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Mutations in the Phosphatase Domain of the 6-phosphofructo-2-kinase/fructose
2,6-bisphosphatase Result in the Transcriptional Activation of the Alternative
Oxidase and Gluconeogenic Pathways in *Podospira anserina*

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1 **Abstract**

2 By screening suppressors of a respiratory mutant lacking a functional cytochrome pathway in
3 the filamentous fungus *Podospora anserina*, we isolated a mutation located in the
4 phosphatase domain of the bi-functional enzyme 6-phosphofructo-2-kinase/fructose 2,6-
5 bisphosphatase (PFK-2/FBPase-2). We show that the inactivation of the phosphatase but not
6 of the kinase domain is responsible for the suppressor effect that results from the activation of
7 the RSEs transcription factors that control expression of AOX, an alternative oxidase able to
8 bypass the mitochondria cytochrome pathway of respiration. Remarkably, activation of the
9 RSEs also stimulates the expression of the gluconeogenic enzymes, fructose-1,6 bi-
10 phosphatase (FBPase-1) and phosphoenolpyruvate carboxykinase (PCK-1). We thus reveal in
11 *P. anserina* an apparently paradoxical situation where the inactivation of the phosphatase
12 domain of PFK-2/FBPase-2, supposed to stimulate glycolysis, is correlated with the
13 transcriptional induction of the gluconeogenic enzymes. Phylogenic analysis revealed the
14 presence of multiple presumed PFK-2/FBPase-2 isoforms in all the species of tested
15 Ascomycetes.

16 Key words: glycolysis and gluconeogenesis, PFK-2/FBPase-2, alternative oxidase regulation,
17 *Podospora anserina*

18

19

20 **1. Introduction**

21 Fungi have a very flexible metabolism that enables them to live in a variety of environments
22 and to use diverse carbon sources. They can grow on a range of carbohydrates using
23 glycolysis but are also able to grow on two-carbon compounds, fatty acids, or other sources of
24 tricarboxylic acid (TCA) cycle intermediates, thereby requiring gluconeogenesis. Glycolysis
25 and gluconeogenesis are two opposite pathways for glucose metabolism (degradation or
26 biosynthesis of glucose), and fungi have to adapt their carbon metabolism according to the

1 available carbon source. Regulation of the enzymes involved is of great importance to avoid
2 futile cycling between the degradation (glycolysis) and biosynthesis (gluconeogenesis) of
3 sugars. Phosphoenolpyruvate carboxykinase (PCK-1) and fructose-1,6-bisphosphatase
4 (FBPase-1) are the key enzymes of the gluconeogenic pathway. PCK-1 catalyzes an early step
5 converting oxaloacetate to phosphoenolpyruvate. FBPase-1 catalyzes the final step in hexose
6 monophosphate formation by dephosphorylating fructose-1,6-biphosphate to yield fructose-6-
7 phosphate. It has been shown that the expression of these two enzymes is controlled by two
8 zinc cluster transcription factors called RSE2 and RSE3 in *P. anserina* (Bovier et al., 2014;
9 Sellem et al., 2009), ACUK and ACUM in *A. nidulans* (Hynes et al., 2007; Suzuki et al.,
10 2012). In *N. crassa*, the orthologs of these transcription factors (AOD2 and AOD5) also
11 control the expression of *pck-1* but not of *fbpase-1* (Qi et al., 2017). Surprisingly, in these
12 three species, these transcription factors that are highly conserved, are also required for the
13 expression of the alternative oxidase, AOX (Chae et al., 2007b; Sellem et al., 2009; Suzuki et
14 al., 2012). AOX is a mitochondrial non-proton-pump enzyme of nuclear origin that is able to
15 receive electrons from reduced ubiquinone and to catalyze the reduction of oxygen to water.
16 AOX is found in plants, many fungal species, several protists, some animal phyla and certain
17 bacteria (McDonald, 2008). In most fungi *aox* transcripts are undetectable, or present at very
18 low levels, under classical growth conditions but become expressed when the cytochrome
19 respiratory chain is restricted by inhibitors or by mutations (Chae et al., 2007a; Lorin et al.,
20 2001; Suzuki et al., 2012). In response to such mitochondrial electron transport chain
21 inhibition, AOX bypasses respiratory complexes III and IV. It is believed to contribute to the
22 maintenance of the tricarboxylic acid (TCA) cycle turnover and to act as an overflow for
23 electron transport, preventing the deleterious oxidative stress associated with the increased
24 generation of mitochondrial reactive oxygen species (ROS). In *N. crassa* and *A. nidulans* the
25 orthologs of RSE2 and RSE3 have been shown to bind constitutively to DNA, as a

1 heterodimer, by their N-termini (Chae et al., 2007a; Qi et al., 2017; Suzuki et al., 2012). Thus,
2 it appears that the activation of these transcription factors is achieved by communication of an
3 unknown signal(s) to the nucleus when cells are exposed to specific conditions. The identity
4 of the signal(s) is crucial to the understanding of these regulatory pathways, as is the potential
5 biological significance of this unexpected co-regulation of AOX and gluconeogenic enzymes.

6 In the current study, we report the properties of *P. anserina* mutants carrying
7 mutations in a gene encoding the bi-functional enzyme 6-phosphofructo-2-kinase/fructose-
8 2,6-bisphosphatase (PFK-2/FBPase-2). This enzyme catalyzes two opposite reactions (Pilkis
9 et al., 1995; Rider et al., 2004): synthesis of Fru-2,6-P₂ (fructose 2,6-biphosphate) from Fru-
10 6-P (fructose-6-phosphate) and ATP *via* its phosphofructose-2-kinase activity (PFK-2), and
11 degradation of Fru-2,6-P₂ to Fru-6-P and inorganic phosphate *via* its fructose-2,6-
12 bisphosphatase activity (FBPase-2). A mutant carrying a single substitution in a conserved
13 motif of the phosphatase domain was first isolated as a suppressor of a respiratory-deficient
14 mutant. Null mutations of the kinase and phosphatase domains were then constructed. Their
15 analysis showed that only mutations affecting the phosphatase domain are able to suppress the
16 respiratory mutant phenotype and that this suppression is correlated with a strong expression
17 of the AOX due to the activation of the RSEs transcription factors. Moreover we show that
18 the key enzymes of gluconeogenesis are also strongly overexpressed. The phenotype of the
19 phosphatase mutants was intriguing since two other genes predicted to each also encode a
20 PFK-2/FBPase-2 are present in all the tested Ascomycetes, one of them being probably mono-
21 functional, lacking FBPase-2 activity.

22 **2. Materials and methods**

23 *2.1. Strains and growth conditions*

24 The knockout of the COX5 subunit of complex IV has deleterious consequences

1 (Dufour et al., 2000). This respiratory mutant (Δcox) carries a phleomycin resistant (*Phleo^R*)
2 cassette in the *cox5* gene and exhibits a typical low growth phenotype that enabled the
3 identification of suppressors (Bovier et al., 2014; Humbert et al., 2015; Sellem et al., 2009).
4 The new suppressor mutation described in this work (momentarily named *su*) was obtained in
5 the $\Delta cox \Delta med13$ strain. Inactivation of the *med13* gene by insertion of a nourseothricin
6 resistant (*Nat^R*) cassette (Humbert et al., 2015) was already shown to be a suppressor of the
7 Δcox phenotype and its weak suppressor effect allowed us to select a sector of more aerial and
8 faster growing mycelium from a culture. The fast growing sector, containing three mutant
9 alleles Δcox (*Phleo^R*), $\Delta med13$ (*Nat^R*) and a new one (*su*), was crossed with the wild-type
10 strain. The presence of an extragenic suppressor was indicated by the recovery in the progeny
11 of two types of $\Delta cox \Delta med13$ ascospores (*Phleo^R*, *Nat^R*): those giving rise to a mycelium
12 identical to that of $\Delta cox \Delta med13$ strain, and those giving rise to an improved fast-growing
13 mycelium, $\Delta cox \Delta med13 su$. Two types of Δcox ascospores (*Phleo^R*, *Nat^S*) were also
14 identified in the progeny: again, those giving rise to a mycelium identical to the Δcox strain,
15 and those giving rise to an improved fast-growing mycelium, $\Delta cox su$. There is no additive
16 effect of the $\Delta med13$ and *su* mutations, since the $\Delta cox \Delta med13 su$ and $\Delta cox su$ strains showed
17 the same phenotype. Construction of the strains with $\Delta pfkfbase-1$, *pfkfbase-1^{L24A, D103A}* and
18 *pfkfbase-1^{H233A}* was done by a gene replacement strategy (El-Khoury et al., 2008) with several
19 PCR amplification steps, and a final integration with a *Nat^R* cassette in the *Podospora* $\Delta Ku70$
20 strain (Details of the constructions are given in supplemental figure S1A et S1B for
21 constructions, supplemental Table S1A for list of primers). The M2 medium is a minimal
22 medium in which carbon is supplied as dextrin (0.5%) and nitrogen as urea (0.05%). Acetate
23 medium is a minimal medium in which dextrin is replaced by sodium acetate (1.4%). When
24 necessary, 50 μ g/ml nourseothricin, 10 μ g/ml phleomycin, 20 μ g/ml antimycin A and 150 μ g/ml
25 salicylhydroxamic acid (SHAM) were added to the medium. The germination medium (G)

1 contains ground corn meal (5%) and ammonium acetate (0.5%).

2 *2.2. Fertility tests*

3 As each strain produces spermatia (male cells) and proto-perithecia (female cells)
4 regardless of its mating type, the male and female fertility of a strain was determined by
5 growing this strain with the wild-type strain of opposite mating type on the same plate of M2
6 medium. When the two cultures were confluent, sterile water was poured and dispersed on the
7 surface of the mycelium previously grown for 3 days in the light to allow formation of
8 gametes. Reciprocal fertilization of the two strains took place and revealed whether the strain
9 was fertile as a male (donor) and/or as a female (receptor).

10 *2.3. Deep sequencing*

11 A pool of five DNA preparations extracted from five ascospores carrying the *su* allele
12 (wild-type phenotype with pigmented mycelium) and a pool of five DNA preparations
13 extracted from five wild-type progenies were sequenced on Illumina genome analyzer
14 (platform IMAGIG, CNRS Gif-sur-Yvette). The sequence reads were mapped together and
15 the differences recorded.

16 *2.4. Database searches and phylogenetic analysis*

17 The *Podospora anserina* genome, EST and protein databases are available at
18 <http://podospora.i2bc.paris-saclay.fr/>. Sequences in different species of Ascomycetes of the
19 homologues of the three presumed PFK-2/FBPase-2 proteins of *P. anserina* were obtained
20 from the JGI fungal genomics resource (<http://genome.jgi.doe.gov/>). Sequences were aligned
21 using MUSCLE (<https://www.ebi.ac.uk/Tools/msa/muscle/>) and the resulting alignments
22 manipulated using JALVIEW. Trees were constructed with PhyML 3.0. We used SH-like for
23 approximate like hood-ratio test for statistical tests for branch support. The tree was

1 visualized with the iTOL server (<http://itol.embl.de/>)

2 *2.5. Real-time RT-PCR experiments*

3 For RNA extraction, the different strains were grown on cellophane sheets (Bio-Rad)
4 overlaid on Petri dishes containing M2 medium for 48 h at 27 °C. Mycelia were harvested,
5 flash frozen in liquid nitrogen, and ground in a Fastprep apparatus as described (Bovier et al.,
6 2014). Total RNA was extracted using the RNAeasy plant kit (Qiagen). For quantitative
7 reverse transcription-PCR (qRT-PCR) analysis, 2 or 4µg of freshly extracted RNA were
8 reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and oligo (dT)₁₅ as a
9 primer. The qRT-PCR amplification mixture of cDNAs was performed with the Light Cycler
10 SYBR green I master kit (Roche). Reactions were run on and read in a Light Cycler 480 real-
11 time PCR system (Roche) as described. Amplification efficiencies were measured for each
12 pair of primers based on standard curves established using four serial dilution points. The
13 couples of gene-specific primers used are listed in supplemental Table S1A. For all genes, a
14 non-reverse-transcribed control was performed on each biological replicate to control the
15 absence of genomic DNA contamination. The level of expression of each gene was estimated
16 relative to that of two normalization genes used for qRT-PCR in *P. anserina*: Pa_3_5110
17 (gene *gpd*, high level of expression, encoding the glyceraldehyde phosphate dehydrogenase)
18 and Pa_4_8980 (low level of expression, encoding a transcription factor). For a given strain
19 and a given gene, the abundance of transcripts is expressed relative to the level of expression
20 of this gene in the wild-type strain. It is a mean value obtained from two or three biological
21 and two or three technical replicates for each strain.

22

23 **3. Results**

24 *3.1. A mutation in a gene encoding PFK-2/FBPase-2 partially corrects the phenotype of the*
25 *respiratory mutant Δcox*

1 The great majority of the suppressors of the respiratory mutant phenotype *cox5::ble*
2 (Δ *cox*) obtained in a previous screen fell in the regulatory genes *rse2* and *rse3* (Bovier et al.,
3 2014) encoding two zinc cluster proteins, RSE2 and RSE3 (Sellem et al., 2009), required for
4 the expression of the alternative oxidase (AOX) and of the gluconeogenic enzymes, fructose-
5 1,6 bi-phosphatase (FBPase-1) and phosphoenolpyruvate carboxykinase (PCK-1). All of them
6 are responsible for a strong constitutive expression of the AOX, shown previously to suppress
7 the detrimental effects (slow growth, thinness of the mycelium) due to the loss of function of
8 the cytochrome pathway of respiration in *P. anserina* (Lorin et al., 2001; Sellem et al., 2009).
9 Two other loss of functions mutations are able to partially suppress the Δ *cox* phenotype, one
10 in the kinase module of the Mediator (Δ *med13* or Δ *cdk8*) with a weak suppressor effect
11 proceeding *via* an AOX-independent pathway (Humbert et al., 2015) and one in the gene
12 described in this work (see Materials and Methods for selection). Transiently called *su*, this
13 suppressor has a stronger effect than that of Δ *med13* but less strong than that of the *rse*
14 mutations. As shown in Figure 1 it improves the aspect of the growing mycelium that appears
15 aerial and colored and increases the growth rate (150% compared to that of Δ *cox* strain).
16 Conversely it does not restore female fertility and, in contrast to the *rse* mutations, it does not
17 improve the germination rate to the Δ *cox* ascospores. It does not either restore a wild type
18 longevity: the Δ *cox pfkfb-1*^{R284C} strain is still alive after two months whereas the longevity of
19 the wild type strain is about three weeks and that of the Δ *cox* strain is more than two years
20 (Dufour et al., 2000). Sequencing of the *rse2* and *rse3* genes revealed that the *su* strain does
21 not harbor mutation in any of these two genes. Crosses between the Δ *cox su* and the wild-
22 type (wt) strains led to the recovery of the *cox*⁺*su*⁺ (wt) and *cox*⁺ *su* progenies that can be
23 distinguished thanks to the more pigmented aspect of the mycelium of the *su* strain compared
24 to the wild type (see Table 1). Deep sequencing of the two strains revealed a C-to-T
25 substitution in gene Pa_3_11390 (*pfkfb-1*) located on chromosome III in the mutant compared

1 to the wild-type strain. The gene *pfkfb-1* encodes a protein of 443 amino acids
2 (PFK2/FBPase-2) and the substitution is predicted to change an arginine into a cysteine codon
3 at position 284 (*pfkfb-1^{R284C}*). PFK-2/FBPase-2, the 6-phosphofructose-2-kinase/fructose-2,6-
4 bisphosphatase is a bi-functional enzyme (Pilkis et al., 1995; Rider et al., 2004) whose kinase
5 and phosphatase activities determine the steady-state level of fructose-2,6-biphosphate (Fru-2,
6 6-P2). The former is a potent positive allosteric activator of the glycolytic enzyme PFK-1 (6-
7 phosphofructo-1-kinase) and the latter a negative effector of the gluconeogenic enzyme
8 FBPase-1 (fructose-1-6, bisphosphatase), and thus a key regulator metabolite of glucose
9 homeostasis (supplemental Fig. S2). PFK-2/FBPase-2 isoenzymes have been identified in
10 animals, plants, fungi and Trypanosomatidae with an N-terminal PFK-2 domain and a C-
11 terminal FBPase-2 domain, the functional form of the enzyme being a dimer ((Rider et al.,
12 2004) for a review).

13

14 *3.2. Mutations in the phosphatase but not in the kinase domain of PFK-2/FBPase-2 alter* 15 *sexual development and lead to suppression of the Δ cox phenotype*

16 Mutagenesis experiments and the resolution of crystal structures of the rat testis
17 (Hasemann et al., 1996) and the human liver (Lee et al., 2003) PFK-2/FBPase-2 enzymes
18 have led to the characterization of key catalytic residues for each PFK-2 and FBPase-2
19 activities and a better understanding of the catalytic mechanisms of this enzyme. The R284
20 amino acid is conserved in all known FBPase-2 sequences and is part of a large conserved
21 structural motif that defines the catalytic center of all the FBPase-2 enzymes (Fig.2). This
22 suggests that the *pfkfb-1^{R284C}* mutant could be a null mutant for the FBPase-2 activity that has
23 conserved the PFK-2 activity. This conclusion prompted us to construct a null mutant for the
24 two activities by deletion of the complete gene and two mutants carrying non-conservative
25 substitutions in key catalytic residues of the PFK-2 or FBPase-2 domains (other than R284).

1 The FB Pase-2 domain presents structural homology with the cofactor-dependent
2 phosphoglycerate mutases and acid phosphatases. These enzymes share a common catalytic
3 mechanism and use the histidine of a conserved RHG motif as a crucial component of the
4 catalytic machinery (Okar et al., 2001; Pilkis et al., 1995; Rigden, 2008). Mutation of this
5 histidine in the rat liver enzyme (Tauler et al., 1990; Yuen et al., 1999) or in the yeast PFK26
6 enzyme (Kretschmer et al., 1991) abolishes any FB Pase-2 activity. We therefore changed the
7 corresponding histidine codon of the RHG motif (H233) in the phosphatase domain of *pfkfb-1*
8 into an alanine codon to obtain a *pfkfb-1*^{H233A} mutant allele. The PFK-2 domain is related to a
9 superfamily of mononucleotide binding proteins with motifs that present some similarity with
10 the “Walker A” (GLPXXGKS/T) and “Walker B” motifs (Rider et al., 2004) for review).
11 Mutagenesis in the Walker A motif (G, K or T residues) of the rat liver enzyme (Li et al.,
12 1992; Vertommen et al., 1996) drastically decreased the PFK-2 activity. Also, mutagenesis of
13 a conserved D residue in the Walker B motif of the rat muscle enzyme abolishes the PFK-2
14 activity (Rider et al., 1994). In view of these data, we introduced two mutations into the
15 kinase domain of *pfkfb-1*, one in the GLP conserved “Walker A” motif (L24A), the other one
16 in the “Walker B” motif (D103A) of the enzyme. We also constructed a *pfkfb-1* null allele by
17 gene replacement (Δ *pfkfb-1*) with a cassette conferring resistance to nourseothricin (*Nat*^R) (see
18 Materials and Methods and supplemental information).

19 As shown in Table 1, the null Δ *pfkfb-1* allele and the two *pfkfb-1*^{H233A} and *pfkfb-1*^{R284C}
20 mutations in the phosphatase domain, associated with a functional cytochrome pathway,
21 confer slight vegetative defects: the growth rate is reduced by 12%, compared to the wild-type
22 and, as previously noticed, the pigmentation of the mycelium is increased. Furthermore,
23 germinating thalli derived from ascospores carrying these alleles appeared less dense than
24 those of the wild-type strain. These mutant strains also show a delay in the development of
25 the fruiting body (perithecium) when used as female and the expulsion of ascospores takes

1 place from the 7th day after fertilization instead of the 4th day for the wild-type strain. In
2 contrast, the *pfkfb-1*^{L24A, D103A} mutant strain in the kinase domain does not lead to any visible
3 vegetative or sexual development defects. Associated with the *cox+* allele, none of these
4 different mutant alleles significantly modify the longevity of the strain, a parameter that is
5 often addressed when the metabolism of this fungus is perturbed (Lorin et al., 2006;
6 Scheckhuber and Osiewacz, 2008). To determine whether, as *pfkfb-1*^{R284C}, the other generated
7 alleles are able to suppress the Δ *cox* phenotype they were associated by genetic crosses with
8 the Δ *cox* allele. The phenotype of the strains, presented in Figure 3, revealed that the null
9 allele Δ *pfkfb-1* and the mutation *pfkfb-1*^{H333A} located into the phosphatase domain showed the
10 same suppressor effect as the mutation *pfkfb-1*^{R284C} when associated with Δ *cox*. As *pfkfb-*
11 *1*^{R284C}, these two alleles lead to a wild-type-like aspect of the mycelium and improve the
12 growth rate (150-175%). By contrast, the *pfkfb-1*^{L24A, D103A} mutant allele located into the kinase
13 domain does not confer any improvement to the Δ *cox* phenotype. All these data (Fig. 3 and
14 Table 1) indicate therefore that only the mutations expected to inactivate the phosphatase
15 activity, and not those expected to inactivate the kinase activity, are able to partially suppress
16 the phenotype of the Δ *cox* respiratory mutant strain and lead to vegetative and sexual defects.

17

18 *3.3. The inactivation of the phosphatase domain of pfkfb-1 causes the transcriptional*
19 *activation of genes positively regulated by the transcription factors RSE2 and RSE3*

20 In *P. anserina*, the major way to improve the phenotype of cytochrome deficient
21 mutants is to constitutively overexpress the alternative oxidase (AOX). This has been
22 performed by the introduction of an *aox* gene under the control of a strong constitutive
23 promoter (Lorin et al., 2001) or gain-of-function mutations in the *rse2* or *rse3* genes (Bovier
24 et al., 2014; Sellem et al., 2009). Genes *rse2* and *rse3* encode transcription factors highly
25 conserved in the filamentous Ascomycetes *P. anserina*, *N. crassa* and *A. nidulans* and

1 required for the expression of the *aox* gene in these three species (Bovier et al., 2014; Chae et
2 al., 2007b; Van Schaftingen, 1987). They have also been shown to be responsible for the
3 activation of the gluconeogenic genes encoding fructose-1,6 bisphosphatase (FBPase-1) and
4 phosphoenolpyruvate carboxykinase (PCK-1) in *P. anserina* (Bovier et al., 2014; Sellem et
5 al., 2009) and *A. nidulans* (Van Schaftingen, 1987). We thus addressed the question of the
6 level of expression of the *aox*, *fbpase-1* and *pck-1* genes in the strains carrying the different
7 *pfkfb-1* mutations in a functional respiratory context. As shown on Figure 4A real-time
8 quantitative RT-qPCR experiments reveal that the expression of the three genes is strongly
9 activated in the null ($\Delta pfkfb-1$) and in the mutants of the phosphatase domain (*pfkfb-1*^{R284C} and
10 *pfkfb-1*^{H333A}) alleles. The strongest induction was observed for *pck-1* (about 40-100X). For
11 *fbpase-1*, it was about 10-20X and for *aox* about 10X. By contrast, in the mutant of the kinase
12 domain (*pfkfb-1*^{L24A,D103A}) no change in the expression levels of the three genes was observed.
13 We therefore propose that the suppressor effect of the *pfkfb-1*^{R284C} and *pfkfb-1*^{H333A} mutant
14 alleles in the Δcox strain results from the high expression level of the alternative oxidase
15 (AOXp) in presence of these alleles. The amount of AOXp was estimated by Western blot
16 and also by the growth resistance to salicylhydroxamic acid (SHAM), a specific inhibitor of
17 the alternative respiratory pathway. These 2 parameters examined in the Δcox *pfkfb-1*^{R284C} and
18 Δcox strains (Fig. S4 and Fig. 3, respectively) reveal a higher amount of AOXp in the Δcox
19 *pfkfb-1*^{R284C} strain. Our data also suggest that 1) the strong constitutive expression of AOXp
20 depends on the RSEs since there is co-activation of the three RSE-regulated genes tested, 2)
21 inactivation of the phosphatase activity of PFK-2/FBPase-2 results in the activation of the
22 RSEs and consequently in the transcriptional activation of the RSE-target genes. Genes other
23 than *aox*, *fbpase-1* and *pck-1* were also recently shown to be regulated by the RSEs in *P.*
24 *anserina*: Pa_7_2610 encoding a Zn₂Cys₆ transcription factor, Pa_5_1700 encoding a
25 flavohemoglobin and various hydrolases specially Pa_4_3860 (Bovier et al., 2014). So, to

1 further investigate the role of the RSEs in the *pfkfb-1* mutant strains, the expression level of
2 these genes was determined in the *pfkfb-1*^{R284C} mutant and compared to the wild-type strain.
3 As a control, we also investigated the level of expression of 2 glycolytic enzymes, hexokinase
4 (Pa_1_6760) and 6-phosphofructo-1-kinase (Pa_1_14910). Figure 4B shows that the
5 expression of two of the three RSE-regulated genes are induced (about 3-10X) in this mutant,
6 supporting the hypothesis that the RSEs are under an active form in the *pfkfb-1* phosphatase-
7 dead mutants. No modification of expression of the glycolytic genes was detected.

8 The nature of the signal(s) responsible for the activation of the RSEs and the
9 mechanism(s) of this activation are not currently understood. As previously shown (Bovier et
10 al., 2014) in the wild type strain, some genes whose expression is controlled by the RSEs are
11 differently induced according to the growth culture conditions: in the presence of antimycin A
12 (a strong inhibitor of complex III) the *aox*, Pa_5_1700, Pa_7_2610 and Pa_4_3860 genes are
13 induced more strongly than when acetate is used as the only source of carbon. The opposite is
14 observed for genes *fbpase-1* and *pck-1*. These observations suggest that the inducer-mediated
15 activation of RSEs is different on acetate and antimycin A. The expression level of these
16 different genes was estimated in the *pfkfb-1*^{R284C} mutant and compared to the wild-type grown
17 on non-inducer or various inducer conditions (Fig. 5). Consistent with our previous results,
18 the tested genes can be classified in two categories, differing by their induction level
19 according to the inducer medium. Interestingly, the expression level of these two categories of
20 genes in the *pfkfb-1*^{R284C} mutant is close to that observed in the wild-type strain grown on
21 acetate. These data suggest that in this mutant, the RSEs are under an active form similar or
22 close to that they adopt in the wild-type strain grown on acetate. In agreement with this
23 hypothesis is the fact that acetate has only a very poor, or no inducer effect in the *pfkfb-1*^{R284C}
24 mutant (Fig. 6A). In contrast to acetate, antimycin A still acts as an inducer on the genes that
25 it strongly induced in the wild-type strain (Fig. 6B). However the induction factor on

1 antimycin A is decreased in the *pfkfb-1*^{R284C} mutant compared to the wild-type strain.

2

3 3.4. Three genes are predicted to each encode PFK-2/FBPase-2 in all the tested Ascomycetes

4 The lack of distinguishable phenotype of strains with a mutant kinase domain, and the
5 relatively small phenotypic defects of strains with a mutant phosphatase domain and with
6 loss-of-function mutations (Table 1) is a slightly surprising. A search for *pfkfb-1*
7 (Pa_3_11390) homologous genes in the *Podospora anserina* protein database revealed two
8 other possible homologs: Pa_2_10590 and Pa_3_6610 (supplemental Fig. S3). However, the
9 RHG motif is not conserved in Pa_2_10590 due to the change of H274 to A. In view of the
10 importance of this motif (Kretschmer et al., 1991; Tauler et al., 1990; Yuen et al., 1999), it is
11 highly likely that the Pa_2_10590 protein lacks FBPase-2 activity. Deep sequencing of cDNA
12 libraries constructed from various stages of the life cycle of the fungus was undertaken to help
13 the genome annotation (Espagne et al., 2008). A search for transcripts corresponding to each
14 of the three possible enzymes in various *Podospora* ESTs database revealed that only
15 Pa_3_11390 (*pfkfb-1*) is expressed all along the life cycle of the fungus (supplemental Table
16 S2). Transcripts for Pa_2_10590 and Pa_3_6610 are only found during the sexual cycle (in
17 the fruiting bodies where meiosis take place). Using the JGI fungal genomics resource, we
18 carried out searches for orthologs in the genomes of at least one species of each of the ten
19 classes of Ascomycetes. Four sequences were found in *S. pombe* but were omitted due to their
20 low level of conservation and long evolutionary distance. For the same reason, we also
21 omitted the *S. cerevisiae* mono-functional PFK27 protein that only contains a PFK-2 domain
22 and a highly aberrant C-terminal domain (Boles et al., 1996). For all the other tested
23 Ascomycetes, we found one ortholog of each of the three *Podospora* proteins. We attributed
24 number 1 to Pa_3_11390 orthologs, (subfamily 1) the number 2 to Pa_2_10590 orthologs,
25 (subfamily 2) and number 3 to Pa_3_6610 orthologs, (subfamily 3). To explore the

1 phylogenetic relationships among these different proteins, we generated a maximum
2 likelihood phylogenetic tree shown in Figure 7. Inside each of the 3 subfamilies, the
3 topological relationship of these proteins is highly consistent with the evolutionary
4 relationships between the different species considered. Furthermore it can be seen that the
5 three subfamilies are well supported in the tree, as indicated by high statistical values for tree
6 branch supports. As said previously, the RHG motif is important for the catalytic function of
7 the FBPase-2 domain but is not conserved in Pa_2_10590. Interestingly, like Pa_2_10590, all
8 the proteins of the subfamily 2 lack this motif that is replaced by RAG, except in *A.*
9 *oligospora* (RSG). This suggests that no protein of subfamily 2 acts as fructose-2,6-
10 bisphosphatase. In contrast, all the proteins of subfamilies 1 and 3 possess the RHG motif
11 except *S. cerevisiae* PFK26 (subfamily 3) where it is replaced by RSG.

12

13 **4. Discussion**

14 *4.1. Abolishing the phosphatase activity of PFK-2/FBPase-2 partially suppresses the growth* 15 *defect of the respiratory deficient Δ cox mutant*

16 The null mutation in the COX5 subunit of complex IV of *P. anserina* (Dufour et al.,
17 2000) leads to a slow growth rate and a thin mycelium in the corresponding Δ cox mutant
18 strain and offers a positive selection for suppressor screening. We previously reported a large-
19 scale screening of suppressors of this mutation and found only two ways to improve the
20 growth rate of this respiratory mutant: The strong constitutive expression of the alternative
21 oxidase (more than 20 gain-of-function mutations in *rse2* and *rse3* genes that control the
22 expression of the AOX) and, to a lesser extent, the loss-of-function of the kinase module of
23 the Mediator (Humbert et al., 2015). The new suppressor described in this paper encodes a
24 mutated PFK-2/FBPase-2 enzyme. In mammals, plants and fungi, PFK-2/FBPase-2 is a bi-
25 functional enzyme that catalyzes both the synthesis of Fru-2,6-P₂ *via* its PFK-2 activity and

1 its degradation *via* its FBPase-2 activity. The two activities of this enzyme therefore
2 determine the steady-state level of Fru-2,6-P₂ that is a cellular signaling molecule found in all
3 eukaryotes (Rousseau and Hue, 1993). The crystal structure of the rat testis (Hasemann et al.,
4 1996), of the rat liver (Lee et al., 1996) and of the human liver isozyme (Lee et al., 2003)
5 indicated that the FBPase-2 domain is related to the phosphoglycerate mutases and to the
6 histidine phosphatases. These enzymes possess a common catalytic center involving a
7 covalent phosphohistidine intermediate (H233 in *P. anserina*) located in a RHG motif whose
8 importance for the FBPase-2 activity was confirmed when histidine was mutated in the rat
9 liver (Tauler et al., 1990) and testis (Yuen et al., 1999) isoenzyme or in the yeast PFK26
10 enzyme (Kretschmer et al., 1991). The RHG motif is itself located in a large conserved
11 structural region that includes an arginine corresponding to R284 in *P. anserina*. This residue
12 was shown to be important for substrate binding in the rat liver enzyme (Lee et al., 1996), and
13 this is reinforced by the fact that this residue is conserved in other members of the histidine
14 phosphatase family (Bazan et al., 1989; Fothergill-Gilmore and Watson, 1989). Furthermore,
15 mutagenesis studies in the liver isozyme have confirmed the functional importance of this R
16 residue in the catalysis reaction (Lin et al., 1992).

17 These data suggested that the R284C suppressor is a loss of function mutation of the
18 FBPase-2 part of the protein. To confirm this hypothesis, we mutated the other critical
19 position, H233 of the RHG motif into alanine. We also constructed a strain inactivated for the
20 two activities (kinase and phosphatase) by replacement of the gene with a resistance cassette.
21 In agreement with our hypothesis, these two alleles (*pfkfb-1^{H233A}* and Δ *pfkfb-1*), that *a priori*
22 confer a loss of phosphatase activity, act as suppressors of the Δ *cox* phenotype similarly to the
23 R284C allele. In contrast, mutations in the kinase domain do not behave as suppressors. The
24 kinase domain consists of a central six-stranded β sheet surrounded by seven α -helices. A
25 Walker A motif or nucleotide-binding fold (G48LPARG53KT in rat liver) is located at the C-

1 terminus of the first β -sheet (Hasemann et al., 1996; Rider et al., 2004). This motif is
2 conserved in the Podospora gene (G23LPARG28KS). Mutagenesis of G48 (Li et al., 1992;
3 Vertommen et al., 1996) in the rat liver isozyme abolished PFK-2 activity without affecting
4 bisphosphatase activity (Li et al., 1992) and a comparable result was obtained when D130
5 (D103 in Podospora) of the Walker B motif (Z-Z-Z-Z-D where Z is any hydrophobic residue)
6 was mutated into alanine (Rider et al., 1994). We therefore constructed a strain whose *pfkfb-1*
7 gene carried both a mutated Walker A (L24A) and a mutated Walker B (D103A) motif. These
8 two mutations were expected to drastically reduce the kinase activity without affecting the
9 phosphatase activity of the corresponding protein. We show that this mutated allele does not
10 suppress the Δ *cox* phenotype.

11

12 *4.2. How do null alleles in the phosphatase domain of pfkfb-1 partially suppress the Δ cox*
13 *phenotype?*

14 To our knowledge, the R284C mutation of gene *pfkfb-1* encoding a PFK-2/FBPase-2
15 in *Podospora anserina* is the first mutation affecting this enzyme that has been obtained in a
16 genetic screen. All the mutations described in the mammalian isozymes have been obtained in
17 site-directed mutagenesis studies and their effects analyzed *in vitro*. In *S. cerevisiae*, the
18 strains *PFK26* and *PFK27*, devoid of FBPase-2 activity and the strain *FBP26* devoid of PFK-
19 2 activity are supposed to result from crucial substitutions or major deletions that occurred
20 during evolution in one of the two domains (Chevalier et al., 2005; Rider et al., 2004). The
21 selection of R284C as a genetic suppressor of the Δ *cox* respiratory mutant strain is interesting.
22 Identification of genetic suppressors of a given mutant is a powerful way to discover
23 unexpected interactions between functions affected in the mutant on one hand and the
24 suppressor on the other hand.

25 As mentioned above, the only mutations having a robust suppressor effect on the Δ *cox*

1 phenotype identified so far were those leading to the constitutive overexpression of the
2 alternative oxidase (Lorin et al., 2001; Sellem et al., 2009; Bovier et al., 2014). We show here
3 that both mutations in the phosphatase domain (R284C and H233A) of the PFK-2/FBPase-2,
4 lead to a constitutive expression of the *aox* gene and to a suppressor effect when associated
5 with Δcox mutation. The double mutation in the kinase domain has no effect on the *aox* gene
6 expression and no suppressor effect on the Δcox phenotype. The correlation between a strong
7 constitutive induction of the alternative pathway and the suppressor effect was also attested at
8 the protein level showing an increased resistance to SHAM, an inhibitor of the AOXp, and a
9 higher steady state level of AOXp of the Δcox strains when they carry the PFK-2/FBPase-2
10 mutated in the phosphatase domain. The *aox* gene expression is nevertheless lower in *pfkfb-*
11 *I^{R284C}* and $\Delta pfkfb-1$ (7-12 x more than in wild type, Fig. 4) than in the *rse* mutant strains (25-
12 50x more than in wild type, (Sellem et al., 2009)) and correlates with a lower suppression
13 effect. Previous results strongly suggest that RSE2 and RSE3 are the main, if not the only,
14 transcriptional regulators of the *aox* gene (Bovier et al., 2014). They are highly conserved in
15 the filamentous ascomycetes *P. anserina*, *N. crassa* and *A. nidulans*. In addition to being
16 required for the expression of the *aox* gene in the three species (Bovier et al., 2014; Chae et
17 al., 2007b; Suzuki et al., 2012), they have been shown to control the expression of the
18 gluconeogenic genes encoding *fbpase-1* and *pck-1* in *P. anserina* (Bovier et al., 2014; Sellem
19 et al., 2009) and *A. nidulans* (Suzuki et al., 2012) and of a number of other genes that
20 contribute to various adaptative strategies and defense mechanisms in *P. anserina* (Bovier et
21 al., 2014) or involved in energetic metabolism in *N. crassa* (Qi et al., 2017). We demonstrate
22 here that the mutations in the phosphatase domain of *pfkfb-1* not only confer higher
23 expression of the *aox* gene but also of *fbpase-1* and *pck-1* and of two other RSE-target genes
24 supporting the hypothesis that these mutations lead to the activation of the RSEs transcription
25 factors. Various mechanisms that might control the activation of transcription factors are

1 conceivable: nuclear localization, ligand binding, post-translational modification.... The RSEs
2 orthologs in *N. crassa* (Chae et al., 2007b; Qi et al., 2017) and *A. nidulans* (Suzuki et al.,
3 2012) have been shown to be constitutively localized in the nucleus and a large body of data
4 suggest that heterodimerization of the two proteins is essential for activation. These data
5 support the idea that the activation of these transcription factors is determined by signal(s) to
6 the heterodimer when there are modifications of the respiratory function or availability of
7 carbon sources. The nature of the signal(s) is not currently known though malate has been
8 suggested as an inducing molecule for the RSEs orthologs in *A. nidulans* (Suzuki et al., 2012).
9 We have proposed that the activation of the RSEs is different according to inducer (acetate or
10 antimycin A containing medium). In the presence of antimycin A, induction of genes such as
11 *aox* is strong whereas that of genes such as *fbpase-1* and *pck-1* is weak. On acetate as the sole
12 carbon source, induction of *fbpase-1* and *pck-1* is strong and that of *aox* much less ((Bovier et
13 al., 2014) and this work). These data strongly suggest that different signals arising from
14 different growth conditions can differentially affect the transcription of the RSE-target genes.
15 We show here that in the mutants of the phosphatase domain of *pfkfb-1*, the level of
16 expression of the RSE-target genes is comparable to that in the wild-type grown on acetate. In
17 addition, we show that in these mutants, acetate has only a slight, or no inducer effect on the
18 expression of genes of the second category, whereas antimycin A is still able to induce the
19 expression of genes of the first category. Altogether, these observations support the
20 hypothesis that in the phosphatase mutants of *pfkfb-1*, the RSEs are under an activated form
21 similar or close to which they adopt in the wild-type strain grown on acetate with the
22 transcriptional activation of the gluconeogenic enzymes.

23 In mammalian tissues, the balance between the kinase and the bisphosphatase
24 reactions of PFK-2/FBPase-2 determines the cellular content of Fru-2,6-P₂ that is a potent
25 allosteric positive effector of PFK-1 and inhibitor of FBPase-1, and thus stimulates glycolysis

1 (Rousseau and Hue, 1993; Van Schaftingen, 1987). Bisphosphatase-dead mutants are
2 therefore expected to increase the level of Fru-2,6-P2 and stimulate glycolysis (Fig. 2). This
3 effect could, in addition of the overexpression of the AOX, participate to the suppressor effect
4 of the phosphatase mutants, by compensating partially the ATP deficit of the Δcox mutant.
5 However, if glycolysis is in fact, activated in the phosphatase-dead mutants, there is then an
6 apparent discrepancy between this stimulation and the activation of the RSEs that, under their
7 active form, increase the expression of the gluconeogenic gene *fbpase-1*. In contrast to
8 mammals, where the level of Fru-2,6-P2 was shown to be the main control element in
9 carbohydrate metabolism (Rousseau and Hue, 1993; Van Schaftingen, 1987), the
10 physiological importance of Fru-2,6-P2 in yeast is not clear and contradictory results have
11 been presented for the regulation of glycolytic flux rates by Fru-2, 6-P2 (Boles et al., 1996;
12 Müller et al., 1997; Navas et al., 1993). Mutants affected in the amount of Fru-2,6-P2 or in the
13 control of FBPase-1 are able to grow normally on glucose indicating that in this organism, the
14 concentration of Fru-2,6-P2 does not regulate glycolytic flux rates during growth on glucose
15 and that Fru-2,6-P2 does not play a major role in the prevention of futile cycling of the
16 FBPase-1/PFK-1 pair (Boles et al., 1996; Müller et al., 1997). Unfortunately, data in fungi
17 other than *S. cerevisiae* is missing. In *P. anserina*, consistently with a decrease in intracellular
18 ATP, the phosphatase-dead mutants present a reduction of the growth rate, a delay in the
19 development of the fruiting bodies (Table 1) and alterations of the germinating mycelium. It
20 is tempting to attribute these phenotypes to futile cycling, what focus on the unsolved and
21 exciting question: why does abolishing the phosphatase activity of PFK-2/FBPase-2 lead to
22 the activation of the RSE and the transcriptional activation the FBPase-1 gluconeogenic
23 enzyme while we rather expected an increase of Fru-2,6-P2 level and thus a negative effect on
24 gluconeogenesis? Although experimental data for this functional link are missing so far, we
25 are convinced that the elucidation of this intriguing metabolic network will allow gaining new

1 insights into the control of carbohydrate metabolism in fungi.

2

3 4.3. Evolution of PFK-2/FBPase-2 in Ascomycota

4 In mammals, multiple tissue-specific isoforms encoded from four genes, designated
5 *PFKFB1-4*, have been identified whereas in plants, molecular data suggest that only one gene
6 encodes the bi-functional enzyme (Nielsen et al., 2004; Okar et al., 2001). In fungi, data on
7 PFK-2/FBPase-2 have only been reported in *S. cerevisiae* in which three isoforms are present,
8 but each displays only one activity, two for kinase (PFK26, PFK27) and one for phosphatase
9 (FBP26, (Boles et al., 1996; Kretschmer and Fraenkel, 1991; Paravicini and Kretschmer,
10 1992). Searching Ascomycota databases, we found that all the tested Ascomycetes, except *S.*
11 *pombe* that is evolutionary distant, contain three homologous sequences that fall into 3 sub-
12 families (subfamilies 1, 2 and 3, Fig.7). FBP26 and PFK27 of *S. cerevisiae* can be clustered in
13 subfamilies 1 and 3 respectively. Noticeably, all the isoforms of subfamily 2 lack the key
14 catalytic residue RHG that is replaced by RAG (or RSG in *A. oligospora*). These data
15 strongly suggest that all these isoforms are mono-functional PFK-2s. In contrast the RHG is
16 conserved in all the isoforms of subfamily 1 and in most of the isoforms of subfamily 3
17 except *PFK26* of *S. cerevisiae*. Trypanosomatidae have also been shown to contain four
18 isoenzymes for PFK-2/FBPase-2. Two of them are predicted to be mono-functional PFK-2s
19 and the two others mono-functional FBPase-2 enzymes respectively (Chevalier et al., 2005).
20 As mentioned earlier, it is clear that the FBPase-2 domain is homologous to the
21 phosphoglycerate mutases, and the PFK-2 domain to several nucleotide-binding proteins. The
22 most plausible hypothesis for the origin of PFK-2/FBPase-2 is a gene fusion followed by
23 duplication (Rider et al., 2004). Why the bi-functional PFK-2/FBPase-2 evolved into several
24 mono-functional enzymes in *S. cerevisiae* and Trypanosomatidae is unclear. It has been
25 proposed that expression of multiple mono-functional enzymes offers additional flexibility of

1 metabolic regulation in the trypanosomes whose life cycles involve dramatic environmental
2 shifts (Chevalier et al., 2005). We show here that all the Ascomycetes contain several PFK-
3 2/FBPase-2 isoenzymes. Aside from the yeasts (Saccharomycotina), two are probably bi-
4 functional and one a mono-functional PFK-2 enzyme. Our data do not permit to draw any
5 conclusions as to the reason why Ascomycetes contain these different isoenzymes. However,
6 as said in the introduction, a remarkable feature of fungi is their important flexible
7 metabolism that enables their adaptation to the available carbon source. As proposed for the
8 Trypanosomatidae, the presence of multiple FBK-2/FBPase-2 isoforms could participate to an
9 as yet not understood complex system of regulation of carbohydrate metabolism in these
10 organisms.

11

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16

17

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19 **References**

- 20 Bazan, J.F., Fletterick, R.J., Pilgis, S.J., 1989. Evolution of a bifunctional enzyme: 6-
21 phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Proc. Natl. Acad. Sci. U.S.A.* 86,
22 9642–9646.
- 23 Boles, E., Göhlmann, H.W., Zimmermann, F.K., 1996. Cloning of a second gene encoding 5-
24 phosphofructo-2-kinase in yeast, and characterization of mutant strains without
25 fructose-2,6-bisphosphate. *Mol. Microbiol.* 20, 65–76.
- 26 Bovier, E., Sellem, C.H., Humbert, A., Sainsard-Chanet, A., 2014. Genetic and functional
27 investigation of Zn(2)Cys(6) transcription factors RSE2 and RSE3 in *Podospora*
28 *anserina*. *Eukaryotic Cell* 13, 53–65. <https://doi.org/10.1128/EC.00172-13>
- 29 Chae, M.S., Lin, C.C., Kessler, K.E., Nargang, C.E., Tanton, L.L., Hahn, L.B., Nargang, F.E.,
30 2007a. Identification of an alternative oxidase induction motif in the promoter region
31 of the *aod-1* gene in *Neurospora crassa*. *Genetics* 175, 1597–1606.
32 <https://doi.org/10.1534/genetics.106.068635>

- 1 Chae, M.S., Nargang, C.E., Cleary, I.A., Lin, C.C., Todd, A.T., Nargang, F.E., 2007b. Two
2 zinc-cluster transcription factors control induction of alternative oxidase in
3 *Neurospora crassa*. *Genetics* 177, 1997–2006.
4 <https://doi.org/10.1534/genetics.107.078212>
- 5 Chevalier, N., Bertrand, L., Rider, M.H., Opperdoes, F.R., Rigden, D.J., Michels, P.A.M.,
6 2005. 6-Phosphofructo-2-kinase and fructose-2,6-bisphosphatase in
7 *Trypanosomatidae*. Molecular characterization, database searches, modelling studies
8 and evolutionary analysis. *FEBS J.* 272, 3542–3560. <https://doi.org/10.1111/j.1742-4658.2005.04774.x>
- 10 Dufour, E., Boulay, J., Rincheval, V., Sainsard-Chanet, A., 2000. A causal link between
11 respiration and senescence in *Podospora anserina*. *Proc. Natl. Acad. Sci. U.S.A.* 97,
12 4138–4143. <https://doi.org/10.1073/pnas.070501997>
- 13 El-Khoury, R., Sellem, C.H., Coppin, E., Boivin, A., Maas, M.F.P.M., Debuchy, R., Sainsard-
14 Chanet, A., 2008. Gene deletion and allelic replacement in the filamentous fungus
15 *Podospora anserina*. *Curr. Genet.* 53, 249–258. <https://doi.org/10.1007/s00294-008-0180-3>
- 17 Espagne, E., Lespinet, O., Malagnac, F., Da Silva, C., Jaillon, O., Porcel, B.M., Couloux, A.,
18 Aury, J.-M., Ségurens, B., Poulain, J., Anthouard, V., Grossetete, S., Khalili, H.,
19 Coppin, E., Déquard-Chablat, M., Picard, M., Contamine, V., Arnaise, S., Bourdais,
20 A., Berteaux-Lecellier, V., Gautheret, D., de Vries, R.P., Battaglia, E., Coutinho, P.M.,
21 Danchin, E.G., Henrissat, B., Khoury, R.E., Sainsard-Chanet, A., Boivin, A., Pinan-
22 Lucarré, B., Sellem, C.H., Debuchy, R., Wincker, P., Weissenbach, J., Silar, P., 2008.
23 The genome sequence of the model ascomycete fungus *Podospora anserina*. *Genome*
24 *Biol.* 9, R77. <https://doi.org/10.1186/gb-2008-9-5-r77>
- 25 Fothergill-Gilmore, L.A., Watson, H.C., 1989. The phosphoglycerate mutases. *Adv.*
26 *Enzymol. Relat. Areas Mol. Biol.* 62, 227–313.
- 27 Hasemann, C.A., Istvan, E.S., Uyeda, K., Deisenhofer, J., 1996. The crystal structure of the
28 bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase reveals
29 distinct domain homologies. *Structure* 4, 1017–1029.
- 30 Humbert, A., Bovier, E., Sellem, C.H., Sainsard-Chanet, A., 2015. Deletion of the MED13
31 and CDK8 subunits of the Mediator improves the phenotype of a long-lived
32 respiratory deficient mutant of *Podospora anserina*. *Fungal Genet. Biol.* 82, 228–237.
33 <https://doi.org/10.1016/j.fgb.2015.07.010>
- 34 Hynes, M.J., Szewczyk, E., Murray, S.L., Suzuki, Y., Davis, M.A., Sealy-Lewis, H.M., 2007.
35 Transcriptional control of gluconeogenesis in *Aspergillus nidulans*. *Genetics* 176,
36 139–150. <https://doi.org/10.1534/genetics.107.070904>
- 37 Kretschmer, M., Fraenkel, D.G., 1991. Yeast 6-phosphofructo-2-kinase: sequence and mutant.
38 *Biochemistry* 30, 10663–10672.
- 39 Kretschmer, M., Tempst, P., Fraenkel, D.G., 1991. Identification and cloning of yeast
40 phosphofructokinase 2. *Eur. J. Biochem.* 197, 367–372.
- 41 Lee, Y.-H., Li, Y., Uyeda, K., Hasemann, C.A., 2003. Tissue-specific structure/function
42 differentiation of the liver isoform of 6-phosphofructo-2-kinase/fructose-2,6-
43 bisphosphatase. *J. Biol. Chem.* 278, 523–530.
44 <https://doi.org/10.1074/jbc.M209105200>
- 45 Lee, Y.H., Ogata, C., Pflugrath, J.W., Levitt, D.G., Sarma, R., Banaszak, L.J., Pilkis, S.J.,
46 1996. Crystal structure of the rat liver fructose-2,6-bisphosphatase based on
47 selenomethionine multiwavelength anomalous dispersion phases. *Biochemistry* 35,
48 6010–6019. <https://doi.org/10.1021/bi9600613>
- 49 Li, L., Lin, K., Kurland, I.J., Correia, J.J., Pilkis, S.J., 1992. Site-directed mutagenesis in rat
50 liver 6-phosphofructo-2-kinase. Mutation at the fructose 6-phosphate binding site

- 1 affects phosphate activation. *J. Biol. Chem.* 267, 4386–4393.
- 2 Lin, K., Li, L., Correia, J.J., Pilkis, S.J., 1992. Arg-257 and Arg-307 of 6-phosphofructo-2-
3 kinase/fructose-2,6-bisphosphatase bind the C-2 phospho group of fructose-2,6-
4 bisphosphate in the fructose-2,6-bisphosphatase domain. *J. Biol. Chem.* 267, 19163–
5 19171.
- 6 Lorin, S., Dufour, E., Boulay, J., Begel, O., Marsy, S., Sainsard-Chanet, A., 2001.
7 Overexpression of the alternative oxidase restores senescence and fertility in a long-
8 lived respiration-deficient mutant of *Podospora anserina*. *Mol. Microbiol.* 42, 1259–
9 1267.
- 10 Lorin, S., Dufour, E., Sainsard-Chanet, A., 2006. Mitochondrial metabolism and aging in the
11 filamentous fungus *Podospora anserina*. *Biochim. Biophys. Acta* 1757, 604–610.
12 <https://doi.org/10.1016/j.bbabi.2006.03.005>
- 13 McDonald, A.E., 2008. Alternative oxidase: an inter-kingdom perspective on the function and
14 regulation of this broadly distributed ‘cyanide-resistant’ terminal oxidase. *Functional*
15 *Plant Biol.* 35, 535–552. <https://doi.org/10.1071/FP08025>
- 16 Müller, S., Zimmermann, F.K., Boles, E., 1997. Mutant studies of phosphofructo-2-kinases do
17 not reveal an essential role of fructose-2,6-bisphosphate in the regulation of carbon
18 fluxes in yeast cells. *Microbiology (Reading, Engl.)* 143 (Pt 9), 3055–3061.
19 <https://doi.org/10.1099/00221287-143-9-3055>
- 20 Navas, M.A., Cerdán, S., Gancedo, J.M., 1993. Futile cycles in *Saccharomyces cerevisiae*
21 strains expressing the gluconeogenic enzymes during growth on glucose. *Proc. Natl.*
22 *Acad. Sci. U.S.A.* 90, 1290–1294.
- 23 Nielsen, T.H., Rung, J.H., Villadsen, D., 2004. Fructose-2,6-bisphosphate: a traffic signal in
24 plant metabolism. *Trends Plant Sci.* 9, 556–563.
25 <https://doi.org/10.1016/j.tplants.2004.09.004>
- 26 Okar, D.A., Manzano, A., Navarro-Sabatè, A., Riera, L., Bartrons, R., Lange, A.J., 2001.
27 PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-
28 bisphosphate. *Trends Biochem. Sci.* 26, 30–35.
- 29 Paravicini, G., Kretschmer, M., 1992. The yeast FBP26 gene codes for a fructose-2,6-
30 bisphosphatase. *Biochemistry* 31, 7126–7133.
- 31 Pilkis, S.J., Claus, T.H., Kurland, I.J., Lange, A.J., 1995. 6-Phosphofructo-2-kinase/fructose-
32 2,6-bisphosphatase: a metabolic signaling enzyme. *Annu. Rev. Biochem.* 64, 799–835.
33 <https://doi.org/10.1146/annurev.bi.64.070195.004055>
- 34 Qi, Z., Smith, K.M., Bredeweg, E.L., Bosnjak, N., Freitag, M., Nargang, F.E., 2017.
35 Alternative Oxidase Transcription Factors AOD2 and AOD5 of *Neurospora crassa*
36 Control the Expression of Genes Involved in Energy Production and Metabolism. *G3*
37 (Bethesda) 7, 449–466. <https://doi.org/10.1534/g3.116.035402>
- 38 Rider, M.H., Bertrand, L., Vertommen, D., Michels, P.A., Rousseau, G.G., Hue, L., 2004. 6-
39 phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional
40 enzyme that controls glycolysis. *Biochem. J.* 381, 561–579.
41 <https://doi.org/10.1042/BJ20040752>
- 42 Rider, M.H., Crepin, K.M., De Cloedt, M., Bertrand, L., Hue, L., 1994. Site-directed
43 mutagenesis of rat muscle 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: role
44 of Asp-130 in the 2-kinase domain. *Biochem. J.* 300 (Pt 1), 111–115.
- 45 Rigden, D.J., 2008. The histidine phosphatase superfamily: structure and function. *Biochem.*
46 *J.* 409, 333–348. <https://doi.org/10.1042/BJ20071097>
- 47 Rousseau, G.G., Hue, L., 1993. Mammalian 6-phosphofructo-2-kinase/fructose-2,6-
48 bisphosphatase: a bifunctional enzyme that controls glycolysis. *Prog. Nucleic Acid*
49 *Res. Mol. Biol.* 45, 99–127.
- 50 Scheckhuber, C.Q., Osiewacz, H.D., 2008. *Podospora anserina*: a model organism to study

1 mechanisms of healthy ageing. *Mol. Genet. Genomics* 280, 365–374.
2 <https://doi.org/10.1007/s00438-008-0378-6>
3 Sellem, C.H., Bovier, E., Lorin, S., Sainsard-Chanet, A., 2009. Mutations in two zinc-cluster
4 proteins activate alternative respiratory and gluconeogenic pathways and restore
5 senescence in long-lived respiratory mutants of *Podospora anserina*. *Genetics* 182, 69–
6 78. <https://doi.org/10.1534/genetics.109.100834>
7 Suzuki, Y., Murray, S.L., Wong, K.H., Davis, M.A., Hynes, M.J., 2012. Reprogramming of
8 carbon metabolism by the transcriptional activators AcuK and AcuM in *Aspergillus*
9 *nidulans*. *Mol. Microbiol.* 84, 942–964. <https://doi.org/10.1111/j.1365->
10 [2958.2012.08067.x](https://doi.org/10.1111/j.1365-2958.2012.08067.x)
11 Tauler, A., Lin, K., Pilkis, S.J., 1990. Hepatic 6-phosphofructo-2-kinase/fructose-2,6-
12 bisphosphatase. Use of site-directed mutagenesis to evaluate the roles of His-258 and
13 His-392 in catalysis. *J. Biol. Chem.* 265, 15617–15622.
14 Van Schaftingen, E., 1987. Fructose 2,6-bisphosphate. *Adv. Enzymol. Relat. Areas Mol. Biol.*
15 59, 315–395.
16 Vertommen, D., Bertrand, L., Sontag, B., Di Pietro, A., Louckx, M.P., Vidal, H., Hue, L.,
17 Rider, M.H., 1996. The ATP-binding site in the 2-kinase domain of liver 6-
18 phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Study of the role of Lys-54 and
19 Thr-55 by site-directed mutagenesis. *J. Biol. Chem.* 271, 17875–17880.
20 Yuen, M.H., Mizuguchi, H., Lee, Y.H., Cook, P.F., Uyeda, K., Hasemann, C.A., 1999.
21 Crystal structure of the H256A mutant of rat testis fructose-6-phosphate,2-
22 kinase/fructose-2,6-bisphosphatase. Fructose 6-phosphate in the active site leads to
23 mechanisms for both mutant and wild type bisphosphatase activities. *J. Biol. Chem.*
24 274, 2176–2184.
25

1 LEGENDS OF FIGURES

2 **Fig. 1.** Phenotypic characteristics of the different Δcox suppressors. For each strain
3 picture represents the mycelium obtained after 6 days of incubation at 27°C from an explant
4 laid in the center of M2 medium containing Petri dishes. ^a The growth rate is a mean value
5 expressed in mm/day (\pm standard deviation). ^b Female fertility is determined according to the
6 number of fruiting bodies or ascospores produced in a cross with the wild type strain used as a
7 male. – corresponds to sterility and + to wild type fertility. ^c Ascospore germination rate is
8 given as ++ for wild-type thalli, \pm for sparse and slow-growing thalli, and + for thalli of
9 intermediate phenotype on germination medium.

10 **Fig. 2.** Alignment of PFK-2/FBPase-2 in *R. norvegicus* (R), *H. sapiens* (H), *N. crassa*
11 (NCUO9347TO), *P. anserina* (Pa_3_11390) and in *S. cerevisiae* (PFK26). The PFK-2 kinase
12 domain is framed in black and the FBPase-2 phosphatase domain in grey. Locations of
13 mutations are indicated by * and arrows.

14 **Fig. 3.** Characteristics of the suppressor effect of mutations in *pfkfb-1*. Strains Δcox
15 carry either the *pfkfb-1*⁺ wild type allele or the *pfkfb-1*^{R384C} (called “su” in Fig. 1) suppressor
16 allele. The deleted $\Delta pfkfb-1$ and mutated *pfkfb-1*^{H233A} constructed alleles in the phosphatase
17 domain or the double mutated (*pfkfb-1*^{L24A, D103A}) constructed allele in the kinase domain were
18 also associated with Δcox . Δcox *pfkfb-1*^{R384C} and Δcox *pfkfb-1*^{H233A} having the same
19 phenotype only Δcox *pfkfb-1*^{R384C} was shown. ^a The growth rate is given as a mean value
20 expressed in mm/day (\pm standard deviation). The resistance to salicylhydroxamic acid
21 (SHAM^R), reflecting the level of AOX expression, is given as the percentage of growth in the
22 presence of 150 μ g/ml SHAM compared to the growth in absence of SHAM.

23 **Fig. 4.** Level of transcripts in the *pfkfb-1* mutants relative to the wild type strain. The
24 amount of *aox*, *fbpase-1*, *pck-1* transcripts (A) and of transcripts for Pa_5_1700, Pa_7_2610
25 and Pa_4_3860 (other RSE-target genes) and Pa_1_6760 and Pa_1_14910 (genes encoding

1 glycolytic enzymes) (B) was determined by real-time RT-PCR in each strain. The histograms
2 represent the increase amount of transcripts in each strain relative to the wild type.

3 **Fig. 5.** Level of transcripts in the *pfkfb-1^{R284C}* mutant strain grown on non inducer
4 medium and in the wild type strain in presence of inducer relative to wild type in absence of
5 inducer. The amount of transcripts was determined, as in figure 4. AA: Antimycin A
6 supplemented medium; Ac: medium acetate as the sole source of carbon.

7 **Fig. 6.** Level of transcripts in the *pfkfb-1^{R284C}* mutant and wild type strains grown on
8 acetate medium (A) or on antimycin A (B). The amount of transcripts was given relative to
9 wild type strain and *pfkfb-1^{R284C}* mutant grown on medium in absence of inducer,
10 respectively.

11 **Fig. 7.** Phylogenetic tree of the PFK-2/FBPase-2 proteins in Ascomycetes.
12 Abbreviations: Sordariomycetes : *Podospora anserina* (Pa), *Neurospora crassa* (Neucr) ;
13 *Fusarium graminearum* (Fusgr), *Magnaporthe grisea* (Maggr) ; Xylonomycetes :
14 *Symbiotaphrina kochii* (Symko) ; Leotiomycetes : *Botrytis cinerea* (Botci),
15 Dothideomycetes: *Stagonospora nodorum* (Stano) ; Eurotiomycetes : *Aspergillus nidulans*
16 (Aspni) ; Orbiliomycetes *Arthrobotrys oligospora* (Artol) ; Pezizomycetes: *Ascobolus*
17 *immersus* (Ascim) ; Saccharomycotina: *Saccharomyces cerevisiae* (Sacce), *Yarrowia*
18 *lipolytica* (Yarli), Lecanoromycetes: *Cladonia grayi* (Clargr). The tree was rooted using the
19 human PFKB2 (HumPFKB2) as an out-group. Approximate likelihood-ratio test (SH-like)
20 was used for statistical tests. Only the scores between 0.995 and 1 are shown.

21

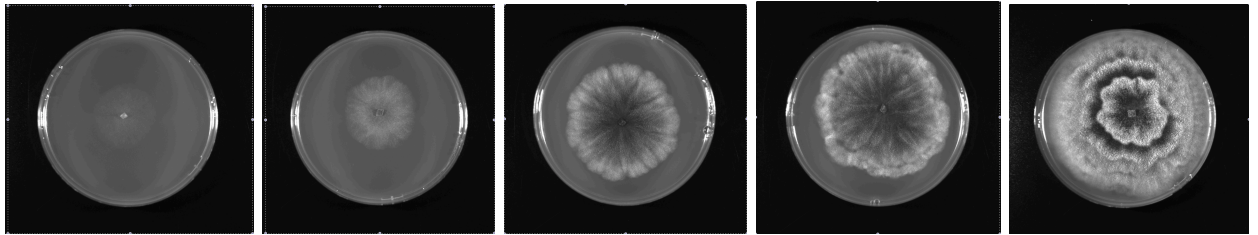
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Figure 1



	Δcox	$\Delta\text{cox } \Delta\text{med13}$	$\Delta\text{cox } \llbracket \text{su} \rrbracket$	$\Delta\text{cox } rse$	wt
GROWTH ^a	2.8±0.3	3.6±0.3	4.2±0.2	5.1±0.2	6.7±0.2
FERTILITY ^b	-	-	-	-	+
GERMINATION ^c	±	±	±	+	++

Figure 2

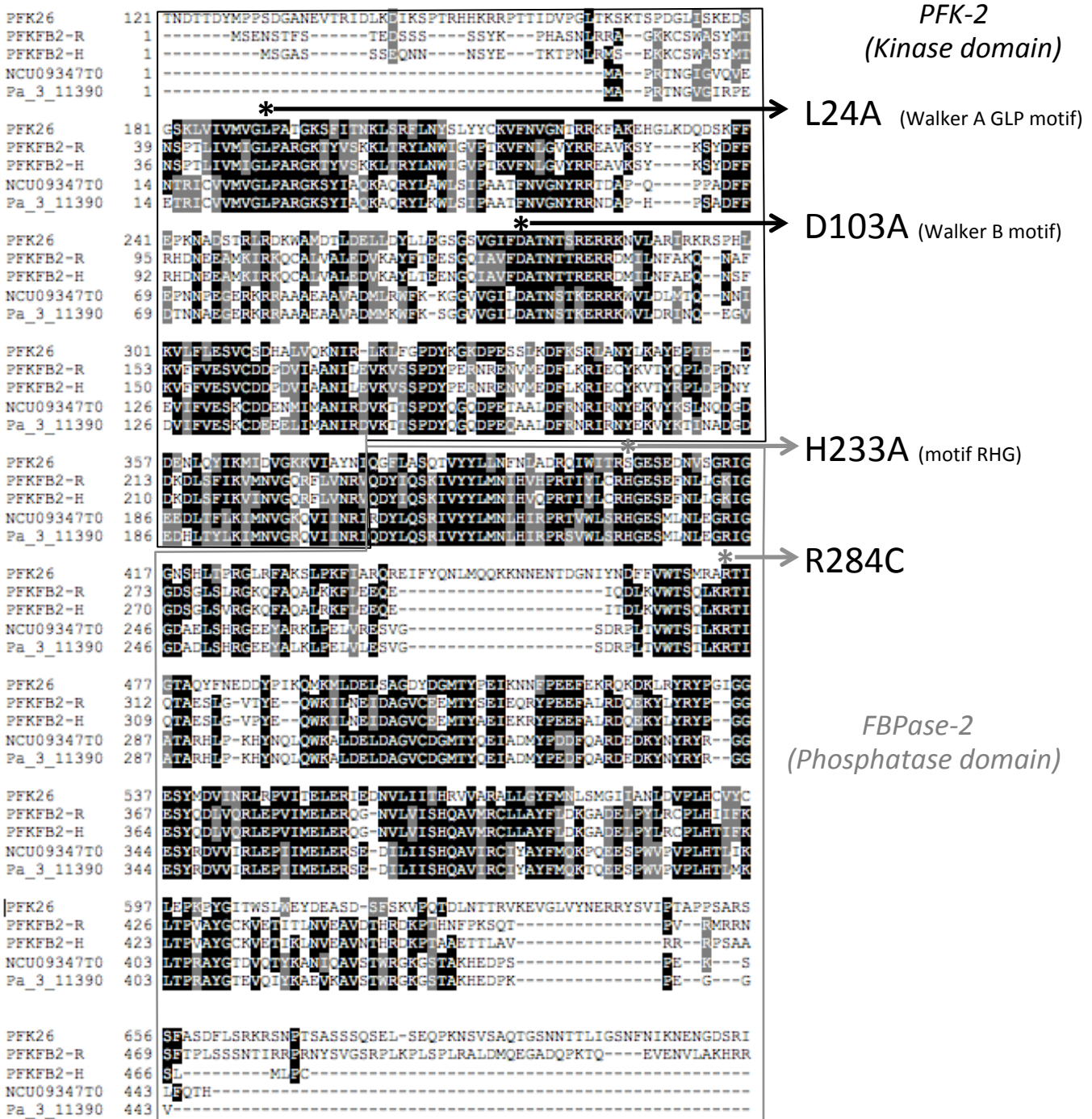
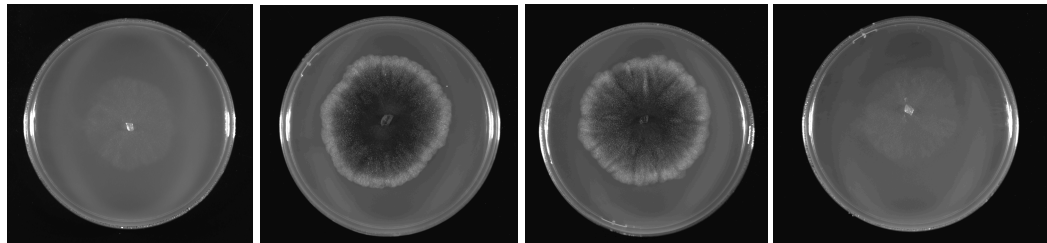


Figure 3



	<i>Δcox pfkfb-1⁺</i>	<i>Δcox Δpfkfb-1</i>	<i>Δcox pfkfb-1^{R384C}</i>	<i>Δcox pfkfb-1^{L24A, D103A}</i>
GROWTH ^a	2.8±0.3	4.7±0.2	4.2±0.2	2.9±0.3
SHAM ^R	8.9±1.2	63.2±6.0	65.3±2.5	10.0±3.1

Figure 4

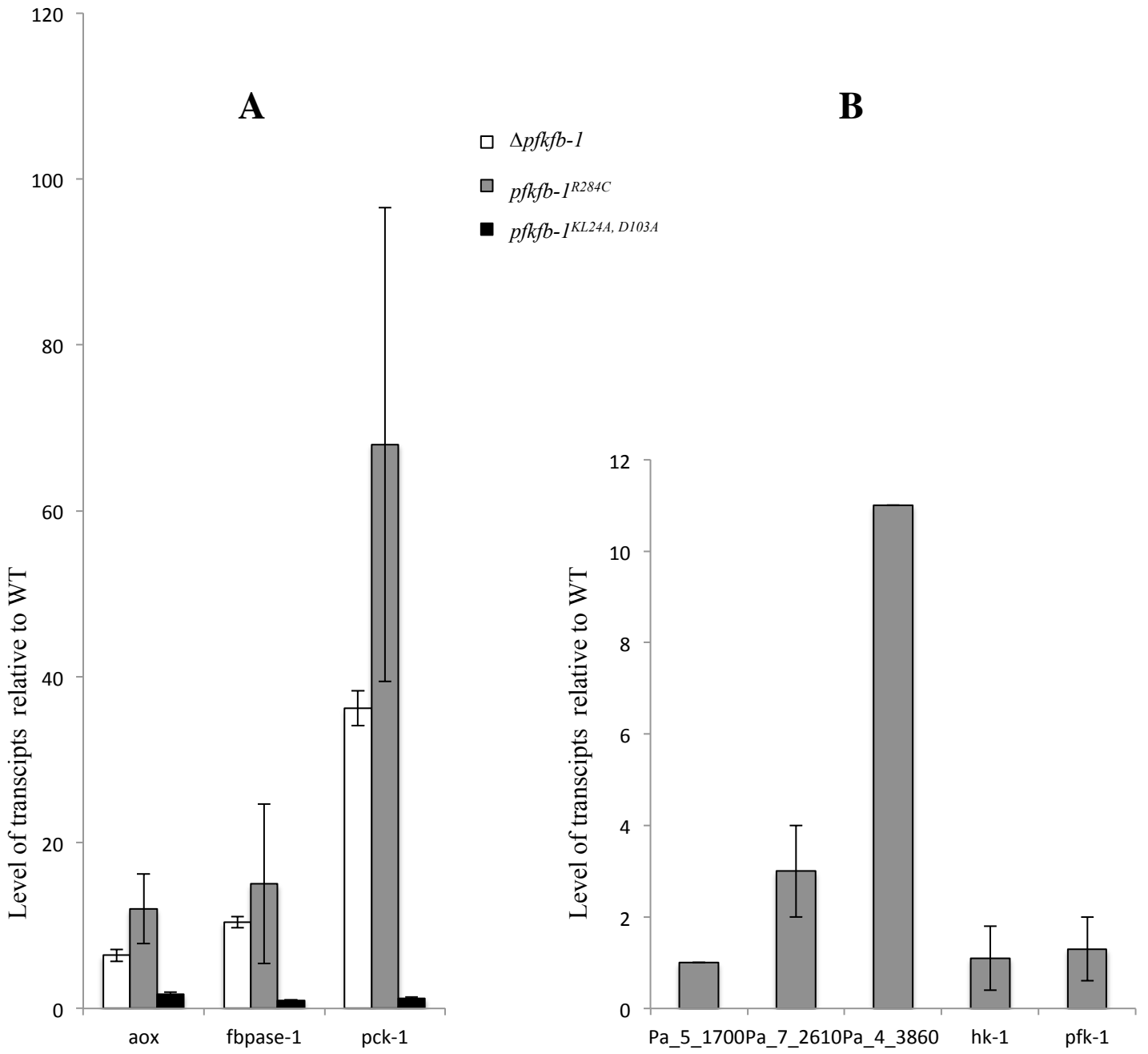


Figure 5

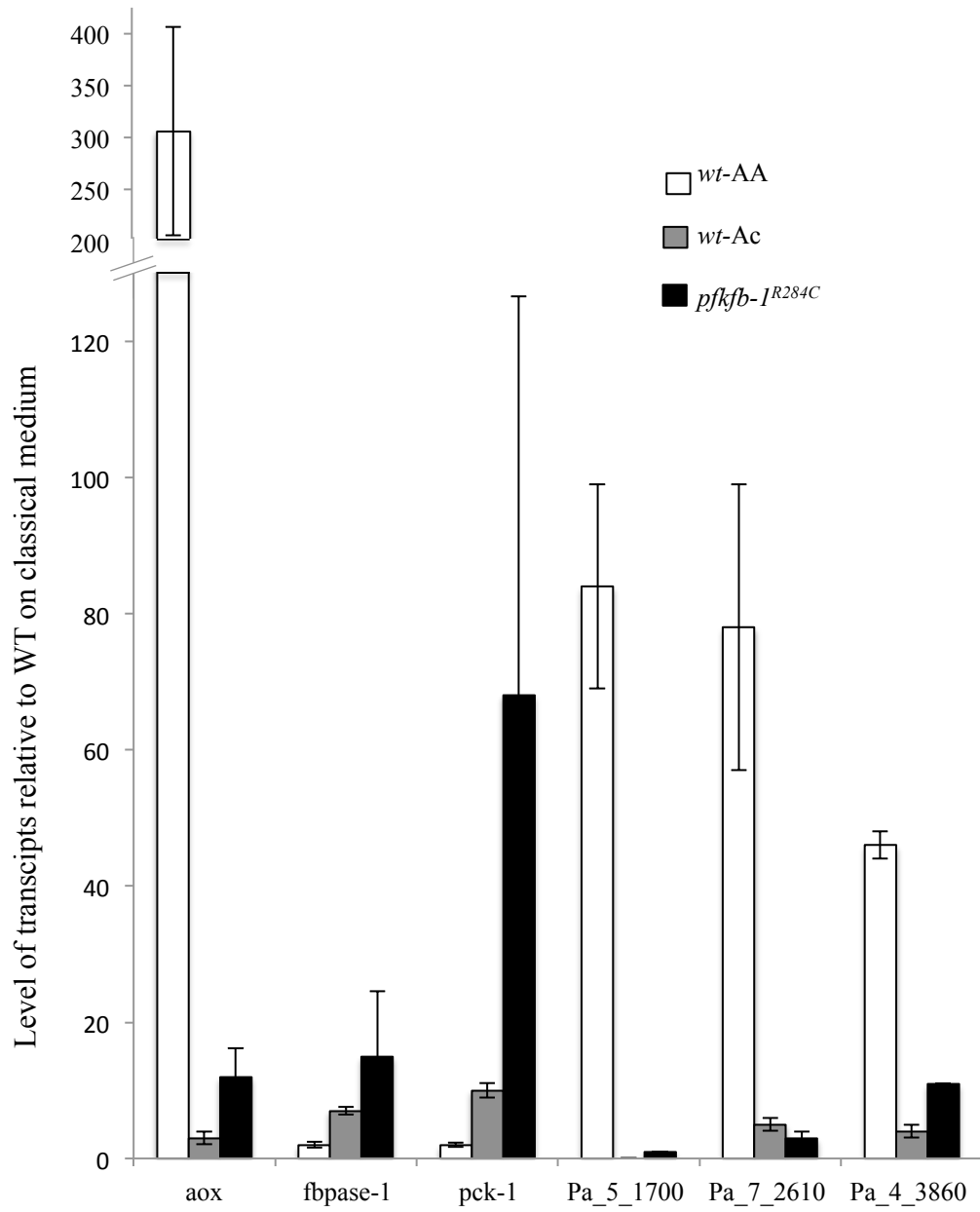


Figure 6

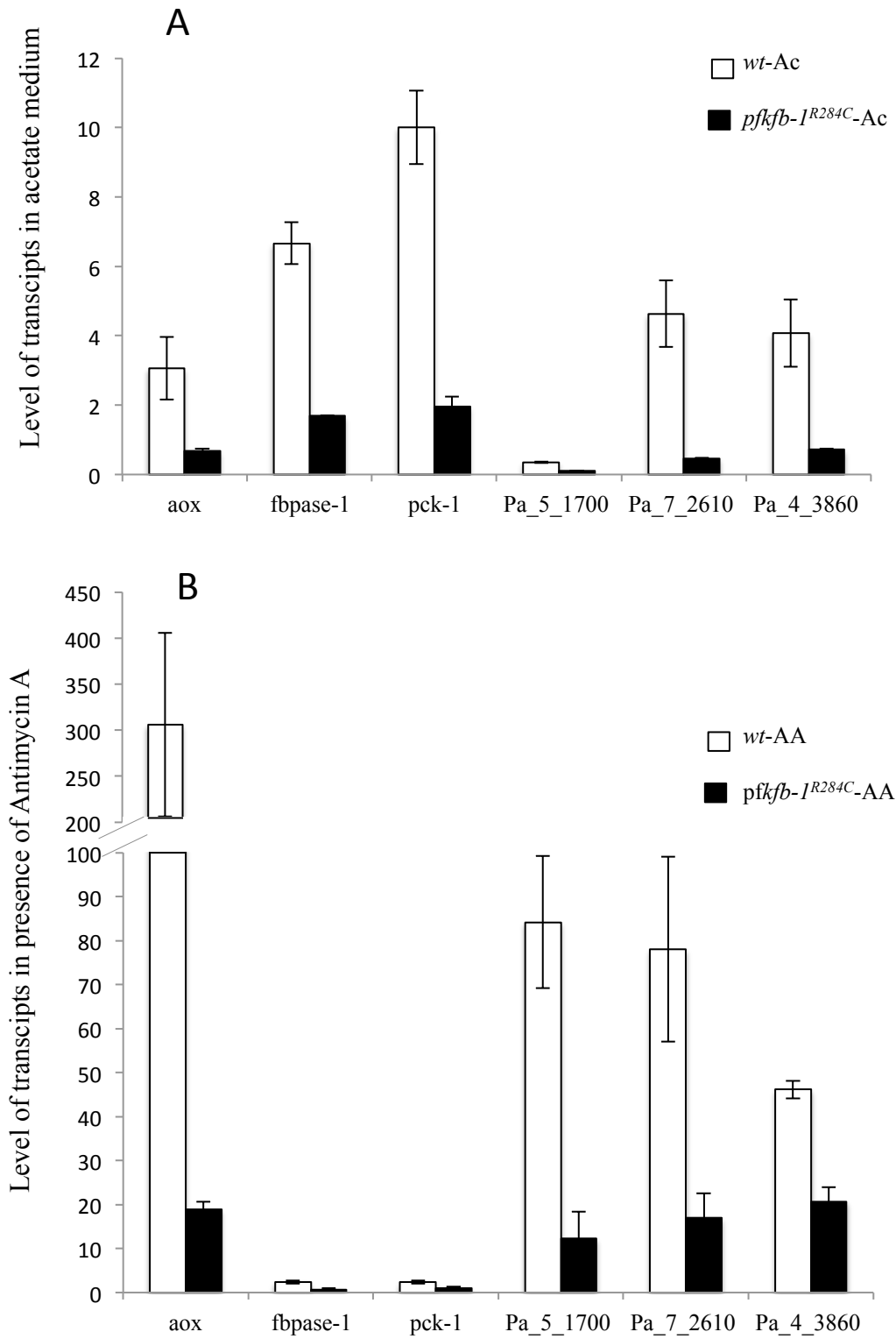


Figure 7

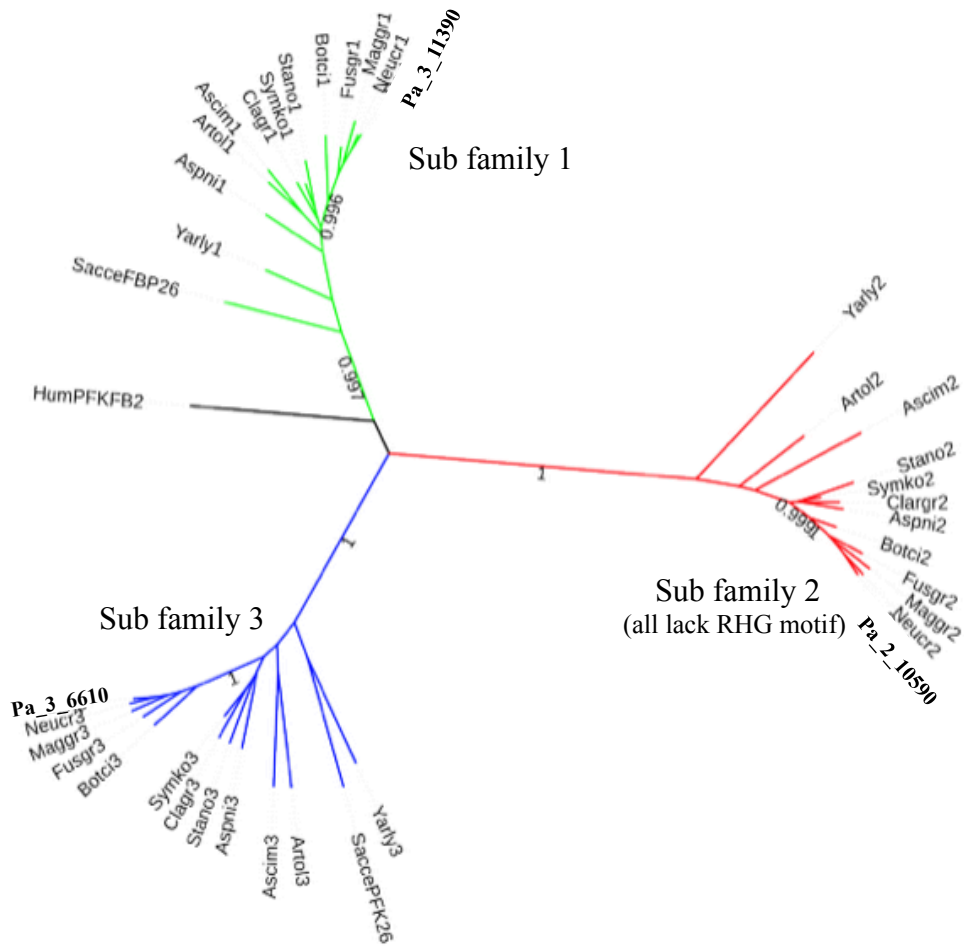


TABLE 1 Phenotypic characterization of the *pfkfb-1* mutant strains

	<i>wt</i>	Δ <i>pfkfb-1</i>	<i>pfkfb-1</i> ^{R284C}	<i>pfkfb-1</i> ^{H233A}	<i>pfkfb-1</i> ^{L23A D103A}
GROWTH ^a	6.9±0.2	6.1±0.1	6.1±0.1	6.2±0.1	7.0±0.1
PIGMENTATION ^b	+	++	++	++	+
GERMINATION	+	+/-	+/-	+/-	+
FERTILITY ^c	WT	delayed	delayed	delayed	WT
ANTIMYCIN ^d	+/-	++	++	++	+/-
LONGEVITY ^e	11.3±0.8	9.7±0.8	9.7±1.9	11.2±1.8	13.8±1.8

^a The growth rate is a mean value expressed in mm/day (\pm standard deviation).

^b ++ referred to an increased pigmentation of the mycelium compared to that of the wt (+)

^c Female fertility (F) is determined according to the number of fruiting bodies or ascospores produced in a cross with the wild type strain used as a male. – corresponds to sterility and + to wild type fertility.

^d Growth on antimycin reflects a constitutive expression of AOX

^e Longevity is expressed in centimeters of growth (\pm standard deviation), measured on M2 medium on three parallel subcultures derived from two to five independent ascospores with a given genotype as previously described (Sellem et al., 2009)