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Shahi, S.; Fokkens, L.; Houterman, P.M.; Rep, M.

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Suppressor of fusion, a *Fusarium oxysporum* homolog of Ndt80, is required for nutrient-dependent regulation of anastomosis

Shermineh Shahi, Like Fokkens, Petra M. Houterman, Martijn Rep *

Molecular Plant Pathology, University of Amsterdam, Amsterdam, The Netherlands

ABSTRACT

Heterokaryon formation is an essential step in asexual recombination in *Fusarium oxysporum*. Filamentous fungi have an elaborate nonself recognition machinery to prevent formation and proliferation of heterokaryotic cells, called heterokaryon incompatibility (HI). In *F. oxysporum* the regulation of this machinery is not well understood. In *Neurospora crassa*, Vib-1, a putative transcription factor of the p53-like Ndt80 family of transcription factors, has been identified as global regulator of HI. In this study we investigated the role of the *F. oxysporum* homolog of Vib-1, called Suf, in vegetative hyphal and conidial anastomosis tube (CAT) fusion and HI. We identified a novel function for an Ndt80 homolog as a nutrient-dependent regulator of anastomosis. Strains carrying the *SUF* deletion mutation display a hyper-fusion phenotype during vegetative growth as well as germling development. In addition, conidial paring of incompatible *SUF* deletion strains led to more heterokaryon formation, which is independent of suppression of HI. Our data provides further proof for the divergence in the functions of different members Ndt80 family. We propose that Ndt80 homologs mediate responses to nutrient quality and quantity, with specific responses varying between species.

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1. Introduction

Filamentous fungi grow by hyphal tip expansion and branching. Hyphae at the growing edge of the colony display a growth pattern described as avoidance, in which hyphal tips show negative auto-tropism towards each other (Prosser, 1995). Behind the growth front ongoing fusions, or anastomoses, between hyphae build a three dimensional network, the mycelium (Glass et al., 2000). In any given habitat different individuals not only of the same species but also of different species meet and can undergo vegetative hyphal fusion to form heterokaryons (Glass et al., 2000; Saupe, 2000).

In heterokaryotic cells one or more genetically distinct nuclei from each individual share a common cytoplasm (Glass et al., 2000). Although there are potential benefits to heterokaryon formation, such as functional polyploidy and mitotic genetic recombination, filamentous ascomycetes display an elaborate nonself recognition system, by which cells with genetically distinct nuclei are compartmentalized and subject to programmed cell death, a process referred to as heterokaryon incompatibility (HI) (Glass and Dementhon, 2006; Glass and Kaneko, 2003; Pontecorvo, 1956). It has been proposed that HI plays a role in restricting the

transmission of pathogenic elements such as double-stranded RNAs and the exploitation by aggressive genotypes (Cortesi et al., 2001; Debets and Griffiths, 1998).

Nonself recognition during HI is genetically regulated by allelic specification at *het* (for heterokaryon) loci and individuals that differ at one or more *het* loci are incompatible with each other (Glass et al., 2000; Saupe, 2000). In *Neurospora crassa* 11 unlinked *het* loci have been described to be involved in nonself recognition and HI, demonstrating the elaborate character of this immune system (Dementhon et al., 2006; Glass et al., 2000). The *N. crassa* HET-c/ PIN-c system has been studied as a model and it has been shown that incompatible interactions at these loci lead to severe growth reduction, decreased conidiation, and programmed cell death of the fusion cell and surrounding cells (Glass and Dementhon, 2006; Glass and Kaneko, 2003; Glass et al., 2000). *het* loci have been shown to encode a variety of products, however the HET domain (Pfam PF06985) is conserved among proteins involved in HI in *N. crassa* and *Podospora anserina* (Espagne et al., 2002; Smith et al., 2000). Predicted HET domain genes are common in, and specific to, filamentous ascomycete and basidiomycete genomes (Fedorova et al., 2005; Van der Nest et al., 2014).

In the past decade, extensive studies have been carried out in *N. crassa* to elucidate molecular mechanisms and genetic regulation of HI. Vib-1 was first identified as a regulator for *HET-c* mediated HI. In incompatible interactions between strains carrying the

* Corresponding author.
E-mail address: m.rep@uva.nl (M. Rep).

VIB-1 deletion the phenotype associated with *HET-c/PIN-c* HI is suppressed (Xiang and Glass, 2002). Further investigation revealed that Vib-1 is required for the expression of genes involved in HI, and these results collectively lead to the conclusion that Vib-1 is a global mediator of HI in *Neurospora* (Dementhon et al., 2006; Xiang and Glass, 2002). Furthermore it was established that Vib-1, like its *A. nidulans* homolog XprG, is a positive regulator of extracellular protease production under carbon and nitrogen starvation (Dementhon et al., 2006; Katz et al., 2006). *VIB-1* encodes a putative transcription factor of the *p53*-like Ndt80/PhoG DNA binding family. It was shown that the Ndt80/PhoG DNA binding domain (Pfam PF05224) is conserved among ascomycete fungi (Dementhon et al., 2006; Hutchison and Glass, 2010; Katz et al., 2013; Lamoureux et al., 2002; Montano et al., 2002; Sopko and Stuart, 2004).

The filamentous ascomycete *Fusarium oxysporum* is an important plant pathogen and has been studied as a model organism for plant microbe interactions (for reviews see (de Sain and Rep, 2015; Takken and Rep, 2010). Although no apparent sexual recombination is known, the genomes of *F. oxysporum* species show a high degree of genetic variability (Rep and Kistler, 2010). Additional comparative genome studies have demonstrated that *Fusarium* species share a conserved core genome, however, *F. oxysporum* and *F. solani* also carry lineage-specific chromosomes (Coleman et al., 2009; Ma et al., 2010). In the case of *F. oxysporum* it was demonstrated that small lineage specific chromosomes harboring essential virulence genes, also known as pathogenicity chromosomes, can be horizontally transferred to a non-pathogenic strain, and thereby conferring pathogenicity towards a specific host (Ma et al., 2010). We have recently described a mechanism in *F. oxysporum* that is likely to enable horizontal chromosome transfer under nutrient-limiting conditions (Shahi et al., 2016). Although this study has added to our knowledge of the mechanics of asexual recombination, the genetic regulation as well as the molecular mechanics of nonself recognition and HI in *F. oxysporum* and their suppression during CAT fusion remain unclear. Given the similarity in phenotypes it was suggested that HI might share a common machinery between different fungal systems (Glass and Kaneko, 2003). The reference *F. oxysporum* genome encodes around 80 putative HET domain proteins (http://www.fungidb.org), yet, to our knowledge, none has been characterized. In this species vegetative compatibility groups (VCG) are determined using nitrate non-utilizing (NIT) mutants (Correll et al., 1987; Puhalla, 1985).

Here we investigate the *F. oxysporum* Vib-1 homolog, encoded by *FOXG_01644* (*f. sp. lycopersici*) and *FOMG_05487* (*f. sp. melonis*), for its role in vegetative fusion and nonself recognition. We show that the *F. oxysporum* homolog of Vib-1 is a nutrient-dependent negative regulator of hyphal and CAT fusion. In addition, conidial paring of strains carrying a deletion mutant yielded higher numbers of heterokaryotic cells. However, in contrast to *N. crassa*, the deletion mutant does not overcome HI nor is secretion of proteases affected. We suggest that the increase in heterokaryon formation is a result of increased CAT fusion and propose to rename FOXG_01644/FOMG_05487 <u>su</u>ppressor of <u>f</u>usion (*SUF*).

2. Material and methods

2.1. Strains and culture conditions

Fusarium oxysporum f. sp. lycopersici strain 4287 (*Fol*4287, FGSC9935), *Fusarium oxysporum f. sp. melonis* strain 001 (*Fom*001, FGSC10441), and the non-pathogenic *Fusarium oxysporum* strain 47 (*Fo*47, FGSC10445) were used as the parental strains for fungal transformation. They were stored as a monoconidial culture at −80 °C and revitalized on potato dextrose agar (PDA, Difco) at

25 °C. *Agrobacterium tumefaciens* EHA105 (Hood et al., 1993) was used for *Agrobacterium*-mediated transformation of *F. oxysporum* and was grown in either Luria broth (LB) or 2YT medium (Sambrook and Russel, 2001) containing 20 μg/ml rifampin at 28 °C. Introduction of the plasmids into the *Agrobacterium* strain was performed as previously described (Mattanovich et al., 1989). *Escherichia coli* DH5α (Invitrogen) was used for construction, propagation, and amplification of the plasmid and was grown at 37 °C in LB medium containing 50 μg/ml kanamycin.

2.2. Construction of vectors and Agrobacterium-mediated Fusarium transformation

2.2.1. SUF deletion

To generate *SUF* (FOXG_01644) deletion (Δ*suf*) mutants, we used plasmids pRW2h-H1-GFP and pRW2p-H1-RFP (Shahi et al., 2016, 2015). These plasmids have either the hygromycin resistance cassette (hph) and histone-GFP under the control of the xylanase promoter or the phleomycin resistance cassette (tested with zeocin, ble) and histone-RFP under the control of the xylanase promoter as selection marker. We introduced an ~1 kb sequence upstream (left flank) and downstream (right flank) of *SUF* open reading frame (ORF) for homologous recombination. For this we PCR amplified the left flank with primer combination FP4278 (5′-cggaattcGCCGTGCCGTTCACTCC-3′) and FP4279 (5′-ggggtaccGCC TACGAAGTAGAAGATGAGCTT-3′) and the right flank with primer combination FP4280 (5′-tgctctagaCGATATACAAAAGTTGCCAT CAAA-3′) and FP4281 (5′-cccaagcttGAACAGATCTATTAACAAAGC CATCC-3′). The right flank was cloned in the *Xba*I site upstream the right border and the left flank in the *Pac*I site downstream the left border.

2.2.2. SUF complementation

Binary vectors pRW2h (hph) and pRW2p (ble) were used as a backbone for vector construction (Houterman et al., 2008; Shahi et al., 2016). We introduced an ~1 kb sequence upstream (left flank) plus the ORF and an ~1 kb sequence downstream (right flank) of *SUF* for homologous recombination. For this we PCR amplified the left flank plus *SUF* ORF with primer combination FP6455 (5′-ttaattaaGCCGTGCCGTTCACTC-3′) and FP6456 (5′-ttaat taaCTAGTTCCCATTCCAGGAATATTG-3′) and the right flank as described before. The right flank was cloned in the *Xba*I site upstream the right border and the left flank plus *SUF* ORF in the *Pac*I site downstream the left border. pRW2h-*SUF* (hph) was used to complement H1-RFP Δ*suf* (ble) and pRW2p-*SUF* (ble) to complement H1-GFP Δ*suf* (hph, for plasmid maps see Fig. S1).

2.2.3. Agrobacterium-mediated Fusarium transformation

The obtained plasmids H1-GFP Δ*suf* (hph), H1-RFP Δ*suf* (ble), pRW2h-*SUF* (hph) and pRW2p-*SUF* (ble) were transformed into *Agrobacterium tumefaciens* EHA105 and the transformants were used for subsequent *A. tumefaciens*-mediated *Fusarium* transformation. *Agrobacterium*-mediated transformation of *F. oxysporum* was performed as previously described (Shahi et al., 2015).

2.3. CAT and hyphal fusion assays

For CAT fusion assay, conidia of *Fol*4287 H1-RFP Δ*suf*, *Fom*001 H1-GFP Δ*suf*, and *Fo*47 H1-GFP Δ*suf* were collected from one-week-old PDA plates in 2 ml of the medium to be tested and filtered through one layer of sterile Miracloth (Calbiochem). 200 μl 7.5×10^5 conidia per ml were incubated in an 8-well microscope chamber slide (Nunc) for 15–18 h in CAT medium and observed with the AMG Evos FL digital inverted microscope. 1000–2500 conidia were counted in two biological replicates. CAT fusion

frequency was calculated as the percentage of CAT fusions per germinated conidia.

For hyphal fusion assays, *Fol*4287 H1-RFP Δ*suf*, *Fom*001 H1-GFP Δ*suf*, and *Fo*47 H1-GFP Δ*suf* were grown on PDA, CDA, or CAT medium supplemented with 1.5% agarose for one week at 25 °C. Side-to-side fusion between hyphae behind the growth front were counted per area (0.5 mm^2). 15 technical in 3 biological replicates were performed.

2.4. Co-cultivation

Conidia of *Fol*4287 wild type (wt), *Fol*4287 H1-RFP Δ*suf*, *Fom*001 wt, and *Fom*001 H1-GFP Δ*suf* were collected from one-week-old PDA plates in 2 ml water, filtered through one layer of sterile Miracloth (Calbiochem), and washed with water. To select heterokaryotic cells, 1 ml with 10^6 conidia from each parental strain were incubated in a 1-well microscope chamber slide (Nunc). After two days conidia were collected and 50 µl was plated on PDA and incubated for additional two days. Again conidia were collected and washed and 10^4 conidia per ml were incubated on PDA buffered with 0.1 M Tris (pH 8.0) and supplemented with 100 µg/ml hygromycin (Duchefa) and 100 µg/ml zeocin (Invivogen) for five days. The number of double drug-resistant colonies was counted.

2.5. Selection of Nit mutations and complementation testing

The screen was performed as previously described (Puhalla, 1985). We selected different nitrate non-utilizing (NIT) mutants for each strain (*Fol*4287 wt, *Fol*4287 H1-RFP Δ*suf*, *Fom*001 wt, and *Fom*001 H1-GFP Δ*suf*) and tested compatibility in different combinations.

2.6. Analysis of culture supernatant

Conidia of *Fol*4287 wt, *Fol*4287 H1-RFP Δ*suf*, *Fom*001 wt, and *Fom*001 H1-GFP Δ*suf* were collected from 3-day old liquid culture, filtered through one layer of sterile Miracloth (Calbiochem), and washed with water. Conidia concentration was adjusted to 10^8 conidia/ml. 20 ml NO$_3$ (0.17% YNB, 100 mM KNO$_3$, 3% sucrose) were inoculated with 5×10^7 conidia and grown at 25 °C shaking. After 2 days the mycelium was pelleted and washed with CAT medium. One pellet each was transferred to 20 ml NO$_3$, minimal medium (MM, 0.17% YNB, 100 mM KNO$_3$), CAT, and CAT +3% sucrose media, with (induction for protease activity assay) or without (for SDS-PAGE) 1% BSA and incubated for another 2 days. The culture was again filtered through one layer of sterile Miracloth and the filtrate was centrifuged. The supernatant used for further testing. The mycelial pellet was dried and the dry weight was measured. Protease activity in the culture supernatant was determined by measuring the release of the trichloroacetic acid (TCA)-soluble orange sulfanilamide component of azocasein upon proteolysis, as previously described (Dementhon et al., 2006).

Further, SDS-PAGE was conducted to visualize protein patterns of culture supernatant. For this 20 µl of culture supernatant was loaded on a 15% sodium dodecyl sulfate (SDS) polyacrylamide gel followed by protein silver staining (Shevchenko et al., 1996).

2.7. Carbon utilization assay

To analyze carbon utilization of the *Fol*4287 *SUF* deletion mutant, BIOLOG FF MicroPlates containing in each well a different carbon source were used, as described (Michielse et al., 2009).

2.8. Phylogenetic analysis

Complete proteomes of *F. oxysporum f. sp. lycopersici* 4287, *F. oxysporum f. sp. melonis* 26406, *F. graminearum* PH1, *Magnaporthe oryzae* 70-15 and *N. crassa* OR74a were obtained *via* the Broad institute and the genome of *Aspergillus nidulans* was obtained from GenBank. Proteins that have a Ndt80 domain were identified by searching all proteomes with a hmmer model for PF05224 (Finn et al., 2014) using hmmsearch from the Hmmer package (Eddy, 2009). The domain sequences were cut out using a custom python script and a multiple sequence alignment for the domain sequences was constructed using Clustal Omega with default settings (Sievers et al., 2011). The alignment was inspected but no changes were performed. The alignment was trimmed using trimAl (-strictplus) (Capella-Gutiérrez et al., 2009). Finally, PhyML (Guindon et al., 2010) with 4 substitution rate categories and estimated proportion of invariable sites and gamma distribution was used to infer the phylogeny.

3. Results

3.1. Fusarium oxysporum has four Ndt80 homologs

It was previously shown that the number of Ndt80 homologs can vary between different filamentous ascomycetes (Dementhon et al., 2006; Katz and Cooper, 2015). The reference genome of *F. oxysporum* encodes four predicted proteins with a Ndt80/PhoG binding domain that fall into three clades described earlier, a Vib-1 (vegetative incompatibility blocked, NCU03725) clade, a Fsd-1 (female sexual development, NCU09915) clade, and a NCU04729 clade (Dementhon et al., 2006). *VIB-1* and *NCU04729* each have one ortholog in *F. oxysporum*. Interestingly, even though a sexual cycle is not known for *F. oxysporum*, there is a duplication of *FSD-1* in *F. oxysporum* but not *F. graminearum* (Fig. 1). With the aim to better understand the molecular underpinnings of vegetative fusion and nonself recognition in *F. oxysporum*, we decided to investigate the role of *N. crassa* Vib-1 homolog Suf, encoded by *FOXG_01644* and *FOMG_05487*, in these processes.

3.2. SUF deletion strains display a hyper-fusion phenotype under nutrient-limiting conditions

To understand the role of *F. oxysporum* homologs of Vib-1, we first studied the phenotype of *SUF* deletion mutants. We used a construct based on the flanking regions of *F. oxysporum f. sp. lycopersici* strain 4287 (*Fol*4287) *FOXG_01644* to obtain deletion mutants in *Fol*4287, *F. oxysporum f. sp. melonis* strain 001 (*Fom*001), and *F. oxysporum* strain 47 (*Fo*47). None of the deletion mutants showed altered colony morphology or conidiation (data not shown). With around 6.5×10^6 conidia/ml the number of conidia produced after three days incubation in NO$_3$ medium was similar in all strains.

We next investigated the microscopic phenotype of the deletion mutants. For this we grew the wild type strain, two independent *SUF* deletion (Δ*suf*) mutants, and a complemented deletion strain from each background on PDA, CDA, and CAT medium supplemented with 1.5% agarose. PDA is rich in various carbon and nitrogen sources, CDA offers sucrose as the sole carbon and nitrate as the sole nitrogen source, and CAT medium offers no carbon source and limited nitrate (25 mM). Strains grown on PDA did not show any morphological differences between deletion mutant and wild type strains. However, under nutrient-limiting conditions (CDA and CAT medium), hyphae of the *SUF* deletion mutants of *Fol*4287 and *Fom*001, but not *Fo*47 *SUF* deletion strains nor the wild type, exhibited a strong increase in side-to-side fusions.
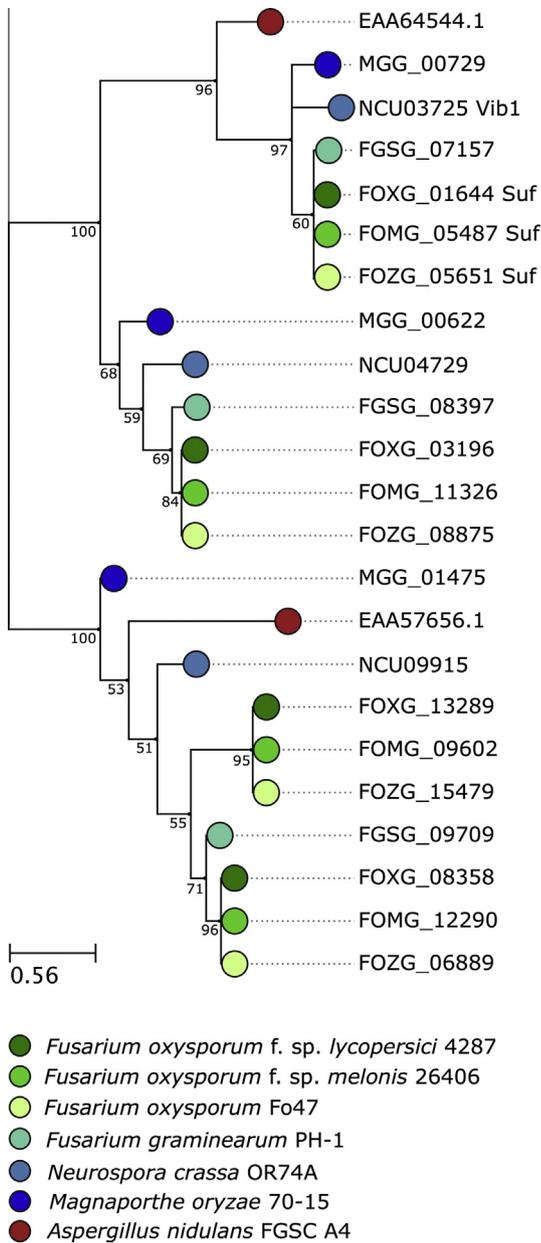
Fig. 1. Phylogenetic tree displaying the relationship of *F. oxysporum* Ndt80 homologs to homologs in several other ascomycetes. The tree is based on alignments of Ndt80/PhoG DNA binding domains (PF05224). Each colored circle represents a species. The appearance of the branch illustrates bootstrap values: grey – bootstrap values 50–80, black – bootstrap values 80–90, bold black: bootstrap values >90. Scale bar indicates number of substitutions per amino acid site. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Complemented strains, in which the *SUF* gene was reintroduced *in locus*, displayed a similar phenotype as the wild type strains (Fig. 2). Interestingly, not all hyphae that came into close proximity showed side-to-side fusion nor did we observe a specific pattern, *e.g.* in the distance between the fusions.

We examined 15 areas of 0.5 mm² in three biological replicates. In PDA, all strains showed very little or no hyphal fusion (Fig. 2a, PDA). In CDA, *Fol*4287 *SUF* deletion mutants peaked at 8 and 10 fusions/area, and *Fom*001 *SUF* deletion mutants at 6 and 7 fusions/area (Fig. 2a, CDA). In CAT medium the situation was reversed: *Fol*4287 mutants showed lower numbers of fusion/area (2 and 4) than *Fom*001 mutants (9 and 10 fusions/area, Fig. 2a,

CAT). *Fo*47 did not show any differences in number of fusions between the different media – the number was low in all cases. This hyper-fusion phenotype is distinct from what has been described for any of the Ndt80 homologs so far (Chu and Herskowitz, 1998; Dementhon et al., 2006; Katz and Cooper, 2015; Xiong et al., 2014).

We next decided to investigate another type of fusion, CAT (for conidial anastomosis tube) fusion. In contrast to vegetative hyphal fusion that can occur throughout the colony, CAT fusion is restricted to the developmental stage of colony initiation (Roca et al., 2005a, 2005b). We have observed that CAT fusion in *F. oxysporum* is restricted to carbon starvation and nitrogen limitation and obtained the highest frequency of CAT fusion in a medium with no carbon source and 25 mM nitrate, thus naming this medium CAT medium (Shahi et al., 2016). To test whether *SUF* deletion also has an effect on CAT fusion, we incubated spores of each strain (wild type, two independent deletion mutants, and a complemented strain in each background) in CAT medium for 18 h. We observed that *Fol*4287 *SUF* deletion mutants exhibit not only an increase CAT fusion, but also an increase in number of conidia that are interconnected (Fig. 3). In the wild type strain we mostly found two conidia and to a much lesser extent three conidia that are connected, whereas in the *SUF* deletion mutant more than five conidia could be part of an interconnected network (Fig. 3b). We calculated the percentage of CAT fusions connecting 2, 3, 4, 5 and >5 conidia per germinated conidia based on two biological replicates and 1000–2500 conidia. Overall, with *Fol*4287 *SUF* deletion strains the percentage of CAT fusions doubled compared to wild type. About half of the CAT fusions connected more than 3 conidia in the mutants. In the complemented strain the wild type phenotype was partially restored. The percentage of total CAT fusion was not much reduced, but the number of conidia that were connected decreased to wild type level. In *Fom*001 *SUF* deletion mutants the phenotype was less severe. No increase in percentage of total CAT fusion was detected. However, there was a tendency that more than two conidia are connected through CAT fusions (Fig. 3a). *Fo*47 did not show any CAT fusions and *SUF* deletion did not have an effect on this (Fig. 3). We decided to continue our further investigations with *Fol*4287 and *Fom*001.

3.3. SUF deletion strains display increased heterokaryon formation but no suppression of HI-associated cell death

Under nutrient-limiting conditions CAT fusion between incompatible strains of *F. oxysporum* allows heterokaryon formation (Shahi et al., 2016). *N. crassa* Vib-1 has been described as a global regulator of HI (Dementhon et al., 2006). We investigated the effect of *SUF* deletion on heterokaryon formation between *Fol*4287 and *Fom*001 during conidial pairing. For this we co-cultivated *Fol*4287 expressing phleomycin resistance and *Fom*001 expressing hygromycin resistance and allowed for conidium formation. 10^4 conidia emerging from the mixed incubation were plated on double-selection PDA plates (*i.e.* containing both hygromycin and zeocin) and the percentage of emerging colonies was calculated. Co-cultivation of *Fol*4287 and *Fom*001 wild type (at the *SUF* locus) yielded no double-selective colonies. We tested four different combinations of *Fol*4287 Δ*suf* and *Fom*001 Δ*suf* strains. In all combinations double drug-resistant colonies emerged: *Fol*4287 Δ*suf* #2 and *Fom*001 Δ*suf* #1 (9.4 ± 2.1%), *Fol*4287 Δ*suf* #2 and *Fom*001 Δ*suf* #5 (2.5 ± 1.3%), *Fol*4287 Δ*suf* #4 and *Fom*001 Δ*suf* #1 (14.1 ± 3.5%), and *Fol*4287 Δ*suf* #4 and *Fom*001 Δ*suf* #5 (1.4 ± 0.9%, average and standard deviation based on 10 replicates). This demonstrates that, similar to *N. crassa*, deletion of *SUF* increases heterokaryon formation.
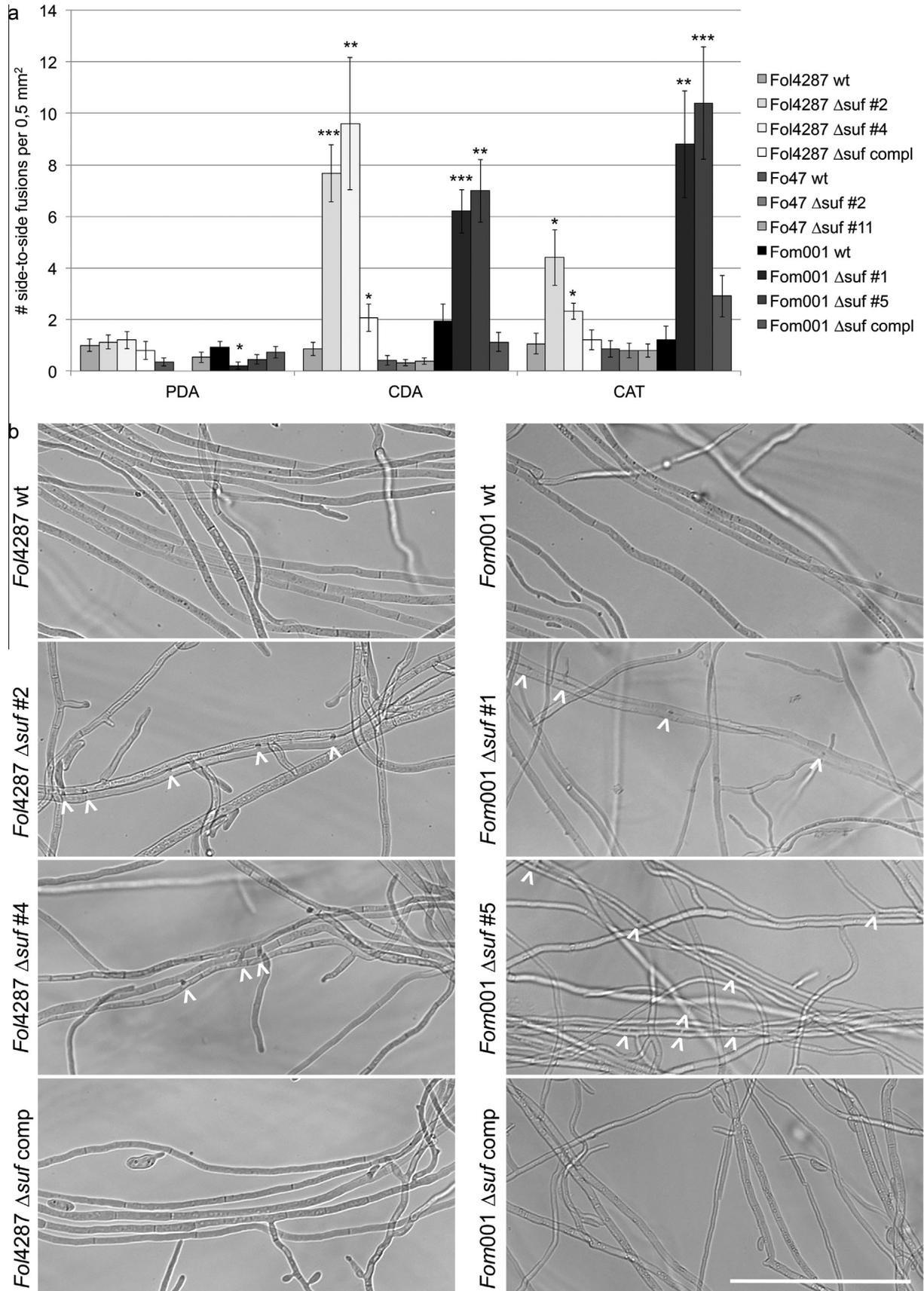
Fig. 2. *SUF* deletion strains of *Fol*4287 and *Fom*001, but not *Fo47*, display increased vegetative hyphal fusion. (a) In CDA and CAT medium, *Fol*4287 and *Fom*001 strains carrying the *SUF* deletion mutation exhibit higher numbers of hyphal fusion. This phenotype is reversed in the respective complementation strains. *Fo*47 does not undergo vegetative hyphal fusion. Depicted are average and standard errors of hyphal fusion per 0.5 mm². The calculation is based on 15 areas from 3 biological replicates. An unpaired *t*-test between wild type and mutant strains was performed for each medium; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. (b) *Fol*4287 and *Fom*001 phenotypes on CDA. Arrowheads mark side-to-side fusions. Scale bar: 100 μm.
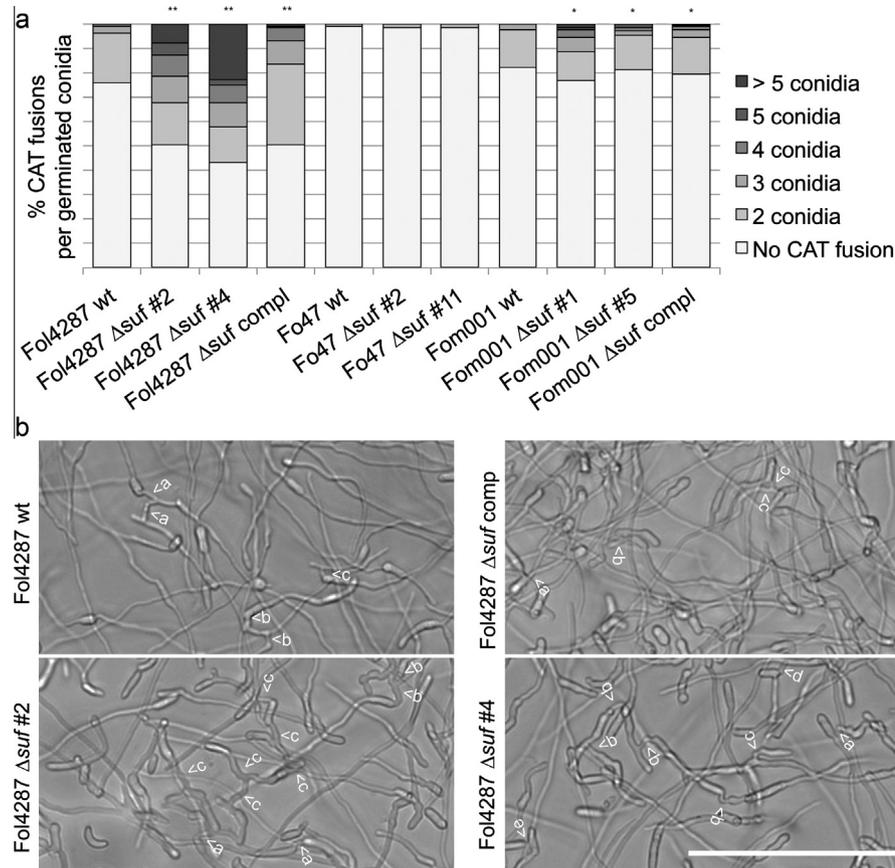
Fig. 3. *Fol*4287 *SUF* deletion strains exhibit higher fusion rates during germling development. (a) *Fol*4287 *SUF* deletion strains grown in CAT medium show higher conidial anastomosis tube (CAT) fusion rates. In addition, the number of conidia that are connected increase. *Fom*001 *SUF* deletion strains do not exhibit a significant difference to the wild type strain, although a tendency towards more connected conidia was observed. *Fo*47 does not undergo CAT fusion. Presented are percentages of CAT fusions connecting 1, 2, 3, 4, 5, and >5 conidia per germinated spores. Calculations are based on 1000–2500 conidia and 2 biological replicates. An unpaired *t*-test comparing CAT fusions including more that two conidia between wild type and mutant strains was performed; [*] $p < 0.05$, [**] $p < 0.0001$. (b) CAT fusion in *Fol*4287 wild type (wt) and *SUF* deletion strain (Δsuf #2). Arrowheads mark CAT fusions. A letter indicates conidia that are interconnected. Scale bar: 100 μm.

We next tested whether the increase in heterokaryon formation is caused by a general suppression of HI-mediated cell death, as was described for *N. crassa*. As mentioned earlier, the mechanisms underlying nonself recognition and HI are not well understood in *F. oxysporum*. In species in which allelic interactions of HI are not known, heterokaryon formation can be tested using nitrate non-utilizing (NIT) mutants. NIT mutants display thin growth on media containing nitrate as a sole nitrogen source. Strains with different NIT mutations are able to complement each other only when they are able to form heterokaryons and are thus compatible with each other (Correll et al., 1987; Puhalla, 1985). Complementation is apparent from aerial hyphae formation where hyphae of the two strains meet. We used the VCG testing system to determine whether *SUF* deletion mutants can overcome HI during vegetative growth. For this we selected at least one NIT mutant per strain (*Fol*4287 wt, *Fol*4287 Δsuf #2, *Fom*001 wt, and *Fom*001 Δsuf #1, for phenotype on nitrate medium see Fig. S2). As was expected, the positive control, *i.e.* interaction between compatible strains or selfing, resulted in formation of aerial hyphae (Fig. 4, upper left panel). Neither
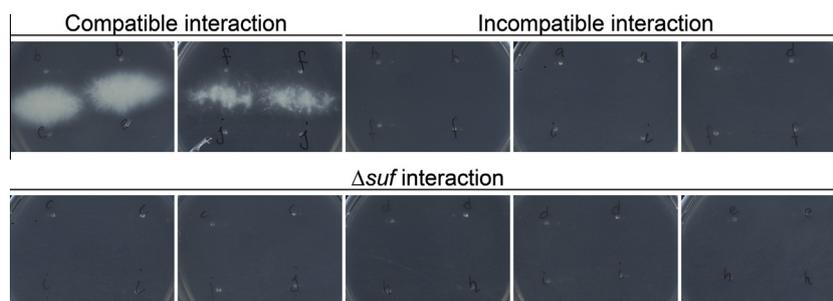
Fig. 4. *SUF* deletion does not overcome heterokaryon incompatibility. In *F. oxysporum* vegetative compatibility is tested using nitrate non-utilizing (NIT) mutants. Compatible strains carrying different NIT mutations can complement the NIT phenotype (thin growth on nitrate medium) resulting in formation of aerial hyphae. Pairing of complementing *Fol*4287 and *Fom*001 NIT mutants resulted in production of aerial hyphae (compatible interaction), whereas the pairing of *Fol*4287 with *Fom*001 did not complement the NIT phenotype (incompatible interaction). The same results were obtained in different pairings between *Fol*4287 and *Fom*001 *SUF* deletion strains (Δsuf interaction). a: *Fol*4287 Nit1, b: *Fol*4287 NitM, c: *Fol*4287 Δsuf Nit1, d: *Fol*4287 Δsuf NitM, e: *Fol*4287 Δsuf Nit3, f: *Fom*001 Nit1, h: *Fom*001 Δsuf Nit1, i: *Fom*001 Δsuf Nit3, j: *Fom*001 Δsuf NitM.

the interaction between *Fol*4287 and Fom001 wild type nor *SUF* deletion strains resulted in formation of aerial hypha, thus marking these strains as incompatible (Fig. 4, upper right and bottom panel). We conclude that the increase in heterokaryon formation observed upon mixing of conidia is caused by the increase in CAT fusion and that, unlike *N. crassa Vib*-1, Suf is likely not involved in regulation of HI.

3.4. Suf is not important for secretion of proteases or for carbon utilization

Another phenotype that has been associated with Ndt80 homologs Vib-1 and XprG is absence of extracellular proteases in the culture medium in the respective deletion mutants. In *N. crassa* and *A. nidulans* extracellular protease production is induced upon carbon or nitrogen starvation (Dementhon et al., 2006; Katz et al., 2006). We examined culture supernatants of *Fol*4287 and Fom001 wild type and *SUF* deletion mutants for protease activity in different media (NO₃, minimal medium (MM), CAT, and CAT +3% sucrose). Similar to *N. crassa* and *A. nidulans*, extracellular protease production is strongly induced under nutrient-limiting condition in *F. oxysporum* (MM and CAT). However, *SUF* deletion had no effect on protease production (Fig. 5). We also found no major differences in culture supernatant protein profiles using SDS-PAGE between wild type and *SUF* deletion mutants in any of the media (Fig. S3). We conclude that Suf does not play a major role in protease production in *F. oxysporum* in response to carbon or nitrogen starvation.

In another study a link between Vib-1 and carbon utilization was demonstrated (Xiong et al., 2014). We analyzed carbon source utilization using BIOLOG FF MicroPlates, which contain a different carbon source in each of the 96 wells. The ratios between *Fol*4287 *SUF* deletion strain and wild type strain growth rates were calculated. Values higher than 1.5 or lower than 0.5 are considered a substantial difference in growth rate (Michielse et al., 2009). In our assay, the *SUF* deletion strain performed similar to the wild type strain on all tested carbon sources (Fig. S4). Thus, Suf is not required for utilization of these carbon sources.

4. Discussion

In this study we present a novel role for the *F. oxysporum* Ndt80 homolog suppressor of fusion (Suf) as a nutrient-dependent negative regulator of vegetative hyphal and CAT fusion. *Fol*4287 and *Fom*001 strains carrying a *SUF* deletion show increased vegetative hyphal fusion and in case of *Fol*4287 also increased CAT fusion (Figs. 2 and 3). Genes negatively regulating fusion have rarely been characterized. To our knowledge only two such genes have been identified, *N. crassa* NCU006362, a predicted GTPase activating protein, and *SPR7*, a secreted serine protease (Palma-Guerrero et al., 2013). In both cases the deletion strain exhibits higher CAT fusion frequencies and higher numbers of connected conidia. It was suggested that both genes are involved in fungal communication prior to fusion (Palma-Guerrero et al., 2013).

Despite (almost) identical protein sequences of the Suf homologs in the three *F. oxysporum* strains analyzed in this study, the deletion strains show different phenotypes. We have previously observed that *Fo*47 has a fusion defect and does not undergo vegetative fusion in form of hyphal or CAT fusion and confirmed this phenotype in this study (Shahi et al., 2016 and Figs. 2 and 3). *SUF* deletion does not overcome this fusion defect, indicating that either the two processes are unlinked or that the fusion defect of *Fo*47 is caused by a protein downstream of Suf, maybe even a target of Suf. The differences in phenotype between *Fol*4287 and *Fom*001 *SUF* deletion strains could also be caused by differences in Suf targets.

We show that *SUF* deletion increases heterokaryon formation but does not suppress HI. The increase in heterokaryon formation could be caused by the observed increase in CAT fusion frequency. In other ascomycete fungi, Ndt80 homologs play different roles. *Saccharomyces cerivisiae* Ndt80, the founder of this family of p53-like transcription factors, is a transcriptional activator of ~150 genes involved in completion of meiosis (Chu and Herskowitz, 1998). In *Candida albicans* one Ndt80 homolog has been characterized, which is involved in drug resistance, biofilm formation, and virulence (Chen et al., 2004; Nobile et al., 2012; Sellam et al., 2010; Wang et al., 2006). The filamentous ascomycete *A. nidulans* has two Ndt80 homologs. The one most similar to Ndt80 is involved in sexual reproduction. The second, XprG, which is more
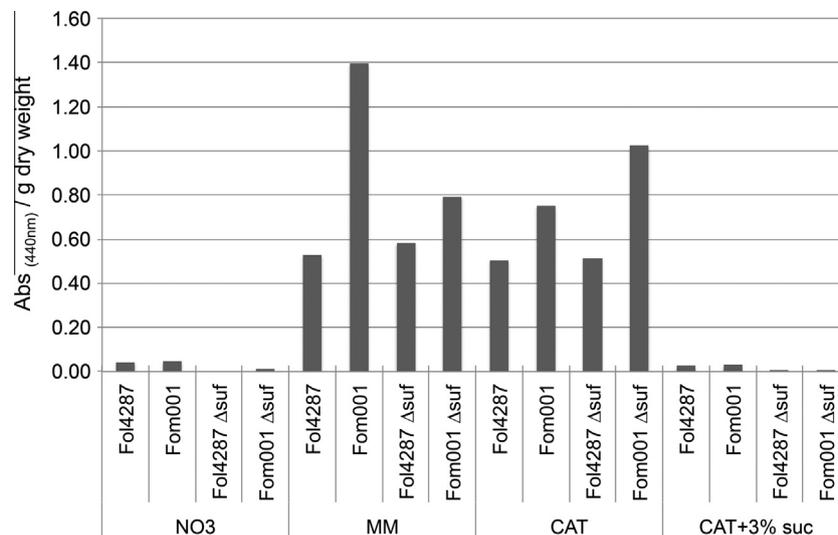
Fig. 5. In *F. oxysporum* the production of extracellular proteases in response to carbon and nitrogen starvation is independent of Suf. *Fol*4287 and *Fom*001 wild type and *SUF* deletion strains were assessed for extracellular protease activity. Protease activity was determined by measuring the release of the trichloroacetic acid (TCA)-soluble orange sulfanilamide component of azocasein upon proteolysis at 440 nm. Results are displayed as absorbance per gram dry weight. Measurements were done once, precluding calculation of standard deviation. Nevertheless, each pair of strains behaves similarly and it is clear that *F. oxysporum* extracellular protease production is, similar to *N. crassa* and *A. nidulans*, triggered by carbon source starvation (MM and CAT medium) but deletion of the *F. oxysporum NDT80* homolog *SUF* does not suppress the induction of protease production.

closely related to *N. crassa* Vib-1, is a transcriptional activator of a large number of genes in response to carbon limiting conditions (Katz and Cooper, 2015). *N. crassa* Vib-1 was characterized as a global regulator of heterokaryon incompatibility (HI) and a positive regulator of extracellular protease production under carbon and nitrogen starvation (Dementhon et al., 2006; Xiong et al., 2014). A second Ndt80 homolog in *N. crassa*, Fsd-1, is involved in female sexual development, a process that is initiated under nitrogen limiting conditions (Hutchison and Glass, 2010). The one apparent commonality across these diverse functions is response to nutrient-limiting conditions. Although this has not been tested for *N. crassa* Vib-1, in *P. anserina* HI is associated with the response to starvation (Pinan-Lucarré et al., 2005). It was suggested that nutrient sensing might represent one of the ancestral roles for Ndt80 family of proteins (Dementhon et al., 2006; Katz and Cooper, 2015; Katz et al., 2013). Our data provides further support for this hypothesis.

Phylogenetic analyses revealed that the Ndt80 DNA-binding domain (DBD) is conserved among ascomycete fungi, but the proteins have diverged outside this domain (Fig. 1). The question that arises is how these different Ndt80 homologs can respond to the same environmental cues. It is possible that the proteins are activated through a conserved mechanism despite sequence divergence. Alternatively, the expression of *NDT80* and its homologs could be triggered by nutritional cues. In *S. cerivisiae*, Ime1 and Ime2 are positive regulators of expression of *NDT80* (Winter, 2012). *N. crassa* and *A. nidulans* both have a homolog of Ime2, which have been shown to be negative regulators of Vib-1 and XprG, respectively (Hutchison and Glass, 2010; Hutchison et al., 2012; Katz and Cooper, 2015). It was suggested that the regulation of *NDT80* homologs by Ime2 homologs might be conserved among ascomycete fungi (Hutchison et al., 2012). *F. oxysporum* also has a *IME2* homolog, *FOXG_13813* (http://www.fungidb.org), and it will be interesting to study its function in regulation of *SUF*.

We have shown that the deletion mutant phenotype is triggered in CDA. CDA contains sucrose as sole carbon source and nitrate as sole nitrogen source but both in adequate quantities, thus the medium is not causing starvation. Apparently, then, it is the absence of complex compounds in CDA (present in PDA) that triggers increased hyphal fusion (in the absence of Suf). That may also hold true for other Ndt80 homologs, possibly regulated by Ime2 homologs. Depending on the fungal species, different developmental processes are then activated. In addition, conidial pairing of strains with a *SUF* deletion show an increase in heterokaryon formation, the first step towards horizontal chromosome transfer (Shahi et al., 2016).

Continued work will be required to elucidate the role of Ndt80 homologs in fungal development as well as asexual recombination and horizontal chromosome transfer in *F. oxysporum*. This might include the identification of downstream targets of Suf by transcriptome analysis, characterization of other *F. oxysporum* Ndt80 homologs and regulators of Suf.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2016.08.005.

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