FcRav2, A GENE WITH ROGDI DOMAIN INVOLVED IN FUSARIUM HEAD BLIGHT AND CROWN ROT ON DURUM WHEAT CAUSED BY *FUSARIUM CULMORUM*

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SUMMARY

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

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).

Here we report on the identification of a new *F. culmorum* gene (*FcRav2*), ~~not yet described in Leotiomycetes for the first time while our paper was under revision in *Neurospora crassa* (Tu et al 2017)in Leotiomycetes~~, by *mimp1*-mediated insertional mutagenesis. The putative role of *FcRav2* was determined in *F. culmorum* and, as a comparison, in *F. graminearum* by analyzing the effect of deletion on the fungal phenotype.

Formattato: Tipo di carattere: Corsivo

RESULTS

Molecular characterization of the *mimp1*-tagged mutant R38

PCR and Southern blot analyses confirmed that the excision event in the revertant strain R38 was followed by the reinsertion of the *mimp1/impala* construct into a different genome site (not shown). Based on the left flanking sequence of the *mimp1* element (BLASTn results revealed that *mimp1* had reinserted at the position 6542003 of the UK99 genome draft (LT598662.1), in the gene FCUL_11566.1, located in the 4th chromosome of *F. culmorum* (Supplementary Figure 1).

Characterization of the *FcRav2* gene

FcRav2 (in *F. graminearum FgRav2* corresponding to Fg09428/FGSG_17209) has two introns and 3 exons and codes for a hypothetical protein of 318 amino acids, with a molecular weight of 34.8 kDa, and an isoelectric point of 6.43. The amino acid sequence contains a ROGDI leucine zipper domain (pfam10259), including a region of 30 amino acids with leucine repeats every seven or eight residues (Supplementary Figure 2). Homologous genes, with unknown function, were found in *F. oxysporum* (FOX_06114) and *F. verticillioides* (FVEG_03978) with sequence homologies of up to 83%, as well as in other filamentous fungi (Supplementary Figure 3). Orthology conservation

is strongly confirmed (e-value Eggnog e-145 in the Class *Leotiomycetes*). Orthology classification from NCBI allocates the gene within the Subclass Sordariomycetidae including *Neurospora crassa* (NCU08091) and *Magnaporthe oryzae* (MGG_02604) ROGDI containing group. Lower level of conservation was found within the Class *Saccharomycetes* using both Eggnog and Inparanoid. Being the domain unique and the homology along the overall protein length, we hypothesize that orthology is also occurring with *Rav-2* homologue in *Saccharomyces cerevisiae* as well with higher eukaryotes (Dawson *et al*., 2008). Protein localization is likely nuclear according to WoLF-PSORT analysis (http://www.genscript.com/wolf-psort.html; Horton *et al*., 2007).

According to TMpred (http://www.ch.embnet.org/software/TMPRED_form.html; Hoffmann and Stoffel, 1993) the protein may contain a transmembrane domain (AA 178-201) collocating its N-terminal in the internal membrane part. Hypothetical structure obtained with RaptorX (Källberg et al 2012) is reported in Supplementary Figure 4.

Obtainment of *FcRav2/FgRav2* deletion mutants in *F. culmorum* UK99 and in *F. graminearum* PH1 and annotation confirmation

A total of seven hygromycin B resistant transformants were obtained from *F. culmorum* UK99, 5 showing a distinct deletion, and 2 being ectopic transformants (Figure 1). One ectopic strain (*Fc*B6) and two *ΔFcRav2* strains (Δ*FcRav2* B24, Δ*FcRav2* B51) were used for all the following assays and characterizations. In addition, five FGSG_17209 deletion mutants were obtained from *F. graminearum* PH1, among which Δ*FgRav2* G8 and Δ*FgRav2* G10 (Figure 1) were selected for further analyses.

To verify the *in silico* annotation of the gene the wild type strain UK99 was treated with 11 μmolar bafilomycin that is known to inhibit specifically V-ATPase complex (Faraco *et al.*, 2014). Indeed the phenotype of the wild type treated with bafilomycin reproduced the same morphological features of the mutants including colony morphology, hyphal hyperbranching and pigmentation

(Figure 2), allowing to confirm that at least part of the phenotypic effect of the *FcRav2* mutation can be mimicked by a vATPase inhibiting drug.

Deletion of the *FcRav2/FgRav2* gene involves significant changes in physiological and metabolic profiles of *F. culmorum* and *F. graminearum*

All *FcRav2* deletion mutants as well as the revertant strain R38 showed significantly decreased growth compared to the recipient strains FcM7, *F. culmorum* UK99, and *F. graminearum* PH1 when coping with multiple osmotic stresses, while the ectopic transformant strain *Fc*B6 displayed the same responsiveness as the *F. culmorum* wild-type strain UK99 (Table 1; Supplementary Figures 5-8). Addition of 2 M sorbitol or 0.02% [w/v] SDS led to more incisive growth reductions than 1 M NaCl. *F. graminearum* was completely inhibited at 0.02% [w/v] SDS, and therefore PH1, Δ*FgRav2* G8 and Δ*FgRav2* G10 were characterized in a second test series on 0.01% [w/v] SDS, where deletion mutants displayed a significant growth reduction compared to the wild type PH1 (Table 1).

Only *F. culmorum* strain FcM7 and its revertant R38 were able to grow in the presence of 30 mM $K_2S_2O_8$, with R38 being significantly inhibited (over 70%) compared to FcM7. In the presence of 20 mM $K_2S_2O_8$ all *F. culmorum* and *F. graminearum* deletion mutants were significantly inhibited compared to the respective wild-type (Table 1).

Conidial germination was significantly impaired in the *F. culmorum* and *F. graminearum* deletion mutants compared to the wild-type strains. On the contrary, germination capability of the revertant strain R38 was not reduced compared to co-transformant strain FcM7 (Table 1).

FcRav2 deletion dramatically reduced mycelium hydrophobicity: while for both the wild type strain UK99 and the ectopic transformant *Fc*B6 the time required to absorb a 20 µL drop of water was > 15 min, in Δ*FcRav2* B24, Δ*FcRav2* B51 the water droplet was immediately adsorbed upon deposition (data not shown).

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

phenological stage of infection, but that these changes depend on various conditions and therefore depict a high variability in experimental repetition *in planta* (Figure 4).

FcRav2 is involved in type-B trichothecene production

In *in vitro* experiments, only the *F. culmorum mimp1*-tagged mutant R38 has lost completely its ability to produce type B trichothecenes, while trichothecene production by deletion mutants Δ*FcRav2* B24 and Δ*FcRav2* B51 did not differ significantly from the wild type strain UK99 and from the ectopic transformant *Fc*B6 (Table 2). In the case of *F. graminearum*, mutant Δ*FgRav2* G8 was not significantly affected by gene deletion in its ability to produce deoxynivalenol (DON) and its acetylated form 3-ADON, while trichothecene production by mutant Δ*FgRav2* G10 was reduced by approximately 70% (Table 2).

In accordance to the development of FRR symptoms, type B trichothecenes levels reached 1,000-2,000 ng/g in seedling stem tissue infected by *F. culmorum* strains FcM7, UK99 and *Fc*B6 and by *F. graminearum* strain PH1. On the contrary, trichothecene mycotoxins were not detected in wheat seedlings upon inoculation with the *mimp1*-tagged mutant R38 and with deletion mutants of both *F. culmorum* UK99 and *F. graminearum* PH1 (Table 2).

When the *F. culmorum* FcM7 was spray-inoculated, multiple infection sites in the spikes resulted in far higher levels of trichothecene content in the infected kernels, whereas the *mimp1*-tagget mutant R38 produced approximately 15% mycotoxin compared to the co-transformant strain FcM7 (Table 2). Infected kernels collected from spikes that were spray-inoculated with *F. culmorum* ectopic transformant *Fc*B6 contained unusually high trichothecene content (approximately 12,000 ng/g tissue), whereas the wild type strain UK99 and both deletion mutants Δ*FcRav2* B24 and Δ*FcRav2* B51 did not differ significantly in their deoxynivalenol production upon kernel colonization (approximately 1,700-2,600 ng/g tissue; Table 2).

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 Δ*FcRav2* 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.

Further investigations are needed to experimentally confirm the role of the gene in filamentous fungi. Given that conservation of the *RAV2* gene is lower compared to ATPase components (Dawson *et al*., 2008), or that it may be present only in fungi (Parra *et al.*, 2014), it would be feasible to focus on molecules able to interfere with RAV2 *in planta* in order to impair the toxigenic process as well as fostering the activity of known fungicides, as suggested already for human fungal pathogens (Hayek *et al*., 2014). For this reason we propose that RAV2 in *F. graminearum* and in *F. culmorum* may represent a new target for developing integrative approaches against fungicide adaptation and resistance or low sensitivity phenomena that are becoming important against different fungicides in Fusaria (Dubos *et al*., 2011, 2013; Talas and Mc Donald, 2015; Becher *et al*., 2011; Serfling and Ordon, 2014; Chen and Zhou, 2009).

FcRav2 appears deeply involved in a number of morphological and physiological traits, while no definite phenotypes were identified in *Candida albicans* in a large genomic screening of mutants (Noble *et al.,* 2010). Spore germination and hydrophobicity are significantly altered in deletion mutants, as well as resistance to various stresses: Δ*FcRav2* inactivation resulted in increased sensitivity to osmotic and oxidative stress, and to the sterol biosynthesis inhibitor tebuconazole. The higher susceptibility to tebuconazole observed for the *RAV2* mutant is a further confirmation of the importance that alternative mechanisms related to the extrusion of the compound can play in azole resistance. Zhang *et al*. (2010) already hypothesized that azole resistance mechanism involves also acidification of the medium.

The gene transcription seems to be influenced by azole treatments. Indeed, by analysing gene expression patterns of *F. graminearum* after treatment with sublethal concentrations of tebuconazole, Becher *et al*. (2011) found a decreased expression of FGSG_17209. This leads to the hypothesis that FGSG_17209 (*FgRav2*) may play an indirect role in controlling the expression of genes involved in compensating the azole stress, or that the gene is underexpressed as growth is decreased.

A putative role of *FcRav2* in stress resistance may be also inferred by reduced growth of the deletion mutant on some of the tested carbon sources. For instance, L-sorbose is a well-known inhibitor of cell wall biosynthesis in fungi and showed a paramorphogenic action on *Neurospora crassa* (Mishra and Tatum, 1971; Trinci and Collinge, 1973). Ethanolamine (2-amino ethanol) is commonly used as wood preservative (Humar *et al.,* 2003). Also, weak acids such as bromosuccinic acid and β-hydroxy-butyric acid may represent a source of stress on an impaired deleted mutant. These data suggest that *FcRav2* is, as *RAV2* in yeast, involved in stress, including the stress *F. culmorum* is exposed to by being confronted with its victim plant.

The lower capacity of *FcRav2* deletion mutant to metabolize different mono-, di- and trisaccharides in comparison to the wild type is also concordant with the hypothesis that it represents the *RAV2* homologue. Indeed the RAVE complex in yeast is important for the reassembly of the vATPAse machinery under sugar starvation (Smardon *et al.,* 2015). Our Biolog experiment confirms the major role that *FcRav2* plays in sugar metabolism. This hypothesis is corroborated by expression data obtained from independent experiments, highlighting under-expression of *FgRav2* in *F. graminearum* during glucose starvation (Figure 4). In particular, among the sugars that determine the most significant differences in growth between the mutant and the wild type, xylose and arabinose belong to the pathway of pentose and glucuronate interconversions pathway (Fungipath) that is important in conditions of glucose starvation. Noteworthy, RAV2 is overexpressed in *S. cerevisiae* exposed to high sugar stress (Erasmus *et al.,* 2003), and RAV2-overexpressing *Saccharomyces pastorianus* bottom fermenting strains exhibited increased ethanol tolerance and increased fermentation rates in high-sugar medium (Hasegawa *et al.,* 2012).

As mentioned, phenotype microarray analysis highlighted a significantly reduced ability of the *FcRav2* deletion mutant in the utilization of 2-amino-ethanol as carbon source. Quite interestingly, a behavioral screening of *P*-transposon-generated memory mutants coupled to DNA microarray analysis highlighted a *Drosophila melanogaster* ROGDI mutant and suggested its role in long-term olfactory memory (Dubnau *et al.,* 2003) and reduced ethanol tolerance (Berger *et al.,* 2008). This

would support indirectly the hypothesis that homologous function are maintained by the gene with ROGDI domain also in higher eukaryotes, although further evidences are needed to corroborate such functional homology.

FcRav2/FgRav2 has a major impact on fungal virulence in *F. culmorum* and *F. graminearum*, respectively, and influenced the severity of crown rot and Fusarium head blight on durum wheat. This role was clearly demonstrated by different inoculation methods (contact between mycelium and caryopsis, spray-inoculation of conidia), and was confirmed in *F. graminearum*.

Infection experiments with *F. graminearum* (Lysoe *et al.*, 2011; Güldener *et al.*, 2006) showed a minimal, albeit not significant, modulation of gene expression during the different stages of interaction with the plant. Indeed, expression profiles suggest that the gene modifies its expression according to the phenological stage of infection, but that these changes depend on different conditions likely related to the variability in experimental repetitions *in planta*.

Somehow less clear was the implication of *FcRav2* in mycotoxin production. The effect of *FcRav2* disruption is likely dependent on the substrate: while the *F. culmorum mimp1*-tagged mutant R38 had lost completely its ability to produce type B trichothecenes *in vitro*, deletion mutants did not differ significantly from their wild type strain. In seedling stem tissue, mycotoxin biosynthesis was completely inhibited upon *FcRav2* deletion, while DON and 3-ADON were still abundantly produced by deleted strains colonizing spray-inoculated wheat spikes. This suggests that specific inducers of DON by-passing *FcRav2* regulation exist in the spike that are not able to activate the production of toxin in the stem or *in vitro*. Therefore, a plausible hypothesis is that some molecules in the spike may favor the synthesis of the toxin independently from *FcRav2* control.

Interestingly, we observed a diverse DON production *in vitro* and *in planta* for some of the strains. The phenomenon has been observed also for the transcription factor in FG Atf1 (Nguyen *et al.*, 2013), which is also a stress regulator (Lawrence *et al.*, 2007) with opposite behavior compared to *FcRav2*. To better elucidate the mechanisms that lead to high toxin contamination there is a need to further characterize the specific molecules that the fungus may encounter *in planta*.

In yeast the lack of vacuolar acidification in the RAVE mutants is possibly compensated by other mechanisms of acidification of other compartments (Smardon *et al.*, 2014). This would explain the relatively mild phenotype of the *rav1*Δ and *rav2*Δ mutants. We would anticipate that this acidification process is likely under the control of FgAtf1, in the HOG pathway. It is therefore plausible that the HOG pathway and the RAVE complex play two different but complementary roles in toxin production, linking toxin production and stress response pathways (Ponts *et al.*, 2015). To further verify this hypothesis we are currently investigating the cellular components of the assembly.

Expression profiles obtained from different array studies carried out on FG PH1 strain confirms indirectly the homology of *FcRav2* with *RAV-2* gene from *S. cerevisiae*. Indeed, major oscillations of the gene (higher expression) are observed in agmatine medium that is a strong toxin inducer (Gardiner *et al.*, 2009a; Pasquali *et al.*, 2016a). Given the need for vacuole assembly in condition of high toxin production (toxisomes, Menke *et al.*, 2013) it is plausible that a vacuole acidification is highly induced when high level of toxins should be produced.

From a more practical perspective, FcRav2 is proposed as a suitable target for new antifungal drug development or plant-mediated resistance response, given its pivotal role affecting hydrophobicity, sugar metabolism, secondary metabolism, virulence, and resistance to various stresses. Our work is also the confirmation that untargeted methods for the identification of genes involved in fitness and pathogenicity are useful for the identification of genes that are not directly identified as homologous with normal orthology search programs. The further exploration of the *mimp1* reinsertion mutant library will possibly allow in the future the identification of further genes involved in the fitness of the pathogen.

EXPERIMENTAL PROCEDURES

360

361 **Fungal strains and storage conditions**

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

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

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

373

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

375 The *mimp1/impala* revertant strains were selected on Minimal Medium Agar supplemented with
376 50µg/mL hygromycin (MMH50) containing sodium nitrate as sole nitrogen source (Spanu *et al.*,
377 2012). Briefly, selection was performed with a phenotypic assay by inoculating 10 µL of a spore
378 suspension (10^6 CFU/mL) of the co-transformant strain FcM7 on MMH50 plates and by incubating
379 at 25°C up to 1 month. First *nia*$^+$ colonies (referred to as "revertants") appeared 15 days after
380 inoculation upon reacquisition of the nitrate-reductase function, and consisted in patches of aerial
381 mycelium with a wild-type phenotype. A single-spore culture was obtained from each collected
382 revertant strain.

383

384 **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

available in the BIOLOG web site (http://www.biolog.com/products-static/microbial_identification_literature.php).

Spore suspensions were prepared in carboxy-methyl cellulose liquid medium (CMC, Pasquali *et al.*, 2013) and after two subsequent washing steps with sterile distilled water, spores were suspended in "Inoculation fluid FF" (Biolog) at a final concentration of $1x10^4$ CFU/mL. One-hundred µL of spore suspension were pipetted into each of the 96 wells. Plates were incubated at 25°C for 90h and OD was recorded at 750 nm every 15 min using the Microarray Omnilog Reader (Biolog, Inc. Hayward, CA). Samples were tested in triplicate.

Pathogenicity assay on durum wheat

The *F. culmorum* co-transformant FcM7 and its revertant strain R38, wild-type strain UK99, deletion mutants Δ*FcRav21* B24 and Δ*FcRav2* B51, and the ectopic transformant strain *Fc*B6; *F. graminearum* strain PH1, deletion mutants Δ*FgRav2* G8 and Δ*FgRav2* G10 were tested for their virulence *in planta*. Both FRR and FHB disease were evaluated.

Mycelium plugs, each bearing one durum wheat seed (*Triticum durum* cv. Iride, kindly provided by Unità di Ricerca per la Valorizzazione Qualitativa dei Cereali, CRA-QCE, Rome, Italy) were placed in a plastic sowing pot and covered by sterile soil. Durum wheat seeds placed onto sterile PDA plugs (1 seed/plug) served as negative control. FRR severity was assessed after 21 days of incubation at 25°C in a greenhouse and evaluated using the McKinney index (McKinney, 1923; Spanu *et al.*, 2012). Three independent tests were carried out, each consisting of 3 replicates of 10 seedlings for each fungal strain.

The ability to cause FHB was tested on durum wheat (cv. Iride) at mid-anthesis stage wheat heads. Spray inoculation was carried out with 4 mL of each spore suspension ($1x10^5$ spores/mL) covering the head until runoff. Inoculated heads were coated with a transparent plastic bag for 48 hours to 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 $1x10^6$ 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**

A one-way analysis of variance, followed by multiple comparison using the Dunnett's test was performed on all data obtained from phenotypic, pathogenicity and trichothecene production assays by using the Minitab® for Windows release 12.1 software.

Phenetic patterns were acquired with OmniLog-OL_PM_FM/Kin 1.30 and OmniLog-OL_PM_Par 1.30 software. Data were analyzed separately by well with a mixed linear model that included the fixed effects of strain, sampling time, their interaction, and the random effect of the replicates. The model was solved using the PROC MIXED of SAS software (SAS Institute, 2008).

Sequences for alignment were obtained from FungiDB (http://FungiDB.org). Sequence analyses and expression profiles were analysed using CLC Main Workbench v 7.0. Expression profiles were obtained from PLEXdb database (www.plexdb.org; Dash *et al.*, 2012). Orthology was calculated with Eggnog 4.5 (Jenses *et al.*, 2008) and Inparanoid 8 (Sonnhammer and Östlund, 2015). The putative protein structure was generated with RaptorX structure (raptorx.uchicago.edu).

Note by the authors: while our paper was under revision a paper from Nguyen et al 2017 showed the effect of deletion of the homologous of FcRav2 in *Neurospora crassa*, consisting in a decreased growth of hyphae. The gene was selected as it is supposed to be part of a set of candidate genes with a role in multicellular complexity, being conserved in complex eukaryotes. Our orthology search confirmed the work by Nguyen et al 2017, suggesting that FcRAv2 homologs in Pezizomycotina may have many roles in governing cellular complexity that are partially lost in yeast. Moreover, the gene localization in *Neurospora crassa* showed by Nguyen et al 2017 is consistent with our bioinformatic predictions that suggest a cytoplasmic localization with a transmembrane domain.

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SUPPORTING INFORMATION LEGENDS

TABLES

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.

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.

Table 3: Primer sequences used in this study.

FIGURE LEGENDS

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

Supplementary Figure 2: Protein domains identified in FcRav2 and ~~T~~the typical ROGDI leucin zipper pfam domain aligned to FcRav2 aminoacid sequence.

Supplementary Figure 3: Muscle alignment of FcRAV2 homologues.

Supplementary Figure 4: RaptorX putative 3D structure of FcRAV2 protein and summarized results of prediction probabilities for the modelling~~.~~ Likely, only one part of the protein is correctly modelled given the low GDT value (<50)

Supplementary Figure 5. Phenotype of *Fusarium culmorum mimp1* revertant R38, nit⁻ recipient and co-transformant M7 on different stress-inducing media.

Supplementary Figure 6. Phenotype of *Fusarium culmorum mimp1* revertant R38, nit⁻ recipient and co-transformant M7 on Czapek's medium amended with $K_2S_2O_8$ (30 mM).

Supplementary Figure 7. Phenotype of *Fusarium culmorum* UK99, FcB6 (ectopic transformant), *ΔFcRav2* B24, and *ΔFcRav2* B51 on different stress-inducing media after 10 d growth at 25°C.

Supplementary Figure 8. Phenotype of *Fusarium graminearum* PH1, *ΔFgRav2* G8, and *ΔFgRav2* G10 on different stress-inducing media after 10 d growth at 25°C.

Supplementary Figure 9. Left: FRR symptoms on 21-d old durum wheat cv Iride seedlings Iride mock inoculated (A) or infected with *Fusarium culmorum mimp1* revertant R38 (B), co-transformant M7 (C), and nit⁻ recipient strain (D). Right: Spike of durum wheat cv Iride mock inoculated (A) or infected with *mimp1* revertant R38 (B), co-transformant M7 (C), and nit⁻ recipient strain (D) 21 days post inoculation.

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Formattato: Tipo di carattere: Grassetto

Formattato: Allineato a sinistra

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