Characterization of *CbCyp51* from Field Isolates of *Cercospora beticola*

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Accepted for publication 12 October 2011.

ABSTRACT

Bolton, M. D., Birla, K., Rivera-Varas, V., Rudolph, K. D., and Secor, G. A. 2012. Characterization of *CbCyp51* from field isolates of *Cercospora beticola*. *Phytopathology* 102:298-305.

The hemibiotrophic fungus *Cercospora beticola* causes leaf spot of sugar beet. Leaf spot control measures include the application of sterol demethylation inhibitor (DMI) fungicides. However, reduced sensitivity to DMIs has been reported recently in the Red River Valley sugar beet-growing region of North Dakota and Minnesota. Here, we report the cloning and molecular characterization of *CbCyp51*, which encodes the DMI target enzyme sterol P450 14 α -demethylase in *C. beticola*. *CbCyp51* is a 1,632-bp intron-free gene with obvious homology to other fungal *Cyp51* genes and is present as a single copy in the *C. beticola* genome. Five nucleotide haplotypes were identified which encoded three amino acid sequences. Protein variant 1 composed 79% of the sequenced

isolates, followed by protein variant 2 that composed 18% of the sequences and a single isolate representative of protein variant 3. Because resistance to DMIs can be related to polymorphism in promoter or coding sequences, sequence diversity was assessed by sequencing >2,440 nucleotides encompassing *CbCyp51* coding and flanking regions from isolates with varying EC₅₀ values (effective concentration to reduce growth by 50%) to DMI fungicides. However, no mutations or haplotypes were associated with DMI resistance or sensitivity. No evidence for alternative splicing or differential methylation of *CbCyp51* was found that might explain reduced sensitivity to DMIs. However, *CbCyp51* was overexpressed in isolates with high EC₅₀ values compared with isolates with low EC₅₀ values. After exposure to tetraconazole, isolates with high EC₅₀ values responded with further induction of *CbCyp51*, with a positive correlation of *CbCyp51* expression and tetraconazole concentration up to 2.5 μ g ml⁻¹.

Leaf spot, caused by the hemibiotrophic fungus *Cercospora beticola* (Sacc.), is one of the most destructive foliar pathogens of sugar beet (*Beta vulgaris* L.) worldwide. *C. beticola* is able to overwinter as stromata in infected sugar beet leaf residue on or directly below the soil surface (14). Sporulation can occur from overwintered stromata and the resulting conidia are delivered to sugar beet leaf surfaces primarily by wind and water splash. The fungus penetrates stomata to gain access to the apoplast (34), where effectors are produced by the invading hyphae that facilitate disease establishment (3). Conidia are produced in necrotic tissue and can be dispersed by wind and water splash to start another disease cycle. This cycle can be repeated several times in a growing season, particularly during conditions of protracted warm and humid weather when crop canopies close (15). Control measures for leaf spot include resistant sugar beet varieties and crop rotation but the disease is managed effectively only when combined with timely fungicide applications (10,13,31).

An important group of fungicides used to manage leaf spot is the sterol demethylation inhibitors (DMIs). These fungicides act by inhibiting cytochrome P450-dependent sterol 14 α -demethylase (CYP51), which is required for biosynthesis of the cell membrane component ergosterol in fungi (11). DMIs have been popular

because of their broad-spectrum antifungal activities, protective and curative properties, and low levels of phytotoxicity. Although DMI-resistant isolates were initially thought to exhibit a fitness penalty that would preclude DMI resistance becoming widespread in nature, resistance to DMIs has been reported in many fungal pathogens (18,20,39), including *C. beticola* (12,31).

Resistance mechanisms to DMI fungicides include (i) mutations in the *Cyp51* gene that maintain appropriate protein function while avoiding inhibition by the DMI fungicide, (ii) overexpression of the *Cyp51* gene leading to an excess of the CYP51 DMI target, and (iii) reduction of the intracellular fungicide concentration by means of increased ATP-binding cassette (ABC) or major facilitator superfamily (MSF) transporter efflux activity (4,19). Regardless of the molecular mechanism employed, DMI-resistant field isolates of *C. beticola* can be identified by the concentration of fungicide that reduces growth or spore germination of the fungus on fungicide-amended media by 50% compared with growth on nonamended media (EC₅₀ value) (M. Bolton et al., unpublished). There has been a significant increase in EC₅₀ values to the DMI fungicide tetraconazole in field isolates of *C. beticola* collected in recent years compared with the baseline EC₅₀ values of isolates collected in 1998, the year before tetraconazole was first used to control leaf spot in the Red River Valley (31).

The identification of mutations that confer resistance to fungicides has led to high-throughput means of fungicide resistance detection in several pathosystems (7,8,24,26). As part of our ongoing work on fungicide resistance management in this pathosystem, we are interested in studying the molecular basis of resistance to DMI fungicides in *C. beticola* to lay the foundation for polymerase chain reaction (PCR)-based fungicide resistance detection. Therefore, the objectives of this study were to (i) clone the full-length *C. beticola Cyp51* (*CbCyp51*) coding sequence and flanking regions, (ii) sequence *CbCyp51* from DMI-resistant and

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<http://dx.doi.org/10.1094/PHYTO-07-11-0212>

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DMI-sensitive isolates to assess whether mutations correlate with DMI resistance, and, if mutations in *CbCyp51* do not correlate with DMI-resistance, (iii) carry out *CbCyp51* gene expression and related analyses to help gain a better understanding of DMI resistance in *C. beticola*.

MATERIALS AND METHODS

Sample collection and fungal isolation. Sugar beet leaves with Cercospora leaf spot were collected from commercial fields of the Red River Valley region of Minnesota and North Dakota. Conidia were harvested by adding 50 µl of T-water (0.06% [vol/vol] Tween 20 [Sigma-Aldrich] and 0.02% [wt/vol] filter-sterilized ampicillin added after the solution had been autoclaved) to the surface of a *C. beticola* leaf spot lesion. The lesion was gently scraped with a pipette tip to liberate conidia into the T-water. The conidia-laden T-water was collected and transferred to water agar plates (1.5% [wt/vol] agar [Difco Laboratories] and 0.02% [wt/vol] filter-sterilized ampicillin added after the solution had been autoclaved) for 24 h at 22°C (±1°C) to initiate conidia germination. One germinated conidium was transferred to a clarified V8-medium plate (10% [vol/vol] clarified V8 juice [Campbell's Soup Co.], 0.5% [wt/vol] CaCO₃, and 1.5% [wt/vol] agar [Difco Laboratories]) and was incubated at 25°C for 2 weeks. This single spore-derived colony was the source inoculum plate for subsequent fungicide sensitivity assays and *CbCyp51* characterization experiments.

Fungicide sensitivity assays. EC₅₀ values were calculated using the active ingredient of each fungicide, which included tetraconazole (Eminent 125SL; Sipcam Agro), prothioconazole

(Proline 480 SC; Bayer CropScience), and difenoconazole (Inspire XT 2.08 EC; Syngenta). Difenoconazole and propiconazole are both active ingredients in Inspire XT 2.08 EC but only difenoconazole was assessed. EC₅₀ values were calculated for all three fungicide using the method described by Secor and Rivera (30). Briefly, agar plugs (5 mm each) were taken from the leading edge of colony growth of the original nonamended V8-agar plates described above and placed on V8-agar plates amended with serial 10-fold dilutions of active ingredient at 0.001 to 1.0 µg ml⁻¹. After 15 days, inhibition of radial growth was measured and compared with growth on nonamended medium and was used to calculate an EC₅₀ value for each isolate.

***Cyp51* cloning, rapid amplification of cDNA ends, and sequence analysis.** Primers CYP51FS and CYP51RS (Table 1) were used to amplify a fragment of the *Cyp51* gene as previously described (23). Using the DNA Walking SpeedUp Kit II (Seegene, Rockville, MD) and primers (Table 1) designed on the derived *Cyp51* sequence, two rounds of DNA walking were used to identify sequences upstream and one round of DNA walking was used to identify downstream sequence following the manufacturer's instructions.

To obtain the *C. beticola Cyp51* 5' untranslated region (UTR) sequence, primers 364 and 365 (Table 1) were used with the GeneRacer kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Template total RNA was extracted from an in-vitro-grown culture of 09–234 (as shown below) using the RNeasy Plant Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

Based on EC₅₀ value determination, isolates were characterized as having low (EC₅₀ < 0.01 µg ml⁻¹), medium (EC₅₀ of 0.01 to

TABLE 1. Primers used in this study

Primer	Specificity/role ^a	Sequence (5' to 3')	Amplicon size (bp) ^b	Reference
CYP51FS	<i>CbCyp51</i>	GTGTTCCGCAAGGACGTCG	1,229	23
CYP51RS	<i>CbCyp51</i>	CTCTCCCACTTCACAACAGC	...	23
348	<i>CbCyp51/5'</i> walk round I	CCCATATCGAGGTAGTG	NA	This study
349	<i>CbCyp51/5'</i> walk round I	AGCAGTGTAATGGTGAGCTC	NA	This study
350	<i>CbCyp51/5'</i> walk round I	GCTGGTCGAAGCGAATCGCTT	NA	This study
372	<i>CbCyp51/5'</i> walk round II	GTCGCTGTTTGCCAAAGTG	NA	This study
371	<i>CbCyp51/5'</i> walk round II	GGAAGTGCAGGTGTTCAAAAGC	NA	This study
370	<i>CbCyp51/5'</i> walk round II	CTCCATCGTACGGTTTCAACA	NA	This study
351	<i>CbCyp51/3'</i> walk	TTACGGCTACGGTCTGGTCA	NA	This study
352	<i>CbCyp51/3'</i> walk	TACCTACCATTTGGTGCTGG	NA	This study
353	<i>CbCyp51/3'</i> walk	CGGATGGAAGCAAGAAGGTC	NA	This study
364	<i>CbCyp51/5'</i> RACE GSP1	GGCGAAAGCAGGCGGGAGGTCGAGAGT	NA	This study
365	<i>CbCyp51/5'</i> RACE Nested	CGGTGATCAATGTGACATAGG	NA	This study
422	<i>CbCyp51/fragment I</i> For	GTATGGGATGCGCATTGTG	935	This study
546	<i>CbCyp51/fragment I</i> Rev	ATCTCCTCGGCATTGACATC	...	This study
565	<i>CbCyp51/fragment II</i> For	ACCGTATGCCTGGGCACCAA	890	This study
566	<i>CbCyp51/fragment II</i> Rev	GGAGATTGTACTTTGCGAAG	...	This study
426	<i>CbCyp51/fragment III</i> For	TGCTCAGCCAAGTTGTCAAG	592–598	This study
532	<i>CbCyp51/fragment III</i> Rev	GCGAATGCTTCAATACTGGATGC	...	This study
426	<i>CbCyp51/fragment III</i> For	TGCTCAGCCAAGTTGTCAAG	786	This study
427	<i>CbCyp51/fragment III</i> Rev	GGGTCGAGTGTACCTCCT	...	This study
CYP51RT-F	<i>CbCyp51/qPCR</i> For	AACTCCAAATTGATGGAGCA	200	23
CYP51RT-R	<i>CbCyp51/qPCR</i> Rev	CGGCTAGCAGTGTAATGGT	...	23
CbactinRT-F	Actin/qPCR For	ACATGGCTGGTCGTGATTTG	202	23
CbactinRT-R	Actin/qPCR Rev	TGTCGGTCAGGAAGCTCGTA	...	23
530	<i>CbCyp51/spice</i> variation	TGCTTTGAACACCGCACTTCCA	1,745–1,751	This study
532	<i>CbCyp51/spice</i> variation	GCGAATGCTTCAATACTGGATGC	...	This study
511	<i>CbCyp51/methylation</i>	GGATTTTGTGTTAAAAATGTTGAAAT	242	This study
512	<i>CbCyp51/methylation</i>	AAAAAAATAATAAAACCAACAACAC	...	This study
523	<i>CbCyp51/methylation</i>	AGTTGGAGATATGGGGATTTT	340	This study
524	<i>CbCyp51/methylation</i>	TTCACATTTTAAACAAAAATCCA	...	This study
525	<i>CbCyp51/methylation</i>	GTATGTTTGGGTATTAAGGGTAA	433	This study
526	<i>CbCyp51/methylation</i>	CCCAAAACAACATAAAATTAATC	...	This study
544	<i>CbCyp51/methylation</i>	ATATGGGTTTTATTYTGATTAATT	252	This study
545	<i>CbCyp51/methylation</i>	CCATAAACAAAAACAATCATCAT	...	This study
549	<i>CbCyp51/methylation</i>	GTTTGTAGTAAATTTGATTTG	293	This study
550	<i>CbCyp51/methylation</i>	AAAACAATCATCATATATACC	...	This study

^a RACE = rapid amplification of cDNA ends and qPCR = quantitative polymerase chain reaction.

^b N/A = not applicable.

1.0 $\mu\text{g ml}^{-1}$), or high ($\text{EC}_{50} > 1.0 \mu\text{g ml}^{-1}$) resistance to DMI fungicides. To assess *CbCyp51* sequence variation between isolates with low, medium, and high EC_{50} values, liquid cultures of isolates were initiated by placing an agar plug taken from the leading edge of the clarified V8-medium plates (described above) into a 250-ml flask containing 50 ml potato dextrose broth (BD Diagnostics, Sparks, MD). Isolates were allowed to grow for ≈ 4 days, after which time mycelium was harvested using a Büchner funnel and DNA was isolated using the cetyltrimethylammonium bromide (CTAB) method (35). For each isolate, the *CbCyp51* gene and flanking sequences were generated with three PCR reactions using primer combinations 422/546, 565/566, and 426/532 (Table 1) that produced overlapping sequence fragments. Additional sequence was obtained using primer combination 426/427 that amplified 3' flanking sequence from *CbCyp51* protein variants 1 and 3 but was not successful in producing amplicons from protein variant 2 (see Results). PCR was conducted using GoTaq Green PCR kit (Promega Corp., Madison, WI) following the manufacturer's instructions. PCR conditions were as follows: an initial 95°C denaturation step for 5 min followed by denaturation for 15 s at 95°C, annealing for 30 s at 50°C, and extension for 60 s at 72°C for 30 cycles. PCR amplicons were

sequenced directly (McLab, South San Francisco, CA). Consensus sequences were generated and analyzed for polymorphisms using Vector NTI (Invitrogen) software. In total, the *CbCyp51* gene and flanking sequences were obtained from 20 isolates with EC_{50} values $> 1.0 \mu\text{g ml}^{-1}$ to tetraconazole and either prothioconazole or difenoconazole and in 13 isolates that exhibited EC_{50} values $< 0.09 \mu\text{g ml}^{-1}$ to tetraconazole and either prothioconazole or difenoconazole (Table 2).

***Cyp51* gene expression.** Three experiments were conducted to quantify *CbCyp51* gene expression. In the first experiment, a strategy similar to that employed by Stammler et al. (33) was utilized where *CbCyp51* gene expression in isolates with high and medium EC_{50} values was compared with a single calibrator isolate with a low EC_{50} value. Isolate 09-36 was chosen as the calibrator because it was shown to have a low expression of *CbCyp51* and was highly sensitive to tetraconazole, prothioconazole, and difenoconazole (Table 2). Liquid cultures of 09-36, 09-52, 09-157, 09-267, 10-270, 10-75-4, 10-78-5, 10-257, 10-411, and 10-451 (Table 2) were initiated by placing an agar plug taken from the leading edge of the clarified V8-medium plates (described above) into a 250-ml flask containing 100 ml of potato dextrose broth (BD Diagnostics). Isolates were allowed to grow for 96 h, after

TABLE 2. *Cercospora beticola* isolates used in this study

Isolate	EC_{50} values ($\mu\text{g ml}^{-1}$) ^a			Protein variant number ^b	GenBank number ^c
	Tetraconazole	Prothioconazole	Difenoconazole		
04-924	>1.0	...	...	1	JN399024
07-796	>1.0	>1.0	>1.0	1	JN399025
07-836	>1.0	>1.0	>1.0	1	JN399026
07-857	>1.0	>1.0	>1.0	1	JN399027
07-970	>1.0	>1.0	>1.0	1	JN399028
08-203	>1.0	>1.0	...	3	JN399029
08-240	>1.0	>1.0	...	1	JN399030
08-642	>1.0	>1.0	...	1	JN399031
08-885	>1.0	>1.0	...	1	JN399032
09-24	>1.0	>1.0	>1.0	...	...
09-28	0.068	0.313	0.036	2	JN399033
09-32	0.006	0.007	0.005	2	JN399034
09-36	0.006	0.006	0.005	1	JN399035
09-52	0.006	0.006	0.005	...	...
09-57	0.005	0.006	0.005	1	JN399036
09-71	0.066	0.071	0.057	1	JN399037
09-88	>1.0	>1.0	0.915	1	JN399038
09-93	0.069	0.080	0.008	2	JN399039
09-95	0.060	0.093	0.008	1	JN399040
09-99	>1.0	>1.0	0.364	1	JN399041
09-110	0.062	0.084	0.023	1	JN399042
09-131	0.067	0.072	0.032	1	JN399043
09-151	>1.0	>1.0	0.699	1	JN399044
09-157	0.070	0.075	0.033	1	JN399045
09-159	>1.0	>1.0	0.566	1	JN399046
09-173	>1.0	>1.0	0.244	1	JN399047
09-183	>1.0	>1.0	0.701	1	JN399048
09-237	0.065	0.074	0.037	2	JN399049
09-267	0.095	...	0.053	2	JN399050
10-59	0.525	...	...	...	...
10-74-2	>1.0	...	>1.0	1	JN399051
10-75-4	>1.0	...	>1.0	1	JN399052
10-76-4	>1.0	...	>1.0	...	...
10-78-5	>1.0	...	>1.0	1	JN399053
10-219	0.088	...	0.064	...	...
10-257	>1.0	>1.0	>1.0	1	JN399054
10-270	>1.0	...	0.748	...	...
10-274	>1.0	...	...	...	...
10-407	0.632	...	...	...	...
10-411	>1.0	>1.0	>1.0	1	JN399055
10-451	0.069	...	0.061	2	JN399056
10-496	0.091	...	...	...	...

^a Effective concentration to reduce growth by 50% (EC_{50}) values were calculated for tetraconazole, prothioconazole, and difenoconazole as previously described (30); ... indicates not assessed.

^b Five nucleotide haplotypes were identified which encoded three amino acid variants (see Results).

^c GenBank accession number for *CbCyp51* sequence and deduced amino acid sequence, if submitted.

which mycelium was harvested using a Büchner funnel and immediately frozen in liquid nitrogen. Total RNA was isolated from mycelium using the RNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions that included an on-column DNase treatment (Qiagen). Total RNA (5 µg) was used for cDNA synthesis using an oligo-(dT) primer and the SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. The resulting cDNA was used as a template for quantitative PCR (qPCR). qPCR was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) using a PTC-200 thermal cycler (MJ Research, Hercules, CA) outfitted with a Chromo4 Real-Time PCR Detector (Bio-Rad, Hercules, CA) and MJ Opticon Monitor analysis software (version 3.1; Bio-Rad). Real-time PCR conditions were as follows: an initial 95°C denaturation step for 10 min followed by denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C for 40 cycles. No-reverse-transcriptase (NRT) and no-template (NT) controls were included for each sample during every qPCR experiment.

In the second experiment, *CbCyp51* gene induction was assessed in isolates with high EC₅₀ values after being treated with tetraconazole at the equivalent of an EC₅₀ of 1.0 µg ml⁻¹. To that end, liquid cultures of 10-76-4, 10-78-5, 10-257, 10-270, and 10-411 (Table 2) were initiated as described above using a 125-ml flask containing 50 ml of potato dextrose broth (BD Diagnostics). Each isolate was allowed to grow for 96 h, after which 5 µl of tetraconazole stock solution (10 µg ml⁻¹ in acetone) was added to each flask for a final concentration of 1.0 µg ml⁻¹. For comparison, each isolate was also grown as shown above, except 5 µl of acetone was added in place of tetraconazole. Flasks were shaken (125 rpm) for 48 h at room temperature, after which mycelium was harvested using a Büchner funnel and immediately frozen in liquid nitrogen. All subsequent nucleic acid manipulations and qPCR parameters were as described above.

In the third experiment, the effect of fungicide dose on *CbCyp51* gene expression was assessed in isolate 10-78-5 after being treated with tetraconazole at 0.50, 0.75, 1.0, 2.5, or 5.0 µg ml⁻¹. Liquid cultures of 10-78-5 were initiated as described above using a 125-ml flask containing 50 ml of potato dextrose broth (BD Diagnostics). Isolate 10-78-5 was allowed to grow for 96 h, after which tetraconazole stock solution (10 µg ml⁻¹ in acetone) was added to each flask for a final concentration of 0.50, 0.75, 1.0, 2.5, or 5.0 µg ml⁻¹. For comparison, 10-78-5 was also grown as shown above, except that the equivalent volume of acetone was added in place of tetraconazole. Flasks were shaken (125 rpm) for 48 h at 22°C (±1°C), after which mycelium was harvested using a Büchner funnel and immediately frozen in liquid nitrogen. All subsequent nucleic acid manipulations and qPCR parameters were as described above.

All qPCR reactions were conducted in triplicate for each cDNA sample and the respective NRT control, and once for each respective NT control. The primer pair CYP51RT-F/CYP51RT-R (Table 1) was used to determine the relative expression of *CbCyp51* in isolates 09-36, 09-52, 09-157, 10-270, 10-411, 10-75-4, 10-76-4, 10-257, and 10-78-5. Because *Cyp51* protein variant 2 had three single-nucleotide polymorphisms (SNPs) in the CYP51RT-F primer-binding region, primer pair 352/CYP51RS was used to determine the relative expression of *CbCyp51* in 09-267 and 10-451. The *C. beticola* actin gene was amplified with the primer pair CbactinRT-F/CbactinRT-R (Table 1) and served as the endogenous control. The abundance of *CbCyp51* gene transcripts was calculated relative to actin using the method of Pfaffl (25). Efficiencies for primers CYP51RT-F/CYP51RT-R, 352/CYP51RS, and CbactinRT-F/CbactinRT-R were 1.892, 1.962, and 1.996, respectively. All qPCR experiments were repeated twice.

***CbCyp51* Southern analysis.** To assess whether any variation in *CbCyp51* copy number exists between isolates with high EC₅₀ values versus isolates with low EC₅₀ values, we utilized the dig-

oxigenin (DIG) nonradioactive nucleic acid labeling and detection system (Roche Diagnostics, Indianapolis, IN). DNA from three isolates with low and medium EC₅₀ values (09-36, 10-496, and 10-219) and seven isolates with high EC₅₀ values (10-74-2, 10-78-5, 10-76-4, 10-257, 10-411, 09-24, and 10-274) (Table 2) was isolated using the CTAB method as described above. The resulting DNA was subjected to an overnight restriction digest using *EcoRI* or *HindIII* (Promega Corp.). The digested DNA and a DIG-labeled marker (Roche Diagnostics) were electrophoresed in an agarose gel and transferred to a membrane using standard Southern hybridization techniques (28). Low-EC₅₀ isolates and high-EC₅₀ isolates were transferred to separate membranes and were probed separately. To generate a *Cyp51* probe, primer combination 426/427 (Table 1) was used in a standard PCR reaction using the GoTaq Green PCR kit (Promega Corp.) following the manufacturer's instructions. The resulting amplicon was gel purified using the QIAquick Gel Extraction kit (Qiagen) following the manufacturer's instructions and was used as the template for a second PCR using the same primers and reaction conditions above, except that DIG-labeled UTPs from the PCR DIG Probe Synthesis kit (Roche Diagnostics) were utilized. The DIG-labeled *Cyp51* probe was used in a standard Southern hybridization utilizing DIG Easy Hyb (Roche Diagnostics) following the manufacturer's instructions. A hybridization temperature of 54°C and washes with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate were carried out at 68°C. Chemiluminescent probe-target hybrids were visualized using CDP-Star (Roche Diagnostics) following the manufacturer's instructions and were exposed to x-ray film for ≈3 min.

Splice variation analysis. To investigate whether alternative splicing may be involved with DMI resistance, primer combination 530/532 (Table 2) was used to amplify the mature *CbCyp51* mRNA region using cDNA derived from isolates 09-36, 09-157, 09-267, 10-75-4, 10-78-5, 10-257, 10-411, and 10-451 as described above for *Cyp51* gene expression experiments. PCR reactions were conducted using GoTaq Green PCR kit (Promega Corp.) following the manufacturer's instructions. PCR amplicons were sequenced directly (McLab) using primers 530, 349, 566, 426, and 532. Consensus sequences were generated and analyzed for alternative splicing using Vector NTI (Invitrogen) software.

***Cyp51* methylation.** Because induced expression of *Cyp51* was found in isolates with high EC₅₀ values compared with those with low EC₅₀ values, we were interested whether differential methylation patterns may explain *Cyp51* gene expression in these isolates. To investigate this, genomic DNA was isolated from 09-52 and 09-36 (low EC₅₀ group), 10-59 and 10-407 (medium EC₅₀ group), and 10-411 and 10-257 (high EC₅₀ group) as described above. The resulting DNA was used as template in the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA). After bisulfate conversion, primer combinations 511/512, 523/524, 525/526, 544/545, and 549/550 (Table 1) were used to amplify 5' UTR and coding sequence regions of the *Cyp51* gene. PCR reactions were conducted using GoTaq Green PCR kit (Promega Corp.) following the manufacturer's instructions. PCR amplicons were sequenced directly (McLab). Consensus sequences were generated and analyzed for differential methylation patterns using Vector NTI (Invitrogen) software.

RESULTS

***CbCyp51* cloning and sequence analysis.** Using the previously published primer set (23) that amplifies a partial fragment of *CbCyp51* coding sequence, we used a primer walking strategy to clone more than 945 bases on the 5' end of the fragment, which included 558 bases upstream of the predicted start codon, as well as 290 bases on 3' end of the fragment. The predicted start codon is contained within the eukaryotic translation initiation consensus sequence (16) and 5' rapid amplification of cDNA ends was used

to identify 48 nucleotides of UTR upstream of the putative start codon. Because resistance to DMI fungicides can be related to polymorphisms in promoter or coding regions of *Cyp51* (9,20), sequence diversity was assessed by sequencing up to 2,449 nucleotides encompassing *CbCyp51* coding and flanking regions. In total, sequence data were obtained from 13 *C. beticola* isolates with low and medium EC₅₀ values and 20 isolates with high EC₅₀ values to at least one DMI fungicide. With regard to the *CbCyp51* coding region, five nucleotide haplotypes were identified which encoded three amino acid sequences. Protein variant 1 composed 79% of the sequenced isolates, followed by protein variant 2 that composed 18% of the sequences (Table 2). Amino acid alignment between protein variants 1 and 2 revealed 97% identical residues (524 of 543) and 99% positives (536 of 543) with no gaps. Protein variant 3 was represented by a single isolate (08-203) that encoded a single amino acid change (Ala185Pro) compared with protein variant 1 (Table 2). There were additional SNPs outside of the *CbCyp51* coding region, none of which could be found in either all isolates with low EC₅₀ values or all isolates with high EC₅₀ values. Although primer pair 426/532 reliably produced an amplicon of the expected size from all isolates tested, we were unable to produce an amplicon using primer pairs 426/427 on any protein variant 2 haplotype isolate. Moreover, additional reverse primers designed on protein variant 1 sequence data and used in conjunction with primer 426 also did not provide an amplicon (not shown). Taken together, no SNP or haplotype correlated with DMI resistance in this study. The DNA and deduced protein sequences described in this article have been deposited in the GenBank database under accession numbers JN399024 to JN399056.

A conserved domain search of the deduced 543-amino-acid sequence of the putative CYP51 protein variant 1 revealed hits to pfam00067, cytochrome P450 (*E* value = 1.55e-49). Top BLASTP hits of the putative *C. beticola* CYP51 protein were to eburicol 14 α -demethylase of *Mycosphaerella graminicola* (*E* value = 0.0; 83% identities, 90% positive matches), lanosterol 14 α -demethylase from *Coccidioides posadasii* (*E* value = 0.0; 67% identities, 78% positive matches), 14 α sterol demethylase Cyp51B from *Aspergillus fumigatus* (*E* value 0.0; 66% identities, 78% positive matches), lanosterol 14 α -demethylase from *Ajellomyces capsulatus* (*E* value 0.0; 66% identities, 77% positive matches), and 14 α sterol demethylase Cyp51B from *Neosartorya fischeri* (*E* value = 0.0; 66% identities, 77% positive matches).

Fig. 1. Relative quantification (RQ) of *CbCyp51* to actin in *Cercospora beticola* isolates with varying effective concentration to reduce growth by 50% (EC₅₀) values compared with the demethylation inhibitor (DMI)-sensitive calibrator isolate 09-36. The low-EC₅₀ value group is represented by 09-52; the medium-EC₅₀ value group is represented by 09-157, 10-451, and 09-267; and the high-EC₅₀ value group is represented by 10-270, 10-411, 10-75-4, 10-257, and 10-78-5. Values and error bars represent the mean and standard error, respectively, of triplicate cycle threshold values from both repeats of the experiment.

***CbCyp51* gene expression.** Because analysis of sequence data did not indicate that mutations in the *CbCyp51* gene correlated with resistance to DMI fungicides, we asked whether enhanced expression of the *CbCyp51* gene might be involved with this phenotype. We designed three experiments to quantify *CbCyp51* expression. In the first experiment, isolates with varying EC₅₀ values were compared against the DMI-sensitive isolate 09-36 (Table 2). Isolates with low or medium EC₅₀ values had lower expression of *CbCyp51* compared with isolates with EC₅₀ values > 1.0 $\mu\text{g ml}^{-1}$ (Fig. 1). For example, *CbCyp51* fold change for 09-52 (low EC₅₀) and 09-157 (medium EC₅₀) was 1.1 and 1.9, respectively. In contrast, fold change for isolates with high EC₅₀ values was 4.2 to 27.2 (Fig. 1). Isolates 10-451 and 09-267, which have medium EC₅₀ values, also had lower *CbCyp51* expression (2.7- and 2.8-fold change, respectively) compared with isolates with high EC₅₀ values.

In a second experiment, we quantified the induction of *CbCyp51* in isolates with high EC₅₀ values after treatment with tetraconazole at 1.0 $\mu\text{g ml}^{-1}$ compared with the same isolate after treatment with acetone. Although there was variability in expression level, *CbCyp51* was induced in all five isolates upon tetraconazole treatment (Fig. 2). Isolate 10-76-4 had the least amount of *CbCyp51* induction (3.7-fold change) while isolate 10-78-5 had the greatest induction (10.7-fold change). Finally, *CbCyp51* expression level was quantified in isolate 10-78-5 (Table 2) after treatment with tetraconazole at varying concentrations to study the effect of fungicide dose on *CbCyp51* expression. There was a general correlation between fungicide dose and *CbCyp51* expression until 2.5 $\mu\text{g ml}^{-1}$ (Fig. 3). In all experiments above, no amplification was found in either NRT or NT controls.

***CbCyp51* copy number.** Because copy number of the *Cyp51* gene may be related to overall *CbCyp51* gene expression, we performed Southern analyses to assess whether isolates with high EC₅₀ values had additional copies of the *Cyp51* gene. Using a partial fragment of the *CbCyp51* gene, we probed three isolates with low EC₅₀ values (Fig. 4A) and 7 isolates with high EC₅₀ values (Fig. 4B). No variation in copy number was seen between isolates (Fig. 4). An additional band of less intensity was seen in all isolates tested, suggesting that the *Cercospora beticola* genome contains a P-450 gene with some sequence similarity to *CbCyp51* (Fig. 4).

***CbCyp51* splice variation and methylation.** The putative *CbCyp51* coding sequence amplified from cDNA derived from in vitro grown cultures was 1,632 nucleotides in length and lacked any introns. There was no evidence of alternative splicing of *CbCyp51* in any of the isolates tested under these conditions. Because methylation status may have a significant impact on gene

Fig. 2. Relative quantification (RQ) of *CbCyp51* to actin in *Cercospora beticola* isolates with high effective concentration to reduce growth by 50% (EC₅₀) values after treatment with tetraconazole at 1.0 $\mu\text{g ml}^{-1}$. Values and error bars represent the mean and standard error, respectively, of triplicate cycle threshold values from both repeats of the experiment.

expression and activity in a heritable fashion (17), we looked for differential methylation patterns that may explain the DMI resistance phenotype. Although methylation is typically associated with reduced or no transcription (36), there was no evidence of increased methylation in isolates with low EC₅₀ values (not shown). Indeed, sequence analysis of 1,184 nucleotides of bisulfite-converted *Cyp51* coding and 5' flanking regions showed no variation in methylation between the selected isolates, and no methylated cytosines were found.

DISCUSSION

DMI fungicides are widely used to control leaf spot of sugar beet. The reliance on DMI fungicides has led to reduced sensitivity to DMIs in *C. beticola* populations (12,31). A predominant mechanism for reduced sensitivity to DMI fungicides in fungi are mutations in the *Cyp51* gene encoding amino acid alterations in the DMI target enzyme, sterol P450 14 α -demethylase (1,2,5, 6,8,26,38). To investigate whether this type of mechanism explains reduced DMI sensitivity in *C. beticola*, we cloned and analyzed the full-length *CbCyp51* gene and flanking sequence from isolates with varying sensitivities to DMI fungicides. Although there was a degree of sequence variation in the coding and flanking regions, no polymorphisms could be attributed to DMI resistance or susceptibility. Nikou et al. (23) performed sequence analysis of a fragment of the *C. beticola* *Cyp51* gene and identified a silent mutation at position Glu169 that was present in all four analyzed DMI-resistant isolates but was lacking in sensitive and moderately resistant isolates. Although we identified this mutation in our library of isolates as well, it was found in isolates with low EC₅₀ values as well as those with high EC₅₀ values. Moreover, analysis of the deduced amino acid sequence identified three protein variants, which also did not correlate with DMI resistance or sensitivity. Therefore, it does not appear likely that polymorphisms in or near *CbCyp51* explain the enhanced resistance to DMI fungicides in *C. beticola* isolates collected from the Red River Valley sugar-beet-growing region. Similarly, several haplotypes of *M. graminicola* *Cyp51* were found and were not correlated to in vitro DMI sensitivity (32). In contrast, preliminary evidence in the peanut leaf spot pathogen, *C. arachidicola*, has indicated mutations in the *Cyp51* gene associated with reduced sensitivity to DMI fungicides (27).

Because members of the *Cyp* gene family are known to encode alternative splicing variants (22) that may be responsible for protein alternations leading to DMI resistance, we assessed whether there was any splice variation in *CbCyp51* between isolates with different EC₅₀ values. However, no evidence of alternative splicing was found in any isolate. Analysis of genomic DNA revealed

a Met start codon upstream and in-frame with the putative *Cyp51* coding region but no amplification products could be produced with primers designed on this region using cDNA as a template (not shown). Moreover, the presence of a strong Kozak sequence (16) further suggests that the predicted start codon is the point of translation initiation.

Mutations in the target protein did not correlate with DMI resistance; therefore, *CbCyp51* gene expression was assessed in three gene expression experiments. In the first experiment, overexpression of *CbCyp51* gene expression was found in all isolates with high EC₅₀ values (Fig. 1). Overexpression of *Cyp51* has been associated with DMI resistance in a number of pathosystems. Hamamoto et al. (9) correlated constitutive expression of *Cyp51* in *Penicillium digitatum* with a tandemly repeated 126-bp sequence in the *Cyp51* promoter region in DMI-resistant strains. Similarly, Ma et al. (20) identified various lengths of a retrotransposon in the *Cyp51* promoter region of resistant isolates of *Blumeriella jaapii* that correlated with a 5- to 12-fold increase in *Cyp51* expression. Likewise, a 553-bp sequence upstream of *Cyp51* correlated with enhanced resistance in some isolates from *Venturia inaequalis* (29). Despite cloning 558 bp upstream of the *CbCyp51* ATG start codon, no promoter differences were found that correlate with DMI sensitivity in *C. beticola*. This suggests that regulation of *trans*-acting factors may be involved with the resistance phenotype. Further studies are needed to address this aspect.

To characterize *CbCyp51* expression further, we asked whether *CbCyp51* would be induced in isolates with high EC₅₀ values upon treatment with tetraconazole at 1.0 μ g ml⁻¹. All isolates responded with significant *CbCyp51* gene induction (Fig. 2). Interestingly, it appeared that tetraconazole dose was positively correlated to *CbCyp51* gene expression up to and including 2.5 μ g ml⁻¹ but dropped at the 5.0 μ g ml⁻¹ level (Fig. 3), which may represent the fungicide dose at which growth is arrested in this isolate. Although these results suggest that overexpression of *CbCyp51* plays a role in reduced sensitivity to DMI fungicides, gene expression results should be interpreted with care because conclusive evidence is difficult to achieve in this respect. Moreover, other resistance mechanisms such as drug transporters (37) may operate simultaneously with overexpression of *Cyp51*.

Because gene copy number can influence overall mRNA levels, we performed a Southern blot using a *CbCyp51* probe on 10 *C. beticola* isolates exhibiting a range of EC₅₀ values. However, we found no variation in *CbCyp51* banding patterns between any of the isolates (Fig. 4). This is in contrast to other studies in which *Cyp51* copy number correlated with overall *Cyp51* expression and with DMI resistance (21). We then assessed whether differential methylation may be involved with DMI resistance. DNA methyl-

Fig. 3. Relative quantification (RQ) of *CbCyp51* to actin in *Cercospora beticola* isolate 10-78-5 after treatment with tetraconazole at varying rates. Values and error bars represent the mean and standard error, respectively, of triplicate cycle threshold values from both repeats of the experiment.

Fig. 4. Southern hybridization of *Cercospora beticola* using a *CbCyp51* probe. **A**, Southern hybridization of isolates with low or medium effective concentration to reduce growth by 50% (EC₅₀) values to tetraconazole. Lane 1, lambda *Hind*III DNA ladder; lanes 2 to 4, *C. beticola* isolates 09-36, 10-496, and 10-219, respectively, digested with *Eco*RI; lanes 5 to 7, the same isolate order but digested with *Hind*III. **B**, Southern hybridization of isolates with high EC₅₀ values to tetraconazole. Lane 1, lambda *Hind*III DNA ladder; lanes 2 to 8, *C. beticola* isolates 10-74-2, 10-78-5, 10-76-4, 10-257, 10-411, 09-24, and 10-274, respectively, digested with *Eco*RI; lanes 9 to 15, the same isolate order but digested with *Hind*III.

ation occurs exclusively at cytosine residues and is a heritable, post-synthetic modification typically associated with repressed transcription (36). After development of primers to amplify bisulfite-converted *CbCyp51* coding and 5' flanking regions, we sequenced PCR amplicons originating from isolates with varying EC₅₀ values. However, no methylated cytosines were found in any of the isolates tested. Although we were not able to locate literature that associated differential DNA methylation with fungicide resistance, recent evidence demonstrating methylation of active genes in *Uncinocarpus reesii* (40) highlights the complexity of gene expression in fungi.

In conclusion, we cloned and characterized *CbCyp51* and flanking regions in isolates with a wide range of EC₅₀ values. One of the main objectives of this study was to assess whether molecular-based detection of DMI resistance was feasible; therefore, the results of this study essentially preclude PCR-based identification of DMI-resistant isolates using genomic DNA. Nonetheless, overexpression of *CbCyp51* appears to play a strong role in DMI resistance, as was evident by the increased expression in all isolates with high EC₅₀ values compared with isolates with low and medium EC₅₀ values. The finding that *C. beticola* isolates with high EC₅₀ values do not have a fitness penalty compared with DMI-sensitive isolates in the absence of DMI selection pressure (M. Bolton and G. Secor, *unpublished data*) suggests that close monitoring of DMI sensitivity in *C. beticola* populations is essential for long-term control of this important disease.

ACKNOWLEDGMENTS

This research was supported by United States Department of Agriculture–Agricultural Research Service CRIS project 5442-22000-042-00D and grants from the Sugarbeet Research and Education Board of Minnesota and North Dakota and the Beet Sugar Development Foundation. We thank X. Wang for excellent technical assistance.

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