

## Analysis

# Symbiotic sequencing for the *Populus* mesocosm

**Sequencing the endomycorrhizal fungus *Glomus intraradices* and the ectomycorrhizal basidiomycete *Laccaria bicolor* – United States Department of Energy Joint Genome Institute (JGI)**

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Completion of the genome sequence of the model tree *Populus trichocarpa*, the first perennial plant to be tackled, will paradoxically prove to be a flagship project for symbiosis research – in its wake, the United States Department of Energy Joint Genome Institute (JGI) is now planning on sequencing several known *Populus* associates, the endomycorrhizal fungus *Glomus intraradices* and the ectomycorrhizal basidiomycete *Laccaria bicolor*. Why focus on organisms associated with *Populus*? Why these organisms? What do *G. intraradices* and *L. bicolor* have to contribute to our knowledge of the function of symbiotic fungi?

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### Mycorrhizal genomics

In order to gain a predictive understanding of the complex biological systems that evolve from mycorrhizal interactions, a surge of studies based on functional genomics (large scale EST sequencing, cDNA array analysis of gene expression, proteomics) have allowed an assessment of the development and functioning of arbuscular endomycorrhizal (AM) and ectomycorrhizal (ECM) symbioses on a larger scale (Martin, 2001). If there is a basic repertoire of fungal symbiotic genes (which surely there is), it can be accessed only by comparing

whole genomes of saprobic (e.g. *Coprinus cinereus*) and pathogenic (e.g. *Magnaporthe grisea* and *Ustilago maydis*) species with mycorrhizal genomes. The availability of genome sequences from ecologically and taxonomically diverse fungi will not only allow ongoing research on those species, but will enhance the value of other sequences through comparative studies of gene evolution, genome structure, metabolic and regulatory pathways, and symbiosis/pathogenesis. One of the major strengths of rhizosphere studies for addressing these issues is that realistic ecological interactions can be investigated in a restricted micro or mesocosm under environmentally controlled conditions with organisms whose genomes have been completely defined (Phillips *et al.*, 2003) and/or genetically modified.

In the next few years, numerous fungal genomes are scheduled to be sequenced, owing largely to the Fungal Genome Initiative at the Whitehead Institute (Cambridge, MA, USA – <http://www-genome.wi.mit.edu/annotation/fungi/fgi/>). This initiative has proposed to sequence up to 44 fungal genomes that include well studied models important to human health, plant pathogens, as well as mutualistic species (e.g. the ectomycorrhizal *Paxillus involutus* and *Tuber borchii*). However, no mycorrhizal genomes have been sequenced yet, and none are on the final Whitehead list. The resolve of the JGI sequence onto the *Populus*-associated *G. intraradices* and *L. bicolor* is therefore especially welcome.

### *Populus* – the perennial favorite

A relatively small number of forest tree species have been subjected to intensive molecular genetic analysis. Trees in general are difficult experimental organisms, because of their large size and long generation times, and so attention has been focused on those species of greatest commercial importance, such as *Populus* and loblolly pine. Over the past decade, *Populus* has been advanced as a model woody plant because of its relatively modest genome size, extensive genetic resources, rapid early growth, ease of clonal propagation, and routine transformation protocols (Bradshaw *et al.*, 2000; Taylor, 2002; Wullschlegel *et al.*, 2002). The sequencing of the *Populus trichocarpa* genome to an approximately 7X depth adds to a long list of important attributes for research. Several groups in the USA, Canada, Sweden and France contributed significant technical resources, EST sets, fundamental *Populus* genome map information and data analysis to this international project (see the International *Populus* Genome Consortium site at <http://www.ornl.gov/ipgc/home.htm>). As a result of its

advanced silvicultural system, *Populus* has been developed for pulp and paper manufacturing, biofuels production, deployed for phytoremediation, and considered for its role in carbon management/sequestration settings (Tuskan, 1998; Tuskan & Walsh, 2001). The successful deployment of *Populus*-based systems for any of the abovementioned purposes depends not only on the ability of *Populus* as a genome to address the demands placed upon it by environmental stresses and stimuli unique to each scenario, but also by the suite of microorganisms that exist around and within the deployed genotypes. It is this consortium of endophytic symbionts that in part determines the effectiveness of the deployed tree system.

### **Populus as an ecosystem**

There is growing evidence that the microfauna and flora associated with a plant's rhizosphere influence the 'host' genotype's ability to respond to fluctuations in the environment (Daniell *et al.*, 1999; van der Heijden *et al.*, 1998; Staddon *et al.*, 2003). The same may be true for the hundreds of as yet unclassified endophytes: symbiotic, commensal and parasitic microorganisms that are continuously being discovered in the leaves and stems of *Populus* (G. Newcombe, unpublished). When viewed in total, a single large perennial plant – a tree – may be considered as an ecosystem in itself, where organisms interact beneficially or antagonistically over time, creating structure and function for further interactions to occur, to the point where the future of the host genotype lays in the balance. For example, the effectiveness of a *Populus* plantation on a contaminated site depends on the inherent ability of the *Populus* genotype to remove the contaminant from the soil and to transport the contaminant to the stem and leaves (Di Baccio *et al.*, 2003). The rate of transport is determined primarily by the transpiration stream, which is affected by mycorrhizal infection (Allen, 1991). This in turn affects overall water uptake and plant health, which influences susceptibility to leaf and stem pathogens, such as foliar rust fungi. Pathogens may ultimately kill the genotype and prevent timely site remediation if insufficient resistance responses occur.

Unlike ephemeral herbaceous annuals, it is the perennial nature of *Populus*, and other tree species, that evolutionarily facilitate the development of consortia of microorganisms that help shape responses to global climate change or stresses from environmental contaminants. The response of these systems to elevated CO<sub>2</sub> and temperature is virtually unknown, and could be an important component of overall ecosystem responses to climate change (Fitter *et al.*, 2000; Gielen *et al.*, 2002). Characterization of the *Populus* mesocosm would allow in-depth exploration of the coordinated community response to these abiotic stresses, thus adding a needed dimension to climate change research and providing

another step in the quest for mechanistic modeling of ecosystem responses.

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*'The challenge is to use the sequenced genomes to determine how mycorrhizal fungi evolve and function'*

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### **Glomus intraradices**

The AM symbiosis between fungi in the Glomeromycota and plants involves around two thirds of all plant species, and is of great ecological significance (Van der Heijden *et al.*, 1998; Daniell *et al.*, 1999; Fitter *et al.*, 2000). The key process in the symbiosis is the acquisition of the immobile phosphate ion from soil by the fungi, greatly enhancing plant P uptake (Smith & Read, 1997). The fungi are obligate symbionts, but there appears to be a low degree of specificity. The fungi may supply other nutrients (NH<sub>4</sub><sup>+</sup>) and may provide defense against pathogens, alter plant water relations and affect palatability to herbivores (Smith & Read, 1997). There are around 150 described species in the Glomeromycota, and about 200 000 plant species involved in the symbiosis. Despite the lack of specificity and the promiscuity of some of the fungi, the association is not in practice random (Sanders, 2003). Colonization of plants by mycorrhizal fungi results in a 5%–20% net increase in photosynthesis (Smith & Read, 1997). Thus AM fungi make a very large, if poorly understood, contribution to the global carbon cycling budget. The Glomeromycota have extremely unusual biological characteristics, being coenocytic, multinucleate, asexual, obligate symbionts with very wide host ranges. Arbuscular mycorrhizal fungi have strategic importance for environmental research (Fitter *et al.*, 2000). For example, between 4% and 20% more photosynthate goes to root systems in arbuscular mycorrhizal plants than in nonsymbiotic plants and a substantial fraction of photosynthate allocated below-ground is transferred to the fungus (Smith & Read, 1997). Because the large majority of land plants are AM and because net photosynthesis of mycorrhizal plants is higher than nonmycorrhizal plants, this represents a substantial fraction of photosynthetically assimilated carbon worldwide (Fitter *et al.*, 2000). There is also experimental evidence that when CO<sub>2</sub> levels are elevated, significantly more carbon is likely to move below ground under the influence of the AM symbiosis, and it may be that this will have a significant role in carbon

sequestration as global CO<sub>2</sub> levels rise (Lussenhop *et al.*, 1998). Mycorrhiza are also important for heavy metal remediation (Rufyikiri *et al.*, 2002). From an evolutionary standpoint, the AM fungi are unique obligate symbionts with coenocytic hyphae (lacking cellular structure) that transport organelles and nutrients over long distances. The regulation of gene expression in such a system with multiple nuclei migrating long distances is completely unexplored. Further, the concept of an individual does not apply, raising substantial questions about the natural selection and population genetics of these highly unusual organisms (Kuhn *et al.*, 2001). There is no known sexual cycle in AM fungi, although anastomosis between hyphae has been described. However, examination of EST sequences from a *G. intraradices* germinating spore cDNA library revealed three cDNAs with significant homology to fungal meiosis-specific proteins (Jun *et al.*, 2002). This raises intriguing questions and suggests there is much still to learn about the basic biology of AM fungi.

Genome sequencing of *G. intraradices* will have a tremendous impact on the scientific community as this, together with *L. bicolor*, will be the first mycorrhizal fungi to be sequenced. While no individual species is ideally suited to be a representative of an entire phylum, a number of factors make the choice of a model species particularly attractive for the AM fungi: there is a small number of species; the distinguishing characteristics of structure, life cycle, and life style are common to all AM fungi; and individual species such as *G. intraradices* occupy very wide ranges of hosts and ecosystems. Thus it may reasonably be concluded that insights and tools obtained from the genome sequence of *G. intraradices* will be very widely applied in AM research.

*G. intraradices* is a widespread mycorrhizal fungus found in different ecosystems throughout the world, including temperate and tropical locations. As a symbiont, *G. intraradices* is highly effective in mobilizing, taking up and transferring mineral nutrients from soils to plants, and it readily colonizes many plant species including agriculturally important species such as maize, wheat, alfalfa, rice, and key model plants such as *Medicago truncatula*, *Lotus japonicum*, and, most importantly in this context, *P. trichocarpa*. For these reasons *G. intraradices* is among the most studied AM fungi and is the prime ingredient in several commercially available inocula. As a representative of the AM fungi (Glomeromycota) *G. intraradices* is a close relative of two other highly studied AM fungal species: *G. etunicatum* and *G. mosseae*. *G. intraradices* can also be grown *in vitro* in dual culture with transformed carrot roots, and of particular importance is the development of a split plate system in which a separate fungal compartment allows a range of manipulations and observations to be made on the extraradical mycelium in the absence of other organisms (St.-Arnaud *et al.*, 1996). *G. intraradices* is also the only species whose spores are available commercially in pure form in large

quantities (Premier Tech, Quebec Canada), which is a resource for researchers worldwide and is mandatory for sequence-quality DNA preparation and construction of BAC libraries.

*G. intraradices* has a very small genome of *c.* 11–12 Mbases (I. Sanders, U. Lausanne, pers. comm.). In addition, random genomic survey sequencing (GSS) in the Lammers lab suggests a gene density comparable to, or higher than, yeast. Nearly 600 000 bp of GSS is now available, spread among 680 sequences (<http://darwin.nmsu.edu/~plammers/>). Fully 24% of these sequences yield a high quality BLAST match to the nonredundant GenBank databases. Analysis of the GSS data did not reveal any highly repetitive sequences that might make assembly of random shotgun sequence data into draft assemblies a difficult task. Nearly 3000 EST sequences from *G. intraradices* have been deposited in GenBank dbEST, along with nearly 1500 from other *Glomus* species (Jun *et al.*, 2002). Biolistic transformation has been achieved for AM fungi (Harrier & Millam, 2001), and efforts are under way to develop protocols for *G. intraradices* (Y. Shachar-Hill, pers. comm.).

### *Laccaria bicolor*

*Laccaria bicolor* is a member of the Tricholomataceae, a large order of ectomycorrhizal and saprobic basidiomycetes. The order is evolutionarily significant because switches between saprobic and ectomycorrhizal lifestyles have occurred several times within the order (Hibbett *et al.*, 2000). *Laccaria bicolor* is a common ectomycorrhizal fungus (ECM) that is a mutualist with many northern temperate forest trees including populus. It provides a useful system for studying the evolution of host and ecological specificity (Kropp & Mueller, 1999). In nature and in the laboratory, *L. bicolor* usually grows as a heterokaryon composed of two different nuclei, each of a different mating type, but axenic haploid strains isolated from spores are available (Di Battista *et al.*, 1996). Completion of the sexual cycle in the laboratory is possible (Godbout & Fortin, 1990). *Laccaria* has been used extensively in both basic and applied research. The physiological ecology of *L. bicolor* is well studied among ectomycorrhizal taxa, because it grows rapidly in culture and its mycorrhizas are easily established with tree roots under laboratory conditions (Kim *et al.*, 1998). *L. bicolor* is commonly used in microcosms and *in vitro* experiments in dual culture with *Populus* or conifer seedlings (Tagu *et al.*, 2001). These systems have been used to study carbon metabolism, nitrogen and phosphorous acquisition and transport, and the ability of this fungus to scavenge nutrients from soil. Finally, this species is used in large-scale commercial inoculation programs in forest nurseries worldwide to enhance growth of tree seedlings (Selosse *et al.*, 2000).

The haploid genome size of *L. bicolor* is estimated at *c.* 25 Mb, in the range of other basidiomycetes, such as

*Paxillus involutus* (20 Mb, Le Quéré *et al.*, 2002), *Ustilago maydis* (20 Mb), and *C. cinereus* (38 Mb). The gene density is estimated to be one gene every 2.5 kb (F. Martin, unpublished). Several cDNA libraries of vegetative mycelium grown under different growth conditions (e.g. N-depleted and C-rich media) (Peter *et al.*, 2003) and from *Populus* ectomycorrhizas (A. Kohler & F. Martin, unpublished) have been constructed. Currently, there is sequence from approx. 2000 ESTs and random genomic fragments deposited in GenBank dbEST (Podila *et al.*, 2002; Peter *et al.*, 2003). Ongoing EST projects (see EctomycorrhizaDB at: <http://mycor.nancy.inra.fr/ectomycorrhizadb/index.html>) will increase the number of sequences available for genome annotation. The 5000, 4000 and 2500 ESTs from *H. cylindrosporium* (H. Sentenac & D. Wipf, unpublished), *P. involutus* (T. Johansson, unpublished) and *Pisolithus microcarpus* (Peter *et al.*, 2003), respectively, could likely be used for annotation. A cosmid library, a partial genetic map, cDNA arrays and transformation systems are also available (Bills *et al.*, 1999; Podila *et al.*, 2002; Peter *et al.*, 2003). As a result, it should be possible to identify a gene of interest, alter its expression, and test the impact on biology and symbiosis formation. Comparison of the genomes of the different plant fungal pathogens with the *Laccaria* genome will be of interest to a wide range of genome and evolutionary scientists outside those working directly on this organism. The comparative genomics of *Laccaria* and *Glomus* will provide critical insights into the genetic makeup of free living vs obligate symbiotic fungi, insights into host range limitations in the ectomycorrhiza, comparisons of metabolism and nutrient exchange, and facilitate the study of host/symbiont signaling processes as well. Comparative genomics between *L. bicolor* and *G. intraradices* will also provide evolutionary and functional genetic clues about the endo- and ectomycorrhizal habits.

There is a network of cooperating laboratories working on *L. bicolor*, *P. involutus*, *Pisolithus* spp., *A. muscaria* and *Tuber borchii* that have organized efforts to share sequence information within the International Ectomycorrhiza Genome Consortium (<http://mycor.inra.nancy.fr/IEGC/>). The *Laccaria* sequence will be of broad interest to fungal researchers and to biologists working on plant–microbe interactions. Groups in the United States, UK, Sweden, France and Germany work on *L. bicolor* and related *Laccaria* species. The overall purpose of the initiative is to build on available resources and to provide a comprehensive understanding of the symbiotic process and related fungal biology and ecology.

## Outcomes

What will the whole genome sequences of *G. intraradices* and *L. bicolor* bring? Together with the *Populus* genome sequence, researchers will have in hand the genetic blueprints for the mycobionts and their host tree. This will

provide the ability to take a holistic approach in understanding how the symbionts interact with the tree host. We have already taken substantial steps in this direction. Combined genomics research on *Populus* and its associated mycorrhizal symbionts, together with the Fungal Genome Initiative, will not only allow the complete genetic blueprints of important fungal species to be determined and compared, but also to greatly accelerate such research on plant–microbe interactions. It should provide a platform for detailed comparative genomic analysis across the fungal taxa, including a comparison of saprotrophic, pathogenic and mutualistic species. The challenge is to use these sequenced genomes to determine how mycorrhizal fungi evolve and function.

Given the diversity within the mycorrhizal lineages, the availability of the *G. intraradices* and *L. bicolor* genome sequences should fuel interest in the study of additional symbiotic fungal genomes. EST programs of mycorrhizal fungi (Peter *et al.*, 2003) have revealed a large proportion of unknown sequences, so-called orphans, which might represent genes that rapidly diverge between closely related species. Defining their functions is a major undertaking, and any functional clues from sequence comparisons will help guide experimental design for studying their functions. Whole genome availability will certainly allow an in-depth analysis of these rapidly evolving genes that may code for specific functions, such as symbiosis. Analysis of this wealth of information is certain to provide breakthroughs in understanding of the molecular and cellular mechanisms involved in the development and biochemical pathways in symbiotic partners. In addition, it will allow us to answer fundamental questions about whether parasitic and symbiotic habits evolved through gene acquisition and loss, or gene regulation. The promoter analysis of the current compendium of mycorrhiza-regulated genes will provide the basis for a more precise molecular dissection of the complex genetic networks that control symbiosis development and function. Determination of entire genome sequences, however, is only the first step in understanding the inner workings of an organism. The next critical step is to elucidate the functions of these sequences and give biochemical, physiological and ecological meaning to this information. This will require an efficient integration of bioinformatics tools and genome-wide functional analyses, including gene disruption, transcriptomics and proteomics, to determine gene function (Tunlid & Talbot, 2002).

The *New Phytologist* Trust is organizing a symposium (October 2004 – see <http://www.newphytologist.org/popgen/>) for plant genomics researchers to explore applications of the *Populus* genome sequence and gain insights into the molecular bases of adaptation in natural populations. Genes are key players in ecological function, and genome-wide identification of plant and fungal genes that are transcribed in response to alteration in trophic webs within symbiotic associations and in the rhizosphere offers ecologists optimum

opportunities to understand soil and rhizosphere ecology. *Populus* eco-genomics will deepen our understanding of tree biology, particularly the genetic responses to stresses, pests and the environment. It may also provide insights and potential solutions to protecting and maximizing the value of forest ecosystems, hopefully leading to new sustainable strategies for breeding, nurturing and better utilization of trees.

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