungal dienes have not been reported to involve in the defense mechanism in mango tissue. So, we hypothesize that laccase may play other roles than biotransformation of flavonoid epicatechin, in *C. gloeosporioides* pathogenicity on mango fruit. However, no laccase gene has been identified to be related to virulence and other biological characters in C. gloeosporioide. In the previous study, a laccase gene, designated *LAC1* (JQ762259.1) and encoding a putative secreted laccase, was isolated in *C. gloeosporioides* from mango leaves [24]. Here, we obtained the *LAC1* gene disrupted mutant and its analysis revealed that the *LAC1* is essential for *C. gloeosporioides* pathogenesis. Notably, a phenotypic analysis of the *lac1* mutants indicated that LAC1 is involved in vegetative growth, conidiation, appressorium formation, melanization, effects of this disruption on mycelial growth, development, differentiation, production of cell wall-hydrolytic enzymes of *C. gloeosporioides*.

2. Materials and methods

2.1. Strains and culture conditions

A wild-type *C. gloeosporioides* isolate (A2) was isolated from diseased mango leaf and kept at the Environment and Plant Protection Institute, Chinese Academy of Tropical Agricultural Sciences (Hainan province, China). The pure cultures of A2 were obtained via single-spore isolation. The wild-type and mutant strains were cultured on Potato Dextrose Agar (PDA) containing 200 g potato, 20 g dextrose, and 18 g agar per 1000 ml H_2O (pH 7.0). Mycelial plugs (6 mm diameter) were cut from the margin of a 5-day-old *C. gloeosporioides* culture on PDA grown under dark conditions at 28 °C.

2.2. DNA manipulation

Genomic DNA and RNA were isolated from *C. gloeosporioides* mycelia with a HP Fungal DNA Kit (OMEGA Bio, America) and the RNAprep Pure Plant Kit (TIANGEN Bio, China), respectively. Gene fragments were amplified using TransStart Fast Pfu DNA Polymerase (TaKaRa, Dalian, China). The cDNA of the fungal strains was synthesized with the TIANScript cDNA First-Stand Kit (TIANGEN Bio, China). Amplified PCR fragments were purified from 1% TAE agarose gels using a DNA Fragment Purification Kit (TaKaRa, Dalian, China). DNA samples were sequenced by the Beijing Genomics Institute Biotechnology (Shenzhen Co. Ltd., China) and plasmids were purified using an EZgeneTM Plasmid Miniprep Kit (Biomiga, San Diego, CA). Sequence alignments were performed with DNAssist1.0 software. A vector pGH14 was constructed by our lab, which carries both the hygromycin phosphotransferase (*hygB*) gene and green fluorescent protein (*sGFP*) gene originated from plasmid pCT74 donated by Lorang et al. [25].

2.3. Plasmid construction and transformation

To investigate *C. gloeosporioides LAC1* gene function, a construct was designed to replace the *LAC1* coding region with the *hygB* gene and *sGFP* from pGH14. Primer pairs corresponding to the 5′ and 3′ flanking regions of the *LAC1* gene were used to construct the replacement vector: A2LF-F (5′-tatgaccatgattaccacctctacgcacacctc -3′) and A2LF-R (5′-gcccttgctcaccatagcaaggaaaagtccagc -3′), as well as A2LB-F (5′-ccttcaatatcagttcaagaccgacaacccagg-3′) and A2LB-R (5′- aaaacgacggccagtggagaggagagaacccca-3′). Underlined and framed bases indicate left and right sequences corresponding to the cloning site on pUC19 (Clontech, Mountain View, CA, USA) and *gfp-hygB* gene, respectively. The *gfp-hygB* gene was amplified from pGH14 using primers GH-F (5′-atggtgagcaagggcgag-3′) and GH-R(5′- aactgatattgaaggagcatttttt-3′). Resulting 5′ and 3′ *LAC1* PCR products and *gfp-hygB* gene were cloned into pUC19 via T-cloning site with a In-FusionR HD Cloning Kit (Clontech, Mountain View, CA, USA), yielding the recombinant vector p*Δlac1*, in which the *gfp-hygB* gene is flanked by *LAC1* fragments (Fig. 1A). pΔ_{lac1} was transformed into A2 using the polyethylene glycol-mediated transformation of fungal protoplasts method [26]. Hygromycin-resistant transformants were selected and purified on PDA media containing 150 μg/ml hygromycin B (Wako Pure Chemicals, Osaka, Japan).

Putative hygromycin-resistant transformants were initially confirmed by PCR using primers FFG-F (5′-gctttgttgttcccctccatgtca-3′) and FFG-R (5′-ctgggtgctcaggtagtggttgt-3′), HHB-F (5′-gaacccgctcgtctggctaagat-3′) and HHB-R (5′- aacgtcccaaacgcccaaataac-3′) (Fig. 1A). All true transformants were purified by monoconidial isolation. Southern blot analysis was conducted with a Digoxigenin (Dig) High Primer DNA Labeling Kit (Roche, Mannheim, Germany). The *LAC1* probe was amplified from gDNA with primers A2LProbel-F (5′-gcacattcaccatgcgttac-3′) and A2LProbel-R (′-gtgggcgatttcgtcctt-3′), and labeled with alkali-labeled DIG–dUTP. Mutant transformant genomic DNA samples were digested with *Xab*1 and *Pst*1.

2.4. RT-PCR analysis of gene expression

To confirm whether *LAC1* was transcribed in the wild-type isolate (A2) and the *Δlac1* mutant, and the disruption influenced the expression of melanin synthesis related genes polyketide synthase gene (*PKS*), tetra-HN reductase scytalone gene (*THR*), scytalone dehydratase gene (*SCD*), as well as exo-glucocanase gene

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