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Key words: canopy photosynthesis, carbon balance of leaves, global change research, leaf senescence, optimisation models.

The complete *Glomus intraradices* mitochondrial genome sequence – a milestone in mycorrhizal research

Introduction

Arbuscular mycorrhizal fungi (AMF) belong to the Zygomycota, a phylogenetically inhomogeneous (paraphyletic) taxon. Arbuscular mycorrhizal fungi are ubiquitous and most common in soil, forming symbioses with the roots of approx. 80% of all vascular plant species (Smith & Read, 1997). They offer a variety of benefits to their host, including improved phosphate and water uptake, drought tolerance and resistance to root pathogens. Arbuscular mycorrhizal fungi reproduce by asexual spores that contain hundreds of nuclei and mitochondria (Fig. 1, Supporting Information Video S1). Arbuscular mycorrhizal fungi cannot be cultivated without a host plant (i.e. they are obligate biotrophs) and are therefore notoriously elusive to molecular biology and genetics research. A common culturing technique is to grow AMF together with T-DNA-transformed carrot roots on solid synthetic media (Bécard & Fortin, 1988). This technique usually permits the purification of AMF spores *en masse*, but collecting adequate amounts of the fragile vegetative cells (hyphae) remains challenging. Therefore, many fundamental questions bearing on AMF biology remain difficult to investigate. In similar cases, nuclear genome sequencing has been used to kick-start the development of molecular genetics and biochemical research. Not so in AMF. The nuclear genome project has turned into a sequence assembly nightmare (Martin *et al.*, 2008). Evidently, and contrary to previous views (e.g. Pawlowska & Taylor, 2004), gene sequences differ substantially within these populations of nuclei (Hijri & Sanders, 2005; Croll & Sanders, 2009; Croll *et al.*, 2009). This implies that nuclear genome sequencing is not a simple matter of deciphering ~15 Mbp (the estimated average genome size within *Glomus intraradices* nuclear populations; Hijri & Sanders, 2004), but two to three orders of magnitude more. Despite all these obstacles, it has been shown that genetic exchange between distinct isolates of *G. intraradices* does in fact occur via cellular connections, or anastomoses (Croll *et al.*, 2009). There is also early evidence for limited recombination among nuclear genes (Croll & Sanders, 2009).

'... mycorrhizal research enters a phase in which many open questions on its biology, genome organization and segregation can finally be addressed.'

Complete mitochondrial genome sequence from *G. intraradices*

One might expect, then, that sequencing the multicopy mitochondrial DNA (mtDNA) of AMF also ends up at an assembly impasse. Instead, initial studies of individual

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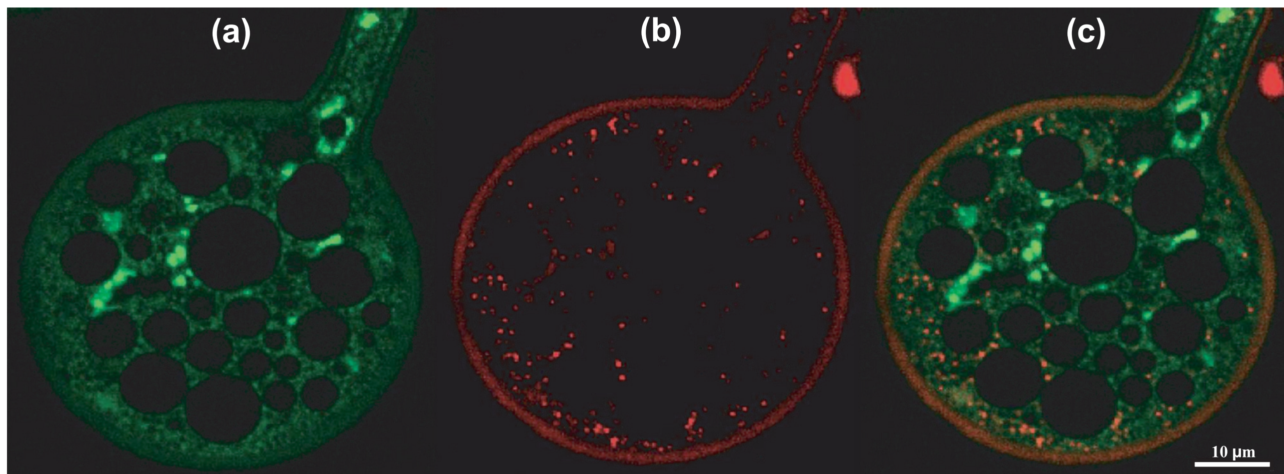

Fig. 1 Confocal microscopy of live *Glomus diaphanum.* (a) Nuclei stained with SytoGreen, green spots; (b) mitochondria stained with MitoTracker, small red spots; (c) merged image. Spore walls autofluoresce in both the green and red channels. See also Supporting Information Video S1.

mitochondrial genes have shown a minor degree of gene sequence variation within AMF species and isolates (Raab *et al.*, 2005), although subsequently corrected to be more substantial (Börstler *et al.*, 2008). In this issue of *New Phytologist* (pp. 200–211), Jaikoo Lee and J. Peter W. Young address this issue, by reporting the first complete mtDNA sequence of a glomeromycete, *G. intraradices* (isolate 494). To overcome difficulties in preparing sufficient cellular material for mtDNA extraction, the authors amplified total DNA from 24 spores by whole-genome amplification and randomly sequenced this mixture of mitochondrial and nuclear DNA using 454 FLX pyrosequencing. Sequences were assembled into a few mitochondrial contigs (among the bulk of difficult-to-assemble nuclear sequences), which were then joined by Sanger sequencing of PCR products into a single, circular-mapping genome of 70 606 bp.

Limited mtDNA polymorphism – a basis for AMF molecular genetics

The mitochondrial genome sequence appears homogenous, except for a small contig that did not assemble with the main, circular-mapping mtDNA. Lee & Young interpret this sequence as an mtDNA insertion in the nuclear genome. Transfer of mitochondrial sequences to nuclear genomes is indeed observed in several eukaryotes, including plants and humans, but is less common in fungi. It remains to be demonstrated whether the postulated nuclear DNA copy is indeed flanked by nuclear genes – which, as discussed in the previous section, relies on resolving the nuclear genome assembly issues (Martin *et al.*, 2008).

The authors are careful in accepting as true polymorphisms only those sequence differences that are supported by at least two independent reads, which may appear overly cautious. Here, however, limitations of the new sequencing technology come into play: the quality assessment of base calling from 454 reads, and the use of quality information in assembly, are still in the early phases of development. Assembly software providing interpretation of base call qualities would help in such cases, but is not readily available to end users. In fact, misinterpretation may not only arise from sequence interpretation, but from real changes that have been introduced during whole-genome amplification. In any case, confirmation of potential polymorphic sites by independent resequencing will be in order. On the other hand, naturally occurring AMF are expected to contain more mitochondrial sequence variation for the following reasons: the material used here for mtDNA sequencing was amplified from only 24 spores; the AMF culture was inbred over a long period of time; and, in nature, anastomoses between distant AMF strains are expected to add to sequence variation.

Knowing that mtDNA sequences are virtually homogenous provides an unprecedented opportunity to answer many questions in mycorrhizal research – simply by sequence analysis. First, mitochondrial markers will permit us to identify and quantify AMF strains by PCR, even at the single-spore level. Second, new and more effective mycorrhizal strains may be selected using mitochondrial markers. However, making a distinction between AMF isolates and species would require the identification of reliable mitochondrial sequence differences, preferably insertions, deletions or genome re-arrangements. These remain to be identified by comparing mtDNAs from several species of AMF. Preliminary data indicate a wealth of highly specific PCR markers, even at short evolutionary distance (B. F. Lang & M. Hijri, unpublished).

The availability of mitochondrial markers will also help to investigate why mtDNA is homogenous in sequence while nuclear DNA is not. Lee & Young mention two probable mechanisms: segregation of mitochondria through a genetic

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bottleneck; or, as in yeast (Ling & Shibata, 2004), an active process of transmitting homogenous mtDNA to descendants. To test these alternatives, population genetics and the mitochondrial segregation process could be monitored using mtDNA; such an experiment could employ anastomosis between cell lines carrying distinct mitochondrial markers.

On the merits of developing molecular markers based on mtDNA

The advantages of using PCR markers based on mtDNA are multifold. Similarly to nuclear ribosomal DNA (rDNA), mtDNA occurs in multiple copies (Fig. 1, Video S1; note the scattering of mitochondrial signals throughout the spore) and is easily amplified from minute amounts of cellular material. In addition, interpretation of results is straightforward as mitochondrial gene duplications are rare and, if they exist, copies are usually identical. Not so in nuclear genomes carrying large gene families, or in variable gene copies caused by ancient whole-genome duplications. Finally, mtDNA is usually small, with a predictable gene set. Indeed, Lee & Young report a mitochondrial gene set that is comparable to that of zygomycete fungi, except that *rps3* (ribosomal protein encoding) and *rnpB* (coding for the RNA subunit of mitochondrial RNase P) are lacking. That these two genes have not been identified is unsurprising; their distribution is spurious and quite variable (Bullerwell *et al.*, 2000; Seif *et al.*, 2003, 2005). The situation is similar for introns and intronic open-reading frames (ORFs), which are abundant in *G. intraradices* and in zygomycetes and have a high degree of variability as a result of frequent loss, and re-introduction by intron homing. Such variability is even observed in *rnl* sequences (coding for the large rRNA subunit) of *G. intraradices* isolates (Börstler *et al.*, 2008) that provide the first reliable molecular markers for AMF identification.

Lateral *cox1* intron transfer from AMF to plants?

As previously suggested, one of the plant *cox1* introns has been acquired by lateral transfer from fungi, probably via AMF that are in intimate contact with their plant host. This interpretation is based on the high sequence similarity between plant and fungal introns (Vaughn *et al.*, 1995), including one in the zygomycete *Rhizopus oryzae* (Seif *et al.*, 2005). Lee & Young reason that, accordingly, this intron should also be detected in the *cox1* gene of AMF, yet it does not exist in *G. intraradices*. The authors go on to conclude that 'a more promising donor may of course, be found once the mitochondrial sequences of more AM fungi become known.' Indeed, according to preliminary sequence data (B. F. Lang & M. Hijri, unpublished), the mtDNA of *Glomus diaphanum* does contain this *cox1* intron, in the same sequence position and with high sequence similarity to its plant and *R. oryzae* counterparts.

The phylogenetic position of Glomeromycota, told by mitochondrial genes

Like previous phylogenomic analyses with nuclear and mitochondrial genes (e.g. Seif *et al.*, 2006; Liu *et al.*, 2009), analyses including the new *Glomus* mitochondrial data neither provide a consistent picture nor are backed by satisfactory statistical support. There is, however, a consensus that AMF are not a sister taxon of Dikarya (ascomycetes plus basidiomycetes), as previously suggested (Schüßler *et al.*, 2001; James *et al.*, 2006), and that they belong instead to one of the lineages of the paraphyletic Zygomycota, a currently disputed taxon (Seif *et al.*, 2006; Hibbett *et al.*, 2007; Liu *et al.*, 2009). In fact, an affinity of *Mortierella* with *Glomus* is observed with a large nuclear data set (Liu *et al.*, 2009), although based on poor taxon sampling and without support by a strict statistical test such as AU (Shimodaira, 2002). Analysis of the mitochondrial data set, including the new *Glomus* sequences, hints in the same direction, but because of the smaller data size, statistical support is even less certain. It seems unlikely that better taxon sampling with mitochondrial data alone will resolve the question of zygomycete monophyly and the positioning of Glomeromycota. A final assessment is more likely to require substantially larger data sets, broadly sampled from a multitude of zygomycete nuclear genomes.

Conclusion and perspectives

The first complete AMF mitochondrial sequence is now available, others are in preparation and partial nuclear genome assemblies will soon be released. With these new resources, mycorrhizal research enters a phase in which many open questions on its biology, genome organization and segregation can finally be addressed. Furthermore, comparative mitochondrial genomics will soon provide an abundance of molecular markers for the timely application of mycorrhizal research in sustainable agriculture.

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Key words: arbuscular mycorrhizal fungi, genetic segregation, intron evolution.

Supporting Information

Additional supporting information may be found in the online version of this article.

Video S1 Confocal microscopy of live *Glomus diaphanum* spore.

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Letters

Depolarization-activated calcium channels shape the calcium signatures induced by low-temperature stress

In their excellent Tansley Review, Martin McAinsh & Jon Pittman (2009) describe how the cellular complement of Ca^{2+} channels, Ca^{2+}-ATPases and Ca^{2+}/H^+-antiporters can interact to produce defined spatial and temporal changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in response to specific environmental and developmental stimuli. Here, I describe how the unique pharmacological characteristics of specific depolarization-activated calcium channels (DACCs) in the plasma membrane of plant cells have provided evidence for their involvement in shaping the changes in $[Ca^{2+}]_{cyt}$ in response to low-temperature stress.

Pharmacological studies indicate that acclimation to low temperatures requires Ca^{2+} influx across the plasma membrane and a transient increase in $[Ca^{2+}]_{cyt}$ (White & Broadley, 2003). Cooling plant cells rapidly to low, non-freezing temperatures

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