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1 **Old Yellow Enzyme homologues in *Mucor circinelloides*: expression profile and**
2 **biotransformation.**

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22 **Abstract**

23 The reduction of C=C double bond, a key reaction in organic synthesis, is mostly
24 achieved by traditional chemical methods. Therefore, the search for enzymes capable of
25 performing this reaction is rapidly increasing. Old Yellow Enzymes (OYEs) are flavin-
26 dependent oxidoreductases, initially isolated from *Saccharomyces pastorianus*.
27 In this study, the presence and activation of putative OYE enzymes was investigated in
28 the filamentous fungus *Mucor circinelloides*, which was previously found to mediate
29 C=C reduction. Following an *in silico* approach, using *S. pastorianus* OYE1
30 aminoacidic sequence as template, ten putative genes were identified in the genome of
31 *M. circinelloides*. A phylogenetic analysis revealed a high homology of McOYE1-9
32 with OYE1-like proteins while McOYE10 showed similarity with thermophilic-like
33 OYEs.

34 The activation of *mcoyes* was evaluated during the transformation of three different
35 model substrates. Cyclohexenone, α -methylcinnamaldehyde and methyl cinnamate were
36 completely reduced in few hours and the induction of gene expression, assessed by
37 qRT-PCR, was generally fast, suggesting a substrate-dependent activation. Eight genes
38 were activated in the tested conditions suggesting that they may encode for active
39 OYEs. Their expression over time correlated with C=C double bond reduction.

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45 **Keywords**

46 α,β -unsaturated compounds, C=C double bond reduction, gene expression, *Mucor*
47 *circinelloides*, Old Yellow Enzymes.

48 **1. Introduction**

49 The reduction of C=C double bonds is a key reaction in organic chemistry but it is
50 usually carried out by metal catalysts with a strong impact on the technical and
51 economic feasibility of the process [1, 2]. For instance, Yang et al. [3] reported that
52 toxic traces of heavy metals remained in the reaction products and needed to be
53 removed before pharmaceutical use.

54 Since the major challenges of bulk and fine chemicals synthesis are the reduction of the
55 environmental impact and process costs, biocatalysis became one of the most intriguing
56 alternative to traditional processes. The use of microorganisms or their enzymes has
57 recently found room in the industrial production of pharmaceuticals, flavors, aromas,
58 etc. [2, 4]. The biological reduction of activated C=C double bonds may be carried out
59 by flavin-dependent oxidoreductases, namely ene reductases (ERs), belonging to the
60 Old Yellow Enzyme (OYE) family (**EC 1.6.99.1**) [5]. They catalyze the asymmetric
61 hydrogenation of C=C double bond conjugated with electron withdrawing groups
62 (EWGs) in the presence of NAD(P)H as cofactor [2]. In contrast with heavy metals,
63 which are capable of mediating *cis*-hydrogenation, OYEs can catalyze this reaction
64 *trans*-fashion with high stereo-selectivity [1].

65 The reactions catalyzed by OYEs are very interesting and have strong application
66 outcomes. Robinson and Panaccione [6] showed the involvement of OYE homologues
67 involvement in the biosynthetic pathway of ergot alkaloids, commonly used to treat
68 disorders such as Alzheimer's disease, dementia, type 2 diabetes, and
69 hyperprolactinemia or to induce labor and reduce bleeding (lysergic acid-derived
70 drugs). OYE1 from *S. pastorianus* transformed methyl 2-hydroxymethylacrylate in (*R*)-
71 3-hydroxy-2-methylpropanoate, known as "Roche-Ester", which is a chiral building
72 block for the synthesis of vitamins (vitamin E) [4]. Some fragrance compounds
73 (muscone), antibiotics (rapamycin), and natural products have been obtained by OYE-

74 mediated reduction [2]. The 12-oxophytodienoate reductase enzymes (OPRs, EC
75 **1.3.1.42**), OYE homologues from plants, are involved in the biosynthesis of jasmonic
76 acid, which is implicated in the regulation of plant responses to abiotic and biotic
77 stresses as well as plant growth and development [7]. Pentaerythritol tetranitrate
78 reductase (PETNR) from *Enterobacter cloacae* successfully degraded tri nitro toluene
79 (TNT) [8].

80 OYEs have been ubiquitously described in yeasts, bacteria, animals and plants, and
81 recently in filamentous fungi [9]. Fungi are perfect candidates to set up biocatalysis
82 processes: they combine operative versatility to simple growth conditions and they are a
83 well-known enzymatic machinery [1, 2, 4, 10]. For instance, a homologue of OYE has
84 been discovered in *Aspergillus fumigatus* and *Claviceps purpurea* and associated to the
85 ergot biosynthesis [6, 11]. To date, most of the literature evidences focused on
86 Ascomycetes and Basidiomycetes [9, 12] but the presence of OYE homologue within
87 Zygomycota phylum has never been assessed.

88 Despite the potential application in several biotechnological fields, microorganisms and
89 enzymes are still scarcely used in manufacturing processes, mostly due to the lack of
90 suitable biocatalysts. Novel enzymatic activities with strong catalytic potential could be
91 achieved with traditional functional screening or advanced molecular approaches [2, 4].
92 Genome-wide analysis is a useful tool to identify OYEs homologues among the
93 available fungal genomes. For instance, Nizam et al. [9] by analysing 60 Ascomycota
94 and Basidiomycota genomes identified 424 OYEs homologues and provided a first
95 classification of these enzymes within the fungal kingdom. They also explored the
96 evolutionary significance of fungal OYEs. Unfortunately, this data can be considered
97 just a first step, and the actual capability of strains to transform target compounds by
98 reducing C=C double bond need further validation.

99 In this work, we aimed to fill the lack of information about the occurrence of OYEs in
 100 fungi belonging to the Zygomycota phylum. *Mucor circinelloides* was selected due to
 101 its ability of converting several substrates [13]. Despite those interesting results, the
 102 enzymatic pattern responsible for the reactions has never been investigated before. The
 103 availability of *M. circinelloides* complete genome sequence (Joint Genome Institute,
 104 JGI: <http://jgi.doe.gov>) allowed a genome-mining approach to investigate the presence
 105 of putative OYEs homologues.

106 2. Results

107 2.1 Identification of putative OYEs in the genome of *M. circinelloides*

108 In order to identify OYE encoding genes in the filamentous fungus *M. circinelloides*, a
 109 BlastP analysis (Basic Local Alignment Search Tool, NCBI, USA) on the complete
 110 genome of *M. circinelloides* using *S. pastorianus* OYE1 as query was performed. Ten
 111 putative sequences were retrieved and named McOYE1-McOYE10 (Table 1). The 10
 112 amino acid sequences and the amino acid sequence of OYE1 were aligned to evaluate
 113 sequence similarities (Table 1). Nine McOYEs showed a similarity with *S. pastorianus*
 114 OYE1 of about 40 % while McOYE10 showed a lower similarity (25.33 %; Table 1).

115 **Table 1**

116 Putative OYE homologues of *M. circinelloides* - McOYE1, McOYE2, McOYE3,
 117 McOYE4, McOYE5, McOYE6, McOYE7, McOYE8, McOYE9 and McOYE10 - with
 118 sequence ID according to JGI database and identity percentage with *S. pastorianus*
 119 OYE1.

McOYE	Sequence ID	ID matrix (%) with OYE1	ID matrix (%) with McOYE1	ID matrix (%) with McOYE2	ID matrix (%) with McOYE3	ID matrix (%) with McOYE4	ID matrix (%) with McOYE5	ID matrix (%) with McOYE6	ID matrix (%) with McOYE7	ID matrix (%) with McOYE8	ID matrix (%) with McOYE9	ID matrix (%) with McOYE10
1	160302	44.14	97.50	89.60	70.40	75.00	67.80	66.10	60.60	67.00	58.40	37.00
2	137297	43.99	89.60	97.50	69.70	75.30	68.70	66.10	61.10	67.00	59.50	36.40
3	177510	42.19	71.20	70.50	96.40	76.10	63.30	63.60	57.10	61.60	60.50	40.70
4	155592	41.30	73.40	73.70	73.60	100.00	65.30	65.70	57.80	64.80	60.10	41.60
5	110873	43.41	65.80	66.70	60.40	65.30	100.00	64.50	61.60	65.90	59.10	39.10
6	144573	42.30	64.40	64.30	61.10	64.90	64.60	100.00	67.70	66.10	58.20	38.20
7	153280	41.80	63.70	64.20	62.10	62.20	66.30	68.80	90.50	60.50	56.60	45.10
8	76836	42.19	65.70	65.70	59.50	64.80	65.90	66.10	56.70	100.00	59.20	-

9	134845	38.19	56.90	57.90	57.90	59.50	57.70	57.50	54.90	58.50	97.00	-
10	152500	25.33	35.90	35.20	39.20	41.60	39.10	40.30	48.40	41.60	41.80	100.0

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122 Three conserved domains typical of OYEs were found in all the 10 sequences: the FMN
123 binding site, the active site and the substrate binding site (Supp. Fig. 1).

124 Specific primer pairs were designed on the nucleotide sequences of the 10 putative
125 *mcoyes* (Supp. Table 1) and tested by conventional PCR on genomic DNA. Amplicons
126 of the expected size (about 200 bp) were obtained (Supp. Fig. 2). PCR products were
127 sequenced confirming the specificity of the primers pairs and the authenticity of the
128 DNA sequences.

129 A phylogenetic analysis was performed by implementing sequence data analyzed by
130 Nizam et al. [9], who divided OYEs proteins into three groups: Class I, Class II and
131 Class III. Nine out of 10 McOYEs clustered together in Class I showing a specie-
132 specific clade whereas McOYE10 was located within Class II (Fig. 1).

133 *2.2 Biotransformation of conventional substrates and gene expression*

134 The expression profile of the 10 putative OYEs homologues was monitored on the RNA
135 extracted from the mycelium grown in liquid culture during the biotransformation of
136 three conventional substrates presenting different EWGs. For each substrate, data on
137 biotransformation and gene expression pattern are presented. Since the substrate was
138 dissolved in dimethyl sulfoxide (DMSO), *mcoyes* activation was also evaluated in the
139 presence of this solvent to exclude artifacts. None of the genes was activated in the
140 presence of DMSO (data not shown). The 10 genes showed a basal activity in the
141 absence of substrates (data not shown).

142 *2.2.1 Cyclohexenone (CE)*

143 *M. circinelloides* completely reduced the substrate CE into cyclohexanol within 24 h;
144 the reaction process is well known: first an OYE reduces the C=C double bond of CE

145 producing cyclohexanone, then the keto group is reduced by an alcohol dehydrogenase
146 (ADH) into cyclohexanol (Fig. 2 A) [13]. As shown in Fig. 3 A, the reaction began 30
147 min after the addition of CE to the medium and at 3.5 h the C=C double bond was
148 completely reduced producing cyclohexanone which was continuously converted in its
149 corresponding alcohol, cyclohexanol.

150 The transcripts level of the 10 *mcoye* homologues was monitored both in the presence
151 and absence of CE at 30 min, 1 h, 2 h and 5 h (Fig. 4 A). With the exception of *mcoye7*
152 and *mcoye8* that did not show activation upon CE exposure (data not shown), all the
153 other genes were activated within the first two hours. In particular *mcoye2*, *mcoye1* and
154 *mcoye10* displayed a fast and strong induction of gene expression: 730, 111 and 76 fold
155 compared to the control sample without CE at 1 h and at 30 min for *mcoye10* (Fig. 4 A).
156 *Mcoye4* and *mcoye5* showed an activation of 30-50 fold, while for *mcoye3*, *mcoye6* and
157 *mcoye9* the induction of gene expression compared to the control sample remained
158 below 20 fold at the different time points. Noteworthy, expression levels of these genes
159 decreased to the control values after 5 h.

160 A clear relation between *mcoyes* expression profile and the biotransformation of CE
161 was observed (Fig. 3 A). *Mcoye2* transcript levels were strongly induced at the
162 beginning of the reductive process reaching the maximum at 1 h when 20 % of CE had
163 been converted into cyclohexanone.

164 2.2.2 α -Methylcinnamaldehyde (MCA)

165 *M. circinelloides* completely reduced the C=C double bond of MCA within 20 h. Both
166 the C=C double bond (OYE) and the aldehydic group (ADH) of the substrate were
167 reduced (Fig. 3 B). One hour after MCA addition, α -methylcinnamyl alcohol
168 represented 50 % of the substrates in the culture medium. The concentration of this first
169 product increased until 8 h and then dropped down before 20 h. The production rate of

170 the saturated alcohol was constant, starting from 2 h until 20 h, when it was the
171 remaining metabolite detected (Fig. 3 B).

172 According to literature data [2], OYEs are able to catalyze the reduction of the C=C
173 double bonds of unsaturated aldehydes, whereas they are usually inactive on allylic
174 alcohols. In the case of *M. circinelloides*, α -methylcinnamyl alcohol seemed to be the
175 intermediate of the conversion of MCA into the corresponding saturated alcohol. Thus,
176 α -methylcinnamyl alcohol was added directly to *M. circinelloides* cultures, and indeed
177 its conversion into α -methyldihydrocinnamyl alcohol was observed to be complete after
178 48 h. In order to elucidate this reduction pathway, the dideuterated α -methylcinnamyl
179 alcohol, showing two deuterium atoms linked to the carbon atom bearing the OH group,
180 was prepared (Figure 2 B). This compound was submitted to bioreduction with *M.*
181 *circinelloides* and a monodeuterated saturated alcohol was recovered. The formation of
182 this compound could be explained only admitting the formation of the unsaturated
183 aldehyde as an intermediate, because the two deuterium atoms should have been
184 preserved in the direct reduction of the starting allylic alcohol. The alcohol
185 dehydrogenases, which are present in the fermentation medium, catalyse the oxidation
186 of the allylic alcohol to the unsaturated aldehyde, which is easily reduced by ERs and
187 removed from the equilibrium. Then, the saturated aldehyde is further reduced by
188 ADHs to afford the corresponding saturated alcohol. The intermediate aldehydes did not
189 accumulate in the reaction medium and it was not possible to detect them during the
190 reaction course by GC/MS analysis. On the basis of these results, the reaction sequence
191 shown in Figure 2 C can be hypothesized for MCA.

192 As for CE, the transcripts level of the 10 putative *mcoyes* was monitored both in the
193 presence and absence of MCA at 30 min, 1 h, 2 h and 4 h (Fig. 4 B). *Mcoye2* showed
194 the highest gene activation level with about 2,880 fold compared to the control sample,
195 followed by *mcoye1* that displayed 1,860 fold induction (Fig. 4 B). An induction of

196 gene expression of about 500 fold was observed for *mcoye4*, *mcoye5* and *mcoye10*.
197 Remarkably, for these 5 genes the highest activation was reached 1 h after the addition
198 of the substrate. For *mcoye3* and *mcoye6* the highest induction levels (138 and 243,
199 respectively) were observed at 2 h. *Mcoye9* displayed moderate gene activation (about
200 14 fold) only at 4 h, *mcoye7* and *mcoye8* did not show activation, as it was observed
201 with CE (data not shown).

202 In this case too, a relation between *mcoyes* gene expression activation and MCA
203 biotransformation was observed (Fig. 3 B). The strongest activation of *mcoye1* in
204 presence of MCA was reached at 1 h, well before the beginning of the C=C reduction,
205 represented by the formation of the saturated alcohol.

206 2.2.3 Methyl cinnamate (MCI)

207 The MCI substrate was completely reduced by *M. circinelloides* within 66 h; both the
208 C=C double bond and the ester group were reduced producing cinnamyl alcohol and
209 phenylpropanol. The exact reaction profile is unknown; however, the one reported in
210 Figure 2 D can be hypothesized on the basis of what has been observed for MCA,
211 starting from the enzymatic hydrolysis of the ester moiety followed by the biocatalysed
212 reduction of the COOH group to primary alcohol. As shown in Fig. 3 C, MCI decreased
213 slightly but constantly until 66 h, when all the substrate was transformed. The detected
214 amount of cinnamyl alcohol was never more than 20 %; also the level of
215 phenylpropanol remained low (< 20 %) until 9 h, after which the concentration reached
216 100 % within 66 h.

217 The transcripts level of the 10 putative *mcoyes* was analyzed in presence and absence of
218 MCI at 1, 2, 3 and 6 h (Fig. 4 C). *Mcoye1* was the most induced gene (about 300 fold).
219 An activation of about 60 fold was observed for *mcoye2*, *mcoye5*, and *mcoye10*, while
220 *mcoye3*, *mcoye4*, *mcoye6*, and *mcoye9* showed 20 fold induction. *Mcoye1* and *mcoye6*
221 displayed the fastest activation, with the maximum within the first 2 h; after that time

222 point, their transcripts level rapidly decreased. *Mcoye10* showed a peak between 2 and 3
223 h (Fig. 4 C). Also in this case, neither *mcoye7* nor *mcoye8* were activated upon substrate
224 exposure (data not shown).

225 A relation between OYE activation and the biotransformation of MCI was observed
226 (Fig. 3 C). The transcription of *mcoye1* started early, when the substrate was still the
227 only detectable compound in the reaction mixture.

228 **3. Discussion**

229 Hydrogenation of C=C double bonds is an important reaction in several manufacturing
230 processes for the production of bulk and fine chemicals; researchers and industries are
231 moving towards more sustainable approaches as biocatalysis and in recent years, several
232 research groups have focused on the identification of OYEs homologues to be exploited
233 in different processes [1]. In the last few years the attention was given to OYEs from
234 filamentous fungi; nevertheless only few studies report their occurrence and their
235 physiological role in this group of organisms [9, 13].

236 An *in silico* approach allowed to identify in the genome of the zygomycete fungus *M.*
237 *circinelloides* 10 gene sequences that shared similarity and conserved domains with
238 known OYEs. The presence of multiple OYE genes appears to be a common feature not
239 only among Ascomycetes and Basidiomycetes [7, 12] but also in Zygomycetes: indeed
240 with a similar approach we found from 4 to 10 putative OYE sequences within some of
241 the completely sequenced genomes (Suppl. Table 2).

242 A phylogenetic analysis grouped the McOYEs in two classes: nine proteins (McOYE1-
243 9) were placed in Class I, including most of the OYE1-like proteins [5, 9, 12], while
244 McOYE10 clustered with Class II. Genome sequence data allowed to hypothesize that a
245 number of Class I McOYEs are located within the same chromosome; this information
246 may suggest duplication events for some of these genes, as suggested by Corrochano et
247 al. [14]. Class II gathers OYEs originally identified from different thermophilic bacteria

248 [5, 9]; however, the recent work by Nizam et al. [9, 12] demonstrated that a number of
249 sequences, although not yet characterized, from filamentous fungi (Ascomycota and
250 Basidiomycota) also clusters within Class II. To the best of our knowledge, this is the
251 first report of an OYE homologue from a Zygomycota belonging to this Class.

252 The fungal enzymatic activity was analyzed in the presence of three different substrates
253 while previous works considered only one substrate or a series of compounds belonging
254 to the same chemical class [15]. *M. circinelloides* showed a strong enzymatic activity
255 being able to completely reduce the C=C double bond of the three substrates. CE was
256 converted very fast (3.5 h), followed by MCA (20 h) and MCI (66 h), suggesting an
257 increasing recalcitrance of the molecules. These results are in line with those obtained
258 by Gatti et al. [2], who demonstrated that the carbonyl moiety acts as a strong activator,
259 while the ester group is a weak EWG. Being able to convert compounds with different
260 EWGs, *M. circinelloides* was very versatile; during the biotransformation the EWG
261 influenced only the timing of the reaction; the ester group of MCI was the weakest
262 EWG as the reaction was accomplished in 66 h.

263 The reduction of α,β -unsaturated ketones has been extensively studied using either the
264 whole microorganism or the purified enzymes [5, 15, 16]. Generally CE is a well
265 reduced compound; in fact *M. circinelloides* completely reduced the C=C double bond
266 (100 %) in only 3.5 h. Comparable yields were achieved with other filamentous fungi: a
267 previous study, which examined 28 filamentous fungi for the reduction of three
268 different conventional compounds, showed that CE was the easiest to reduce for almost
269 all the fungi (96.4 %); in particular, 19 fungi completely reduced this molecule [13].

270 Stueckler et al. [7] reported that purified OYE1 (*S. pastorianus*) reduced 92 % of CE
271 and purified YqjM (OYE from *Bacillus subtilis*) reduced 85 % of CE.

272 The reduction of α -substituted cinnamaldehydes is very important at industrial level [2].
273 Aldehyde is considered a good EWG and MCA was completely reduced within 20 h;

274 Fardelone et al. [17] obtained comparable yields using a commercial strain of *S.*
275 *cerevisiae* in the biotransformation of cinnamaldehyde derivatives. Other authors
276 reported that MCA is not always an easily reduced compound. For instance, Goretti et
277 al. [18] analyzed different non conventional yeasts in the reduction of MCA and found
278 that only *Kazachstania spenceroum* was able to convert this substrate with a yield of 60
279 %. Romagnolo et al. [13] reported that, among 19 fungi tested, only two, belonging to
280 the *Mucor* genus were able to completely convert the C=C double bond of this
281 substrate.

282 The bioreduction of MCI and its derivatives is not frequently reported in the literature,
283 suggesting a possible recalcitrance of this molecule to OYE-mediated
284 biotransformation. A biotransformation study performed on 7 bacterial, yeast and plant
285 OYEs homologues showed a conversion rate of MCI < 1 % [19]. Therefore, the ability
286 of *M. circinelloides* to completely reduce MCI is remarkable, since unsaturated esters
287 with no other EWG are rarely converted by OYEs.

288 BlastP analysis, using OYE1 of *S. pastorianus* as query, allowed the identification of 10
289 putative genes coding for OYEs, confirmed by PCR amplification and sequencing. The
290 high versatility found in the reduction of different compounds by *M. circinelloides* may
291 depend on its enzymatic pattern and on the possibility to activate distinct genes
292 specifically in the presence of different molecules or in defined environmental
293 conditions. In a recent paper, Nizam et al. [9] performed a genome-wide analysis on
294 available genomes of filamentous fungi: 60 species were investigated leading to the
295 identification of 424 OYE homologues. Surprisingly, some species were shown to
296 possess up to 22 OYEs homologues in their genome, while, in other microorganisms the
297 number of OYEs homologues number was more exiguous: only two homologues are
298 present in *S. cerevisiae*, while there are four in *Shewanella oneidensis* [20, 21].

299 Gene activation upon exposure to CE and MCA was extremely high (i.e. up to 2,900
300 fold for *mcoye2* in presence of MCA) and occurred soon after substrate addition. Nizam
301 et al. [9, 12] monitored the expression profile of 6 OYEs homologues from the
302 *Ascochyta rabiei* in two different conditions reporting an increase of 80 fold in
303 transcript levels during plant infection and a weaker activation during oxidative stress.
304 Among the 10 genes identified in *M. circinelloides*, *mcoye1* and *mcoye2* showed the
305 highest degree of gene activation (70-2,900 fold), followed by *mcoye4*, *mcoye5* and
306 *mcoye10* (20-800 fold). *Mcoye3*, *mcoye6* and *mcoye9* were poorly activated, while
307 transcripts of *mcoye7* and *mcoye8* were never activated in each condition. On the basis
308 of these results it seems reasonable to conclude that 8 out of 10 putative OYEs
309 homologues are rapidly activated in response to the substrates addition.

310 A relation between the biotransformation of each substrate and the expression profile of
311 the eight putative OYEs homologues has been observed. Generally, the transcript levels
312 reached the maximum peak before the beginning of the C=C double bond reduction. For
313 example, during CE analysis, the maximum peak of expression of *mcoye2* was reached
314 after 1 h when 20 % of substrate was reduced.

315 The biological role of these enzymes as well as their cell localization is still an open
316 question. By *in silico* analysis Nizam et al. [9], found that the majority of the OYE
317 homologues were allegedly located in the cytoplasm and in the cytoskeleton, although
318 some of them were associated to other cell compartments such as nucleus, peroxisomes,
319 plasma membrane. Only three OYE seemed to be extracellular. A preliminary
320 experiment carried out on *M. circinelloides* during the biotransformation of CE, showed
321 that ene reductase activity was detected only in presence of cell debris indicating that
322 these enzymes may be intracellular (data not shown); further and deeper experiments
323 are needed to confirm this hypothesis.

324 Studies are in progress to analyze the secondary and tertiary structure of these enzymes
325 by *in silico* approaches [22]. In order to purify and catalytically characterize McOYEs,
326 efforts will concentrate on the production of the homologues of *M. circinelloides* by
327 heterologous expression systems.

328 4. Materials and methods

329 4.1 Fungal strain

330 *Mucor circinelloides* 277.49 was obtained from CBS (CBS-KNAW fungal biodiversity
331 centre) and was selected due to its capability of reducing C=C double bonds [13]. The
332 strain is preserved as MUT 44 at the *Mycotheca Universitatis Taurinensis* (MUT),
333 Department of Life Sciences and Systems Biology, University of Turin.

334 4.2 Chemicals

335 CE, MCA and MCI were purchased from Sigma-Aldrich. Stock solutions of 500 mM of
336 each substrate were prepared in DMSO (Sigma-Aldrich).

337 (*E*)-2-methyl-3-phenylprop-2-en-1,1-*d*₂-1-ol (dideuterated α -methylcinnamyl alcohol)
338 was prepared by reduction of ethyl (*E*)-2-methyl-3-phenylacrylate (0.50 g, 2.6 mmol)
339 with DIBAL-D (7.9 mmol, 0.7 M in toluene) in THF. After the usual work-up, the
340 dideuterated compound was obtained (0.41 g, 2.3 mmol, 89 %). ¹H NMR (CDCl₃, 400
341 MHz): δ = 7.39 - 7.19 (5H, m, aromatic hydrogens), 6.53 (1H, q, *J* = 1.5 Hz, CH=C),
342 1.91 (3H, d, *J* = 1.5 Hz, CH₃); GC-MS (EI) *t*_R = 14.1 min: *m/z* (%) = 150 (M⁺, 92), 107
343 (68), 91 (100). 2-Methyl-3-phenylpropan-1-*d*-1-ol (monodeuterated α -
344 methyl-dihydrocinnamyl alcohol) was isolated from the reaction medium and
345 characterized by NMR and GC/MS analysis: ¹H NMR (CDCl₃, 400 MHz): δ = 7.37 –
346 7.13 (5H, m, aromatic hydrogens), 3.45 (1H, m, CHDOH), 2.75 (1H, dd *J* = 13.5 and
347 6.4 Hz, CHHPh), 2.43 (1H, dd *J* = 13.5 and 8.0 Hz, CHHPh), 1.97 (1H, m, CHCH₃),
348 0.92 (3H, s, CH₃); GC-MS (EI) *t*_R = 12.6 min: *m/z* (%) = 151 (M⁺, 10), 133 (23), 118
349 (27), 91 (100).

350 4.3 Genome mining and phylogenetic analyses

351 BlastP analysis was performed on the complete genome of *M. circinelloides* strain
352 277.49 (Joint Genome Institute, JGI: <http://jgi.doe.gov>) using the sequence of OYE1 of
353 *Saccharomyces pastorianus* (UniProtKB accession no. **Q02899**) as query. Primer pairs
354 for qRT-PCR assays were designed by using Primer 3 (<http://primer3.ut.ee/>) (Supp.
355 Table 1). Total genomic DNA was extracted from the mycelium grown in MEA liquid
356 medium (20 g/l glucose, 20 g/l malt extract, 2 g/l peptone) for 24 h using the CTAB
357 method [23]. Oligonucleotides were tested by conventional PCR on genomic DNA. The
358 PCR mixture included distilled water, PCR buffer (10 X), 1 mM deoxynucleotide
359 triphosphates (dNTPs), 10 mM of each primer, 0.5 U of DNA polymerase (Taq DNA
360 polymerase, Qiagen) and 100 ng of genomic DNA in a total volume of 20 µl.
361 Amplifications were performed using a T100 Thermal Cycler (BIORAD). For the
362 validation of *mcoye1* F-R, *mcoye2* F-R, *mcoye3* F-R, *mcoye5* F-R, *mcoye6* F-R, *mcoye9*
363 F-R e *mcoye10* F-R, the amplification protocol was as follows: 95 °C (5 min), 34 cycles
364 of 95 °C (40 sec), 60 °C (50 sec) and 72 °C (50 sec), 72 °C (8 min). For the detection of
365 *mcoye4* F-R, *mcoye7* F-R e *mcoye8* F-R the amplification protocol was as follows: 95
366 °C (5 min), 34 cycles 95 °C (40 sec), 56 °C (50 sec) and 72 °C (50 sec), 72 °C (8 min).
367 PCR products were loaded on a 1.5 % agarose electrophoresis gel stained with ethidium
368 bromide; the molecular weight marker used was the GelPilot 1 kb Plus Ladder (cat. no.
369 239095, Qiagen). Products were purified and sequenced at Macrogen (The
370 Netherlands). Newly generated sequences were analyzed using Sequencher 5.4 (Gene
371 Code Corporation).
372 To perform the phylogenetic analyses, over 400 OYEs aminoacidic sequences of fungi
373 were aligned with MUSCLE (<http://www.ebi.ac.uk/Tools/msa/muscle/>) using default
374 conditions for gap openings and gap extension penalties and trimmed by TrimAl (v 1.2)
375 (<http://trimal.cgenomics.org>) with the AUTOMATED 1 setting. The analysis was

376 performed using two approaches. First, a phylogenetic tree was derived by Bayesian
377 Inference (BI) implemented in MrBayes (v 3.2.2) (<http://mrbayes.sourceforge.net>)
378 under a mixed amino acid substitution model. The alignment was run over 10 million
379 generations with two independent runs each containing four Markov Chains Monte
380 Carlo (MCMC) and sampling frequency of every 300 iterations. The first 2,500 trees
381 were discarded as “burn-in” (25 %). Using the Sumt function of MrBayes a consensus
382 tree was generated and posterior probabilities were estimated. In a second approach,
383 Maximum Likelihood (ML) was performed using RAxML GUI (v 1.5 b) [24] with
384 WAG+I+G model. Statistical reliability was determined by Bootstrap analysis. All the
385 phylogenetic trees were visualized using FigTree (v 1.4)
386 (<http://tree.bio.ed.ac.uk/software/figtree>).

387 *4.4 Biotransformation by whole cell system*

388 A conidia suspension of *M. circinelloides* was made from pre-growth mycelium in
389 MEA solid medium (same composition of MEA liquid with the addition of 20 g/l of
390 agar). 10⁶ conidia were inoculated in 100 ml flasks containing 40 ml of MEA liquid
391 medium. Flasks were incubated at 25 °C in agitation. After 2 days, substrates were
392 added (5 mM final concentration), each cultural line was run in triplicate. In addition,
393 biotic controls (in absence of substrates) were set up.
394 According to previous results (unpublished data), the conversion of CE, MCA and MCI
395 was followed for 24 h, 48 h and 7 d, respectively. Every 2 h, 1 ml of broth and 100 mg
396 of biomass were collected to perform chemical analysis and RNA extraction,
397 respectively. The mycelium was frozen in liquid nitrogen and stored at - 80 °C until the
398 analysis.
399 At any collection time point, pH and glucose content were measured. The concentration
400 of reducing sugars was obtained following the reaction with 3,5-dinitrosalicylic acid
401 assay (DNS) [25], using a modified protocol as described by Spina et al. [26]. At each

402 time point and at the end of the experiment, fungal biomasses were separated from the
403 culture medium by filtration and dried in oven at 60 °C for 24 h to calculate the dry
404 weight.

405 *4.5 Chemical analyses*

406 Samples taken at the different time points were extracted by two-phase separation using
407 0.4 ml of methyl *t*-butyl ether (MTBE) as solvent; the organic phase was dried over
408 anhydrous Na₂SO₄ and analyzed by GC/MS.

409 GC/MS analyses were performed on an Agilent HP 6890 gas chromatograph equipped
410 with a 5973 mass detector and an HP-5-MS column (30 m × 0.25 mm × 0.25 μm,

411 Agilent), employing the following temperature program: 60 °C (1 min) / 6 °C min⁻¹ /
412 150 °C (1 min) / 12 °C min⁻¹ / 280 °C (5 min). The end products of the

413 biotransformations were identified by GC/MS analysis, using authentic commercial

414 samples as reference compounds: i) cyclohexenone $t_R = 5.40$ min m/z 96 (M⁺, 33), 81

415 (19), 68 (100); cyclohexanone $t_R = 4.65$ min m/z 98 (M⁺, 47), 83 (13), 55 (100);

416 cyclohexanol $t_R = 4.45$ min m/z 100 (M⁺, 2), 82 (35), 57 (100), ii) α-

417 methylcinnamaldehyde $t_R = 14.7$ min m/z 146 (M⁺, 64), 145 (100), 117 (79), 91 (43);

418 α-methylcinnamyl alcohol $t_R = 15.5$ min m/z 148 (M⁺, 50), 115 (63), 91 (100); α-

419 methyl dihydrocinnamyl alcohol $t_R = 13.7$ min m/z 150 (M⁺, 12), 117 (62), 91 (100); iii)

420 methyl cinnamate $t_R = 16.03$ min m/z 162 (M⁺, 58), 131 (100), 103 (72); cinnamyl

421 alcohol $t_R = 12.80$ min m/z 134 (M⁺, 53), 115 (65), 92 (100) ; phenylpropanol $t_R = 12.36$

422 min m/z 136 (M⁺, 21), 117 (100), 91 (84).

423 *4.6 RNA extraction, first strand cDNA synthesis and quantitative Real-Time PCR*

424 *experiments*

425 The extraction of RNA was performed from about 100 mg of fungal biomass using the

426 RNeasy Plant Mini Kit (Qiagen). Quantity and quality of RNA samples were checked

427 spectrophotometrically (Tecan Infinite 200, i-control software). After DNase treatment

428 (TURBO DNA-free, Ambion), RNA quality has been tested again and for all the
429 samples, the ratios of absorbance 260/280 were between 1.8 and 2.2. Subsequently they
430 were processed to obtain cDNA with the use of the Super-Script II Reverse
431 Transcriptase (Invitrogen), following instructions.
432 qRT-PCR were performed with an iCycler iQTM Real-Time PCR Detection System
433 (BIORAD); reactions were carried out in a final volume of 15 μ l by using iTaq
434 Universal SYBR GREEN Supermix (BIORAD), specific primers (3 μ M; Table 1) and
435 cDNA . For the detection of *mcoye1*, *mcoye2*, *mcoye3*, *mcoye5*, *mcoye6*, *mcoye9* and
436 *mcoye10*, the amplification protocol was as follows: 95 °C (1.5 min), 40 cycles of 95 °C
437 (15 sec), 60 °C (30 sec) and 72 °C (50 sec), 72 °C (8 min). For the detection of *mcoye4*,
438 *mcoye7* and *mcoye8* the amplification protocol was as follows: 95 °C (1.5 min), 40
439 cycles 95 °C (15 sec), 56 °C (30 sec) and 72 °C (50 sec), 72 °C (8 min). The *M.*
440 *circinelloides* β -actin encoding gene was used as internal control [27]. The relative
441 expression was calculated using the $2^{-\Delta\Delta C_t}$ method [28]. One-way ANOVA and Tukey's
442 tests ($p < 0.05$) were performed to assess the statistical significance of the gene
443 expression data (IBM SPSS Statistics for Macintosh, Version 22.0).

444 *4.7 Availability of materials and data*

445 Authors confirm that all relevant data are included in the article and its supplementary
446 information file.

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539 **Author Contribution Statement**

540 **A.R., F.S.:** wrote the manuscript

541 **A.P.:** performed phylogenetic analysis

542 **A.R., F.S., S.R., B.S.:** performed lab experiments

543 **L.L.:** gene expression experimental design

544 **M.C., D.M., E.B.:** performed chemical analysis and data curation

545 **G.C.V:** project administration and supervision

546 All authors reviewed the manuscript.

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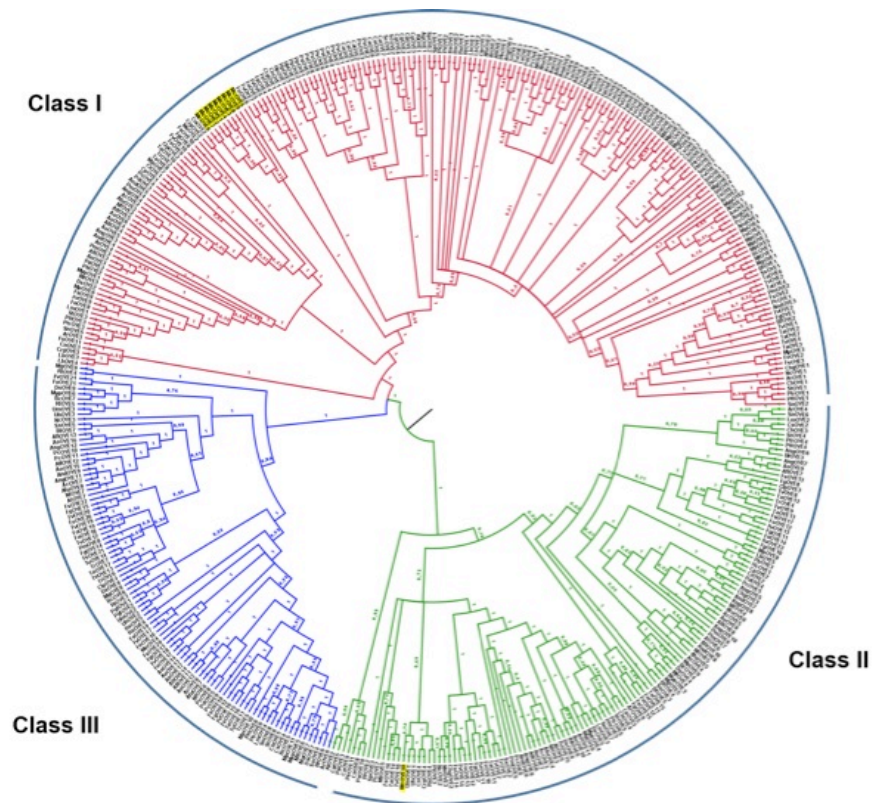
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553 The authors declare no competing financial interests.

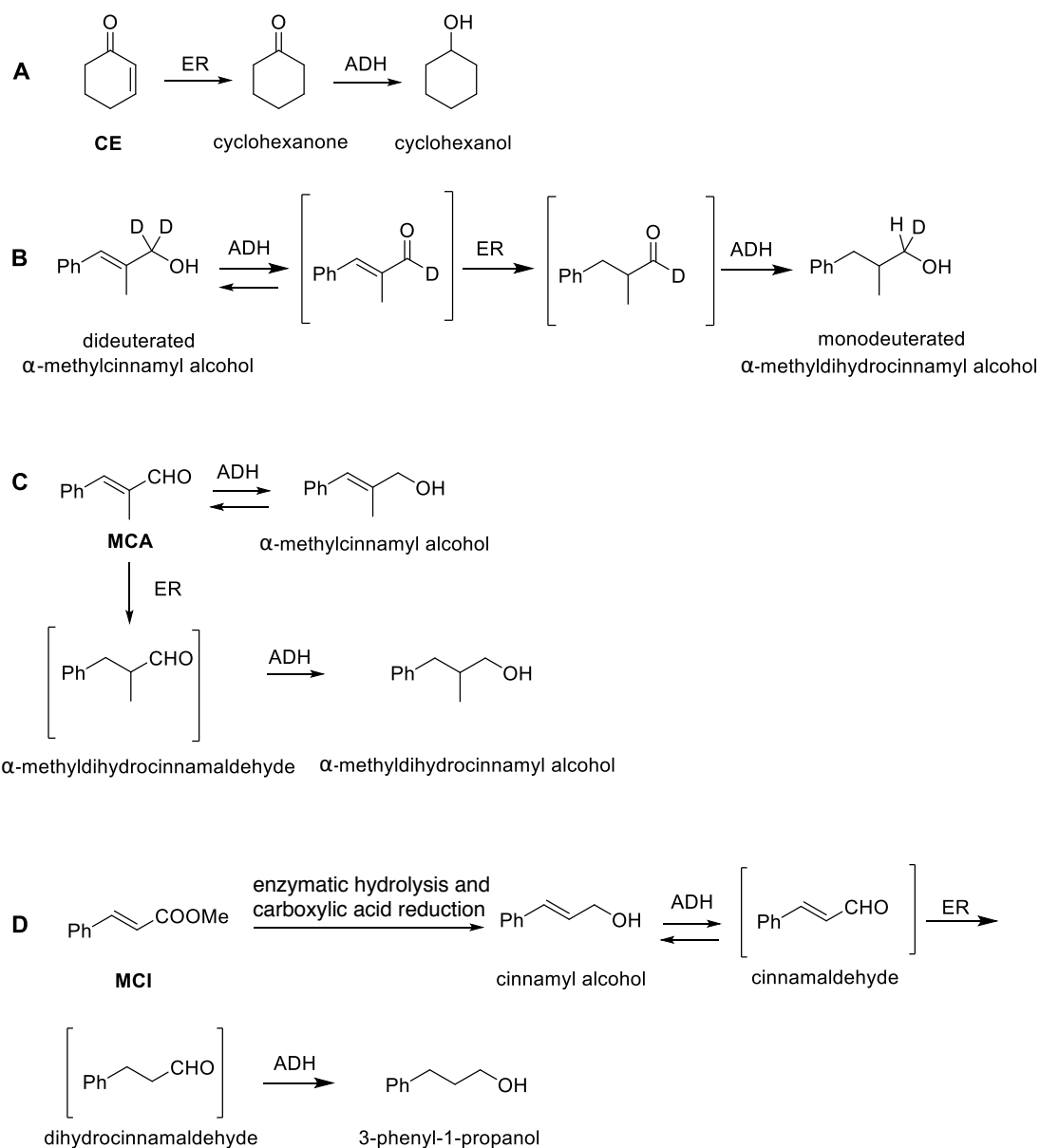
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555 **Figure and figure legends**



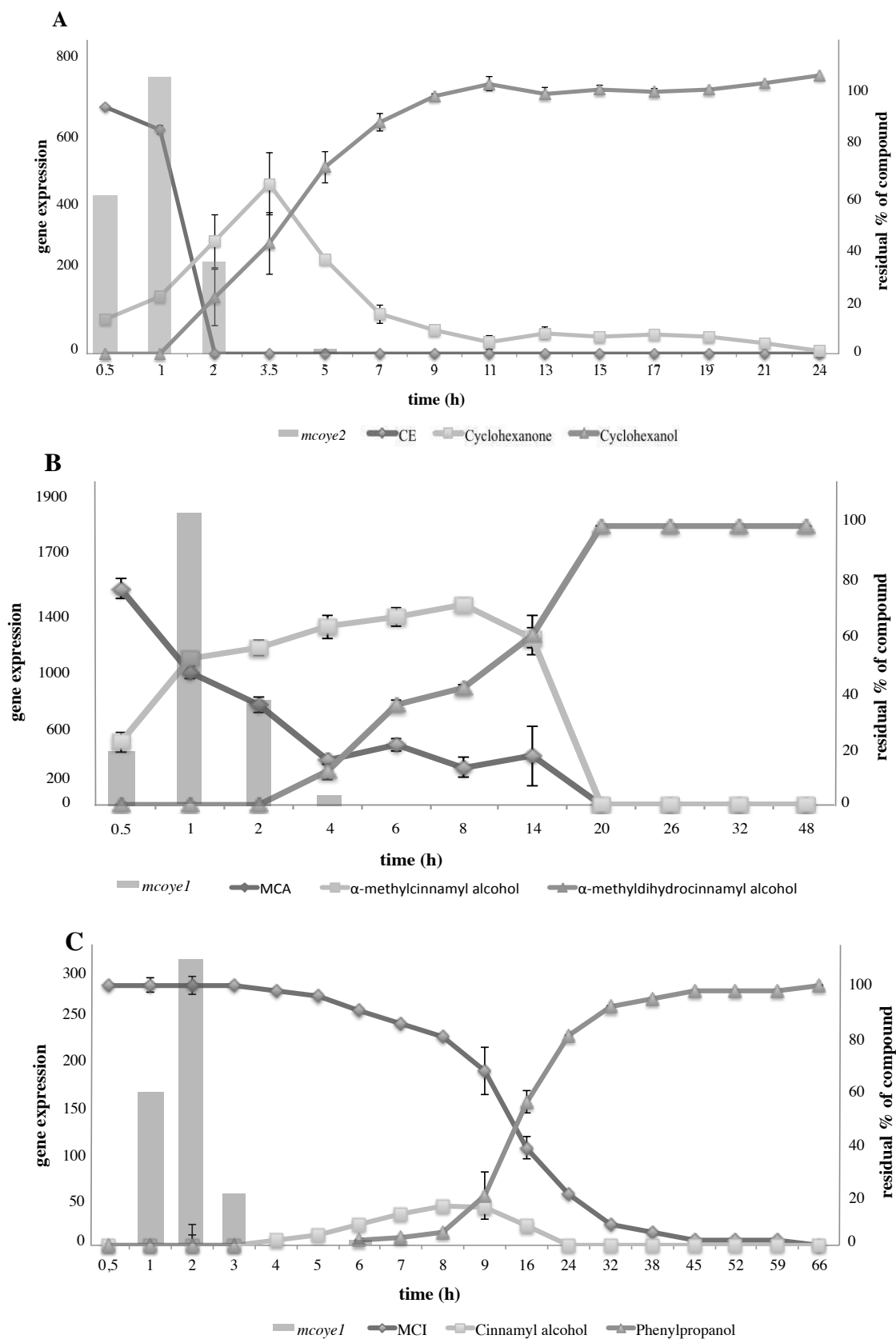
556

557 **Figure 1.** Evolutionary relationship of deduced OYE proteins based on Bayesian
558 inference analysis of the structure-based amino acid sequence alignment. The numbers
559 at the nodes indicates Bayesian posterior probabilities. The phylogenetic tree was
560 implemented from Nizam et al., 2014 [9].



561

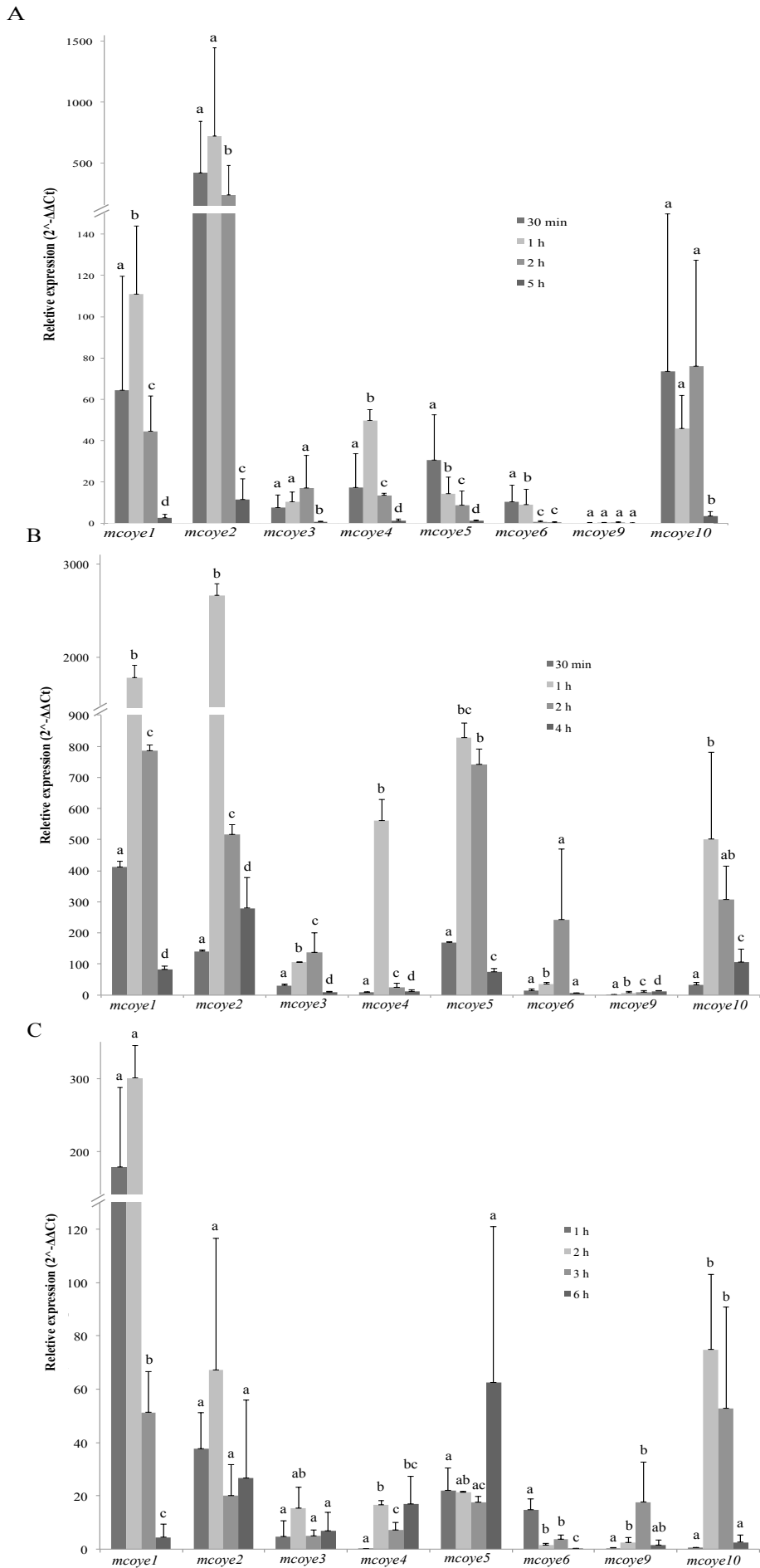
562 **Figure 2.** Reaction profiles of (A) CE, (B, C) MCA and (D) MCI biotransformations.



563

564 **Figure 3.** Graph combining the data of expression of (A) *mcoy2* in presence of CE
 565 (bars) with the biotransformation data of CE (lines); (B) *mcoy1* in presence of MCA
 566 (bars) with the biotransformation data of MCA (lines); (C) *mcoy1* in presence of MCI

567 (bars) with the biotransformation data of MCI (lines). Data are the averages \pm standard
568 deviations (error bars) of the results of at least three different biological replicates.



570 **Figure 4.** Gene expression of OYE homologues in presence of (A) CE, (B) MCA and
571 (C) MCI during the time course experiments. The relative gene expression was
572 calculated with the $2^{-\Delta\Delta C_t}$ method according to Livak & Schmittgen [27] using the β -
573 actin as housekeeping gene [26] and the control (non treated) as reference sample.
574 Different letters indicate statistically significant difference ($p < 0.05$, ANOVA and
575 Tukey's tests) for each gene at the different time points.