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1 **Mitochondrial complex III Q_i-site inhibitor resistance mutations**
2 **found in laboratory selected mutants and field isolates.**

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14 **Running title:** Target site Q_i resistance mutations

15 **Keywords:** Q_iL, bc₁ complex, yeast model, resistance, target site mutation, fungicides

17 **Abstract**

18 **BACKGROUND:** Complex III inhibitors targeting the Q_i-site have been known for decades;
19 some are used or are being developed as anti-microbial compounds. Target site resistance
20 mutations have been reported in laboratory-selected mutants and in field isolates. Here we
21 presented a brief over-view of mutations found in laboratory selected resistant mutants; and a
22 study of mutations observed in field isolates of *Plasmopara viticola*, in particular the
23 ametoctradin resistance mutation, S34L that we analyzed in the yeast model.

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2
3 25 **RESULTS:** The survey of laboratory mutants showed that resistance could be caused by a
4
5 26 large number of substitutions in the Q_i-site. Four residues seemed key in term of resistance,
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7 27 N31, G37, L198 and K228. Their mutations caused resistance to several QiIs, in different
8
9 28 organisms with, in yeast, no or moderate loss of complex III function. Two mutations have
10
11 29 been reported in the field, in *P.viticola* resistant to either cyazofamid or ametoctradin. Using
12
13 30 the yeast model, we analyzed the effect of the ametoctradin resistance mutation S34L. We
14
15 31 showed that S34L caused a high level of resistance combined with a loss of complex III
16
17 32 activity, resulting in a decreased growth competence.

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19
20 33 **CONCLUSION:** The use of QiIs that target a single site encoded by a mitochondrial gene,
21
22 34 presumably prone to mutations is expected to result in the selection of resistant mutants.
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24 35 However if the mutation is associated with a fitness penalty, as it seems the case with S34L,
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26 36 the development of the resistance might not be an insuperable obstacle but careful monitoring
27
28 37 is required.
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33 39 **1 INTRODUCTION**

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35 40 The respiratory chain complex III (or *bc*₁ complex) with its two quinol binding sites, termed
36
37 41 the Q_o- and Q_i-sites, is a proven target for anti-microbial compounds. The first generation of
38
39 42 antimicrobial drugs targeted the Q_o-site: the antimalarial atovaquone and a large series of
40
41 43 fungicides used against plant pathogen fungi, such as azoxystrobin.¹ Target site resistance
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43 44 mutations have often been reported, for instance Y279C/S in *Plasmodium falciparum* (see for
44
45 45 example ^{2,3}) or G143A in many species of plant pathogen fungi that confers cross-resistance
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47 46 to all the >20 Q_o-site targeting fungicides (termed QoIs) on the market (see FRAC web site).
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49 47 The structural basis of the resistance has been well studied.^{4,5}
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52 48 New generation of inhibitors, still in development, target the Q_i-site, such as the anti-
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54 49 malarial ELQs⁶ and the agrochemical fungicide fenpicoxamid.⁷ The only Q_i-site inhibitors
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(or QiIs) already on the market are cyazofamid⁸ and amisulbrom, used in field against plant pathogen oomycetes (see www.frac.info). A third anti-oomycete in use, ametoctradin, was listed as QoSI (see www.frac.info) i.e. binding at the Q_o-site in a manner similar to the Q_o-site inhibitor stigmatellin but distinct to QoIs.⁹ On the basis of further spectroscopic studies, the compound would target both Q_o- and Q_i- sites.¹⁰ Other Qi-site inhibitors have been known for a long time, such as funiculosin, diuron, HQNO and antimycin, or described more recently, such as ilicicolin H¹¹ or HDQ.¹² These compounds have proven useful in structural and mechanistic studies of complex III.

Q_i-site mutations conferring resistance (or suspected to cause resistance) to inhibitors have been observed after treatment of pathogens in laboratory assays or in the field, and also through the use of model organisms (mainly *Saccharomyces cerevisiae*).

Here, we briefly review these reported Q_i-site mutations and study (using the yeast model) a mutation (S34L) found in the plant pathogen oomycete *Plasmopara viticola* field isolates after ametoctradin treatment.¹³

2 MATERIALS AND METHODS

2.1 Materials and growth media

Ametoctradin was kindly provided by BASF. The following media were used for yeast growth: YPD (1% yeast extract, 2% peptone, 3% glucose), YPG (1% yeast extract, 2% peptone, 2% glycerol), YPEth (1% yeast extract, 2% peptone, 2% ethanol), YPGal (1% yeast extract, 2% peptone, 0.2% glucose, 3% galactose)

2.2 Yeast strains

Strains derived from AD1-9 that lacks several membrane transporters (α *ura3 his1*, *yor1Δ::hisG*, *snq2Δ::hisG*, *pdr5Δ::hisG*, *pdr10Δ::hisG*, *pdr11Δ::hisG*, *ycf1Δ::hisG*,

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2
3 75 pdr3 Δ ::hisG, pdr15 Δ ::hisG, pdr1 Δ ::hisG) and kindly provided by M. Ghislain, UCL,
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5 76 Belgium. The mutations were introduced in yeast cytochrome *b* as described in ^{14,15}. In all
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7 77 experiments, control and mutants have identical nuclear and mitochondrial genomes with the
8
9 78 exception of the cytochrome *b* mutations.
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13 80 **2.3 O₂ consumption activity of cells**

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15 81 Activities were measured using a Clarke-type oxygen electrode. Cells were grown in YPGal
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17 82 medium for 24h. The cells were then harvested. O₂ consumption rates in presence of 4 μ M
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19 83 CCCP were recorded for two minutes. Ametoctradin was then added and the rate measured
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21 84 after two minutes. The data, normalised per cell count, are presented as % of control rates
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23 85 (without ametoctradin). The measurements were repeated at least three times and averaged.
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28 29 87 **2.4 Complex III activity**

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31 88 Activities were determined spectrophotometrically by measuring the reduction of 20 μ M
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33 89 cytochrome *c* at 550 *minus* 540 nm over 1-minute time-course in 1 ml of 10 mM potassium
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35 90 phosphate pH 7.0, 0.01% (w/v) lauryl-maltoside and 1 mM KCN. Mitochondria prepared as
36
37 91 in¹⁶ were added to obtain a final concentration of 2 - 10 nM complex III. Complex III
38
39 92 concentration was determined from dithionite-reduced optical spectra, using $\epsilon=28.5$ mM-
40
41 93 1.cm-1 at 562nm *minus* 575nm. The reaction was initiated by the addition of 20 μ M
42
43 94 decylubiquinol and initial rates were measured. Each measurement was repeated three to five
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45 95 times and the values obtained were averaged. Complex III activity was determined as the
46
47 96 cytochrome *c* reduction rate per complex III per second. Mid-point inhibition concentrations
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49 97 were determined by inhibitor titration.
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54 55 99 **2.5 Molecular modelling**

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3 100 *In silico* model of *P.viticola* Q_i-site was built by comparative modelling using MODELLER
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5 101 9v7¹⁷, with the yeast structure (PDB 3CX5) as template. Three-dimensional structure of
6
7 102 ametoctradin was generated using CORINA (Molecular Networks GmbH, Erlangen,
8
9 103 Germany, <http://www.molecular-networks.com>). The docking of ametoctradin on the
10
11 104 *P.viticola* model of the Q_i-site was performed with GOLD version 5.0.¹⁸ The binding site was
12
13 105 defined as a sphere with a 15 Å radius around the antimycin in the PDB structure 1PPJ.
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15 106 GoldScore was used as a scoring function and all other parameters had default values. UCSF
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17 107 CHIMERA¹⁹ was used for generating the molecular modelling images.
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22 109 **3 RESULTS AND DISCUSSION**

23 110 **3.1 Laboratory selected mutants with Q_i-site mutations causing inhibitor resistance: An** 24 25 26 27 111 **over-view**

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29 112 The Q_i-site is formed from the cytochrome *b* C-terminal region of surface helix a, the N-
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31 113 terminal region of transmembrane helices A and E and the C-terminal regions of helix D
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33 114 (Fig.1).

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35 115 Mutations reported to cause (or suspected to cause) inhibitor resistance have been found at
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37 116 positions I17, S20, Q22, I27, W30, N31, G33, S34, L35, G37, L198, H204, M221, F225,
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39 117 K228, G232 (Table 1). Note that we used *S.cerevisiae* residues and positions all through
40
41 118 except if specified.
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44 119 Of these, four positions seem to be particularly important in term of inhibitor
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46 120 resistance, namely N31, G37, L198 and K228. Mutations of these residues have been reported
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48 121 in different studies and organisms and/or after selection with different inhibitors. N31K have
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50 122 been found in *S.cerevisiae* and *Kluyveromyces lactis* after selection on the structurally-related
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52 123 antimycin and fenpicoxamid, but also on the unrelated compound diuron, (or DCMU). L198F
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54 124 causes resistance to antimycin, fenpicoxamid, ilicicolin H and funiculosin in *S.cerevisiae*;
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3 125 resistance to antimycin and GNF7686 in *Trypanosoma cruzi* the causative agent of Chagas
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5 126 disease. K228M confers resistance to antimycin in *S.cerevisiae* and *K.lactis*. Replacement of
6
7 127 the threonine by a proline at the equivalent position in *Toxoplasma gondii* causes resistance to
8
9 128 antimycin and ELQ-316. The most striking are mutations of residue G37. Five substitutions
10
11 129 have been reported: G37A,C,D,S,V. Acquired resistance mutations at that position have been
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13 130 found in fungi *S.cerevisiae*, *Schizosaccharomyces pombe* and *Zymoseptoria tritici*, in the
14
15 131 human malaria parasite *P.falciparum* and in mouse cells in culture. They confer resistance to
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17 132 antimycin and fenpicoxamid, diuron or the macrocyclic compound ML238 and its derivative,
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19 133 BRD6323.

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21
22 134 The residues N31, G37, L198 and K228, might have a key role for the binding of
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24 135 inhibitors and accommodate substitutions without a severe loss of complex III activity. In
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26 136 yeast, the decrease in complex III activity resulting from the substitution L198F was only of
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28 137 10%²⁰, and of around 50%, for G37S, K228M and N31S^{12,21}, with no or mild effect on
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30 138 respiratory growth competence. G37 and K228 are very highly conserved residues, although
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32 139 N31 is often replaced by glycine in plants and isoleucine in prokaryotic cytochrome *b*
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34 140 sequence data. N31 does not show binding interactions with bound quinone in the atomic
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36 141 structure of yeast complex III while L198 forms a stabilizing hydrophobic interaction with the
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38 142 benzoquinone headgroup of bound quinone (PDB 1EZV), and presumably a similar class of
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40 143 interaction is maintained in the L198F substituent. The sidechain amino group of K228 has
41
42 144 been suggested to be involved in proton transfer to bound quinone during redox chemistry at
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44 145 the Q_i-site.²² As the mutation of this residue to methionine is well tolerated by yeast, it is
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46 146 likely that nearby protonatable residues H202 and D229 can rescue.

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50 147 Inhibitor resistant mutants harbouring mutations at position N31, G37, L198 and K228
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52 148 have been selected (and maintained) in laboratory conditions. In the field, a moderate loss of
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54 149 complex III activity, such as observed with the yeast G37S, K228M and N31S mutants, may

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3 150 result in decreased organismal fitness, which could be protective against resistance evolution
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5 151 or at least delay resistance evolution. Nevertheless the observed cross-resistance to unrelated
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7 152 compounds caused by mutations of those residues calls for a close monitoring when anti-
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9 153 microbial Q_i-site targeting compounds will be on the market. On the other hand, monitoring
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11 154 of resistance might –and likely will- detect other possible resistance mutations. Studies with
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13 155 model organisms and laboratory strains would come useful, as illustrated above especially for
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15
16 156 the ametoctradin resistance mutation, S34L.
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19 20 158 **3.2 Anti-oomycetes resistance mutations found in the fields and the study in the yeast** 21 22 159 **model**

23 24 160 **3.2.1 Cyazofamid resistance mutations**

25
26 161 The cyazofamid resistance mutation, observed in field isolates of *P. viticola* is intriguing. It is
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28 162 a short sequence duplication of six nucleotides resulting in the insertion of two residues E203-
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30 163 DE-V204.²³ The Q_i-site region between the conserved residues H202 and S206 differs
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32 164 between species (Fig.2). However, structures of the enzyme with quinone bound at the Q_i-site
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34 165 showed that the conserved residues H202 and S206 act as hydrogen bond donors to the
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36 166 benzoquinone headgroup, orientating the quinone correctly. Mutations H202F,N and S206L
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38 167 resulted in growth defect (unpublished data and ²⁴). Therefore it would not be unexpected that
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40 168 a major modification of that short region between H202 and S206 could have a deleterious
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42 169 effect the on respiratory competence.
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46 170 In order to test such effect, we replaced, in yeast cytochrome *b*, the sequence I203-
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48 171 H204 by the sequence E-D-E-V (Fig.2). We observed that the mutation had no effect on
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50 172 respiratory growth, measured as the cell density reached after three day cultures in YPEth (not
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52 173 shown), which indicated that the region could accommodate such modifications without a
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54 174 severe loss of function. Unfortunately, as yeast complex III is insensitive to cyazofamid, the
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3 175 effect of the mutation on the fungicide sensitivity could not be analysed in our model. A
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5 176 cyazofamid binding model has been reported.²⁵ It can be hypothesized that the mutation
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7 177 E203-DE-V204 (Fig.2) found in *P.viticola* would affect the compound binding or access into
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9 178 the Q_i-site. The insertion is in the linker region between helices D and E (Fig.1), which is
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11 179 proximal to the benzoquinone headgroup of Q_i-site bound quinone (PDB 1EZV), at
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13 180 approximately 7 Å separation at the closest approach. This is likely to perturb the local fold
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15 181 around this region, and/or interfere directly with cyazofamid binding.

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17
18 182 In order to pursue the study of the effect of that mutation on complex III activity and
19
20 183 reactivity to cyazofamid, a yeast mutant sensitive to the compound needs to be found. It might
21
22 184 be possible to generate such mutant by genetically replacing the yeast residues of and around
23
24 185 the Q_i-site by the oomycetes equivalents, providing the multiple changes would not cause a
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26 186 too severe loss of function.

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30 31 188 **3.2.2 The ametoctradin resistance mutation, S34L**

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33 189 Ametoctradin, first listed as a QoSI, was shown to be a dual Q_o- and Q_i-site binding
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35 190 compound based on spectroscopic analysis.¹⁰ The approach, however, could not determine
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37 191 whether the compound would bind at the Q_o- and Q_i-sites with similar affinity. If
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39 192 ametoctradin affinity for one of the sites is much higher than its affinity for the other, resistant
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41 193 isolates harbouring target site mutation could be selected. Indeed a mutation in the Q_i-site,
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43 194 S34L, has been reported in *P. viticola* after treatment¹³, suggesting that ametoctradin have *in*
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45 195 *vivo* a higher affinity for the Q_i-site. In order to confirm the effect of S34L on ametoctradin
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47 196 resistance, we studied the mutation in the yeast model.

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49
50 197 Yeast complex III is naturally highly resistant to ametoctradin (and to the two other
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52 198 anti-oomycetes compounds amisulbrom and cyazofamid). As we have previously shown¹⁰,
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54 199 the respiratory growth of the WT strain can be inhibited by a high dose (300 µM) of

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3 200 ametoctradin which binds at the Q_o-site, and not at the Q_i-site, as Q_o-site mutations I147V and
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5 201 L275F increased the resistance. Thus, as expected, the introduction of S34L in yeast Q_i-site
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7 202 had no effect on the ametoctradin sensitivity (not shown).
8

9 203 However a mutant highly sensitive to ametoctradin, namely PFQi3, was identified in a
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11 204 collection of yeast mutants carrying point and multiple mutations in the Q_i-site. PFQi3
12
13 205 complex III was inhibited by ametoctradin in the low μ M range and behaved in a similar
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15 206 manner to the oomycete enzyme in inhibitor binding assays.¹⁰ PFQi3 seemed thus a pertinent
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17 207 model to study the effect of S34L. In addition of PFQi3, we tested here two other mutants,
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19 208 PFQi1 and PFQi2 (PFQi2 was already described in ²⁶). These mutants, and three other mutant
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21 209 strains described below, namely PFQi8, PFQi9 and PFQi10, combined several modifications
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23 210 at the Q_i-site (Table 2).
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27 211 First we tested the ametoctradin sensitivity and resistance of the respiratory growth of
28
29 212 the PFQi1,2 and 3. To that end, cell suspensions were spotted on respiratory medium
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31 213 containing increasing concentrations of ametoctradin and the plates were incubated for three
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33 214 to four days (Fig.3). PFQi3 was highly sensitive to ametoctradin as the respiratory growth was
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35 215 fully inhibited at 1 μ M while the growth of PFQi2 and PFQi1 was blocked at 10 μ M and 100
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37 216 μ M, respectively.
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40 217 PFQi1 harbours six mutations in the Q_i-site; PFQi2 has two additional changes and
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42 218 PFQi3 has two more changes and the substitution L198F instead of L198I present in PFQi1
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44 219 and 2 (Table 2). L198F has been reported as resistance mutation (Table 1). We considered
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46 220 whether that change could be responsible for the increased sensitivity of PFQi3. Therefore
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48 221 mutant PFQi8 was produced that combined the six mutations of PFQi1 with L198F. As
49
50 222 shown in Fig.3, PFQi8 was also highly sensitive to ametoctradin, suggesting that L198F could
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52 223 stabilize ametoctradin in the modified Q_i-site.
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3 224 We then introduced the mutation S34L in PFQi1 and PFQi3. As shown in Fig.3, the
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5 225 resulting mutants, PFQi9 and 10, were resistant to ametoctradin. By comparison, we
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7 226 monitored the sensitivity of the PFQi strains to cyazofamid and amisulbrom. The strains
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9 227 remained fully insensitive to cyazofamid (not shown) while a moderate increase in sensitivity
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11 228 to amisulbrom could be observed with PFQi1 and 2. Their growth was inhibited at 100 μ M
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13 229 amisulbrom (Fig.4). Interestingly, the substitution L198F that renders the cells more sensitive
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15 230 to ametoctradin seems to have the opposite effect with amisulbrom as the respiratory growth
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17 231 of PFQi3 and PFQi8 was not inhibited at 100 μ M amisulbrom. This suggests that the three
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19 232 anti-oomycete compounds would not share the same binding mode in the Q_i -site. However the
20
21 233 introduction of the ametoctradin resistance mutation S34L in PFQi1 (Fig.4, mutant PFQi9)
22
23 234 conferred also resistance to amisulbrom, suggesting some overlap between ametoctradin and
24
25 235 amisulbrom binding sites. However to pursue the study of amisulbrom sensitivity and
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27 236 resistance, other yeast mutants with modified Q_i -site highly reactive to the compound would
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29 237 be required.
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33 238 PFQi10, while clearly resistant to ametoctradin as compared to PFQi3, presented a
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35 239 weaker respiratory growth as seen on YPG plate without inhibitor (Figs. 3 and 4), suggesting
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37 240 a deleterious impact of S34L on the respiratory competence. In order to confirm the effect of
38
39 241 S34L, we first monitored the respiratory growth in YPEth liquid cultures with or without
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41 242 ametoctradin at increasing concentrations. We compared PFQi1, PFQi9, PFQi3 and PFQi10.
42
43 243 The cell densities reached after three day culture in absence of ametoctradin are shown in
44
45 244 Fig.5A. PFQi9 presented a moderate defect as compared to PFQi1 (that reached the same cell
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47 245 density than control (not shown)). The growth yield of PFQi3 was slightly decreased whereas
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49 246 a severe defect was observed for PFQi10. Thus the introduction of S34L in PFQi1 and in
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51 247 PFQi3 had an impact on the growth competence, which was more deleterious for PFQi3 /
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53 248 PFQi10.
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3 249 Growth assays in presence of ametoctradin confirmed that S34L conferred resistance.
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5 250 A three-fold increase in resistance was observed in PFQi9 as compared to PFQi1. Mid-point
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7 251 inhibitory concentrations were around 15 and 45 μM for PFQi1 and PFQi9, respectively
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9 252 (Fig.5B). A much more dramatic difference was observed between PFQi3 and PFQi10. Mid-
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11 253 point inhibitory concentration was around 0.3 μM for PFQi3 while PFQi10 growth was not
12
13 254 affected at 20 μM ametoctradin (Fig.5C). The high level of resistance of PFQi10 was further
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15 255 confirmed by monitoring the rates of O_2 consumption of cells in presence of increasing
16
17 256 concentrations of ametoctradin. The mid-point inhibitory concentration obtained for PFQi3
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19 257 was around 0.7 μM while PFQi10 respiration could not be inhibited at 50 μM ametoctradin
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21 258 (not shown).
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25 259 Finally we prepared mitochondria and monitored complex III activity (measured as
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27 260 cytochrome *c* reduction rates) as a function of ametoctradin concentration. As expected,
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29 261 PFQi3 complex III was sensitive and 50% inhibition was reached at around 15 μM
30
31 262 ametoctradin while PFQi10 complex III was highly resistant as only 10-15% inhibition was
32
33 263 observed at 100 μM , in agreement with the growth and O_2 consumption assays.
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36 264 The deleterious effect of S34L was also clearly observed in the enzymatic assays.
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38 265 Complex III activities of control, PFQi3 and PFQi10, in absence of ametoctradin, were
39
40 266 compared (Fig.6). PFQi3 enzyme had a two-fold decreased activity as compared to control. In
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42 267 PFQi10, a further two-fold decrease as compared to PFQi3 was observed. Thus the
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44 268 introduction of S34L in the Q_i -site of PFQi3 impaired the activity of complex III. That loss of
45
46 269 activity can explain the observed growth defect. The remaining 20-25% complex III activity
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48 270 in PFQi10 (as compared to control) could only support a weak respiratory growth while the
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50 271 50% activity in PFQi3 had only a mild effect on respiratory growth competence. This was in
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52 272 agreement with our previous observations with cytochrome *b* mutants. For comparison, we
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3 273 also tested the effect of S34L introduced in unmodified Q_i-site on complex III activity. The
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5 274 same assay was performed. We observed 25% loss of activity (not shown).
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7 275 To summarize, the assays performed here showed that S34L conferred a high level of
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9 276 ametoctradin resistance when introduced in the most sensitive surrogate model, PFQi3. The
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11 277 resistance was associated with a severe loss of complex III activity and respiratory growth
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13 278 competence. Introduction of S34L in the unmodified (and naturally highly resistant) complex
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15 279 III resulted in a mild decrease in activity. S34L introduced in PFQi1 that presented a moderate
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17 280 ametoctradin sensitivity caused a moderate loss of respiratory competence.
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21
22 282 Finally, *in silico* docking of ametoctradin into a homology model of the Q_i-site of
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24 283 *P.viticola* cytochrome *b* was performed (Fig.7). The analysis of the docking results suggests a
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26 284 binding mode similar to that of native quinone in the atomic structure of yeast complex III
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28 285 (PDB 1EZV). The aliphatic octyl- and ethyl substituents of ametoctradin are predicted to form
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30 286 stabilizing hydrophobic interactions with the sidechains of I17 (helix A, which is L17 in
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32 287 *P.viticola*), V194 and L198 (C-terminal region of helix D). Accordingly, we note that the
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34 288 L198F substitution in PFQi3 and PFQi8 may further strengthen the interaction with
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36 289 ametoctradin, explaining the increased sensitivity of these mutants to this inhibitor. In
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38 290 contrast, the interactions between the amino-substituted triazolo-pyrimidinyl headgroup of
39
40 291 ametoctradin and the Q_i-site are predicted to be more hydrophilic in nature, with a putative H-
41
42 292 bonding interaction (2.5 Å separation) between the amino group of the headgroup and the
43
44 293 carboxylate sidechain of D229. This may take the form of a salt bridge, depending upon the
45
46 294 local dielectric microenvironment. Significantly, we also predict a weaker H-bonding
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48 295 interaction between this amino moiety of ametoctradin and the serinyl sidechain of S34 (3.1 Å
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50 296 separation), which may explain the resistance conferred by the S34L mutation. In any case,
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3 297 introduction of the bulky leucyl sidechain at this position may be expected to be sterically
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5 298 destabilizing with respect to ametoctradin binding.
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8 9 300 **4. CONCLUSION**

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11 301 The brief overview of the reported Q_i-site resistance mutations found in laboratory models or
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13 302 field isolates demonstrates the mutational plasticity of cytochrome *b* in the presence of QiIs,
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15 303 single site competitive inhibitors that function as Q_i-site antagonists. Cytochrome *b* is
16
17 304 mitochondrially encoded, and it is apparent that mitochondrial genes seem to accumulate
18
19 305 mutations at a higher frequency than nuclear genes. Target site resistance mutations are hence
20
21 306 expected to arise under selective pressure. Dual Q_o- and Q_i-site inhibitors would be effective
22
23 307 as mutation of both quinone binding sites would be expected to be a very infrequent event
24
25 308 whilst still maintaining organismal fitness. However such dual acting inhibitors are rare²⁶. In
26
27 309 their absence, a combination of a QoI and a QiI could be used. Unfortunately, at least for
28
29 310 *P.viticola*, the Q_o-site mutation G143A is already present in field population and isolates
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31 311 harbouring both G143A and S34L have been observed¹³, which hinders that strategy. Other
32
33 312 combined treatments might need to be devised, possibly targeting sites away from complex
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35 313 III.
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40 314 A fitness cost associated with the resistance mutations would also be protective, as the
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42 315 mutants would be counter-selected in absence of treatment, as might be the case for the
43
44 316 ametoctradin resistance mutation S34L as reported by Fontaine et al.¹³. A fitness penalty
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46 317 associated with the resistance was confirmed in our yeast model. PFQi3 with a modified Q_i-
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48 318 site rendered sensitive to ametoctradin seemed thus a useful model. However its pertinence
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50 319 should be further confirmed by studying the effect of S34L on the oomycete complex III
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52 320 activity and inhibitor sensitivity.
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22 455 **Table 1. Inhibitor resistance mutations in complex III Q_i-site**

Mutation ^a	Resistant to	Organism	References ^b
I17F	Diuron	<i>Saccharomyces cerevisiae</i>	27
S20L, T	Ilicicolin H	<i>Saccharomyces cerevisiae</i>	11
Q22E, T	Ilicicolin H	<i>Saccharomyces cerevisiae</i>	11
I26L	ELQ-300	<i>Plasmodium falciparum</i>	6
W30C ^c	Ilicicolin H, Funiculosin	<i>Saccharomyces cerevisiae</i>	20
N31K	Diuron, Antimycin, Fencicoxamid	<i>Saccharomyces cerevisiae</i>	27 7
N31K	Antimycin	<i>Kluyveromyces lactis</i>	28
G33A ^c	Funiculosin, HQNO HDQ	<i>Saccharomyces cerevisiae</i>	29 12
S34L	Ametoctradin	<i>Plasmopora viticola</i>	13
S(L)35I	Antimycin	<i>Leishmania tarentola</i>	30
G37C	Fencicoxamid, Antimycin	<i>Saccharomyces cerevisiae</i>	7
G37D, S	Ilicicolin H	<i>Saccharomyces cerevisiae</i>	11
G37V	Antimycin	<i>Saccharomyces cerevisiae</i>	31

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3	G37A	ML238, BRD6923	<i>Plasmodium falciparum</i>	32
4	G37V	Antimycin	<i>Zymoseptoria tritici</i> .	9
5	G37V	ML238, BRD6323, Antimycin	<i>Plasmodium falciparum</i>	32
6	G37V	Antimycin	<i>Mus musculus</i>	33
7	A(G)37V	Antimycin	<i>Schizosaccharomyces pombe</i>	34
8	A(G)37G	Diuron	<i>Schizosaccharomyces pombe</i>	34
9	L198F	Funiculosin,	<i>Saccharomyces cerevisiae</i>	35
10		Ilicolin H,		11
11		Fenpicoxamid, Antimycin		7
12	L198F	GNF7686, Antimycin	<i>Trypanosoma cruzi</i>	36
13	E203-DE-V204	Cyazofamid	<i>Plasmopora viticola</i>	23
14	(I203--H204)			
15	H204Y ^c	HDQ	<i>Saccharomyces cerevisiae</i>	12
16	M221Q ^c	Antimycin,	<i>Saccharomyces cerevisiae</i>	37
17		HQNO		29
18		Ilicolin H		20
19		ELQ-271, ELQ-319		26, 38
20	M221E ^c	Antimycin	<i>Saccharomyces cerevisiae</i>	37
21		HQNO		29
22		Ilicolin H,		20
23	F225S, L	Diuron	<i>Saccharomyces cerevisiae</i>	27, 31
24	K228M	Antimycin	<i>Saccharomyces cerevisiae</i>	31
25	K228M ^c	HDQ	<i>Saccharomyces cerevisiae</i>	12
26	K228I/M	Antimycin	<i>Kluyveromyces lactis</i>	28
27	T228P	ELQ-316, Antimycin	<i>Toxoplasma gondii</i>	39
28	G(T)232D	Antimycin, HQNO	<i>Mus musculus</i>	33

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3 457 ^a Yeast numbering; (), equivalent residue in yeast cytochrome *b* if different
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5 458 ^b Cited references are the first report or first study on the mutations
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8 459 ^c These mutations were not identified in mutants selected after inhibitor treatment but
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10 460 originally were found in “revertants” i.e. respiratory growth competent clones selected from
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12 461 respiratory deficient mutants (the defect was due to a point mutation in cytochrome *b*)
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463 **Table 2. Mutations introduced in yeast Q_i-site**

position	WT	PFQi1	PFQi2	PFQi3	PFQi8	PFQi9	PFQi10
34	S					L	L
40	L		F				
41	V		F				
190	L			G			G
191	A	L	L	L	L	L	L
192	A			C			C
195	I	F	F	F	F	F	F
198	L	I	I	F	F	I	F
207	S			T			T
221	M	F	F	F	F	F	F
225	F	L	L	L	L	L	L
226	I	L	L	L	L	L	L

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465 The PFQi mutants were constructed using the mitochondrial transformation technique. PFQi1,
 466 2, 3 and 8: yeast amino-acids were replaced by their equivalents observed in the protozoan
 467 *Plasmodium falciparum* (with the exception of L198F). PFQi9 and 10: the ametoctradin
 468 resistance mutation S34L was introduced in the PFQi modified Q_i-site.

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3 473 **Figures legends**
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7 475 **Figure 1.** A) Catalytic core of complex III showing the redox-groups, haems and the 2Fe2S
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9 476 cluster, formed by three of the complex subunits: cytochrome *c*, the Rieske Iron-Sulphur
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11 477 Protein and cytochrome *b*, which is mitochondrially-encoded and provides the Q_o- and Q_i-
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13 478 sites.

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15 479 B) Schematic representation of cytochrome *b*. Positions of residues involved in inhibitor
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17 480 resistance are shown: Q_o-site G143 and Y279; Q_i-site I17, N31, S34, G37, L198, H204 and
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19 481 K228.
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24 483 **Figure 2.** Sequence comparison of the Q_i-site region of the cyazofamid resistance mutation,
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26 484 from H197 to S207 (*S.cerevisiae* residues and numbering). The sequences of ten fungal
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28 485 cytochrome *b* are compared. In bold, the sequence of the cyazofamid resistant isolate of
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30 486 *P.viticola*. Four residues are indicated, the conserved residues L198, H202 and S206, and the
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32 487 variable residue H204.
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37 489 **Figure 3.** Effect of mutations in complex III Q_i-site on the growth sensitivity to ametoctradin.
38
39 490 Suspensions in water of cells pre-grown on YPD plates were spotted, at three different cell
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41 491 concentrations, on plates containing the respiratory medium YPG with increasing
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43 492 concentrations of ametoctradin.
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48 494 **Figure 4.** Growth sensitivity to amisulbrom of PFQ_i mutants. Suspensions in water of cells
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50 495 pre-grown on YPD plates were spotted, at three different cell concentrations, on plates
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52 496 containing the respiratory medium YPG with or without 100 µM amisulbrom.
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3 497 **Figure 5.** Growth competence and ametoctradin sensitivity of mutants PFQi1, 3, 9 and 10.
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5 498 Cells freshly grown on YPD plates are inoculated at an initial OD_{600nm} of 0.2 in YPEth
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7 499 without or with ametoctradin at increasing concentrations. The cultures were incubated three
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9 500 days at 28°C with vigorous agitation. The OD_{600nm} were then recorded. The growth
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11 501 experiments were repeated at least twice and the data averaged. The error bars represent
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13 502 standard deviation A) Cell densities reached in absence of ametoctradin. B and C) Effect of
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15 503 ametoctradin. The data are presented as percentage of the OD_{600nm} of the untreated cultures.
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17 504 B) Comparison between PFQi1 (solid line) and PFQi9 (dotted line); C) Comparison between
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19 505 PFQi3 (solid line) and PFQi10 (dotted line).
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24 507 **Figure 6.** Complex III activity of control, PFQi3 and PFQi10. Cytochrome *c* reduction was
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26 508 monitored at 550-540 nm. The reaction was initiated by the addition of 20 µM decylubiquinol
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28 509 to the reaction mixture containing 20 µM cytochrome *c* and 2-10 nM complex III. Initial rates
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30 510 were measured. Each measurement was repeated three to five times and the values obtained
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32 511 were averaged. The activities are presented as the cytochrome *c* reduction rate per complex III
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34 512 per second
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40 514 **Figure 7.** Docking conformation of ametoctradin in the Q_i-site of *P.viticola*. Ametoctradin is
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42 515 represented as sticks with partially transparent surface. Residues that are interacting with the
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44 516 ligand are shown as sticks. The haem is also visible in the background.
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Figure 1. Catalytic core of complex III and schematic representation of cytochrome b

69x29mm (300 x 300 DPI)

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 3 *S.cerevisiae* HLMALHI--HGSS
 4 *Z.tritici* HLIALHDT-AGSG
 5 *B.cinerea* HLIALHDS-AGSG
 6 *P.viticola* HLILLHE--VGSN
 7 **Cyazo-Res** HLILLHEDEVGSN
 8 *C.albicans* HLMALHV--HGSS
 9 *A.fumigates* HLIAMHDT-VGSG
 10 *S.pombe* HLIALHT--NGSS
 11 *P.anserina* HLIALHDT-AGSS
 12 *U.maydis* HLLALHE--HGSS
 13 *P.graminis* HLLTLHE--HGSN

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 17 L198 H202 H204 S206
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23 **Figure 2.** Sequence comparison of the Q_i-site region of the cyazofamid resistance
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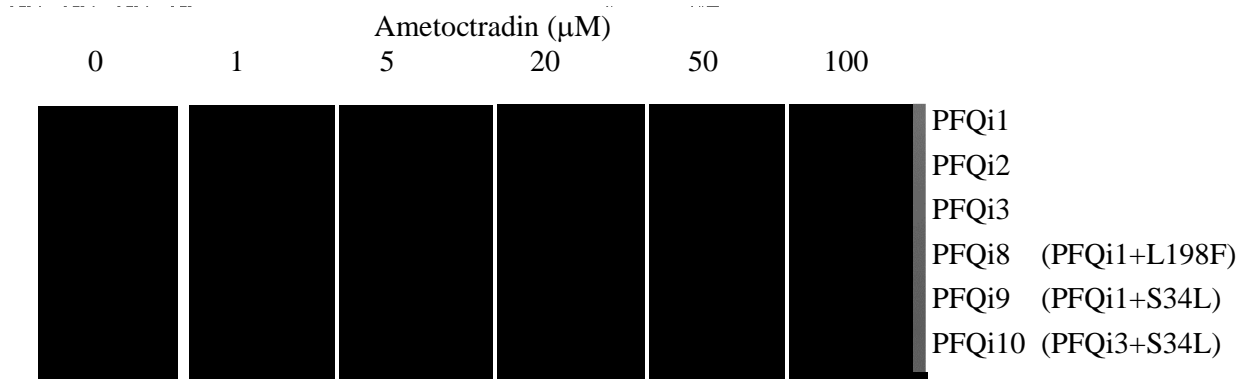
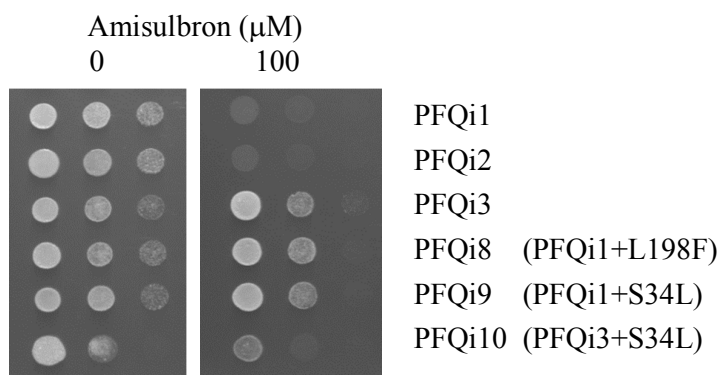
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Figure 3. Effect of mutations in complex III Q_i-site on the growth sensitivity to ametoctradin

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17 **Figure 4.** Growth sensitivity to amisulbrom of PFQI mutants
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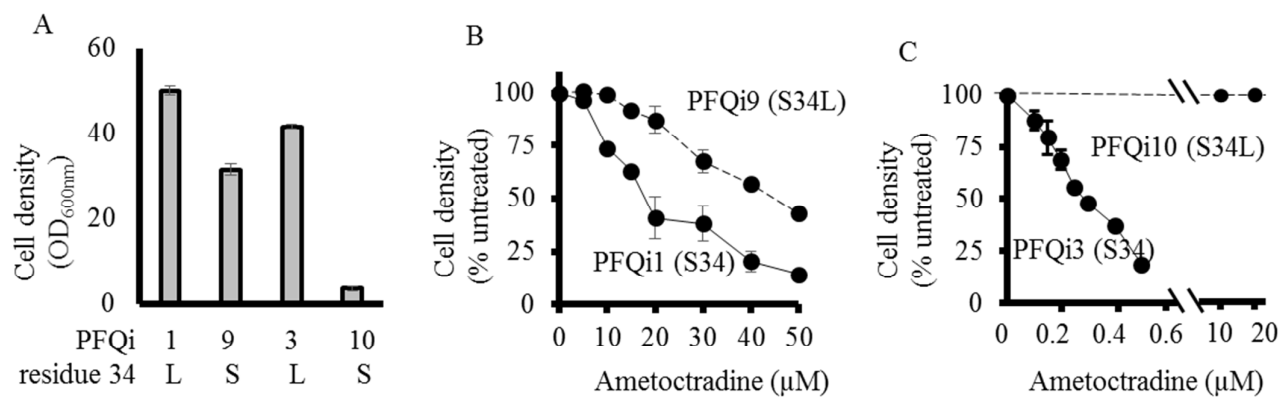


Figure 5. Growth competence and ametoctradin sensitivity of mutants PFQi1, 3, 9 and 10

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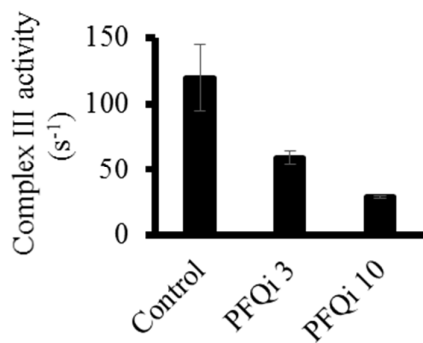


Figure 6. Complex III activity of control, PFQi3 and PFQi10

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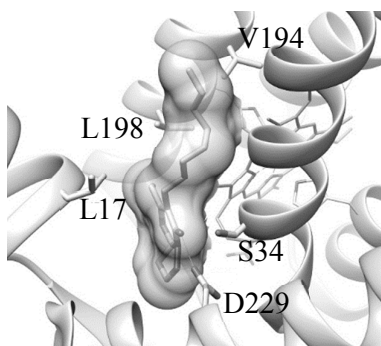


Figure 7. Docking conformation of ametoctradin in the Q_i -site of *P.viticola*

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