ASSEGNO DI RICERCA XXI TORNATA

Dipartimento di Scienze della Vita e Biologia dei Sistemi

Relazione finale sull'attività di ricerca svolta

# *"Caratterizzazione di polichetide sintasi in un fungo endomicorrizico ericoide tramite un approccio molecolare"*

Dr. Simone BELMONDO

Dipartimento di Scienze della Vita e Biologia dei Sistemi

Orto Botanico di Torino

Viale P.A. Mattioli, 25 10125 Torino

Responsabile scientifico: Dott.sa Elena MARTINO

# DESCRIZIONE DELL'ATTIVITA' DI RICERCA

## INTRODUZIONE

Fungi produce a wide variety of biologically active secondary metabolites, a large proportion of them being polyketides, a group of compounds characterized by a great complexity and structural diversity, and commercially important due to their therapeutic properties (Agarwala & Moore, 2014). Polyketides are produced by polyketide synthases (PKSs), very large multifunctional iterative enzymes that operate as "assembly lines" to bind together basic units of acyl-coenzyme A. Several functions have been attributed to fungal polyketides. Most of them play a role in the ecological and evolutionary adaptation of fungi (Keller, 2019): they can be toxin precursors, pigments important for virulence or for resistance to abiotic stresses, they are also important for growth, sexual development and spore production.

So far, the study of fungal polyketides has been limited by the difficulty of identifying and characterising the polyketide itself. Only recently a more in-depth analysis of the genetic potential of fungi for polyketide production became possible thanks to the rapid increase of the fungal genome sequencing projects. Now selective cloning of genes encoding fungal PKSs may precede the identification of the produced metabolite, contributing to the diversity and functional analysis of fungal polyketides. To date, while there is an extensive literature on the role of PKS in pathogenic interactions *(Böhnert et al.*, 2004) or in biological control (Yao et al., 2016), no data are available concerning the role of PKSs and the produced polyketides in the symbiotic interactions. Over 90% of plants form a symbiotic relationship with soil fungi. These associations are present in almost all ecosystems, having therefore a strong impact on plant growth and health and playing a key role in element cycles (Van der Heijden et al., 2015). The ericoid mycorrhizal symbiosis is established between soil fungi, mainly Ascomycetes, and plant species belonging to the Ericaceae family (Perotto et al., 2012). Ericaceous plant habitats feature acidic soils, very poor nutrient status and considerable edaphic stress. The success of Ericaceae in these harsh environments has been attributed to the unique abilities of their fungal partners (Cairney & Meharg, 2003). The ericoid fungal strain used in this project belongs to the species *Oidiodendron maius* (class Leotiomycetes). *O. maius* represents a good study model as it can be easily grown in vitro, it can be stable genetically transformed, an in vitro mycorrhization protocol is available, its genome was sequenced (Kohler et al., 2015) and transcriptomic data are available.

A recent comparative genomic study of 60 fungi with different taxonomy and ecology showed that O.maius (genome sequenced, Kohler et al., 2015), contains so far the largest number of genes encoding PKSs (Martino et al., 2018). In addition, the available transcriptomic data provided a list of the most induced genes during the symbiosis between *O. maius* and its host plant, including several genes encoding PKSs.

The general objective of this project is to characterize several polyketide synthases of the fungus *O.maius* in order to understand their role in the molecular signaling between the fungus and the host plant, and to identify new molecules with a potential interest for pharmacology, ecological engineering, biocontrol and stress response. In particular, the aim of the project include: i) in silico prediction of Biosynthetic Gene Clusters in the O. maius genome and analysis of the expression in symbiosis; ii) domain prediction, phylogenetic analysis and comparison with other fungal functionally characterized enzymes of O. maius PKSs and iii) generation of mutants lacking PKSs genes highly regulated in symbiosis, in order to analyse their mycorrhizal phenotype.

## MATERIALI E METODI

### *Fungal Strains and Growth Conditions*

Oidiodendron maius strain Zn (hereafter O. maius) was isolated from the roots of V. myrtillus growing in the Niepolomice Forest (Poland), and first described by Martino et al. (2000). This O. maius strain is deposited at the Mycotheca Universitatis Taurinensis collection (MUT1381; University of Turin, Italy) and at the American Type Culture Collection (ATCC MYA4765; Manassas, VA, US), and was maintained on CzapekDox solid medium (NaNO3 2 g L−1, KCl 0.5 g L−1 , glycerol phosphate∗H2O 0.5 g L−1 , K2HPO4 0.35 g L−1 , FeSO4 0.01 g L−1 , sucrose 30 g L−1 , agar 10 gL−1 , adjusted to pH 6). OmPKS197601-null mutants were also grown on the same conditions.

### *Construction of the OmPKS197601-Disruption Vector and Agrobacterium-Mediated Transformation*

*OmPKS197601*-null mutants were obtained through *Agrobacterium tumefaciens*-mediated (ATM) homologous recombination. PCR reactions were used to produce the 5′ upstream flanking region (1040 bp) and the 3′ downstream flanking region (1040 bp) of the OmSSP1 gene. PCR reactions were carried out in a final volume of 50 µl containing: 50 ng of genomic DNA of O. maius Zn, 1 µl dNTPs 10 mM, 2.5 µl of each primer (10µM

stock concentration), 10 µl of 5× Phusion HF Buffer and 0.5 units of Phusion Hot Start II High-Fidelity (Thermo Scientific). The PCR program was as follows: 30 s at 98◦C for 1 cycle, 10 s at 98◦C, 30 s at 60◦C, 45 s at 72◦C for 30 cycles, 10 min at 72◦C for 1 cycle. Amplicons were then purified with Wizard R SV gel and PCR clean-up system (PROMEGA) following the manufacturer's instructions. PCRs amplicons were cut with XmaI-HindIII (for the 5′) and BglII-HpaI (for the 3′) and cloned into the pCAMBIA0380_HYG vector (Fiorilli et al., 2016) in order to obtain the pCAMBIA0380_HYG_PKS197601-KO2 vector. The restriction reactions were performed in 30 µl final volume containing 0.5 µg of DNA (1 µg for the plasmid), 0.5 µl of each enzyme (from PROMEGA), 0.3 µl of BSA 100X and 3 µl of buffer 10X, overnight at 37◦C. The ligase reaction was carried out in 20 µl final volume containing 50 ng of vector, 18 ng of the amplicon, 2 µl of buffer 10X and 1 µl of T4 enzyme (In-Fusione HD Cloning Kit), overnight at 4◦C. The vector sequence was checked by PCR and DNA sequencing. The vector was cloned into Agrobacterium tumefaciens LBA1100, that was used to transform ungerminated O. maius conidia according to the protocol described in Abbà et al. (2009).

*Phylogenetic and Bioinformatic Analyses*

*Prediction of biosynthetic gene clusters*

We used antiSMASH 4.1 for prediction of biosynthetic gene clusters (Madema et al., 2011). The calculation was performed on the computing cluster at INRA, Nancy, France.

*Estimation of global up/down regulated gene clusters*

We determined global up and down regulations of the gene clusters by applying a simple criterion. The majority (at least 5 of 10 genes in a single cluster) should be either up or down-regulated (i.e. Ratio of 0.5 = Number of up/down regulated genes / total gene number per gene cluster). The assumption is the majority of genes in gene clusters are co-regulated for synthesising compounds. A heatmap was made with the normalised log2 reads of the genes using a custom R script. We merged two tables; i) normalised log2 reads; and ii) Protein IDs for the PKS genes selected.

*Integration of multi-omics data and visualisation*

First, Raw reads were normalised and differential expression of genes was calculated from RNA-seq data (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63922). Transcript read counts from biological replicates from the mycorrhizal roots were used to calculate log2 fold changes against the free living mycelia with DESeq2 (Love et al., 2014). We excluded genes showing either very low raw reads or adjusted p value (FDR) more than 0.05. Normalised count per gene was generated with the package. We examined the distribution of normalised read counts between the replicates for quality assurance. Secondly, secreted proteins were predicted using the method described previously (Pellegrin et al., 2015). CAZy annotations were provided from CAZy team (www.cazy.org). Thirdly, transposable element (TE) identification was performed with Transposon Identification Nominative Genome Overview (TINGO; Morin et al., 2019). Finally, output files obtained from the various analyses above and functional annotations from JGI Mycocosm were cleaned, sorted, combined and visualised with R package karyoploteR (Gel & Serra 2017) using a set of custom R scripts, Visually Integrated Numerous Genres of Omics (VINGO).

*PKS domain description and Phylogenetic Analyses*

A phylogenetic tree was created with all of the 47 KS protein domain sequences from O. maius, as well as the protein sequences of some well-characterized PKS enzymes from other fungal species. KS domain sequences were identified and than extracted using the University of Maryland PKS/NRPS Analysis Web Server. Sequences of the 47 O. maius PKS sequences predicted were aligned separately with MAFFT using the E-INS-i strategy (Katoh et al. 2002) and viewed in BioEdit 7.0.9 (Hall 1999). After alignment, poorly aligned positions and divergent regions of these aligned matrices were eliminated using Gblocks 0.91b (Castresana 2000). Maximum likelihood analysis (Guindon and Gascuel, 2003) was conducted using www.phylogeny.fr in advanced mode (Dereeper et al., 2008). The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT). iTOL tree of life was used to visualise the phyylogenetic tree (https://itol.embl.de).

## RISULTATI

### In silico prediction of Biosynthetic Gene Clusters (BGCs) in the genome of *O. maius* and analysis of the expression in symbiosis

Using antiSMASH a total of 59 gene clusters were predicted on the genome of *O. maius* (Table 1). Out of these, 25 PKS and 11 PKS-NRPS hybrid were identified. On the basis of the criterion (see Methods), we identified a total of 3 gene clusters co-up/down-regulated (1 co-upregulated and 2 co-downregulated) under the conditions of mycorrhizal formation according to the RNA-seq data set analysed.

| Fungal ID | Number Gene Cluster | NRPS | Other | Type1 PKS | Type1 PKS + NRPS | Terpene | Indole + Type1PKS | Type 3 PKS + Type1 PKS | Siderophore |
|---|---|---|---|---|---|---|---|---|---|
| Oidma1 | 59 | 4 | 10 | 25 | 11 | 4 | 3 | 1 | 1 |

**Table 1**. Number of backbone genes and gene clusters. **PKS**: Polyketide synthase. **NRPS**: Non-ribosomal peptide synthetase cluster.

*Global genomic view of predicted gene clusters*

We combined and visualised multiple omics results (Fig 1) containing predicted biosynthetic gene clusters, genomic coordinates of genes, transposable elements, differential transcriptions of mycorrhizal roots against free living mycelia, predicted secretome (i.e. CAZymes, proteases, lipases, others, a subcategory for small secreted proteins). The transposable/ repeat elements identified are summarised (Table 2).

| Fungal ID | Unknown | Copia | Gypsy | Mariner. Tc1 | MuDR | Tad1 | Harbinger | piggyBac | DNA | rRNA | Helitron |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Oidma1 | 5874 | 1312 | 478 | 414 | 311 | 303 | 187 | 45 | 30 | 14 | 10 |

**Table 2**. Number of transposable elements and unknown repeats identified in the genome.

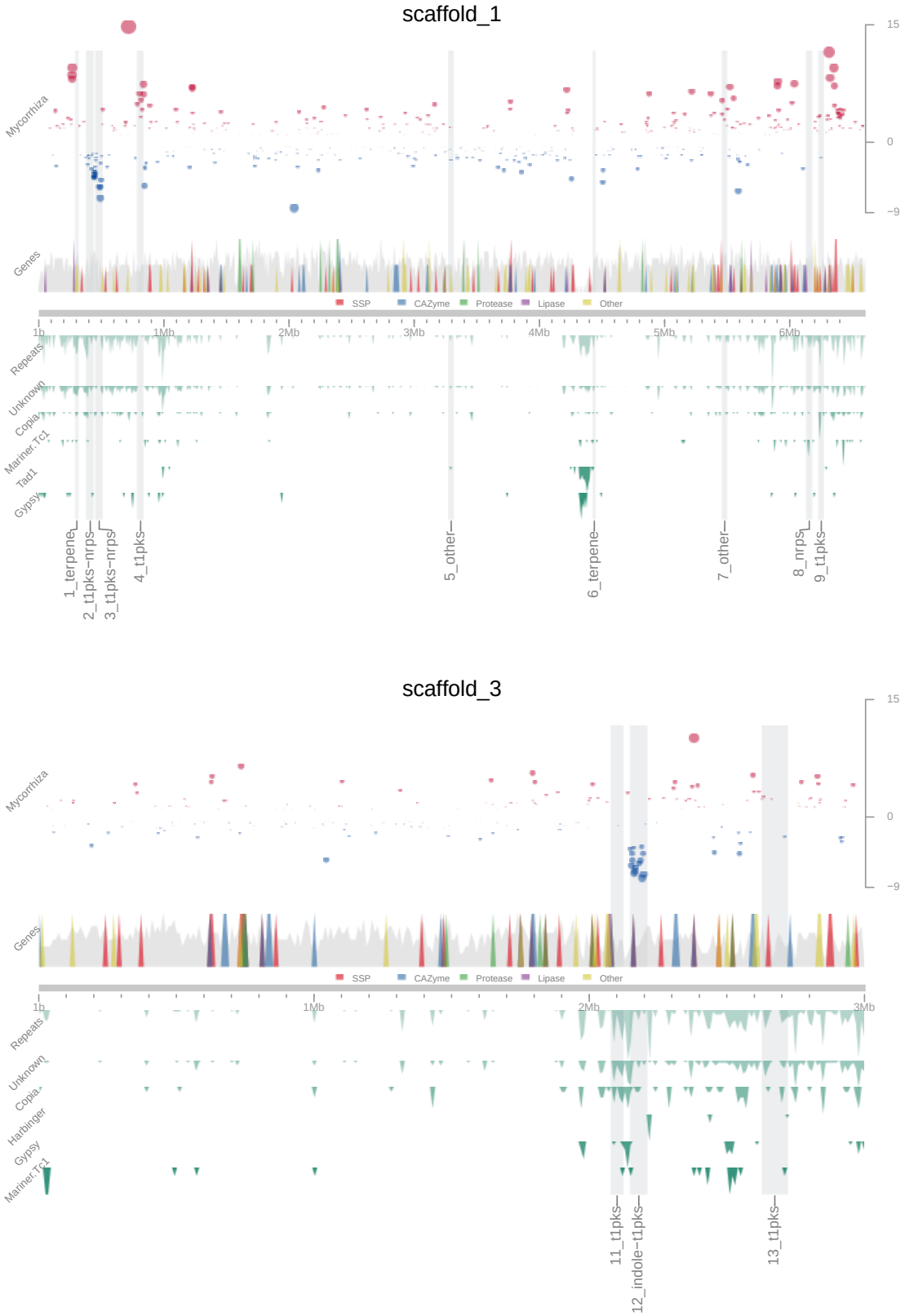


***Figure 2**. Visualisation of scaffolds with gene clusters (grey vertical bars). Scaffold 1 and 3 are shown. **Top panel**: Up and down regulations of genes. The size of circles represents differential transcription levels in log2. **Middle panel**: Density of all genes (grey) and gene for secreted proteins (colours) in a scaffold (grey bar). **Bottom panel**: Density of total and individual TE families.*

RNA-seq quality control and normalised reads

Log2 transformed normalised read counts were examined (Fig. 2). Normalised reads seem to be consistent and comparable between the conditions. Differential transcription of genes between two conditions (Mycorrhizal roots vs Free living mycelia) was calculated and showed using hierarchical clustering analysis (Fig. 3).

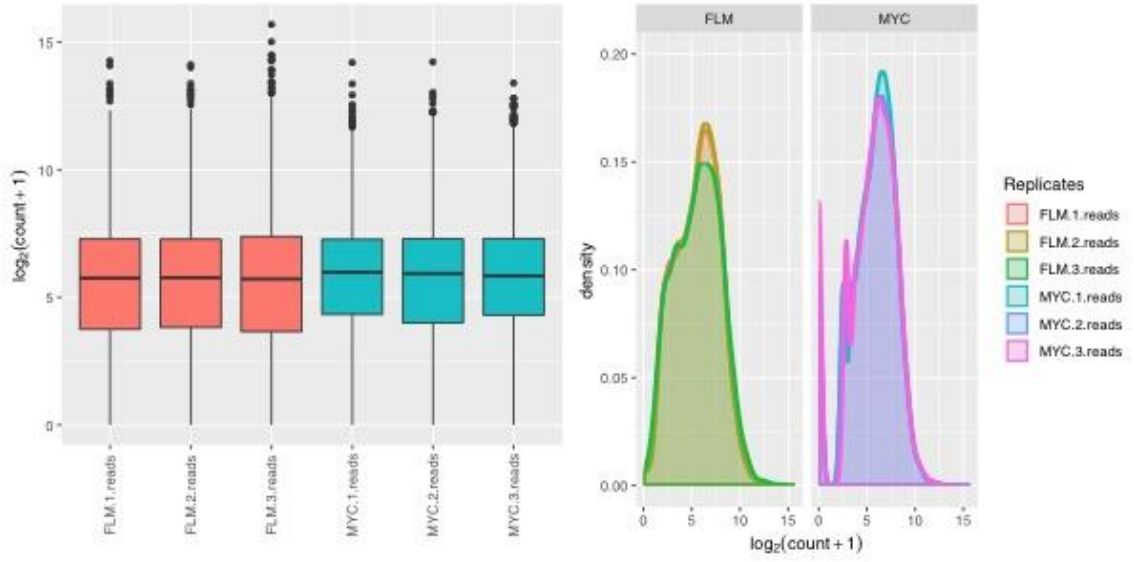


**Figure 2**. Distributions of normalised read count from the RNA-seq data. **Myc**: Mycorrhizal roots. **FLM**: Free living mycelia.

A total of 47 PKS genes were combined with the normalised reads of the genes using DESeq2. Three PKS coding genes were highly regulated under the mycorrhizal condition. PKS 197601 and PKS200887 were upregulated and PKS174030 was highly downregulated during the mycorrhizal condition compared to the free living mycelium. PKSs 107268, 39626, 32168 are excluded for visualisation due to the insufficient transcript reads.

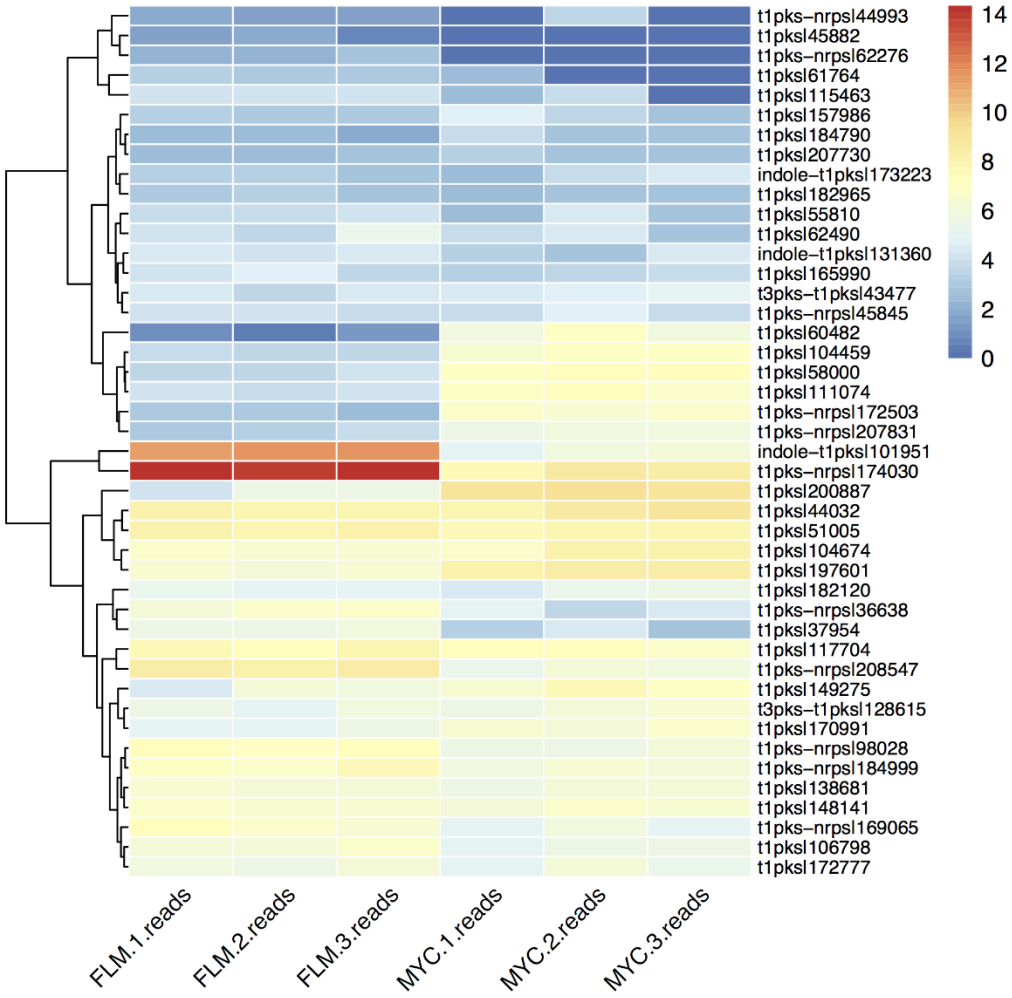


**Figure 3. Hierarchical clustering analysis of symbiosis regulated (*p<0.05*) *O. maius* PKS coding genes**. The expression level is shown as normalized log2 reads. PSK type and protein IDs are reported on the right side of the figure. **MYC**: Mycorrhizal samples. **FLM**: Free living mycelium.

*Domain prediction and phylogenetic analysis*

Prediction of which domains are encoded in each PKS gene can provide clues as to whether the PKS enzyme has the necessary domains to be functional, and whether the PKS enzyme produces a reduced or a non-reduced product. Blastp analysis was done for all of the 45 *O. maius* predicted PKSs and PKS-NRPS hybrids protein sequences, using the NCBI database. In order to further characterize the *O. maius* PKS sequences a phylogenetic tree was created with all of the 45 KS protein domain sequences from *O. maius*, as well as the protein sequences of some well-characterized PKS enzymes from other fungal species (Fig.4)

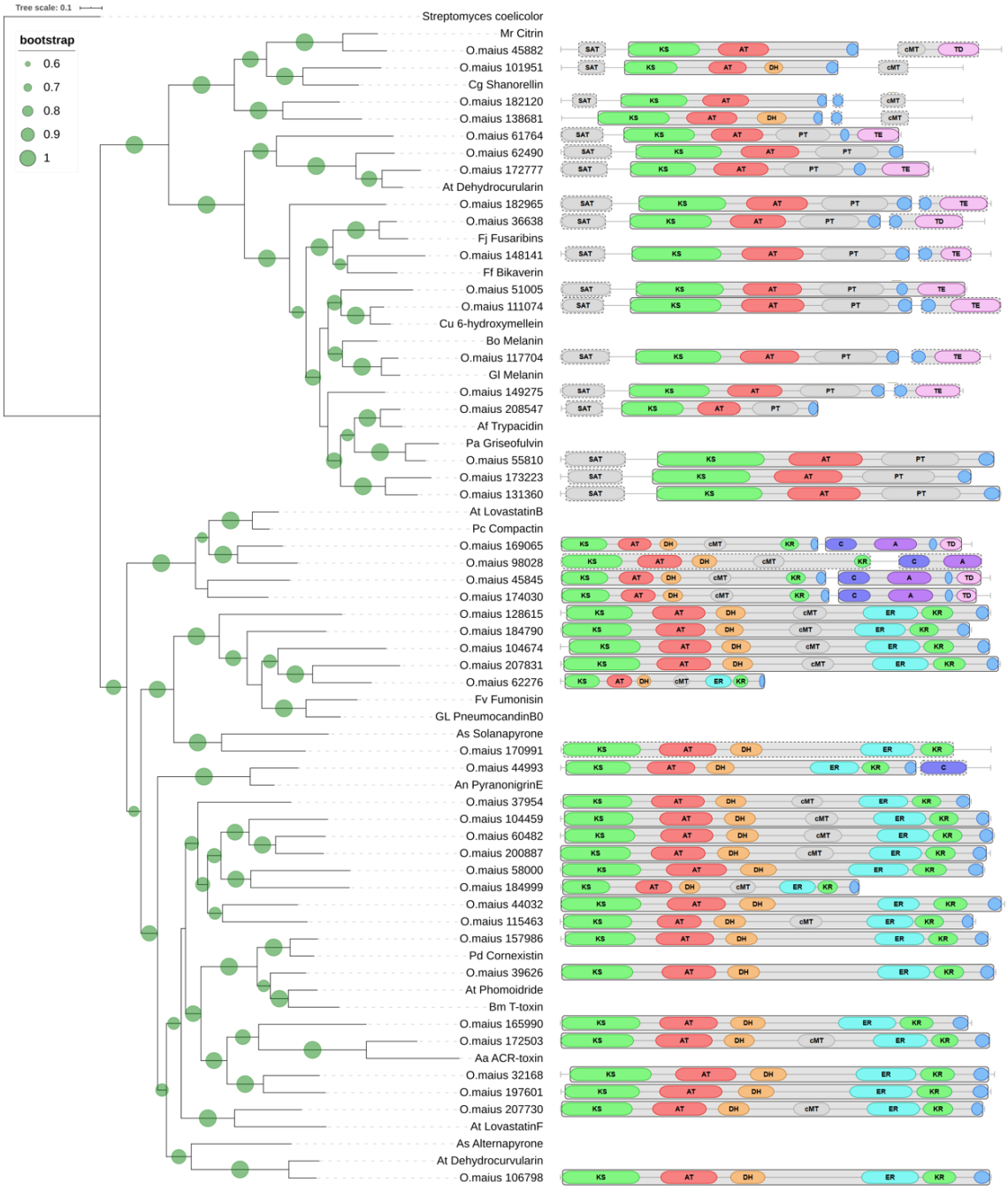


**Figure 4. Maximum Likelihood phylogenetic tree of O. maius PKS and sequences as well as protein sequences from well-characterized PKS genes from other species.** Genealogy of PKSs and PKS-NRPSs was inferred by Maximum Likelihood (PhyML) of the aligned amino acid sequences of the KS domains. Products of each well-characteized PKS enzyme are indicated on the tree. Branch length indicates number of inferred amino acid changes. Only branch nodes with >50% support are shown. PKS and PKS-NRPS domains from O. maius were annotated using the anti-SMASH 5.0 tools.

*Generation of mutants lacking PKSs genes highly regulated in symbiosis, in order to analyze their mycorrhizal phenotype*

From the expression analysis of *O. maius* PKS coding genes, three gene candidates were selected, whose expression is highly regulated during the interaction with the host plant and phylogenetically distant from other characterised PKSs of which the produced poliketides are known. In order to obtain the knock-out mutants, plasmids were designed for the three PKS genes selected by bioinformatics analysis and cloned in the pCAMBIA0380_HYG vector.

The pCAMBIA0380_HYG_PKS197601 vector was cloned into *Agrobacterium tumefaciens* LBA1100, and used to transform ungerminated *O. maius* conidia according to the protocol described in Abbà et al. (2009). 700 mutants were collected in single culture in multiwell (medium: Czapek-dox-hyg), and a PCR-screening was performed, by using specific primers drawn on the gene sequence of the putatively deleted gene. The negative result of the PCR confirmed the knock-out of the PKS 197601 gene in three *O. maius* PKS 197601 knock-out mutants (Fig. 5). A second PCR was performed with primers designed on the hph resistance cassette confirming that the resistance box is inserted in the right place and that the gene has been deleted as expected (Fig. 5).

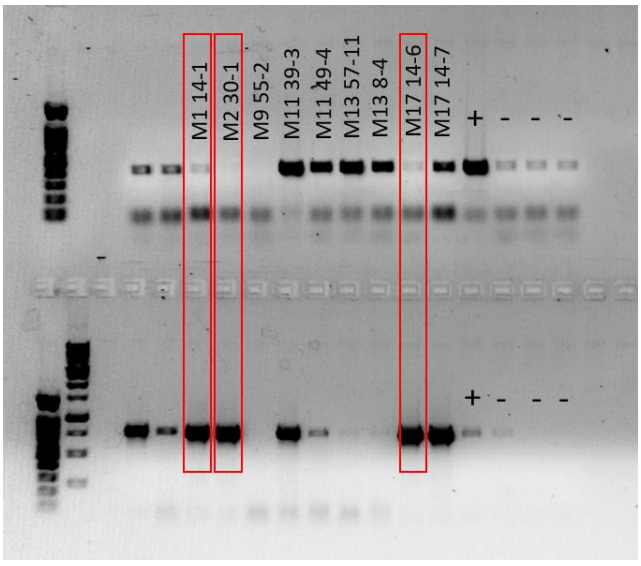


**Figure 5.** PCR-screening on the DNA extracts of the three candidate mutants. a) PKS-r2/PKS-f2 primers designed on the AT domain of 197601 PKS b) Hyg2r/Hyg4f primers designed on the hygromycin resistance gene cassette.

Since *A. tumefaciens*-mediated recombination can lead (with low frequency) to multiple insertions in the genome, Southern blot analysis will be performed to validate the mutants obtained. The three *O. maius* candidate mutants selected will be used for the in vitro preparation of mycorrhizal syntheses to verify whether the deletion of the PKS encoding gene has affected their ability to establish mycorrhizal symbiosis. As literature data mention a biocontrol activity among the functions of polyketides, experiments will also be carried out to collect information on possible biocontrol activities for the *O. maius* strain used.

## CONCLUSIONI E PROSPETTIVE

As *O. maius* is one of the very few genetically tractable mycorrhizal fungi, it represents an interesting model system to investigate with a molecular approach the role of polyketides in the mycorrhizal symbiosis. No data are currently available on the polyketides produced by this symbiotic fungus, and this investigation should provide a better picture of its genetic potential in the polyketide biosynthetic pathway, as well as to the characterization of some of these compounds and potential role in symbiosis, biocontrol and stress response.

**BIBLIOGRAFIA**

Abbà, S., Khouja, H. R., Martino, E., Archer, D. B., and Perotto, S. (2009). SOD1-targeted gene disruption in the ericoid mycorrhizal fungus *Oidiodendron maius* reduces conidiation and the capacity for mycorrhization. Mol. Plant Microbe Int. 22, 1412–1421. doi: 10.1094/MPMI-22-11-1412

Agarwala V., Moore BS. 2014. Fungal polyketide engineering comes of age. Proceedings of the National Academy of Sciences USA, 111: 12278–12279.

Böhnert HU., Fudal I., Dioh W., Tharreau D., Notteghem JL., Lebrun MH. 2004. A putative polyketide synthase/peptide synthetase from Magnaporthe grisea signals pathogen attack to resistant rice. Plant Cell, 16: 2499-513.

Cairney J.W.G., Meharg A.A. 2003. Ericoid mycorrhiza: a partnership that exploits harsh edaphic conditions. European Journal of Soil Science, 54: 735–740.

Fiorilli, V., Belmondo, S., Khouja, H.-R., Abb,à, S., Faccio, A., Daghino, S., et al. (2016). RiPEIP1, a gene from the arbuscular mycorrhizal fungus *Rhizophagus irregularis*, is preferentially expressed in planta and may be involved in root colonization. Mycorrhiza 26, 609–621. doi: 10.1007/s00572-016-0697-0

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. doi:10.1186/PREACCEPT-8897612761307401.

Keller N.P., 2019. Fungal secondary metabolism: regulation, function and drug discovery. Nat Rev Microbiol 17, 167–180

Kohler A., Kuo A., Nagy L.G., Morin E., Barry K.W., Buscot F., Canbäck B., Choi C., Cichocki N., Clum A., et al. 2015. Covergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. Nature Genetics, 47: 410-415.

Medema, M. H. et al. antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res. 39, W339-46 (2011).

Martino E., et al. 2018. Comparative genomics and transcriptomics depict ericoid mycorrhizal fungi as versatile saprotrophs and plant mutualists. New Phytologist, 217, 1213–1229.

Martino, E., Turnau, K., Girlanda, M., Bonfante, P., and Perotto, S. (2000). Ericoid mycorrhizal fungi from heavy metal polluted soils: their identification and growth in the presence of zinc ions. Mycol. Res. 104, 338–344.

Pellegrin, C., Morin, E., Martin, F. M. & Veneault-Fourrey, C. Comparative Analysis of Secretomes from Ectomycorrhizal Fungi with an Emphasis on Small-Secreted Proteins. Front. Microbiol. 6, 1278 (2015).

Perotto S., Martino E., Abbà S., Vallino M. 2012. Genetic diversity and functional aspects of ericoid mycorrhizal fungi. In: Hock, B. (ed.), The Mycota, IX: Fungal Associations. 2nd ed. Springer, Berlin 255-285.

Van der Heijden M.G.A., Martin F.M., Selosse M-A., Sanders I.R. 2015. Mycorrhizal ecology and evolution: the past, the present and the future. New Phytologist, 205: 1406-23.

Yao L., Tan C., Song J., Yang Q., Yu L., Li X. 2016. Isolation and expression of two polyketide synthase genes from Trichoderma harzianum 88 during mycoparasitism. Brazilian Journal of Microbiology, 47: 468-79.

## ALTRE ATTIVITA' SVOLTE

### CONVEGNI NAZIONALI E INTERNAZIONALI

1. Belmondo S., Daghino S., Miyauchi S., Wu G., Meloni D., Aiello C., Collin S., Kohler A., Perotto S., Martino E., Jacob C.: "Polyketide synthases in the ericoid endomycorrhizal fungus *Oidiodendron maius*" 15th European Conference on Fungal Genetics (ECFG15); 17 – 20 February 2020, Rome, Italy. **Poster**

2. Belmondo S., Daghino S., Miyauchi S., Wu G., Meloni D., Aiello C., Collin S., Kohler A., Perotto S., Martino E., Jacob C.: "Characterization of polyketide synthases in an ericoid endomycorrhizal fungus using a molecular approach" 1st Conference for Young Botanists (CYBO); 6 – 7 February 2020, Genova, Italy. **Oral presentation**

3. Martino E., Daghino S., Belmondo S., Meloni D., Miyauchi S., Collin S., Kohler A., Jacob C., Perotto S.:"Polyketide synthases in the ericoid endomycorrhizal fungus *Oidiodendron maius*" 114° Congresso S.B.I. (IPSC); 4 – 7 September 2019, Padova, Italy. **Poster**

### MISSIONI PRESSO ALTRI ENTI DI RICERCA

1. Project meeting with the french partners involved on the PKS project from UMR IAM 1136 INRA/UL and UMR 7365 CNRS/UL presso UMR IAM 1136 INRA/Lorraine University. 11-15 January 2020.
2. Analisi informatiche e costruzione di alberi filogenetici presso UMR IAM 1136 INRA/Lorraine University, Champenoux (France). 28-31 August 2019.
3. Bioinformatics analysis and construction of phylogenetic trees in collaboration with Dr. Shingo Miyauchi and Dr. Wu Gang from the UMR 1136 IAM bioinformatic unit at UMR IAM 1136 INRA/Lorraine University, Champenoux (France). 1 June – 4 July 2019.

### ALTRE ATTIVITA' SVOLTE

1. Remote collaboration with Ekaterina Sheelest, Researcher at the head of the Bioinformatics Unit at the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig (Leipzig, Germany). 1 March – 31 May 2020.

2. Tutoring of 1 Master and 1 Bachelor students during internships and thesis activities (LM in Molecular Biotechnology and LT in Biological Science). Department of Life Sciences and Systems Biology, University of Turin, Italy.

Data Firma

Torino, 30/07/2020

Presa visione Dott.ssa Elena Martino

Data Firma

Torino, 03/08/2020