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Molecular insights into fungicide resistance in sensitive and resistant *Penicillium digitatum* strains infecting citrus

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ABSTRACT

The continuous use of chemical fungicides on citrus postharvest has led to the development of resistant strains against the fungicides in use, representing a considerable threat because the control systems are no longer effective. Evaluation of the sensitivity of 75 *Penicillium digitatum* strains to seven different fungicides revealed the presence of a significant number of TBZ- (84%) and IMZ-resistant (77%) strains, i.e., those fungicides most used in citrus postharvest. Molecular characterization of different *P. digitatum* genes involved in fungicide resistance was carried out. All *P. digitatum* genes were selected based on particular mechanisms of resistance due to fungicide target or mode of action. TBZ-resistance was characterized by a unique point mutation in the β-tubulin gene sequence corresponding to amino acid 200, confirming previous work on this subject. Moderate to low resistance to strobilurins did not reveal any mutation in the cytochrome *b* gene. DMI-resistance was evaluated by examining the *CYP51* gene and four different ABC transporters *PMR1*, *PMR3*, *PMR4* and *PMR5*. The *CYP51* gene did not exhibit any mutation relating to DMI-resistance, but a five tandem repeat sequence previously described was found in the *CYP51* promoter in 3 of the 75 isolates examined, whereas DMI-sensitive isolates and the other DMI-resistant isolates of *P. digitatum* had only one tandem repeat. Of all the ABC transporters studied, only *PMR1* and *PMR5* appear to be involved in fungicide resistance and several mutations were found in the promoter and the coding region for *PMR5* in resistant strains compared to sensitive ones. In all cases, the resistance mechanism was consistent in both orchard or packing-house isolates and no differences conferred by either origin or fungicide pressure were observed.

Consequently, since different processes have been described that confer fungicide resistance to the same compounds, such as DMIs, the hypothesis that multiple mechanisms could be acting simultaneously gains strength.

1. Introduction

Decay accounts for the most significant postharvest losses of citrus worldwide. Green mold caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. is most commonly responsible for postharvest decay of citrus, contributing up to 90% of total losses, especially in arid and sub-tropical climates (Eckert and Wild, 1983; Eckert and Eaks, 1989; Li, 2001). Good practices such as avoiding injury during harvest and transportation, as well as sanitation of packing- and store-houses, can reduce postharvest decay. However, the application of fungicides after harvest has been shown to provide the most effective control. Thiabendazol (TBZ), Imazalil (IMZ) and sodium o-phenylphenate (SOPP) are the most commonly used fungicides worldwide for managing green and blue molds of citrus (Bus et al., 1991; Bus, 1992; Smilanick et al., 2005, 2006), but resistance to these fungicides is very common and compromises their efficacy (Bus et al., 1991; Eckert et al., 1994; Holmes and Eckert, 1995, 1999; Zhu et al., 2006). Therefore, resistance has become an important factor in limiting the effectiveness and useful lifetime of fungicides, which are being developed at increasingly high costs (Kinay et al., 2007).

Extensive molecular studies have been reported to explain mechanisms of fungicide resistance and to develop effective, rapid methods for the detection of resistant genotypes in pathogens (Ma and Michialides, 2005). Different mechanisms for fungicide resistance have been described, not only in *P. digitatum* but in other phytopathogenic fungi (Gisi et al., 2000; Ma and Michialides, 2005; De Waard et al., 2006). Resistance to benzimidazole fungicides has been detected in many fungal species, and in most cases, resistance is correlated with point mutations in the β-tubulin gene that result in altered amino acid sequences at the benzimidazole-binding site (Koenraadt et al., 1992; Holloman et al., 1998; Albertini et al., 1999; Baraldi et al., 2003; Ma et al., 2003; Schmidt et al., 2006; Banno et al., 2008).

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Resistance arising from the use of different demethylation inhibitors (DMIs) has been reported in several phytopathogenic fungi (Délye et al., 1998; Köller and Wilcox, 1999; Luo and Schnabel, 2008; Ma et al., 2006) and a range of different resistance mechanisms have been identified. The main mechanism involves mutations or over-expression of the *CYP51* gene that encodes sterol 14α-demethylase, the target enzyme of DMI fungicides (van Nistelrooy et al., 1996). A substitution of phenylalanaine for tyrosine at position 136 (Y136F) was found in *Uncinula necator* (Délye et al., 1997) and also in *Erisiphe graminis* f.sp. *hordei* (Délye et al., 1998). Different mutations have also been found in *Tapesia* sp. (Albertini et al., 2003), *Penicillium italicum* (Joseph-Horne and Hollomon, 1997) and *Ustilago maydis* (Butters et al., 2000). But changes in levels of expression of *CYP51* have also been found to be responsible for DMI-resistance. In *P. digitatum*, a unique 126-bp sequence in the promoter region of *CYP51* is tandemly repeated five times in resistant isolates, but is represented only once in sensitive isolates. This provides a quick and easy method to detect DMI-resistant strains of *P. digitatum* (Hamamoto et al., 2001a). Insertions in the promoter have also been reported in *Blumeriella jaapii* (Ma et al., 2006), *Venturia inaequalis* (Schnabel and Jones, 2001) and in *Monilinia fructicola* (Luo et al., 2008), while recently, another insertion was described in *P. digitatum* associated with resistant phenotypes (Ghosoph et al., 2007).

Currently, the most widely accepted mechanism for preventing or reducing the accumulation of DMIs inside cells and, hence, the avoidance or reduction of their toxic action, is based on active efflux of these compounds by transporters such as ABC (ATP binding cassette) or MFS (Major Facilitator Superfamily) that have a remarkably broad substrate specificity, although they are also capable of transporting specific compounds (Stergiopoulos et al., 2002). Both these transporter families are involved in multiple resistance against anti-microbial compounds that protect the organism against a wide variety of toxic compounds of synthetic and natural origins, which can include the defence compounds of the plant (Del Sorbo et al., 2000; Andrade et al., 2000; Hayashi et al., 2001, 2002a,b; Schoonbeek et al., 2001; Zwiers et al., 2002; De Waard et al., 2006).

Two genes from *P. digitatum*, identified as ABC transporters, have been cloned and characterized (*PMR1* and *PMR5*). Both genes are involved in fungicide resistance and increased expression in the presence of different toxic compounds has been reported (Nakaune et al., 1998, 2002).

New fungicides have been registered in the USA for postharvest disease control of citrus (Smilanick et al., 2006; Kanetis et al., 2007, 2008), of which Azoxystrobin has proven to be very effective in controlling green mold. Nevertheless, the risk of resistance remains and its molecular mechanism has been recently reported for *P. digitatum* UV mutants (Zhang et al., 2009), involving a G143A mutation.

Taking account of these mechanisms, we aim to uncover the factors relevant to fungicide resistance in *P. digitatum* by analysing different genes involved in fungicide resistance at the molecular level to try to establish the putative differences between resistant and sensitive strains of *P. digitatum* that infect citrus.

2. Material and methods

2.1. Collection of P. digitatum isolates

All isolates of *P. digitatum* strains were collected from different orchards and from packing-houses. The isolates were taken from citrus fruit with typical green mold symptoms. A mass of conidia was picked from a lesion with a sterile toothpick and streaked onto potato-dextrose agar (PDA) with 100 mg/mL streptomycin (Sigma). Following incubation at 24 °C for 24 h, a single colony was excised and cultured on a fresh PDA plate.

Conidia were collected from 1-week-old plates by scraping them with a sterile spatula, and transferring them to sterile water. Conidia were then filtered, and titrated with a hemacytometer and adjusted to the desired final concentration.

2.2. Determination of fungicide sensitivity

The activities and efficacy of the following fungicides were tested: Azoxystrobin (Quadris; Syngenta Crop Protection), Fludioxonil (Scholar230; Syngenta Crop Protection), Imazalil (Textar I; Tecnidex), Myclobutanil (Thiocur 12; Rohmand Haas Italia SRL), Prochloraz (Ascurit; Tecnidex), and Trifloxystrobin (Flint; Bayer CropScience) all at concentrations of 0, 0.25, 0.5, 1.0, 2.0, 5.0, and 10.0 μg/mL and Thiabendazol (Textar 60 T; Tecnidex) at concentrations of 0, 0.5, 1.0, 5.0, 10.0, 20.0 and 40.0 μg/mL.

To assess the effect of different fungicides, antifungal activity was determined using a microtiter plate assay as described previously (López-García et al., 2002) using 2.5×10^5 conidia/mL. Growth was determined by OD at 492 nm in a Multiskan spectrum microplate spectrophotometer (Thermo Electron Corporation, Finland). The growth medium was PDB (Liofilchen) containing 50 μg/mL streptomycin (Sigma). Three replicates were prepared for each treatment.

2.3. Polymerase chain reaction (PCR) and DNA sequencing

PCR was used for gene amplification using fungal genomic DNA as a template. Fungal genomic DNA isolation was carried out according to Lee et al. (1988) and utilizing specific primers (Table 1) for *PMR1* (AB010442), *PMR3* (AB164459), *PMR4* (AB164460), *PMR5* (AB060639), *CYP51* (AB030179), β-tubulin (D78154) and cytochrome *b* (FM177883) for PCR amplification.

Standard PCR conditions consisted of an initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 45 s

Table 1
Sequence of olignucleotides used in this study.

Oligo name	Gene	Sequence 5′–3′
Tub1F	*β-Tubulin*	CTGACTCGTCACCGGTTCTC
Tub4R	*β-Tubulin*	GCGCTCAAACAAATTGGGTTC
Tub3F	*β-Tubulin*	GATTGTCTTGAGAAATACTG
Tub3R	*β-Tubulin*	TGTACCAGTGCAAGAAAGCC
PMR1-1F	*PMR1*	GAGGCTGCGACCGACATGTC
PMR1-1R	*PMR1*	CTTCCAGCACGGTCACTGTC
PMR1-cdF	*PMR1*	CCCCACTTAATCAGCCCCACCT
PMR1-cdR	*PMR1*	AGGAAGGATCAGGTATGGCGGTCT
PMR1-1967F	*PMR1*	ACATGCTTGGTTGGTCTCGCTGG
PMR1-2239R	*PMR1*	TAATTCCCATGTTCCTCCACCTGT
ProPMR3F	*PMR3*	CATCTTAGCTCTTTTCCGGC
ProPMR3R	*PMR3*	TGTGGACCACCTAGTGC
PMR3c-For	*PMR3*	AGCTTCATTTTCTTGTTGTG
PMR3-1-Rev	*PMR3*	ATTTCTCCTTTGCCTACGTC
PMR3-1-For	*PMR3*	ATTTCTCTGACATGCGCTAT
PMR3c-Rev	*PMR3*	TACATATCTGTCTCTCACTGC
ProPMR4F	*PMR4*	ACATCCACAGTTGGTAAGGC
ProPMR4R	*PMR4*	GAGCAGGATTTGGGTTGATTG
PMR4c-For	*PMR4*	AATCTAGGATTTGAAAGGTTG
PMR4-1-Rev	*PMR4*	TGGACTTCTTCTCGGAGATG
PMR4-1-For	*PMR4*	CATGTCCACCTATCTTTTTG
PMR4c-Rev	*PMR4*	TAGGCACTGGCTCTGGTCTA
PMR5-F	*PMR5*	CTCTACCTTCCACCGCGATG
PMR5-N-R	*PMR5*	TTTCACCCATCCATGGACGTT
PMR5-H-F	*PMR5*	AGGCGCATCAAGCTTGTGCAG
PMR5-R	*PMR5*	TCTAAAATCGTTAGAGAGGAT
CYP1F	*CYP51*	ACATCTGGGGATGGCCTGAC
CYP2F	*CYP51*	GAGGAAATCTACGGCAAG
CYP3R	*CYP51*	CAGGTTCGAGATCATGTCGG
CYP3F	*CYP51*	CCAACGACAACGGCAGGGAC
CYP-426R	*CYP51*	CGAAGACGGGGGTTGTAAGCT
CYP-1875R	*CYP51*	CTCGCGTGAACCTATGATCGT