Identification of Genes Potentially Involved in the Biocontrol Activity of *Pseudozyma flocculosa*

G. Marchand, W. Rémus-Borel, F. Chain, W. Hammami, F. Belzile, and R. R. Bélanger

First, second, third, fourth, and sixth authors: Département de Phytologie, Centre de recherche en horticulture, Pavillon de l'Environnement, and fifth author: Département de Phytologie, Pavillon C. E.-Marchand, Université Laval, Québec, Québec, G1V 0A6, Canada.
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ABSTRACT

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Flocculosin is an antifungal cellobiose lipid linked to the biocontrol activity of *Pseudozyma flocculosa* and whose structure is very similar to that of ustilagic acid produced by *Ustilago maydis*. In this work, homologs of the *U. maydis cyp1* gene, involved in the biosynthesis of ustilagic acid, were isolated and sequenced from *P. flocculosa* and *P. fusiformata*, the latter species being also known to produce ustilagic acid. Interestingly, no homologs were found in four other closely related *Pseudozyma* spp. from which no evidence of ustilagic acid production has ever been obtained, thus supporting the specificity of *cyp1* with ustilagic acid synthesis. In addition, a homolog of the *U. maydis uat1* gene involved in the acetylation of the molecule and located next to the *cyp1*

gene was partially sequenced from *P. flocculosa*. All three newly sequenced genes showed strong sequence similarity to their counterparts in *U. maydis*. *Cyp1* expression was monitored in conditions that were either conducive or repressive to flocculosin production. Expression increased markedly (>100×) when *P. flocculosa* was inoculated in a growth medium conducive to flocculosin production but was rapidly downregulated in a repressive medium (in vitro) or on powdery mildew-infected cucumber leaves (in vivo). This suggests that the molecule was preferentially synthesized early in the process of searching for a growth substrate. This study provides the first identification of genes involved in the production of flocculosin, a molecule potentially associated with the biocontrol properties of *P. flocculosa*.

Additional keywords: biocontrol agent, gene expression, quantitative reverse-transcription PCR.

Pseudozyma flocculosa (Traquair, L. A. Shaw & Jarvis) Boekhout & Traquair (syn.: *Sporothrix flocculosa*) is a biocontrol agent (BCA) with particular activity against powdery mildews. This yeast-like epiphyte was first described as an ascomycete (26) and later reclassified as a basidiomycete related to anamorphs of the Ustilaginales (3,6). The intrinsic biocontrol properties of *P. flocculosa* have led to the study of its mode of action against powdery mildews. From the very beginning, it was evident that *P. flocculosa* was neither a strong competitor in the phyllosphere nor a direct hyperparasite, and that direct contact between the biocontrol agent and target powdery mildew was not required (10). Powdery mildew cells exposed to *P. flocculosa* were plasmolyzed rapidly and, thus, antibiosis was proposed as the main mode of action (9). Isolation and characterization of active compounds were then instigated (2,4,5,11), which led to the discovery of flocculosin (7). This molecule, an unusual glycolipid with strong antimicrobial activity (17), is believed to be a key factor in the biocontrol properties of *P. flocculosa*.

In an effort to identify genes involved in the biocontrol activity (or flocculosin synthesis) of *P. flocculosa*, random insertional mutagenesis was attempted (7). Even though mutants altered in their antagonistic properties and flocculosin production were obtained, genetic analysis of insertions remained inconclusive due to the high number of tandem insertions.

One breakthrough in efforts to understand the role of flocculosin in the biocontrol activity of *P. flocculosa* came from

the discovery that its chemical structure was nearly identical to that of ustilagic acid produced by the model plant pathogen *Ustilago maydis* (DC.) Corda (Fig. 1). *U. maydis* is one of the first fungal pathogens to be sequenced and its complete genome is now publicly available (14). Using a reverse genetics approach, Hewald et al. (13) identified a gene essential for the production of ustilagic acid. This gene, *cyp1*, codes for a putative P450 monooxygenase that is believed to catalyze the terminal hydroxylation of hexadecanoic acid to generate the rare 15-16-dihydroxy palmitic acid, the essential precursor for ustilagic acid biosynthesis, and, thus, would be the first active enzyme in the biochemical pathway for ustilagic acid synthesis (24). Expression studies of genes located near *cyp1* revealed a cluster of coregulated genes involved in ustilagic acid synthesis, including *uat1*, located downstream of *cyp1* and believed to code for an acyl/acetyltransferase involved in acetylation of ustilagic acid.

Given that *P. flocculosa* and *U. maydis* are very closely related genetically and given that flocculosin and ustilagic acid share the same unusual chemical structure, we surmised that the production of flocculosin in *P. flocculosa* must share a common biochemical pathway with that of ustilagic acid in *U. maydis*. If this hypothesis holds true, this could lead to the identification of genes involved in flocculosin synthesis and, by extension, to the biocontrol activity of *P. flocculosa*. In addition, considering that another species of *Pseudozyma*, *P. fusiformata* (Buhagiar) Boekhout, also produces ustilagic acid (15), we were interested in assessing the genetic basis for synthesis of these unusual molecules by screening other species within the genus. Accordingly, the objective of this study was to search for homologs of *U. maydis* genes involved in ustilagic acid synthesis in related *Pseudozyma* spp., as well as to monitor expression of key genes in the biosynthetic pathway should a homolog be found in the

Corresponding author: R. R. Bélanger;
E-mail address: richard.belanger@plg.ulaval.ca

BCA *P. flocculosa* in conditions of flocculosin production and biocontrol activity.

MATERIALS AND METHODS

Fungal material. *P. flocculosa* (CBS 167.88) and *U. maydis* (DSM 4500) were maintained on potato dextrose agar (PDA) at 4°C. In addition, *P. fusiformata*, reported to produce ustilagic acid, and four other *Pseudozyma* spp. presumed not to produce the molecule—*P. rugulosa* (Traquair, L. A. Shaw & Jarvis) Boekhout & Traquair (CBS 170.88), *P. antarctica* (Goto, Sugiyama & Iizuka) Boekhout (CBS 516.83), *P. tsukubaensis* (Onishi) Boekhout (CBS 6389), and *P. aphidis* (Henninger & Windisch) Boekhout (CBS 517.83)—were tested for the presence of the *cyp1* gene and maintained under the same conditions. Unless indicated otherwise, fungi were cultured in YMPD broth. All culture media ingredients were supplied by Difco (BD Biosciences, Mississauga, Ontario, Canada). *Podosphaera xanthii* (Castagne) U. Braun & Shishkoff was grown and maintained on cucumber plants (cv. Corona) in a greenhouse as described by Neveu et al. (19).

Culture conditions for *cyp1* gene expression analysis and flocculosin production experiments. Primary cultures of *Pseudozyma flocculosa* were obtained by inoculating 100 ml of YMPD broth in a 500-ml baffled flask with one bottle of lyophilized culture previously hydrated with 3 ml of sterile water. Seed cultures were prepared in triplicate by adding 5 ml of a 3-day-old primary culture to either 100 ml of YMPD broth repressive to flocculosin production or 100 ml of buffered basic culture medium (MOD medium) (12) conducive to flocculosin production. Two 1-ml aliquots were taken at 0, 4, 8, 12, and 24 h after inoculation in repressive conditions and at 0, 4, 8, 12, 24, 48, 72, 96, and 120 h after inoculation in conducive conditions. The first 1-ml aliquot was centrifuged at $3,000 \times g$ for 15 min, the supernatant was removed, and cells were frozen in liquid nitrogen until RNA extraction. The second 1-ml aliquot was used to quantify flocculosin production.

For the experiment on infected cucumber leaf disks, primary cultures of *P. flocculosa* were prepared as previously described. After centrifugation, cells were resuspended in saline (NaCl 0.9%) and cell titer was adjusted to 5×10^6 cells/ml with a hemacytometer. As described by Neveu et al. (19), the cell suspension was then sprayed evenly over 20-mm discs excised from leaves of cucumber plants infected with *Podosphaera xanthii*. The treated leaf discs were placed in petri dishes, which were sealed with parafilm and incubated in a growth chamber with a photoperiod of 16 h at 22 to 20°C with a photosynthetic photon flux of $\approx 275 \mu\text{mol}/\text{m}^2 \text{ s}$ as described by Hajlaoui and Bélanger (9). Three foliar discs were harvested at 0, 2, 4, 6, 24, 48, 72, and 96 h after being sprayed with the fungal suspension. Individual discs were placed in microtubes, frozen in liquid nitrogen, and then stored at -80°C until RNA extraction.

DNA and RNA extraction. Fungal genomic DNA was isolated from 3- to 5-day-old cultures in potato dextrose broth using the Genomic Tip 20/G or 100/G kits (Qiagen, Mississauga, Ontario, Canada). Total RNA was isolated from the fungal biomass of *Pseudozyma flocculosa* and *P. fusiformata* grown in flocculosin-conducive MOD medium (12) and from infected cucumber leaf discs inoculated with *P. flocculosa* using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Bacteriophage DNA was isolated as described by Santos (22) based on phage precipitation with zinc chloride.

Southern blot analysis of the *cyp1* gene in *Pseudozyma* spp. Genomic DNA (10 μg) was digested with *Bam*HI, separated electrophoretically on a 1% agarose gel, and transferred onto Hybond N+ nylon membrane (Amersham Biosciences UK Ltd., Little Chalfont Bucks, UK) using a VacuGene XL vacuum blotting system (Amersham) according to the manufacturer's

instructions. The open reading frame (ORF) of the cloned *U. maydis cyp1* gene (donated by M. Böcker, Universitaet Marburg, Germany) was labeled with ^{32}P using the Rediprime II random prime labeling system (Amersham); hybridized to the Southern blot overnight at 65°C in $2\times \text{SSC}$ ($1\times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate), 0.25% dry milk, and 0.5% sodium dodecyl sulfate (SDS); and washed at 65°C in $2\times \text{SSC}$ and 0.1% SDS.

Construction of a *P. flocculosa* genomic DNA library. As described by Neveu et al. (18), a *P. flocculosa* genomic DNA library was constructed using *Bam*HI-digested genomic DNA in the lambda Fix-II replacement vector (Stratagene, La Jolla, CA) following the manufacturer's protocol. For screening of this library, plate lifts were performed on Hybond N+ nylon membrane (Amersham) according to the manufacturer's instructions. The cloned *U. maydis cyp1* gene ORF was used as a probe as above. A lambda clone containing a 15-kb insert was selected for sequencing. This clone was digested with *Sac*I and *Sal*I and restriction fragments were subcloned in pBluescript II KS(+) (Stratagene) and sequenced using an ABIPrism 3130XL sequencer (Applied Biosystems, Foster City, CA).

Northern transfer and hybridization. Total RNA (3 μg) extracted from *P. flocculosa* grown in MOD medium conducive to flocculosin production was separated electrophoretically on a 1% (wt/vol) agarose gel containing 5.4% formaldehyde in MOPS $1\times$ buffer. Total RNA was transferred onto Hybond N+ nylon membrane as above. A 412-bp polymerase chain reaction (PCR) product of the *P. flocculosa cyp1* gene obtained with primers 5'-ACGGCTACGGCAACACGC-3' and 5'-GGCTGGTCGGGCTTGGAG-3' was labeled and hybridized to membranes as above.

Primer extension. To map the transcription start site, non-radioactive automated primer extension analysis was used as described by Altermann et al. (1). Extension reactions were carried out with 9 pmol primer (5'-CCCAGAAGAGGTTGC-CGAGC-3', with IRD700 at the 5' end) annealed to 1 μg of total RNA isolated from *P. flocculosa*. Reference sequencing reactions

Fig. 1. Chemical structures of A, ustilagic acid and B, flocculosin.

were initiated using the same primer on a *SalI* genomic subclone using a primer-labeled cycle sequencing kit (LI-COR, Lincoln, NE). Products of the primer extension and sequencing reactions were separated on a 4200L IR2 system DNA sequencer (LI-COR).

3' Rapid amplification of cDNA ends on the *P. flocculosa* *cyp1* transcript. 3' Rapid amplification of cDNA ends (RACE) PCR was performed as follows: oligo-dT-adaptor-primer 5'-GGAATCAGTCAGTAATTGGAGGTTTTTTTTTTTTTTTTTT-3' was used for first-strand cDNA synthesis using SuperScriptIII reverse transcriptase (Invitrogen) on 1.5 µg of total RNA. Second-round amplification was performed with adaptor-primer 5'-GGAATCAGTCAGTAATTGGAGG-3' and gene-specific primer 5'-GACGCGAGGTCATCAACTCG-3' using Phusion DNA polymerase (Finnzymes, Espoo, Finland), yielding a PCR product of ≈440 bp that was gel purified using the QIAquick gel extraction kit (Qiagen) and sequenced.

Phylogenetic analysis. To further establish whether the putative *P. flocculosa* *cyp1* gene was a functional homolog of the *U. maydis* *cyp1* gene, the amino acid sequence of *P. flocculosa* *cyp1* transcript was submitted to the National Center for Biotechnology Information (NCBI) Position-Specific Iterated Basic Local Alignment Search Tool (PSI-BLAST) using a non-redundant protein sequence database (nr). The multiple sequence alignment was obtained using ClustalW software (25). A neighbor-joining phylogenetic tree was then constructed using the MEGA software version 4.1 (23) based on the previously obtained alignment of amino acid sequences. Reliability of internal nodes was assessed using 1,000 bootstraps and the amino acid scoring matrix Jones-Taylor-Thornton (JTT) was used to calculate the evolutionary distance between each sequence pair.

PCR-based cloning of partial sequences of the *P. fusiformata* *cyp1* gene. Degenerate primers 5'-AGAAGTACGGNTACGG-3' and 5'-CCATGGCCCAGTCCGANTAGAG-3' were designed using consensus regions in the alignment between the *P. flocculosa* and *U. maydis* *cyp1* genomic sequences, and used to amplify a fragment of ≈1,100 bp of the *P. fusiformata* *cyp1* gene from genomic DNA, yielding 1,033 bp of sequence data. The 3' RACE PCR reactions were performed as follows: oligo-dT-adaptor-primer 5'-GGAATCAGTCAGTAATTGGAGGTTTTTTTTTTTTTTTTTT-3' was used for first-strand cDNA synthesis using SuperScriptIII reverse transcriptase (Invitrogen) on 1.5 µg of total RNA. Second-round amplification was per-

formed with adaptor-primer 5'-GGAATCAGTCAGTAATTGGAGG-3' and gene-specific primer 5'-GTTGGCGGACGATGTCTGC-3' using HotMaster DNA polymerase (Eppendorf), yielding a PCR product of ≈530 bp that was cloned into the pGEM-T easy vector (Promega Corp., Madison, WI) and sequenced.

Quantitative reverse-transcription PCR analysis of *cyp1* expression. Differential expression of *P. flocculosa* *cyp1* was assessed by quantitative (q)PCR using a Mastercycler EP Realplex 2S (Eppendorf). Total RNA was extracted from fungal biomass grown in media either conducive (MOD) or repressive (YMPD) to flocculosin production, or from infected cucumber leaf discs using 1 ml of Trizol reagent (Invitrogen) and then quantified using a Nanodrop spectrophotometer (Nanodrop, Delaware). For each of the three biological replicates, 1 µg of total RNA was first treated with RQ1 RNase-free DNase (Promega Corp.) and then reverse transcribed into first-strand cDNA using OligodT(18) and Superscript II reverse transcriptase (Invitrogen) in a 20-µl reaction volume. Primer pairs for selected genes were designed with Oligo Explorer software (Genelink) in order to amplify 100- to 150-bp fragments with similar GC and Tm and were as follows: *P. flocculosa* *actin* (20): 5'-TCTGCTTCGAGACCTTCAAC-3' and 5'-GAGTAGCCCTCGTAGATGG-3' and *P. flocculosa* *cyp1*: 5'-TGTCAGATCGCCTTTAGCC-3' and 5'-CGGTCTCGTTGTACTTTTCG-3'. PCR amplifications were performed in a 20-µl reaction volume using the Quantitect SYBR green PCR kit (Qiagen) with 1 µl of sample cDNA. PCR was performed as follows: 15 min at 95°C plus 50 cycles of 20 s at 95°C, 20 s at 58°C, and 20 s at 72°C. Each sample from each biological replicate was analyzed twice. The specificity of primer pairs was verified both by gel and melting curve analysis. The *P. flocculosa* *actin* gene was used as an internal control for normalization. Efficiencies of all primer pairs used were determined and the quantification of the relative changes in gene expression was performed using the Pfaffl method (21).

Measurement of flocculosin concentration. Quantification of flocculosin was carried out as described by Hammami et al. (12). Briefly, each 1-ml aliquot of liquid culture of *P. flocculosa* was centrifuged at 10,000 × g for 5 min. The supernatants were removed and 1 ml of CH₃OH was added to the pellets. Tubes were vortexed and centrifuged at 10,000 × g for an additional 5 min. Supernatants were kept at -20°C prior to high-performance liquid chromatography (HPLC) analysis. An HPLC (Agilent, 1100 series) coupled with an ELSD detector (Sedere, Sedex 75, France) was used for flocculosin quantification. Flocculosin was eluted on a C8 column (ZORBAX, Eclipse XDB-C8; 4.6-by-150 mm) with the following linear gradient: 40:60 CH₃OH/water to 80:20 CH₃OH/water at a flow rate of 0.8 ml/min for 20 min. Under these conditions, flocculosin was eluted at ≈13 min.

Sequence deposition. Sequence data have been deposited in GenBank under the following accession numbers: *P. flocculosa* *cyp1* and partial *uat1*: EU556541, partial *P. fusiformata* *cyp1*: EU553488.

RESULTS

Southern blot analysis of the *cyp1* gene in *Pseudozyma* spp.

Given our interest in understanding the relationship between glycolipid production and antibiosis in related *Pseudozyma* spp., we attempted to screen different species with known or reported properties for glycolipid production (or lack thereof) for the presence of *cyp1* gene homologs using the *U. maydis* ORF as probe (Fig. 2). Among the seven strains of the six *Pseudozyma* spp. tested, a signal was detected only in lanes corresponding to *P. flocculosa* and *P. fusiformata* genomic DNA. In both species, a single *Bam*HI fragment was observed as in the *U. maydis*-positive control, suggesting that the putative *cyp1* gene homolog is present in a single copy.

Fig. 2. Southern transfer of *Bam*HI-digested genomic DNA from *Pseudozyma* spp. and hybridization with the ³²P-labeled *Ustilago maydis* cloned *cyp1* open reading frame probe. Genomic DNA from *U. maydis* was used as a positive control. *Pseudozyma flocculosa*: CBS 167.99, *P. rugulosa* and *P. rugulosa* ONT: CBS 170.88, *P. fusiformata*: CBS 6951, *P. tsukubaensis*: CBS 6389, *P. aphidis*: CBS 517.83, *P. antarctica*: CBS 516.83, and *U. maydis*: DSM 4500.

Sequencing of a *P. flocculosa* genomic clone. A *P. flocculosa* genomic DNA library was screened with the *U. maydis* *cyp1* probe. Among several positive clones, one was selected for sequencing. Sequenced subclones revealed an ORF showing strong sequence similarity (70% identity) to the *U. maydis* CYP1 protein (Fig. 3). Fortuitously, sequencing of genomic plasmid subclones from the lambda clone identified using the *U. maydis* *cyp1* probe revealed the presence of another partial ORF (1,076 nucleotides [nt]) at the extremity of the 15-kb genomic insert. When the sequence was compared against the *U. maydis* database (14), this partial ORF showed sequence similarity (88% identity) to the *U. maydis* *uat1* gene (1,542 nt), located next to *cyp1* and coding for an acyl/acetyltransferase believed to be involved in acetylation and possibly also acylation of ustilagic acid (24).

Molecular characterization of *cyp1* gene. In order to further characterize the putative *cyp1* gene in *P. flocculosa*, total RNA was extracted from the fungus grown in a medium conducive to flocculosin production (MOD). Following a Northern blot analysis with a fragment of the native *cyp1* gene used as a probe, a predominant band of ≈ 2.2 kb was detected (Fig. 4). Primer extension indicated a transcription initiation site located 74 bp upstream of the start codon in the *P. flocculosa* *cyp1* gene homolog, and no alternative transcription initiation site was detected (Fig. 5). The 3' RACE yielded a 3' untranslated region (UTR) of 212 nt. Thus, the predicted ORF (1,602 nt) along with the two UTRs (74 + 212 nt) and the polyA tail corresponds well with the ≈ 2.2 -kb band observed in the Northern analysis. Analysis of the ORF indicates that the predicted *P. flocculosa* CYP1 protein (534 amino acids [aa]) is missing 148 aa relative to the N-terminal portion of the *U. maydis* CYP1 protein (Fig. 3).

PCR-based cloning of partial sequences of the *P. fusiformata* *cyp1* gene and phylogenetic analysis. A partial sequence of a *cyp1* gene homolog was amplified from *P. fusiformata* using degenerate primers based on an alignment of the DNA sequences of the putative *P. flocculosa* and *U. maydis* *cyp1* genes, followed by 3' RACE on cDNA. This putative *P. fusiformata* *cyp1* gene showed strong sequence similarity (88% identity with *U. maydis* *cyp1*) to homologs in *U. maydis* and *P. flocculosa* (Fig. 3).

When comparing the amino acid sequence of the *P. flocculosa* putative CYP1 protein to that of other fungi or plants found in the

NCBI databank, the search indicated that all retrieved sequences shared a conserved domain typical of the P450 superfamily, and most of these were annotated as cytochrome P450 monooxygenases. This conserved domain extended approximately between residues 180 and 630 of the *U. maydis* sequence. Among the obtained sequences, the phylogenetic tree revealed a tight and specific clustering between the CYP1 protein of *U. maydis* and its nearest homologs found in *P. fusiformata* and *P. flocculosa* (Fig. 6).

Quantitative reverse-transcription PCR analysis of *cyp1* expression and flocculosin production. The expression of *cyp1* was assessed in situ through quantitative reverse-transcription (qRT)-PCR. First, the expression of *cyp1* was compared in *P. flocculosa* cultures grown in a medium conducive (MOD medium) or repressive (YMPD medium) to flocculosin production. In conducive conditions, expression of the *cyp1* gene increased rapidly and was closely associated with the accumulation of flocculosin in the medium (Fig. 7). At the end of the sampling period, the expression of *cyp1* decreased while flocculosin accumulation started to stabilize. In repressive conditions, both the expression of *cyp1* and the concentration of flocculosin were undetected over the sampling period (data not shown).

When expression of the *cyp1* gene was monitored following the inoculation of *P. flocculosa* on powdery mildew-infected cucum-

Fig. 4. Northern transfer of total RNA from *Pseudozyma flocculosa* and hybridization with the 32 P-labeled *Ustilago maydis* cloned *cyp1* open reading frame probe.

Fig. 3. Sequence alignment of the putative *Pseudozyma flocculosa* CYP1 protein (EU556541), partial *P. fusiformata* CYP1 predicted proteins (EU553488), and the *Ustilago maydis* CYP1 protein (UM06463.1). GenBank accession numbers are between parentheses. The alignment was performed using the ClustalW application. Similar and identical amino acid residues are shadowed in gray and black, respectively.

ber leaves, it decreased rapidly (Fig. 8) as the fungus expanded over the powdery mildew colonies.

DISCUSSION

Considering the number of fungal BCAs identified in the literature, there is very little information about potential genes that confer, even partially, an antagonistic activity to such fungi. In this work, we report on the identification and the study of expression of genes in the powdery mildew BCA *P. flocculosa* that present characteristics consistent with the production of the antifungal glycolipid flocculosin, associated with the biocontrol properties of the fungus.

Fig. 5. Mapping of *cyp1* transcript from *Pseudozyma flocculosa* by nonradioactive automated primer extension. Lanes A, C, G, and T correspond to the reference sequencing primer reactions. The sequence of the coding strand encompassing the 3' end of the extension product is shown to the right, with an asterisk marking the transcription initiation site.

Our results revealed that homologs of the *U. maydis cyp1* gene, a putative P450 monooxygenase involved in ustilagic acid synthesis in *U. maydis*, could be found in only two of the six species of *Pseudozyma* tested, *P. flocculosa* and *P. fusiformata*. Although this may seem surprising from a phylogenetic point of view, it is biologically relevant because these are the only two species reported to produce cellobiose-type lipids similar to ustilagic acid (7,15,17). Thus, this observation supports the proposed functional tie and specificity between the *cyp1* gene and the production of ustilagic acids (including flocculosin). It also indicates that the other species of *Pseudozyma* have either lost the need for the production of ustilagic acid, such that the gene sequence has been lost or is free to mutate and diverge considerably over the course of evolution, or simply never carried the gene. Considering that the *Pseudozyma* genus has been recently created from an aggregate of more or less disparate species (3,6), the latter hypothesis seems more probable. The apparent absence of a close homolog of *cyp1* (based on the results of the Southern analysis) is consistent with the loss or absence of such homologs in species known not to produce ustilagic acids. Finally, the finding that a single copy of the gene was present in *P. flocculosa* and *P. fusiformata* suggests that the cloned sequences are truly orthologous to the *U. maydis cyp1* gene.

Another argument which ties the function of the *cyp1* gene from *U. maydis* with that of the putative *cyp1* cloned from *P. flocculosa* and *P. fusiformata* is the tight clustering of these sequences observed in the phylogenetic tree. All three sequences shared a common node, with robust bootstrap values indicating that these proteins share a close genetic and functional relatedness compared with other P450 proteins. This supports their specific role in the unique ability of those three fungi to synthesize ustilagic acids.

Three other putative P450 monooxygenases were found in *U. maydis* (UM01863, UM01723, and UM06473) but were located on other nodes, suggesting that these proteins are closer to other P450 sequences than to CYP1. If there was another P450 monooxygenase in *P. flocculosa* more closely related to the *U. maydis cyp1* gene, it would likely have been detected in the Southern analysis.

In spite of their relatedness, an intriguing difference between the *U. maydis* and *P. flocculosa* CYP1 proteins is the additional 148 aa reported in the N terminus of the former (13). Based on our sequence alignment, it appears that *U. maydis* would be exceptional in this regard because all the other proteins lacked this segment.

Although identification of a *cyp1* homolog in *P. flocculosa* was the central objective of this work, we stumbled upon a partial sequence of the *P. flocculosa uat1* gene from the genomic clone containing the *cyp1* gene. This finding could have important implications because these two genes are also positioned next to each other in the *U. maydis* genome. Incidentally, Teichman et al. (24) reported that *U. maydis* contained a gene cluster with 12 ORFs that probably accounted for all the enzymes necessary for ustilagic acid synthesis. With their model system, the latter group was able to ascertain the role of some ORFs in ustilagic acid synthesis by mutational analysis. For our part, we have been unable to apply the same approach with *cyp1* in *P. flocculosa*, because only a low number of transformants have been obtained in our attempts to generate a *cyp1* deletion mutant by gene knockout via homologous recombination (16). Thus, whether the same cluster exists in *P. flocculosa* or *P. fusiformata* still remains to be determined but our results do support this possibility. It would then be interesting to determine whether the small structural differences between flocculosin and ustilagic acid can be explained by the presence of a few different ORFs within the cluster.

If ustilagic acids appear to be produced by only a few ustilagineous fungi, it is unclear, based on the reported ecology of

these fungi, what role, if any, the molecule plays in their respective ecological fitness. The creation of specific $\Delta cyp1$ *P. flocculosa* mutant strains would undoubtedly prove very useful in assessing with precision the role of flocculosin in biocontrol but this approach is currently hampered by our inability to transform

the fungus via homologous recombination as explained earlier. However, by measuring *cyp1* expression by qPCR in controlled conditions, we were able to shed some light on its possible role, and to gather additional evidence linking it to the production of flocculosin in *P. flocculosa*. For instance, it was clear that *cyp1*

Fig. 6. Unrooted phylogenetic tree of cytochrome P450 monooxygenases from various plants and fungi. Sequences were obtained from a BLASTp search using the putative *Pseudozyma flocculosa* CYP1 protein as query. Accession number for each amino acid sequence is indicated in parentheses. The numbers on the branches are the percentage values based on 1,000 bootstraps. Only bootstrap values >50% are shown. The bar represents 0.5 estimated amino acid change per sequence position.

Fig. 7. Quantitative reverse-transcription polymerase chain reaction analysis of *Pseudozyma flocculosa* actin-normalized *cyp1* expression and flocculosin quantification within a growth medium conducive to flocculosin production. Error bars represent the standard error for each treatment.

Fig. 8. Quantitative reverse-transcription polymerase chain reaction analysis of *Pseudozyma flocculosa* actin-normalized *cyp1* expression after inundative applications of conidia on powdery mildew-infected cucumber leaf discs. Error bars represent the standard error for each treatment.

expression was closely associated with the production of flocculosin in culture media under conditions conducive or repressive to its synthesis. Based on this observation, our qRT-PCR results of *cyp1* expression on infected cucumber suggest that flocculosin production is not synchronized, or strictly inherent, to the development of *P. flocculosa* on powdery mildew. This is in agreement with a recent report by Clément-Mathieu et al. (8), who found that, even if *P. flocculosa* possesses the ability to induce collapse of powdery mildews when sharing the same environment, its biocontrol activity, if modulated by flocculosin, is highly dependent on other factors stimulating its ability to develop and grow in the presence of powdery mildew colonies. Indeed, in the presence of powdery mildew, *cyp1* expression would be quickly downregulated because *P. flocculosa* expands rapidly on the pathogen colonies, a process similar to the rapid biomass development in a culture medium, conditions shown here and previously (12) to be repressive to flocculosin production. It may seem counterintuitive that the studied gene was downregulated in the presence of the target plant pathogen, but this relates to our knowledge of the ecology of *P. flocculosa*. An interesting parallel can also be drawn from the reported production of ustilagic acid by *U. maydis*. It has been hypothesized that anamorphs of the pathogen produce ustilagic acid as they search for a sporidium of a compatible mating type. It is likely that the infectious dikaryon then formed does not need to produce ustilagic acid in order to infect the plant, because knockout mutants of *cyp1* showed no alteration in pathogenicity (13). Furthermore, Teichmann et al. (24) demonstrated the antagonistic activity of ustilagic acid against *Botrytis cinerea*. Therefore, it is very plausible that this antagonistic activity helps protect the ecological niche of the haploid sporidia awaiting their compatible mating type in the same way that flocculosin protects the ecological niche of *P. flocculosa* against would-be invaders such as powdery mildews.

In conclusion, this study provides the identification of two genes, *cyp1* and *uat1*, along with transcriptional studies of *cyp1* that support its involvement in the production of flocculosin, a molecule associated with the biocontrol properties of *P. flocculosa*. A homolog of *cyp1* was also discovered in the closely related fungus *P. fusiformata*, known to produce ustilagic acid. Both these genes share considerable sequence similarity with those found in the gene cluster responsible for ustilagic acid production in the plant pathogen *U. maydis*. Although both *P. flocculosa* and *U. maydis* are phylogenetically related, they have evolved different ecological roles that seem nonetheless enhanced in similar ways by the production of their respective glycolipid.

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