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Characterization of the mitochondrial cytochrome *b* gene from *Venturia inaequalis*

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Abstract A new class of agricultural fungicides derived from the group of antifungal strobilurins acts as specific respiration inhibitors by binding to mitochondrial cytochrome *b*. The cytochrome *b* gene was cloned and sequenced from the mitochondrial genome of *Venturia inaequalis*, the causal agent of apple scab. The gene was 10.65 kbp in size and contained seven exons and six introns. The exons encoded a protein of 393 amino acids. Comparison of the deduced amino-acid sequence with cytochrome *b* proteins from other fungi revealed highest homologies to the respective proteins of *Aspergillus nidulans*, *Podospora anserina* and *Neurospora crassa*. All amino acids of the *V. inaequalis* cytochrome *b* at positions altered in mutants of *Saccharomyces cerevisiae* resistant to strobilurins, and other fungi with reduced sensitivities to strobilurins, were identical to wild-type isolates of several fungi. The cloning and characterization of the *V. inaequalis* cytochrome *b* gene is the initial step in the assessment of resistance risks inherent to the strobilurin fungicides.

Key words *Venturia inaequalis* · Cytochrome *b* · Strobilurin fungicides

Introduction

Cytochrome *b* is an integral membrane protein and forms the core of the mitochondrial bc_1 complex (complex III; ubiquinol-cytochrome *c* oxidoreductase, EC 1.10.2.2) as part of the respiratory chain (Brandt and Trumpower 1994). The protein plays a central role in the electrogenic electron transfer through the membrane between an electropositive ubiquinol oxidase proton-ejector site [center Q_P (or Q_o)] and an electronegative ubiquinone reductase proton-acceptor site [center Q_N (or Q_i)] (Brandt and Trumpower 1994). Each of the two sites is the target for specific inhibitors that block the electron-transfer process (von Jagow and Link 1986). Of the proteins assembled in the bc_1 complex, only cytochrome *b* is encoded by mitochondrial DNA (Hatefi 1985; Hudspeth 1992).

Several related natural products with antifungal activities, which act as center Qp inhibitors (myxothiazol, oudemansin and the strobilurins), contain methoxyacrylate as a common structural element and were, thus, named MOA inhibitors (von Jagow and Link 1986; Weber and Anke 1990; Weber et al. 1990; Geier et al. 1994; Engler et al. 1995). The chemical structures of these MOAs were sufficiently simple to provide chemical leads for a new class of fully synthetic fungicides (Beautement et al. 1991; Clough et al. 1995; Sauter et al. 1995). Thus far, the strobilurin analogues kresoxim-methyl (BAS 490F) (Gold et al. 1996), azoxystrobin (ICI A5504) (Baldwin et al. 1996), SSF-126 (Mizutani et al. 1995, 1996), and the structurally different famoxadone (DPX-JE 874) (Joshi and Sternberg 1996), have been introduced as commercial or developmental fungicides acting as inhibititors of the bc_1 complex.

Point mutations of the cytochrome *b* gene resulting in single amino-acid exchanges were found responsible for resistance to either center Q_P or Q_N inhibitors in laboratory mutants of *Saccharomyces cerevisiae* (Di Rago and Colson 1988; Di Rago et al. 1989) and *Chlamydomonas reinhardtii* (Bennoun et al. 1991). Furthermore, the analysis of the cytochrome *b* sequences of the two strobilurin-producing basidiomycetes *Strobilurus tenacellus* and *Mycena galopoda*, the naturally strobilurin-resistant basidiomycete *Mycena viridimarginala* and the naturally resistant yeast *Schizosaccharomyces pombe* revealed that their resistance to center Q_P inhibitors was most likely caused by similar point mutations of the target protein (Kraiczy et al. 1996).

Because strobilurin analogues have been developed as fungicides for commercial use in agriculture, the analysis

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of cytochrome *b* genes of plant pathogenic fungi became important in the evaluation of future resistance risks inherent to this new class of fungicides. The current study describes the analysis of the cytochrome *b* gene of *Venturia inaequalis* (Cooke) G. Wint., a plant pathogen commercially controlled with one of the strobilurin fungicides, kresoxim-methyl (Gold et al. 1996).

Materials and methods

Fungal isolate and preparation of mycelium. The isolate of *V. inaequalis* used in this study was S-56–88. It was collected from an experimental orchard at the New York State Agricultural Experiment Station in 1988 and was stored as described before (Köller et al. 1997). The orchard had never been treated with strobilurin fungicides, and the ED_{50} of kresoxim-methyl for the formation of mycelial colonies from germinating conidia was 0.01 μg ml^{-1}. The isolate was, thus, rated sensitive to strobilurin fungicides. Fungal cultures were grown on potato-dextrose agar (PDA; Difco, Detroit, Mich.). A colony of actively growing mycelium was cut from PDA plates, fragmented in 5 ml of potato dextrose broth (PDB; Difco, Detroit, Mich.) with a tissue grinder and added to 50 ml of PDB in 250-ml flasks. The cultures were incubated at 20°C on a rotary shaker at 150 rpm for 10–14 days. The mycelium was harvested by filtration through Miracloth (Calbiochem Corp., La Jolla, Calif.), washed with water 2–3 times and dried between paper towels. Chemicals were purchased from the Sigma Chemical Company, St. Louis, Mo., if not stated otherwise.

Isolation of total DNA and mtDNA. Total DNA was isolated by freezing the mycelium (15–20 g wet weight) in liquid nitrogen, grinding the frozen mycelium in a mortar, suspending the powder by vortexing in 50 ml of extraction buffer (100 mM LiCl, 50 mM Na_2 EDTA, 1% SDS, 10 mM Tris-HCl, pH 7.4) amended with 20 mg/ml of proteinase K, extracting the suspension with phenol and precipitating DNA with ethanol (Koenraadt et al. 1992). The DNA pellet was resuspended in 2–4 ml of water or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Mitochondrial DNA (mtDNA) was obtained by centrifugation of the total DNA in a CsCl density gradient containing the fluorescent dye bisbenzimide as described by Garber and Yoder (1983).

Polymerase chain reaction (PCR). Based on four highly conserved regions of cytochrome *b* amino-acid sequences, the following degenerate PCR primers were designed:

P1-F 5′-TGAAA(CT)(TA)T(CG)GGTTC(TA)TTATTA-3′
P2-F 5′-GC(TC)AC(AT)GC(TC)TT(TC)TT(AG)GGTTAT-3′
P2-R 5′-ATAACC(CT)AA(GA)AA(GA)GC(AT)GT(GA)GC-3′
P3-F 5′-TG(AG)GGTGC(AT)AC(AT)GT(TA)ATTAC-3′
P3-R 5′-GTAAT(AT)AC(AT)GT(AT)GCACC(CT)CA-3′
P4-R 5′-GGTAATAAATATCATTC(AT)GG(TC)ACAAT-3′

P1-F, P2-F and P3-F were used as 5′ primers, while P2-R, P3-R and P4-R were used as 3′ primers. The primer P1-F was designed according to the amino-acid positions 30–36, primers P2-F and P2-R to positions 126–132, primers P3-F and P3-R to positions 142–148 and primer P4-R to position 269–276 of the *S. cerevisiae* cytochrome *b* (Nobrega and Tzagoloff 1980). The primers were sythesized with an ABI instrument, model 394 DNA Sythesizer, at DNA Services, Cornell University. PCR amplifications employing various primer pairs were carried out in 50 μl of reaction buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton-X 100) containing 100 ng of mtDNA or 500 ng of total DNA, 2.5 mM of $MgCl_2$, 1 μg of each primer, 0.2 mM of each dNTP and 2.5 units of *Taq* polymerase (Promega, Madison, Wisc.). PCR reactions were performed in a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer, Norwalk, Conn.) programed for 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, a final extension of 5 min at 72°C and holding at 4°C. PCR products were separated by electrophoresis on 0.7% agarose gels in 0.5 × Tris-Borate EDTA (TBE) buffer as described by Sambrook et al. (1989).

Southern hybridization. Total DNA and mtDNA were digested with restriction enzymes, separated on a 0.7% agarose gel in 0.5 × TBE buffer and then transferred onto a nylon membrane (MSI, Westora, Mass.) according to Sambrook et al. (1989). The DNA blots were incubated in prehybridization solution (50% formamide, 0.5% SDS, 5 × Denhardt's solution, 6 × SSC and 100 μg/ml of denatured salmon-sperm DNA) at 42°C for 3–5 h and then incubated in hybridization solution (50% formamide, 0.5% SDS, 10% dextran sulfate, 6 × SSC and 50 μg/ml of denatured salmon-sperm DNA) at 42°C overnight in the presence of random-primed probes ([α-^{32}P] dCTP; DuPont-NEN, Boston, Mass.) prepared according to the protocol of the Prime-a-Gene System Kit (Promega, Madison, Wis.). The blots were washed in 0.5% SDS, 2 × SSC at room temperature for 5 min, in 1% SDS, 1 × SSC at room temperature for 15 min and in 1% SDS, 0.1 × SSC at 68°C twice for 30 min with one buffer change, and then exposed to Fuji Medical X-Ray Film (Fuji Photo Film Co., Tokyo). Some of the DNA blots were stripped by soaking the membranes in a solution containing 1% SDS and 0.2 N NaCl by gently shaking the membranes at room temperature for 20 min with one intermediate-solution exchange. The stripped membranes were re-probed with alternative probes.

Size-enriched mtDNA library construction and colony hybridization. Restriction fragments of mtDNA were separated on agarose gels and stained with ethidium bromide. Gel pieces containing desired sizes of mtDNA fragments, chosen according to the results of Southern analyses, were eluted by using the GeneClean Kit III (Bio 101, La Jolla, Calif.) or the QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, Calif.) following manufacturer's protocols. The mtDNA libraries were prepared by ligating the size-enriched fragments to the corresponding sites of plasmid vector pUC18/19 (Life Technologies, Grand Island, N. Y.) or pBluescript KS (Stratagene, La Jolla, Calif.), respectively, and then transformed into *Escherichia coli* competent cells (Max Efficiency DH5α competent cells, Life Technologies, Grand Island, N. Y.). Screening of plasmids containing the respective inserts were carried out by colony hybridization according to Sambrook et al. (1989).

DNA sequence analysis. Depending on the size of the mtDNA fragments selected, subclonings were performed in order to generate inserts with sizes suitable for sequencing. Plasmids containing inserts of desired sizes were sequenced by the dideoxynucleotide chain-termination method (Sanger et al. 1977) using the Sequenase version 2.0 sequencing kit (Amersham, Cleveland, Ohio) and an ABI automated DNA sequencer with fluorescent-dye-labeled dideoxy terminators at DNA Services, Cornell University. DNA sequence homology searches were performed with the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1990) provided by the National Center for Biotechnology Information (Bethesda, Md.). Analysis of DNA sequences was performed with DNASTAR software. The complete DNA sequence of the cytochrome *b* gene of *V. inaequalis* has been deposited GenBank under the accession number AF004559.

Results

Cloning of the cytochrome *b* gene

Different combinations of 5′ and 3′ primer pairs were used in initial PCR experiments. A fragment of 0.4 kbp in size was amplified with the primer pair P2-F and P3-R. Separation of the PCR products yielded a single band, while either multiple bands or no products were obtained with other

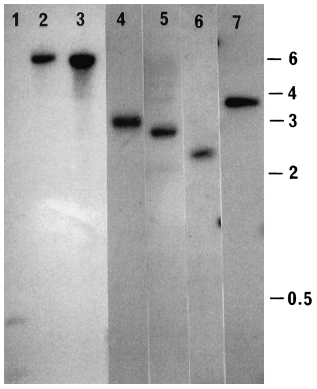

Fig. 1 Southern analysis of total DNA and mtDNA of *V. inaequalis*. Total DNA (*lane 1*) digested with *Hin*dIII, total DNA (*lane 2*) and mtDNA (*lane 3*) digested with *Eco*RI all hybridized with P1; mtDNA digested with *Hin*dIII and hybridized with P2 (*lane 4*) and re-probed with P3 (*lane 5*); mtDNA digested with *Eco*RI-*Pst*I and hybridized with P4 (*lane 6*); mtDNA digested with *Hin*dIII-*Xba*I and hybridized with P5 (*lane 7*). The probes P1–P5 were derived from the partial gene fragments described in Fig. 2. Molecular weights (right) are in kbp

primer pairs. The sequence of the 0.4-kbp PCR product revealed highly significant homologies for 30 bp at the 5′ end and 36 bp at the 3′ end with the coding sequences of other cytochrome *b* genes. The 358-bp middle region of the 0.4-kbp fragment was considered to represent an intron. Southern analysis of either mtDNA or total DNA digested with *Eco*RI and probed with the 0.4-kbp PCR product revealed a single positive band of 6.0 kbp, while two bands around 0.5 kbp and 0.3 kbp were observed after digestion of total DNA with *Hin*dIII (Fig. 1). The latter result was expected, because a *Hin*dIII, but no *Eco*RI, site was present in the 0.4-kbp PCR product.

Based on these results, a size-enriched mtDNA library was prepared by cloning *Eco*RI fragments of mt DNA with sizes of around 6.0 kbp. Employing the 0.4-kbp PCR product as probe 1 (Fig. 2), the screening of 3000 colonies resulted in the identification of two positive colonies. The plasmid DNA isolated from these colonies showed identical restriction patterns, and plasmid DNA from one of the clones was chosen for amplification, subcloning, and sequencing. Six subclones with 1.2-kbp, 0.3-kbp, 0.5-kbp, 0.9-kbp, 1.0-kbp and 1.9-kbp inserts, respectively, were obtained by *Hin*dIII digestion of the 6.0-kbp *Eco*RI fragment and cloning of the respective fragments. The clone with a 1.9-kbp insert was further subcloned by digestion with *Xba*I.

Sequencing of all subclones revealed that the 6.0-kbp *Eco*RI fragment contained the 0.4-kbp PCR product within a region of four exons separated by three introns (Fig. 2). The exons encoded 207 amino acids corresponding to positions 54–260 of the cytochrome *b* amino-acid sequences of *Aspergillus nidulans* (Waring et al. 1981), *Podospora anserina* (Cummings et al. 1989) and *Neurospora crassa* (Citterich et al. 1983). In order to clone the missing 5′ part of the *V. inaequalis* cytochrome *b* gene, a 1.2-kbp *Eco*RI-*Hin*dIII fragment as part of the original 6.0-kbp fragment (Fig. 2) was used as probe 2 to screen a mtDNA library prepared from a 3-kbp size range as indicated by Southern analysis of mtDNA (Fig. 1). The 3.0-kbp *Hin*dIII fragment contained a 1.2-kbp region overlapping with the 6.0-kbp *Eco*RI fragment (Fig. 2). Sequencing of this fragment revealed that the 5′ coding sequence of the cytochrome *b* gene of *V. inaequalis* had been completed.

For cloning the missing 3′ part of the cytochrome *b* gene, a similar approach of preparing probes according to known restriction sites and cloning appropriate size-enriched DNA libraries was employed. Probe 3, a 1.9-kbp *Hin*dIII-*Eco*RI fragment, probe 4, a 0.7-kbp *Eco*RI-*Hin*dII fragment, and probe 5, a 0.4-kbp *Hin*dIII-*Pst*I fragment (Fig. 2), were used to screen size-enriched mtDNA libraries prepared from 2.7-kbp HinDIII fragments, 2.3-kbp *Hin*dIII-*Pst*I fragments and 3.7-kbp *Hin*dIII-*Xba*I fragments, respectively, based on the results of Southern analyses of mt-DNA (Fig. 1). The region of the cytochrome *b* gene at the 3′ end had been completed after cloning and sequencing the 3.7-kbp *Hin*dIII-*Xba*I fragment (Fig. 2).

Structure of the cytochrome *b* gene

The combination of all sequences from the different and overlapping fragments (Fig. 2) revealed that the cytochrome *b* gene of *V. inaequalis* is 10.65 kbp in size. The gene has seven exons and six introns (Fig. 2). The exon/intron junctions were identified by comparison with other

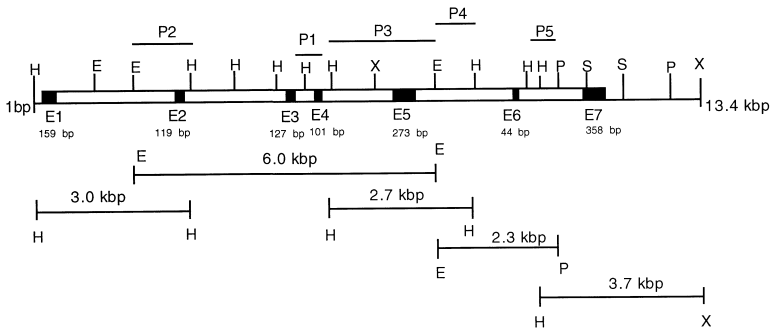

Fig. 2 Restriction map and organization of the mitochondrial cytochrome *b* gene of *V. inaequalis*. *Filled boxes* indicate exons (*E1–E7*), *open boxes* indicate intron regions. The positions of restriction sites used for subcloning are indicated by *vertical lines* (*H*, *Hin*dIII; *E*, *Eco*RI; *P*, *Pst*I; *S*, *Sau*3AI; *X*, *Xba*I). The positions of probes P1–P5 (Fig. 1) are indicated by *horizontal bars*. The locations and sizes of all five overlapping fragments cloned and sequenced are indicated

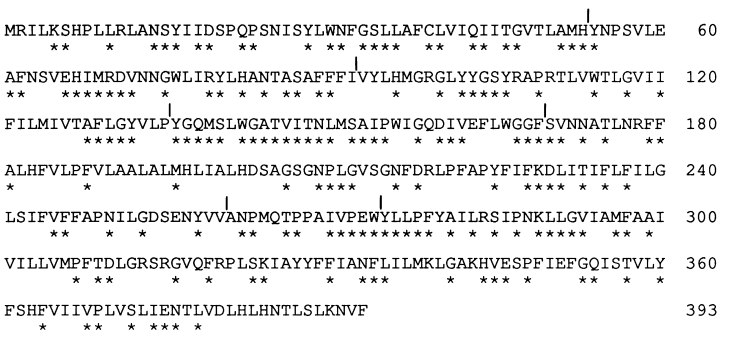

Fig. 3 Amino-acid sequence of *V. inaequalis* cytochrome *b* deduced from exon DNA sequences. Amino acids conserved in the respective proteins of *A. nidulans* (Waring et al. 1981), *P. anserina* (Cummings et al. 1989), *N. crassa* (Citterich et al. 1983), *K. lactis* (Brunner and Coria 1989), and *S. cerevisiae* (Nobrega and Tzagoloff 1980) are marked by an *asterix*. Intron locations are indicated by *vertical bars*

cytochrome *b* gene sequences. Both 5′-and 3′-splice consensus sequences for introns were present. The sequence GUGCG immediately downstream from the 5′ splice site and UUCAC preceding the 3′ splice site observed for intron 1 is characteristic of group-II introns (Cech 1988). The exon base U immediately upstream of the 5′ splice site and the base G preceding the 3′ splice indicate that group-I introns (Cech 1988) are present in the remaining five introns.

Amino-acid sequence of cytochrome *b*

The exons of the cytochrome *b* gene of *V. inaequalis* encode a protein of 393 amino acids. The amino-acid sequence deduced from all coding nucleotide sequences is shown in Fig. 3. Comparison with other fungal amino-acid sequences of cytochrome *b* revealed that the similarities of cytochrome *b* sequences were high between *V. inaequalis* and the filamentous ascomycetes *A. nidulans* (81.9%), *P. anserina* (80.1%), and *N. crassa* (73.0%). Similarities were lower for the ascomycetous yeasts *Kluyveromyces lactis* (61.9%), *S. cerevisiae* (61.0%) and *Sch. pompe* (55.8%), and for the two strobilurin-producing basidiomycetes *S. tenacellus* (62.2%) and *M. galopoda* (60.5%)

All amino acids reported to be changed in cytochrome *b* of *S. cerevisiae* laboratory mutants resistant to center Q_P inhibitors (Di Rago et al. 1989) and in several fungi naturally resistant to the inhibitors (Kraiczy et al. 1996) are clustered within positions 127–153 and 255–276 (Table 1). Both regions are part of amino-acid sequences highly preserved in fungal cytochrome *b* proteins, including *V. inaequalis* (Fig. 3). The critical amino acids of cytochrome *b* that are replaced in center Q_P inhibitor-resistant mutants or naturally resistant species were identical between *V. inaequalis* and several wild-type fungal isolates sensitive to center Q_P inhibitors (Table 1).

Codon usage in the cytochrome *b* gene

Because the cytochrome *b* gene is the first gene sequenced from the *V. inaequalis* mitochondrial genome, the codon usage was analysed (Table 2). A significant A and T bias was apparent. Of the 393 amino-acid codons, 144 and 161 ended in A and T, respectively, while only 37 and 51 ended in G and C, respectively. This codon bias was similar to the cytochrome *b* gene of *S. cerevisiae* and other mitochondrial genes (Hudspeth 1992). As reported before (Hudspeth 1992), TGA was the codon for tryptophan instead of being used as the termination codon. Instead, the open reading frame terminated with the codon TAA.

Table 1 Comparison of amino acids in cytochrome *b* of *V. inaequalis*with wild-type fungi sensitive to center Q_P inhibitors and with mutants of *S. cerevisiae* and fungi naturally resistant to center Q_P inhibitors. Amino acid exchanges are italic

Position[a]	*V. i.* [b]	Sensitive [c]	*S. c.*[d]	*S.t.*[e]	*M.g.*[f]	*M.v.*[f]	*S.p.*[g]
127	Thr	Thr (Ala)[h]	Thr	*Ile*	*Ile*	*Ile*	Thr
129	Phe	Phe	*Leu*	Phe	Phe	Phe	Phe
137	Gly	Gly	*Arg*	Gly	Gly	Gly	*Asn*
143	Gly	Gly	Gly	Gly	*Ala*	Gly	Gly
147	Ile	Ile	*Phe*	Ile	Ile	Ile	Ile
153	Ala	Ala	Ala	Ala	*Ser*	Ala	Ala
255	Ser	Ser (Pro)[i]	Pro	*Gln*	Ser	Pro	Pro
257	Asn	Asn	*Tyr*	Asn	Asn	Asn	*Cys*
262	Asn	Asn	Asn	*Asp*	Asn	Asn	*Asp*
275	Tyr	Tyr	*Asn*	Tyr	Tyr	Tyr	Tyr
276	Leu	Leu	*Ser* *Phe* *Thr*	Leu	Leu	Leu	Leu

[a] The amino-acid position relates to cytochrome *b* of *V. inaequalis* (Fig. 3)
[b] *V. inaequalis* isolate S-56–88
[c] *A. nidulans* (Waring et al. 1981), *P. anserina* (Cummings et al. 1989), *N. crassa* (Citterich et al. 1983), *K. lactis* (Brunner and Coria 1989), and *S. cerevisiae* (Nobrega and Tzagoloff 1980)
[d] Mutants of *S. cerevisiae* resistant to center Q_P inhibitors (Di Rago et al. 1989)
[e] Strobilurin-producing basidiomycetes *S. tenacellus, M. galopoda* (*M.g.*) (Kraiczy et al. 1996)
[f] Strobilurin-resistant basidiomycete *M. viridimarginaa* (*M.v.*) (Kraiczy et al. 1996)
[g] *Sch. pombe* (Lang et al. 1985; Kraiczy et al. 1996)
[h] *K. lactis* (Brunner and Coria 1989)
[i] Wild-type *S. cerevisiae* (Nobrega and Tzagoloff 1980) and *K. lactis* (Brunner and Coria 1989)

Discussion

In this study, we describe the first cytochrome *b* gene cloned and sequenced from a plant pathogenic fungus. The

Table 2 Codon usage in the cytochrome *b* gene of *V. inaequalis*

Amino acid	Codon	Number	Amino acid	Codon	Number
Gly	GGT	15	Cys	TGT	1
Gly	GGA	7	Cys	TGC	0
Gly	GGC	1	Trp	TGA	5
Gly	GGG	1	Trp	TGG	2
Asp	GAT	5	Tyr	TAT	11
Asp	GAC	4	Tyr	TAC	6
Glu	GAA	4	End	TAA	1
Glu	GAG	4	End	TAG	0
Val	GTT	13	Phe	TTT	23
Val	GTA	7	Phe	TTC	11
Val	GTC	3	Leu	TTA	37
Val	GTG	7	Leu	TTG	3
Ala	GCT	14	Ser	TCT	8
Ala	GCA	11	Ser	TCA	5
Ala	GCC	3	Ser	TTC	2
Ala	GCG	1	Ser	TTG	1
Ser	AGT	9	Arg	CGT	0
Ser	AGC	1	Arg	CGA	3
Arg	AGA	10	Arg	CGC	0
Arg	AGG	0	Arg	CGG	0
Asn	AAT	13	His	CAT	8
Asn	AAC	9	His	CAC	4
Lys	AAA	7	Gly	CAA	7
Lys	AAG	0	Gly	CAG	0
Ile	ATT	11	Leu	CTT	7
Ile	ATA	24	Leu	CTA	8
Ile	ATC	5	Leu	CTC	0
Met	ATG	11	Leu	CTG	3
Thr	ACT	11	Pro	CCT	12
Thr	ACA	3	Pro	CCA	6
Thr	ACC	0	Pro	CCC	2
Thr	ACG	1	Pro	CCG	2

gene was cloned by screening size-enriched mtDNA libraries. Although a similar cloning approach for genes of nuclear origin has been described (e.g., Ohnishi et al. 1995), the strategy appears of particular advantage in the cloning of mitochondrial genes. The mitochondrial genome size is much smaller than genomic DNA. Digestion fragments of interest are highly enriched in the particular size ranges of interest, and this facilitates the screening of such size-enriched mtDNA libraries.

The size of fungal cytochrome *b* genes is primarily determined by the number and size of intron regions. Intron numbers reported thus far ranged from no intron in the gene of two *K. lactis* trains (Brunner and Coria 1989) to five introns in a long form of the *S. cerevisiae* cytochrome *b* gene (Lazowska et al. 1980; Nobrega and Tzagoloff 1980). Six introns, as identified for the cytochrome *b* gene of the *V. inaequalis* isolate S-56–88, have not been reported before. Regardless of the unusually high number of introns, homologies of amino-acid sequences of the *Venturia* cytochrome *b* were highest for respective proteins of the three filamentous ascomycetes *A. nidulans*, *N. crassa* and *P. anserina*.

The amino-acid exchanges reported for mutants of *S. cerevisiae* resistant to center Q_P inhibitors (Di Rago et al. 1989) and for several fungi naturally resistant to the inhibitors (Kraiczy et al. 1996) are located in highly conserved cytochrome *b* regions. Using the mitochondrial genetic code, we deduce that the cytochrome *b* protein of *V. inaequalis* would not contain any of these altered amino acids, but rather would be identical to the cytochrome *b* from other fungi sensitive to the MOA group of Q_P inhibitors.

In contrast to all previous target sites for agricultural fungicides (Köller 1992), cytochrome *b* as the target of the strobilurins is encoded by mitochondrial, rather than nuclear, DNA. The impact of this difference on the risk of resistance development has not been evaluated. In contrast to nuclear genes, the consequences of a mutation within the genome of a single mitochondrion would not only depend on the nature of the mutation, but also on the speed of displacements of the wild-type population of mitochondria by the mutated mitochondrion. For *S. cerevisiae*, stable laboratory mutants with target-site mutations in the mitochondrial cytochrome *b* gene were found resistant to Q_P inhibitors (Di Rago et al. 1989). However, some of these mutations were also found to cause a severe disorder in the binding of the iron-sulfur subunit as part of the bc_1 complex (Geier et al. 1992) or else slow growth of the mutant on non-fermentable carbon sources (Bruel et al. 1995). Displacement of the wild-type by other mutated mitochondrial populations has also been reported to impose penalties in *S. cerevisiae* (Bonjardim 1996) and *N. crassa* (Bertrand 1995). Both the severity of potential fitness penalties imposed by mutations of the cytochrome *b* gene and the speed of displacement of wild-type by mutated mitochondria in field populations of plant pathogenic fungi will determine the risk of strobilurin resistance development. Work on this aspect is in progress, with *V. inaequalis* as the model system.

In addition to the assessment of resistance risks inherent to the new class of strobilurin fungicides, the structure of the cytochrome *b* gene of *V. inaequalis* might also be useful as a molecular tool in population studies. For example, cytochrome *b* of *S. cerevisiae* occurs in a long- and a short-form distinguished by the number of introns (Lazowska et al. 1980; Nobrega and Tzagoloff 1980). The cytochrome *b* gene might also become a tool in the phylogenetic analysis of *Venturia* spp., similar to approaches described recently for *Saccharomyces* spp., (Cardazzo et al. 1997), mammals (Arnason and Gullberg 1996; Yoder et al. 1996) and plants (Hiesel et al. 1994).

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