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GiFRD encodes a protein involved in anaerobic growth in the arbuscular mycorrhizal fungus *Glomus intraradices*

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ABSTRACT

Fumarate reductase is a protein involved in the maintenance of redox balance during oxygen deficiency. This enzyme irreversibly catalyzes the reduction of fumarate to succinate and requires flavin cofactors as electron donors. Two examples are the soluble mitochondrial and the cytosolic fumarate reductases of *Saccharomyces cerevisiae* encoded by the *OSM1* and *FRDS1* genes, respectively.

This work reports the identification and characterization of the gene encoding cytosolic fumarate reductase enzyme in the arbuscular mycorrhizal fungus, *Glomus intraradices* and the establishment of its physiological role. Using a yeast expression system, we demonstrate that *G. intraradices GiFRD* encodes a protein that has fumarate reductase activity which can functionally substitute for the *S. cerevisiae* fumarate reductases. Additionally, we showed that *GiFRD* transformants are not affected by presence of salt in medium, indicating that the presence of this gene has no effect on yeast behavior under osmotic stress. The fact that *GiFRD* expression and enzymatic activity was present only in asymbiotic stage confirmed existence of at least one anaerobic metabolic pathway in this phase of fungus life cycle. This suggests that the AMF behave as facultative anaerobes in the asymbiotic stage.

1. Introduction

Maintaining redox balance in hypoxia and anoxia, is a crucial challenge for many organisms. To achieve this, they oxidize reduced cofactors that are generated during the substrate uptake and catabolism, using alternative pathways that function without oxygen. For example, reoxidation of the cytosolic NADH created during glycolysis by glycerol-3-phosphate dehydrogenase (Gpd2) and alcohol dehydrogenase (Adh3) may serve as redox sink (Albertyn et al., 1994; Ansell et al., 1997; Bakker et al., 2000). In anaerobic respiration, an alternative respiratory terminal electron acceptor such as fumarate is required (Tielens and van Hellemond, 1998). Fumarate reductase is a key enzyme in fumarate respiration in both anaerobic and facultative anaerobic organisms such as the bacteria *Wolinella succinogenes*, *Escherichia coli* and the yeast *Saccharomyces cerevisiae* (Arikawa et al., 1998; Cecchini et al., 2002; Enomoto et al., 1996, 2002; Lauterbach et al., 1990; Lemire, 1986). This enzyme irreversibly catalyzes the reduction of fumarate to succinate and requires $FADH_2$, $FMNH_2$ or reduced riboflavin as electron donors.

Fumarate reductases can be divided into two classes, depending on the electron transfer mechanism. One of the classes is the membrane-bound enzymes which are covalently linked to flavin cofactors (FAD or FMN). They transfer electrons from a quinol to fumarate and are involved in the production of ATP by oxidative phosphorylation in anaerobic bacteria (Dickie and Weiner, 1979) and lower eukaryotes (Tielens and van Hellemond, 1998). The second class of fumarate reductases has been identified in only two organisms, the yeast *S. cerevisiae* and the protozoan *Trypanosoma brucei* (Besteiro et al., 2002). In contrast to the first class, these enzymes are soluble and can catalyze the reduction of fumarate independently from the electron transport chain (Camarasa et al., 2007).

Two members of the soluble enzymes in *S. cerevisiae* are the mitochondrial and cytosolic fumarate reductases, encoded by the *OSM1* and *FRDS1* genes, respectively (Enomoto et al., 1996; Muratsubaki and Enomoto, 1998). Camarasa et al. (2007) demonstrated the potential role of Frds1p as the main fumarate reductase in the regeneration of the FAD-prosthetic group of essential flavoproteins under anaerobic conditions. It can be functionally replaced by Osm1p, which contributes less than the cytoplasmic form to the total fumarate reductase activity.

Genomic sequencing projects have enabled the identification of homologues of *S. cerevisiae* Frds1p in some fungal species, such as

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Laccaria bicolor, *Neurospora crassa* and *Coprinus cinereus* (Galagan et al., 2003; Martin et al., 2008; Stajich et al., 2010) but this has not been attempted in the arbuscular mycorrhizal fungi.

The arbuscular mycorrhizal fungi (AMF) are organisms that have a symbiotic relationship with 70–90% of known land plant species (Smith and Read, 2008) and play a crucial role in agricultural systems (Kiers et al., 2011). They increase plant growth by improving nutrient uptake e.g. of phosphorus and nitrogen (Bücking and Shachar-Hill, 2005; Govindarajulu et al., 2005; Smith and Read, 1997). They also improve salt and drought tolerance (Evelin et al., 2009; Marulanda et al., 2006, 2007; Smith and Read, 2008), provide the host with tolerance to pathogens (Hata et al., 2010; Liu et al., 2007) and potentially improve heavy metal tolerance (Ricken and Hofner, 1996; Zhang et al., 2005). The extraradical AMF hyphae network with the plant root systems makes a significant in improvement of soil structure and water relations (Rillig and Mummey, 2006). To date little is known about the redox homeostasis in these fungi. In this study, we report the identification and characterization of the gene encoding fumarate reductase enzyme (*GiFRD*) and the determination of its physiological role in the arbuscular mycorrhizal fungus, *Glomus intraradices* (Sędzielewska et al., 2011). The gene cloning, measurement of RNA accumulation in different developmental stages and enzymatic activity were performed with AMF material. However, since there is currently no transformation technology available for AMF, we have used a *S. cerevisiae* expression system to functionally characterize the Gifrdp and to determine its subcellular localization.

2. Materials and methods

2.1. AMF monoxenic cultures

AMF *in vitro* monoxenic cultures were established as described by Declerck (2005). A carrot Ri-T DNA transformed root organ culture (*Daucus carota*, Gatersleben, Germany) was cultivated with single-spore isolate of the industrial AMykor AMF, *G. intraradices* (AMykor GmbH, Bitterfeld-Wolfen, Germany) in three-compartment Petri dishes which contained MRS medium gelled with 0.5% Phytagel (Sigma–Aldrich, Germany). Fungal hyphae, but not carrot roots, were allowed to grow over the plastic barrier to the other compartments. Plates were incubated in the dark at 26 °C for 3–6 months.

Extraradical mycelia and secondary spores were recovered from the root-free plate compartments by dissolving the culture medium in 10 mM citrate buffer (Declerck, 2005). The fungal material was collected with a pipette tip under sterile conditions (Bago et al., 1999) and stored at −20 °C or −80 °C until used.

2.2. Cell cultures

E. coli TOP10 cells from Invitrogen (USA) were used for cloning experiments and were cultivated as previously described (Sambrook et al., 1989). The cells were grown on LB medium supplemented with ampicillin to provide selection pressure (100 μg ml^{-1}; AppliChem, Germany).

The *S. cerevisiae* strains used in this study are listed in Table 1. Yeast strains were maintained in YPD medium (1% yeast extract, 2% Difco Bacto Peptone, 2% glucose) or minimal SD medium (1% glucose or 2% galactose, supplemented with 0.67% Difco yeast nitrogen base), and complemented when necessary with vitamins and amino acids (Sherman, 1991). Yeast cells were pre-cultured in YPD or 1% glucose SD media for 24 h or 48 h. Main cultures inoculated at a density of 0.1–0.2 OD_{600}, were grown in YPD or 2% galactose SD medium.

Aerobic cultures were grown in Erlenmeyer flasks at 30 °C with shaking (180× rpm). The anaerobic cultures were prepared as follows. Anaerobic conditions were created by bubbling nitrogen through the medium, for 30 min at 70 °C. The 12 ml plastic tubes were completely filled with the deoxygenated medium containing 2 mg/l resazurin to monitor anaerobiosis. The tubes were closed with plastic stoppers and covered with Parafilm®. Inoculation and collection of the samples were done through a needle placed in the stopper for the complete incubation period. The needle also allowed CO_2 to escape and prevented the entry of air to the tube. Cells were grown at 30 °C without shaking.

2.3. Double *Δosm1Δ frds1* mutant construction

To construct the *S. cerevisiae Δosm1 Δfrds1* mutant, strains Y06849 and Y10288 (Table 1) were allowed to mate and sporulate (Spencer et al., 1989). The haploid sporulation product of *S. cerevisiae* S0010 *Δosm1 Δfrds1* (BY4741 MATα*his3Δ1; leu2Δ0; met15Δ0; lys2Δ0; ura3Δ0; osm1*::*kanMX4*; *frds1*::*kanMX4*) was identified by PCR of the genomic DNA, using primers complementary to regions upstream and downstream of the *OSM1* gene (OSM1_delet_fwd and OSM1_delet_rev) and *FRDS1* gene (FRDS1_delet_fwd and FRDS1_delet_rev) (Table 2).

2.4. Gene isolation and DNA constructs

A fragment containing the *GiFRD* sequence was identified on the *G. intraradices* cDNA. The cDNA was synthesised using RNA isolated from spores and extraradical hyphae (First Strand cDNA Synthesis Kit, Thermo Scientific, Germany; RNeasy Mini Kit, Qiagen, Germany) and digested by restriction enzymes. The DNA fragments were cloned into a bacterial plasmid pCR4 using the TOPO TA Cloning Kit for Sequencing (Invitrogen, USA) and then transformed into *E. coli* TOP10 cells. DNA inserts were sequenced with primers in the kit. *GiFRD* partial sequence was identified using similarity searches in BLAST.

The 3′- and 5′-ends of *GiFRD* ORF were obtained using a TOPO Walker Kit on cDNA and then confirmed by PCR Taq polymerase amplification of the genomic DNA using the specific primers FR_Eco and FR_Not (Table 2).

Table 1
Bacteria and yeast strains involved in this study.

Strains	Genotype	Source
Escherichia coli		
TOP10	F′ *mcrAΔ* (*mmr-hsdRMS-mcrBC*) *F80ΔlacZDM15 DlacX74 deoR recA1 araD139 Δ*(*ara leu*) *7697 galU galK I*⁻ *rspL andA1 nupG F*′	Invitrogen, Germany
Saccharomyces cerevisiae		
Y06849	Mata, *his3D1*; *leu2D0*; *lys2D0*; *ura3D0*; *osm1*::*KanMX4*	Euroscarf, Germany
Y10288	Matα, *met15D0*; *leu2D0*; *lys2D0*; *ura3D0*; *frds1*::*KanMX4*	Euroscarf, Germany
S0010	*his3D1*; *leu2D0*; *lys2D0*; *met15D0*; *ura3D0*, *osm1*::*kanMX4*; *frds1*::*kanMX4*	Created in our lab
Y15271	Matα, *his3D1*; *leu2D0*; *lys2D0*; *ura3D0*; *YLR362w*::*kanMX4*	Euroscarf, Germany
S288C	Matα, *SUC2*; *gal2*; *mal*; *mel*, *flo1*; *flo8-1*; *hap1*; *ho*, *bio1*; *bio6*	Euroscarf, Germany

Table 2
Oligonucleotides used in this study.

Name	Sequence
Deletion mutant construct	
OSM1_delet _fwd	ATCATCCCGAGTCTTAGG
OSM1_delet_rev	CAGTATATCCTATCATGTCGCA
FRDS1_delet_fwd	GATACGAAGCAAGGCCTCAA
FRDS1_delet_rev	GAGTCTAAAAAACGTTCACTTT
Gene amplification	
FR_Eco (fwd)	GAATTCATGGCTAGTCAAATTATT
FR_Not (rev)	GCGGCCGCTTATTTTGTGACAGAAATACCACCC
RT-PCR	
RT_GiFRD_fwd	GAA TTC GGT GAA GAA GTT ATT GGT GTA G
RT_GiFRD_rev	GTC GAC ATC TTT TAC TTC AGA ATC AGG
RT_Tub_fwd	TAC CAT GGA CTC CGT TCG T
RT_Tub_rev	GAC GTG GAA AAG GCA CCA TA
RT_TEF_fwd	GCC CTT ACT TGA TTT ACA AGT
RT_TEF_rev	TTG ACC GTC CTT GGA GAT A
Protein detection and localization	
Hind_GiFRD (fwd)	AAGCTTATGGCTAGTCAAATTATTGTCG
Sac_linker_GiFRD (rev)	CCGCGG**TTCTGGACCTGGTAGTTCTGGACCTGGTAG**TTTTGTGACAGAAATACCACCC
SacI_GFP (fwd)	GAGCTCATGGTGAGCAAGGGCGAGGAG
NotI_GFP* (rev)	GCGGCCGCTTACTTGTACAGCTCGTCCATGCC

Underlined letters – restriction enzymes site; bold type letters – linker.

The amplified genomic *GiFRD*, flanked by *Eco*RI and *Not*I restriction sites, was cloned into pCR4 TOPO cloning vector (Invitrogen, USA). The complete ORF was released by restriction digestion and cloned into *Eco*RI/*Not*I linearized yeast expression vector pYES2 (including a uracil marker gene) with an inducible galactose promoter (Invitrogen, USA).

To obtain the pYES2-GiFRD::GFP construct, a variant of *GiFRD* was fused to the N-terminus of green fluorescence protein gene (*GFP*) in pYES2. The *GiFRD* was PCR amplified from genomic DNA using the primers Hind_GiFRD (fwd) and a Sac_linker_GiFRD with the 30 bp linker (rev) (Table 2). The full length of *GiFRD* with the 30 bp linker flanked by *Hin*dIII and *Sac*I sequences was then subcloned into a pCR4 vector, released from the vector by digestion and cloned into a linearized pYES2 vector. Next, *GFP* was PCR amplified using the primers SacI_GFP (fwd) and NotI_GFP* (rev) (Table 2). The *GFP*, flanked by *Sac*I and *Not*I sequences was subcloned into pCR4 vector, released from it by digestion and cloned into the *Sac*I/*Not*I linearized vector pYES2-GiFRD. The control vector pYES2-GFP was constructed in analogous way.

All vectors were constructed and amplified by transformation in *E. coli* TOP10 cells by following standard procedures and purified using the method for plasmid isolation based on the QIAprep Spin Miniprep kit (Qiagen, Germany), except that column isolation was replaced by precipitation of the plasmid DNA with isopropanol. All constructs were checked by sequencing and restriction digestion before further use.

2.5. RNA and DNA extraction and cDNA synthesis

RNA and DNA from fungal material were extracted using the RNeasy and DNeasy Plant Mini Kit (Qiagen, Germany) following manufacturer's instructions. cDNA synthesis was performed following the method described in the handbook for the First Strand cDNA Synthesis Kit (Thermo Scientific, Germany) using random hexamer primers.

2.6. Gene expression

GiFRD expression was studied by RT-PCR on previously prepared cDNA, isolated from *G. intraradices*. PCR primers used in this study are listed in Table 2 and were designed to amplify gene fragments of the following sizes: 735 bp for *GiFRD*, 575 bp for *β-tubulin*, and 326 bp for *TEF*. 1 μg of extracted cDNA was used as a template in a 25 μl final reaction volume. The amplification was done using the following cycling conditions: 5 min at 94 °C, followed by 25 cycles of 30 s at 94 °C, 30 s at T_m °C, 1 min at 72 °C, and a final extension phase of 5 min at 72 °C. T_m was dependent on the pair of primers in use. All PCR amplifications were performed with Ampli*Taq* DNA polymerase (Roche, USA).

2.7. DNA sequencing

Sequencing was performed using an automatic laser fluorescence DNA sequencer (Pharmacia, Sweden). The nucleotide sequence reported in this paper has been entered into the GenBank/EMBL data libraries (JN835295).

Computer database comparisons were performed using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990). An amino acid sequence comparison was made using the ClustalW software (www.ebi.ac.uk/ClustalW).

2.8. Heterologous expression assay in yeast

The *S. cerevisiae* S0010 (*Δosm1 Δfrds1*) mutant was transformed with the constructed pYES2-GiFRD, pYES2-GFP, pYES2-GiFRD::GFP construct or with the empty vector (negative control) according to Rösel and Kunze (1998). Yeast positive transformants were selected by prototrophy for uracil in SD medium with 1% glucose as a carbon source. Additionally, unmodified pYES2 vector was transformed into Y15271 strain (Table 1) and used as a positive control.

2.9. Enzymatic assay

Permeabilized cells, prepared by the procedure described by Miozzari et al. (1978), were used for the detection of intracellular fumarate reductase activities in *S. cerevisiae* S288C, S0010/pYES2, Y15271/pYES2 and S0010/pYES2-GiFRD transformants. Cells were grown at 30 °C in 2% galactose SD medium under anaerobic conditions, harvested and then permeabilized. The concentration

of permeabilized cells was determined from the OD_{600} value. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

The activity of fumarate reductase was assayed according to Camarasa et al. (2007). The assay mixture contained 0.1 M potassium phosphate buffer, pH 7.5, 0.4 mM FMN and 10 mM fumarate. FMN was completely reduced by the addition of sodium hydrosulfite (70 mM) before addition of the cell extracts. The activity was measured spectrophotometrically at 445 nm after 20 min incubation at 30 °C.

The fumarate reductase activity in fungal material was measured in a similar way.

2.10. Immunoblot analysis

Protein samples were boiled in sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer, separated on a 10% SDS–Page gel and blotted to a polyvinylidene difluoride (PVDF, Roth) membrane (Kunze et al., 1998). The membrane was incubated for one hour in blocking solution (1× PBS, 0.05% Tween 20, 1% milk powder) and, after washing, was incubated for one hour at room temperature in 1:2000 dilution of an antibody raised against GFP protein in rabbits (Invitrogen, USA). After washing away the incubation mixture, the bound antibodies were reacted for one hour with alkaline phosphatase goat anti-rabbit conjugated immunoglobulin G (IgG) (1:3000 dilution, Promega, Germany) and detected by incubation with Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega, Germany).

2.11. Fluorescence microscopy

S. cerevisiae S0010 mutants, transformed with pYES2-GiFRD::GFP or with pYES2-GFP vector were pre-cultured in 1% glucose SD media for 24 h and used to inoculate 5 ml of 2% galactose SD medium to a density of 0.1 OD_{600}. The cells were incubated for 24 h at 30 °C. Cells from both transformants were examined with Zeiss Axioskop fluorescence microscope equipped with Zeiss AxioCam HRc camera system.

3. Results

3.1. Isolation and characterization of GiFRD

A 743 bp cDNA fragment corresponding to a gene with homology to the fumarate reductase family members was identified in a *G. intraradices* cDNA library. The full-length cDNA, named *GiFRD* was obtained by the Genome Walking method. *GiFRD* is 1536 bp long and codes for a protein with 512 amino acid residues with theoretical molecular weight of 55.1 kDa. The genomic sequence was obtained by PCR amplification on *G. intraradices* genomic DNA using primers based on the first and last 20 nucleotides of the *GiFRD* coding sequence. The genomic *GiFRD* sequence was identical to the cDNA sequence, indicating that the open reading frame of *GiFRD* consists of only one exon and no introns.

The predicted protein sequence of Gifrdp has a similarity to the fumarate reductase of the yeast *Arxula adeninivorans* Afrd1p of 45%

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S. cerevisiae_Osm1      MIRSVRRVFIYVSIFVLIIVLKRTLSGTDQTSMKQPVVVIGSGLAGLTTSNRLISKY-RI 59
S. cerevisiae_Frds1     ----------------------MSLS---------PVVVIGTGLAGLAAANELVNKY-NI 28
A. adeninivorans        --------------------MPHTEN----------VIVVGAGLAGLSAAHQILQKSPKI 30
G. irregulare_AMykor    -----------------MAS--------------QIIVVGGGLSGLSAAHTVLEHG--A 26
                                                             ::*:* **:**:::: ::.:
S. cerevisiae_Osm1      PVVLLDKAASIGGNSIKASSGINGAHTDTQQNLKVMDTPELFLKDTLHSAKGRGVPSLMD 119
S. cerevisiae_Frds1     PVTILEKASSIGGNSIKASSGINGACTETQRHFHIEDSPRLFEDDTIKSAKGKGVQELMA 88
A. adeninivorans        KVFVLEKMKSTGGNSIKASSGINGAGTPQQERANIHDSPSLFYEDTRKSAKQLGDESLAQ 90
G. irregulare_AMykor    NVLVIDKNSFFGGNSTKATSGINGALTKTQIALGIKDSAALFQEDTTRSARDLARPDLIK 86
                         * :::*    **** **:****** *  *    : *:. ** .** :**:  .  .*
S. cerevisiae_Osm1      KLTKESKSAIRWLQTEFDLKLDLLAQLGGHSVPRTHRSSGKLPPGFEIVQALSKKLKDIS 179
S. cerevisiae_Frds1     KLANDSPLAIEWLKNEFDLKLDLLAQLGGHSVARTHRSSGKLPPGFEIVSALSNNLKKLA 148
A. adeninivorans        TLSERSANAVKWLEQSFGIDLSVVSMLGGHSVARTHRGGGKIPPGFAIMKALGDKLGTYA 150
G. irregulare_AMykor    VLAGNSASAVEWLQDKFNLDLSLVSRLGGHSQPRTHRGK-EMFPGMTITYALMERLEDIA 145
                          *:  *  *:.**: .*.:.*.::: ***** .****.  :: **: *  ** ..*   :
S. cerevisiae_Osm1      SKDSNLVQIMLNSEVVDIELDNQGHVTGVVYMDENGNRKIMKSHHVVFCSGGFG---YSK 236
S. cerevisiae_Frds1     ETKPELVKINLDSKVVDIHEKD-GSISAVVYEDKNGEKHMVSANDVVFCSGGFG---FSK 204
A. adeninivorans        NCQLITEAKVLNLITKEHAGPIGKKVIGVNYLKDNKEESLFG--NVILATGGFS---ASD 205
G. irregulare_AMykor    ENQPNRARIIKKARVTNLIKEG-EEVIGVEYEKDGQTLKEYG--PVILATGGYAADFTEN 202
                        . .       .  . :       : .* * ...          *::.:**:.    ..
S. cerevisiae_Osm1      EMLKEYSPNLIHLPTTNGKQTTGDGQKILSKLGAELIDMDQVQVHPTGFIDPNDRENNWK 296
S. cerevisiae_Frds1     EMLKEYAPELVNLPTTNGQQTTGDGQRLLQKLGADLIDMDQIQVHPTGFIDPNDRSSSWK 264
A. adeninivorans        QLVSKYRPDLVGLPTTNSEGTLGEGLEMCQAVNAELIDADQVQVHPTGFVDPKDPASQSK 265
G. irregulare_AMykor    SLLKKYRPDIYDLPTTNGDHCTGDGHKMVLAIGGKAIDLEKVQVHPTGLVDPKDPDSKIK 262
                        .::.:* *::  *****..   *:* .:   :... ** :::******::**:*  .. *
S. cerevisiae_Osm1      FLAAEALRGLGGILLHPTTGRRFTNELSTRDTVTMEIQSKCPKNDNR--ALLVMSDKVYE 354
S. cerevisiae_Frds1     FLAAESLRGLGGILLNPITGRRFVNELTTRDVVTAAIQKVCPQEDNR--ALLVMGEKMYT 322
A. adeninivorans        FLAGEALRREGGILL--IGGKRFTDELQTRDFVTAKVLESCKRANVSPGSVIALNPAGYD 323
G. irregulare_AMykor    FLAAEALRGVGGLLIN-AEGKRFCDELGHRDYVTGEIWKT--KGPVR----LVLNSKASK 315
                        ***.*:**  **:*:    *:** :**  ** **  : .   :        :.:.
S. cerevisiae_Osm1      NYTNNINFYMSKNLIKKVSIN-DLIRQYDLQTTASELVTELKSYSDVNTK-DTFDRPLII 412
S. cerevisiae_Frds1     DLKNNLDFYMFKKLVQKLTLS-QVVSEYNLPITVAQLCEELQTYSSFTTKADPLGRTVIL 381
A. adeninivorans        KIKHHVDFYVFKGLMKKGTLT-EMCTELNWDVDLVRAEFESYNKVVEGQEADSKGRNILV 382
G. irregulare_AMykor    EIEWHCKHYAGRGLMKKINSGEELAKEIGVSVAQLKATFDEYNDIASGKKKDPYGKKFFQ 375
                        .   : ..*  : *::* .   ::  : .      .   :  .      : *. .: .:
S. cerevisiae_Osm1      NAFDKDISTESTVYVGEVTPVVHFTMGGVKINEKSQVIKKNSESVLSNGIFAAGEVSGGV 472
S. cerevisiae_Frds1     NEFGSDVTPETVVFIGEVTPVVHFTMGGARINVKAQVIGKNDERLLK-GLYAAGEVSGGV 440
A. adeninivorans        LAS-SQPANDGELFWGLTTPVLHFTMGGVHINTSAQVLGAGGNAIEG--LYAAGEVSGGV 439
G. irregulare_AMykor    NAP---MSINDNFHVSLMSPVLHYTMGGVEVTPDSEVKDVQGKTIPG--LYASGEIAGGV 430
                              : :  .. .  :**:*:****..:. .::*    .: :    ::*:**::***
S. cerevisiae_Osm1      HGANRLGGSSLLECVVFGKTAADNIAKLY------------------------------ 501
S. cerevisiae_Frds1     HGANRLGGSSLLECVVFGRTAAESIANDRK----------------------------- 470
A. adeninivorans        HGANRLGGSSLLECVVFGRIAADSSTGAGSSL--------------------------- 471
G. irregulare_AMykor    HGANRLGGSSLLGCVVFGRVAGDSASRHLLQNLSNATATRRLGQIAGQLAPYQATVNVDP 490
                        ************ *****: *.:. :
S. cerevisiae_Osm1      ----------------------
S. cerevisiae_Frds1     ----------------------
A. adeninivorans        ----------------------
G. irregulare_AMykor    TNQKVHLEIYWGQQGGISVTK 511
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Fig. 1. Amino acid sequence alignment of Gifrdp with fumarate reductase protein sequences from other organisms. The alignment was performed using ClustalW software (www.ebi.ac.uk/ClustalW). Identical residues in all sequences are marked with an asterix. The GenBank accession numbers of FR proteins are as followed: Gifrdp (JN835295), *S. cerevisiae* Osm1p (NP_012585.1), *S. cerevisiae* Frds1p (P32641.1), *A. adeninivorans* Afrd1p (JN835294).

and to both FR genes of *S. cerevisiae* (Osm1p and Frds1p) of 38%. Alignment of the fumarate reductase protein sequences from those organisms showed a low degree of conserved domains along the entire length of the proteins (Fig. 1). Further examinations (InterProScan, EMBL-EBI; Marchler-Bauer et al., 2011) showed that the Gifrdp sequence contains the consensus motif which is proposed to be FAD/NAD(P) binding domain (SSF51905) at amino acids position 1–461 (1–1383 bp) which is typical for all the known fumarate reductases of yeast and fungi. Additionally, a domain of the redox protein, flavocytochrome c (IPR010960), was present at amino acid position 4–453 (12–1359 bp) (InterProScan, EMBL-EBI).

3.2. Transcript analysis

GiFRD RNA accumulation was studied by RT-PCR with the gene specific primers, RT_GiFRD_fwd and RT_GiFRD_rev (Table 2). The transcript analysis was established in three AMF developmental stages: asymbiotic ASY (spores from *in vitro* culture cultivated for 14 days in the absence of roots), presymbiotic Pre-SY (spores from *in vitro* culture cultivated for 14 days in the presence of root exudates in medium but without physical contact with roots) and symbiotic SY (secondary spores with extra- and intraradical mycelium and roots from an *in vitro* culture).

The expression of *GiFRD* was detected only in the asymbiotic stage, ASY (Fig. 2). Expression of the fumarate reductase gene was not observed in stages with roots or root exudates present. The expression of two housekeeping genes encoding β-tubulin (Olsson et al., 2005) and TEF (Gianinazzi-Pearson et al., 2009), was used as a control for cDNA synthesis. The expression level was similar in all stages and no expression was observed in control sample with *D. carota* cDNA only.

3.3. Role of Gifrdp during anaerobiosis

To assess whether *GiFRD* encodes a functional enzyme and to verify the physiological role of the *G. intraradices* fumarate reductase, a *S. cerevisiae* assay was used. A double fumarate reductase mutant strain (S0010) was constructed by mating single knockout mutants of *S. cerevisiae* fumarate reductase genes (*OSM1* and *FRDS1*). The *S. cerevisiae* S0010 double mutant was transformed with the basic vector pYES2 (S0010/pYES2) and the *GiFRD* expression vector pYES2-GiFRD (S0010/pYES2-GiFRD). Strain Y15271, which contains both *S. cerevisiae* fumarate reductase genes, was transformed with an empty pYES2 vector, and used as a positive control for all experiments. All selected strains were pre-cultured for 48 h at 30 °C, with or without shaking, to obtain aerobic and anaerobic conditions respectively. Cultures were inoculated with

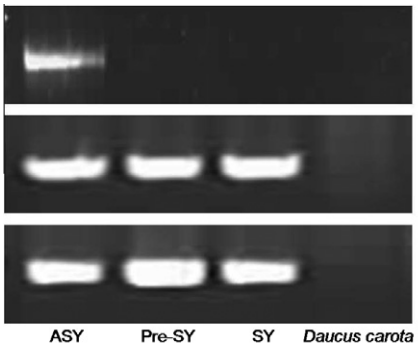

Fig. 2. *GiFRD* expression shown by RT-PCR in three developmental stages of *G. intraradices* (ASY-spores from *in vitro* culture cultivated for 14 days without root presence, Pre-SY-spores cultivated for 14 days with root presence but with no physical contact, SY-spores with extraradical hyphae and roots). The expression of two housekeeping genes, encoding β-tubulin and TEF, was used as a control to monitor cDNA synthesis.

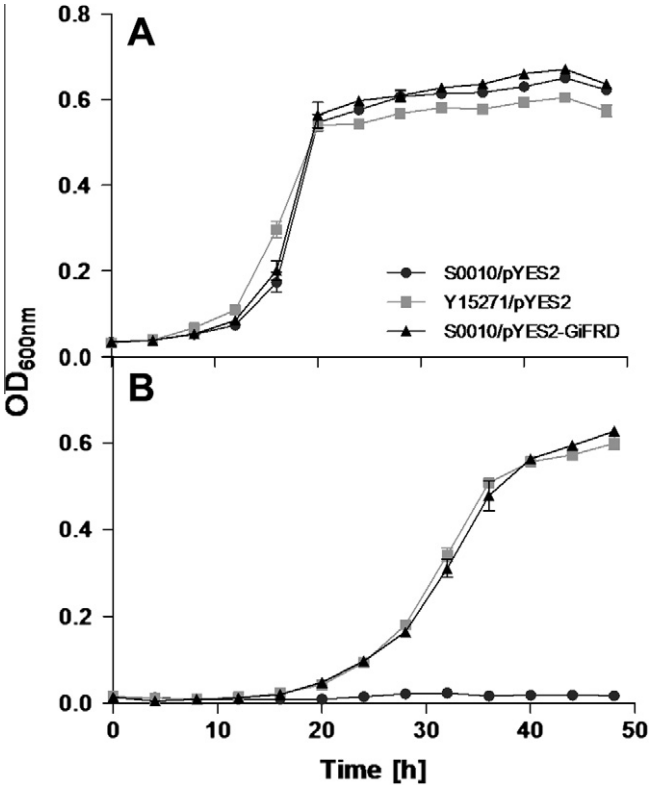

Fig. 3. Contribution of *G. intraradices* fumarate reductase to yeast aerobic and anaerobic growth. The *S. cerevisiae* Y15271/pYES2 control strain (■), the S0010/pYES2-GiFRD transformant (▲) and the S0010/pYES2 mutant (●) were grown in 2% galactose SD medium under aerobic (A) and anaerobic (B) conditions. Standard deviations were calculated from three independent experiments.

cells from the pre-cultures, and incubated in corresponding conditions and grown for analysis.

Wild type-like behavior was observed when all transformants were grown under aerobic conditions (Fig. 3A). However, the S0010 mutant (*Δosm1 Δfrds1*) was unable to grow under anaerobic conditions in 2% galactose SD medium, which is in agreement with previous reports (Camarasa et al., 2007; Enomoto et al., 2002). The transformant S0010/pYES2-GiFRD was able to grow under anaerobic conditions as well as the control strain Y15271/pYES2 (Fig. 3B). Thus, it was demonstrated that functional substitution of *S. cerevisiae* enzymes by *GiFRD* in yeast cells does occur.

3.4. Enzymatic activity

Enzymatic activity in *S. cerevisiae GiFRD* transformants was measured under semi-anaerobic conditions using aerobically grown cells. Measurement of the *GiFRD* transformants showed reduced fumarate reductase activity compared to control strains, which confirms the functional substitution of *S. cerevisiae* genes with the *G. intraradices* gene (Table 3). The pH optimum of the Gifrdp enzyme reaction was 7.2–8.0 and the Michaelis constant (km) measured with fumarate as substrate was 1.1 mM.

The fumarate reductase activity was also analyzed in fungal material, in three AMF developmental stages. Permeabilized cells were incubated for 20 min and the enzyme activity was measured as the rate of oxidation of $FMNH_2$. Only in the asymbiotic stage fumarate reductase activity was detected in range of 24 nmol of oxidized $FMNH_2$ per mg of wet weight.

3.5. Localization of Gifrdp

The localization of fumarate reductase in yeast has been reported to be in the cytoplasm and mitochondria (Besteiro et al.,

Table 3
Fumarate reductase activities in control, mutant and transformants of *S. cerevisiae* strains and in three AMF developmental stages.

S. cerevisiae	Fumarate reductase activity ($OD_{445/600}$)
S288C	0.34
Y15271/pYES2	0.35
S0010/pYES2	0.0
S0010/pYES2-GiFRD	0.13

Permeabilized cells from semi-anaerobic cultures were incubated for 20 min at 30 °C and the enzyme activity was measured as $OD_{445/600}$. Data is given as the mean of three experiments, and the standard error of the mean is less than 20%.

G. intraradices stage	Fumarate reductase activity (nmol/mg of wet weight)
ASY	24.0
Pre-SY	0.0
SY	0.0

Permeabilized cells from AMF material were incubated for 20 min at 30 °C and the enzyme activity was measured as amount of oxidized $FMNH_2$ per mg of wet weight. Data is given as the mean of three experiments.

2002; Enomoto et al., 1996; Muratsubaki and Enomoto, 1998). Sequence analysis of Gifrdp detected no transmembrane domains in the protein sequence (TMHMM Server v.2.0 provided by the Centre for Biological Sequence Analysis at the Technical University of Denmark). SOSUI sequence analysis (Hirokawa et al., 1998) indicates that Gifrdp is a soluble protein.

Prediction of subcellular localization, using the fuzzy *k*-NN method, showed a 47.8% possibility that Gifrdp is a cytoplasmic protein (Huang and Li, 2004). To confirm that prediction, we fused *GiFRD* genomic sequence with *GFP* gene at its C terminus in pYES2 vector. We expressed GFP-tagged Gifrdp in *S. cerevisiae* S0010 mutant (*Δosm1 Δfrds1*). The yeast cells expressing Gifrd-GFP fused protein and GFP protein alone showed general cytoplasmic fluorescence, which indicated that Gifrdp was targeted to the cytoplasm in *S. cerevisiae* cells (Fig. 4).

To confirm presence of GFP protein in the transformants, western blotting was performed on a total protein extract of transformant yeast cells using a specific antibody raised against GFP. Predicted sizes of proteins are ~82 kDa for Gifrd-GFP and ~28 kDa for GFP. Bands of expected size were observed in all GFP transformants (Fig. 5).

3.6. Influence of fumarate reductase activity on cell growth under osmotic stress

There are contradictory reports about possible regulation of fumarate reductase expression by osmotic stress. It was reported that deletion of *OSM1* increases the sensitivity of *S. cerevisiae* cells to hypertonic media (Singh and Shermann, 1978). In contrast to those results, Camarasa et al. (2007) showed that expression of the *OSM1* gene is not controlled by osmotic stress or by oxygen availability. In an attempt to clarify, an experiment was performed to determine whether fumarate reductase presence or absence increases osmosensitivity of *S. cerevisiae* cells. Additionally, AMF are often used for increasing the plant growth on dry and salty soils. The experiments were performed to verify if *GiFRD* gene product might be involved in the AMF adaptation mechanism under salt stress.

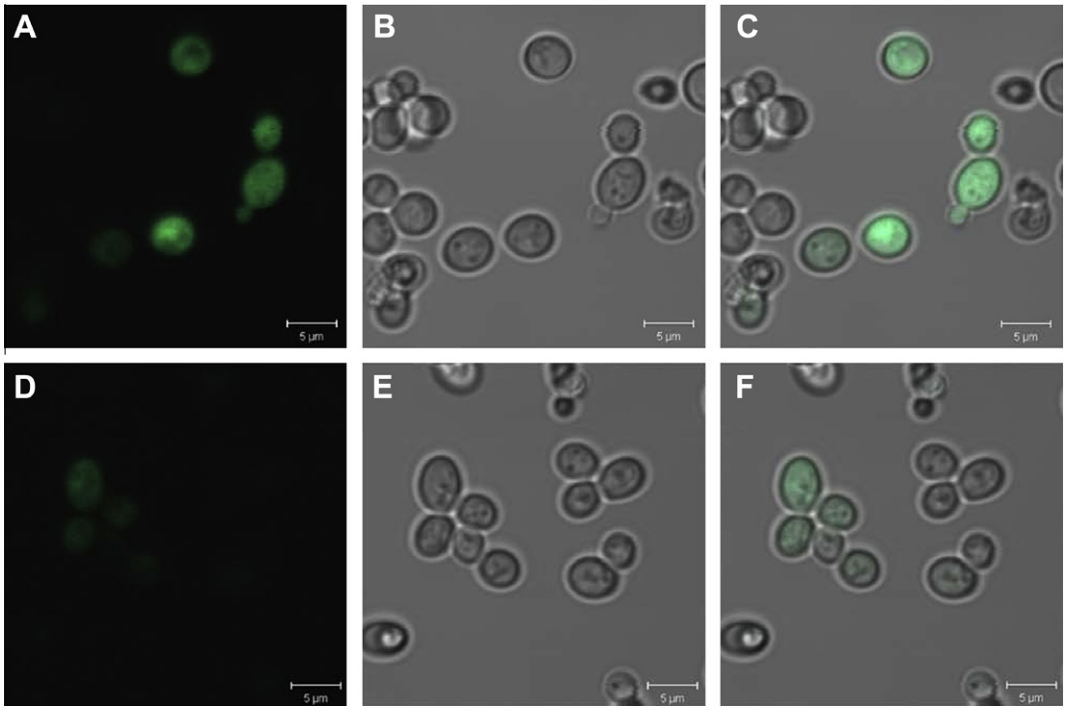

Fig. 4. Subcellular localization of Gifrdp. The *S. cerevisiae* S0010 mutant (*Δosm1 Δfrds1*) transformed with pYES2-GiFRD::GFP (Clon 7) (A–C) or with pYES2-GFP (D–F) were visualized by epifluorescence microscopy (A and D), bright field (B and E) and merged image (C and F). The Gifrdp was targeted to the cytoplasm in *S. cerevisiae* cells.

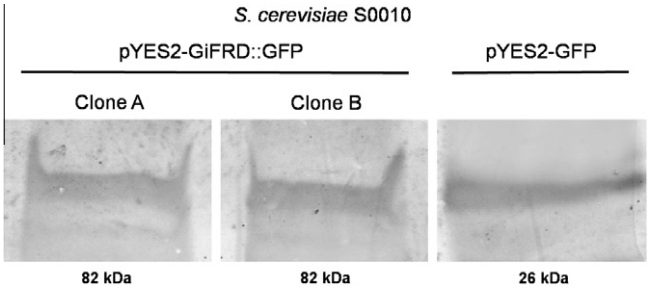

Fig. 5. Immunodetection of GFP protein and fused Gifrd-GFP in *S. cerevisiae* S0010 (*Δosm1 Δfrds1*) mutant transformed with pYES2-GiFRD::GFP or with pYES2-GFP. Predicted sizes of target proteins are ~82 kDa for Gifrd-GFP and ~28 kDa for GFP. Total protein of yeast cells was separated by 10% SDS–Page gel electrophoresis, blotted to PVDF membrane and reacted to rabbit anti-GFP.

The growth of the *S. cerevisiae* S0010 mutant with empty pYES2 vector (negative control), and with a vector containing *GiFRD* and *S. cerevisiae* Y15271 mutant with empty pYES2 vector (positive control) was monitored in presence of different concentrations of sodium chloride (0–8%) on selective 2% galactose SD medium plates. After 4 days of incubation at 30 °C, all strains were able to grow up to 7% NaCl with the growth rate correlating with the NaCl concentration. Increasing the NaCl concentration to 8% NaCl however caused a cessation of growth (Fig. 6). Also, no difference was observed in the growth of the negative (S0010/pYES2) and positive (Y15271/pYES2) controls and the *GiFRD* transformant i.e. the transformant expressing Gifrdp did not have greater sensitivity to a hypertonic medium than the negative control (empty pYES2).

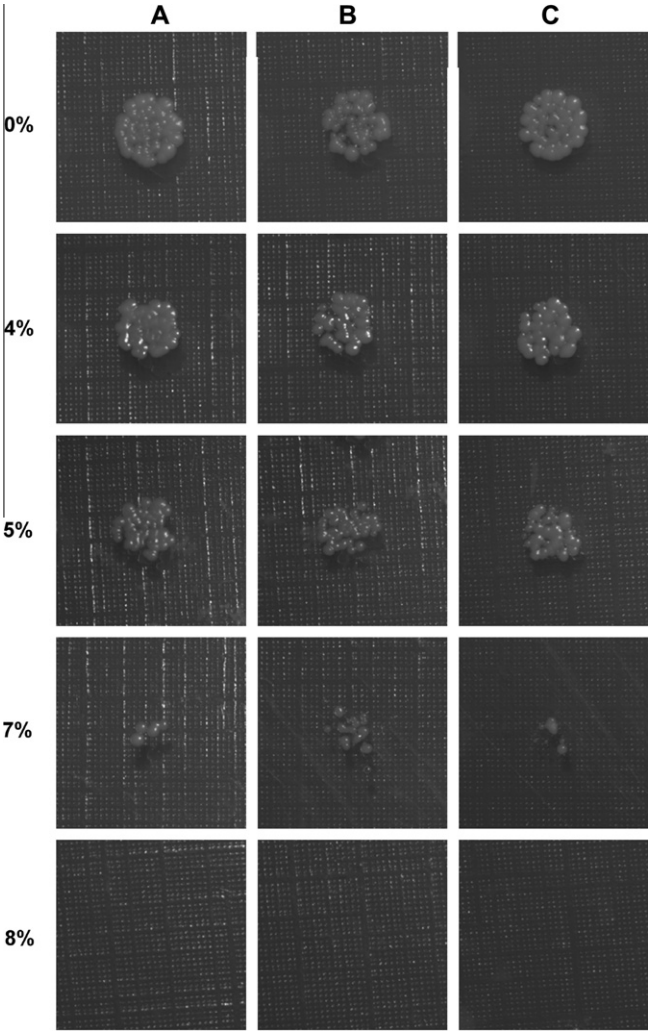

Fig. 6. Influence of Gifrdp on the osmotolerance in *S. cerevisiae*. Aliquots (ca. 10^3 cells) of *S. cerevisiae* S0010/pYES2 (A), Y15271/pYES2 (B) and S0010/pYES2-GiFRD (C) grown on 2% galactose SD agar plates supplemented with the indicated NaCl concentrations and cultured for 4 days at 30 °C. Pictures were taken on a gray checkered background at the end of the growth period.

4. Discussion

Growth in the absence of oxygen requires adaptation of the cell. This appears to be mainly at the regulatory level, in particular, gene transcription. Of the more than 500 genes that are up- or downregulated under anaerobic conditions in *S. cerevisiae*, only 23 are considered essential in oxygen deficiency (Snoek and de Steensma, 2007).

One of these genes encodes a fumarate reductase enzyme which irreversibly catalyzes the reduction of fumarate to succinate with the concomitant oxidation of the cofactors involved in glycolysis. It has been shown that in *S. cerevisiae* cells, this enzyme is encoded by two genes *FRDS1* and *OSM1*, and that it is essential for the reoxidation of $FADH_2$ during anaerobiosis. Several publications show that anaerobic growth inhibition is caused by deficiency in the regeneration of the intracellular pool of FAD^+, rather than an inability to regenerate NAD^+ (Arikawa et al., 1998; Camarasa et al., 2007; Enomoto et al., 2002).

Other ways of maintaining the redox balance during oxygen deficiency in yeast is via the action of Gpd3, a glycerol-3-phospate dehydrogenase, and Adh2, a mitochondrial alcohol dehydrogenase (Snoek and de Steensma, 2007). Both of these enzymes, together with fumarate reductase, may function cooperatively to maintain the redox conditions during hypoxia or anoxia.

For AMF, Miller and Bever (1999) suggested a mechanism by which the fungus could be concentrated near the plant root, obtaining oxygen directly from the root or as oxygen diffuses from the root into the rhizosphere. Brown and Bledsoe (1996) observed AMF in the plant aerenchyma, tissue with large intercellular spaces, the formation of which is associated with plant adaptation to hypoxia. This suggests that AMF are adapted to life in oxygen-deficient soil during symbiotic stages of their life cycle. However, it is not known how hypoxic conditions are managed during the asymbiotic stage without presence of plant roots. Additionally, none of the genes that have been characterized in other organisms and demonstrated to be involved in redox balance preservation during absence of oxygen, have yet been identified in AMF.

This work characterizes a new fumarate reductase gene (*GiFRD*) from an AMF *G. intraradices*. The sequence analysis demonstrates the presence of a gene encoding a fumarate reductase protein with high homology to both of the *S. cerevisiae* isoenzymes (38%). Based on amino acid identity, cytoplasmic localization and biochemical characteristics, the *G. intraradices* fumarate reductase (Gifrdp) was classified as an equivalent of *S. cerevisiae's* Frds1p, the most abundant isoform. This indicates that Gifrdp belongs to the second class of fumarate reductases, which regenerate the reduced cofactors independently from the electron transport chain.

Using a yeast expression system, it was demonstrated that the protein encoded by *GiFRD* has fumarate reductase activity. Detection of enzymatic activity in *S. cerevisiae Δosm1 Δfrds1* mutant expressing the *GiFRD* gene confirmed that *GiFRD* encodes an enzyme able to functionally substitute for the *S. cerevisiae* fumarate reductases.

We have shown that the *S. cerevisiae Δosm1 Δfrds1* mutant is not able to grow under anaerobic conditions, which was expected because fumarate reductase is essential for maintaining an intra-

cellular redox balance during oxygen deficiency. The presence of *GiFRD* gene however restored the ability of the *Δosm1 Δfrds1* mutant to grow under oxygen deficiency conditions, confirming that the *G. intraradices* fumarate reductase is an active enzyme capable of functional complementation of the missing *S. cerevisiae* genes. However the lack of *S. cerevisiae* fumarate reductase genes or their complementation with the *G. intraradices* gene was not shown to have any influence on growth in aerobic conditions indicating that the fumarate reductase enzyme is only essential in anaerobic conditions.

Additionally, it was shown that *GiFRD* transformants were not affected by presence of salt in the medium because the *S. cerevisiae* negative (S0010/pYES2) and positive (Y15271/pYES2) controls and the *GiFRD* transformant showed no difference in their growth in hypertonic media. This indicates that the presence of fumarate reductase enzyme is not involved in yeast growth under osmotic stress.

Transcription analysis in the developmental stages of the fungus and the presence of enzymatic activity in *S. cerevisiae Δosm1 Δfrds1* mutant expressing *GiFRD* gene, confirms the existence in *G. intraradices* of a functional fumarate reductase which reoxidises cellular $FMNH_2/FADH_2$ generated by anaerobic metabolism. Analysis of *GiFRD* expression and fumarate reductase activity in the life cycle revealed that it was only expressed in the asymbiotic phase of *G. intraradices* life cycle, where spores exist without physical contact with roots and roots exudates.

It has been suggested that root exudates influence fungal respiratory activity (Tamasloukht et al., 2003; Besserer et al., 2006) and O_2 consumption clearly showed higher fungal respiratory activity after the addition of root exudates. The gene encoding pyruvate carboxylase showed 10-fold RNA levels after addition of the root exudates. This could be caused by the need to more effectively incorporate additional carbon sources obtained from the plant into oxaloacetate, which is required for a range of processes important to maintain fungal growth, such as gluconeogenesis. Tamasloukht et al. (2003) showed that during asymbiotic growth, AMF spores demonstrate a low respiratory activity. Probably, they are using an alternative electron transport pathway to minimize carbon consumption from their own resources such as glycogen and lipids.

The fact that *GiFRD* expression was present only in the asymbiotic stage (Fig. 2) demonstrates existence of at least one metabolic pathway involved in anaerobic metabolism in the only plant-independent phase of fungus life. Further, the absence of *GiFRD* expression in the presymbiotic stage indicates that the switch from the asymbiotic to the symbiotic phase coincides with the change from anaerobic to aerobic metabolism. This suggests that the AMF behave as facultative anaerobes in the asymbiotic stage.

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