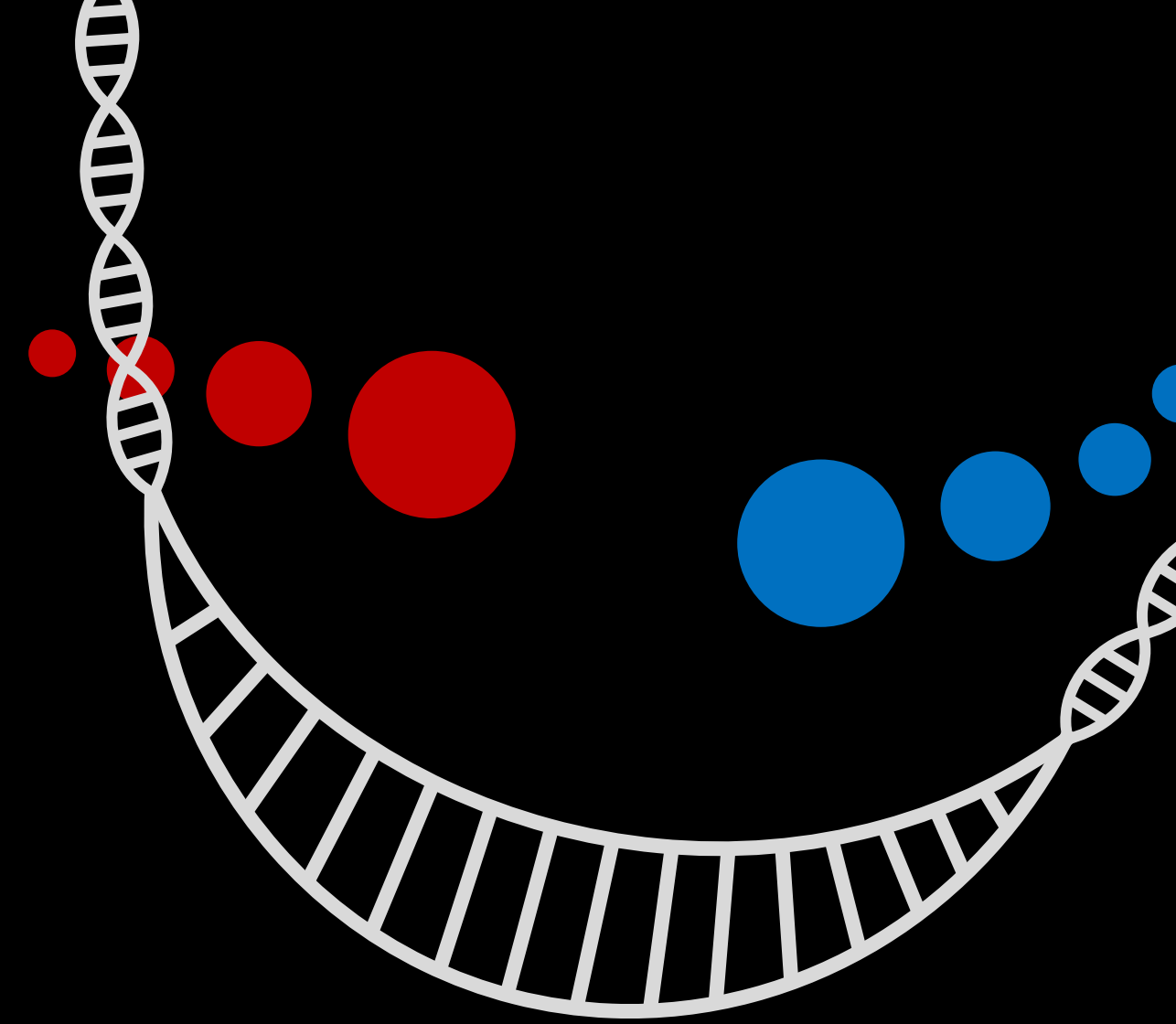

Dynamic genome organization in an arbuscular mycorrhizal fungus

Jelle van Creij

Propositions

1: Isolates of *Rhizophagus irregularis* cannot strictly be categorized as either homokaryotic or dikaryotic.
(this thesis)

2: Nuclei of *Rhizophagus irregularis* isolate C3 can undergo genetic recombination.
(this thesis)

3: Working with model organisms is a necessary evil that should be avoided if possible.

4: The desire to provide definitive answers to long-standing questions can leave us blind for alternative theories.

5: Lab-grown food production will benefit the environment at the cost of social equity.

6: Debating the uninformed is as unpleasant as it is necessary.

Propositions belonging to the thesis, entitled

Dynamic genome organization in an arbuscular mycorrhizal fungus

Jelle van Creij
Wageningen, October 3rd, 2023

Dynamic genome organization in an arbuscular mycorrhizal fungus

Jelle van Creij

Thesis committee

Promotor
Prof. Dr Ton Bisseling
Professor at the Laboratory of Molecular Biology
Wageningen University and Research

Co-promotor
Dr Erik Limpens
Assistant professor at the Laboratory of Molecular Biology
Wageningen University and Research

Other members
Prof. Dr Duur Aanen, Wageningen University and Research
Prof. Dr Daniel Croll, University of Neuchâtel, Switzerland
Dr Sandra Smit, Wageningen University and Research
Prof. Dr Maarja Öpik, University of Tartu, Estonia

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Thesis

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Thesis Committee appointed by the Academic Board

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Jelle van Creij

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Chapter 1

General introduction

Genetic variation is a key driver of evolution. It allows organisms to adapt differently to environmental factors, and to pass on these adaptations to their progeny. As such, it is the precursor to speciation. Fundamentally, evolution can only occur when a selective pressure can act on a diverse population, and thereby favor those within that population that are better adapted to that pressure [Darwin, 1859]. Adaptation requires the acquisition of new traits through genetic variation that increase the fitness of an organism in a certain environment.

Genetic variation can be induced within an individual by small scale, random mutations such as single nucleotype polymorphisms (SNPs), insertions, or deletions. The effects of such mutations depend on where in the genome they occur: mutations disrupting coding sequences can functionally knock out important genes, while mutations in non-coding or repetitive regions may go unnoticed. Genetic variation can also be induced on a larger scale, resulting for example from genome rearrangements. All these mechanisms contribute to evolution of genomes, that can help an organism to adapt to varying selection pressures [Seidl and Thomma, 2014].

However, to avoid the accumulation of deleterious mutations and to generate novel genetic combinations, horizontal exchange of genetic information between organisms is typically required [Muller, 1932]. This allows for the implementation of novel genetic variants in different genomic backgrounds. For example, bacteria can exchange genetic material through conjugation, involving direct cell-to-cell connections between two cells. Through conjugation, genes that are involved in antibiotic resistance or virulence can be transferred at a high rate within populations of bacteria [Waldor and Mekalanos, 1996; Friesen *et al.*, 2006]. Horizontal gene transfer also occurs in eukaryotes, although its contribution to genome evolution is generally underappreciated compared to that in prokaryotes [Keeling and Palmer, 2008]. This is in part because the most common method for exchanging genetic material in eukaryotes is through sexual reproduction [Goddard, 2016]. The majority of eukaryotes reproduce sexually, providing an advantage over organisms that purely reproduce asexually [McDonald *et al.*, 2016]. Besides enriching the population for beneficial mutations, it also purges the genome of detrimental mutations [Birky, 1996]. Meiosis is the driving mechanism behind sexual reproduction [Schurko *et al.*, 2008]. During meiosis, somatic cells divide and split into haploid daughter cells, called gametes. Gametes from different parental somatic cells can subsequently fuse, after which the cell returns to a diploid stage. During meiosis, homologous chromosomes pair with each other and can recombine to exchange genetic information. As a result the gametes will be genetically different from their parents and novel allelic effects can be introduced. As such, meiotic recombination is a vital process for the introduction of genetic variation within a species.

While in the kingdoms of Animalia and Plantae the classical process of sexual reproduction and meiotic recombination is found in most species, the Fungal kingdom shows a wider variety of types of sex [Nieuwenhuis and James, 2016]. In a considerable number of fungi, possibly up to 20%, no sexual cycle has ever been observed [Heitman *et al.*, 2007; Seidl and Thomma, 2014]. In the absence of sex and meiotic recombination, different types of exchange of genetic information are needed to renew genetic variation and purge the genome of deleterious mutations.

An important group of fungi for which no sexual cycle has ever been observed are the arbuscular mycorrhizal (AM) fungi [Smith and Read, 2003]. These fungi form symbiotic interactions with the majority of vascular land plants, and primarily reproduce by forming asexual spores. For a long time, they were considered to be ancient asexual scandals, as a lack of sexual reproduction would be expected to lead to the accumulation of deleterious mutations. However, recent advancements in genetic analysis have revealed that some kind of sexual reproduction may be possible in these fungi. In this chapter, I will introduce the genetic composition of AM fungi, focusing on the model species *Rhizophagus irregularis*, and illustrate some of the mysteries surrounding this species that formed the basis of this thesis.

Multinucleate fungi

A characteristic of most filamentous fungi is their ability to host multiple nuclei within a common cytoplasm [Weld *et al.*, 2006]. Depending on the species, some fungi, like Ascomycota and Basidiomycota, form hyphae that contain septa, separating the hyphae into cell-like compartments. These septa can be closed or open, allowing the flow of organelles and nuclei between compartments [Roper *et al.*, 2011]. Fungi in the Dikarya subkingdom contain two nuclei per cell (dikaryons) during several parts of their life cycle [Hibbett *et al.*, 2018]. In others, such as Mucoromycotina and Chytridiomycota, septa are (mostly) lacking, resulting in highly coenocytic hyphae with many nuclei coexisting in a shared cytoplasm [Bonfante and Venice, 2020; Mela *et al.*, 2020]. Furthermore, fungal hyphae can fuse, or anastomose (also called plasmogamy), to create a highly interconnected network. This can allow the horizontal transfer of nuclei, or other genetic material, between individuals. As a result genetically different nuclei can occupy the same cell for extended times. The presence of two or more genetically distinct nuclei within a cell is referred to as heterokaryosis [Strom and Bushley, 2016].

Heterokaryosis has fascinated fungal biologists since its first discovery [Burgeff, 1913] because of its implications for fungal adaptation, competition or co-operation between nuclei, as well as the biotechnological applications of heterokaryons [Strom and Bushley, 2016]. Containing multiple genetically different nuclei has been shown to enhance the phenotypic plasticity of fungi [Jinks *et al.*, 1952; Caten and Jinks, 1966; Strom and Bushley, 2016] and may increase the performance of strains via heterosis. Furthermore, it is thought to contribute to fungal virulence and host range of fungal pathogens [Roper *et al.*, 2011]. Selection might act at different levels, ranging from individual nuclei, to groups of nuclei or to the whole mycelium level [Lakovic and Rillig, 2022].

Upon heterokaryosis, several things can happen when two compatible nuclei fuse (karyogamy). In a sexual cycle the two nuclei fuse and undergo meiosis, resulting in recombined haploid spores. In asexual fungi, instead of meiosis a so-called parasexual cycle can occur. First described by Pontecorvo *et al.* (1956), the parasexual cycle involves mitotic recombination between chromosomes followed by repeated chromosome loss through mitotic non-disjunction to return to the haploid, or aneuploid, stage. Like meiotic recombination, mitotic recombination increases the genetic diversity among the nuclei [Seidl and Thomma, 2014]. Other mechanisms that increase genetic variation, in addition to mutations, may involve the exchange of chromosomes between nuclei [Dutcher, 1981; Ma *et*

al., 2010; Vlaardingerbroek *et al.*, 2016] or (inter)chromosomal rearrangements due to the activity of transposable elements [Daboussi, 1997; Torres *et al.*, 2020]. It should be noted that several fungal species that were initially considered to be asexual were later found to have hidden or cryptic sexual cycles [De Jonge *et al.*, 2013]. These fungi may only undergo a sexual cycle under specific environmental conditions, that are not easily reproducible in the lab. This complicates the distinction between sexual and truly asexual fungi.

Arbuscular mycorrhizal fungi

One group of intriguing fungi for which a heterokaryotic nature may be especially relevant and whose genetic make-up has been a matter of debate for several decades, are the AM fungi [Smith and Read, 2010]. These fungi are part of the Glomeromycota subphylum of the Mucuromycota [Spatafora *et al.*, 2016], sister clade to the Dikarya, and are arguably the most successful plant symbionts [Van der Heijden *et al.*, 1998].

These soil fungi form symbiotic interactions with the majority of vascular land plants [Smith and Read, 2010; Öpik *et al.*, 2013]. This interaction is ancient, as traces of AM fungi have been found in fossils of the earliest land plants [Remy *et al.*, 1994; Redecker *et al.*, 2000; Martin *et al.*, 2017]. It has therefore been hypothesized that AM fungi played an important role in the colonization of the land by plants approximately 450 million years ago, by providing their hosts access to scarce nutrients in the bare soil environment [Redecker *et al.*, 2000; Martin *et al.*, 2017]. AM fungi form a vast mycelial network, which scavenges the soil for nutrients. It transports these nutrients (most importantly phosphorus and nitrogen) to the host plants, in return for lipids that the fungus cannot make itself and sugars [Smith and Smith, 2011; Luginbuehl *et al.*, 2017]. This obligate biotrophic nature of AM fungi makes them more difficult to study as they cannot be grown without a host plant.

The AM symbiosis allows plants to grow in otherwise nutrient poor environments, as the fine fungal mycelium can more effectively collect nutrients from the soil than plant roots. In addition, AM fungi can help plants to deal with numerous stresses, such as abiotic (for example salinity or drought) or biotic stresses both above and below ground [Smith and Read, 2010]. Nutrient exchange takes place in specialized hyphal structures called arbuscules, from which these fungi get their name. Arbuscules are tree-like highly branched fungal hyphae that form within root cortex cells [Luginbuehl *et al.*, 2017; Wang *et al.*, 2017; Wipf *et al.*, 2019]. They are surrounded by a specialized host-derived membrane, the peri-arbuscular membrane, where specific transporter proteins accumulate to facilitate the exchange of nutrients with the host [Ivanov *et al.*, 2019]. In Chapter 2, I give a more detailed overview of the symbiotic interaction, with particular focus on the fungal side of the interaction [van Creij *et al.*, 2020].

Genomic variation in AM fungi

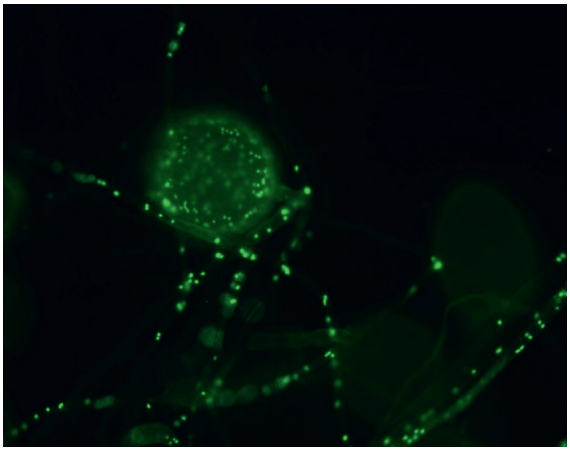

Figure 1: *Nuclei, stained with SytoxGreen, in the mycelium and spores of AM fungus* Rhizophagus irregularis.

In line with the genetic organization of other Mucoromycota, the coenocytic mycelium of a single AM fungus consists of one vast, interconnected body of cytoplasm, containing thousands to millions of nuclei that migrate freely through the mycelium [Marleau *et al.*, 2011, Figure 1]. Upon damage, AM fungi have the capacity to close off branches of their hyphae by forming septa, but usually the whole mycelium is connected by the same continuous cytoplasm. In contrast to some well-studied Mucoromycota species such as Mucor or Rhizopus that have a sexual cycle [Blakeslee, 1904; Lee and Idnurm, 2017], no known sexual stage has ever been observed for AM fungi. Moreover, no stage during the life cycle of AM fungi has been found where only one nucleus generates the next generation. Therefore, AM fungi were considered to be ancient asexual scandals for a long time [Judson and Normark, 1996]. They propagate primarily by forming multinucleate chlamydospores. These spores are formed by protruding from the cytoplasm, after which they are filled with nearby haploid nuclei that flow in from the mycelium [Jany and Pawlowska, 2010; Marleau *et al.*, 2011]. Depending on the AM species, the number of nuclei per spore can vary from several hundred to thousands of nuclei [Bécard and Pfeffer, 1993; Kokkoris *et al.*, 2021]. The spores can separate from the parental colony to find new hosts or they may reconnect to the parental colony by hyphal fusion [Giovanetti *et al.*, 2001; Sbrana *et al.*, 2011]. After the spore germinates, nuclei multiply through mitosis and occupy the newly formed mycelium [Sward *et al.*, 1981; Bécard and Pfeffer, 1993; Bianciotto and Bonfante, 1993]. In the life cycle of AM fungi, the spore is the stage where the least amount of nuclei are present in an individual, making spore formation currently the strongest known genetic bottleneck in these fungi. The large number of nuclei present in the communal cytoplasm of AM fungi has been the subject of interest and heavy debate in the fungal genetics community [Pawlowska and Taylor, 2004; Croll and Sanders, 2009; Sanders and Croll, 2010; Corradi and Brachmann, 2017]. Measurement of the DNA content of AM fungi initially generated widely variable values, ranging from 15Mbp to over 1000Mbp per nucleus [Hosny *et al.*, 1998; Hijri and Sanders, 2004]. This led to the speculation that some AM genomes may be highly polyploid [Pawlowska and Taylor, 2004]. This was further fueled by the observation of high levels of genetic variation between fungal individuals and their offspring [Sanders *et al.*, 1995; Lanfranco *et al.*, 1999; Bever *et al.*, 2001; Jansa *et al.*, 2002; Young, 2005; Stockinger *et al.*, 2010; Boon *et al.*, 2015; Wyss *et al.*, 2016]. Such variation was observed in rDNA sequences, which in other species are typically highly homologous due to concerted evolution, as well as in some protein coding genes [Sanders *et al.*, 1995; Pringle *et al.*, 2000; Sanders and Croll,

2010]. However, the polyploidic nature was debated by others, who suggested that the genetic variation might be explained by a high level of heterokaryosis [Kuhn *et al.*, 2001; Hijri *et al.*, 2005]. Such a situation also occurs for example in the heterokaryotic Basidiomycete pathogen *Rhizoctonia solani*, which was reported to maintain 6 to 15 genetically divergent nuclei within its mycelium [Sneh *et al.*, 1991; Hane *et al.*, 2014].

A highly diverse population of nuclei may carry enough genetic variation to maintain a healthy mycelium. After all, a shared cytoplasm may also mean a shared transcriptome, where a subset of nuclei maintain the fungus, giving other nuclei the chance to differentiate and diverge. Moreover, AM fungi can exchange nuclei through anastomosis, which would allow for more genetic diversity to be introduced into the population [Bever, 2008]. Hyphal fusion and exchange of nuclei has indeed been observed between closely related, yet genetically distinct, AM fungi [Croll *et al.*, 2009; Jany and Pawlowska, 2010; Barreto de Novais *et al.*, 2017]. However, the genetic factors that determine the genetic compatibility between AM fungi are not well understood and the fate of nuclei that are exchanged is also unknown.

***Rhizophagus irregularis*, an AM fungal model species**

In order to investigate whether genetic variation exists between lineages of AM fungi, many species and strains have been isolated in the past. The ability to culture these fungi axenically on Ri T-DNA transformed root cultures (perpetually developing roots caused by *Agrobacterium rhizogenes* transformation) [Becard *et al.*, 1988] allowed the *in vitro* culturing of pure colonies [Figure 2]. With this setup AM fungi could be propagated axenically, which made crossing and co-culturing experiments possible in a controlled laboratory environment. Among the first to be isolated was *Rhizophagus irregularis* (previously called *Glomus intraradices* or *Glomus irregulare*), which proved to be a suitable model organism for the study of AM fungi, since it grew relatively quickly and developed many spores in this culture system [Chabot *et al.*, 1992]. The first isolate, DAOM197198 (previously called DAOM181602), was isolated in Pont Rouge, Canada, and has been in culture since then [Stockinger *et al.*, 2009]. Besides serving as a model isolate, it is also commercially available as a biological fertilizer. Later, several other isolates of *R. irregularis* were obtained from a field in Tänikon, Switzerland [Koch *et al.*, 2004]. These isolates were obtained as single spores, and named after the locations in the grid used to isolate them. Interestingly, these isolates were shown to differ in the amount of plant growth response they induced on carrot (*Daucus carota*) [Koch *et al.*, 2006].

Co-culturing of these different isolates of *R. irregularis* can lead to successful anastomosis and the production of offspring spores that showed biparental inheritance of molecular markers [Croll *et al.*, 2009]. Progeny spores also showed traits that differed from their parental lines, such as variation in hyphal growth or spore density, suggesting that nuclear exchange may have led to novel phenotypes. In general 1-5% of hyphal contacts between genetically different isolates resulted in perfect fusion events, while ~50% of fusions appeared successful among individuals of the same isolate [Croll *et al.*, 2009]. Under natural conditions plants are typically colonized by multiple different AM fungal strains, which opens up the possibility of novel interconnections. However, how often hyphal fusions and exchange of nuclei truly occurs in natural settings between genetically different strains is currently not known.

Figure 2: *Hyphae and spores of* Rhizophagus irregularis *growing on* Daucus carota *root cultures [published in "Le peuple microbien", Laurent Palka, 2002].*

This work was followed up by Angelard *et al.* (2010), who studied the effect of axenically propagated single spore lines derived from crossing two genetically different parental lines (isolates D1, C2 and C3 [Koch *et al.*, 2004]) on plant growth upon symbiosis. Individual single spore lines varied in the ratio of different molecular markers, based on amplified fragment length polymorphism (AFLP) analyses, suggesting that segregation of genetically different nuclei, called nucleotypes, occurred upon spore formation. However, the use of AFLP analyses could not show the origin of the polymorphisms. For example, appearance or disappearance of specific AFLP markers could also be caused by chromosomal rearrangements due to activity of transposable elements or due to technical limitations. The simple sequence repeat marker Bg112 showed similar shifts in frequencies, although the reliability of this marker has also been questioned [Lin *et al*., 2014; Kokkoris *et al*., 2020]. Later, the same group generated single spore lines originated from isolate C3, and confirmed segregation of the Bg112 marker, suggesting the segregation of alleles partitioned on different nuclei [Ehinger *et al.*, 2012; Masclaux *et al*., 2018]. Different progeny lines showed difference in growth traits and effects on plant growth in *Plantago lanceolata* and rice [Angelard *et al.,* 2010; Ehinger *et al*., 2012]. Intriguingly, some progeny lines could even increase rice growth five-fold compared to the parental lines under mycorrhized conditions. This result highlights the importance of understanding the genetic composition of AM fungi. Understanding and exploiting AM genetics has the potential to unravel and steer the still largely enigmatic mechanisms that control mycorrhizal growth responses in plants.

If nucleotype ratios play a role in adaptation to different environments, it could be expected that different environments, such as a different host plant species, may affect these ratios either through selection or genetic drift [Angelard *et al.*, 2014; Limpens and Geurts, 2014]. Indeed, upon a shift in host plant identity from carrot to potato, significant changes in AFLP profiles were observed in parental lines as well as single spore progeny lines [Angelard *et al.*, 2014]. Since allelic variants of AM fungi are located on different haploid nuclei, these observations suggest a shift in the frequency of nucleotypes. Such shifts in nucleotype frequencies have the potential to increase the phenotypic plasticity and thereby the adaptability of the fungus to different environments or hosts. Shifts in nuclear ratios have also been reported in other heterokaryotic fungi in response to the environment [James *et al.*, 2009; Zhang *et al.*, 2019] and have even been observed in different parts of fungal mycelium [Lakovic and Rillig, 2022]. Therefore heterokaryons are thought to be advantageous in variable environments, which may be relevant for the extremely wide host range of AM fungi [Scott *et al.*, 2019].

Insights from AM fungal genome sequencing

The highly heterokaryotic view on genetic variation within AM fungi was challenged with advances in genome sequencing technologies. Initially, efforts to assemble the genome of the model fungus *R. irregularis* DAOM197198 by a large international consortium failed [Martin, 2008]. The use of genetic material from multiple sources likely contributed to the introduction of unwanted variation, making the assembly or a genome difficult. A new effort, using clean fungal material from axenic root cultures, resulted in the first whole genome sequence of an AM fungus [Tisserant *et al.*, 2013]. Around the same time, a single nucleus sequencing strategy was used by Lin *et al.* (2014), resulting in a parallel genome sequence of this isolate. Both studies revealed a total genome size of ~150 Mb, which was well in line with genome size measurements based on flow cytometry [Sędzielewska *et al.*, 2011] , though the assemblies were still fragmented. The genome size was among the largest known in all fungal species [Dallaire and Paszkowski, 2022]. Transposable elements (TEs) made up a significant amount (~40%) of the genome.

Functional annotation of the genome revealed a large gene repertoire, one of the highest in the Fungal kingdom, a trait possibly linked to the large amount of hosts AM fungi can colonize. Later, RNAseq studies on DAOM197198 colonizing different hosts, and showed that the fungus expressed genes in a host-dependent manner [Kamel *et al*., 2017; Zeng *et al*., 2018]. These genes included putative secreted proteins, with potential functions as effector proteins mediating host defense mechanisms to facilitate mycorrhization [Aparicia Chacon *et al.*, 2023; Chapter 2]. Furthermore, Zeng *et al*. (2018) showed stage-specific gene expression in DAOM197198, with different genes being active in germinating spores, extraradical mycelium, intraradical mycelium, and arbuscules. These findings showed a remarkable spatial regulation of gene expression, especially considering the free movement of nuclei and the lack of individual cells. Such spatial regulation has been shown in other heterokaryotic fungi [Roper *et al.*, 2011; Mela *et al.*, 2020]. In *Aspergillus niger*, for example, mRNA was shown to accumulate heterogeneously in different parts of the cytoplasm [Levin *et al.*, 2007; de Bekker *et al.*, 2011]. In the heterokaryotic fungus *Agaricus bisporus* nucleus-specific regulation of

transcription was shown that changed during the development of different tissues [Gehrmann *et al*., 2018]. The spatial regulation of nuclei activity is likely instrumental in orchestrating the simultaneous interaction of AM fungi with different hosts and environments.

The DAOM197198 genome assemblies detected a surprisingly low level of polymorphisms in the genome. Ultimately, both studies concluded that DAOM197198 was homokaryotic, meaning they contained a largely homogeneous population of genetically highly similar haploid nuclei. SNPs were found at a frequency of 0.43 SNPs/kb, much lower than would be expected in heterokaryotic fungi [Tisserant *et al.*, 2013]. In comparison, known true heterokaryotic fungi such as *R. solani* and *Coccidioides immitis* have SNP frequencies of 15.9/kb and 23.7/kb, respectively [Hane *et al.*, 2014, Neafsey *et al.*, 2010]. Therefore, DAOM197198 lacked the inter-nucleus variation that was expected for a true asexual organism that could depend on its nuclear variety for adaptation.

Significant genetic variation was however found in the polymorphic ITS regions of the 45S rDNA locus between nuclei [Lin *et al.*, 2014], consistent with previous analysis of these loci [Sanders *et al.*, 1995; Pawlowska and Taylor, 2004; Pringle *et al.*, 2000; Jansa *et al.*, 2002; Thiéry *et al.*, 2016]. An improved genome assembly of isolate DAOM197198 later revealed a unique organization of rDNA loci [Maeda *et al.*, 2018]. In the vast majority of eukaryotes, ribosomal DNA copies are highly homologous due to concerted evolution, and are often used as universal barcoding marker for fungi [Schoch *et al.*, 2012]. Typically, ribosomal DNA copies can be found in long stretches of tandem repeats, containing hundreds of copies [Eickbush *et al.*, 2007]. Remarkably, DAOM197198 was shown to contain only 10 or 11 copies, located as single copies throughout the genome [Maeda *et al.*, 2018]. This type of organization has also been seen in the malaria parasite *Plasmodium falciparum*, which, like AM fungi, interact with multiple, taxonomically distantly related host species [Gardner *et al.*, 2002]. The heterogenetic nature of the rDNA copies raises the possibility for variation at the translational level. However, the effect of genetic variation within these loci on ribosomal activity is still unclear.

Besides novel insights into the biology of the symbiotic interaction, genome sequencing efforts also changed the view on the presumed asexual nature of AM fungi. Halary *et al.* (2011) and Tisserant *et al.* (2013) found a complete set of meiosis-machinery encoding genes in the genome of *R. irregularis*. The conservation of these genes suggests that they are still active, and may have a role in the sensing of compatible nuclei and meiosis, after a sexual or parasexual cycle [Corradi and Brachmann, 2017; Sanders, 2018; Reinhardt *et al.*, 2021]. Soon after, the genomes of more isolates of *R. irregularis* were characterized, and these revealed a higher level of intraspecies variety in some isolates [Wyss *et al.*, 2016]. Genome wide restriction site associated DNA sequencing (RAD-seq) of the isolates obtained by Koch *et al.* (2006) showed high amounts of isolate specific SNPs, illustrating the divergence between different strains of *R. irregularis*.

Ropars *et al*. (2016) assembled the genomes of five these isolates, which revealed that these indeed differed in genetic variation. Whereas isolates A1, B3 and C2 had low SNP contents similar to DAOM197198, isolates A4 and A5 had a higher SNP content (~0.8 SNPs/kb). The isolate A4 was highly similar to isolate C3, both of which were isolated from the same field in Switzerland [Koch *et al*., 2004]. RAD-seq could not distinguish these two isolates, suggesting that they might be clonal lines, possibly even being two spores derived from the same parental mycelium [Wyss *et al.*, 2016]. Furthermore, the frequencies of SNPs in the A4 and A5 isolates suggested a bi-allelic distribution, where the majority of alleles were found in equal ratios [Ropars *et al*., 2016]. This indicated a dikaryotic-like organization. Conversely, the other isolates showed patterns corresponding to a largely homozygous, homokaryotic genome. In dikaryons, nuclei can carry one of either mating type (MAT) loci [Casselton and Olesnicky, 1998]. Indeed, nuclei of the two *R. irregularis* dikaryote-like isolates were found to carry a divergent MAT locus [Ropars *et al*., 2016]. In other fungi, MAT loci each encode for genes involved in nuclear pairing and maintenance of dikaryosis [Casselton and Olesnicky, 1998; Meltin *et al.*, 2021; Wang *et al.*, 2021]. The MAT locus in *R. irregularis* contained two homeodomain (HD) genes with homology to HD genes that are typically found in the MAT locus of basidiomycetes [Ropars *et al.*, 2016]. Divergent MAT-alleles encoded different homeodomain (HD) proteins, the pairing of which is a precursor to sexual development [Banham *et al.*, 1995]. This suggested the existence of a potentially heterothallic, bipolar mating system in AM fungi. Together with the conserved meiotic genes and suggestions of recombination signatures in population studies [Vandenkoornhuyse *et al.*, 2001; Croll and Sanders, 2009], this strongly hints to the existence of a hidden reproductive cycle. At which point this could occur, and whether it would be sexual or parasexual, is still unknown.

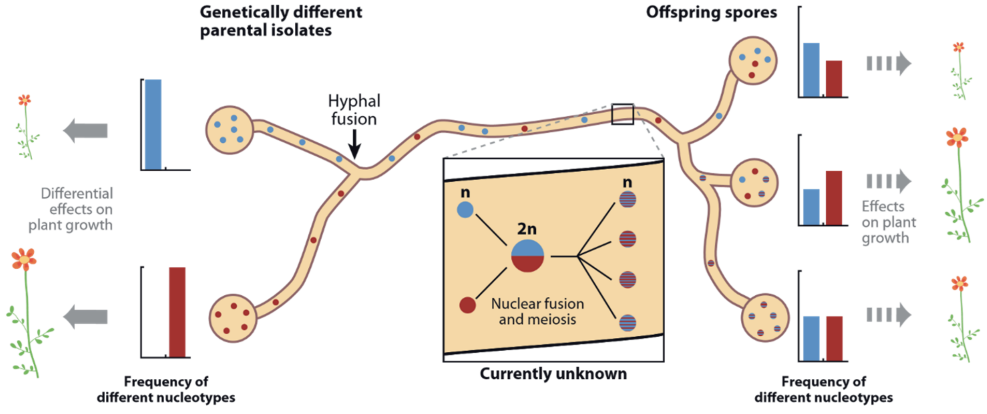

Figure 3: *Proposed model for exchange of nuclei between different AM fungal isolates (Figure from Sanders and Croll, 2010). Two homokaryotic parental isolates undergo anastomosis, and form a dikaryotic colony, with dikaryotic spores. Parental lines and their offspring may induce different MGR, depending on nuclear ratios of the fungus. After exchanging nuclei, nuclear fusion and meiosis may happen, although this has never been observed.*

Further comparisons of the genomes of the different isolates revealed surprisingly large differences in genome content [Chen *et al.*, 2018; Morin *et al.*, 2018]. Isolates were found to encode for different amounts of genes, and contained many lineage specific genes, next to the shared "core" genes that all isolates had. Strikingly, only around 50% of the gene repertoire was shared between isolates. Furthermore, TE content and families were also highly divergent between isolates. These two observations may be linked, as TE activity is known to contribute to large re-arrangements of genomes [Bowen and Jordan, 2002]. They also beg the question: can these isolates, many of which originated from the same field in Switzerland, even still be considered the same species [Bruns *et al.*, 2018]? It was postulated that they perhaps should be viewed as a species complex, much like fungal pathogens like *Fusarium*, where closely related members are similar in appearance yet at the genomic level large differences can be found [Sumerell, 2019; Reinhardt *et al.*, 2021].

These discoveries illustrate two vastly different types of genomic composition in *R. irregularis*. Homokaryons like DAOM197198 possess only one type of nucleus, with one type of MAT locus and low levels of genetic polymorphisms. Dikaryotes, like A4, C3, and A5, contain two main nucleotypes marked by different MAT loci. They might be derived from the fusion of two compatible homokaryons to form a stable dikaryotic offspring [Figure 3]. However, while nuclear exchange has been seen after anastomosis of different isolates [Croll *et al.*, 2009], these did not yield stable novel lineages. These findings suggest that AM fungi cannot solely depend on plasmogamy and nuclei exchange as sources of genetic variation. Conversely, concrete evidence of mating in dikaryotic isolates like C3 and A4 is also lacking. However, the conservation of core meiosis genes and the existence of a bipolar MAT-locus system in some isolates suggest that mating can happen in these species. No stage in the lifecycle of AM fungi has been found where mating may occur, and the only observed form of reproduction is still the formation of asexual spores. This raises the question of where in the developmental cycle of *R. irregularis* mating could happen. Does it occur in a particular, uncommon occasions upon the meeting of two compatible homokaryons? Or can it happen within a dikaryon, between nuclei with compatible MAT-loci? If karyogamy ever occurred clear signs of recombination between co-existing genotypes should be expected. Furthermore, the effects of nuclei dynamics on fitness and host response of AM fungi are still poorly understood. Since individual nuclei of *R. irregularis* are haploid, they may encode for allelic variants of genes that allow them to adapt to different hosts more efficiently than homokaryons that lack such allelic variants.

Outline of the thesis

The role of the two nucleotypes of dikaryote-like *R. irregularis* isolates is still poorly understood. Previous research suggested that nuclei ratios may shift during development, but the causes and effects of these shifts on the genomic composition, transcriptional activity, and their contribution to the adaptation to different host plants or environments are largely unknown.

In this thesis, I use whole genome, mycelium metagenome, single spore, single nucleus, and transcriptome sequencing to study the genomic architecture of the dikaryotic-like *R. irregularis* isolate C3 and the influence of different host plants on its nuclear organization. C3

was chosen as a research isolate, because it was shown to have a relatively high biallelic SNP content compared to other isolates. Furthermore, previous work indicated nuclear drift between lineages. These traits make it an ideal model isolate to study changes in nuclei ratios between lineages.

In **Chapter 2,** I present an overview of the molecular mechanisms behind mycorrhizal symbiosis [van Creij *et al.*, 2020]. There, I focus on the fungal side of the interaction, with an emphasis on signaling molecules that trigger symbiotic signaling, fungal effectors influencing host immune responses, and nutrient exchange with the host. Furthermore, I summarize several findings in the field of AM fungal genetics that were published since the beginning of this thesis.

When this research started, the closest genome assembly for C3 was a fragmented, Illumina-based assembly of isolate A4. Using PacBio SMRT Sequel II sequencing, we set out to produce a new genome assembly specific for C3 in **Chapter 3** [van Creij *et al.*, 2023], which allowed us to more accurately study genetic variation in this isolate. I used this assembly to study genetic variation in different C3 lineages, including single spores and single spore lines. There, I found an unusually dynamic genetic organization, where nuclei ratios varied between lineages and even more between individual spores in a seemingly stochastic manner. Sequencing of single nuclei of C3 provided evidence of internucleus genetic recombination between the two nucleotypes. With strict filtering, I found evidence of recombination events, based on the exchange of haploblocks that were correlated to MAT-loci in other nuclei.

Besides shifts in nuclei ratios based on genomic DNA, we also observed host-dependent shifts in total mRNA allele ratios in C3 grown with different hosts. In **Chapter 4**, I used mRNA sequencing of C3 colonizing four hosts to functionally analyze these shifts. The included host species were monocot chives (*Allium schoenoprasum*), legume Medicago (*Medicago truncatula*), and Solanaceae model species Nicotiana (*Nicotiana benthamiana*) and tomato (*Solanum lycopersicum*). I complemented this dataset with mRNA sequencing data from DAOM197198 from the same hosts, in order to make intraspecies comparisons. There, I found that the two isolates differ in their host-dependent gene expression. Measurement of host growth responses showed that chives benefited more from interacting with DAOM197198 than with C3. Furthermore, C3 was shown to express a variety of alleles in a host-dependent manner, indicating different contributions of both nucleotypes depending on the local environment of the fungus. Moreover, I found evidence of host-dependent expression of rRNA alleles in DAOM197198, but not in C3.

Finally, in **Chapter 5** (the general discussion), I discuss our results in the light of the considerable advances in the field of AM fungal genomics in the last five years. I highlight the remarkably dynamic nuclei ratios and activity of C3 compared to other AM fungal isolates, and discuss the implications of genetic recombination in the life cycle of these species. Together, this thesis highlights that even within the same species, AM fungi may utilize a variety of different strategies to adapt and evolve.

Chapter 2

Arbuscular mycorrhiza, a fungal perspective

Jelle van Creij[1], Peng Wang[1,2], Erik Limpens[1]

1. Laboratory of Molecular Biology, Department of Plant Sciences, Wageningen University & Research, Droevendaalsesteeg 1, Wageningen, The Netherlands
2. Current affiliation: Hunan Normal University, Changsha, China

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Introduction

Arbuscular mycorrhizal (AM) fungi are arguably the most successful endosymbionts of plants, forming an integral part of the life of most land plants under natural conditions [Brundrett and Tedersoo, 2018]. They offer a range of benefits to their plant partners such as improved access to scarce nutrients in the soil (especially phosphate and nitrate), enhanced water uptake and water-use efficiency, resistance to abiotic (drought, salinity), protection from pathogens and herbivores, and general soil improvement including effects on the soil microbiome. This symbiosis originated >400 million years ago and has since been maintained in the majority of land plants. It is therefore of major ecological importance and potential for sustainable agriculture [Rodriguez and Sanders, 2015; Strullu-Derrien *et al.*, 2018].

AM fungi are soil fungi belonging to the Glomeromycotina, a subphylum of Mucoromycota [Spatafora *et al.*, 2016]. Currently, ~300 AM fungal species have been described based on morphological characteristics, although molecular data suggest that this number may be vastly underestimated [Öpik and Davison, 2016]. AM fungi are globally distributed and in general there is very little host-specificity in their interaction with plants, although host preferences may exist [Savary *et al.*, 2018].

Most insights into the molecular aspects of AM symbiosis to date have been obtained from the plant side of this interaction. Particularly, molecular genetic studies in the model legumes *Medicago truncatula* and *Lotus japonicus* have provided a wealth of information on the plant components controlling this key symbiosis. Compared to this, insight into the fungal aspects has largely fallen behind. This was mostly caused by the lack of genomic information of these fungi and the inability to genetically manipulate them. In recent years however, major progress in this direction has been made, with the first elucidations of an AM genome sequence from *Rhizophagus irregularis* strain DAOM197198 [Tisserant *et al.*, 2013; Lin *et al.*, 2014]. Currently, genome assemblies are available for 6 isolates of *R. irregularis* as well as for the AM species *R. clarus*, *R. diaphanus*, *R. cerebriforme* (Glomerales) and *Diversispora epigaea* (*Glomus versiforme*) and *Gigaspora rosea* (Diversisporales) [Ropars *et al.*, 2016; Chen *et al.*, 2018a,b; Maeda *et al.*, 2018; Kobayashi *et al.*, 2018; Sun *et al.*, 2019; Morin *et al.*, 2019].

These genome data have started to provide insight into the molecular components governing AM biology. In this chapter, we will highlight and address several of these recent molecular insights, specifically from the model AM fungus *R. irregularis*, such as communication with the plant host, (reciprocal) nutrient exchange, and the genetic make-up of AM fungi.

The *R. irregularis* life cycle

AM fungi are obligate biotrophs that need a living plant to be able to fulfill their life cycle. They are globally present in the soil both as spores and vast mycelium networks [Figure 20.1A]. Although AM fungal spores can germinate in the absence of a plant host, hyphal growth is only limited and rapidly stops if the fungus does not sense the presence of a plant. Upon the perception of plant-derived signals, the fungal hyphae show enhanced branching and growth, thereby enhancing their chance to reach a plant root. After contact, the fungus forms a hyphopodium on atrichoblasts of the root epidermis from which it penetrates the root.

In response to signal exchange (see below), the plant cell reorganizes its cytoskeleton to form a specialized secretory structure, called the pre-penetration apparatus (PPA), that facilitates the entry of the fungus into the cell [Genre *et al.*, 2011]. Genome annotation has shown that AM fungi lack most cell wall degrading enzymes [Tisserant *et al.*, 2013; Morin *et al.*, 2019]. Therefore, they rely on the plant to weaken and break down the cell wall, after which they become enveloped by the plant plasma membrane. When the fungal hyphae reach the root cortex, they mainly grow in between the cells (Arum-type colonization), and/or spread in a cell-to-cell manner (Paris-type) depending on the plant species [Kubota *et al.*, 2005]. Ultimately, they reach the inner cortical cells where they enter and form highly branched hyphal structures, called arbuscules [Figure 20.1B and C]. There they become surrounded by a specialized plant membrane (the peri-arbuscular membrane) creating a symbiotic interface where primary exchange of nutrients takes place [Gutjahr and Parniske, 2013; Harrison and Ivanov, 2017]. Recently, detailed electron microscopy combined with tomography showed that the interface is more complex than previously thought. Extensive tubular and vesicular extracellular membranes, either secreted as vesicles or extrusions of the plasma membranes, were observed in the peri-arbuscular space. Furthermore, extensive, interconnected tubular fungal plasma membrane extensions were observed in the paramural space of arbuscules and intraradical hyphae [Ivanov *et al.*, 2019; Roth *et al.*, 2019]. Such paramural structures are not restricted to AM fungi, as similar structures have been observed in a wide range of fungi, where they are called lomasomes or plasmalemmasomes and thought to play a role in nutrient absorption. Extracellular vesicles have also been associated with defense responses [Limpens, 2019].

Arbuscules are relatively short lived structures that degenerate after a couple of days. It is currently unknown why arbuscules degenerate or which of the two partners initiates their degeneration. It has been shown that the life-time of arbuscules can be controlled by the host plant [Kobae *et al.*, 2010, 2014; Floss *et al.*, 2017], which likely gives the plant a means to prevent the interaction from becoming parasitic if the fungus does not provide sufficient nutrients. This hypothesis is supported by the observation that a lack of phosphate delivery, due to the mutation of an arbuscule-specific plant phosphate transporter or a proton pump that fuels it, leads to the premature collapse of arbuscules [Krajinski *et al.*, 2014; Wang *et al.*, 2014; Figure 20.1D].

After the first establishment of arbuscules, fungal hyphae continue to spread through the root and form (lipid-filled) storage structures called vesicles. Colonization of the root continuously occurs via intraradical mycelium (IRM) that go on to make new arbuscules, as well as extraradical mycelium (ERM) and spores that establish new infection sites. As the fungus grows it can be in contact with multiple host plant species at the same time. Similarly, a plant is often colonized by several different AM fungi [Berruti *et al.*, 2013], creating an underground network through which plants are interconnected and nutrients and signals can be exchanged [van der Heijden *et al.*, 2015]. Generally, the hyphal network forms a continuous cytoplasm through which millions of nuclei migrate, although the fungus can make septa to seal off stressed or damaged parts of the hypha. It was shown that nuclei fill up the spores by migrating from the hypha into the developing spores as they are being formed [Marleau *et al.*, 2011]. As a result, the spore typically contains several hundreds of nuclei, and there is never

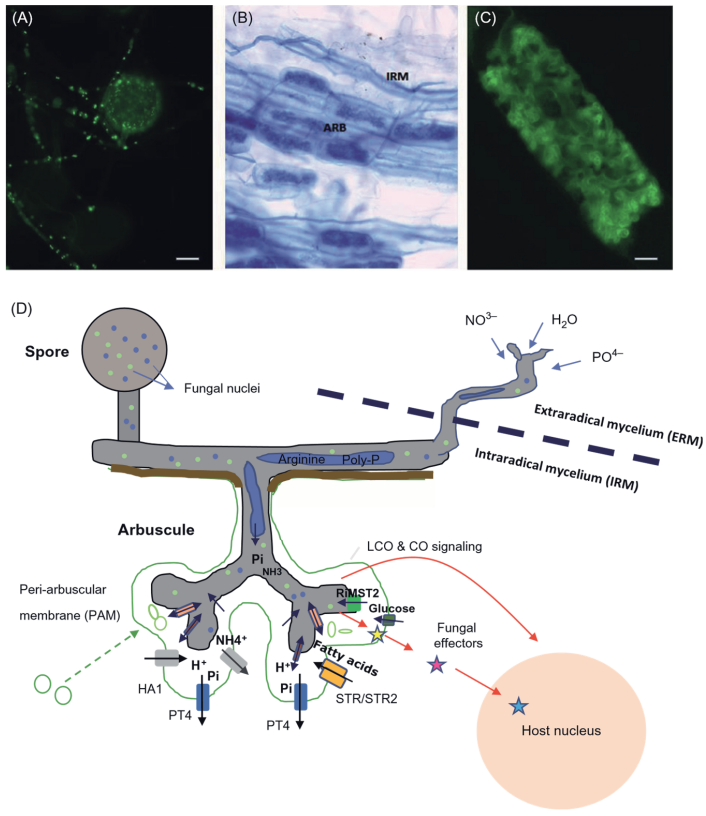

FIGURE 20.1 Arbuscular mycorrhizal lifestyle. (A) *Rhizophagus irregularis* spores and mycelium stained with SytoxGreen to mark the nuclei. A single spore typically contains hundreds of nuclei and the coenocytic mycelium forms a shared cytoplasm in which millions of nuclei migrate. (B) Trypan blue stained *Medicago truncatula* root colonized by *R. irregularis*. IRM = intraradical mycelium, i.e. fungal hyphae growing mostly in between cortical cells. ARB = arbuscules. (C) Confocal image of an Arbuscule where the peri-arbuscular membrane is marked with a green-fluorescent plasma membrane marker. (D) Schematic drawing of various aspects of Arbuscular mycorrhiza, for details see main text.

a single nucleus stage that generates the next generation [Sanders and Croll, 2010; Fig. 20.1A]. Genetically related AM fungi have been shown to be able to undergo anastomosis (hyphal fusion) and exchange nuclei, however a sexual stage or conclusive evidence for meiosis has not yet been found. The current ideas on the genetic make-up of AM fungi are discussed further on in this chapter.

Communication with the host plant

Communication between both partners starts before the fungus contacts the plant root through the exchange of signal molecules. The plant root secretes among others flavonoids, 2-hydroxy fatty acids and strigolactones, that are perceived by the fungus [Bécard *et al.*, 1995; Nagahashi and Douds, 2011; Nadal and Paszkowski, 2013]. Best studied is the role of strigolactones, which stimulate the germination and branching of hyphae and trigger the activation of mitochondria and metabolism in the fungus [Akiyama *et al.*, 2005; Besserer *et al.*, 2006, 2008]. In addition to their role as symbiotic signals perceived by AM fungi, strigolactones are also endogenous plant hormones that control plant architecture. Besides strigolactones, the plant hormones ethylene and cytokinin may also be perceived by AM fungi. Ethylene was shown to promote AM spore germination and hyphal growth and homologs of plant ethylene and cytokinin receptors have been identified in the genome of *R. irregularis* [Herivaux *et al.*, 2017]. However, the involvement of these receptors in plant hormone perception still needs to be assessed.

An N-acetylglucosamine transporter called NOPE1 has been identified in rice and maize and was shown to be required for the induction of symbiotic transcriptome changes in the fungus by root exudates [Nadal *et al.*, 2017]. NGT1, a homolog of this transporter in *Candida albicans*, is required for the uptake of N-acetylglucosamine to control proliferation, signaling, and virulence [Alvarez and Konopka, 2007]. Therefore, it is hypothesized that NOPE1 may transport a still-to-be-identified molecule that is perceived by AM fungi to prime them for symbiosis [Nadal *et al.*, 2017].

(Lipo-)chitooligosaccharides

In turn, AM fungi secrete diffusible molecules that trigger responses in the plant, such as the induction of calcium oscillations in and around the nucleus (known as calcium spiking), stimulation of lateral root formation, activation of symbiosis-specific genes, and formation of the PPA. The best studied AM signals are chitin-like lipo-chitooligosaccharides (myc-LCOs) and short chain chitooligosaccharides (COs), together called myc-factors [Maillet *et al.*, 2011; Genre *et al.*, 2013]. LCOs consisting of β-1-4-linked N-acetyl glucosamine residues with an acyl chain at the non-reducing residue were first identified in rhizobium bacteria. These are called Nod factors and are essential to initiate a nitrogen-fixing endosymbiosis with legume plants. Several decades of research have led to the identification of a plant signaling pathway that is activated by Nod factors and required for both the rhizobium as well as AM symbiosis. Therefore, this pathway has been called the common symbiotic (SYM) signaling pathway [Parniske, 2008; Oldroyd, 2013]. In line with its essential role in establishing an AM symbiosis, the common SYM pathway is conserved in all AM host plants and has typically been lost in non-host plants such as *Arabidopsis thaliana* [Delaux *et al.*, 2014; Bravo *et al.*, 2016].

R. irregularis produces a mix of sulfated and non-sulfated tetrameric and pentameric LCOs mostly acylated with either an oleic acid (C18:1) or palmitic acid (C16:0) [Maillet *et al.*, 2011]. Although various genes encoding chitin synthases and deacetylases are present in the fungal genome, it is currently unknown how lipid-modified LCOs are made. Direct homologs of the rhizobial *nod* genes, such as *nodC* which encodes an enzyme that attaches the fatty acyl chain to the GlcNac backbone, have not been found in the fungal genome.

In addition to LCOs, short-chain chito-oligosaccharides (COs) lacking an acyl chain have been proposed to play a role in AM symbiosis [Genre *et al.*, 2013]. Tetrameric and pentameric COs were identified in germinated spore exudates at markedly higher concentrations than myc-LCOs, and their secretion was induced by the application of strigolactones [Genre *et al.*, 2013]. Such short-chain COs are also able to trigger calcium oscillations in epidermal cells of legume as well as in non-legume roots. Like myc-LCO signaling, CO-induced signaling is dependent on the SYM pathway.

Myc-LCOs can stimulate mycorrhization in a variety of plant species at sub-nanomolar concentrations, indicating that they are efficiently perceived by a broad range of plants. Myc-LCOs are thought to be perceived by LysM domain containing receptor-like kinases (LysM-RLK), in analogy to the perception of rhizobial Nod factors. In the model legumes *M. truncatula* and *L. japonicus*, Nod factors are perceived by a complex of a LysM-domain containing receptor containing a functional kinase domain (MtLYK3/LjNFR1) and one with a non-

functional kinase domain (MtNFP/LjNFR5). Although these receptors have been implicated in AM symbiosis [Maillet *et al.*, 2011; Czaja *et al.*, 2012; Zhang *et al.*, 2014a,b], knock-out mutants are still able to establish an AM interaction. This suggests that additional (redundant) receptors are involved or that additional fungal signals are able to activate the SYM pathway. In rice, a single LysM-RLK, called OsCERK1, was shown to be essential for establishing an AM interaction [Miyata *et al.*, 2014; Zhang *et al.*, 2014a,b]. Interestingly, OsCERK1, like its ortholog Arabidopsis CERK1, is also required for chitin-triggered immune responses. This raises the question how a plant distinguishes between beneficial AM fungi and fungal pathogens [van Zeijl *et al.*, 2015; Zipfel and Oldroyd, 2017]. OsCERK1 likely forms a complex with an NFP/NFR5type LysM receptor and it is the specificity of the complex that may determine whether symbiotic responses or defense is activated. In line with this, it was recently shown in *M. truncatula* and *L. japonicus* that paralogues of the Nod factor receptors, MtLYK9/MtLYR4 and LjLYS6, are required for chitin/chitooligosaccharide-triggered immune responses [Bozsoki *et al.*, 2017]. This indicates that the duplication and subsequent divergence of LysM-RLKs led to the emergence of distinct roles in symbiosis [De Mita *et al.*, 2014; Gough *et al.*, 2018]. Double mutants of MtNFP and MtLYR4 show a much more drastic reduction in mycorrhization [Giles Oldroyd, personal communication], which suggests that both myc-LCOs and short-chain COs are used symbiotic signals. Additionally, AM fungi can actively suppress the chitin-triggered immune response through the secretion of effector proteins (see below).

Effectors

Another class of signal molecules released by AM fungi are (small) secreted proteins that act as potential effector proteins. In plant-pathogen interactions 'effectors' are defined as pathogen-secreted proteins that modulate host plant defense response and enable colonization of plant tissue [Hogenhout *et al.*, 2009]. In nature, plants are constantly exposed to multiple micro-organisms, ranging from pathogenic and commensalistic to symbiotic. The cell wall of AM fungi contains several molecules, such as chitin and β-glucans, that are well characterized general fungal molecule-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs), which induce host defense responses called PAMP-triggered immunity [Lo Presti *et al.*, 2015; Fesel and Zuccaro, 2016; Rovenich *et al.*, 2016]. This raises the question how AM fungi manage to intracellularly colonize such an extremely broad range of plants (>80% of land plants).

The first evidence that AM fungi secrete effector proteins to manipulate the immune system of the host was provided in 2011, when it was shown that *R. irregularis* secretes a protein, called Secreted Protein 7 (SP7), that was able to translocate to the plant cell nucleus [Kloppholz *et al.*, 2011]. It was shown to interact with a pathogenesis-related transcription factor, ERF19, in *M. truncatula* roots, and could interfere with ERF19-dependent defense gene induction. In line with this, expression of RiSP7 in the hemibiotrophic fungal rice pathogen *Magnaporthe oryzae* was able to increase the biotrophic phase of this interaction. Cell- and stage-specific transcriptome analyses have shown that SP7 is mainly expressed in the extracellular hyphae in close contact with the root, but not or hardly when the fungus grows inside the root [Zeng *et al.*, 2018]. This suggests that it mainly plays a role at the first contact at the epidermis. The occurrence of KEX2 protease cleavage sites in SP7 have led to the

suggestion that it may in fact be cleaved before secretion, raising the hypothesis that the resulting peptides may be biologically active [Kamel *et al.*, 2017], however evidence is currently lacking. Intriguingly, follow-up work on the role of SP7 is AM symbiosis has suggested that SP7 may affect the phosphate starvation response in plants [Natalia Requena, personal communication]. It will be interesting to see whether this would be a strategy used by the fungus to make the plant more reliant on the fungus to obtain phosphate.

With the release of the first *R. irregularis* genome sequence, the number of potential effector candidates was increased significantly, with >600 predicted secreted proteins (SPs) [Tisserant *et al.*, 2013; Lin *et al.*, 2014; Toro and Brachmann, 2016; Kamel *et al.*, 2017; Zeng *et al.*, 2018]. It should be noted that the number of SPs varies between studies because the genome assemblies (gene models) and prediction pipelines that were used differ. A detailed analysis of the stage- and host species-dependent expression profile of secreted *R. irregularis* proteins was performed by Zeng *et al.* (2018). Laser microdissection combined with RNA-seq was used to monitor the expression of SP encoding genes in germinating spores, ERM, IRM, and arbuscules. Furthermore, expression was analyzed in three different host plants (*Allium schoenoprasum, M. truncatula* and *Nicotiana benthamiana*) to determine whether the fungus uses a distinct set of effectors in the interaction with different host plants. This work revealed the expression of ~300 RiSPs that could be reliably confirmed by RNA-seq data. Several of these SPs were predicted to be targeted to the nucleus, suggesting that they may be translocated to the host nucleus similarly to SP7. The majority (85%) of the SPs was more or less equally expressed in the interaction with all three plant hosts, however a subset was expressed in a host-dependent manner. A similar observation of putative host-dependent expression of effectors was made by Kamel *et al.* (2017), who monitored SP expression in *M. truncatula*, *Brachypodium distachyon* and the liverwort *Lunularia cruciata*. Host-dependent expression of such putative effectors raises the hypothesis that they may play a role in controlling symbiotic efficiency and adaptability to different hosts. A comparison of the genome sequences of 6 different *R. irregularis* isolates and 5 AM species further indicated substantial variation in the repertoire of candidate effector proteins [Chen *et al.*, 2018a,b; Morin *et al.*, 2019].

Unraveling the function of the putative effectors in AM symbiosis is a major challenge. This is especially hampered by the fact that AM fungi currently cannot be stably transformed [Helber and Requena, 2008]. As an alternative, host-induced gene silencing (HIGS; Nowara *et al.*, 2010) has been successfully used in some studies to silence AM fungal genes, although the efficiency of this method appears to vary significantly. Using this method, to date three additional effectors have been identified that influence AM symbiosis.

Tsuzuki *et al.* (2016) searched for effector proteins that are up-regulated in germinating spores in response to strigolactone perception. This identified 11 putative effectors among which strigolactone-induced putative secreted protein 1 (SIS1). HIGS of SIS1 in *Agrobacterium rhizogenes* transformed root cultures of *M. truncatula* resulted in suppression of colonization and the formation of stunted arbuscules. A role in efficient intraradical colonization fits with its high expression in IRM as well as in arbuscules [Zeng *et al.*, 2018]. The molecular mechanism by which SIS1 controls fungal colonization is currently not known.

Another effector from *R. irregularis* that showed induction upon strigolactone perception consisted of a signal peptide followed by a single LysM domain, and was named secreted LysM protein (RiSLM) [Schmitz *et al.*, 2019; Zeng *et al.*, 2019 unpublished]. Stage-specific expression analyzes showed that it is most highly expressed in the IRM. LysM domain containing effectors have mostly been studied in pathogenic fungi, where they were shown to bind chitinoligosaccharides [Sáchez-Vallet *et al.*, 2015]. Such effectors, containing one to three LysM domains, were reported to protect fungal hyphae from plant chitinases and/or to interfere with chitin-triggered immunity. Purification of RiSLM in combination with binding studies confirmed that it can also bind both long-(CO7/8) and short-chain COs (CO4) and that it also binds both sulphated and non-sulphated myc-LCOs [Zeng *et al.*, 2019]. Furthermore, bioassays showed that it can both protect fungal hyphae against hydrolysis by plant chitinases and interfere with chitin-triggered immunity, analogous to pathogenic LysM effectors. HIGS of RiSLM in *M. truncatula* hairy roots caused a significant reduction in fungal colonization [Zeng *et al.*, 2019, unpublished]. This indicates that LysM effectors are a common strategy used by both symbiotic and pathogenic fungi to colonize plants. Despite its ability to bind short-chain COs or myc-LCOs, co-application of RiSLM did not significantly block the induction of symbiotic gene expression. This is likely caused by a higher affinity of the symbiotic receptor complexes for myc-LCOs. The affinity of RiSLM for COs is in the same range as reported for chitin immune-receptors, raising the question how it can efficiently interfere with chitin-triggered immunity. A comparison of RiSLM homologs in different AM species and *R. irregularis* isolates showed a strikingly high level of variation in the protein sequence and indications of positive selection [Schmitz *et al.*, 2019; Zeng *et al.*, 2019]. Besides LysM effectors, Crinkler (CRN) effectors represent a well-known class of effectors that was first identified in pathogenic oomycetes and is also found in AM fungi [Lin *et al.*, 2014; Voß *et al.*, 2018]. These effectors are characterized by a highly conserved N-terminal LFLAK domain, consisting of a highly conserved LxLFLAK motif and a neighboring DWL domain [Torto *et al.*, 2003]. The LFLAK domain is required to translocate CRNs into the plant cell where they exert their function in the host nucleus [Schornack *et al.*, 2010]. Different functions have been reported for CRNs, which may be derived from the various C-terminal domains, ranging from induction of cell death to protection form biotic and abiotic stresses [Torto *et al.*, 2003; Liu *et al.*, 2011; Rajput *et al.*, 2015; Zhang *et al.*, 2015]. In *R. irregularis* at least 42 putative CRN homologs were identified [Lin *et al.*, 2014; Voß *et al.*, 2018]. Of these only a small set contains a signal peptide. Similarly, in oomycetes only a small subset of CRNs contain a signal peptide, and the ones without one are believed to be subject to alternative secretion pathways [Haas *et al.*, 2009; Gaulin *et al.*, 2018]. One of the *R. irregularis* CRNs containing a signal peptide, RiCRN1, was shown to be significantly more highly expressed in IRM and arbuscules. It was shown to form homodimers and to localize to nuclear bodies suggesting that, similarly to other CRNs, it functions in the plant nucleus [Voß *et al.*, 2018]. Suppression of RiCRN1 expression via HIGS caused a reduction of root colonization. Surprisingly, ectopic expression of RiCRN1 led to a drastic reduction in arbuscule number and size, indicating a role in arbuscule formation and development. Therefore, it was suggested that a tight regulation of RiCRN1 expression is critical for proper arbuscule development. Clearly, many more exciting insights into the role of effector proteins in AM symbiosis are expected to be revealed in the future.

Nutrient exchange

AM fungi are obligate biotrophs that need a living plant to full-fil their life cycle. By providing mineral nutrients to their host they obtain fixed carbon in return. This exchange of nutrients has been compared to a market setting, where reciprocal rewarding between both partners is thought to stabilize the symbiosis [Kiers *et al.*, 2011]. In *M. truncatula* it was shown that a fungus that is less cooperative, i.e. provides less phosphate, gets less carbon from the plant than a more cooperative fungus. In turn, when the plant gives less carbon to the fungus, the fungus provides less phosphate to the plant [Kiers *et al.*, 2011; Fellbaum *et al.*, 2014]. Modeling approaches have suggested that reciprocal exchange may be the result of the two partners competing for the same nutrient sources [Schott *et al.*, 2016]. Subsequent modeling provided a basic thermodynamic model that may explain the underlying market forces, with a key role for proton pumps to fuel the nutrient exchanges [Dreyer *et al.*, 2019]. How this reciprocal rewarding works in (complex) natural settings is a major question in AM ecology [Walder and van der Heijden, 2015]. A study where a shared AM hyphal network was established between flax and sorghum showed that depending on the AM fungal species, the plant that provided most carbon to the network did not obtain the most phosphate/nitrogen in return [Walder *et al.*, 2012]. A proper understanding of the molecular components controlling nutrient exchange in both plant and fungus as well as colonization levels will therefore be required to better understand this key aspect of the symbiosis.

From fungus to plant

Phosphate

Fungal hyphae form a vast and dense mycelial network and are much better able to explore the soil for scarce mineral sources than the relatively large plant roots. Plants can only take up soluble inorganic orthophosphate (Pi), but most phosphate in the soil in present in bound forms, with large parts as organic P (i.e. phytate). Extraradical hyphae of AM fungi have been shown to exhibit both phytase and acid phosphatase activity to mineralize organic P [Wang *et al.*, 2017a,b]. Although acid phosphatases are expressed in the extraradical hyphae, phytase-encoding genes have not been reported in AM fungi. It is thought that fungal hyphae-associated bacteria are responsible for the enhanced phytase activity. Indeed, it was shown that AM hyphal exudates can promote the growth of phytase-producing bacteria [Zhang *et al.*, 2014a,b, 2016].

Mineralized Pi is subsequently taken-up by the fungus through the activity of Pi transporters. Proton(H^1/Pi)- and sodium(Na1/Pi)-coupled Pi transporters have been identified in AM fungi [Harrison and van Buuren, 1995; Maldonado-Mendoza *et al.*, 2001; Tisserant *et al.*, 2012, 2013; Fiorilli *et al.*, 2013]. The first AM H^1/Pi transporter was identified in *G. versiforme* and it was shown to be primarily expressed in extraradical hyphae [Harrison and van Buuren, 1995]. Corresponding H^1-ATPases, providing the force to drive the uptake of Pi across the extraradical hyphal membrane, have been subsequently identified [Ferrol *et al.*, 2000; Requena *et al.*, 2003]. Many of the fungal Pi transporters are also expressed in the arbuscules. This may indicate a competition between plant and fungus at the interface to control the amount of Pi delivered, or a role in phosphate secretion from the arbuscules [Xie *et al.*, 2016; Ferrol *et al.*,

2019]. The functional involvement of a high-affinity fungal Pi transporter, expressed throughout the IRM and ERM, was only recently revealed in *Gi. margarita* [Xie *et al.*, 2016]. HIGS of phosphate transporter GigmPT led to reduced colonization and premature collapse of arbuscules. Interestingly, the same study revealed that GigmPT can function both as Pi transporter as well as Pi sensor, forming a transceptor in analogy to the phosphate transceptor Pho84 in yeast. Silencing of GigmPT affected PHO- and PKA-dependent Pi-signaling. Furthermore, it was shown that increased carbon availability induced the expression of GigmPT in extraradical hyphae under low phosphate conditions, corresponding with increased P transfer to the host.

Once taken up, Pi is polymerized into polyphoshate (poly-P) in the vacuoles [Ezawa *et al.*, 2002; Figure 20.1D]. This likely occurs by the vacuolar transporter chaperon complex (VTC), which controls poly-P synthesis in yeast. Several VTC components are upregulated when Pi is applied to extraradical AM hyphae [Kikuchi *et al.*, 2014]. The poly-P is then transported to the intraradical hyphae via the mobile vacuolar system and cytoplasmic streaming [Uetake *et al.*, 2002]. Recently it was shown that water transport across the plasma membrane in the intraradical hyphae by the aquaporin RcAQP3 of *R. clarus* influences poly-P transport [Kikuchi *et al.*, 2014]. Virus-induced silencing of RcAQP3 as well as the reduction of host transpiration reduced the rate of poly-P translocation from extraradical to intraradical hyphae. This suggests that host transpiration drives water transport through the hyphae.

A major question that remains is how phosphate is released by the fungi to be taken up by the host plant at the arbuscules. It is thought that poly-P is hydrolyzed inside by the fungus inside the root [Kikuchi *et al.*, 2014], after which it is transported across the vacuolar membrane, possibly by the vacuolar Pi transporter PHO91 [Tisserant *et al.*, 2012]. Subsequently the Pi is transported across the fungal plasma membrane into the peri-arbuscular space from which it is taken up by specific phosphate transporters into the plant [Ferrol *et al.*, 2019; Fig. 20.1D]. The identity of the proposed candidate Pi efflux transports however is still unknown. Relevant in this aspect, a H1-coupled Pi transporter HcPT2 from the ectomycorrhizal fungus *Hebeloma cylindrosporum* was shown to be involved in the export of Pi towards *Pinus pinaster* roots [Becquer *et al.*, 2018; Dreyer *et al.*, 2019]. Alternatively, a role for a plasma membrane localized VTC complex and export of poly-P followed by hydrolysis by a plant acid phosphatase in the peri-arbuscular space has been proposed [Ezawa and Saito, 2018]. Additionally, the exchange of vesicles may be involved in the transport of nutrients [Ivanov *et al.*, 2019; Roth *et al.*, 2019].

Nitrogen

Besides providing phosphate, AM fungi also play a key role in nitrogen (N) supply to host plants [Garcia *et al.*, 2016; Wipf *et al.*, 2019]. The fungal ERM can take up inorganic ($NH4^1$ or $NO3^2$) or organic nitrogen (amino acids/short peptides). AM fungi contain a few high affinity nitrate transporters (NRTs) [Tian *et al.*, 2010; Koegel *et al.*, 2015] as well as ammonium transporters [López-Pedrosa *et al.*, 2006; Pérez-Tienda *et al.*, 2011; Calabrese *et al.*, 2016]. Several of these transporters, such as RiNRT2, are expressed in both the ERM and IRM, suggesting that competition for nitrogen sources at the symbiotic interface may occur as was suggested for phosphate, or that bidirectional transfer can occur. Once taken up, nitrogen ions are

converted via glutamine into arginine through the GS/GOGAT cycle [Wipf *et al.*, 2019]. Arginine is then transported to the IRM [Figure 20.1D], where it is broken down into urea and ornithine [Govindarajulu *et al.*, 2005; Jin *et al.*, 2005]. To be transported, the positively charged arginine may form complexes with the negatively charged poly-P granules. Hydrolysis of urea then generates ammonium which is released into the peri-arbuscular space to be taken up by the plant by specific ammonium transporters [Kobae *et al.*, 2010; Breuillin-Sessoms *et al.*, 2015]. Interestingly, it was found that premature arbuscule degeneration in the arbuscule-specific *M. truncatula* phosphate transporter (PT4) mutant is suppressed when plants are starved for nitrogen. This suppression requires the ammonium transporter MtAMT2;3 [Breuillin-Sessoms *et al.*, 2015]. Because MtAMT2;3 failed to complement a yeast mutant impaired in ammonium transport, it was suggested that it may have an N-sensing and signaling function.

Organic nitrogen transporters, such as di- and tripeptide transporters (PTRs) or amino acid permease, such as GmosAAP1 from *Funneliformis mosseae* [Cappellazzo *et al.*, 2008] have been found to be induced in AM fungi. One of the dipeptide transporters, RiPTR2 in *R. irregularis*, was shown to be more highly expressed in IRM compared to ERM, and to be expressed in arbuscules as well [Belmondo *et al.*, 2014]. Again, this finding suggests that the fungus may competitively reabsorb nutrients released in the peri-arbuscular space and thus exert a control over the amount of nutrients delivered to the host.

Potassium transport

The third mineral, next to P and N, that is often most limiting plant growth under natural conditions is potassium (K). Several studies indicate also a positive effect of AM symbiosis on plant K acquisition [Garcia *et al.*, 2016]. Uptake of K from the soil may be dependent on the high affinity K uptake transporter (RiHAK) that has been identified in *R. irregularis* [Garcia *et al.*, 2016]. How the K is subsequently transferred to the host plant is not known.

Metals and other minerals

In addition to the main mineral nutrients phosphate, nitrate, and potassium, numerous additional transporters for metals and minerals such as sulfate can be identified in the *R. irregularis* genome [Tisserant *et al.*, 2013; Garcia *et al.*, 2016]. With the availability of cell-specific transcriptome analyzes, putative transporters can now be mined to identify key candidates for a role at the arbuscule interface [Zeng *et al.*, 2018]. Furthermore, the role of these transporters in the symbiosis remains to be investigated.

From plant to fungus

Hexoses

It was long thought that sugars (hexoses) were the main carbon source provided by the plants across the peri-arbuscular membrane to the AM fungus [Roth and Paszkowski, 2017]. This was mainly based on studies with isotope-labeled sugars added to mycorrhized roots in combination with detailed NMR analyzes. These indicated that glucose can be directly transferred from the host to the fungus [Shachar-Hill *et al.*, 1995; Solaiman & Saito, 1997; Pfeffer *et al.*, 1999; Bago *et al.*, 2000; 2003]. Furthermore, the ERM was shown to be largely

unable to take up sugars. In agreement with a role for plant-derived hexoses, a fungal proton-coupled monosaccharide transporter, RiMST2 from *R. irregularis*, was shown to be required for the proper development of arbuscules and fungal colonization through HIGS [Helber *et al.*, 2011; Figure 20.1D]. MST2 is most highly expressed in arbuscules but also showed expression in the IRM and ERM [Helber *et al.*, 2011; Zeng *et al.*, 2018]. This led to the proposition that AM fungi may also take up sugars from the apoplast through their intraradical hyphae. Furthermore, cell wall derived xylose was shown to trigger the upregulation of RiMST2 expression, suggesting that cell wall monosaccharides may also be taken up by AM fungi [Helber *et al.*, 2011]. Several MST members show preferential expression in the ERM, where they may play a role in the uptake of organic C [Roth and Paszkowski, 2017]. At present, the plant sugar transporters that export hexoses across the peri-arbuscular membrane are unknown, however it is likely that SWEET (Sugars Will Eventually be Exported) transporters are involved as several SWEET members are induced during the AM symbiosis [Manck-Götzenberger and Requena, 2016; Kafle *et al.*, 2019]. A recent study in *M. truncatula* identified a single SWEET gene (MtSWEET1b) that is significantly expressed in arbuscule cells and localizes to the peri-arbuscular membrane [An *et al.*, 2019]. It was shown to transport glucose. Furthermore, expression of dominant-negative forms of this SWEET caused an early collapse of arbuscules. This suggests that glucose transport to the arbuscule plays a role in the maintenance of the interface, although a role for MtSWEET1b in transport of other molecules, such as gibberellic acid [Kanno *et al.*, 2016] cannot be excluded. After uptake of hexoses from the plant, the sugars can be converted into glycogen as storage form and transported to the ERM. However, the absence of a gene encoding glucoamylase in the genome of AM fungi, suggest they may not be able to use glycogen effectively [Kobayashi *et al.*, 2018].

Fatty acids

The view of hexoses as major sole carbon source for AM fungi recently changed when it was found that AM fungi lack a key enzyme, type-I multidomain fatty acid synthase (FAS), in their genome, required to efficiently synthesize longer chain (C16:0) fatty acids [Wewer *et al.*, 2014; Chen *et al.*, 2018a,b]. This was striking as lipids are in fact the major carbon storage form in AM fungi [Rich *et al.*, 2017; Wang *et al.*, 2017a,b]. It suggested that AM fungi are fatty acid auxotrophs that need to obtain fatty acid precursors from the plant [Figure 20.1D]. Correspondingly, the plant induces a whole set of genes involved in fatty acid biosynthesis specifically in the arbuscule-containing cells [Bravo *et al.*, 2017; Jiang *et al.*, 2017; Luginbuehl *et al.*, 2017; Limpens and Geurts, 2018; An *et al.*, 2018]. Mutations in these genes typically cause the premature collapse or underdevelopment of arbuscules and overall reductions in fungal colonization. By expressing a fatty acylACP thioesterase (UcFatB) that produces lauric acid (C12:0), which is normally not or very lowly present in mycorrhizal roots or AM fungi, it was shown that indeed the plant provides fatty acids to the AM fungi [Jiang *et al.*, 2017; Luginbuehl *et al.*, 2017]. The fatty acid precursor that is likely transported is β-monacylglycerol (β-MAG). Inside the fungus β-MAG is converted into the storage form triacylglycerol (TAG) to be transported to the ERM and stored in spores and vesicles. TAG can be converted back into sugars through the glyoxylate cycle and gluconeogenesis [Bago *et al.*, 2003], and therefore fatty acids are likely a major nutritive carbon source for the fungus. Currently, the different contributions of sugars and fatty acids to feed the fungus remain to be addressed.

Micronutrients

Besides the inability to efficiently synthesize fatty acids, the available AM genome sequences further indicate that AM fungi are not able to synthesize essential micronutrients such as thiamine (vitamin B1) or vitamin B6 [Tisserant *et al.*, 2013; Kobayashi *et al.*, 2018; Morin *et al.*, 2019]. Therefore, these micronutrients are likely also provided by the plant host.

Intriguing in this respect is the report by Hildebrandt *et al.* (2002, 2006), who found that co-culturing of the AM fungus *R. irregularis* Sy167 with a mixture of two isolates of the bacterium *Paenibacillus validus* allowed the growth of the fungus even up to the formation of fertile spores. It was found that the *P. validus* isolates secrete raffinose which could stimulate the growth of fungal hyphae, but not the formation of spores. Given the fatty auxotrophy of AM fungi, it must mean that the *P. validus* cultures also contained fatty acids, and micronutrients, at sufficient levels to sustain the development of hyphae and spores.

Genetic make-up of AM fungi

AM fungi used to be classified with Mucoromycotina under the phylum Zygomycetes based on shared phenotypical traits [Spatafora *et al.*, 2016; Orchard *et al.*, 2017]. However, molecular phylogenetic analyzes have shown that these groups form two separate subphyla. AM fungi are now considered to form a subphylum of the Mucoromycota, called Glomeromycotina [Spatafora *et al.*, 2016], which share characteristics such as the development of a coenocytic mycelium and phylum-specific gene families [Lin *et al.*, 2014; Morin *et al.*, 2019]. AM fungi diverged quite early (>450 Mya) and have a striking genetic make-up that may be related to their early endosymbiosis with plants. The genomes of the six currently sequenced AM fungal species are among the largest genomes in the fungal kingdom, ranging from 126 to 598Mb [Ropars *et al.*, 2016; Chen *et al.*, 2018a, b; Maeda *et al.*, 2018; Kobayashi *et al.*, 2018; Sun *et al.*, 2019; Morin *et al.*, 2019]. These substantial differences are mainly due to the expansion of transposable elements (TEs, 30-60% of the genome). Furthermore, several gene families are strikingly expanded in the Glomeromycotina. Among these are for example proteins with a kinase domain, especially tyrosine kinases often associated with Sel1-repeats involved in protein-protein interactions [Mittl and Schneider-Brachert, 2007], and BTB domain (BTB-POZ and BTB-Kelch) containing proteins, a protein-protein interaction domain found in proteins involved in a wide range of cellular functions such as transcriptional regulation and chromatin remodeling, protein degradation and cytoskeletal regulation [Chaharbakhshi and Jemc, 2016]. Characteristic for AM fungi is also the absence of most plant cell wall degrading genes, genes required for degradation of sucrose and glycogen (invertase, glucoinvertase, and glucoamylase), non-ribosomal peptides and genes required for the biosynthesis of polyketides, thiamine and fatty acids [Morin *et al.*, 2019]. These metabolic adaptations are likely causally related to their obligate biotrophy and intimate interaction with plants.

AM fungi propagate asexually by forming spores. A sexual cycle and related structures have never been observed in AM fungi. Therefore, they have been called “ancient asexual scandals” [Judson and Normark, 1996]. This view has recently been challenged with the identification of all core meiotic genes in AM fungi, suggesting that they may have the capacity to undergo

meiosis [Halary *et al.*, 2011]. In line with this, Ropars *et al.* (2016) identified a putative mating type locus in *R. irregularis* by sequencing 5 different isolates [Koch *et al.*, 2004]. This locus consists of two homeobox domains containing proteins that are structurally and evolutionarily similar to the mating-type locus in some Dikarya. Intriguingly, the same study observed the presence of two different mating type loci in two *R. irregularis* isolates, while others contained only a single mating type locus. Variant calling combined with single nucleus sequencing and flow cytometry analyzes suggested the presence of two dominant genetically different haploid nuclei, called nucleotypes, in the strains with two mating type loci [Ropars *et al.*, 2016]. The other isolates appeared to be homokaryotic, similar to the DAOM197198 isolate [Tisserant *et al.*, 2013; Lin *et al.*, 2014]. Interestingly, two of the homokaryotic strains, A1 and B3, contained mating type loci that were found together in the dikaryotic strain A5 [Ropars *et al.*, 2016]. This led the authors to propose a mechanism where two homokaryotic AM fungal strains with compatible mating type loci have the ability to fuse, exchange nuclei and form a stable dikaryotic strain, similar to other heterothallic, bipolar Dikarya in the fungal kingdom. The fusion of hyphae (anastomosis) followed by the exchange of nuclei has indeed been reported for genetically closely related AM fungi [Giovannetti *et al.*, 1999, 2015; Croll *et al.*, 2009]. However, meiosis or a sexual stage have still not been observed in AM fungi. Many other fungi that were previously thought to propagate only asexually, were later shown to have a cryptic sexual cycle. It can therefore be possible that meiosis is rare in AM fungi or that the right conditions for it to occur (in a natural setting) have not yet been found. Alternatively, the meiosis related genes may be involved in processes other than meiosis. For example, several meiotic genes have been implicated in processes such as chromatin repair.

If a sexual cycle would not occur, it raises the question how AM fungi have been able to maintain themselves for such a long time. Nuclear fusion and recombination (most notably during meiosis) are generally required to purge the genome of deleterious mutations and to generate genetic variation, creating offspring that would be better adapted to varying environmental conditions. Population genetics approaches suggested the occurrence of (cryptic) recombination in AM fungi [Croll and Sanders, 2009; den Bakker *et al.*, 2010]. Additional proof for the occurrence of internuclear recombination was recently claimed by Chen *et al.* (2018a,b) through single nucleus sequencing of the dikaryotic *R. irregularis* isolate SL1. The recombination in this strain strikingly contrasted with the (almost complete) lack of observed recombination among nuclei of the dikaryons A4 and A5 [Chen *et al.*, 2018a,b]. However, it should be noted that the genome coverage of the SL1 strain was very low, and therefore conclusions based on this data set should be handled with care.

Several other hypotheses have been raised as to how genetic variation in AM fungi may contribute to their evolutionary success and ability to adapt to so many environments and plants. One of these hypotheses is based on the level of genetic variation observed within AM fungal individuals [Figure 20.1D]. This is continuing to be a matter of great debate in the AM research community [Kuhn *et al.*, 2001, Sanders and Croll, 2010; Limpens and Geurts, 2014, 2010, Ropars and Corradi, 2015; Young, 2015]. Genome sequencing has revealed a relatively low level of genetic variation (0.10.4 SNP/Kb) within the presumed “homokaryotic” strains and somewhat higher (varying from 0.8-2.5SNP/Kb, depending on strain and method) in the “dikaryotic” strains [Ropars *et al.*, 2016; Wyss *et al.*, 2016; Chen *et al.*, 2018a,b]. This variation

is significantly lower than the variation reported for true heterokaryotic fungi such as *Rhizoctonia solani*, containing approximately 6 to 15 genetically divergent nuclei within a common cytoplasm [Sneh *et al.*, 1991; Hane *et al.*, 2014], and *Coccidioides immitis*, which have SNP frequencies of 15.9 and 23.7 SNP/Kb, respectively [Neafsey *et al.*, 2010]. Nevertheless, reports of phenotypic variation between single spores, derived from single AM individual, have been published [Angelard *et al.*, 2010; 2014; Ehinger *et al.*, 2012; Wyss *et al.*, 2016]. Single spore lines derived from two anastomosed *R. irregularis* isolates caused up to five-fold differences in host plant performance when colonizing rice, and showed differences in symbiotic gene expression [Angelard *et al.*, 2010, 2014; Colard *et al.*, 2011]. Because spore formation is currently the strongest known genetic bottleneck in AM fungal development, this has led to the suggestion that segregation or drift of (more than two genetically different) nucleotypes may occur as spores are formed. Thereby, different offspring spores may obtain different allele frequencies that could cause the observed differences in phenotypes [Sanders and Croll, 2010]. In line with this, several approaches such as SSR markers, AFLP or amplicon sequencing, suggested that shifts in allele frequencies among individual spores do occur [Wyss *et al.*, 2016, Masclaux *et al.*, 2018]. It was even suggested that the host plant may induce a shift in allele frequencies [Wyss *et al.*, 2016]. It was claimed that transfer of AM lines to a different host plant species induced a higher genome plasticity, and changes appeared to be non-random [Wyss *et al.*, 2016]. This led to the proposal that changes in nucleotype frequency might play an important role in the adaptation to a different (plant) environments as an alternative to sexual reproduction. Frequent exchange of nuclei between AM individuals would ensure the maintenance of genetic variation within the populations. However, the AFLP approach used to monitor the genetic changes in this study makes it difficult to corroborate these findings. A suggestion that the host plant may "select" for preferred alleles or nucleotypes was also put forward after comparing two genome assemblies of the same *R. irregularis* DAOM197198 isolate cultured for over a decade on two different host plants. This revealed slight but significant genetic changes [Tisserant *et al.*, 2013; Lin *et al.*, 2014]. Building on this assumption, a model of how selection at the nuclei-level could be involved was proposed [Limpens and Geurts, 2014]. In this model different nuclei occupying arbuscules would be rewarded or sanctioned depending on the host plant, for example depending on the effectors being expressed, which would in turn influence the allele frequencies in the mycelium and resulting spores. Of course this nuclei-focussed model could also apply to other selection pressures such as conditions in the soil, potentially leading to segregation in the hyphal network.

In line with this model, it was shown that *R. irregularis* can adjusts its transcriptome in a host and stage-dependent manner [Zeng *et al.*, 2018]. It was shown that even in a shared hyphal network, the fungus can express different secreted proteins in the intraradical hyphae and/or arbuscules in a host-dependent manner. It would therefore now be interesting to determine whether this also leads to host-dependent allele expression. Combined with single nucleus sequencing this could reveal whether segregation in the hyphal network indeed occurs. Furthermore, it would be highly informative to determine the level of genetic variation in spores obtained directly from the field, as all the AM fungi sequenced to date came from only

two sources in the world and have been in culture with a single host for several decades [Koch *et al.*, 2004]. This could reveal whether prolonged culture on the same host leads to a loss of genetic diversity in the fungus.

In true asexually propagating species it has been suggested that mutations (due to replication errors or repeat-induced), horizontal gene transfer and chromatin rearrangements can cause sufficient genetic variation to replace the lack of sex [Seidl and Thomma, 2014]. Especially the action of transposable elements (TEs) may promote genome evolution. The excision of transposable elements can create double stranded breaks that need to be repaired by either nonhomologous end-joining or homologous recombination. This in turn can lead to inversions, duplications and deletions, and TE insertions can affect neighboring gene expression or knock genes out. The relatively low amount of genetic variation among individual nuclei in AM fungi seems to argue against a prominent role for mutations or genome rearrangements. However, it should be noted that single nucleus sequencing in particular can lead to highly fragmented assemblies [Chen *et al.*, 2018b] by which genome rearrangement could be underestimated.

Bdelloid rotifers are a notable example of asexual organisms that survived evolution through horizontal gene transfer [Gladyshev *et al.*, 2008, Debortoli *et al.*, 2016]. By intra- and interspecific allele exchange and gene conversion, these organisms can maintain genetic variation without meiotic recombination. Frequent occurrence of anastomosis between different individuals could facilitate this in AM fungi. However, genomic evidence of these processes has not been found.

Comparing the genomes of different *R. irregularis* isolates showed strikingly high levels of genomic variation. Large variation in gene families numbers, variation in active TE numbers, large numbers of isolate-specific genes and even isolate-specific TEs were observed [Chen *et al.*, 2018a]. This indicated that different isolates collected from the same field can vary substantially with respect to their function and potential to adapt to different environments. Furthermore, evidence for inter-isolate exchange was observed, potentially involving anastomosis. After anastomosis, AM fungi may exchange mitochondria in addition to nuclei, however stable inheritance of two mitochondrial genomes has not been found [Providencia *et al.*, 2013]. Another theory on the ability of AM fungi to adapt to different environments was raised by the observations that rDNA sequences in AM fungi are highly variable, and possibly encode for different ribosome profiles [Sanders *et al.*, 1995; Pawlowska and Taylor 2004; Maeda *et al.*, 2018]. Maeda *et al.* (2018) found that the genome of *R. irregularis* lack the typical highly tandem organization of the rDNA loci in the genome, as observed in most eukaryotes [Gibbons *et al.*, 2015]. Instead, *R. irregularis* was found to only have ten non-repeat rDNAs spread over its genome. Furthermore, SNP frequency in rDNAs was significantly higher than in the rest of the genome, at over 4SNPs/kb. The lack of tandem repeat structures causes AM fungi to not amplify rDNA through unequal sister chromatid recombination, allowing mutations in these sequences to remain. Consequently, variation in rDNA sequences would produce different rRNA profiles per nucleus. Maeda *et al.* (2018) speculate that AM fungi may rapidly adapt to new environments or hosts by translational regulation through alterations in their ribosome compositions. A similar mechanism has been reported in malaria parasites, a species that also switches hosts during its life cycle [Vembar *et al.*, 2016].

AM-associated microbes

In nature, AM fungi are surrounded by a plethora of other microbes. Often these microbial communities modulate the mycorrhizal symbiosis and may range from parasitic to beneficial associations, and from endosymbionts to loose hyphal associations.

Several AM fungal species, especially ones belonging to the Gigasporaceae, have been shown to contain endosymbiotic bacteria. Such ancient endosymbiotic bacterial associations with fungi are strikingly prominent in Mucoromycota [Bonfante and Desirò, 2017; Pawlowska *et al.*, 2018]. Bacterium-like organisms were originally found by electron microscopy in different AM species, such as *G. calidonium*, *Acaulospora laevis* and *Gi. margerita* [Bianciotto *et al.*, 1996]. Subsequent molecular studies in Gigaspora have identified these endosymbiotic bacteria as beta-proteobacteria, closely related to the Burholderia genus, called *Candidatus Glomeribacter gigasporeae* [Bonfante and Desirò, 2017]. CaGg is vertically transmitted via the spores, but is not an essential partner as AM fungal individuals with or without these endosymbionts can be found. CaGg cannot be cultured outside of the fungus indicating their obligate symbiotic nature, which is also in line with their streamlined genomes ranging from 1.34 to 2.36Mb. CaGg has been shown to affect the metabolism of its host. AM fungi cured for CaGg endosymbionts showed reduced hyphal elongation and branching of germinating spores, which correlates with a reduced amount of lipid droplets and fatty acids in the fungus [Lumini *et al.*, 2007; Salvioli *et al.*, 2010]. This is accompanied by a higher expression of genes related to beta-oxidation of fatty acids and the pentose phosphate pathway, and lower respiration rates, indicating a role for CaGg in enhancing AM fungal energy metabolism to fuel pre-symbiotic growth [Vannini *et al.*, 2016]. Interestingly, strigolactones activate proliferation of both mitochondria and CaGg, suggesting a link between the two. A second endosymbiotic bacterium found in AM fungi is an uncultivatable mollicute in the taxon of Mollicutes/Mycoplasma-related endobacteria [Naumann *et al.*, 2010; Toomer *et al.*, 2015]. It has been placed in the novel genus *Candidatus Moeniiplasma* and named *C. Moeniiplasma glomeromycotorum* (CaMg) [Naito *et al.*, 2017]. Both CaGg and CaMg can be found within the same AM fungal individual. Like CaGg, CaMg cannot be cultured outside of its host, however its role in the biology of its host is less well characterized. Endosymbiotic bacteria closely related to CaMg are also found in other Mucoromycota, where in some cases they have been reported to have a negative impact on their host depending on environmental conditions [Pawlowska *et al.*, 2018; Desirò *et al.*, 2018]. The high genetic diversity in the CaMg populations within AM fungal individuals could be in line with such facultative parasitic behavior of CaMg.

In addition to endosymbiotic bacteria, AM fungi contain bacteria that live on, or close to, the fungal hyphae [Bonfante and Anca 2009; Olsson *et al.*, 2017]. This is not surprising as a large amount of carbon fixed by plants ends up in the soil through AM fungi. Several bacteria have been found to have a beneficial effect on the AM symbiosis with plants and they have therefore been termed mycorrhiza-helper bacteria [Frey-Klett *et al.*, 2007]. These bacteria belong to diverse bacterial taxa and groups, and the mechanisms by which they stimulate AM symbiosis vary, although in most cases the exact mechanisms are not well known. Such mechanisms can include among others: stimulation of spore germination, promotion of

hyphal growth, mineral solubilization, detoxification of soil, inhibition of pathogens and modulation of host root architecture or metabolism (through production or modulation of plant hormones). Given their key role in the plants rhizosphere it can be expected that AM fungi in turn may actively (through the secretion of metabolites and nutrients) or passively (through effects on soil structure and pH) modulate the rhizosphere microbiome [Frey-Klett *et al.*, 2007]. For example, as mentioned before, it was shown that AM hyphal exudates can promote the growth of phytase-producing bacteria to increase the access to bound P forms [Zhang *et al.*, 2014a,b, 2016]. Fructose was shown to be one of the fungal exudates that could activate phosphatase expression and activity of the phosphate solubilizing bacterium *Rahnella aquatilis* [Zhang *et al.*, 2018]. However, due to its difficult accessibility and complex nature, the interplay of AM fungi with other microbes and its environment under natural conditions is still largely understudied.

Conclusion and future directions

In summary, our insight into fungal aspects of AM symbiosis and their molecular components have significantly increased in recent years, with the availability of the first AM fungal genomes. These now offer a stepping stone to further unravel the molecular workings of this key symbiosis. One of the major areas to be studied is to understand what determines the symbiotic efficiency of different plant-fungus combinations in natural settings. Understanding at a molecular level how (epi)genetic variation within and among AM fungi, and between different plant species or accessions, contribute to the observed phenotypic variation among AM fungi in the effects on plant performance remains one of the key mysteries in AM biology that remain to be resolved. This will also be of paramount importance to select or breed for optimal plant-fungal combinations for use in sustainable agriculture and to realize the widely recognized potential of AM fungi as sustainable biofertilizers.

Chapter 3

Stochastic nuclear organization and host-dependent allele contribution in *Rhizophagus irregularis*

Jelle van Creij[1], Ben Auxier[2], Jianyong An[1,3], Raúl Y. Wijfjes[4,5], Claudia Bergin[6], Anna Rosling[7], Ton Bisseling[1,3], Zhiyong Pan[8], Erik Limpens[1]

1. Laboratory of Molecular Biology, Department of Plant Sciences, Wageningen University & Research, Droevendaalsesteeg 1, Wageningen, The Netherlands
2. Laboratory of Genetics, Department of Plant Sciences, Wageningen University & Research, Droevendaalsesteeg 1, Wageningen, The Netherlands
3. Beijing Advanced Innovation Center for Tree Breeding by Molecular Design, Beijing University of Agriculture, Beijing, 102206, China.
4. Laboratory of Bioinformatics, Department of Plant Sciences, Wageningen University & Research, Droevendaalsesteeg 1, Wageningen, The Netherlands
5. Current affiliation: Faculty of Biology, Ludwig Maximilian University of Munich, München, Germany
6. Department of Cell and Molecular Biology, Uppsala University, and Microbial Single Cell Genomics Facility, Science for Life Laboratory, Uppsala, Sweden.
7. Department of Ecology and Genetics, Uppsala University, Norbyvägen 18D, SE-75236, Uppsala, Sweden.
8. Key Laboratory of Horticultural Plant Biology (Ministry of Education), Key Laboratory of Horticultural Crop Biology and Genetic Improvement (Central Region, Ministry of Agriculture), College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan, P.R. China.

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Abstract

Background

Arbuscular mycorrhizal (AM) fungi are arguably the most important symbionts of plants, offering a range of benefits to their hosts. However, the provisioning of these benefits does not appear to be uniform among AM fungal individuals, with genetic variation between fungal symbionts having a substantial impact on plant performance. Interestingly, genetic variation has also been reported within fungal individuals, which contain millions of haploid nuclei sharing a common cytoplasm. In the model AM fungus, *Rhizophagus irregularis*, several isolates have been reported to be dikaryotes, containing two genetically distinct types of nuclei recognized based on their mating-type (MAT) locus identity. However, their extremely coenocytic nature and lack of a known single nucleus stage has raised questions on the origin, distribution and dynamics of this genetic variation.

Results

Here we performed DNA and RNA sequencing at the mycelial individual, single spore and single nucleus levels to gain insight into the dynamic genetic make-up of the dikaryote-like *R. irregularis* C3 isolate and the effect of different host plants on its genetic variation. Our analyses reveal that parallel spore and root culture batches can have widely variable ratios of two main genotypes in C3. Additionally, numerous polymorphisms were found with frequencies that deviated significantly from the general genotype ratio, indicating a diverse population of slightly different nucleotypes. Changing host plants did not show consistent host effects on nucleotype ratios after multiple rounds of subculturing. Instead, we found a major effect of host plant-identity on allele-specific expression in C3.

Conclusion

Our analyses indicate a highly dynamic/variable genetic organization in different isolates of *R. irregularis*. Seemingly random fluctuations in nucleotype ratios upon spore formation, recombination events, high variability of non-tandemly repeated rDNA sequences and host-dependent allele expression all add levels of variation that may contribute to the evolutionary success of these widespread symbionts.

Background

Fungi belonging to the Glomeromycotina subphylum of the Mucoromycota are globally distributed soil fungi that form an endosymbiosis with the vast majority of land plants [Spatafora *et al.*, 2016]. These so-called arbuscular mycorrhizal (AM) fungi rely on their interaction with plants to complete their life cycle. During colonization of plant roots, they form highly branched structures called arbuscules inside root inner cortex cells, where mineral nutrients such as phosphate and nitrogen are exchanged for sugars and fatty acids from the plant [Luginbuehl *et al.*, 2017]. This symbiosis originated more than 400 million years ago and has since been maintained in the vast majority of plants, highlighting its importance in natural ecosystems [Redecker *et al.*, 2000]. Currently, around 315 AM fungal species have been described, however the species concept for these enigmatic fungi is not well defined [Bruns *et al.*, 2018]. Significant intraspecific genetic variation has been observed but evidence for sexual reproduction remains elusive. How the genetic organization of these important fungi contributes to the evolutionary success of this key symbiosis is an important and highly debated question [Sanders and Croll, 2010; Kokkoris *et al.*, 2020]. Large variations in the symbiotic performance, often referred to as mycorrhizal growth response, of different isolates or even among strains derived from single spores from a fungal individual has been reported [Koch *et al.*, 2006; Angelard *et al.*, 2010; Mensah *et al.*, 2015; Sanders and Rodriguez, 2016]. What determines this variation in mycorrhizal growth response, i.e. how much growth benefit a plant has from interacting with a certain fungus, remains unknown. An important first step to understanding the mycorrhizal response is understanding if the genetic organization of AM fungi adapts to different environments and plant hosts, impacting their growth.

Among fungi, AM fungi have relatively large genome sizes (~150–750 Mb) and are rich in transposable elements [Kokkoris *et al.*, 2020]. They form mycelia with a shared cytoplasm containing many nuclei, ranging from hundreds in spores to millions in grown mycelial networks [Balestrini *et al.*, 1992; Sanders and Croll, 2010; Kokkoris *et al.*, 2020]. Such coenocytic hyphae generally lack cross-walls and nuclei can flow freely from the hyphae into the spores as they form and grow [Jany *et al.*, 2010; Marleau *et al.*, 2011]. As a result, spores contain hundreds of nuclei and there is no known single nucleus stage that generates the next generation. Although other fungi with multinucleate hyphae and spores are known [Roper *et al.*, 2010; Roberts *et al.*, 2015], to our knowledge the extremely large coenocytic nuclei number and apparent lack of a single nucleus stage is unique to AM fungi.

Another confounding aspect about the genetics of AM fungi is that sexual structures have never been observed [Kokkoris *et al.*, 2020]. Therefore, historically AM fungi were thought to propagate asexually, raising questions about their ability to purge deleterious mutations and to generate genetic variation required for adaptation. One mechanism proposed a large variety of genetically diverse nuclei in fungal individuals, and subsequent selection on individual nuclei [Sanders and Croll, 2010]. However, the availability of various whole genome sequences from different AM fungi has somewhat challenged this view, revealing much lower intra-organismal genetic variation than previously assumed [Martin *et al.*, 2008; Tisserant *et al.*, 2013; Lin *et al.*, 2014; Ropars *et al.*, 2016; Maeda *et al.*, 2018; Chen *et al.*, 2018; Venice *et al.*, 2020]. Furthermore, AM fungi were found to contain a full complement of the core genes required for meiosis [Tisserant *et al.*, 2012, 2013; Halary *et al.*, 2011]. A putative mating-type (MAT) locus, consisting of two HD-like genes, has been identified in *Rhizophagus irregularis*, consistent with a bipolar mating system [Ropars *et al.*, 2016]. Whole genome sequencing

together with single nucleus sequencing of various *R. irregularis* isolates revealed that some were in fact monokaryotic (ie. containing genetically very similar nuclei representing one nucleotype with a single MAT allele), while others (such as isolates A4 and A5) appeared to be dikaryotic (ie. two different nucleotypes carrying two distinct MAT alleles). Furthermore, allele frequency analyses indicated a mostly 1:1 ratio of the two nucleotypes in the two dikaryotic strains studied [Ropars *et al.*, 2016]. A recent study by the same group suggested that in dikaryotic strains the ratio of the two nucleotypes may shift in response to host plant identity [Kokkoris *et al.*, 2021]. Interestingly, recent RAD sequencing of the dikaryote-like C3 isolate, which is closely related to the A4 isolate [Croll *et al.*, 2008; Wyss *et al.*, 2016], showed that progeny lines grown on the same host can already vary substantially in the ratio of the two nucleotypes [Robbins *et al.*, 2021]. Similar observations were previously made based on a polymorphic genetic marker [Ehinger *et al.*, 2012; Masclaux *et al.*, 2018] and AFLP analyses [Angelard *et al.*, 2010].

To get more detailed insight into the organization of intra-strain genetic variation and especially the impact of different host plants at the genome and transcriptome level, we focused on the putative dikaryote-like *R. irregularis* C3 isolate, because of its presumed high level of intra-genomic variation [Croll *et al.*, 2008; Wyss *et al.*, 2016]. By using a combination of culture meta-genome, single spore and single nucleus sequencing as well as RNA sequencing in different host plants we reveal a highly dynamic genomic organization.

Results

Characterizing intragenomic variation

Genome assembly

The *R. irregularis* C3 isolate was initially chosen because of its reported relative high level of genetic variation, based on RADseq data [Wyss *et al.*, 2016], which in hindsight was overestimated due to lack of an appropriate reference genome [Masclaux *et al.*, 2019]. As no reference genome for C3 was yet available we first generated a C3 reference assembly, using a combination of PacBio and Illumina sequencing on genomic DNA extracted from a large number of spores and hyphae from axenic *Daucus carota* root cultures, to be able to characterize the genetic variation in this isolate [Figure 1; Table 1]. This resulted in an assembly [RirC3; Genbank BioProject ID PRJNA747641] comprising 1380 contigs and representing a total length of 155Mbp [Table 1, Figure 1]; a genome size similar to previous estimates for the genome length in *R. irregularis* strains [Kokkoris *et al.*, 2020]. A representation of the 10 longest contigs covering 9.9 Mb, depicting the distribution of repeats, coding regions and SNP density, is shown in Figure 1.

Table 1: *RirC3 genome assembly overview.*

Total assembly length	155,051,422
Contigs	1380
Gaps	2
GC content	27.94%
Longest contig length	1,364,683
Average contig length	112,356
Contig N50 length	263,815
Contig L50	174
Contig N90 length	63,299
Contig L90	605
Total repeat length	71,617,038
Percentage of genome composed of repeats	46.19%
Total number of biallelic SNPs in the meta genome	121,109
Non-synonymous SNPs	10,677
Stop codon gains	381
Frameshift gains	487
SNP frequency	0.78/kb
SNP density outside repeats	0.59/kb
Number of predicted genes	27,181
PFAM domains	3,714
BUSCO (fungi_odb10)	84.3% (639/758)
Complete and single-copy BUSCO genes	82.6% (626/758)
Complete and duplicated BUSCO genes	1.7% (13/758)
Fragmented BUSCO genes	1.2% (9/758)
Missing BUSCO genes	14.5% (110/758)

Repeated regions, including transposable elements, represented 46% of the genome (71 Mb). These repeat regions appear to be randomly distributed over the genome and the majority remain unclassified [Figure 1]. The genome assembly contained 85% of the BUSCO (fungi_odb10) gene set, which is similar to the completeness observed for the high quality *R. irregularis* DAOM197198 genome [Rir17 [Maeda *et al.*, 2018]; Additional File 1] and closely related isolate A4 [Ropars *et al.*, 2016]. BUSCO genes that were not found include FATTY ACID SYNTHASE I and other genes reported to be consistently lost in the *R. irregularis* genome [Additional File 1], correlating with their obligate biotrophic lifestyle [Miyauchi *et al.*, 2020]. Nearly all BUSCO genes were found in a single copy, indicating a low level of contig duplication from the two nucleotypes. The RirC3 assembly was further annotated using the FunAnnotate pipeline, adapted for fungal genome annotation [Palmer and Stajich, 2018], resulting in 27181 predicted gene models.

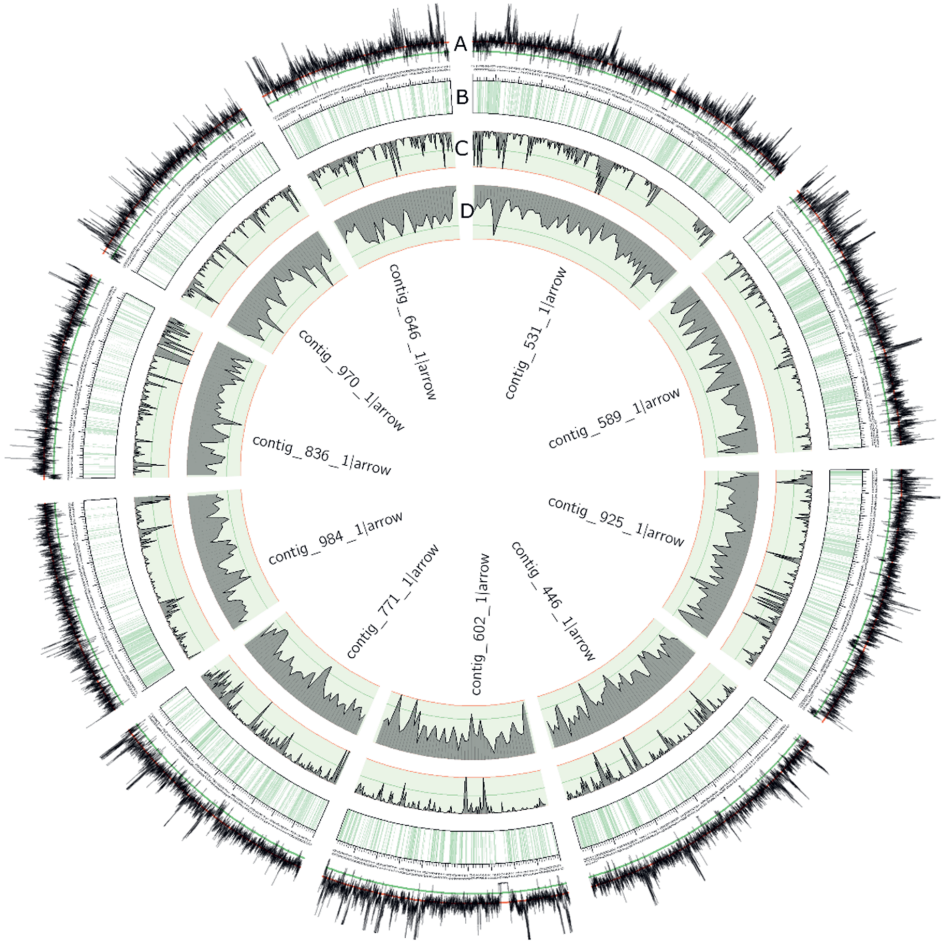

Figure 1: *Circos diagram of the ten largest contigs of the RirC3 assembly, representing 9.9 Mb.* ***A****: Mapping depth of C3 Illumina reads, green line = 50, red line = 100.* ***B****: Physical map of the contigs, with coding regions coloured green.* ***C****: SNP density, green line = 10, red line = 20.* ***D****: Repeat density, green line = 75, red line = 100.*

45S rDNA organization

R. irregularis DAOM197198 (Rir17) was reported to contain an atypical non-tandemly repeated organization of the 45S rDNA locus, consisting of 10 or 11 copies [Maeda *et al.*, 2018]. Similarly, RirC3 contains only eight 45S rDNA copies that also lack a tandem organization. Four of these copies were located on separate contigs; the other four were found in two pairs, separated over 50 kb apart on separate contigs.

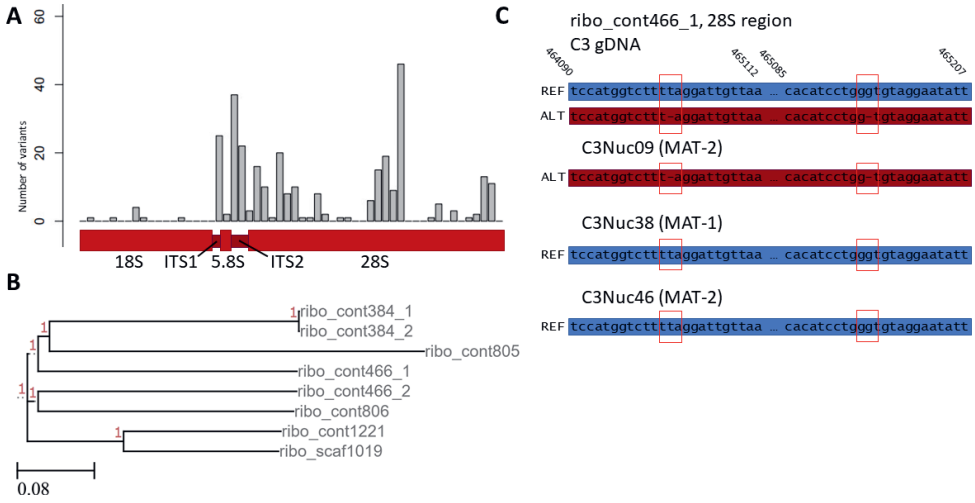

Figure 2: *Polymorphisms found in the RirC3 45S rDNA locus.* ***A****: Graph showing the amount of genetic variation among 45S rDNA copies.* ***B****: Phylogenetic representation based on of multiple sequence alignment (1000 bootstraps) of the eight 45S rDNA copies. No copies were identical. The names of the samples correspond to which contig they were found on (e.g. ribo_cont466_1 was the first copy on contig_466 of the RirC3 assembly). Red numbers indicate support values.* ***C****: Example of biallelic SNPs in the rDNA sequence distributed over different nuclei. The reference and alternate alleles for a 28S subregion of ribo_contig466_1 in the C3 gDNA and 3 individually sequenced nuclei (C3Nuc9, C3Nuc38 and C3Nuc46) are shown.*

Alignment of the sequences of these copies showed significant variation among the different loci, each consisting of 18S rDNA, intergenic spacer region 1 (ITS1), 5.8S rDNA, ITS2, and 28S rDNAs [Figure 2a,b]. When assessing the sequencing depth at these 45S rDNA sequences, we found no increased coverage that would suggest a collapse of assembled sequences as would be expected in the case of many highly conserved copies. Upon analyzing the number of polymorphisms among the 45S rDNA copies, we identified 31 SNPs within four of the 45S rDNA contigs [Additional File 2]. These data support the relative high heterogeneity of *R. irregularis* 45S rDNA copies, which has been suggested to potentially modulate the translational activity of different ribosomes [Maeda *et al.*, 2018]. Single nucleus sequencing (see below) showed that different nuclei indeed encode distinct rDNA alleles [Figure 2c], confirming the observed heterogeneity in the assembly.

Allelic variation

To investigate the genome-wide level of genetic variation, SNP calling was performed using freebayes based on Illumina sequencing reads from DNA isolated from a large collection of root culture plates, referred to as meta-genome (C3gDNA). SNPs were filtered based on a coverage within the 25th percentile from the average mapping depth (between 80-135x), and at least 10 observations of both alleles. With these settings 121,109 SNPs were found [Additional Files 3 and 4], giving a SNP density of 0.79 SNPs/kb. After removing SNPs that were located inside repetitive regions 0.59 SNPs/kb remained. 10,677 SNPs represented non-synonymous SNPs in the predicted protein coding genes. To compare the observed allele

frequencies, similar analyses were performed on previously published Illumina data of DAOM197198 and A4 [Maeda *et al.*, 2018; Ropars *et al.*, 2016]. Allele frequency distribution analyses confirmed the homokaryotic nature of the DAOM197198 isolate and the reported 50:50 distribution of allelic variations in the A4 isolate [Figure 3a,b]. However, the allele frequency distribution in the C3 meta-genome sample showed two peaks corresponding to 33% and 67% allele frequencies for C3 [Figure 3c,d]. Such an allele frequency distribution is typically found in triploid genomes [Viruel *et al.*, 2019]. The observed 2:1 SNP ratio had a consistent genome-wide distribution, ruling out that this distribution was caused by local aneuploidy.

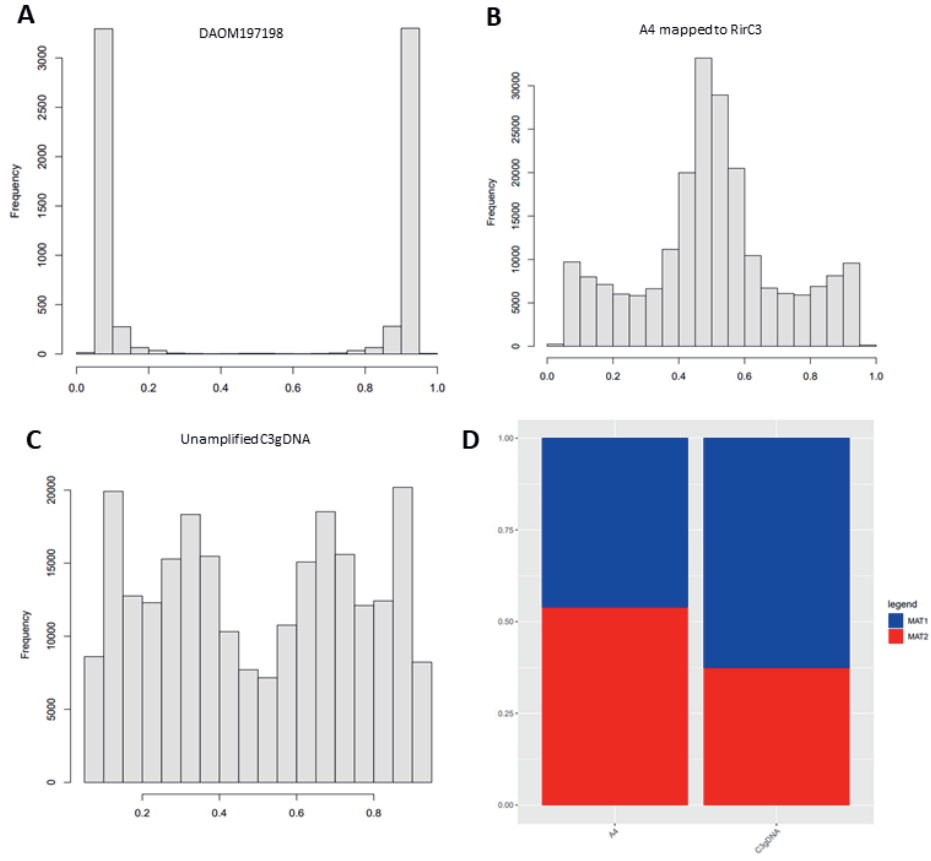

Figure 3: *Allele frequencies in different* Rhizophagus irregularis *isolates. Only biallelic SNPs were considered in all samples, where both reference and alternative alleles were observed at least 10 times.* ***A****: DAOM197198, mapped to Rir17 [Maeda* et al., 2018*] (coverage between 355 and 455). B: A4 reads [Ropars* et al., 2016*] mapped to the RirC3 assembly (coverage between 75 and 125). RirC3 was chosen as the reference genome since the mapping rate of A4 Illumina reads was higher with this assembly.* ***C****: C3 Illumina reads mapped to the RirC3 assembly (coverage between 85 and 135).* ***D****: MAT locus proportions based on coverage of the MAT loci in A4 and C3 Illumina reads mapped against the RirC3 assembly.*

To determine whether such unequal allele frequencies were consistent between C3 cultures, gDNA of another batch of C3 root culture plates was also sequenced. Whole genome amplification (WGA) was used to generate sufficient DNA before sequencing of this sample [C3_WGA1, Figure 4a]. To rule out any artefacts introduced by WGA, the original C3gDNA sample used for the assembly was also amplified (C3_WGA2) and sequenced [Figure 4b]. To further monitor the reproducibility of the whole genome amplification procedure with respect to SNP frequencies, multiple WGA replicates were included for both meta gDNA samples (meta refers to the use of a large number of spores and mycelium) [Figure 4c]. Principal component analysis (PCA) of allele frequencies showed that the whole genome amplification did not introduce much variation in allele frequencies between technical replicate samples as seen by the tight clustering of these samples [Figure 4d]. This indicated that whole genome amplification did not cause a significant bias in the allele frequencies of the respective samples. However, it also showed that the two meta DNA samples, isolated from different batches of root culture plates, differed in allele frequencies. The C3_WGA2 samples showed allele frequency peaks at ~33% and 67% in line with the allele frequency distribution in the unamplified C3gDNA, while the others (C3_WGA1 replicates) showed a rather broad peak around 50% suggestive of a 1:1 nucleotype ratio [Figure 4a,b, Figure S1].

These analyses suggested that different batches can differ in their nucleotype ratios, in line with the ddRADseq data from Robbins *et al.* (2021). Two additional DNA samples, each from 50 spores collected from two other root culture plates (labelled C3_spores_1 and C3_spores_2), were sequenced after whole genome amplification. These again showed divergent allele frequency distributions based on both genome-wide allele frequencies and MAT allele ratios [Figure 4c,e].

To further investigate the nucleotype ratios we searched for the presumed MAT loci in RirC3. We identified two MAT loci identical to the MAT-1 and MAT-2 sequences reported for A4 [Ropars *et al.*, 2016]. Read mapping to these loci showed similar ratios consistent with the genome wide SNP analyses; approximately 1:1 in the C3-WGA2 reads and 2:1 in the C3_WGA1 reads [Figure 4e]. Variable nucleotype frequencies were also observed based on MAT allele ratios determined by ddPCR on multiple unamplified DNA samples collected from different root culture plates [Figure 4f], further confirming that the observed variation was not caused by the whole genome amplification.

The presence of the same MAT loci and near 100% mapping of the A4 Illumina reads to the RirC3 assembly [Table S1] confirmed the very close relationship between these two isolates. Both strains were harvested as single spores from different parts of the same field in Switzerland and axenic root cultures using *Daucus carota* as host plant were initiated ~20 years ago [Jansa *et al.*, 2002; Koch *et al.*, 2004]. Many SNPs, even low frequency SNPs, were found to be conserved between A4 and C3, i.e. being variable sites in both, although not necessarily at similar frequencies [Additional File 4].

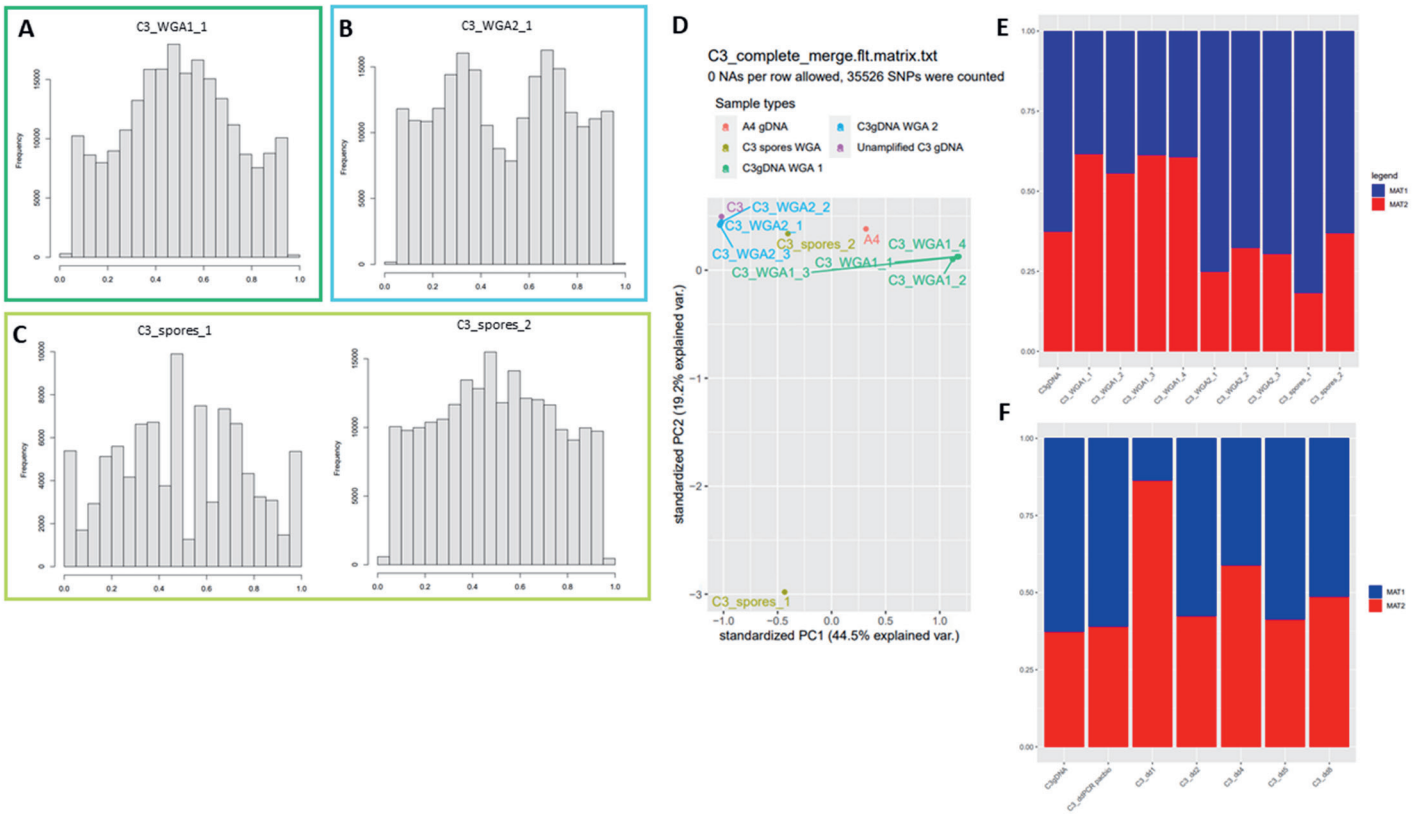

Figure 4: *Allele frequencies of different C3 colonies.* ***A****: Allele frequency distribution of C3 genomic DNA that was amplified from the sample used for Illumina sequencing.* ***B****: Allele frequency distribution of amplified C3 genomic DNA from a previously isolated sample.* ***C****: Allele frequencies of two C3 colonies, of which ~50 spores and mycelium were isolated and amplified.* ***D****: Principal component analysis of different C3 DNA samples and A4, based on allele frequencies of shared SNPs.* ***E****: MAT locus proportions based on whole genome Illumina sequencing data of different C3 DNA samples and A4.* ***F****: ddPCR results of other, newly isolated C3 colonies. Note, C3_ddPCR pacbio refers to the WGA amplified version of the DNA shown in lane 1. C3_dd1,2,4,5 and 8 represent unamplified DNA samples for 5 different root culture plates.*

In most basidiomycete fungi, despite migration of nuclei, exchange of mitochondria does not occur during hyphal anastomosis. Previous studies suggested that anastomoses between closely related AM fungi could lead to exchange of genetically divergent mitochondria [de la Providencia *et al.*, 2013]. However, only one mitochondrial parental haplotype was found in derived single spore cultures [Montoliu-Nerin *et al.*, 2020]. This has been suggested to occur through an active segregation mechanism by which one mitochondrial haplotype dominated the other. We observed only a single mitochondrial haplotype in C3. Although several low frequency SNPs were found, their number was much lower compared to the SNP frequencies observed in the genomic DNA [Additional File 5]. This indicated that the mitochondrial population in this heterogenic strain is also largely homogeneous.

In summary, the characterization of the intragenomic variation showed that there can be substantial variation in allele frequencies among individual cultures of the same strain. To investigate the reasons behind such variation we next looked for signs of inter-nucleus recombination and the (partial) segregation of nuclei during sporogenesis, as well as the effect of different host plants on the genetic variation.

Mechanisms behind the observed differences in allele distribution

Potential inter-nucleus recombination

To determine to which extent the observed allele ratios in the genome correlated with the two MAT loci, we sequenced 10 individual nuclei and matched allele variants with their respective MAT locus for each nucleus. Individual nuclei were collected using a fluorescence activated cell sorter (FACS) and subsequently whole-genome amplified (WGA) before Illumina sequencing [Montoliu-Nerin *et al.*, 2020]. The MAT locus identity of the individual nuclei was determined by PCR analyses [Figure S2].

Sequencing reads of individual nuclei were mapped against RirC3 and variants were called in parallel using freebayes [Additional File 6]. To avoid confounding effects of putative repetitive sequences or potential mapping/assembly artifacts we only considered SNPs in uniquely mapped reads that were outside genomic regions annotated as repeats. Any loci where two alleles were found in a single nucleus were omitted. Furthermore, SNPs within 500bp of these heterozygous SNPs in single nuclei or based on non-paired reads only were omitted. The same analysis was done using Illumina reads of A4 nuclei [Chen *et al.*, 2019] mapped against RirC3 [Additional File 7]. These analyses showed that C3 nuclei clustered together based on MAT locus identity [Figure 5a; Figure S3].

PCA analyses showed that the 6 nuclei containing MAT-1 clustered more closely together with the meta-genomic DNA (C3gDNA), which suggests that this nucleotype mostly contributed the alleles in the assembly [Figure S3a]. These analyses indicated that most SNPs that were found in the MAT-1 nuclei carried the reference allele called in the assembly, while the MAT-2 nuclei mostly carried the alternative alleles [Figure 5a; Additional File 6]. These analyses further suggested that MAT-2 nuclei are more divergent from each other than the MAT-1 nuclei, which is evident from their clustering less together in the PCA plot [Figure S3a]. Nuclei with matching MAT loci showed a high level of similarity.

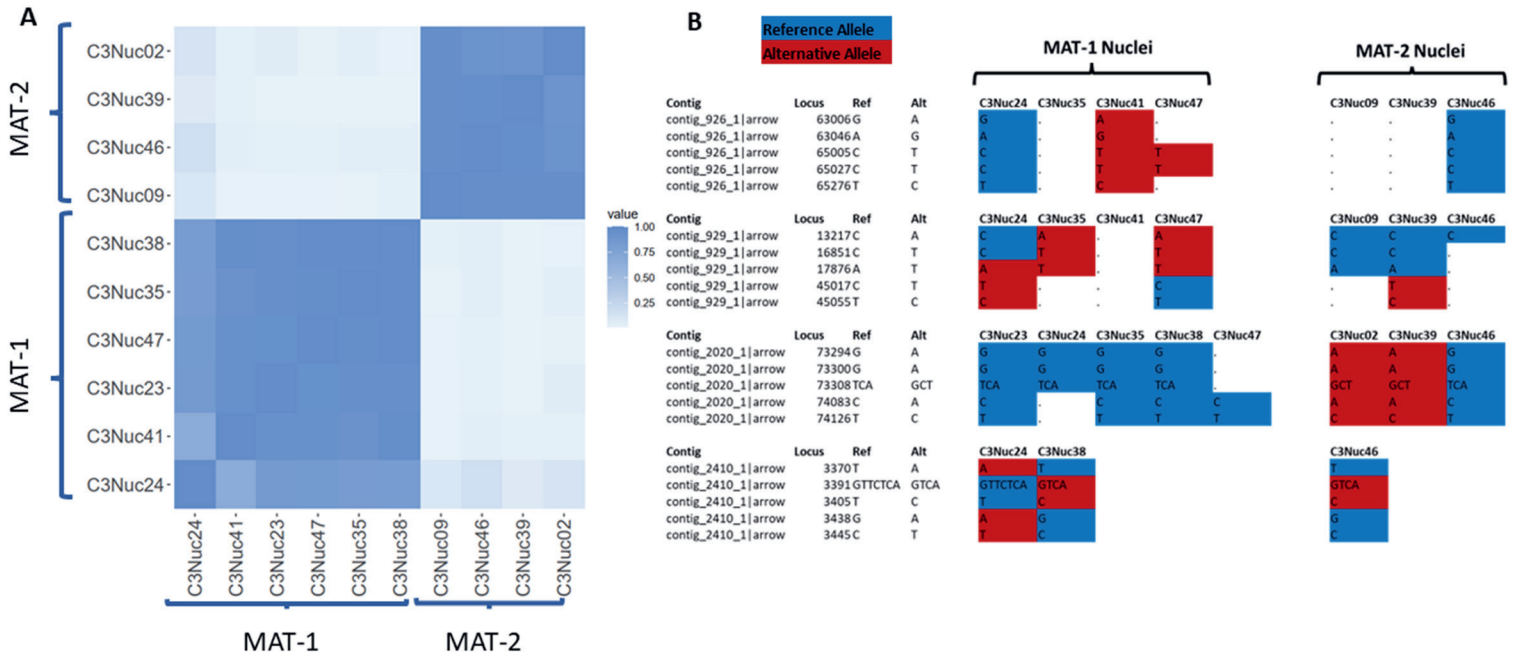

Figure 4: A. *Similarity plots of C3 nuclei based on single nucleus sequencing data. Color coding indicates level of relatedness among the nuclei. A sharper contrast between the groups means that the nuclei are more different, while patches of differing colors within the groups indicate similarities to nuclei of the other group (meaning the other MAT locus). Nuclei are grouped based on which MAT locus they contain.* ***B****: Examples of genotypes of C3 nuclei not consistent with mating type. Indicated in blue the allele typically found in MAT-1 nuclei (ie. the reference allele called in the assembly), in red the allele typically found in MAT-2 nuclei (alternate allele). The MAT locus identity of the different nuclei is indicated on top. In a true dikaryotic division, all MAT-1 nuclei should have blue alleles, while all MAT-2 nuclei should have red alleles. A complete list of putative recombination sites is given in Additional file 8.*

Overall, 95% of the SNPs matched the corresponding/expected MAT locus identity, while 5% of the SNPs did not; of the 9947 total SNPs, 503 were represented by both alleles among nuclei with the same MAT allele [Additional File 8]. After ignoring contigs where only one SNP was found, 408 SNPs remained covering 89 contigs. Blocks of at least 5 consecutive non-matching SNPs (of 244 total SNPs) were found on 25 contigs (examples of some shown in Figure 5B). Such non-matching sites may point to recombination events between nuclei, as previously suggested for A4 [Chen *et al.*, 2018, 2019; Auxier and Bazzicalupo, 2019].

Partial segregation of nuclei during sporogenesis

Our observed variation in allele frequency distribution between different root culture plates [Figure 4] raised the suspicion that allele frequencies might be subject to stochastic drift effects. It was previously suggested that varying assortment of genetically different nuclei into newly formed spores can lead to different allele ratios between individual offspring spores [Sanders and Croll, 2010; Angelard *et al.*, 2010, 2014; Ehinger *et al.*, 2012; Masclaux *et al.*, 2018; Robbins *et al.*, 2021]. This so-called partial segregation of nuclei could bestow individual single spore offspring lines the ability to differentially affect plant performance. For example, it was shown that some single spore lines could increase rice growth by a factor of five compared to other lines from the same starting strain [Angelard *et al.*, 2010]. To test for signals of nuclei segregation at spore formation, three single spore lines (root cultures named SS1, SS3 and SS6) were generated from a single ancestral C3 root culture plate. These single spore lines were re-sequenced together with single spores derived from these lines [Figure 6a]. For example, for SS3 one of its single spores was used to generate a second-round single spore line (SS3-1) and a single spore (SS3-1-3) is derived from it. To obtain sufficient material for sequencing all DNA samples were whole genome amplified.

Patterns of allele frequency distribution varied across single spore lines [Figure 6b-d] and derived individual spores [Figure S4], again indicating that nucleotype composition varies between spores within strains. MAT allele ratio was also variable between these samples, showing that MAT locus based nucleotype composition differs between spores [Figure 6e]. Differential MAT allele proportions were also supported by ddPCR analyses of the MAT alleles in the same samples [Figure S5]. PCA analyses based on allele frequencies showed that individual spores varied significantly and no signs of convergence of allele frequencies in next generation spores was observed [Figure 6f,g]. Intriguingly, DNA isolated from the single spore line SS1 showed an almost exclusively presence of MAT-2 nuclei, with very little MAT-1 nuclei. Nevertheless, a single spore derived from this line (SS1-4) showed a MAT-1:MAT-2 ratio of 8:1, indicating that individual spores can vary widely in their nucleotype composition. Similar, but less extreme variation was also observed in second- and third round progeny spores of SS3 and SS6.

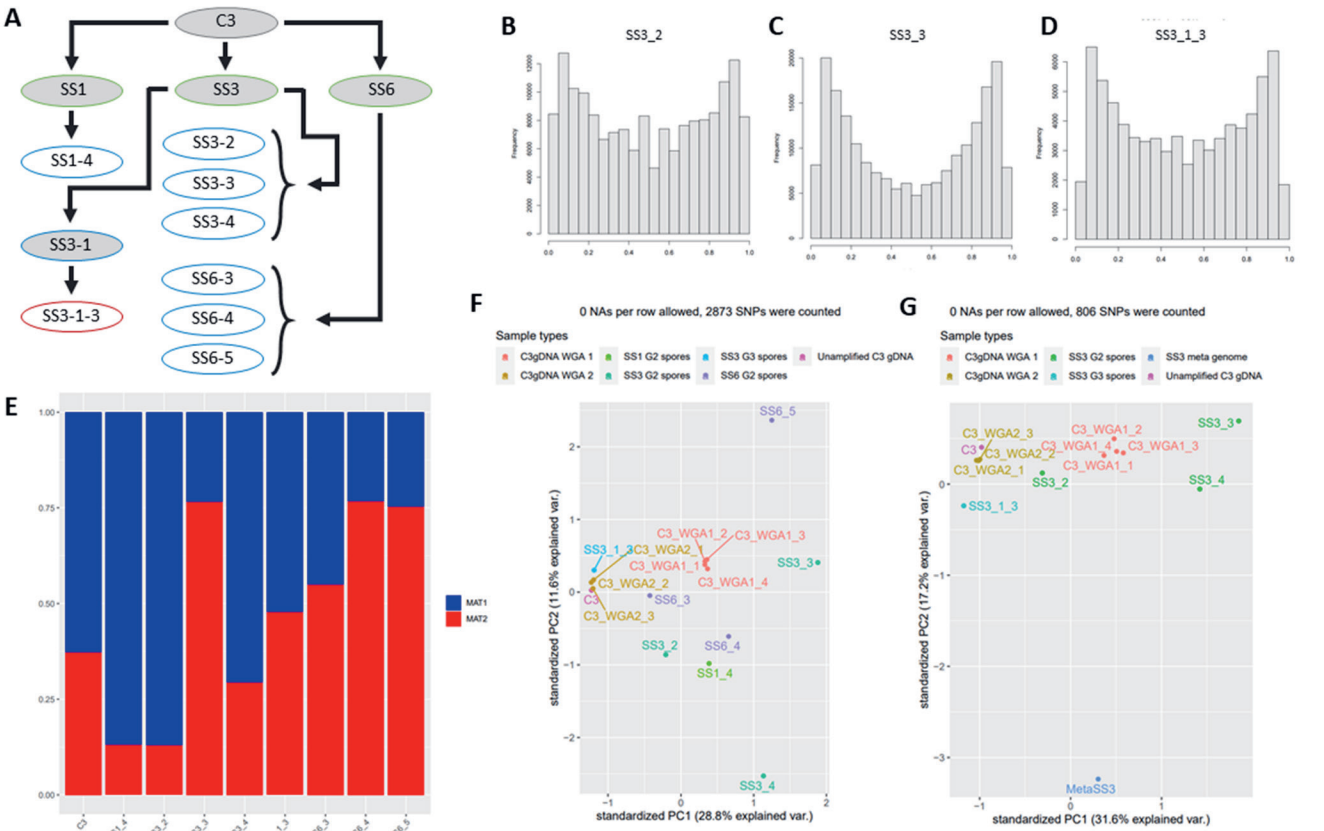

Figure 6: *Single spore line variant analysis.* ***A****: Schematic overview of relatedness of all single spore lines. Lines were created by inoculating* D. carota *root cultures with a single C3 spore. Subsequent generations were made by inoculating a new root culture with a single spore derived from the previous single spore line. Generation (G) number is indicated by color: Black = parental C3 colony, green = G1, blue = G2, red = G3. White circles indicate single spores that were amplified and sequenced, grey circles indicate an established colony producing spores.* ***B-D****: Allele frequency distributions of several amplified single spores.* ***E****: MAT loci frequencies of amplified single spores, based on sequencing data.* ***F****: Principal component analysis of single spores based on allele frequencies of shared SNPs. WGA samples were included as additional control samples.* ***G****: Principal component analysis of single spores derived from SS3, including metagenomic DNA from SS3:*

Host-dependent differential expression of alleles

Variation in nucleotype ratios can lead to variation in allele expression [Robbins *et al.*, 2021]. To investigate whether different host plants affect the expression of specific alleles we performed RNAseq analyses of C3 after colonization of *Medicago truncatula* (Medicago), *Nicotiana benthamiana* (Nicotiana), *Allium schoenoprasum* (Chives) and *Solanum lycopersicum* (Tomato) roots. One batch of spore suspension used for inoculation of the different plants was prepared from a separate host, *D. carota* root culture plates. Strikingly, these analyses revealed two different allele frequency distributions in the fungal mRNA populations depending on host plant identity. In the colonized Medicago and Chive roots the C3 mRNA allele frequencies of biallelic SNPs expressed in all four host species showed a clear peak at 50%, while in after colonizing Nicotiana and Tomato there was no peak at 50%, and slight allele frequency peaks at ~33% and 67% were observed [Figure 7a]. The same observation in three biological replicates of each plant-fungus combination negates batch effects for each inoculation [Figure S6]. After filtering on SNPs that were expressed (at least 20 reads) in all four hosts, similar differences in allele frequency distribution were visible, confirming that the allele frequencies of the same genes changed in different hosts [Figure 7b; Figure S6]. Since all plants were inoculated with the same spore batch, these data indicated that alleles contributed differently to the mRNA pool when colonizing Medicago and Chives, compared to when colonizing Nicotiana or Tomato. Since alleles are distributed over different (haploid) nuclei the genome-wide shift in allele frequencies it suggests that expression activity varies between nucleotypes.

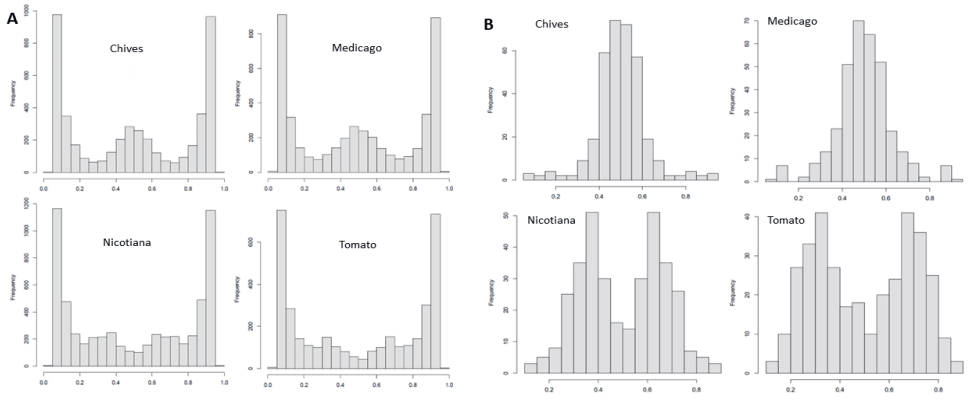

Figure 7: *Allele frequency histograms of C3 RNA on different hosts. SNPs were filtered on a minimum sequencing depth of 20 reads, with a of 10 observed reference and alternative alleles.* ***A****: Transcriptome-wide allele frequencies per host.* ***B****: Allele frequencies of SNPs that were sufficiently expressed in all hosts.*

Unfortunately, we did not have fungal DNA available from the corresponding mycorrhized root samples used for RNAseq. This prevented us from testing whether the observed allele frequency distribution in the RNA reflected already a shift in nucleotype ratios at the DNA level due to the different hosts, as suggested for the A4 isolate [Kokkoris *et al.*, 2021]. We have previously hypothesized that genetically different nuclei could have different abilities/efficiencies to interact with distinct plant species [Limpens and Geurts, 2014]. For example, certain nuclei could be more adapted to interact with plant species A, whereas other nuclei could be more adapted to interact with plant species B.

This could in theory lead to a plant effect on the allele frequencies in the offspring when cultured for a longer time on different plant hosts. To determine whether prolonged growth of C3 on Medicago as a host would lead to a consistent shift in of nucleotype ratios, we performed a selection experiment where we subcultured C3 for four rounds, spanning >2 years, on axenic Medicago root cultures. This resulted in three independent Medicago selection lines that were subsequently sequenced after DNA extraction and whole genome amplification (referred to as MetaMB-D samples in Figure 8). Unlike the observed 1:1 allele frequencies in the mRNA populations, the prolonged co-culturing of C3 with Medicago did not lead to a consistent shift in nucleotype ratios, based on both genome wide allele frequency distributions and MAT allele ratios [Figure 8a,b; Figure S4]. PCA analyses based on allele frequencies did not indicate a closer relatedness of Medicago selection lines compare to different batches of *D. carota* root cultures [Figure 8b]. Furthermore, MAT loci frequencies of these lines showed similar variation [Figure 8c].

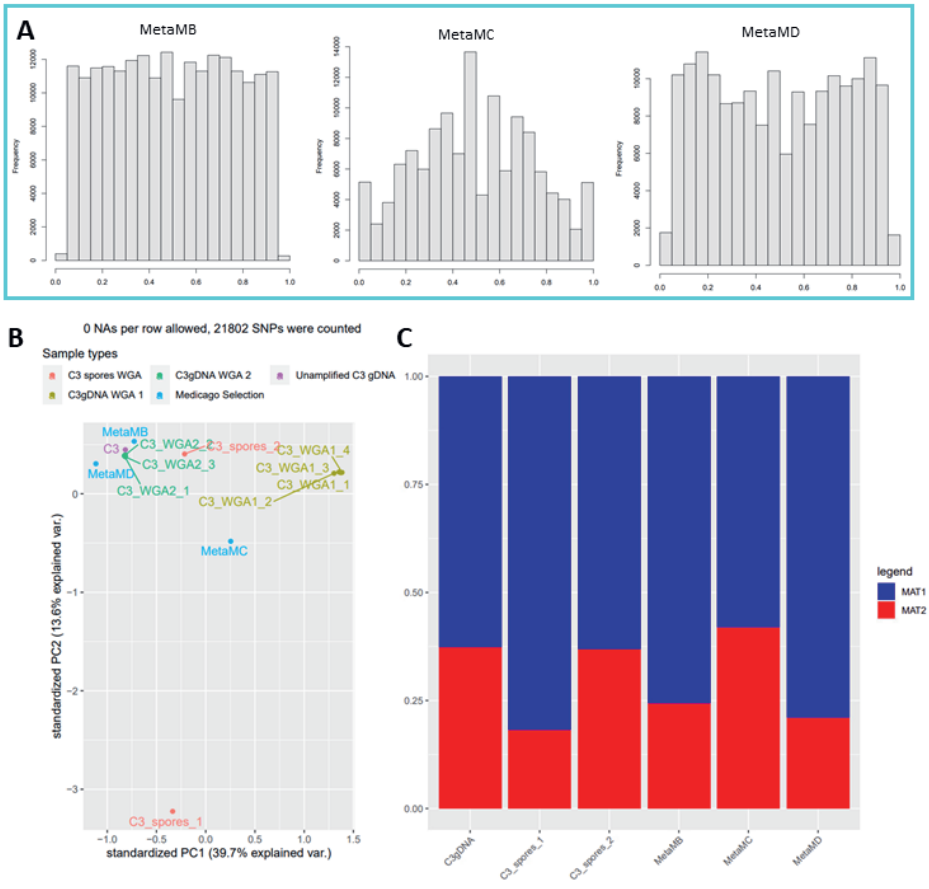

Figure 8: *Selection line variant analysis.* ***A****: Allele frequency distributions of three Medicago selection lines.* ***B****: Principal component analysis of C3 and selection lines, based on allele frequencies of shared SNPs. Additional WGA and C3_spores samples were included as additional reference samples.* ***C****: MAT loci frequency of C3 and selection lines based on read mapping.*

Discussion

Heterokaryosis is common aspect in fungal biology and is hypothesized to play an important role in the ability of fungi to adapt to a continuously changing environment [Strom *et al.*, 2016]. In case of the extremely coenocytic AM fungi, it was proposed that changes in nucleotype ratio could be adaptive in the colonization of different host plants [Angelard *et al.*, 2014; Wyss *et al.*, 2016; Sanders and Rodriguez, 2016; Kokkoris *et al.*, 2021]. Here, we confirm that the distribution of genetically divergent nuclei in the *R. irregularis* C3 isolate is highly variable, with seemingly random fluctuations of nucleotype ratio. Large variations in allele frequencies were observed between individual (progeny) spores and single spore lines and even between different root culture plates/batches of the same spore line. This is in line with the recent findings of Robbins *et al.* (2021) based on ddRAD sequencing. No consistent effect of host plant identity on the distribution of nucleotypes was observed after 2 years of subculturing on a different host. Interestingly, host identity did have a reproducible effect on allele-specific expression as observed for C3 colonizing four different host plants.

The extent of genetic diversity within AM fungal individuals has been highly debated and the current view is that *R. irregularis* strains are either homokaryotic or dikaryotic [Tisserant *et al.*, 2013; Lin *et al.*, 2014; Ropars *et al.*, 2016; Kokkoris *et al.*, 2020]. Our analyses show that numerous low frequency polymorphisms are not just mere sequencing artefacts, as suggested by Ropars *et al.* (2016), but real components of the genetic variation within AM individuals that is distributed over different nuclei, in line with Masclaux *et al.* (2019). Furthermore, sequencing of multiple replicate amplifications showed that, although some minor fluctuation in allele frequencies was observed, the whole genome amplification procedure did not introduce significant biases. These results show that the term “dikaryotic” does not fully capture the breadth of genetic variation in *R. irregularis* [Masclaux *et al.*, 2019], as the coenocytic nature allows for the population of nuclei to accumulate and retain polymorphisms within the nuclear population. This is similar to other fungi, where somatic mutations within an individual lead to polymorphisms that can be maintained through nuclear selection [Grum-Grzhimaylo *et al.*, 2021]. Intriguingly, multiple low frequency SNPs (occurring between in 10-25% of the reads mapping) were even conserved between the C3 isolate and its presumed clone A4 [Additional File 4]. Both strains originated as single spores from different locations in a field in Switzerland and have been individually grown in root cultures for ~20 years [Jansa *et al.*, 2002; Koch *et al.*, 2004]. Given their very high sequence similarity it therefore seems likely that these isolates once originated from the same parental line(s) in the field. Despite all these years of separation, many SNPs have been maintained within the two isolates, even though their allele ratios can vary substantially. In contrast to the variable nucleotype ratio in C3, A4 was reported to show stable nucleotype ratio among different root cultures and individual spores [Ropars *et al.*, 2016; Kokkoris *et al.*, 2021], while in that same study another *R. irregularis* isolate called SL1, the MAT allele ratio was also found not to be stable across spores and subcultures like in C3 [Kokkoris *et al.*, 2021]. Furthermore, in the very recent work by Cornell *et al.* (2022) it was found that distinct abiotic factors could affect nucleotype ratios. In our case, all root cultures were grown under the same environmental conditions, but still we noticed large variations between spores and batches in C3. What determines this rather different behavior of nuclei remains to be determined.

Different siblings of the same parental line have been shown to differentially affect plant growth [Angelard *et al.*, 2010]. Recent field trials with different C3 progeny lines in Cassava revealed large differences in cassava growth [Ceballos *et al.*, 2019]. Intriguingly, in this work also progeny lines of homokaryotic strains, with much less intra-genomic variation, showed similar strong differential symbiotic effects. This might suggest that in addition to the genetic composition additional factors, such as possible epigenetic effects, contribute to variation in symbiotic performance of lines [Ceballos *et al.*, 2019].

C3 contains two main nucleotypes that can be distinguished based on the sequence diversity of two presumed MAT loci. Single nucleus sequencing revealed at least 503 SNPs in C3 that occurred in different nuclei marked by the same MAT locus. Such SNPs could be the result of somatic mutations and/or point to potential inter-nucleus recombination events between nuclei containing opposing MAT loci. Especially those cases where multiple consecutive SNPs occurred in a single contig and whose allele frequencies in the genome were similar, are strongly suggestive for recombination events. This might be facilitated by the high level of repetitive regions in the genome. The number of putative recombination events was significantly higher than that reported for the closely related A4 isolate [Ropars *et al.*, 2016]. To rule out that this might be due to a different genome assembly, single nuclei data for the A4 strain [Ropars *et al.*, 2016] were analyzed with the same settings using the RirC3 assembly [Additional Files 8 and 14]. We still found a much lower number of putative recombination events in the A4 data, although the coverage of the A4 single nuclei reads was also lower than those of the C3 nuclei [Figure S7]. It should be noted that the C3 and A4 data were generated in different labs with potentially different environmental conditions. A variety of external factors such as growing conditions, temperature, starvation or (biotic) stress, as well as intrinsic genetic or epigenetic mechanisms have been linked to recombination rate plasticity [Kamel *et al.*, 2017]. To which extent different recombination rates in *R. irregularis* are conditional remains to be determined.

The allele frequencies varied wildly among progeny spores as well as compared to their parental lines in a seemingly random fashion. Currently, spore formation is thought to represent the most narrow genetic bottleneck in the AM fungal life cycle, where the fewest nuclei (ranging from ~60 to thousands) will start a new generation [Marleau *et al.*, 2011]. Single spores that were derived from a previously generated single spore line therefore underwent two genetic bottlenecks compared to the original root culture from which the single spore lines were derived. This could lead to a reduction in genetic variation in subsequent progeny spores. Yet, these second generation single spores were not more similar to each other, but instead varied as much from each other as single spores derived from a different single spore line [Figure 7]. These results illustrate that the genetic composition of a spore is not necessarily representative of the colony that develops from it, in line with data from Ehinger *et al.* (2012) and Masclaux *et al.* (2018). We even found an extreme case where the single spore line 1 contained a large majority of MAT-2 nuclei, with very little MAT-1 nuclei (SS1 in Figure 4). Although we cannot completely rule out that such an extreme ratio is due to the whole genome amplification, we did see large variation in multiple unamplified samples as well. This may suggest that this line would be on its way to a homokaryotic state, however individual progeny spores derived from SS1 again showed a completely different ratio.

If segregation of nuclei into developing spores would be a truly random process, modelling suggested that this should lead to a loss of diversity and eventual reversion to a homokaryotic state over time [Bever *et al.*, 2005, 2008]. However, the long-term conservation of multiple nucleotypes in C3 indicates that there must be mechanisms that counteract this drift effect. One of these mechanisms may involve continuous nuclear mixing as a result of hyphal fusion/anastomosis, which can occur quite frequently in AM fungi [Giovanetti *et al.*, 1999, 2001; de La Providencia *et al.*, 2005]. Modelling showed that such mixing could be sufficient to offset the drift effect [Rayner, 1991]. Currently, the dynamics of nuclei are not well understood in AMF. Live cell imaging of hyphae found no evidence for synchronized divisions but showed that nuclei can move in "pulses" in a bi-directional manner, seemingly independent form cytoplasmic streaming [Bago *et al.*, 1999]. How this movement is regulated or coordinated in different parts of the mycelium is not known, but such pulsed movements could ensure the constant mixing of nuclei facilitating the maintenance of the dikaryotic-like status.

Fluctuation in nuclear ratios can also be caused by competition between nuclei [Rayner, 1999]. However, also in this case, modelling suggested that it would lead to a loss of diversity in favor of the most dominant nucleotype. Therefore, it was proposed that cooperation, or division of labor, between nuclei could lead to the long-term and stable coexistence of distinct genotypes [Kokkoris and Hart, 2019]. Also in other fungi, variation in the ratio of nuclear populations have been observed and suggested to be influenced by nuclear selection [Roper *et al.*, 2013]. The observed stochastic behavior of nuclei in C3 would argue against a strong interdependence of nucleotypes. In the absence of varying selection pressures inter-nucleus recombination would be expected to reduce diversity in the long term and lead to the fixation of a single nucleotype. This might explain why most of the current AMF cultures appear to be homokaryotic, since the axenic root cultures represent a more or less homogeneous artificial environment with very little variation [Bécard *et al.*, 1988]. In nature, AM fungi will be exposed to continuously changing environments, such as multiple different host plants and soil characteristics with fluctuations in pH, nutrient sources, water availability or other microbes. All these factors may impose different selection pressures which could favor a heterokaryotic state. It would therefore now be interesting to apply single spore sequencing to spores collected directly from the field to determine the prevalence of dikaryotic-like states, or possibly higher levels of genetic variation.

Upon colonization of different host plants we found that, using the same batch of spores, shifts in allele frequency distribution occurred at the transcriptome level. C3 colonizing Medicago and Chives showed a dominant allele frequency distribution around 50% at the mRNA level for the two MAT nucleotypes, while in the same C3 batch colonizing Nicotiana and Tomato slight allele frequency peaks at ~33% and 67% were observed. Observing such reproducible shifts in expressed allele-frequency distributions using the same batch of spores suggests that host-identity not only affects the expression of different genes [Kamel *et al.*, 2017; Zeng *et al.*, 2018; Mateus *et al.*, 2019] but also different alleles of the same genes. Robbins *et al.* (2021) showed that allele frequencies in the transcriptome of extraradical mycelium mostly resembled the frequency of the two nuclear genotypes in axenic cultures. We cannot completely rule out that the shift in expressed alleles that we observed was caused

by a similar host-dependent shift in nucleotype ratios, as was shown by [Kokkoris *et al.*, 2021] for the A4 isolate. However, in our host-selection experiment, which spanned over two years, we did not observe reproducible effects on nucleotype ratios in C3 upon a host shift from Carrot to Medicago [Figure 8], indicating that if a shift in host-induced nucleotype ratios initially occurred it does not seem to be stably maintained. It further remains to be determined whether nuclear ratios in the extraradical mycelium and intraradical stages are similar.

Nucleotype-specific expression was recently reported for the multinucleate mushroom *Agaricus bisporus*, which contains two to 25 nuclei of two nuclear types per cell. Widespread transcriptome variation was observed between the two nucleotypes in relation to the development of various *A. bisporus* tissues [Gehrmann *et al.*, 2018]. This was found to be correlated with differential methylation states, suggesting that epigenetic factors may be important regulators of nucleus-specific expression. An additional level of variation may involve the nucleus-specific expression of distinct ribosomal RNAs. Like DAOM197198, C3 lacked a tandem repeat organization of the 45S rDNA [Maeda *et al.*, 2018]. Eight 45S copies were identified in C3 that showed significant sequence variation and additional polymorphisms were found to be distributed over different nuclei. This may lead to ribosomes with different translational activities in different spores or even different parts of the mycelium [Maeda *et al.*, 2018].

Conclusions

In conclusion, our analyses show that nuclear behavior in *Rhizophagus irregularis* can be highly dynamic. The C3 isolate showed inter-nucleus genetic variation and putative recombination, seemingly stochastic (partial) nuclear segregation, root culture batches with varying nucleotype ratios, significant variation in rDNA variants and host-dependent, nucleotype-specific expression. As the combined output of this genetic variation ultimately determines the effect on plant growth promotion [Sanders and Rodriguez, 2016; Kokkoris *et al.*, 2020], further insight into the regulation of such nuclear dynamics will be important to understand their distribution and contribution in ecological settings and to exploit their potential as sustainable biofertilizers in agriculture.

Methods

Fungal material

Rhizophagus irregularis isolate C3 was originally isolated from a Tänikon, Switzerland as described in [Koch *et al.*, 2004]. The fungus was propagated on *Agrobacterium rhizogenes*-transformed *Daucus carota* root cultures on M medium [Bécard *et al.*, 1988; St-Arnaud *et al.*, 1996].

Single spore lines were generated by placing a single C3 spore next to a fresh *D. carota* root culture (initial culture provided by dr. Toby Kiers, University of Amsterdam). Spores were selected from spore clusters from the same source plate, and single spore lines were named after their respective cluster.

Medicago selection lines (MedSel) were made by inoculating *Medicago truncatula* (Jemalong A17) root cultures with ~50 C3 spores. When these cultures produced enough spores, these spores moved to fresh *M. truncatula* root cultures to start a new round. Three of these subsequent transfers were made. For DNA sequencing, ~50 spores were isolated from the M medium and crushed in 2µL DNA free mQ water. Total genomic DNA was then amplified using the Repli-G WGA kit (Qiagen).

DNA isolation for genome assembly

Four square plates, six round plates and four split plates containing fully C3 mycorrhized *D. carota* root cultures were harvested and pooled. Upon harvesting the fungal material, roots were removed from root culture plates with pliers and scalpel, after which the medium was liquidized by adding ½ volumes 100mM Citrate buffer (40mM sodium citrate dihydrate, 60mM citric acid, pH = 6.5) to each volume of M medium and gently shaking at RT for at least 30 minutes. The dissolved medium was then poured into an empty square petri dish, from which the mycelium and spores were collected with a sterile disposable inoculator loop, while taking care to avoid any pieces of the root culture. Collected spores and mycelium were washed in sterile milli-Q water, collected in a 2mL Eppendorf tube and centrifuged at 5000rpm. As much water as possible was removed from the tube, after which the sample was weighed and flash-frozen in liquid nitrogen.

Samples were thoroughly (>20x 20s) pulverized with a metal bead in a TissueLyser LT (Qiagen). All materials were kept at minimal temperatures to avoid thawing of the sample. For the isolation of high molecular weight genomic DNA, a protocol from Fauchery *et al.* (2018) was adapted. The lysis buffer was made of five stock solutions [Table S2] that were combined shortly before the isolation. 1.5mL of the lysis buffer was added to the frozen fungal material. The sample was mixed by gently shaking until the sample was completely suspended in the buffer. Lysis was performed at 65°C for 30 minutes, gently shaking every 10 minutes. The lysis was stopped by adding 492µL 5M Kac (pH 7.5) and gently inverting. The sample was incubated on ice for 30 minutes and centrifuged at 5000g at 4C for 20 minutes. The supernatant was transferred to a 15mL Falcon tube, and cleaned by adding 1 volume chloroform–isoamyl alcohol (24:1 v/v), gently but thoroughly shaking until completely mixed, and pipetting the upper layer to a fresh tube. This step was repeated twice to remove all residual proteins. 10µL

RNAse A (10mg/mL) was added and the sample was incubated at 37°C for 1 hour. Next, 20µL 3M NaAc (pH = 5.2) was added, the sample was mixed, and then precipitated by adding 1 volume of isopropanol. The sample was incubated at RT for 15 minutes before centrifuging at 4°C for 30 minutes at max speed. The supernatant was discarded and the pellet was washed with ice cold 70% ethanol. The sample was then dried at RT and resuspended in 55µL 20mM Tris-HCl at 65% for 30 minutes. 5µL of the solution was diluted 4x for quality control, the rest was immediately stored at -70°C. Yield was measured by Qubit 2.0 fluorometer via the Qubit dsDNA HS Assay (Life Technologies) and DNA integrity was checked on 0.8% agarose gel. 900ng of high molecular weight genomic DNA was collected for PacBio sequencing.

PacBio assembly

PacBio SMRT Sequel II subreads were generated at GenomeScan B.V. (Leiden, The Netherlands). The subreads were assembled using Flye (2.7.1-b1590) [Kolmogorov *et al.*, 2019] with the following command: flye --pacbio-raw C3_PacBio_subreads.fastq.gz -g 156m --out-dir C3_assembly --threads 30. Duplicated regions were removed with purge_dups [Guan *et al.*, 2020]. Genome polishing was performed in two steps: first with the PacBio subreads using Arrow (Pacific Biosciences), then with Illumina reads of C3 using two iterations of Racon (v1.4.13) [Vaser *et al.*, 2017]. C3 Illumina reads were produced by sequencing 300ng of C3 genomic DNA, isolated from the same cultures as the PacBio sample, at NovoGene B.V. (Hong Kong). Genome completeness was assessed with BUSCO [Simão *et al.*, 2015], using database fungi_db10. Repeats were modelled *de novo* with RepeatModeler and subsequently masked with RepeatMasker (v. open-4.0.9) [68]. The genome was annotated using Funannotate (v1. 6.0) [Smit *et al.*, 2015], using predicted gene models and C3 RNAseq reads from C3 grown on multiple hosts (see RNAseq section). The mitochondrial genome was found by blasting RhiirA4 mitochondrial markers [Ropars *et al.*, 2016] against the raw RirC3 assembly (before purge_dups). All markers were found on a single contig covering the entire predicted mitochondrial genome. Ribosomal DNA copies were found by blasting Rir17 rDNA sequences in the RirC3 assembly. Contigs of the assembly were visualized with Circos [Krzywinski *et al.*, 2009].

Variant calling

Illumina reads of C3 were mapped against the RirC3 assembly using Hisat2 [Kim *et al.*, 2019], and sorted with samtools sort. Variant calling was performed on mapped reads using Freebayes (v1.3.2) [Garrison and Marth, 2012], setting ploidy level to 1 with the pooled-discrete -J option. Only SNPs located outside of repeated regions were counted. Variants were filtered using bcftools filter (v 1.10.2) [Li *et al.*, 2009]. SNPs for C3 were filtered on coverage between 85 and 135 (mean coverage 110), and both reference and alterative allele observation of at least 10. Allele frequency distributions were plotted in R (v 4.0.3) using the hist() command. Principal component analysis was performed by merging vcf files with bcftools merge, and creating a dataframe of all allele frequencies in R. Next, only SNPs with coverage in all samples were selected. Principal component analysis was performed using prcomp(df, center = TRUE, scale. = TRUE), and plotted using ggbiplot().

During the preparation of this manuscript another genome assembly was published for the C3 isolate, named CHRIC3 [Robbins *et al.*, 2021]. As the CHRIC3 assembly appeared to contain more duplicated genome regions [Table S3], we continued analyses using our own RirC3 assembly for which the unique read mapping rate was higher. As a comparison, we performed preliminary analyses with both assemblies, showing that either assembly produces similar results regarding the distribution of genetic variation [Figure S8].

Single nuclei isolation and sequencing

Spores of C3 were suspended in 1xPBS buffer (pH 7.4) and crushed with a pestle. Nuclei were selected by fluorescent associated cell sorting (FACS) [Montoliu-Nerin *et al.*, 2020] and whole genome amplified (WGA) through MDA using Phi29 polymerase. An 80x dilution of the reactions was used for genotyping. The remaining reaction mixture was purified using ethanol precipitation and dissolved in 30µL 10mM Tris-HCl. Genotyping was done using primers targeting the ITS region (AM1 + NS31) [Simon *et al.*, 1992; Helgason *et al.*, 1998] or MAT loci (Forward: ACTATCTGACTTGCTATTGTTGA, Reverse: CAGGGCCTGCATCGGATTA). Ten of the nuclei were sent for Illumina sequencing (NovoGene, Hong Kong). Reads were mapped against RirC3 using HiSat2 [Kim *et al.*, 2019] with standard settings, and variant calling was performed with freebayes (ploidy = 1). Variants were selected by first intersecting the vcf file with the filtered RirC3 gDNA vcf file, where only SNPs that were found in the whole genome data were selected. SNPs inside repeated regions were ignored. Next loci where any nucleus contained a heterozygous SNP (ie. both alleles were found in a single nucleus) were filtered out as well. Heterozygous SNPs were found using the Awk utility in bash, and were defined as having both alleles at a frequency above 10% (RO/DP > 0.1 || AO/DP > 0.1) in any of the nuclei. Finally, sites in nuclei with a coverage <10 were ignored. The same selection was done using the A4 single nucleus data [Ropars *et al.*, 2016]. Similarity plots were made in R using ggplot(). Individual nuclear genomes were assembled using Spades [Montoliu-Nerin *et al.*, 2020; Bankevich *et al.*, 2012].

To find potential recombination sites, vcf files were grouped based on MAT identity. Only uniquely mapping reads were considered (samtools view -f 2). SNPs within 500bp upstream and downstream of heterozygous loci in and/or based on non-paired reads only, or with a coverage below 10, were ignored. If genotypes of any SNP in the genome were linked to MAT identity of the nuclei, all nuclei sharing a MAT locus should have the same genotype on that SNP. Therefore, any SNP where both the reference and alternative allele were found in nuclei with the same MAT locus, was considered as a non-matching region representing a potential recombination event.

Single spore amplification and analysis

Single spores were isolated by excising M medium containing spores from root cultures, and subsequently dissolving them in citrate buffer. The spores were thoroughly rinsed with sterile mQ water and collected in 2µL of mQ in 200µL PCR strips. Spores were manually crushed using pipette tips of which the tips were briefly melted in an open flame, to create a “pestle”. After crushing, the samples were flash-frozen in liquid nitrogen and incubated at 95°C for 10 minutes to further lyse the nuclei. WGA was performed using the Repli-G Single Cell kit

(Qiagen) following manufacturer's instructions. The samples were purified by ethanol precipitation and dissolved in 30µL 10mM Tris-HCl buffer. Samples were sent for Illumina sequencing in NovoGene (Hong Kong).

Plant inoculation for RNAseq

C3 inoculum

Spores of C3 were released from root cultures by disrupting the root cultures in a blender with 2x volume water and filtering with a 40µm mesh to capture the spores and mycorrhized root fragments. Spore suspensions were stored at 4°C.

Nicotiana

Nicotiana benthamiana seeds were sterilized in 20% bleach solution for 12 minutes, thoroughly washed with sterile water and germinated on water agar with a filter for 72h at RT, in 16/8 light dark cycle. Pots (9x11x11cm) with 2:1 sterilized clay:silver sand mix were prepared, and ~200 C3 spores were added in the middle of the pot, ~4cm below surface. The middle of the surface was covered with a small amount of 1:1 vermiculite/silver sand mix, to act as a more stable soil to plant the small seedling. After germination, seedlings were planted with a fine brush in a 1mm hole in the vermiculite/silver sand mix. To increase initial growth rate, 2mL of high phosphate half-strength Hoagland solution [Hoagland *et al.*, 1950] (1mM K2PO4) was added to the seedlings. The pots were covered with plastic foil for the first week to maintain soil humidity, and plants were watered twice a week with adjusted low phosphate half-strength Hoagland solution (50µM K2PO4). Plants were grown at 25°C in a 16/8 light dark cycle. Mycorrhized roots were harvested after six weeks by gently submerging the pot in water, removing soil and clay from the roots under water and rinsing carefully with tap water.

Medicago

Medicago truncatula Jemalong A17 seeds were scarified in 96% sulfuric acid for 10 minutes, thoroughly rinsed with water, sterilized with 50% bleach for 10 minutes and washed again with sterilized water. Seeds were then incubated on a water agar plate with filter at 4°C in the dark, and then incubated at 21°C in the dark. Pots (9x11x11cm) with 1:1 sterilized clay:silver sand mix were prepared, and ~200 C3 spores were added in the middle of the pot, ~4cm below the surface. After germinating, seedlings were planted in the pots. Plants were grown at 21°C in a 16/8 light dark cycle, and were watered with adjusted half-strength Hoagland solution (20µM K2PO4). Mycorrhized roots were harvested after six weeks and gently washed with tap water.

Chives

Allium schoenoprasum seeds were soaked in mQ at 4°C for 8h, disinfected with 20% bleach for 12 minutes and thoroughly rinsed with sterile water. Seeds were germinated on agar plates with a filter for 48h at 21°C (16/8 light dark cycle). Pots (9x11x11cm) with 1:1 sterilized clay:silver sand mix were prepared, and ~200 C3 spores were added in the middle of the pot, ~4cm below the surface. After germinating, seedlings were planted in the pots. Plants were grown at 21°C (16/8 light dark cycle), and were watered with adjusted half-strength Hoagland

solution (20µM K2PO4). Mycorrhized roots were harvested after six weeks and gently washed with tap water.

Tomato

Solanum lycopersicum (MoneyMaker) seeds were soaked in 0.03M HCl for 6h and sterilized in 50% bleach for 5 minutes. Seeds were then germinated at 25°C for 72h in a 16/8 light dark cycle. Large pots (18x11x11cm) were filled with a 2:1:1 clay:silver sand:vermiculite mix. ~200 C3 spores were placed ~8cm below surface. After germination, seedlings were planted and watered with adjusted half-strength Hoagland solution (50µM K2PO4). Mycorrhized roots were harvested after eight weeks by gently submerging the pot in water, removing vermiculite and clay from the roots under water and rinsing carefully with tap water.

Medicago truncatula (Jemalong A17), *Nicotiana benthamiana*, *Allium schoenoprasum* and *Solanum lycopersicum* (MoneyMaker) were all propagated in-house under greenhouse conditions at Wageningen University (Wageningen, The Netherlands).

RNA isolation and sequencing

RNA from colonized roots was isolated by flash-freezing colonized roots and destroying the tissue with a cold mortar and pestle. RNA isolation was performed using the RNeasy Mini kit (Qiagen), according to manufacturer's instructions including an on-column RNAse free DNAse (Qiagen) treatment. Three biological replicates of each treatment were sent for Illumina sequencing (BGI, Denmark). RNAseq reads were mapped to the RirC3 assembly with Hisat2 using the --dta option. Variant calling was performed with freebayes as described above; only biallelic SNPs found in the genome were used. Variants were filtered based on a minimal coverage in all hosts of 20, with at least 10 observations of both alleles.

Digital droplet PCR

Digital droplet PCR was performed using 80 ng/µL each of MAT-1 and MAT-2 specific primers [Kokkoris *et al.*, 2021] and QX200™ ddPCR™ EvaGreen Supermix (BioRad) in a total volume of 20µL. For unamplified (meta-) samples 2 µl of a 1:10 dilution was used per reaction. For WGA amplified samples, 2 µL of a 1:100 dilution was used as template. The PCR mix was suspended in oil for EvaGreen using the QX200 Droplet Generator (Biorad), following manufacturer's instructions. PCR was performed for 40 cycles, annealing and elongation at 58°C. Subsequently, the absolute number of positive droplets was counted using a QX200 Droplet Reader and analyzed via QuantaSoft Software (BioRad).

Availability of data

The RirC3 assembly with annotation is available at https://zenodo.org/record/7037960. All C3 sequencing data generated in this work are available from Genbank under BioProject ID PRJNA747641 and SRR15179489 - SRR15179534. Sequencing data for A4 were retrieved from BioProject ID PRJNA299206 and PRJNA477348.

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Supplementary Information

Additional file 1: RirC3 BUSCO output and comparison.

Additional file 2: Additional C3 45S rDNA polymorphisms (.vcf file).

Additional file 3: Allele variant (SNP) list based on C3 gDNA reads (.vcf file).

Additional file 4: Allele variant (SNP) comparison of C3 and A4 (.vcf file).

Additional file 5: C3 mitochondrial DNA variants (.vcf file).

Additional file 6: Allele variant (SNP) table of 10 single nuclei from C3 (.vcf file).

Additional file 7: Allele variant (SNP) table of 14 single nuclei from A4 (reads from Chen *et al.*, 2018) (.vcf file).

Additional file 8: Potential inter-nucleus recombination events in C3 and A4.

Additional files are available online with the publication on:

https://link.springer.com/article/10.1186/s12864-023-09126-6#additional-information

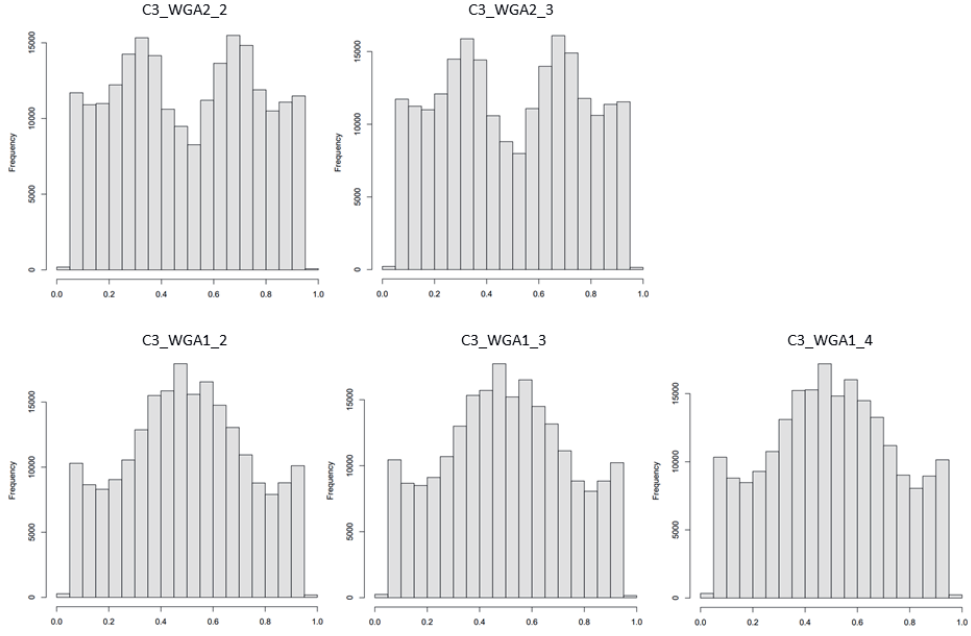

Figure S1: *Allele frequency distribution of replicate, independently WGA-amplified, C3 gDNA samples; corresponding to main Figure 4A,B. Two replicates for C3-gDNA2 and 3 replicates for C3-gDNA1 (used for genome assembly).*

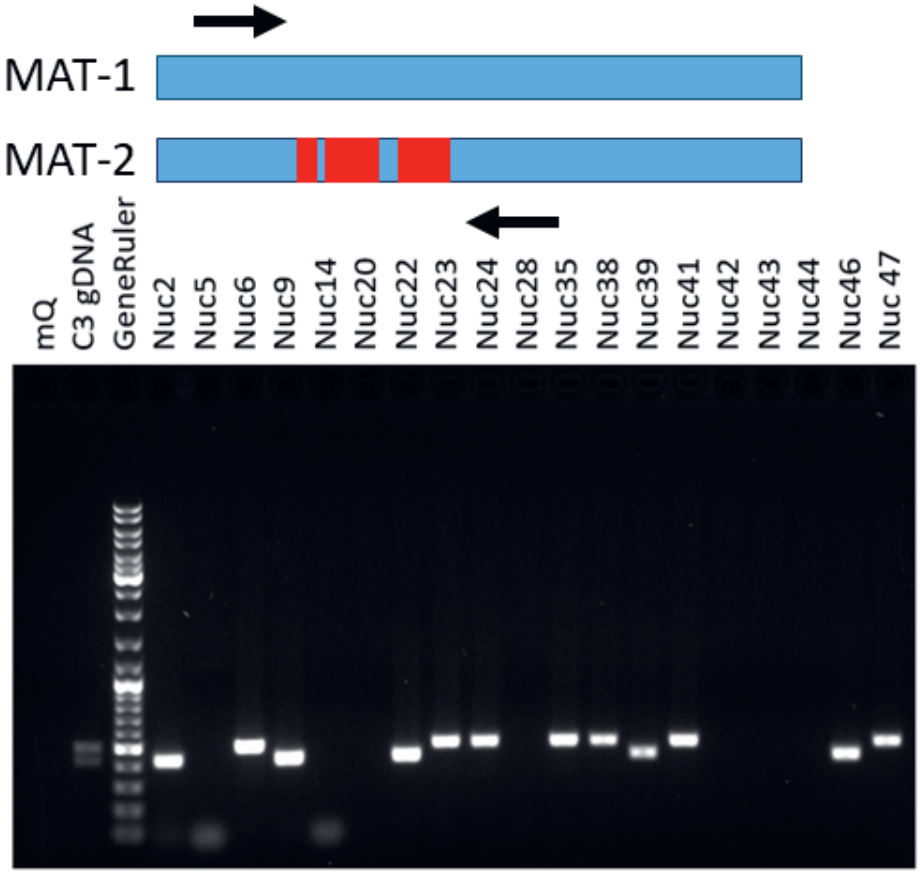

Figure S2: *PCR analysis of MAT locus identity in C3 single nuclei. The upper band corresponds to MAT-1, the lower band to MAT-2.*

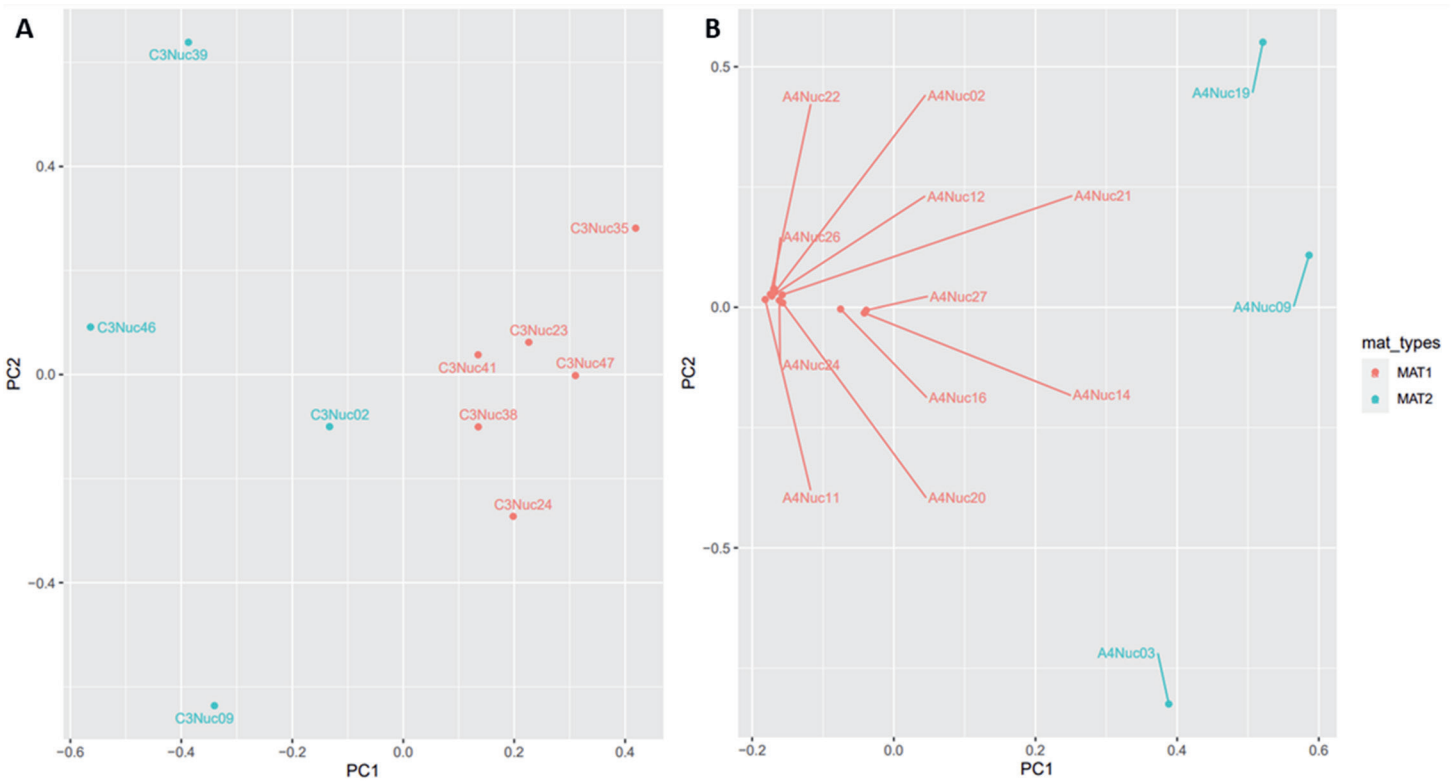

Figure S3: *Principal component analysis of C3 single nuclei (A) and A4 single nuclei (B) based on allele frequencies when mapped to the RirC3 assembly. The MAT locus identity of the individual nuclei is indicated by color: red = MAT-1, blue = MAT-2.*

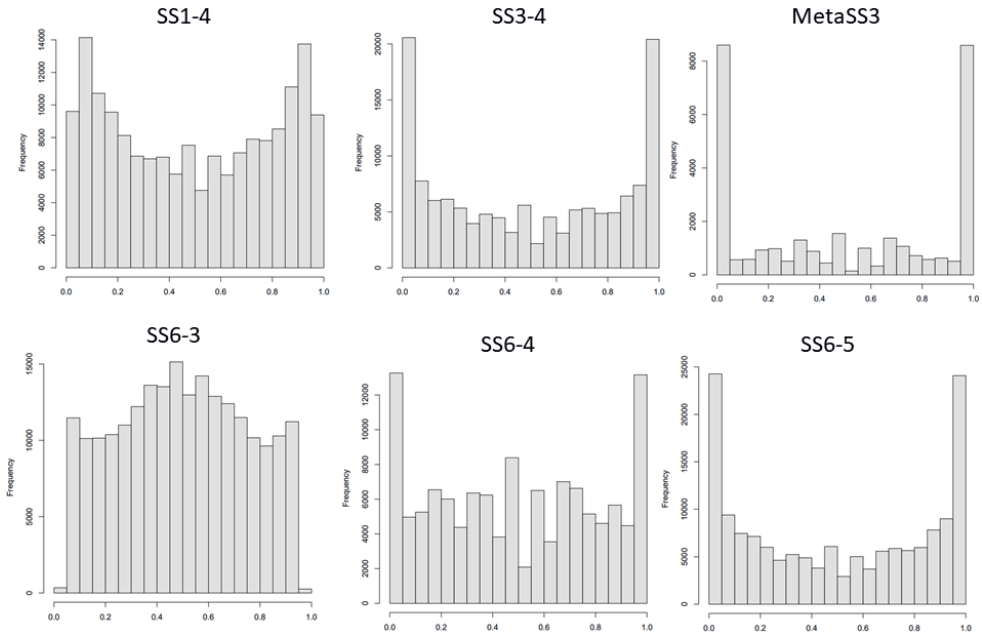

Figure S4: *Allele frequency analysis of (WGA amplified) C3 single spores derived from single spore lines.*

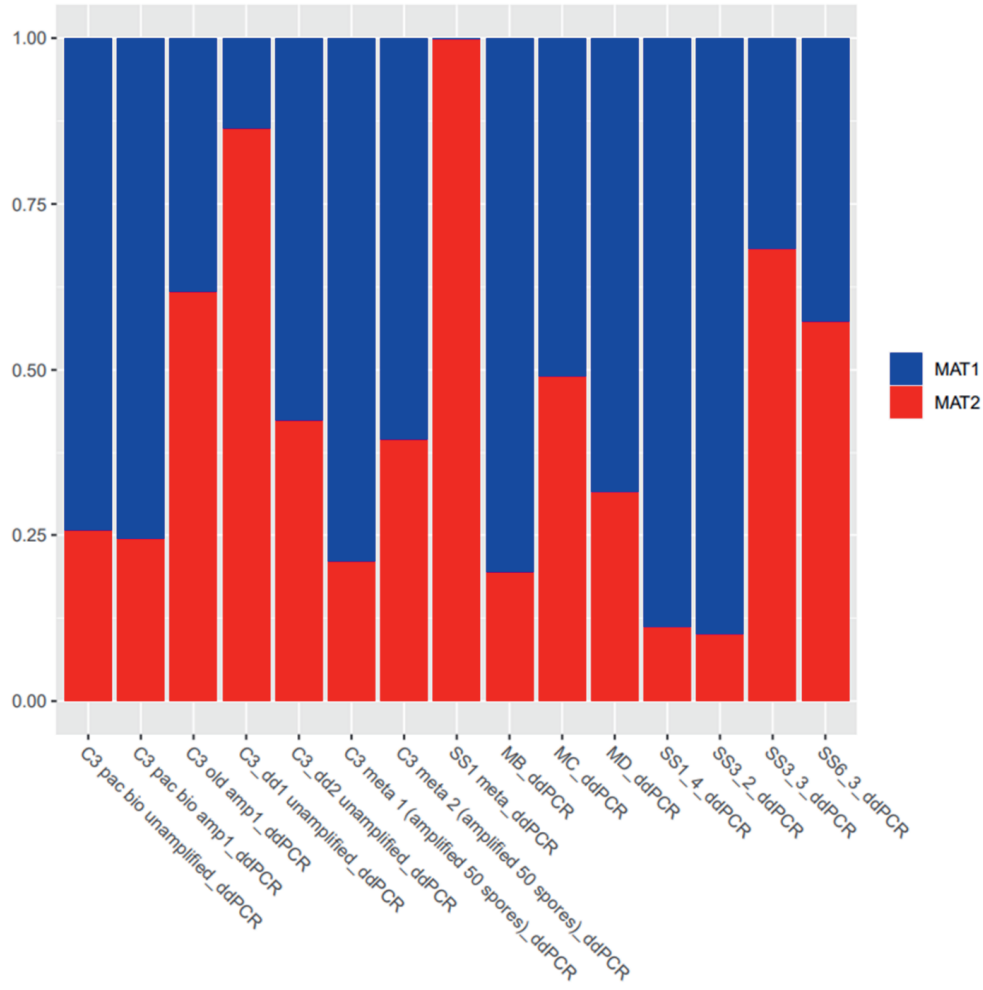

Figure S5: *MAT ratio based on digital droplet PCR of different root culture batches/lines. C3 pac bio refers to DNA sample C3 gDNA2 used for genome assembly, either unamplified or WGA amplified (Amp1). C3 old refers to the independent DNA sample C3 gDNA1. C3_dd1 and _dd2 refer to WGA amplified DNA from two additional independent C3 carrot root culture batches. C3 meta1 and meta2 refer to DNA extracted and WGA amplified from groups of 50 spores from two different root culture plates. SS1 refers to non-amplified DNA from single spore line 1. MB, MC and MD refer to non-amplified DNA from three Medicago selection lines. SS1_4, SS3_2, SS3_3 and SS6_3 refer to DNA samples from 2nd generation single spore lines.*

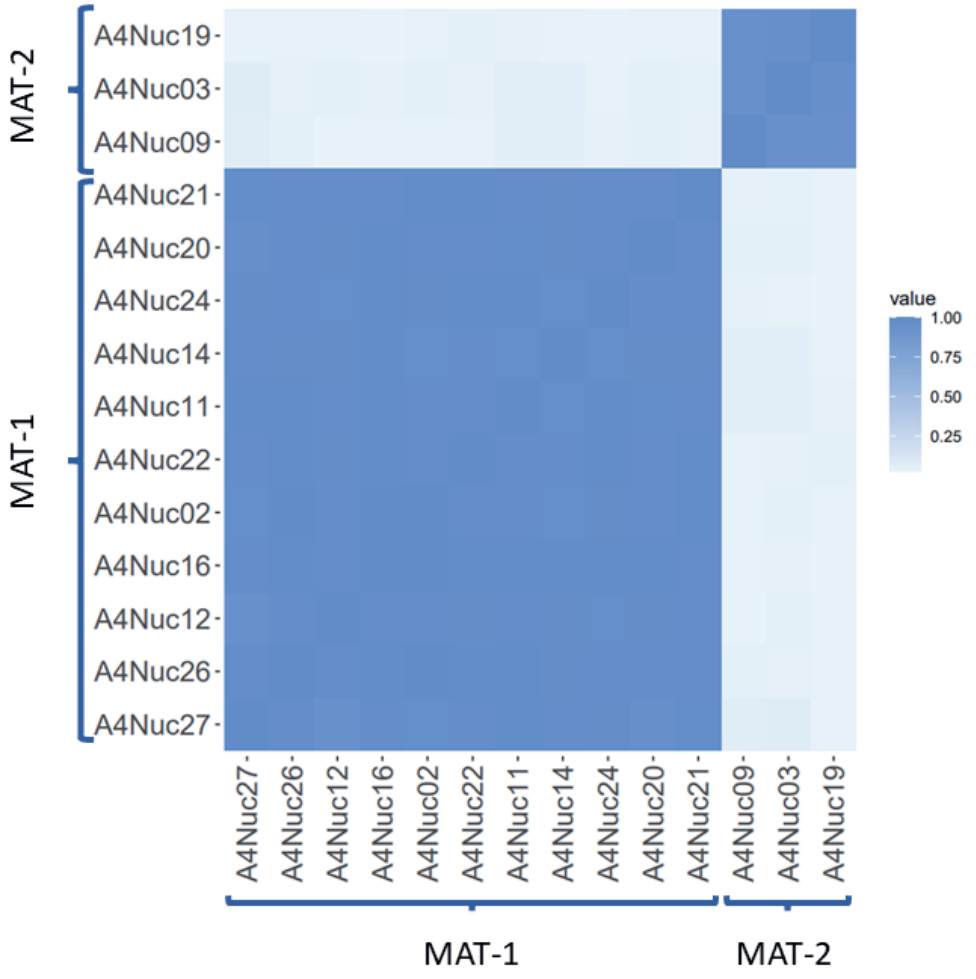

Figure S6: *Similarity plot (heat maps) of A4 nuclei based on single nucleus sequencing data from [Chen* et al.*, 2018]. Color coding indicates level of relatedness between among the nuclei. A sharper contrast between the groups means that the nuclei are more different, while patches of differing colors within the groups indicate similarities to nuclei of the other group (meaning the other MAT locus). Nuclei are grouped based on which MAT locus they contain.*

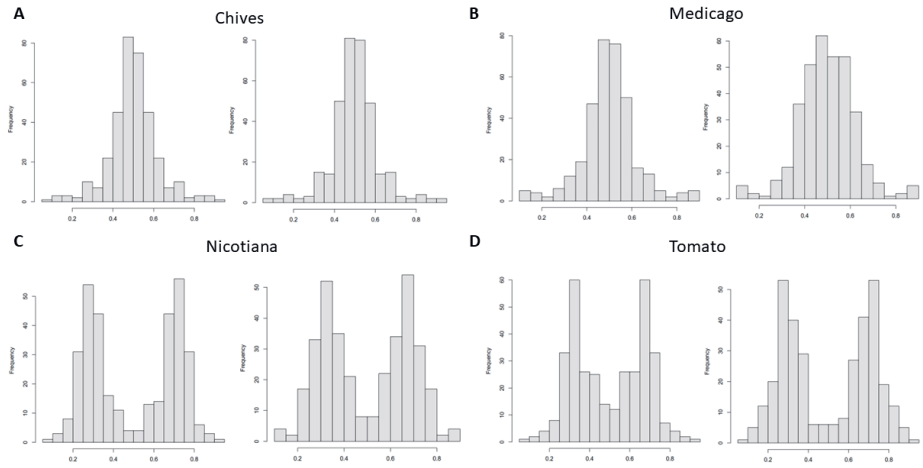

Figure S7: *Allele frequency analysis based on RNAseq data from two additional biological replicate samples of C3 colonizing Chives, Medicago, Nicotiana and Tomato; corresponding to main Figure 7.*

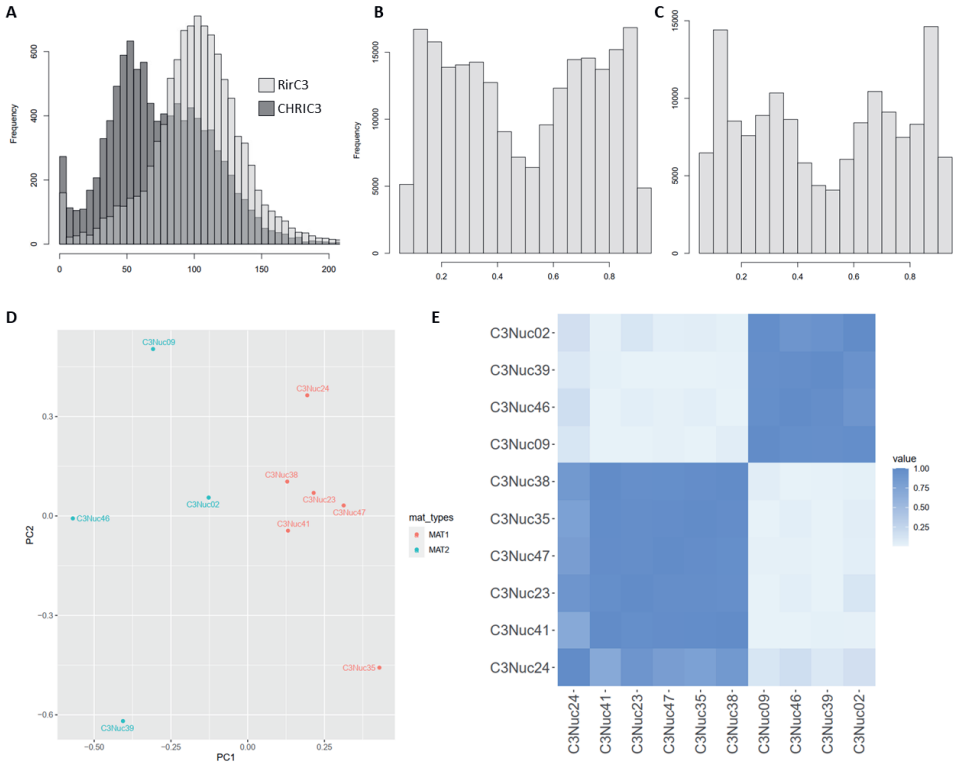

Figure S8: *Comparison of the CHRIC3 (Robbins* et al.*, 2021) assemblies on the distribution of genetic variation, showing comparable results as with the RirC3 assembly (this study).* ***A****: Mapping depth of C3 Illumina reads against RirC3 (light) and CHRIC3 (dark) assemblies.* ***B****: Allele frequencies of SNPs in C3 Illumina reads (C3gDNA) mapped against the CHRIC3 assembly. SNPs were filtered on coverage between 35 and 135x, and both alleles being found at least 10 times.* ***C:*** *Allele frequencies of SNPs in C3 Illumina reads mapped against the RirC3 assembly. Only SNPs were included that were also found when using CHRIC3 as a reference (shown in (B)).* ***D****: PCA of C3 nuclei, filtered on SNPs that were commonly found when using both the CHRIC3 and RirC3 assembly.* ***E****: Simplot of C3 nuclei (as in figure 5A), based on SNPs that were commonly found when using both the CHRIC3 and RirC3 assembly.*

Table S: *Comparison of mapping rate and genome coverage of A4 gDNA and single nuclei data (from [Chen* et al*., 2018]), mapped against the RhiiA4 assembly [Ropars* et al*., 2016] and RirC3. As comparison, the mapping rate of C3 nuclei to RirC3 is included.*

	Mapping rate (%)		Genome Coverage (%)	
	To RirC3	To RhiirA4	To RirC3	To RhiirA4
A4 gDNA	79.05	65.98	98.66	95.13
A4Nuc02	70.72	62.65	22.25	18.71
A4Nuc03	66.61	61.54	15.97	13.61
A4Nuc09	67.6	62.68	22.14	18.86
A4Nuc11	69.4	60.78	20.65	17.03
A4Nuc12	70.84	63.76	19.73	16.60
A4Nuc14	66.45	60.83	6.79	5.75
A4Nuc16	66.87	60.65	10.53	9.02
A4Nuc19	67.04	61.76	21.03	17.78
A4Nuc20	67.68	61.39	25.67	21.24
A4Nuc21	65.47	59.28	20.11	16.77
A4Nuc22	68.84	62.09	20.77	17.18
A4Nuc24	70.38	61.15	19.78	16.24
A4Nuc26	66.34	59.32	20.69	17.17
A4Nuc27	67.29	59.4	6.12	4.95
C3Nuc02	73.40		22.19	
C3Nuc09	78.68		35.85	
C3Nuc23	77.32		24.40	
C3Nuc24	74.91		27.46	
C3Nuc35	79.39		43.55	
C3Nuc38	74.88		16.16	
C3Nuc39	74.40		36.05	
C3Nuc41	76.78		41.43	
C3Nuc46	77.76		42.43	
C3Nuc47	79.49		27.91	

Table S2: *Composition of the lysis buffer mix used for gDNA extraction, used for PacBio sequencing.*

Solution	Ingredients	Amount in final lysis buffer
A	0.35M sorbitol, 0.1M Tris-HCl pH 9, 5mM EDTA	6.5mL
B	0.2M Tris-HCl pH 9, 2M NaCl, 2% CTAB	6.5mL (pre-heated at 65C)
C	5% Sarkosyl	2.6mL
D	20mg/mL Proteinase K	125µL
E	10% PVP	1.75mL

Table S3: *Assembly stats of RirC3 and CHRIC3 assemblies.*

	RirC3	**CHRIC3**
Assembly size	155Mbp	222Mbp
N count	200	743399
Gaps	2	4646
Illumina read unique mapping rate	83.95%	66.99%
Illumina read multiple mapping rate	6.01%	25.66%
BUSCO (fungi_odb10)	84.3% (639/758)	94.6% (717/758)
Complete and single-copy	82.6% (626/758)	64.1% (486/758)
Complete and duplicated	1.7% (13/758)	30.5% (231/758)
Fragmented	1.2% (9/758)	1.1% (8/758)
Missing	14.5% (110/758)	4.3% (33/758)

Chapter 4

Host-dependent expression in *Rhizophagus irregularis* isolates C3 and DAOM197198

Jelle van Creij[1], Peng Wang[1,2], Casper van Dam[1], Ton Bisseling[1,3], Erik Limpens[1]

1. Laboratory of Molecular Biology, Department of Plant Sciences, Wageningen University & Research, Droevendaalsesteeg 1, Wageningen, The Netherlands
2. Current affiliation: Hunan Normal University, Changsha, China
3. Beijing Advanced Innovation Center for Tree Breeding by Molecular Design, Beijing University of Agriculture, Beijing, 102206, China

Abstract

Arbuscular mycorrhizal (AM) fungi can colonize a wide variety of distantly related plant hosts, including important food crops like rice, wheat, maize and tomato. These fungi can regulate this interaction by expressing host-dependent genes. *Rhizophagus irregularis*, the most well studied AM fungal species, has been found to differ in its gene repertoire between isolates. Furthermore, some isolates are distinct in that they carry two types of nuclei, with much higher amount of genetic variation, compared to the more common homokaryons with one type of nuclei. While genetic differences between these isolates have been reported, how these differ in host interactions is not well known. Here, we compared the transcriptomes of the dikaryon-like isolate C3 and homokaryon DAOM197198 in four hosts: chives (*Allium schoenoprasum*), Medicago (*Medicago truncatula*), Nicotiana (*Nicotiana benthamiana*) and tomato (*Solanum lycopersicum*). Both isolates expressed genes in a host-dependent manner, although the amount and identity of such genes varied a lot. Furthermore, they caused different mycorrhizal growth responses in hosts, with DAOM197198 particularly improving the shoot fresh weight of chives compared to C3. Isolate C3 showed biallelic expression of genes, of which a subset of alleles was host-dependent. Many of the chives-dependent genes in DAOM appeared to be lacking in C3. A small subset of biallelic genes showed mono-allelic expression in all hosts, demonstrating haplotype-specific gene expression. Furthermore, C3 was found to express distinct patterns of allelic variants in chives and Medicago, compared to Nicotiana and tomato. DAOM197198, on the other hand, expressed ribosomal RNA variants in a host-dependent manner. Our results show that these AM fungal isolates show distinct host-dependent gene expression, and that both adapt their allele expression in different manners.

Background

Arbuscular mycorrhizal (AM) fungi are known for their extremely wide host range. They can colonize and form symbioses with up to 85% of all vascular land plant species [Van der Heijden *et al.*, 1998; Öpik *et al.*, 2006]. AM fungi use their wide mycelial network to scavenge the soil for nutrients (most importantly phosphate and fixed nitrogen) and transport these to the plant in return for photosynthetically derived carbon [Smith and Read, 2008; Smith and Smith, 2011]. Typically, these fungi can colonize multiple hosts simultaneously and have the ability to regulate their transcriptomes to adapt to the nearest host [Zeng *et al*, 2018; Kamel *et al.*, 2017]. AM fungi facilitate this plasticity by carrying one of the largest amount of genes of any fungal species [Mohanta and Bae, 2015; Kokkoris *et al.*, 2020].

In general low to no host specificity has been observed in AM fungi [Sanders, 2003]. Host preferences have been observed in wild environments, where AM fungi preferentially colonized certain host species, even when other suitable hosts were present [Martínez-Garcia & Pugnaire, 2011; Torrecillas *et al.*, 2012]. Furthermore, variation in mycorrhizal growth response (MGR) from hosts to different AM fungal species and isolates has been widely reported [Klironomos, 2003; Munkvold *et al.*, 2004; Eo and Eom, 2009; Angelard *et al*., 2014; Ceballos *et al.* 2019; Peña *et al.*, 2020]. The molecular basis for host preferences and variation in mycorrhizal growth response (MGR) are still largely unknown.

Among AM fungi, the most well-studied is *Rhizophagus irregularis* (formerly known as *Glomus intraradices*). It was the first AM fungal species to have its genome sequenced [Tisserant *et al.*, 2013; Lin *et al.*, 2014] and is now also known for its high intra-species variation [Wyss *et al.*, 2016; Chen *et al.*, 2018; Reinhardt *et al.*, 2021]. Previous research has shown that *R. irregularis* expresses host-dependent genes, that are thought to help the fungus adapt to particular hosts [Zeng *et al.*, 2018; Kamel *et al.* 2017]. These genes include among others transporters, protein kinases and effectors, with functions in nutrient exchange and suppression of host immune responses [Chapter 2; Zeng *et al.*, 2020; Aparicio Chacón *et al.*, 2023]. Different isolates of *R. irregularis* are known to have significant differences in gene repertoire [Chen *et al.* 2018; Morin *et al.*, 2019], to a point where it is difficult to recognize them as the same species, and might be better classified as a species complex [Bruns *et al.*, 2018; Mathieu *et al.*, 2018; Reinhardt *et al.*, 2021].

The lab isolate DAOM197198 (hereafter referred to as DAOM) is homokaryotic, meaning it has only a single type of nuclei with a low amount of inter-nucleus genetic variation [Tisserant *et al*, 2013; Lin *et al.*, 2014; Maeda *et al.*, 2018]. The isolate C3, however, has a much higher amount of genetic variation, and has two main types of nuclei marked by divergent MAT loci [Wyss *et al.*, 2006; Ropars *et al.*, 2016; van Creij *et al.*, 2023]. Five dikaryote-like isolates have been described so far [Sperschneider *et al.*, 2023], and these have been shown to outcompete homokaryotic isolates like DAOM in traits like hyphal development [Serghi *et al.*, 2021]. Furthermore, isolates have been observed to differ in colonization rate and induced MGR in cassava [Ceballos *et al.*, 2019; Pena *et al.*, 2020; Savary *et al.*, 2020].

In Chapter 3, I have shown that the nuclear ratios within C3 are dynamic, and can differ between different lineages. Moreover, whole transcriptome sequencing revealed that allele frequency distributions of the total mRNA expressed by C3 changed consistently between hosts [Chapter 3]. Since all C3 material used in that study came from the same batch of spores, these results suggested that variation in mRNA allele frequency could have been caused by differences in transcriptomic activity between nuclei.

To get a more detailed view on the host effects on the fungal transcriptome and the contribution of different nuclei, we compared the performances and transcriptomes of *R. irregularis* isolates DAOM and C3 on four hosts: chives (*Allium schoenoprasum*), Medicago (*Medicago truncatula*), Nicotiana (*Nicotiana benthamiana*) and tomato (*Solanum lycopersicum*). These analyses indicate that DAOM and C3 differ in host-dependent gene repertoires, and utilize their genetic variation in different ways to adapt to different hosts.

Results

Strain differences in symbiotic efficiency with different hosts

To compare potential differences in symbiotic performance between the homokaryotic *R. irregularis* DAOM197198 (hereafter referred to as DAOM) and the dikaryotic-like C3 isolate, we quantified plant fresh weight and colonization levels of the two isolates on four different host species: chives, Medicago, Nicotiana, and tomato.

As expected, all plants grew better under high phosphate than under low phosphate conditions. Treatments with AMF generally resulted in a slight increase in both shoot and root fresh weight compared to low phosphate conditions [Figure 1a]. In most host plant species, DAOM and C3 had comparable effects on plant development. However, DAOM showed a significantly stronger enhancing effect on the fresh shoot weight of chives compare to C3. While substantial variation in shoot weight was visible, in some cases shoot weights were similar as under high phosphate conditions, effectively rescuing the phosphate starvation phenotype. Although C3 treatment did show an increase in both root and shoot weight of chives, the effect was not nearly as strong as that of DAOM [Figure 1a].

Mycorrhization levels of the two strains were also quantified [Figure 1b]. Overall, DAOM showed a higher level of colonization in Medicago and Nicotiana compared to C3, with higher numbers of arbuscules and intraradical hyphae. On the other hand, DAOM showed lower intraradical colonization levels and arbuscule numbers than C3 in chives and tomato. Interestingly, vesicle levels were higher in all plants hosts colonized by C3. Together, these results show that despite *R. irregularis* being a generalist symbiont, isolates vary in their performance on different hosts, and provide different isolate-specific effects on host development that are not necessarily reflected in the level of colonization.

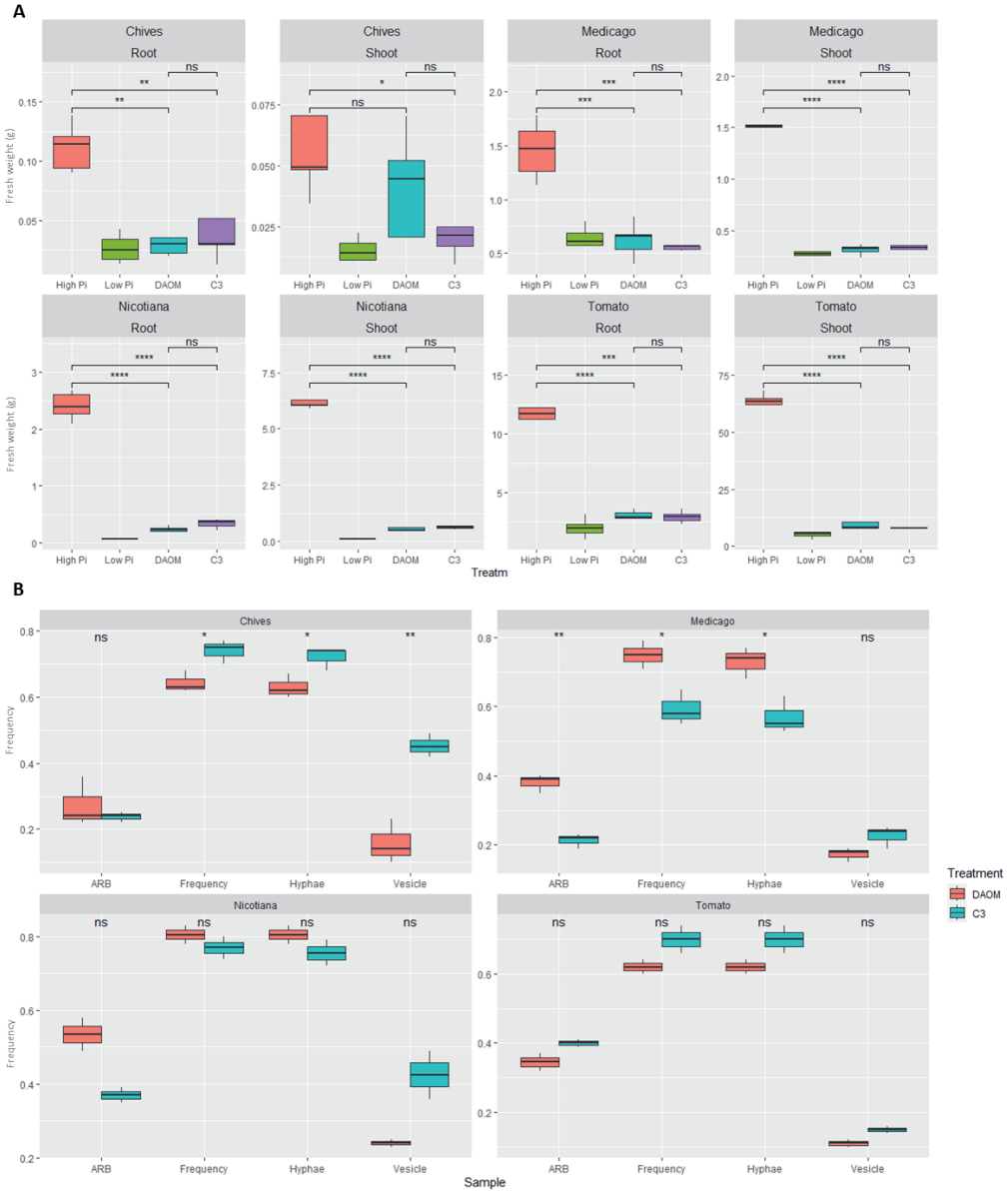

Figure 1: *Differences in colonization rates between DAOM and C3 on chives, Medicago, Nicotiana and tomato.* ***A****: Fresh root and shoot weight of the host plants under high phosphate, low phosphate, low phosphate + DAOM and low phosphate + C3.* ***B:*** *Differences in arbuscule counts (ARB), frequency (reflecting overall colonization level), hyphae levels and vesicle counts between DAOM and C3 on different hosts.*

DAOM and C3 transcriptome comparison

The observed differences in MGR between DAOM and C3 are likely reflected in their transcriptomes. In order to find how these isolates differ in their host-dependent gene expression, we sequenced mRNA of C3 growing in chives, Medicago, Nicotiana, and tomato. In addition, mRNA of DAOM growing in tomato was sequenced. The tomato-DAOM reads were combined with mRNA reads from DAOM in chives, Medicago and Nicotiana, which were previously generated in our group [Zeng *et al.*, 2018].

Since all RNA samples were isolated from colonized plant roots, the majority of RNA came from the plant host, and mapping rates against the fungal reference were variable [Table S1]. Principal component analysis of both sets of transcriptomes showed strong grouping based on the source of the samples [Figure 2a,b]. For DAOM, the three tomato samples separated on the second principle component from the other three hosts, which were derived from Zeng *et al.* (2018). This may be related to differences in growth conditions, since these reads were generated in separate studies. DAOM colonizing chives clustered away from the other hosts among the first principle component, corresponding with an evolutionary more distant relationship, except for one Nicotiana sample which also deviated from the rest [Figure 3a]. Medicago and Nicotiana samples clustered together quite strongly, suggesting that the DAOM transcriptome was more similar in these hosts. For C3, all hosts formed individual clusters, separated evenly by the first and second principle components, each explaining around 20% of the variation [Figure 2b].

For a more thorough functional comparison between the two isolates, a reciprocal blast was performed to identify putative orthologous genes. A match with at least 90% identity was considered an ortholog (or close homolog). DAOM shared only 39% of its predicted genes with C3. For C3, 56% has at least one DAOM putative ortholog. These differences are likely influenced by the fact that different annotation methods were used to predict genes in these two assemblies. The annotation of Rir17 contained a total of 41572 genes, encoding 43672 transcripts [Maeda *et al.*, 2018]. RirC3, on the other hand, contained 27181 predicted genes, encoding a total of 28824 transcripts [Chapter 3]. When only taking expressed genes into account (ie. transcripts with average normalized counts >10 in any of the four hosts), the proportions were a lot more similar. DAOM expressed a total of 13813 transcripts in any of the hosts, of which 9409 (68%) had a putative C3 ortholog. 2178 DAOM transcripts had multiple C3 homologs (16%). For C3, a total of 14171 expressed transcripts were found, of which 10205 (72%) had DAOM putative orthologs; 1859 (13%) had multiple DAOM homologs. C3 contained 1062 genes that encoded multiple active splice variants and 849 DAOM genes contained active splice variants. In terms of activity, the numbers were also similar; out of 14171 expressed C3 genes, 8727 (62%) had DAOM orthologs that were also expressed (average count >10). For the 13813 DAOM expressed transcripts, 9254 (67%) had C3 homologs that were expressed.

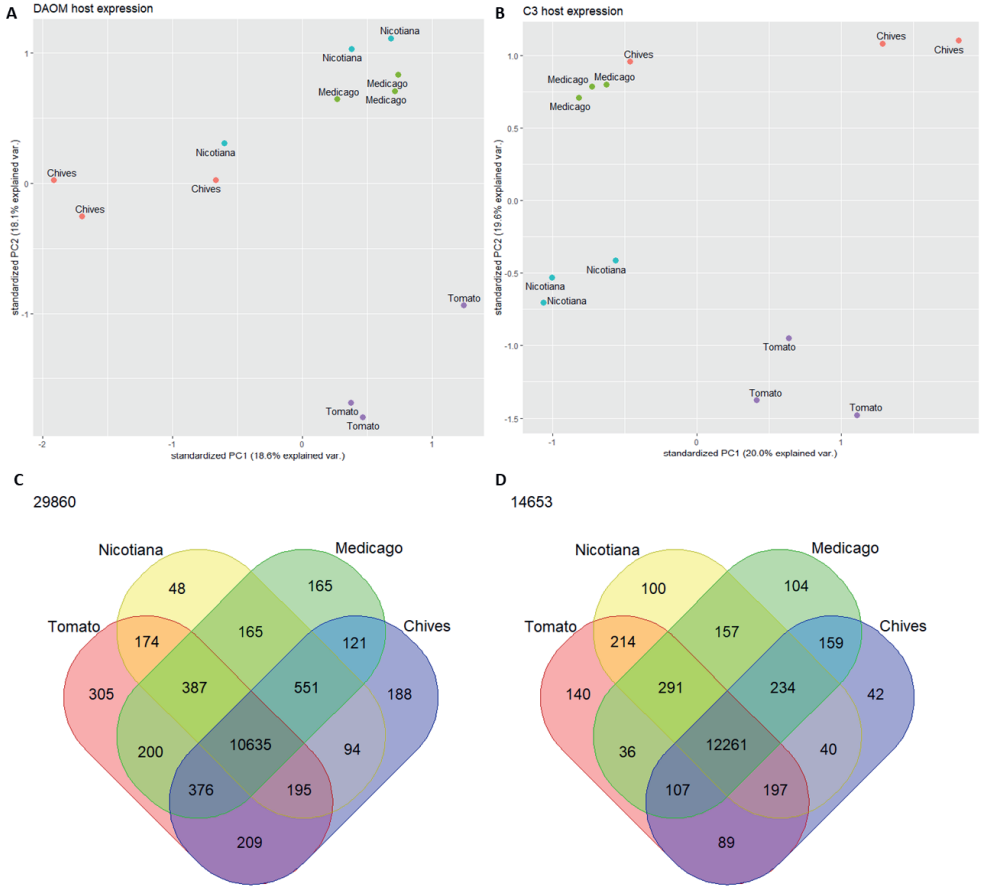

Figure 2: *Host-dependent gene expression in the four different hosts.* ***A:*** *PCA analysis of transcripts in DAOM.* ***B:*** *PCA analysis of transcripts in C3.* ***C:*** *Venn diagram showing host-dependent gene expression in DAOM.* ***D:*** *Venn diagram showing host-dependent gene expression in C3.*

Making direct comparisons between both transcriptomes is difficult, as two different functional annotation methods were used. However, based on reciprocal blasts, we saw that compared to DAOM, C3 is missing 1002 orthologues of serine/threonine protein kinases. Other strikingly missing gene families included RNA-directed DNA polymerases, BED-domain zinc-finger containing proteins, calmodulin-dependent protein kinases and reverse transcriptases, which lacked 254, 241, 207 and 183 C3 orthologs, respectively. RirC3 contained 51 predicted genes with retrotransposon Gag domains characteristic of LTR retrotransposons [Wang and Han, 2021], of which 34 did not have a putative ortholog in Rir17.

Host-dependent gene expression

In order to identify host-dependent genes, we compared each host-fungus combination and selected fungal genes that showed a significant (adjusted p<0.05) at least 4 fold difference between hosts. To do this automatically and in a way that can be up-scaled if more hosts are added, we developed an R script that can make these comparisons. The main concept of this script is to categorize each expression in a binary categorical manner, with 1 being (highly) expressed, and 0 being significantly lower expressed (see Materials and methods for a full description). This analysis showed that both isolates expressed multiple genes with significantly higher (or lower) expression in specific hosts. These genes are considered to be host-dependent. Annotated lists of host dependent genes per isolate can be found in Supplementary Files 1 and 2.

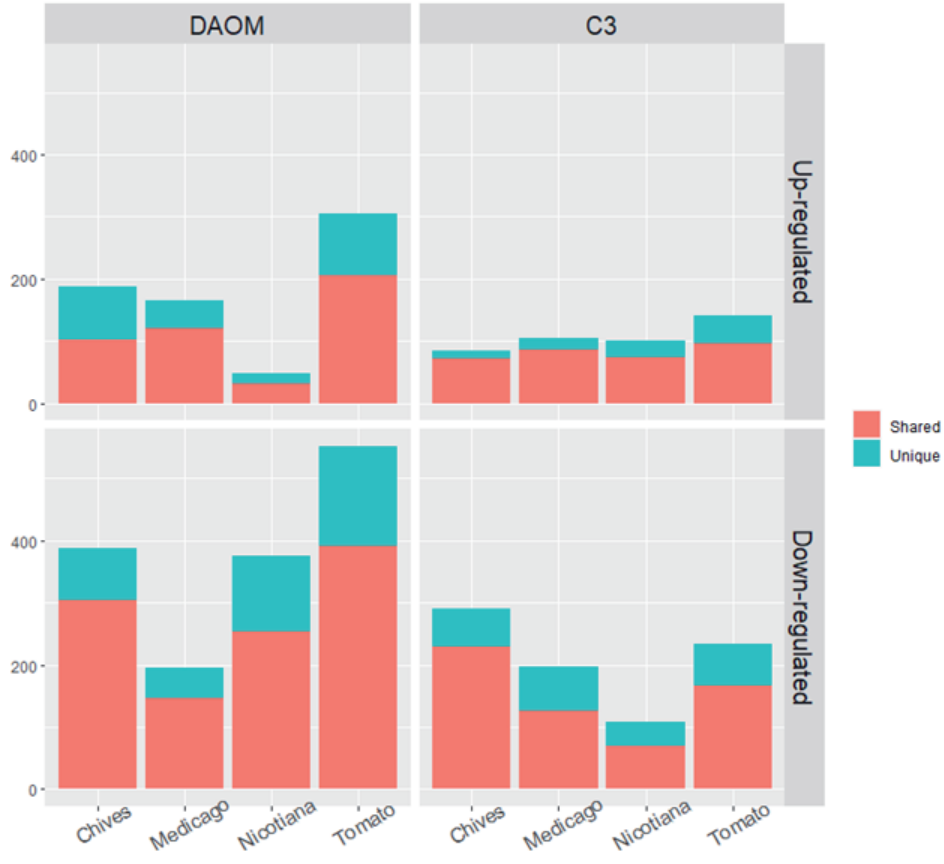

Figure 3: *Number of host-dependent genes in each isolate. Each bar shows the total number of host-dependent genes per isolate that are up (upper row) or down (lower row) regulated. In each graph, the proportion of genes that have at least one putative ortholog in the other isolate are colored in orange, while genes that are unique to that isolate are blue.*

DAOM showed strikingly more variation in host-dependent genes, both up- and down-regulated, between the 4 hosts compared to C3 [Figure 3]. Such variation was highest between Nicotiana and tomato when looking at up-regulated genes in DAOM, and between Medicago and tomato for host-dependent down-regulated genes. This indicates that more closely related plant species do not necessarily show a more comparable transcriptional response in the fungus.

Overall the number of host-dependent down-regulated genes was higher than the number of host-dependent up-regulated genes [Figure 2c,d; Figure 3]. For DAOM the amount of host dependent up-regulated genes was the highest in tomato (305), compared to Medicago (165), Nicotiana (48) and chives (188). For host-dependent down-regulated genes, DAOM had 551 in tomato, 195 in Medicago, 376 in Nicotiana, and 387 in chives. C3 showed less variation in the host-dependent upregulated genes: 42 in chives, 104 in Medicago, 100 in Nicotiana, and 140 in tomato. For host-dependent down-regulated genes, C3 had 291 for chives, 197 for Medicago, 107 for Nicotiana, and 234 for tomato [Figure 3].

Chives

Chives-dependent genes in DAOM included multiple serine/threonine kinases, cytochrome p450 monooxygenases, kelch-like protein 7 homologs and UMTA. Interestingly, DAOM expressed about two times more upregulated genes in chives compared to C3, of which almost half of the genes did not have a C3 ortholog [Figure 3]. Two chives-dependent DAOM secreted proteins (with a signal peptide but without a transmembrane domain) were also found to be missing in C3: gene12671 (an ornithine decarboxylase antizyme, which inhibits the biosynthesis of polyamine, resulting in the inhibition of cell growth [El-Sayed *et al.*, 2019]) and gene19720 (containing lipoprotein attachment sites). These secreted proteins may have effector functions unique to DAOM. Chives-dependent down-regulated genes in DAOM included cytochrome p450 monooxygenases, phosphodiesterases and ER to Golgi transport genes. C3 chives-dependent up-regulated genes included multiple Piwi-domain containing genes, Ribonuclease H genes and genes involved in Argonaute activity. The activity of Argonaute family genes has been linked to the defense against activity of retrotransposons [Chang *et al.*, 2012]. Many genes also showed GO-terms related to protein, nucleic acid and GTP binding. Chives down-regulated genes in C3 contained Argonaute related genes as well, but also genes involved in oxidation reduction processes and transmembrane transport.

Medicago

DAOM expressed multiple cytochrome p450 monooxygenases in Medicago, along with multiple HSP70 heat shock family genes and several serine/threonine protein kinases. Lipases, on the other hand, were strongly down-regulated compared to the other hosts, as was a homlog of an 88 kDa immunoreactive mannoprotein (involved in eliciting immune responses in human pathogenic fungi [Levits *et al.*, 2006]) and FAD-binding domain containing proteins). C3 also expressed several cytochrome p450 genes, as well as several iron binding and protein binding genes. In addition, C3 expressed multiple glutathione S-transferases, which function in protecting the fungus against reactive oxygen species. C3 genes down-regulated in Medicago included multiple (endo)peptidases and lipid metabolism genes.

Nicotiana

DAOM expressed a relatively low number of Nicotiana-dependent genes compared to the other hosts. These included several lipases, a small glutamine-rich TPR-containing protein (SGT1, involved in kinetochore assembly [Bansal *et al.*, 2009]), and a kelch-repeat protein. The kelch protein (involved in protein ubiquitination) was particularly highly expressed in Nicotiana, and C3 interestingly lacked a putative ortholog of this gene. An aldo/keto reductase showed a particularly high difference in expression between Nicotiana and the other hosts. Gene4160_T3 was particularly down-regulated in Nicotiana, but unfortunately no GO terms could be found in this gene, nor did it have any clear blast hits. Nicotiana dependent genes in C3 were characterized by encoding protein kinase and ATP binding domains. Moreover, multiple SEL1-like repeat proteins (which inhibit the Notch signaling pathway) and serine/threonine kinases were highly expressed in Nicotiana, as well as oxidoreductases and glutathione S transferases. GO terms for heme and ion binding were also enriched. Conversely, several genes with transmembrane activity were down-regulated, as well as DNA transcription-related genes and helicases.

Tomato

Tomato was the only host in our experimental design for which RNA of both isolates was sequenced in the same study under the same circumstances, allowing for more direct comparisons. In tomato, DAOM showed the highest number of host-dependent genes, with 35 serine/threonine protein kinases and several SEL1 like genes. Other genes encoded for cobalamin biosynthesis proteins, calmodulin-dependent protein kinases, the known *R. irregularis* effector SP7 [Kloppholz *et al.* 2011] and cyclin binding proteins. BSC1 (Upiquinol-cytochrome c reductase synthesis), a mitochondrial chaperone protein, had highly tomato-dependent expression as well, but C3 lacked a putative ortholog for this gene. The same holds for a predicted chitin synthase. Tomato down-regulated DAOM genes included two crinkler family protein (presumed effectors [Voß *et al.*, 2018]), several RNA binding proteins and one adenosylhomocysteinase. C3 expressed multiple methyltransferases in a tomato-dependent manner, as well as several high mobility group box proteins. Cupredoxin stood out as a gene with high tomato-dependent expression, as well as peptidase S8. GO terms related to zinc ion binding and proteolysis were found in multiple tomato-dependent genes. Tomato down-regulated C3 genes contained GO terms involved in oxidoreductase activity and iron ion binding.

The highest number of host-dependent genes in both isolates were either up- or down-regulated in Nicotiana and tomato, the two most taxonomically related hosts in our study. DAOM expressed several peptidases, nitrilases, and protein kinases in these hosts, as well as benzoate 4-monooxygenases. Conversely, GTPases and isoflavone reductases were down-regulated in Nicotiana and tomato. HMP1, a mismatched base pair and cruciform DNA recognition protein [Burleigh and Harrison, 1998], also showed significantly lower expression in these hosts compared to chives and Medicago. In C3, Nicotiana and tomato up-regulated genes were enriched for protein phosphorylation, ATP binding and protein kinase activity. FUN_000112-T2, a transmembrane protein of unknown function, was particularly highly expressed in both Nicotiana and tomato. Furthermore, multiple genes involved in heme

binding, oxidoreductase activity and iron, zinc and calcium ion binding were observed, as well as transmembrane transporters and UDP-glycosyltransferases. Similar GO terms were also found in multiple chives and Medicago up-regulated genes, but C3 genes involved in proteolysis were particularly enriched/upregulated in Nicotiana and tomato.

Overall, putative orthologs of host-dependent genes in the two isolates were rarely expressed in the same host dependent manner. Most C3 orthologs of host-dependent DAOM genes were expressed in all, or multiple hosts. The same was observed vice versa. In general, most orthologs of host-dependent genes (in either DAOM or C3) were also active in the other isolate, just not in the same host-dependent manner. The only host where this was not the case was tomato. For tomato-dependent down-regulated C3 genes, over 40% of their putative DAOM homologs was not expressed in the interaction with any host.

Host-dependent allele expression in C3

Besides the difference in gene repertoire, another clear genetic difference between the DAOM and C3 isolates is that C3 has a substantially higher amount of genetic variation distributed over two main nucleotypes [Chapter 3], while DAOM is monokaryotic with a low level of internucleus variation. To investigate whether host-dependent allelic expression occurred in either of the isolates, we performed variant calling on the RNAseq data and searched for alleles that were differentially regulated in a host-dependent manner.

Host-dependent allelic expression was determined by analyzing the allele frequencies of SNPs that were found in transcripts. Only SNPs that were previously confirmed in the genomic DNA [Chapter 3] were taken into account. To allow a more reliable analysis of allele frequencies, SNPs were only included if they had coverage of at least 20 in all three replicates.

Principle component analysis of the allelic expression of DAOM transcripts showed overall grouping based on host identity, with chives and Nicotiana separating from tomato and Medicago along the first principle component (accounting for 93.6% of all variation), though the samples did not cluster very strongly [Figure 4a]. C3, on the other hand, did show very strong clustering of hosts [Figure 4b], with the hosts forming two distinct groups separated among the first principle component, accounting for over 50% of the variance. Chives and Medicago formed a cluster separate from Nicotiana and tomato, except for one of the tomato samples which deviated along the second principle component (explaining just 7% of the variation).

To determine host-dependent allelic activity, the same method as for host-dependent gene expression was used, except that for this analysis, the allele frequency of the SNPs was used rather than transcript count. Furthermore, the difference in allele frequency had to be at least 0.1. If a SNP was not expressed in a host, it was assumed to not be different from the host with the highest allele frequency (i.e., a SNP that was not expressed in tomato, but similarly expressed in the other three hosts, was classified in the binary matrix as “1 1 1 1”, instead of “1 1 1 0”).

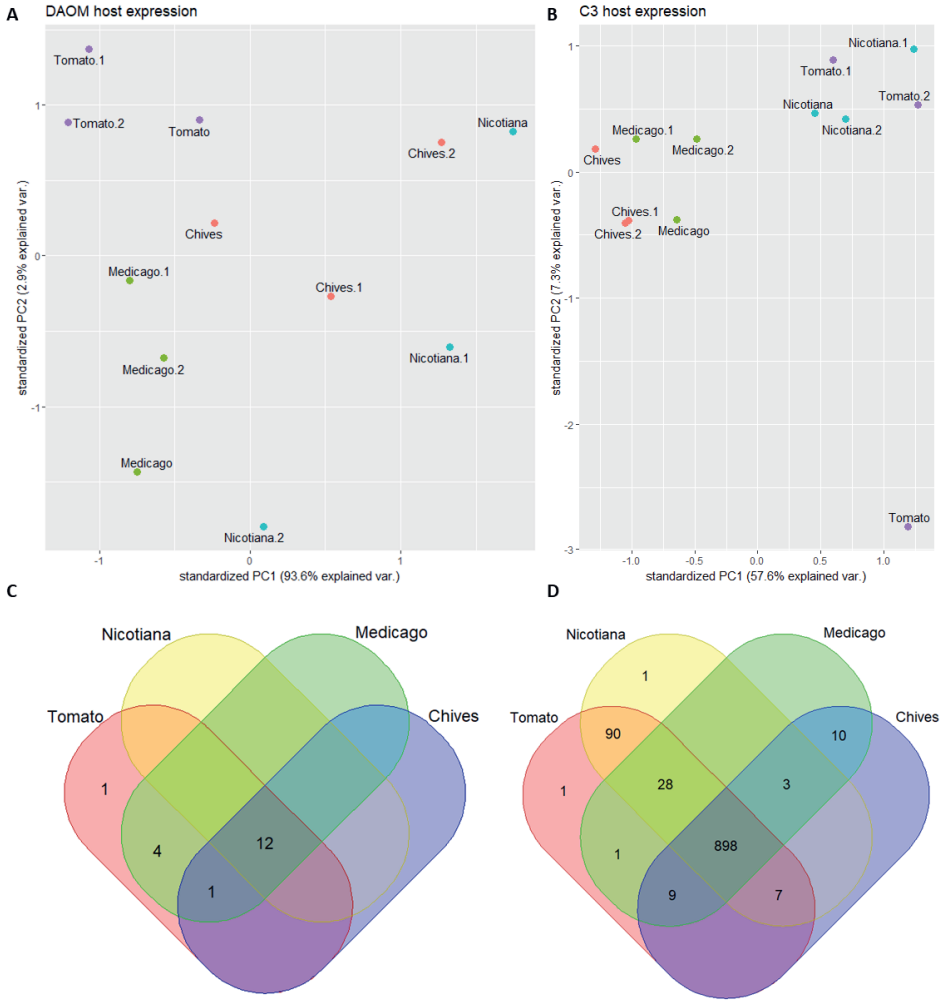

Figure 4: *Host dependent allele expression. Only SNPs that were expressed with DP>20 in all three replicates of at least two hosts were included.* ***A:*** *PCA analysis of DAOM alleles.* ***B:*** *PCA analysis of C3 alleles.* ***C:*** *Venn diagram of host-dependent DAOM alleles.* ***D:*** *Venn diagram of host-dependent C3 alleles.*

After filtering for SNPs that had enough coverage, 1048 out of 9395 SNPs remained, in a total of 626 genes. The main cause of this low number is that SNPs were only considered when present at a minimum read depth of 20 in at least two hosts. In particular, around two thirds of the SNPs in this dataset were not sufficiently expressed (DP < 20) in tomato (657 out of 1048 SNPs). In comparison, the number of SNPs that did not reach the threshold of 20 in chives, Medicago and Nicotiana were 102, 99 and 112, respectively. The majority of the SNPs (898 out of 1048) were not differentially expressed between any of the hosts, while the remaining 150 showed differential expression between hosts [Figure 4b]. The number of alleles that were affected by only a single host was very low, with only one SNP being exclusively up-regulated for Nicotiana, and one for tomato [Figure 4d]. For chives and Medicago, no host-dependent SNPs were found. Instead, the majority of differentially

expressed alleles (100 out of 150) showed similar allele frequencies in Nicotiana and tomato. For 90 of these SNPs, the allele frequencies (measured here as the fraction of reference alleles in the transcripts) was usually above 0.7 in Nicotiana and tomato, and around 0.5 for chives and Medicago [Supplementary File S3]. This difference matches the previously observed allele frequency patterns in the mRNA in these host species [Chapter 3]. Following this observation, the 10 other SNPs were found where the frequency in chives and Medicago was 0.5, and 0.33 in Nicotiana and tomato.

In total, 100 SNPs (in 83 transcripts) showed host dependent expression in either chives and Medicago, or Nicotiana and tomato [Supplementary File S3]. These genes contained GO terms for protein binding, transmembrane transporter activity, catalytic activity, ATP binding and nuclei acid binding. 56 of these SNPs were synonymous SNPs [Supplementary File 3]. 31 missense SNPs caused an single amino acid change, one introduced a premature stop codon and one caused a frame shift. The remaining SNPs were located in the UTR of genes. SNPs causing a missense variant were found in genes involved in protein binding, phospholipase activity and ubiquitin conjugation. The stop codon was introduced in FUN_015326, a hypothetical gene with 89% homology to TATA binding protein-associated factor 1 (TAF1), the biggest subunit of the multiprotein transcription factor TFIID essential for gene transcription [Sanders *et al.*, 2002]. The frameshift variant was induced in FUN_002263, another hypothetical gene encoding a CRAL-TRIO lipid binding domain [Panagabko *et al.*, 2003].

Closer examination of alleles that were similarly expressed in all hosts revealed a wide range of allele frequencies in the transcriptome. However, in 109 SNPs, the reference allele frequency in the mRNA was on average above 0.95, indicating that only one haplotype was transcriptionally active. Out of these 109 SNPs, 47 were missense variants. Conversely, only 15 SNPs had the alternative allele at over 0.95. These results are remarkable, as they suggest that nuclei carrying the reference allele of a gene (usually MAT-1 carrying nuclei [Chapter 3]) contributed more to the transcriptome in most cases. Expressed genes carrying only the reference allele included protein kinases, ATPases, different peptidases and ion binding proteins. Genes with only the alternative allele included GO terms for peptidases, as well as oxidoreductases and protein phosphorylation genes. Roughly two thirds of these SNPs represented missense variants.

In summary, these results demonstrate that alleles in C3 can be expressed in host-dependent manners. Instead of being dependent on single host species, allelic expression showed distinct patterns in chives and Medicago versus Nicotiana and tomato. Host-dependent alleles were found in genes with a variety of functions, including protein binding, transmembrane transport, protection against oxidative stress, and gene transcription. Moreover, we found that in the four hosts included in this study, some alleles were expressed in a haplotype specific manner.

Host-dependent rRNA expression

DAOM expressed very few allelic variants in its transcripts, an unsurprising result given its low level of genetic variation. Only 93 SNPS were expressed by DAOM. After filtering on coverage in at least two hosts, only 18 remained [Figure 4c]. Remarkably, all of these SNPs were located in the ribosomal DNA copies of the genome. As published by Maeda *et al.* (2018), DAOM has a unique ribosomal DNA organization. While in most eukaryotes ribosomal DNA copies are organized in thousands of tandem repeat structures, DAOM only has 10 single copy loci. All SNPs considered in the RNA analysis were found in the DAOM genomic DNA as well, ruling out potential sequencing artifacts. Significant host dependent expression of allelic rRNA variants was visible [Table 1]. The coverage of rRNA was relatively low, likely due to the enrichment for poly-adenylated RNAs in the RNA sequencing method, but still sufficient to reliable assign variants to their corresponding loci. To our knowledge, this is the first experimental evidence of expression of host-dependent rRNA alleles in plant-fungal interactions.

Table 1: *Allele frequencies of SNPs within rRNA copies in DAOM and C3. SNPs are listed as "contig_location". Frequencies were calculated as the percentage of reference alleles of the total amount of alleles that were observed. Frequencies are color coded from red (low) to green (high).*

	Chives	Medicago	Nicotiana	Tomato
DAOM				
BDIQ01000091.1_832284	0.46	0.62	0.38	0.73
BDIQ01000051.1_1264691	0.56	0.79	0.43	0.77
BDIQ01000051.1_1264701	0.45	0.66	0.33	0.74
BDIQ01000122.1_1092897	0.51	0.68	0.36	0.73
BDIQ01000190.1_321876	0.47	0.63	0.38	0.74
BDIQ01000190.1_321886	0.58	0.78	0.49	0.77
C3				
contig_384_1|arrow_57632	0.99	0.97	0.97	NA
contig_384_1|arrow_58529	0.94	0.91	0.93	0.80
contig_384_1|arrow_59230	0.89	0.90	0.96	0.89
contig_384_1|arrow_109915	0.92	0.90	0.93	0.87
contig_384_1|arrow_110166	0.88	0.85	0.86	0.84
contig_384_1|arrow_110484	0.91	0.92	0.92	0.87
contig_466_1|arrow_463525	0.96	0.94	0.91	0.92
contig_466_1|arrow_465101	0.86	0.83	0.85	0.79
contig_466_1|arrow_465195	0.96	0.95	0.97	0.96
contig_466_1|arrow_465419	0.92	0.89	0.90	0.88
contig_806_1|arrow_168902	0.81	0.79	0.84	0.88
contig_806_1|arrow_170591	0.90	0.93	0.93	0.91
contig_806_1|arrow_171249	0.92	0.87	0.92	0.94

The SNPs were all located in the 28S region of the locus, which is also where the most intra-copy sequence variants are found [Maeda *et al.*, 2018]. Since 28S ribosomal RNA form the large 60S subunit of ribosomes, variations in this region may affect ribosome folding and activity. This suggests that DAOM may be able to adapt to different hosts by translational regulation, with the expression of specific allelic rRNA variants.

While C3 also has SNPs in its rDNA copies [Chapter 3], with four rRNA copies having sufficient coverage in all hosts to be included in our analysis, no significant host effects on these SNPs were found in the RNAseq data. Instead, the reference allele of these copies was significantly more expressed in all hosts, with frequencies between 0.8 and 1 [Table 1]. These allele frequencies were similar to those found in rDNA copies in the genome [Figure S1]

Discussion

The many differences between *Rhizophagus irregularis* isolates have been subject to much debate, particularly in relation to its genetic variation. The most striking difference between isolates DAOM and C3 is that the former is homokaryotic (with one nucleotype), while the latter is more dikaryotic-like, with two main nucleotypes resulting in a higher level of genetic variation [Chapter 3].

Here we report highly dynamic transcriptional regulation in both C3 and DAOM in response to different host plants. Both isolates have inherently different gene repertoires and expressed many genes in host-dependent manners, which likely contributed to differences in performance and symbiotic abilities. Furthermore, C3 was shown to express specific alleles and to shift allelic expression according to host identity. DAOM, in contrast to C3, only showed host-dependent variation in rRNA allele expression.

Growth effect and colonization rate

Co-inoculation experiments showed that DAOM and C3 differ in the extent by which they increased MGR, as well as in their level of colonization of the host roots. In particular, chives showed a significantly more positive growth response upon interaction with DAOM compared to C3. This did not appear to be due to a higher colonization rate, as mycorrhization levels were even slightly lower as compared to C3. However, it should be noted that we only measured the colonization levels at a single time point. It would be relevant to perform a time course analyses to examine potential differences in colonization rates more carefully. Nevertheless, inoculation with DAOM resulted in an increase in shoot weight in chives, to a level similar as grown under high phosphate conditions. Since shoot yield is a relevant commercial trait for this crop, such isolate specific effects on MGR may be of particular interest for use as biological fertilizers.

Although dikayotic-like strains have been shown to outcompete homokaryotic strains in terms of hyphal development [Serghi *et al.*, 2021], these results indicate that more variation exists between and within isolates in terms of life traits. Indeed, homokaryons have been shown to outcompete dikaryotic isolates on Cassava, with both a higher colonization rate and MGR [Ceballos *et al.*, 2019]. Within C3, considerable differences in MGR between lineages have

been found as well [Ehinger *et al.*, 2012]. Although significant variation in MGR has been reported in literature [Klironomos, 2003; Eo and Eom, 2009; Angelard *et al.*, 2014; Berger and Gutjarhr, 2021; Thirkell *et al.*, 2022], the mechanisms behind the variation in MGR are still largely elusive. Overall, there seems to be no correlation between the level of colonization or the amount of arbuscules and the extent of plant growth promotion [Baon *et al.*, 1993; Kaya *et al.*, 2009; Thirkell *et al.*, 2022]. Differences in MGR may be due to variation in efficiency of nutrient exchange, different effects on plant metabolism, and host immune or stress responses [Smith *et al.*, 2003; Sawers *et al.*, 2017]. A recent study by Savary *et al.* (2020) found that genetic variation among twelve *R. irregularis* isolates affected the regulation of the fatty acid pathway in the host plant cassava. Since fatty acids are essential plant carbon sources that feed the fungus [Luginbuehl *et al.*, 2017], this suggests that different effects on this important currency may have a significant effect on trade in the symbiosis. In Medicago it was shown that more carbon supply by the plant can lead to more Pi supply by the fungus, and vice versa [Kiers *et al.*, 2011]. However, isolates that induced the fatty acid pathway most strongly in cassava were among the lowest colonizers [Savary *et al.*, 2020], questioning whether this would affect the plant growth response. It could be worthwhile to perform a *de novo* transcriptome assembly using the chives reads to examine whether DAOM and C3 differentially affect the plant fatty acid regulation or other pathways, which may explain the observed difference in MGR. Since *Allium* species are known to be especially reactive to mycorrhization [Galván *et al.*, 2011], making the same comparison with other species may also reveal whether this difference in MGR is conserved between other species of this family.

Transcriptome differences between DAOM and C3

In their respective genome annotations DAOM contained a much higher amount of predicted genes than C3. However, the number of actively transcribed genes, expressed in at least one of the hosts, was much more comparable. This is likely caused by the fact that different methods were used for gene prediction in DAOM and C3 [Maeda *et al.*, 2017; Weismann *et* al., 2022; van Creij *et al.*, 2022]. The high number of DAOM genes that were not active in any of the four host plants suggests that the predicted Rir17 transcriptome may contain many inactive, theoretical genes. Although, it cannot be ruled out that these are active in other hosts or conditions that were not included in this study.

In both isolates, around 30% of their active transcriptome was lineage specific, with the other 70% having at least one potential ortholog in the other isolate. This is in line with a previous study, where variation in gene repertoire between multiple *R. irregularis* isolates was demonstrated [Chen *et al.*, 2018; Morin *et al.*, 2018].

Host-dependent gene expression

Both DAOM and C3 showed host-dependent gene expression in all four hosts that were included in this study. PCA of the C3 data showed that all hosts (with the exception of one of the chives samples) clustered separately among the first two principle components, forming four distinct groups. For DAOM, on the other hand, tomato samples were separated from the other hosts. The RNAseq reads from the other hosts came from a different study in our group [Zeng *et al.*, 2018], where (currently unknown) differences in growth conditions may have

caused differences in the transcriptomes. Moreover, the lower mapping rates of these samples against the DAOM reference genome reflect a lower amount of RNA in these samples, which likely affected the number of transcripts that could be reliably quantified. Therefore, some caution must be used to make direct comparisons between DAOM and C3 samples in these hosts.

Mechanistic reasons behind differences in MGR between *R. irregularis* isolates are likely reflected in the transcriptomes of these fungi. However, as mentioned above the large difference in gene repertoire makes it challenging to functionally link variation at the transcriptome level with processes that regulate the symbiosis. Furthermore, many transcripts do not have homologs in other organisms (outside of the Glomeromycota) and functional annotation is still largely based on predicted function, rather than experimental evidence.

Our analysis showed that C3 lacks orthologs of nearly half of the chives dependent genes found in DAOM. Among the host-dependent genes were a few predicted secreted proteins that could act as effectors, that are intriguing candidates to affect host responses [Aparicio Chacón *et al.*, 2023]. One of the putative DAOM effectors that are highly expressed specifically in chives is a homolog of the beta-glucan binding effector FGB1 of *Pirformaspora indica* [Wawra *et al.*, 2016], and therefore called RiFGB1 (gene2644 in our data, Supplementary File S1). Wang *et al.* (2021) showed that RiFGB1 could bind glycans such as β-glucan, chitin and xylan and interfere with a wide range of MAMP-induced defense responses to facilitate AM colonization. The closest orthologs of RiFGB1 in C3 did not show host-dependent expression. RiFGB2 (gene2645 in our data), a DAOM homolog of RiFGB1, was up-regulated for both chives and tomato. Its closest C3 ortholog (FUN_007615) was not expressed in any host. Two additional chives-dependent DAOM genes encoding secreted proteins were found to be missing in C3. These encoded an ornithine decarboxylase antizyme and a protein containing lipoprotein attachment sites, possibly involved in the regulation of secretion. It is tempting to speculate that such proteins may affect the observed difference in symbiotic-efficiency between the two isolates.

Two tomato-dependent effector proteins are the RiSP7 [Kloppholz *et al.* 2011] and a putative CRINKLER-type effector. DAOM SP7 was higher expressed in tomato compared to the other hosts, and proposed to play a role in the suppression of host defense responses. The C3 ortholog of SP7 (FUN_027289-T1), was up-regulated in Medicago and Nicotiana, compared to chives and tomato. The CRINKLER effector on the other hand was down-regulated specifically in tomato, suggesting differential effects related to host-specific immune responses. Interestingly, RiCRN1 [Voβ *et al.*, 2018] did not have a putative ortholog in C3.

Considering their extremely broad host range, it is unlikely that generalists like *R. irregularis* possess genes adapted to a single host species. The two most closely related host species in our experimental setup were Nicotiana and tomato, both part of the Solanaceae family. In both isolates, the amount of genes that were up- and down-regulated in these two hosts was higher than in the others. Many Nicotiana and tomato up-regulated genes in both DAOM and C3 encoded for protein kinases and other genes involved in protein processing, phosphorylation proteins, suggesting important changes in signaling pathways for these host

species [Dickman and Yarden, 1999; Turrà *et al.*, 2014]. Furthermore, metal ion binding and transport genes were up-regulated in both isolates, which may signify that ion regulation is also of particular importance in these hosts.

Host-dependent allele expression

Besides host dependent gene expression we also observed differences in allele expression in the dikaryote-like C3 isolate. The nuclei from C3 are divided in two main nucleotypes marked by divergent MAT loci [Chapter 3]. Since each nucleus carries a haploid genome, different allelic variants are divided over different nuclei. Therefore, increased expression of certain alleles points to either differential nucleotype ratios or differences in allelic activity between nuclei. Robbins *et al.* (2021) reported that shifts in nucleotype ratios found in single spore lines derived from C3, correlated strongly with biallelic expression at the RNA level. Unfortunately, the DNA samples corresponding to our different RNAseq samples were lost during the sequencing process. Therefore, we cannot directly compare the nucleotype ratios to the expressed allele ratios. However, since we used a single batch of C3 spores for the inoculation of all the four host plants at the same time, our data imply that either a very fast shift in nucleotype ratios occurred in specific hosts or that the different nucleotypes have different activity in different hosts. Given the lack of a consistent shift in nuclei ratios in our Medicago selection lines [Chapter 3], changes in transcriptional activity between nuclei seem more likely. One important difference between our analyses and those of Robbins *et al.* (2021) is the fact that they examined the transcriptome in the extraradical hyphae and spores, while we focused on colonized roots. Nuclei-specific allele expression has also been reported in other multinucleate fungi, such as *Agaricus bisporus* and *Neurospora tetrasperma* [Gehrmann *et al.*, 2018; Meunier *et al.*, 2022]. In *A. bisporus*, nucleotype-specific expression varied in different parts of the mycelium. The authors used methylation sequencing to determine differences methylation states between the two nucleotypes, and found that differential expression of alleles may be caused by methylation patterns [Gehrmann *et al.*, 2018]. Little is known about epigenetic regulation in AM fungi, although recent work from several groups has indicated differences in methylation levels in different parts of the genome [Chaturvedi *et al.*, 2021; Yildiri *et al.*, 2021; Manley *et al.*, 2023].

Recently, Sperschneider *et al.* (2023) demonstrated host-dependent allele expression as well in *R. irregularis* dikaryote-like isolates A4, A5 and G1. They created fully phased chromosome level genome (haplotype) sequences for these lines that distinguish the main nucleotypes. For A4 they noted a correlation between the shift in nucleotype ratios and allele expression when comparing chicory and carrot root cultures. For example, for A4 ~70% of the alleles represented by the MAT-2 haplotype dominated in carrot, while ~80% of the MAT-1 haplotype dominated in chicory (*Cichorium intybus*). Also in *Lotus japonicus* (a legume like Medicago) and the monocot *B. distachyon*, the MAT-2 nucleotype of A4 was more active [Sperschneider *et al.*, 2023]. The higher activity of this nucleotype is in line with our observations of increased activity in Medicago and chives, suggesting that the MAT-2 haplotype may contain genes that are important for the symbiosis of these host types. Our results show that a large proportion of C3 alleles were either chives and Medicago dependent, or Nicotiana and tomato dependent. This grouping is remarkable, since chives and Medicago are phylogenetically

distantly related. It suggests that certain conditions that the fungus encounters when colonizing both Medicago and chives are more similar than when it is colonizing Nicotiana and tomato. The alleles that were chives/Medicago or Nicotiana/tomato dependent were found in genes with functions involved in protein binding and transmembrane activity.

Nicotiana and tomato also differ from chives and Medicago in that they were grown under different conditions in our setup. AM fungal inoculated Nicotiana and chives grew on 150% higher phosphate levels, and at a warmer temperature, than chives and Medicago. These growth conditions were based on different temperature and nutrient requirements for these hosts. The phosphate levels of the environment are a particularly important factor controlling mycorrhization [Chapter 2]. Therefore, we cannot rule out that differences in growth conditions may have contributed to allele expression in C3 [Cornell *et al.*, 2021].

In our data, we found monoallelic expression of SNPs as well, where only one allele of a transcript was found, rather than both. This was previously observed in single spore lines of C3 by Robbins *et al.* (2021), where such genes were related to energy production, transcription, and signal transduction. These results are unexpected, since suppression of only one allele of these housekeeping genes could reduce the energy production and overall health of the fungus. Perhaps this mono-allelic expression is also conditional, and the other allele can be expressed under yet-to-identify conditions. Since most of these SNPs did not result in a knockout of a gene, it is unlikely that the other allele of these genes is not functional. More interestingly, mono-allelic genes that contain the alternative allele (likely encoded by the MAT-2 locus carrying nucleotype) had a much higher proportion of missense variants. These genes were enriched for peptidases, protein kinases and protein phosphorylases, suggesting that the variants of these alleles may possibly be more advantageous for the fungus. However, more functional analysis of these genes is needed to draw any conclusions about the different functions of these variants.

With recent advancements in single cell RNA sequencing technologies, it would be interesting to study whether variation in allelic expression also occurs at different locations or stages of the fungal mycelium. Since AM fungi are known to express different genes in different stages and regions of their mycelium [Zeng *et al.*, 2018], such local changes in allele expression may allow for another level of transcriptome regulation.

Host-dependent rRNA expression

DAOM is an isolate with much lower genetic variation than C3, and as such, the amount of SNPs in its transcriptome was low. However, host-dependent allele analysis in DAOM revealed that 18 SNPs that were consistently expressed in at least two hosts were located in the rDNA copies. Interestingly, several of these ribosomal RNA variants were expressed in a host dependent manner. This builds upon a theory that was proposed by Maeda *et al.* (2018). There, the authors showed that due to the unique non-tandem organization of ribosomal DNA copies in *R. irregularis*, genetic variation is maintained in these loci. Later, the same group showed that different rDNA loci are expressed in the different hosts *Lotus japonicus* and *Marchantia paleacea*, demonstrating a role of ribosome heterogeneity in host adaptation [Maeda *et al.*, 2020]. We saw no differences in rRNA copy expression between hosts, which

may have been caused by our use of different host species. A similar rDNA organization can be found in the malaria parasite *Plasmodium falciparum* [Gardner *et al.*, 2002]. Like AM fungi, malaria interact with a variety of distantly related hosts, albeit parasitically rather than symbiotically. The expression of different ribosomal profiles is believed to allow the parasite to alter translation rates of its transcriptome, making it more versatile in switching hosts.

The same may hold true for AM fungi. The SNPs were all located in the 28S RNA, which forms the large 60S subunit. Therefore, allelic variants of 28S may affect ribosome function. The exact effects these SNPs may have on translation rate, however, are not clear, and definitely warrants further investigation.

Like DAOM, C3 also contains a low number of non-tandemly organized ribosomal DNA copies (eight instead of ten). In our RNAseq data for C3, we found that four copies were expressed in our hosts, containing a total of 13 SNPs. However, while SNPs were found within these copies [Chapter 3; van Creij *et al.*, 2023], allelic expression of these copies did not differ between hosts. Instead, the reference allele was preferred in all hosts, with the average allele frequency of all SNPs being around 0.9. These allele frequencies were similar to the allele frequencies in the genome sample that was used as a reference. To further investigate the potential role of rRNA variants in host adaptation of either DAOM or C3, more focused rRNA sequencing will be required.

Conclusion

Together, our results highlight the diversity within *R. irregularis* isolates, and the effect that genetic variation may have on adaptive strategies of these fungi. Both DAOM and C3 showed host-dependent gene expression, but also appeared to have unique methods to respond to different hosts. While C3 showed strong host-dependent effects on its allele expression, DAOM demonstrated a host-dependent effect on its rRNA variant expression. In particular, the growth promoting effects that DAOM had on chives were reflected in a relatively high number of isolate-specific differentially expressed genes, missing in C3, that may have host dependent effects, although their biological function remains to be studied.

Materials and methods

Biological material

Spores of *Rhizophagus irregularis* isolate DAOM197198 were obtained from Agronutrition (France). C3 was grown on *Daucus carota* root cultures on M medium [Bécard *et al.*, 1988]. Spores were isolated by manually isolating spores, extraradical mycelium and mycorrhized roots with forceps and suspending them in water. Next, the mycorrhized roots and spores were processed in a blender, separating the spores from the hyphae and roots without damaging the spores, and filtered with a 40µm mesh to collect spores. The spores were suspended in water, counted using a stereo microscope and diluted to a density of ~100 spores per mL.

The four plant host species were chives (*Allium schoenoprasum*), Medicago (*Medicago truncatula* Jemalong A17), Nicotiana (*Nicotiana benthamiana*) and tomato (*Solanum lycopersicum*, cv. MoneyMaker). Protocols for cleaning, germinating and growing the seeds for each plant host species are described in Chapter 3. Five biological replicates per treatment were used, with ~200 spores per plant. All plants were grown using half Hoagland medium [Hoagland *et al.*, 1950] with lower phosphate conditions (20µM for chives and Medicago, 50µM for Nicotiana and tomato). In addition to mycorrhizal inoculation, plants were grown at low phosphate conditions without AMF as well as under high phosphate conditions (1mM). Plants were watered three times per week. Chives and Medicago were grown at 21°C, while Nicotiana and tomato were grown at 25°C. All plants were exposed to 16/8 hour light/dark cycles. After six (chives, Medicago, Nicotiana) or eight (tomato) weeks, plants were harvested.

Upon harvesting, the roots were rinsed of soil using tap water, and fresh root and shoot weight was measured. Half of the roots were flash frozen in liquid nitrogen for RNA extraction, while the other half was stained in 2% Trypan Blue for mycorrhiza quantification [Phillips and Hayman, 1970; Trouvelot *et al.*, 1986].

RNA sequencing

RNA from colonized roots was isolated by flash-freezing colonized roots and grinding the tissue with a cold mortar and pestle. RNA isolation was performed using the RNeasy Mini kit (Qiagen), according to manufacturer's instructions including an on-column RNAse free DNAse (Qiagen) treatment. The RNA was eluted in 20mM Tris-HCl buffer, quantified using the QuBit fluorometric quantification (Invitrogen) and sent for Illumina sequencing (BGISEQ-500 platform at BGI, Denmark), see Chapter 3. Three biological replicates per treatment were used. RNA from C3 in chives, Medicago, Nicotiana and tomato was sequenced, as well as RNA from DAOM in tomato. Reads from DAOM grown on chives, Medicago and Nicotiana were obtained from Zeng *et al.*, 2018. Reference transcriptomes were obtained from annotated reference genomes Rir17 [Maeda *et al.*, 2017] and RirC3 [van Creij *et al.*, 2023; Chapter 3] with gffread with the -x option [Pertea and Pertea, 2020]. Illumina reads were mapped to the reference transcriptomes using Kallisto [Bray *et al.*, 2016].

Host-dependent gene analysis in R

Host dependent gene expression was determined as follows: count tsv files produced by Kallisto were imported in R, and normalized using quantile normalization. Then, a binary matrix was generated dependent on the number of hosts. Within this matrix, any possible combination of lower expression (0) and higher expression (1) was made. For example, with three hosts, the following matrix would be generated:

Host 1	Host 2	Host 3	Category
0	0	0	Not expressed
1	0	0	Host 1 up-regulated
0	1	0	Host 2 up-regulated
1	1	0	Host 3 down-regulated
0	0	1	Host 3 up-regulated
1	0	1	Host 2 down-regulated
0	1	1	Host 1 down-regulated
1	1	1	Expressed, no difference between hosts

The average transcript count of each gene per host was calculated, and for each transcript the host with the highest average read count was determined. If the highest average count was under the threshold of 10, this gene was categorized as not expressed. If the highest count was above the threshold, an ANOVA test was done for that gene, to determine whether there was significant variation in expression of this gene between hosts. If the p score of that test was above 0.05, the gene was classified as expressed, with no difference between hosts.

For genes that passed the ANOVA test, t-tests were performed, comparing the expression in the highest expressed host with all the other hosts. For example, if Host 3 had the highest average expression for gene A, then the script would compare host 3 vs host 1, host 3 vs host 2 and host 3 vs host 3. P values of these t tests were stored in a vector, and p values of comparisons of the host against itself were replaced with NA. Then, the remaining p values were adjusted using the Benjamin-Hochberg method [Benjamini and Hochberg, 1995]. Next, genes were classified according to the binary matrix. First, the host in which the gene was expressed the highest was classified as “1”. If the expression in another host was significantly lower (meaning the adjusted p-value of that t-test was below 0.05), and the difference in average expression was more than 4-fold (FD > 4), than the host showing significantly lower expression was classified as “0”. If the adjusted p value was above 0.05, the expression was not considered significantly different from the highest, and the host was categorized as “1”. After making all comparisons, the gene was categorized according to the binary matrix. For example, if a gene was classified as “0 0 1”, expression in hosts 1 and 2 was significantly (at least 4-fold) lower than in host 3. Genes that were expressed below the aforementioned threshold were classified as “0” in all hosts, while genes that did not pass the ANOVA test were classified as “1” in all hosts.

The annotated R script can be found on:
[https://github.com/jellevancreij/scripts/blob/main/RNA_binary_comp.R]

Identification of putative orthologs

Orthologs of C3 genes in DAOM19798 (and vice versa) were identified using reciprocal BLAST [Altschul *et al.*, 1990; Zhang *et al.*, 2000]. Blast databases were made of the reference transcriptomes of both isolates. The C3 transcripts were BLASTed against the DAOM blast database, and hits with at least 90% identity over the entire sequence length were counted as orthologs. The same was done for DAOM197198 using the C3 blast database.

Variant calling

Illumina reads were mapped against their reference genomes using HiSat2 with the –dta option [Kim *et al.*, 2019]. Variant calling was performed using freebayes [Garrison and Marth, 2012], only utilizing SNPs that were also found in the genomic data [Chapter 3]. The synonymous vs non-synonymous effects of SNPs on amino acid sequence were determined using Ensembl Variant Effect Predictor [McLaren *et al.*, 2016].

To determine host-dependent allele expression, allele frequencies (reference allele observation divided by the sum of the reference allele observations and alternative allele observations) were calculated for all SNPs. To make comparisons between hosts possible, SNPs were only counted if they were expressed (DP > 20) in all three replicates of at least two of the host species. Next, the same method as in host-dependent gene expression was used to determine whether the average allele frequency of any of the SNPs in one or more hosts was significantly different from the rest. However, two changes were made in the method: the adjusted p value cut-off for the initial ANOVA test was 0.1, rather than 0.05 (to find more host-dependent differences), and allele frequencies were determined to be different if they passed the t-test (adjusted $p < 0.05$), with an allele frequency difference of at least 0.1. SNPs that failed the ANOVA test were categorized as equally expressed in all hosts.

Data availability

RirC3 genome and annotation can be found at https://zenodo.org/record/7037960. RNA sequencing data generated in this work are available from Genbank under BioProject ID PRJNA747641.

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Supplementary Information

Additional file 1: Host dependent gene overview for DAOM197198. Per tab, genes are listed that fit in the category listed in the tab name, with average expression per host. Genes are sorted on average difference in expression compared to the other hosts. Transcripts with a signal peptide are highlighted in yellow.

Additional file 2: Host dependent gene overview C3. Per tab, genes are listed that fit in the category listed in the tab name, with average expression per host. Genes are sorted on average difference in expression compared to the other hosts. Transcripts with a signal peptide are highlighted in yellow.

Additional file 3: Host dependent allele expression in C3. Per tab, SNPs are listed that fit in the category listed in the tab name, with average allele frequency per host.

Additional files are available on: https://zenodo.org/record/8083928

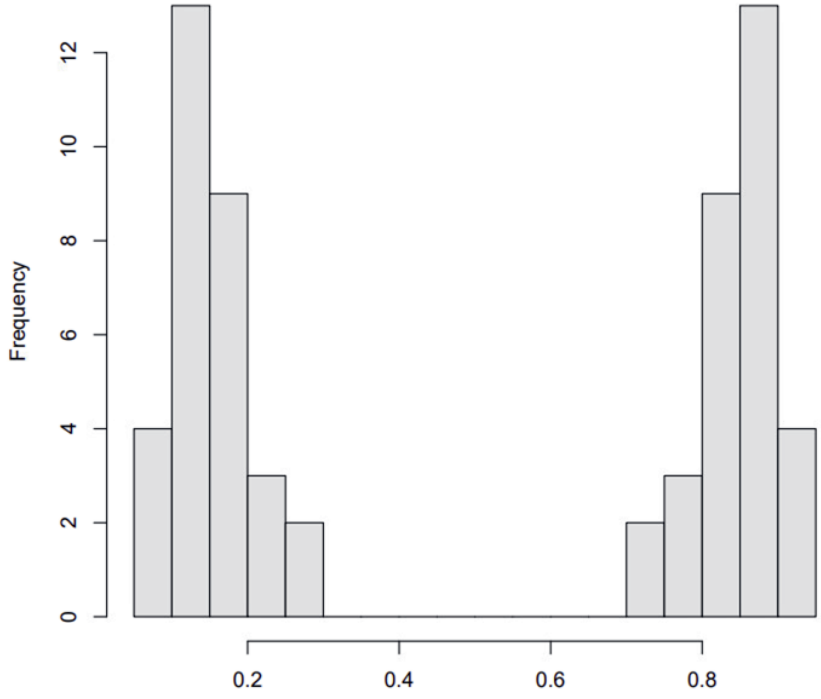

Figure S1: *Allele frequencies of rDNA copies in C3 genomic DNA.*

Table S1: *Mapping rates of RNAseq reads. DAOM197198 reads were mapped against Rir17 [Maeda* et al.*, 2018]. C3 reads were mapped against RirC3 [van Creij* et al.*, 2023; Chapter 3].*

DAOM197198		**C3**	
Sample	**Mapping rate**	**Sample**	**Mapping rate**
Chives 1	6.92%	Chives 1	20.17%
Chives 2	6.71%	Chives 2	26.20%
Chives 3	11.86%	Chives 3	30.30%
Medicago 1	8.66%	Medicago 1	20.46%
Medicago 2	9.85%	Medicago 2	19.90%
Medicago 3	9.92%	Medicago 3	14.77%
Nicotiana 1	5.59%	Nicotiana 1	24.53%
Nicotiana 2	2.80%	Nicotiana 2	19.27%
Nicotiana 3	7.62%	Nicotiana 3	21.63%
Tomato 1	14.84%	Tomato 1	7.89%
Tomato 2	19.41%	Tomato 2	15.51%
Tomato 3	19.35%	Tomato 3	9.90%

Chapter 5

General discussion

The multinucleate nature of arbuscular mycorrhizal (AM) fungi, together with the lack of an observed sexual cycle, has intrigued researchers for decades. This thesis focused on a key question, namely how genetic variation is distributed over and used by different nuclei, and how dynamically it functions in the interaction with different host plants. To study this, I investigated the genetic organization of the dikaryote-like *Rhizophagus irregularis* isolate C3 by assembling a new reference genome, and sequencing mycelium, single spores and individual haploid nuclei. Furthermore, the transcriptome of C3 was compared to that of homokaryon DAOM197198 when grown with four different host plants.

This revealed a highly dynamic nuclear organization where different nucleotypes showed seemingly stochastic segregation among individual spores as well as unequal (variable) nucleotype ratios in single spore derived lines and even among different batches of propagated cultures. Furthermore, we found evidence for past inter-nucleus recombination events in C3. Upon a shift to a different host plant, no consistent changes in nucleotype ratios were observed. However, at the transcriptome level host-dependent changes in allelic expression were observed. In contrast to C3, the homokaryotic DAOM197198 expressed different allelic variants of its rRNA in different hosts.

Here, I will discuss our novel findings on the genetic composition of the C3 isolate and place them in the light of recent findings in the field of AM genomics that arose during the duration of this thesis.

Genetic variation in *Rhizophagus irregularis*

The genetic composition of AM fungi is characterized by its multinucleate nature. Where most eukaryotes contain one nucleus per cell, AM fungi contain thousands to millions of nuclei in one interconnected body of cytoplasm [Smith and Read, 2010]. These fungi propagate primarily asexually by forming chlamydospores. This step constitutes the strongest known genetic bottleneck in the lifecycle of AM fungi, since all nuclei from the adult mycelium (barring those that are exchanged horizontally through anastomosis) derive from that initial sub-population. Instead of a single nucleus filling the spore through mitotic divisions, hundreds of nuclei migrate from the mycelium into the spores as they develop [Jany and Pawlowska, 2010; Marleau *et al.*, 2011]. The nuclei of *R. irregularis* are haploid, but contain conserved SNPs that act as allelic variants in its genome. Whole genome sequencing of multiple isolates of *R. irregularis* revealed two types of genome organization. While the majority of isolates, including model isolate DAOM197198, where homokaryotic with low amounts of genetic variation, others contained two distinct types of nuclei [Ropars *et al.*, 2016]. The latter types were called “dikaryons”, with allelic variation equally spread out between the two nucleotypes. The different nucleotypes were characterized by a divergent mating-type (MAT) locus [Ropars *et al.*, 2016]. To date, only five dikaryotic isolates have been described: SL1, A4, A5, G1, and C3 [Sperschneider *et al.*, 2023].

Initially, isolate C3 was reported to have the highest level of intragenomic polymorphisms of all the isolates studied by Wyss *et al.* (2016), based on RADseq data. However, this turned out to be an overestimation due to the use of an inappropriate reference genome [Masclaux *et al.*, 2019]. Moreover, A4 and C3 were found to be genetically highly similar to each other

[Wyss *et al.*, 2016]. Both strains were isolated from the same field in Switzerland, ~50 meters apart from each other, suggesting that they could be derived from sibling spores from the same maternal AM fungal colony, or a common anastomosis event of compatible homokaryons [Koch *et al.*, 2004].

Advancements in *R. irregularis* genomics

Since a suitable reference genome of C3 was missing at the start of this project, we first set out to assemble a new genome from our own C3 material. The resulting assembly, RirC3, provided a higher mapping rate for Illumina reads of both C3 and A4, compared to the more fragmented RhiirA4 assembly [Ropars *et al.*, 2016]. Variant calling on these mapped reads showed that C3 and A4 share many biallelic SNPs, although isolate specific SNPs were also found.

Compared to the best contemporary assembly of DAOM197198, Rir17 [Maeda *et al.*, 2018], RirC3 differed substantially in the amount of predicted genes [Chapter 3]. This difference may in part have been caused by the use of different annotation methods. In Chapter 4 I showed that around 1/3rd of the predicted genes in Rir17 were not active in any of the four hosts from which we generated RNA. The number of active genes in the four hosts combined was more similar between Rir17 and RirC3, indicating that many of the predicted genes in Rir17 were either inactive in the conditions we tested, or they might be inactive, theoretical gene models. Like DAOM and other *R. irregularis* isolates, RirC3 contained a high number of repeats, covering nearly half (46%) of the assembly size.

Around the time that RirC3 was finished, Robbins *et al.* (2021) also published a C3 reference genome, called CHRIC3. Compared to RirC3, this assembly had a slightly lower contig number and nearly four times higher N50. However, the total size of this assembly was 50% longer than that of RirC3 and the predicted genome size based on flow cytometry [Sędzielewska *et al.*, 2011]. This observation, along with a high duplicated BUSCO score and rate of C3 Illumina reads that mapped on multiple locations in the genome, showed that the assembly contained many duplicated contigs, potentially belonging to different haplotypes. RirC3 provided a higher unique mapping rate for C3 Illumina reads than CHRIC3. Therefore, we decided to continue using RirC3 for further analyses.

Recently, Yildirir *et al*. (2022) and Manley *et al.* (2023) used Oxford Nanopore sequencing to produce the first chromosome level genome assembly for the model isolate DAOM197198, assembling a total of 32 chromosomes. Combined with chromatin conformation capture (Hi-C) sequencing, this revealed a two compartment genome. Based on gene density, DNA methylation signatures and phylostratigraphy analysis, the authors proposed that the different compartments serve different purposes in fungal activity, with the transcriptionally active A compartment being gene rich and encoding for the conserved core genome, containing 90% of all BUSCO genes. The B compartment, on the other hand, was less transcriptionally active and contained evolutionarily younger genes, including more *in planta* induced and secreted proteins. Such a division somewhat resembles the “two-speed genome” model that can be found in filamentous plant pathogens with large gene repertoires [Dong *et al.*, 2015; Reinhardt *et al.*, 2021]. In this model, rapidly evolving (lineage-specific) effector

genes are mostly found in repetitive and rearranging genome regions that are separate from more gene-rich areas containing most core genes. Comparing the genome conservation between *R. irregularis* isolates showed higher levels of (structural) variation in the B compartment [Yildrir *et al.*, 2022; Manley *et al.*, 2023], suggesting that it may indeed contribute more to the differentiation of these isolates. This genomic organization would allow AM fungi to be able to evolve its gene repertoire to constantly adapt to their hosts.

Shortly after Yildirir *et al.* (2022), the same group assembled a fully phased genome for dikaryotic isolate A4 [Sperschneider *et al.*, 2023]. The authors found similar A and B compartments in A4, and highlighted the high gene density in the A compartments. Unfortunately, C3 was not included in the dataset of the authors, and at the moment of writing this thesis, the phased A4 reference genome has not been made publicly available. Upon its publication it would be interesting to see if it is also a suitable reference genome for C3. If it is, we could see if the many host-dependent genes we found in Chapter 4 are indeed located in the B compartments. Furthermore, a more detailed comparison between these two very closely related isolates may reveal more variation in the B compartment, supporting the theory that this compartment evolves more quickly. Finally, the chromosome-wide phased haplotypes may allow us to more accurately find genetic recombination, and predict in which region such recombination has happened.

In summary, our understanding of intraspecies genomic variation in *R. irregularis* has increased with every improvement in sequencing technology. The generation of chromosome level genome assemblies and phased haplotypes make for impressive resources. However, these are all based on whole metagenome data, meaning these were made with the combined genomic DNA of millions of nuclei. In phasing a dikaryotic (or diploid) genome, potential variation between haplotypes can be missed, as consensus haplotypes are made based on the assumption that loci are linked [Marchini *et al.*, 2006; Browning *et al.*, 2011]. To truly understand how much intra-species and intra-genomic variation exists, individual nuclei must also be investigated.

Recombination between nuclei

In order to study inter-nucleus variation in C3, we sequenced ten individual haploid nuclei of this isolate. The nuclei could clearly be distinguished based on their MAT-locus, with six carrying MAT-1 and four carrying MAT-2. The same MAT-1 and MAT-2 loci occur in the A4 isolate [Ropars et al., 2016]. Since the nuclei were haploid, they also contain either allele of the numerous SNPs that can be found in the genome. In a strictly dikaryotic non-recombining genome, each haploid nucleotype would contain either of two haplotypes. For the majority of SNPs, this was indeed the case: the reference allele for SNPs in the genome was usually found on nuclei with the MAT-1 locus. In other word, nuclei with the same MAT-locus did not carry different alleles for most SNPs. However, for about 5% of all SNPs, variation within nucleotypes was observed that did not match the predicted haplotype. These non-matching patterns were based on SNPs that were confirmed in the metagenome using whole genome Illumina reads, indicating that these are real SNPs and not artefacts due to mapping issues. All ten nuclei we sequenced contained such non-matching haploblocks. These results indicate that recombination between nuclei must have occurred.

Remarkably, doing the same analysis on single nuclei of A4 (data from Ropars *et al.*, 2016), which is highly similar to C3, revealed no such non-matching haploblocks despite earlier claims [Chen *et al*., 2018b, 2020; commented on by Auxier and Bazzicalupo, 2019]. This illustrates one of several differences we noticed between the supposedly clonal isolates A4 and C3. It is unclear why only C3 nuclei show signs of genetic recombination. Variation in recombination rate has been shown to be dependent on growing conditions, temperature, starvation or (biotic) stress, in addition to intrinsic genetic or epigenetic mechanisms [Stapley *et al.*, 2017]. Therefore, C3 and A4 may have encountered different stresses in their life history that led to differences in recombination levels. It should be noted that both isolates have been maintained in different labs, potentially under different circumstances, and nuclei were isolated independently. Repeating the nuclei sequencing of both isolates within the same study can provide a more direct comparison. It would now also be highly relevant to study individual spores from *R. irregularis* collected directly from the field, to determine whether they may reveal more recombination events and genetic variation [Eshgi Sahraei *et al.*, 2022].

Meiosis or not?

The existence of non-matching haploblocks appears to point to a past recombination event in the lifecycle of C3. However, mating is unlikely to occur constantly in these fungi. With constant mating and recombination, the two haplotypes of the genome would be reshuffled until most allelic variants occur independently from the MAT-loci [Slatkin, 2008]. Such disassociation of loci is not visible in any dikaryotic *R. irregularis* isolate, suggesting that if genetic recombination happens, it is not common [Savary *et al.*, 2018]. The conservation of a full set of meiosis related genes in the *R. irregularis* genome suggests that they are utilized by the fungus [Halary *et al.*, 2010]. Furthermore, the MAT loci in *R. irregularis* are similar of those found in bipolar basidiomycetes that do have a sexual cycle [Casselton and Olesnicky, 1998; Seidl and Thomma, 2004; Raudaskoski and Kothe, 2010; Ropars *et al.*, 2016]. The MAT-loci of *R. irregularis* contain two homeodomain (HD) proteins are encoded in opposite directions, and contain different dimerization helices [Ropars *et al.*, 2016]. The heterodimerization of compatible HD proteins of opposite MAT-loci is a precursor to nuclear pairing and mating in fungi [Banham *et al.*, 1995]. Since C3, and the other known dikaryotic *R. irregularis* isolates, have a population of nuclei containing either of two MAT-loci that co-exist in the same cytoplasm, meiotic pairing could occur. However, the reported extensive divergence of gene order and structure between the two haplotypes in A4 could argue against a meiotic cycle [Sperschneider *et al.*, 2023]

The existence of conserved meiotic genes and heterothallic MAT-loci has led to the discovery of cryptic sexual stages in other fungi that were previously thought to be asexual [Schurko *et al.*, 2008]. For example, the Ascomycete pant pathogen *Magnaporthe oryzae* was found to reproduce sexually under rare circumstances [Saleh *et al.*, 2012]. The same is seen in saphrotropic cheese fungus *Penilillium roqueforti* [Ropars *et al.*, 2014]. Another presumed asexual Ascomycete plant pathogen is *Verticilium dahlia*. This was in part because the vast majority of isolates carry the same MAT-locus, and so far only one isolate is known to carry

the other idiomorph [Short *et al.*, 2014]. However, no sexual stage has ever been observed for these fungi. Nevertheless, the genome of *V. dahlia* shows signs of multilocus linkage disequilibrium, similar to what we observed in single nuclei of C3, suggesting that recombination between lineages also occurs in these species [Atallah *et al.*, 2010].

An alternative for genetic recombination during meiosis, one which does not require the formation of sexual structures, is parasexual reproduction [Pontecorvo *et al.*, 2011]. In a parasexual cycle, fungi exchange nuclei via anastomosis. After the fusion of two different nuclei, cross-overs between homologous chromosomes or gene conversions, as a mechanism for repair of double-strand breaks caused by DNA damage, can occur during mitosis. The resulting diploid nucleus then returns to a haploid stage through aneuploidization, as a result of chromosome loss. This has been observed in other dikaryotic fungi, such as *Agaricus bisporus*, where it occurs frequently after hyphal fusion [Xu *et al.*, 1996; Anderson and Kohn, 2007]. Such transfer between nuclei can also occur through degrading nuclei (nucleophagy), which have been observed in the cytoplasm of AM fungi [Kokkoris *et al.*, 2020]. Intriguingly, many of the proteins involved meiosis may also be involved in the parasexual cycle [Reinhardt *et al.*, 2021]. If this process would occur at a significant rate, a population of diploid and aneuploid nuclei should be present in the fungus. However, so far flow cytometry analyses of AM nuclei has not found evidence for such a population [Sędzielewska *et al.*, 2011]. Attempting to test this with single nuclei sequencing is difficult due to technical limitations of whole genome sequencing of single nuclei, as chromosome losses may not be distinguishable from incomplete genome amplification. Instead, measuring DNA content by flow cytometry at different developmental stages, or performing this analysis on wild isolates, may reveal more signs of ploidy changes.

Another hypothesis proposed by Yildirir *et al.* (2020) is that horizontal transfer of genetic information can occur through transposable element (TE) activity. Indeed, the genome of *R. irregularis* has a high amount of TEs, much higher than most fungi [Chen *et al.*, 2018a; Dallaire *et al.*, 2021]. Methylation analyses showed that the TEs in the B compartment of DAOM197198 had significantly less CpG methylation, which could indicate more transposition in these parts of the genome [Manley *et al.*, 2023]. TE activity is a known driver of genomic variation, but can be highly detrimental for the genome as well. Intragenomic proliferation of TEs can be limited, by pathways that are associated with meiosis [Arkhipova and Meselson, 2005]. In other words, TE activity is less restrained in asexual organisms. Other ancient asexual eukaryotes have evolved different strategies to constrain TE activity [Ågren *et al.*, 2014]. Bdelloid rotifers, a microscopic animal that reproduces through parthenogenesis and environmental uptake of DNA, lacks active retrotransposons altogether [Arkhipova and Meselson, 2000; Arkhipova and Meselson]. The same was seen in asexual protozoan *Giardia lamblia* [Arkhipova and Morrison, 2001]. This is in stark contrast with the high TE content of *R. irregularis*, which also contains multiple retrotransposases, enhancing retrotransposon activity. Without meiosis occurring in AM fungi, deleterious TEs would accumulate in the genome. This observation argues for the activity of meiosis related genes to keep TE activity in check.

Recently, more potential sources of horizontal gene transfer have been discovered in AM fungi. Endobacteria harbored by *R. irregularis* have been shown to have transferred genes to their fungal host [Torres-Cortés *et al.*, 2015]. AM fungi have likely transferred their genes to their plant hosts early in evolution, based on paraphyletic groups of fungal genes within land plant genomes [Li *et al.*, 2018]. Foreign genes coming from plants or bacteria have been described in the *R. irregularis* genome, that were likely introduced through horizontal gene transfer. Recently, even a contiguous viral genome was discovered in one *R. irregularis* isolate [Zhao *et al.*, 2023]. While such horizontal gene transfers appear to be uncommon based on the low number of external genes described in the AM genome, they could contribute to an increased intra-species genomic variation.

The conservation of meiosis genes and the traces of genetic recombination all point to cryptic sexual or parasexual reproduction occurring in *R. irregularis*. Co-inoculation experiments of two homokaryotic isolates with divergent MAT loci revealed the transcriptional activation of genes presumed to be involved in fungal mating, though the MAT-loci themselves were not up-regulated [Mateus *et al.*, 2020]. These findings suggested that other environmental factors can activate (parts of) the sexual reproductive pathway. However, the results of that study were contested by Malar *et al.* (2021), who argued that the up-regulated genes have other functions, unrelated to mating [Malarkey and Churchill, 2012], and that the two isolates that were used (DAOM197198 and B1) are likely incompatible, based on MAT-locus and overall genome divergence.

In conclusion, inter-nucleus exchange of genetic material through recombination seems possible in *R. irregularis*. However, if (para)sexual reproduction happens in *R. irregularis* it is likely to be rare, as haplotypes appear to be relatively stable across different isolates, even in dikaryotic isolates such as C3. The technical limitations of single nuclei analysis complicate these investigations, but recent advancements in single nuclei sequencing may allow us to find more evidence of inter-nucleus recombination, particularly in changing environments [Eshgi Sahraei *et al.*, 2022]. It does however, leave us with the question how *R. irregularis* manages to efficiently purge the genome from accumulating somatic mutations. If recombination events would occur at a much higher frequency between nuclei of the same haplotype, it could possibly explain the relative low SNP density. Alternatively, a still unknown mutation suppression mechanism as proposed for the fairy-ring mushroom *Marasmius oreades* may be at work [Hiltunen *et al*., 2019; Sperschneider *et al*., 2023].

Nuclei ratios in C3 are highly dynamic

Variant calling in our C3 data revealed an unusual, unequal distribution of the two nucleotypes. The MAT-1 nucleotype in C3 was sometimes dominant, being twice as common as the MAT-2 nucleotype. In other lineages, the ratios were more equal. Changes in nuclei ratios are seen more often in heterokaryons and signify competition between nucleotypes [Leslie, 1993; Strom and Bushley, 2016].

Both whole genome sequencing of single spores and ddPCR of MAT-loci ratio showed that individual spores had even more highly variable nuclei ratios [Chapter 3], suggesting that nuclei fill up spores in a stochastic manner [Marleau *et al.*, 2011]. Spores derived from multiple

generations of single spore lines, having gone through multiple successive genetic bottlenecks, did not converge. Instead of sibling single spore lines (derived from the same single spore line parent) becoming more similar, they again showed random nuclei ratios. This illustrates how nuclei ratios change during development. This is not in line with earlier single spore line analysis by Ehinger *et al.* (2012), who also generated single spore lines of C3. Based on a single locus, the Bg112 marker, the authors concluded that sibling lines became more convergent, though the validity of this marker has been questioned [Kokkoris *et al.*, 2021b]. The differences between their results and ours may be because of different growth conditions, media or other abiotic effects (since the same host species were used). Cornell *et al.* (2021) showed that nuclei ratios is A4 can change depending on abiotic factors. Such shifts have been observed in other dikaryotic fungal species, such as Basidiomycete *Heterobasidion parviporum*, which shifts its nuclei ratios upon environmental changes in pH [James *et al.*, 2008]. Heterokaryotic ascomycete *Penicillium cyclopium* also adapts its nucleotype ratios to adapt to changing environments [Rees and Jinks, 1952]. Such environmental effects may also apply to AM fungi [Kokkoris *et al.*, 2021b]. These shifts in nuclei ratios seem deliberate and consistent, and a lack of these factors in laboratory conditions may result in more uneven nuclei distributions in the fungi. This could explain the high amount of variation that was observed between spores.

It is important to note that single spore lines came from spores that were viable in the first place. In our data, we found that nuclei ratios were more variable between amplified spores than between adult single spore lines. Since AM fungal spores appear to be filled with nuclei in a seemingly stochastic manner, local differences in nuclear ratios could lead to highly skewed ratios in the spores, compared to the rest of the fungus. Perhaps spores with a nuclear composition that is too divergent will have such a reduced fitness, if viable at all, that they are outcompeted by spores with a more balanced composition. Based on ddPCR of the two MAT-loci, we did find one C3 spore that appeared to be homokaryotic. However, since we had to sacrifice that spore in order to analyze it, we do not know if it was viable, and if it could have indeed resulted in a homokaryotic lineage. Besides unequal proliferation during development, nuclei shifts may also change due to anastomosis with other fungi [Croll *et al.*, 2009]. In wild environments, this could be particularly relevant, since nuclei ratios could be balanced by constant mixing with other AM fungi [Rayner, 1991].

Besides genetic changes, single spore lines are also known to differ in mycorrhizal growth response (MGR) they induce on their host. For example, Angelard *et al.* (2014) showed up to 5-fold differences in rice MGR between C3 single spore lines. Later, Ceballos *et al.* (2019) showed differences in MGR of Cassava for different C3 lineages. These findings suggest that genetic bottlenecking does have an effect on AM fungi, even if nuclei ratios shift during later development. The genetic consequences of successive bottlenecks could include an over-representation of nucleotypes with advantageous (or deleterious) mutations. Through this selection, AM fungi may also be able to purge deleterious nuclei from their population, since spores with a relatively high amount of deleterious SNPs may have reduced viability. Intriguingly, similar variable effects of different single spore lines on the MGR in cassava were

observed for the homokaryotic C2 isolate [Ceballos *et al*., 2019]. As this isolate only has a single nucleotype with less genetic variation, it was proposed that epigenetic variation between siblings can also contribute significantly to the observed variation in host growth.

Biotic factors such as host identity have been proposed to influence nuclei ratios as well. Limpens and Geurts (2014) proposed that plant hosts could "select" for advantageous nuclei, causing them to proliferate more than other ("sanctioned") nuclei. However, during the span of our Medicago selection experiment, no consistent shifts in nucleotype ratios were observed. This appears to be in contrast with Kokkoris *et al.* (2021a), who found that nuclei ratios of A4 did shift after a host switch. However, even if local shifts in nucleotype ratios occur, these do not (necessarily) reflect ratios in the spores. Any change in nuclei ratios may be offset by stochastic nuclei shuffling in spores. We only analysed the nuclear ratios after three rounds of subculturing. It is possible that potential initial effects on nucleotype ratios got reshuffled during the different subculturing events.

The exact mechanisms behind the dynamic changes in nuclei ratios are not clear, as the individual roles of the two nucleotypes are not well understood. The extended conservation of both nucleotypes in dikaryotic-like strains suggests that they may cooperate [Kokkoris and Hart, 2019]. Dikaryotic AM fungi have been observed to outperform homokaryons in the past with respect to certain traits [Serghi *et al.*, 2021], so the maintenance of heterokaryosis seems to be advantageous to the fungus in the conditions that were tested. This is however difficult to reconcile with the fact that to date more homokaryotic isolates than dikaryotic isolates have been identified. Sequencing of more spores directly from the field should be done to show whether this also hold true in wild isolates, or if it is due to the long maintenance of lines as root cultures.

Host adaptation on a transcriptomic level

While we found no evidence of host adaptation through consistent changes in nucleotype ratios, comparison of allele frequency distributions of mRNA of C3 grown on different hosts did show a consistent shift in different hosts. While C3 grown on Nicotiana and tomato expressed alleles in the same 2:1 ratio as SNPs found in the genome, the expression in chives and Medicago followed a 1:1 ratio. Unfortunately, we lacked the data to make a direct comparison between genomic DNA and RNA allele frequencies C3 grown on the same host, because the corresponding DNA samples were unfortunately lost during the shipping to the sequencing company. The spores and mycelium that were used to colonize these plants came from the same batch of C3 colonized carrot root cultures, so initial nucleotype ratios must have been similar. Since mRNA was isolated after 6 to 8 weeks, the nuclei ratio would have to have shifted within this time period, but no consistent shift in nucleotype ratios was observed in the selection experiment, after over two years of subculturing on Medicago root cultures as host. If a shift in host-induced nucleotype ratios initially did occur it does not seem to be stably maintained by the fungus.

Instead, it is more likely that C3 adapts its nuclear transcriptional activity to its hosts. Zeng *et al.* (2018) showed that AM fungi are able to locally regulate their transcriptome, as different genes were expressed in different parts of the fungus. Furthermore, different genes were

found to be expressed under different hosts even when connected by a shared mycelium. Differential nuclei gene expression has been observed in other multinucleate fungi as well, such as basidiomycete *A. bisporus* [Gehrmann *et al*., 2018] and ascomycete *Neurospora tetrasperma* [Meunier *et al.*, 2022]. Sperschneider *et al.* (2023) revealed differences in haplotype specific gene expression by A4 in different hosts. As host species for non-root culture hosts, the authors used legume *Lotus japonicus* and monocot *Brachypodium distachyon*. In both, the MAT-2 locus carrying haplotype was more transcriptionally active than the MAT-1 haplotype. This appears to be in line with our observations in legume Medicago and monocot chives, where both allelic RNA variants were equally present, though the MAT-2 nuclei was only half as common in the genome of most of our C3 lines.

For transcripts with similar allele ratios between hosts, we also observed mono-allelic expression of several genes. Although the gene itself contained biallelic SNPs, only one allele was transcribed. We saw such monoallelic expression for both the reference (MAT-1) and alterative (MAT-2) alleles. Similar expression patterns were observed in single spore lines of C3 [Robbins *et al.*, 2021], and is known to occur in other heterokaryons as well [Liu *et al.*, 2017]. The molecular mechanisms behind nucleotype-specific expression are still unclear, but may involve interactions between nuclei, where one may be able to silence the other.

Besides host dependent expression of genes and alleles, our study also revealed a remarkable difference in rRNA expression between DAOM197198 and C3. DAOM was found to express alleles of ribosomal 45S DNA operons in a host-dependent manner. Host-dependent variants particularly occurred in the 28S unit. While genetic variation within the genome of DAOM is overall quite low, the rDNA operons have a higher SNP rate [Pawlowska and Taylor, 2004], and different rRNA alleles are believed to effect translation efficiency of the transcriptome [Parks *et al.*, 2018]. Differential rRNA expression has been found for example in the malaria-causing parasite *Plasmodium falciparum*, which, like AM fungi, interacts with genetically distant hosts in their life cycle [Gardner *et al.*, 2002]. Maeda *et al.* (2018) first proposed that *R. irregularis* may use differential ribosomal expression to rapidly adapt their proteome to a new host. Later, the same group found that specific rDNA copies are expressed in different hosts [Maeda *et al.*, 2020]. However, our work is as far as we know the first report showing that allelic variants of rRNA copies are expressed in a host-dependent manner.

Interestingly, while C3 has a much higher amount of genetic variation than DAOM, we found no evidence that it also expressed different alleles of rRNA. It is tempting to speculate that DAOM might use its ribosomal RNA variants to adapt to different hosts at a post-transcriptional level, while C3 can make more use of its differential allelic expression. However, these results are based on only four species of host plants. AM fungi can be found all over the world, and can colonize the majority of vascular land plants [Van der Heijden *et al.*, 1998]. Expanding this dataset by adding more (taxonomically divergent) hosts as well as fungal isolates will certainly reveal more novel host-dependent effects.

Concluding remarks

In recent years major insight into the genetic make-up of AMF fungi has increased significantly, especially due to developments in sequencing technologies. Part of the great mysteries concerning these presumed asexual scandals [Sanders and Croll, 2010; Corradi and Brachmann, 2017] are thereby starting to be demystified. While comparison of the sequences of single nuclei has disproven the theory of vast genetic inter-nucleus differences, the discovery of MAT loci and conserved genes involved in genetic pairing and meiosis have hinted towards a still unobserved cryptic sexual cycle or parasexuality. Our analyses of the C3 isolate provide evidence for the occurrence of inter-nucleus genetic recombination between nuclei of C3. Classifying this isolate as a mere dikaryote seems an oversimplification, as no two nuclei we sequenced were identical. Furthermore, nucleotype ratios were found to be highly variable between C3 batches, spores and possibly even within different parts of the mycelium. On top of that, we observed allelic variation at the transcriptome level that may add another layer of potential adaptive responses to different hosts and environments.

The study of different isolates of the model fungus *Rhizophagus irregularis* has shown the importance of not relying solely on a single isolate. Even within the species large differences between isolates have emerged in terms of gene content, inter-nucleus recombination, the level and dynamic distribution of genetic variation, and their associated (allelic) transcriptomes. “Evolutionary scandals” such as these are often the most interesting, as they help us better understand the many aspects of evolution. However, the picture that is being unveiled is still far from complete and many important questions remain to addressed. To answer these, it is important to remember the incredibly complex network of interactions that AM fungi are a part of in their natural environment. Solely analyzing these fungi in isolated axenic cultures will doubtlessly miss many of their intricacies, and while C3 was a relatively new isolate, it has now been in monoculture for nearly two decades. The isolation of new wild strains of AM fungi, combined with advancements in low input and single cell sequencing will help us characterize these enigmatic organisms. To accomplish this, we can surely be inspired by the symbiotic nature of AM fungi, and conduct such research with healthy collaborations and pleasant, respectful attitudes. There is still much to discover about the genome of AM fungi, and studying unique genomic compositions like these allows us to gain a deeper understanding of genetic variation, and the many mechanisms that shape evolution.

References

Ågren JA. Evolutionary transitions in individuality: insights from transposable elements. Trends in ecology & evolution. 2014 Feb 1;29(2):90-6.

Akiyama, K., Matsuzaki, K.I., Hayashi, H., 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435 (7043), 824.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of molecular biology. 1990 Oct 5;215(3):403-10.

Alvarez, F.J., Konopka, J.B., 2007. Identification of an N-acetylglucosamine transporter that mediates hyphal induction in Candida albicans. Mol. Biol. Cell. 18 (3), 965975.

An, J., Sun, M., van Velzen, R., Ji, C., Zheng, Z., Limpens, E., et al., 2018. Comparative transcriptome analysis of Poncirus trifoliata identifies a core set of genes involved in arbuscular mycorrhizal symbiosis. J. Exp. Bot. 69 (21), 52555264.

An, J., Zeng, T., Ji, C., de Graaf, S., Zheng, Z., Xiao, T.T., et al., 2019. A Medicago truncatula SWEET transporter implicated in arbuscule maintenance during arbuscular mycorrhizal symbiosis. New Phytol.

Anderson JB, Kohn LM. Dikaryons, diploids, and evolution. Sex in fungi: molecular determination and evolutionary implications. 2007 Aug 27:333-48.

Anderson MZ, Thomson GJ, Hirakawa MP, Bennett RJ. A 'parameiosis' drives depolyploidization and homologous recombination in Candida albicans. Nature communications. 2019 Sep 26;10(1):4388.

Angelard, C., Colard, A., Niculita-Hirzel, H., Croll, D., Sanders, I.R., 2010. Segregation in a mycorrhizal fungus alters rice growth and symbiosis specific gene transcription. Curr. Biol. 20 (13), 12161221.

Angelard, C., Tanner, C.J., Fontanillas, P., Niculita-Hirzel, H., Masclaux, F., Sanders, I.R., 2014. Rapid genotypic change and plasticity in arbuscular mycorrhizal fungi is caused by a host shift and enhanced by segregation. ISME J. 8 (2), 284.

Aparicio Chacón MV, Van Dingenen J, Goormachtig S. Characterization of Arbuscular Mycorrhizal Effector Proteins. International Journal of Molecular Sciences. 2023 May 23;24(11):9125.

Arkhipova I, Meselson M. Transposable elements in sexual and ancient asexual taxa. Proceedings of the National Academy of Sciences. 2000 Dec 19;97(26):14473-7.

Arkhipova IR, Morrison HG. Three retrotransposon families in the genome of Giardia lamblia: two telomeric, one dead. Proceedings of the National Academy of Sciences. 2001 Dec 4;98(25):14497-502.

Arkhipova I, Meselson M. Deleterious transposable elements and the extinction of asexuals. Bioessays. 2005 Jan;27(1):76-85.

Atallah ZK, Maruthachalam K, du Toit L, Koike ST, Davis RM, Klosterman SJ, Hayes RJ, Subbarao KV. Population analyses of the vascular plant pathogen Verticillium dahliae detect recombination and transcontinental gene flow. Fungal Genetics and Biology. 2010 May 1;47(5):416-22.

Auxier B, Bazzicalupo A. Comment on 'Single nucleus sequencing reveals evidence of inter-nucleus recombination in arbuscular mycorrhizal fungi'. Elife. 2019 Oct 25;8:e47301.

Auxier B, Scholtmeijer K, van Peer AF, Baars JJ, Debets AJ, Aanen DK. Cytoplasmic mixing, not nuclear coexistence, can explain somatic incompatibility in basidiomycetes. Microorganisms. 2021 Jun 8;9(6):1248.

Bago B, Zipfel W, Williams RM, Piché Y. Nuclei of symbiotic arbuscular mycorrhizal fungi as revealed by in vivo two-photon microscopy. Protoplasma. 1999 Mar;209(1):77-89.

Bago, B., Pfeffer, P.E., Shachar-Hill, Y., 2000. Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiol. 124 (3), 949958.

Bago, B., Pfeffer, P.E., Abubaker, J., Jun, J., Allen, J.W., Brouillette, J., et al., 2003. Carbon export from arbuscular mycorrhizal roots involves the translocation of carbohydrate as well as lipid. Plant Physiol. 131 (3), 14961507.

Balestrini R, Bianciotto V, Bonfante-Fasolo P. Nuclear architecture and DNA location in two VAM fungi. Mycorrhiza. 1992 Jun 1;1(3):105-12.

Banham AH, Asante-Owusu RN, Gottgens B, Thompson S, Kingsnorth CS, Mellor E, Casselton LA. An N-terminal dimerization domain permits homeodomain proteins to choose compatible partners and initiate sexual development in the mushroom Coprinus cinereus. The Plant Cell. 1995 Jun;7(6):773-83.

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. Journal of computational biology. 2012 May 1;19(5):455-77.

Bécard G, Fortin JA. Early events of vesicular–arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New phytologist. 1988 Feb;108(2):211-8.

Bécard G, Pfeffer PE. Status of nuclear division in arbuscular mycorrhizal fungi during in vitro development. Protoplasma. 1993 Mar;174:62-8.

Bécard, G., Taylor, L.P., Douds, D.D., Pfeffer, P.E., Doner, L.W., 1995. Flavonoids are not necessary plant signal compounds in arbuscular mycorrhizal symbioses. MPMI-Mol. Plant Microbe Interact. 8 (2), 252258.

Becquer, A., Garcia, K., Amenc, L., Rivard, C., Doré, J., Trives-Segura, C., et al., 2018. The Hebeloma cylindrosporum HcPT2 Pi transporter plays a key role in ectomycorrhizal symbiosis. New Phytol. 220 (4), 11851199.

Belmondo, S., Fiorilli, V., Pérez-Tienda, J., Ferrol, N., Marmeisse, R., Lanfranco, L., 2014. A dipeptide transporter from the arbuscular mycorrhizal fungus Rhizophagus irregularis is upregulated in the intraradical phase. Front Plant Sci. 5, 436.

Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal statistical society: series B (Methodological). 1995 Jan;57(1):289-300.

Berger F, Gutjahr C. Factors affecting plant responsiveness to arbuscular mycorrhiza. Current Opinion in Plant Biology. 2021 Feb 1;59:101994.

Berruti, A., Borriello, R., Lumini, E., Scariot, V., Bianciotto, V., Balestrini, R., 2013. Application of laser microdissection to identify the mycorrhizal fungi that establish arbuscules inside root cells. Front. Plant Sci. 4, 135.

Besserer, A., Puech-Pagès, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A., Roy, S., et al., 2006. Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. PLoS Biol. 4 (7), e226.

Besserer, A., Bécard, G., Jauneau, A., Roux, C., Séjalon-Delmas, N., 2008. GR24, a synthetic analog of strigolactones, stimulates the mitosis and growth of the arbuscular mycorrhizal fungus Gigaspora rosea by boosting its energy metabolism. Plant Physiol. 148 (1), 402413.

Bever JD, Schultz PA, Pringle A, Morton JB. Arbuscular mycorrhizal fungi: more diverse than meets the eye, and the ecological tale of why: the high diversity of ecologically distinct species of arbuscular mycorrhizal fungi within a single community has broad implications for plant ecology. Bioscience. 2001 Nov 1;51(11):923-31.

Bever JD, Wang M. Hyphal fusion and multigenomic structure. Nature. 2005 Jan;433(7022):E3-4.

Bever JD, Kang HJ, Kaonongbua W, Wang M. Genomic organization and mechanisms of inheritance in arbuscular mycorrhizal fungi: contrasting the evidence and implications of current theories. In Mycorrhiza 2008 (pp. 135-148). Springer, Berlin, Heidelberg.

Bianciotto V, Bonfante P. Evidence of DNA replication in an arbuscular mycorrhizal fungus in the absence of the host plant. Protoplasma. 1993 Sep;176(3-4):100-5.

Bianciotto, V., Bandi, C., Minerdi, D., Sironi, M., Tichy, H.V., Bonfante, P., 1996. An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. Appl. Environ. Microbiol. 62 (8), 30053010.

Birky Jr CW. Heterozygosity, heteromorphy, and phylogenetic trees in asexual eukaryotes. Genetics. 1996 Sep 1;144(1):427-37.

Blakeslee AF. Sexual reproduction in the Mucorineae. In Proceedings of the American Academy of Arts and Sciences 1904 Aug 1 (Vol. 40, No. 4, pp. 205-319). American Academy of Arts & Sciences.

Bonfante, P., Anca, I.A., 2009. Plants, mycorrhizal fungi, and bacteria: a network of interactions. Annu. Rev. Microbiol. 63, 363383.

Bonfante, P., Desirò, A., 2017. Who lives in a fungus? The diversity, origins and functions of fungal endobacteria living in Mucoromycota. ISME J. 11 (8), 1727.

Bonfante P, Venice F. Mucoromycota: going to the roots of plant-interacting fungi. Fungal Biology Reviews. 2020 Jun 1;34(2):100-13.

Boon E, Halary S, Bapteste E, Hijri M. Studying genome heterogeneity within the arbuscular mycorrhizal fungal cytoplasm. Genome Biology and Evolution. 2015 Feb 1;7(2):505-21.

Bowen NJ, Jordan IK. Transposable elements and the evolution of eukaryotic complexity. Current issues in molecular biology. 2002 Jul;4(3):65-76.

Bozsoki, Z., Cheng, J., Feng, F., Gysel, K., Vinther, M., Andersen, K.R., et al., 2017. Receptor-mediated chitin perception in legume roots is functionally separable from Nod factor perception. Proc. Natl. Acad. Sci. 114 (38), E8118E8127.

Bravo, A., York, T., Pumplin, N., Mueller, L.A., Harrison, M.J., 2016. Genes conserved for arbuscular mycorrhizal symbiosis identified through phylogenomics. Nat. Plants 2 (2), 15208.

Bravo, A., Brands, M., Wewer, V., Dörmann, P., Harrison, M.J., 2017. Arbuscular mycorrhiza-specific enzymes FatM and RAM 2 fine-tune lipid biosynthesis to promote development of arbuscular mycorrhiza. New Phytol. 214 (4), 16311645.

Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq quantification. Nature biotechnology. 2016 May;34(5):525-7.

Breuillin-Sessoms, F., Floss, D.S., Gomez, S.K., Pumplin, N., Ding, Y., Levesque-Tremblay, V., et al., 2015. Suppression of arbuscule degeneration in Medicago truncatula phosphate transporter4 mutants is dependent on the ammonium transporter 2 family protein AMT2; 3. Plant Cell 27 (4), 13521366.

Browning SR, Browning BL. Haplotype phasing: existing methods and new developments. Nature Reviews Genetics. 2011 Oct;12(10):703-14.

Brundrett, M.C., Tedersoo, L., 2018. Evolutionary history of mycorrhizal symbioses and global host plant diversity. New Phytol. 220 (4), 11081115.

Bruns TD, Corradi N, Redecker D, Taylor JW, Öpik M. Glomeromycotina: what is a species and why should we care?. New Phytologist. 2018 Dec;220(4):963-7.

Burgeff, H., Über Sexualität, Variabilität, und Vererbung bei Phyconyces nitens. Ber. Deutsch. Bot. Gesells. 30:679-685. I9I3.

Burleigh SH, Harrison MJ. A cDNA from the arbuscular mycorrhizal fungus Glomus versiforme with homology to a cruciform DNA-binding protein from Ustilago maydis. Mycorrhiza. 1998 May;7:301-6.

Calabrese, S., Pérez-Tienda, J., Ellerbeck, M., Arnould, C., Chatagnier, O., Boller, T., et al., 2016. GintAMT3a low-affinity ammonium transporter of the Arbuscular mycorrhizal Rhizophagus Irregularis. Front. Plant Sci. 7, 679.

Cappellazzo, G., Lanfranco, L., Fitz, M., Wipf, D., Bonfante, P., 2008. Characterization of an amino acid permease from the endomycorrhizal fungus Glomus mosseae. Plant Physiol. 147 (1), 429437.

Casselton LA, Olesnicky NS. Molecular genetics of mating recognition in basidiomycete fungi. Microbiology and molecular biology reviews. 1998 Mar 1;62(1):55-70.

Caten CE, Jinks JL. Heterokaryosis: its significance in wild homothallic ascomycetes and fungi imperfecti. Transactions of the British Mycological Society. 1966 Mar 1;49(1):81-93.

Ceballos I, Mateus ID, Peña R, Peña-Quemba DC, Robbins C, Ordoñez YM, Rosikiewicz P, Rojas EC, Thuita M, Mlay DP, Masso C. Using variation in arbuscular mycorrhizal fungi to drive the productivity of the food security crop cassava. BioRxiv. 2019 Nov 4:830547.

Chabot S, Bécard G, Piché Y. Life cycle of Glomus intraradix in root organ culture. Mycologia. 1992 May 1;84(3):315-21.

Chaharbakhshi, E., Jemc, J.C., 2016. Broad-complex, tramtrack, and bric-à-brac (BTB) proteins: critical regulators of development. Genesis 54 (10), 505518.

Chang SS, Zhang Z, Liu Y. RNA interference pathways in fungi: mechanisms and functions. Annual review of microbiology. 2012 Oct 13;66:305-23.

Chaturvedi A, Cruz Corella J, Robbins C, Loha A, Menin L, Gasilova N, Masclaux FG, Lee SJ, Sanders IR. The methylome of the model arbuscular mycorrhizal fungus, Rhizophagus irregularis, shares characteristics with early diverging fungi and Dikarya. Communications Biology. 2021 Jul 22;4(1):901.

Chen EC, Mathieu S, Hoffrichter A, Sedzielewska-Toro K, Peart M, Pelin A, Ndikumana S, Ropars J, Dreissig S, Fuchs J, Brachmann A. Single nucleus sequencing reveals evidence of inter-nucleus recombination in arbuscular mycorrhizal fungi. Elife. 2018 Dec 5;7:e39813.

Chen EC, Morin E, Beaudet D, Noel J, Yildirir G, Ndikumana S, Charron P, St-Onge C, Giorgi J, Krüger M, Marton T. High intraspecific genome diversity in the model arbuscular mycorrhizal symbiont Rhizophagus irregularis. New Phytologist. 2018 Dec;220(4):1161-71.

Chen EC, Mathieu S, Sedzielewska-Toro K, Peart M, Pelin A, Ndikumana S, Ropars J, Dreissig S, Fuchs J, Brachmann A, Corradi N. Correction: Single nucleus sequencing reveals evidence of inter-nucleus recombination in arbuscular mycorrhizal fungi. Elife. 2019 Mar 21;8:e46860.

Chen EC, Mathieu S, Hoffrichter A, Ropars J, Dreissig S, Fuchs J, Brachmann A, Corradi N. More filtering on SNP calling does not remove evidence of inter-nucleus recombination in dikaryotic arbuscular mycorrhizal fungi. Frontiers in Plant Science. 2020 Jul 7;11:912.

Colard, A., Angelard, C., Sanders, IR., 2011. Genetic exchange in an arbuscular mycorrhizal fungus results in increased rice growth and altered mycorrhiza-specific gene transcription. Appl. Environ. Microbiol. 77 (18), 65106515.

Cornell C, Kokkoris V, Turcu B, Dettman J, Stefani F, Corradi N. The arbuscular mycorrhizal fungus Rhizophagus irregularis harmonizes nuclear dynamics in the presence of distinct abiotic factors. Fungal Genetics and Biology. 2022 Jan 1;158:103639.

Corradi N, Brachmann A. Fungal mating in the most widespread plant symbionts?. Trends in Plant Science. 2017 Feb 1;22(2):175-83.

Croll D, Wille L, Gamper HA, Mathimaran N, Lammers PJ, Corradi N, Sanders IR. Genetic diversity and host plant preferences revealed by simple sequence repeat and mitochondrial markers in a population of the arbuscular mycorrhizal fungus Glomus intraradices. New Phytologist. 2008 May;178(3):672-87.

Croll D, Giovannetti M, Koch AM, Sbrana C, Ehinger M, Lammers PJ, Sanders IR. Nonself vegetative fusion and genetic exchange in the arbuscular mycorrhizal fungus Glomus intraradices. New Phytologist. 2009 Mar;181(4):924-37.

Croll D, Sanders IR. Recombination in Glomus intraradices, a supposed ancient asexual arbuscular mycorrhizal fungus. BMC Evolutionary Biology. 2009 Dec;9(1):1-1.

Croll, D., Sanders, IR., 2009. Recombination in Glomus intraradices, a supposed ancient asexual arbuscular mycorrhizal fungus. BMC Evolut. Biol. 9 (1), 13.

Croll D, Lendenmann MH, Stewart E, McDonald BA. The impact of recombination hotspots on genome evolution of a fungal plant pathogen. Genetics. 2015 Nov 1;201(3):1213-28.

Czaja, L.F., Hogekamp, C., Lamm, P., Maillet, F., Martinez, E.A., Samain, E., et al., 2012. Transcriptional responses toward diffusible signals from symbiotic microbes reveal MtNFP-and MtDMI3-dependent reprogramming of host gene expression by arbuscular mycorrhizal fungal lipochitooligosaccharides. Plant Physiol. 159 (4), 16711685.

Daboussi MJ. Fungal transposable elements and genome evolution. Genetica. 1997 Mar;100:253-60.

Dallaire A, Manley BF, Wilkens M, Bista I, Quan C, Evangelisti E, Bradshaw CR, Ramakrishna NB, Schornack S, Butter F, Paszkowski U. Transcriptional activity and epigenetic regulation of transposable elements in the symbiotic fungus Rhizophagus irregularis. Genome Research. 2021 Dec 1;31(12):2290-302.

Dallaire A, Paszkowski U. Genomes of Arbuscular Mycorrhizal Fungi. In Plant Relationships: Fungal-Plant Interactions 2022 Dec 8 (pp. 67-81). Cham: Springer International Publishing.

Darwin C. On the origin of species, 1859. Routledge; 2004 Jun 1.

Daubois L, Beaudet D, Hijri M, de la Providencia I. Independent mitochondrial and nuclear exchanges arising in Rhizophagus irregularis crossed-isolates support the presence of a mitochondrial segregation mechanism. BmC microbiology. 2016 Dec;16(1):1-2.

de Bekker C, van Veluw GJ, Vinck A, Wiebenga LA, Wösten HA. Heterogeneity of Aspergillus niger microcolonies in liquid shaken cultures. Applied and environmental microbiology. 2011 Feb 15;77(4):1263-7.

De Jonge R, Bolton MD, Kombrink A, van den Berg GC, Yadeta KA, Thomma BP. Extensive chromosomal reshuffling drives evolution of virulence in an asexual pathogen. Genome research. 2013 Aug 1;23(8):1271-82.

De La Providencia IE, De Souza FA, Fernández F, Delmas NS, Declerck S. Arbuscular mycorrhizal fungi reveal distinct patterns of anastomosis formation and hyphal healing mechanisms between different phylogenic groups. New Phytologist. 2005 Jan;165(1):261-71.

De la Providencia IE, Nadimi M, Beaudet D, Rodriguez Morales G, Hijri M. Detection of a transient mitochondrial DNA heteroplasmy in the progeny of crossed genetically divergent isolates of arbuscular mycorrhizal fungi. New Phytologist. 2013 Oct;200(1):211-21.

De Mita, S., Streng, A., Bisseling, T., Geurts, R., 2014. Evolution of a symbiotic receptor through gene duplications in the legumerhizobium mutualism. New Phytol. 201 (3), 961972.

Debortoli, N., Li, X., Eyres, I., Fontaneto, D., Hespeels, B., Tang, C.Q., et al., 2016. Genetic exchange among bdelloid rotifers is more likely due to horizontal gene transfer than to meiotic sex. Curr. Biol. 26 (6), 723732.

Delaux, P.M., Varala, K., Edger, P.P., Coruzzi, G.M., Pires, J.C., Ané, J.M., 2014. Comparative phylogenomics uncovers the impact of symbiotic associations on host genome evolution. PLoS Genet. 10 (7), e1004487.

Den Bakker, H.C., VanKuren, N.W., Morton, J.B., Pawlowska, T.E., 2010. Clonality and recombination in the life history of an asexual arbuscular mycorrhizal fungus. Mol. Biol. Evol. 27 (11), 24742486.

Desirò, A., Hao, Z., Liber, J.A., Benucci, G.M.N., Lowry, D., Roberson, R., et al., 2018. Mycoplasma-related endobacteria within Mortierellomycotina fungi: diversity, distribution and functional insights into their lifestyle. ISME J. 12 (7), 17431757.

Dickman MB, Yarden O. Serine/threonine protein kinases and phosphatases in filamentious fungi. Fungal Genetics and Biology. 1999 Mar 1;26(2):99-117.

Dong S, Raffaele S, Kamoun S. The two-speed genomes of filamentous pathogens: waltz with plants. Current opinion in genetics & development. 2015 Dec 1;35:57-65.

Dreyer, I., Spitz, O., Kanonenberg, K., Montag, K., Handrich, M.R., Ahmad, S., et al., 2019. Nutrient exchange in arbuscular mycorrhizal symbiosis from a thermodynamic point of view. New Phytol. 222 (2), 10431053.

Dutcher SK. Internuclear transfer of genetic information in kar1-1/KAR1 heterokaryons in Saccharomyces cerevisiae. Molecular and cellular biology. 1981 Mar;1(3):245-53.

Ehinger MO, Croll D, Koch AM, Sanders IR. Significant genetic and phenotypic changes arising from clonal growth of a single spore of an arbuscular mycorrhizal fungus over multiple generations. New Phytologist. 2012 Nov;196(3):853-61.

Eickbush TH, Eickbush DG. Finely orchestrated movements: evolution of the ribosomal RNA genes. Genetics. 2007 Feb 1;175(2):477-85.

El-Sayed AS, George NM, Yassin MA, Alaidaroos BA, Bolbol AA, Mohamed MS, Rady AM, Aziz SW, Zayed RA, Sitohy MZ. Purification and characterization of ornithine decarboxylase from Aspergillus terreus; Kinetics of inhibition by various inhibitors. Molecules. 2019 Jul 29;24(15):2756.

Eo JK, Eom AH. Differential growth response of various crop species to arbuscular mycorrhizal inoculation. Mycobiology. 2009 Mar 1;37(1):72-6.

Eshghi Sahraei S, Sánchez-García M, Montoliu-Nerin M, Manyara D, Bergin C, Rosendahl S, Rosling A. Bringing uncultured AM fungi to the genomic era–a case study with field spores of Funneliformis geosporum.

Eyres I, Boschetti C, Crisp A, Smith TP, Fontaneto D, Tunnacliffe A, Barraclough TG. Horizontal gene transfer in bdelloid rotifers is ancient, ongoing and more frequent in species from desiccating habitats. BMC biology. 2015 Dec;13(1):1-7.

Ezawa, T., Smith, S.E., Smith, F.A., 2002. P metabolism and transport in AM fungi. Plant Soil 244 (12), 221230.

Ezawa, T., Saito, K., 2018. How do arbuscular mycorrhizal fungi handle phosphate? New insight into fine-tuning of phosphate metabolism. New Phytol. 220 (4), 11161121.

Fauchery L, Uroz S, Buée M, Kohler A. Purification of fungal high molecular weight genomic DNA from environmental samples. In Fungal Genomics 2018 (pp. 21-35). Humana Press, New York, NY.

Fellbaum, C.R., Mensah, J.A., Cloos, A.J., Strahan, G.E., Pfeffer, P.E., Kiers, E.T., et al., 2014. Fungal nutrient allocation in common mycorrhizal networks is regulated by the carbon source strength of individual host plants. New Phytol. 203 (2), 646656.

Ferrol, N., Barea, J.M., Azcón-Aguilar, C., 2000. The plasma membrane H1-ATPase gene family in the arbuscular mycorrhizal fungus Glomus mosseae. Curr. Genet. 37 (2), 112118.

Ferrol, N., Azcón-Aguilar, C., Pérez-Tienda, J., 2019. Arbuscular mycorrhizas as key players in sustainable plant phosphorus acquisition: an overview on the mechanisms involved. Plant Sci. 280, 441447.

Fesel, P.H., Zuccaro, A., 2016. β-Glucan: crucial component of the fungal cell wall and elusive MAMP in plants. Fungal Genet. Biol. 90, 5360.

Fiorilli, V., Lanfranco, L., Bonfante, P., 2013. The expression of GintPT, the phosphate transporter of Rhizophagus irregularis, depends on the symbiotic status and phosphate availability. Planta 237 (5), 12671277.

Floss, D.S., Gomez, S.K., Park, H.J., MacLean, A.M., Müller, L.M., Bhattarai, K.K., et al., 2017. A transcriptional program for arbuscule degeneration during AM symbiosis is regulated by MYB1. Curr. Biol. 27 (8), 12061212.

Flot JF, Hespeels B, Li X, Noel B, Arkhipova I, Danchin EG, Hejnol A, Henrissat B, Koszul R, Aury JM, Barbe V. Genomic evidence for ameiotic evolution in the bdelloid rotifer Adineta vaga. Nature. 2013 Aug 22;500(7463):453-7.

Formey, D., Molès, M., Haouy, A., Savelli, B., Bouchez, O., Bécard, G., et al., 2012. Comparative analysis of mitochondrial genomes of Rhizophagus irregularis syn. Glomus irregulare reveals a polymorphism induced by variability generating elements. New Phytol. 196 (4), 12171227.

Fraser JA, Hsueh YP, Findley KM, Heitman J. Evolution of the mating-type locus: the basidiomycetes. Sex in fungi: Molecular determination and evolutionary implications. 2007 Aug 27:19-34.

Frey-Klett, P., Garbaye, J.A., Tarkka, M., 2007. The mycorrhiza helper bacteria revisited. New Phytol. 176 (1), 2236.

Friesen TL, Stukenbrock EH, Liu Z, Meinhardt S, Ling H, Faris JD, Rasmussen JB, Solomon PS, McDonald BA, Oliver RP. Emergence of a new disease as a result of interspecific virulence gene transfer. Nature genetics. 2006 Aug 1;38(8):953-6.

Galván GA, Kuyper TW, Burger K, Keizer LP, Hoekstra RF, Kik C, Scholten OE. Genetic analysis of the interaction between Allium species and arbuscular mycorrhizal fungi. Theoretical and applied genetics. 2011 Mar;122:947-60.

Garcia, K., Doidy, J., Zimmermann, S.D., Wipf, D., Courty, P.E., 2016. Take a trip through the plant and fungal transportome of mycorrhiza. Trends Plant Sci. 21 (11), 937950.

García-Garrido JM, Ocampo JA. Regulation of the plant defence response in arbuscular mycorrhizal symbiosis. Journal of experimental Botany. 2002 Jun 1;53(373):1377-86.

Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT. Genome sequence of the human malaria parasite Plasmodium falciparum. Nature. 2002 Oct 3;419(6906):498-511.

Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing. arXiv preprint arXiv:1207.3907. 2012 Jul 17.

Gaulin, E., Pel, M.J., Camborde, L., San-Clemente, H., Courbier, S., Dupouy, M.A., et al., 2018. Genomics analysis of Aphanomyces spp. identifies a new class of oomycete effector associated with host adaptation. BMC Biol. 16 (1), 43.

Gehrmann T, Pelkmans JF, Ohm RA, Vos AM, Sonnenberg AS, Baars JJ, Wösten HA, Reinders MJ, Abeel T. Nucleus-specific expression in the multinuclear mushroom-forming fungus Agaricus bisporus reveals different nuclear regulatory programs. Proceedings of the National Academy of Sciences. 2018 Apr 24;115(17):4429-34.

Genre, A., Chabaud, M., Balzergue, C., Puech-Pagès, V., Novero, M., Rey, T., et al., 2013. Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca21 spiking in Medicago truncatula roots and their production is enhanced by strigolactone. New Phytol. 198 (1), 190202.

Genre, A., Ivanov, S., Fendrych, M., Faccio, A., Žárský, V., Bisseling, T., et al., 2011. Multiple exocytotic markers accumulate at the sites of perifungal membrane biogenesis in arbuscular mycorrhizas. Plant Cell Physiol. 53 (1), 244255.

Gibbons, J.G., Branco, A.T., Godinho, S.A., Yu, S., Lemos, B., 2015. Concerted copy number variation balances ribosomal DNA dosage in human and mouse genomes. Proc. Natl. Acad. Sci. 112 (8), 24852490.

Giovannetti M, Azzolini D, Citernesi AS. Anastomosis formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi. Applied and environmental microbiology. 1999 Dec 1;65(12):5571-5.

Giovannetti M, Fortuna P, Citernesi AS, Morini S, Nuti MP. The occurrence of anastomosis formation and nuclear exchange in intact arbuscular mycorrhizal networks. New Phytologist. 2001 Sep;151(3):717-24.

Giovannetti, M., Avio, L., Sbrana, C., 2015. Functional significance of anastomosis in arbuscular mycorrhizal networks. Mycorrhizal Netw. Springer, Dordrecht, pp. 4167.

Gladyshev, E.A., Meselson, M., Arkhipova, IR., 2008. Massive horizontal gene transfer in bdelloid rotifers. Science 320 (5880), 12101213.

Goddard MR, Godfray HC, Burt A. Sex increases the efficacy of natural selection in experimental yeast populations. Nature. 2005 Mar 31;434(7033):636-40.

Goddard MR. Sex accelerates adaptation. Nature. 2016 Mar 10;531(7593):176-7.

Gough, C., Cottret, L., Lefebvre, B., Bono, J.J., 2018. Evolutionary history of plant LysM receptor proteins related to root endosymbiosis. Front. Plant Sci. 9, 923.

Govindarajulu, M., Pfeffer, P.E., Jin, H., Abubaker, J., Douds, D.D., Allen, J.W., et al., 2005. Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435 (7043), 819.

Grum-Grzhimaylo AA, Bastiaans E, van den Heuvel J, Berenguer Millanes C , Debets AJM, Aanen DK. Somatic deficiency causes reproductive parasitism in a fungus. Nat Commun 202;12(1):783. doi: 10.1038/s41467-021-21050-5.

Guan D, McCarthy SA, Wood J, Howe K, Wang Y, Durbin R. Identifying and removing haplotypic duplication in primary genome assemblies. Bioinformatics. 2020 May 1;36(9):2896-8.

Gutjahr, C., Parniske, M., 2013. Cell and developmental biology of arbuscular mycorrhiza symbiosis. Annu. Rev. Cell Develop. Biol. 29, 593617.

Haas, B.J., Kamoun, S., Zody, M.C., Jiang, R.H., Handsaker, R.E., Cano, L.M., et al., 2009. Genome sequence and analysis of the Irish potato famine pathogen Phytophthora infestans. Nature 461 (7262), 393.

Halary S, Daubois L, Terrat Y, Ellenberger S, Wöstemeyer J, Hijri M. Mating type gene homologues and putative sex pheromone-sensing pathway in arbuscular mycorrhizal fungi, a presumably asexual plant root symbiont. pLoS One. 2013 Nov 19;8(11):e80729.

Halary S, Malik SB, Lildhar L, Slamovits CH, Hijri M, Corradi N. Conserved meiotic machinery in Glomus spp., a putatively ancient asexual fungal lineage. Genome biology and evolution. 2011 Jan 1;3:950-8.

Hane JK, Anderson JP, Williams AH, Sperschneider J, Singh KB. Genome sequencing and comparative genomics of the broad host-range pathogen Rhizoctonia solani AG8. PloS genetics. 2014 May 8;10(5):e1004281.

Harrison, M.J., van Buuren, M.L., 1995. A phosphate transporter from the mycorrhizal fungus Glomus versiforme. Nature 378 (6557), 626.

Heitman J. Sexual reproduction and the evolution of microbial pathogens. Current Biology. 2006 Sep 5;16(17):R711-25.

Helber, N., Requena, N., 2008. Expression of the fluorescence markers DsRed and GFP fused to a nuclear localization signal in the arbuscular mycorrhizal fungus Glomus intraradices. New Phytol. 177 (2), 537548.

Helber, N., Wippel, K., Sauer, N., Schaarschmidt, S., Hause, B., Requena, N., 2011. A versatile monosaccharide transporter that operates in the arbuscular mycorrhizal fungus Glomus sp. is crucial for the symbiotic relationship with plants. Plant Cell 23 (10), 38123823.

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JP. Ploughing up the wood-wide web?. Nature. 1998 Jul;394(6692):431-.

Herivaux, A., De Bernonville, T.D., Roux, C., Clastre, M., Courdavault, V., Gastebois, A., et al., 2017. The identification of phytohormone receptor homologs in early diverging fungi suggests a role for plant sensing in land colonization by fungi. MBio 8 (1), e01739-16.

Hibbett DS, Blackwell M, James TY, Spatafora JW, Taylor JW, Vilgalys R. Phylogenetic taxon definitions for fungi, dikarya, ascomycota and basidiomycota. IMA fungus. 2018 Dec;9:291-8.

Hijri M, Sanders IR. The arbuscular mycorrhizal fungus Glomus intraradices is haploid and has a small genome size in the lower limit of eukaryotes. Fungal Genetics and Biology. 2004 Feb 1;41(2):253-61.

Hijri, M., Sanders, IR., 2005. Low gene copy number shows that arbuscular mycorrhizal fungi inherit genetically different nuclei. Nature 433 (7022), 160.

Hildebrandt, U., Janetta, K., Bothe, H., 2002. Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Appl. Environ. Microbiol. 68 (4), 19191924.

Hildebrandt, U., Ouziad, F., Marner, F.J., Bothe, H., 2006. The bacterium Paenibacillus validus stimulates growth of the arbuscular mycorrhizal fungus Glomus intraradices up to the formation of fertile spores. FEMS Microbiol. Lett. 254 (2), 258267.

Hiltunen M, Grudzinska-Sterno M, Wallerman O, Ryberg M, Johannesson H. Maintenance of high genome integrity over vegetative growth in the fairy-ring mushroom Marasmius oreades. Current Biology. 2019 Aug 19;29(16):2758-65.

Hoagland DR, Arnon DI. The water-culture method for growing plants without soil. Circular. California agricultural experiment station. 1950;347(2nd edit).

Hogenhout, S.A., Van der Hoorn, R.A., Terauchi, R., Kamoun, S., 2009. Emerging concepts in effector biology of plant-associated organisms. Mol. Plant-Microbe Interact. 22 (2), 115122.

Hosny M, Gianinazzi-Pearson V, Dulieu H. Nuclear DNA content of 11 fungal species in Glomales. Genome. 1998 Jun 1;41(3):422-8.

Ivanov S, Austin J, Berg RH, Harrison MJ. Extensive membrane systems at the host–arbuscular mycorrhizal fungus interface. Nature Plants. 2019 Feb;5(2):194-203.

James TY, Stenlid J, Olson Å, Johannesson H. Evolutionary significance of imbalanced nuclear ratios within heterokaryons of the basidiomycete fungus Heterobasidion parviporum. Evolution. 2008 Sep 1;62(9):2279-96.

Jansa J, Mozafar A, Anken T, Ruh R, Sanders I, Frossard E. Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza. 2002 Oct;12(5):225-34.

Jansa J, Mozafar A, Banke S, McDonald BA, Frossard E. Intra-and intersporal diversity of ITS rDNA sequences in Glomus intraradices assessed by cloning and sequencing, and by SSCP analysis. Mycological Research. 2002 Jun;106(6):670-81.

Jany JL, Pawlowska TE. Multinucleate spores contribute to evolutionary longevity of asexual glomeromycota. The American Naturalist. 2010 Apr;175(4):424-35.

Jiang, Y., Wang, W., Xie, Q., Liu, N., Liu, L., Wang, D., et al., 2017. Plants transfer lipids to sustain colonization by mutualistic mycorrhizal and parasitic fungi. Science 356 (6343), 11721175.

Jin, H., Pfeffer, P.E., Douds, D.D., Piotrowski, E., Lammers, P.J., Shachar-Hill, Y., 2005. The uptake, metabolism, transport and transfer of nitrogen in an arbuscular mycorrhizal symbiosis. New Phytol. 168 (3), 687696.

Jinks JL. Heterokaryosis: a system of adaptation in wild fungi. Proceedings of the Royal Society of London. Series B-Biological Sciences. 1952 Aug 27;140(898):83-99.

Judson OP, Normark BB. Ancient asexuals: scandal or artifact? Reply. Trends in Ecology & Evolution. 1996 Jul 1;11(7):297-.

Judson, O.P., Normark, B.B., 1996. Ancient asexual scandals. Trends Ecol. Evol. 11 (2), 4146.

Kafle, A., Garcia, K., Wang, X., Pfeffer, P.E., Strahan, G.D., Bücking, H., 2019. Nutrient demand and fungal access to resources control the carbon allocation to the symbiotic partners in tripartite interactions of Medicago truncatula. Plant, Cell Environ. 42 (1), 270284.

Kamel L, Tang N, Malbreil M, San Clemente H, Le Marquer M, Roux C, Frei dit Frey N. The comparison of expressed candidate secreted proteins from two arbuscular mycorrhizal fungi unravels common and specific molecular tools to invade different host plants. Front. Plant Sci.; 8: 1– 18.

Kanno, Y., Oikawa, T., Chiba, Y., Ishimaru, Y., Shimizu, T., Sano, N., et al., 2016. AtSWEET13 and AtSWEET14 regulate gibberellin-mediated physiological processes. Nat. Commun. 7, 13245.

Kaya C, Ashraf M, Sonmez O, Aydemir S, Tuna AL, Cullu MA. The influence of arbuscular mycorrhizal colonisation on key growth parameters and fruit yield of pepper plants grown at high salinity. Scientia horticulturae. 2009 Jun 2;121(1):1-6.

Keeling PJ, Palmer JD. Horizontal gene transfer in eukaryotic evolution. Nature Reviews Genetics. 2008 Aug;9(8):605-18.

Kiers ET, Duhamel M, Beesetty Y, Mensah JA, Franken O, Verbruggen E, Fellbaum CR, Kowalchuk GA, Hart MM, Bago A, Palmer TM. Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. Science. 2011 Aug 12;333(6044):880-2.

Kikuchi, Y., Hijikata, N., Yokoyama, K., Ohtomo, R., Handa, Y., Kawaguchi, M., et al., 2014. Polyphosphate accumulation is driven by transcriptome alterations that lead to near-synchronous and near-equivalent uptake of inorganic cations in an arbuscular mycorrhizal fungus. New Phytol. 204 (3), 638649.

Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nature biotechnology. 2019 Aug;37(8):907-15.

Klironomos JN. Variation in plant response to native and exotic arbuscular mycorrhizal fungi. Ecology. 2003 Sep;84(9):2292-301.

Kloppholz, S., Kuhn, H., Requena, N., 2011. A secreted fungal effector of Glomus intraradices promotes symbiotic biotrophy. Curr. Biol. 21 (14), 12041209.

Kobae, Y., Tamura, Y., Takai, S., Banba, M., Hata, S., 2010. Localized expression of arbuscular mycorrhiza-inducible ammonium transporters in soybean. Plant Cell Physiol. 51 (9), 14111415.

Kobae, Y., Gutjahr, C., Paszkowski, U., Kojima, T., Fujiwara, T., Hata, S., 2014. Lipid droplets of arbuscular mycorrhizal fungi emerge in concert with arbuscule collapse. Plant Cell Physiol. 55 (11), 19451953.

Kobayashi Y, Maeda T, Yamaguchi K, Kameoka H, Tanaka S, Ezawa T, Shigenobu S, Kawaguchi M. The genome of Rhizophagus clarus HR1 reveals a common genetic basis for auxotrophy among arbuscular mycorrhizal fungi. BMC genomics. 2018 Dec;19(1):1-1.

Koch AM, Croll D, Sanders IR. Genetic variability in a population of arbuscular mycorrhizal fungi causes variation in plant growth. Ecol Lett. 2006 Feb;9(2):103-10.

Koch AM, Kuhn G, Fontanillas P, Fumagalli L, Goudet J, Sanders IR. High genetic variability and low local diversity in a population of arbuscular mycorrhizal fungi. Proceedings of the National Academy of Sciences. 2004 Feb 24;101(8):2369-74.

Koegel, S., Brulé, D., Wiemken, A., Boller, T., Courty, P.E., 2015. The effect of different nitrogen sources on the symbiotic interaction between Sorghum bicolor and Glomus intraradices: expression of plant and fungal genes involved in nitrogen assimilation. Soil Biol. Biochem. 86, 159163.

Kokkoris V, Hart M. In vitro propagation of arbuscular mycorrhizal fungi may drive fungal evolution. Frontiers in microbiology. 2019 Oct 22;10:2420.

Kokkoris V, Stefani F, Dalpé Y, Dettman J, Corradi N. Nuclear dynamics in the arbuscular mycorrhizal fungi. Trends in Plant Science. 2020 Aug 1;25(8):765-78.

Kokkoris V, Chagnon PL, Yildirir G, Clarke K, Goh D, MacLean AM, Dettman J, Stefani F, Corradi N. Host identity influences nuclear dynamics in arbuscular mycorrhizal fungi. Current Biology. 2021 Apr 12;31(7):1531-8.

Kolmogorov M, Yuan J, Lin Y, Pevzner PA. Assembly of long, error-prone reads using repeat graphs. Nature biotechnology. 2019 May;37(5):540-6.

Krajinski, F., Courty, P.E., Sieh, D., Franken, P., Zhang, H., Bucher, M., et al., 2014. The H1-ATPase HA1 of Medicago truncatula is essential for phosphate transport and plant growth during arbuscular mycorrhizal symbiosis. Plant Cell 26 (4), 18081817.

Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA. Circos: an information aesthetic for comparative genomics. Genome research. 2009 Sep 1;19(9):1639-45.

Kubota, M., McGonigle, T.P., Hyakumachi, M., 2005. Co-occurrence of Arum-and Paris-type morphologies of arbuscular mycorrhizae in cucumber and tomato. Mycorrhiza 15 (2), 7377.

Kuhn G, Hijri M, Sanders IR. Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. Nature. 2001 Dec 13;414(6865):745-8.

Lakovic M, Rillig MC. A nuclei-based conceptual model of (eco) evolutionary dynamics in fungal heterokaryons. Frontiers in Microbiology. 2022;13.

Lanfranco L, Delpero M, Bonfante P. Intrasporal variability of ribosomal sequences in the endomycorrhizal fungus Gigaspora margarita. Molecular Ecology. 1999 Jan 9;8(1):37-45.

Lee SC, Idnurm A. Fungal sex: the Mucoromycota. The Fungal Kingdom. 2017 Oct 1:177-91.

Leslie JF. Fungal vegetative compatibility. Annual review of phytopathology. 1993 Sep;31(1):127-50.

Levin AM, de Vries RP, Conesa A, de Bekker C, Talon M, Menke HH, van Peij NN, Wösten HA. Spatial differentiation in the vegetative mycelium of Aspergillus niger. Eukaryotic cell. 2007 Dec;6(12):2311-22.

Levitz SM, Specht CA. The molecular basis for the immunogenicity of Cryptococcus neoformans mannoproteins. FEMS yeast research. 2006 Jun 1;6(4):513-24.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. The sequence alignment/map format and SAMtools. Bioinformatics. 2009 Aug 15;25(16):2078-9.

Li M, Zhao J, Tang N, Sun H, Huang J. Horizontal gene transfer from bacteria and plants to the arbuscular mycorrhizal fungus Rhizophagus irregularis. Frontiers in Plant Science. 2018 May 25;9:701.

Lilja T, Eklöf D, Jaenson TG, Lindström A, Terenius O. Single nucleotide polymorphism analysis of the ITS2 region of two sympatric malaria mosquito species in Sweden: Anopheles daciae and Anopheles messeae. Medical and Veterinary Entomology. 2020 Sep;34(3):364-8.

Limpens, E., 2019. Extracellular membranes in symbiosis. Nat.Plants 5 (2), 131.

Limpens, E., Geurts, R., 2014. Plant-driven genome selection of arbuscular mycorrhizal fungi. Mol. Plant Pathol. 15 (6), 531534.

Limpens, E., Geurts, R., 2018. Transcriptional regulation of nutrient exchange in arbuscular mycorrhizal symbiosis. Mol. Plant 11 (12), 14211423.

Lin K, Limpens E, Zhang Z, Ivanov S, Saunders DG, Mu D, Pang E, Cao H, Cha H, Lin T, Zhou Q. Single nucleus genome sequencing reveals high similarity among nuclei of an endomycorrhizal fungus. PloS genetics. 2014 Jan 9;10(1):e1004078.

Liu, T., Ye, W., Ru, Y., Yang, X., Gu, B., Tao, K., et al., 2011. Two host cytoplasmic effectors are required for pathogenesis of Phytophthora sojae by suppression of host defenses. Plant Physiol. 155, 490501.

Liu T, Li H, Ding Y, Qi Y, Gao Y, Song A, Shen J, Qiu L. Genome-wide gene expression patterns in dikaryon of the basidiomycete fungus Pleurotus ostreatus. brazilian journal of microbiology. 2017 Apr;48:380-90.

Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., et al., 2015. Fungal effectors and plant susceptibility. Annu. Rev. Plant Biol. 66, 513545.

López-Pedrosa, A., González-Guerrero, M., Valderas, A., Azcón-Aguilar, C., Ferrol, N., 2006. GintAMT1 encodes a functional high-affinity ammonium transporter that is expressed in the extraradical mycelium of Glomus intraradices. Fungal Genet. Biol. 43 (2), 102110.

Luginbuehl LH, Menard GN, Kurup S, Van Erp H, Radhakrishnan GV, Breakspear A, Oldroyd GE, Eastmond PJ. Fatty acids in arbuscular mycorrhizal fungi are synthesized by the host plant. Science. 2017 Jun 16;356(6343):1175-8.

Luginbuehl LH, Oldroyd GE. Understanding the Arbuscule at the Heart of Endomycorrhizal Symbioses in Plants. Curr Biol. 2017;27(17):R952-R963. doi: 10.1016/j.cub.2017.06.042.

Lumini, E., Bianciotto, V., Jargeat, P., Novero, M., Salvioli, A., Faccio, A., et al., 2007. Presymbiotic growth and sporal morphology are affected in the arbuscular mycorrhizal fungus Gigaspora margarita cured of its endobacteria. Cell. Microbiol. 9 (7), 17161729.

Ma LJ, Van Der Does HC, Borkovich KA, Coleman JJ, Daboussi MJ, Di Pietro A, Dufresne M, Freitag M, Grabherr M, Henrissat B, Houterman PM. Comparative genomics reveals mobile pathogenicity chromosomes in Fusarium. Nature. 2010 Mar 18;464(7287):367-73.

Maeda T, Kobayashi Y, Kameoka H, Okuma N, Takeda N, Yamaguchi K, Bino T, Shigenobu S, Kawaguchi M. Evidence of non-tandemly repeated rDNAs and their intragenomic heterogeneity in Rhizophagus irregularis. Communications biology. 2018 Jul 10;1(1):1-3.

Maeda T, Kobayashi Y, Nakagawa T, Ezawa T, Yamaguchi K, Bino T, Nishimoto Y, Shigenobu S, Kawaguchi M. Conservation and host-specific expression of non-tandemly repeated heterogenous ribosome RNA gene in arbuscular mycorrhizal fungi. bioRxiv. 2020 May 15:2020-05.

Maillet, F., Poinsot, V., André, O., Puech-Pagès, V., Haouy, A., Gueunier, M., et al., 2011. Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature 469 (7328), 58.

Malar C M, Roux C, Corradi N. Regulation of mating genes during arbuscular mycorrhizal isolate co-existence—where is the evidence?. The ISME Journal. 2021 Aug;15(8):2173-9.

Malarkey CS, Churchill ME. The high mobility group box: the ultimate utility player of a cell. Trends in biochemical sciences. 2012 Dec 1;37(12):553-62.

Maldonado-Mendoza, I.E., Dewbre, G.R., Harrison, M.J., 2001. A phosphate transporter gene from the extra-radical mycelium of an arbuscular mycorrhizal fungus Glomus intraradices is regulated in response to phosphate in the environment. Mol. Plant-Microbe Interact. 14 (10), 11401148.

Manck-Götzenberger, J., Requena, N., 2016. Arbuscular mycorrhiza symbiosis induces a major transcriptional reprogramming of the potato SWEET sugar transporter family. Front. Plant Sci. 7, 487.

Manley BF, Lotharukpong JS, Barrera-Redondo J, Llewellyn T, Yildirir G, Sperschneider J, Corradi N, Paszkowski U, Miska EA, Dallaire A. A highly contiguous genome assembly reveals sources of genomic novelty in the symbiotic fungus Rhizophagus irregularis. G3: Genes, Genomes, Genetics. 2023 Jun;13(6):jkad077.

Marchini J, Cutler D, Patterson N, Stephens M, Eskin E, Halperin E, Lin S, Qin ZS, Munro HM, Abecasis GR, Donnelly P. A comparison of phasing algorithms for trios and unrelated individuals. The American Journal of Human Genetics. 2006 Mar 1;78(3):437-50.

Marleau J, Dalpé Y, St-Arnaud M, Hijri M. Spore development and nuclear inheritance in arbuscular mycorrhizal fungi. BMC evolutionary biology. 2011 Dec;11(1):1-1.

Martin F, Aerts A, Ahrén D, Brun A, Danchin EG, Duchaussoy F, Gibon J, Kohler A, Lindquist E, Pereda V, Salamov A. The genome of Laccaria bicolor provides insights into mycorrhizal symbiosis. Nature. 2008 Mar;452(7183):88-92.

Martin F, Gianinazzi-Pearson V, Hijri M, Lammers P, Requena N, Sanders IR, Shachar-Hill Y, Shapiro H, Tuskan GA, Young JP. The long hard road to a completed Glomus intraradices genome. New Phytologist. 2008 Dec 1:747-50.

Martin FM, Uroz S, Barker DG. Ancestral alliances: plant mutualistic symbioses with fungi and bacteria. Science. 2017 May 26;356(6340):eaad4501.

Martínez-García LB, Pugnaire FI. Arbuscular mycorrhizal fungi host preference and site effects in two plant species in a semiarid environment. Applied soil ecology. 2011 Jul 1;48(3):313-7.

Masclaux FG, Wyss T, Mateus-Gonzalez ID, Aletti C, Sanders IR. Variation in allele frequencies at the bg112 locus reveals unequal inheritance of nuclei in a dikaryotic isolate of the fungus Rhizophagus irregularis. Mycorrhiza. 2018 May;28(4):369-377.

Masclaux FG, Wyss T, Pagni M, Rosikiewicz P, Sanders IR. Investigating unexplained genetic variation and its expression in the arbuscular mycorrhizal fungus Rhizophagus irregularis: A comparison of whole genome and RAD sequencing data. PloS one. 2019 Dec 27;14(12):e0226497.

Mateus ID, Masclaux FG, Aletti C, Rojas EC, Savary R, Dupuis C, Sanders IR. Dual RNA-seq reveals large-scale non-conserved genotype × genotype-specific genetic reprograming and molecular crosstalk in the mycorrhizal symbiosis. ISME J. 2019 May;13(5):1226-1238.

Mateus ID, Rojas EC, Savary R, Dupuis C, Masclaux FG, Aletti C, Sanders IR. Coexistence of genetically different Rhizophagus irregularis isolates induces genes involved in a putative fungal mating response. The ISME Journal. 2020 Oct;14(10):2381-94.

Mathieu S, Cusant L, Roux C, Corradi N. Arbuscular mycorrhizal fungi: intraspecific diversity and pangenomes. New Phytologist. 2018 Dec;220(4):1129-34.

McDonald MJ, Rice DP, Desai MM. Sex speeds adaptation by altering the dynamics of molecular evolution. Nature. 2016 Mar 10;531(7593):233-6.

McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GR, Thormann A, Flicek P, Cunningham F. The ensembl variant effect predictor. Genome biology. 2016 Dec;17(1):1-4.

Mela AP, Rico-Ramírez AM, Glass NL. Syncytia in fungi. Cells. 2020 Oct 8;9(10):2255.

Mensah JA, Koch AM, Antunes PM, Kiers ET, Hart M, Bücking H. High functional diversity within species of arbuscular mycorrhizal fungi is associated with differences in phosphate and nitrogen uptake and fungal phosphate metabolism. Mycorrhiza. 2015 Oct;25(7):533-46.

Metin B, Findley K, Heitman J. The mating type locus (MAT) and sexual reproduction of Cryptococcus heveanensis: insights into the evolution of sex and sex-determining chromosomal regions in fungi. PLoS Genetics. 2010 May 20;6(5):e1000961.

Meunier C, Darolti I, Reimegård J, Mank JE, Johannesson H. Nuclear-specific gene expression in heterokaryons of the filamentous ascomycete Neurospora tetrasperma. Proceedings of the Royal Society B. 2022 Aug 10;289(1980):20220971.

Mittl, P.R., Schneider-Brachert, W., 2007. Sel1-like repeat proteins in signal transduction. Cell. Signal. 19 (1), 2031.

Miyata, K., Kozaki, T., Kouzai, Y., Ozawa, K., Ishii, K., Asamizu, E., et al., 2014. The bifunctional plant receptor, OsCERK1, regulates both chitin-triggered immunity and arbuscular mycorrhizal symbiosis in rice. Plant Cell Physiol. 55 (11), 18641872.

Miyauchi S, Kiss E, Kuo A, Drula E, Kohler A, Sánchez-García M, Morin E, Andreopoulos B, Barry KW, Bonito G, Buée M. Large-scale genome sequencing of mycorrhizal fungi provides insights into the early evolution of symbiotic traits. Nature communications. 2020 Oct 12;11(1):1-7.

Montoliu-Nerin M, Sánchez-García M, Bergin C, Grabherr M, Ellis B, Kutschera VE, Kierczak M, Johannesson H, Rosling A. Building de novo reference genome assemblies of complex eukaryotic microorganisms from single nuclei. Scientific reports. 2020 Jan 28;10(1):1-0.

Morin E, Chen EC, Kuo A, Beaudet D, Grigoriev IV, Henrissat B, Roux C, Corradi N, Martin FM. Exploring genome diversity of 6 strains of the model arbuscular mycorrhizal symbiont Rhizophagus irregularis. InJOBIM 2018 Jul 3.

Morin, E., Miyauchi, S., San Clemente, H., Chen, E.C., Pelin, A., de la Providencia, I., et al., 2019. Comparative genomics of Rhizophagus irregularis, R. cerebriforme, R. diaphanus and Gigaspora rosea highlights specific genetic features in Glomeromycotina. New Phytol.

Morrow CA, Fraser JA. Sexual reproduction and dimorphism in the pathogenic basidiomycetes. FEMS yeast research. 2009 Mar 1;9(2):161-77.

Muller HJ. Some genetic aspects of sex. The American Naturalist. 1932 Mar 1;66(703):118-38.

Munkvold L, Kjøller R, Vestberg M, Rosendahl S, Jakobsen I. High functional diversity within species of arbuscular mycorrhizal fungi. New Phytologist. 2004 Nov;164(2):357-64.

Muzzey D, Sherlock G, Weissman JS. Extensive and coordinated control of allele-specific expression by both transcription and translation in Candida albicans. Genome research. 2014 Jun 1;24(6):963-73.

Nadal, M., Paszkowski, U., 2013. Polyphony in the rhizosphere: pre-symbiotic communication in arbuscular mycorrhizal symbiosis. Curr. Opin. Plant Biol. 16 (4), 473479.

Nadal, M., Sawers, R., Naseem, S., Bassin, B., Kulicke, C., Sharman, A., et al., 2017. An N-acetylglucosamine transporter required for arbuscular mycorrhizal symbioses in rice and maize. Nat. Plants 3, 17073.

Nagahashi, G., Douds Jr., D.D., 2011. The effects of hydroxy fatty acids on the hyphal branching of germinated spores of AM fungi. Fungal Biol. 115 (45), 351358.

Naito, M., Desirò, A., González, J.B., Tao, G., Morton, J.B., Bonfante, P., et al., 2017. 'Candidatus Moeniiplasma glomeromycotorum', an endobacterium of arbuscular mycorrhizal fungi. Int. J. System. Evolution. Microbiol. 67 (5), 11771184.

Naumann, M., Schüßler, A., Bonfante, P., 2010. The obligate endobacteria of arbuscular mycorrhizal fungi are ancient heritable components related to the Mollicutes. ISME J. 4 (7), 862.

Neafsey DE, Barker BM, Sharpton TJ, Stajich JE, Park DJ, Whiston E, Hung CY, McMahan C, White J, Sykes S, Heiman D. Population genomic sequencing of Coccidioides fungi reveals recent hybridization and transposon control. Genome research. 2010 Jul 1;20(7):938-46.

Nieuwenhuis BP, James TY. The frequency of sex in fungi. Philosophical Transactions of the Royal Society B: Biological Sciences. 2016 Oct 19;371(1706):20150540.

Novais CB, Pepe A, Siqueira JO, Giovannetti M, Sbrana C. Compatibility and incompatibility in hyphal anastomosis of arbuscular mycorrhizal fungi. Scientia Agricola. 2017 Sep;74:411-6.

Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., et al., 2010. HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen Blumeria graminis. Plant Cell 22 (9), 31303141.

Oldroyd, G.E., 2013. Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat. Rev. Microbiol. 11 (4), 252.

Olsson, S., Bonfante, P., Pawlowska, T.E., 2017. Ecology and evolution of fungal-bacterial interactions. In: Dighton, J., White, J.F. (Eds.), The Fungal Community: Its Organization and Role in the Ecosystem, fourth ed. Taylor & Francis, Boca Raton, FL, pp. 563583.

Öpik M, Zobel M, Cantero JJ, Davison J, Facelli JM, Hiiesalu I, Jairus T, Kalwij JM, Koorem K, Leal ME, Liira J. Global sampling of plant roots expands the described molecular diversity of arbuscular mycorrhizal fungi. Mycorrhiza. 2013 Jul;23:411-30.

Öpik, M., Davison, J., 2016. Uniting species- and community-oriented approaches to understand arbuscular mycorrhizal fungal diversity. Fungal Ecol. 24, 106113.

Orchard, S., Standish, R.J., Dickie, I.A., Renton, M., Walker, C., Moot, D., et al., 2017. Fine root endophytes under scrutiny: a review of the literature on arbuscule-producing fungi recently suggested to belong to the Mucoromycotina. Mycorrhiza 27 (7), 619638.

Palmer J, Nextgenusfs SJ. Funannotate: Funannotate v1. 6.0.

Panagabko C, Morley S, Hernandez M, Cassolato P, Gordon H, Parsons R, Manor D, Atkinson J. Ligand specificity in the CRAL-TRIO protein family. Biochemistry. 2003 Jun 3;42(21):6467-74.

Parks MM, Kurylo CM, Dass RA, Bojmar L, Lyden D, Vincent CT, Blanchard SC. Variant ribosomal RNA alleles are conserved and exhibit tissue-specific expression. Science advances. 2018 Feb 28;4(2):eaao0665.

Parniske, M., 2008. Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat. Rev. Microbiol. 6 (10), 763.

Pawlowska TE, Taylor JW. Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. Nature. 2004 Feb 19;427(6976):733-7.

Pawlowska, T.E., Gaspar, M.L., Lastovetsky, O.A., Mondo, S.J., Real-Ramirez, I., Shakya, E., et al., 2018. Biology of fungi and their bacterial endosymbionts. Annu. Rev. Phytopathol. 56, 289309.

Peña R, Robbins C, Corella JC, Thuita M, Masso C, Vanlauwe B, Signarbieux C, Rodriguez A, Sanders IR. Genetically different isolates of the arbuscular mycorrhizal fungus Rhizophagus irregularis induce differential responses to stress in cassava. Frontiers in Plant Science. 2020 Dec 2;11:596929.

Pérez-Tienda, J., Testillano, P.S., Balestrini, R., Fiorilli, V., Azcón-Aguilar, C., Ferrol, N., 2011. GintAMT2, a new member of the ammonium transporter family in the arbuscular mycorrhizal fungus Glomus intraradices. Fungal Genet. Biol. 48 (11), 10441055.

Pertea G, Pertea M. GFF utilities: GffRead and GffCompare. F1000Research. 2020 Sep 9;9:304.

Pfeffer, P.E., Douds, D.D., Bécard, G., Shachar-Hill, Y., 1999. Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. Plant Physiol. 120 (2), 587598.

Phillips JM, Hayman DS. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British mycological Society. 1970 Aug 1;55(1):158-IN18.

Pontecorvo G. The parasexual cycle in fungi. Annual review of microbiology. 1956 Oct;10(1):393-400.

Pringle A, Moncalvo JM, Vilgalys R. High levels of variation in ribosomal DNA sequences within and among spores of a natural population of the arbuscular mycorrhizal fungus Acaulospora colossica. Mycologia. 2000 Mar 1;92(2):259-68.

Providencia, I.E., Nadimi, M., Beaudet, D., Rodriguez Morales, G., Hijri, M., 2013. Detection of a transient mitochondrial DNA heteroplasmy in the progeny of crossed genetically divergent isolates of arbuscular mycorrhizal fungi. New Phytol. 200 (1), 211221.

Rajput, N.A., Zhang, M., Shen, D., Liu, T., Zhang, Q., Ru, Y., et al., 2015. Overexpression of a Phytophthora cytoplasmic CRN effector confers resistance to disease, salinity and drought in Nicotiana benthamiana. Plant Cell Physiol. 56 (12), 24232435.

Raudaskoski M, Kothe E. Basidiomycete mating type genes and pheromone signaling. Eukaryotic cell. 2010 Jun;9(6):847-59.

Raudaskoski M. Mating-type genes and hyphal fusions in filamentous basidiomycetes. Fungal Biology Reviews. 2015 Dec 1;29(3-4):179-93.

Rayner AD. The challenge of the individualistic mycelium. Mycologia. 1991 Jan 1;83(1):48-71.

Redecker D, Morton JB, Bruns TD. Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). Molecular phylogenetics and evolution. 2000 Feb 1;14(2):276-84.

Rees H, Jinks JL. The mechanism of variation in Penicillium heterokaryons. Proceedings of the Royal Society of London. Series B-Biological Sciences. 1952 Aug 27;140(898):100-6.

Reinhardt D, Roux C, Corradi N, Di Pietro A. Lineage-specific genes and cryptic sex: parallels and differences between arbuscular mycorrhizal fungi and fungal pathogens. Trends in plant science. 2021 Feb 1;26(2):111-23.

Remy W, Taylor TN, Hass H, Kerp H. Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proceedings of the National Academy of Sciences. 1994 Dec 6;91(25):11841-3.

Requena, N., Breuninger, M., Franken, P., Ocón, A., 2003. Symbiotic status, phosphate, and sucrose regulate the expression of two plasma membrane H1-ATPase genes from the mycorrhizal fungus Glomus mosseae. Plant Physiol. 132 (3), 15401549.

Rich, M.K., Courty, P.E., Roux, C., Reinhardt, D., 2017. Role of the GRAS transcription factor ATA/RAM1 in the transcriptional reprogramming of arbuscular mycorrhiza in Petunia hybrida. BMC Genomics 18 (1), 589.

Riley R, Corradi N. Searching for clues of sexual reproduction in the genomes of arbuscular mycorrhizal fungi. Fungal Ecology. 2013 Feb 1;6(1):44-9.

Robbins C, Cruz Corella J, Aletti C, Seiler R, Mateus ID, Lee SJ, Masclaux FG, Sanders IR. Generation of unequal nuclear genotype proportions in Rhizophagus irregularis progeny causes allelic imbalance in gene transcription. New Phytologist. 2021 Sep;231(5):1984-2001.

Roberts SE, Gladfelter AS. Nuclear autonomy in multinucleate fungi. Current opinion in microbiology. 2015 Dec 1;28:60-5.

Rodriguez, A., Sanders, IR., 2015. The role of community and population ecology in applying mycorrhizal fungi for improved food security. ISME J. 9 (5), 1053.

Ropars J, López-Villavicencio M, Dupont J, Snirc A, Gillot G, Coton M, Jany JL, Coton E, Giraud T. Induction of sexual reproduction and genetic diversity in the cheese fungus Penicillium roqueforti. Evolutionary applications. 2014 Apr;7(4):433-41.

Ropars, J., Corradi, N., 2015. Homokaryotic vs heterokaryotic mycelium in arbuscular mycorrhizal fungi: different techniques, different results? New Phytol. 208 (3), 638641.

Ropars J, Toro KS, Noel J, Pelin A, Charron P, Farinelli L, Marton T, Krüger M, Fuchs J, Brachmann A, Corradi N. Evidence for the sexual origin of heterokaryosis in arbuscular mycorrhizal fungi. Nature microbiology. 2016 Mar 21;1(6):1-9.

Roper M, Ellison C, Taylor JW, Glass NL. Nuclear and genome dynamics in multinucleate ascomycete fungi. Current biology. 2011 Sep 27;21(18):R786-93.

Roper M, Simonin A, Hickey PC, Leeder A, Glass NL. Nuclear dynamics in a fungal chimera. Proceedings of the National Academy of Sciences. 2013 Aug 6;110(32):12875-80.

Roth, R., Paszkowski, U., 2017. Plant carbon nourishment of arbuscular mycorrhizal fungi. Curr. Opin. Plant Biol. 39, 5056.

Roth, R., Hillmer, S., Funaya, C., Chiapello, M., Schumacher, K., Presti, L.L., et al., 2019. Arbuscular cell invasion coincides with extracellular vesicles and membrane tubules. Nat. Plants 5 (2), 204.

Rovenich, H., Zuccaro, A., Thomma, B.P., 2016. Convergent evolution of filamentous microbes towards evasion of glycan-triggered immunity. New Phytol. 212 (4), 896901.

Saleh D, Xu P, Shen Y, Li C, Adreit H, Milazzo J, Ravigné V, Bazin E, NOTTÉGHEM JL, Fournier E, Tharreau D. Sex at the origin: an Asian population of the rice blast fungus Magnaporthe oryzae reproduces sexually. Molecular ecology. 2012 Mar;21(6):1330-44.

Salvioli, A., Chiapello, M., Fontaine, J., Hadj-Sahraoui, A.L., Grandmougin-Ferjani, A., Lanfranco, L., et al., 2010. Endobacteria affect the metabolic profile of their host Gigaspora margarita, an arbuscular mycorrhizal fungus. Environ. Microbiol 12 (8), 20832095.

Sánchez-Vallet, A., Mesters, J.R., Thomma, B.P., 2015. The battle for chitin recognition in plant-microbe interactions. FEMS Microbiol. Rev. 39 (2), 171183.

Sanders IR, Alt M, Groppe K, Boller T, Wiemken A. Identification of ribosomal DNA polymorphisms among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytologist. 1995 Jul;130(3):419-27.

Sanders SL, Garbett KA, Weil PA. Molecular characterization of Saccharomyces cerevisiae TFIID. Molecular and cellular biology. 2002 Aug 15;22(16):6000-13.

Sanders IR. Specificity in the arbuscular mycorrhizal symbiosis. Mycorrhizal ecology. 2003:415-37.

Sanders IR, Croll D. Arbuscular mycorrhiza: the challenge to understand the genetics of the fungal partner. Annual review of genetics. 2010 Dec 1;44:271-92.

Sanders IR, Rodriguez A. Aligning molecular studies of mycorrhizal fungal diversity with ecologically important levels of diversity in ecosystems. ISME J. 2016 Dec;10(12):2780-2786.

Sanders IR. Sex, plasticity, and biologically significant variation in one Glomeromycotina species. New Phytologist. 2018 Dec 1;220(4):968-70.

Savary R, Masclaux FG, Wyss T, Droh G, Cruz Corella J, Machado AP, Morton JB, Sanders IR. A population genomics approach shows widespread geographical distribution of cryptic genomic forms of the symbiotic fungus Rhizophagus irregularis. The ISME journal. 2018 Jan;12(1):17-30.

Savary R, Dupuis C, Masclaux FG, Mateus ID, Rojas EC, Sanders IR. Genetic variation and evolutionary history of a mycorrhizal fungus regulate the currency of exchange in symbiosis with the food security crop cassava. The ISME journal. 2020 Jun;14(6):1333-44.

Sawers RJ, Svane SF, Quan C, Grønlund M, Wozniak B, Gebreselassie MN, González-Muñoz E, Chávez Montes RA, Baxter I, Goudet J, Jakobsen I. Phosphorus acquisition efficiency in arbuscular mycorrhizal maize is correlated with the abundance of root-external hyphae and the accumulation of transcripts encoding PHT1 phosphate transporters. New Phytologist. 2017 Apr;214(2):632-43.

Sbrana C, Fortuna P, Giovannetti M. Plugging into the network: belowground connections between germlings and extraradical mycelium of arbuscular mycorrhizal fungi. Mycologia. 2011 Mar 1;103(2):307-16.

Schmitz, A.M., Pawlowska, T.E., Harrison, M.J., 2019. A short LysM protein with high molecular diversity from an arbuscular mycorrhizal fungus, Rhizophagus irregularis. Mycoscience 60 (1), 6370.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Fungal Barcoding Consortium, Fungal Barcoding Consortium Author List, Bolchacova E, Voigt K. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the national academy of Sciences. 2012 Apr 17;109(16):6241-6.

Schornack, S., van Damme, M., Bozkurt, T.O., Cano, L.M., Smoker, M., Thines, M., et al., 2010. Ancient class of translocated oomycete effectors targets the host nucleus. Proc. Natl. Acad. Sci. 107 (40), 1742117426.

Schott, S., Valdebenito, B., Bustos, D., Gomez-Porras, J.L., Sharma, T., Dreyer, I., 2016. Cooperation through competition—dynamics and microeconomics of a minimal nutrient trade system in arbuscular mycorrhizal symbiosis. Front. Plant Sci. 7, 912.

Schurko AM, Logsdon Jr JM. Using a meiosis detection toolkit to investigate ancient asexual "scandals" and the evolution of sex. Bioessays. 2008 Jun;30(6):579-89.

Scott TW, Kiers ET, Cooper GA, Dos Santos M, West SA. Evolutionary maintenance of genomic diversity within arbuscular mycorrhizal fungi. Ecology and evolution. 2019 Mar;9(5):2425-35.

Sędzielewska KA, Fuchs J, Temsch EM, Baronian K, Watzke R, Kunze G. Estimation of the Glomus intraradices nuclear DNA content. The New Phytologist. 2011 Dec 1;192(4):794-7.

Seidl MF, Thomma BP. Sex or no sex: evolutionary adaptation occurs regardless. Bioessays. 2014 Apr;36(4):335-45.

Serghi EU, Kokkoris V, Cornell C, Dettman J, Stefani F, Corradi N. Homo-and dikaryons of the arbuscular mycorrhizal fungus Rhizophagus irregularis differ in life history strategy. Frontiers in Plant Science. 2021 Aug 5;12:715377.

Shachar-Hill, Y., Pfeffer, P.E., Douds, D., Osman, S.F., Doner, L.W., Ratcliffe, R.G., 1995. Partitioning of intermediary carbon metabolism in vesicular-arbuscular mycorrhizal leek. Plant Physiol. 108 (1), 715.

Short DP, Gurung S, Hu X, Inderbitzin P, Subbarao KV. Maintenance of sex-related genes and the co-occurrence of both mating types in Verticillium dahliae. PLoS One. 2014 Nov 10;9(11):e112145.

Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics. 2015 Oct 1;31(19):3210-2.

Simon L, Lalonde M, Bruns TD. Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Applied and environmental microbiology. 1992 Jan;58(1):291-5.

Slatkin M. Linkage disequilibrium—understanding the evolutionary past and mapping the medical future. Nature Reviews Genetics. 2008 Jun;9(6):477-85.

Smit AF, Hubley R, Green P. RepeatMasker Open-4.0. 2013–2015.

Smith SE, Smith FA, Jakobsen I. Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. New phytologist. 2004 May;162(2):511-24.

Smith SE, Read DJ. Mycorrhizal symbiosis. Academic press; 2010 Jul 26.

Smith SE, Smith FA. Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. Annual review of plant biology. 2011 Jun 2;62:227-50.

Sneh B, Burpee L, Ogoshi A. Identification of Rhizoctonia species. APS press; 1991.

Solaiman, M.Z., Saito, M., 1997. Use of sugars by intraradical hyphae of arbuscular mycorrhizal fungi revealed by radiorespirometry. New Phytol. 136 (3), 533538.

Song R, Li J, Xie C, Jian W, Yang X. An overview of the molecular genetics of plant resistance to the Verticillium wilt pathogen Verticillium dahliae. International journal of molecular sciences. 2020 Feb 7;21(3):1120.

Spatafora JW, Chang Y, Benny GL, Lazarus K, Smith ME, Berbee ML, Bonito G, Corradi N, Grigoriev I, Gryganskyi A, James TY. A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. Mycologia. 2016 Sep 1;108(5):1028-46.

Spatafora, J.W., Chang, Y., Benny, G.L., Lazarus, K., Smith, M.E., Berbee, M.L., et al., 2016. A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. Mycologia 108 (5), 10281046.

Sperschneider J, Yildirir G, Rizzi Y, Malar C M, Sorwar E, Chen EC, Iwasaki W, Brauer EK, Bosnich W, Gutjahr C, Corradi N. Resolving the haplotypes of arbuscular mycorrhizal fungi highlights the role of two nuclear populations in host interactions. bioRxiv. 2023 Jan 18:2023-01.

Stapley J, Feulner PG, Johnston SE, Santure AW, Smadja CM. Variation in recombination frequency and distribution across eukaryotes: patterns and processes. Philosophical Transactions of the Royal Society B: Biological Sciences. 2017 Dec 19;372(1736):20160455.

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA. Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus Glomus intraradices in an in vitro system in the absence of host roots. Mycological research. 1996 Mar 1;100(3):328-32.

Stockinger H, Walker C, Schüßler A. 'Glomus intraradices DAOM197198', a model fungus in arbuscular mycorrhiza research, is not Glomus intraradices. New phytologist. 2009 Sep;183(4):1176-87.

Stockinger H, Krüger M, Schüßler A. DNA barcoding of arbuscular mycorrhizal fungi. New Phytologist. 2010 Jul;187(2):461-74.

Strom NB, Bushley KE. Two genomes are better than one: history, genetics, and biotechnological applications of fungal heterokaryons. Fungal Biology and Biotechnology. 2016 Dec;3(1):1-4.

Strullu-Derrien, C., Selosse, M.A., Kenrick, P., Martin, F.M., 2018. The origin and evolution of mycorrhizal symbioses: from palaeomycology to phylogenomics. New Phytol. 220 (4), 10121030.

Summerell BA. Resolving Fusarium: Current status of the genus. Annual Review of Phytopathology. 2019 Aug 25;57:323-39.

Sun, X., Chen, W., Ivanov, S., MacLean, A.M., Wight, H., Ramaraj, T., et al., 2019. Genome and evolution of the arbuscular mycorrhizal fungus Diversispora epigaea (formerly Glomus versiforme) and its bacterial endosymbionts. New Phytol. 221 (3), 15561573.

Sward RJ. The structure of the spores of Gigaspora margarita: II. Changes accompanying germination. New Phytologist. 1981 Aug;88(4):661-6.

Thiéry O, Vasar M, Jairus T, Davison J, Roux C, Kivistik PA, Metspalu A, Milani L, Saks Ü, Moora M, Zobel M. Sequence variation in nuclear ribosomal small subunit, internal transcribed spacer and large subunit regions of Rhizophagus irregularis and Gigaspora margarita is high and isolate-dependent. Molecular Ecology. 2016 Jun;25(12):2816-32.

Thirkell TJ, Grimmer M, James L, Pastok D, Allary T, Elliott A, Paveley N, Daniell T, Field KJ. Variation in mycorrhizal growth response among a spring wheat mapping population shows potential to breed for symbiotic benefit. Food and Energy Security. 2022 May;11(2):e370.

Tian, C., Kasiborski, B., Koul, R., Lammers, P.J., Bücking, H., Shachar-Hill, Y., 2010. Regulation of the nitrogen transfer pathway in the arbuscular mycorrhizal symbiosis: gene characterization and the coordination of expression with nitrogen flux. Plant Physiol. 153 (3), 11751187.

Tisserant E, Kohler A, Dozolme-Seddas P, Balestrini R, Benabdellah K, Colard A, Croll D, Da Silva C, Gomez SK, Koul R, Ferrol N. The transcriptome of the arbuscular mycorrhizal fungus Glomus intraradices (DAOM 197198) reveals functional tradeoffs in an obligate symbiont. New Phytologist. 2012 Feb;193(3):755-69.

Tisserant E, Malbreil M, Kuo A, Kohler A, Symeonidi A, Balestrini R, Charron P, Duensing N, Frei dit Frey N, Gianinazzi-Pearson V, Gilbert LB. Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. Proceedings of the National Academy of Sciences. 2013 Dec 10;110(50):20117-22.

Toomer, K.H., Chen, X., Naito, M., Mondo, S.J., den Bakker, H.C., VanKuren, N.W., et al., 2015. Molecular evolution patterns reveal life history features of mycoplasma-related endobacteria associated with arbuscular mycorrhizal fungi. Mol. Ecol. 24 (13), 34853500.

Toro, K.S., Brachmann, A., 2016. The effector candidate repertoire of the arbuscular mycorrhizal fungus Rhizophagus clarus. BMC Genomics 17 (1), 101.

Torres DE, Oggenfuss U, Croll D, Seidl MF. Genome evolution in fungal plant pathogens: looking beyond the two-speed genome model. Fungal Biology Reviews. 2020 Sep 1;34(3):136-43.

Torres-Cortés G, Ghignone S, Bonfante P, Schüßler A. Mosaic genome of endobacteria in arbuscular mycorrhizal fungi: Transkingdom gene transfer in an ancient mycoplasma-fungus association. Proceedings of the National Academy of Sciences. 2015 Jun 23;112(25):7785-90.

Torto, T., Li, S., Styer, A., Huitema, E., Testa, A., Gow, N.A., et al., 2003. EST mining and functional expression assays identify extracellular effector proteins from Phytophthora. Genome Res. 13, 16751685.

Trouvelot A, Kough JL, Gianinazzi-Pearson V. Estimation of vesicular arbuscular mycorrhizal infection levels. Research for methods having a functional significance. In Physiological and genetical aspects of mycorrhizae= Aspects physiologiques et genetiques des mycorhizes: proceedings of the 1st European Symposium on Mycorrhizae, Dijon, 1-5 July 1985 1986. Paris: Institut national de le recherche agronomique, c1986..

Tsuzuki, S., Handa, Y., Takeda, N., Kawaguchi, M., 2016. Strigolactone-induced putative secreted protein 1 Is required for the establishment of symbiosis by the arbuscular mycorrhizal fungus Rhizophagus irregularis. Mol. Plant Microbe Interact. 29 (4), 277286.

Turrà D, Segorbe D, Di Pietro A. Protein kinases in plant-pathogenic fungi: conserved regulators of infection. Annual Review of Phytopathology. 2014 Aug 4;52:267-88.

Uetake, Y., Kojima, T., Ezawa, T., Saito, M., 2002. Extensive tubular vacuole system in an arbuscular mycorrhizal fungus, Gigaspora margarita. New Phytol. 154 (3), 761768.

Van Creij J, Auxier B, An J, Wijfjes RY, Bergin C, Rosling A, Bisseling T, Pan Z, Limpens E. Stochastic nuclear organization and host-dependent allele contribution in Rhizophagus irregularis. BMC genomics. 2023 Jan 28;24(1):53.

Van Der Heijden MG, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature. 1998 Nov 5;396(6706):69-72.

Van Der Heijden, M.G., Martin, F.M., Selosse, M.A., Sanders, IR., 2015. Mycorrhizal ecology and evolution: the past, the present, and the future. New Phytol. 205 (4), 14061423.

van Zeijl, A., den Camp, R.H.O., Deinum, E.E., Charnikhova, T., Franssen, H., den Camp, H.J.O., et al., 2015. Rhizobium lipo-chitooligosaccharide signaling triggers accumulation of cytokinins in Medicago truncatula roots. Molecular Plant 8 (8), 12131226.

Vannini, C., Carpentieri, A., Salvioli, A., Novero, M., Marsoni, M., Testa, L., et al., 2016. An interdomain network: the endobacterium of a mycorrhizal fungus promotes antioxidative responses in both fungal and plant hosts. New Phytol. 211 (1), 265275.

Vaser R, Sović I, Nagarajan N, Šikić M. Fast and accurate de novo genome assembly from long uncorrected reads. Genome research. 2017 May 1;27(5):737-46.

Vembar, S.S., Droll, D., Scherf, A., 2016. Translational regulation in blood stages of the malaria parasite Plasmodium spp.: systems-wide studies pave the way. Wiley Interdiscip. Rev.: RNA 7 (6), 772792.

Venice F, Ghignone S, Salvioli di Fossalunga A, Amselem J, Novero M, Xianan X, Sędzielewska Toro K, Morin E, Lipzen A, Grigoriev IV, Henrissat B. At the nexus of three kingdoms: the genome of the mycorrhizal fungus Gigaspora margarita provides insights into plant, endobacterial and fungal interactions. Environmental microbiology. 2020 Jan;22(1):122-41.

Viruel J, Conejero M, Powell RF, Forest F, Kantar MB, Soto Gomez M, Graham SW, Gravendeel B, Wilkin P. A target capture-based method to estimate ploidy from herbarium specimens. Frontiers in plant science. 2019 Jul 24;10:937.

Vlaardingerbroek I, Beerens B, Rose L, Fokkens L, Cornelissen BJ, Rep M. Exchange of core chromosomes and horizontal transfer of lineage-specific chromosomes in Fusarium oxysporum. Environmental Microbiology. 2016 Nov;18(11):3702-13.

Voß S, Betz R, Heidt S, Corradi N, Requena N. RiCRN1, a crinkler effector from the arbuscular mycorrhizal fungus Rhizophagus irregularis, functions in arbuscule development. Frontiers in microbiology. 2018 Sep 4;9:2068.

Voβ, S., Betz, R., Heidt, S., Corradi, N., Requena, N., 2018. RiCRN1, a crinkler effector from the arbuscular mycorrhizal fungus Rhizophagus irregularis, functions in arbuscule development. Front. Microbiol. 9, 2068.

Walder, F., Niemann, H., Natarajan, M., Lehmann, M.F., Boller, T., Wiemken, A., 2012. Mycorrhizal networks: common goods of plants shared under unequal terms of trade. Plant Physiol. 159 (2), 789797.

Walder, F., van der Heijden, M.G., 2015. Regulation of resource exchange in the arbuscular mycorrhizal symbiosis. Nat. Plants 1 (11), 15159.

Waldor MK, Mekalanos JJ. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science. 1996 Jun 28;272(5270):1910-4.

Wang G, Wang Y, Chen L, Wang H, Guo L, Zhou X, Dou M, Wang B, Lin J, Liu L, Wang Z. Genetic structure and evolutionary diversity of mating-type (MAT) loci in Hypsizygus marmoreus. IMA fungus. 2021 Dec;12:1-6.

Wang J, Han GZ. Unearthing LTR retrotransposon gag genes co-opted in the deep evolution of eukaryotes. Molecular Biology and Evolution. 2021 Aug;38(8):3267-78.

Wang P. Partner control in arbuscular mycorrhizal symbiosis (Doctoral dissertation, Wageningen University and Research).

Wang W, Shi J, Xie Q, Jiang Y, Yu N, Wang E. Nutrient exchange and regulation in arbuscular mycorrhizal symbiosis. Molecular plant. 2017 Sep 12;10(9):1147-58.

Wang, E., Yu, N., Bano, S.A., Liu, C., Miller, A.J., Cousins, D., et al., 2014. A H1-ATPase that energizes nutrient uptake during mycorrhizal symbioses in rice and Medicago truncatula. Plant Cell. 26 (4), 18181830.

Wang, W., Shi, J., Xie, Q., Jiang, Y., Yu, N., Wang, E., 2017a. Nutrient exchange and regulation in arbuscular mycorrhizal symbiosis. Mol. Plant 10 (9), 11471158.

Wang, X.X., Hoffland, E., Feng, G., Kuyper, T.W., 2017b. Phosphate uptake from phytate due to hyphae-mediated phytase activity by arbuscular mycorrhizal maize. Front. Plant Sci. 8, 684.

Wawra S, Fesel P, Widmer H, Timm M, Seibel J, Leson L, Kesseler L, Nostadt R, Hilbert M, Langen G, Zuccaro A. The fungal-specific β-glucan-binding lectin FGB1 alters cell-wall composition and suppresses glucan-triggered immunity in plants. Nature communications. 2016 Oct 27;7(1):13188.

Weisman CM, Murray AW, Eddy SR. Mixing genome annotation methods in a comparative analysis inflates the apparent number of lineage-specific genes. Current Biology. 2022 Jun 20;32(12):2632-9.

Weld RJ, Plummer KM, Carpenter MA, Ridgway HJ. Approaches to functional genomics in filamentous fungi. Cell research. 2006 Jan;16(1):31-44.

Wewer, V., Brands, M., Dörmann, P., 2014. Fatty acid synthesis and lipid metabolism in the obligate biotrophic fungus Rhizophagus irregularis during mycorrhization of Lotus japonicus. Plant J. 79 (3), 398412.

Wipf D, Krajinski F, van Tuinen D, Recorbet G, Courty PE. Trading on the arbuscular mycorrhiza market: from arbuscules to common mycorrhizal networks. New Phytologist. 2019 Aug;223(3):1127-42.

Wyss T, Masclaux FG, Rosikiewicz P, Pagni M, Sanders IR. Population genomics reveals that within-fungus polymorphism is common and maintained in populations of the mycorrhizal fungus Rhizophagus irregularis. The ISME journal. 2016 Oct;10(10):2514-26.

Xie, X., Lin, H., Peng, X., Xu, C., Sun, Z., Jiang, K., et al., 2016. Arbuscular mycorrhizal symbiosis requires a phosphate transceptor in the Gigaspora margarita fungal symbiont. Mol. Plant 9 (12), 15831608.

Xu J, Horgen PA, Anderson JB. Somatic recombination in the cultivated mushroom Agaricus bisporus. Mycological Research. 1996 Feb 1;100(2):188-92.

Xue, S., Barna, M., 2012. Specialized ribosomes: a new frontier in gene regulation and organismal biology. Nat. Rev. Mol. Cell Biol. 13 (6), 355.

Yildirir G, Kokkoris V, Corradi N. Parasexual and sexual reproduction in arbuscular mycorrhizal fungi: room for both. Trends in microbiology. 2020 Jul 1;28(7):517-9.

Yildirir G, Sperschneider J, Malar C M, Chen EC, Iwasaki W, Cornell C, Corradi N. Long reads and Hi-C sequencing illuminate the two-compartment genome of the model arbuscular mycorrhizal symbiont Rhizophagus irregularis. New Phytologist. 2022 Feb;233(3):1097-107.

Young JP. Genome diversity in arbuscular mycorrhizal fungi. Current Opinion in Plant Biology. 2015 Aug 1;26:113-9.

Zeng T, Holmer R, Hontelez J, te Lintel-Hekkert B, Marufu L, de Zeeuw T, Wu F, Schijlen E, Bisseling T, Limpens E. Host-and stage-dependent secretome of the arbuscular mycorrhizal fungus Rhizophagus irregularis. The Plant Journal. 2018 May;94(3):411-25.

Zeng, T., Rodriguez-Moreno, L., Mansurkhodzaev, A., Wang, P., van den Berg, W., Gasciolli, V., et al., 2019. A LysM effector subverts chitin-triggered immunity to facilitate arbuscular mycorrhizal symbiosis. New Phytol. Available from: https://doi.org/10.1111/nph.16245.

Zhang Z, Schwartz S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences. Journal of Computational biology. 2000 Feb 1;7(1-2):203-14.

Zhang, X., Dong, W., Sun, J., Feng, F., Deng, Y., He, Z., et al., 2014a. The receptor kinase CERK1 has dual functions in symbiosis and immunity signalling. Plant J. 81 (2), 258267.

Zhang, L., Fan, J., Ding, X., He, X., Zhang, F., Feng, G., 2014b. Hyphosphere interactions between an arbuscular mycorrhizal fungus and a phosphate solubilizing bacterium promote phytate mineralization in soil. Soil Biol. Biochem. 74, 177183.

Zhang, M., Li, Q., Liu, T., Liu, L., Shen, D., Zhu, Y., et al., 2015. Two cytoplasmic effectors of Phytophthora sojae regulate plant cell death via interactions with plant catalases. Plant Physiol. 167, 164175.

Zhang, L., Xu, M., Liu, Y., Zhang, F., Hodge, A., Feng, G., 2016. Carbon and phosphorus exchange may enable cooperation between an arbuscular mycorrhizal fungus and a phosphate-solubilizing bacterium. New Phytol. 210 (3), 10221032.

Zhang, L., Feng, G., Declerck, S., 2018. Signal beyond nutrient, fructose, exuded by an arbuscular mycorrhizal fungus triggers phytate mineralization by a phosphate solubilizing bacterium. ISME J. 12 (10), 23392351.

Zhang J, Snelders EE, Zwaan BJ, Schoustra SE, Kuijper EJ, Arendrup MC, Melchers WJ, Verweij PE, Debets AJ. Relevance of heterokaryosis for adaptation and azole-resistance development in Aspergillus fumigatus. Proceedings of the Royal Society B. 2019 Feb 13;286(1896):20182886.

Zhao H, Zhang R, Wu J, Meng L, Okazaki Y, Hikida H, Ogata H. A 1.5 Mb continuous endogenous viral region in the arbuscular mycorrhizal fungus Rhizophagus irregularis. bioRxiv. 2023 Apr 17:2023-04.

Zipfel, C., Oldroyd, G.E., 2017. Plant signalling in symbiosis and immunity. Nature 543 (7645), 328.

Scientific summary

Background

Arbuscular mycorrhizal (AM) fungi are widespread plant symbionts that can colonize the majority of vascular land plants. AM fungi originated over 450 million years ago, forming the phylum of Glomeromycota. They effectively consist of a single cell, with a continuous body of cytoplasm harboring millions of haploid nuclei. AM fungi primarily reproduce by forming asexual spores, filled with a sub-selection of hundreds of nuclei, that can germinate and form new colonies. While hyphal fusion (anastomosis) has been observed, and nuclei can be exchanged, no sexual stage has ever been observed in these fungi. This has led to a long lasting debate on how these ancient fungi managed to be so evolutionary successful and are able adapt to an extreme wide range of environments and hosts.

Recent advancements in genetic analysis of the AM fungi model species *Rhizophagus irregularis* revealed large intraspecies differences, where different isolates have varying genomic compositions. Model isolate DAOM197198 was found to be homokaryotic, having one nucleotype and low genetic diversity, while isolate C3 contained two distinct nucleotypes with a higher amount of biallelic SNPs. Furthermore, these isolates differed significantly in gene repertoire, and induced different mycorrhizal growth responses in the same hosts.

The discovery of distinct nucleotypes in some AM fungal isolates led to the hypothesis that nuclei ratios may be subject to shifts in their lifecycle. Furthermore, the existence of conserved meiosis genes and a presumed mating type locus in AMF hinted to the existence of a hidden (para)sexual cycle.

How different nucleotypes interact and contribute to the adaptation to different host plants is largely unknown. To start addressing this question I utilized a combination of whole genome, single spore, single nucleus, and transcriptome sequencing to follow the nuclear dynamics of the dikaryote-like C3 isolate in its interaction with different plant hosts.

Chapter 2 consists of a review of the molecular mechanisms behind the symbiosis between AM fungi and their plant hosts, with a focus on the fungal side of the interaction. Besides providing a general overview of the different stages in the AM fungal life cycle, it also summarizes several advancements into the genetic composition of *R. irregularis* since the beginning of this PhD project. Furthermore, this chapter provides an in-depth review of signaling pathways that induce and regulate the symbiosis, the role of secreted effector proteins, and nutrient exchange mechanisms. The chapter also briefly touches on recent emerging knowledge on AM-associated microbes, living both on the surface of the fungus and within the cytoplasm as endosymbionts, that may form a “second genome” regulating fungal physiology.

In **Chapter 3** I present RirC3, a genome assembly for *R. irregularis* isolate C3, which was obtained using PacBio SMRT Sequel II sequencing. The annotation of the genome revealed that it contained two variants of the mating type (MAT) locus, identical to the ones found in isolate A4. Sequencing of individual nuclei of C3 showed that they mostly matched either of the two nucleotypes. However, all nuclei that we sequenced also showed non-matching

haploblocks on nuclei with the same MAT-locus, indicating past recombination events between the two nucleotypes. Illumina sequencing of C3 cultures revealed a remarkable unequal distribution of allele frequencies, where the reference allele of biallelic SNPs was twice as common as the alternative allele, meaning the MAT-1 carrying nucleotype was twice as common as the MAT-2 nucleotype. However, this nuclei ratio varied between lineages, i.e. different batches or single spore lines, both based on allele frequency distributions and ddPCR of the MAT-locus. Even higher variation in nucleotype ratios were observed in individual spores, indicating a stochastic segregation upon spore formation. Successive genetic bottlenecks through single spore lines did not cause these lines to converge, suggesting a constant reshuffling of nuclei during development. Host identity did not appear to have a consistent effect on nuclei ratios in C3, as they were equally variable after a host shift from carrot to Medicago followed by successive rounds of subculturing. However, consistent shifts in allele frequencies were visible in the transcriptome of C3, which likely reflects host-dependent allele expression.

To examine the host-dependent transcriptome contributions of the different nucleotypes in more detail, I used RNA sequencing in **Chapter 4** to compare the transcriptome of C3 colonizing four host plant species: chives (*Allium schoenoprasum*), Medicago (*Medicago truncatula*), Nicotiana (*Nicotiana benthamiana*), and tomato (*Solanum lycopersicum*). The dataset was complemented with reads from DAOM197198 on the same hosts. To determine which genes were expressed in a host-dependent manner, I developed an R script that makes crosswise comparisons between all treatments. I found that both isolates express host-dependent genes, but differ substantially in their host-dependent gene repertoire. Furthermore, variant calling revealed that C3 also expresses alleles in a host-dependent manner, signifying differential nuclear contributions to the transcriptome. Remarkably, shifts in RNA allele frequencies were mostly visible between groups of hosts, with expression in chives and Medicago being distinct from expression in Nicotiana and tomato. Moreover, transcripts were found where only one allelic variant was expressed, showing haplotype-specific expression. Finally, I discovered that DAOM197198, in contrast to C3, expresses its rRNA variants in a host-dependent manner. Together, this chapter reveals distinct, dynamic nuclei contributions to the transcriptomes of both isolates in the interaction with different hosts.

In conclusion, this thesis illustrates the highly dynamic nuclear organization of *R. irregularis* isolate C3. Nuclei ratios were discovered to shift between C3 lineages, and contribute differentially to the transcriptome depending on the host they are colonizing. Furthermore, sequencing individual nuclei revealed signs of genetic recombination events, hinting at a hidden (para)sexual cycle. In **Chapter 5** these results are discussed in the light of recent advances in the field of AM genomics. The results presented in this thesis illustrate the immense genomic diversity in AM fungi, and highlight the importance of studying unusual genomic compositions to improve our understanding of evolution.

Acknowledgements

For those of you who just skipped to this part of the thesis: Hi! I'm Jelle and I worked on fungi in my PhD. You can find a convenient summary of my work in the previous two pages.

Saying that this thesis project would not have been possible without the support of many people would be a vast understatement. There are many people whom I would like to thank, and I will try not to forget anyone. During my stay at Molecular Biology I've had the chance to do a variety of interesting labwork, but also to develop bioinformatics skills. It's safe to say that I've grown a lot as a scientist, and this would not have been possible without the supportive environment in (and outside of) the chair group.

First and foremost I would like to thank **Erik**, for his guidance, insights, and most of all his patience for my occasional stubbornness. I've had the pleasure of working in your team even as an MSc student during the toolbox course many years ago. Your involved style of supervision allowed me to pick up many experimental skills in a short time. I'm very happy that you chose me for this challenging PhD project. This project has certainly seen many changes in its duration, and perhaps if our attempts at genetically editing *Rhizophagus irregularis* had worked, this thesis could have looked very different. Your support for me to broaden my horizons and pick up skills outside of the initial scope of my project has shaped the rest of my career, and that's something I make use of every day. Secondly, I want to thank **Ton**, for being my promoter and for hiring me in the first place. Even before my PhD you saved my MSc internship by stepping in as my WUR based supervisor when I couldn't find one, even though the project was not related to the research done in your group. Furthermore, I want to thank **Duur**, for being my external PhD supervisor. Your advice during my PhD has helped me make it through some very stressful moments, and I'm very happy I could always knock on your door for support.

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This PhD has taught me many life lessons that could have benefitted me on earlier stages in life, like applying for group projects in time for my first course in my BSc. I'm happy I wasn't the only person who forgot though, and that this is how I met **René**. Our weekly Friday lunches (and Friday afternoon drinks) were a nice constant factor throughout my MSc and PhD. The spontaneous trip to Malaysia was a definite highlight of my PhD, since traveling there does not require a visa. **Abel**, we struck a nice balance of time spent cooking together and weekend-long gaming marathons. I appreciate all the effort you make to help me whenever I need it. I'm very happy to have the two of you with me as my paranymphs today. Besides that, I'd like to thank my other friends in Wageningen, who I've known since my student time: **Judith**, **Joren**, **Casper**, **Reinier** and **Jeroen**, thanks for the Friday night drinks, get-togethers and weekends away.

The change from labwork to bioinformatics was the most important change in my PhD. In a way, I blame **Mara** who first introduced me to the bioinformatics department. Thanks for the many laughs, dinners and movie nights. I was honored you picked me as your maid of honor for your wedding, though after our wildly successful lab pubquiz that decision kind of makes

sense. **Kevin**, setting up Trash Movie Thursday was a game changer, and I truly can't believe that we're still doing this after so many years. Watching a bad movie every week with **Henry**, **Marco**, **Jonathan**, **Emilie**, **Gregoire** and **Violet** during the Covid lockdown was a blast, and probably the most productive we could have spent it.

Learning bioinformatics as an experimentalist was challenging, but I was fortunate enough to have had a lot of help. **Ben**, I enjoyed our many discussions on the topic of mycorrhizal genetics, and your interest in this field, even though it was not your main focus. I don't care what other people say, I think you're a pleasant guy. **Raúl**, in one video call you outlined the entire workflow that lead to my first genome assembly. **Ronald**, thank you for teaching me the first basics of command line, and for letting me join your DnD campaign. Meeting with you, **Carlos**, **Barbara**, **Jay**, **Mehmet**, **Miguel** and **Elvira** on a weekly basis was lots of fun, and must have made me a bit smarter. And the fact that we managed to finish our campaign is also a miracle.

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I would also like to thank my family: **Mom**, **Twan**, **Iris**, **Jeffrey**, **Amber**, **Robin**, **Sophie** and **Mitch** for supporting me throughout this whole period. During this thesis our family has also grown a lot, so I would also like to welcome **Doris**, **Neel**, **Mila**, **Aukje**, **Ruby**, **Bart** and **Mijntje** to the family.

Finally, I would like to highlight the importance of work that does not lead to the expected results. Though much of my work did not end up in this thesis, one experiment did lead me to meet **Laura**, *amor de mi vida*. Thank you for your care, your patience, and your support during my PhD.

About the author

Jelle van Creij was born on October 11th, 1992 in Landhorst, the Netherlands. He received a BSc and MSc in Biology (specialization Molecular Development and Gene Regulation) at Wageningen University and Research, which included a six month internship at the National University of Singapore, where he set up CRISPR/Cas9 transformation protocols in butterflies. In 2016, he started his PhD at the Laboratory of Molecular Biology in Wageningen, supervised by Dr. Erik Limpens and Prof. Ton Bisseling. In his PhD he combined labwork with bioinformatics approaches to study the genetic composition of *Rhizophagus irregularis*. Following the completion of this thesis, he will continue his work as a genetics/bioinformatics specialist at Hudson River Biotechnology in Wageningen.

List of publications

van Creij J, Wang P, Limpens E. Arbuscular mycorrhiza, a fungal perspective. Molecular aspects of plant beneficial microbes in agriculture. 2020 Jan 1:241-58.

van Creij J, Auxier B, An J, Wijfjes RY, Bergin C, Rosling A, Bisseling T, Pan Z, Limpens E. Stochastic nuclear organization and host-dependent allele contribution in Rhizophagus irregularis. BMC genomics. 2023 Jan 28;24(1):53.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Jelle van Creij**
Date: **03 October 2023**
Group: **Laboratory of Molecular Biology**
University: **Wageningen University & Research**

1) Start-Up Phase	*date*	*cp*
► **First presentation of your project**		
Effector-driven selection of Arbuscular Mycorrhiza	04-11-2016	1.5
► **Writing a review or book chapter**		
van Creij, J., Wang, P., & Limpens, E. (2020). Arbuscular mycorrhiza, a fungal perspective. Molecular aspects of plant beneficial microbes in agriculture, 241-258. doi.org/10.1016/B978-0-12-818469-1.00021-3	10-03-2020	6.0
Subtotal Start-Up Phase		7.5

2) Scientific Exposure	*date*	*cp*
► **EPS PhD days**		
EPS PhD days Get2Gether 2017, Soest, NL	09-02-2017 - 10-02-2017	0.6
EPS PhD days Get2Gether 2018, Soest, NL	15-02-2018 - 16-02-2018	0.6
EPS PhD days Get2Gether 2020, Soest, NL	11-02-2020 - 12-02-2020	0.6
► **EPS theme symposia**		
EPS Theme 2 Symposium & Willie Commelin Scholten Day "Interactions between plants and biotic agents", Wageningen, NL	23-01-2017	0.3
EPS Theme 3 Symposium "Metabolism and Adaptation", Wageningen, NL	14-03-2017	0.3
EPS Theme 1 Symposium "Developmental Biology of Plants", Wageningen, NL	30-01-2018	0.3
EPS Theme 2 Symposium & Willie Commelin Scholten Day "Interactions between plants and biotic agents", Wageningen, NL	01-02-2019	0.3
► **Lunteren Days and other national platforms**		
Annual Meeting Experimental Plant Sciences, Lunteren, NL	10-04-2017 - 11-04-2017	0.6
Annual Meeting Experimental Plant Sciences, Lunteren, NL	09-04-2018 - 10-04-2018	0.6
Annual Meeting Experimental Plant Sciences, Lunteren, NL	08-04-2019 - 09-04-2019	0.6
Annual Meeting Experimental Plant Sciences, online	12-04-2021	0.3
► **Seminars (series), workshops and symposia**		
Jean-Francois Arrighi: "Evolution of Nod factor-independent rhizobium symbiosis"	18-10-2017	0.1
Giles Oldroyd: "Recognition of symbiotic microorganisms by plants"	18-10-2017	0.1
Michael Djordjevic: "CLE peptide dependent autoregulation of nodulation in Medicago truncatula"	15-08-2018	0.1
Ronald de Jongh: "Redesigning-cells-organs-and-communities"	01-10-2018	0.1
David Adelson: "From TE to TCM" (Uppsala)	24-05-2019	0.1
Daniel Croll: "Parasites within parasites"	21-06-2019	0.1
Public Lecture: Alga Zuccaro and Ikram Blilou (WUR)	09-09-2019	0.2
Public Lecture: Jürgen Kleine-Vehn (WUR)	28-09-2019	0.1
B-Wise Seminar – Eliana Papoutsoglou	01-10-2019	0.1
B-Wise Seminar – Roeland Voorrips	01-10-2019	0.1
Jian Xu: "The root of single cell CAPability"	02-12-2019	0.1
Pascal Ratet: "Suppression of defense during Medicago root nodule formation"	03-12-2019	0.1
Duur Aanen: "Science and Myth in Evolution"	06-02-2020	0.1

Vasilis Kokkoris: "Nuclear dynamics in the arbuscular mycorrhizal fungus Rhizophagus irregularis"	29-07-2020	0.1
Daniel Croll: "WEES Seminar: Drivers and brakes of pathogen emergence"	18-02-2021	0.1
Symposium "The underground labyrinth: roots, friends and foes", Wageningen, NL	08-02-2017	0.2
Molecular Biology Lunch & Meet Mini-Symposium with Janet Sprent, Euan James & Sofie Goormachtig, Wageningen, NL	23-01-2018	0.2
Wageningen PhD Symposium (WPS) 2019 "Science with impact", Wageningen, NL	25-10-2019	0.3
Mini-symposium "Genetic and genomic analysis of polyploids", Wageningen, NL	11-6-2019	0.2
► **International symposia and congresses**		
International Molecular Mycorrhiza Meeting (iMMM) 2017, Toulouse, FR	26-07-2017 - 28-07-2017	0.9
International Molecular Mycorrhiza Meeting (iMMM) 2019, Torino, IT	06-02-2019 - 07-02-2019	0.6
Adam Kondorosi "Beneficial Plant-Microbe Interactions" Symposium, Gif sur Yvette, FR	26-11-2019 - 27-11-2019	0.6
► **Presentations**		
iMMM2019 Poster "Effects of nuclei selection on symbiotic efficiency of Rhizophagus irregularis"	06-02-2019	1.0
WPS 2019 Poster "Effects of genetic diversity in arbuscular mycorrhizal fungi on host plant development benefits and symbiotic efficiency"	25-10-2019	1.0
Adam Kondorosi Symposium Poster "Genetic drift, dispersal, and host adaptation in Rhizophagus irregularis"	26-11-2019	1.0
EPS Lunteren 2021 Poster "Genome, ribosome and transcriptome SNPs in Rhizophagus irregularis isolate C3 show unique allele distributions"	12-04-2021	1.0
Subtotal Scientific Exposure		13.7

3) In-Depth Studies	*date*	*cp*
► **Advanced scientific courses & workshops**		
SURF Research Bootcamp - Analytics using R, Utrecht, NL	02-11-2018	0.3
Workshop & Symposium Advances in Plant Metabolomics, Wageningen, NL	10-12-2019 - 11-12-2019	0.9
BioSB course Algorithms for Genomics, Delft, NL	14-10-2019 - 18-10-2019	3.0
Workshop Snakemake, online	13-05-2020	0.3
► **Journal club**		
Molecular Biology Literature Discussion	2017-2020	3.0
► **Individual research training**		
Training with Anna Rosling, Uppsala University, SE	23-05-2019 - 24-05-2019	0.6
Training with Anna Rosling, Uppsala University, SE	17-09-2019 - 20-09-2019	1.2
Subtotal In-Depth Studies		9.3

4) Personal Development	*date*	*cp*
► **General skill training courses**		
WGS PhD Competence assessment, Wageningen, NL	07-02-2017	0.3
WGS course Career Orientation, Wageningen, NL	02-10-2018 - 23-10-2018	1.5
Lorentz workshop - Life Sciences with Industry 2019, Leiden, NL	11-11-2019 - 15-11-2019	1.5
Subtotal Personal Development		3.3

TOTAL NUMBER OF CREDIT POINTS*	**33.8**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.	

** A credit represents a normative study load of 28 hours of study.*

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