1 *FcRav2,* A GENE WITH ROGDI DOMAIN INVOLVED IN FUSARIUM HEAD BLIGHT

2 AND CROWN ROT ON DURUM WHEAT CAUSED BY *FUSARIUM CULMORUM*

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29 **SUMMARY**

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F. culmorum is a soil-borne fungal pathogen able to cause foot and root rot and Fusarium head blight on small grain cereals, particularly on wheat and barley. It causes significant yield and quality loss and results in the contamination of kernels with type B trichothecene mycotoxins. Knowledge on pathogenicity factors of this fungus is still limited. A transposon tagging approach based on the *mimp1/impala* double component system has allowed us to select a mutant altered in multiple metabolic and morphological processes, trichothecene production and virulence. The flanking regions of *mimp1* were used to seek homologies in the *F. culmorum* genome and revealed that *mimp1* had reinserted within the last exon of a gene encoding a hypothetical protein of 318 amino whichthe *FcRav2* gene, encoding a hypothetical protein of 318 amino acids, and containsing a ROGDI like leucine zipper domain, supposidelysupposedly playing a protein- protein interaction or a regulatory role. By functional complementation and bioinformatic analysis we characterize the gene as yeast Rav2 homologue, acknowledging the high level of divergence of the gene in Pezizomycotinain multicellular fungi. Deletion of *FcRav2* or its orthologous gene in *F. graminearum* highlighted its ability to influence a number of functions including virulence, trichothecene type B biosynthesis, resistance to azoles and resistance to osmotic and oxidative stress. Our results indicate that the FcRav2 protein (and possibly the RAVE complex on the whole) may become a suitable target for new antifungal drug development or plant-mediated resistance response also in filamentous fungi of agricultural interest.

KEY WORDS: Fusarium head blight, *Fusarium graminearum*, virulence genes, molecular target, transposon tagging, fungicide, fungal pathogens.

INTRODUCTION

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Fusarium culmorum (W.G. Smith) Sacc, along with *F. graminearum* Schwabe and *F. pseudograminearum* O'Donnell & T. Aoki, are considered the most devastating fungal pathogens on small-grain cereals (soft and durum wheat, barley, oat, rye and triticale). The interest in these species is justified by their role in the onset of two distinct diseases, namely foot and root rot (FRR, also known as "crown rot") and Fusarium head blight (FHB) (Goswami and Kistler, 2004; Wagacha and Muthomi, 2007; Xu and Nicholson, 2009; Kazan *et al.,* 2012; Scherm *et al.,* 2013). FRR infection results in pre- or post- emergence seedling death, brown discoloration on coleoptiles and formation of whiteheads, leading to significant yield losses. However, yield and grain quality is particularly affected when these pathogens induce FHB, infecting the heads at anthesis and colonizing tissues until grain harvest. FHB infection causes contamination of the grain with mycotoxins, such as type B trichothecenes, i.e., sesquiterpene epoxides that are able to inhibit eukaryotic protein synthesis, induce apoptosis, and may play an important role as virulence factors. Significant progress has been made during the last years towards a better understanding of the processes involved in FHB, especially in *F. graminearum* (Kazan *et al.,* 2012; Jiang *et al.,* 2010; Lysøe *et al.,* 2011; Fu *et al.,* 2013; Liu *et al.,* 2014; Sperschneider *et al.,* 2015, Ma *et al.,* 2013). On the contrary, knowledge on *F. culmorum* pathogenicity is still poorly understood, and despite genes are reported from whole genome sequencing projects (Urban *et al.,* 2016; Moolhuijzen *et al.,* 2013) only a few new potential fungicide targets in this species have been reported so far (Skov *et al.,* 2004; Baldwin *et al.,* 2010; Scherm *et al.,* 2011; Pasquali *et al.,* 2013).

 Aiming at the identification of new *F. culmorum* genes playing a role in both FHB and FRR on durum wheat, we have undertaken a transposon-mediated mutagenesis approach based on the heterologous element *mimp1* (Hua-Van *et al.,* 2000). *mimp1* does not code a transposase gene, but can be trans-mobilized by the transposase of the *impala* element (Dufresne *et al*., 2007, 2008; Spanu *et al.,* 2012).

81 Here we report on the identification of a new *F. culmorum* gene (*FcRav2*), ~~not yet described in~~

82 ~~Leotiomycetes for the first time while our paper was under revision in~~ *~~Neurospora crassa~~* ~~(Tu et al~~

83 ~~2017)in Leotiomycetes~~, by *mimp1*-mediated insertional mutagenesis. The putative role of *FcRav2*

84 was determined in *F. culmorum* and, as a comparison, in *F. graminearum* by analyzing the effect of

85 deletion on the fungal phenotype.

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89 **RESULTS**

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91 **Molecular characterization of the *mimp1*-tagged mutant R38**

92 PCR and Southern blot analyses confirmed that the excision event in the revertant strain R38 was

93 followed by the reinsertion of the *mimp1/impala* construct into a different genome site (not shown).

94 Based on the left flanking sequence of the *mimp1* element (BLASTn results revealed that *mimp1*

95 had reinserted at the position 6542003 of the UK99 genome draft (LT598662.1), in the gene

96 FCUL_11566.1, located in the 4th chromosome of *F. culmorum* (Supplementary Figure 1).

97

98 **Characterization of the *FcRav2* gene**

99 *FcRav2* (in *F. graminearum* *FgRav2* corresponding to Fg09428/FGSG_17209) has two introns and

100 3 exons and codes for a hypothetical protein of 318 amino acids, with a molecular weight of 34.8

101 kDa, and an isoelectric point of 6.43. The amino acid sequence contains a ROGDI leucine zipper

102 domain (pfam10259), including a region of 30 amino acids with leucine repeats every seven or

103 eight residues (Supplementary Figure 2). Homologous genes, with unknown function, were found

104 in *F. oxysporum* (FOX_06114) and *F. verticillioides* (FVEG_03978) with sequence homologies of

105 up to 83%, as well as in other filamentous fungi (Supplementary Figure 3). Orthology conservation

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106 is strongly confirmed (e-value Eggnog e-145 in the Class *Leotiomycetes*). Orthology classification

107 from NCBI allocates the gene within the Subclass Sordariomycetidae including *Neurospora crassa*

108 (NCU08091) and *Magnaporthe oryzae* (MGG_02604) ROGDI containing group. Lower level of

109 conservation was found within the Class *Saccharomycetes* using both Eggnog and Inparanoid.

110 Being the domain unique and the homology along the overall protein length, we hypothesize that

111 orthology is also occurring with *Rav-2* homologue in *Saccharomyces cerevisiae* as well with higher

112 eukaryotes (Dawson *et al.*, 2008). Protein localization is likely nuclear according to WoLF-PSORT

113 analysis (http://www.genscript.com/wolf-psort.html; Horton *et al.*, 2007).

114 According to TMpred (http://www.ch.embnet.org/software/TMPRED_form.html; Hoffmann and

115 Stoffel, 1993) the protein may contain a transmembrane domain (AA 178-201) collocating its N-

116 terminal in the internal membrane part. Hypothetical structure obtained with RaptorX (Källberg et

117 al 2012) is reported in Supplementary Figure 4.

118

119 **Obtainment of *FcRav2/FgRav2* deletion mutants in *F. culmorum* UK99 and in *F. graminearum***

120 **PH1 and annotation confirmation**

121 A total of seven hygromycin B resistant transformants were obtained from *F. culmorum* UK99, 5

122 showing a distinct deletion, and 2 being ectopic transformants (Figure 1). One ectopic strain (*Fc*B6)

123 and two *ΔFcRav2* strains (Δ*FcRav2* B24, Δ*FcRav2* B51) were used for all the following assays and

124 characterizations. In addition, five FGSG_17209 deletion mutants were obtained from *F.*

125 *graminearum* PH1, among which Δ*FgRav2* G8 and Δ*FgRav2* G10 (Figure 1) were selected for

126 further analyses.

127 To verify the *in silico* annotation of the gene the wild type strain UK99 was treated with 11 μmolar

128 bafilomycin that is known to inhibit specifically V-ATPase complex (Faraco *et al.,* 2014). Indeed

129 the phenotype of the wild type treated with bafilomycin reproduced the same morphological

130 features of the mutants including colony morphology, hyphal hyperbranching and pigmentation

131 (Figure 2), allowing to confirm that at least part of the phenotypic effect of the *FcRav2* mutation

132 can be mimicked by a vATPase inhibiting drug.

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134 **Deletion of the *FcRav2/FgRav2* gene involves significant changes in physiological and**

135 **metabolic profiles of *F. culmorum* and *F. graminearum***

136 All *FcRav2* deletion mutants as well as the revertant strain R38 showed significantly decreased

137 growth compared to the recipient strains FcM7, *F. culmorum* UK99, and *F. graminearum* PH1 when

138 coping with multiple osmotic stresses, while the ectopic transformant strain *Fc*B6 displayed the

139 same responsiveness as the *F. culmorum* wild-type strain UK99 (Table 1; Supplementary Figures 5-

140 8). Addition of 2 M sorbitol or 0.02% [w/v] SDS led to more incisive growth reductions than 1 M

141 NaCl. *F. graminearum* was completely inhibited at 0.02% [w/v] SDS, and therefore PH1, Δ*FgRav2*

142 G8 and Δ*FgRav2* G10 were characterized in a second test series on 0.01% [w/v] SDS, where

143 deletion mutants displayed a significant growth reduction compared to the wild type PH1 (Table 1).

144 Only *F. culmorum* strain FcM7 and its revertant R38 were able to grow in the presence of 30 mM

145 $K_2S_2O_8$, with R38 being significantly inhibited (over 70%) compared to FcM7. In the presence of

146 20 mM $K_2S_2O_8$ all *F. culmorum* and *F. graminearum* deletion mutants were significantly inhibited

147 compared to the respective wild-type (Table 1).

148 Conidial germination was significantly impaired in the *F. culmorum* and *F. graminearum* deletion

149 mutants compared to the wild-type strains. On the contrary, germination capability of the revertant

150 strain R38 was not reduced compared to co-transformant strain FcM7 (Table 1).

151 *FcRav2* deletion dramatically reduced mycelium hydrophobicity: while for both the wild type strain

152 UK99 and the ectopic transformant *Fc*B6 the time required to absorb a 20 μL drop of water was >

153 15 min, in Δ*FcRav2* B24, Δ*FcRav2* B51 the water droplet was immediately adsorbed upon

154 deposition (data not shown).

155

Tebuconazole sensitivity is significantly increased in the *mimp1*-tagged mutant and in deletion mutants

Addition of 0.5 μg/mL of tebuconazole to Czapek dox agar determined a significant reduction of the colony growth in all deletion mutants. In *F. culmorum*, growth of the *mimp1*-tagged mutant R38 decreased by 70% compared to the co-transformant FcM7, while *FcRav2* deletion resulted in 25-35% growth inhibition on fungicide-amended medium. Similarly, *F. graminearum* deletion mutants grew less than 50% compared to the *F. graminearum* wild-type strain PH1 in the presence of tebuconazole (Table 1).

FcRav2 **gene plays a major role in sugar metabolism**

Using a phenotype microarray approach, *F. culmorum* wild type strain UK99, its deletion mutant Δ*FcRav2* B24, and the ectopic transformant strain *Fc*B6 were further screened for their putative phenotype differences connected to low molecular weight carbon uptake and metabolism. The complete set of triplicate OD_{750} readings recorded every 15 min from 0 to 72 h of incubation on 96 different carbon sources is reported in Supplementary Table 1 (available at: https://drive.google.com/file/d/0B2zMAlgHF40RWE5jX3lsTFdsY0k/view?usp=sharing).

Based on the analysis of the growth curves, the interval comprised between 60 and 72 h was selected as the most informative to highlight differences in growth among the three tested strains. The heat map depicted in Figure 3 reports the average OD_{750} readings recorded during the 60-72 h interval on the 30 most differentiating carbon sources, listed in order of decreasing proportional growth difference between deletion mutant Δ*FcRav2* B24 compared to its wild type strain UK99.

The deletion mutant Δ*FcRav2* B24 was impaired in its ability to catabolize different mono-, di- and trisaccharide carbon sources (particularly L-sorbose, D-xylose, α-methyl-D-glucoside, lactulose, L-arabinose, L-fucose, palatinose, D-ribose, D-mannose, D-fructose, D-raffinose, D-melezitose), with percent growth rates between 45 and 70% compared to the wild type UK99 and the ectopic strain

181 *Fc*B6 (Figure 3). Moreover, the deletion mutant grew at a reduced rate on 2-amino ethanol and on

182 weak acids such as bromosuccinic acid and β-hydroxy-butyric acid (Figure 3).

183

184 ***FcRav2* influences the severity of crown rot and Fusarium head blight on durum wheat**

185 In a preliminary screening, the *mimp1*-tagged mutant R38 was compared to the co-transformant

186 strain FcM7 and to the wild-type strain UK99 to evaluate the effect on seed germination. Abundant

187 mycelium formation by all tested strains was observed after 24-48 h. However, only mutant R38 did

188 not hamper the emergence of the primary root from the caryopsis, while FcM7 and the highly

189 virulent strain UK99 killed 100% of the inoculated durum wheat seeds before germination (data not

190 shown).

191 Greenhouse experiments further confirmed the role of *FcRav2/FgRav2* in both crown rot and

192 Fusarium head blight. Insertion of the *mimp1* transposable element or deletion of the

193 *FcRav2/FgRav2* gene in *F. culmorum* and in *F. graminearum* caused a highly significant ($P < 0.01$)

194 reduction of crown rot symptoms on durum wheat seedlings for all tested mutants, while the ectopic

195 transformant *Fc*B6 behaved similarly to the wild-type reference strains UK99 and PH1, which

196 caused disease incidences of 95 and 100, respectively (Table 2; Supplementary Figure 9).

197 In spray-inoculation tests, *FcRav2* inactivation by *mimp1* insertion (R38) or deletion (Δ*FcRav2* B51

198 and Δ*FcRav2* B24) reduced FHB symptoms on durum wheat heads at 21 dpi to 10 and 5-20%

199 compared to *F. culmorum* control strains FcM7 and UK99, respectively (Table 2; Supplementary

200 Figure 9). On the contrary, the ectopic transformant *Fc*B6 was not significantly affected in its

201 virulence towards the host plant.

202 In *F. graminearum*, *FgRav2* deletion mutants have completely lost their pathogenicity, hence

203 differing significantly from the wild-type reference strain PH1 (Table 2).

204 Infection experiments showed a minimal, albeit not significant, modulation of gene expression

205 during the different stages of interaction with the plant. Indeed, expression profiles obtained in *F.*

206 *graminearum* from array studies suggest that the gene modifies its expression according to the

207 phenological stage of infection, but that these changes depend on various conditions and therefore

208 depict a high variability in experimental repetition *in planta* (Figure 4).

209

210 **_FcRav2_ is involved in type-B trichothecene production**

211 In *in vitro* experiments, only the *F. culmorum mimp1*-tagged mutant R38 has lost completely its

212 ability to produce type B trichothecenes, while trichothecene production by deletion mutants

213 Δ*FcRav2* B24 and Δ*FcRav2* B51 did not differ significantly from the wild type strain UK99 and

214 from the ectopic transformant *Fc*B6 (Table 2). In the case of *F. graminearum*, mutant Δ*FgRav2* G8

215 was not significantly affected by gene deletion in its ability to produce deoxynivalenol (DON) and

216 its acetylated form 3-ADON, while trichothecene production by mutant Δ*FgRav2* G10 was reduced

217 by approximately 70% (Table 2).

218 In accordance to the development of FRR symptoms, type B trichothecenes levels reached 1,000-

219 2,000 ng/g in seedling stem tissue infected by *F. culmorum* strains FcM7, UK99 and *Fc*B6 and by *F.*

220 *graminearum* strain PH1. On the contrary, trichothecene mycotoxins were not detected in wheat

221 seedlings upon inoculation with the *mimp1*-tagged mutant R38 and with deletion mutants of both *F.*

222 *culmorum* UK99 and *F. graminearum* PH1 (Table 2).

223 When the *F. culmorum* FcM7 was spray-inoculated, multiple infection sites in the spikes resulted in

224 far higher levels of trichothecene content in the infected kernels, whereas the *mimp1*-tagget mutant

225 R38 produced approximately 15% mycotoxin compared to the co-transformant strain FcM7 (Table

226 2). Infected kernels collected from spikes that were spray-inoculated with *F. culmorum* ectopic

227 transformant *Fc*B6 contained unusually high trichothecene content (approximately 12,000 ng/g

228 tissue), whereas the wild type strain UK99 and both deletion mutants Δ*FcRav2* B24 and Δ*FcRav2*

229 B51 did not differ significantly in their deoxynivalenol production upon kernel colonization

230 (approximately 1,700-2,600 ng/g tissue; Table 2).

231

232

DISCUSSION

Within the course of an extensive transposon-mediated mutagenesis program in the wheat pathogen *F. culmorum* (Spanu *et al.,* 2012), a pathogenicity mutant was identified among over 2,000 *niaD* revertants on minimal medium containing nitrate as the sole nitrogen source. In this mutant, the *mimp1* transposable element was integrated within the last exon of the *FcRav2* gene, encoding a hypothetical protein of 318 amino acids, and containing a ROGDI like leucine zipper domain known to have a regulatory role (pfam 10259). The ROGDI domain is conserved in eukaryotic genomes from *Drosophila* to yeasts and is mostly unique. Our protein presents some low level of similarity with the *RAV2* gene from yeast (Seol *et al*., 2001), part of the RAVE complex involved in the assembly and disassembly of vATPase complex in yeast (Kane, 2006).

Based on the analysis of phenotype, expression and function we hypothesize that the gene is also in *F. graminearum* and in *F. culmorum* a *rav2* homologue. As *RAV2* genes homologues have not been investigated before in other fungi, apart from *Schizosaccharomyces pombe*, where the gene is important for sugar response and Ca stress (Dawson *et al*., 2008), our work represents the first ~~description~~ characterisation of the role of RAV2 in filamentous fungi. More importantly, we showed that the protein affects the fitness of the fungus including the ability to grow on different sugars as well as the sensitivity to pH and fungicides such as tebuconazole.

Treatment with the specific vATPase inhibitor bafilomycin reproduced the same morphological features of ΔFcRav2 mutants, including the hyphal hyperbranching observed upon deletion of the *vmaA1* gene, which encodes one of the subunits of the vATPase in *Aspergillus nidulans* (Melin *et al*., 2004). The vATPase has been shown to play a role in virulence for different fungi (Patenaude *et al*., 2013; Chen *et al*., 2013; Hilty *et al*., 2008). The impairment of pathogenicity observed in *F. graminearum* and in *F. culmorum* confirms indirectly the putative function of *FcRav2/FgRav2* as the homologue of *RAV2*, a controller of vacuolar and endosomal processing.

258 Further investigations are needed to experimentally confirm the role of the gene in filamentous

259 fungi. Given that conservation of the *RAV2* gene is lower compared to ATPase components

260 (Dawson *et al*., 2008), or that it may be present only in fungi (Parra *et al.,* 2014), it would be

261 feasible to focus on molecules able to interfere with RAV2 *in planta* in order to impair the toxigenic

262 process as well as fostering the activity of known fungicides, as suggested already for human fungal

263 pathogens (Hayek *et al*., 2014). For this reason we propose that RAV2 in *F. graminearum* and in *F.*

264 *culmorum* may represent a new target for developing integrative approaches against fungicide

265 adaptation and resistance or low sensitivity phenomena that are becoming important against

266 different fungicides in Fusaria (Dubos *et al*., 2011, 2013; Talas and Mc Donald, 2015; Becher *et al*.,

267 2011; Serfling and Ordon, 2014; Chen and Zhou, 2009).

268 *FcRav2* appears deeply involved in a number of morphological and physiological traits, while no

269 definite phenotypes were identified in *Candida albicans* in a large genomic screening of mutants

270 (Noble *et al.,* 2010). Spore germination and hydrophobicity are significantly altered in deletion

271 mutants, as well as resistance to various stresses: Δ*FcRav2* inactivation resulted in increased

272 sensitivity to osmotic and oxidative stress, and to the sterol biosynthesis inhibitor tebuconazole. The

273 higher susceptibility to tebuconazole observed for the *RAV2* mutant is a further confirmation of the

274 importance that alternative mechanisms related to the extrusion of the compound can play in azole

275 resistance. Zhang *et al*. (2010) already hypothesized that azole resistance mechanism involves also

276 acidification of the medium.

277 The gene transcription seems to be influenced by azole treatments. Indeed, by analysing gene

278 expression patterns of *F. graminearum* after treatment with sublethal concentrations of

279 tebuconazole, Becher *et al*. (2011) found a decreased expression of FGSG_17209. This leads to the

280 hypothesis that FGSG_17209 (*FgRav2*) may play an indirect role in controlling the expression of

281 genes involved in compensating the azole stress, or that the gene is underexpressed as growth is

282 decreased.

283 A putative role of *FcRav2* in stress resistance may be also inferred by reduced growth of the

284 deletion mutant on some of the tested carbon sources. For instance, L-sorbose is a well-known

285 inhibitor of cell wall biosynthesis in fungi and showed a paramorphogenic action on *Neurospora*

286 *crassa* (Mishra and Tatum, 1971; Trinci and Collinge, 1973). Ethanolamine (2-amino ethanol) is

287 commonly used as wood preservative (Humar *et al.,* 2003). Also, weak acids such as bromosuccinic

288 acid and β-hydroxy-butyric acid may represent a source of stress on an impaired deleted mutant.

289 These data suggest that *FcRav2* is, as *RAV2* in yeast, involved in stress, including the stress *F.*

290 *culmorum* is exposed to by being confronted with its victim plant.

291 The lower capacity of *FcRav2* deletion mutant to metabolize different mono-, di- and trisaccharides

292 in comparison to the wild type is also concordant with the hypothesis that it represents the *RAV2*

293 homologue. Indeed the RAVE complex in yeast is important for the reassembly of the vATPAse

294 machinery under sugar starvation (Smardon *et al.,* 2015). Our Biolog experiment confirms the

295 major role that *FcRav2* plays in sugar metabolism. This hypothesis is corroborated by expression

296 data obtained from independent experiments, highlighting under-expression of *FgRav2* in *F.*

297 *graminearum* during glucose starvation (Figure 4). In particular, among the sugars that determine

298 the most significant differences in growth between the mutant and the wild type, xylose and

299 arabinose belong to the pathway of pentose and glucuronate interconversions pathway (Fungipath)

300 that is important in conditions of glucose starvation. Noteworthy, RAV2 is overexpressed in *S.*

301 *cerevisiae* exposed to high sugar stress (Erasmus *et al.,* 2003), and RAV2-overexpressing

302 *Saccharomyces pastorianus* bottom fermenting strains exhibited increased ethanol tolerance and

303 increased fermentation rates in high-sugar medium (Hasegawa *et al.,* 2012).

304 As mentioned, phenotype microarray analysis highlighted a significantly reduced ability of the

305 *FcRav2* deletion mutant in the utilization of 2-amino-ethanol as carbon source. Quite interestingly, a

306 behavioral screening of *P*-transposon-generated memory mutants coupled to DNA microarray

307 analysis highlighted a *Drosophila melanogaster* ROGDI mutant and suggested its role in long-term

308 olfactory memory (Dubnau *et al.,* 2003) and reduced ethanol tolerance (Berger *et al.,* 2008). This

309 would support indirectly the hypothesis that homologous function are maintained by the gene with

310 ROGDI domain also in higher eukaryotes, although further evidences are needed to corroborate

311 such functional homology.

312 *FcRav2/FgRav2* has a major impact on fungal virulence in *F. culmorum* and *F. graminearum*,

313 respectively, and influenced the severity of crown rot and Fusarium head blight on durum wheat.

314 This role was clearly demonstrated by different inoculation methods (contact between mycelium

315 and caryopsis, spray-inoculation of conidia), and was confirmed in *F. graminearum*.

316 Infection experiments with *F. graminearum* (Lysoe *et al.,* 2011; Güldener *et al.,* 2006) showed a

317 minimal, albeit not significant, modulation of gene expression during the different stages of

318 interaction with the plant. Indeed, expression profiles suggest that the gene modifies its expression

319 according to the phenological stage of infection, but that these changes depend on different

320 conditions likely related to the variability in experimental repetitions *in planta*.

321 Somehow less clear was the implication of *FcRav2* in mycotoxin production. The effect of *FcRav2*

322 disruption is likely dependent on the substrate: while the *F. culmorum mimp1*-tagged mutant R38

323 had lost completely its ability to produce type B trichothecenes *in vitro*, deletion mutants did not

324 differ significantly from their wild type strain. In seedling stem tissue, mycotoxin biosynthesis was

325 completely inhibited upon *FcRav2* deletion, while DON and 3-ADON were still abundantly

326 produced by deleted strains colonizing spray-inoculated wheat spikes. This suggests that specific

327 inducers of DON by-passing *FcRav2* regulation exist in the spike that are not able to activate the

328 production of toxin in the stem or *in vitro*. Therefore, a plausible hypothesis is that some molecules

329 in the spike may favor the synthesis of the toxin independently from *FcRav2* control.

330 Interestingly, we observed a diverse DON production *in vitro* and *in planta* for some of the strains.

331 The phenomenon has been observed also for the transcription factor in FG Atf1 (Nguyen *et al.*,

332 2013), which is also a stress regulator (Lawrence *et al.*, 2007) with opposite behavior compared to

333 *FcRav2*. To better elucidate the mechanisms that lead to high toxin contamination there is a need to

334 further characterize the specific molecules that the fungus may encounter *in planta*.

335   In yeast the lack of vacuolar acidification in the RAVE mutants is possibly compensated by other

336   mechanisms of acidification of other compartments (Smardon *et al.,* 2014). This would explain the

337   relatively mild phenotype of the *rav1Δ* and *rav2Δ* mutants. We would anticipate that this

338   acidification process is likely under the control of FgAtf1, in the HOG pathway. It is therefore

339   plausible that the HOG pathway and the RAVE complex play two different but complementary

340   roles in toxin production, linking toxin production and stress response pathways (Ponts *et al.,*

341   2015). To further verify this hypothesis we are currently investigating the cellular components of

342   the assembly.

343   Expression profiles obtained from different array studies carried out on FG PH1 strain confirms

344   indirectly the homology of *FcRav2* with *RAV-2* gene from *S. cerevisiae*. Indeed, major oscillations

345   of the gene (higher expression) are observed in agmatine medium that is a strong toxin inducer

346   (Gardiner *et al.,* 2009a; Pasquali *et al.,* 2016a). Given the need for vacuole assembly in condition of

347   high toxin production (toxisomes, Menke *et al.,* 2013) it is plausible that a vacuole acidification is

348   highly induced when high level of toxins should be produced.

349   From a more practical perspective, FcRav2 is proposed as a suitable target for new antifungal drug

350   development or plant-mediated resistance response, given its pivotal role affecting hydrophobicity,

351   sugar metabolism, secondary metabolism, virulence, and resistance to various stresses. Our work is

352   also the confirmation that untargeted methods for the identification of genes involved in fitness and

353   pathogenicity are useful for the identification of genes that are not directly identified as homologous

354   with normal orthology search programs. The further exploration of the *mimp1* reinsertion mutant

355   library will possibly allow in the future the identification of further genes involved in the fitness of

356   the pathogen.

357

358

359   **EXPERIMENTAL PROCEDURES**

360

Fungal strains and storage conditions

A *mimp1*-mediated revertant (see below) strain collection was generated from the *F. culmorum* FcM7 co-transformant strain, integrating a single copy of the *niaD::mimp1* construct and the *impalaE* transposase gene under constitutive control of the *gpdA* promoter (Spanu *et al.,* 2012, Dufresne *et al*., 2007).

Homologous recombination experiments were carried out with *F. culmorum* wild type strain UK99 (Baldwin *et al.,* 2010; kindly provided by Dr. Kim Hammond-Kosack, Rothamsted Research, UK) and with *F. graminearum* strain NRRL 31084 (syn. PH1), obtained from H.C. Kistler and deposited in the Luxembourg Microbial Culture Collection (Pasquali *et al.,* 2016b).

All fungal strains were routinely cultured on potato dextrose agar (PDA, Sigma-Aldrich, St. Louis, MO, USA). For long-term storage, plugs colonized by mycelium were transferred to 50% [v/v] glycerol and stored at -80°C.

Isolation of revertants originating by *mimp1/impala* double component system

The *mimp1/impala* revertant strains were selected on Minimal Medium Agar supplemented with 50µg/mL hygromycin (MMH50) containing sodium nitrate as sole nitrogen source (Spanu *et al.,* 2012). Briefly, selection was performed with a phenotypic assay by inoculating 10 µL of a spore suspension (10^6 CFU/mL) of the co-transformant strain FcM7 on MMH50 plates and by incubating at 25°C up to 1 month. First nia^+ colonies (referred to as "revertants") appeared 15 days after inoculation upon reacquisition of the nitrate-reductase function, and consisted in patches of aerial mycelium with a wild-type phenotype. A single-spore culture was obtained from each collected revertant strain.

Bioassay screening of the revertant strain collection

385   Single-spore revertant strains were preliminarily screened for virulence during the first steps of

386   kernel colonization by using an *in vitro* bioassay described by Pasquali *et al.* (2013). Ten PDA-

387   mycelium plugs of each revertant bearing one durum wheat seed (cv. Simeto) were placed into a 90-

388   mm diam sterile Petri dish and incubated at 25°C for 3-5 days in the dark. Control assays included

389   FcM7, *F. culmorum* strain UK99 and sterile PDA plugs. Inhibition of seed germination and kernel

390   death were visually observed.

391

392   **Molecular characterization of selected revertants**

393   Excision of the *mimp1* transposable element from the *niaD* gene was evaluated by Southern blot

394   using a *niaD*-specific probe, whereas a 120-bp fragment of the *mimp1* transposable element was

395   used as probe to verify the reinsertion events (Table 3). Identification of the reinsertion site of

396   *mimp1* was achieved by Splinkerette-PCR (Potter *et al*., 2010) according to the protocol described

397   by Spanu *et al.* (2012). Flanking sequences were blasted to the *Fusarium culmorum* genome (Urban

398   *et al.,* 2016) in order to identify *mimp1* locations.

399

400   **Creation of deletion mutants and mutant screening**

401   *FcRav2* deletion mutants in *F. culmorum* strain UK99 and deletion mutants for the FGSG_17209

402   gene homologue in *F. graminearum* strain PH-1 (*FgRav2*) were obtained by split-marker

403   recombination as described by Breakspear *et al*. (2011). First screening for deletion mutants and

404   ectopic strains was carried out by PCR applying several primer combinations (Table 3; 1F-

405   FGSG17209/4R-FGSG17209,   NF-FGSG17209/NR-FGSG17209,   NF-FGSG17209/4R-

406   FGSG17209, and 1F-FGSG17209/NR-FGSG17209). PCR mix was made up of 0.5 μM of each

407   primer, 1X Phusion[®] High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs) and 20

408   ng of DNA in a final volume of 20 μL. A subset of putative mutants was then analyzed by Southern

409   blot with 2 different probes, (i) gene upstream probe obtained with primers 1F/2R (916 bp), and (ii)

410   internal gene probe NF/NR (456 bp) (Table 3). Probe labeling, hybridization and detection reactions

411   were carried out using the Dig High Prime DNA labeling kit and detection starter II® (Roche

412   Applied Science, Basel, Switzerland) following the manufacturer's protocol.

413

414   **Phenotypic analysis of transposon-tagged and deletion mutants of *Fusarium culmorum***

415   The following strains were screened for altered growth under osmotic and oxidative stress or

416   sensitivity to the fungicide tebuconazole: *mimp1*-tagged *F. culmorum* revertant strain R38, co-

417   transformant strain FcM7; wild-type *F. culmorum* strain UK99, deletion mutants Δ*FcRav2* B24 and

418   Δ*FcRav2* B51, ectopic transformant strain *Fc*B6; *F. graminearum* strain PH1, deletion mutants

419   Δ*FgRav2* G8 and Δ*FgRav2* G10. Assays were performed on Czapek dox agar (Oxoid Limited,

420   Hampshire, UK) and Czapek dox agar supplemented with either 2 M sorbitol, 1 M NaCl, 0.01-

421   0.02% [v/w] sodium dodecylsulphate (osmotic stress), 20-30 mM $K_2S_2O_8$ (oxidative stress), and 0.5

422   μg/mL tebuconazole (fungicide sensitivity). Ten μL of a titrated conidial suspension ($1x10^6$

423   CFU/mL) were spotted on the center of each plate (60 mm Ø). Colony diameter growth was

424   measured after 3 days of incubation at 25°C in the dark, and compared to the respective controls.

425   Conidiogenesis and spore germination were evaluated by inoculating 150 mL of Czapek dox broth

426   with 5 mL of conidial suspension ($1x10^6$ CFU/mL). At 0, 2, 4, and 8 hpi (25°C, 150 rpm), 100

427   conidia were examined with a hematocytometer using a light microscope (Olympus BX41). Three

428   independent tests were performed for each assay, with three replicate plates for each test.

429   Mycelium hydrophobicity was evaluated according to Pasquali *et al.* (2013). Briefly, a 20- μL

430   droplet of sterile H_2O was pipetted onto the surface of colonies grown on solid Vogel's medium

431   (Vogel, 1956) for 5 days. The time (in seconds) needed to get complete droplet absorption by

432   deletion mutants Δ*FcRav2* B24 and Δ*FcRav2* B51, and by the ectopic transformant strain *Fc*B6 was

433   compared to the wild-type strain UK99.

434   The wild-type *F. culmorum* strain UK99 and its deletion mutant Δ*FcRav2* B24 were further

435   screened for phenetic differences applying BIOLOG FF MicroPlates (Biolog Inc., Hayward, CA,

436   USA). The plate panel composition, which contains 95 low molecular weight carbon sources, is

437 available   in   the   BIOLOG   web   site   (http://www.biolog.com/products-

438 static/microbial_identification_literature.php).

439 Spore suspensions were prepared in carboxy-methyl cellulose liquid medium (CMC, Pasquali *et al.,*

440 2013) and after two subsequent washing steps with sterile distilled water, spores were suspended in

441 "Inoculation fluid FF" (Biolog) at a final concentration of $1x10^4$ CFU/mL. One-hundred μL of

442 spore suspension were pipetted into each of the 96 wells. Plates were incubated at 25°C for 90h and

443 OD was recorded at 750 nm every 15 min using the Microarray Omnilog Reader (Biolog, Inc.

444 Hayward, CA). Samples were tested in triplicate.

445

446 **Pathogenicity assay on durum wheat**

447 The *F. culmorum* co-transformant FcM7 and its revertant strain R38, wild-type strain UK99,

448 deletion mutants Δ*FcRav21* B24 and Δ*FcRav2* B51, and the ectopic transformant strain *Fc*B6; *F.*

449 *graminearum* strain PH1, deletion mutants Δ*FgRav2* G8 and Δ*FgRav2* G10 were tested for their

450 virulence *in planta*. Both FRR and FHB disease were evaluated.

451 Mycelium plugs, each bearing one durum wheat seed (*Triticum durum* cv. Iride, kindly provided by

452 Unità di Ricerca per la Valorizzazione Qualitativa dei Cereali, CRA-QCE, Rome, Italy) were placed

453 in a plastic sowing pot and covered by sterile soil. Durum wheat seeds placed onto sterile PDA

454 plugs (1 seed/plug) served as negative control. FRR severity was assessed after 21 days of

455 incubation at 25°C in a greenhouse and evaluated using the McKinney index (McKinney, 1923;

456 Spanu *et al.,* 2012). Three independent tests were carried out, each consisting of 3 replicates of 10

457 seedlings for each fungal strain.

458 The ability to cause FHB was tested on durum wheat (cv. Iride) at mid-anthesis stage wheat heads.

459 Spray inoculation was carried out with 4 mL of each spore suspension ($1x10^5$ spores/mL) covering

460 the head until runoff. Inoculated heads were coated with a transparent plastic bag for 48 hours to

461 maintain high humidity conditions. Disease incidence was evaluated every 7 days and a final score

462   was carried out at 21 dpi using the McKinney index. Each strain was tested in four replicates of five

463   durum wheat heads each.

464

465   ***In vitro* and *in planta* mycotoxin production**

466   *In vitro* production of deoxynivalenol and its acetylated forms was determined in Vogel's medium

467   (Vogel, 1956) as described by Pani *et al.* (2014) and expressed in ng/mL of culture filtrate.

468   The presence of trichothecenes in durum wheat seedlings (cv. Iride; 3 replicates of 10 seedlings

469   each) was determined by inoculating seedlings at emergence with a conidial suspension (100 μL of

470   1×10^6 CFU/mL) of each strain. After 15 dpi, the basal portions of 10 seedlings were pooled, dried at

471   80°C for 24 h, finely ground in a mortar and weighed. Samples were purified by MycoSep® 227

472   Trich+ columns (Romer Labs, Tulln, Austria), as described by the manufacturer, previous to LC-MS

473   spectrum analysis. Quantitative determinations were carried out as described previously (Pani *et al.,*

474   2014), using a model HP 1100 liquid chromatography and mass spectrophotometric detector

475   (Agilent Technologies, Palo Alto, USA).

476   The content of trichothecenes in durum wheat seeds (cv. Iride) harvested from the spray-inoculated

477   heads was determined by using the ROSA® FAST5 DON assay (Rapid One Step Assay, Charm

478   Sciences, Inc. Lawrence, MA, USA), according to the manufacturer's protocol. Total trichothecene

479   content is expressed in ng/g plant tissue.

480

481   **Growth on H(+)-ATPase inhibitor bafilomycin**

482   *F. culmorum* wild type strain UK99 and his mutant Δ*FcRav2* B51 were grown at 25 °C on Czapek

483   dox agar amended with 6.7 μg/mL (equivalent to 11 μmol) of Vo H(+)-ATPase inhibitor

484   bafilomycin and on Czapek dox agar, respectively. After 48 hours, colony diameters were measured

485   and the phenotype of treated wild type and the untreated mutant were compared.

486

487   **Bioinformatic and statistical analysis**

488 A one-way analysis of variance, followed by multiple comparison using the Dunnett's test was

489 performed on all data obtained from phenotypic, pathogenicity and trichothecene production assays

490 by using the Minitab® for Windows release 12.1 software.

491 Phenetic patterns were acquired with OmniLog-OL_PM_FM/Kin 1.30 and OmniLog-OL_PM_Par

492 1.30 software. Data were analyzed separately by well with a mixed linear model that included the

493 fixed effects of strain, sampling time, their interaction, and the random effect of the replicates. The

494 model was solved using the PROC MIXED of SAS software (SAS Institute, 2008).

495 Sequences for alignment were obtained from FungiDB (http://FungiDB.org). Sequence analyses

496 and expression profiles were analysed using CLC Main Workbench v 7.0. Expression profiles were

497 obtained from PLEXdb database (www.plexdb.org; Dash *et al.,* 2012). Orthology was calculated

498 with Eggnog 4.5 (Jenses *et al.,* 2008) and Inparanoid 8 (Sonnhammer and Östlund, 2015). The

499 putative protein structure was generated with RaptorX structure (raptorx.uchicago.edu).

500

501 **Note by the authors:** while our paper was under revision a paper from Nguyen et al 2017 showed

502 the effect of deletion of the homologous of FcRav2 in *Neurospora crassa,* consisting in a decreased

503 growth of hyphae. The gene was selected as it is supposed to be part of a set of candidate genes

504 with a role in multicellular complexity, being conserved in complex eukaryotes. Our orthology

505 search confirmed the work by Nguyen et al 2017, suggesting that FcRAv2 homologs in

506 Pezizomycotina may have many roles in governing cellular complexity that are partially lost in

507 yeast. Moreover, the gene localization in *Neurospora crassa* showed by Nguyen et al 2017 is

508 consistent with our bioinformatic predictions that suggest a cytoplasmic localization with a

509 transmembrane domain.

510

520

521

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SUPPORTING INFORMATION LEGENDS

719

720

TABLES

722

Table 1: Phenotype assay of the strains used in this study. Colony growth was measured after 3 d incubation at 25°C on Czapek dox agar supplemented with either 2M sorbitol, 1M NaCl, 0.01-0.02% [v/w] sodium dodecylsulphate (osmotic stress) 20-30 mM $K_2S_2O_8$ (oxidative stress), 0.5 µg/mL tebuconazole (fungicide sensitivity). Percent spore germination was evaluated after 4 h incubation in Czapek dox broth. Three independent tests were performed with 3 replicates for each test.

729

Table 2: Production of type B trichothecenes *in vitro* and *in planta* and virulence on durum wheat (cv Iride) of the strains used in this study. Values are expressed as mean of three or four replicates ± standard deviation.

733

Table 3: Primer sequences used in this study.

735

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FIGURE LEGENDS

738

Figure 1: Southern blot analysis of: **A**, *Fusarium culmorum* and **B**, *F. graminearum* trasformants. Upper series: *Eco*RV-digested DNAs were blotted and hybridized with a gene upstream probe obtained with primers 1F/2R (expected size for wild type and ectopic transformant: 3800 bp;

742   expected size for deletion mutants: 4068 bp). Lower series: *Bgl*III-digested DNAs were blotted and

743   hybridized with an internal *FcRav2* gene upstream probe obtained with primers NF/NR (expected

744   size for wild type and ectopic transformant: 2409 bp).

745

746   **Figure 2:** Comparison of *F. culmorum ΔFcRav2* mutant phenotype and *in vitro* effect of the

747   vacuolar type H(+)-ATPase inhibitor bafilomycin A1. **A**, *F. culmorum* wild type strain UK99 colony

748   border after 48 growth on Czapek dox agar; **B**, UK99 grown on Czapek dox agar containing 11

749   μmol bafilomycin A1; **C**, *F. culmorum* mutant *ΔFcRav2* B51 grown on Czapek dox agar.

750   Microscopic detail (40 X) shows hyperbranching and thickening of the hyphal tips in the *ΔFcRav2*

751   mutant and in the wild type exposed to bafilomycin A1.

752

753   **Figure 3:** Phenetic heat map of carbon utilization patterns of *Fusarium culmorum* deletion mutant

754   Δ*FcRav2* B24 compared to its wild type strain UK99 and to ectopic transformant strain *Fc*B6.

755   Average OD_{750} readings recorded during the 60-72 h interval on the 30 most differentiating carbon

756   sources are listed in order of decreasing proportional growth difference between Δ*FcRav2* B24 and

757   UK99.

758

759   **Figure 4:** Expression pattern of different Affymetrix array experiments carried out on *Fusarium*

760   *graminearum* in different environmental conditions obtained from plexdb.org platform. The results

761   are the average of 3 repetitions, with bars indicating standard error.

762

763

764   **Supplementary Figure 1:** *FcRav2* gene structure with sequence from ENSEMBLE modified to

765   show insertion site of *mimp1* in the third exon of the gene. UTR, introns and exons are reported.

766

767 **Supplementary Figure 2: Protein domains identified in FcRav2 and T**the typical ROGDI leucin

768 zipper pfam domain aligned to FcRav2 aminoacid sequence.

769

770 **Supplementary Figure 3:** Muscle alignment of FcRAV2 homologues.

771

772 **Supplementary Figure 4:** RaptorX putative 3D structure of FcRAV2 protein and summarized

773 results of prediction probabilities for the modelling. Likely, only one part of the protein is correctly

774 modelled given the low GDT value (<50)

775

776 **Supplementary Figure 5.** Phenotype of *Fusarium culmorum mimp1* revertant R38, nit⁻ recipient

777 and co-transformant M7 on different stress-inducing media.

778

779 **Supplementary Figure 6.** Phenotype of *Fusarium culmorum mimp1* revertant R38, nit⁻ recipient

780 and co-transformant M7 on Czapek's medium amended with $K_2S_2O_8$ (30 mM).

781

782 **Supplementary Figure 7.** Phenotype of *Fusarium culmorum* UK99, FcB6 (ectopic transformant),

783 *ΔFcRav2* B24, and *ΔFcRav2* B51 on different stress-inducing media after 10 d growth at 25°C.

784

785 **Supplementary Figure 8.** Phenotype of *Fusarium graminearum* PH1, *ΔFgRav2* G8, and

786 *ΔFgRav2* G10 on different stress-inducing media after 10 d growth at 25°C.

787

788 **Supplementary Figure 9.** Left: FRR symptoms on 21-d old durum wheat cv Iride seedlings Iride

789 mock inoculated (A) or infected with *Fusarium culmorum mimp1* revertant R38 (B), co-

790 transformant M7 (C), and nit⁻ recipient strain (D). Right: Spike of durum wheat cv Iride mock

791 inoculated (A) or infected with *mimp1* revertant R38 (B), co-transformant M7 (C), and nit⁻ recipient

792 strain (D) 21 days post inoculation.

793

Supplemetary Table 1: Phenotypic growth raw data (OD_{750} readings) of *F. culmorum* wild type strain UK99, its deletion mutant Δ*FcRav2* B24, and the ectopic transformant strain *Fc*B6 recorded in triplicate every 15 min from 0 to 72 h of incubation on 96 different carbon sources. The complete set of data is available at:

https://drive.google.com/file/d/0B2zMAlgHF40RWE5jX3lsTFdsY0k/view?usp=sharing