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Intra-species genetic variability drives carbon use strategies and symbiotic host interactions in the ectomycorrhizal fungus *Pisolithus microcarpus*.

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Running title: Intra-species genetic variability in *P. microcarpus*

Originality-Significance Statement

Summary (200 words)

Introduction

Ectomycorrhizal (ECM) fungi form beneficial symbiotic associations with the roots of approximately 3% of land plant species (Smith and Read, 2008), however, as their hosts include the majority of tree species dominating boreal and temperate forests, the influence of ECM fungi on ecosystem function and nutrient cycling is substantial (Read and Perez-Moreno, 2003; Zak *et al*., 2019). Mycelium of ECM fungi form a mycorrhizal root tip by growing around the fine roots of the host plant and then penetrating in-between root epidermal cells to create the complex symbiotic structure known as the Hartig net, which acts as the site of nutrient exchange between the two organisms. These fungi acquire the majority of their carbon resources from their host, with as much as one third of the carbon fixed by the tree transferred belowground to mycorrhizal symbionts in exchange for host growth-limiting nutrients (Nehls *et al*., 2010). Additionally, ECM fungi are increasingly seen as forest decomposers, breaking down soil organic matter to acquire nutrients (Lindahl and Tunlid, 2015; Shah *et al*., 2016), although this decomposing activity typically does not result in sufficient amounts of carbon for extensive growth or the formation of reproductive structures, leaving the fungus reliant on its host (Lindahl *et al*., 2007; Le Tacon *et al*., 2015). The impact of ECM fungi on host tree nutrition, coupled with their role in soil organic matter decomposition, highlights the need to properly integrate ECM fungi into global ecosystem models (Orwin *et al*., 2011; Sulman *et al*., 2017; Fry *et al*., 2019). One factor complicating this goal, however, stems from the complex evolutionary history of ECM fungi. Rather than descending from one common ancestor, ECM fungi are highly diverse, having evolved independently 60-80 times from saprotrophic ancestors (Hibbett *et al*., 2000; Tedersoo and Smith, 2013; Martin *et al*., 2016). Thus, while all ECM fungi form the same symbiotic

structures and exchange nutrients with their hosts, they exhibit a high level of variability in areas such as host range and nutritional benefit, growth characteristics and the ability to decay soil organic matter (Ekblad *et al*., 2013; Clemmensen *et al*., 2015; Pellitier and Zak, 2018).

While the importance of considering ECM function in light of fungal lineage is becoming increasingly apparent (Pellitier and Zak, 2018; Stuart and Plett, 2020), the role of intra-species genetic variation and the extent to which it may impact ECM fungal growth and function has received less focus. Previous studies have demonstrated that intra-species variation can have significant impacts on the ability of ECM fungi to scavenge N from organic sources (Anderson *et al*., 1999; Sawyer *et al*., 2003; Guidot *et al*., 2005) or colonize and interact with their hosts (Plett *et al*., 2015; Hazard *et al*., 2017; Hortal *et al*., 2017). Intra-species diversity in natural ecosystems may, in fact, be equally as important as inter-species diversity in ecosystem functionality and outcomes (Johnson *et al*., 2012, Hazard *et al*., 2017). Given these increasingly apparent effects of intra-species variation on ECM fungi and their interactions with their hosts, more research is required to better understand what is “core” to the ECM-host relationship and what is individual specific.

We chose to use the model interaction between the ECM fungus *Pisolithus microcarpus* and its host *Eucalyptus grandis* to better understand the impact of intra-specific variability on fungal traits within altered abiotic conditions. Along with the ability of *P. microcarpus* to grow in culture conditions, the availability of fully sequenced fungal (Kohler *et al*., 2015) and host (Myburg *et al*., 2014) genomes make this an excellent model system. Using four isolates of *P. microcarpus* collected from a similar geographical area, we investigated the influence of intra-species variability on gene regulation, growth, carbon metabolism, and symbiotic

interaction with *E. grandis* at two different levels of exogenous carbon availability. Our results uncovered larger than expected variation between these closely related isolates with substantial differences in how they behave transcriptomically, metabolically, and in symbiosis with a host. Together, these results highlight the importance of using a wider range of individuals within a species to understand the broader ecological roles of ECM fungi and their host interactions.

Results

Isolates of P. microcarpus show distinct patterns of gene expression

Four isolates of *Pisolithus*, originally harvested from various locations in Eastern New South Wales, Australia and maintained in pure culture (Plett *et al*., 2015), were determined by ITS-based phylogenetic analysis to all be *P. microcarpus* (MW3, Si9, R10 and R4; Figure 1A). To assess the level of transcriptomic variability between the isolates, MW3, Si9, R10 and R4 were grown for four weeks axenically and RNA from three biological replicates for each was sequenced (Figure 1B). Principle components analysis (PCA) of the resulting normalized and regularized-log transformed transcriptomes demonstrated that gene expression within replicates of each *P. microcarpus* isolate was quite consistent, but that there were distinct differences in gene expression profiles between isolates (Figure 1C, Supplemental Figure 1A). The first three principle components together account for 96.46% of the variation. The genes most implicated in this separation (820 genes total; loading coordinates with $r > 0.75$ in a loading plot for PC1, 2 and 3; Supplemental Figure 1B) are listed in Supplemental Table 1. A GO enrichment analysis of these genes highlights that processes involved in protein modification (i.e. methylation, alkylation) and in DNA integration were what most differentiated the transcriptome between the isolates (Table 1).

P. microcarpus isolates have similar biomass accumulation but different carbon use strategies depending on exogenous glucose concentration

Given the differences in gene expression between the four *P. microcarpus* isolates, we investigated whether this resulted in similar alterations to isolate growth and biomass accumulation, carbon metabolism and ability to colonize a host. These traits were assessed at two concentrations of exogenous glucose (1.0 g/L and 0.1 g/L) to assess potential differential responses to the abiotic constraint of carbon limitation. Following two weeks of growth at the reduced glucose condition, the mycelium of all isolates formed colonies that were both smaller and less dense than those grown with higher levels of glucose (Figure 2A, B), resulting in significantly less biomass (approximately 10% of the biomass obtained in the higher glucose condition; Figure 2C). Isolate was not a significant factor in biomass accumulation, although there was a significant isolate x glucose level effect based on two-way ANOVA ($p<0.05$). Following an additional two-weeks of growth on the media, the sugar composition of the mycelium was analysed by GC-MS (Figure 2D). While the macroscopic growth rate of each isolate was similar at a given glucose concentration, the sugar composition in the mycelium of the *P. microcarpus* isolates showed distinct differences. MW3 and Si9 were the most distinct in their carbon metabolism at a glucose concentration of 1.0 g/L exhibiting high levels of free glucose and myo-inositol (Figure 2D,E). At 0.1 g/L, this profile changed to primarily high trehalose accumulation. The sugar profiles of R10 and R4 were impacted to a lesser degree by the glucose concentration, with sugar alcohols making up a large proportion of the free sugars, along with trehalose and relatively little glucose. R4 was unique in containing substantial amounts of xylitol/arabitol when grown under 0.1g/L glucose conditions.

Given the impact of both fungal genotype and glucose concentration on the composition of free sugars in the fungal mycelium, we considered the relative expression of key enzymes in

fungal sugar metabolic pathways. *P. microcarpus* encodes homologues of key carbon metabolic enzymes as found in other ectomycorrhizal basidiomycetes (Deveau et al., 2008). While the relative expression (TPM) of these enzyme genes showed different patterns between isolates and media glucose concentration (Figure 3; Supplemental Figure 2) it is interesting to note that the differences in gene expression are not as distinct as those found in the sugar metabolic profiles. This may indicate another level of regulation controlling the activity of these enzymes.

Finally, we determined the impact of *P. microcarpus* isolate and exogenous glucose concentration on the ability to colonize *E. grandis* roots. All four isolates of *P. microcarpus* colonized *E. grandis* roots to varying extents, with R10 and R4 having the highest level of colonization under regular glucose conditions (Figure 4A). When fungal isolates were grown on 0.1g/L glucose, the percentage of *E. grandis* root tips colonized decreased for all isolates, except for R4, which was able to colonize a greater percentage of root tips at reduced glucose (Figure 4A). Microscopic observation of cross sections of mycorrhizal root tips demonstrated that all isolates were able to form mycorrhizas with normal formation of the Hartig net. The depth of penetration of the Hartig net into the root tissue varied with both isolate and media glucose concentration with MW3 penetrating into the root apoplast the deepest under 1.0g/L glucose conditions (Figure 4B). The amount of glucose in the media, the fungal isolate and their interaction were all significant factors in determining the outcome of both mycorrhization and Hartig net depth (Two-way ANOVA analysis; $p < 0.05$).

Fungal gene expression during colonization identifies both fungal isolate-independent and dependent expression patterns

Differential gene expression analysis was considered for the four *P. microcarpus* isolates after they had successfully colonized a host plant to determine if the isolate-related transcriptomic variations seen in free living mycelium would continue or if genes induced by the presence of a host were fairly uniform. RNA was isolated and sequenced from each *P. microcarpus* isolate after the formation of mature colonized root tips with *E. grandis*. Tissue was taken from the colonized root tips (ECM condition) and from attached extra-matricial mycelium (EMM condition) when grown on 1.0 g/L glucose and gene expression was compared to the free-living mycelium grown axenically without a plant present (FLM condition). All genes differentially regulated in the ECM condition as compared to FLM ($p < 0.05$, $|\log2FC| > 1$) across all four isolates were considered for a weighted gene co-expression network analysis (WGCNA; Figure 5A; Supplemental Figure 3). Relative gene expression (TPM) for all isolates and tissue types was used in the generation of the network (34 samples in total). The network analysis demonstrates that isolate identity was more relevant in describing gene co-regulation and network modularity than tissue type, with R10 and R4 responding the most similarly to each other. A comparison of all significantly differentially regulated genes in ECM versus FLM tissues across the four isolates (Supplemental Table 2) showed that most were isolate-specific (78.9% and 72.7% of up- and down-regulated genes, respectively), although there was also a core set of isolate-independent genes (103 up-regulated, 170 down-regulated) that are similarly expressed in at least three of the four isolates (Figure 5B,C; Supplemental Figure 3,4). While almost half of these genes are unknown in function, a large portion fall under the categories of small secreted proteins (SSPs), nutrient transporters, and carbohydrate active enzymes (CAZymes; Table 2).

A large percentage of host genes regulated during P. microcarpus *colonization are fungal isolate-specific*

Given the differences in gene expression seen among the four *P. microcarpus* isolates, we considered the transcriptomic response of the host plant in colonized root tissues. Between 5,787 (Si9 colonized) and 9,708 (R4 colonized) genes were significantly differentially regulated in *E. grandis* colonized root tips as compared to axenic control roots ($p<0.05$, $|log2FC|>1$; Supplemental Table 3). Of the many differentially regulated genes in the plant roots, a large proportion (18.7-34.2%) were fungal isolate-specific – that is, significantly differentially regulated when in contact with one isolate of *P. microcarpus*, but not with any of the others (Figure 6A, B). In order to determine if these unique genes represented different parts of the same plant pathways/processes or were truly unique plant responses, we performed GO enrichment analyses on each set of fungal isolate specific genes (Supplemental Table 4). Comparison of the enriched host GO terms influenced by each fungal isolate showed that there was very little overlap in function (Figure C, D). Many of the host genes showed fungal isolate-specific enrichment in GO terms pertaining to hormone and other signalling pathways or defence responses.

198 **Discussion**

199

200 Using four isolates of *P. microcarpus*, all collected from eastern New South Wales, Australia,
201 we examine the impacts of intra-species variation and show that isolate identity affects gene
202 expression, carbon use and sugar metabolism and host interactions. This variation was
203 observed most clearly in the substantially different sugar metabolic profiles in the mycelium
204 of the fungal isolates and in their varied transcriptomic responses to interactions with host
205 plant roots. These isolate-specific differences have the potential for more wide-spread effects
206 as they also influenced gene regulation in the host tree in an isolate-responsive manner.

207

208 Carbon obtained by ECM fungi can be assimilated into biomass promoting fungal growth or
209 stored as free sugars or glycogen within the fungal hyphae. Eventually this stored carbon will
210 be returned to the atmosphere via fungal respiration or decomposition. When in symbiotic
211 association with a host tree, carbon acquired by the ECM fungus is primarily in the form of
212 glucose (López *et al*., 2008; Nehls *et al*., 2010), while free living mycelium may be reliant on
213 carbon from other sources. Previous work in *P. microcarpus* has shown that glucose taken up
214 into the mycelium is quickly assimilated into other forms, primarily trehalose, mannitol and
215 other sugar alcohols such as arabitol or erythritol (Martin *et al*., 1998). It is hypothesized that
216 this fast conversion of glucose into sugar alcohols creates a greater sink strength for the
217 mycelium to maximize glucose uptake from the host (López *et al*., 2007; Wiemken 2007). In
218 our study, isolates R4 and R10 followed this pattern with free sugars being made up primarily
219 of trehalose, mannitol, and other sugar alcohols. Isolates Si9 and MW3, however, had only
220 trace amounts of mannitol, and at higher levels of exogenous carbon input, contained large
221 percentages of free glucose and myo-inositol. In another study using isolates of the ECM

fungus *Hebeloma* spp., Tibbett *et al*. (2002) also showed a range of sugar alcohol ratios in isolates of the same species across a temperature gradient, however, generally the same types of sugars were present within a species. Mannitol, in addition to being an important C storage compound, has demonstrated roles in quenching reactive oxygen species and improving host colonization in pathogenic fungi (Vélëz *et al*., 2008; Meena *et al*., 2015). While it is currently not known if a similar pattern exists in mutualistic fungi, it would be interesting to see if the higher levels of mannitol seen in other isolates of *Pisolithus* correspond to higher rates of *E. grandis* root colonization as was found in R4 and R10.

A comparison of genes differentially regulated in mycorrhizal root tips as compared to free living mycelium demonstrated that about three quarters of all genes with altered expression were isolate-specific – that is differentially regulated in one isolate of *P. microcarpus*, but not in any of the others. Despite this surprisingly high level of genotype driven gene expression, we were able to identify a core set of genes that were consistently regulated across the fungal isolates in response to host colonization. This core set of genes included a number of small secreted proteins, nutrient transporters and CAZymes, all of which are classes of genes that have been previously highlighted as putative keystone genes involved in the establishment of ECM colonization (Kohler *et al*., 2015). Small secreted proteins induced by the presence of a host (called Mycorrhizal induced Small Secreted Proteins (MiSSPs)) have been shown to be crucial in ECM fungal-host interactions with several characterized MiSSPs linked to the ability of the fungus to colonize its host. Within *P. albus*, PaMiSSP10b was recently shown to impact root colonization through interacting with an enzyme in the plant polyamine pathway (Plett *et al*., 2020b). While the equivalent PmMiSSP10b was not in our core data set (while up-regulated in all isolates, it did not meet our cut off criteria of log2FC > 1 for all

246 isolates), several other SSPs were consistently induced in the presence of a host suggesting
247 important involvement in the colonization process. There were also a number of nutrient
248 transporters included in the core set of genes, including the up-regulation of sugar
249 transporters and an ammonium (Amt) transporter, potentially involved in the symbiotic
250 transfer of nutrients between partners. Finally, CAZymes are involved in the degradation of
251 cell wall constituents and most lineages of ECM fungi have a reduced complement of these
252 enzymes as compared to their saprotrophic ancestors, highlighting their importance in the
253 evolution of the symbiotic lifestyle (Martin *et al*., 2016). In our data set, a number of
254 CAZymes were down-regulated in symbiotic associations. In contrast, during the
255 colonization of *Populus* roots, the ECM fungus *Laccaria bicolor* has increased expression of
256 CAZymes, some of which are linked to the ability of the fungus to colonize its host
257 (Veneault-Fourrey *et al*., 2014, Zhang *et al*., 2018). As these enzymes are required to loosen
258 cell wall connections and allow for penetration of fungal hyphae between the plant cells and
259 the formation of the Hartig net, it is unusual that these enzymes are down-regulated in *P.*
260 *microcarpus*. As we tested fully colonized roots, it is possible that the up-regulation of these
261 genes would have occurred at an earlier stage of symbiosis when mycorrhizal root tips were
262 not yet fully formed.
263
264 In addition to the isolate-specific gene regulation seen in the four fungal isolates, we observed
265 a similar isolate-specific variation in the transcriptomic response in the host *E. grandis*. This
266 is significant as it points to the potential larger scale consequences of mycorrhizal intra-
267 species variability on plant function, nutrition and carbon sequestration. In another study
268 considering nutrient trading (Hortal *et al*., 2017), the *P. microcarpus* isolates R10 and Si9
269 exhibited opposing nutrient trading strategies, with R10 donating very little nitrogen to the

plant while consuming more carbon and Si9 donating more nitrogen for less carbon. Experiments with arbuscular mycorrhizal fungi have demonstrated that different isolates within a species have significant effects on both the resulting biomass and nutritional status of the host plant (Koch *et al*., 2006; Mensah *et al*., 2015). Altered gene regulation in colonized roots can also have systemic effects on the whole plant and affect how the plant interacts with other soil micro-organisms, including other competing mycorrhizal fungi (Johnson and Gehring, 2007; Hortal *et al*., 2017).

The availability of exogenous glucose to the fungal mycelium also produced variable responses between isolates. While reduced glucose generally resulted in fewer mycorrhizal root tips formed across the isolates tested, R4 was an exception. In another study considering the effect of glucose availability on the colonization of *E. grandis* by the closely related *P. albus*, reduced glucose availability resulted in more mycorrhizal root tips formed, similar to R4 (Plett et al., 2020a). Similarly, the depth of the Hartig net in individual root tips was significantly affected by the interaction between glucose availability and isolate. These findings demonstrate that conclusions about the function of an ECM species based on the reaction of a single isolate to altered environmental conditions may need to be verified using a variety of genotypes within that species.

Much of the research regarding the genetic basis of how ECM symbiosis functions is reliant on studies in model systems. While these studies have provided us with invaluable information on ECM function there is a need to complement them with studies across a wider variety of species to understand the extent of species-induced variability (Stuart and Plett, 2020; Pellitier and Zak, 2018). Further to this, our present study expands upon previous

294 research on intra-species variation to demonstrate that multiple members of a given species
295 should also be studied as different isolates of the same species may have substantially
296 different traits and affect their hosts and environment in correspondingly distinct ways. The
297 impact of this observed intra-species variability on plant performance in natural ecosystems is
298 understudied, however, indications are that genetic diversity within an ECM species is an
299 important mediator of plant performance (Johnson *et al*., 2012; Hazard *et al*., 2017). The
300 contribution of intra-species variation may be amplified where the physiological traits of
301 individuals are more diverse, allowing the ecosystem to respond more readily to change
302 (Hughes *et al.*, 2008). Thus, an understanding of the range of physiological traits present
303 within a species can assist in predicting the resilience of an ecosystem and how it may adapt
304 to change, as well as contributing to a greater understanding of the mechanisms behind ECM
305 function and responses to their abiotic and biotic environment.
306
307

Experimental Procedures

Biological materials

E. grandis seeds were obtained from the CSIRO Australian Tree Seed Centre (seedlot #20974) and surface sterilized in 30% hydrogen peroxide for ten minutes and rinsed three times in sterile water prior to germinating on 1% agar plates. After one month of growth (25°C, 16-hour photoperiod) the seedlings were transferred onto half-strength modified Melin Norkrans (MMN) media (1.0 g/L glucose) covered with a sterile cellophane membrane to prevent ingrowth of the root into the media. Seedlings were allowed to grow for another month under the same conditions prior to contact with *P. microcarpus*.

P. microcarpus isolates used in this experiment were MW3, Si9, R10 and R4 as described in Plett *et al*. (2015). Isolates were sub-cultured onto full-strength MMN media (10g/L glucose) and grown for one month and then sub-cultured again onto half-strength MMN media covered in a sterile cellophane membrane containing either 1.0 g/L (regular glucose level) or 0.1 g/L (reduced glucose level) glucose. These colonies were allowed to grow to four weeks for sugar composition analysis or RNA-sequencing of free-living mycelium. The perimeter of the colony was marked at 2 and 4-weeks for growth rate.

Mycorrhization experiments

The roots of *E. grandis* seedlings were placed in contact with colonies of two-week old pre-grown *P. microcarpus* isolates. Plates containing plants only or fungus only were included as controls. The plates were sealed with Micropore tape to allow for gas exchange and placed at a 45° angle in a growth chamber (Conviron, Winnipeg, MB, Canada; 30°C daytime/22°C night, 16 hour photoperiod). The experiment was harvested at 14 days after contact.

At harvest, the percentage of mycorrhization for each root system was calculated (number of mycorrhizal root tips/total lateral roots x 100%) based on a visual assessment of the lateral roots in contact with fungus. The perimeter of the fungal colonies at harvest were marked and photographed. The total areas of the fungal colonies at two and four weeks were calculated using ImageJ software. Mycorrhizal root tips were excised and placed in 4% paraformaldehyde (in 1xPBS) for microscopic analysis of the Hartig net. Three biological replicates of free-living mycelium (FLM; axenically grown), extra-matricial mycelium (EMM) and ectomycorrhizal roots (ECM) were collected for each fungal isolate and glucose concentration for RNA and immediately frozen in liquid nitrogen. Roots of axenically grown *E. grandis* were also collected for RNA. FLM tissue was also collected for sugar analysis and frozen immediately in liquid nitrogen. Finally, radial portions of the fungal FLM colonies were excised (area excised determined by ImageJ analysis), dried and weighed to determine the density of the fungal colonies. Fungal dry weight colony biomass data was calculated using each colony area multiplied by its density.

Microscopic analysis

Excised root tips in 4% paraformaldehyde were rinsed 3 times in 1 x PBS and then embedded in 6% agarose. Embedded roots were sectioned on a vibratome (30 μm thick), stained with wheat germ agglutinin (WGA) -FITC and propidium iodide to visualize the plant and fungal cells, rinsed in 1 x PBS and mounted on microscope slides. Cross sections were visualized on a confocal microscope (Leica type) and ImageJ software was used to calculate the Hartig net depth. A minimum of three root tips from each sample type were observed.

Sugar analysis

Fungal mycelium from five biological replicates for each of the four *P. microcarpus* isolates, at both normal and low glucose conditions, was collected and snap frozen in liquid nitrogen. The sample was transferred to a 2-mL screw top vial with a glass bead and 250 μL methanol and homogenized using a bead mill (FastPrep24, MP Biomedicals). Samples were incubated at 30ºC for 15 minutes with shaking and then centrifuged. The supernatant was collected and the remaining pellet was extracted again with 250 μL of water at room temperature. Following centrifugation, this aqueous extract was added to the methanol extract. 25-200 μL of metabolite containing extract (depending on input mass) from above was added into a 0.1mL micro-insert for a standard 2.0 mL GC crimp top sample vial. The metabolite solution was dried down completely using a vacuum concentrator (SpeedVac, Thermo Fisher Scientific) set at ambient temperature. The micro-inserts were sealed into 2.0 mL GC sample vials and 20 μL of pyridine (Sigma-Aldrich) and 20 μL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA; Sigma-Aldrich) was injected into each sample. Samples were incubated with shaking for 2 hours at 37ºC and then 1 μL of the solution was injected onto a GC-MS instrument using a splitless injection (Agilent Technologies 7890A GC system with a 5975C-XL MSD and a Gerstel Maestro Prep autosampler). Separation was achieved using a Agilent HP-5MS column (30 m length; 0.250 mm diameter; 0.25 μm film) with a flowrate of 1.2 mL/min and an initial oven temperature of 70ºC, held for one minute and then increased at 7 degrees per minute to 300ºC and held at that temperature for 5 minutes. Standards for identified sugars were also run using the same derivatization procedure to ensure correct identification of sugars based on retention times and mass spectra. Ratios between integrated peak areas were used to calculate the percent composition of each sample.

Generation of Pisolithus *phylogenetic tree*

RNA sequencing

RNA was extracted from three biological replicates of frozen FLM, EMM, ECM and plant control tissues using either the Bioline miRNA extraction kit or the Qiagen RNeasy Plant RNA kit. All samples for RNA sequencing were harvested 4 weeks after inoculation of the fungal isolate onto the test plate and, for plates with plants present, after the formation of mature mycorrhizal root tips (about two weeks after physical contact between the two organisms). RNA was sequenced at the Western Sydney University Next Generation Sequencing facility (FLM and ECM samples, 1.0 g/L glucose) or at the Joint Genome Institute (*E. grandis* controls, EMM and 0.1 g/L glucose FLM samples). …. (JGI please insert details of library preparation and sequencing here).

Transcriptomic analysis

Raw reads were trimmed for low quality (quality score 0.05), Illumina adapters and sequences shorter than 15 nucleotides and aligned to the reference transcripts available at JGI (*Pisolithus microcarpus* 441; https://mycocosm.jgi.doe.gov/Pismi1/Pismi1.home.html) using CLC Genomics Workbench v12 (Qiagen). The following CLC genomic workbench parameters were used for read mapping: minimum length fraction 0.9, minimum similarity fraction 0.8, Mismatch cost = 2, insertion cost = 3, deletion cost = 3, and the maximum number of hits for a read was set to 10. The unique and total mapped reads number for each

transcript were determined and then normalized to transcripts per million (TPM). The mapping for the different isolates is summarized in Supplemental table 5.

DESeq2 (version 1.24) was used to determine which genes were differentially expressed between pairs of conditions (Love *et al*., 2014). The parameters used to call a gene differentially expressed between conditions were log2 fold change > 1 and FDR $p < 0.05$. For each isolate the unique mapped reads from FLM, EMM and ECM were normalised together and the log2 fold change (log2 FC) in gene expression level between conditions was calculated for ECM versus FLM and EMM versus FLM identified.

Protein sequences for sugar biosynthetic enzymes from the yeast (*Saccharomyces cerevisiae*) proteome were obtained from www.uniprot.org and a protein-protein BLAST was performed against the *P. microcarpus* 441 proteome at mycocosm.jgi.doe.gov to identify the corresponding *P. microcarpus* homologues. The normalized expression of each *P. microcarpus* sugar biosynthetic enzyme homologue was determined in the four isolates from the TPM data calculated as above.

Network generation

We performed semi-supervised weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008), including only differential regulated transcripts (Log2 FC>1, $p < .05$, ECM vs FLM, EMM vs ECM, normal glucose condition) and using TPM normalised values as input. The resulting 8,761 genes were binned into 20 colour-coded modules with a minimum module size of 150. Pearson correlation values were calculated for all genes across all samples, correlation matrix was transformed into connection strengths (connectivity) matrix using a power adjacency function, clusters of highly correlated genes were identified

by coupling linkage hierarchical clustering with topological overlap matrix and sample information were related to network properties (Supplemental Figure 3). Cytoscape (Cline *et al*., 2007) was used to visualize the gene network.

Statistical and enrichment analyses

GO enrichment of *P. microcarpus* genes was performed in R Studio (v.1.2.1335; R Core Team, 2016) using the Bioconductor topGO package (Alexa and Rahnenfuhrer, 2020) for terms under Biological Process using the *P. microcarpus* GO annotations available at https://mycocosm.jgi.doe.gov/Pismi1/Pismi1.home.html. GO terms were considered as enriched with a corrected p-value of <0.05 based on Fisher's Exact tests.

GO enrichment analysis for isolate-specific differentially regulated genes in *E. grandis* was conducted using the online platform PlantRegMap (http://plantregmap.cbi.pku.edu.cn/ ; Tian *et al*., 2020) using dcGO and Fisher's Exact tests for Biological Process using the *E. grandis* genome as the species background. GO terms were considered as enriched with a corrected p-value of <0.001.

Statistical analyses were performed in R. For PCA analysis of RNA sequencing data, mapped count data from the RNA sequencing for the free living mycelium of the four *P. microcarpus* isolates (three biological replicates per isolate) at 1.0 g/L glucose was trimmed to remove non-expressed or lowly expressed genes (<5 counts on average across the three replicates across all conditions). Gene counts were normalized and rlog transformed using the "DeSEQ2" package in R (Love *et al*., 2014) and the resulting data frame was used for generation of the PCA plots (prcomp). The "plotly" package was used for 3D PCA visualization. Loadings data for each gene for PC1, PC2 and PC3 were acquired and those genes falling outside of a sphere of radius 0.75 on the corresponding loadings plot were

considered to contribute most to the separation observed on the PCA plot. Two-way ANOVAs (unbalanced, Type III) were used to determine the effect of isolate or glucose availability and their interaction on experimental outcomes using the "car" package (Fox and Weisberg, 2011; R Core Team, 2016). Significant differences between two experimental conditions were calculated using a single tailed Student's T-test. Clustering of the sugar composition data used hierarchical clustering using a one minus Pearson correlation metric to calculate distances between observations. Heatmaps were generated using the Morpheus online tool at https://software.broadinstitute.org and Venn diagrams were generated using the online tool found at http://bioinformatics.psb.ugent.be/webtools/Venn/.

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655 Table 1

656

GO ID	Term	P value (Fisher)
GO:0015074	DNA integration	0.015
GO:0006479	protein methylation	0.025
GO:0006481	C-terminal protein methylation	0.025
GO:0008213	protein alkylation	0.025
GO:0018410	C-terminal protein amino acid modification	0.025
GO:0006558	L-phenylalanine metabolic process	0.037
GO:0006559	L-phenylalanine catabolic process	0.037
GO:0006829	zinc ion transport	0.037
GO:0009439	cyanate metabolic process	0.037

GO:19022 21	erythrose 4-phosphate/phosphoenolpyruvate family amino acid metabolic process	0.037
GO:19022 22	erythrose 4-phosphate/phosphoenolpyruvate family amino acid catabolic process	0.037

Table 1 Gene ontology (GO) terms significantly enriched in genes most contributing to the separation of the transcriptomes of Si9, R10, R4 and MW3 in principal components analysis. Gene ontology (GO) enrichment was performed with dcGO on 820 *P. microcarpus* genes having loading coordinates with $r > 0.75$ in a loading plot for PC1, 2 and 3 from PCA analysis of the transcriptomes of Si9, R10, R4 and MW3 (Free-living mycelium, 1.0 g/L glucose). Significantly enriched terms have $p < 0.05$ based on a Fisher's exact test.

Table 2 List of *P. microcarpus* genes significantly up- or down- regulated in the core response of *P. microcarpus* to colonization of *E. grandis* root tips. Genes listed are similarly regulated in at least 3 of the 4 isolates considered and are from the gene categories of small-secreted proteins (SSPs), transporters, or cell-wall active enzymes (CAZymes). A complete list of all core genes and the expression level of each gene can be found in Supplemental Figures 4 and 5.

Up-regulated Core Genes		Down-regulated Core Genes	
Small Secreted Proteins (SSPs)			
Protein ID	**Description**	**Protein ID**	**Description**
685288	Carbonic anhydrase	22402	SSP Cerato-platanin
683008	SSP	672065	Fungal hydrophobin
685028	SSP	676962	Fungal hydrophobin
687566	SSP	676970	Fungal hydrophobin
689601	SSP	677031	Fungal hydrophobin
689604	SSP	278092	SSP
683015	SSP Cerato-platanin	439320	SSP
682669	SSP Conotoxin	674112	SSP
674767	Unknown	675746	SSP
676755	Unknown SSP	688218	SSP
		688221	SSP
		679157	SSP
		97211	SSP
		676273	SSP
		672747	SSP, contains WSC domain
		690542	Unknown SSP
Transporters			
Protein ID	**Description**	**Protein ID**	**Description**
244660	ABC transporter	673253	Ca^{2+}:Cation Antiporter (CaCA) Family
690664	H^+ or Na^+ translocating ATPase	422614	Major Facilitator Superfamily (MFS)
22082	Major Facilitator Superfamily (MFS)	10179	Monovalent Cation:Proton Antiporter-2
680085	Major Facilitator Superfamily	678674	MOP Flippase Superfamily

91885 59181 670385 113068 675820	(MFS) Major Facilitator Superfamily (MFS) Mitochondrial Carrier (MC) Family Oligopeptide Transporter (OPT) Family Sugar transporter, (MFS) Ammonia Transporter Amt Family	261938	Oligopeptide Transporter (OPT) Family
CAZymes			
Protein ID	**Description**	**Protein ID**	**Description**
102510 351590	Laccase Laccase	680524 102842 104390 91890 684101 672122 678857 611459 651808 28092 636179	AA1_1 Laccase AA1_1 Laccase AA9 AA9 GH16 GH16 GH16 GH18 Chitinase GH5_15 GH71 GH79

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674

675 **Figure legends**

676

677 **Figure 1. Genomic and transcriptomic variation between *P. microcarpus* isolates MW3,**
678 **Si9, R10 and R4.** (A) Phylogenetic tree based on ITS sequence showing the relationship of
679 the four studied *P. microcarpus* isolates (in blue) with other *Pisolithus* isolates including
680 Australian isolates (in red) and *P. microcarpus* isolate 441 (in green). (B) Photos of the
681 studied *P. microcarpus* isolates following two weeks of growth on ½ strength MMN media
682 with 1.0 g/L glucose. (C) 3D PCA plot of normalized and rlog transformed transcriptomic
683 data (3 biological replicates per condition) for MW3, Si9, R10 and R4 grown for four weeks
684 on ½ strength MMN media.

685

686 **Figure 2. Glucose availability affects fungal biomass accumulation and sugar**
687 **metabolism.** Two-week old colonies of *P. microcarpus* isolate R10 growing on media
688 containing 1.0 g/L glucose (A) or 0.1 g/L glucose (B). (C) Dry weight biomass of *P.*
689 *microcarpus* isolates after two weeks of growth with normal glucose levels (1.0 g/L; light
690 grey bars) or low glucose levels (0.1 g/L; dark grey bars). +/- SE. Results from two-way
691 ANOVA assessing the impact of glucose availability, fungal isolate and their interaction on
692 experimental outcomes are indicated. (D) Percent composition of free sugars in *P.*
693 *microcarpus* mycelium after four weeks of growth on normal (N; 1.0 g/L) or low (L; 0.1 g/L)
694 glucose media. (E) Hierarchical clustering of the *P. microcarpus* isolates at different sugar
695 concentrations based on the percent composition of free sugars in mycelium from (D).

696

697 **Figure 3. Expression of key enzymes in *P. microcarpus* sugar metabolic pathways.** (A)
698 Simplified sugar metabolic pathway for *P. microcarpus* with key enzymes indicated with a

number corresponding to enzymes in (B). Bolded sugars are those analysed for and detected in fungal mycelium samples. (B) *P. microcarpus* gene ID, closest yeast homologue and identity of the numbered enzymes from (A). The average normalized expression (TPM; n = 3) of each enzyme in each condition is indicated by heat map (heat map with individual replicates given in Supplemental Figure 2).

Figure 4. Extent of root colonisation is dependent on both glucose availability and fungal isolate. A) Percentage of lateral roots colonised after two weeks of fungal contact on media with either 1.0 g/L (light grey bars) or 0.1 g/L (dark grey bars) glucose; n=7-11. B) Depth of Hartig net in colonised root tips after two weeks of fungal contact on media with either 1.0 g/L (light grey bars) or 0.1 g/L (dark grey bars) glucose. +/- standard error, *= significant difference between media types. Results from two-way ANOVA assessing the impact of glucose availability, fungal isolate and their interaction on experimental outcomes are indicated.

Figure 5 Differentially expressed genes in *P. microcarpus* when colonizing *E. grandis* are isolate dependent but preserve a common core. (A) Visualization of weighted gene co-expression network analysis (WGCNA) for P. microcarpus gene expression. WGCNA was generated using all significantly differentially regulated genes (ECM vs FLM condition, $p<0.05$) from MW3, R10, R4 and Si9 transcriptomes of FLM, EMM and ECM tissue types at normal levels of glucose (1.0 g/L). Major gene clusters are identified with the represented *P. microcarpus* isolate (Bold text) and tissue type (Italic text). (B, C) Venn diagrams describing overlap of significantly differentially up- (B) and down- (C) regulated genes for each *P. microcarpus* isolate in the ECM condition versus FLM. Genes describing the core response

of *P. microcarpus* to its host (regulated in at least 3 of the 4 isolates) are outlined. FLM = Free living mycelium; EMM = Extra-matricial mycelium; ECM = Ectomycorrhizal.

Figure 6 Colonized *E. grandis roots* differentially express genes assigned to unique GO processes depending on *P. microcarpus* isolate identity (A, B) Venn diagrams showing the overlap of genes significantly up- (A) or down- (B) regulated in E. grandis when colonized by P. microcarpus isolates Si9, R4, R10 or MW3. Isolate specific genes are indicated with black circles. (C, D) Venn diagrams showing the overlap of GO terms (Biological Function) significantly enriched ($p < 0.05$) in the up- (C) and down- (D) regulated genes uniquely regulated by each *P. microcarpus* isolate. Main functions found enriched within the GO terms for each isolate are indicated in italics.

Supplemental Figure 1 PCA analysis and loadings graphs for transcriptomes of *P. microcarpus* isolates.

Supplemental Figure 2 Heatmap of expression (TPM) of key genes in sugar biosynthesis pathways in isolates of *P. microcarpus*.

Supplemental Figure 3 WGCNA module trait relationships and p- values for *P. microcarpus* isolates.

Supplemental Figure 4 Up-regulated Core Genes in *P. microcarpus* colonizing *E. grandis*.

Supplemental Figure 5 Down-regulated Core Genes in *P. microcarpus* colonizing *E. grandis*.

Supplemental Table 1 List of genes, with their annotations, contributing to the separation of *P. microcarpus* isolate transcriptomes in PCA analysis.

Supplemental Table 2 List of all genes significantly differentially expressed in *P. microcarpus* isolates Si9, R10, R4 or MW3 colonizing *E. grandis* roots as compared to axenic controls.

Supplemental Table 3 List of all genes significantly differentially expressed in *E.grandis* roots when colonized by *P. microcarpus* isolates Si9, R10, R4 or MW3 as compared to axenic controls.

Supplemental Table 4 List of significantly enriched GO terms resulting from genes uniquely up- or down- regulated in *E. grandis* roots when colonized by *P. microcarpus* isolates

Supplemental Table 5 *P. microcarpus* mapping statistics