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1 Title: Characterization of mutants with single and combined Q_i and Q_o site mutations in *Saccharomyces*
2 *cerevisiae* reveals interactions between the picolinamide fungicide CAS-649 and azoxystrobin

3

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8

9 ABSTRACT

10 Picolinamide and strobilurin fungicides bind to the Q_i and Q_o sites on cytochrome *b*, respectively, and
11 target many of the same plant pathogens. Using *Saccharomyces cerevisiae* as a model system, we explore
12 effects of amino acid changes at each site on sensitivity to a fungicide acting at the opposite site and
13 examine the relationship between altered sensitivity and growth penalty. In addition, double mutants
14 containing the G143A or F129L mutations responsible for strobilurin resistance in combination with Q_i
15 site mutations that confer resistance to picolinamides are characterized in terms of their sensitivity to QiI
16 and QoI fungicides and growth rate. Mutants containing amino acid changes at the Q_o site varied in their
17 growth rate and sensitivity to the picolinamide CAS-649, and increased sensitivity was associated with a
18 greater growth penalty. Conversely, changes at the Q_i site affected sensitivity to azoxystrobin and also
19 showed a correlation between increased sensitivity and reduced growth. There was no overall correlation
20 between resistance to azoxystrobin and CAS-649 among mutants, however negative cross-resistance
21 occurred in the case of mutations which conferred resistance to either compound and also carried a
22 growth penalty. These results suggest the use of QoI fungicides to delay the emergence of pathogen
23 resistance to QiIs, and vice versa. Double mutants containing G143A or F129L in combination with Q_i
24 site changes N31K, G37C/V or L198F that cause resistance to picolinamides generally exhibited lower
25 resistance factors for both azoxystrobin and CAS-649 than corresponding resistant strains with a single
26 mutation. Reduced growth was observed for all F129L-containing double mutants, whereas the growth

27 rate of double mutants containing G143A was significantly reduced only by the Q_i site mutations N31K
28 and G37V that confer a larger growth penalty. Our results suggest that resistance to picolinamides in
29 pathogens could emerge more readily in a strobilurin-sensitive genetic background than in a strobilurin-
30 resistant one.

31

32 Keywords:

33 Picolinamide fungicide

34 CAS-649

35 Florylpicoxamid

36 Strobilurin

37 Azoxystrobin

38 Resistance mutation

39

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43

44 1. Introduction

45 The cytochrome bc_1 complex (complex III) of the mitochondrial electron transport chain represents
46 one of the most successful biochemical targets for commercial fungicides and contains two distinct
47 binding sites, known as the Q_o and Q_i sites, which are located on cytochrome b (cyt b) on opposite sides
48 of the inner mitochondrial membrane and correspond to the quinol oxidation and reduction sites,
49 respectively. Compounds are classified as QoI (Quinone outside Inhibitor) or QiI (Quinone inside
50 Inhibitor) fungicides based on their site of action (Sierotzki, 2015). QoIs originated from the strobilurin
51 class of natural products and are commonly referred to as “strobilurin” fungicides. First introduced in
52 1996, the strobilurin fungicides represent a large class of active ingredients and control a broad spectrum
53 of diseases caused by ascomycete, basidiomycete and oomycete pathogens. Resistance to QoIs is now
54 widespread and associated primarily with two cyt b mutations at the Q_o site, G143A and F129L. The
55 G143A mutation produces a very high level of resistance and has caused a loss in field efficacy in at least
56 42 pathogens (Sierotzki, 2015). Although less common than G143A and associated with lower resistance
57 factors, the F129L mutation is the main cause of strobilurin resistance in *Phakopsora pachyrhizi* (soybean
58 rust), *Pyrenophora teres* (net blotch in barley), *Alternaria solani* (potato/tomato early blight) and *Pythium*
59 *aphanidermatum* (Pythium blight of turfgrass) (Sierotzki, 2015; Klosowski et al., 2016).

60 The picolinamide fungicides fenpicoxamid, florylpicoxamid and metarylpicoxamid represent a new
61 chemical class and are the first QiIs to target ascomycete and basidiomycete pathogens. Fenpicoxamid is
62 derived from the natural product UK-2A and was recently introduced to control Septoria wheat leaf
63 blotch (*Zymoseptoria tritici*) and black Sigatoka (*Pseudocercospora fijiensis*) in banana (Owen et al.,
64 2017). Inspired by UK-2A, florylpicoxamid is a fully synthetic molecule which shares the same key
65 binding interactions at the Q_i site as the natural product (Meyer et al., 2021a, 2021b). Florylpicoxamid
66 will extend the use of picolinamides to control additional pathogens, including *Botrytis*, *Colletotrichum*
67 and *Alternaria* species, as well as powdery mildew diseases (Yao et al., 2021). The development of a
68 third picolinamide, metarylpicoxamid, was recently announced for control of asian soybean rust (Meyer
69 et al., 2022).

70 It can be assumed that target site-based resistance to picolinamides will eventually emerge in the field
71 as it has for other complex III inhibitors, and the particular mutations will be determined by the level of
72 resistance and any associated fitness penalty. Lab mutants with resistance to picolinamides have been
73 identified in yeast and *Z. tritici*. In yeast, N31K, G37L/I/C/V/M and L198F conferred resistance (Young
74 et al., 2018, 2022) while G37V was isolated in *Z. tritici* (Fouché et al., 2022). Most diseases which will be
75 managed by application of picolinamide fungicides involve pathogens in which strobilurin resistance is
76 already present in the population. In this context, for resistance risk assessment and management strategy
77 it is important to consider the phenotype associated with Q_i site mutations in both QoI sensitive and
78 resistant genetic backgrounds. Since the Q_o and Q_i sites are spatially separated, a mutation at one site
79 cannot directly impact binding of an inhibitor at the opposite site. However, the two sites are functionally
80 linked and actively coupled such that ligand binding at one site might impact the opposite site (Cooley et
81 al., 2009). Intriguingly, negatively-correlated cross-resistance between picolinamides and strobilurins has
82 been reported in *Zymoseptoria tritici* (Fouché et al., 2022). Also, double mutants of *Z. tritici* containing
83 both the G37V Q_i site mutation and the G143A Q_o site mutation showed a reduced level of resistance to
84 both QiI and QoI fungicides as compared to the corresponding single mutants.

85 The commercial use of QoI and QiI fungicides to control the same diseases presents a unique situation
86 among fungicides in which two inhibitors act at different sites on the same target protein. In this study,
87 using the yeast *Saccharomyces cerevisiae* as a model organism, we explore the relationship between
88 effects of mutations at the Q_i and Q_o sites on growth rate, as a measure of “fitness”, and altered sensitivity
89 to fungicides. To assess the phenotype of Q_i site mutants in strobilurin-resistant genetic backgrounds we
90 also examine the levels of resistance and growth rate of strains containing combinations of Q_o and Q_i site
91 mutations. Possible implications of our findings for resistance development and strategies for use of QiI
92 and QoI fungicides in resistance management are discussed.

93

94

95 2. Materials and methods

96 2.1. Strains and media

97 The following media were used for the growth of *S. cerevisiae*: YPD (1% yeast extract, 2% peptone,
98 3% glucose), YPG (1% yeast extract, 2% peptone, 3% glycerol), and transformation medium (0.7% yeast
99 nitrogen base, 3% glucose, 2% agar, 1 M sorbitol and 0.8 g of a complete supplement mixture minus
100 uracil per litre, supplied by Anachem).

101 Mutants containing the single *cyt b* changes G37C, L198F and N31K were isolated by selection on
102 fenpicoxamid or antimycin A (Young et al., 2018). All other mutants were generated by site-directed
103 mutagenesis and mitochondrial transformation. The following *S. cerevisiae* strains were used for
104 transformation and screening: i) W303-1B MAT alpha *ura3 his3 leu2 ade2 trp1 rho*⁰ (lacking
105 mitochondrial DNA), used as recipient strain for biolistic transformation; ii) CKPF3 MAT a *leu1 kar1-1*
106 with an intronless mitochondrial DNA and a short deletion (Δ 158-161) in *CYTB*, the *cyt b* encoding gene,
107 rendering the cells unable to growth on respiratory medium. The strain used for the direct selection of
108 fungicide resistant mutants (Young et al., 2018) was a diploid issued from the crossing between W303-1B
109 MAT alpha *ura3 his3 leu2 ade2 trp1 rho*⁰ and CKWT MAT a *leu1 kar1-1* with an intronless
110 mitochondrial DNA (and wild-type *CYTB*), thus isogenic to CKPF3 with the exception of the *CYTB*
111 deletion.

112 2.2. Site-directed mutagenesis, biolistic transformation, and selection of mitochondrial mutants

113 Wild-type intronless *CYTB* with its 350 bp upstream sequence was cloned in the pCRscript vector
114 (Stratagene). The chosen *CYTB* mutations were then introduced using the Quickchange site-directed
115 mutagenesis method (Stratagene) according to the manufacturer's recommendations. After verification of
116 the sequence, the plasmids carrying the mutated *CYTB* were used for biolistic transformation.

117 Mitochondrial transformation by microprojectile bombardment was adapted from Bonnefoy et al., 2007.
118 Approximately 3 μ g of the plasmids carrying mutated *CYTB* and 0.5 μ g of plasmid YEP352 (which
119 contains the *URA3* gene, allowing the selection of Ura⁺ nuclear transformants) were mixed with 50 μ l of
120 0.7- μ m tungsten particles (at a concentration of 60 mg ml⁻¹). The particles (supplied by Bio-Rad) were

121 prepared and coated with DNA according to the manufacturer's protocol. Aliquots of the coated particles
122 were used for transformation of the recipient strain W303-1B rho⁰. Around 10⁷ cells of the recipient strain
123 were evenly spread on transformation medium (selective for Ura⁺ transformants). The particle
124 bombardments were performed with a Biolistic PDS-1000/He particle delivery system (Bio-Rad)
125 according to the manufacturer's recommendations. Colonies of Ura⁺ transformants (which have received
126 plasmid YEP352) appeared after 4 to 5 days of incubation. Ura⁺ colonies (>1,000) were then crossed with
127 the respiratory-deficient CKPF3 and the diploids replica-plated on respiratory medium (YPG).
128 Respiration-competent diploids (usually 1/500 diploids tested) were selected and subcloned. Their
129 respiratory competence resulted from homologous recombination between *CYTB* carrying Δ158-161 and
130 plasmid-derived *CYTB* carrying the chosen mutations. The sequencing of *CYTB* from the respiratory
131 competent diploids showed that the new mutations were incorporated while Δ158-161 was replaced by
132 the wild-type sequence.

133 2.3. *Evaluation of kinetic growth rate*

134 The method used to measure the growth rate of yeast strains in 96-well microtiter plates was based on
135 the technique described by Held in an application note from Agilent Technologies
136 (<https://www.agilent.com/cs/library/applications/monitoring-growth-of-saccharomyces-cerevisiae-5994-3284EN-agilent.pdf>). Cells from overnight cultures grown on YPD medium at 30 °C were suspended in
137 YPEth broth (10 g of yeast extract, 20 g of Bacto peptone and 2% (v/v) of ethanol per liter) and adjusted
138 to A₆₀₀ = 0.3 in a 1 cm cuvette. Cell suspensions (100 μl) were added to wells of a 96-well flat-bottom
139 tissue culture plate (Cat. No. 655180, Greiner Bio-One, Monroe, NC, USA) with 8 replicate wells per
140 strain. The plate was sealed with Breathe-EasyTM sealing film (Cat. No. BEM-1, Diversified Biotech,
141 Dedham, MA, USA). Growth curves were measured in a Cytation 3 instrument (BioTek Instruments,
142 Winooski, VT, USA) at 30 °C using continuous double orbital shaking at 425 cpm with 3 mm amplitude
143 and A₆₀₀ measurements taken at 1 h intervals. Growth rates were determined during exponential growth
144 over the 6 - 11 h period after start of the experiment using Excel as described by Bio-Resource
145 (<https://www.youtube.com/watch?v=adYWnTPPWIM>) and used to calculate doubling time (Dt), as a

147 more intuitive expression of growth, using the equation $Dt = \ln(2)/\text{growth rate}$. All experiments were
148 repeated at least twice for each strain with 8 replicates per experiment and the mean growth rates \pm SD
149 were determined across all replicates.

150 2.4. *Fungicide sensitivity of cytochrome b mutants*

151 The experimental system used to measure kinetic growth rate was also employed in fungicide
152 sensitivity tests so that relationships between the two parameters could be compared under the same
153 growth conditions. CAS-649, the active metabolite of florylpicoxamid, and azoxystrobin were chosen to
154 represent the QiI and QoI fungicide classes, respectively. CAS-649 (Chemical Abstracts Service Registry
155 Number: 1961312-07-1) was preferred in this study over the natural product UK-2A, the active
156 metabolite of fenpicoxamid, due to its greater stability in culture medium (unpublished results).
157 Azoxystrobin was selected as a strobilurin with similar *in vitro* fungitoxicity to CAS-649 against yeast.
158 CAS-649 was synthesized at Corteva Agriscience and azoxystrobin was obtained from Chem Service,
159 Inc., West Chester, PA. CAS-649 or azoxystrobin were dissolved in DMSO and diluted 1000-fold into
160 YPEth broth to the desired concentration. Two-fold dilutions were prepared in YPEth broth containing
161 0.1% DMSO then 50 μ l aliquots were dispensed into a 96-well plate. Wells were inoculated with 50 μ l of
162 cell suspension prepared at $A_{600} = 0.6$ with 3 replicate wells at each concentration per strain. The plate
163 was sealed and incubated for 19 h in the Cytation 3 instrument, as described above. 50% Effective
164 concentration (EC_{50}) values for growth inhibition were determined based on initial (1 h measurement) and
165 final A_{600} readings using the CALCUSYN program (Biosoft, Cambridge, UK). Strains were tested in two
166 or more independent experiments and average EC_{50} values \pm SD determined across all replicates.
167 Sensitivity to cycloheximide (product number C7698, Sigma-Aldrich, Inc., St. Louis, MO) was
168 determined similarly except that each strain was tested in one experiment with 3 replicate wells at each
169 concentration.

170 2.5. *Statistical Analysis*

171 The SigmaStat module of SigmaPlot v 14.5 (Inpixon) was used for all analyses. The Holm-Sidak multiple
172 comparison procedure was used to compare the effects of mutations on doubling time and fungicide

173 sensitivity. A nonlinear regression model of exponential growth was used to examine the relationship
174 between fungicide sensitivity and growth rate. Differences in mean doubling time and fungicide
175 resistance factors between single Qi site mutants and double mutants containing Qo and Qi site mutations
176 were analyzed using the Student's *t*-test.

177

178 **3. Results**

179 *3.1. Effect of amino acid exchange at the Q_o site on growth rate and sensitivity to CAS-649*

180 Doubling time (Dt) for the parent strain was 4.26 h and Dt values for the Q_o site mutants ranged from
181 4.04 to 15.53 h (Fig. 1A). Strains with the G143A and F129L mutations showed the smallest changes in
182 growth rate as compared to the parent strain (Dt = 4.26 h); Dt of the F129L mutant increased to 5.24 h
183 and did not change significantly for the G143A mutant (Dt = 4.04 h).

184 Amino acid changes at the Q_o site increased sensitivity to CAS-649 by 1.5 to 7.5-fold (Fig. 1B, Table
185 1), and the increase in sensitivity was correlated ($R^2 = 0.793$) with a reduction in growth rate (Fig. 2). The
186 F129L mutant showed a 2.9-fold increase in sensitivity compared to the parent strain and, interestingly,
187 the G143A mutant was also slightly more sensitive (1.5-fold) than the parent although its growth rate was
188 not reduced (Fig. 1B, Table 1).

189 For Q_o site mutants, the correlation between growth penalty and increased sensitivity to CAS-649
190 resulted in negatively correlated cross-resistance between azoxystrobin and CAS-649 in the case of
191 mutations which cause strobilurin-resistance and also carry a growth penalty, e.g. F129L and G143S
192 (Table 1). However, there was no overall correlation between sensitivity of the mutant set to azoxystrobin
193 and to CAS-649 (Table 1). This is consistent with discrete binding sites for QoI and QiI fungicides which
194 implies that a mutation at one site which causes resistance by interfering with fungicide binding should
195 not affect ligand binding interactions at the opposite site.

196 *3.2. Effect of amino acid exchange at the Qi site on growth rate and sensitivity to azoxystrobin*

197 Since the mutations known to affect resistance to the picolinamide fungicides involve N31, G37 and
198 L198, a set of mutants with changes at these positions and different growth rates (Young et al., 2022) was
199 chosen for study.

200 Dt values for the Q_i site mutants ranged from 4.46 to 7.14 h (Fig. 3A), and mutants displaying a
201 growth penalty showed substantially increased sensitivity to azoxystrobin, ranging from 5.8 (N31G) to
202 15.2-fold (G37V) (Fig. 3B, Table 2). Mutants which did not show a statistically significant change in
203 growth rate exhibited only small changes in sensitivity to azoxystrobin, ranging from 0.8 (L198V) to 3.2-
204 fold (L198G). As in the case of Q_o site mutants, a significant correlation ($R^2 = 0.800$) was observed
205 between the increase in sensitivity of Q_i site mutants to the inhibitor acting at the opposite site
206 (azoxystrobin) and a reduction in growth rate (Fig. 2). Also, similar to the Q_o site mutation effects, while
207 there was no overall correlation between sensitivity to CAS-649 and to azoxystrobin among the Q_i site
208 mutants (Table 2), those mutations which conferred resistance to CAS-649 and also carried a growth
209 penalty (e.g. G37V/C and N31K) caused negatively correlated cross-resistance to azoxystrobin.

210

211 3.3. *Growth rate and fungicide sensitivity of double mutants with amino acid exchange at both Q_i and* 212 *Q_o sites*

213 L198F, G37C, G37V and N31K changes at the Q_i site confer resistance to picolinamide fungicides
214 (Young et al., 2018, 2022). Strains containing these single changes were compared with corresponding
215 double mutant strains which also contained the G143A or F129L changes at the Q_o site.

216 All double mutants containing a Q_i site mutation in combination with F129L showed a significant
217 increase in Dt as compared to corresponding single Q_i site mutants (Fig. 4). With the exception of the
218 N31K/F129L double mutant, the level of resistance to CAS-649 was lower in all F129L double mutants
219 (2.0 to 4.6-fold) than in corresponding single mutants (Table 3), and the levels of resistance to
220 azoxystrobin were lower by 1.5 to 6.4-fold (Table 4).

221 G143A-containing double mutants with Q_i site changes G37V and N31K showed an increase in Dt
222 relative to the respective single Q_i mutant strains, but the combination of G143A with G37C or L198F did

223 not significantly affect Dt (Fig. 4). As in the case of F129L double mutants, with the exception of the
224 N31K/G143A double mutant, the level of resistance to CAS-649 was lower in all G143A double mutants
225 (1.6 to 2.6-fold) than in corresponding single mutants (Table 3), and levels of resistance to azoxystrobin
226 were lowered by 1.5 to 13.5-fold (Table 4).

227 Single and double mutants were also tested for sensitivity to cycloheximide to assess whether the
228 slower-growing mutants might show a general increase in sensitivity to fungicidal compounds. No
229 relationship was found between sensitivity to cycloheximide and Dt (Supplemental Fig. S1).

230

231 **4. Discussion**

232 Analysis of Q_o site mutations showed an increase in sensitivity to CAS-649 for all mutants and a clear
233 correlation between growth penalty and sensitivity. These data raise the question of whether the
234 introduction of picolinamide fungicides might alter the proportion of strobilurin-resistant and sensitive
235 genotypes in pathogen populations. Sensitivity of the G143A mutant to CAS-649 increased only slightly,
236 by 1.5-fold. If a similar increase in sensitivity occurred in a pathogen containing G143A, this would be
237 unlikely to affect the size of the resistant population given the general fitness of G143A genotypes and
238 very high resistance factors, which provide a very strong selection pressure in the presence of a
239 strobilurin. Unlike G143A, the F129L mutation did cause a significant growth penalty in yeast and a
240 greater increase (2.9-fold) in sensitivity to CAS-649. Consistent with the increased Dt of the yeast mutant,
241 in pathogens it is generally assumed that F129L carries a greater fitness penalty than G143A (Sierotzki,
242 2015). Doubling time of the yeast F129L mutant increased to 5.24 h as compared to 4.26 h for the parent
243 strain. Over multiple generations in the absence of fungicide selection pressure, a similar growth penalty
244 could translate to a considerable difference in population size; in yeast for example this difference in
245 doubling time would translate to a 3.6-fold difference in population size between the parent and resistant
246 strains over 10 generations of the parent, when growing exponentially. Given the much lower resistance
247 factors associated with F129L-containing pathogen populations, which imply lower selection pressure
248 than for G143A, and reduced fitness, even a small increase in sensitivity to picolinamide fungicides might

249 reduce the size of F129L strobilurin-resistant populations and perhaps help to maintain longer term utility
250 of strobilurins. It would be interesting to test this hypothesis in field experiments against pathogens where
251 F129L causes strobilurin resistance.

252 Similar to the effect of Q_o site mutations on sensitivity to CAS-649, there was a clear correlation
253 between reduced growth of Q_i site mutants and increased sensitivity to azoxystrobin. Earlier studies
254 (Young et al., 2018, 2022) showed that certain amino acid changes involving N31, G37 and L198 were
255 associated with resistance to picolinamides in yeast and since changes to N31 were not tolerated without a
256 large growth penalty, ones involving G37 and L198F seem to be of greatest potential concern for
257 resistance development (Young et al., 2022). In the case of *Z. tritici*, lab studies suggest that the G37V
258 mutation may be the most likely resistance mutation to develop in the field (Fouché et al., 2022). Unlike
259 in yeast, the G37V mutation did not cause a growth penalty in *Z. tritici*, highlighting the fact that a given
260 mutation will not necessarily produce the same fitness phenotype in different organisms, and that
261 resistance mutations which emerge in the field might differ between pathogens.

262 The use of fungicide mixtures, or alternation, is a common strategy to delay resistance development
263 and the negative cross-resistance between QiI and QoI fungicides observed for target site mutants which
264 carry a growth penalty suggests that strobilurins could be particularly effective in delaying resistance to
265 picolinamides. However, a QiI-QoI combination would be most advantageous in delaying resistance
266 caused by Q_i site mutations which confer a larger fitness penalty and might not apply in the case of a
267 mutant with no fitness penalty. A recent development in QoI chemistry has been the discovery of
268 molecules like metarylpicoxamid which are almost unaffected by the G143A and F129L mutations
269 (Matsuzaki et al., 2019). Many recent patent applications also reveal ongoing interest from Ag companies
270 in new QoIs, particularly in compounds which control resistant soybean rust strains containing the F129L
271 mutation. As novel QoIs which overcome existing resistance are introduced, resistance is likely to
272 develop via new Q_o site mutations. The higher efficacy of picolinamides against Q_o site mutants suggests
273 their utility for resistance management in fungicide programs to slow the emergence of such mutants.

274 The introduction of picolinamides to control pathogens where a proportion of the population is already
275 resistant to strobilurins could lead to Q_i site resistance mutations in QoI-sensitive, QoI-resistant or both
276 genotypes. Analysis of yeast mutants containing both Q_o and Q_i site changes showed an increased growth
277 penalty for most double mutants as compared to single mutants. This was true for all combinations of Q_i
278 site mutation with F129L and for the combination of N31K or G37V with G143A. L198F and G37C had
279 little effect on Dt in single mutants, and were the only Q_i site changes which in combination with G143A
280 did not produce a statistically significant growth penalty. Double mutants containing G37C/V or L198F
281 were more sensitive to both CAS-649 and azoxystrobin than corresponding single mutants. The combined
282 effects of increased growth penalty for most double mutants and lower resistance factors suggest that
283 resistance to picolinamides may be more likely to emerge in strobilurin-sensitive than in resistant
284 populations. Nevertheless, strains resistant to both picolinamides and strobilurins could develop if the
285 individual Q_i and Q_o site mutations carried little or no fitness penalty. Assuming a greater fitness penalty
286 for F129L than for G143A, double mutants resistant to both QoI and QiI fungicides seem less likely to
287 appear in pathogens where F129L is the cause of strobilurin resistance than in those where it results from
288 the G143A change.

289 The mechanism by which a mutation at the Q_i or Q_o site increases sensitivity to an inhibitor acting at
290 the opposite site is unclear. However, the observed correlation between increased sensitivity and growth
291 penalty, and lack of such correlation for cycoheximide, strongly suggests the effect is specific to
292 cytochrome bc_1 inhibitors. The simplest explanation is that the increased sensitivity of Q_o mutants to
293 CAS-649, and of Q_i mutants to azoxystrobin, results from decreased activity of the cytochrome bc_1
294 complex making the cell more dependent on the residual activity and consequently more sensitive to
295 inhibitors. An alternative explanation is based on interactions between the Q_o and Q_i sites (Cooley et al.,
296 2009; Cooley, 2010). It was reported that in yeast, when stigmatellin was bound at the Q_o site, only one of
297 the two Q_i sites was accessible to the Q_i site inhibitor antimycin and that a conformational change was
298 needed to enable binding to the remaining Q_i site (Covian and Trumpower, 2006). Also, inhibitor binding
299 or mutations at the Q_i site of *Rhodobacter capsulatus* have been shown to increase susceptibility of the

300 Rieske iron-sulfur protein to proteolysis (Valkova-Valchanova et al., 2000) and affect the interaction
301 between the Rieske protein and the Q_o site (Cooley et al., 2005). Thus, despite their locations on opposite
302 sides of the membrane there is communication between the Q_o and Q_i sites involving conformational
303 change which could be affected by mutations and result in altered inhibitor sensitivity. These two possible
304 explanations are not mutually exclusive and detailed biochemical studies will be needed to explore the
305 underlying mechanism(s) responsible for the observed mutation effects.

306 An important question is the extent to which results from a yeast model will translate to plant
307 pathogens. Cytochrome *bc*₁ is highly conserved across eukaryotic organisms and although some
308 mechanistic details remain unclear, it can be assumed to function similarly in yeast and in fungal
309 pathogens. Consequently, in pathogens similar effects of mutations at the Q_i or Q_o site on sensitivity to
310 inhibitors acting at the opposite site are expected. This is supported by analysis of *Z. tritici* lab mutants
311 containing the G37V Q_i site change, where negative cross-resistance was found between azoxystrobin
312 and the picolinamide UK-2A for G37V and G143A mutants (Fouché et al., 2022). Also, as in the current
313 yeast work, *Z. tritici* double mutants with G37V and G143A had lower resistance factors for both Q_iIs
314 and Q_oIs. The fact that this could be observed in *Z. tritici* where G143A is not generally believed to carry
315 a fitness penalty in the field is noteworthy. The slight increase in sensitivity to picolinamides in G143A
316 mutants of both yeast (Fig. 1B) and *Z. tritici* (Fouché et al., 2022) may reflect a small effect of the G143A
317 mutation on cytochrome *bc*₁ activity which did not result in a detectable effect on growth.

318 QoI and QiI fungicides are also used to control Oomycete diseases and resistance has emerged to both
319 fungicide classes. In grape downy mildew, resistance to the QiI cyazofamid is associated with L201S and
320 with two insertions, E203-DE-V204 and E203-VE-V204 (R4P, 2020). Another Q_i site mutation, S34L,
321 confers resistance to ametoctradin (Fontaine et al., 2019), which is believed to act at both the Q_o and Q_i
322 sites (Dreinert et al., 2018). These mutations are found in some of the same populations of *Plasmopara*
323 *viticola* where strobilurin-resistance exists due to the G143A mutation. In *P. viticola*, the G143A mutation
324 does appear to cause some loss in fitness since QoI-resistant strains have been shown to revert to
325 sensitivity in the absence of selection pressure from strobilurins (Genet et al., 2006). To our knowledge,

326 the combination of G143A with Q_i site resistance mutations in the same strain has not yet been reported
327 in *P. viticola*. Considering the lower growth rates observed for Q_o/Q_i double mutants in yeast, it will be
328 interesting to see whether or not double mutants do eventually contribute to resistance problems in grape
329 downy mildew in the field since a greater fitness penalty could be expected.

330 The picolinamide Q_i fungicides provide a new mode of action against many ascomycete and
331 basidiomycete pathogens and it is imperative to preserve their efficacy for as long as possible by
332 implementing appropriate resistance management strategies. One element of resistance management is the
333 use of mixtures or alternation with fungicides which have a different mode of action to reduce selection
334 pressure. The present studies in yeast reveal specific interactions between Q_o and Q_i site mutations which
335 could be exploited through the use of picolinamide-strobilurin mixtures or alternation for resistance
336 management.

337

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341

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345

346 **Supplementary material**

347 Supplementary data may be found in the online version of this article.

348

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- 422

423 **FIGURE LEGENDS**

424

425 **Fig. 1.** Effect of Q_o site amino acid exchange on doubling time (A) and sensitivity to CAS-649 (B). Data
426 are mean values \pm SD. Different letters next to bars indicate a significant difference between strains ($\alpha =$
427 0.05).

428

429 **Fig. 2.** Correlation between growth rate of Q_o and Q_i site mutants and their sensitivity to fungicides
430 acting at the opposite site. Circles represent growth rate of Q_o site mutants and their sensitivity to CAS-
431 649; crosses represent growth rate of Q_i site mutants and their sensitivity to azoxystrobin. R² values were
432 determined using an exponential growth nonlinear regression model.

433

434 **Fig. 3.** Effect of Q_i site amino acid exchange on doubling time (A) and sensitivity to azoxystrobin (B).
435 Data are mean values \pm SD. Different letters next to bars indicate a significant difference between strains
436 ($\alpha = 0.05$).

437

438 **Fig. 4.** Doubling time of mutants containing Q_i site mutations, with and without the Q_o site mutations
439 G143A and F129L. Data are mean values \pm SD. Asterisks indicate statistically significant differences
440 between double mutants with G143A or F129L and strains with no Q_o site mutation and the
441 corresponding Q_i site allele (Student's t-test, $P = < 0.001$).

442

443

444 **Table 1**445 Sensitivity of cytochrome *b* mutants with Q_o site amino acid exchange to CAS-649 and azoxystrobin.

446

Amino Acid Change	CAS-649 EC50 ± SD (µg ml ⁻¹)	Azoxystrobin EC50 ± SD (µg ml ⁻¹)
None	0.0082 ± 0.0020	0.0076 ± 0.001
G143A	0.0054 ± 0.0008	16.30 ± 1.95
F129L	0.0028 ± 0.0002	0.38 ± 0.05
F129S	0.0022 ± 0.0001	0.052 ± 0.005
L275S	0.0017 ± 0.0005	0.071 ± 0.003
G143S	0.0013 ± 0.0006	>20
Y132C	0.0011 ± 0.0005	0.036 ± 0.01
Y279S	0.0011 ± 0.0003	0.042 ± 0.007

447

448

449 **Table 2**450 Sensitivity of cytochrome *b* mutants with Q_i site amino acid exchange to azoxystrobin and CAS-649.

451

Amino Acid Change	Azoxystrobin EC50 ± SD (µg ml ⁻¹)	CAS-649 EC50 ± SD (µg ml ⁻¹)
None	0.0076 ± 0.0010	0.0082 ± 0.0020
L198V	0.0095 ± 0.0017	0.0089 ± 0.0006
L198F	0.0059 ± 0.0011	0.22 ± 0.05
G37A	0.0058 ± 0.0011	0.069 ± 0.006
G37C	0.0027 ± 0.0002	0.21 ± 0.02
L198G	0.0024 ± 0.0008	0.0063 ± 0.0006
N31G	0.0013 ± 0.0001	0.047 ± 0.015
N31K	0.0009 ± 0.0001	1.96 ± 0.22
N31S	0.0008 ± 0.0001	0.022 ± 0.003
G37V	0.0005 ± 0.0001	0.45 ± 0.12

452

453

454

455 **Table 3**

456 Resistance factors for CAS-649 in single Qi mutants and corresponding double (Qi + Qo) mutants

457 containing G143A or F129L.

458

Qi site change	Qo site change	Resistance Factor \pm SD
none	none	1.00 \pm 0.24
none	G143A	0.67 \pm 0.10*
none	F129L	0.35 \pm 0.03*
L198F	none	26.63 \pm 6.00
L198F	G143A	13.30 \pm 3.08*
L198F	F129L	5.81 \pm 0.78*
G37C	none	26.32 \pm 2.57
G37C	G143A	16.05 \pm 2.38*
G37C	F129L	12.90 \pm 4.43*
G37V	none	54.72 \pm 14.48
G37V	G143A	21.04 \pm 6.05*
G37V	F129L	21.60 \pm 6.95*
N31K	none	240.3 \pm 26.5
N31K	G143A	220.1 \pm 41.3 NS
N31K	F129L	260.9 \pm 56.2 NS

Asterisks indicate statistically significant differences in resistance factor between the double mutants with G143A or F129L and the corresponding strain with no Qo site mutation (Student's t-test, $P = < 0.001$). NS = not significant.

459

Table 4

Resistance factors for azoxystrobin in single Qo mutants and corresponding double (Qo + Qi) mutants containing L198F, G37C/V and N31K.

Qo site change	Qi site change	Resistance Factor \pm SD
none	none	1.00 \pm 0.13
none	L198F	0.77 \pm 0.14 NS
none	G37C	0.35 \pm 0.03*
none	G37V	0.061 \pm 0.01*
none	N31K	0.12 \pm 0.02 *
G143A	none	2139.4 \pm 255.9
G143A	L198F	1412.4 \pm 250.9*
G143A	G37C	487.5 \pm 101.3*
G143A	G37V	157.9 \pm 35.4*
G143A	N31K	427.5 \pm 174.3*
F129L	none	49.80 \pm 6.76
F129L	L198F	33.61 \pm 3.98*
F129L	G37C	28.61 \pm 6.19*
F129L	G37V	7.80 \pm 5.17*
F129L	N31K	17.95 \pm 7.16*

460

461 Asterisks indicate statistically significant differences in resistance factor between the double mutants with

462 L198F, G37C/V and N31K and the corresponding strain with no Qi site mutation (Student's t-test, P = <

463 0.001).

464