PCR cloning and detection of point mutations in the eburicol l4 alpha-demethylase (CYP51) gene from Erysiphe graminis f. sp.hordei, a "recalcitrant" fungus

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PCR cloning and detection of point mutations in the eburicol 14 a-demethylase (CYP51) gene from *Erysiphe graminis* f. sp. *hordei*, a "recalcitrant" fungus

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Abstract
Molecular studies of some micro-organisms are hampered by the difficulty of obtaining sufficient amounts of nucleic acids. A cloning strategy based on PCR has therefore been used to clone the eburicol 14a-demethylase (*CYP51*) gene of the obligate fungus *Erysiphe graminis* f. sp. *hordei* (*Egh*) using minute amounts of genomic DNA. The *CYP51* gene encodes the enzymatic target of a major group of fungicides. Sequencing *CYP51* from different *Egh* isolates revealed the occurrence of two alleles for this gene. An allele-specific PCR assay was developed to detect each *CYP51* allele.

Keywords: *Erysiphe graminis,* Polymerase chain reaction, Cytochrome P450, Fungicide

Introduction
Genome sequencing programs, as well as cloning and sequencing of particular genes, has facilitated a number of studies concerning the cell biochemistry and physiology, taxonomy, phylogeny and/or genetic diversity of numerous micro-organisms. However, the vast majority of known sequences have been obtained by screening genomic libraries. This time-consuming approach can be used on micro-organisms such as fungi capable of saprophytic growth, in which it is easy to obtain large amounts of pure fungal material. It has, however, only rarely been employed for "recalcitrant" species such as slow-growing or obligate biotrophic phytopathogenic fungi (Sherwood and Somerville 1990; Justesen et al. 1996; Délye et al. 1997a), mostly because of the difficulty of obtaining adequate amounts of nucleic acids. This indeed is the major bottleneck for molecular and biochemical studies of many phytopathogenic fungi of major economic importance, such as rusts, downy mildews and powdery mildews. The availability in the literature of numerous sequences coding for genes of taxonomic or metabolic importance now makes it possible to clone a variety of genes from "recalcitrant" micro-organisms species, without having any sequence data from these species. The purpose of the present work was to clone the single-copy gene (*CYP51*) encoding cytochrome P450 eburicol 14a-demethylase from the major cereal pathogen *Erysiphe graminis* DC. ex M rat f. sp. *hordei* Em. Marchal (*Egh*), a haploid, obligate biotrophic fungus. CYP51 is a key enzyme of the sterol biosynthesis pathway (for a review see Yoshida 1993) which is the enzymatic target for a major class of antifugals called DeMethylase Inhibitors (DMIs) (Gadher et al. 1983). Although *Egh* has developed field resistance towards DMIs for many years (Walmsey-Woodward et al. 1979), the molecular basis of this resistance is still unknown.

To clone the *Egh CYP51* gene, we successfully used a PCR cloning procedure consisting of the three following steps: (1) production of a fragment of the gene using PCR with degenerated primers, (2) digestion of 100–200 ng of genomic DNA and ligation into a plasmid vector, and (3) cloning of the remainder of the gene sequence using two sets of primer pairs. In each of these pairs, one primer is based on the plasmid sequence, and the other is based on the sequence of the gene fragment obtained in (1).

Materials and methods
Fungal material.

A total of seven single-spore *Egh* isolates has been used for this work. Isolates AL1, Tr2, JEH11 and Ge3 are reference isolates sensitive to DMIs (Limpert 1987). They were kindly provided by Dr. E. Limpert (Swiss Institute of Technology, Zürich, Switzerland). Isolates GV1-22 and AP2-19 were collected in 1995 in Châlons en Champagne (France) and in Amiens (France), respectively. Isolate 92-18 was collected in 1992 in Grignon (France). The collection of these three isolates has been done in DMI-treated areas before the beginning of the spraying program. Isolates were mass-produced by blowing conidia on detached leaves from the susceptible barley (*Hordeum vulgare*) cultivar Igri, which were then placed on a 0.4% water agar medium, to which 30 mg/l of benzimidazole was added, in 9-cm-diameter Petri dishes. Inoculated leaves were incubated for 14 days at 16°C under 24 h/day illumination (10 mE/m2/s). Conidia were then dislodged onto a glass slide and collected in a microcentrifuge tube using a razor blade. Conidia were kept at –20°C and freeze-dried before nucleic-acid extraction. *DNA extraction and PCR assays*. For PCR-based experiments, DNA was extracted from about 5 mg dry weight of conidia as described by Délye et al. (1995). PCR conditions were also as described (Délye et al. 1997 b). Amplified fragments were visualised on 1% (w/v) agarose gels run in 0.5 ´ tris-borate EDTA buffer and stained with ethidium bromide (0.4 mg/ml gel).

Obtaining a fragment of the Egh CYP51 gene.

The three published *CYP51* sequences from filamentous fungi were aligned. These sequences are from *Penicillium italicum* (Van Nistelrooy et al. 1996), *Ustilago maydis* (Hargreave and Keon 1996) and *Uncinula necator* (Délye et al. 1997a). Degenerate primers D-CR1 (5'-TAYGGIGAYRTITTYWSITT, sense strand, degeneracy = 64) and D-CR4 (5'-ATCATCATIYSIGCDATYTC, antisense strand, degeneracy=24), which correspond to filamentous fungi conserved CYP51 aminoacid sequences Y-G-D-(I,V)-F-(T,S)-F and E-I-A-(H,G)-M-M-I, respectively, were used to amplify a fragment with an expected size of about 700 bp. The annealing temperature was 50 °C and the final primer concentration was 2.0 mM for primer D-CR1 and 0.6 mM for primer D-CR4. Cloning and sequencing procedures were as described (Délye et al. 1997 a).

Obtaining the complete Egh CYP51 sequence. DNA was extracted from 15 mg dry weight of conidia from isolate AL1 using a CTAB protocol (Murray and Thompson 1980). Approximately 200 ng of DNA were digested to completion with *Xba*I (MBI Fermentas) following the manufacturer's instructions, purified using the Cleanmix purification kit (Talent) and ligated into the *Xba* I site of the pBS+ plasmid vector (Stratagene). Ligation was performed overnight at 15°C using T4 DNA ligase (MBI Fermentas) in a total volume of 10 ml. Primer pairs PBS-U (5¢-GCCAGTGAATTGTAATACGACTCACTATAGG)/ C51-700R (5'-TCGACCTAGTATATCCGTCGTCTTCTTACCC) and PBS-R (5'-CCATGATTACGCCAAGCTCGAAATTAACCC)/ C51-700 (5'-TGCGCTCTTCCTACAAAGATGGCACGCCCG) were used to amplify DNA fragments encompassing sequences located upstream of (primers PBS-U and C51-700R) and downstream from (primers PBS-R and C51-700) the sequence obtained using the degenerate primers D-CR1 and D-CR4. Primers PBS-U and PBS-R targeted sequences flanking the polylinker site of the pBS+ plasmid vector. Primers C51-700 and C51-700R targeted sequences located on the D-CR1/D-CR4 *CYP51* fragment. Primers were used at a final concentration of 0.1 mM each. Amplifications were performed on 1/10 dilutions of the ligation mix. The cycling program consisted of 37 cycles with 30 s denaturation at 94°C and 2 min annealing and extension at 72°C. Primers C51Egh (5'-CCGTCCTTATCGCAAGATTTG) and C51EghR (5'-CATAGTAGCCTGTAATCTAAGC), targeting sequences located 10 bp upstream of the ATG initiation codon and 66 bp downstream from the final TGA codon respectively, were used at a final concentration of 0.2 mM each with an annealing temperature of 60°C to amplify a 1791-bp fragment encompassing the whole *CYP51* sequence from the *Egh* isolates. *CYP51 cDNA cloning*. Reverse-transcription PCR, followed by cloning and sequencing, was used to confirm that the putative introns found within the *Egh CYP51* sequence were readily excised from transcripts. Total RNA was extracted from 5 mg dry weight of conidia from isolate AL1 using the RNAble™ reagent (Eurobio). Reverse transcription was performed using a first-strand cDNA synthesis kit (Pharmacia). A cDNA fragment was amplified from 1/10 dilutions of the reverse transcription mix using primers C51Egh and C51EghR. Cloning and sequencing procedures were as above.

Detection of CYP51 alleles: allele-specific PCR.

Primers MUT-T (5'-AATTAGGACAGTCAA) and MUT-A (5'-AATTAGGACAGTCAT) were designed specifically for the priming of *Egh* CYP51 sequences exhibiting respectively a T or a A at nucleotide 458, considering that a 3¢ mismatch does not prime in a PCR reaction under specific annealing temperatures (Sommer and Tautz 1989). Each of these primers was used in PCR amplifications, together with primer C51Egh, to amplify a 503-bp fragment. Both primers in each primer pair were used at a final concentration of

0.1 mM. The annealing temperature was 47°C for primer pair C51Egh/MUT-T and 49 °C for primer pair C51Egh/MUT-A.

Results

Cloning the Egh CYP51 gene

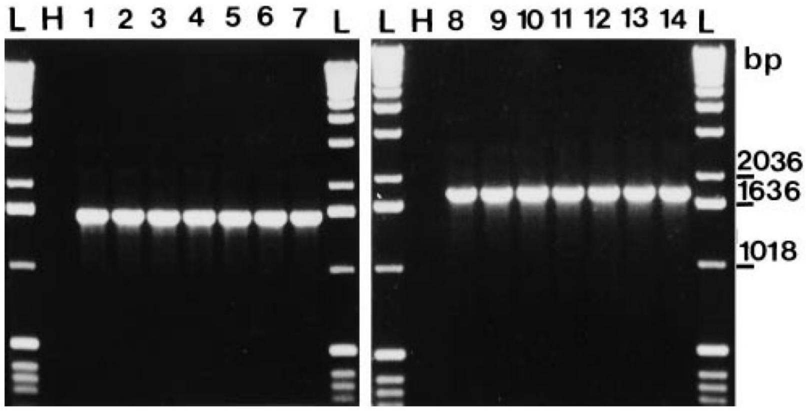
Amplifications with primers D-CR1 and D-CR4 yielded a single major PCR product of the expected size (data not shown). This 714-bp fragment potentially encoded a 220 amino-acid polypeptide interrupted by a putative 52-bp intron. This polypeptide displayed 76.4% identity and 80.9% similarity with the corresponding region of *U. necator* CYP51.

Amplification of *Egh XbaI*-digested DNA ligated into the pBS+ plasmid vector using primer pairs PBS-U/C51- 700R and PBS-R/C51-700 yielded a single major PCR product of approximately 1100 and 1400 bp, respectively (data not shown). The 1100-bp fragment encompassed a partial open reading frame of 357 bp including a putative 51-bp intron and the first 60 bp of the D-CR1/D-CR4 fragment. The 1400-bp fragment contained a partial open reading frame of 681 bp terminated by a TGA codon and including the last 58 bp of the D-CR1/D-CR4 fragment. The nucleotide sequence of the *Egh CYP51* gene, obtained after joining the overlapping sequences included in the 1100-bp fragment, the 714-bp D-CR1/D-CR4 fragment and the 1400-bp fragment was 1672-bp long.

Cloning the Egh CYP51 cDNA

The *Egh CYP51* gene was interrupted by two putative introns at nucleotides 247–297 and 496–547, respectively. A comparison of the size of the PCR fragments amplified with primers C51Egh and C51EghR from genomic DNA or cDNA (Fig. 1), as well as the sequencing of the cDNA obtained from isolate AL1, revealed that the two putative introns were readily excised. The remainder of the cDNA coding sequence was identical to that of the genomic DNA.

Figure 1 *PCR products obtained by amplification of cDNA (tracks 1–7) and genomic DNA (tracks 8–14) from 1 mg dry weight of conidia from Egh isolates using primers C51Egh and C51EghR. Tracks: L molecular-weight marker (1-kb DNA ladder, Gibco-BRL); H H2O negative control (no DNA); 1 and 8 isolate GV1-22; 2 and 9 isolate AP2-19; 3 and 10 isolate 92-18; 4 and 11 isolate AL1; 5 and 12 isolate Ge3; 6 and 13 isolate Tr2; 7 and 14 isolate JEH11*



Analysis of the Egh CYP51 sequence

The inferred 522 amino-acid protein encoded by the 1569-bp coding sequence of the 1672-bp *Egh CYP51* gene was compared to the known complete CYP51 sequences. The strongest homology (72.2% identity and 77.8% similarity) was with *U. necator* CYP51. This is much more than the 40% homology required for two cytochrome P450 genes to belong to the same family (Nelson et al. 1993). The weakest homology (28.4% identity and 42.1% similarity) was with *Sorghum bicolor* CYP51 (Bak et al. 1997). Alignment of the predicted amino-acid sequence of CYP51 from *Egh* with those of the filamentous fungus *U. necator*, the yeast *Saccharomyces cerevisiae* (Kalb et al. 1987), the human *Homo sapiens* (Strömstedt et al. 1996) and the plant *S. bicolor* is given in Fig. 2. The alignments highlight the conserved domains CR1–CR6. Domains CR1–CR4 are believed to be involved in substrate specificity (Aoyama et al. 1996). Domains CR5 (k-helix) and CR6 (heme-binding domain) are hallmarks of cytochrome P450 (Gotoh 1993).

The two introns in the *Egh CYP51* gene interrupted nucleotide sequences encoding domain CR1 and a region located eight amino-acids downstream from CR2 (Fig. 2). The position of the two introns in the *Egh CYP51* gene is exactly the same as that of the two introns in the *U. necator CYP51* gene and of the two first introns in the *P. italicum CYP51* gene. These are the only other known fungal *CYP51* sequences for which introns have been identified to-date. The *Egh CYP51* sequence will appear in Genbank under the accession number AF052515.

Figure 2 *Alignment of predicted CYP51 amino-acid sequences from Egh (Genbank: AF052515) with representative CYP51 sequences from the fungal (U. necator, Genbank: U72657, S. cerevisiae, Genbank: M18109), animal (H. sapiens, Genbank: U23942) and plant (S. bicolor, Genbank: U74319) kingdoms. The alignment was generated using the GCG program pileup (Devereux et al. 1984). Gaps have been introduced to maximise the alignment (gap weight: 12.0, gap length weight: 4.0). Identical residues are marked by an asterix. CR1–CR6 regions are in bold, and underlined in the Egh sequence. The position of introns is arrowed. Numbers on the right refer to amino-acid positions in Egh CYP51. Identity and similarity percentages are given at the left bottom of the figure*

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                                                                          46
E. graminis    ~~~~~~~~~~ ~~~~MGISES FMFPYLQPLL QLGFGIALAS GILSLLLLLT FLNVLKQLLF
U. necator     ~~~~~~~~~~ ~~~~MYIADI LSDLLTQQTT RYGWIFMVTS IAFSIILLAV GLNVLSQLLF
S. cerevisiae  ~~~~~~~~~M SATKSIVGEA LE..YVNIGL SHFLALPLAQ RISLIIIIPF IYNIVWQLLY
H. sapiens     MAAAAGMLLL GLLQAGGSVL GQAMEKVTGG NLLSMLLIAC AFTLSLVYLI RLAAGHLVQL
S. bicolor     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~MDLA DIPQQQRLMA GLALVVATVI FLKLLLSFRS

                                            intron ↓ CR1                 105
E. graminis    K.NPNEPPIV FHWIPIIGST ISYGMNPYKF FHESQAKYGN IFTFILLGKK TTVYLGRQGN
U. necator     R.RPYEPPVV FHWFPIIGST ISYGIDPYKF YFDCRAKYGD IFTFILLGKK VTVYLGLQGN
S. cerevisiae  SLRKDRPPLV FYWIPWVGSA VVYGMKPYEF FEECQKKYGD IFSFVLLGRV MTVYLGPKGH
H. sapiens     PAGVKSPPYI FSPIPFLGHA IAFGKSPIEF LENAYEKYGP VFSFTMVGKT FTYLLGSDAA
S. bicolor     GGGKKRLPPT IPGAPVVGGL VKFMRGPIPM IREQYAALGS VFTVPIITRR ITFLIGPEVS

                                  CR2            intron ↓                165
E. graminis    NFILNGKLRD VNAEEIYTVL TTPVFGTDVV YDCPNSKLME QKKFMKAALT TEAFRSYVPI
U. necator     NFILNGKLKD VNAEEIYTNL TTPVFGRDVV YDCPNSKLME QKKFMKTALT IEAFHSYVTI
S. cerevisiae  EFVFNAKLAD VSAEAAYAHL TTPVFGKGVI YDCPNSRLME QKKFVKGALT KEAFKSYVPL
H. sapiens     ALLFNSKNED LNAEDVYSRL TTPVFGKGVA YDVPNPVFLE QKKMLKSGLN IAHFKQHVSI
S. bicolor     AHFFKGNEAE MSQQEVY.RF NVPTFGPGVV FDVDYSVRQE QFRFFTEALR ANKLRSYVDQ

                                                                         222
E. graminis    IQNEVKSFIE KCDDFR...K SKGIINIDAV MAEITIYTAS HTLQGKEVRD RFDSSLAVLY
U. necator     IQNEVEAYIN NCVSFQ...G ESGTVNISKV MAEITIYTAS HALQGEEVRE NFDSSFAALY
S. cerevisiae  IAEEVYKYFR DSKNFRLNER TTGTIDVMVT QPEMTIFTAS RSLLGKEMRA KLDTDFAYLY
H. sapiens     IEKETKEYFE S.......WG ESGEKNVFEA LSELIILTAS HCLHGKEIRS QLNEKVAQLY
S. bicolor     MVAEAEEYFS K.......WG ESGTVDLKYE LEHLIILTAS RCLLGREVRE KLFDDVSALF

                CR3                                                      281
E. graminis    HDLDMGFTPI NFMLH.WAPL PHNRARDHAQ RTVAKIYMEI INSRRTQKET DDSNLDIMWQ
U. necator     HDLDMGFTPI NFTFY.WAPL PWNRARDHAQ RTVARTYMNI IQARREEKRS GENKHDIMWE
S. cerevisiae  SDLDKGFTPI NFVFP.NLPL EHYRKRDHAQ KAISGTYMSL IKERR..KNN DIQDRDLIDS
H. sapiens     ADLDGGFSHA AWLLPGWLPL PSFRRRDRAH REIKDIFYKA IQKRRQSQEK IDD....ILQ
S. bicolor     HDLDNGIQPI SVLFP.YLPI PAHKRRDKAR ARLAEIFATI IKSRKASGQS EED....MLQ

                                   CR4                                   340
E. graminis    LMR.SSYKDG TPVPDKEIAH MMIALLMAGQ HSSSSSSTWI MLWLAARPDI TEELYQEQLE
U. necator     LMR.STYKDG TPVPDREIAH MMIALLMAGQ HSSSSTSSWI MLWLAARPDI MEELYEEQLR
S. cerevisiae  LMKNSTYKDG VKMTDQEIAN LLIGVLMGGQ HTSAATSAWI LLHLAERPDV QQELYEEQMR
H. sapiens     TLLDATYKDG RPLTDDEVAG MLIGLLLAGQ HTSSTTSAWM GFFLARDKTL QKKCYLEQKT
S. bicolor     CFIDSKYKNG RPTTEGEVTG LLIAALFAGQ HTSSITSTWT GAYMLRFKQY FAEAVEEQKD

                                       CR5                               395
E. graminis    LLGSE..LPP LKYEDLSKLS LHQNVLKEVL RLHAPIHSIL RKVKNPMPV. ..PGTSYVIP
U. necator     IFGSEKPFPP LQYEDLSKLQ LHQNVLKEVL RLHAPIHSIM RKVKNPMIV. ..PGTKYVIP
S. cerevisiae  VLDGGKK..E LTYDLLQEMP LLNQTIKETL RMHHPLHSLF RKVMKDMHV. ..PNTSYVIP
H. sapiens     VCG..ENLPP LTYDQLKDLN LLDRCIRETL RLRPPIMIMM RMARTPQTVA .....GYTIP
S. bicolor     VMK..RHGDK IDHDILAEMD VLYRCIKEAL RLHPPLIMLL RQSHSDFTVT TKEGKEYDIP

                                                                         455
E. graminis    KTHSLLAAPG WTSRDASYFP NPLKWDPHRW DTGSGGVIGT DMEDEKFDYG YGLISTGAAS
U. necator     TSHVLISSPG CTSQDATFFP DPLKWDPHRW DIGSGKVLGN DAVDEKYDYG YGLTSTGASS
S. cerevisiae  AGYHVLVSPG YTHLRDEYFP NAHQFNIHRW NKDSA...SS YSVGEEVDYG FGAISKGVSS
H. sapiens     PGHQVCVSPT VNQRLKDSWV ERLDFNPDRY ..LQDNP... .......... ....ASGEKF
S. bicolor     KGHIVATSPS FANRLPHIYK NPDSYDPDRF GPGREED... .......... ....KAAGAF

                         CR6                                             515
E. graminis    PYLPFGAGRH RCIGEQFATV QLVTIMATMV RSFKFHNLDG RNSVAETDYS SMFSRPMQPA
U. necator     PYLPFGAGRH RCIGEQFATL QLVTIMATMV RFFRFRNIDG KQGVVKTDYS SLFSMPLGTP
S. cerevisiae  PYLPFGGGRH RCIGEHFAYC QLGVLMSIFI RTLKWHYPEG K.TVPPPDFT SMVTLPTGPA
H. sapiens     AYVPFGAGRH RCIGENFAYV QIKTIWSTML RLYEFDLIDG ...YFPTVNY TTMIHTPENP
S. bicolor     SYISFGGGRH GCLGEPFAYL QIKAIWTHLL RNFEFELVSP ...FPENDWN AMVVGIKGEV

                    522                           Identity   Similarity
E. graminis    TIAWEKR~~~ ~~~~
U. necator     LIGWEKR~~~ ~~~~                    72.2%      77.8%
S. cerevisiae  KIIWEKRNPE QKI~                    47.0%      56.5%
H. sapiens     VIRY.KRRSK~ ~~~                    37.7%      46.4%
S. bicolor     MVNY.KRRKLV VDN                    28.4%      42.1%
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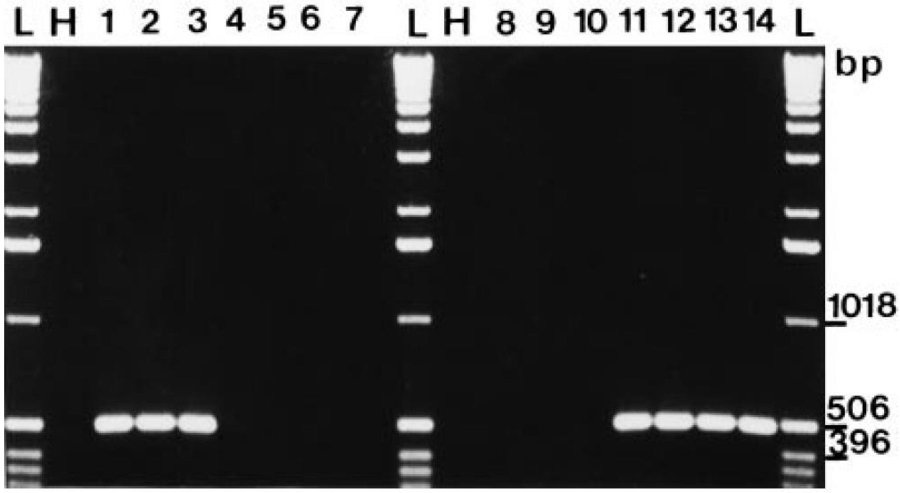
Variability of the Egh CYP51 gene and allele-specific PCR amplifications

The *CYP51* gene was cloned and sequenced from *Egh* isolate GV1-22 using primers C51Egh and C51EghR. Comparison with the sequence from isolate AL1 revealed a single A-to-T change at nucleotide 458, resulting in the presence of a phenylalanine residue at position 136 in CYP51 of isolate GV1-22 instead of the tyrosine residue in CYP51 of isolate AL1. The amino acid at position 136 is located within the highly conserved CR2 domain, which is presumably involved in substrate recognition (Aoyama et al. 1996).

Allele-specific PCR amplifications using primer pairs C51Egh/MUT-T and C51Egh/MUT-A revealed that isolates AP2-19 and 92-18 exhibited a T at nucleotide position 458, whereas isolates Tr2, Ge3 and JEH11 displayed a A at the same position (Fig. 3). Amplifications were reproducibly obtained using DNA extracted from less than 1 mg dry weight of conidia.

Figure 3 *PCR products obtained by allele-specific amplification of DNA from 1 mg dry weight of conidia from Egh isolates using primer pairs C51Egh/MUT-T (tracks 1–7) and C51Egh/MUT-A (tracks 8–14). Tracks: L molecular-weight marker (1-kb DNA ladder, Gibco-BRL); H H2O negative control (no DNA); 1 and 8 isolate GV1-22; 2 and 9 isolate AP2-19; 3 and 10 isolate 92-18; 4 and 11 isolate AL1; 5 and 12 isolate Ge3; 6 and 13 isolate Tr2; 7 and 14 isolate JEH11*



Discussion

Cloning the Egh CYP51 gene using PCR

By using degenerate primers designed on the basis of the highly conserved amino-acid sequences of CYP51 from the only three known filamentous fungi, we were able to amplify a portion of the *Egh CYP51* gene. The remainder of the *Egh CYP51* sequence was also obtained by PCR from less than 200 ng of *Egh* genomic DNA, without the need to establish and screen a genomic library in bacteria. We used only 50 ml of the 100 ml of the *Xba*I-digested DNA ligation mix to achieve this result. The PCR cloning strategy may thus be considered as very "DNA-sparing". PCR primers C51Egh and C51EghR, derived from *Egh CYP51* flanking sequences, enabled us to verify that the two putative introns identified within this gene were readily excised. Successful amplification of a DNA fragment encompassing *CYP51* was obtained from DNA extracted from less than 1 mg dry weight of *Egh* conidia (data not shown). Successful amplification of a cDNA fragment encompassing *CYP51* was obtained from RNA extracted from 1–2 mg dry weight of *Egh* conidia. Cloning and sequencing *CYP51* from a variety of *Egh* isolates is thus a task that does not require massive production of conidia from this fungus. The availability of the *Egh CYP51* gene now paves the way for molecular and biochemical studies of resistance to DMIs in this fungus.

PCR detection of a point mutation

Two *CYP51* alleles were identified in *Egh*. The DMI-sensitive reference isolate AL1 exhibited a TAT (tyrosine) codon at position 136. Isolate GV1-22, which was collected from DMI-treated fields, exhibited a TTT (phenylalanine) codon at position 136. The occurrence of a phenylalanine residue at this position has been reported for a laboratory mutant of *P. italicum* (De Waard 1996), for field isolates of *U. necator* (Délye et al. 1997b), and for clinical isolates of *Candida albicans* (Sanglard et al. 1998) that were all highly resistant to DMIs. DMI-sensitive isolates from these three fungi all exhibited a tyrosine residue instead of a phenylalanine residue at this position.

A PCR assay was developed for specific detection of each *Egh CYP51* allele. Allele-specific PCR was preferred to PCR-RFLP because of (1) the ommission of the digestion step and (2) the lack of a suitable restriction site at nucleotide 458. We found that *CYP51* sequences from DMIsensitive reference isolates AL1, Tr2, Ge3 and JEH11 all exhibited a A at nucleotide 458. Isolates GV1-22, AP2-19 and 92-18, which were collected from DMI-treated fields, all exhibited a T at that position. It is thus possible that, as found in *P. italicum*, *U. necator* and *C. albicans*, substitution of a phenylalanine residue for a tyrosine residue may cause resistance to DMIs in *Egh*. Allele-specific PCR will enable us to follow the distribution of the two identified *CYP51* alleles in field populations of *Egh* using only minute amounts of fungal material. Should a correlation between the presence of the phenylalanine/tyrosine at position 136 and resistance to DMIs be established, then allele-specific PCR may be used to monitor resistance of *Egh* to such compounds in the field.

The PCR gene-cloning strategy we used has proven efficient for the cloning of a gene from an organism in which obtaining significant amounts of DNA is a long and difficult procedure. This strategy may thus be recommended for other similar systems, provided that (1) a few hundred ng of DNA can be obtained from the organism in question, and (2) a few sequences of the investigated DNA exist in the literature and display conserved regions. For microorganisms such as fungi or bacteria living exclusively inside their host tissues, a PCR cloning strategy targeting genes that are either not present in the host genome, or are not conserved between the host and the micro-organism, can be considered.

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