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# Nuclear dynamics and genetic rearrangement in heterokaryotic colonies of *Fusarium oxysporum*

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A B S T R A C T

Recent studies have shown horizontal transfer of chromosomes to be a potential key contributor to genome plasticity in asexual fungal pathogens. However, the mechanisms behind horizontal chromosome transfer in eukaryotes are not well understood. Here we investigated the role of conidial anastomosis in heterokaryon formation between incompatible strains of *Fusarium oxysporum* and determined the importance of heterokaryons for horizontal chromosome transfer. Using live-cell imaging we demonstrate that conidial pairing of incompatible strains under carbon starvation can result in the formation of viable heterokaryotic hyphae in *F. oxysporum*. Nuclei of the parental lines presumably fuse at some stage as conidia with a single nucleus harboring both marker histones (GFP- and RFP-tagged) are produced. Upon colony formation, this hybrid offspring is subject to progressive and gradual genome rearrangement. The parental genomes appear to become spatially separated and RFP-tagged histones, deriving from one of the strains, *Fol*4287, are eventually lost. With a PCR-based method we showed that markers for most of the chromosomes of this strain are lost, indicating a lack of *Fol*4287 chromosomes. This leaves offspring with the genomic background of the other strain (*Fo*47), but in some cases together with one or two chromosomes from *Fol*4287, including the chromosome that confers pathogenicity towards tomato.

## 1. Introduction

Genome plasticity has been described for a variety of plant-pathogenic fungi, and is considered a driving force in the "arms-race" between pathogen and host. Mutation and meiotic recombination are well-known processes underlying genomic variation. With the increasing availability of genome sequences it has become clear that asexual fungal pathogens also show a high degree of genetic variability (Gladieux et al., 2014; Karasov et al., 2014; Perez-Nadales et al., 2014; Takken and Rep, 2010). This has, for example, been demonstrated for *Fusarium oxysporum* (*Fo*) in comparative genome studies using different *Fusarium* species. *Fusarium graminearum* (*Fg*), *Fusarium verticillioides* (*Fv*), *Fusarium solani* (*Fs*), and *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) share a highly conserved core genome harboring all essential housekeeping genes. In addition, *Fs* and *Fol* also carry lineage specific (LS) chromosomes enriched in genes specialized to niche adaptation, such as pathogenicity-related genes (Coleman et al., 2009; Ma

et al., 2010). In the *Fusarium oxysporum* species complex each *forma specialis* is pathogenic towards a specific plant species. Strains pathogenic towards the same host can be polyphyletic and it has been suggested that horizontal transfer of genetic material might facilitate the emergence of new pathogenic strains (Lievens et al., 2009; Ma et al., 2010; Michielse and Rep, 2009). Horizontal transfer is defined as non-meiotic transfer of genetic material and the stable integration into the recipient genome. Horizontal chromosome transfer (HCT) is a special case of horizontal transfer where entire chromosomes or sets of chromosomes are transferred between strains (Mehrabi et al., 2011; Rosewich and Kistler, 2000; Soanes and Richards, 2014; van der Does and Rep, 2012). In the case of *Fo*, it was demonstrated that the two smallest LS chromosomes of strain *Fol*007 can be transferred to a non-pathogenic strain, *Fo*47, leading to the acquirement of pathogenicity towards tomato plants (Ma et al., 2010).

HCT has also been demonstrated for *Colletotrichum gloeosporioides* and *Alternaria alternata* (Akagi et al., 2009; He et al., 1998), but the underlying mechanisms are not well understood. In the absence of extracellular DNA transfer mechanisms in fungi it is likely that HCT occurs through hyphal fusion (Fitzpatrick, 2012; Mehrabi et al., 2011). In filamentous ascomycetes vegetative hyphal fusion or anastomosis occurs frequently within the same mycelium (self

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fusion) and presumably ensures the equal distribution of nutrients and facilitates signaling across the mycelium (Read et al., 2009; Simonin et al., 2012). Hyphal fusion between genetically distinct individuals results in formation of heterokaryotic cells, where one or more nuclei from each individual share a continuous cytoplasm. However, genetically distinct fungi differ at vegetative or heterokaryon incompatibility loci and a fusion generally leads to the heterokaryon incompatibility (HI) reaction. The HI reaction includes extreme growth reduction and in most cases compartmentalization of the fused cells and programmed cell death (Glass and Dementhon, 2006; Glass and Kaneko, 2003; Glass et al., 2000; Manners and He, 2011). It has been suggested that the HI reaction is at least partially suppressed during conidial anastomosis tube (CAT) fusion. CATs are specialized hyphae interconnecting conidia or germ tubes during early colony initiation (Ishikawa et al., 2010; Read and Roca, 2000; Roca et al., 2005a, 2005b).

In recent years, studies have been conducted to investigate formation of viable heterokaryons. For example, conidial pairing and antidrug resistance testing in *C. gloeosporioides* showed that CAT fusion could generate heterokaryotic colonies with severe growth retardation and it was suggested that slow-growing heterokaryons might act as an intermediate step towards HCT (Manners and He, 2011). In the bean pathogen *C. lindemuthianum* fluorescently labeled nuclei were used to monitor nuclear fates in heterokaryotic cells. It was demonstrated that heterokaryotic cells formed by CAT fusion are viable and produce uninucleate conidia, which formed colonies with distinct phenotypes from the parental lines (Ishikawa et al., 2012). The grass endophyte *Epichloë* lacks the HI reaction and vegetative hyphal fusion resulted in stable heterokaryotic cells. Protoplast fusion produced hybrids with genetic markers from both parental lines (Shoji et al., 2015). In addition to their role during colony establishment, nutrient distribution, and signaling, it has been suggested that fungal anastomosis might mediate parasexual or non-meiotic recombination, which could contribute to the high level of genetic variation in the apparent absence of sexual recombination and possibly also enables HCT (Castro-Prado et al., 2007; Clutterbuck, 1996; Milgroom et al., 2009; Teunissen et al., 2002).

The aim of this study was to investigate the role of anastomosis in heterokaryon formation between different strains of *F. oxysporum* and determine the importance of heterokaryons for HCT. We observed the fate of fluorescently labeled nuclei during co-cultivation of vegetatively incompatible strains of *F. oxysporum* (Puhalla, 1985), anastomosis, and early development of heterokaryons, as well as during long-term development of hybrid offspring. In addition, the chromosomal composition of the hybrid offspring during the long-term study was determined using a PCR-based method to detect chromosome-specific markers.

We show that CAT fusion in *F. oxysporum* is greatly increased during carbon starvation and nitrogen limitation and that conidial pairing of the incompatible strains *Fol*4287 and *Fo*47 results in formation of viable heterokaryons. We conclude that CAT fusion of incompatible conidia leads to nuclear fusion and micronuclei formation in the hybrid offspring. During this process *Fol*4287 chromosomes are generally lost, but some chromosomes or parts of chromosomes can apparently be integrated into the *Fo*47 genome, including the pathogenicity chromosome that was previously shown to be horizontally transferrable.

## 2. Material and methods (1200/2250)

### 2.1. Strains and culture conditions

*Fusarium oxysporum* f. sp. *lycopersici* strain 4287 (*Fol*4287, FGSC9935) and the tomato 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 histone H1 fusion protein-expressing vector and Agrobacterium-mediated Fusarium transformation

Construction of pRW2h-H1-GFP and pRW2h-H1-REF was described previously (Shahi et al., 2015; Vlaardingerbroek et al., 2015). To generate an H1-RFP plasmid with the phleomycin resistance cassette, plasmids pRW2h-H1-RFP and pRW1p (Houterman et al., 2008) were both digested with *Mfe*I and *Blp*I. The resulting fragment from pRW1p containing the phleomycin resistance gene was then ligated into the vector to create pRW2p-H1-RFP. The obtained plasmids pRW2h-H1-GFP, pRW2h-H1-RFP and pRW2p-H1-RFP were transformed into *Agrobacterium tumefaciens* EHA105 and the transformants 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 fusion assay

*Fol*4287 conidia 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 $7.5 \times 10^5$ conidia per ml were incubated in an 8-well microscope chamber slide (Nunc) for 15–18 h. CAT fusion was tested in PDB (Difco), CDL (Oxoid), minimal medium (0.17% yeast nitrogen base (YNB, Difco) without amino acids and ammonium sulfate, 100 mM $KNO_3$), CAT medium (0.17% YNB, 25 mM $KNO_3$), PDB supplemented with 10 μg/ml glutamic acid, minimal medium and CAT medium supplemented with 1 or 10 μg/ml tryptophan, CAT medium supplemented with 3% sucrose, and water. Observations were performed, if not otherwise stated, with the AMG Evos FL digital inverted microscope equipped with transmitted light, DAPI (357/44 to 447/60 nm), GFP (470/22 to 510/42 nm), and Texas Red (585/29 to 624/40 nm) light cubes, and driven by built-in software for image acquisition. Images were analyzed with the Fiji software from imageJ (http://fiji.sc/Fiji). CAT fusion frequency was calculated as the percentage of CAT fusions per germinated conidium. 300–3000 conidia were counted in two to four biological replicates.

### 2.4. Co-cultivation

Conidia of *Fol*4287 H1-RFP (*ble*), *Fol*4287 H1-GFP (*hph*), and *Fo*47 H1-GFP (*hph*) 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 detect viable heterokaryons in *F. oxysporum*, $10^6$ conidia of *Fol*4287 H1-GFP (*hph*) and *Fo*47 H1-GFP (*hph*) were co-incubated in PDB, minimal medium or water for two to five days. After one round of monosporing, single spore colonies were tested for presence of yellow nuclei (i.e. red and green) and incubated for four to six months to monitor nuclear composition (inverted agar block method, Hickey et al., 2004).

### 2.4.1. Conidial pairing

To test CAT fusion frequency, 100 µl 7.5 × 10⁵ conidia per ml from each parental strain were incubated in an 8-well microscope chamber slide (Nunc) for 15–18 h. Cat fusion was tested in CAT medium containing 0.25%, 0.5%, or 1% xylose. CAT fusion frequency was calculated based on three to four biological replicates and ∼300 conidia per replicate. To select heterokaryotic cells, 500 µl 10⁶ conidia per ml from each parental strain were incubated in a 1-well microscope chamber slide (Nunc) and after two days 50 µl of the mix was plated on PDA and incubated for two days. Again conidia were collected and washed and 10 µl 10⁶ conidia per ml were incubated on PDA buffered with 0.1 M Tris (pH 8) and supplemented with 100 µg/ml hygromycin (Duchefa) and 100 µg/ml zeocin (Invivogen) for five days.

### 2.4.2. Hyphal tip pairing

Conidia of *Fol*4287 H1-RFP (*ble*), and *Fo*47 H1-GFP (*hph*) were collected from one-week-old PDA plates in 2 ml water, filtered through one layer of sterile Miracloth (Calbiochem), and washed with water. Conidia of each parental line were applied on CAT medium supplemented with 1% agarose and 0.25% xylose in lines 2 cm apart from each other and incubated until the hyphae met. An agarose block from this area was transferred to PDA containing hygromycin and zeocin. Outgrowth of double-selective hyphae was monitored.

### 2.5. Observation of heterokaryotic colonies

#### 2.5.1. Live-cell imaging of CAT fusion and early development of heterokaryotic colonies

To investigate CAT formation and fusion, conidia of *Fol*4287 H1-RFP (*ble*), *Fol*4287 H1-GFP (*hph*), and *Fo*47 H1-GFP (*hph*) were collected from one-week-old PDA plates in 2 ml water, filtered through one layer of sterile Miracloth (Calbiochem), and washed with water. 100 µl 10⁶ conidia per ml from each parental line were mixed and 20 µl was mounted on CAT medium supplemented with 1% agarose and 0.25% xylose in the shape of a microscope slide. These were incubated conidia phase down in a 1-well microscope chamber slide (Nunc) and observed after 15 h. To monitor early development of heterokaryotic cells conidia of the parental lines were co-incubated in CAT medium containing 0.25% xylose for two days prior to mounting on agarose slides.

Live-cell imaging was performed using an Eclipse Ti inverted microscope (Nikon) equipped with an EM-CCD iXon DU897 camera (Andor), and a plan apo VC 40X 1.4 oil objective (Nikon). GFP was excited with a 488-nm light (emission 525–50 nm BP filter) and RFP with a 561-nm light (emission 600–37 nm BP filter). Pictures were analyzed with the Nikon NIS and Fiji software from imageJ (http://fiji.sc/Fiji).

#### 2.5.2. Development of hybrid offspring

Double-selective colonies were transferred on CDL plates supplemented with 1% agarose and 2% xylose. After one week nuclear composition of young hyphae from the edge of the colony was determined. Nuclear localization was confirmed by DNA counterstaining. For this the mycelium was treated for 1 min with 1 mg/ml Hoechst 33342 (Life Technologies) and washed with water before microscopy. To test chromosomal composition, DNA extraction was performed, and an agarose block from the edge of the colony was transferred to a fresh plate for the next time point. This was repeated until only one of the two fluorescent signals was detected.

### 2.6. Chromosomal composition

To test for the presence of *Fol*4287 and *Fo*47 chromosomes primers based on FOXY insertion sites in *Fol*4287 were used. FOXY transposons are enriched in pathogenic strains of *F. oxysporum*. For each chromosome of *Fol* a locus-specific primer was used together with a FOXY specific primer (Ma et al., 2010). Similarly, for the same region two specific primers were designed for *Fo*47 (Table S1; for an alignment of *Fol*4287 and *Fo*47 chromosomes see Fig. S1)

## 3. Results

### 3.1. Starvation-induced CAT fusion enables heterokaryon formation

In a first attempt to find viable heterokaryotic cells of *F. oxysporum*, we performed a simple co-cultivation experiment. We mixed conidia of the tomato pathogen *F. oxysporum* f. sp. *lycopersici* strain 4287 (*Fol*4287) expressing histone H1 fused with red fluorescent protein (H1-RFP) and the hygromycin resistance cassette (*hph*) with conidia of the tomato non-pathogenic *F. oxysporum* strain *Fo*47 expressing histone H1 fused with green fluorescent protein (H1-GFP) and *hph* (Fig. S2). The conidial mixture was incubated in nutrient rich medium (PDB), minimal (i.e. low carbon) medium, or water for three to five days and then examined microscopically. While we were not able to detect heterokaryotic cells after co-cultivation in rich medium, co-cultivation under carbon starvation resulted in heterokaryotic conidia, germlings, and hyphae. We observed three different types of heterokaryons. In Type I one red nucleus and green cytosolic fluorescence are present in conidia, but the green fluorescence does not continue into the germ tube. This was the most abundant Type. In Type II both red and green nuclei are present in the conidia, but only red nuclei are present in the germ tube. In Type III both red and green nuclei are found in conidia as well as the hyphae (Fig. 1a). Type III was the least frequently detected. Interestingly, in all cases observed the dominant (always propagating) nucleus was derived from *Fol*4287, showing as red nuclei.

It has been established in *Colletotrichum* species that heterokaryon incompatibility is suppressed during conidial anastomosis tube (CAT) fusion (Ishikawa et al., 2012; Manners and He, 2011). To find out whether the same holds true for *F. oxysporum* we studied CAT fusion in *Fol*4287 in water, czapek dox liquid (CDL), potato dextrose broth (PDB), and minimal medium with no carbon source. Although in our initial experiment co-cultivation of *Fol*4287 and *Fo*47 in water resulted in heterokaryotic cells, we were not able to find CAT fusion in water. This inability may be explained partly by a reduction of germination in water to 33% and partly by aggregation of the conidia to a degree that it was not possible to distinguish whether any CATs had formed. In CDL and PDB CAT fusions occurred in <1% of germinated conidia. However, when incubated in minimal medium 9% of all germinated conidia showed CAT fusion. Further reduction of nitrate in the minimal medium to 25 mM (referred to as CAT medium from hereon) yielded 47% CAT fusion (Fig. S3). Addition of 3% sucrose to CAT medium reduced the CAT fusion frequency to 6% ($N = 300$–3000, Fig. 1b and Fig. S3). Neither glutamic acid nor tryptophan had any effect on CAT formation or fusion, unlike as described for *N. crassa* (Fischer-Harman et al., 2012). These observations led us to the conclusion that CAT fusion in *F. oxysporum* is stimulated by carbon starvation and limited access to nitrogen. Another interesting observation is that for CAT fusion the formation of a CAT from one of the conidia is sufficient and is in fact the case in the majority of events (85%, $N = 95$).
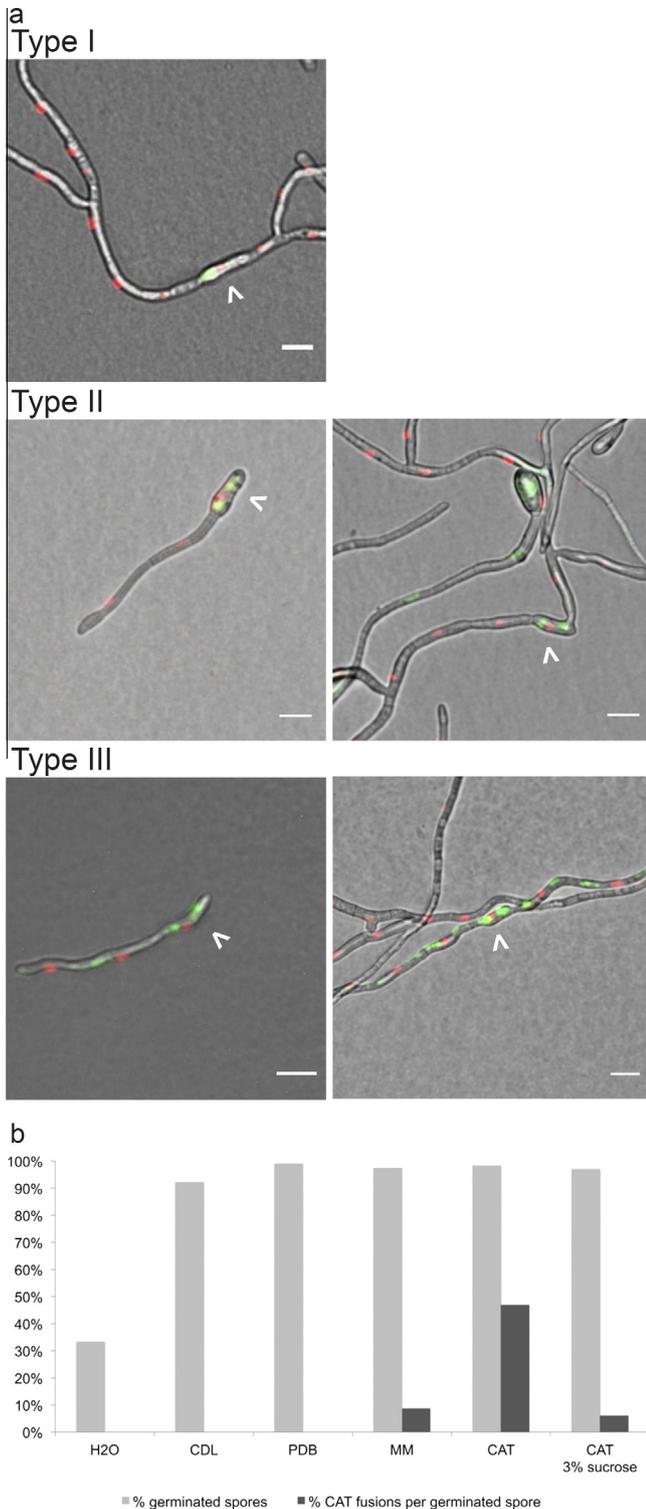
**Fig. 1.** Heterokaryon formation and CAT fusion. (a) Under carbon starvation conditions (H2O, minimal medium) incompatible strains of *F. oxysporum* can produce heterokaryotic conidia, which develop in three distinct ways. Type I: colonies show a red nucleus from the pathogenic strain *Fol*4287 with green cytosolic signal deriving from the non-pathogenic strain *Fo*47 in the conidia. Type II: colonies show both red and green nuclei in the conidia, but only the red nucleus proliferates. Type III: colonies harbor red and green foci in the conidia as well as the hyphae. In heterokaryotic colonies nuclei derived from *Fol*4287 always proliferate and seem dominant. Arrowhead indicates heterokaryotic conidia, scale bar: 10 μm. (b) CAT fusion in *Fol*4287 is restricted carbon and nitrogen starvation. Addition of a carbon source decreases the number of CATs formed per germinated conidia. Calculations were based on 300–3000 conidia from two to four biological replicates. MM, minimal medium; CAT, CAT medium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Given that heterokaryon formation and CAT fusion were both detected in medium without a carbon source, we next tested whether an increase of CAT fusion frequency has an effect on heterokaryon formation. For this we introduced H1-RFP with a phleomycin resistance cassette (*ble*) in *Fol*4287 to be paired with the above-mentioned *Fo*47 expressing H1-GFP (*hph*). Conidia from both strains were mixed and co-cultivated for 15–18 h. We compared CAT fusion frequencies in self-fusion (*Fol*4287-*Fol*4287 and *Fo*47-*Fo*47) and in the incompatible interaction between *Fol*4287 and *Fo*47, the products of which we consider as heterokaryons. Because the expression of the histone H1 fusion protein is under the control of an inducible xylanase promoter, we also tested different concentrations of xylose in the CAT medium, looking for the lowest effective concentration for expression of the H1 fusion genes. We found that CAT fusion frequency between *Fol*4287 conidia is highest in CAT medium with 0.5% xylose (37 ± 11%). The highest CAT fusion frequency between *Fol*4287 and *Fo*47 we detected was in CAT medium with 0.25% xylose (5 ± 4%) and we therefore decided to use this medium for further co-cultivation studies. CAT fusion between *Fo*47 conidia was virtually absent.

In addition to conidial pairing we also performed hyphal tip pairing to test the effect of hyphal fusion on heterokaryon formation. Hyphal fusion connects vegetative hyphae throughout the colony, independent of developmental stage. For this we grew *Fol*4287 H1-RFP (*ble*) and *Fo*47 H1-GFP (*hph*) in separate lines side by side on CAT medium supplemented with 0.25% xylose and 1% agarose. We then transferred an agarose block from the area where the hyphae of both strains met onto double selective plates and monitored for hyphal growth. However, no double-resistant outgrowth was observed. Form this we conclude that CAT medium does facilitate heterokaryon formation and that CAT fusion is the main source for heterokaryon formation under the conditions used.

*3.2. Heterokaryotic germlings show aberrant nuclear behavior during early development*

To better understand the process of CAT fusion we observed nuclear behavior in *Fol*4287 after 15 h (CAT formation and fusion) and after two days (early development). We followed fusion of *Fol*4287 expressing H1-RFP (*ble*) either with itself or with *Fol*4287 expressing H1-GFP (*hph*). Both CAT fusion and hyphal fusion were frequently observed when cultivated in CAT medium. Again we found that in the majority of cases (85%) the CAT is formed by only one of the conidia (Fig. 2a and Movie M1). Furthermore, we observed that migration of the nucleus through a CAT did not result in the degradation of either nucleus, as was observed during hyphal fusion in an earlier study (Fig. 2b and Movie M2, Ruiz-Roldán et al., 2010). However, nuclear migration did not always occur in the process of CAT fusion. In fact in most cases the nuclei remained in the original conidia. After fusion had been established the fluorescent signal traveled through the CAT and it is unknown whether transport of mRNA or protein was responsible. In the hypha of the fusion partner the fluorescent protein was then taken up into the nuclei, showing as yellow nuclei (i.e. red and green). This indicates that after completion of CAT fusion between compatible strains septal pores are open and allow for cytoplasmic continuity (Fig. 2c and Movie M3).

Next we investigated the incompatible interaction between *Fol*4287 H1-RFP (*ble*) and *Fo*47 H1-GFP (*hph*) by live-cell imaging. Unfortunately, due to the low frequency of CAT fusion in this interaction we were unable to capture a CAT fusion event by visual prediction of where such an event might occur at 15 h after co-cultivation. Nonetheless, we detected an apparent attempt and failure of *Fol*4287 CAT to fuse with *Fo*47 conidia. Movie M4 shows how the CAT attempts several times to fuse with the *Fo*47 conidia
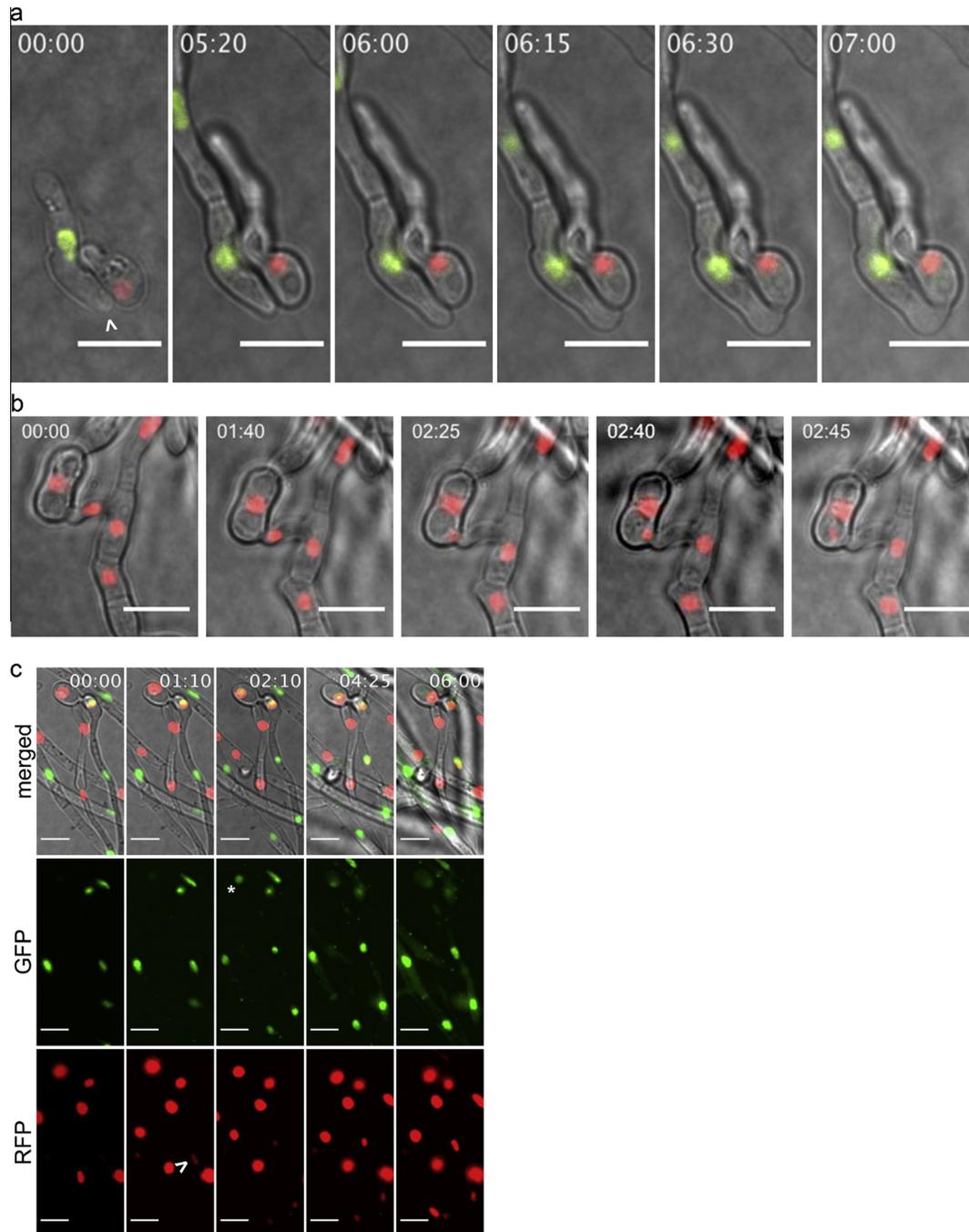
**Fig. 2.** CAT fusion in *Fol*4287. (a) Time-lapse sequence of CAT formation and fusion. In *F. oxysporum* generally one conidium initiates a CAT and homes toward another conidium. It is neither necessary nor often observed that both conidia form a CAT. Arrowhead marks area where CAT is formed. (b) Time-lapse sequence of nuclear migration between two conidia after CAT fusion, showing survival of both nuclei in the same compartment. (c) Time-lapse sequence of diffusion of fluorescent signal through CAT. Over time in all nuclei downstream the fused conidia red and green fluorescent signal is detected, showing as yellow nuclei. Top panel: merged, middle panel: GFP, bottom panel: RFP. Arrowhead marks first occurrence of red signal in an originally green nucleus and asterisk first occurrence of green signal in an originally red nucleus. Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

at different spots, but the fusion never takes place. We were, however, able to find fused CATs after the process had been completed after 24 h (Fig. 3a). In all five cases in which a clear distinction was possible, *Fol*4287 formed the CAT. Remarkably, even after two days we did not observe yellow nuclei (i.e. red and green) in the fused CATs or the hyphae as a result of transmission of fluorescent signal, contrary to the self-fusion observed in *Fol*4287. However, the co-cultivation had allowed for the production of viable heterokaryotic conidia harboring at least one nucleus of each parental line, visible as distinct red and green nuclei. Further observation of nuclear dynamics in heterokaryotic germlings using live-cell imaging revealed that the red nucleus, originating from *Fol*4287, appeared intact, whereas the green nuclei from *Fo*47 appeared fractionated (two or more) and sometimes blurry. Nonetheless, nuclei from both parental lines proliferated during germination and hyphal growth, but in an asynchronous manner. Surprisingly, exchange of fluorescent protein did not take place in either direction (Fig. 3b and Movie M5).
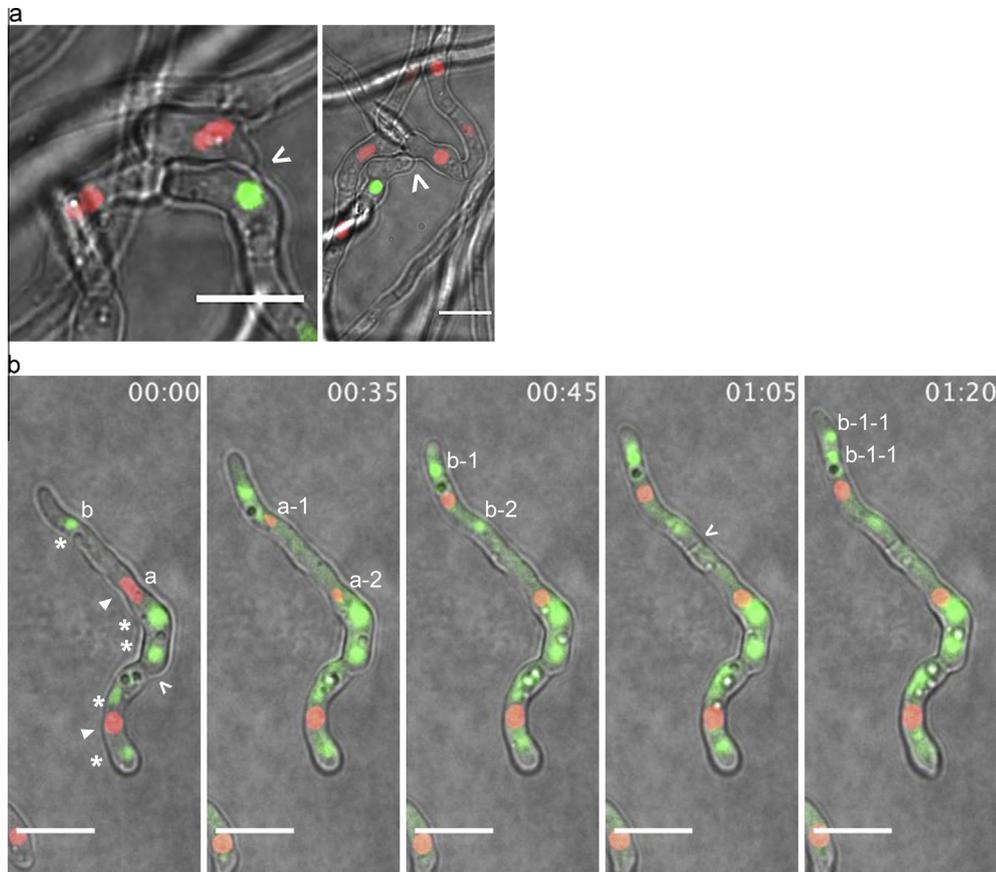
**Fig. 3.** Incompatible interaction. (a) CAT fusion between heterokaryotic conidia. Arrowhead marks CAT fusion. (b) Proliferation of red and green nuclei in a hybrid offspring. Time-lapse sequence showing mitosis in a heterokaryotic germling. Red nuclei originating from *Fol*4287 appear intact, showing as regular shaped nuclei (filled arrow heads). Green nuclei originating from *Fo*47 appear fractionated (two or more) and/or bleary (asterisks). Open arrow heads mark septi. Both red (a) and green (b) nuclei undergo mitosis in an asynchronous manner. Scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### 3.3. Horizontal transfer occurs after heterokaryon formation

To select heterokaryons for subsequent analysis we allowed the co-cultivation mix to produce conidia for two to five days. Since we did not make use of antifungal drug selection, the majority of the hyphae in the mix were either *Fol*4287 or *Fo*47, with only red or green nuclei, respectively. However, we also found heterokaryotic hyphae. Interestingly, these hyphae produced conidia with a single yellow nucleus, indicating that at some point during development the nuclei of the parental lines must have fused. To better monitor the yellow-nuclei progeny of the heterokaryotic colonies, conidial monosporing was performed. Out of 40 tested single spore colonies, four contained yellow nuclei. Conidia with a yellow nucleus germinated and formed germ tubes with no perceivable morphological differences to either of the parental lines (Fig. S4a). However, in the mature colonies after one to two weeks nuclei seemed fractionated and formed micronuclei (Fig. S4b). We went on to test the stability of the hybrid colonies and incubated the plates for four to six months. Interestingly, the fluorescent signal redistributed over time and a range of green, red and yellow nuclei of different sizes was observed (Fig. S4c). To follow this process further, we transferred an agar block from such plates to a new plate and incubated for two additional weeks. At this time only green nuclei remained (i.e. with histone-GFP from the Fo47 parent). To find out what had happened to the parental genomes during this process, we examined the chromosomal composition of the colonies at different stages. For each chromosome of both parental lines a specific primer pair was designed. Similar to what was found in an earlier study investigating HCT (Ma et al., 2010), after

redistribution of the fluorescent signal and the disappearance of *Fol*4287-derived nuclei, the colonies showed the markers for all chromosomes of *Fo*47 plus the marker for *Fol*4287 chromosome 14. In two cases, the marker for *Fol*4287 chromosome 12 was also detected. In these colonies the marker for the homologous chromosome from *Fo*47 (chromosome 10) was either missing or showed a weaker band (Fig. S4d). Taken together, these results indicate that heterokaryon formation, nuclear fusion in heterokaryons and (mostly uniparental) chromosome loss can lead to apparent horizontal transfer of genetic material from *Fol*4287 to *Fo*47.

We then wished to inspect this process in more detail by observing more examples and gaining a better time resolution. For this purpose we decided to simplify the selection process by using the anti-fungal drug resistance markers. We co-cultivated conidia from each line for two days in CAT medium supplemented with 0.25% xylose and incubated the mix on a PDA plate to allow for conidium formation for additional two days (=t0). Heterokaryotic conidia were then selected by growth on PDA plates supplemented with both hygromycin and zeocin for five days. About 1 in 300 conidia were double drug resistant. For microscopical analysis, up to ten colonies were transferred to CDL plates and nuclear composition was determined after one week (=t1). To ensure that always the youngest part of the colony was studied we monitored hyphae from the edge of the colony and transferred an agarose block from this region to a new plate and incubated for one additional week (=t2, t3...). Sixty percent of the double drug resistant colonies showed both red and green signals, either separately or co-localizing to the same nuclei to various degrees (*N* = 90, eight biological replicates). These were selected for further study. We

monitored nuclear and chromosomal composition at each time point until only one single fluorescent signal was observed.

One thing all the offspring had in common, in spite of growing on medium without anti-fungal drugs, is that especially during the first weeks of development the hyphae appeared stressed, showing high levels of vacuolization. In addition, many nuclei appeared fractionated and seemed to form micronuclei (Fig. 4, first and second panel). We performed Hoechst 33343 DNA staining at t1 to determine the localization of the H1-RFP and H1-GFP signals. Interestingly, we found that H1-RFP and H1-GFP sometimes co-localized with the DNA stain (Fig. 4, *1), sometimes only H1-GFP and rarely only H1-RFP localized with the DNA stain (Fig. 4, *2 and *3), and sometimes both co-localized to what seems to be a vacuole (Fig. 4, arrow head).

The progeny of heterokaryotic cells displayed a large variety of colony phenotypes. The growth rate of the colonies varied between 1 mm to >3 cm per week (Fig. S5a). In addition, some colonies had a patchy phenotype caused by higher branching, accelerated growth, or increased sporulation (Fig. S5b, from left to right). These characteristics are all distinct from the colony morphology of the parental lines. We followed the nuclear composition in 24 individual hybrid colonies on a weekly basis. As described earlier, at time point t1 all colonies showed red and green fluorescence, which either completely or partially co-localized (Fig. 4). Surprisingly, this image changed during development of the colonies. Red nuclei derived from Fol4287 became less abundant and the red fluorescent signal decreased in intensity. Also, over time H1-RFP co-localized less with DNA and more to what are likely to be vacuoles (Fig. 5). By t4 93% of the colonies contained only green nuclei, i.e. with H1-GFP derived from Fo47. Interestingly, this development was not always uniform across the colony. Where in one hypha there could still be a mix of red and green nuclei, in other hyphae only green nuclei were present (Fig. 5a t2–t5). Once red nuclei had disappeared, the hyphae of the colonies looked healthier, showing regularly shaped nuclei and vacuoles (Fig. 5a t6 and b t2).

To investigate whether the above-described development of heterokaryotic colonies can indeed lead to horizontal transfer of genetic material, we tested the chromosomal composition of these colonies at different time points by chromosome-specific PCR. Our hypothesis was that during the shift from red to green dominance chromosomes from Fol4287 would gradually disappear along with the H1-RFP marker. We tested colonies with only green nuclei and colonies with both red and green nuclei at time point t1. In general, if any were detected at all, bands for Fol4287 chromosomes were weaker than bands for Fo47 chromosomes (Fig. S4d). In two of the three tested colonies harboring only green nuclei the marker for Fol4287 chromosome 14 or both 12 and 14 was detected (colonies #2 and #4, Table 2). This suggests that transfer had already been completed. Alternatively, these chromosomes were still being eliminated at that moment.

We examined five individual colonies from three different pairings showing mixed nuclei at time point t1. In one of these colonies, despite detecting both red and green nuclei at all time points no markers for Fol4287 chromosomes were detected (colony #1, Table 2). Two other colonies contained mixed nuclei at time point t1, but only green nuclei were found at time points t2 and t3. Again no Fol4287 markers were detected (colonies #5 and #6, Table 2). The other two colonies both showed mixed nuclei at time points after t1. In these colonies markers for Fol4287 chromosomes 12 and 14 and chromosomes 10, 12 and 14 were found (colonies #3 and #7, Table 2). In colony #3 the marker for Fo47 chromosome 10 was not detected, and was possibly being replaced by the homologous Fol4287 chromosome 12. Interestingly, horizontal transfer always took place in one direction, with Fol4287 being the donor. Furthermore, horizontal transfer appears to always include at least parts chromosome 14.
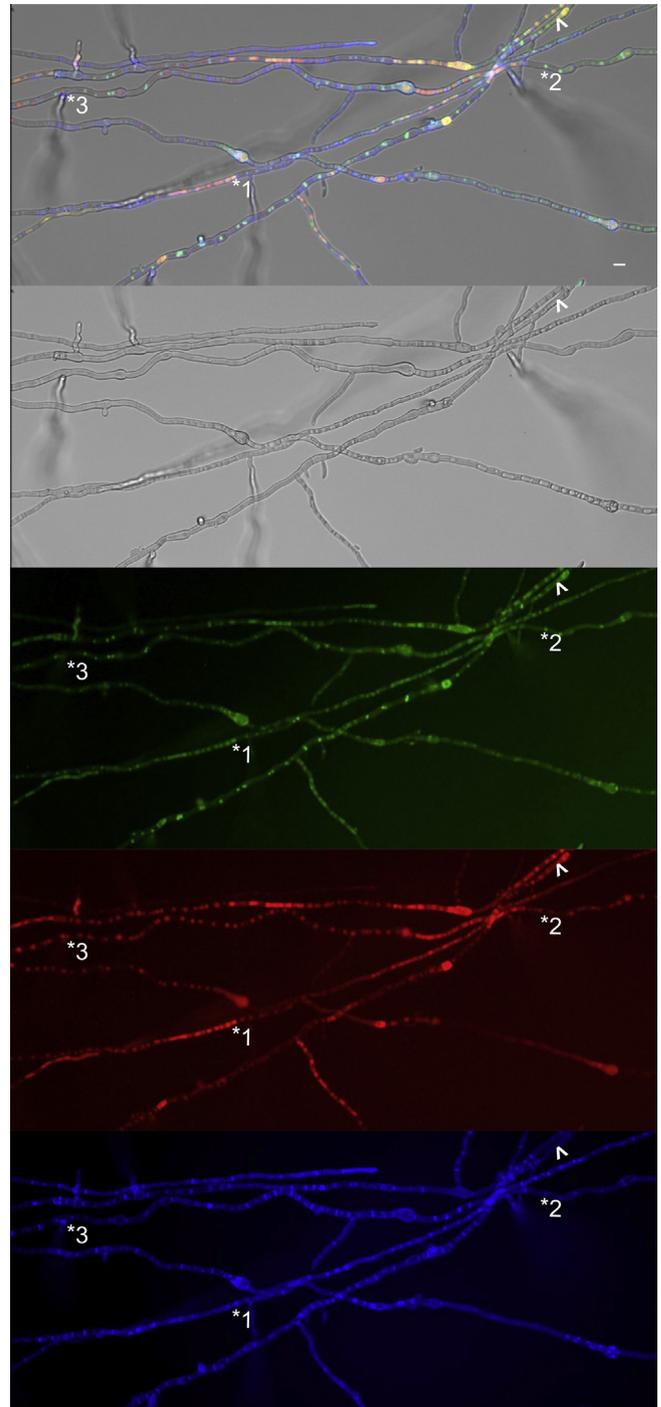


Fig. 4. Phenotype and localization of fluorescent histones in a heterokaryotic colony. In heterokaryotic colonies rearrangement of genomes and nuclear shuffling causes stress in the fungus, indicated by highly vacuolated hyphae. During this process nuclei often are smaller and appear as micronuclei. Red and green fluorescent histones co-localize in some cases either into the nucleus (*1) or a vacuole (arrowhead), in other cases only GFP (*2) or rarely RFP (*3) localizes to nuclei. From top to bottom: merged, bright field, GFP, RFP, Hoechst 33342 (DNA) staining. Colony at t1, scale bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 4. Discussion

This study was aimed to improve our understanding of the processes underpinning horizontal chromosome transfer in filamentous ascomycetes. Using live-cell imaging we demonstrated that conidial pairing of incompatible strains under carbon starvation
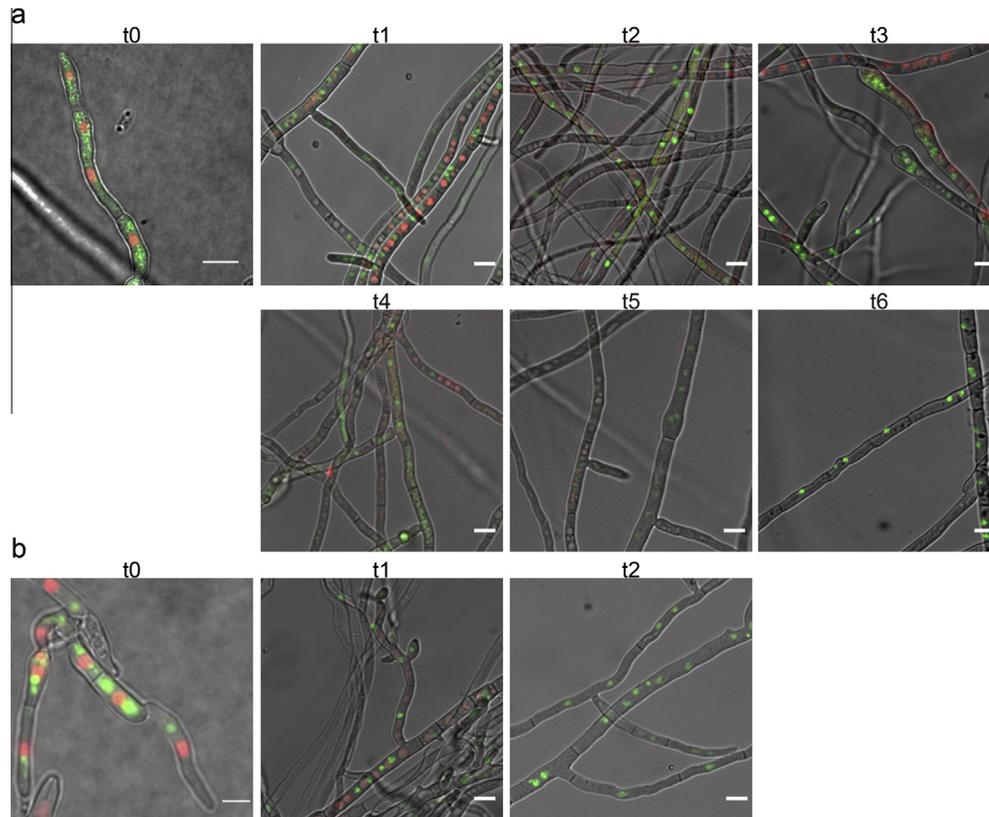
**Fig. 5.** Examples of development of hybrid offspring. Slow (a) and fast (b) transition from heterokaryotic cells with red dominant nucleus to colonies only harboring green nuclei. During this transition often micronuclei are formed (a t1 to t5). After completion green nuclei show a regular shape known from the parental lines (a t5 and b t2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 1**
CAT fusion and heterokaryon formation of *F. oxysporum* strains in different media.

| Parameter | Results [%] ($n$) in | | | |
|---|---|---|---|---|
| | H$_2$O | CAT (0.25% xyl) | CAT (0.5% xyl) | CAT (1% xyl) |
| *Fol*428 | | | | |
| Conidia | 91 ± 5 (1731) | 96 ± 4 (2283) | 95 ± 3 (718) | 99 ± 1 (302) |
| CATs | 8 ± 4 | 21 ± 10 | 37 ± 10 | 4 ± 2 |
| *Fol-Fol* CATs | 7 ± 4 | 19 ± 11 | 37 ± 11 | 4 ± 2 |
| *Fol-Fo* CATs | 1 ± 2 | 5 ± 4 | 2 ± 2 | 0 ± 0 |
| *Fo*47 | | | | |
| Conidia | 87 ± 6 (1265) | 97 ± 4 (1091) | 98 ± 2 (250) | 100 ± 0 (350) |
| CATs | 2 ± 3 | 6 ± 5 | 2 ± 2 | 1 ± 1 |
| *Fo-Fo* CATs | 1 ± 1 | 1 ± 2 | 0 ± 0 | 0 ± 1 |
| *Fol-Fo* CATs | 1 ± 2 | 5 ± 4 | 2 ± 2 | 0 ± 0 |

and nitrogen limitation can produce viable heterokaryotic cells in *F. oxysporum*. During development of heterokaryotic hyphae, nuclei of the parental lines presumably fuse as conidia with a single yellow nucleus are produced. This hybrid offspring then undergoes a progressive and gradual genome rearrangement, during which markers for most chromosomes of one parental strain (*Fol*4287) are lost, leaving hybrid offspring with the genomic background of *Fo*47 with the addition of markers for (a) transferred chromosome(s) from *Fol*4287.

We demonstrated that CAT fusion in *Fol* is restricted to carbon starvation and limited access to nitrogen (Fig. 1b). On the other hand, we did not observe self-anastomosis in the form of either hyphal or CAT fusion in *Fo*47 (Table 1), possibly caused by a loss-of-function mutation in a gene essential for anastomosis in this strain. These observations suggest that during vegetative growth

CAT fusion does not play an important role in colony initiation, as is the case for other fungi such as *N. crassa* (Roca et al., 2010, 2005a, 2005b; Simonin et al., 2012). CAT fusion may be a survival strategy of *Fol* to adapt to limited nutrient availability. One advantage could be a better distribution of nutrients through interconnected germlings. Alternatively, CAT fusion might have an additional and more specialized role in *Fo* by forming heterokaryotic cells to facilitate non-meiotic recombination, from which hybrids can emerge with new properties (e.g. the ability to colonize a certain plant species). Our results support the second role. First, CAT fusion is rarely detected between more than two conidia, which would not add much to the distribution of nutrients. Second, heterokaryon formation is observed during conidial but not hyphal tip pairing experiments (Table 1). Third, we show that new genotypes can emerge from heterokaryons.

**Table 2**
Presence of chromosome markers in heterokaryotic colonies at one-week intervals.

| Fol4287 | × Fo47 | Colony # | Time point | Growth diameter (cm) | GFP | RFP | 1 | 2/entry> | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Presence/absence of Fol4287 marker of chromosomes | | | | | | | | | | | | | | | Presence/absence of Fo47 marker of chromosomes | | | | | | | | | | | |
| Fol4287 H1-RFP (ble) #3 | Fo47 H1-GFP (hph) #10 | | t0 | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | |
| | | 7 | t1 | 2.8 | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | t2 | | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | 9 | t1 | 2.0 | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | t2 | | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | t3 | 1.4 | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| Fol4287 H1-RFP (ble) #3 | Fo47 H1-GFP (hph) #15 | | t0 | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | |
| | | 4 | t1 | 3.9 | + | + | – | – | – | – | – | – | – | – | – | – | – | + | – | – | – | + | + | + | + | + | + | + | + | + | – | + | + |
| | | | t2 | | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | – | + | + |
| | | | t3 | 2.9 | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | – | + | + |
| | | | t4 | 2.8 | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | – | + | + |
| | | | t5 | 3.4 | + | – | – | – | – | – | – | – | – | – | – | + | – | + | – | + | – | + | + | + | + | + | + | + | + | + | – | + | + |
| | | 6 | t1 | 1.6 | + | – | – | – | – | – | – | – | – | + | – | + | – | + | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | t2 | | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | t3 | 2.1 | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | – |
| Fo47 H1-gFP (hph) #10 | Fo47 H1-GFP (hph) #15 | | t0 | | | | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | | 1 | t1 | 2.7 | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | t2 | | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | 4 | t1 | 3.5 | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | t2 | | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | t3 | 3.9 | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | 5 | t1 | 3.9 | + | + | – | – | – | – | – | – | – | – | – | + | – | + | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | t2 | | + | – | – | – | – | – | – | – | – | + | – | + | – | + | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | t3 | 3.5 | + | – | – | – | – | – | – | – | – | – | – | + | – | + | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | t4 | 3.7 | + | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |
| | | | t5 | 3.8 | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | + | + | + | + | + | + | + | + | + | + | + | + |

In contrast to *Fol-Fol* self interactions, after CAT fusion between *Fol*4287 and *Fo*47 the fluorescently tagged histone proteins were not taken up into the nucleus of the fusion partner and nuclei from each parental strain remained distinct from one another (Figs. 2c and 3). We were surprised that despite cytoplasmic continuity, in an incompatible interaction histones encoded in one nucleus are apparently not taken up and integrated into nuclei of the fusion partner. Since during self-anastomosis exchange of nuclear proteins does take place, it appears that having two genetically different nuclei somehow prevents cross-uptake of histones. Asynchronous cell division and asynchronous nucleoprotein synthesis have been proposed as mechanisms for uniparental chromosome elimination in interspecific plant hybrid cells (Bennett et al., 1976; Gupta, 1969; Laurie and Bennett, 1989). In this study we have seen that in heterokaryotic conidia and germlings nuclei derived from the two parental lines undergo mitosis in an asynchronous manner (Fig. 3b). Similar to interspecies plant hybrids, this might either be the first step towards *Fol*4287 chromosome degradation or alternatively directed degradation of *Fol*4287 chromosomes might be initiated by the asynchronous cell division and/or nucleoprotein synthesis. Supportive of this idea are the differences in nuclear morphology we observed. Where red nuclei deriving from *Fol*4287 at this stage appeared healthy, green nuclei of *Fo*47 were fractionated (two or more nuclei) and sometimes blurry (Figs. 1a and 3b). This process may be related to a heterokaryon incompatibility reaction, but the germlings are viable and form colonies that can produce conidia in turn. However, these conidia contain a single yellow (i.e. red and green) nucleus (Fig. S4a), indicating that nuclei must have fused sometime during development. In a previous study we show that *F. oxysporum* essentially follows a multinucleate state after colony initiation but returns to a uninucleate state during sporulation (Shahi et al., 2015). Consistent with these findings, we suggest that prior to conidiation nuclei of *Fol*4287 and *Fo*47 can fuse in phialides to produce uninucleate conidia. Nuclear fusion did not take place during sporulation following CAT fusion, perhaps because the suppression of heterokaryon incompatibility reaction allows for multinuclearity also during conidiation. In the mature hyphae emerging from uninucleate spores (with histones from both parents), the multinucleate state is again 'activated' and genome rearrangements are apparently initiated.

In human-mouse hybrid cells spatial separation of the parental genomes takes place prior to the directed elimination of human chromosomes (Cieplinski et al., 1983; Sengupta et al., 2008; Wang et al., 2014). We observed formation of micronuclei in mature hyphae of colonies that emerged from yellow (i.e. red and green) nucleus conidia (Fig. S4d). In the beginning these micronuclei remain yellow, possibly still having copies of both parental chromosomes or taking up both histone variants. However, during the course of two to six weeks, a redistribution of the red and green fluorescent histones was observed (Fig. 5), suggesting that in hybrid offspring of *F. oxysporum* strains spatial separation of parental genomes may also precede chromosome degradation. Supportive of this idea is the decrease of the red signal over time until only green nuclei remain (Fig. 5). An interesting question that then arises is how the distinction between core chromosomes highly similar in DNA sequence can be made. As mentioned previously, *Fol*4287 and *Fo*47 share a core genome, which is highly conserved (Ma et al., 2010, Ma, personal communication). We have demonstrated that in the incompatible interaction *Fol*4287 always forms the CAT. The distinction between a "fuser" as the active and a "fusee" as the inactive partner could induce different signaling pathways, one of which might modify DNA or chromatin, allowing subsequent discrimination between chromosomes from different parents.

We used a PCR-based approach to identify markers for each chromosome of the two parental strains. Although we were able to detect markers for *Fol*4287 chromosome 14 and sometimes also 10 and 12 in the *Fo*47 background, the bands are weak compared to bands for *Fo*47 or *Fol*4287 chromosomes at time point t0 (Fig. S4f). We performed a single round of double drug resistant selection to prevent parental conidia from growing into colonies. Without further selection pressure, however, probably only a minority of the nuclei have incorporated genetic material of *Fol*4287 into the *Fo*47 genome and the detection of the markers appears close to the detection threshold. This would explain the apparent 'loss and gain' of *Fol*4287 markers during development of heterokaryotic colonies. Detection of a marker does not necessarily mean that the entire chromosome is present, but nevertheless indicates horizontal transfer of genetic material. However, in an earlier study, only transfer of entire chromosomes has been observed and chromosome transfer detected by markers was always confirmed by karyotyping (Ma et al., 2010).

As mentioned earlier, a parasexual cycle has been suggested to contribute to the high level of genetic variation in asexual fungi (Castro-Prado et al., 2007; Clutterbuck, 1996; Ishikawa et al., 2012; Milgroom et al., 2009). To our knowledge a parasexual cycle in *Fusarium* sp. has only been achieved by protoplast fusion and results in equal distribution of chromosomes from each parental line and recombination between them (Molnár et al., 1990; Teunissen et al., 2002). Our results however, demonstrate that HCT likely occurs through nuclear fusion before conidiation followed by gradual degradation of most chromosomes from the 'donor' parental strain. This suggests that mechanisms underlying HCT are distinct from parasexual recombination.

Heterochromatinization has been demonstrated to play a major role in uniparental chromosome elimination in interspecies plant hybrid cells (Gernand et al., 2005; Sanei et al., 2011) and directed DNA elimination in *Tetrahymena* sp. (Chalker, 2008). A future challenge will be to identify the different steps involved in directed chromosome elimination in *F. oxysporum*. For example, it will be interesting to study the nature and role of micronuclei and chromatin marks in HCT.

## Acknowledgments

## 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.2016.03.003.

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