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CHARACTERIZATION OF THE CYTOCHROME *B* GENE IN PLANT PATHOGENIC BASIDIOMYCETES AND CONSEQUENCES FOR QoI RESISTANCE

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CHARACTERIZATION OF THE CYTOCHROME *B* GENE IN PLANT PATHOGENIC BASIDIOMYCETES AND CONSEQUENCES FOR QoI RESISTANCE

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Der Dekan
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To my family

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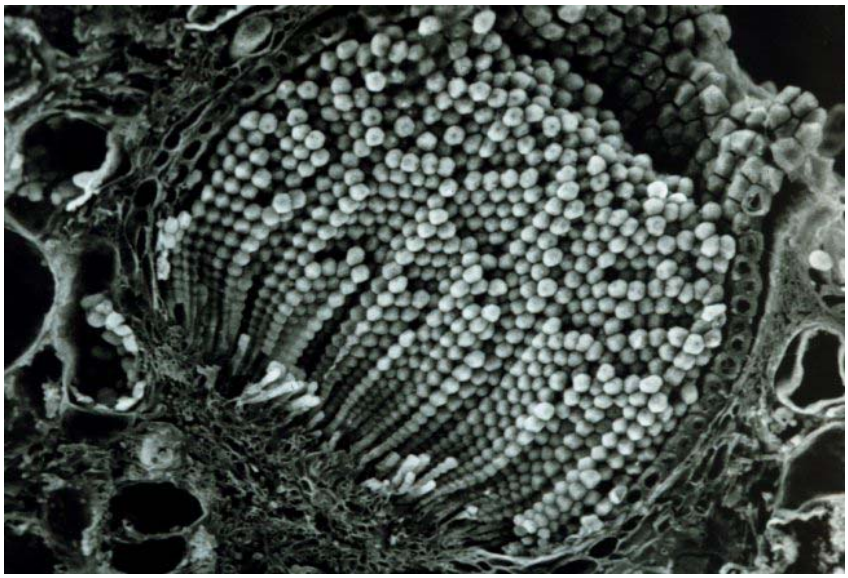
CHAPTER 1

Introduction



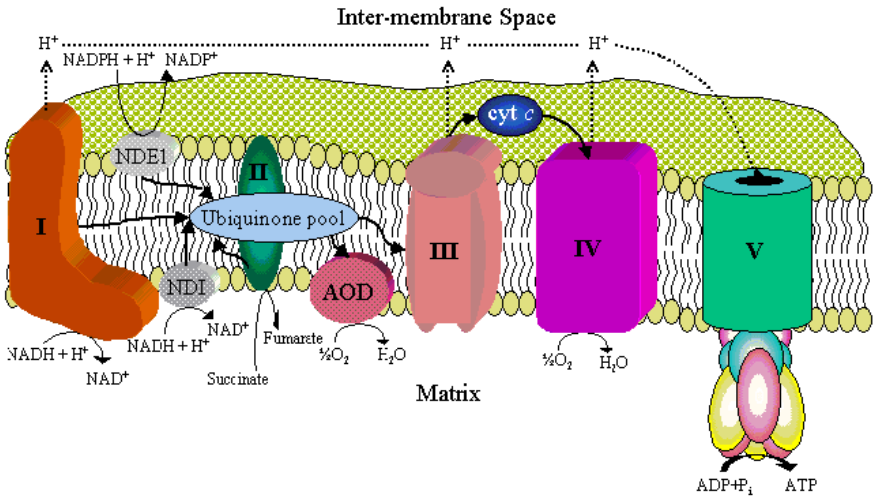
Strobilurins are an important class of agricultural fungicides and their invention was inspired by a group of fungicidally active natural products. These compounds have a single-site mode of action. They are known as Qo inhibitors (QoIs) because they inhibit mitochondrial respiration by binding to the Qo site (the outer, quinone oxidizing pocket) of the cytochrome bc_1 enzyme complex (complex III), thus blocking electron transfer in the respiration pathway and leading to energy deficiency due to a lack of ATP (Bartlett *et al.*, 2002). QoIs were first introduced to the market in 1996 and now several molecules are commercially available (azoxystrobin, kresoxim-methyl, metominostrobin, trifloxystrobin, picoxystrobin, pyraclostrobin, fluoxastrobin, orysastrobin, dimoxystrobin). In addition, famoxadone and fenamidone, chemically distinct from the strobilurins but in the same cross-resistance group, are also commercially available. QoI fungicides represent over 10% of the global fungicide market and are now registered in 72 countries for use on more than 80 different crops, including cereals, turfgrass, grapevines, potatoes, fruit, nut and vegetable crops (Phillips McDougall, 2001).

Discovery and mode of action of QoI fungicides

The discovery of the strobilurin fungicides was inspired by a group of natural fungicidal derivatives of β-methoxyacrylic acid (Becker *et al.*, 1981). These natural compounds, such as strobilurin A, are produced by a range of Basidiomycete wood-rotting fungi, such as *Strobilurus tenacellus*. Strobilurins are able to inhibit mitochondrial respiration by binding at the so-called Qo site of cytochrome *b*. Cytochrome *b* is part of the cytochrome bc_1 complex (or complex III), located in the inner mitochondrial membrane of fungi and other eukaryotes (Fig. 1). When one of the inhibitors binds, it blocks electron transfer between cytochrome *b* and cytochrome c_1 and leads to energy deficiency due to a lack of ATP. The natural strobilurins are unsuitable for use as agricultural fungicides because of their photochemical instability and volatility. However, the knowledge of their structures and physical properties provided the starting point for many researches, which led to the

development of synthetic analogues, known as QoI fungicides (Sauter *et al.*, 1996 and 1999).

Figure 1. The mitochondrial respiratory chain. (from http://pages.slu.edu/faculty/kennellj/)



The first commercially available strobilurins were azoxystrobin (Godwin *et al.*, 1992; Clough and Godfrey, 1998), kresoxim-methyl (Ammermann *et al.*, 1992; Sauter *et al.*, 1999) and metominostrobin (Clough and Godfrey, 1998; Hayase *et al.*, 1995). Few years later trifloxystrobin (Margot *et al.*, 1998), picoxystrobin (Godwin *et al.*, 2000) and pyraclostrobin (Ammerman *et al.*, 2000) were announced. During the 1990s also famoxadone (Joshi and Sternberg, 1996) and fenamidone (Mercer *et al.*, 1998) were discovered. The various QoI fungicides have very different physicochemical properties which confer different behaviours in the plant. For example, picoxystrobin is the most rapidly absorbed into plant tissue and the most xylem-systemic (Godwin *et al.*, 2000). Azoxystrobin (Goodwin *et al.*, 1999) and metominostrobin (Masuko *et al.*, 1993) are also xylem-systemic. In contrast, kresoxim-methyl (Ammermann *et al.*, 1992), trifloxystrobin (Margot *et al.*, 1998, Reuvini, 2001) and pyraclostrobin (Ammermann *et al.*, 2000) are all non-systemic.

Spectrum of disease control

The major reasons for the success of QoI fungicides have to be found in their broad-spectrum activity, control of fungal isolates resistant to other fungicide modes of action, low use-rates and excellent preventative activity (Gullino *et al.*, 2000). QoIs are generally active against all four major groups of plant pathogens, namely Ascomycetes, Basidiomycetes, Deuteromycetes and Oomycetes. They are active against most of the foliar diseases of cereals and rice, such as *Mycosphaerella graminicola*, *Pyrenophora teres*, *Erysiphe graminis*, *Puccinia recondita*, *Puccinia hordei*, *Rhynchosporium secalis* and *Pyricularia grisea*. On grapevine, for the first time, a single active ingredient can control both powdery (*Uncinula necator*) and downy (*Plasmopara viticola*) mildews. QoI fungicides are also largely used on apple, banana, potato, tomato, turfgrass against pathogens such as *Venturia inaequalis*, *Podosphaera leucotricha*, *Mycosphaerella fijiensis*, *Phytophthora infestans*, *Alternaria solani*, *Pythium* spp., *Rhizoctonia solani, Microdochium nivale*. These fungicides have been shown to demonstrate high levels of preventative activity, mainly because of their potent inhibiting effect on spore germination and zoospores motility (Godwin *et al.*, 1994; Godwin *et al.*, 1997¸ Leinhos *et al.*, 1997; Stark *et al.*, 1996; Stierl *et al.*, 2000; Margot *et al.*, 1998; Ammermann *et al.*, 2000). Therefore, QoI fungicides are best applied prior to infection or in the early stages of disease development. However, curative activity has also been observed (Bartlett *et al.*, 2002). In addition, clear benefits, particularly in terms of yield and grain size, were observed following treatments with strobilurins (Bartlett *et al.*, 2002).

Mode of resistance to QoI fungicides

The risk of resistance developing in plant pathogens to fungicides is related to the mode of action of compounds as well as the biology of the pathogen species. In general, multi-site inhibitors confer a lower risk of resistance than inhibitors with a single site of action. Since the mode of action of QoI fungicides is highly specific, the risk of resistance is also

high. In addition, all the strobilurin compounds, and also famoxadone and fenamidone, belong to the QoI cross-resistance group. Cross-resistance means simultaneous resistance to all QoI compounds, since they have the same mode of action.

In 1998 QoI resistant isolates of *E. graminis* f.sp. *tritici* (Sierotzki *et al.*, 2000b) were first discovered in cereals. Since then, QoI resistance has been detected in other pathogens, including *Venturia inaequalis* (Steinfeld *et al.*, 2001 and 2002), *P. viticola* (Gullino *et al.*, 2004; Gisi *et al.*, 2002; Heaney *et al.*, 2000), *M. fijiensis* (Sierotzki *et al.*, 2000a), *M. graminicola* (Gisi *et al.*, 2005), *Pseudoperonospora cubensis* and *Sphaerotheca fuliginea* (Ishii *et al.*, 2001). The molecular mode of resistance to QoI fungicides is understood to an advanced level and at least 15 different point mutations have been described in the *cytb* gene leading to resistance (Brasseur *et al.*, 1996). In field isolates of different pathogen species, the major mechanism is the amino acid substitution of glycine with alanine at position 143 (G143A) of the cytochrome *b* protein (Gisi *et al.*, 2002; Fig.2). This amino acid substitution confers high levels of resistance.

Figure 2. Detail of the nucleotide and amino acid sequences of the *cyt b* gene from a sensitive and a resistant isolate of *Erysiphe graminis* f. sp. *tritici*. The codon for the glycine or alanine at position 143 is underlined. The nucleotide and amino acid residues involved in the mutation G143A conferring resistance to QoI fungicides are represented in bold.

	Nucleotide sequence	Amino acid sequence
Sensitive:	5' … TGG G**G**T GCA … 3'	… W**G**A …
Resistant:	5' … TGG G**C**T GCA … 3' (143)	… W**A**A … (143)

Nevertheless, in other species, including *V. inaequalis*, additional mechanisms leading to resistance were described (Steinfeld *et al.*, 2001 and 2002; Köller *et al.*, 2004; Kraiczy *et al.*, 1996; Brasseur *et al.*, 1996). In addition, a change in phenylalanine to leucine at position 129,

F129L, also confers resistance to QoIs in species such as *P. grisea* (Kim *et al.*, 2003) and *Pythium aphanidermatum* (Gisi *et al.*, 2002), although the level of resistance is lower than that conferred by the G143A substitution. The amino acid exchanges in mutants of *Saccharomyces cerevisiae* resistant to QoIs (Di Rago *et al.*, 1989) and in fungi naturally resistant to these compounds (Kraiczy *et al.*, 1996) are located in two highly conserved cytochrome *b* regions, so called 'hot spot' regions. For plant pathogenic fungi the first hot spot is at amino acid residues 120-160, containing the two main point mutations responsible for QoI resistance, G143A and F129L.

How to monitor and manage resistance to QoI fungicides

One of the key components of fungicide resistance management is the risk assessment of resistance development (Brent and Hollomond, 1998). A continuous and accurate sensitivity monitoring is necessary to provide information regarding the sensitivity of field populations in certain key crop-pathogen combinations and to track the evolution of resistance genes (Olaya and Köller, 1999; Wong and Wilcox, 2000 and 2002). Conventional monitoring methods are based on sensitivity tests *in vitro* or on leaf discs (for obligate pathogens such as downy and powdery mildews). Bioassays provide essential information on sensitivity to fungicides. However, these methods can be slow and costly, especially for obligate pathogens that cannot be grown on artificial media. DNA-based methods targeted at specific resistance genes offer rapid, cost-effective alternatives. Molecular methods can provide especially powerful tools to detect the early appearance of resistant isolates or to follow populations where resistance already exists. However, these detection methods are only of value if there is a very high correlation with the resistant phenotype. To develop appropriate molecular methods for the detection of resistance, it is important to know the gene(s) coding for the altered target protein and to characterize the alterations.

In the case of QoI fungicides, the characterization of the *cyt b* gene is essential for the development of diagnostic tools to easily isolate

the *cyt b* gene and detect point mutations conferring resistance to these compounds. In pathogens such as *E. graminis*, *M. fijiensis* and *V. inaequalis*, extensive sequence data for the *cyt b* gene were produced, mostly after the detection of isolates resistant to QoI fungicides (Sierotzki *et al.*, 2000a and b; Steinfeld *et al.*, 2001 and 2002; Zheng and Köller, 1997). Based on these sequences, specific primers and several PCR methods were developed to monitor resistance in field populations (Wille *et al.*, 2002; McCartney *et al.*, 2003). PCR linked with allele-specific probes, PCR-restriction fragment length polymorphisms and allele-specific PCR have been used to detect resistance based on point mutations in the target enzymes of different pathogens (Wille *et al.*, 2002; Sierotzki and Gisi, 2003). In addition, the quantification of resistance due to a point mutation, such as G143A or F129L, can be done with novel and powerful molecular technologies, such as the quantitative polymerase chain reaction (Q-PCR). Tests that measure the frequency of resistant alleles offer opportunities to improve the assessment of the risk of resistance development, optimize resistance management and support product development.

Although resistance occurred in the pathogens described above, QoI fungicides continue to be a key component of disease-management programmes. The evolution of resistance is driven by the selection process imposed by the fungicide and is strongly influenced by factors such as number, timing and type of applications (e.g. solo use, mixtures, alternations). In order to safeguard the efficacy of these compounds, specific guidelines for the proper use of QoI compounds are published every year (FRAC, http://www.frac.info/). These guidelines are based mainly on reducing the QoI selection pressure by limiting the number of applications of compounds of the QoI cross-resistance group. Alternation with effective compounds from different cross-resistance groups and, where appropriate, use of mixtures with effective partners are also strongly recommended. In addition, manufacturers' recommendations should be followed to ensure appropriate use-rates and correct spray intervals.

Why plant pathogenic Basidiomycetes in this study?

Among the most devastating plant diseases caused by fungal pathogens, rusts (*Uredinales*) are a large and important group of obligate biotrophic organisms that exhibit a complex life cycle (Littlefield and Heath, 1979; McLaughlin *et al.*, 1995). Severe damages on graminaceous crop plants, such as wheat, oat, barley, rye and corn, can be caused by several *Puccinia* species, including *P. recondita* f.sp. *tritici*, *P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis* and *P. sorghi*. Other rust species, such as *Uromyces appendiculatus*, *Phakopsora pachyrhizi* and *Hemileia vastatrix*, can cause serious losses on bean, soybean and coffee, respectively. Especially soybean rust is a serious disease in South America since 2001 (Pivonia and Yang, 2004) and an emerging pathogen in North America since 2004 (Klag, 2005). In spite of cultural practices and breeding for resistant cultivars, chemical control is the most effective and economic way currently used to protect crop plants from these diseases. The most important classes of chemical products against these pathogens are the demethylation inhibitors (DMIs) and the strobilurin fungicides.

In plant pathogenic Basidiomycetes such as the rusts *(Puccinia* spp.), resistance to QoIs has surprisingly not been reported until now, even though these pathogens have been treated with QoIs as frequent as powdery mildews in cereals. The strobilurin-producing Basidiomycetes *S. tenacellus* and *Mycena galopoda* exhibit 'natural resistance' to inhibitors of the ubiquinol oxidation centre of the cytochrome bc_1 complex and the molecular mechanisms of this 'natural resistance' are known to be point mutations in the *cyt b* gene (Kraiczy *et al.*, 1996). Since resistant isolates to QoIs were detected in several Ascomycetes and Oomycetes soon after the commercial introduction of these fungicides, extensive sequence data were produced for the *cyt b* gene of these pathogens (e.g. *E. graminis*, *P. viticola*, *M. fijiensis* and *M. graminicola*) in order to elucidate the molecular basis of resistance. For plant pathogenic Basidiomycetes, no *cyt b* gene sequences and,

consequently, no specific primers for this gene were available in the GenBank.

Aim of the work

The main goal of the present study was to amplify and sequence the *cyt b* gene fragment responsible for the binding site of QoI fungicides in different plant pathogenic Basidiomycetes. This fragment includes all possible mutations known to confer resistance to QoI fungicides in many plant pathogens (the two 'hot spot' regions, amino acid residues 120-160 and 250-300). Based on this information, specific primers for the *cyt b* gene of *Puccinia* spp. were designed to detect point mutations that eventually could evolve. The *cyt b* gene fragment was characterized in nine different *Puccinia* species, including *P. recondita* f.sp. *tritici*, *P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis*, *P. sorghi*, *P. arachidis* and *P. horiana*. The *cyt b* gene fragment of *Phakopsora pachyrhizi*, *Uromyces appendiculatus*, *Hemileia vastatrix* and *Rhizoctonia solani* was also sequenced.

Based on the new molecular data the relatedness within *Uredinales* and other Basidiomycetes, such as *S. tenacellus*, *Mycena viridimarginata* and *M. galopoda*, was investigated and compared with some Ascomycetes and Oomycetes. The purpose was to investigate the degree of relatedness among agronomically important pathogens by analysing mitochondrial DNA and ITS sequences.

The *cyt b* gene structure of these plant pathogens was characterized including the two 'hot spot' regions and compared with other organisms already described in literature, such as the strobilurin producing basidiomycetes, *S. cerevisiae*, *M. fijiensis*, *V. inaequalis* and *Alternaria alternata* (Ascomycetes). The gene structure of rusts and the other agronomically important plant pathogens was investigated especially in regard to the presence and absence, location and length of introns, and whether it may influence the occurrence of point mutations in the *cyt b* gene. Of special importance was the recognition of the

exon/intron junctions and their relevance for splicing of the cytochrome *b* gene.

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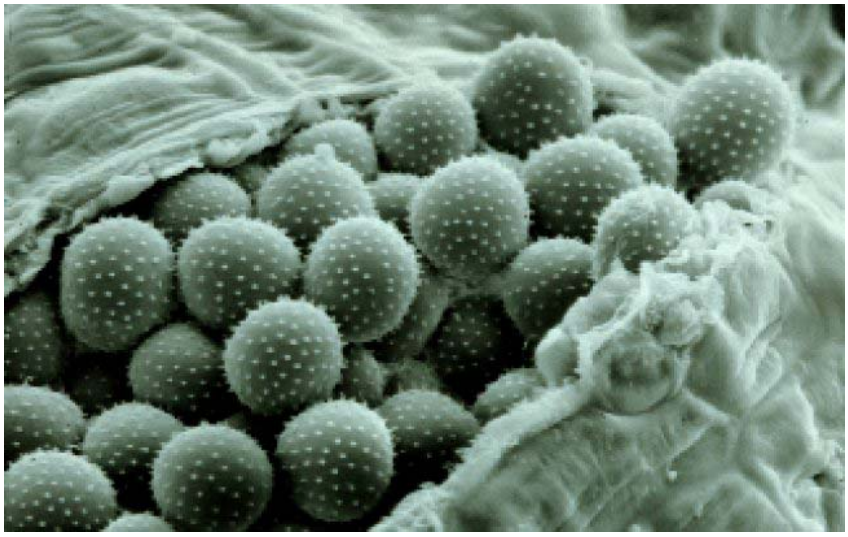
CHAPTER 2

Characterization of the cytochrome *b* gene fragment of *Puccinia* species responsible for the binding site of QoI fungicides



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Abstract

The fragment of the cytochrome *b* (*cyt b*) gene responsible for the binding site of QoI fungicides was sequenced for different *Puccinia* species by using DNA and RNA as template for PCR and RT-PCR, respectively. Degenerated primers for the *cyt b* gene amplified in *P. recondita* f.sp. *tritici* a 450 bp fragment, which was cloned and sequenced. At cDNA level, several *T*hermal *A*symmetric *I*nter*L*aced (*TAIL*)-PCR cycles were needed to produce a 996 bp long fragment, which corresponded to almost the whole *cyt b* gene (about 1160-1180 bp, without introns). This fragment was sequenced and specific primers were designed. Amplification with *cyt b* specific primers using genomic DNA as template revealed the presence of an intron of about 1500 bp length after the codon for glycine at amino acid position 143. By using the same primer pair, the *cyt b* gene fragment was amplified and sequenced both at cDNA and genomic DNA level also for other rust species, including *P. graminis* f.sp. *tritici* (length: 506 bp), *P. striiformis* f.sp. *tritici* (755 bp), *P. coronata* f.sp. *avenae* (644 bp), *P. hordei* (660 bp), *P. recondita* f.sp. *secalis* (687 bp), *P. sorghi* (709 bp) and *P. horiana* (478 bp). At the same position as for *P. recondita* f.sp. *tritici*, an intron of about 1500-1600 bp length was detected also in all other *Puccinia* species. High homologies were observed among all *Puccinia* species for both the exonic and intronic fragments of the *cyt b* gene. Specific primers for the *cyt b* gene of all eight *Puccinia* species were developed, which easily amplified the fragment of the gene including all possible mutations known to confer resistance to QoIs in several plant pathogens. However, in all tested isolates of the *Puccinia* species included in this study, the sequence of *cyt b* gene fragment did not contain any point mutations.

Keywords: Basidiomycetes, rust pathogens, mitochondrial respiration, strobilurin fungicides, point mutation, fungicide resistance

1. Introduction

Fungicides such as kresoxim-methyl, azoxystrobin, trifloxystrobin and pyraclostrobin ('strobilurins') have a single-site mode of action. They are known as Qo inhibitors (QoIs) because they inhibit mitochondrial respiration by binding to the Qo site (the outer, quinone oxidizing pocket) of the cytochrome bc_1 enzyme complex (complex III), thus blocking electron transfer in the respiration pathway and leading to energy deficiency due to a lack of ATP [1]. Part of this complex is the cytochrome *b*, which is encoded by the cytochrome *b* (*cyt b*) gene located in the mitochondrial genome. QoIs were first introduced to the market in 1996 and, after a short time period, resistant isolates were detected in field populations in several plant pathogens including *Blumeria graminis* f. sp. *tritici* [2], *Mycosphaerella fijiensis* [3], *Venturia inaequalis* [4, 5] and *Plasmopara viticola* [6, 7]. In most cases, resistance was conferred by a single point mutation in the *cyt b* gene leading to a change at amino acid position 143 from glycine to alanine (G143A) [6, 8]. Nevertheless, in other species, including *V. inaequalis*, additional mechanisms leading to resistance were described [4, 5, 9, 10]. In addition, a change in phenylalanine to leucine at position 129, F129L, also confers resistance to QoIs in species such as *Pythium aphanidermatum* and *Pyricularia grisea*, although the level of resistance is lower than that conferred by the G143A substitution [6, 11]. In Basidiomycetes such as *Puccinia* species, resistance to QoIs has surprisingly not been reported until now, even though these pathogens have been treated with QoIs as frequent as powdery mildews in cereals. The strobilurin-producing Basidiomycetes *Strobilurus tenacellus* and *Mycena galopoda* exhibit 'natural resistance' to inhibitors of the ubiquinol oxidation centre of the cytochrome bc_1 complex and the molecular mechanisms of this 'natural resistance' are known to be point mutations in the *cyt b* gene [9].

The main goal of the present study was to amplify and sequence the *cyt b* gene fragment responsible for the binding site of QoI fungicides in the Basidiomycete genus *Puccinia*. This fragment includes all possible mutations known to confer resistance to QoI fungicides in

many plant pathogens (amino acid residues 120-160 and 250-300). Based on this information, specific primers for the *cyt b* gene of *Puccinia* spp. were designed to detect point mutations that eventually could evolve. The *cyt b* gene fragment was characterized in eight different *Puccinia* species, including *P. recondita* f.sp. *tritici*, *P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis*, *P. sorghi* and *P. horiana*. In rusts, resistance to QoIs has never been reported until now, except for *P. horiana* [12], although the mechanisms were not elucidated in more detail.

2. Materials and Methods

2.1. Pathogen isolates

This study was carried out with eight different species of *Puccinia* with a total of 22 different isolates, of which DNA and RNA were extracted and a part of the *cyt b* gene was sequenced (Table 1). These isolates are stored in the Syngenta strain collection in Stein (Switzerland). For *P. horiana*, infected leaves of *Chrysanthemum* plants were collected in the UK in 2001 and kindly provided by Kelvin Hughes, Central Science Laboratory MAFF, Sand Hutton, York. One of these isolates, Phor4, showed disease symptoms after repeated treatments with azoxystrobin, therefore it was claimed to be resistant to QoI fungicides [12].

2.2. DNA extraction

Genomic DNA (gDNA) was extracted from spores and infected leaves, which were lyophilised overnight and stored at – 80 °C before extraction. Frozen samples, amended with pure sea sand, were ground in liquid nitrogen in a mortar with a pestle. 600 µl extraction buffer (1.4 ml NaCl 5 M, 0.5 ml Tris 1 M, 0.2 ml EDTA 0.5 M, 2 ml CTAB 5%, 0.1 ml mercaptoethanol 100 %, 5.8 ml deionised water to obtain a final volume of 10 ml) were added to approximately 30 mg of ground material. The samples were mixed by vortexing and incubated for 1-2 hours in a 65 °C

water bath. Vortexing was repeated every 15 minutes. Subsequently, 400 μl chloroform/isoamylalcohol (24:1) were added in each tube. After 15 minutes shaking on the 'Roto-Torque' heavy duty rotator (Cole-Parmer Instrument Company, USA), samples were centrifuged at 16000 rcf for 10 minutes. The supernatant was transferred in 2 ml microcentrifuge tubes and mixed with 500 μl extraction buffer and 500 μl chloroform/isoamylalcohol (24:1). The 'Roto-Torque' shaking and centrifugation were repeated again. The supernatant was transferred in 1.5 ml microcentrifuge tubes, 500 μl isopropanol were added, well mixed and centrifuged for 10 minutes at 16000 rcf. The supernatant was discarded and 500 μl of cold 70% ethanol were added to the pellet. After 10 minutes of centrifugation at 16000 rcf, the supernatant was discarded again and tubes were dried overnight at room temperature. The DNA was suspended in 30 μl TE buffer pH 8 (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) and stored at – 20 °C. The amount of gDNA was measured by the Eppendorf Biophotometer and its quality checked by electrophoresis in a 1% agarose gel in Tris-acetate (TAE) buffer.

2.3. RNA extraction

The total RNA was extracted by using the kit 'SV Total RNA Isolation System' (Promega). 30 mg of ground spores or leaves, prepared in the same way as for DNA extraction, were used as starting material for the extraction. The amount of RNA was measured by the Eppendorf Biophotometer and its quality was checked by electrophoresis in a 1% agarose gel.

2.4. PCR and RT-PCR with degenerated primers

As a first step, *P. recondita* f.sp. *tritici* gDNA was used as template for PCR reactions using degenerated primers based on the *cyt b* gene sequence from basidiomycetous yeasts [13]. The five degenerated primers were used in all possible combinations to amplify a product covering the amino acid residues 149-280.

Table 1. Isolates of *Puccinia* species used for DNA and RNA extraction.

Species[a)]	Isolate name	Host	Origin	Material
P. recondita f.sp. *tritici*	Prec376	Wheat	Unknown	spores
	BR95-01	Wheat	France, 1995	infected leaves
	BR95-91	Wheat	Germany, 1995	infected leaves
P. graminis f.sp. *tritici*	Pgra65	Wheat	Switzerland, 1984	spores
	Pgra375	Wheat	Unknown	spores
P. striiformis f.sp. *tritici*	Pstr66	Wheat	Unknown	spores
	Pstr105	Wheat	Unknown	spores
P. coronata f.sp. *avenae*	Pcor16	Oat	Germany, 1985	spores
	Pcor167	Oat	Switzerland	spores
P. hordei	04A001	Barley	Austria, 2004	infected leaves
	04D002	Barley	Germany, 2004	infected leaves
P. recondita f.sp. *secalis*	04UK002	Rye	UK, 2004	infected leaves
P. sorghi	Psor1	Corn	Indiana, USA, 2004	spores
	Psor2	Corn	Indiana, USA, 2004	spores
	Psor4	Corn	Indiana, USA, 2004	spores
	Psor5	Corn	Indiana, USA, 2004	spores
P. horiana	Phor1	Chrysanthemum	UK, 2001	infected leaves
	Phor2	Chrysanthemum	UK, 2001	infected leaves
	Phor3	Chrysanthemum	UK, 2001	infected leaves
	Phor4	Chrysanthemum	UK, 2001	infected leaves
	Phor5	Chrysanthemum	UK, 2001	infected leaves
	Phor6	Chrysanthemum	UK, 2001	infected leaves

[a)] Nomenclature according to EPPO (Bayer) Code for diseases

A touch-down PCR approach was employed for amplification consisting of: 94 °C for 5 min, followed by 20 cycles at 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 30 sec with a decrease in the annealing temperature of 0.5 °C each cycle, followed by further 20 cycles at 94 °C for 30 sec, 40 °C for 30 sec, 72 °C for 30 sec, and a final extension step at 72 °C for 7 min. 1 μl aliquots of template (10-20 ng/μl) were added to 0.2 ml PCR reaction tubes containing 19 μl of PCR mixture. This mixture consisted of 1X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 4 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM each primer, 0.025 U/μl *Taq* DNA Polymerase. The primer pairs E1m4/E2mr4 and E1mr2/E2mr4 were also used in reverse transcriptase-PCR (RT-PCR) experiments, where RNA

was employed as template. The RT reaction was combined with the touch-down approach and the thermal cycling conditions consisted of: 48 °C for 45 min (for complementary DNA, cDNA, synthesis using reverse transcriptase), 94 °C for 2 min, followed by 20 cycles at 94 °C for 30 sec, 50 °C for 1 min, 68 °C for 2 min with a decrease in annealing temperature of 0.5 °C each cycle, followed by further 20 cycles at 94 °C for 30 sec, 40 °C for 1 min, 68 °C for 2 min, and a final extension step at 68 °C for 7 min. 1μl aliquots of template (approximately 5-10 ng/μl) were added to 0.2 ml PCR reaction tubes containing 19 μl of RT-PCR mixture, prepared according to the 'Access RT-PCR System' protocol (Promega). PCR and RT-PCR products were examined by electrophoresis in a 1.5 % agarose gel in Tris-acetate (TAE) buffer.

2.5. PCR product cloning and sequencing

The PCR products of the expected size (450 bp), obtained both from *P. recondita* f.sp. *tritici* gDNA and cDNA, were cloned into the pCR 2.1 vector using the 'TA Cloning Kit' from Invitrogen. Ten white transformants of each original PCR product were picked and analysed for the presence of insert by a standard PCR with primers M13 forward and M13 reverse, that anneal to the plasmid DNA. The original PCR products that contained the insert, were purified by 'NucleoSpin Extract 2 in 1' kit (Macherey-Nagel). For sequencing reaction, 1 μl aliquots of purified PCR products (3-10 ng/μl) were added to 19 μl sequencing mixtures prepared following the 'BigDye Terminator v3.1' protocol (Applied Biosystems) and containing, the first, 3.2 pmol of a primer that anneals to the T7 promoter in the vector, and the second, the same amount of primer M13 reverse. The thermal cycling conditions consisted of: 96 °C for 10 sec, followed by 25 cycles at 96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min. Products from this reaction were purified with the 'DyeEx 2.0 Spin Kit' (Qiagen). Sequencing was carried out by using the DNA sequencer ABI Prism 377 (Applied Biosystems) according to the instructions provided with the instrument. The sequences were analysed using the Bioinformatics software Chromas 1.45 (1996-1998 Conor McCarthy), EditSeq 5.03 (1989-2002 DNASTAR Inc.), MegAlign 5.03

(1993-2002 DNASTAR Inc.) and SeqMan 4.00 (1989-1999 DNASTAR Inc.). Related sequences were obtained by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

2.6. PCR and RT-PCR with cyt b gene specific primers

On the basis of the *cyt b* gene sequence of *P. recondita* f.sp. *tritici* obtained from the sequencing reactions, the specific primers Prcytb2C (TGAGTGGGTGGGGATCTAGT) and Prcytb2Br (CGAAGCTGGTGTCTGTATCG) were designed to amplify a product covering the amino acid residues 156-268. Both *P. recondita* f.sp. *tritici* gDNA and RNA were used as template in PCR and RT-PCR, respectively. The PCR consisted of 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 sec, 60 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. For the RT-PCR the thermal conditions were: 48 °C for 45 min, 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 sec, 60 °C for 1 min, 68 °C for 2 min, and a final extension step at 68 °C for 7 min. The amplified products (338 bp) were purified and sequenced directly, following the same protocol as already described. The sequencing mixtures contained 3.2 pmol of the specific primers used for the PCR amplification. The primer pair Prcytb2C/Prcytb2Br was tested also on the gDNA and RNA of the other seven *Puccinia* species (*P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis*, *P. sorghi* and *P. horiana*), by using the same mix and thermal conditions as used for *P. recondita* f.sp. *tritici*, and the amplified products were sequenced.

2.7. TAIL-PCR procedure

Specific primers, complementary to the *cyt b* gene sequence previously obtained, were designed for *T*hermal *A*symmetric *I*nter*L*aced (*TAIL*)-PCR (primer sequences not shown). Each primer was tested using RNA of *P. recondita* f.sp. *tritici* in RT-PCR in combination with four arbitrary degenerate (AD) primers [14]. In addition, in some cases, the specific primers were tested in combination with OPERON primers. The

three *TAIL*-PCR mixtures and reactions were prepared and carried out according to the protocol described by Liu and Whittier [14], except that the starting template was RNA and not gDNA. Thus, it was necessary to add a 45 minutes step at 48 °C at the beginning of the primary reaction to allow the retrotranscription of RNA into cDNA.

2.8. New specific primers for the cyt b gene of Puccinia spp.

On the basis of the new information obtained by carrying out different *TAIL*-PCR reactions, new specific primers for *P. recondita* f.sp. *tritici cyt b* gene were designed: Prcytb2D (GCACGTTGGAAGAGGGCTAT) and, subsequently, Prcytb2G (TCTTAAGACGCACCCGATTC) and Prcytb2Dr (TTCGAGCCAGAACTCAGAAAA), covering the gene fragment encoding for the amino acid residues 4-332. The gDNA of all mentioned *Puccinia* species was used as template for PCR, consisting of: 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, and a final extension step at 72 °C for 7 min. 1 μl aliquots of gDNA (10-20 ng/μl) were added to 0.2 ml PCR reaction tubes containing 19 μl of PCR mixture. This mixture consisted of 1X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM each primer and 0.025 U/μl *Taq* DNA Polymerase. Moreover, for each species RT-PCR was carried out with RNA (5-10 ng/μl) as template by using the same mixture and thermal conditions as already described. The amplified products obtained from these reactions were sequenced, according with the same protocol previously mentioned. The *cyt b* gene sequence of *P. recondita* f.sp. *tritici* was aligned to other *cyt b* genes belonging to different fungal species and the percentage of identity was calculated by Clustal W analysis (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html). Publicly available sequences were taken from GenBank. Moreover, ClustalW analysis was carried out to calculate the percentage of identity in the nucleotide and amino acid residues of the eight different *Puccinia* species on the basis of their *cyt b* gene sequence.

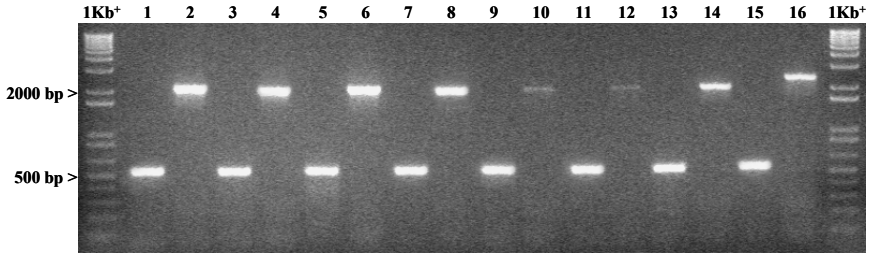
3. Results

The two degenerated primer pairs E1m4/E2mr4 and E1mr2/E2mr4 amplified a 450 bp long fragment in *P. recondita* f. sp. *tritici*, which was cloned and sequenced. On the basis of this sequence, new *cyt b* gene specific primers (Prcytb2C and Prcytb2Br) were designed. This primer pair produced a specific 338 bp long amplicon at both gDNA and cDNA level. This fragment was sequenced. By comparing to other known *cyt b* gene sequences, it was demonstrated that the primers effectively annealed to this gene. The same primer pair was tested also for *P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis*, *P. sorghi* and *P. horiana* gDNA and RNA, and it yielded the same amplified product of 338 bp.

A *TAIL*-PCR approach was carried out for *P. recondita* f.sp. *tritici* RNA in order to characterize the two hot spot regions which include possible mutations that can confer resistance to QoIs (amino acid residues 120-160 and 250-300). The *TAIL*-PCR reactions yielded two specifically amplified fragments of 300 and 200 bp, respectively. These fragments were sequenced and a new forward primer (Prcytb2D), which annealed to this sequence, was designed and used for all *Puccinia* species in combination with the reverse primer Prcytb2Br in order to amplify a longer *cyt b* gene sequence. The amplification yielded a 517 bp fragment with RNA as template in a RT-PCR reaction and a more than 2000 bp fragment using gDNA as template in PCR reactions (Fig. 1). The alignment between gDNA and cDNA sequences revealed the presence of an intron of about 1500 bp length in *P. recondita* f.sp. *tritici, P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis*, *P. sorghi* and about 1600 bp or more in *P. horiana*. In all species the intron was found to start exactly after the codon that encodes for glycine at position 143 (Fig. 2). This intron was partially sequenced: about 750 nucleotides at the beginning and 600 nucleotides at the end of the intron. The ClustalW sequence alignment of the 750 nucleotides at the beginning of the intron revealed a percentage of nucleotide identity ranging between 89.2 and 96.4% among the different rust species. BLAST search with each of these

intronic sequences revealed 38% identical and 59% strongly similar amino acid residues to 200 amino acid residues of the *Saccharomyces cerevisae* mRNA maturase (AAA32150). On the other hand, the alignment of the 600 nucleotides at the end of the intron revealed a percentage of nucleotide identity among *Puccinia* species ranging from 75.1 to 92.8%, but there were no similarities with any sequences in the GenBank.

Figure 1. Agarose gel with amplified products obtained by using the primer pair Prcytb2D/Prcytb2Br, specific for the *cyt b* gene of the genus *Puccinia*. *P. recondita* f.sp. *tritici* cDNA (1) and gDNA (2); *P. graminis* f.sp. *tritici* cDNA (3) and gDNA (4); *P. striiformis* f.sp. *tritici* cDNA (5) and gDNA (6); *P. coronata* f.sp. *avenae* cDNA (7) and gDNA (8); *P. hordei* cDNA (9) and gDNA (10); *P. recondita* f.sp. *secalis* cDNA (11) and gDNA (12); *P. sorghi* cDNA (13) and gDNA (14); *P. horiana* cDNA (15) and gDNA (16). $1Kb^+$: 1Kb Plus DNA Ladder (Invitrogen).



Further *TAIL*-PCR experiments carried out with *P. recondita* f.sp. *tritici* RNA permitted to gain new information on the *cyt b* gene sequence. The secondary reaction with the specific primer Prcytb3Hr combined with an Operon primer (E5) resulted in a specific product that was about 650 bp long. After sequencing, this product was confirmed to be part of the *cyt b* gene, partially overlapping up-stream to the known sequence. The tertiary reaction with the primer combination Prcytb4C/Operon primer C1 amplified a 166 bp long fragment located 26 nucleotide residues down-stream to the known sequence. Finally, the *TAIL*-PCR experiments allowed to sequence a 996 bp long fragment (at cDNA level) in *P. recondita* f.sp. *tritici*, starting from the codon for methionine at the beginning of the gene (Fig. 2). This fragment

corresponds almost to the whole gene, usually about 1160-1180 bp long at cDNA level, e.g. 1170 bp in *Strobilurus tenacellus* (X88000), 1182 bp in *Venturia inaequalis* (AF047029), 1167 bp in *Mycosphaerella graminicola* (AY247413), 1158 bp in *Aspergillus niger* (D63375) and *Neurospora crassa* (M37324).

Figure 2. Partial *cyt b* gene sequence (996 bp) deduced by sequencing the cDNA of *P. recondita* f.sp. *tritici*. In gray are highlighted the annealing points for the five specific primers, starting with Prcyt2G, then Prcyt2D, Prcyt2C, Prcyt2Br and Prcyt2Dr. In bold are indicated the two most common amino acid substitutions (F129L and G143A) and, in addition, a compilation of mutations known to confer resistance to QoIs [9, 10]: X127I, G137R, I147F, A153S, X254Q, N256C, N261D. The arrow indicates the position of the intron.

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      M  R  I  T  K  T  H  P  I  T  G  T  V  N  S  Y  M  G  D  S
1     atgagaattcttaagacgcacccgattctaggtctagtaaattcctatataggggattca  60
      P  Q  P  A  N  I  S  Y  M  W  N  F  G  S  T  T  G  C  C  T
61    ccacaaccagcaaatattagttatatatggaactttgggagtcttctaggatgttgtcta  120
      I  I  Q  M  I  T  G  V  T  T  A  M  H  Y  T  P  S  V  D  T
121   atcattcagataattacaggggtaactctagcgatgcattatacacctagtgtagatctg  180
      A  F  I  S  V  E  H  I  M  R  D  V  E  Y  G  W  T  I  R  Y
181   gcatttatttcagtagagcatatcataagggatgtagaatatggttgactgattaggtat  240
      T  H  A  N  V  A  S  F  F  F  I  F  V  Y  T  H  V  G  R  G
241   cttcacgctaatgtagcctctttttttttcatttttgtatatctacacgttggaagaggg  300
      T  Y  Y  G  S  Y  K  S  P  R  T  T  T  W  A  I  G  V  I  I
301   ctatactacggttcttacaagtcaccaaggacacttctatgagccattggtgtaattatt  360
      T  I  V  M  M  A  T  A  F  M  G  Y  V  T  P  Y  G  Q  M  S
361   ctaattgtaataatagcgactgcctttatgggttacgttctaccttacggacagatgtca  420
      T  W  G  A  T  V  I  T  N  T  M  S  A  I  P  W  V  G  G  D
421   ctatgaggtgcaacagtaattacgaatctaataagtgcgattccatgagtgggtggggat  480
      T  V  E  F  I  W  G  G  F  S  V  N  N  A  T  T  N  R  F  F
481   ctagtagaatttatttgaggagggtttagtgtaaacaatgcgactctgaacagatttttt  540
      S  T  H  F  V  T  P  F  I  T  A  A  T  V  V  M  H  T  T  T
541   agtcttcattttgtactacctttcattctagctgcactagtagtaatacatcttctcacg  600
      T  H  E  H  G  S  N  N  P  T  G  V  T  G  N  A  D  R  T  P
601   ctacatgagcacggttcaaataatcctctaggggtgacagggaatgcggataggctgcca  660
      M  A  P  N  F  I  F  K  D  T  V  T  I  F  T  F  T  T  V  T
661   atggctcctaatttttattttcaaagacctagtaaccattttccttttccttctagttcta  720
      A  I  F  V  M  Y  A  P  N  M  M  G  H  S  D  N  Y  I  P  A
721   gctattttcgttatgtatgcacctaacataatagggcattcagataattatatcccagct  780
      N  P  M  Q  T  P  A  S  I  V  P  E  W  Y  T  T  P  F  Y  A
781   aatccgatacagacaccagcttcgatcgtacctgagtggtatcttcttccattctatgct  840
      I  T  R  S  I  P  N  K  T  T  G  V  M  A  M  F  A  S  T  T
841   attctacgttcaattcctaataagctactaggggtgatagccatgtttgcaagtcttcta  900
      I  T  T  A  M  P  V  M  D  R  S  R  M  R  G  S  Q  F  R  P
901   atcctgctagccataccggtaatagaccggagcagaatgagagggagtcaatttagacct  960
      T  N  R  F  I  F  W  V  T  A  R  N
961   cttaatagatttattttctgagttctggctcgaaac  996
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On the basis of this sequence new specific primers (Prcytb2G and Prcytb2Dr) were designed, which were able to amplify the region of the gene that includes the two hot spots of point mutations (amino acid residues 120-160 and 250-300). At nucleotide level the *cyt b* gene of *P. recondita* f.sp. *tritici* showed high homologies with *Strobilurus tenacellus* (65.77 % identity), *Mycena galopoda* (64.62 %), *Venturia inaequalis* (68.21%), *Mycosphaerella graminicola* (66.98 %), *Aspergillus niger* (66.86 %) and *Neurospora crassa* (64.43 %). By comparing the amino acid sequences, similarities were also observed with *Strobilurus tenacellus* (65.26 % identical residues and 12.98 % strongly similar), *Mycena galopoda* (61.54 % and 12.24 %), *Venturia inaequalis* (65.03 % and 12.94 %), *Mycosphaerella graminicola* (63.29 % and 16.43 %), *Aspergillus niger* (63.64 % and 13.99 %) and *Neurospora crassa* (58.04 % and 14.69 %). The forward primers Prcytb2G and Prcytb2D were tested in combination with the two reverse primers Prcytb2Br and Prcytb2Dr on gDNA and RNA also for the isolates of the other seven rust species and for two additional isolates of *P. recondita* f.sp. *tritici*. PCR products were sequenced. All isolates of the same species showed identical nucleotide sequences. The longest consensus sequence deduced for each species was submitted to the GenBank with the following accession numbers: DQ009925 (*P. recondita* f.sp. *tritici*, length: 996 bp), DQ009927 (*P. graminis* f.sp. *tritici*, 506 bp), DQ009932 (*P. striiformis* f.sp. *tritici*, 755 bp), DQ009926 (*P. coronata* f.sp. *avenae*, 644 bp), DQ009928 (*P. hordei*, 660 bp), DQ009930 (*P. recondita* f.sp. *secalis*, 687 bp), DQ009931 (*P. sorghi*, 709 bp) and DQ009929 (*P. horiana*, 478 bp). The cDNA sequence alignments among the different *Puccinia* species revealed high percentages of identity both at nucleotide and amino acid level (Table 2). Alignments between the QoI-sensitive and the putative QoI-resistant genotypes of *P. horiana* showed no differences in their sequences. In the latter isolate, the glycine residue at position 143 was not replaced by alanine that is involved in resistance to QoIs in pathogens such as *M. graminicola*. In addition, no other point mutations conferring resistance were detected (e.g. F129L).

Table 2. Percentage of identity in the nucleotide residues of the *cyt b* gene and in the deduced amino acid sequence of eight *Puccinia* species [a)].

		Percent identity in amino acid residues							
		P. rec.	***P. gra.***	***P. str.***	***P. cor.***	***P. hdi.***	***P. sec.***	***P. sor.***	***P. hor.***
Percent identity in nucleotide residues	***P. rec.***	-	100.0	98.0	99.1	100.0	99.6	99.6	99.4
	P. gra.	100.0	-	98.2	98.2	94.0	100.0	99.4	99.4
	P. str.	94.8	95.1	-	76.2	71.2	72.8	71.2	97.5
	P. cor.	97.8	96.8	73.8	-	94.9	98.6	99.5	99.4
	P. hdi.	99.4	93.3	69.1	93.9	-	99.1	99.5	98.7
	P. sec.	99.1	99.0	70.6	97.5	99.4	-	99.1	99.4
	P. sor.	98.4	98.2	69.0	99.1	98.5	98.3	-	100.0
	P. hor.	98.3	98.1	93.9	98.7	98.3	98.5	99.4	-

[a)] *P. rec.: Puccinia recondita* f.sp. *tritici, P. gra.: P. graminis* f.sp. *tritici, P. str.: P. striiformis* f.sp. *tritici, P. cor.: P. coronata* f.sp. *avenae, P. hdi.: P. hordei, P. sec.: P. recondita* f.sp. *secalis, P. sor.: P. sorghi, P. hor.: P. horiana*

4. Discussion

Since resistant isolates to QoIs were detected in several Ascomycetes and Oomycetes soon after the commercial introduction of these fungicides, extensive sequence data were produced for the *cyt b* gene of these pathogens in order to elucidate the molecular basis of resistance. In pathogens such as *Blumeria graminis*, *Plasmopara viticola*, *Mycosphaerella fijiensis* and *M. graminicola*, resistance is conferred by a single point mutation in the *cyt b* gene leading to a change at amino acid position 143 from glycine to alanine (G143A) [6, 8]. A change in phenylalanine to leucine at position 129, F129L, also confers resistance

to QoIs in species such as *Pythium aphanidermatum* and *Pyricularia grisea*, although the level of resistance is lower than that conferred by the G143A substitution [6, 11]. In addition, other mutations were described [4, 5, 9, 10, 15]. However, in Basidiomycetes resistance to QoIs has surprisingly never been reported until now, even though these pathogens have been treated with QoIs as frequent as other pathogens, e.g. powdery mildews in cereals. However, the strobilurin-producing Basidiomycetes *Strobilurus tenacellus* and *Mycena galopoda* exhibit 'natural resistance' to QoIs and the molecular basis of this 'resistance' is known to be point mutations in the *cyt b* gene [9]. For other Basidiomycetes, no *cyt b* gene sequences and consequently, no specific primers for this gene were available in the GenBank. Therefore, the first goal of this work was to sequence the *cyt b* gene in *P. recondita* f.sp. *tritici* and to design specific primers able to amplify the region of the gene that includes all known mutations that can confer resistance to QoIs, particularly the G143A substitution. The starting point was to amplify *P. recondita* f.sp. *tritici* gDNA and RNA by using degenerated primers. Since only a 360 bp fragment of the gene was sequenced, it was necessary to 'walk' on the genome with a *TAIL*-PCR approach, a technique that allows to isolate an unsequenced segment bordering a known sequence [14]. Nested sequence-specific primers, designed on the known *cyt b* gene sequence, were utilized together with shorter arbitrary degenerated primers and Operon random primers in three serial PCR reactions. By the interspersion of high stringency PCR cycles with reduced-stringency PCR cycles, the targeted sequence (*cyt b* gene) was preferentially amplified over non-targeted ones. Different *TAIL*-PCR experiments allowed to sequence almost the whole *cyt b* gene (996 bp at cDNA level) starting from the codon for methionine at the beginning of the gene.

Specific primers for the *cyt b* gene of *Puccinia* species were designed (the forward Prcytb2C, Prcytb2D and Prcytb2G, and the reverse Prcytb2Br and Prcytb2Dr) to amplify different parts of the gene. Particularly, the primer pair Prcytb2G/Prcytb2Dr was able to amplify specifically the *cyt b* gene fragment from cDNA of rust species encoding for the amino acid residues 4-332. This fragment is responsible for the

binding site of QoI fungicides and includes all possible mutations known to confer resistance to QoI fungicides in many plant pathogens (amino acid residues 120-160 and 250-300). Therefore, these primers represent a valuable diagnostic tool to easily isolate the *cyt b* gene fragment of *Puccinia* species, including *P. recondita* f.sp. *tritici, P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis*, *P. sorghi* and *P. horiana*, and detect point mutations that eventually may evolve. The different rust species showed 69 to 100% identity in the nucleotide sequence and 71 to 100% in the amino acid residues of cytochrome *b*. The lowest percentages of identity were found in *P. striiformis* f.sp. *tritici* compared to *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis* and *P. sorghi*. The sequence alignments demonstrated that the *cyt b* gene is highly conserved among species within the same genus. This result explains why the specific primers, developed at first for *P. recondita* f.sp. *tritici*, worked also for the other *Puccinia* species. Alignments between the sensitive and the QoI-resistant genotypes of *P. horiana* showed no differences in the sequences, indicating that the 'resistant' isolate did not carry any point mutations in its *cyt b* gene. Therefore, in this isolate alternative resistance mechanisms, such as bypass reactions (alternative oxidase) [16], efflux (ABC transporters) [17] or mechanisms other than the most common amino acid substitutions (e.g. G143A or F129L) [15] may be involved. However, they were not investigated in more detail in the frame of this work.

The alignment of gDNA and cDNA provided essential information to elucidate the gene structure. In all *Puccinia* species, the presence of an intron, at least 1500 bp long, was observed, and the sequence alignment revealed high similarities among each other (between 75.1 and 96.4% identity of the nucleotide residues). This intron starts exactly after the codon that encodes for glycine at position 143. It is interesting to investigate the role of this intron in pre-mRNA splicing or other transcriptional and post-transcriptional mechanisms and its possible effect on the occurrence of point mutations. Mitochondrial DNA of mammals does not have any introns, whereas in many lower eucaryotic mitochondrial genomes, introns are present. In general, certain specific

sequences within introns are essential for the excision of the intron itself from the mitochondrial transcripts. Splicing in mitochondria is due to the activity of some nuclear-encoded proteins or sometimes by polypeptides encoded by the intron itself (or by the intron of another gene). For example, the *cyt b* gene mRNA in species such as *Aspergillus nidulans* and *Saccharomyces cerevisiae* is produced by the action of a maturase, encoded by an intronic sequence [18]. In *M. fijiensis*, 303 amino acid residues encoded by an intron showed 75 % identity with the *A. nidulans* mRNA maturase [19]. *S. cerevisiae* splicing-deficient mutants, carrying mis-sense mutations in the maturase gene, were shown to completely abolish maturase activity and thus leading to a respiratory-deficient phenotype [20]. Blastx analysis of *Puccinia* intronic sequences revealed similarities with 200 amino acid residues of the *S. cerevisae* mRNA maturase.

Since the structure of introns and exons alters from organism to organism, it is an interesting question whether the same gene structure is present in other rusts and also in other fungal *taxa* and whether it may influence the occurrence of point mutations at position 143 in the *cyt b* gene. The number of introns in the *cyt b* gene ranges from zero in *E. graminis* [2], to one in *M. fijiensis* [3], two in *S. cerevisiae* [21], four to eight in the strobilurin-producing Basidiomycetes [9], and six in *V. inaequalis* [22]. As a consequence of the different number and sizes of introns, the total length of the *cyt b* gene varies significantly from 1.1 kb (e.g. in *E. graminis* without introns) to >10 kb (e.g. in *V. inaequalis* with six introns). Since the introns of *P. recondita* f.sp. *tritici* and *P. horiana* are highly homologous in the first 750 bp and in the last 600 bp, the difference in size must be located in the unsequenced part in the middle of the intron. Therefore, it is necessary to sequence the entire *cyt b* gene of *P. recondita* f.sp. *tritici, P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis*, *P. sorghi* and *P. horiana*, and to investigate whether the same intron is present in other rusts and plant pathogenic Basidiomycetes. Genus specific primers will facilitate the sequencing process in order to determine the intron/exon structure in *Puccinia* species and to develop diagnostic tools for QoI resistance [23] in this group of pathogens.

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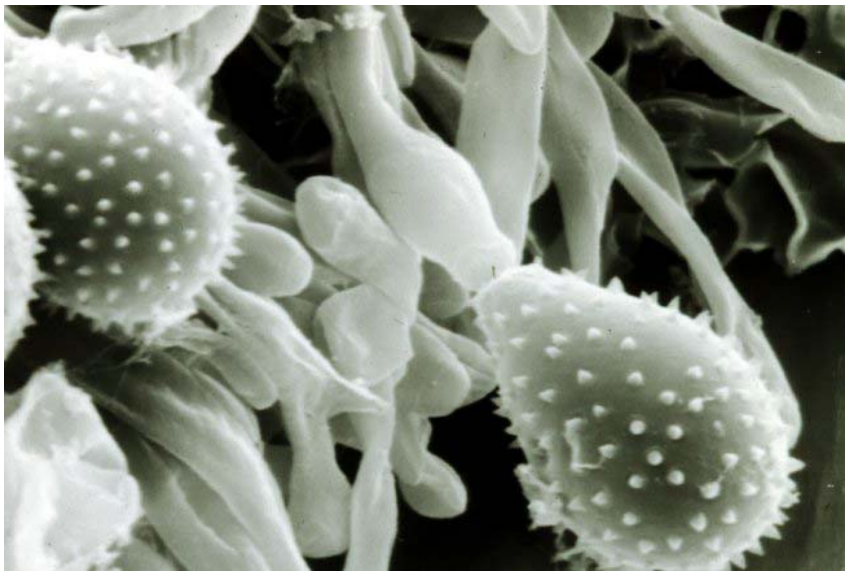
CHAPTER 3

Relatedness among agronomically important rusts based on mitochondrial cytochrome *b* gene and ribosomal ITS sequences



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Abstract

A fragment of the mitochondrial cytochrome *b (cyt b)* gene of 13 agronomically important plant pathogenic Basidiomycetes was sequenced, including several *Puccinia* spp., *Uromyces appendiculatus*, *Phakopsora pachyrhizi*, *Hemileia vastatrix* and *Rhizoctonia solani*. The deduced amino acid sequences (residues 142-266) were used to study the relatedness of these pathogens as compared to other species of the Basidiomycetes, Ascomycetes and Oomycetes. The relatedness was also studied at nuclear level using the Internal Transcribed Spacers (ITS) in the ribosomal DNA. Phylogenic trees were constructed with the maximum parsimony (MP) and the neighbour-joining (NJ) methods. On the basis of both cytochrome *b* and ITS sequences, the *Puccinia* species pathogenic to graminaceous crop plants, such as *P. recondita* f.sp. *tritici*, *P. graminis* f.sp. *tritici, P. striiformis* f.sp. *tritici, P. coronata* f.sp. *avenae, P. hordei, P. recondita* f.sp. *secalis* and *P. sorghi*, together with *P. horiana* from *Chrysanthemum*, were very closely related to each other, whereas *P. arachidis* (from peanut) was closely related to *U. appendiculatus* (from beans) but more distant from the other *Puccinia* species. Both rusts on soybean (*P. pachyrhizi*) and coffee (*H. vastatrix*) were outside the *Puccinia* cluster. All rusts were separated from other Basidiomycetes such as *R. solani* and the strobilurin producing species *Strobilurus tenacellus* and *Mycena galopoda*. Our results demonstrate that the amino acid sequence of the mitochondrial cytochrome *b* is a valid tool to study phylogenic relatedness among plant pathogenic Basidiomycetes and supports taxonomic grouping based on morphological structures and host specificity. Because of their high variability, ITS sequences were able to discriminate *Puccinia* species which were identical on the basis of the cytochrome *b* amino acid sequence. Thus, ITS sequences could better show differences among species or within a species, whereas cytochrome *b* is more suitable than ITS for phyologenic inference at family or genus level. In addition, the sequence data obtained during this study represent essential information for easy isolation of the *cyt b* gene and detection of point

mutations conferring resistance to QoI fungicides that eventually may evolve.

Keywords: Basidiomycetes, *Puccinia*, QoI fungicides, point mutation, phylogeny

Introduction

Among the most devastating plant diseases caused by fungal pathogens, rusts (*Uredinales*) are a large and important group of obligate biotrophic organisms that exhibit a complex life cycle (Littlefield and Heath, 1979; McLaughlin *et al.*, 1995). Severe damages on graminaceous crop plants, such as wheat, oat, barley, rye and corn, can be caused by several *Puccinia* species, including *P. recondita* f.sp. *tritici* (Eriksson) C.O. Johnston, *P. graminis* f.sp. *tritici* Erikss. and Henning, *P. striiformis* f.sp. *tritici* Erikss., *P. coronata* f.sp. *avenae* P. Syd & Syd., *P. hordei* G. H. Otth, *P. recondita* f.sp. *secalis* Roberge and *P. sorghi* Schwein.. Other rust species, such as *Uromyces appendiculatus* F. Strauss, *Phakopsora pachyrhizi* Syd. & P. Syd. and *Hemileia vastatrix* Berk. & Broome, can cause serious losses on bean, soybean and coffee, respectively.

In spite of cultural practices and breeding for resistant cultivars, chemical control is the most effective and economic way currently used to protect crop plants from these diseases. The most important classes of chemical products are the demethylation inhibitors (DMIs) and the strobilurin fungicides. The latter fungicides, also known as Qo Inhibitors (QoIs), interfere with mitochondrial respiration at the Qo site (the 'outer', quinone oxidizing pocket) of the cytochrome bc_1 enzyme complex by blocking electron transfer in the respiration pathway and leading to energy deficiency (Bartlett *et al*., 2002). Cytochrome *b* is part of this enzyme complex and is encoded by the cytochrome *b* (*cyt b*) gene. Since resistant isolates to QoI fungicides were detected in several Ascomycetes (Sierotzki *et al.*, 2000a, b; Steinfeld *et al.*, 2001, 2002; Kim *et al.*, 2003) and Oomycetes (Gisi *et al.*, 2002), extensive sequence

data were produced for the *cyt b* gene of these pathogens in order to elucidate the molecular basis of resistance. On the other hand, in Basidiomycetes resistance to QoIs has surprisingly not been reported until now, and no *cyt b* gene sequences were available in literature.

Therefore, the *cyt b* gene fragment encoding for the binding site of QoI fungicides was sequenced in a range of plant pathogenic Basidiomycetes (only complementary DNA, cDNA), such as several *Puccinia* species (Grasso *et al.,* 2005), *Puccinia arachidis* Speg., *U. appendiculatus*, *P. pachyrhizi*, *H. vastatrix* and *Rhizoctonia solani* Kühn (present study). The purpose of this study was to investigate the degree of relatedness among agronomically important pathogens within the order of *Uredinales* based on these molecular data. These species were also compared with some Ascomycetes, Oomycetes and other Basidiomycetes, such as *Strobilurus tenacellus* (Pers.) Singer, *Mycena viridimarginata* P. Karst. and *M. galopoda* (Pers.) P. Kumm.. Mitochondrial genes such as the *cyt b* gene have never been used before for similar studies in rusts.

In addition, the phylogenic relatedness based on the mitochondrial *cyt b* gene was compared to that obtained with the nuclear ITS (Internal Transcribed Spacer) regions (White *et al.*, 1990) and to taxonomic grouping based on morphology of fungal structures and host specificity (Littlefield and Heath, 1979; McLaughlin *et al.*, 1995).

Materials and Methods

RNA extraction

RNA of different Basidiomycetes species was extracted from lyophilised spores, infected leaves (*P. arachidis*, *P. hordei*, *P. horiana* Henn. and *P. recondita* f.sp. *secalis*) or lyophilised mycelium (*R. solani*) (Table 1). 30 mg of each sample were ground with extra pure sea sand under liquid nitrogen and the total RNA was extracted by using the kit "SV Total RNA Isolation System" (Promega Corporation, Madison, WI, USA). The total RNA was treated with DNAse-I provided with the kit. The amount of RNA was determined using the Eppendorf Biophotometer

(Eppendorf AG, Hamburg, Germany) and its quality was checked by loading 1 μl of RNA sample together with 5 μl of Orange 6X Loading Dye in a 1% agarose gel.

Table 1. List of species, sequence origin and number of isolates.

		Source of sequences[a] and number of isolates			
Species [b]	**Abbreviation**	***cyt b***		**ITS**	
Basidiomycetes					
Puccinia arachidis	Puc.ara.	DQ022194 [c]	3	- [d]	
Puccinia coronata f.sp. *avenae*	Puc.cor.	DQ009926	2	AY114290	1
Puccinia graminis f.sp. *tritici*	Puc.gra.	DQ009927	2	AF468044	2
Puccinia hordei	Puc.hdi	DQ009928	2	AY187089	1
Puccinia horiana	Puc.hor.	DQ009929	6	- [d]	
Puccinia recondita f.sp. *tritici*	Puc.rec.	DQ009925	3	AY187088	1
Puccinia recondita f.sp. *secalis*	Puc.sec.	DQ009930	1	L08705	1
Puccinia sorghi	Puc.sor.	DQ009931	4	AY114291	1
Puccinia striiformis f.sp. *tritici*	Puc.str.	DQ009932	2	AY114292	1
Uromyces appendiculatus	Uro.app.	DQ022196 [c]	2	AB115741	2
Hemileia vastatrix	Hem.vas.	DQ022192 [c]	2	DQ022191 [c]	2
Phakopsora pachyrhizi	Pha.pac.	DQ022193 [c]	1	AF333491	1
Rhizoctonia solani	Rhi.sol.	DQ022195 [c]	1	AY684924	1
Strobilurus tenacellus	Str.ten.	X88000	1	- [d]	
Mycena galopoda	Myc.gal.	X87997	1	AY805614	1
Mycena viridimarginata	Myc.vir.	X87998	1	- [d]	
Ascomycetes					
Blumeria graminis	Blu.gra.	AF343441	1	AF073352	1
Fusarium culmorum	Fus.cul.	X87996	1	AY147338	1
Magnaporthe grisea	Mag.gri.	X87999	1	U17328	1
Mycosphaerella fijiensis	Mcs.fij.	AF343070	1	AY752150	1
Mycosphaerella graminicola	Mcs.gra.	AY247413	1	AY152603	1
Saccharomyces cerevisiae	Sac.cer.	V00696	1	AY525600	1
Venturia inaequalis	Ven.ina.	AF047029	1	AF065839	1
Oomycetes					
Phytophthora infestans	Phy.inf.	U17009	1	AY829469	1
Phytophthora megasperma	Phy.meg.	L16863	1	AY848930	1

[a] GenBank accession numbers
[b] Nomenclature according to EPPO (Bayer) Code for diseases
[c] Sequence data produced during this study
[d] No data

Amplification of the cyt b gene

Since RNA was used as template, reverse transcription was coupled with PCR amplification (RT-PCR). The thermal cycling conditions consisted of 48 °C for 45 min (for first strand cDNA synthesis), 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 sec, 60 °C for 1 min, 68 °C for 2 min, and a final extension step at 68 °C for 7 min. 1μl aliquots of template (approximately 5-10 ng/μl) were added to 0.2 ml PCR reaction tubes containing 19 μl of RT-PCR mixture, prepared according to the "Access RT-PCR System" protocol (Promega Corporation, Madison, WI, USA). The *cyt b* gene fragment of *Puccinia* and *Uromyces* isolates was amplified with the forward primer Prcytb2D (5'-GCACGTTGGAAGAGGGCTAT-3') and the reverse Prcytb2Dr (5'-TTCGAGCCAGAACTCAGAAAA-3'), both designed on the basis of the *P. recondita* f.sp. *tritici cyt b* gene sequence (Grasso *et al.*, 2005). Since *cyt b* specific primers for *R. solani*, *P. pachyrhizi* and *H. vastatrix* were not available, the degenerated primers E1mr2 and E2mr4, which are based on the *cyt b* gene sequence of basidiomycetous yeasts (Biswas *et al.*, 2001), were used to amplify a 400 bp long product covering the amino acid residues 142-266. The PCR products (5 μl) were loaded together with 1 μl Orange 6X Loading Dye on a 1.5 % agarose gel. The amplicons of the expected size were subsequently cloned into the pCR 2.1 vector using the "TA Cloning Kit" (Invitrogen Corporation, Carlsbad, CA, USA). Ten white transformants of each original PCR product were picked and analysed for the presence of the insert by a standard PCR with the primers M13-forward and M13-reverse, that anneal to the plasmid DNA.

Amplification of the ITS regions

The rDNA region containing ITS1 and ITS2 and the intervening 5.8S rRNA gene was amplified by using the universal primers ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'; White *et al.*, 1990). 1μl aliquots of RNA template (approximately 5-10 ng/μl) were added to 0.2 ml PCR reaction

tubes containing 19 μl of RT-PCR mixture, prepared according to the "Access RT-PCR System" protocol (Promega Corporation, Madison, WI, USA). The amplification program consisted of: 48 °C for 45 min, 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 sec, 50 °C for 1 min, 68 °C for 2 min, and a final extension step at 68 °C for 7 min.

Sequencing

All PCR products were previously purified by using the "NucleoSpin Extract 2 in 1" kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). For sequencing reactions, 1 μl aliquots of these purified products (10-20 ng/μl) were added to 19 μl of sequencing mixture prepared following the "BigDye Terminator v3.1" protocol (Applied Biosystems, Foster City, CA, USA). For all *Puccinia* and *Uromyces* samples, two sequencing mixtures were prepared, the first containing 3.2 pmol of the forward primer Prcytb2D, and the second containing the same amount of the reverse primer Prcytb2Dr. For *R. solani*, *P. pachyrhizi* and *H. vastatrix* samples, the first sequencing mixture contained 3.2 pmol of the primer T7 that anneals to the T7 promoter in the vector and, the second mixture, the same amount of the primer M13-reverse. The thermal cycling conditions consisted of 96 °C for 10 sec, followed by 25 cycles at 96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min. The final products were purified with the "DyeEx 2.0 Spin Kit" (Qiagen GmbH, Hilden, Germany). Sequencing was carried out on the DNA sequencer ABI Prism 377 (Applied Biosystems, Foster City, CA, USA) according to the instructions provided with the instrument. The sequences were analysed using the Bioinformatics software, Chromas 1.45 (1996-1998 Conor McCarthy), EditSeq 5.03 (1989-2002 DNASTAR Inc.), MegAlign 5.03 (1993-2002 DNASTAR Inc.) and SeqMan 5.03 (1989-2001 DNASTAR Inc.).

Sequence alignment and analysis

For the construction of the phylogenic tree, a fragment of 125 amino acids (residues 142-266) encoded by the *cyt b* gene was chosen. Sequence translation was made with the Biology WorkBench 3.2 web-

based tool (http://workbench.sdsc.edu/) and by using the yeast mitochondrial code. For the phylogenic analysis based on the ITS sequences, a fragment including the ITS1, the 5.8S rRNA and the ITS2 was used (the length of the fragment depended on the species). Publicly available sequences were taken from the GenBank and the sequence accession numbers are reported in Table 1. Sequence alignments were performed with the Clustal W programme (Thompson *et al.*, 1994; http://www.ebi.ac.uk/clustalw/). Cladistic analyses were performed with the MEGA version 2.1 program (Kumar *et al.*, 2001), using the maximum parsimony (MP) methods for nucleotide and amino acid sequence data (Eck and Dayhoff, 1966; Fitch, 1971) with confidence values for individual branches determined by bootstrap test (500 replicates). To search for the MP trees, the close-neighbour-interchange heuristic search was used. A second type of phylogram was generated by using the neighbour-joining (NJ) method (Saitou and Nei, 1987) and it was presented as a radial tree.

Results

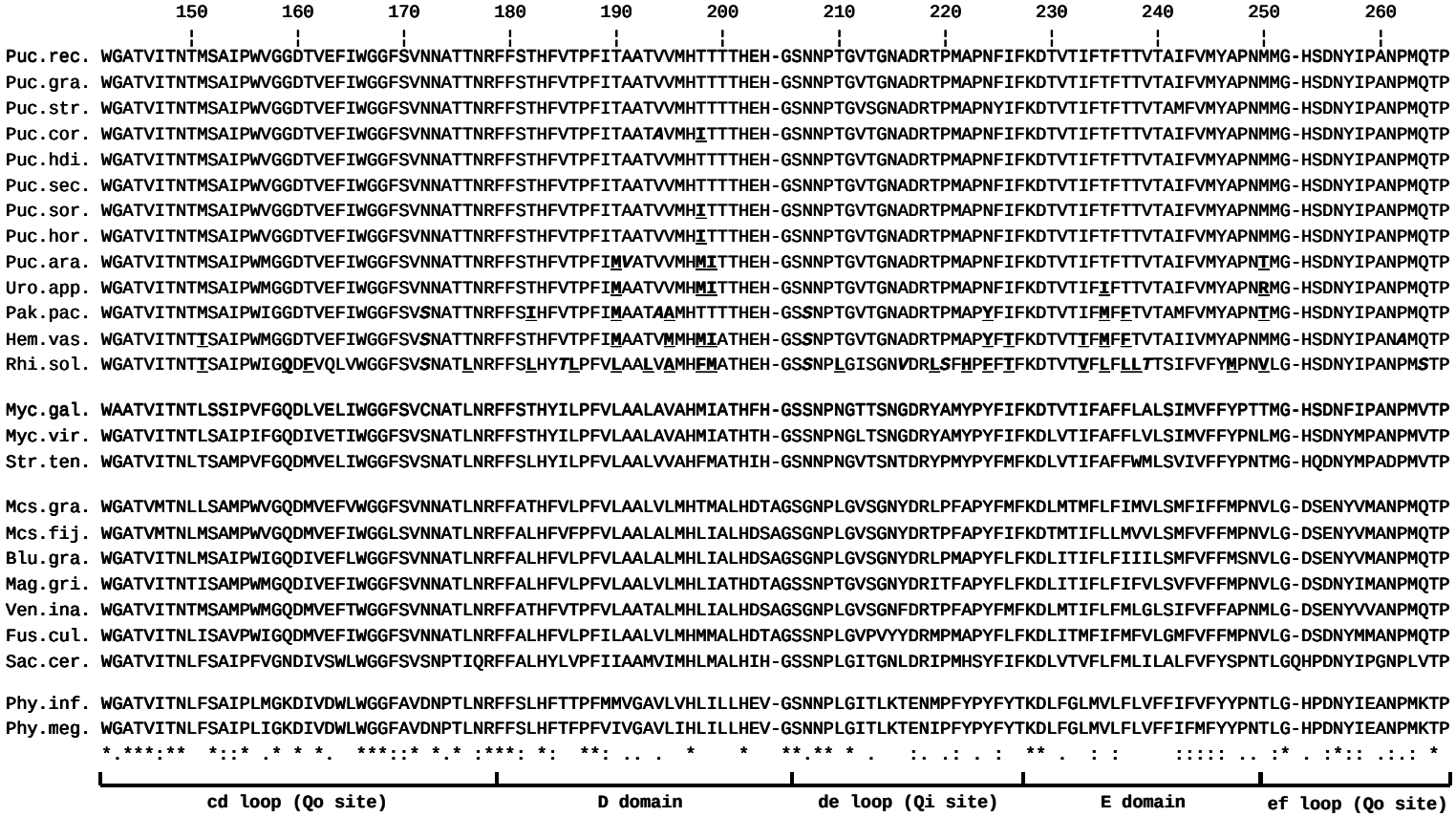
The use of RNA as template for the *cyt b* gene amplification excluded the presence of a >1500 bp long intron (Grasso *et al.*, 2005) in the amplified products, and made the alignments and phylogenic analyses easier than with genomic DNA. The Clustal W multiple alignment of the deduced amino acid sequence of the *cyt b* gene fragment (Figure 1) revealed that this gene is strongly conserved among all analysed species, which belong to the three different *taxa* of Basidiomycetes, Ascomycetes and Oomycetes. For the rust species with more than one analysed isolate, identical fragments were found. Therefore, consensus sequences were used to construct the phylograms for these species.

Based on amino acid sequence analyses, Degli Esposti *et al.* (1993) deduced the likely secondary structure of the cytochrome *b* protein, including eight trans-membrane domains (from 'A' to 'H') and seven cytoplasmic domains (from 'ab' to 'gh'). The fragment used for our phylogenic study included five of these regions (Figure 1): the 'cd'

loop (Qo site, amino acid residues 142-178); the trans-membrane domain 'D' (179-205); the 'de' loop (Qi site, 206-227); the trans-membrane domain 'E' (228-249); the 'ef' loop (Qo site, 250-266). The comparison of the cytochrome *b* amino acid sequences of Basidiomycetes, Ascomycetes and Oomycetes revealed that the Qo site, 'cd', is the most conserved region among the different taxonomic groups (only 24.3% of the amino acids are 'non-consensus' residues – Figure 1), followed by the other part of the Qo site, 'ef' (33.3% 'non-consensus' residues). The Qi site, 'de', showed 45.5% 'non-consensus' residues. The most variable regions were the two trans-membrane domains 'D' and 'E' (about 50% 'non-consensus' residues). Some amino acid residues are typical for particular taxonomic groups, e.g. in Ascomycetes there is an additional alanine (A) between the amino acid residues 204 and 205 (using the amino acid numbering of the Basidiomycetes cytochrome *b* sequence), whereas in *Saccharomyces cerevisiae* Meyen ex Hansen there is an additional glutamine (Q) between the amino acid residues 252 and 253.

Among the *Puccinia* species, most of the 125 analysed amino acid residues of the cytochrome *b* were fully conserved in all rust species on cereals and Chrysanthemum and just few residues were strongly similar (Figure 1). *U. appendiculatus* and *P. arachidis* were more similar to each other and differed from the previous group in 5 amino acid residues. All 5 were not conserved residues (in bold and underlined in Figure 1) in *U. appendiculatus*, whereas *P. arachidis* showed 4 not conserved amino acid residues and a weakly conserved one (in bold and italics in Figure 1) compared to the *Puccinia* cluster. *P. pachyrhizi* and *H. vastatrix* differed from the *Puccinia* cluster in 10 and 13 amino acid residues, respectively (Figure 1), and were found to be more related to *R. solani* and to the strobilurin producing Basidiomycetes, *S. tenacellus*, *M. galopoda* and *M. viridimarginata*. In *R. solani*, 22 of the 125 analysed amino acid residues were not conserved and 7 weakly conserved residues (Figure 1).

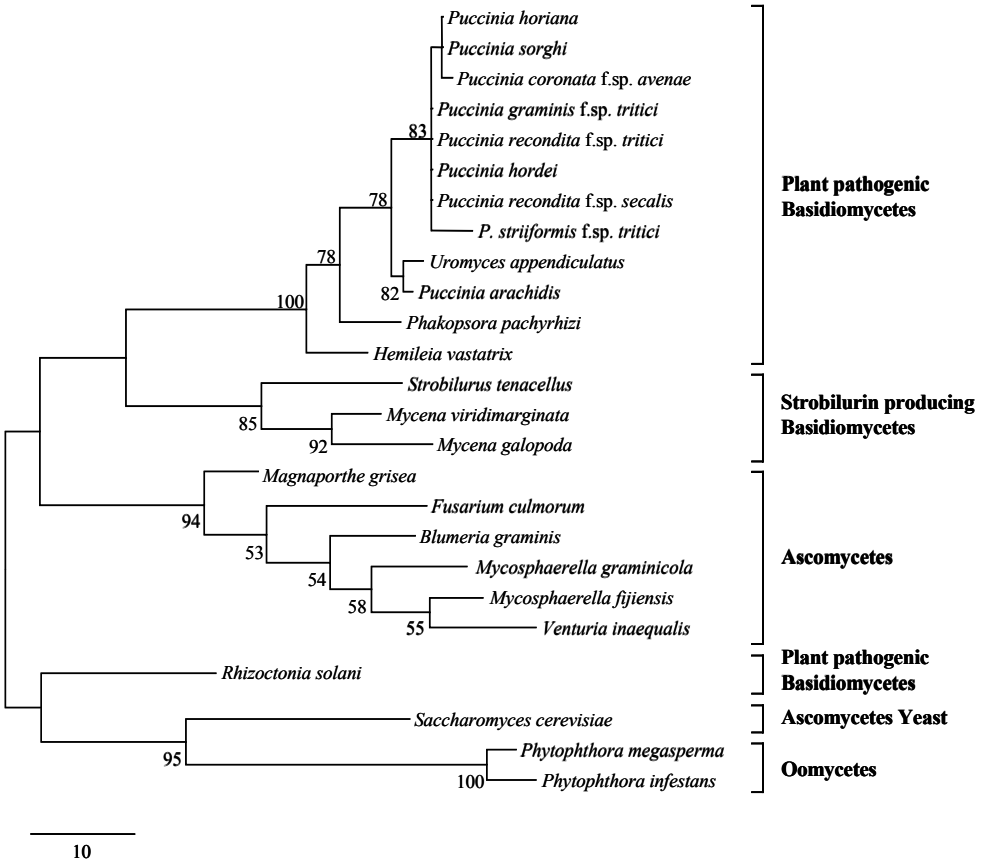
Figure 1. ClustalW multiple sequence alignment of a fragment of 125 amino acids (residues 142-266) of the cytochrome *b* of different species of the Basidiomycetes, Ascomycetes and Oomycetes. Abbreviations of species names are explained in Table 1. Consensus key: '*' means single, fully conserved amino acid residues; ':' and '.' mean conservation of strong and weak groups, respectively. The lack of symbols represents 'no consensus' in the sequence alignment. In the Basidiomycetes group, weakly conserved amino acid residues are in bold and italics, whereas not conserved residues are in bold and underlined.



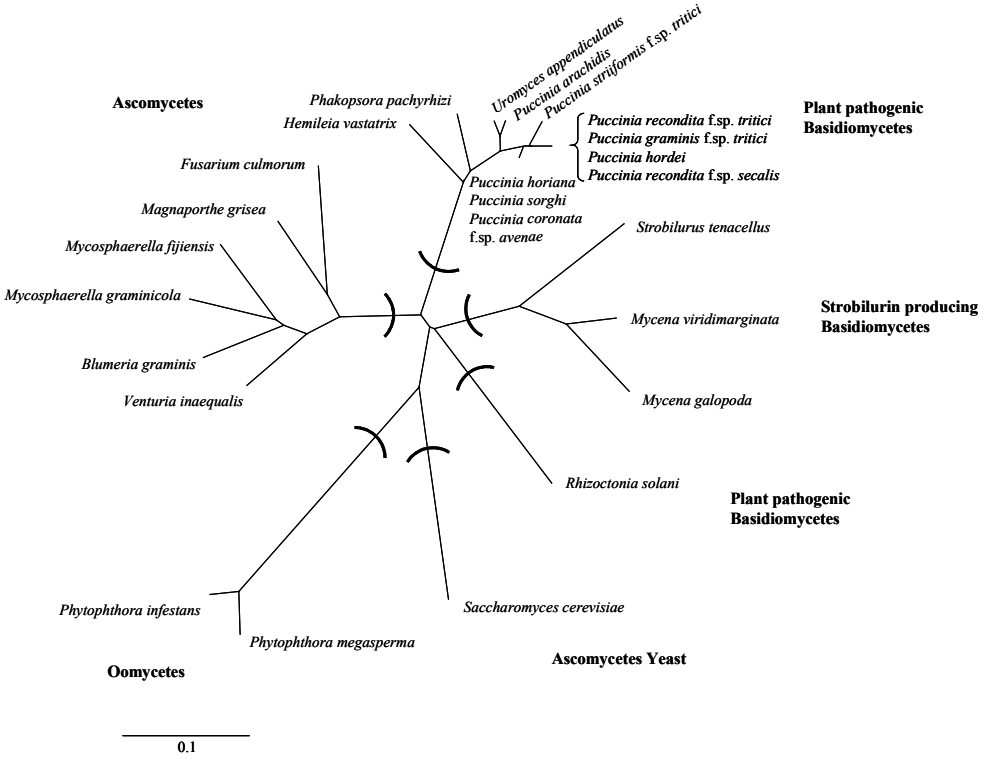
Based on the cytochrome *b* sequence of these different species, the ClustalW sequence alignment analysis revealed the presence of well-represented clusters in both the MP (Fig. 2A) and the NJ (Fig. 2B) phylograms.

Figure 2. Phylogenic relatedness of plant pathogenic and strobilurin producing Basidiomycetes and selected Asco- and Oomycetous pathogens, as derived from maximum parsimony (A) and neighbour-joining (B) analysis of the mitochondrial *cyt b* gene. Bootstrap values from 500 replications are reported on branches. Values lower than 50 are not shown. Branch lengths are scaled in terms of evolutionary distances.

(A)



(B)



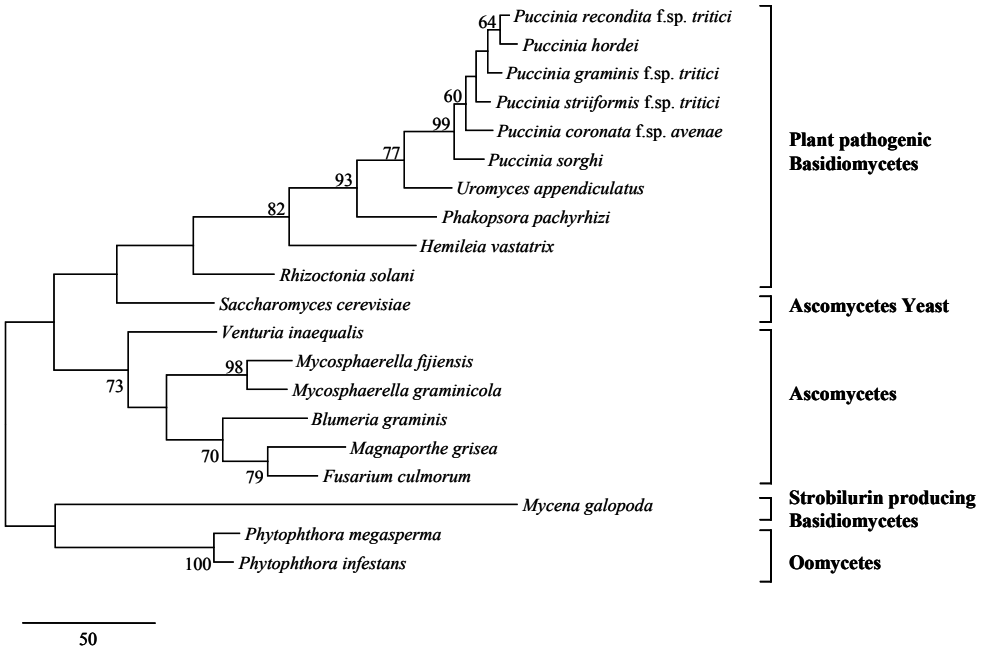
They clearly formed three clusters, representing the three *taxa* Ascomycetes, Basidiomycetes and Oomycetes. In addition, all analysed *Uredinales* were, as already observed in the sequence alignment, closely related to each other or even identical (mostly the *Puccinia* species). Among the nine *Puccinia* species included in this study, all species pathogenic to graminaceous crop plants (*P. recondita* f.sp. *tritici*, *P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis* and *P. sorghi*), together with *P. horiana* from *Chrysanthemum* were very closely related to each other, whereas the rust on peanut (*P. arachidis*) was closely related to *U. appendiculatus* (on beans) but separate from the other *Puccinia* species. Both rusts on soybean (*P. pachyrhizi*) and coffee (*H. vastatrix*) were outside the *Puccinia* cluster. The MP and the NJ trees provided almost

the same cluster representation. The only difference between the two phylograms was the position of *R. solani* which, in the MP tree, seemed to be closer to *S. cerevisiae* and the two *Phytophthora* species than to the Basidiomycetes cluster. On the contrary, in the NJ tree *R. solani* was close to the strobilurin producing Basidiomycetes cluster. Therefore, the NJ tree provided a clear clustering of the Basidiomycetes.

The same species were also studied by analysing the ribosomal ITS. The RNA of the different plant pathogenic Basidiomycetes was amplified with the primer pair ITS5/ITS4. The RT-PCR products were sequenced and they resulted in most cases in identical ITS sequences as already reported in the GenBank. However, the rRNA sequence of *H. vastatrix* including the ITS1-5.8S-ITS2 was not available in the GenBank and for this reason, it was generated during this study (data not shown) and submitted under the number DQ022191. Five species were not included in the ITS phylogram either because it was not possible to sequence the ribosomal RNA (for *P. arachidis* and *P. horiana*, because of the presence of a big amount of plant RNA) or because these sequences were partially (*P. recondita* f.sp. *secalis*) or not at all available in the GenBank (*S. tenacellus* and *M. viridimarginata*). For *P. recondita* f.sp. *secalis*, the fragment of the rRNA sequence from the GenBank (418 residues) was aligned to *P. recondita* f.sp. *tritici* rRNA revealing a 98.6% identity for the nucleotide residues. For all the other species, the Clustal W multiple alignment of the ITS1–5.8S–ITS2 fragments showed more differences than those observed by analyzing the cytochrome *b* amino acid sequence. Not only the nucleotide residues, but also the length of the ITS1 and ITS2 differed among species. However, the 5.8S rRNA was generally highly conserved. The differences were well represented in both the MP (Fig. 3) and NJ trees (not shown), which clearly distinguished the three taxonomic groups Basidiomycetes, Ascomycetes and Oomycetes. In the MP phylogram, as well as in the trees based on the cytochrome *b* amino acid sequence, the species within the *Uredinales* were highly related to each other: *P. recondita* f.sp. *tritici*, *P. graminis* f.sp. *tritici*, *P. hordei* and *P. striiformis* f.sp. *tritici* formed an unique cluster. Closely related to this group were also *P. sorghi* and *P. coronata* f.sp. *avenae*. Slightly more distant was, as in the cytochrome *b*

phylogram, *U. appendiculatus*, followed by *P. pachyrhizi* and *H. vastatrix*. The latter two species were more related to *R. solani* and to the strobilurin producing Basidiomycetes, *S. tenacellus*, *M. galopoda* and *M. viridimarginata*.

Figure 3. Phylogenic relatedness of plant pathogenic and strobilurin producing Basidiomycetes and selected Asco- and Oomycetous pathogens, as derived from maximum parsimony analysis of the ribosomal ITS1-5.8S-ITS2 region. Bootstrap values from 500 replications are reported on branches. Values lower than 50 are not shown. Branch lengths are scaled in terms of evolutionary distances.



Discussion

Rusts (*Uredinales*) are an important group of obligate plant pathogens (Littlefield and Heath, 1979; McLaughlin *et al.*, 1995) causing severe damages on cereals, as well as on other important crops, such as

beans, soybean and coffee. Brown rust on wheat (*P. recondita* f.sp. *tritici*) is a continuous threat for wheat growing in many European countries, whereas soybean rust (*P. pachyrhizi*) is a serious disease in South America since 2001 (Pivonia and Yang, 2004) and an emerging pathogen in North America since 2004 (Klag, 2005).

The use of QoI fungicides is one of the most effective strategies to protect crop plants from these diseases. However, a continuous resistance monitoring is essential to prolong the use of these chemicals. The mechanism and evolution of resistance to QoIs was described recently for several Ascomycetes and Oomycetes by characterizing their *cyt b* gene sequence (Gisi *et al.*, 2000; Sierotzki *et al.*, 2002). In most cases, resistance was conferred by single point mutations in the *cyt b* gene, all located in two highly conserved cytochrome *b* regions, so called 'hot spot' regions (Gisi *et al.*, 2002; Sierotzki *et al.*, 2002; Kim *et al.*, 2003; Steinfeld *et al.*, 2001, 2002; Brasseur *et al.*, 1996). For Basidiomycetes, except for the strobilurin producing species (Kraiczy *et al.*, 1996), no *cyt b* gene data were available in literature. For the first time, the *cyt b* gene of different plant pathogenic Basidiomycetes including several *Puccinia* species was sequenced by Grasso *et al.* (2005), supplemented by *P. arachidis*, *U. appendiculatus*, *P. pachyrhizi*, *H. vastatrix* and *R. solani* (present study). These sequences were used to study the relatedness among Basidiomycetes and other taxonomic groups (Ascomycetes and Oomycetes). For this purpose, a fragment of the *cyt b* gene encoding for 125 amino acids (residues 142-266) was chosen and analysed by using the MP and the NJ methods for phylogenesis.

The structure and function of mitochondrial cytochrome *b* protein are well conserved, and its substitution rates are in proportion to evolutionary time. Therefore, this gene has been used for identification, classification, and phylogenic analysis of animals and plants (Irwin *et al.*, 1991; Kocher *et al.*, 1989), for some Ascomycetes yeasts and fungi (Yokoyama *et al*., 2000, 2001; Wang *et al.,* 2001) and *R. solani* (Sierotzki *et al.*, 2002), but never for rusts. The *cyt b* gene is generally highly conserved among species belonging to different *taxa*. The comparison of the cytochrome *b* amino acid sequences of different

Basidiomycetes, Ascomycetes and Oomycetes confirmed this observation. The amino acid sequences of pathogen species showed highly conserved domains, particularly in the 'cd' and 'ef' domains, both involved in the formation of the Qo site (Degli Esposti *et al.*, 1993).

Since mitochondrial genes, such as the *cyt b* gene, have another inheritance and repair mechanisms than nuclear genes, the evolutionary processes may be different in mitochondria from those in the nucleus. For this reason, the nuclear ITS sequences in the ribosomal DNA (rDNA) were used to construct a different phylogenic tree. The rDNA sequences of many organisms have been characterized and are able to provide valid information about phylogenic relatedness (Hillis and Dixon, 1991). Particularly the ITS sequences were extensively used to study phylogenic relatedness among closely related *taxa* because ITS evolve much more rapidly than other conserved regions. Therefore, the sequences of ITS may vary among species within a genus or even among isolates within the same species (Iwen *et al.*, 2002). For fungal pathogens, species-specific variations within ITS regions have been determined for a broad range of *Phytophthora* species (Cooke and Duncan, 1997), for several phytopathogenic and saprotrophic *Colletotrichum* species (Sreenivasaprasad *et al*., 1996), *Penicillium* species (Skouboe *et al.*, 1999) and for some mycorrhizal Basidiomycetes (Gardes and Bruns, 1993). Phylogenic studies were performed also for *Uredinales* by using the ribosomal DNA sequences (Maier *et al.*, 2003; Wingfield *et al.*, 2004). The ITS regions were used to study the phylogenic relatedness among closely related species of *Puccinia* and *Uromyces* (Zambino and Szabo, 1993; Kropp *et al.*, 1997; Roy *et al.*, 1998; Pfunder *et al.*, 2001; Weber *et al.*, 2003).

In the present study, the relatedness among plant pathogenic Basidiomycetes was similar on the basis of both mitochondrial *cyt b* gene and nuclear ITS sequences. In both cases, all *Puccinia* species pathogenic to graminaceous crops, together with *P. horiana* from *Chrysanthemum*, were very closely related to each other, whereas *P. arachidis* (from peanut) was closely related to *U. appendiculatus* (on beans) but separate from the other *Puccinia* species. However, *P. recondita* f.sp. *secalis*, *P. horiana* and *P. arachidis* were not included in

the ITS phylogram because sequence data were not available. Both rusts on soybean (*P. pachyrhizi*) and coffee (*H. vastatrix*) were outside the *Puccinia* cluster and more related to the strobilurin producing Basidiomycetes. These results confirmed the taxonomy based on morphological characteristics, which groups *Puccinia* and *Uromyces* in the family of the *Pucciniaceae* within the *Uredinales* and the two genera *Phakopsora* and *Hemileia* in the families of *Phakopsoraceae* and *Chaconiaceae*, respectively (Wingfield *et al.*, 2004). The rather weak relatedness of *R. solani* with the other plant pathogenic Basidiomycetes confirms the traditional taxonomy, which does not allocate this species within the Urediniomycetes, but to the Hymenomycetes. Although the phylogenic relatedness based on the cytochrome *b* amino acid sequences resulted in a reliable clustering of *Uredinales*, it did not clearly differentiate between species of *Erysiphales* (Sierotzki *et al.*, 2002). Molecular phylogeny may be improved by including geographically different isolates of a species, additional species and gene sequences.

Only few discrepancies between the phylograms deduced from cytochrome *b* and ITS sequences were found. On the basis of the ITS sequence, *M. galopoda* was closely related to the Oomycetes rather than the Basidiomycetes cluster. On the contrary, the MP and the NJ trees, inferred from the cytochrome *b* amino acid sequence, demonstrated that this species belongs to the Basidiomycetes cluster. We have no explanation for this discrepancy. However, Clustal W alignment of *M. galopoda* ITS with other Basidiomycetes such as *Armillaria mellea* (Vahl) Kumm., *Agaricus campestris* L. and *Amanita muscaria* (L.) Pers. showed many differences with these fungi (data not shown). On the other hand, *M. galopoda* revealed strong similarities with the ITS sequences of other *Mycena* spp. and other Basidiomycetes, such as *Nolanea conferenda* (Britzelm.) Sacc., *Panellus stipticus* (Bull.) Karst. and *Gymnopus* spp.. By comparing the trees deduced from *cyt b* gene and rRNA sequences, also *R. solani* and *S. cerevisiae* behaved differently. Both species resulted more related to the Oomycetes in the MP tree based on the cytochrome *b* sequence, however they were closer to Ascomycetes and Basidiomycetes in the ITS tree. For this reason, the NJ radial tree,

inferred from the cytochrome *b* sequence, provided obviously a better clustering of the three different *taxa* than the MP phylogram.

The results obtained demonstrated that both *cyt b* gene and ITS sequences are useful markers for phylogenic studies of plant pathogenic fungi and they are able to distinguish the main taxonomic groups (Ascomycetes, Basidiomycetes, Oomycetes) as well as different genera and species within the same genus. The ITS sequences, as non-coding regions, evolve more rapidly than coding regions such as the *cyt b* gene, and are able to show more differences among species and within a species. For this reason, the ITS sequences were able to discriminate *Puccinia* species which were identical on the basis of the cytochrome *b* amino acid sequence. On the other hand, since ITS sequences are highly variable, they are not suitable for phylogenic inference among families. Nevertheless, our results demonstrate for the first time that the mitochondrial cytochrome *b* sequence is a valid tool to study phylogenic relatedness among plant pathogenic Basidiomycetes and support the classical taxonomy based on morphological structures and host specificity (Littlefield and Heath, 1979; McLaughlin *et al.*, 1995). Moreover, the phylogenic clusters generated by analysing the cytochrome *b* sequence confirm previous results based on the analysis of rRNA of the *Uredinales* (Maier *et al.*, 2003, Wingfield *et al.*, 2004) and more specifically, on the ITS regions of closely related species of *Puccinia* (Zambino and Szabo, 1993). In addition, these sequence data represent essential information for the development of diagnostic tools to easily isolate the *cyt b* gene and detect point mutations conferring resistance to QoI fungicides that eventually may evolve.

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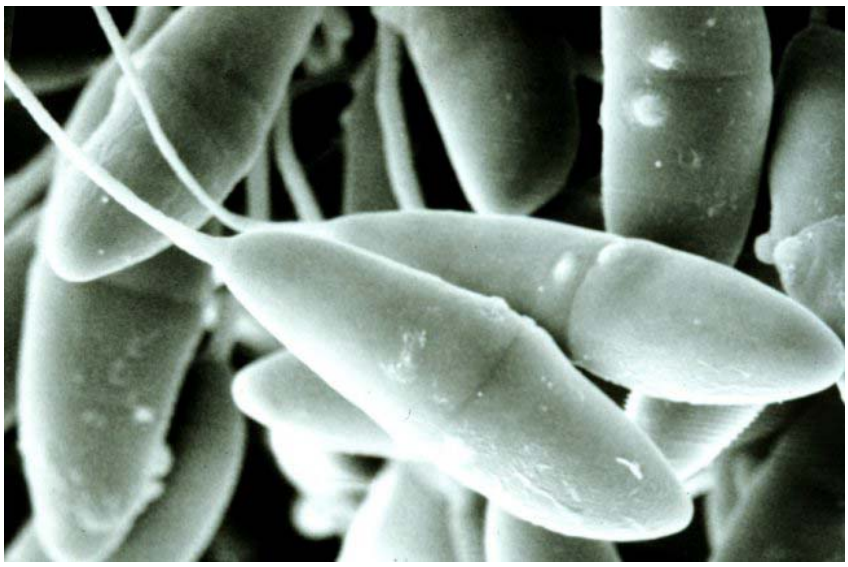
CHAPTER 4

Cytochrome *b* gene structure and consequences for resistance to QoI fungicides in plant pathogens



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Abstract

A cytochrome *b* (*cyt b*) gene coding fragment (about 950 bp long) was sequenced at genomic DNA level in a range of plant pathogens, including *Puccinia recondita* f sp *tritici* (Erikss) CO Johnston, *P graminis* f sp *tritici* Erikss and Hennings, *P striiformis* f sp *tritici* Erikss, *P coronata* f sp *avenae* P Syd & Syd, *P hordei* GH Otth, *P recondita* f sp *secalis* Roberge, *P sorghi* Schwein, *P horiana* Henn and *Uromyces appendiculatus* F Strauss. A shorter coding fragment (about 600-700 bp) was sequenced in *Phakopsora pachyrhizi* Syd & P Syd, *Hemileia vastatrix* Berk & Broome, *Alternaria solani* Sorauer, *A alternata* (Fr) Keissl and *Plasmopara viticola* (Berk & MA Curtis) Berl & de Toni. The *cyt b* gene structure of these agronomically important plant pathogens was characterized, especially in the two hot spot regions (amino acid residues 120-160 and 250-300), in which mutations conferring resistance to QoI fungicides may occur. The *cyt b* gene structure was compared with that of other species, including the strobilurin producing fungus *Mycena galopoda* (Pers) P Kumm, *Saccharomyces cerevisiae* Meyen ex Hansen, *Venturia inaequalis* (Cooke) Winter and *Mycosphaerella fijiensis* Morelet. The exon/intron organization was characterized and its possible role investigated for the occurrence of point mutations in the *cyt b* gene, especially the amino acid substitutions G143A and F129L, which are mainly responsible for resistance to QoI fungicides. In all rust species included in this study as well as in *A solani*, the mutation G143A was not detected, but an intron was observed starting exactly after the codon GGT for glycine at position 143. By comparing this intron with other sequences in the GenBank, high homologies were found to the group I introns, which are able to splice themselves. In pathogens such as *A alternata*, *Blumeria graminis* (DC) Speer, *Pyricularia grisea* Sacc, *Mycosphaerella graminicola* (Fuckel) J Schröt, *M fijiensis*, *V inaequalis* and *P viticola*, resistance to QoI fungicides is known and the glycine (triplet GGT) is replaced by alanine (triplet GCT) at position 143 in the resistant genotype. In those pathogen species, no intron was observed after the codon 143. In the rust species as well as in *A solani*, the codon GGT at position 143 is

located exactly at the exon/intron boundary and is likely part of the signal sequences essential for the recognition of the intronic RNA to be excised. We predicted that a nucleotide substitution in the codon 143 (GGT → GCT), which is two nucleotides upstream from the exon/intron junction, may strongly affect the splicing process, leading to a deficient cytochrome *b*, which is lethal. Therefore, individuals carrying the G143A substitution and an intron at this position will not survive. As a consequence, the evolution of resistance to QoI fungicides based on G143A is not likely to evolve in plant pathogens such as *Puccinia* spp, *U appendiculatus*, *P pachyrhizi*, *H vastatrix* and *A solani*. The relevance of the splicing process is discussed in the light of resistance to QoI fungicides.

Keywords: *Puccinia recondita*, *Phakopsora pachyrhizi*, *Alternaria solani*, strobilurins, exon/intron organization, pre-mRNA splicing, point mutation.

1 INTRODUCTION

Cytochrome *b* is a membrane protein forming the core of the mitochondrial bc_1 complex (complex III) in the respiratory chain.[1] Cytochrome *b* is encoded by the cytochrome *b* (*cyt b*) gene located in the mitochondrial genome. The fungicides known as Qo Inhibitors (QoIs) are able to inhibit mitochondrial respiration by binding to the Qo site (the outer, quinone oxidizing pocket) of the cytochrome bc_1 enzyme complex. This inhibition blocks the electron transfer process in the respiration pathway and leads to energy deficiency due to a lack of ATP.[2] In the strobilurin producing basidiomycetes *Strobilurus tenacellus* (Pers) Singer and *Mycena galopoda* (Pers) P Kumm several point mutations are present in the *cyt b* gene causing 'natural resistance' to their own metabolite.[3] Single amino-acid exchanges in the cytochrome *b* were found conferring resistance to QoI fungicides in different plant pathogens including *Blumeria graminis* f sp *tritici* (DC) Speer,[4] *Mycosphaerella fijiensis* Morelet,[5] *Venturia inaequalis* (Cooke) Winter[6,7]

and *Plasmopara viticola* (Berk & MA Curtis) Berl & de Toni.[8,9] In most cases, resistance was conferred by a single point mutation in the *cyt b* gene leading to a change at amino acid position 143 from glycine to alanine (G143A).[8,10] In species such as *Pythium aphanidermatum* (Edson) Fitzp and *Pyricularia grisea* Sacc, a change from phenylalanine to leucine at position 129, F129L, also confers resistance to QoIs, although the level of resistance is lower than that conferred by the G143A substitution.[8,11] In other species, including *V inaequalis*, additional mechanisms leading to resistance were described.[3,6,7,12] The amino acid exchanges in mutants of *Saccharomyces cerevisiae* Meyen ex Hansen resistant to QoIs[13,14] and in fungi naturally resistant to these compounds[3] are located in two highly conserved cytochrome *b* regions, so called 'hot spot' regions (amino acid residues 120-160 and 250-300). In plant pathogenic fungi the first hot spot contains the two point mutations responsible for QoI resistance, G143A and F129L, detected so far. For the evaluation of inherent resistance risk to QoI fungicides, the *cyt b* gene of several pathogens, such as *V inaequalis*,[15] *B graminis*,[4] or *M fijiensis*,[5] was sequenced. Based on these sequences, specific primers and Q-PCR methods were developed to quantify resistance in field populations.[16,17]

The *cyt b* gene fragment responsible for the binding site of QoI fungicides was partially sequenced at cDNA level for several basidiomycetes species.[18,19] In the present study, the *cyt b* gene was sequenced at genomic DNA level in a range of plant pathogens, including *Puccinia recondita* f sp *tritici* (Erikss) CO Johnston, *P graminis* f sp *tritici* Erikss and Hennings, *P striiformis* f sp *tritici* Erikss, *P coronata* f sp *avenae* P Syd & Syd, *P hordei* GH Otth, *P recondita* f sp *secalis* Roberge, *P sorghi* Schwein, *P horiana* Henn, *Uromyces appendiculatus* F Strauss, *Phakopsora pachyrhizi* Syd & P Syd and *Hemileia vastatrix* Berk & Broome. The fragment of the *cyt b* gene including the hot spot regions was also characterized (both at cDNA and gDNA level) in *Alternaria solani* Sorauer, *A alternata* (Fr) Keissl and *P viticola*. Based on the sequence data, specific primers for the *cyt b* gene were designed to permit an easy amplification of the *cyt b* gene fragment responsible for the binding site of QoI fungicides and for the detection of point

mutations that eventually may evolve. The sequence data are essential for the development of diagnostic tools to monitor resistance to QoI fungicides in important pathogens, such as cereal rusts and *Phakopsora pachyrhizi*, the causal agent of rust on soybean, which is a serious disease in South America since 2001[20] and an emerging problem also in North America since 2004.[21]

The *cyt b* gene structure of these plant pathogens was characterized including the two hot spot regions and compared with other organisms already described in literature, such as the strobilurin producing basidiomycetes,[3] *S* cerevisiae,[22] *M fijiensis*[5] and *V inaequalis*.[15] The aim of this study was to characterize the gene structure in rust species and other agronomically important plant pathogens and investigate whether it may influence the occurrence of point mutations in the *cyt b* gene. In rust species and *A solani*, the presence of an intron, at least 1500 bp long, was observed in the *cyt b* gene, starting exactly after the triplet GGT that encodes for glycine at position 143.[18] Since this codon is located exactly at the exon/intron boundary, it is likely part of the signal sequences essential for the recognition of the intronic RNA to be excised during the pre-mRNA splicing process.[23,24] In the current study this intron was completely sequenced. It was investigated what role it may play in pre-mRNA splicing and occurrence of point mutations conferring resistance to QoI fungicides. Additional introns at different positions in the *cyt b* gene were also characterized and sequenced during this study.

2 MATERIALS AND METHODS

2.1 Fungal isolates

This study was carried out with different species of plant pathogens (Table 1). The isolates are stored in the Syngenta strain collection in Stein (Switzerland).

Table 1. List of species, sequence origin and number of introns present in the *cyt b* gene.

Species	GenBank Accession Number	Reference Number	Number of introns	Position and size (bp) of introns
Basidiomycetes				
Puccinia coronata f.sp. *avenae*	DQ209272	Present work	2 [a]	G143 (1477); I269 (>1000) [h]
Puccinia graminis f.sp. *tritici*	DQ209273	Present work	2 [a]	G143 (1477); I269 (>1000) [h]
Puccinia hordei	DQ209274	Present work	2 [a]	G143 (1480); I269 (>1000) [h]
Puccinia horiana	DQ209275	Present work	2 [a]	G143 (1734); I269 (>1000) [h]
Puccinia recondita f.sp. *tritici*	DQ209276	Present work	2 [a]	G143 (1492); I269 (>1000) [h]
Puccinia recondita f.sp. *secalis*	DQ209277	Present work	2 [a]	G143 (1480); I269 (>1000) [h]
Puccinia sorghi	DQ209278	Present work	2 [a]	G143 (1474); I269 (>1000) [h]
Puccinia striiformis f.sp. *tritici*	DQ209279	Present work	2 [a]	G143 (1546); I269 (>1000) [h]
Uromyces appendiculatus	DQ209280	Present work	3 [b]	H67 (1021); G143 (1458); I269 (1167)
Phakopsora pachyrhizi	DQ209281	Present work	1 [c]	G143 (1337)
Hemileia vastatrix	DQ209282	Present work	3 [d]	I68-H82 (>1500) [h]; Y132 (1396); G143 (1657)
Strobilurus tenacellus	X88000	3	3	A95 (272); Y274 (170); L289 (5)
Mycena galopoda	X87997	3	2	G131 (238); Y274 (344)
Mycena viridimarginata	X87998	3	5	V122 (12); G131 (479); A200 (470); Y274 (159); L289 (87)
Ascomycetes				
Alternaria alternata	DQ209283	Present work	0 [e]	-
Alternaria solani	DQ209284/5	Present work	4 [f]	A126 (1140); G143 (2157); V146 (1760); F164 (1292)
Blumeria graminis	AF343441	4	0	-
Magnaporthe grisea	AY245424/7	11	0	-
Mycosphaerella fijiensis	AF343070	5	1	L169 (1064)
Mycosphaerella graminicola	AY247413	Not published	0	-
Venturia inaequalis	AF004559	15	6	H53 (2432); I92 (2172); P135 (360); F169 (1202); V260 (2009); W274 (1302)
Saccharomyces cerevisiae	AJ011856	22	5	M139 (768); G143 (1404); F169 (1623); G252 (1417); I269 (738)
Oomycetes				
Plasmopara viticola	DQ209286	Present work	0 [g]	-

[a] In the region of the gene between the amino acid residues 1 – 332; [b] 12 – 317; [c] 50 – 284; [d] 50 – 276; [e] 110 – 286; [f] 110 – 274; [g] 79- 296
[h] Intronic sequence not completely sequenced

2.2 DNA extraction

Genomic DNA (gDNA) was extracted mainly from spores, but also mycelium (*A solani* and *A alternata*) or infected leaves (*P hordei*, *P recondita* f sp *secalis* and *P horiana*), which were lyophilised overnight and stored at – 80 °C before extraction. Frozen samples, amended with pure sea sand, were ground in liquid nitrogen in a mortar with a pestle. Extraction buffer (600 µl, composed of 1.4 ml NaCl 5 M, 0.5 ml Tris 1 M, 0.2 ml EDTA 0.5 M, 2 ml CTAB 5%, 0.1 ml mercaptoethanol 100 %, 5.8 ml deionised water to obtain a final volume of 10 ml) was added to the ground material (approximately 30 mg). The samples were mixed by vortexing and incubated for 1-2 hours in a 65 °C water bath. Vortexing was repeated every 15 minutes. Subsequently, chloroform+isoamylalcohol (24+1 by volume, 400 µl) were added in each tube. After 15 minutes shaking on the 'Roto-Torque' heavy duty rotator (Cole-Parmer Instrument Company, USA), samples were centrifuged at 16000 rcf for 10 minutes. The supernatant was transferred in 2 ml microcentrifuge tubes and mixed with extraction buffer (500 µl) and chloroform+isoamylalcohol (24+1 by volume, 500 µl). The 'Roto-Torque' shaking and centrifugation were repeated. The supernatant was transferred in 1.5 ml microcentrifuge tubes, isopropanol was added (500 µl), well mixed and centrifuged for 10 minutes at 16000 rcf. The supernatant was discarded and cold 70% ethanol (500 µl) was added to the pellet. After 10 minutes of centrifugation at 16000 rcf, the supernatant was discarded again and tubes were dried at least 1 hour at room temperature. The DNA was suspended in TE buffer pH 8 (30 µl, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) and stored at – 20 °C. The amount of gDNA was measured by the Eppendorf Biophotometer and its quality checked by electrophoresis in a 1% agarose gel in Tris-acetate (TAE) buffer.

2.3 RNA extraction

The total RNA was extracted by using the kit 'SV Total RNA Isolation System' (Promega). Ground spores, mycelium or infected

leaves (30 mg), prepared in the same way as for DNA extraction, were used as starting material for the extraction. The amount of RNA was measured by the Eppendorf Biophotometer and its quality was checked by electrophoresis in a 1% agarose gel.

2.4 PCR amplification of the *cyt b* gene fragment

2.4.1 Puccinia spp and Uromyces appendiculatus

The gDNA of the eight *Puccinia* species and *U appendiculatus* was used as template for PCR using all possible combinations of the forward primers Prcytb2F (5'-ATGAGAATTCTTAAGACGCA-3'), Prcytb2D (5'-GCACGTTGGAAGAGGGCTAT-3'), Prcytb2C (5'-TGAGTGGGTGGGGATCTAGT-3') and Prcytb4A (5'-TCCCAGCTAATCCGATACAGA-3') with the reverse Prcytb3Hr (5'-GGCTCATAGAAGTGTCCTTGG-3'), Prcytb3Dr (5'-ACTAGATCCCCACCCACTCA-3'), Prcytb2Br (5'-CGAAGCTGGTGTCTGTATCG-3'), Prcytb2Cr (5'-CTCTCATTCTGCTCCGGTCT-3') and Prcytb2Dr (5'-TTCGAGCCAGAACTCAGAAAA-3'). These primers were designed on the basis of the *cyt b* gene cDNA sequence of *P recondita* f sp *tritici*.[18] In addition, the primer pair Phor2fw (5'-CGCCAAAGATACAGACACGA-3')/Phor3rev (5'-TTCGCTCTCGCTACTTCTCA-3') was used to amplify the central part of the intron present after the codon for the glycine 143. Genomic DNA (1 μl, 10-20 ng μl^{-1}) was added to a PCR mixture (19 μl) containing 1X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM each primer and 0.025 U μl^{-1} *Taq* DNA Polymerase (Invitrogen). The thermal conditions consisted of: 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, and a final extension step at 72 °C for 7 min.

2.4.2 Phakopsora pachyrhizi and Hemileia vastatrix

For *P pachyrhizi* and *H vastatrix* the degenerated primers cytbA (5'-TAGCDATGCAYTAYAHHCCT-3') and cytbB (5'-

CATATHATRMGRGATGTWRA-3') were designed and used as forward primers in combination with the reverse primers Ppcytb1rev (5'-GCATTCCCTGTTACACCCAG-3'), Ppcytb2rev (5'-GCCGGTGTCTGTATTGGATT-3') and Ppcytb3rev (5'-TAGCACGTAAAATAGCATAAAA-3'), Hvcytb1rev (5'-ATGCATTATGACTAGGGCCG-3'), Hvcytb2rev (5'-TTGTATGGCGTTAGCTGGAA-3') and Hvcytb3rev (5'-TCGACGGTGGTGTTTGTATG-3'), previously designed on the basis of the partial *cyt b* gene sequence of *P pachyrhizi* and *H vastatrix*, respectively.[19] In these reactions RNA was used as template in a RT-touchdown PCR previously described[18] and the amplified products were sequenced. On the basis of the new sequences specific forward primers for the *cyt b* gene were designed for both species: Ppcytb4fw (5'-AAGGGATGTAGAGTACGGGTGA-3') and Pp5fw (5'-CATGCGAATGTAGCATCATTTT-3') for *P pachyrhizi* and Hvcytb4fw (5'-AGCTTTTATTTCAGTGGAGCA-3') and Hvcytb5fw (5'-TGCTAATGTGGCTTCATTTTTC-3') for *H vastatrix*. These primers were used in combination with the specific reverse primers already available. The gDNA of these two species was amplified by using the BD Advantage™ 2 PCR Kit (BD Biosciences), following the instruction provided with the kit for the mixture preparation. The thermal conditions consisted of: 95 °C for 1 min, followed by 35 cycles at 95 °C for 30 sec, 60 °C for 30 sec, 68 °C for 3 min, and a final extension step at 68 °C for 3 min.

2.4.3 Alternaria solani and *Alternaria alternata*

RNAs of *A solani* and *A alternata* were retrotranscribed to cDNA and then amplified by using the forward primer DTRcytb2 (5'-CTAGTATGAACTATTGGTAC-3') in combination with the reverse primers DTRcytb2r (5'-GAGCAAAAGATATTCTTTCA-3') and Pteres5r (5'-AATATTAGAATAGCTGCAAACATCG-3'), designed on the basis of the *cyt b* gene cDNA sequence of *Pyrenophora tritici-repentis* (Died) Drechsler and *Pyrenophora teres* Drechsler, two species closely related to

Alternaria spp. The reactions were performed by a RT-touchdown PCR previously described[18] and the amplified products were sequenced.

On the basis of the cDNA sequences obtained as above, two new reverse primers, Asint143b_r (5'-TTGTCCTACTCAAGGGATAGCA-3') and Asint4d_r (5'-TCATTCTGGCACGATAGCTG-3'), specific for the *cyt b* gene of *A solani*, were designed and used in combination with the forward primer AS-5F.[25] The amplification of gDNA was performed by using the BD Advantage™ 2 PCR Kit as for *P pachyrhizi* and *H vastatrix*. The thermal conditions consisted of: 95 °C for 1 min, followed by 25 cycles at 95 °C for 30 sec, 60 °C for 1 min, 68 °C for 3 min, and a final extension step at 68 °C for 3 min. The long fragments (approximately 5200 and 6800 bp long, respectively) obtained using such reaction were partially sequenced and new specific primers were designed to amplify the rest of the sequence. The forward primers Asint143a_f (5'-GGGCAAATGTCTTTATGAGG-3'), Asint143c_f (5'-TGAGGTGAACACGAATTAGTTGA-3'), Asint143e_f (5'-GCTCGTTTAAATGATGGTAAACCT-3'), Asint143h_f (5'-GGGTAGCTGAAATGCTGCTT-3'), Asint143i_f (5'-TTTCACTTGTCCCTACTTCGGT -3') and the reverse primers Asint129_r (5'-GGCAAAAATTTACCTCATAAAGACA-3'), Asint143a_r (5'-CACAGTGGCTATGTGCTTGG-3'), Asint143h_r (5'-AAGCAGCATTTCAGCTACCC-3'), Asint143g_r (5'-ACCGAAGTAGGGACAAGTGAAA-3') were designed and used to amplify gDNA in PCR reactions performed with the BD Advantage™ 2 PCR Kit. The thermal conditions were those described above for AS-5F /Asint143b_r and AS-5F /Asint4d_r primer pairs.

Genomic DNA of *A alternata* was amplified with the same primer pairs used to amplify RNA, DTRcytb2 as forward primer and DTRcytb2r and Pteres5r as reverse primers. Genomic DNA (1 μl, 200 ng μl^{-1}) was added to a PCR mixture (19 μl) containing 1X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.25 mM each dNTP, 0.5 μM each primer and 0.05 U μl^{-1} *Taq* DNA Polymerase (Invitrogen). The thermal conditions consisted of: 94 °C for 5 min, followed by 25 cycles at 94 °C for 1 min, 57 °C for 1 min, 72 °C for 2 min, and a final

extension step at 72 °C for 5 min. RNA was amplified by using a RT-PCR previously described.[18]

2.4.4 Plasmopara viticola

The *cyt b* gene of *P viticola* has been amplified at gDNA level using the primers Pvcytb 1f (5'-TATACATGCGAATGGTGCATCT-3') and Pvcytb 4r (5'-GCGATCACACCACCAATTTTAT-3') with the same reaction conditions described for *A alternata*. In the reactions in which RNA was used as template, a RT-PCR approach was used.[18]

2.5 Sequencing and sequence analysis

All the amplified products obtained from these reactions were purified by 'NucleoSpin Extract 2 in 1' kit (Macherey-Nagel). For sequencing reaction, purified PCR products (1 μl, 20-50 ng μl^{-1}) were added to the sequencing mixture (19 μl) prepared following the 'BigDye Terminator v3.1' protocol (Applied Biosystems). The thermal cycling conditions consisted of: 96 °C for 10 sec, followed by 25 cycles at 96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min. Products from this reaction were purified with the 'DyeEx 2.0 Spin Kit' (Qiagen). Sequencing was carried out by using the DNA sequencer ABI Prism 3130 Genetic Analyser (Applied Biosystems) according to the instructions provided with the instrument. The sequences were analysed using the Bioinformatics software Chromas 1.45 (1996-1998 Conor McCarthy), EditSeq 5.03 (1989-2002 DNASTAR Inc.), MegAlign 5.03 (1993-2002 DNASTAR Inc.) and SeqMan 5.03 (1989-2001 DNASTAR Inc.). The *cyt b* gene structure was deduced by comparing the sequences obtained previoulsy from RNA[18,19] with the sequences obtained from gDNA .

3 RESULTS

Several PCR experiments with different combinations of 5' and 3' primers allowed characterizing the exon/intron organization of the *cyt b* gene in fourteen different agronomically important plant pathogens. For

the eight *Puccinia* species and for *U appendiculatus* (Table 1), the exon/intron junctions were identified by comparing the sequences obtained from gDNA with those obtained from mRNA.[18] A coding fragment of 996 bp, previously sequenced for *P recondita* f sp *tritici*,[18] was sequenced also for the other seven *Puccinia* species (sequences submitted to the GenBank with accession numbers reported in Table 1). This part of the gene encodes for the amino acid residues 1-332 and two introns were present at gDNA level. The first intron started exactly after the triplet encoding for glycine 143 (G143), as previously observed for *P recondita* f sp *tritici*,[18] the second intron was identified after the codon for isoleucine 269 (I269). The first intron ranged from 1474 to 1734 bp long for the *Puccinia* species (Table 1 and Fig 1). The first 750 bp of the intron were in frame with the upstream exon (430 bp long), which constituted an exon-intron fusion protein encoding for a maturase. The ORF (open reading frame) of the maturase started with the same methionine as the *cyt b* gene and encoded for about 420 amino acids. The intron at position G143 most likely belongs to the group I intron family,[24] because it encodes for a maturase and because the exon base immediately upstream of the 5' splice site is a T (U, uracile, in pre-mRNA) and the base preceeding the 3' splice site is a G (Fig 2). The maturase encoded in a mosaic organization by the exon-intron sequence of the *Puccinia* species confirmed high homologies with the maturase encoded by the second intron, bi2, in the *cyt b* gene long version of *S cerevisiae*,[18,22] which also starts after the codon 143 and is a type I intron (Fig 2). The second intron of *Puccinia cyt b* gene (at least 1000 bp) started at the same position of the fifth intron, bi5, in *S cerevisiae cyt b* gene[22] and did not reveal any homologies with other known maturases. Also the intron bi5 in *S cerevisiae* does not encode for a maturase.

Figure 1. Comparison of the *cyt b* gene structure in the region of the gene encoding for the amino acid residues 120-170 (first hot spot) in different plant pathogen species. The amino acid changes F129L and G143A responsible for resistance to QoI fungicides are in rectangles. The arrows indicate the position of the introns. *Puc. spp.*: includes *Puccinia recondita* f sp *tritici*, *P graminis* f sp *tritici*, *P striiformis* f sp *tritici*, *P coronata* f sp *avenae*, *P hordei*, *P recondita* f sp *secalis*, *P sorghi*, *P horiana*; *Uro. app.*: *Uromyces appendiculatus*; *Pha. pac.*: *Phakopsora pachyrhizi*; *Hem. vas.*: *Hemileia vastatrix*; *Myc. gal.*: *Mycena galopoda*; *Alt. alt.*: *Alternaria alternata*; *Alt. sol.*: *Alternaria solani*; *Blu. gra.*: *Blumeria graminis*; *Mag. gri.*: *Magnaporthe grisea*; *Mcs. fij.*: *Mycosphaerella fijiensis*; *Mcs. gra.*: *Mycosphaerella graminicola*; *Ven. ina.*: *Venturia inaequalis*; *Pla. vit.*: *Plasmopara viticola*; *Sac. cer.*: *Saccharomyces cerevisiae*.

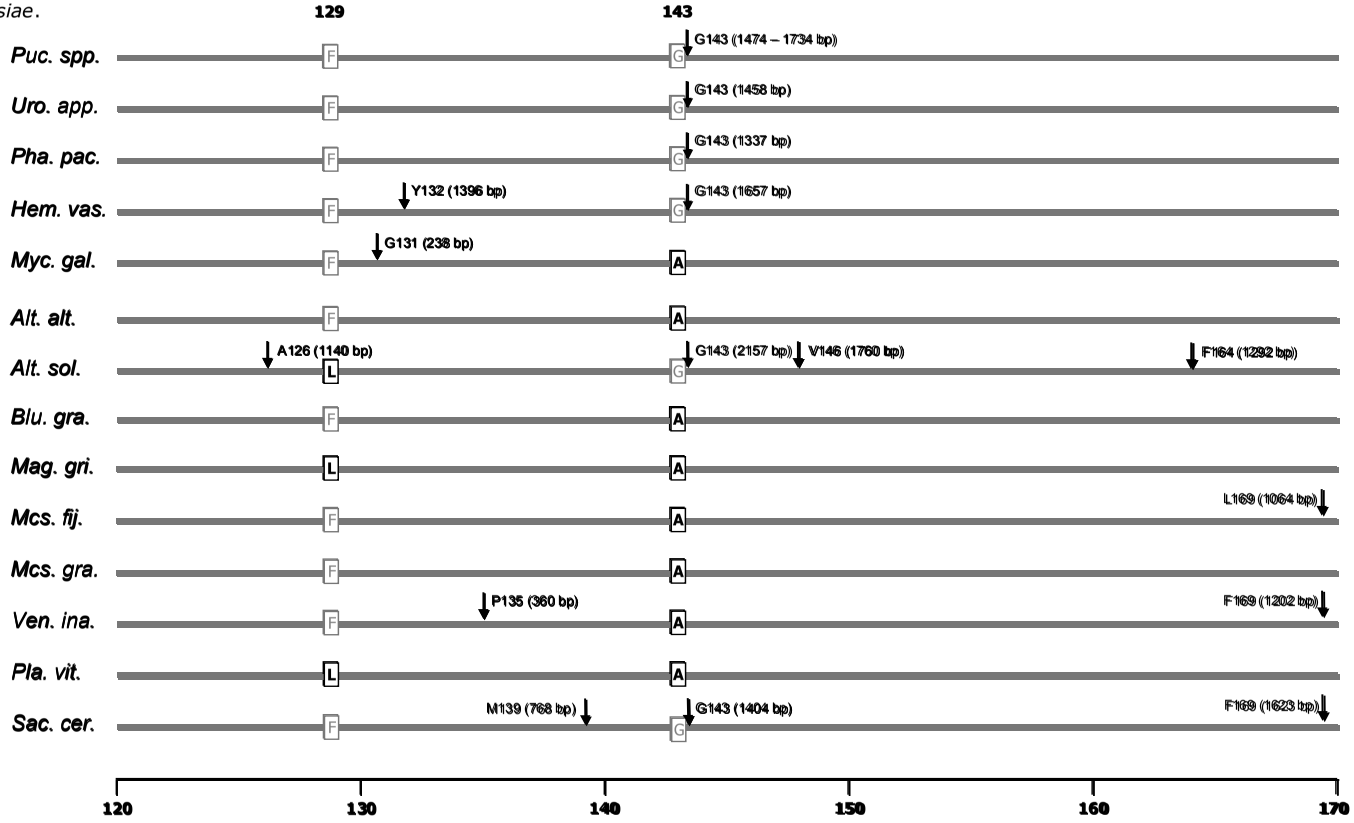
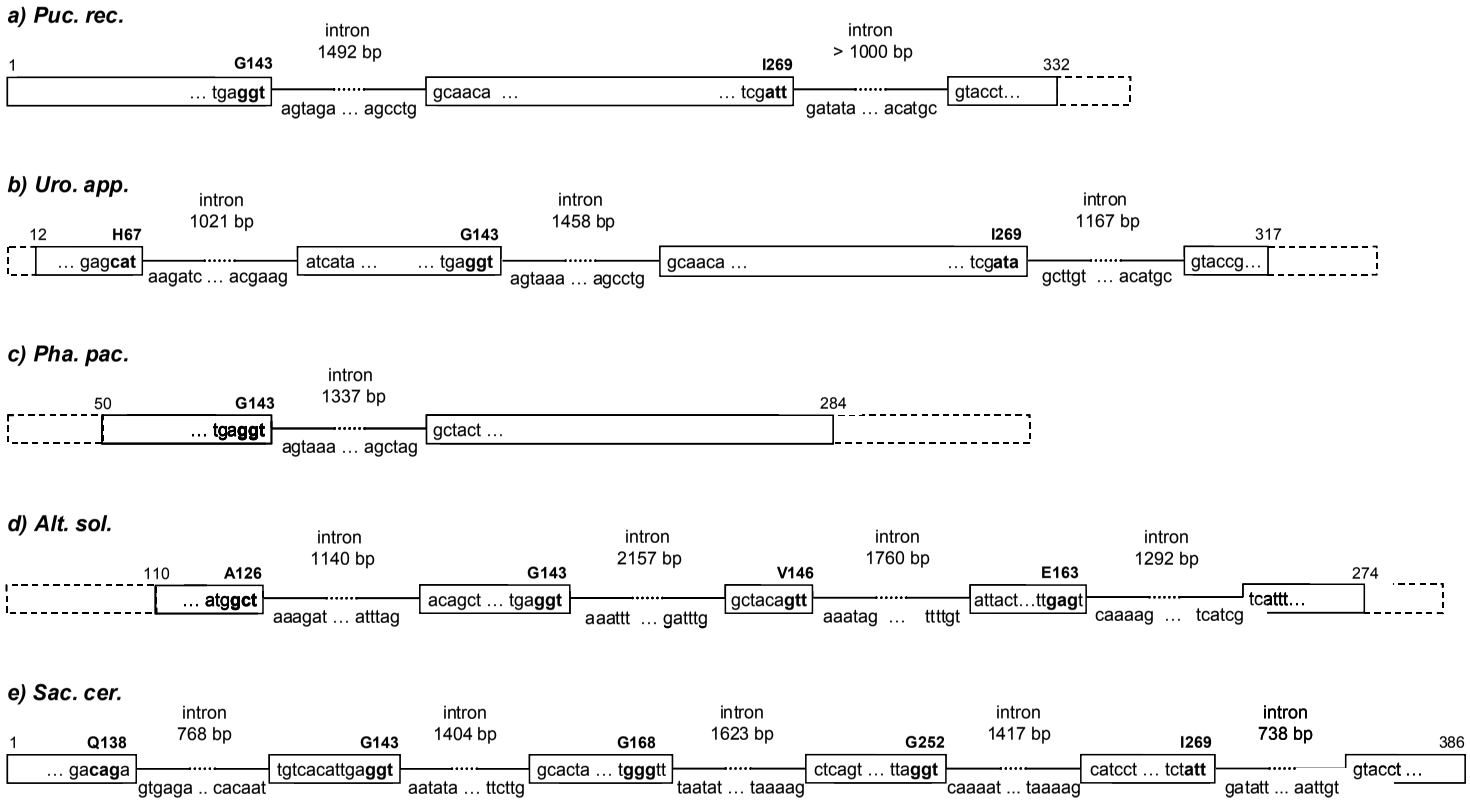


Figure 2. Comparison of the nucleotide sequences at the exon/intron junctions in the *cyt b* gene of different plant pathogen species. Empty boxes indicate exons and lines introns. Dashed boxes represent non sequenced parts of the gene. The length of exons and introns is not in scale. a) *Puc. rec.*: *Puccinia recondita* f sp *tritici*; b) *Uro. app.*: *Uromyces appendiculatus*; c) *Pha. pac.*: *Phakopsora pachyrhizi*; d) *Alt. sol.*: *Alternaria solani*; e) *Sac. cer.*: *Saccharomyces cerevisiae*



For *U appendiculatus*, a coding fragment of 917 bp was sequenced covering the amino acid residues 12-317. In this fragment, two introns were observed at the same positions, G143 and I269, as found in *Puccinia*, and an additional intron after the codon for histidine at position 67 (H67, Table 1, Fig 1 and Fig 2). The intron after the codon for G143 encoded also in this species for a maturase in frame with the upstream exon. The other two introns were non-coding DNA.

In *P pachyrhizi*, only one intron of 1337 bp was found in a coding fragment of 705 bp (Table 1, Fig 1 and Fig 2), which covered amino acid residues 50-284. This intron also started after the codon for G143 and encoded for a maturase in frame with the upstream exon.

A coding fragment of 682 bp, which covered amino acid residues 50-276, was sequenced for *H vastatrix*, it was interrupted by three introns (Table 1 and Fig 1). Again an intron (1657 bp long) was observed starting exactly after the codon for G143 and encoding for a maturase. Another intron started after the codon for tyrosine at position 132 (Y132), it was 1396 bp long. This intron revealed weak similarity with a *cyt b* intron of *Penicillium marneffei* Segretain (NP_943714) and with a putative endonuclease of *Candida metapsilosis* (YP_227555). It is unknown whether this sequence is a coding region in the mitochondrial genome. A third intron (at least 1500 bp) was observed in the *cyt b* gene of *H vastatrix*, but it was not possible to define precisely the exon/intron junction. It started approximately between amino acid residues isoleucine at position 68 and histidine at position 82 (I68-H82).

Among *Puccinia* species the intron starting after the codon for G143 confirmed high homologies,[18] mostly in the first 700 bp, which encode for the maturase. Similarities ranging between 70-80 % were also observed by comparing the intronic sequence of *Puccinia* species with the intron at position G143 of *U appendiculatus*, *P pachyrhizi* and *H vastatrix*.

In *P viticola* sequencing revealed that the fragment (655 bp) spanned the two mutational hot spot regions of *cyt b* gene from amino acid 79 to amino acid 296. Using gDNA and RNA as template no size difference of the amplicon was observed. Therefore, no introns were present in this fragment of the gene (Table 1 and Fig 1).

In *A alternata* no introns were found in a fragment sequenced between amino acid residues 110-286 (Table 1 and Fig 1). However, in *A solani*, the presence of four introns was observed in the fragment of the gene encoding for the amino acid residues 110 – 274 (Table 1, Fig 1 and Fig 2). A first intron (1140 bp long) was observed after the codon for alanine at position 126 (A126). The second one (2157 bp long) started exactly after the codon for glycine 143, as observed for all rust species. A short exon (9 bp) separated this intron from a third intron, which was 1760 bp long. A fourth intron started after the first T of the codon TTC for phenylalanine 164 (F164). The first intron and the last part of the third intron showed low homology to maturase. On the contrary, the fourth intron showed high homology with an intron of *Neurospora crassa* Shear & BO Dodge *cyt b* gene located at the same position as in *A. solani* (F164), which putative product also has some resemblance to other mitochondrial introns and in particular to mRNA maturases.[26]

As already observed for *Puccinia*, by comparing the nucleotide sequences at the exon/intron junctions (Fig 2), the exon base immediately upstream of the 5' splice site is a T and the base preceeding the 3' splice site is a G in most of the analyzed introns. In additon, the codons for glycine, valine and isoleucine are usually the last exonic triplets before an intron starts.

4 DISCUSSION

The *cyt b* gene encodes for cytochrome *b*, which is part of the cytochrome bc_1 enzyme complex (or complex III) in the mitochondrial respiratory chain. This protein is the target enzyme for QoI fungicides, which are able to inhibit mitochondrial respiration by binding to the enzyme complex.[2] The QoI fungicides are widely used for the control of many plant pathogens including rusts. The characterization of the *cyt b* gene is essential for the development of diagnostic tools to easily isolate the *cyt b* gene and detect point mutations conferring resistance to QoI fungicides that eventually may evolve. In pathogens such as *B graminis*, *M fijiensis* and *V inaequalis*, extensive sequence data for the *cyt b* gene

were produced, mostly after the detection of isolates resistant to QoI fungicides.[4,5,6,7,15] Resistance is in most cases conferred by a single point mutation in the *cyt b* gene leading to a change at amino-acid position 143 from glycine to alanine (G143A).[8,10] A change of phenylalanine to leucine at position 129, F129L, also confers resistance to QoIs in species such as *P aphanidermatum*[8] and *P grisea*.[11] In addition, other point mutations[3] and other mechanisms leading to resistance were described.[6,7,12]

In our previous study, a *cyt b* gene fragment was sequenced at cDNA level in a range of plant pathogens, including eight different *Puccinia* species.[18] During this study, a longer fragment was sequenced at gDNA level for the same eight *Puccinia* species including the two hot spot regions. Also for *U appendiculatus*, *P pachyrhizi* and *H vastatrix*, the *cyt b* gene fragment sequenced during this study was longer than the fragment previously sequenced at cDNA level.[19] The *cyt b* gene structure, particularly in the region including the two hot spots, was characterized in fourteen different plant pathogens, including rusts on cereals (*P recondita* f sp *tritici*, *P graminis* f sp *tritici*, *P striiformis* f sp *tritici*, *P coronata* f sp *avenae*, *P hordei*, *P recondita* f sp *secalis*, *P sorghi*), on *Chrysanthemum* (*P horiana*), bean (*U appendiculatus*), soybean (*P pachyrhizi*) and coffee (*H vastatrix*), as well as, leaf spot of *Helianthus annuus* L (*A alternata*), early blight of potato (*A solani*) and grape downy mildew (*P viticola*). The *cyt b* gene structure of these pathogens was compared with other pathogens to examine whether there is any correlation between exon/intron organization and resistance to QoI fungicides, i.e. if the presence of introns influences the occurrence of point mutations responsible for resistance to QoI fungicides. Our observations were mainly concentrated on the introns present in the area of the first hot spot (amino acid residues 120-160), since in this part of the gene the two main point mutations G143A and F129L can occur, which are known to confer resistance to QoIs. Other introns, such as the intron at position I269 in *Puccinia* spp or H67 in *U appendiculatus*, were not investigated in more detail, because they are not in the part of the gene involved in resistance to QoI fungicides. In addition, these introns are non-coding DNA sequences.

The main result of this study is that in all pathogens, in which resistance to QoIs and the mutation G143A were never reported until now (all rusts and *A solani*), an intron was observed starting exactly after the codon of glycine 143. On the other hand, in pathogens such as *A alternata*, *B graminis*, *M grisea*, *M fijiensis*, *M graminicola*, *V inaequalis* and *P viticola*, resistance to QoI fungicides is known and the glycine (triplet GGT) is replaced by alanine (triplet GCT) at position 143 in the resistant genotype. In those pathogen species, no intron was observed after the codon 143. By comparing the intronic sequence after the codon 143 in rusts with other sequences in the GenBank, it was observed that this intron encodes for a maturase, which is involved in folding processes of pre-mRNA in the group I intron family.[23,24,27] In addition, in most of the analysed introns it was observed that the exon base immediately upstream of the 5' splice site is a T (U in pre-mRNA) and the base preceeding the 3' splice site is a G.[23,24] These conserved nucleotide residues at the splice site junctions are typical for the group I introns.

Group I introns are common in fungal mitochondria genes encoding components of the electron transport system (such as cytochrome oxydase and cytochrome *b*) and ribosomal RNA genes. They are usually 68 to over 3000 bp long, mainly over 400 bp. Most of these introns are able to splice themselves in the absence of proteins, thus the RNA itself is catalytic ('ribozymes'), and are often able to propagate themselves in the genome.[28] Their DNA includes an ORF that encodes for a transposase-like protein that can make a copy of the intron and insert it elsewhere at predetermined positions into intronless sites of genes. The intronic ORF also encodes for splicing (RNA maturase) and mobility ('homing endonuclease').[28, 29]

The *cyt b* mRNA in species such as *N. crassa, Aspergillus nidulans* (Eidam) G Winter and *S cerevisiae* is produced by the action of a maturase encoded by intronic sequences.[26,27,30,31] The second intron in the long version of the *cyt b* gene of *S cerevisiae* (Fig 1 and 2) starts exactly after the codon for G143[22] as observed in rusts and *A solani*. This intron encodes for a maturase and is a mobile element in the yeast genome. Splicing-deficient mutants of *S cerevisiae* carrying mis-sense

mutations in the maturase gene were shown to completely abolish maturase activity, thus leading to a respiratory-deficient phenotype.[32] However, this mutation is not at the 143 position. In *M fijiensis*, 303 amino acid residues encoded by the intron at position F169 showed 75 % identity with the *A nidulans* mRNA maturase.[5] Other introns (in addition to the one at position 143) revealed similarities with group I introns and some putative endonucleases or intron proteins (e.g. the intron after Y132 in *H vastatrix* or after A126 and F164 in *A solani*). However, these similarities were too low to predict whether these introns encode for any enzyme; the function of this DNA is not known.

It has been experimentally shown with group I introns that the nucleotide sequence near by the splice junctions has an essential role in the correct mRNA splicing process and therefore, in the correct gene transcription.[24,28,33] Splice site recognition relies on an Internal Guide Sequence (IGS) of the intron, which pairs with exon sequences flanking the 5' and 3' splice sites.[28] Therefore, the only exon sequences required for accurate splicing are those immediately adjacent to the splice sites.[23] Experiments carried out with the fourth intron (I4) of *S. cerevisiae* demonstrated that a mutation 2 nucleotide upstream from the exon/intron junction (B4/I4) in the *cyt b* gene does not permit correct splicing of the pre-mRNA.[23,34] This mutation has been determined as a glycine (GGT) - aspartic acid (GAT) change.

In plant pathogens the triplet GGT encodes for glycine at position 143 in the wild type *cyt b* gene. In resistant genotypes to QoI fungicides, the second guanine in the triplet is replaced by cytosine and the mutated codon GCT encodes for alanine. If an intron is present immediately after the triplet 143, this mutation is supposed to affect the splicing process, since the mutated base is just 2 nucleotide upstream from the exon/intron junction. In the rust species included in this study as well as in *A solani*, the codon GGT at position 143 is located exactly at the exon/intron boundary and is likely part of the signal sequences essential for the recognition of the intronic RNA to be excised. We predicted that a nucleotide substitution in the codon 143 (GGT → GCT) may, therefore, strongly affect the splicing process, leading to a deficient cytochrome *b*. The substitution of guanine to cytosine does

obviously not allow anymore the proper looping of the intron in the pre-mRNA molecule, a process that is essential for correct splicing of the *cyt b* pre-mRNA.[23,33] In pathogens such as *M fijiensis*[5] and *V inaequalis*,[15] the mutation G143A seems to have no influence in the splicing mechanism and maturase activity, since the intron is far apart (78 nucleotides) from the splice site and the triplet 143 is not anymore a signal sequence for pre-mRNA splicing.

Our results generated for rusts are confirmed by the findings on the genus *Alternaria*. In *A solani*, there is no mutation at position 143 but an intron after that codon. However, resistance to QoI fungicides was detected based on the mutation at position 129, F129L,[35] although the level of resistance was lower than that conferred by G143A substitution in other pathogens, probably due to the fact that the F129L substitution can cause a performance reduction during quinol oxidation.[36] A recent screening of many *A solani* isolates[37] demonstrated that none of the isolates resistant to QoI fungicides was found to carry the mutation at the base pair position 143. Only the amino acid substitution F129L was reported. On the other hand, there is no intron after position 143 in *A alternata* and the mutation G143A was detected in resistant isolates[38] (confirmed by this study). In wheat rusts, no resistance to QoI fungicides was detected so far, although thousands of samples were analysed by bioassay over the last eight years. There are no indications until today, that F129L might evolve in rusts, but we cannot rule it out. Therefore, future resistance research in rusts should be oriented to mutations in the *cyt b* gene outside the G143.

In conclusion, we predict that the G143A mutation (GGT → GCT at nucleotide level) will significantly affect the splicing process from pre-mRNA to mature mRNA, if a group I type intron is present after the codon 143 in the *cyt b* gene. In this case, a substitution of guanine by cytosine, which generates the mutated codon GCT, will be lethal and individuals carrying this mutation will not survive. As a consequence, resistance to QoI fungicides based on G143A is not likely to evolve in species such as rusts (*Puccinia* spp, *U appendiculatus*, *P pachyrhizi*, *H vastatrix*) and *A solani*. It can not be excluded that other mechanisms than G143A mutation arise and confer resistance to QoIs. However,

species lacking an intron at position 143 in the *cyt b* gene, may acquire resistance to QoIs quite easily as it was observed in *A alternata*, *B graminis*, *M grisea*, *M fijiensis*, *M graminicola*, *V inaequalis* and *P viticola*.

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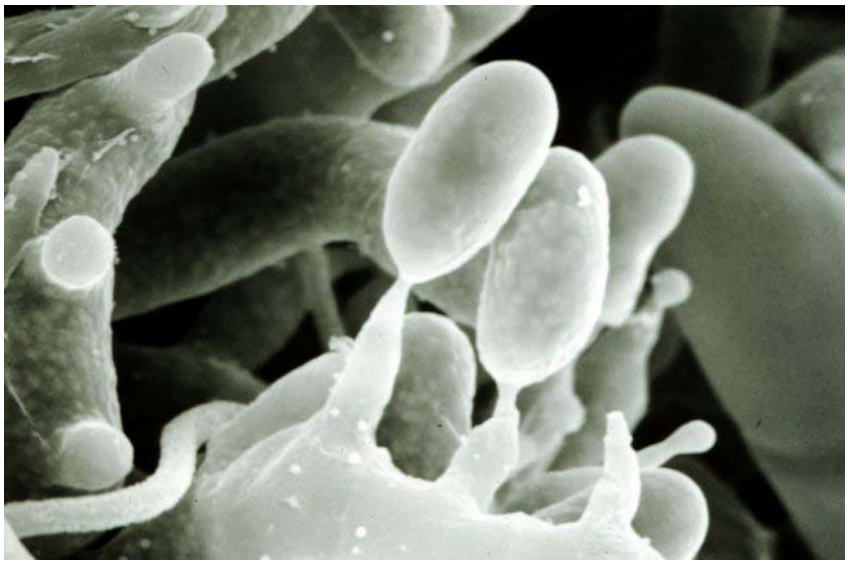
CHAPTER 5

Conclusions



Over the past thirty years, fungicide resistance has been described as one of the most important causes of crop losses due to plant diseases which have not been controlled adequately. The QoI fungicides represent one of the most recent classes of fungicides for which the threat of resistance exists, due to their single-site mode of action (Bartlett *et al.*, 2002). So far, major resistance problems have been observed recently in *Blumeria graminis* f.sp. *tritici* (Sierotzki *et al.*, 2000a, Chin *et al.*, 2000a) and *Mycosphaerella fijiensis* (Sierotzki *et al.*, 2000b; Heaney *et al.*, 2000; Chin *et al.*, 2001b), followed by a number of other important pathogens such as *Plasmopara viticola*, *Pseudoperonospora cubensis*, *Sphaerotheca fuliginea* (Heaney *et al.*, 2000; Ishii *et al.*, 2001) and *Venturia inaequalis* (Steinfeld *et al.*, 2001). However, QoIs remain one of the most important classes of fungicides because they can be used in over 400 crop/disease systems.

To avoid disease control failures depending on the intrinsic resistance risk of QoI fungicides, a continuous sensitivity monitoring, careful risk assessment and implementation of anti-resistance strategies are essential for the successful and long lasting use of these compounds (Brent and Hollomon, 1998). Reliable sensitivity test methods are crucial for the assessment of the resistance risk of pathogens to fungicides. In addition, if the molecular mode of resistance is related to a Single Nucleotide Polymorphism (SNP), DNA-based methods allow the development of molecular diagnostic tools for the detection of SNPs conferring the resistant phenotype (Wille *et al.*, 2002; Sierotzki and Gisi, 2003). The detection of resistance to QoI fungicides based on molecular methods is possible, since the major mechanism of resistance is a nucleotide substitution in the *cyt b* gene, which causes the replacement of glycine with alanine at position 143, G143A (Gisi *et al.*, 2002; Sierotzki *et al.*, 2000a, b), or phenylalanine with leucine at position 129, F129L (Gisi *et al.*, 2002; Kim *et al.*, 2003; Pasche *et al.*, 2002). However, other point mutations were described, all located in two highly conserved cytochrome *b* regions, so called 'hot spot' regions (amino acid residues 120-160 and 250-300; Di Rago *et al.*, 1989; Brasseur *et al.*, 1996; Kraiczy *et al.*, 1996).

Since resistant isolates to QoIs were detected in several Ascomycetes and Oomycetes soon after the commercial introduction of these fungicides, extensive sequence data were produced for the *cyt b* gene of pathogens such as *B. graminis* (Sierotzki *et al.*, 2000a), *V. inaequalis* (Zheng and Köller, 1997) and *M. fijiensis* (Sierotzki *et al.*, 2000b), in order to elucidate the molecular basis of resistance and develop DNA-based techniques for the rapid detection of SNPs. On the other hand, in plant pathogenic Basidiomycetes such as rusts, resistance to QoIs has surprisingly never been reported until now and no molecular data were available in the GenBank for the *cyt b* gene of these fungi.

The main result of the present study was the amplification and sequencing of the *cyt b* gene fragment responsible for the binding site of QoI fungicides in a range of basidiomycete plant pathogens, including *Puccinia recondita* f.sp. *tritici*, *P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis*, *P. sorghi*, *P. horiana*, *P. arachidis*, *Uromyces appendiculatus*, *Phakopsora pachyrhizi*, *Hemileia vastatrix* and *Rhizoctonia solani*. This fragment includes all possible mutations known to confer resistance to QoI fungicides in many plant pathogens (the two 'hot spot' regions). Based on this information, specific primers for the *cyt b* gene of these pathogens were designed to detect point mutations that eventually could evolve, especially the amino acid substitutions G143A and F129L.

Based on these molecular data, the relatedness within rusts (*Uredinales*) and other Basidiomycetes, such as *Strobilurus tenacellus*, *Mycena viridimarginata* and *M. galopoda*, was investigated and compared with some Ascomycetes and Oomycetes. In addition, the phylogenic relatedness based on the mitochondrial *cyt b* gene was compared to that obtained with the nuclear Internal Transcribed Spacers (ITS) in the ribosomal DNA (White *et al.*, 1990). The *cyt b* gene has been used for identification, classification, and phylogenic analysis of animals and plants (Irwin *et al.*, 1991; Kocher *et al.*, 1989), for some Ascomycetes (Yokoyama *et al*., 2001; Wang *et al.,* 2001) and *R. solani* (Sierotzki *et al.*, 2002), but never for rusts. On the other hand, phylogenic studies were already performed for *Uredinales* by using the ribosomal DNA sequences (Maier *et al.*, 2003; Wingfield *et al.*, 2004;

Zambino and Szabo, 1993; Kropp *et al.*, 1997; Roy *et al.*, 1998; Pfunder *et al.*, 2001; Weber *et al.*, 2003).

Our results showed that the relatedness among plant pathogenic Basidiomycetes was similar by using either mitochondrial *cytb* gene or nuclear ITS sequences. *Puccinia* and *Uromyces*, both taxonimically belonging to the family of the *Pucciniaceae* within the *Uredinales*, were the most related genera among the analysed plant pathogenic Basidiomycetes. Separated from the *Pucciniaceae* were the two genera *Phakopsora* and *Hemileia*, which belong to the *Phakopsoraceae* and *Chaconiaceae*, respectively (Wingfield *et al.*, 2004). The rather weak relatedness of *R. solani* with the other plant pathogenic Basidiomycetes confirmed the traditional taxonomy, which does not collocate this species within the Urediniomycetes, but within the Hymenomycetes. We demonstrated that both *cyt b* gene and ITS sequences are useful markers for phylogenic studies of plant pathogenic fungi and they are able to distinguish the main taxonomic groups (Ascomycetes, Basidiomycetes, Oomycetes) as well as different genera and species. The ITS sequences, as non-coding regions, evolve more rapidly than coding regions such as the *cyt b* gene, and are able to show more differences among species and within a species. For this reason, the ITS sequences were able to discriminate *Puccinia* species which were identical on the basis of the cytochrome *b* amino acid sequence. On the other hand, since ITS sequences are highly variable, they are not suitable for phylogenic inference among families.

We demonstrated for the first time that the mitochondrial cytochrome *b* sequence is a valid tool to study phylogenic relatedness among plant pathogenic Basidiomycetes and supports the classical taxonomy based on morphological structures and host specificity (Littlefield and Heath, 1979; McLaughlin *et al.*, 1995). Moreover, the phylogenic clusters generated by analysing the cytochrome *b* sequence confirmed previous results based on the analysis of rRNA of the *Uredinales* (Maier *et al.*, 2003, Wingfield *et al.*, 2004) and more specifically, on the ITS regions of closely related species of *Puccinia* (Zambino and Szabo, 1993).

In our further studies, the *cyt b* gene fragment, including the two 'hot spot' regions, was sequenced at genomic DNA level in a range of plant pathogens, including several *Puccinia* species, *U. appendiculatus*, *P. pachyrhizi*, *H. vastatrix*, and supplemented by *Alternaria solani*, *A. alternata* and *Plasmopara viticola*. By aligning the genomic DNA (gDNA) and the complementary DNA (cDNA) sequences, the *cyt b* gene structure of these agronomically important plant pathogens was characterized and compared with that of other important species. The exon/intron organization was characterized and its possible role investigated for the occurrence of point mutations in the *cyt b* gene, especially the amino acid substitutions G143A and F129L, which are responsible for resistance to QoI fungicides.

The main result of this study was that all pathogens, in which resistance to QoIs and the mutation G143A were never reported until now (all rusts and *A. solani*) were characterized by an intron starting exactly after the codon for glycine at position 143. On the other hand, in pathogens such as *A. alternata*, *B. graminis*, *M. grisea*, *M. fijiensis*, *M. graminicola*, *V. inaequalis* and *P. viticola*, resistance to QoI fungicides is known and the glycine (triplet GGT) is replaced by alanine (triplet GCT) at position 143 in the resistant genotype. In these pathogen species, no intron was observed after the codon 143.

The intron starting after the triplet at position 143 in rusts and *A. solani* revealed high similarities with the group I intron family (Cech, 1988; Burke, 1988). Most of these introns are able to splice themselves in the absence of proteins, thus the RNA itself is catalytic ('ribozymes'), and their DNA often includes an ORF (Open Reading Frame) that encodes for a RNA maturase, which is an enzyme involved in the splicing process of pre-mRNA (Lambowitz and Belfort, 1993). It has been experimentally shown with group I introns that the exon sequences immediately adjacent to the splice sites are required for accurate splicing (Burke, 1988). Splice site recognition relies on an Internal Guide Sequence (IGS) of the intron, which pairs with exon sequences flanking the 5' and 3' splice sites (Lambowitz and Belfort, 1993). Experiments carried out with the fourth intron (I4) of *S. cerevisiae* demonstrated that a mutation 2 nucleotide upstream from the exon/intron junction (B4/I4)

in the *cyt b* gene does not permit correct splicing of the pre-mRNA (Burke, 1988, De La Salle *et al.*, 1982).

In the rust species included in this study, the codon GGT at position 143 is located exactly at the exon/intron boundary and is likely part of the signal sequences essential for the recognition of the intronic RNA to be excised. We predict that a nucleotide substitution in the codon 143 (GGT → GCT) may, therefore, strongly affect the splicing process, leading to a deficient cytochrome *b*. The substitution of guanine to cytosine does obviously not allow anymore the proper looping of the intron in the pre-mRNA molecule, a process that is essential for correct splicing of the *cyt b* pre-mRNA (Bolduc *et al.*, 2003; Burke, 1988). In pathogens such as *M. fijiensis* (Sierotzki *et al.*, 2000b) and *V. inaequalis* (Zheng and Köller, 1997), the mutation G143A seems to have no influence in the splicing mechanism and maturase activity, since the intron is far apart (78 nucleotides) from the splice site and the triplet 143 is not anymore a signal sequence for pre-mRNA splicing.

The results generated for rusts were confirmed by the findings in the genus *Alternaria*. In *A. solani*, there was no mutation at position 143 but an intron after this codon. A recent screening of many *A. solani* isolates (Rosenzweig *et al.*, 2005) demonstrated that none of the isolates resistant to QoI fungicides was found to carry the mutation at the base pair position 143. However, the amino acid substitution F129L was reported (Pasche *et al.*, 2002). On the other hand, there was no intron after position 143 in *A. alternata* and the mutation G143A was detected in resistant isolates (Ma and Michailides, 2004).

We predict that the G143A mutation (GGT → GCT at nucleotide level) significantly affects the splicing process from pre-mRNA to mature mRNA, if a group I type intron is present after the codon 143 in the *cyt b* gene. In this case, a substitution of guanine by cytosine, which generates the mutated codon GCT, will be lethal and individuals carrying this mutation will not survive. As a consequence, resistance to QoI fungicides based on G143A is not likely to evolve in species such as rusts (*Puccinia* spp., *U. appendiculatus*, *P. pachyrhizi*, *H. vastatrix*) and *A. solani*. On the other hand, species lacking an intron at position 143 in the *cyt b* gene, may acquire resistance to QoIs quite easily as it was

observed in *A. alternata*, *B. graminis*, *M. grisea*, *M. fijiensis*, *M. graminicola*, *V. inaequalis* and *P. viticola*.

However, in rusts it cannot be excluded that mutations outside G143A may arise and confer resistance to QoIs, as observed for *A. solani*. Our results cannot predict whether resistance may evolve in these pathogens. However, the characterization of the *cyt b* gene in plant pathogenic Basidiomycetes represents an essential step for the development of diagnostic tools to easily isolate the *cyt b* gene and detect the appearance of resistant isolates in an early stage.

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Summary

Strobilurins are one of the most important classes of agricultural fungicides, which are also known as Qo inhibitors (QoIs) because they inhibit mitochondrial respiration by binding to the Qo ('Quinone outside') site of the cytochrome *b*. Cytochrome *b* is part of the cytochrome bc_1 complex, located in the inner mitochondrial membrane, and it is encoded by the cytochrome *b (cyt b)* gene. Since the mode of action of QoI fungicides is highly specific, the risk of resistance is also high. Single amino-acid exchanges in the cytochrome *b* were found conferring resistance to QoI fungicides in different plant pathogens. The major mechanism of resistance is a point mutation, also called Single Nucleotide Polymorphism (SNP), in the *cyt b* gene leading to a change at amino acid position 143 from glycine to alanine (G143A) or from phenylalanine to leucine at position 129 (F129L). However, other point mutations were described, all located in two highly conserved cytochrome *b* regions, so called 'hot spot' regions (amino acid residues 120-160 and 250-300).

A fragment of the *cyt b* gene of a range of agronomically important plant pathogenic Basidiomycetes was sequenced at cDNA level, including *Puccinia recondita* f.sp. *tritici*, *P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis*, *P. sorghi*, *P. arachidis*, *P. horiana*, *Uromyces appendiculatus*, *Phakopsora pachyrhizi*, *Hemileia vastatrix* and *Rhizoctonia solani*. The sequence data allowed developing specific primers for the *cyt b* gene of all these species, which easily amplified the fragment of the gene including the 'hot spot' regions. Resistance to QoI fungicides has never been reported until now in plant pathogenic Basidiomycetes, and in all tested isolates included in this study, the sequence of the *cyt b* gene fragment did not contain any point mutations.

The deduced amino acid sequences of the cytochrome *b* were used to study the relatedness of these pathogens as compared to other species of the Basidiomycetes, Ascomycetes and Oomycetes. The relatedness was also studied at nuclear level using the Internal Transcribed Spacers (ITS) in the ribosomal DNA. Our results

demonstrated for the first time that the amino acid sequence of the mitochondrial cytochrome *b* is a valid tool to study phylogenic relatedness among plant pathogenic Basidiomycetes and supports taxonomic grouping based on morphological structures and host specificity. Moreover, the phylogenic clusters generated by analysing the cytochrome *b* sequence confirmed previous results based on the analysis of rRNA of the *Uredinales* and more specifically, on the ITS regions of closely related species of *Puccinia*.

The cytochrome *b* gene fragment (coding sequence about 950 bp long) was sequenced also at genomic DNA level in different *Puccinia* species and *U. appendiculatus*. A shorter fragment (coding sequence about 600-700 bp) was sequenced in *P. pachyrhizi*, *H. vastatrix*, *Alternaria solani*, *A. alternata* and *Plasmopara viticola*. The *cyt b* gene structure of these agronomically important plant pathogens was characterized, especially in the two 'hot spot' regions and compared with that of other species. The exon/intron organization was characterized and its possible role investigated for the occurrence of point mutations in the *cyt b* gene, especially the amino acid substitutions G143A and F129.

In all rust species included in this study as well as in *A. solani*, the mutation G143A was not detected, but a group I intron (self-splicing intron) was observed starting exactly after the codon GGT for glycine at position 143. In pathogens such as *A. alternata*, *Blumeria graminis*, *Pyricularia grisea*, *Mycosphaerella graminicola*, *M. fijiensis*, *Venturia inaequalis* and *P. viticola*, resistance to QoI fungicides is known and the glycine (triplet GGT) is replaced by alanine (triplet GCT) at position 143 in the resistant genotype. In those pathogen species, no intron was observed after the codon 143. In the rust species as well as in *A. solani*, the codon GGT at position 143 is located exactly at the exon/intron boundary and is likely part of the signal sequences essential for the recognition of the intronic RNA to be excised. We predicted that a nucleotide substitution in the codon 143, which is two nucleotides upstream from the exon/intron junction, may strongly affect the splicing process, leading to a deficient cytochrome *b*, which is lethal. Therefore, individuals carrying the G143A substitution and an intron at this position

will not survive. As a consequence, the evolution of resistance to QoI fungicides based on G143A is not likely to evolve in plant pathogens such as *Puccinia* spp., *U. appendiculatus*, *P. pachyrhizi*, *H. vastatrix* and *A. solani*.

It cannot be excluded that mutations other than G143A conferring resistance may arise in upcoming rust populations selected by the use of QoI fungicides, as observed for *A. solani,* in which the mutation F129L was reported in resistant isolates. Our results cannot predict whether such mutations may evolve at all in rusts, but the sequence data produced during this study and the understanding of the *cyt b* gene structure represent an essential information for easy isolation of the *cyt b* gene and detection of point mutations conferring resistance to QoI fungicides that eventually may evolve.

Riassunto

Le strobilurine sono un'importante classe di fungicidi, denominata anche 'Qo Inhibitors' (QoI), in riferimento al fatto che la loro azione si esplica a livello del sito Qo ('Quinone outside') dell'ubichinolo. Tali fungicidi hanno un meccanismo d'azione specifico e la loro attività fitoiatrica è determinata dal legame con il citocromo *b* fungino e la conseguente inibizione della respirazione cellulare. Il citocromo *b* fa parte del complesso bc_1 della catena respiratoria mitocondriale ed è codificato dal gene *cyt b*. A causa del meccanismo d'azione estremamente specifico, già dopo due anni dall'immissione in commercio delle prime strobilurine, sono stati osservati in campo i primi casi di resistenza in patogeni quali *Blumeria graminis* f.sp. *tritici*, *Mycosphaerella graminicola*, *M. fijiensis*, *Venturia inaequalis*, *Plasmopara viticola*, *Pyricularia grisea*, *Pseudoperonospora cubensis* e *Sphaerotheca fuliginea*. Nella maggior parte dei casi, la resistenza ai fungicidi QoI è causata da una mutazione puntiforme, anche nota come 'Single Nucleotide Polymorphism' (SNP), a livello del codone 143 del gene *cyt b*, che porta alla sostituzione di una glicina in un'alanina (G143A). Anche altre SNPs nel gene *cyt b* sono state identificate quali responsabili del fenotipo resistente, in particolare la mutazione nella tripletta in posizione 129, che porta alla sostituzione di una fenilalanina con una leucina (F129L). Tali mutazioni sono tutte localizzate in due regioni conservate del gene *cyt b* denominate regioni 'hot spot' e comprese tra i residui amminoacidici 120-160 e 250-300.

Un frammento del cDNA del gene *cyt b* (contenente quindi solo esoni) è stato sequenziato in diversi Basidiomiceti fitopatogeni, tra i quali *Puccinia recondita* f.sp. *tritici*, *P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis*, *P. sorghi*, *P. arachidis*, *P. horiana*, *Uromyces appendiculatus*, *Phakopsora pachyrhizi*, *Hemileia vastatrix* e *Rhizoctonia solani*. Le sequenze ottenute hanno permesso di disegnare primers specifici per il gene *cyt b*, in grado di amplificare mediante PCR il frammento di DNA contenente le due regioni 'hot spot'. I suddetti patogeni non hanno finora mai mostrato

ridotta sensibilità alle strobilurine e tale dato è stato confermato dall'assenza di mutazioni puntiformi nel gene *cyt b*.

Le sequenze amminoacidiche dedotte dal DNA del gene *cyt b* sono state successivamente utilizzate, al fine di studiare il grado di relazione filogenetica tra le specie in studio ed altri funghi fitopatogeni appartenenti ai Basidiomiceti, Ascomiceti e Oomiceti. Tale analisi filogenetica è stata condotta anche utilizzando gli 'Internal Transcribed Spacers' (ITS) presenti nel DNA ribosomale. I risultati ottenuti hanno dimostrato, per la prima volta, che la sequenza amminoacidica del citocromo *b* è un valido strumento per studi filogenetici di Basidiomiceti fitopatogeni e hanno confermato la tassonomia basata sulle caratteristiche morfologiche e la specificità d'ospite di tali specie. Inoltre, i 'clusters' generati dall'analisi del citocromo *b* hanno confermato i risultati basati sull'esame delle sequenze ITS ottenuti sia nel corso di tale studio, sia da altri autori in precedenza.

Successivamente il gene *cyt b* (circa 900 bp di sequenza codificante) è stato sequenziato anche a livello di DNA genomico in diverse specie di *Puccinia* e *U. appendiculatus*. Il DNA genomico contiene sia le sequenze codificanti di un gene, i cosiddetti esoni, sia sequenze non codificanti, gli introni. Un frammento più corto (circa 600-700 bp di sequenza codificante) è stato sequenziato in *P. pachyrhizi*, *H. vastatrix*, *Alternaria solani*, *A. alternata* e *P. viticola*. La struttura del gene *cyt b*, in particolare nelle due regioni 'hot spot', è stata caratterizzata per le suddette specie e confrontata con la struttura genica di altri funghi già nota in letteratura. In particolare, è stata studiata la possibile correlazione tra l'organizzazione di esoni e introni all'interno del gene e la comparsa delle mutazioni G143A e F129L, responsabili del fenotipo resistente ai fungicidi QoI.

In tutte le specie di ruggine in studio, così come in *A. solani*, la mutazione G143A non è mai stata osservata, ma un introne, di circa 1500-2000 bp, è stato osservato subito dopo il codone per la glicina in posizione 143. Tale sequenza intronica ha rivelato elevate omologie con la famiglia degli introni di gruppo I, i quali sono capaci di 'splicing' autonomo (processo che permette la rimozione degli introni dal pre-mRNA di un gene). Al contrario, in patogeni quali *A. alternata*, *B.*

graminis, *P. grisea*, *M. graminicola*, *M. fijiensis*, *V. inaequalis* e *P. viticola*, isolati resistenti ai fungicidi QoI sono stati osservati e, in tali casi, la glicina (tripletta GGT) in posizione 143 è sostituita da un'alanina (GCT). In suddetti patogeni non è stato osservato alcun introne dopo il codone in posizione 143. Nelle ruggini e in *A. solani* il codone 143 si trova esattamente al punto di confine tra esone ed introne. Tale tripletta nucleotidica, pertanto, fa molto probabilmente parte della sequenza segnale essenziale per il riconoscimento della sequenza intronica che va rimossa durante il processo di splicing del gene *cyt b*. Di conseguenza, una mutazione nel codone 143 sembrerebbe impedire il corretto splicing del gene e quindi bloccare la sintesi del citocromo *b*. Tale mutazione, pertanto, risulterebbe letale nel senso che, individui con la mutazione G143A e un introne subito dopo la tripletta 143, non sono in grado di sopravvivere. Conseguentemente, la resistenza ai fungicidi QoI dovuta alla mutazione G143A non sembra in grado di evolvere in patogeni quali *Puccinia* spp., *U. appendiculatus*, *P. pachyrhizi*, *H. vastatrix* e *A. solani*.

I risultati ottenuti non sono in grado di prevedere se e quale altro meccanismo potrà causare la perdita di sensibilità ai fungicidi QoI nelle suddette specie. Non si può escludere che meccanismi diversi dalla mutazione G143A possano evolvere quali, ad esempio, la mutazione F129L, come osservato in *A. solani*. Tuttavia, i risultati ottenuti nel corso di questo studio, in particolare le sequenze nucleotidiche e la struttura del gene *cyt b* di numerose ed importanti specie patogene, rappresentano un traguardo essenziale per lo sviluppo di tecniche molecolari di monitoraggio della resistenza. La conoscenza della sequenza nucleotidica e la presenza di primers specifici per il gene *cyt b* costituiscono, pertanto, un valido strumento per la rapida identificazione delle mutazioni puntiformi, responsabili della resistenza ai fungicidi QoI, che eventualmente potrebbero evolvere in tali patogeni.

Zusammenfassung

Strobilurine sind eine wichtige Klasse kommerzieller Fungizide, die auch als 'Qo Inhibitors' (QoI) bezeichnet werden, da sie an die sogenannte Qo ('Quinone outside') Tasche des Cytochroms *b* anlagern und somit die mitochondriale Zellatmung blockieren. Das Cytochrom *b* Protein wird kodiert vom Cytochrom *b* Gen (*cyt b*) und ist dem Cytochrom bc_1 Enzymkomplex zugeordnet, der an der inneren mitochondrialen Membran lokalisiert ist. Da die Wirkungsweise der QoI Fungizide sehr spezifisch ist, ist auch das Risiko einer Resistenzbildung sehr hoch. Punktmutationen (Single Nucleotide Polymorphisms, SNPs) im mitochondrialen *cyt b* Gen von pilzlichen Pathogenen lösen Resistenz gegenüber QoI Fungiziden aus. Verminderte Sensitivität gegenüber dieser Wirkstoffgruppe wurde bei folgenden Pathogenen beobachtet: *Blumeria graminis* f.sp. *tritici*, *Mycosphaerella graminicola*, *M. fijiensis*, *Venturia inaequalis*, *Plasmopara viticola*, *Pyricularia grisea*, *Pseudoperonospora cubensis* und *Sphaerotheca fuliginea*. Bei fast allen bisher identifizierten resistenten Pathogenisolaten sind die Mutationen bekannt, die zum Austausch von Glycin durch Alanin an Position 143 (G143A), bzw. von Phenylalanin durch Leucin an Position 129 (F129L) führen. Diese Punktmutationen sind in zwei Regionen ('hot spot regions') des *cyt b* Gens, von Position 120-160 und 250-300, lokalisiert und beschrieben worden.

Bei wichtigen Pathogenen der Klasse der Basidiomyceten, wie *Puccinia recondita* f.sp. *tritici*, *P. graminis* f.sp. *tritici*, *P. striiformis* f.sp. *tritici*, *P. coronata* f.sp. *avenae*, *P. hordei*, *P. recondita* f.sp. *secalis*, *P. sorghi*, *P. arachidis*, *P. horiana*, *Uromyces appendiculatus*, *Phakopsora pachyrhizi*, *Hemileia vastatrix* und *Rhizoctonia solani*, ist ein cDNA Fragment des *cyt b* Gens sequenziert worden. PCR-spezifische Primer, die die 'hot spot' Regionen des *cyt b* Gens umspannen, wurden konstruiert, um das *cyt b* Gen dieser Pathogene zu amplifizieren. Eine Resistenz gegenüber QoI Fungiziden ist bei Pathogenen der Klasse der Basidiomyceten bisher noch nicht beobachtet worden. Diesen Ergebnissen bestätigen, dass bei allen analysierten Isolaten keine Punktmutationen identifiziert wurden.

Die Sequenzen des Cytochrom *b* Proteins sind mit verschiedenen anderen Cytochrom *b* Sequenzen verglichen worden, um die phylogenische Verwandtschaft dieser Pathogene in Beziehung zu anderen Basidiomyceten bzw. Ascomyceten und Oomyceten zu bringen. Die phylogenische Verwandtschaft ist zudem auch mit den nukleären 'Internal Transcribed Spacers' (ITS) in der ribosomalen DNA analysiert worden. Unsere Resultate zeigen zum ersten Mal, dass die gefundenen Aminosäuresequenzen des mitochondrialen Cytochroms *b* eine gute Grundlage bilden, um pflanzenpathogene Basidiomyceten taxonomisch einordnen zu können. Diese genetische Einordnung bestätigt die bestehende morphologische bzw. wirtsspezifische Klassifizierung. Weiterhin deckt sie sich mit der Klassifizierung basierend auf rRNA Sequenzen von *Uredinales* und ITS Regionen von *Puccinia*.

Das Fragment des *cyt b* Gens (950 bp lange Exon-Sequenz) ist auch mit genomischer DNA von verschiedenen *Puccinia* Spezies und *U. appendiculatus* amplifiziert und sequenziert worden. Ein kürzeres DNA Fragment (600-700 bp lange Exon-Sequenz) ist in *P. pachyrhizi*, *H. vastatrix*, *Alternaria solani*, *A. alternata* und *P. viticola* sequenziert worden. Die Struktur des *cyt b* Gens dieser Pathogene wurde charakterisiert und mit der Struktur anderer Spezies verglichen. Besonderes Augenmerk wurde hierbei auf die zwei 'hot spot' Regionen gelegt. Die Exon/Intron Organisation wurde charakterisiert und ihre Rolle beim Auftreten des Aminosäurenaustausches G143A und F129L im Cytochrom *b* Protein analysiert.

Bei allen untersuchten Rost Isolaten und *A. solani* ist kein G143A Austausch beobachtet worden. Hingegen konnte ein Intron der Gruppe I ('self-splicing' Intron) nach dem Codon für Glycin an der Position 143 identifiziert werden. Bei Pathogenen wie *A. alternata*, *Blumeria graminis*, *P. grisea*, *M. graminicola*, *M. fijiensis*, *V. inaequalis* und *P. viticola* ist Resistenz gegenüber QoI Fungiziden bekannt. Bei resistenten Genotypen dieser Spezies ist Glycin (Triplett GGT) durch Alanin (Triplett GCT) an Position 143 ausgetauscht. Bei diesen Pathogenen konnte kein Intron nach dem Codon an Position 143 beobachtet werden. Bei Rosten und *A. solani* ist das Triplett GGT an der Schnittstelle des Exon/Introns an Position 143 lokalisiert. Es ist wahrscheinlich, dass dieser Teil des Gens

eine Signalsequenz für die Bestimmung der intronischen RNA ist, die dann herausgetrennt werden kann. Wir nehmen an, dass ein Nucleotidaustauch im Codon 143, welcher sich zwei Nukleotide vor der Exon/Intron Schnittstelle befindet, den Splicing Ablauf verhindert. In diesem Fall kann kein Cytochrom *b* Protein mehr synthetisiert werden und die Mutation G143A ist für den Pilz lethal. Deswegen können Genotypen mit der Mutation G143A und einem Intron an Position 143 nicht überleben. Es ist deshalb unwahrscheinlich, dass sich Resistenz auf Grund des G143A Austausches bei Pathogenen wie *Puccinia* spp., *U. appendiculatus*, *P. pachyrhizi*, *H. vastatrix* und *A. solani* entwickeln kann.

Wir können aber nicht ausschließen, dass sich andere Mutationen außerhalb von G143A bei Rost Isolaten entwickeln werden, wie dies schon bei *A. solani* beobachtet wurde. In diesem Pathogen wurde der F129L Austausch in resistenten Isolaten identifiziert. Unsere Ergebnisse liefern aber auch keine Hinweise ob diese Mutationen sich bei Rosten je entwickeln könnten. Die charakterisierten Sequenzen und das Verstehen der *cyt b* Gen Struktur sind hingegen eine wichtige Information für die einfache Isolierung des *cyt b* Gens und die Entdeckung allfälliger Punktmutationen, die sich entwickeln und Resistenz gegenüber QoI Fungiziden verursachen könnten.

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Curriculum vitae

Valeria Grasso was born in Imperia, Italy, on August 21, 1977.

In July 1996 she finished her studies at the Liceo Classico 'E. De Amicis' of Imperia.

In December 2001 she got at the University of Turin her MS degree in Agricultural Plant Biotechnology with full marks. She won the prize *'The best Thesis in Agricultural Plant Biotechnologies'* of the University of Turin for the year 2000-2001 with the thesis 'Evaluation of the potential antagonistic activity of microorganisms against *Phytophthora cryptogea* on gerbera crops grown in open and closed soilless systems'.

In January 2002 she started a Research Fellow at the Plant Pathology Department – Di.Va.P.R.A., University of Turin, on 'Molecular diagnostics for the identification of fungal plant pathogens', under the supervision of Prof. Maria Lodovica Gullino. The research activity concerned the development of molecular tools for the diagnosis of different pathogenic *Fusarium oxysporum*, particularly f.sp. *dianthi*.

In November 2002 she started her PhD in Plant Pathology at the Centre of Competence for the Innovation in the Agro-environmental Sector (AGROINNOVA), University of Turin, under the tutoring of Prof. Angelo Garibaldi. She spent 18 months of her PhD in the laboratories of Syngenta Crop Protection – Research Biology in Stein AG (Switzerland), under the tutoring of Prof. Ulrich Gisi of the Faculty of Science, University of Basel, and Syngenta. The research activity concerned the molecular mode of resistance of fungal pathogens to fungicides and, particularly, the characterization of the cytochrome *b* gene in plant pathogenic Basidiomycetes and the role of the gene structure in the occurrence of point mutations conferring resistance to QoI fungicides.

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Cover: Visual study of yellow rust spores taken into a 'skyscape' reminiscent of Rene Magritte's '*The voice of the winds*' (http://www.bbsrc.ac.uk/life/zones/rust/rust-2.html).

Photos: Scanning Electron Microscopy (SEM) photos (http://www.bsu.edu/classes/ruch/msa/mims.html), which follow the life cycle of a typical rust: aecial stage (*section of aecium, p. 1*), uredinial stage (*uredinium with urediniospores, p. 15*; *urediniospores with pedicel, p. 35*), telial stage (*teliospores with pedicel, early germination, p. 57*) and basidial stage (*basidiospores, p. 81*).

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