



Identification of Copper-Containing Oxidoreductases in the Secretomes of Three Colletotrichum Species with a Focus on Copper Radical Oxidases for the Biocatalytic Production of Fatty Aldehydes

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David Ribeaucourt, Safwan Saker, David Navarro, Bastien Bissaro, Elodie Drula, et al.. Identification of Copper-Containing Oxidoreductases in the Secretomes of Three Colletotrichum Species with a Focus on Copper Radical Oxidases for the Biocatalytic Production of Fatty Aldehydes. *Applied and Environmental Microbiology*, 2021, 87 (24), pp.e01526-21. <10.1128/AEM.01526-21>. <hal-03619439>

HAL Id: hal-03619439

<https://hal.science/hal-03619439v1>

Submitted on 5 May 2022

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1 **Identification of copper-containing oxidoreductases in the secretomes of three**
2 ***Colletotrichum* species with a focus on copper radical oxidases for the**
3 **biocatalytic production of fatty aldehydes**

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24 ABSTRACT

25 Copper Radical Alcohol Oxidases (CRO-AlcOx), which have been recently discovered among
26 fungal phytopathogens are attractive for the production of fragrant fatty aldehydes. With the
27 initial objective to investigate the secretion of CRO-AlcOx by natural fungal strains, we
28 undertook time-course analyses of the secretomes of three *Colletotrichum* species (*C.*
29 *graminicola*, *C. tabacum* and *C. destructivum*) using proteomics. The addition of a copper-
30 manganese-ethanol mixture in absence of any plant-biomass mimicking compounds to
31 *Colletotrichum* cultures unexpectedly induced the secretion of up to 400 proteins, 29-52% of
32 which were carbohydrate-active enzymes (CAZymes), including a wide diversity of copper-
33 containing oxidoreductases from the auxiliary activities (AA) class (AA1, AA3, AA5, AA7,
34 AA9, AA11-AA13, AA16). Under these specific conditions, while a CRO-glyoxal oxidase from
35 the AA5_1 subfamily was among the most abundantly secreted proteins, the targeted AA5_2
36 CRO-AlcOx were secreted at lower levels, suggesting heterologous expression as a more
37 promising strategy for CRO-AlcOx production and utilization. *C. tabacum* and *C. destructivum*
38 CRO-AlcOx were thus expressed in *Pichia pastoris* and their preference toward both aromatic
39 and aliphatic primary alcohols was assessed. The CRO-AlcOx from *C. destructivum* was further
40 investigated in applied settings, revealing a full conversion of C6 and C8 alcohols into their
41 corresponding fragrant aldehydes.

43 IMPORTANCE

44 In the context of the industrial shift toward greener processes, the biocatalytic production of
45 aldehydes is of utmost interest owing to their importance for their use as flavors and fragrances
46 ingredients. CRO-AlcOx have the potential to become platform enzymes for the oxidation of

47 alcohols to aldehydes. However, the secretion of CRO-AlcOx by natural fungal strains has never
48 been explored, while the use of crude fungal secretomes is an appealing approach for industrial
49 application to alleviate various costs pertaining to biocatalysts production. While investigating
50 this primary objective, the secretomics studies revealed unexpected results showing that under
51 the oxidative-stressful conditions we probed, *Colletotrichum* species can secrete a broad
52 diversity of copper-containing enzymes (laccases, sugar oxidoreductases, LPMOs) usually
53 assigned to “plant-cell wall degradation”, despite the absence of any plant-biomass mimicking
54 compound, and only little amount of CRO-AlcOx were secreted, pointing out at recombinant
55 expression as the most promising path for their biocatalytic application.

56

57 **KEYWORDS:** Copper radical oxidase; CAZymes; Copper-containing enzymes; Alcohol
58 oxidase; Filamentous fungi; Phytopathogen; *Colletotrichum*; Secretomics; Flavors and
59 fragrances.

60

61 INTRODUCTION

62 The development of a new technological paradigm to support the shift from a fossil-
63 based economy to a greener and more circular economy is at the heart of today’s bioeconomic
64 challenges (1). Biotechnology is one of the main levers in this ongoing industrial transformation
65 (2). The development of biotechnology largely relies on biocatalysis and thus require the
66 discovery, control and engineering of a variety of enzymes and microorganisms (3). The fungal
67 kingdom represents a vast and untapped reservoir of biocatalysts (4) but to date, the enzymatic
68 potential of filamentous fungi has mostly been studied in the frame of plant cell wall degradation
69 (5–7). Fungal secretomics is a powerful approach to study the wide diversity of enzymes
70 secreted and to formulate enzymatic cocktails with biotechnological relevance (8–10). Using

71 suitable inducers, some fungal strains can secrete high amounts of oxidoreductases (11)
72 including copper-containing enzymes. For instance, hypersecretory fungal strains from the
73 *Pycnoporus* genus have been selected to produce laccases and use them in applied settings (12,
74 13).

75 Most of the copper-dependent enzymes are classified within the Auxiliary Activities
76 (AA) class (14) in the CAZy database (www.cazy.org) (15). The founding member of copper
77 radical oxidases (CROs), a galactose oxidase (*FgrGalOx* – EC 1.1.3.9), was discovered in 1959
78 from the secretome of *Fusarium graminearum* (16, 17). In 2013, fungal CROs have been
79 classified within the AA5 family and further classified into subfamilies AA5_1 and AA5_2.
80 AA5_1 contains glyoxal oxidases (GLOX - EC 1.2.3.15) that catalyze the oxidation of aldehydes
81 to carboxylic acids, and AA5_2 gathers enzymes that oxidize the alcohol function of diverse
82 substrates (e.g., galactose, galactose-containing polysaccharides, aromatic and aliphatic primary
83 alcohols) to generate the corresponding aldehydes. More recently, the AA5_2 subfamily was
84 shown to be more diverse than expected, encompassing notably various alcohol oxidases (CRO-
85 AlcOx; EC 1.1.3.13) originating from the phytopathogen ascomycete fungi *Colletotrichum* and
86 *Magnaporthe* spp. (18–20). CRO-AlcOx are monocopper, and otherwise organic co-factor free
87 enzymes that catalyze the oxidation of alcohols into aldehydes (with the concomitant reduction
88 of O₂ to H₂O₂). They offer appealing perspectives to produce aldehydes (21), which are
89 indispensable intermediates in many chemical pathways (22, 23) and valuable ingredients for the
90 flavor and fragrance industries (24, 25). In particular, saturated long-chain aldehydes (fatty
91 aldehydes) are widely used for their citrus scent (26, 27), but are currently derived from fossil-
92 based chemistry (28, 29) or extracted from natural plant materials, the supply of which is

threatened by emerging diseases (30, 31). Therefore, alternative green and/or natural production pathways must be investigated (32).

To date, only pure recombinant forms of CRO-AlcOx have been harnessed in biocatalytic applications (18, 19, 21, 33) and no information on their secretion by natural fungal strains is available. Determining whether CRO-AlcOx encoding fungal strains can secrete these enzymes under laboratory conditions is of strong interest for enzyme selection and technological applications. Noteworthy, aside from the scope of pathogenesis (34–36), very few studies investigated the enzymatic biotechnological potential of *Colletotrichum* spp. (37–40). The use of natural fungal strains could be advantageous to identify and then use potential naturally secreted enzyme partners. In particular, catalase and peroxidase are both required to sustain *in vitro* the activity of isolated CRO-AlcOx and other AA5 (21, 33, 41, 42). Catalase acts as a H₂O₂ scavenger while peroxidase is required for CRO-AlcOx activation (20, 33).

In this context, we investigated the ability of several fungal strains from the *Colletotrichum* genus to secrete CRO-AlcOx. While pursuing this primary goal, we observed the unexpected secretion of a large panel of plant-biomass degrading oxidoreductases and chitin-related enzymes under oxidative stress conditions and in absence of any plant-cell wall inducers. We report here an integrated study, starting from fungal time-course secretomics, analysis of copper-containing oxidoreductases secretion profiles, with a final focus on the recombinant CRO-AlcOx production and characterization, and biocatalytic application of these enzymes to produce fatty aldehydes for the flavor and fragrance industries.

RESULTS

Selection of fungal strains and time-course secretomic study

116 To investigate the secretion of copper-containing enzymes including AA5_2 CRO-AlcOx
117 by natural fungal strains and their potential use for bioconversion of fatty alcohols to fragrant
118 aldehydes, we selected three fungal phytopathogens from the *Colletotrichum* genus for which
119 genomic information is available. The first obvious choice was *C. graminicola* because the three
120 AA5_2 it encodes have all been previously biochemically characterized (18, 43, 44), one of them
121 being the first, most studied, and to date, most active CRO-AlcOx, namely *CgrAlcOx* (18, 21,
122 33). By performing a BLAST analysis of the *CgrAlcOx* sequence against the genomes of some
123 other *Colletotrichum* species we had access to, we identified two genes encoding AA5_2 in both
124 *C. tabacum* and *C. destructivum*. For each strain, one gene encoded a putative AlcOx and the
125 other one a putative aryl alcohol oxidase (AAO – based on the recent discovery of an AAO in the
126 AA5_2 family (44)) (Figure S1). These enzymes will thereafter be named *CtaAlcOx/CdeAlcOx*
127 and *CtaAAO/CdeAAO* for *C. tabacum* and *C. destructivum*, respectively. Based on the few
128 studies reporting the detection in some Ascomycota fungal secretomes of a range of copper-
129 containing enzymes from the AA class including the *FgrGalOx* and other AA5 (45–47), we
130 identified copper, manganese and ethanol as possible inducers of AA5. Copper is known to be
131 crucial to activate the *FgrGalOx* and was used previously in combination with manganese to
132 recover active *FgrGalOx* from fungal cultures (47). Ethanol is often used as an inducer of
133 laccases (12) (other copper-containing AAs), and we noticed the presence of CROs in some
134 secretomes induced with ethanol (45). We therefore elaborated two liquid growth media: (i)
135 “YG” (for “Yeast-extract, Glucose”), and (ii) “YG-CME” (for “YG-Copper, Ethanol,
136 Manganese”), with the same composition as YG but supplemented with CuSO₄, manganese and
137 ethanol. A time-course analysis was performed on each strain in both media at three time points
138 (day 3, day 7 and day 14) and proteomic analyses were performed on the 16 secretomes

139 generated (note that no samples were harvested at day 3 for *C. graminicola* in both YG and YG-
140 CME due to insufficient growth).

141 The quantification of total proteins revealed an increasing amount of proteins over time
142 (except for *C. destructivum*) (Figure S2) while capillary electrophoresis analysis revealed distinct
143 profiles over time and between fungal strains (Figure 1).

144 By performing proteomic analyses of the secretomes using LC-MS/MS and mass
145 matching, we detected a high number of secreted proteins (between 150 and over 400 different
146 proteins, depending on the culture conditions; Supplementary Data Set; Figure 2A)
147 encompassing a large diversity of enzymes. CAZymes accounted for ca. 29-52% of the total
148 protein abundance in each secretome (Figure S3), with a notable high diversity of enzyme from
149 the AA class (Figure 2B). Among them, a variety of laccases (AA1), peroxidases (AA2), flavo-
150 oxidases (AA3) including cellobiose dehydrogenases (AA3_1-AA8), CROs (AA5),
151 oligosaccharide oxidases (AA7), lytic polysaccharide monooxygenases (LPMOs; AA9, AA11,
152 AA13, AA16) and pyrroloquinoline quinone-dependent oxidoreductase (AA12) were detected.
153 To determine whether this high number and diversity of proteins detected was the results of cell
154 lysis (7, 48), we evaluated the proportion of proteins predicted as not secreted – i.e., without
155 signal peptide – and checked more specifically the abundance of some known intracellular
156 proteins (i.e., DNA-polymerase, proteasome endopeptidase complex, citrate synthase, malate
157 dehydrogenase, transketolase and ubiquitin-protein ligase) (Figure S4). Despite the significant
158 number of proteins predicted without signal peptide, we did not observe any clear trend that
159 would account for an accumulation of intracellular proteins in the secretomes over time. Notably,
160 some proteins without signal peptide are known to be secreted through unconventional secretion
161 pathways in fungi (48, 49).

Effect of Copper-Manganese-Ethanol supplementation on the secretome profiles

We then looked at the effect of supplementation with CME. At first glance, no clear effect was distinguishable on CAZymes diversity (Figures 2 & S3) but when we focused on copper-containing enzymes (Figure 3), a clear trend became apparent. Indeed, copper-containing enzymes were generally more abundant in the CME medium with a diversity of oxidases from the AA class including AA1 laccases, AA5_1 GLOX and AA9, AA11, AA13 and AA16 LPMOs. Furthermore, AA1_3, AA5_1 and AA9 were among the top-five most abundant enzymes in YG-CME for the *C. tabacum* and *C. destructivum* secretomes, while no AA enzymes were identified in the top-five ranking for the corresponding YG secretomes (Table S1). Remarkably, the presence of some AA9-CBM18 was detected in both the *C. tabacum* and *C. destructivum* secretomes (Figure 3). This association of a chitin-binding domain from the CBM18 family (mainly found associated to chitin-active enzymes) to an AA9 domain is intriguing since AA9 LPMOs have not yet been described to act on chitin substrates. Strikingly AA9 LPMOs were particularly abundant at day 3 in YG medium for *C. destructivum* while drastically decreasing at day 7 and 14 (Figure 3) as already observed in the secretomes of the basidiomycete fungus *Laetisaria arvalis* (50). Interestingly, *C. tabacum* AA9 LPMOs were also found in great abundancy at all time points in the YG-CME conditions.

In addition, a diversity of Glycoside Hydrolases (GH), Polysaccharide Lyases (PL), Carbohydrate Esterase (CE) and AAs were detected exclusively in the CME medium (Figure 4). Among these enzymes, we noticed the intriguing presence of hydrolases (GH18, GH18-CBM18), oxidases (AA7, AA11) and esterases (CE4-CBM18) potentially active on chitin or chito-oligosaccharides. Remarkably, a multimodular protein with five consecutive CBM50

185 chitin-binding domains (“CBM50 (x5)” in Figure 4) but without any catalytic domain was
186 detected in both the *C. tabacum* and *C. destructivum* secretomes. This protein is analogous to
187 other chitin-binding proteins (i.e., LysM effector proteins) (see discussion).

188 **Presence of AA5 in the secretomes**

189 We next focused our analysis on AA5 CROs. Interestingly, a putative GLOX from the
190 AA5_1 subfamily was detected in *C. destructivum* secretomes (Figure 5), being the most
191 abundant protein in the YG-CME secretome at day 7 (and the second most abundant at day 14;
192 Table S1). The *C. tabacum* AA5_1 putative GLOX was also the third most abundant protein in
193 the YG-CME secretome at day 14 (Table S1). We could also detect the occurrence of putative
194 AlcOx and AAO from the AA5_2 subfamily in both *C. graminicola* and *C. tabacum* secretomes
195 (Figure 5). However, their relative abundance was quite low (between 0.05-0.13% of the total
196 Protein Abundance Index (PAI)) and they did not accumulate over time. No correlation with the
197 presence of CME in the medium was seen except for the *C. graminicola* AAO. CROs are known
198 to be activated by peroxidases (41, 51, 52). More specifically, the peroxidase partner responsible
199 for the activation of CRO-AlcOx has been recently unveiled in fungal pathogens (20). Both
200 genes occur as a tandem in the genomes of the *Colletotrichum* and *Magnaporthe* species
201 harboring an AlcOx, and are co-expressed *in vivo* prior to the plant-penetration stage (20). While
202 some peroxidases from the AA2 family (see Figure 2) were found in some of the secretomes, the
203 specific peroxidase partner of the AlcOx was not detected reinforcing the authors claims that
204 interaction with plant is needed for genes co-expression (20).

205 Together, these data provide unexpected information on the induction of secreted copper-
206 enzymes in *Colletotrichum* species. However, the low level (or even the absence for *C.*
207 *destructivum*) of AA5_2 detected under these experimental conditions indicates that the direct

208 implementation of such secretomes for the bioconversion of fatty alcohols is not a viable
209 strategy. This was confirmed by preliminary conversion assays we carried out using the
210 secretomes directly on a range of primary alcohols (data not shown). These tests were
211 inconclusive, most likely due to the low level of AA5_2 secreted and were difficult to interpret
212 given the complexity of the secretome samples (hundreds of enzymes, including various
213 oxidases such as AA3 enzymes – Figure 2B). These results prompted us to produce the
214 *Colletotrichum* CRO-AlcOx enzymes recombinantly in *Pichia pastoris* and to test their ability to
215 convert alcohols of interest for the flavor and fragrance industries. The objective was to find
216 CRO-AlcOx with potential interesting traits not predictable by sequence analysis such as (i)
217 activity on unactivated fatty alcohols, (ii) minimized requirement in accessory enzymes or (iii)
218 reduced overoxidative activity.

219 **Recombinant production and substrate specificity of *Cde*AlcOx and *Cta*AlcOx**

220 Detailed examination of the *Cde*AlcOx and *Cta*AlcOx sequences showed that the two
221 AlcOx amino acid sequences share 90% identity with the *Cgr*AlcOx and likewise comprise a
222 single catalytic module, as with all other characterized CRO-AlcOx from *Colletotrichum* spp.
223 (Figure S1). The two AAO share 85% identity with the recently characterized *Cgr*AAO (44) and
224 possess a N-terminal PAN_1 domain, in addition to the catalytic module, similarly to the PAN_1
225 domain observed in the *Cgr*AAO (Figure S1). The function of the PAN_1 domain in AA5_2-
226 AAOs is unclear but such modules are known to mediate protein-protein or protein-carbohydrate
227 interactions (53). We successfully produced both CRO-AlcOx in *P. pastoris* (Figure S5) with
228 high titers for *Cta*AlcOx in flask culture (200 mg of purified enzyme per liter of culture)
229 compared to *Cde*AlcOx (75 mg.L⁻¹) and *Cgr*AlcOx (35 mg.L⁻¹). Activity screening using the
230 ABTS/HRP assay, with benzyl alcohol (BnOH - often use as benchmark with CRO-AlcOx (18,

231 21, 44)) used as reference, confirmed that both enzymes are indeed AlcOx and that they show
232 similar substrate specificities compared to the canonical *CgrAlcOx* (Figure 6). However,
233 *CdeAlcOx* displayed a slightly higher specific activity on the secondary alcohol butan-2-ol, and
234 on the longer aliphatic substrate decan-1-ol. This latter observation could be related to the
235 M173L substitution compared to *CgrAlcOx*, that could create a slightly more hydrophobic
236 environment in the active site, favoring the accommodation of bulky alcohol substrates (Figure
237 S6). The pH-rate profiles determined for *CdeAlcOx* and *CtaAlcOx* (Figure S7), revealed pH
238 optima around 7.5 – 8 for both enzymes. For subsequent conversion assays and comparison with
239 *CgrAlcOx*, only *CdeAlcOx* was retained because *CtaAlcOx* exhibited no noticeable differences
240 in terms of substrate specificity. Nevertheless, *CtaAlcOx* remains attractive from a
241 biotechnological point of view, being 5.7-fold more highly expressed than the *CgrAlcOx* in flask
242 cultures.

243 **Biocatalytic production of odorant aldehydes using *CdeAlcOx***

244 *CdeAlcOx* was subsequently probed for the conversion of hexan-1-ol and octan-1-ol (two
245 precursors of fragrant aldehydes) using *CgrAlcOx* as a benchmark. For these experiments, we
246 tested the impact of the reaction time (15 minutes *versus* 16 hours conversion) and the addition
247 of accessory enzymes (HRP and catalase) according to the parameters determined in a previous
248 study (21). Conversion results showed that *CdeAlcOx* behave similarly to *CgrAlcOx* with all the
249 tested substrates (Figure 7). As previously observed with *CgrAlcOx* (21, 33), *CdeAlcOx* alone
250 was unable to promote full alcohol conversion. However, when both HRP and catalase were
251 added to the reaction mixture, full conversion was readily achieved in 15 minutes. Strikingly, the
252 sole addition of catalase to the reaction mixture resulted in a significant improvement in substrate

253 conversion that probably cannot be attributed only to its protective effect against H_2O_2 . This may
254 suggest an activating effect of catalase on CRO-AlcOx, similar to that of HRP.

255 We have previously demonstrated that upon long reaction times (16 hours), partial
256 overoxidation of fatty aldehydes $>\text{C}_6$ was observed due to HRP, most likely through a non-
257 enzymatic mechanism (Figure 7A) (21). Carboxylic acids are undesired products in the frame of
258 the F&F application targeted here, but are valuable molecules in other fields (54). In agreement
259 with our previous study (21), we observed here that carboxylic acid was not noticeable with
260 hexan-1-ol as substrate (Figure 7B), but was observed after 16 hours of conversion of octan-1-ol,
261 only when HRP was present in the reaction (Figure 7C).

262 We also observed previously (21) that in the case of benzaldehyde derivatives (i.e. 4-
263 NO_2 -benzyl alcohol) some overoxidation was detected but mediated by the *CgrAlcOx* itself
264 (Scheme S1) and dependent according to the propension of the aldehyde product to undergo
265 hydration. To probe whether *CdeAlcOx* behave in the same way as *CgrAlcOx* with 4- NO_2 -
266 benzyl alcohol, we included this substrate in the panel of compounds assayed (Figure S8). The
267 conversion of 4- NO_2 -benzyl alcohol indeed led to the corresponding acid after 16 hours of
268 reaction even when HRP is omitted from the reaction (Figure S8). Altogether, these results
269 confirm that the behavior observed for the *CgrAlcOx* regarding accessory enzymes and
270 overoxidation (21) applies to other CRO-AlcOx enzymes.

271

272

273 DISCUSSION

274 The time-resolved proteomic analyses performed here on *Colletotrichum* secretomes
275 revealed interesting and unexpected results. We detected up to ca. 400 individual enzymes in the
276 secretomes, with the maximum number found in the YG-CME medium, while former studies by

our group usually yielded ca. 80 – 250 secreted proteins (8, 10, 55, 56). The high proportion of CAZymes in the secretomes is surprising considering the absence of any inducer to mimic plant cell walls. Strikingly, the addition of CME to the medium seemed to boost the secretion of AA enzymes. Moreover, our finding that some LPMOs (especially AA9) preferentially accumulated in the CME medium, in the absence of any plant biomass raises questions about the molecular basis of the regulatory pathways driving their production, and possibly their true biological function (57). In line with this observation, recent work suggests that some fungal LPMOs and LPMO-like proteins may be involved in biological functions distinct from plant biomass conversion, i.e., copper homeostasis and pathogenicity (58, 59) as well as fungal cell fusion (60). Other copper-containing AA enzymes exhibited higher accumulation in YG-CME cultures. Induction of the secretion of laccases could either be due to the addition of ethanol to the medium (12), or a response to oxidative stress (61). Indeed, it is known that at high concentrations, copper contributes to generate reactive oxygen species by Fenton chemistry (62, 63).

The proteins secreted exclusively in YG-CME medium revealed a range of chitin-active enzymes, as well as non-enzymatic chitin-binding proteins (LysM proteins) some of which are released by fungal cells upon interaction with their plant hosts, to conceal their presence from the host immune system by preventing fungal chitin recognition by the plant or protecting fungal hyphae against plant chitinases (64–66). In the absence of the host or of any plant biomass, this observation suggests that the high concentration of CME in the medium could trigger some pathways related to pathogenesis. The presence of chitin active enzymes may also be indicative of a nutrient recycling in older senescing portions of the mycelium or of some fungal cell wall remodeling activity (67).

300 The relatively high abundance of GLOX from the AA5_1 subfamily in some YG-CME
301 secretomes and the low secretion levels of AA5_2 AlcOx suggest distinct biological functions
302 for these two types of CRO. AA5_1 GLOX are often described as enzymes acting on lignin in
303 cooperation with some peroxidases in wood-decaying fungi (68). However, their high abundance
304 here, under CME conditions, despite the absence of lignin points toward a possible additional
305 biological function of GLOX in Ascomycota, related to filamentous growth or pathogenicity, as
306 already demonstrated for the AA5_1 GLOX from the maize pathogen *Ustilago maydis* (69).

307 The primary objective of this study was to investigate the secretion of CRO-AlcOx by
308 natural fungal strains with bioconversion potential. Under our experimental conditions, CRO-
309 AlcOx are only sporadically secreted. Of note, a separate study very recently investigated the
310 biological function of these enzymes and showed that they are co-secreted at low levels, over a
311 short period of time during penetration of the plant host cuticle, and spatially restricted to the
312 puncturing site (20). Thus, with hindsight, the secretion of large amount of CRO-AlcOx might be
313 difficult to achieve without a proper signal to mimic the presence of plant host. Another option
314 would be to identify and engineer the promotor region controlling co-expression of the genes
315 encoding both the AlcOx and the associated peroxidase. However, for biocatalytic purposes, the
316 recombinant expression of CRO-AlcOx remains the most promising alternative. We here
317 confirmed that the knowledge gathered on sequence-structure-function of AA5_2 (18, 21, 44),
318 can predict an AlcOx activity based on bioinformatic analysis and that various CRO-AlcOx can
319 be easily produced heterologously using the yeast *P. pastoris* as host. *CtaAlcOx* was produced to
320 high yield, which is promising for future upscaled production. The conversion assays performed
321 with *CdeAlcOx* revealed that this enzyme can be used as a biocatalyst for the conversion of fatty
322 alcohols. The behavior of *CdeAlcOx* in terms of overoxidation and accessory enzyme

323 requirements suggests that the traits observed here and previously (20, 21, 33) are probably valid
324 for all CRO-AlcOx.

325 In conclusion, the work carried out in this study highlights that *Colletotrichum* species
326 can secrete a range of copper-containing oxidoreductases from the AA class without any plant
327 cell wall inducer. While CRO-AlcOx were detected in some of the secretomes, the direct
328 utilization of fungal strains for bioconversion of fatty alcohols will require further efforts to
329 achieve a high secretion yield of CRO-AlcOx and a deeper understanding of the factors
330 controlling their secretion. The recombinant pathway therefore represents the most promising
331 approach to develop their use in biocatalytic applications for the production of fragrant fatty
332 aldehydes.

333

334 MATERIAL AND METHODS

335 General information

336 All chemicals used for cultivation of fungi and microorganisms were reagent grade or
337 higher. All chemicals used for biochemical assays were $\geq 95\%$ purity. HRP type II and catalase
338 from bovine liver were purchased from Sigma-Aldrich (Hamburg, Germany). Molar
339 concentrations of HRP (MW: 33.89 kDa) and catalase (MW of monomer: 62.5 kDa) were
340 estimated by Bradford assay using BSA as standard. Pre-cast 10% polyacrylamide gel Mini-
341 PROTEAN TGX were purchased from Bio-Rad (USA).

342 Data availability

343 The protein sequences of the *CdeAlcOx* and *CtaAlcOx* were deposited in GenBank under
344 the accession numbers MZ269520 and MZ269521 respectively.

345 Natural and recombinant strains

346 The following fungal strains were used: *C. graminicola* (M1.001, CBS 130836, (35)), *C.*
347 *destructivum* (LARS 709, CBS 520.97, (70)) and *C. tabacum* (N150, CPC 18945, (70)). The
348 DNA sequences of *CdeAlcOx* and *CtaAlcOx* were obtained by blasting against the “raw”
349 unannotated genomes of *C. destructivum* and *C. tabacum* and further intron prediction was done
350 using Augustus web tool (<http://bioinf.uni-greifswald.de/augustus/>). DNA cloning and
351 transformation of *P. pastoris* X33 with codon-optimized genes (for expression in *P. pastoris*)
352 encoding *CdeAlcOx* and *CtaAlcOx* was carried out as described previously for other
353 constructions (71). The recombinant *Pichia pastoris* X33 strains containing *CgrAlcOx* gene
354 (GenBank EFQ30446.1) was recovered from previous work (18, 21). The selection of the most
355 productive recombinant transformants was done through culture and expression in 24-deepwell

plates with subsequent purification from supernatant by affinity chromatography on Ni-NTA resin using an in-house automated procedure (71).

Fungal cultures and secretomes harvesting

Fungal strains were grown and let sporulated on Potato Dextrose Agar medium (PDA) plates in closed Petri dishes, at 23°C for seven days under natural solar light, without controlling the humidity and were stored at 4°C before utilization. Inocula were prepared by aseptically homogenizing the content of a 90 mm petri dish cultures of *Colletotrichum* (containing fungal biomass (both mycelia and spores) and PDA medium) using an Ultra Turrax T25 homogenizer (IKA, Germany) for 1 min at 9,500 rpm in 80 mL of the culture medium (see below). 200 µL of this homogenate was used to inoculate 100 mL of liquid medium placed in 500 mL baffled flask.

Liquid cultures were grown in two different media, namely: “YG” and “YG-CME”. YG medium was composed of glucose (10 g.L⁻¹), ammonium tartrate (2 g.L⁻¹), yeast extract (1 g.L⁻¹), K₂HPO₄ (1 g.L⁻¹), MgSO₄ • 7H₂O (0.5 g.L⁻¹), KCl (0.5 g.L⁻¹) and 0.5 mL.L⁻¹ of trace elements stock solution composed of (NH₄)₆Mo₇O₂₄•4H₂O (20 mg.mL⁻¹), FeSO₄•7H₂O (100 mg.mL⁻¹), ZnSO₄•7H₂O (140 mg.mL⁻¹), B₄O₇Na₂•10H₂O (200 mg.mL⁻¹). Glucose was autoclaved separately as a 10X stock solution. The trace elements stock solution was sterilized by filtration through a 0.22 µm polyether sulfone membrane (Merck-Millipore, Germany). Note that insoluble elements present in this solution were removed by the filtration step. YG-CME medium was prepared exactly as YG but was further supplemented with CuSO₄•5H₂O (500 µM), MnCl₂ (500 µM) and 9 g.L⁻¹ ethanol, filtered sterilized (except for ethanol).

The inoculated cultures were placed in an Innova 42R incubator (New Brunswick, USA) and let grown at 25°C under natural light and shaking (at 150 rpm for *C. destructivum* and 105

rpm for *C. tabacum* and *C. graminicola*). The secretomes were harvested at day 3, day 7 and day 14 after inoculation. All samples were prepared in triplicates which were pooled upon harvesting. Each harvested secretome was submitted to several filtration steps: (i) Miracloth (22-25 μm pore size) (Merck-Millipore), (ii) several glass microfiber filters used sequentially: 47 mm Whatman® grade GF/D, A and F (GE Healthcare, USA), (iii) 0.45 and 0.22- μm polyether sulfone membranes (Merck -Millipore) and finally diafiltered onto a polyether sulfone membrane with a 10kDa cut-off (Vivaspin, Sartorius, Göttingen, Germany) with 50 mM sodium phosphate buffer pH 7.0 to a final volume of 1-7 mL. The secretomes were stored at 4°C until use. Their total protein concentration was evaluated by the Bradford method (Protein Assay, BioRad, France) using a bovine serum albumin (BSA) standard protein.

389

390 **Microfluidic capillary gel electrophoresis**

391 The concentrated secretomes samples were diluted so that 87.5 μL of the initially
392 harvested secretomes (before concentration) were submitted to electrophoresis, except for *C.*
393 *tabacum* YG-CME day 14 which was further diluted by 1/3 to avoid too high protein
394 concentration. Samples were prepared in a 96-well plate using the manufacturer “HT Protein
395 Express Assay Sample Buffer” (PerkinElmer, USA) supplemented with 36.4 mM (final
396 concentration) of dithiothreitol. The samples were heated at 95°C for 10 min and subjected
397 directly to LabChip GX II capillary gel electrophoresis (PerkinElmer) according to the
398 manufacturer’s instructions. The electropherograms were analyzed using the LabChip GX II
399 software (PerkinElmer).

400

401 **LC-MS/MS protein identification**

402 For each secretome, 15 µg of proteins were loaded on a 10% Tris–glycine precast SDS-
403 PAGE gel (Mini-PRO-TEAN TGX, BioRad). After a short migration (0.5 cm) in the stacking
404 gel, the gels were stained with Coomassie blue (BioRad) and each electrophoresis track was cut
405 into two 2-mm-wide strips. Proteomic identification was performed at the Plateforme d'Analyse
406 Protéomique de Paris Sud-Ouest (PAPPSO, INRA, Jouy-en-Josas, France; <http://pappso.inra.fr/>),
407 according to the protocol described in (72). Briefly, the digestion of the proteins contained in the
408 gel strips was carried out according to a standard trypsinolysis process, using modified trypsin
409 (Promega, France). Peptide analysis was performed by a NanoLC Ultra 2D system (Eksigent
410 Technologies, United States) coupled to a Q-exactive mass spectrometer (Thermo Fisher
411 Scientific, France) using electro-spray ionization. Peptide attribution and protein annotation were
412 performed by comparing mass spectrometry data to predicted proteins in the genomes of
413 *Colletotrichum graminicola* M1.001 (<https://mycocosm.jgi.doe.gov/Colgr1/Colgr1.home.html>)
414 and *Colletotrichum higginsianum* IMI 349063
415 (<https://mycocosm.jgi.doe.gov/Colhig2/Colhig2.home.html>) for both *C. destructivum* and *C.*
416 *tabacum* strains. The internal contaminant database X!TandemPipeline software
417 (X!TandemPipeline version 0.2.30, France) was also used. The protein annotation was
418 completed manually using the CAZy database for the CAZymes annotation and using the BlastP
419 tool from the NCBI database (<https://blast.ncbi.nlm.nih.gov>) for other proteins.

420

421 Heterologous expression and purification

422 Recombinant *P. pastoris* X33 strains were streaked on yeast extract peptone dextrose
423 (YPD) agar plates containing Zeocin (100 µg.mL⁻¹) and incubated 3 days at 30°C. Five mL of
424 YPD broth, in 50-mL sterile conical tubes, were inoculated with a *P. pastoris* transformant and

425 incubated for five hours (30°C, 160 rpm in an orbital shaker). The preculture was used to
426 inoculate 0.2% (v/v) of 500 mL buffered complex glycerol medium (BMGY) in a 2-liter flask,
427 incubated (16 hours) until the OD_{600 nm} reached 4-6. Then, the biomass was harvested by
428 centrifugation (10 minutes, 16°C, 5,000 x g), resuspended in 100 mL of buffered complex
429 methanol medium (BMMY) supplemented with CuSO₄ (500 µM) and methanol (1% v/v) in a
430 500 mL flask, and shaken for 3 days in an orbital shaker (200 rpm, 16°C) with daily feeding of
431 methanol (1% v/v). The culture was then centrifuged (10 minutes, 4°C, 5,000 x g) and the
432 supernatant containing the secreted proteins, filtrated on a 0.45-µm cut-off membrane (Millipore,
433 USA) followed by buffer exchange with Tris-HCl (50 mM, pH 8.5), performed by ultrafiltration
434 through a 10 kDa cut-off polyether sulfone membrane (Vivacell 250, Sartorius Stedim Biotech
435 GmbH, Germany). The buffered supernatant was loaded on a DEAE-20 mL HiPrep FF 16/10
436 anion exchange chromatography column (GE Healthcare), equilibrated with Tris-HCl buffer (50
437 mM, pH 8.5) and connected to an Äkta Xpress system (GE Healthcare). Elution was performed
438 by applying a linear gradient from 0 to 500 mM NaCl (in Tris-HCl buffer 50 mM, pH 8.5) with a
439 flow rate set to 5 mL.min⁻¹. The collected fractions were analyzed by SDS-PAGE (10%
440 polyacrylamide precast gel, Bio-Rad) stained with Coomassie blue. Fractions containing the
441 recombinant enzyme were pooled, concentrated and exchanged to sodium phosphate buffer (50
442 mM, pH 7.0). The *CgrAlcOx* ($\epsilon^{280} = 101,215 \text{ M}^{-1}.\text{cm}^{-1}$), *CdeAlcOx* ($\epsilon^{280} = 94,225 \text{ M}^{-1}.\text{cm}^{-1}$) and
443 *CtaAlcOx* ($\epsilon^{280} = 94,225 \text{ M}^{-1}.\text{cm}^{-1}$) concentration was determined by UV absorption at 280 nm
444 using a Nanodrop ND-200 spectrophotometer (Thermo Fisher Scientific). Final solutions (3-5.5
445 mg.mL⁻¹) were flash-frozen using liquid nitrogen and stored at -80°C for long-term storage or
446 stored at 4°C for immediate use.

447

448 **Spectrophotometric enzyme activity assays**

449 AlcOx initial rates were determined by monitoring H₂O₂ released during AlcOx oxidation
450 of various substrates, using the coupled ABTS/HRP assay. Routine assays were performed in 96-
451 wells transparent microtiter plates (Flat bottom, Polystyrene - Greiner Bio One, Austria) in 200
452 μ L final volume containing 0,25 mg/mL ABTS powder, 0,1 mg.mL⁻¹ HRP powder (as provided
453 by the supplier), 3 mM substrate (unless indicated otherwise) and 1 nM AlcOx in sodium
454 phosphate buffer (50 mM, pH 8.0), at 23°C. Reactions were initiated by the addition of substrate
455 to a premix containing all other reagents. Evolution of the absorbance at 414 nm (ABTS cation
456 radical) was measured over time with a Tecan Infinite M200 (Tecan, Switzerland) plate reader.
457 Oxidation of 1 mole of substrate by AlcOx consumes 1 equivalent of O₂ and generates 1
458 equivalent of H₂O₂ which is in turn used by HRP as co-substrate to oxidize 2 equivalents of
459 ABTS. Insoluble substrates were prepared in acetone so that the final acetone concentration in
460 the enzyme assay did not exceed 1% (v/v). Standard curve of known concentrations of H₂O₂ (1 to
461 40 μ M) were made and used to quantify H₂O₂ production. One unit of AlcOx activity was
462 defined as the amount of enzyme necessary to produce 1 μ mole of H₂O₂ per minute.

463

464 **Bioconversion assays**

465 Bioconversions were carried out in 4 mL-clear borosilicate glass vials closed by screw
466 caps with PTFE septum for a total reaction volume of 500 μ L. Routine assay contained 3 mM
467 substrate (prediluted in acetone), 1 μ M AlcOx, various amounts of catalase (0-8 μ M) and/or
468 HRP (0-12 μ M), in sodium phosphate buffer (50 mM, pH 8.0). Final reaction contained 1% (v/v)
469 acetone. Reactions were run at 23°C, under stirring at 190 rpm in an Innova 42R incubator (New
470 Brunswick, USA). Vials were placed lying down in the incubator. The reaction mixture was then

acidified by addition of 10 μ L HCl (12 M) and products and possible remaining substrate were extracted by adding 500 μ L of cyclohexane/ethyl acetate mixture (1:1), followed by shaking and centrifugation for 5 minutes at 1.500 x g. The organic layer was transferred into a new vial by pipetting and analyzed with a GC-2014 apparatus (Shimadzu, Japan) equipped with a flame ionization detector (FID) and an Optima- σ -3 GC capillary column (30 m x 0.25 mm x 0.25 μ m - Macherey-Nagel GmbH & Co, Germany). Nitrogen (200 kPa) was used as carrier gas. The injector and detector temperatures were set at 250°C and temperature programs are described in Table S2. Heptanal or guaiacol were used as internal standards for either aliphatic or aromatic products analyses, respectively.

480

481 AUTHOR CONTRIBUTIONS

482 D.R. carried out most of the experimental work, analyzed the data. S.S. was involved in
483 the culture of fungi and production of the secretomes. L.O.C. and D.N. performed proteomics
484 and analysed data. E.D. and B.H. performed expert annotation of CAZymes. M.H. contributed to
485 enzyme production in *Pichia pastoris*. S.G. performed the microfluidic capillary gel
486 electrophoresis experiments. R.J.O. and N.L. provided the *Colletotrichum* strains and access to
487 the associated genomic data. M.L. and J-G.B conceptualized the study. F.L., B.B., M.L. and J-
488 G.B. supervised the work. D.R. and J-G.B. drafted the manuscript. All authors approved the final
489 version of the manuscript.

490

491 ACKNOWLEDGEMENTS

492 This study was supported by the French National Agency for Research (“Agence
493 Nationale de la Recherche”) through the “Projet de Recherche Collaboratif International” ANR-
494 NSERC (FUNTASTIC project, ANR-17-CE07-0047). We are grateful to MANE & Fils and the

495 “Association Nationale Recherche Technologie” (ANRT) for funding the PhD fellowship of
496 D.R. entitled “Discovery and structure-function study of new fungal copper radicals oxidases
497 used as biocatalysts for the valorisation of alcohols and plant biomass”. This *Convention*
498 *Industrielle de Formation par la Recherche* (CIFRE) grant no. 2017/1169 runs from 1 April
499 2018 to 1 April 2021. Fungal genome sequencing was supported by funding to R.J.O. (grant
500 ANR-17-CAPS-0004-01).

501 We are grateful to Renaud Vincentelli (AFMB – CNRS – Marseille, France) for providing access
502 to the microfluidic capillary gel electrophoresis apparatus.

503

504 **COMPETING INTEREST**

505 The authors declare that they have no competing interests.

506

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- 736

737 **FIGURE LEGENDS**

738

729 **Figure 1. Analysis by capillary electrophoresis of the *Colletotrichum* secretomes samples.**

730 Concentrated secretomes samples were diluted so that the equivalent of 87.5 μ L (prior to
731 concentration) of each secretome sample was injected, except for *C. tabacum* YG-CME day 14
732 that was further diluted 3-fold to avoid a too high protein concentration. The molecular weights
733 (in kDa) of the protein ladder are displayed on the left- and right-hand sides of the image. A darker
734 color corresponds to a higher concentration of protein.

735

736 **Figure 2: Overview of (A) the total number of proteins secreted over time in different media**
737 **and (B) diversity of AA enzymes detected in the secretomes.**

738

739 **Figure 3: Abundance of copper-containing AAs in fungal secretomes from (A) *C.***
740 ***graminicola*, (B) *C. destructivum* and (C) *C. tabacum*.** The Y-axis legend on panel A applies to
741 all panels likewise (note the different scale for *C. tabacum*). For each enzyme family, the bar is
742 subdivided according to the number of enzymes identified; for instance, *C. tabacum* at day 3 in
743 YG-CME displayed six different AA9. PAI: Protein Abundance Index.

744 **Figure 4. Abundance of CAZymes identified exclusively in the YG-CME media from (A) *C.***
745 ***graminicola*, (B) *C. destructivum* and (C) *C. tabacum*.** The Y-axis legend on panel A applies to
746 all panels likewise (note the different scale for *C. tabacum*). For each enzyme family, the bar is
747 subdivided according to the number of enzymes identified. PAI: Protein Abundance Index.

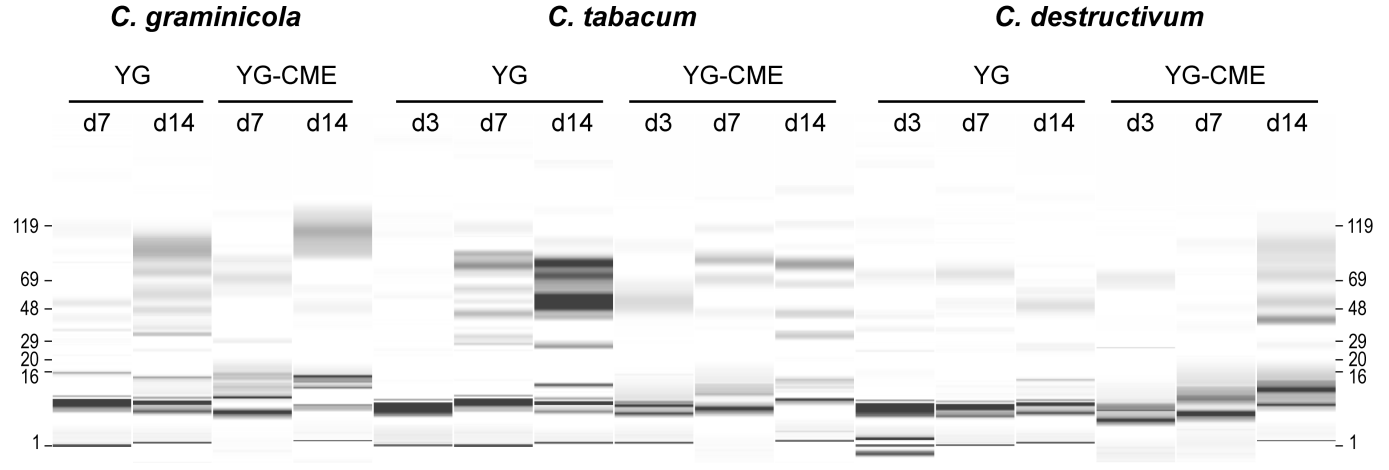
748 **Figure 5. Heatmap of the abundance of AA5 detected in the fungal secretomes.**

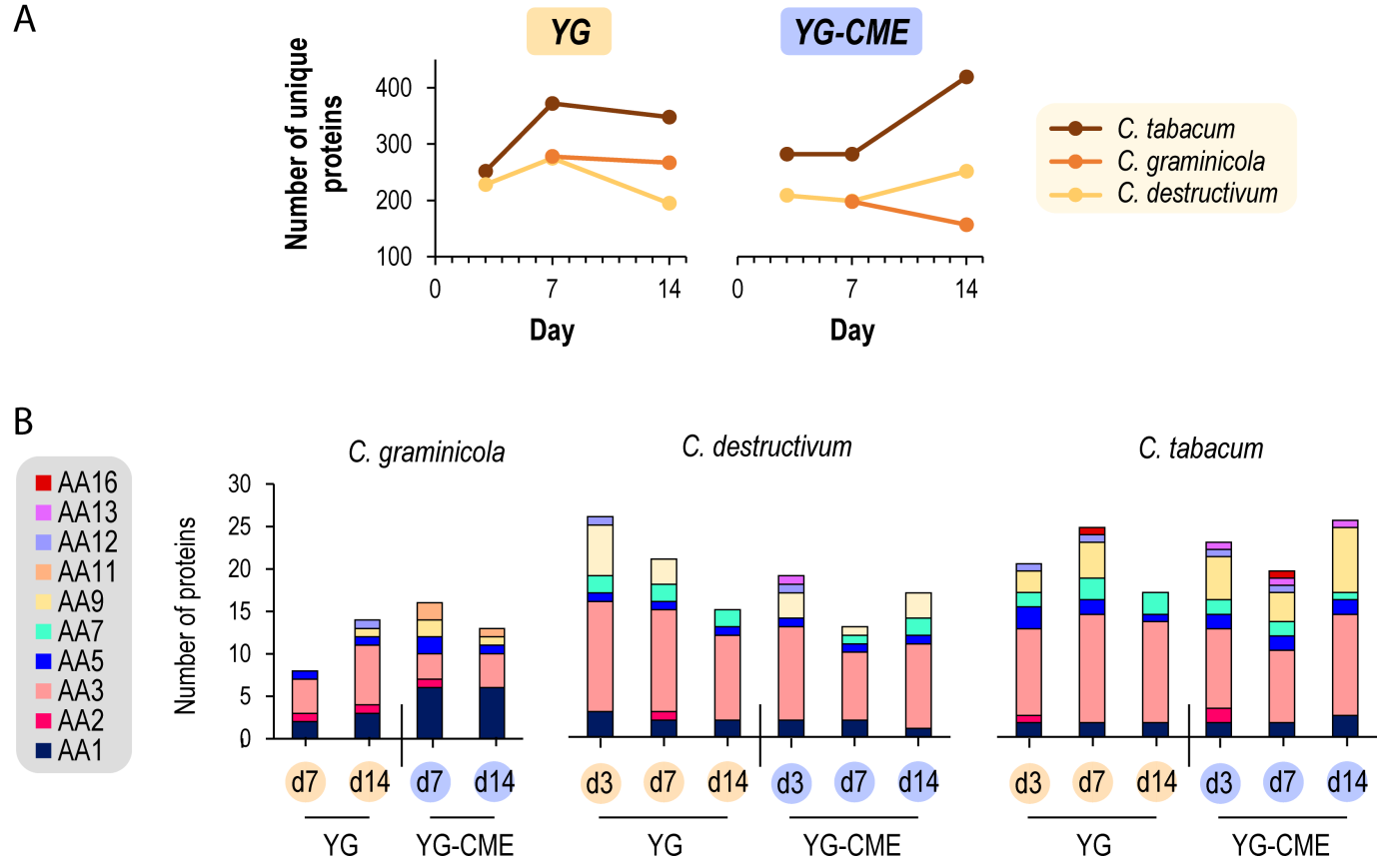
749 **Figure 6: Comparative activity profiles of the three *Colletotrichum* AA5_2 AlcOx, (A)**
750 **Relative activity of the recombinant AlcOx measured by ABTS/HRP coupled assay on 3 mM**

751 BnOH. **(B)** Relative activity profiling of the recombinant AlcOx enzymes on a set of substrates,
752 determined by ABTS/HRP coupled assay, with activity on BnOH set as reference (activity on
753 BnOH set as 100%). Substrate concentrations were as follows: 1% (w/v) xyloglucan, 50 mM for
754 D-glucose, D-galactose, D-raffinose, 3 mM for butan-2-ol, butan-1-ol, benzyl alcohol, cinnamyl
755 alcohol, octan-1-ol, decan-1-ol, and glycolaldehyde dimer. Reactions were performed with 1 nM
756 of *Cgr*AlcOx and *Cta*AlcOx, and 2 nM *Cde*AlcOx. Error bars show s.d. (independent experiments,
757 $n = 3$).

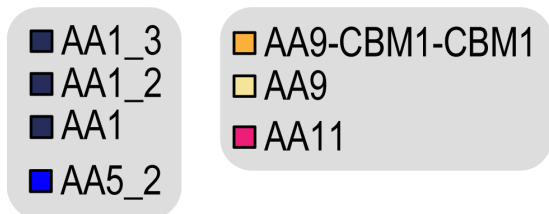
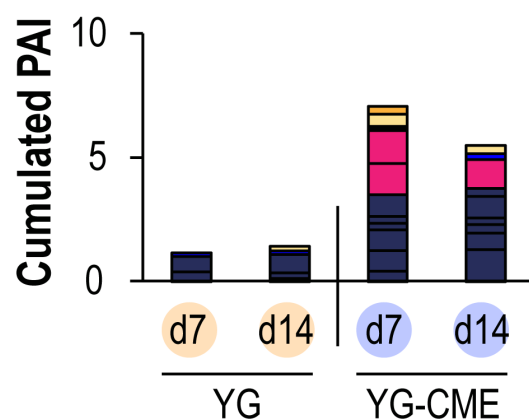
758 **Figure 7: *Cgr*AlcOx and *Cde*AlcOx mediated oxidation of some fatty alcohols. (A)** Reaction
759 scheme proposed (based on (21)) **(B)** conversion of hexan-1-ol **(C)** Conversion of octan-1-ol.
760 Reactions were incubated for either 15 minutes or 16 hours. All reaction mixtures contained: 3
761 mM substrate and 1 μ M AlcOx, in phosphate sodium buffer (50 mM, pH 8.0) and 23°C. Reactions
762 varied as follows: no accessory enzyme added, addition of catalase (8 μ M final), addition of HRP
763 (12 μ M final), addition of both catalase (0.5 μ M) and HRP (12 μ M final). Legend in panel A
764 applies for panel B likewise. Conversion products were analyzed by GC-FID. Error bars show s.d.
765 (independent experiments, $n = 3$).

766

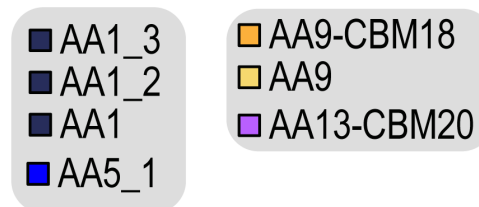
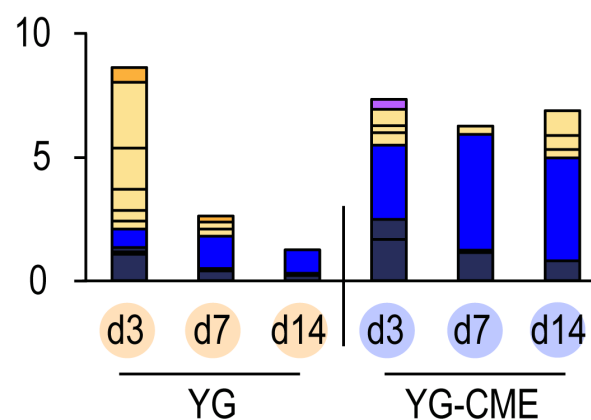




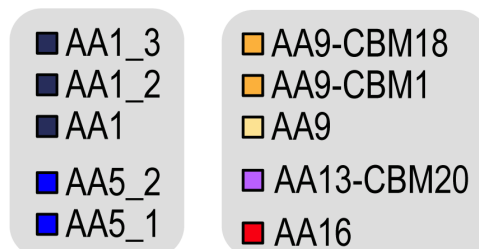
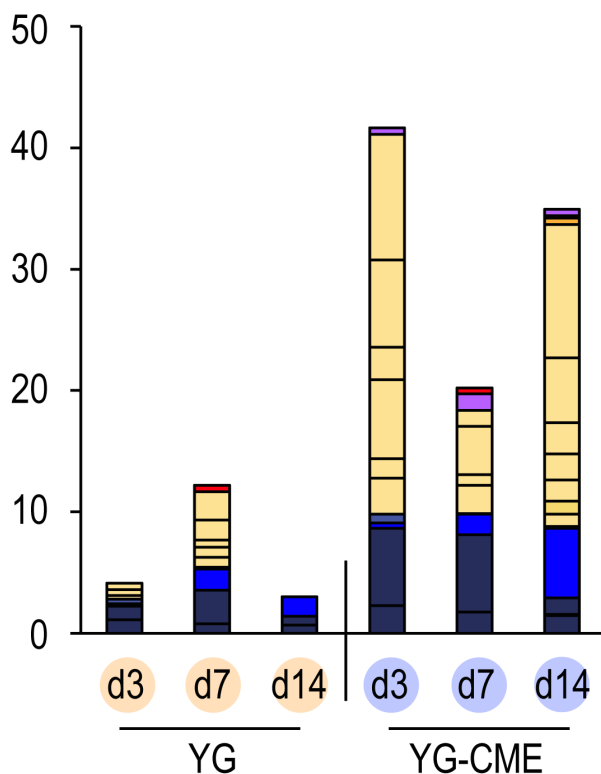
A *C. graminicola*



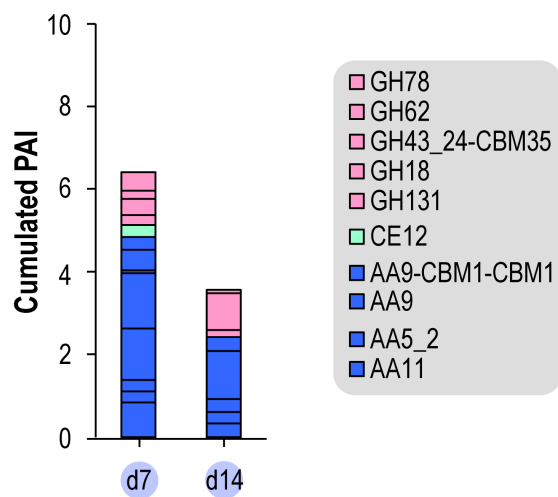
B *C. destructivum*



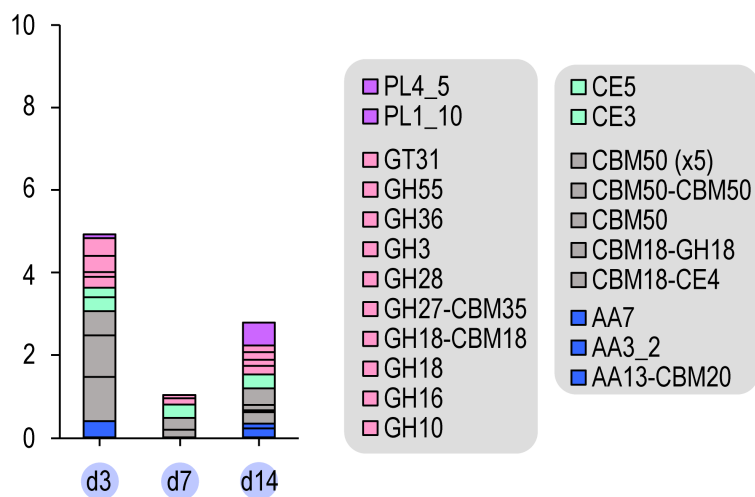
C *C. tabacum*



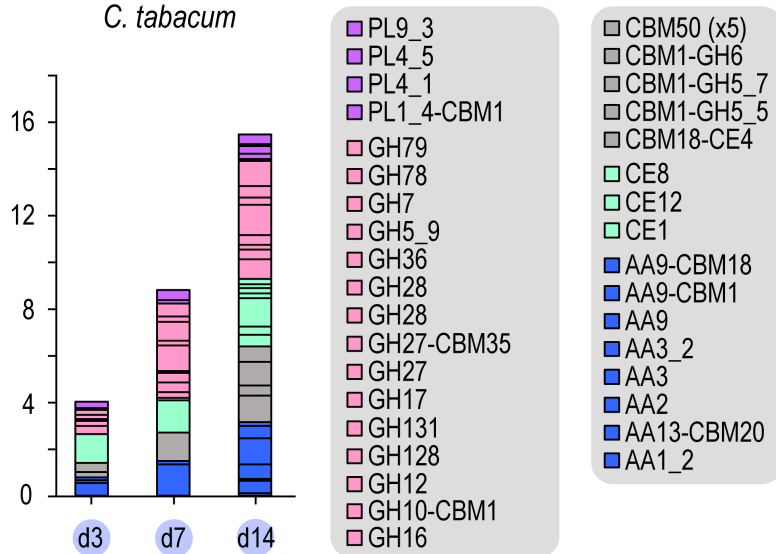
A *C. graminicola*

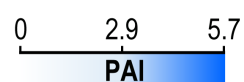


B *C. destructivum*



C *C. tabacum*

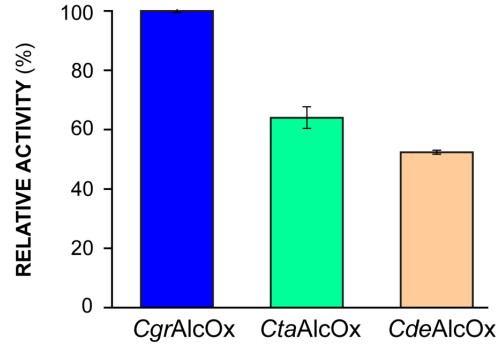




		YG			YG-CME		
		d3	d7	d14	d3	d7	d14
Enzyme							
<i>C. graminicola</i>	AA5_1	nd	-	-	nd	-	-
	AA5_2-AlcOx	nd	0.1	0.1	nd	0.1	0.2
	AA5_2-AAO	nd	-	-	nd	0.1	-
<i>C. destructivum</i>	AA5_1	0.7	1.3	1.0	3.0	4.7	4.2
	AA5_2-AlcOx	-	-	-	-	-	-
	AA5_2-AAO	-	-	-	-	-	-
<i>C. tabacum</i>	AA5_1	0.1	1.8	1.6	0.5	1.7	5.7
	AA5_2-AlcOx	0.1	0.1	-	-	-	-
	AA5_2-AAO	0.4	-	-	0.7	0.1	0.1

"nd": not determined

"-": PAI=0

A**B**