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A Mutation in the 14α-Demethylase Gene of *Uncinula necator* That Correlates with Resistance to a Sterol Biosynthesis Inhibitor

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We investigated the molecular basis of resistance of the obligate biotrophic grape powdery mildew fungus *Uncinula necator* to sterol demethylation-inhibiting fungicides (DMIs). The sensitivity of 91 single-spore field isolates of *U. necator* to triadimenol was assessed by using a leaf disc assay. Resistance factors (RF) ranged from 1.8 to 26.0. The gene encoding the target of DMIs (eburicol 14α-demethylase) from five sensitive and seven resistant isolates was cloned and sequenced. A single mutation, leading to the substitution of a phenylalanine residue for a tyrosine residue at position 136, was found in all isolates exhibiting an RF higher than 5. No mutation was found in sensitive or weakly resistant (RF, <5) isolates. An allele-specific PCR assay was developed to detect the mutation. Among the 91 isolates tested, only isolates with RF higher than 5 carried the mutation. Three of the 19 resistant isolates and all sensitive and weakly resistant isolates did not possess the mutation. The mutation at codon 136 is thus clearly associated with high levels of resistance to triadimenol.

Powdery mildew, caused by *Uncinula necator* (Schw.) Burr., is a major disease of grapes. Disease control relies, in large part, on the use of sterol 14α-demethylation inhibitors (DMIs). DMIs are a major class of fungicides used in both agriculture and medicine. They are systemic compounds displaying a highly protective and curative activity against fungal diseases at low doses but also have selective action against nontarget organisms (plants and animals) (10, 22). They inhibit the cytochrome P-450 sterol 14α-demethylase ($P\text{-}450_{14DM}$), a key enzyme of the sterol biosynthetic pathway (3, 14; for a review, see reference 36). However, because of their site-specific mode of action, the intensive use of DMIs has led to the development of resistance in a number of fungi of medical (18) and agricultural importance (16, 33; see reference 11 for a review). The field resistance of *U. necator* to DMIs arose in the late 1980s in European and Californian vineyards (24, 30). This resistance may lead to severe problems in disease control by considerably reducing treatment efficiency.

Resistance to DMIs is generally thought to be polygenically governed. However, little is known about the molecular and biochemical basis of resistance in field or clinical fungal isolates. The great majority of studies of resistance to DMIs have been conducted with laboratory mutants. In field isolates, resistance to site-specific fungicides is frequently due to reduced affinity of the target site for its inhibitor, as shown for resistance to fungicides that inhibit tubulin polymerization (5). However, in part because of the difficulty of obtaining sufficient amounts of fungal mass from most phytopathogenic fungi for which resistance to DMIs has been reported (11), the possibility of reduced $P\text{-}450_{14DM}$ affinity for DMIs has not been explored in these organisms. The only reports of decreased $P\text{-}450_{14DM}$ affinity for DMIs, to our knowledge, concern fungi more easily grown on artificial media, such as clinical isolates of *Candida albicans* (19) or *Cryptococcus neoformans* (23). The molecular basis of this decreased affinity for DMIs remains unknown.

Economically important crop pathogens such as powdery mildew fungi, for which field resistance to DMIs has been reported (13, 25, 30, 33), are obligate biotrophic fungi and therefore cannot be grown on artificial media (35). For such fungi, cloning and characterization of the gene encoding $P\text{-}450_{14DM}$ is the only way to investigate whether resistance to DMIs correlates with modifications in this gene. This approach became feasible with the availability of $P\text{-}450_{14DM}$ gene sequences from three mammals (1, 26, 31), four yeasts (4, 15, 20, 21), and two filamentous fungi (17, 32). These data are of great help for cloning of the gene encoding $P\text{-}450_{14DM}$ in slow-growing and/or biotrophic fungi.

Recently, we cloned and sequenced the gene encoding eburicol 14α-demethylase in *U. necator* (9). Our objective in this study was to clone and sequence the $P\text{-}450_{14DM}$ gene from isolates differing in sensitivity to triadimenol, a triazole DMI widely used to control *U. necator*, to determine whether mutations in this gene are correlated with resistance to this compound.

MATERIALS AND METHODS

***U. necator* isolates.** Ninety-one clonal isolates of *U. necator* from Europe (62 isolates), India (28 isolates), and Israel (1 isolate) were used in this study. Mildewed grape samples were collected between 1990 and 1996. European and Israeli isolates were collected in vineyards where grape powdery mildew was not fully controlled by fungicide treatments. Indian isolates were from vineyards where DMIs were not used or rarely used. Isolates were collected from grape leaves, shoots, or green berries covered with sporulating powdery mildew.

Isolation. To obtain clonal isolates, a single powdery mildew conidium from contaminated samples was picked under a dissecting microscope under a laminar-flow hood by using an eyelash fastened to a holder. The conidium was placed on the upper surface of a young leaf of grape cultivar "Cinsaut" previously decontaminated by 10 min of immersion in 50 g of calcium hypochlorite per liter, rinsed, and placed on 2% water agar medium in 9-cm-diameter petri dishes. Inoculated leaves were incubated for 14 days at 22°C under 16-h/day illumination (1,000 lx). *U. necator* colonies appeared within 5 to 7 days, and sporulation began 8 to 10 days after inoculation.

Inoculation procedure. Powdery mildew isolates were inoculated under sterile conditions on the upper surface of four decontaminated grape leaves. Petri dishes were placed at the bottom of a Plexiglas settling tower (60 cm high, 25 by

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TABLE 1. Sensitivity of the 91 *U. necator* isolates to the DMI fungicide triadimenol[a]

Origin	Triadimenol sensitivity			
	Sensitive isolates	Resistant isolates		
		RF, <5	10 ≥ RF ≥ 5	RF, >10
France	19	5	9	4
Germany	8	0	0	0
Portugal	5	3	0	2
Switzerland	0	4	2	1
India	27	0	0	1
Israel	0	1	0	0

[a] Resistant isolates (MIC, >3.4 μM triadimenol) were divided into three classes according to their RF.

25 cm square section) previously rinsed with 70% (vol/vol) ethanol and dried. Conidia were blown at the top of the tower from sporulating leaves by using a Pasteur pipette inserted into a flexible plastic tube (3-mm diameter) connected to an aquarium pump and allowed to settle. The plastic tube and the pipette were rinsed with 70% ethanol and allowed to dry before each inoculation to avoid cross contamination between isolates. Inoculum density (600 to 800 conidia per cm^2 of leaf) was determined by placing a hemacytometer among the plates and counting the settled conidia. Petri dishes were sealed with Scel-O-frais (Colgate-Palmolive, Courbevoie, France), and inoculated leaves were incubated for 14 days under the conditions described above. Isolates were subsequently used for fungicide sensitivity testing or fungal material production.

Fungicide testing. The fungicide testing technique used was previously described (29). The triazole DMI used in our experiments was triadimenol (Baytan 5L [triadimenol at 50 g/liter]; kindly supplied by Bayer S. A., Puteaux, France). This fungicide is widely used to control *U. necator*. Nine fungicide concentrations were used: 0.0, 0.3, 1.0, 1.7, 2.7, 3.4, 6.8, 16.9, and 33.8 μM. Receptive grape leaves were decontaminated as described above, and 18-mm-diameter discs were punched out of them. The upper surface of each disc was placed in contact with sterile filter paper soaked with 3 ml of fungicide solution in a petri plate. Ten discs were used per plate. After 24 h of incubation at 22°C, the discs were aseptically transferred upper side up to sterile filter paper soaked with 1.5 ml of sterile water deposited on 2% water agar medium in 9-cm-diameter petri plates. The nine sets of 10 discs were inoculated simultaneously in the same inoculation tower (60 cm high, 35 by 35 cm square section) with *U. necator* conidia and incubated for 14 days as described above. Fungal growth was determined under a dissecting microscope by assessing the percentage of leaf disc surface covered with sporulating powdery mildew for each fungicide concentration. Fungicide testing was performed three times with a range of concentrations adapted to the response of each isolate in order to obtain a dose-response curve (growth inhibition plotted versus fungicide concentration). Triadimenol concentrations inhibiting 50% (50% inhibitory concentration [IC_{50}]) and 100% (MIC) of isolate growth were graphically deduced from these curves. An isolate was considered triadimenol resistant if the MIC of triadimenol was greater than 3.4 μM (30). Resistance factors (RF) were calculated for all of the resistant isolates by using the formula RF = IC_{50} for isolate/IC_{50}S, where IC_{50}S is the mean IC_{50} computed for all sensitive isolates.

P-450_{14DM} gene cloning and sequencing. Mycelium and conidia from selected isolates of *U. necator* resistant or sensitive to triadimenol were harvested and submitted to DNA extraction as previously described (8). Primers C14 (5′-TA AGGTAGTATTGAGGCGGG) and C14R (5′-TTCTAACCCTAACACCTGC C), which correspond to sequences flanking the 1,683-bp gene encoding P-450_{14DM} in *U. necator* (see Fig. 1; GenBank accession no. U72657) were used to generate a 1,756-bp-long PCR fragment. Reaction mixtures of 20 μl containing 70 mM Tris-HCl, 2 mM $MgCl_2$, 17 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 0.05% (wt/vol) polyoxyethylene-ether W1 (Sigma Chemical Co., St. Louis, Mo.), 0.2-mg/ml bovine serum albumin, 200 μM each dATP, dCTP, dGTP and dTTP, approximately 10 ng of template DNA, 0.5 U of Goldstar DNA polymerase (Eurogentec S.A., Seraing, Belgium), and primers C14 and C14R at 0.2 μM each were submitted to 37 cycles of PCR amplification, each consisting of 30 s of denaturation at 94°C, 1 min of annealing at 65°C, and 90 s of extension at 72°C, on a Cetus DNA thermal cycler (Perkin-Elmer, Norwalk, Conn.). Amplified fragments were cloned in *Escherichia coli* XL1 Blue (Stratagene, La Jolla, Calif.) by using the pGEM-T vector system (Promega Corporation, Madison, Wis.). For each *U. necator* isolate used in cloning and sequencing experiments, three DNA inserts were sequenced on both strands by extension of specific primers with a T7 sequencing kit (Pharmacia, Uppsala, Sweden). Sequences were analyzed by using Seqaid II software (24a) for translations.

Allele-specific PCR amplification. Mycelium and conidia from all 91 isolates of *U. necator* studied were harvested and subjected to DNA extraction as previously described (8). Primer MUT1 (5′-AATTTGGACAATCAA) was designed specifically for priming of P-450_{14DM} sequences exhibiting a A-to-T mutation at nucleotide 462, considering that a 3′ mismatch does not prime in an amplification reaction under specific annealing temperatures (27, 28). This primer was used in PCR amplifications together with primer U14DM (5′-ATGTACATTGCTGAC ATTTTGTCGG), which targets the first 25 bases of the *U. necator* P-450_{14DM} coding sequence, to amplify a 476-bp DNA fragment. Amplification was done as described above, except that each primer was used at a final concentration of 0.1 μM. The annealing temperature was 50°C. Amplified fragments were visualized under UV light after electrophoresis in ethidium bromide (0.4 μg/ml)-stained 1.1% agarose gels run in 0.5× Tris-borate-EDTA buffer.

Nucleotide sequence accession number. The nucleotide sequence of the P-450_{14DM} mutant allele (see Fig. 1) will appear in GenBank under accession no. U83840.

RESULTS

Triadimenol sensitivity. Of the 91 isolates tested, 59 were sensitive (MIC, <3.4 μM) and 32 were resistant to triadimenol (Table 1). The MICs for sensitive isolates ranged from 1.3 to 2.9 μM triadimenol. The MICs for resistant isolates ranged from 8.5 μM to more than 50.0 μM triadimenol. The mean IC_{50} calculated for the 59 sensitive isolates was 0.85 μM triadimenol. No growth difference was observed between sensitive and resistant isolates on water-treated leaf discs. Of the European isolates, which came from vineyards where unsatisfactory disease control due to resistance was reported or at least suspected, 32 were sensitive and 30 were resistant to triadimenol. Of 28 Indian isolates, 1 was highly resistant to triadimenol (MIC, 22.0 μM; RF, 10.5). The Israeli isolate was weakly resistant to triadimenol (MIC, 9.5 μM; RF, 2.4). The RF of resistant isolates ranged from 1.8 to 26.0. Resistant isolates were arbitrarily divided into three classes corresponding to weakly resistant isolates (RF, <5), moderately resistant isolates (10 ≥ RF ≥ 5), and highly resistant isolates (RF, >10) (Table 1). Twelve isolates representing the four sensitivity classes were chosen for P-450_{14DM} gene cloning and sequencing (Table 2).

P-450_{14DM} gene cloning and sequencing. PCR amplifications performed on DNAs extracted from the 12 isolates of *U. necator* yielded a single DNA fragment of the expected size (1,756 bp; data not shown). Three DNA inserts containing the gene encoding P-450_{14DM} were sequenced per isolate of *U. necator*. Sequences of P-450_{14DM} genes from sensitive isolates (FIO12, FMA31, FPE11, FPE21, and GUN13) and weakly resistant isolates (IsBD11 and PTV11) were identical. Sequences from moderately resistant (FPE22 and FPE32) and highly resistant (FMA21, PAZ11, and SNO11) isolates all exhibited a single A-to-T change at nucleotide 462, which resulted in the presence of a phenylalanine residue at position 136 in the P-450_{14DM} of these isolates instead of the tyrosine

TABLE 2. Sensitivity of the 12 *U. necator* isolates used for P-450_{14DM} gene cloning and sequencing experiments to the DMI fungicide triadimenol

Isolate	Origin	IC_{50} (μM)	MIC (μM)	RF
FIO12	France	0.7	2.4	S[a]
FMA21	France	11.2	28.7	13.2
FMA31	France	0.3	1.3	S
FPE11	France	0.3	1.7	S
FPE21	France	0.3	1.7	S
FPE22	France	4.4	15.2	5.2
FPE32	France	5.1	18.6	6.0
GUN13	Germany	1.7	2.7	S
IsBD11	Israel	2.0	9.5	2.4
PAZ11	Portugal	21.9	>50.0	26.0
PTV11	Portugal	2.0	7.4	2.4
SNO11	Switzerland	21.3	>50.0	25.2

[a] Sensitive isolate.

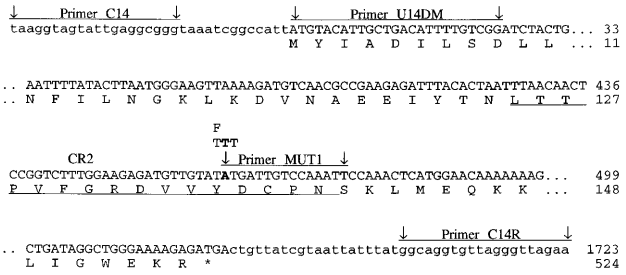

FIG. 1. Partial nucleotide and deduced amino acid sequences of the P-450$_{14DM}$ gene of *U. necator*. The nucleotide sequence is numbered relative to the first base of the ATG initiation codon, and the number on the right of each line represents the position of the last nucleotide or amino acid residue in that line. Sequence truncation is indicated by ellipsis. The CR2 conserved domain of P-450$_{14DM}$ is underlined, and the positions of PCR primers C14, C14R, U14DM, and MUT1 are indicated above the nucleotide sequence. The modified codon and the corresponding deduced amino acid found in the mutant P-450$_{14DM}$ genes from resistant isolates are given above the sequence. The substituted nucleotide is in bold type.

residue in the P-450$_{14DM}$ of the remaining isolates (Fig. 1). This tyrosine residue at position 136 is conserved in all known fungal and animal P-450$_{14DM}$ gene sequences. It is located in a highly conserved domain of P-450$_{14DM}$ called CR2 (consensus amino acid sequence for all known forms of P-450$_{14DM}$: L-T-T-P-V-F-G-X-X-V-X-Y-D-X-P-N), which is presumably involved in substrate recognition (2).

Allele-specific PCR amplification. Primers MUT1 and U14DM (Fig. 1) were used for PCR amplification of DNAs extracted from the 91 isolates of *U. necator* and from various fungi to assess the specificity of amplification (Fig. 2). Amplifications were performed at least twice with two independent DNA extractions. No amplification was obtained with DNAs extracted from other fungi that were present on grape leaves from the field (*Botrytis* sp., *Trichoderma* sp., *Penicillium* spp., *Rhizopus nigricans*, and unidentified yeasts). No amplification occurred with DNAs from sensitive and weakly resistant (RF, <5) *U. necator* isolates. The quality of the extracted DNAs for use as templates was assessed by performing PCR on these samples by using primers C14 and C14R, which readily yielded a DNA fragment of the expected size (data not shown). Allele-specific amplifications performed on DNAs extracted from all 8 highly resistant isolates (RF, >10) and from 9 of the 11 moderately resistant isolates (10 ≥ RF ≥ 5) yielded an amplified DNA fragment of the expected size (Fig. 2). No amplification was obtained with DNAs extracted from three moderately resistant isolates from Portugal (one isolate; RF, 6.4) and Switzerland (two isolates; RF, 5.6 and 6.8). Amplification of these DNA samples was obtained when primers C14 and C14R were used. Sequencing of a 360-bp region ranging from nucleotide 405 to nucleotide 766 in the P-450$_{14DM}$ gene of these isolates revealed no difference from the corresponding region in the gene of sensitive or weakly resistant isolates.

DISCUSSION

Triadimenol sensitivity assays of 91 isolates of *U. necator* revealed a broad range of MICs and RF. This is in agreement with previous work suggesting polygenic control of DMI resistance in this fungus (29).

Our results provided further insight into the molecular basis of resistance to DMIs in *U. necator*. By sequencing the P-450$_{14DM}$ gene of isolates of this fungus, we found a point mutation that was always associated with a resistant phenotype (RF, >5). Allele-specific PCR experiments revealed that the P-450$_{14DM}$ gene of 16 of the 19 isolates with RF of at least 5 contained the same nucleotide substitution at codon 136.

The consequence of this mutation is the substitution of a phenylalanine residue for a tyrosine residue in a P-450$_{14DM}$ conserved putative substrate recognition site (2). These two amino acids have similar molecular structures, except that tyrosine has a hydroxyl group that does not exist in phenylalanine. The loss of a hydroxyl group in the active site of P-450$_{14DM}$, which is the only consequence of the mutation, should increase the hydrophobicity of the active site without affecting its configuration. Increased hydrophobicity at the P-450$_{14DM}$ active site might reduce the affinity of the enzyme for its inhibitor (a polar molecule with a hydrophobic skeleton) without significantly affecting the sterol biosynthetic pathway. This hypothesis is consistent with the fact that similar sterol compositions were found in triadimenol-sensitive and -resistant isolates in previous studies (6, 7).

There is strong evidence that the point mutation found in the P-450$_{14DM}$ gene of resistant isolates of *U. necator* is a major cause of resistance to triadimenol, although complementation experiments are needed to prove this point. The mutation has been found in the P-450$_{14DM}$ gene of most, but not all, isolates with RF higher than 5. It has not been found in isolates with RF lower than 5. This mutation is thus probably sufficient to confer a substantial level of resistance to triadimenol. This conclusion is reinforced by a recent report in which the substitution of a phenylalanine residue for a tyrosine residue was found in the P-450$_{14DM}$ gene of two laboratory mutants of *Penicillium italicum* with high and moderate degrees of resistance to DMIs (12). This substitution occurred at the same position as the mutation found in the *U. necator* P-450$_{14DM}$ gene. A third mutant of *P. italicum* with a low degree of resistance did not carry this mutation. It is possible that some resistant isolates of *U. necator* possess another point mutation(s) in the P-450$_{14DM}$ gene that may result in different levels of DMI resistance, as shown elsewhere for resistance to benzimidazole fungicides (34). In this respect, cloning and sequencing of the gene encoding P-450$_{14DM}$ in additional resistant isolates would be of interest.

Resistance mechanisms independent of P-450$_{14DM}$ are likely to exist in the field. Occurrence of different resistance mechanisms could explain the different RF observed for the resis-

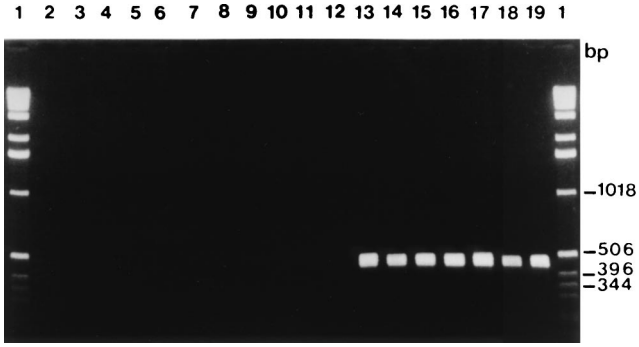

FIG. 2. P-450$_{14DM}$ allele-specific PCR amplification products from genomic DNAs extracted from triadimenol-sensitive and -resistant isolates of *U. necator* by using primers MUT1 and U14DM (Fig. 1). Lanes: 1, molecular weight marker (1-kb DNA ladder; Gibco-BRL); 2, H_2O negative control (no DNA); 3, various fungi, from grape leaves collected in the field, which grew on potato dextrose agar plates; 4 to 8, isolates FIO12, FMA31, FPE11, FPE21, and GUN13 (triadimenol sensitive); 9, isolate IsBD11 (RF, 2.4); 10, isolate PTV11 (RF, 2.4); 11, isolate SSI13 (RF, 4.2); 12, isolate FPE22 (RF, 5.2); 13, isolate FPE32 (RF, 6.0); 14, isolate FPO11 (RF, 6.1); 15, isolate IBA11 (RF, 10.0); 16, isolate FMA21 (RF, 13.2); 17, isolate SNO11 (RF, 25.2); 18, isolate PAZ11 (RF, 26.0).

tant isolates tested with triadimenol. Accumulation of resistance mechanisms may result in an increase in RF. This is probably the case with some highly resistant isolates (RF, >10) in which the P-450_{14DM} gene was sequenced and found to contain only the mutation at codon 136, like the P-450_{14DM} genes of isolates exhibiting significantly lower RF (Table 2).

Systematic cross-resistance to DMIs has not been reported for phytopathogenic fungi. Preliminary fungicide assays conducted with other DMI fungicides on isolates highly resistant to triadimenol showed that isolates very resistant to triadimenol could be sensitive to other DMI molecules. In contrast, some isolates weakly resistant to triadimenol (RF, <5) appeared to be also weakly resistant to other DMIs. The basis of resistance to DMIs in *U. necator* therefore seems to be complex, and additional studies are needed before this resistance is understood. This will be of great help for developing molecular methods for monitoring field resistance to existing fungicides, as well as for determining strategies for the use of new molecules.

Current methods of assessing fungicide resistance in obligate biotrophic phytopathogenic fungi involve the use of host plant leaves soaked with the antifungal agent. Such a procedure is extremely time and labor consuming, especially when the tests need to be repeated. Fungicide testing also requires a sufficient amount of sporulating fungal material. By using a specific PCR assay, such as the one developed here by using DNA extracted from mycelium and/or conidia of grape powdery mildew, it will be possible to reduce the amount of work required for fungicide resistance monitoring and therefore to considerably increase the number of samples analyzed for fungicide resistance.

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