Transcriptome analysis of non-ochratoxigenic *Aspergillus carbonarius* strains and interactions with other ochratoxigenic black aspergilli

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Abstract

Aspergillus carbonarius consistently produces large amounts of ochratoxin A (OTA), a mycotoxin with nephrotoxic effects on animals and humans. In the present study, we analyzed the transcriptional changes associated to OTA production in three atypical non-ochratoxigenic strains of *A. carbonarius*. In addition, *in vitro* interactions between ochratoxigenic strains of *A. carbonarius* and *A. niger* and non-ochratoxigenic strains of *A. carbonarius* and *A. tubingensis* were studied in order to evaluate their potential for controlling OTA production. RNA-seq analysis revealed that there are 696 differentially expressed genes identified in the three non-OTA producing strains, including 280 up-regulated and 333 down-regulated genes. A functional and gene ontology enrichment analysis revealed that the processes related to metabolic and oxidation processes, associated with functions such oxidoreductase and hydrolase activity were down regulated. All the genes related with OTA biosynthesis in *A. carbonarius* were the most down-regulated genes in non-ochratoxigenic strains. We also showed that these strains possess a deleterious mutation in the *AcOTApks* gene required for OTA biosynthesis. Moreover, one of these strains gave the best control of OTA production resulting in an OTA reduction of 98-100% in co-inoculation with an ochratoxigenic strain of *A. niger* and an OTA reduction of 79-89% with an ochratoxigenic strain of *A. carbonarius*. Results of this study provided novel insights into the knowledge of the OTA biosynthetic pathway in these non-ochratoxigenic wild strains, and showed the biocontrol potential of these strains.

Keywords: *Aspergillus carbonarius*, ochratoxin A, transcriptome, fungal interactions, biological control agents.

1. Introduction

Ochratoxin A (OTA) is a nephrotoxic mycotoxin that has been related to human and animal diseases. It is classified as a 2B carcinogen by the International Agency for Research on Cancer (Pfohl-Leszkowicz and Manderville, 2012) and can be found in a variety of common foods and beverages (Pit and Hocking, 2009). This mycotoxin is produced by several species of *Penicillium* and *Aspergillus,* being *Aspergillus carbonarius* the main OTA source on grapes and derived products. This species is also considered a potential source of OTA in coffee and cocoa, and it has been also reported from other foods such as figs, maize, paprika, and peanuts among others (Cabañes and Bragulat, 2018).

The genes involved in the OTA biosynthetic pathway have not been completely characterized, even if many studies have been carried out in several OTA-producing fungi (Wang et al., 2016). Recently, a consensus OTA biosynthetic pathway has been proposed in *Aspergillus ochraceus* (Wang et al., 2018). Key genes of the OTA pathway have been identified by comparative genomic analysis with other OTA-producing fungi as *A. carbonarius*, *A. niger*, *A. steynii*, *A. westerdijkiae* and *P. nordicum*.

In *A. carbonarius* a hypothetical OTA gene cluster (cluster 38) has been described (Gerin et al., 2016). This cluster contained 21 genes and only some have been correlated with OTA biosynthesis. Three genes have been directly related to OTA biosynthesis: the nonribosomal peptide synthetase *AcOTAnrps* (Gallo et al., 2012), the polyketide synthase *AcOTApks* (Gallo et al., 2014) and the halogenase *AcOTAhal* (Ferrara et al., 2016). Two other genes located in the same genomic region have been described: the cytochrome p450 monooxygenase *AcOTAp450* and the transcription factor *AcOTAbZIP*. It has been shown that under OTA induction conditions all these genes were up-regulated and co-expressed (Gerin et al., 2016).

69 OTA production is a very consistent property of *A. carbonarius*. While *A.*
70 *carbonarius* consistently produces large amounts of ochratoxin A, the reported
71 percentages of OTA-producing strains in the *A. niger* aggregate is much lower (Cabañes
72 and Bragulat, 2018). In non-ochratoxigenic strains of *A. niger*, a 21-kb deletion in a
73 remnant of the polyketide sinthase gene of the putative OTA cluster has been identified
74 (Andersen et al., 2011). In the same way, the genome analysis of *A. tubingensis*
75 revealed that it does not contain an orthologue of this gene (Choque et al., 2018). In
76 previous studies, we resequenced the genome of three atypical and unique non-OTA
77 producing strains of *A. carbonarius* (Cabañes et al., 2015; Castellá et al., 2018).
78 Although no large deletions in functional genes related with OTA production were
79 found, some private missense variants of non-ochratoxigneic strains in *AcOTApks* gene
80 were detected (Castellá et al., 2018). The rest of the OTA cluster genes (*AcOTAnrps*,
81 *AcOTAp450*, *AcOTAhal*, and *AcOTAbZIP*) showed no privative mutations in the non-
82 OTA producing strains.
83 On the other hand, one of the more promising strategies for control of aflatoxins
84 in crops involves the use of atoxic strains of *A. flavus* as biological control agents
85 (BCAs) (Ehrlich, 2014). These carcinogenic mycotoxins contaminate worldwide food
86 and feed goods (e.g. maize, cotton seed, and peanuts), which pose a serious health risk
87 to humans and domestic animals, and are the cause of crop losses. It is not difficult to
88 isolate this kind of atoxigenic strains in this species because a high percentage of
89 nonaflatoxigenic strains may be present in some natural environments (Alaniz, 2018;
90 Ehrlich, 2008). On the contrary, OTA production is a very consistent property of *A.*
91 *carbonarius* and for this reason atoxigenic isolates of this species are very rarely found
92 (Cabañes et al., 2013).

93 In this study, we applied the High-throughput Next Generation Sequencing-
94 based RNA Seq method to carry out a global transcriptional analysis on four *A.*
95 *carbonarius* strains, one OTA producer and the three atypical non-OTA producing
96 strains. The aim of this study was to analyze the differentially expressed genes between
97 the OTA producer strain and the three non-ochratoxigenic strains to improve knowledge
98 on OTA-related gene expression and to identify genes directly or indirectly related to
99 OTA biosynthetic pathway. Besides, *in vitro* interactions between ochratoxigenic strains
100 of *A. carbonarius* and *A. niger* and non-ochratoxigenic strains of *A. carbonarius* and *A.*
101 *tubingensis* were assessed in order to evaluate their potential for control of OTA
102 production.
103
104 **2. Material and methods**
105
106 2.1. Strains and growing conditions
107 The OTA-producing strain A-1137 and three non-OTA-producing strains of *A.*
108 *carbonarius* A-2160, A-2579 and A-2594 from our fungal collection were analyzed in
109 this study. Strains were grown in triplicate in Czapek Yeast extract broth medium in the
110 dark at 25ºC for 48 h without shaking. After incubation, six plugs were removed from
111 different points of the colony for OTA determination. The rest of the mycelium was
112 removed and stored frozen at -80 ºC prior to total RNA extraction.
113
114 2.2. OTA quantification
115 OTA production was confirmed using a previously described HPLC screening
116 method designed in our laboratory (Bragulat et al., 2001). Plugs removed were extracted

117 with 0.5 ml of methanol. The extracts were filtered and maintained at 4ºC until their
118 analysis.
119 OTA quantification was made by a Waters 2695 chromatograph with a
120 fluorescence detector Waters 2475 (excitation wavelength: 330 nm/emission
121 wavelength: 460 nm), and with a Sunfire C18 column, 150×4.6 mm, i.d., 3.5 µm.
122 Twenty µl of each extract were applied. The mobile phase was acetonitril/water/acetic
123 acid (57/41/2, v/v/v) eluted at a flow rate of 1 ml/min. The extracts with the same
124 retention time as OTA (around 4.8 min), were considered positive. The limit of
125 quantification of the HPLC technique with the extraction procedure was 0.045 µg/ml for
126 OTA.
127
128 2.3. RNA extraction, RNA-Seq library preparation and sequencing
129 Frozen mycelium (100 mg) was powdered in liquid nitrogen and total RNA was
130 isolated using QIAzol lysis reagent (Qiagen, Madrid, Spain) and purified by using
131 RNeasy Plant Mini Kit (Qiagen, Madrid, Spain), following the manufacturer's
132 protocol.Contaminating genomic DNA was removed with DNAse (DNAse I,
133 amplification grade, Invitrogen, Carlsbad, CA, USA). The amount and quality of total
134 RNA was estimated by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific
135 Inc., Wilmington, Delaware, USA) and a Bioanalyzer 2100 (Agilent Technologies,
136 Santa Clara, CA, USA). Next-generation sequencing including quality control was
137 performed by Sequentia Biotech SL (www.sequentiabiotech.com). Indexed libraries
138 were prepared from 1µg of purified RNA with TruSeq Stranded mRNA Sample Prep
139 Kits (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions.
140 Library size and integrity were determined using the TapeStation 4200 / Agilent
141 Bioanalyzer 2100 (Santa Clara, CA, USA). Libraries were pooled so that each index-

142 tagged sample was present in equimolar amounts, with a final concentration of 2nM.
143 The pooled samples were subject to cluster generation and sequencing using an Illumina
144 NextSeq 500 System in paired-end mode (2x150 bp).
145
146 2.4. RNA-Seq data analysis
147 Sequence analysis was performed using the A.I.R. software
148 (https://transcriptomics.sequentiabiotech.com/) developed by Sequentia Biotech.
149 Briefly, raw sequence files were first subjected to quality control analysis by using
150 FastQC v0.10.1 before trimming and removal of adapters with BBDuk
151 (https://jgi.doe.gov/data-and-tools/bbtools/). Reads were then mapped against the
152 *Aspergillus carbonarius* genome (ITEM 5010 v3 - JGI Genome Portal) with STAR v2.6
153 (Dobin et al., 2013). FeatureCounts v1.6.1 (Liao et al., 2014) was then used to obtain
154 raw expression counts for each annotated gene. The differential expression analysis was
155 conducted with the R package edgeR (Robinson et al., 2010) using the Trimmed mean
156 of M-values (TMM) normalization method. Fragments Per Kilobase Million (FPKM)
157 were obtained with edgeR. Gene Ontology Enrichment Analysis was performed using
158 in-house scripts based on the AgriGO publication (Tian et al., 2017). The analysis of
159 transcription factor binding motifs was performed with the software DMINDA2 (Li et
160 al., 2010) and the database JASPAR (Khan et al., 2017).
161
162 2.5. Validation of RNA-Seq results by RT-qPCR
163 The transcription profiles of five genes of the ocratoxin A biosynthesis cluster of
164 *A. carbonarius* (*AcOTApks*, *AcOTAnrps*, *AcOTAhal*, *AcOTAp450* and *AcOTAbZIP*)
165 were analyzed in all strains by using real-time quantitative reverse transcription-PCR
166 (qRT-PCR) as described previously(Castellá et al., 2018). The RNA samples for each

167 replication and cDNA synthesis were run in triplicate. The comparative $2^{-\Delta\Delta Ct}$ method
168 was used to calculate relative gene expression and all data was normalized to β-tubulin
169 and ubiquitin. Expression difference among strains was assessed for statistical
170 significance using the REST 2009 v2.0.13 software (Pfaffl, Horgan and Dempfle,
171 2002). The calibrator sample corresponded to the value of expression of the OTA-
172 producing strain A-1137.
173
174 2.6. Confirmation of *AcOTApks* gene mutations
175 The OTA-producing strain (A-1137) and three non-OTA-producing strains of *A.*
176 *carbonarius* A-2160, A-2579 and A-2594 were grown on malt extract broth medium in
177 the dark at 25ºC for 48 h. Mycelium was recovered and DNA was extracted using the
178 FastDNA Spin kit (MP Biomedicals, Biolink, Barcelona, Spain). The DNA was kept at
179 -20 °C until used as template for PCR amplification. Primers for partial amplification of
180 *AcOTApks* gene (173482) were designed with Primer3Plus tool (Untergasser et al.,
181 2007). Primers PKS1F (5'-GGATCATCCATGGAGGCCAG-3') and PKS1R (5'-
182 TATTGTGCGGGCCAGATCTC-3') and PKS2F (5'-GCCACAGGGGGAATAGTCTT-
183 3') and PKS2R (5'- CCAGGGAACAAGTCAGACCA-3') were designed to confirm the
184 five common mutations found in non-OTA producing strains and located at nucleotide
185 positions 948986, 949202, 950405, 950501 and 950904 of the *AcOTApks* gene. The
186 Big-Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA,
187 USA) and primers PKS1F/R and PKS2F/R were used for sequencing as specified by the
188 manufacturer. Sequencing was performed using an Applied Biosystems 3730 analyzer.
189 The Clustal X v2.0.12 program was used to perform sequence alignments (Larkin et al.,
190 2007).

191 To predict if the amino acid substitutions have an impact on the biological
192 function of the *AcOTApks* gene, we used the PROVEAN software tool (Choi and Chan,
193 2015). (http://provean.jcvi.org/index.php).
194
195 2.7. Assay of the *in vitro* interactions between ochratoxigenic strains of *A. carbonarius*
196 and *A. niger* and non-ochratoxigenic strains of *A. carbonarius* and *A. tubingensis* and
197 their effects on OTA production
198 In total, four strains of black aspergilli isolated from grapes were selected from
199 our culture collection. All the strains had been confirmed previously for identity by
200 DNA sequencing. An OTA-producing strain of *A.carbonarius* (A-1136) (Ac+) and an
201 OTA-producing strain of *A. niger* (A-1241) (An+) were used in this study. Two non-
202 OTA-producing strains, *A. carbonarius* (A-2579) (Ac-) and *A. tubingensis* (A-1747)
203 (At), used as potential biocontrol agents, were screened for their ability to inhibit or
204 reduce OTA production in co-inoculation with the OTA-producing strains (Ac+ and
205 An+). We designed an adaptation of a technique previously described for
206 ecophysiological studies of toxigenic fungal species using microtiter plates (Abarca et
207 al., 2014; 2019). Briefly, the inoculum suspensions of these strains were prepared in
208 sterile saline (0.85%) containing 0.05% Tween 80 from 7-day-old cultures on malt
209 extract agar (MEA) at 25ºC and spore concentration was adjusted to around 10^6
210 conidia/ml by using a haemocytometer chamber. The inoculum size was confirmed by
211 quantitative colony counts (CFU/ml) on MEA plates.
212 Sterile 96-well flat-bottom microtiter plates were used. The liquid culture
213 medium Yeast Extract Sucrose broth (YESB) (per liter, yeast extract, 20 g; sucrose, 150
214 g; $FeSO_4 \cdot 7 H_2O$, 0.5 g; pH adjusted to 6.5) (Samson et al., 2000) was used. The
215 adjusted fungal suspensions were diluted 1:100 in YESB.

216 The effect of the potential biological agent on OTA production was evaluated
217 using different mixed spore suspensions of OTA-producing strain:non-OTA-producing
218 strain ratios of 100:0, 75:25, 50:50, 25:75 and 0:100 respectively in YESB, following a
219 modification of the technique described by Samsudin and Magan (2016) which used a
220 solid culture medium. In each microplate column, seven wells were inoculated with 200
221 µl of the diluted suspension of each inoculum ratio. One well, used as a blank, was
222 filled with 200 µl of the un-inoculated YESB. OTA production was determined after 5
223 and 11 days of incubation at 25ºC. The entire experiment was repeated twice on
224 different days.
225 OTA production was detected using a previously described HPLC screening
226 method developed in our laboratory for fungi growing in microtiter wells (Abarca et al.,
227 2014). On each sampling occasion, the seven replicate wells inoculated for inoculum
228 ratio were removed and extracted with 0.5 ml of methanol. The extracts were filtered
229 and injected into the HPLC. The limit of quantification was 0.045 µg/ml for this
230 mycotoxin.
231 Data obtained from the different conditions tested were statistically analyzed by
232 means of one-way analysis of variance test. All statistical analyses were performed
233 using Minitab 17 statistical software (Minitab Inc., State College, Pennsylvania, USA).
234
235 **3. Results**
236
237 3.1. OTA production of the *A. carbonarius* strains studied
238 All strains presented good growth with proper sporulation forming typical black
239 colonies. *A. carbonarius* A-1137 produced OTA at detectable levels in the three

240 replicates, with a mean value of 5.73 ± 0.41 μg/ml. The non-OTA producing strains A-
241 2160, A-2579 and A-2594 were not able to produce OTA in none of the three replicates.
242
243 3.2. RNA-Seq analysis
244 A summary of RNA-Seq data is reported in Table 1. In total, 18,510,000,
245 17,383,036, 17,438,795, and 18,276,380 high-quality (QS_30) paired-end reads (150
246 bp) were obtained from A-1137, A-2160, A-2579 and A-2594 transcriptome,
247 respectively, and deposited in the NCBI Sequence Read Archive (SRA) database under
248 study accession number PRJNA550023. Approximately 81% of short reads were
249 successfully mapped on the *A. carbonarius* genome. No significant differences were
250 observed in terms of total number of aligned reads among the replicates and the strains.
251 Most of the mapped reads were uniquely mapped, while the remaining reads were
252 unmapped (12–25%) or showed multi-position matches (6–10%).
253
254 3.3. Differential gene expression and functional analysis
255 Based on the TMM normalized values, differentially expressed genes (DEG)
256 were identified (with FDR ≤ 0.05, $\log_2$ fold change ≥ 1 or ≤ -1) between the OTA
257 producing strain A- 1137 and each of the non-OTA producing strains. Analysis of the
258 transcriptional profiles revealed a total of 1,526 DEGs (710 up-regulated and 816 down-
259 regulated), 2,188 DEGs (1159 up-regulated and 1029 down-regulated), and 1,676 DEGs
260 (860 up-regulated and 816 down-regulated) in A-2160, A-2579, and A-2594,
261 respectively. A total of 696 differentially expressed genes were identified comparing the
262 OTA producing strain *vs*. the three non-OTA producing strains. Among these DEGs,
263 280 genes were up-regulated (Figure 1A) and 333 genes were down-regulated in the

264 three non-OTA producing strains (Figure 1B). The rest of differentially expressed genes
265 (83) were up-regulated or down-regulated depending on the strain.
266 These differentially expressed genes were subjected to GO functional
267 enrichment analysis (Supplementary Table S1). The GO terms over-represented in DEG
268 common in the three non-ochratoxigenic strains were related to metabolic process,
269 oxidation-reduction process and transport. Molecular function was related to catalytic
270 activity, oxidoreductase activity, heme binding, iron ion binding, and monooxygenase
271 activity.
272 Among up-regulated DEGs (Table 2, Figure 2), the GO enrichment analysis
273 revealed that several categories for biological processes were enriched among the DEGs
274 of non-OTA producing strains, including ribosome biogenesis, rRNA and tRNA
275 processing, polysaccharide catabolic process, ion transport, and oxidation-reduction
276 process. The oxidation-reduction processes were the most represented. As to molecular
277 function is concerned, monooxygenase activity, helicase activity, phosphopantetheine
278 binding, oxidoreductase activity, iron ion binding, heme binding, catalytic activity,
279 transaminase activity, and FAD binding (flavin adenine dinucleotide) were over-
280 represented. Among down-regulated DEGs (Table 3, Figure 2), over-represented
281 biological processes categories were oxidation-reduction and metabolic processes. As to
282 molecular function is concerned, oxidoreductase and hydrolase activities were over-
283 represented.
284 Within the most down-regulated genes (Supplementary Table S2), we found a
285 polyketide synthase (ID 173482) and one nonribosomal peptide synthetase (ID 132610).
286 The polyketide synthase ID 173482 corresponded to *AcOTApks* gene and the
287 nonribosomal peptide synthetase ID 1326010 corresponded to *AcOTAnrps* gene, both
288 included in the putative OTA cluster (cluster 38) of *A. carbonarius*. Additional down

289 regulated genes were putatively involved in biosynthesis of mycotoxins and other
290 secondary metabolites such as cytochrome P450 monooxygenases, monoxigenases,
291 dehydrogenases, hydrolases and methyltransferases. Different cytochrome P450
292 monoxigenases were down-regulated, but the most down-regulated in the three non-
293 ochratoxigenic strains was the cytochrome P450 monooxygenase ID 517149 which is
294 *AcOTAp450* gene included in the OTA cluster. Moreover, the bZip transcription factor
295 *AcOTAbZip* (ID 7821) and a pepsin B (ID 7823) located in the same cluster were also
296 down-regulated. The halogenase *AcOTAhal* (ID 209543) was also down-regulated
297 although no significant differences were observed (Table 4).

298 Within the up-regulated genes (Supplementary Table S3), we also find genes
299 putatively involved in biosynthesis of secondary metabolites as a polyketide synthase
300 (ID 166447), some nonribosomal peptide synthetases (ID 133906, ID 503574),
301 cytochrome P450 monooxygenases, monoxigenases, dehydrogenases, hydrolases and
302 methyltransferases.

303 We analyzed the expression of 146 highly up-regulated genes describe in *A.*
304 *carbonarius* strains under OTA induction conditions (Gerin et al., 2016). In the non-
305 ochratoxigenic strains an oxalacetate acetylhidrolase (ID 158065) was down-regulated.
306 Also, three genes related with transport and secretion activities were down-regulated in
307 the non-ochratoxigenic strains. These genes were the MFS transporter (FLU1) (ID
308 135554), the ABC multidrug transporter (ID 171384) and the Formate/nitrate family
309 transporter (ID 132418). One gene related to transferase activity (methyltranferase ID
310 133219) and two genes related to oxidation-reduction processes (cytochrome P450
311 monoxygenase ID 517149 and multicopper oxidase ID 519260) were also down-
312 regulated in these strains.

313 We also analyzed expression of gene clusters co-regulated with cluster 38. In the
314 non-ochratoxigenic strains, no DEGs were found in clusters 24 and 48. In cluster 40
315 only 1 gene was down-regulated, a binding factor (ID 133215). In cluster 42 which
316 contained 17 genes, only two genes were DEG, a down-regulated glutation transferase
317 (ID 175671) and an up-regulated molybdenum cofactor sulfurase (ID 175665).
318
319 3.4. Validation of RNA-Seq results by RT-qPCR
320 RT-qPCR was performed on *AcOTApks*, *AcOTAnrps*, *AcOTAhal, AcOTAp450*
321 and *AcOTAbZIP*. Two genes, β-tubulin and ubiquitin, were used as reference genes.
322 Gene expression patterns of the selected DEGs were consistent with those obtained by
323 RNA-Seq (Figure 3), confirming the reliability and accuracy of the NGS analysis.
324
325 3.5. Transcription factors
326 By using Gene Ontology annotations and information in the OrthoMCL database
327 (https://orthomcl.org/orthomcl/), a total of 341 transcriptions factors were identified in
328 the *Aspergillus carbonarius* genome. Only 14 of them were differentially expressed, of
329 which five were down-regulated and nine were up-regulated (Table 5). Only three
330 transcription factors showed conserved motifs known to act as binding sites specific for
331 the putative OTA cluster genes *AcOTApks* (ID 173482), *AcOTAnrps* (ID 132610),
332 *AcOTAp450* (ID 517149), and pepsin B (ID 7823) and all they were all down-regulated.
333 These transcriptions factors were the bZIP transcription factor *AcOTAbZIP* (ID 7821),
334 located in the OTA cluster, and two Zn_2Cys_6 transcription factors, (ID 131099, ID
335 514084) outside the OTA cluster.
336
337 3.6. *AcOTApks* mutations confirmation by Sanger sequencing

338 In order to confirm the five common missense variants found in *AcOTApks* gene
339 of these atoxigenic strains, we designed the primer pairs PKS1F/R and PKS2F/R. We
340 confirm the mutations located at positions 948986, 949202, 950405, 950501 and
341 950904 in scaffold 12. These missense variants produce amino acid substitutions that
342 have a neutral effect on biological functions of the *AcOTApks* with the exception of
343 mutation at position 949202 (Figure 4, Supplementary Figure S1). This mutation
344 produced an amino acid substitution (Y728H) that can have a deleterious impact on the
345 biological function of the PKS_AT domain of the *AcOTApks*.
346
347 3.7. Assay of the *in vitro* interactions between ochratoxigenic strains of *A. carbonarius*
348 and *A. niger* and non-ochratoxigenic strains of *A. carbonarius* and *A. tubingensis* and
349 their effects on OTA production
350 Non-OTA-producing strains of *A. carbonarius* and *A. tubingensis* (Ac- and At)
351 were screened for their ability to inhibit OTA production by ochratoxigenic strains of *A.*
352 *carbonarius* and *A. niger* (Ac+ and An+). Inocula enumerated with a cell counting
353 haemocytometer provided suspensions from 1.3×10^6 to 1.4×10^6 conidia/ml and
354 colony counts were between 4.0×10^5 and 6.6×10^5 CFU/ml. No statistically significant
355 differences were observed between replicates ($p > 0.05$).
356 The effects of OTA-producing strain:non-OTA-producing strain inoculum ratios
357 on OTA production are detailed in Table 6. The results were expressed as a mean of the
358 14 replicates. No statistically significant differences were observed in OTA production
359 values neither between replicates nor between experiments ($p > 0.05$). In most cases, no
360 significant differences were observed in OTA production values between the two
361 incubation times (5 and 11 days).

362 Regarding OTA production, in all cases of co-inoculation the presence of Ac-
363 and At decreased the production at different percentages, depending on their inoculum
364 load. As more load of the non-ochratoxigenic strains assayed, the higher percentages of
365 OTA reduction were detected.

366 Of the two non-ochratoxigenic species assayed, Ac- gave the best control
367 resulting in practically complete inhibition of OTA production in co-inoculation with
368 An+, at all inoculum ratios at the two incubation times. Figure 5A shows some selected
369 chromatograms of extracts of the interactions between these species at different
370 inoculum ratios. Positive control of OTA production (100 An+:0 Ac-) presented a clear
371 peak of OTA (retention time of 5.2 minutes). A small peak with the same retention time
372 is observed at ratio 75:25. The remaining inoculum ratios showed no signals at the same
373 retention time of OTA.

374 For Ac-, OTA reduction was higher than 74% in co-inoculation with Ac+, at all
375 strain ratios at the two incubation times (see Figure 5B). Percentages of OTA reduction
376 ranged from 96% to 100% when At was co-inoculated with An+ at all inoculum ratios
377 at the two incubation times. On the contrary, co-inoculation of At with Ac+ showed
378 lower percentages of OTA reduction.

379

380 **4. Discussion**

381 Despite some studies have been carried out on molecular aspects of OTA
382 biosynthesis, the OTA cluster remains not completely defined and most of the
383 regulatory aspects underlying OTA production remain unclear. In *A. ochraceus* a cluster
384 that contains four biosynthetic genes and two regulators have been related to OTA
385 production (Wang et al., 2018). The biosynthetic genes were a polyketides sinthase
386 (OtaA), a nonribosomal peptide synthetase (Ota B), a cytochrome P450 (Ota C) and a

387 halogenase (OtaD). Two regulators have been described, a bZIP transcription factor
388 (otaR1), which probably functions as an OTA pathway-specific regulator, and a
389 Zn2Cys6 binuclear DNA-binding protein (ota R2), which is adjacent to biosynthetic
390 genes. All the biosynthetic genes of the OTA cluster of *A. ochraceus* were present in *A.*
391 *carbonarius* in cluster 38, and corresponded to *AcOTApks, AcOTAnrps*, *AcOTAp450*,
392 *AcOTAhal* genes. In *A. carbonarius* the transcription factor *AcOTAbZIP* which is
393 located between *AcOTAp450* and *AcOTAhal* is homologous to otaR1 found in *A.*
394 *ochraceus*. The transcriptome analysis of *A. carbonarius* strains under OTA inducing
395 conditions showed that *AcOTApks*, *AcOTAnrps*, *AcOTAp450*, and *AcOTAhal* were
396 highly up-regulated in OTA inducing conditions and all were co-expressed in addition
397 to the *AcOTAbZIP* (Gerin et al., 2016) supporting the fact that the cluster 38 is likely the
398 OTA gen cluster in *A. carbonarius*.

399 In the present study, we applied the RNA-seq to study the whole transcriptional
400 changes associated to OTA production in three non-OTA producing strains of *A.*
401 *carbonarius*. Functional analysis and enrichment GO terms in DEG revealed that the
402 processes related to metabolic and oxidation processes, associated with functions such
403 oxidoreductase and hydrolase activity were down regulated, indicating that changes in
404 the fungal metabolism occurred in the three non-OTA producing strains. Within the
405 most down-regulated genes, we found the putative OTA genes described in *A.*
406 *carbonarius* in cluster 38. The expression level of all the genes related to OTA
407 biosynthesis was down-regulated. These genes corresponded to *AcOTApks* (ID 173482),
408 *AcOTAnrps* (ID 132610), *AcOTAp450* (ID 517149), *AcOTAhal* (ID 209543), and
409 *AcOTAbZIP* (ID 7821).

410 The *AcOTAbZip* has a high similarity to otaR1 and could function as a cluster
411 specific regulator also in *A. carbonarius*. In fact, this transcription factor showed

412 conserved motifs known to act as binding sites of some of the putative OTA genes in *A.*
413 *carbonarius*. *AcOTAbZip* down-regulation may explain the lack of OTA production in
414 the three non-ochratoxigenic strains. The bZIP transcriptions factors typically form
415 homo or hetero-dimers and bind to the promoter regions to regulate the transcription of
416 their target genes. Some bZIP transcriptions factors have been described to regulate
417 mycotoxin production directly by binding at promoters of genes encoding key elements
418 of the biosynthetic pathway, as shown for RsmA regulating sterigmatocystin in *A.*
419 *nidulans* (Yin et al., 2012) and gliotoxin in *A. fumigatus* (Xiao et al., 2010) and AtfB
420 regulating aflatoxin in *Aspergillus parasiticus* (Roze et al., 2011). In *A. ochraceus*, the
421 bZIP otaR1 control the expression of the four OTA biosynthetic genes, particularly
422 otaA but did not regulate the expression of otaR2, a second regulator described in the
423 OTA cluster of this species (Wang et al., 2018). The regulatory mechanisms of otaR1
424 are not known. The second regulator found in OTA cluster, ota R2, can modulate the
425 expression of otaA, otaC and otaD but did not regulate the expression of otaB, otaE or
426 otaR1. In *A. carbonarius*, toward the edges of the OTA cluster, two genes encoding
427 transcription factors have been described (Gallo et al., 2017). These transcription factors
428 (ID 173470, ID 132614) contain Cys –rich motives which are present in zinc-cluster
429 proteins (Zn_2Cys_6), the most common regulators of fungal secondary metabolism
430 (Brakhage, 2013). In our study, both of them were not differentially expressed in the
431 non-ochratoxigenic strains. We also found two Zn_2Cys_6 transcriptions factors outside
432 the genomic region of the OTA cluster and with binding sites to *AcOTApks*, *AcOTAnrps*
433 and *AcOTAp450*. Both of them were down-regulated in non-ochratoxigenic strains.
434 These transcription factors (ID 131099 and ID 514084) were similar to other
435 transcription factors present in different *Aspergillus* species and their function is
436 unknown.

437 The genome analysis of our non-ochratoxigenic strains revealed five private
438 missense variants in *AcOTApks* gene (Castellá et al., 2018). In the present study these
439 mutations of *AcOTApks* have been confirmed by Sanger sequencing. One of the
440 predicted non-conservative amino acid change was deleterious to enzymatic activity of
441 the *AcOTApks*. OTA biosynthesis begins with the *AcOTApks* gene, utilizing acetyl
442 coenzime A and malonyl-CoA to synthesize 7-methylmellein. This first step could be
443 disrupted in the non-ochratoxigenic strains due to the deleterious mutation found in the
444 acyltransferase domain of the *AcOTApks*. The nucleotide polymorphism (A->G) at nt
445 949202 in *AcOTApks* of non-ochratoxigenic strains is sufficient to account for the lack
446 of OTA production by these strains. A similar situation has been reported in some
447 atoxigenic strains of *A. flavus*, where a nucleotide polymorphism near the beginning of
448 the code sequence of *pksA* gene prevent enzyme production and aflatoxin accumulation
449 (Ehrlich and Cotty, 2004).

450 In the non-ochratoxigenic strains the FLU1 transporter (ID 135554) was also
451 significantly down-regulated. This MFS transporter has been putatively involved in
452 OTA transport in *A. carbonarius* (Crespo-Sempere et al., 2010). It has been shown to be
453 significantly up-regulated under OTA induction conditions (Crespo-Sempere et al.,
454 2010; Gerin et al., 2016) although its role in OTA transport has not been elucidated.
455 Moreover, the transcript ID158065, a homologous to the *A. niger ANI_1_92174* gene
456 encoding oxalacetate acetylhydrolase, was also significantly down-regulatd in non-
457 ochratoxingenic strains. This enzyme converts oxalacetate to oxalate and acetate, which
458 is a potential source of polyketide precursors (Kubicek et al., 1988).

459 *A. carbonarius* is the main responsible source of OTA in wine or dried vine
460 fruits from main viticultural regions worldwide and it is also considered a potential
461 source of OTA in coffee and cocoa, among other foods. Besides, it is very consistent in

462 producing OTA, and non-OTA-producing strains in this species are very rare. On the
463 contrary, *A. niger* and *A. tubingensis* have a worldwide distribution and are very
464 frequently isolated from grapes. However, the reported percentage of OTA-producing
465 strains in *A. niger* is very low whereas *A. tubingensis* is considered a non-
466 ochratoxigenic species (Cabañes and Bragulat, 2018; Frisvad et al., 2011).
467 In our study, Ac- gave the best control resulting in practically complete
468 inhibition of OTA production in co-inoculation with An+, and high percentages of OTA
469 reduction with Ac+. On the other hand, almost complete inhibition of OTA production
470 was also detected by At in co-inoculation with An+.
471 These preliminary results obtained with these no-toxigenic wild strains are very
472 promising for the OTA control. An approach would be the use of these wild atoxigenic
473 black aspergilli to outcompete toxigenic *A. carbonarius* strains in the field and minimise
474 OTA contamination. Biological control using microbial antagonists either alone or as
475 part of an integrated control strategy to reduce pesticide inputs, has emerge as a
476 promising approach for control of mycotoxins in crops, both pre- and post-harvest.
477 However, other studies are needed in order to know if these strains are able to work as
478 effective BCAs in the field. Despite the fact that the use of BCAs has often been
479 successful in the lab, their commercialization largely depends on whether they can
480 consistently control mycotoxigenic fungi in different locations and cultivars and reduce
481 mycotoxin levels below the legislative limits (Ehrlich, 2014). These strains are available
482 and our research group is open for collaboration through a transfer agreement of the
483 material to industry.
484
485 **5. Conclusions**

486 The RNA-seq analysis of the three atypical non-ochratoxigenic strains of *A.*
487 *carbonarius* showed that all the genes described to be related with OTA biosynthesis
488 were down-regulated. Also, the *AcOTApks* gene showed a deleterious mutation that it is
489 sufficient to prevent OTA production. Moreover, one of these strains gave the best
490 control resulting in practically complete inhibition of OTA production in co-inoculation
491 with an ochratoxigenic strain of *A. niger* and high percentages of OTA reduction with
492 an ochratoxigenic strain of *A. carbonarius*. This study represents an important step
493 forward to understand the OTA biosynthetic pathway in these non-ochratoxigenic wild
494 strains, which can lead to the development of improved control strategies to reduce the
495 risk of OTA contamination in food products.
496
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506
507 **Conflict of Interest Statement**
508 RAC was employed by company Sequentia Biotech SL. All other authors
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510

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652

653 **Figure captions**

654

655 **Fig. 1.** Venn diagram of (**A**) up-regulated differentially expressed genes (DEGs) (log2
656 FC ≥1) and (**B**) down-regulated DEGs (log2 FC ≤-1) comparing the ochratoxin A
657 (OTA) producing strain A-1137 *vs*. the three non-OTA producing strains A-2160, A-
658 2579, A-2594.

659

660 **Fig. 2.** Most represented GO terms in functional enrichment analyses for differentially
661 expressed genes down- and up-regulated.

662

663 **Fig. 3.** Relative expression analysis by real time PCR of *AcOTApks*, *AcOTAnrps*,
664 *AcOTAhal*, *AcOTAbZIP* and *AcOTAp450* genes in *A. carbonarius* strains A-2160,
665 A2579 and A-2594 grown on Czapek Yeast extract broth. (*$P < 0.05$).

666

667 **Fig. 4.** Alignment of a portion of the deduced amino acid sequences of *AcOTApks* gene
668 of *Aspergillus carbonarius* strains assayed. Identical residues are indicated by dots. [a]
669 Amino acid sequence of the reference strain *A. carbonarius* ITEM 5010.

670

671 **Fig. 5.** Selected HPLC-FLD chromatograms of the extracts of (**A**) the OTA-producing
672 strain *A. niger* A-1241 in co-inoculation with the non-OTA-producing strain *A.*
673 *carbonarius* A-2579, and (**B**) the OTA-producing strain *A. carbonarius* A-1136 in co-
674 inoculation with the non-OTA-producing strain *A. carbonarius* A-2579 (ND: Not
675 detected. No signals at the same retention time of OTA).

676 **Table 1**.

677 Statistics of the Illumina 150 bp paired-end reads and mapping on *A. carbonarius* genome sequence.

678

Strain	Biological replicates	No. total read pairs	Genome		
				N. mapped reads	
			Total mapped reads (%)	Unique match	Multi-position matches
A-1137	1	17,286,120	82,34%	12,949,113	1,283,919
	2	17,540,106	82,88%	13,375,126	1,161525
	3	20,703,776	83,23%	15,643,222	1,589,570
A-2160	1	18,876,503	87,42%	15,277,532	1,224,674
	2	18,190,280	87,50%	14,731,510	1,184,859
	3	15,082,325	80,67%	11,247,505	919,146
A-2579	1	18,880,195	82,10%	14,141,545	1,360,033
	2	15,033,474	78,00%	10,732,980	992,571
	3	18,402,717	74,30%	11,702,543	1,971,291
A-2594	1	21,905,338	81,87%	16,362,498	1,572,395
	2	15,774,483	76,41%	10,979,552	1,073,423
	3	17,149,319	78,14%	12,258,516	1,142,589

679

680 **Table 2**.

681 Enrichment analysis of GO terms up-regulated in differentially expressed genes common in the three non-ochratoxigenic strains.

682

GO ID	Class	Description	Query Item	p-value	FDR	Enrichment Score
GO:0042254	biological_process	ribosome biogenesis	5	1,01E-03	5,77E-03	4,28
GO:0006364	biological_process	rRNA processing	5	1,42E-03	7,97E-03	4,01
GO:0000272	biological_process	polysaccharide catabolic process	3	2,67E-03	1,17E-02	5,02
GO:0008033	biological_process	tRNA processing	3	1,64E-02	3,63E-02	3,04
GO:0006811	biological_process	ion transport	3	2,12E-02	4,34E-02	2,82
GO:0055114	biological_process	oxidation-reduction process	42	2,13E-02	4,34E-02	1,33
GO:0005730	cellular_component	nucleolus	4	1,09E-02	2,71E-02	2,96
GO:0005886	cellular_component	plasma membrane	3	1,50E-02	3,40E-02	3,12
GO:0004497	molecular_function	monooxygenase activity	13	3,24E-04	4,21E-03	2,70
GO:0004386	molecular_function	helicase activity	7	6,96E-04	4,26E-03	3,59
GO:0031177	molecular_function	phosphopantetheine binding	5	7,88E-04	4,71E-03	4,47
GO:0016705	molecular_function	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	10	2,11E-03	9,59E-03	2,52
GO:0005506	molecular_function	iron ion binding	11	2,21E-03	9,86E-03	2,39
GO:0020037	molecular_function	heme binding	11	3,79E-03	1,44E-02	2,24
GO:0003824	molecular_function	catalytic activity	30	5,69E-03	1,79E-02	1,55
GO:0008483	molecular_function	transaminase activity	3	1,24E-02	2,96E-02	3,30
GO:0071949	molecular_function	FAD binding	5	1,86E-02	3,94E-02	2,38

683
684

685 **Table 3.**

686 Enrichment analysis of GO terms down-regulated in differentially expressed genes common in the three non-ochratoxigenic strains.

687

GO ID	Class	Description	Query Total	Query Item	Background Total	Backgroun d Item	p-value	FDR	Enrichment Score
GO:0055114	biological_process	oxidation-reduction process	283	57	9428	1218	1,94E-04	1,84E-03	1,56
GO:0070941	biological_process	eisosome assembly	283	3	9428	17	1,39E-03	7,43E-03	5,88
GO:0008152	biological_process	metabolic process	283	27	9428	590	1,06E-02	3,00E-02	1,52
GO:0032126	cellular_component	eisosome	283	3	9428	18	1,74E-03	9,12E-03	5,55
GO:0005576	cellular_component	extracellular region	283	4	9428	49	1,53E-02	3,99E-02	2,72
GO:0016407	molecular_function	acetyltransferase activity	283	3	9428	6	1,14E-05	1,17E-04	16,66
GO:0016491	molecular_function	oxidoreductase activity	283	44	9428	894	3,50E-04	3,08E-03	1,64
GO:0016810	molecular_function	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	283	4	9428	36	4,13E-03	1,52E-02	3,70
GO:0016787	molecular_function	hydrolase activity	283	38	9428	858	5,55E-03	1,77E-02	1,48
GO:0010181	molecular_function	FMN binding	283	4	9428	48	1,40E-02	3,71E-02	2,78

688

689 **Table 4.**

690 Down-regulated genes in ochratoxin A (OTA) cluster in the three non-OTA producing strains.

691

Transcript ID	Description	Log2 FC		
		A2160	A2579	A2594
173482	Polyketide synthase	-8,33	-7,55	-8,52
132610	Nonribosomal peptide synthetase	-8,07	-6,30	-7,57
209543	Halogenase	-8,97	-6,47	-7,15
517149	Cytochrome P450	-6,87	-6,12	-6,46
7821	BZIP transcription factor	-5,46	-5,62	-4,48
7823	Pepsin B	-2,60	-2,12	-1,68

692

693 **Table 5.**

694 Transcriptions factors differentially expressed in non-ochratoxigenic strains.

695

Transcript ID	OrthoGroup	Description	A2160	A2579	A2594
7821	OG5_147188	BZIP transcription factor	-5,46	-5,62	-4,48
131099	OG5_155600	Transcription factor	-2,77	-2,05	-2,08
154243	OG5_188739	Transcription factor	-1,78	-1,76	-1,58
13310	OG5_159084	Transcription factor	-1,4	-1,21	-1,87
514084	OG5_175802	C6 zinc finger domain protein	-1,15	-0,92	-0,78
143623	OG5_127437	C2H2 transcription factor	0,5	0,48	0,75
146632	OG5_139332	C6 zinc finger domain protein	0,61	0,57	0,6
144278	OG5_140254	C6 transcription factor (Gal4)	0,99	1,46	1,44
33782	OG5_188739	Transcription factor	1,22	1,59	1,11
513323	OG5_152733	Xylanolytic transcriptional activator xlnR	1,41	1,76	1,62
132388	OG5_176156	BZIP transcription factor	1,56	3,08	2,29
125349	OG5_149802	BZIP family transcription factor	1,66	2,09	2,02
130875	OG5_187896	Transcription factor	1,81	1,81	1,84
145697	OG5_159360	Transcription factor	3,09	1,5	1,91

696

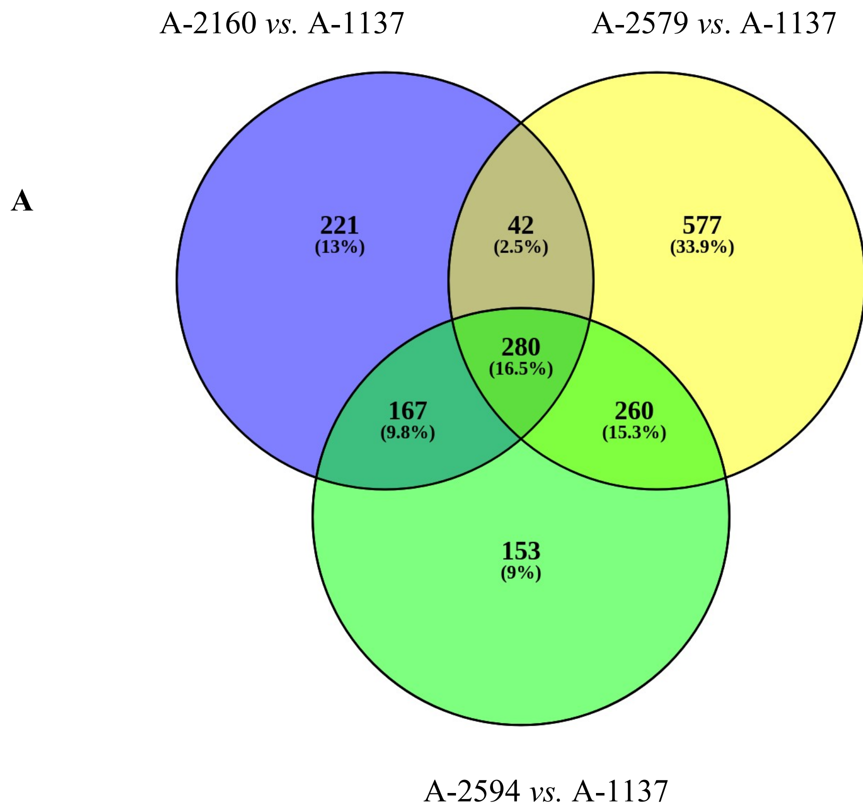
697 **Table 6.**

698 Mean values of ochratoxin A (OTA) concentration in µg / ml produced by the strains assayed in the two experiments.

Co-inoculated strains	days	OTA				
		100 / 0 †	75/25 (%R)	50 / 50 (%R)	25 / 75 (%R)	0 / 100
Ac + / Ac -	5	[a]21.25 ± 11.0	[b]5.42 ± 2.78 (74%)	[b,c]3.3 ± 2.1 (84%)	[b,c]2.4 ± 2.6 (89%)	[c] ND
	11	[a]30.5 ± 4.7	[b]7.64 ± 3.63 (75%)	[c]3.6 ± 3.2 (88%)	[c]3.4 ± 3.4 (89%)	[d]ND
An + / Ac -	5	[a]6.49 ± 2.13	[b]0.10 ± 0.24 (98%)	[b]ND (100%)	[b]ND (100%)	[b]ND
	11	[a]3.42 ± 2.04	[b]0.014 ± 0.027 (99%)	[b]ND (100%)	[b]ND (100%)	[b]ND
An + / At	5	[a]10.54 ± 3.68	[b]0.45 ± 0.57 (96%)	[b]0.023 ± 0.028 (99.8%)	[b]ND (100%)	[b]ND
	11	[a]5.14 ± 2.61	[b]0.13 ± 0.16 (97%)	[b]0.009 ± 0.02 (99.8%)	[b]ND (100%)	[b]ND
Ac + / At	5	[a]8.87 ± 4.46	[a]7.86 ± 3.43 (11%)	[a,b]6.72 ± 3.34 (24%)	[b]4.96 ± 3.35 (44%)	[c] ND
	11	[a]19.76 ± 4.91	[b]10.89 ± 4.59 (45%)	[b]9.51 ± 2.25 (52%)	[c]6.55 ± 3.15 (67%)	[d]ND

†, spore suspension ratios of each strain (in %).
(%R), denotes de percentage of OTA reduction.
[a,b,c,d] In files, values with the same superscript are not significantly different ($p > 0.05$).
Ac, *A. carbonarius*; An, *A. niger*; At, *A. tubingensis*; +, OTA-producing strain; -, non-OTA-producing strain

Figure 1



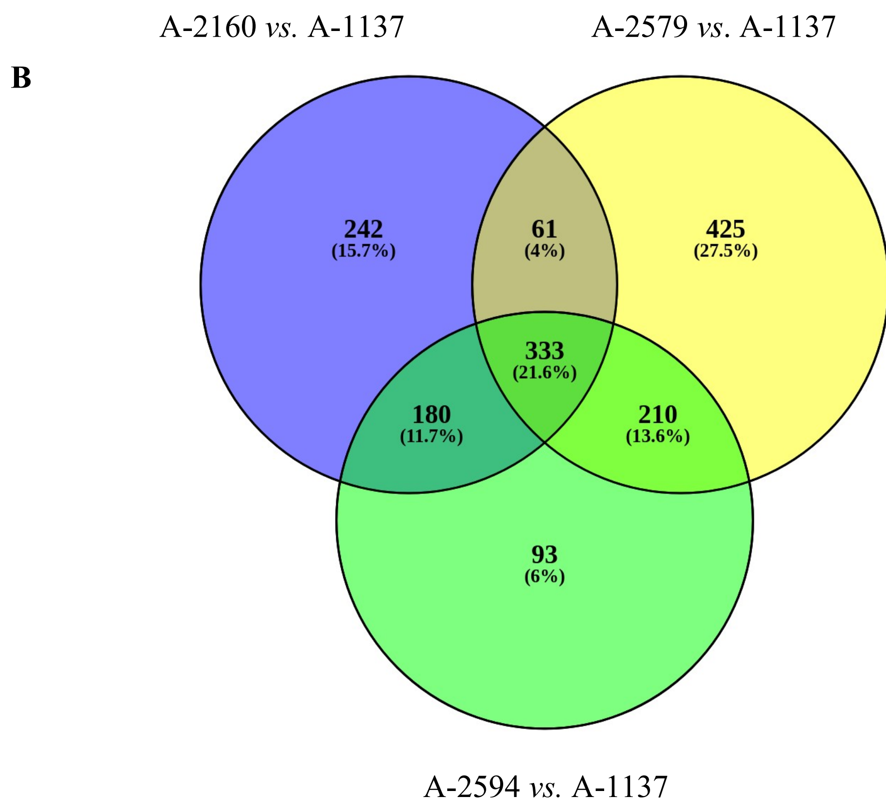


Figure 2

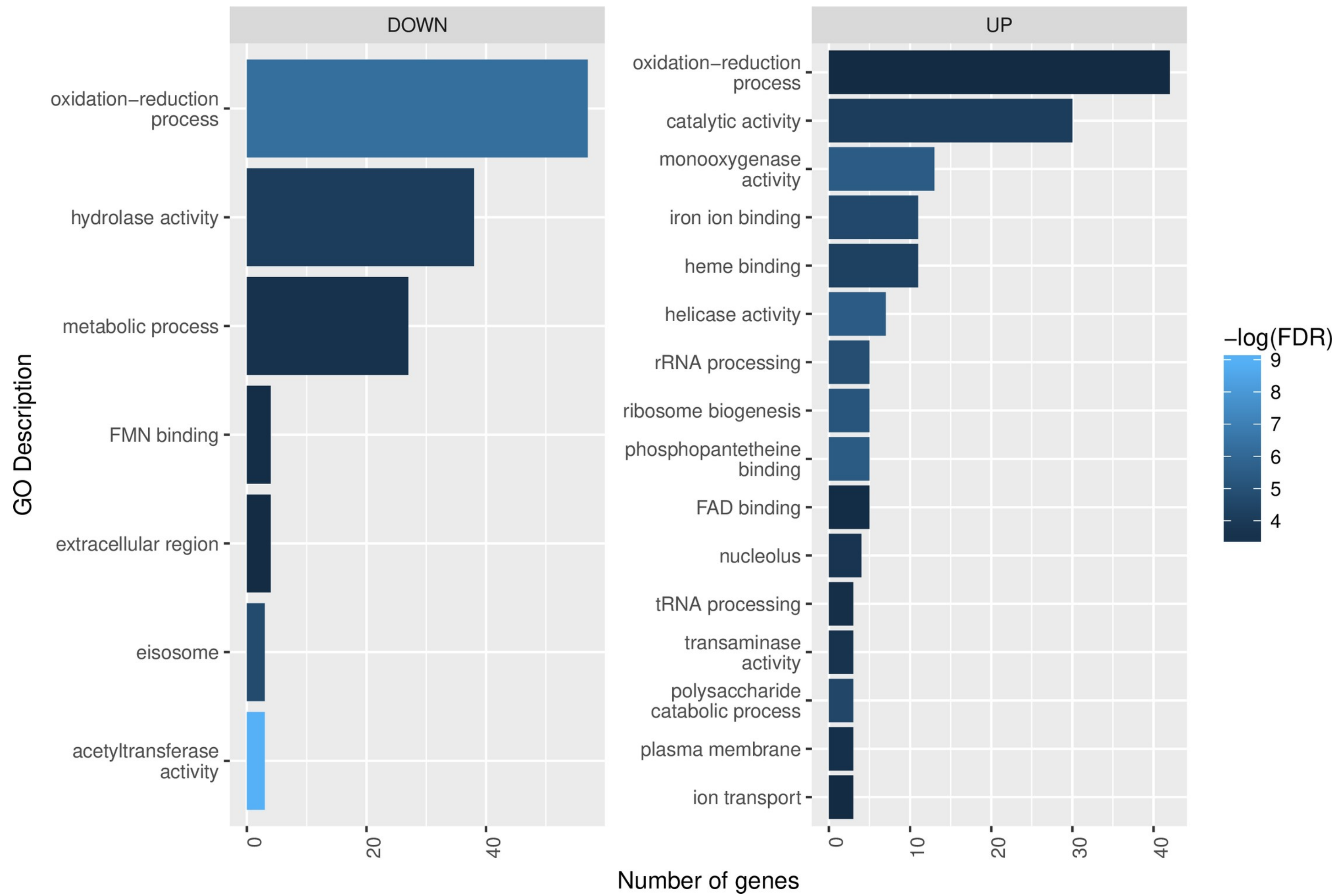


Figure 3

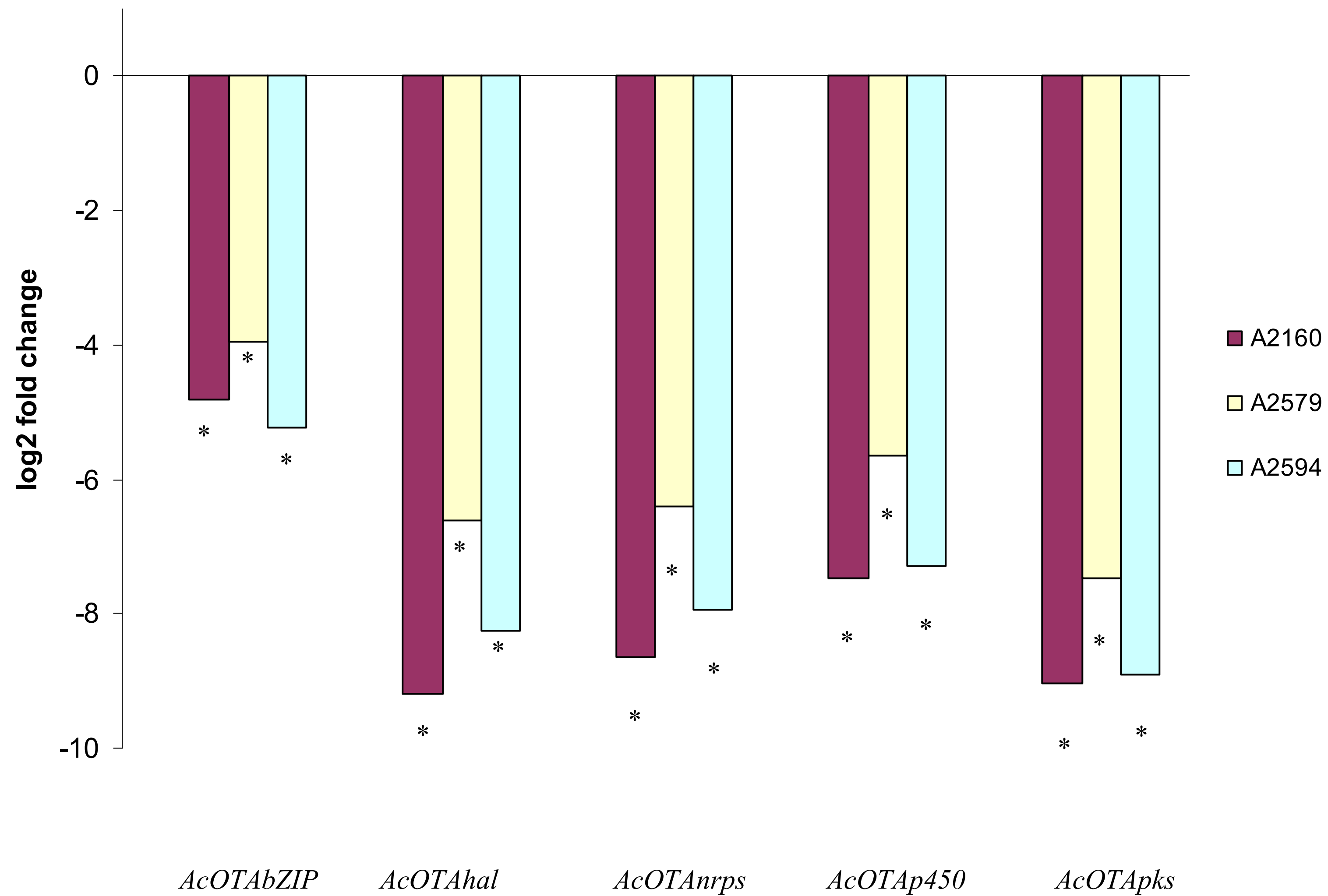


Figure 4

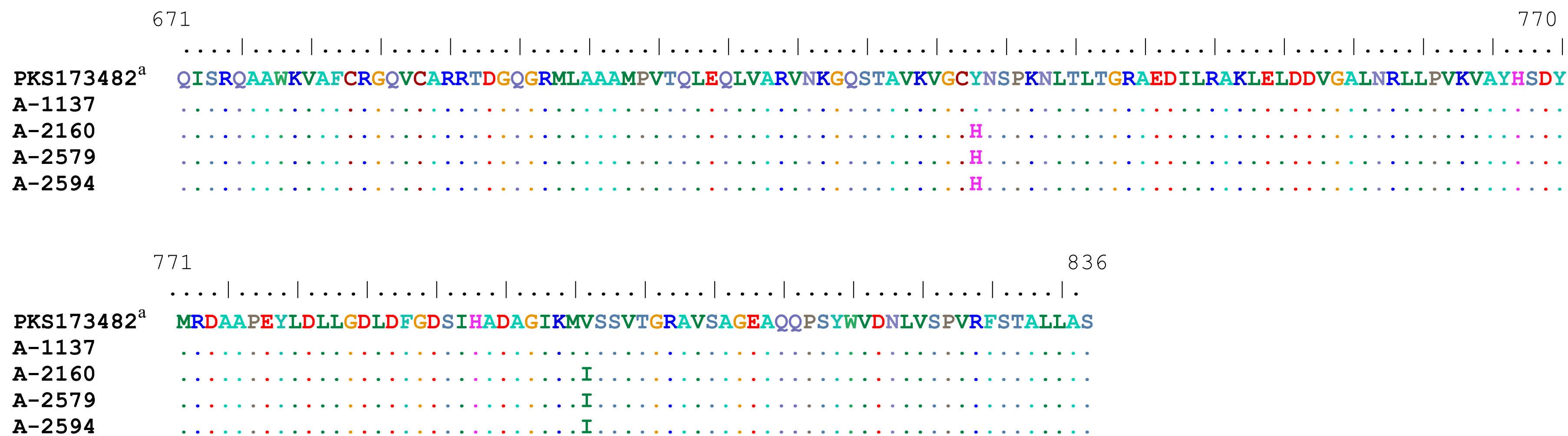
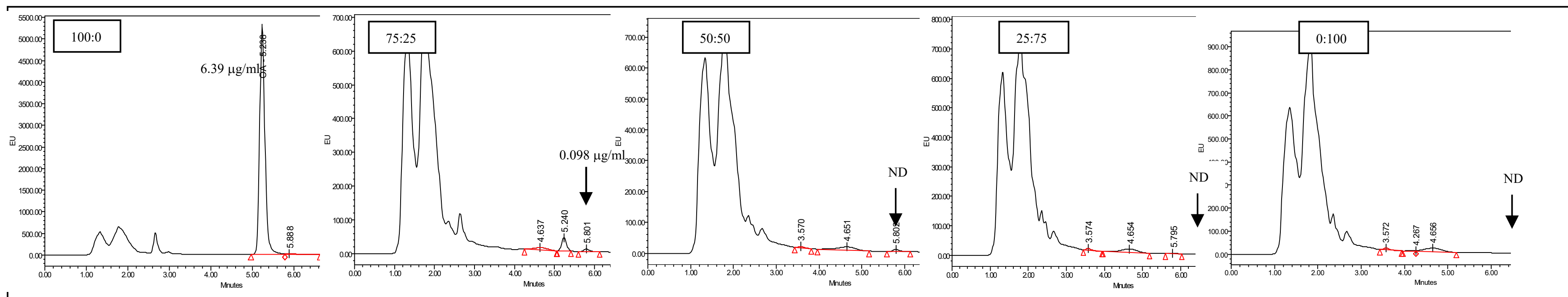


Figure 5

(a)



(b)