Polyketide Synthase Gene Expression in Relation to Chloromonilicin and Melanin Production in *Monilinia fructicola*

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ABSTRACT

Monilinia fructicola is a fungal pathogen of worldwide significance that causes brown rot of stone fruits. There are only few reports related to the production of biologically active polyketides by this pathogen. In this study, we examined an atypical *M. fructicola* strain TW5-4 that shows strong antimicrobial activity against various plant pathogens. TW5-4 also displays sparse growth in culture, low virulence, and higher levels of melanin compared with its albino mutant, TW5-4WM, and a wild-type strain Mf13-81. Antifungal compounds were extracted from TW5-4 and purified by thin-layer chromatography following visualization with an on-the-chromatogram inhibition assay. The principal antifungal compound was identified by linear ion trap mass spectrometry, high-resolution electro-spray ionization mass spectrometry, and proton nuclear magnetic resonance analyses as the polyketide chloromonilicin. Multiple *M. fructicola* polyketide synthase (PKS) sequences were then cloned by degenerate PCR and inverse PCR. Sequence analyses support presence of

a 10-member PKS gene family in the *M. fructicola* genome. Analyses of PKS gene expression found no strong correlation between chloromonilicin production in culture and transcript levels of any of the PKS gene family members in mycelium of strains TW5-4, TW5-4WM, and Mf13-81. However, *MfPKS12*, a homolog of *BcPKS12* involved in biosynthesis of 1,8-dihydroxynaphthalene (DHN)-melanin in *Botrytis cinerea*, was strongly expressed in mycelia of TW5-4 and Mf13-81. An *MfPKS12*-silenced mutant accumulated significantly less melanin in mycelia, had lower resistance to polyethylene glycol-induced osmotic stress, and displayed reduced virulence on nectarine fruit. The results suggest that DHN-melanin is required for tolerance to osmotic stress and full virulence in *M. fructicola*.

Keywords: biochemistry and cell biology, mycology, postharvest pathology and mycotoxins

Monilinia fructicola (G. Winter) Honey is a fungal plant pathogen of worldwide significance, affecting many members of the Rosaceae, and is especially damaging to stone fruits (*Prunus* spp.). *M. fructicola* causes brown rot blossom blight and fruit rot and is a necrotrophic pathogen related to members of the family Sclerotiniaceae, such as *B. cinerea* and *Sclerotinia sclerotiorum* with similar parasitic habits. Necrotrophs produce toxins and cell wall-degrading enzymes to kill host cells during infection and

colonization (Laluk and Mengiste 2010; Pusztahelyi et al. 2016). There is strong evidence for a role of cell wall-degrading enzymes by *M. fructicola* in lesion development and pathogenesis (Chou et al. 2015; Lee et al. 2010). However, the contribution of low molecular weight toxins in brown rot disease is less clear (Byrde and Willetts 1977). Oxalic acid is an important phytotoxin produced by members of the family Sclerotiniaceae. However, gluconic acid appears to be the principal organic acid produced by *M. fructicola*, apparently contributing to host colonization through acidification of the infection court (De Cal et al. 2013). Malic, fumaric, and citric acids are also produced to a lesser degree, and fumaric acid has been shown to be a phytotoxin active in stone fruits (Mirocha et al. 1961). Plant pathogens also produce toxins to help exclude competitors for niche establishment. Production and turnover of such toxins would require tight regulation, and/or effective resistance mechanisms to avoid self-inhibition.

Polyketides are secondary metabolites in fungi important for survival (e.g., melanin), self-defense (e.g., antibiotics), and host attack (e.g., phytotoxins). Their structural complexity confer functional diversity and create many potential applications in pharmacology and biotechnology (Cox et al. 2018; Hussain et al. 2017). Polyketide synthases (PKS) are multifunctional enzymes that initiate polyketide biosynthesis via decarboxylative condensation between a thioesterified malonate derivative and an acyl thioester (Chan et al. 2009). PKS are grouped into three types based on their sequences, primary structures, and catalytic mechanisms (Cox and Simpson 2009). Type I PKS, found in bacteria, fungi, and plants, are large proteins (usually >6 kb) with multiple catalytic

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domains encoded by a single nucleotide sequence. Type II PKS are only found in bacteria and consist of multiple subunit proteins, each with a single catalytic role that coordinate polyketide synthesis. Type III PKS occur in bacteria, fungi, and plants and have a single catalytic function. In fungi, several Type III PKS have been characterized (Funari et al. 2007; Seshime et al. 2005, 2010a, b). However, Type I PKS are most common in fungi (Cox and Simpson 2009; Cox et al. 2018; Kroken et al. 2003).

The biosynthesis of polyketides by a Type I PKS begins with acetate or propionate, with subsequent additions of malonyl-CoA for polymer extension, a process catalyzed by functional domains in the protein that include ketoacyl synthase (KS), acyl transferase (AT), and acyl carrier protein (ACP) (Cox 2007). Nonreducing, partially reducing, and highly reducing PKSs all contain these three catalytic domains. Additional domains in partially and highly reducing PKSs may include ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), and C-methyltransferase (CMeT) to further modify the polymer (Cox 2007). In the absence of an annotated genome sequence, fungal PKSs have been cloned by PCR with primers targeting conserved regions of KS, KR, and CMeT domains (Akamatsu et al. 2010; Amnuaykanjanasin et al. 2005; Bingle et al. 1999; Nicholson et al. 2001). PKSs typically have been found to comprise a large gene family in a fungal genome; members of Pezizomycotina, for example, carry up to 25 PKSs. Still, some fungi do not carry PKS genes in their genome, for instance some members in Saccharomycotina and Taphrinomycotina (Kroken et al. 2003).

Polyketides such as 1,8-dihydroxynaphthalene (DHN)-melanin polymers and various phytotoxins are important as pathogenicity and virulence factors (Pusztahelyi et al. 2016; Teichert and Nowrousian 2011). Fungal cell walls can contain melanin to protect cells from environmental stresses (Akamatsu et al. 2010; Kawamura et al. 1999; Moriwaki et al. 2004). Melanin also plays a critical role in modulating turgor during appressorium development and penetration into host tissue (Kawamura et al. 1997; Ryder and Talbot 2015; Takano et al. 1995). Classic examples of important polyketide phytotoxins include T-toxin (Baker et al. 2006), cercosporin (Choquer et al. 2005), and botcinic acid (Dalmats et al. 2011), among others.

We have collected many *M. fructicola* isolates from different peach orchards in Taiwan. One of the strains, TW5-4, grows slowly, forms dark colonies, and inhibits growth of other plant pathogens. The objective of this study was to further characterize this unusual strain. During the course of our study, we identified a major antifungal compound as a polyketide, which prompted cloning of PKS genes. Ten PKS were fully or partially cloned and sequenced. Gene expression analyses indicate that the 10 PKS genes are differentially expressed in mycelia, with one of these, *MfPKS12*, the most highly expressed. Functional characterization of *MfPKS12* by gene silencing reveals that it contributes to DHN-melanin accumulation and virulence of *M. fructicola*.

MATERIALS AND METHODS

Fungal strains and cultivation. Fungal strains used in this study are listed in Supplementary Table S1. All strains were purified by single-spore isolation following culture from their original hosts or from peach orchard soils in Taiwan, except Mf13-81 (aka MUK-1), which was isolated from infected peach fruit in a California (U.S.A.) orchard (Bostock et al. 1999). Single-spore isolates (TW5-1, TW5-2 and TW5-4) were obtained from isolate TW5 cultured from a mummified peach fruit in Li Mountain, Taiwan. All *M. fructicola* strains used in this study were verified with internal transcribed spacer (ITS) sequences. TW5-4WM was isolated after two serial single-spore isolations, originating from a spontaneous albino sector of strain TW5-4 growing on a potato dextrose agar (PDA) plate (Supplementary Fig. S1). *Trichoderma*-like fungi were isolated from soils of peach orchards in Taiwan and purified by single-spore isolation. They grew and sporulated rapidly on PDA,

forming dark or light green colonies, with conidia produced from phialides on branched conidiophores. Determination as *Trichoderma*-like fungi was based on morphological characteristics, without further characterization to species. Fungal strains were maintained on V8 agar (16.3% V8 juice, 0.08% CaCO₃, and 1.5% agar), potato sucrose agar (PSA; 20% potato extract, 2% sucrose, and 1.5% agar), or modified *Sclerotinia* minimal medium (Lee and Bostock 2006a). All fungal pathogens were cultured at 23 to 24°C, except for *Fusarium oxysporum* f. sp. *phaseoli*, which was cultured at 28°C.

Germination, appressorium formation, and sporulation. Conidia were collected from 7- to 9-day-old fungal cultures on V8 agar (*M. fructicola*) or PSA (*P. digitatum* and *B. cinerea*) and resuspended in 1 ml of sterile water. Conidial suspensions were filtered through a Miracloth membrane (Cabiochem, U.S.A.) and adjusted to a desired concentration for further analysis. For germination and appressorium formation, *M. fructicola* conidial suspensions were washed twice as described previously (Lee and Bostock 2006b). Conidial suspensions containing 1 mM sucrose were placed on a petri dish (5 µl/drop; 1 × 10⁵ spores/ml), and germination and appressorium formation were observed 6 h postincubation under a light microscope. For sporulation, *M. fructicola* strains were cultured on V8 agar, and conidia were collected from each plate at 7 days postincubation and quantified using a hemocytometer.

Melanin quantitation. Melanin was quantified with slight modification of the protocol described by Babitskaya et al. (2000). Briefly, fungal strains were cultured atop cellophane membranes on V8 agar or PDA plates in the dark. Mycelia (100 mg) were collected from the membranes into tubes containing 0.5 N NaOH, and the tubes were placed in a 100°C water bath for 2 h. The supernatant was collected by centrifugation at 12,000 rpm for 10 min, and the pH was adjusted with HCl to 2.0 to precipitate melanin. The melanin pellets were collected by centrifugation at 12,000 rpm for 15 min. Melanin was resuspended in 0.5 N NaOH and measured with a spectrophotometer at 459 nm.

Disease assay. Rose petals (*Rosa* ‘Flaming Peace’), peach (*Prunus persica* ‘Tian Tao’), and nectarine (*P. persica* ‘Fantasy’) fruits were used to assess pathogenicity and virulence as described previously (Chiu et al. 2013; Yu et al. 2017). Lesion size was calculated by Spot image analysis (Spot RT Software version 4.6; Diagnostic Instruments, Inc., U.S.A.). Six rose petals and 5 to 10 fruits were used for one paired inoculation in each experiment. Two separate experiments were performed for the inoculation on each plant species.

Antimicrobial activity assay. To assess antagonism of different *M. fructicola* isolates against *B. cinerea* and *P. digitatum*, a mycelial agar disc (6 mm) of the target pathogen was placed at the center of a PSA plate and four mycelial agar discs (6 mm) of a *M. fructicola* isolate were each placed at four opposite positions at 2 cm distance from a target mycelial disc. In dual cultures, an *M. fructicola* isolate and target pathogen were placed 4 cm opposite each other on a PSA plate. In this assay, mycelial discs of *M. fructicola* isolates were from 8-day-old PSA cultures, while mycelial discs of the target pathogen were from 4-day-old PSA cultures. The plates were incubated at 24°C for 4 or 6 days. The clear zone formed between target pathogen and *M. fructicola* isolate was measured.

In a second assay format, 100 µl of spore suspension (2 × 10⁵ spores/ml) of target pathogen was sprayed on a PSA plate followed by placement of a mycelia-cellophane strip of *M. fructicola* (see below) at the center of the plate. Plates were incubated at 24°C for 4 to 6 days, and the zone of clearing surrounding the strip was measured. Using this same format, we tested mycelial extracts (see below) for antimicrobial activity by spraying a spore suspension of the target pathogen on a PSA plate followed by placement of a filter paper disc (8 mm; Advantec, Japan) containing 40 µl of extract on the plate. Zones of clearing around each paper disk were measured after incubation at 24°C for 4 to 6 days.

Antimicrobial compound extraction. *Monilinia fructicola* isolate TW5-4 was cultured on PSA plates topped with cellophane for various incubation periods. Mycelia-cellophane strips (1 × 5 cm) were excised to assay for antimicrobial activity as described above. Mycelia also were collected from the cellophane and ground to a powder, and immersed overnight in 50 µl of ethyl acetate (EA) per cm² of cellophane-mycelial mat. Insoluble material was then removed by centrifugation at 10,000 rpm for 5 min. The crude extracts were tested for antimicrobial activity on PSA plates. To isolate the antimicrobial compound(s), TW5-4 mycelia cultured on PSA for 10 days were collected and extracted three times with EA. The three EA extracts were combined and thoroughly mixed with an equal volume of deionized water, and the phases allowed to separate at 4°C overnight. The water and EA phases were collected separately and concentrated by freeze drying (FDU-1100, Eyela, Japan) or by a rotary evaporator (Vac V-500 [Switzerland] with Waterbath B-480, Rotavapor R240 and Vacuum controller B-721), respectively. The dried concentrates were weighed and then dissolved in DMSO or EA for bioactivity assays, or in EA for thin-layer chromatography (TLC) analysis.

Inhibitory activity of mycelial extracts on spore germination and growth. For spore germination inhibition of target pathogens, mycelial extracts were dissolved in DMSO and mixed with PDB (Difco) and spores (in water) to make a final suspension containing 2×10^5 spores/ml in 1/4-strength PDB and 0.1% DMSO. Ten microliters of the suspension was placed on a glass slide and incubated in a moist chamber at 25°C. Spore germination was determined after a 16 h incubation. Spores suspended in 1/4-strength PDB and 0.1% DMSO served as a control.

To assess growth inhibition of target pathogens, 100 µl of spore suspension (2×10^5 spores/ml) was sprayed on a PSA plate and an 8-mm filter disk containing 80 µl of EA extract was placed on the PSA plate and incubated at 25°C for 2 days. Growth of target pathogens on PSA plates containing filter disks to which 80 µl of EA solvent had been added served as a control. In both treatments, the EA solvent was allowed to evaporate from the disk prior to placement on the PSA plates.

TLC, HPLC, and liquid chromatography tandem mass spectrometry analyses. The EA extract of *M. fructicola* isolate TW5-4 was analyzed by TLC (Silica gel, 60F₂₅₄, Merck, Germany) with a developing solvent EA, hexane, and methanol (0.192:0.769:0.038; vol/vol/vol). The developed TLC plates were examined under UV (254 nm) or near-UV light (365 nm). For detection of phenolic compounds, plates were sprayed with a solution containing 2% FeCl₃ in 100% ethanol (Exarchou et al. 2002). To identify antimicrobial compounds, an on-the-chromatogram inhibition assay (OCIA) was performed as follows. A developed TLC plate was top-layered with 1 ml of semisolid PSA-spore suspension containing 100 µl of spore suspension (2×10^5 spores/ml) of target pathogen and 0.9 ml of PSA in which agar content was adjusted to 0.7%. After incubation at 24°C for 2 days, the plate was stained with lactophenol cotton blue solution (0.05 g of cotton blue, 20 ml of water, 20 g of phenol crystals, and 40 ml of glycerol). The silica gel subtending a clear zone indicating an area of antimicrobial activity was scraped from the plate and dissolved in EA or acetonitrile. Silica gel powder was removed by filtration through a nylon membrane. When dissolved in EA, usually less than 100 µl, the EA was removed in a dry bath in a fume hood and then resuspended in the same volume of DMSO for phytotoxicity assays. The acetonitrile solution was analyzed by using a LTQ XL linear ion trap mass spectrometer (Thermo Scientific) with electrospray ionization in positive-ion detection mode to identify the active compound. The dry EA extract of 54 mg, prepared from a collection of 440 PSA culture plates of TW5-4, was resolved, fractioned, and purified by silica gel column chromatography (1 × 25 cm, 70 to 230 mesh, ASTM; Merck Co.) in a benzene/EA (80:1) solvent system. The target fractions were collected and evaporated to yield chloromonilicin (8.2 mg), which was identified and verified by

high-resolution electro-spray ionization mass spectrometry (HRESIMS) and nuclear magnetic resonance (NMR) analyses.

Phytotoxicity assay. The EA extracts dissolved in DMSO were diluted with sterile water to bring the final DMSO concentration to 0.5%. The diluted DMSO-EA extracts were applied (5 µl per drop) to unwounded and needle-wounded petals and 0.5% DMSO solution was used as control. The petals were incubated in a moist chamber at 25°C.

Fungal nucleic acid extraction. *M. fructicola* Mf13-81 was cultured on cellophane-topped V8 agar plates (9 cm) at 23°C for 7 days in the dark. An appropriate amount of hyphae was scraped into a 1.5-ml microcentrifuge tube for genomic DNA extraction, as described previously (Lee et al. 2010). For RNA extraction, fungal strains were cultured on cellophane-topped V8 agar plates (9 cm) at 23°C for 6 days in the dark. Hyphae were scraped from the cellophane and RNA was extracted as described previously (Lee et al. 2010).

PKS gene cloning by degenerate PCR and inverse PCR. Partial sequences of polyketide synthase (PKS) genes were cloned by degenerate PCR and genomic DNA of *M. fructicola* Mf13-81 was used as template. Five sets of degenerate primers were designed to correspond to the conserved regions of the β-ketoacyl synthase domain (LC1/LC2c, LC3/LC5c) (Bingle et al. 1999), the C-methyltransferase domain (CMeT1/CMeT 3c) (Nicholson et al. 2001), the β-ketoacyl synthase domain (KAF1/KAR1), and the acyltransferase domain (KAF1/KAR2) (Amnuaykanjanasin et al. 2005). The primer sequences are listed in Supplementary Table S2. PCR conditions were set as follows: an initial reaction at 94°C for 2 min, 34 cycles of 94°C for 30 s, 60°C (varied temperature depending on primers used) for 40 s, and 72°C for 1 min, followed by an additional 5 min extension at 72°C. Amplified products were ligated to the pGEM-T easy vector (Promega, Madison, U.S.A.) and then transferred into *Escherichia coli* XL-1 blue (Stratagene, U.S.A.). Plasmids were isolated and the inserted fragments were sequenced. Approximately 300 to 900 bp sequences were obtained and the flanking sequences were further cloned by inverse PCR (Lee et al. 2010). Briefly, genomic DNA of Mf13-81 was digested with restriction enzymes *Eco*RI, *Eco*RV, *Xho*I, or *Hind*III, and then self-ligated by DNA ligase. After treatment with a PCR cleanup kit, the ligation mixture was diluted for PCR reaction with primers designed from the known sequence to amplify the flanking regions. If necessary, nested PCR was conducted. The PCR products were sequenced and assembled. The 10 sequences were submitted to GenBank (accession number MfPKS-LC1, MT095080; MfPKS12, MT095081; MfPKS-LC3, MT095082; MfPKS-LC4, MT095083; MfPKS-LC5, MT095084; MfPKS-KAF1, MT095085; MfPKS-KAF2, MT095086; MfPKS-KAF3, MT095087; MfPKS-KAF4, MT095088; and MfPKS-KAF5, MT095089).

Semiquantitative reverse transcription PCR (sq-RT-PCR). Fungal RNA was treated with DNase I and converted to cDNA by MMLV High Performance Reverse transcription (Epicentre, Wisconsin, U.S.A.) using Oligo-dT (5'-(T)25VN-3') as primer (Lee et al. 2010). sq-RT-PCR was conducted as described previously (Lee et al. 2010). Briefly, PCR reactions were performed in a total 50-µl reaction mixture, with *TUB2* used as a control to calculate the relative expression. All primers used in this assay are listed in Supplementary Table S3. The PCR program was set as follows: an initial reaction at 95°C for 2 min, 34 cycles of 94°C for 30 s, 60°C for 40 s, and 72°C for 40 s, followed by an additional 5 min extension at 72°C. PCR reaction mixtures were collected after 20, 25, 30, and 35 cycles, 7 µl per collection, and were visualized by electrophoresis using 1.2% agarose gel and staining with ethidium bromide. The band density was determined by Kodak ID Image Analysis Software. The PCR product amplified within the exponential stage was used to calculate band density ratios of PKS genes relative to the β-tubulin gene (*tub2*).

Bioinformatic analysis. The PKS sequences obtained were analyzed with the BLAST program of the National Center for

Biotechnology Information (NCBI) Spot RT Software version 4.6 (Diagnostic Instruments Inc., U.S.A.) to search the database for similar sequences. Protein function domain predictions were analyzed with InterProscan. The phylogenetic analysis was performed by CLUSTALW 1.18 (<http://workbench.sdsc.edu/>) of SDSC Biology Workbench 3.2 based on the highly-retained region C/DPxxR to ExHGHH within the sequence of the KS domain (approximately 222 to 233 amino acids).

Gene silencing and identification of silenced strains. Gene silencing vector pSilent-Dual1, kindly provided by Dr. Nakayashiki (Kobe University, Kobe, Japan) (Nguyen et al. 2008), was used to generate the *MfPKS12* silencing construct. Briefly, a 533-bp *MfPKS12* open reading frame (ORF) fragment was amplified using the primer pair LC2-6-B and LC2-6-R1 (Supplementary Table S3) and cloned into pSilent-Dual1. The *MfPKS12* silencing vector was transferred to *M. fructicola* Mf13-81 by CaCl_2 /PEG-mediated protoplast transformation as described previously (Chou et al. 2015; Lee et al. 2010). Putative transformants were screened by PCR for presence of the *hptII* gene, and five transformants carrying the silencing construct were used for further

analysis, including gene expression and phenotyping. To identify *MfPKS12* gene-silenced strains, transformants were cultured on PDA and V8 medium covered with one layer of cellophane. RNA was isolated from mycelia collected from 6-day-old cultures by using Qiagen RNeasy kit and treated with DNaseI to remove DNA. The RNA was then transferred to cDNA by the iScript cDNA synthesis kit (Bio-Rad Lab, Inc.). Relative expression level of *MfPKS12* was detected by quantitative RT-PCR (qRT-PCR) with the Bio-Rad Fast SYBR Green kit using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Inc.). The *tub2* gene of *M. fructicola* was used as internal control to calculate the relative expression level as described previously (Chou et al. 2015).

Functional characterization of *MfPKS12*-silenced strains. Fruits (mature, stage III) of nectarine and peach were used to assay strains for virulence. Five microliters of conidial suspension (100 conidia/ μl) of wild-type (WT) and *MfPKS12*-silenced transformants was inoculated on the same fruit with the WT strain on the left side and a transformant on the right side. Lesion size was calculated as described above. To assess strains for tolerance to hypertonic solutions, 10 μl of spore suspension (1×10^5 spores/ml) of WT and *MfPKS12*-silenced transformants was placed in a petri dish and incubated in the dark at 25°C for 12 h. The liquid was removed with a piece of filter paper and a 10- μl PEG8000 solution (chilled to 20°C) was applied to the spores. Morphology of fungal structures was observed under a light microscope after 10 min.

Analysis of experimental data. Experiments were conducted at least twice with at least three replicates of each treatment within each experiment, except as noted otherwise. Means comparisons in disease assays were determined by using paired *t* test ($P \leq 0.05$). The significance of differences among treatments in other experiments was determined by analysis of variance and means separation by Tukey's analysis ($P \leq 0.05$) using Statistical Package for the Social Sciences software, version 20 (IBM SPSS software).

RESULTS

***M. fructicola* isolates inhibit growth of other fungi.** In a screen to select *Trichoderma*-like fungi as potential antagonists of *M. fructicola*, we found that *M. fructicola* produces metabolites into the medium that inhibit the growth of other fungi, including *Botrytis cinerea* and *Penicillium digitatum* (Fig. 1, Supplementary Fig. S1). An atypical, darkly pigmented isolate, TW5-4, confirmed to be *M. fructicola* by ITS sequence, exhibited strongest suppression of growth of multiple fungal pathogens (Fig. 1, Table 1), including *M. fructicola* WT isolate Mf13-81 (Fig. 1A). TW5-4 was isolated from a diseased peach fruit from an orchard located at high elevation. The diseased fruit was partially mummified, while a portion of the fruit remained healthy, suggesting that TW5-4 colonization had ceased or was suppressed. TW5-4 and an albino mutant, TW5-4WM, purified from a colony sector of TW5-4, were further characterized and compared with our extensively studied WT Mf13-81 (aka MUK-1) isolate (Chiu et al. 2013; Chou et al. 2015; Lee and Bostock 2006a, b, 2007; Lee et al. 2010; Yu et al. 2017).

Growth, morphology, and pathogenicity of TW5-4. Growth and colony morphology of the three *M. fructicola* isolates were remarkably different on PDA and V8 agar media. Mf13-81 produces light brown aerial hyphae, while TW5-4W forms a pale white colony. However, TW5-4 displayed extremely slow growth and produced dark colonies (Supplementary Fig. S2) and fewer conidia, and had lower germination and appressorium formation rates than the other two isolates (Table 2). Disease assays revealed that TW5-4 produced few or no lesions on rose petals and on peach and apple fruits (Supplementary Fig. S3 and data not shown). TW5-4 accumulated significantly higher amounts of melanin than

Fig. 1. Antagonism of *Monilinia fructicola* isolates to *Botrytis cinerea* strain BT-1 and *Penicillium digitatum* strain DOB-2. **A**, Dual culture of *M. fructicola* isolate TW5-4 (right) showing zone of inhibition against isolate Mf13-81 (left), 10 days postinoculation. **B**, Coculture of *M. fructicola* isolate Mf13-81 (left) or TW5-4 (right) to DOB-2, 6 days postinoculation, in which DOB-2 was inoculated at the center and surrounded with four mycelial plugs of TW5-4 or Mf13-81. **C**, Antagonism of various *M. fructicola* isolates to BT-1 and DOB-2. Zones of inhibition were measured as the average distance between the colony edges of an *M. fructicola* isolate and the target fungus, 6 days postinoculation.

Mf13-81 and TW5-4WM at 6 days postinoculation (Supplementary Fig. S4).

Antimicrobial activity of *M. fructicola* isolates. To further characterize the observed antimicrobial activity, TW5-4, Mf13-81, and TW5-4WM were individually cultured on a cellophane membrane placed atop PSA medium. After different culture periods, the mycelia-cellophane strip was used to assay antibiotic activity against *P. digitatum*, and results showed that 6- to 12-day-old cultures of TW5-4 inhibited *P. digitatum* colony development (data not shown). Cellophane membrane/agar cultures of Mf13-81 and TW5-4WM were only weakly antagonistic to *P. digitatum*. Therefore, antibiotic compounds were extracted by EA from mycelia of cellophane/agar cultures. EA extracts of TW5-4 mycelia collected from 6-day-old cellophane/agar cultures had detectable antimicrobial activity against *P. digitatum* that increased to a maximum in 10-day-old cultures (Fig. 2A and B). EA extracts of Mf13-81 and TW5-4WM mycelia collected from 4- to 12-day-old cultures had no obviously detectable antimicrobial activity against *P. digitatum* (Fig. 2A). *B. cinerea* was less sensitive than *P. digitatum* to TW5-4 EA extracts (Fig. 2B). The TW5-4 EA extracts also inhibited spore germination of *P. digitatum*, with extract from 10-day-old cultures causing more than 70% inhibition at 0.5 ppm (based on dry weight of the crude extract) and 100% inhibition at 5 ppm (Fig. 2C). Spore germination of *B. cinerea* was not inhibited by 50 ppm of the EA extract, but was completely inhibited by 100 ppm of the EA extract (data not shown). TW5-4 mycelia cultured for 10 to 12 days were collected for antimicrobial compound purification.

Antimicrobial compound purification. Compounds in EA extracts were separated by TLC and examined with UV light at 254 and 365 nm. To detect antimicrobial compounds on the TLC plate, OCIA was conducted as described in the Materials and Methods. As shown in Figure 3, the antimicrobial activity localizes to a position with Rf 0.28 when separated with EA/hexane (1:4; vol/vol), or at Rf 0.45 when separated with EA/hexane/methanol (0.192:0.769:

0.038; vol/vol/vol). Phenolic compounds, detected with FeCl₃ reagent, comigrated to the same position with antimicrobial activity (Fig. 3). The antimicrobial compounds were separated and recovered from the TLC plate and analyzed with LTQ XL mass spectrometry. Under positive charge conditions, two dominant peaks were detected with [M+H] at 347 and 351, indicating the two compounds have molecular weights of 346 and 350, respectively (Supplementary Fig. S5).

The two compounds were consistent with chloromonilicin and its methanolysis product (II), as reported previously (Sassa et al. 1989), since the TLC solvent contained methanol. To prevent methanolysis during further compound purification, a solvent system of benzene/EA was used to reconstitute the dry crude EA extract and in the following chromatographic fractioning and purification. Target fractions were collected to afford chloromonilicin, which was further identified and verified with HRESIMS and ¹H NMR analyses (Fig. 4). The two major peaks of M.W. 351 [M+H] and 373 [M+Na] in the HRESIMS were assigned to chloromonilicin while no obvious M.W. peaks, 347 [M+H] or 369 [M+Na], were detected for the proposed methanolysis product observed in the original TLC separation. The obtained NMR assignment was as follows. Chloromonilicin: ¹H NMR (400 MHz, CDCl₃): 2.46 (s, 3H), 3.83 (s, 3H), 6.45 (s, 1H), 6.76 (d, J = 0.8 Hz, 1H), 6.87 (d, J = 0.8 Hz, 1H), 6.90 (s, 1H), 11.70 (s, 1H), and MS (ESI) m/z 373 (M + Na)⁺.

Phytotoxic activity of TW5-4 mycelial extracts. To assess the potential toxicity of TW5-4 extracts on host plants, antimicrobial compounds isolated by TLC-OCIA were applied to intact and wounded rose petals. When treated with 40 µg per 5-µl drop,

TABLE 1. The antagonistic activity of *Monilinia fructicola* strain TW5-4 to various fungal pathogens

Fungal species	Strain	Radius of inhibition zone (mm)
<i>Alternaria solani</i>	AS01	No inhibition
<i>Botrytis cinerea</i>	Bt10	6.5 ± 0.5
<i>Colletotrichum acutatum</i>	Coll-153	No inhibition
<i>Colletotrichum capsici</i>	470	No inhibition
<i>Colletotrichum higginsianum</i>	BC04	6.0 ± 0.6
<i>Colletotrichum gloeosporioides</i>	Gou11	No inhibition
<i>Colletotrichum gloeosporioides</i>	CO-1	2.7 ± 0.9
<i>Fusarium oxysporum</i> f. sp. <i>phaseoli</i>	CYC	No inhibition
<i>Magnaporthe grisea</i>	186	2.6 ± 0.57
<i>Penicillium digitatum</i>	DOB-2	8.0 ± 2.0
<i>Rhizoctonia solani</i>	RN-1	2.7 ± 0.4
<i>Sclerotium rolfsii</i>	JWH	No inhibition

TABLE 2. Sporulation, germination and appressorium formation of three *Monilinia fructicola* strains

Strain	Sporulation (×10 ⁵) ^a	Germination (%) ^b	Appressoria formation (%) ^b
Mf13-81	96.6 ± 3.4 ^c	96.1 ± 0.1	82.5 ± 5.1
TW5-4	0.6 ± 0.1	75.9 ± 5.5	16.2 ± 3.1
TW5-4WM	65.3 ± 0.01	94.0 ± 0.1	73.5 ± 5.5

^a Spores were collected from each strain cultured on a 9-cm V8 agar plate 7 days after inoculation.

^b Spore germination and appressoria formation were measured by placing 500 spores in water containing 1 mM sucrose on a petri dish after 6 h incubation at room temperature.

^c Means and standard errors of three replicates are presented.

Fig. 2. The effect of culture age on antimicrobial activity of *Monilinia fructicola* strains Mf13-81, TW5-4, and TW5-4WM. **A**, Antimicrobial activity against *Penicillium digitatum* of ethyl acetate (EA) extracts of mycelia of *M. fructicola* strains Mf13-81, TW5-4, and TW5-4WM collected 4, 6, 8, 10, and 12 days postinoculation (DPI) on potato sucrose agar (PSA). Eighty microliters of EA extract from 1.6 cm² of cellophane-mycelial mat of each strain was applied to a filter paper disc in the pattern shown to assess antibiotic activity against *P. digitatum*. **B**, Antimicrobial activity against *P. digitatum* and *Botrytis cinerea* of EA extracts of TW5-4 mycelia collected 4, 6, 8, 10, and 12 days postinoculation (DPI) on PSA. Cellophane-mycelial mats were extracted with 50 µl of EA/cm², and 80 µl of the crude extract was applied to the filter paper disc for the assay. **C**, Inhibition of spore germination of *P. digitatum* by EA extract of TW5-4 mycelia from a 10-day culture on PSA. Concentrations are based on the dry weight of the crude extract.

wounded petals developed necrotic lesions within 24 h, while no significant damage was observed on the control treatment (Supplementary Fig. S6). Necrotic lesions had not expanded beyond the site of application at 48 h.

PKS gene cloning and phylogenetic analysis. Production of chloromonilicin, a polyketide, should require a PKS. To better understand regulation of chloromonilicin biosynthesis, PKS genes were amplified from genomic DNA of *M. fructicola* Mf13-81 using degenerate PCR. Twelve PKS partial sequences were cloned, and flanking regions of some of the partial sequences were further identified by inverse PCR. After sequence assembly, 10 gene fragments were obtained and named *LC1*, *LC2*, *LC3*, *LC4*, *LC5*, *KAF1*, *KAF2*, *KAF3*, *KAF4*, and *KAF5* (Supplementary Table S4). Nucleotide sequence query of these fragments in the NCBI database by blastx revealed best hits ranging from 63 to 95% identity to known PKS genes (Supplementary Table S5). The full-length (6,690 bps) ORF of *LC2* contains five introns with sizes 48 to 59 bps and encodes a protein with 2,144 amino acids (Supplementary Table S4). The functional domains of *LC2* were predicted with InterProScan.

LC2 is a nonreducing PKS containing the starter unit ACP (acyl-carrier protein) transacylase (SAT), beta-ketoacyl synthase (KS), acyl transferase (AT), polyketide product template (PT), phosphopantetheine-binding (P), and thioesterase (TE) domains (Supplementary Fig. S7). Amino acid sequence determination using blastp shows that *LC2* is highly similar to BcPKS12 in *Botrytis* and *Sclerotinia* species, with over 87% identity and 100% coverage (Supplementary Fig. S7). Both *LC2* and BcPKS12 appear to have the same functional domain arrangement (Supplementary Fig. S7). Therefore, *LC2* was designated MfPKS12. The complete ketoacyl transferase domain (DxxFF to ExHGTG; KS) was obtained from seven (*LC1*, *LC2*, *LC3*, *LC4*, *LC5*, *KAF2*, and *KAF4*) PKS genes. Based on amino acid sequences of the KS domains, phylogenetic relationships of seven PKSs and PKSs from other fungi were compared. These comparisons reveal that *LC1* and *LC2* are closely related to PKSs involved in melanin biosynthesis, while *LC3*, *LC4*, and *KAF2* are associated with production of toxins (Supplementary Fig. S8).

PKS gene expression. To analyze expression of the 10 PKS genes in the three *M. fructicola* strains, the three strains were cultured on cellophane disks atop V8 agar for 6 days. RNA was extracted from the mycelia and gene expression assessed by sq-RT-

PCR. Mf13-81 and TW5-4 expressed higher levels of *LC1* and *LC2* (*MfPKS12*) than TW5-4WM, while TW5-4 expressed slightly higher levels of *LC5* and *KAF3* than Mf13-81 and TW5-4WM (Fig. 5). These results suggest that *LC5* and *KAF3* are associated with chloromonilicin production.

Functional characterization of MfPKS12 by gene silencing. To begin to identify PKS genes involved in production of chloromonilicin and other polyketides in *M. fructicola*, we attempted to overexpress or knockout PKS genes in TW5-4WM and TW5-4. However, DNA transformation in these strains either by *Agrobacterium tumefaciens*-mediated transformation or by protoplast/PEG-mediated transformation was unsuccessful. Although Mf13-81 (WT) does not display obvious antibiotic activity, we selected it for functional studies because of its well-established transformation system (Chiu et al. 2013; Chou et al. 2015; Lee and Bostock 2006a, b, 2007; Lee et al. 2010; Yu et al. 2017). In our experience, gene disruption by homologous replacement in multinucleate *M. fructicola* results in incomplete or partial phenotypes for the target of interest (Lee and Bostock 2006a; Chou et al. 2015; Yu et al. 2017). However, gene knockdown by silencing in Mf13-81 was performed successfully in a previous study (Yu et al. 2017). We selected the *LC2* (*MfPKS12*) gene for targeting in functional studies because we obtained a complete ORF and it was expressed at relatively higher levels compared with other PKS isoforms in the collection. Although *KAF5* also showed strong expression, we only obtained a partial sequence for this isoform. Putative silenced transformants were screened by PCR for presence of the hygromycin resistance gene (*hptII*) and pSD-MfPKS12. Single-spore isolates of five transformants (*LC2*-6, -8, -11, -15, and -17) carried the *hptII* amplifier. *MfPKS12* expression was further analyzed by qRT-PCR in pSD-MfPKS12 transformants in culture. At 6 and 12 days of growth on PDA and V8 agar, only transformant *LC2*-17 had markedly reduced expression of *MfPKS12* compared with Mf13-81. In the other transformants, *MfPKS12* transcript levels were similar to those of Mf13-81 (Fig. 6).

The color of *LC2*-17 colonies appeared lighter than WT on PDA and SMM (Supplementary Fig. S9). Although melanin accumulated in *LC2*-17 mycelium, the levels were much less than in the WT mycelium or the other *LC*-2 transformants (Fig. 7). To determine if *M. fructicola* spore and appressorium turgor is affected by melanin accumulation, spore germlings were treated with PEG8000. Spores and appressoria of *LC2*-17 collapsed in the presence of 30% PEG,

Fig. 3. Characterization of antimicrobial compound(s) from mycelial extracts of *Monilinia fructicola* TW5-4 by thin layer chromatography (TLC). Mycelia were collected from cellophane topped on potato sucrose agar for 10 days and extracted with ethyl acetate (EA). Three replicate EA extracts were applied to a TLC plate and examined under **a**, UV light (254 nm) and **b**, near-UV light (365 nm) after development. **c**, The plate then was sprayed with 2% FeCl_3 for phenolic compound staining or covered with agarose containing spore suspensions of **d**, *Penicillium digitatum* or **e**, *Botrytis cinerea* to visualize zones of antimicrobial activity. A band ($R_f = 0.28$, indicated with arrows) that stained with 2% FeCl_3 showed antimicrobial activity.

while LC2-15 showed deformation in the presence of 35% PEG (Fig. 8). However, spore/appressorial structures of Mf13-81 and transformants LC2-6 and LC2-8, which had WT melanin levels, maintained turgor and shape up to 35% PEG, but collapsed at 40% PEG (Fig. 8). No notable differences were observed in germination and appressorium formation between WT and the LC2 transformants. In addition, LC2-17 displayed similar growth and sporulation in culture to WT (Supplementary Fig. S10). However,

LC2-17 was less virulent on nectarine and peach fruit compared with Mf13-81 (Table 3).

DISCUSSION

In this study, we describe a *M. fructicola* strain, TW5-4, that displays strong antimicrobial activity, weak growth, and low virulence, but accumulates high levels of melanin. We determined

Fig. 4. High-resolution electro-spray ionization mass spectrometry (HRESIMS) and proton nuclear magnetic resonance (¹H NMR) spectra for the purified chloromonilicin. **A**, Chloromonilicin structure and HRESIMS profile; and **B**, ¹H NMR profile.

that the secreted antibiotic is the polyketide, chloromonilicin, first reported from benomyl-resistant *M. fructicola* strains isolated from cherry fruits (Sassa et al. 1985). In Taiwan, *M. fructicola* is widely distributed in peach orchards at elevations between 400 to 2000 m. During screening of potential biocontrol microorganisms to inhibit growth of *M. fructicola*, we discovered *Monilinia* isolates able to inhibit the growth of various *Trichoderma*-like fungi. TW5-4 is one of these isolates that expresses strong antibiosis toward other fungal plant pathogens as well. In addition, TW5-4 inhibits WT isolates of *M. fructicola*, and grows very slowly compared with strains expressing low antimicrobial activity, suggesting self-inhibition. The principal antimicrobial compound detected by the OCIA using *Penicillium* as the indicator was chloromonilicin. Chloromonilicin and the related phenolic polyketides monilidiol, dechloromonilidiol, and bromomonilicin were shown to inhibit bacterial and fungal human pathogens, including *Candida albicans* and *Trichophyton* sp. (Kachi and Sassa 1986; Sassa et al. 1983). Monilidiol was reported to be phytotoxic on cherry leaves and to inhibit *Staphylococcus aureus*, while bromomonilicin showed a similar antimicrobial spectrum as chloromonilicin. TW5-4 may also produce monilidiol, dechloromonilidiol, and bromomonilicin. However, these were not apparent by OCIA, perhaps overshadowed by the abundance of chloromonilicin, and were not pursued further.

Fig. 5. Semiquantitative RT-PCR analysis of polyketide synthase (PKS) gene expression of *Monilinia fructicola* Mf13-81, TW5-4, and TW5-4WM on V8 agar, 6 days postinoculation. **A**, Colony morphology of the three strains; **B**, antimicrobial activity of the three strains against *Penicillium digitatum* DOB2; and **C**, expression of PKS genes relative to the expression of the *Tub2* gene in each strain. Means and standard errors from two independent experiments are presented.

Compared with Mf13-81 and the spontaneous albino mutant, TW5-4WM, TW5-4 displays reduced growth (Supplementary Fig. S2), sporulation, germination, appressorium formation (Table 2), and virulence (Supplementary Fig. S3), while overproducing both melanin (Supplementary Fig. S4) and chloromonilicin (Figs. 2, 3, and 4). Appressoria are important virulence factors in *M. fructicola* (Lee and Bostock 2006b), and the reduced appressorium formation observed with TW5-4 may contribute to its low virulence. Melanin is a virulence factor in many pathogenic fungi (Butler and Day 1998; Ludwig et al. 2014; Schumacher 2016). The lower virulence of TW5-4WM compared with Mf13-81 is consistent with its low melanin content. Chloromonilicin and DHN-melanin may share the same PKS at their biosynthetic initiation. Misregulation at this early step may explain the overproduction of chloromonilicin and DHN-melanin observed in the TW5-4 mutant. Although we demonstrated

Fig. 6. Semiquantitative RT-PCR analysis for relative gene expression of *Monilinia fructicola* transformants carrying an *MfPKS12* gene-silencing construct (LC2-6, -8, -11, -15, and -17) on **A**, V8 agar medium 6 and 12 days postinoculation (DPI), and **B**, PDA medium 12 DPI. Means and standard errors from two independent experiments are presented.

phytotoxicity of mycelial extracts of TW5-4 on rose petals, the concentrations applied were high and bring into question the physiological relevance in the plant–microbe interaction of the phytotoxic material.

PKS genes are abundant in many pathogenic fungi, such as the family of 16 PKS genes in *B. cinerea*, 22 in *Magnaporthe oryzae*, and 11 to 24 in various *Fusarium* spp. (Amselem et al. 2011; Hansen et al. 2015). Genomes of several *Monilinia* species have been sequenced, and annotation of the *M. fructigena* genome has recently become available in the NCBI database (Landi et al. 2018; Naranjo-

Ortíz et al. 2018; Riveraet et al. 2018). Ten PKS proteins are encoded in the *M. fructigena* genome based on searching the NCBI database with “*Monilinia polyketide synthase*” as key words. Using degenerate and inverse PCR, we identified 10 PKS genes in the Mf13-81 strain of *M. fructicola*. However, it is possible that more than 10 PKS genes are present in the genomes of *M. fructicola* and other *Monilinia* species. Phylogenetic analysis based on amino acid alignments revealed that LC1 and LC2 (MfPKS12) are closely related to BcPKS13 and BcPKS12, respectively. Both BcPKS13 and BcPKS12 have been shown to be involved in DHN-melanin biosynthesis in *B. cinerea* (Schumacher 2016). LC3, LC4, and KAF2 are closely related to PKSs involved in biosynthesis of ACT-toxin and AF-toxin in *Alternaria alternata* and botcinic acid in *B. cinerea* (Dalmais et al. 2011; Meena et al. 2017).

MfPKS12 expression is very high relative to other putative PKS genes in both Mf13-81 and TW5-4. The *MfPKS12*-silenced strain, LC2-17, had very low melanin content on PDA, consistent with a role for *MfPKS12* in DHN-melanin biosynthesis in hyphae of *M. fructicola*. There was no difference in antimicrobial activity between LC2-17 and WT, suggesting that *MfPKS12* is not involved in chloromonilicin biosynthesis. Nonetheless, LC2-17 was less

Fig. 7. Melanin accumulation of *Monilinia fructicola* transformants carrying an *MfPKS12* gene-silencing construct (LC2-6, -8, -15, and -17) and the wild-type Mf13-81. Melanin was extracted from 100 mg of mycelia on potato dextrose agar topped with cellophane at 6, 9, 12, and 15 postinoculation days (DPI) and quantified by a spectrophotometry at 459 nm.

TABLE 3. Lesion sizes in nectarine and peach fruits inoculated with wild-type (WT) strain Mf13-81 of *Monilinia fructicola* and LC2 transformants carrying an *MfPKS12* gene-silencing construct, 72 h postinoculation

		Lesion size (cm ²)				P value ^a
Experiment	Fruit	WT	Means	Transformant	Ave	
1	Nectarine	Mf13-81	4.5	LC2-6	4.0	0.13
		Mf13-81	4.1	LC2-11	3.7	0.12
		Mf13-81	4.6	LC2-15	3.3	0.02
2	Nectarine	Mf13-81	4.3	LC2-17	3.0	<0.01
		Mf13-81	2.7	LC2-6	2.3	0.25
		Mf13-81	3.0	LC2-15	2.0	0.07
3	Peach	Mf13-81	3.9	LC2-17	2.5	<0.01
		Mf13-81	0.9	LC2-11	1.3	0.15
		Mf13-81	1.7	LC2-17	0.2	0.01

^a Reported *P* values are based on a paired *t* test.

Fig. 8. Morphology of spore germlings of *Monilinia fructicola* wild-type strain Mf13-81 and transformants carrying an *MfPKS12* gene-silencing construct under water or varying concentrations of PEG8000. Collapsed cells are indicated with arrows. Bar in each panel indicates 10 μ m.

virulent than WT in disease assays. Melanin contributes to environmental stress tolerance and survival of fungi (Fujii 2010). In our study, hypertonic PEG treatment collapsed and deformed spores and appressoria of *M. fructicola*. However, cells of the WT strain maintained form and function in the presence of higher PEG concentrations than the melanin-deficient LC-2 strains, consistent with the role of DHN-melanin in classic studies of *Magnaporthe oryzae* appressoria (Howard and Ferrari 1989). It follows that MfPKS12, through its putative role in DHN-melanin synthesis, contributes to cell wall integrity and turgor adjustment in spores and appressoria.

M. fructicola continues to be one of the most important fungal pathogens affecting stone fruit production throughout the Americas, Africa, Asia, Oceania, and more recently, Europe. The recently published high-quality draft genome of this pathogen (De Miccolis Angelini et al. 2019) is a resource that will help further understanding of genes and molecules critical for pathogenesis as well as growth and survival during other stages of its disease/life cycle.

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