Mechanisms of gene regulation during the ericoid endomycorrhizal interaction

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Introduction

Mycorrhiza is the most widespread symbiosis between the roots of land plants and some specific soil fungi, and plant-fungus association in mycorrhiza is the result of a long history of co-evolution and relies on an efficient communication system that occurs before and during the fungus colonization of plant roots. In particular, ericoid mycorrhiza involves several soil fungi and the youngest lineages of a single plant family, the Ericaceae. Plants in the Ericaceae are ecologically important because the terrestrial ecosystems where they dominate are estimated to hold about 20% of the earth's terrestrial C stock (Read et al., 2004). Such infertile soils are characterised by acidic conditions and high content of potentially toxic metals and recalcitrant polyphenolic compounds, leading to very slow decomposition of the soil organic matter. The ecological success of ericaceous plants has been ascribed to the saprotrophic capabilities of their mycorrhizal fungal partners to exploit soil organic matter (David Read and Sally Smith, 2008). The first sequenced genomes of ericoid mycorrhizal fungi have indeed revealed an impressive array of degradative enzymes (Martino et al., 2018), suggesting an evolutionary strategy and an interaction with the host plant well distinct from other mycorrhizal fungi.

Whereas the molecular bases of plant-fungus interactions have been extensively investigated in some mycorrhizal model systems (e.g. arbuscular mycorrhiza), little is known on the mechanisms occurring in ericoid mycorrhiza. A mechanism of communication between fungus and plant has been recently discovered in arbuscular mycorrhiza and relies on RNA interference (RNAi) (Silvestri et al., 2019). This mechanism involves short non-coding RNAs molecules (typically 20-30 nt) that can act at transcriptional or post-transcriptional level (Wilson and Doudna, 2013). Small RNAs are incorporated into an RNA-induced silencing complex (RISC) to then base-pair with their target mRNAs, which leads to mRNA cleavage or repression of translation. Silvestri and colleagues demonstrated that *Rhizophagus irregularis* has a functional small RNA-generating pathway and 237 plant genes have been predicted to be a putative target of fungal small RNAs (sRNAs), suggesting a cross-kingdom gene regulation during arbuscular mycorrhizal colonization (Silvestri et al., 2019). There is currently no information on the

possible role of sRNAs in plant-fungus communication in ericoid mycorrhiza, and this aspect will be addressed in the project.

In addition, it has been demonstrated that ericoid mycorrhiza is instrumental in the colonization of heavy metal-enriched soils of either natural or anthropogenic origin (Bradley et al., 1981), but the mechanisms that lead to increased metal tolerance in the host plant are little understood. Due to the effects of HMs on plant production and human health, understanding of the mechanism's plants evolved to cope against the HM pollution is extremely important to prevent damage to our health. Plants evolved several strategies to defend themselves to HMs toxicity, ranging from uptake avoidance by secretion of chelating compounds or immobilization in the cell wall, sequestration in the vacuoles, partitioning HMs between shoots and roots to interacting with soil microorganisms (Casarrubia et al., 2020; Villiers et al., 2011). Arbuscular mycorrhizal fungi seem to have a role in increasing the plant tolerance to HMs by increased nutrient uptake, resulting in improved plant health; sequestration of the HMs, preventing the plant uptake; helping plants to neutralize ROS produced by HMs; and regulating plant metal import-export fluxes (Ferrol et al., 2016; Garg and Bhandari, 2014). However, while progress has been made in the understanding of the components and processes important for metal accumulation, compartmentation, and tolerance in plants, the regulatory mechanisms and network important for the modulation of these components and processes are still poorly understood (Ding et al., 2020). After small RNAs (sRNAs) discovery, increased attention has been focused on the importance of transcriptional and post-transcriptional gene regulation by RNA interference in plants in response to environmental stresses. Recent studied demonstrate that plant sRNAs and their targets play important roles in various abiotic stresses including heavy metals (Ding et al., 2016; Shriram et al., 2016; Zhou et al., 2017).

The possible mechanisms that lead to an increased plant tolerance have only recently been addressed in ericoid mycorrhiza. In particular, Casarrubia and colleagues highlighted a significant reduction, in mycorrhizal roots exposed to Cd, of transcripts known to be targets of plant Cd-responsive microRNAs (Casarrubia et al., 2020). To date, we know on the one hand that mRNA targets in plants could be involved in HM stress; on the other hand, fungal sRNAs can be a mechanism of cross-kingdom communication between mycorrhizal fungi and plants. The question this project aims to address is whether sRNAs produced by ericoid mycorrhizal fungi could be involved not only in communication with the host plants, but also target plant mRNA involved in HM stress.

Project purpose

The final aim of the research proposal is to identify sRNAs in the ericoid mycorrhizal symbiosis in order to understand: 1) whether sRNAs are produced and may play a role during the plant-fungus interaction and 2) whether and how the symbiotic fungus modulates heavy metal accumulation, compartmentation and tolerance in the plant using cross-kingdom RNA silencing strategy.

Due to the limited time of the project (one year), there will be no time to study different systems and compare them, consequently the proposed experimental framework comprises: one plant, one symbiotic fungus and one type of HM.

Studied plant will be *Vaccinium myrtillus*. The reasons to pick this plant among other are:

1. This plant has already been studied in association with fungi in HM polluted areas
2. *V. myrtillus* genome has been recently released (2021/02/23) on NCBI
3. The research group where the project will be developed has experience with this plant

Studied fungus will be *Oidiodendron maius Zn*. The reasons to pick this fungus among others are:

1. *O. maius* tolerance to HM has been the subject of my PhD, so I have experience to work with this fungus
2. *O. maius* genome is available and contains proteins belonging to the core eukaryotic RNAi machinery, in particular 2 Argonaute (AGO), 2 Dicer (DCL) and 3 RNA-directed RNA polymerase (RdRp)
3. *O. maius* is an ericoid mycorrhizal fungus and forms endosymbiosis with *V. myrtillus*
4. *O. maius* is a model system to investigate functional traits involved in fungal HM tolerance

Studied HM will be cadmium. The reasons to pick this HM among other are:

1. Cd is always toxic, even at very low concentrations
2. Cd pollution is a worldwide problem
3. There is an extensive literature on Cd-plant tolerance
4. I have experience in working with Cd

To achieve the project's aim, I want to identify the sRNAs that are produced in general during the symbiosis, and in particular the plant mRNAs - targetted by the symbiotic fungus sRNAs – that may be important for the plant response to symbiosis and to HM. Intermediate steps to achieve the final goal are:

1. Grow *V. myrtillus* on control medium and under different Cd regimes in the presence/absence of the fungus
2. Study the RNAi machinery in *O. maius*
3. Identify the sRNA population (both in roots and in shoots)
4. Characterize *O. maius* sRNA-generating loci
5. Identify *O. maius* sRNAs potentially targeting *V. myrtillus* mRNA
    a. Both already known targets and new targets
    b. Verify the presence of *O. maius* sRNA in *V. myrtillus* leaves
    c. Compare *O. maius* sRNA targets in *V. myrtillus* roots and leaves
6. Publish the findings

Methodology

Experimental design

Plant of *V. myrtillus* are grown on MMN medium as described in (Kohler et al., 2015). For Cd stressed-conditions both *O. maius* and *V. myrtillus* will be grown on the same medium containing different concentrations of Cd (added as $3CdSO_4 * 8H_2O$). A possible experimental design can be:

		Conditions (biological replicates)			
Samples	**Organisms**	**PC**	**ERM**	**MR**	**ML**
CONTROL-OM	*O. maius*	3	-	-	-
CONTROL-SYM	*O. maius* and *V. myrtillus*	-	3	3	3
CD1	*O. maius* and *V. myrtillus*	-	3	3	3
CD2	*O. maius* and *V. myrtillus*	-	3	3	3

PC = pure colture; ERM = extraradical mycelium from colonized roots; MR = mycorrhizal roots without the extraradical mycelium; ML = leaves from mycorrhizal plants

Control samples do not contain Cd. Cd1 contains 1 µM Cd, this concentration is the higher the non-mycorrhizal plant can tolerate. Cd2 will contain higher Cd concentration. RNA samples will be extracted both from roots and from leaves. Since *O. maius* can grow in vitro, Control-OM is the control in pure culture.

A total of 9 biological replicates per condition will be produced plus the fungus pure culture, leading to a total of 30 samples.

Sequencing

Total RNA will be extracted and after quantification and quality control of the nucleic acids, will be send to a company for RNA integrity check, library preparations and sequencing (estimate cost ~10000 euro).

Bioinformatic analysis

Steps of bioinformatic analysis:

1. Quality check with FastQC (Babraham Bioinformatics)
2. Reads cleaning with BBtools
3. Mapping with bowtie2 aligner
4. Genome-guided sRNA-generating loci prediction with ShortStack
5. Differential analysis with R (DESeq2 package)
6. Annotation of genomic coordinates of sRNA-generating loci with ShortStack
7. Statistical analysis with R and R Biocondactor packages
8. Identify fungus sRNAs targeting plant mRNA with sPARTA

Limiting factors

The project is ambitious and the project duration (one year) is not long. I can envisage a couple of steps that can be time consuming like the initial set up of the experiment and the time needed for plant growth and colonization (~ 45 days).

Future developments

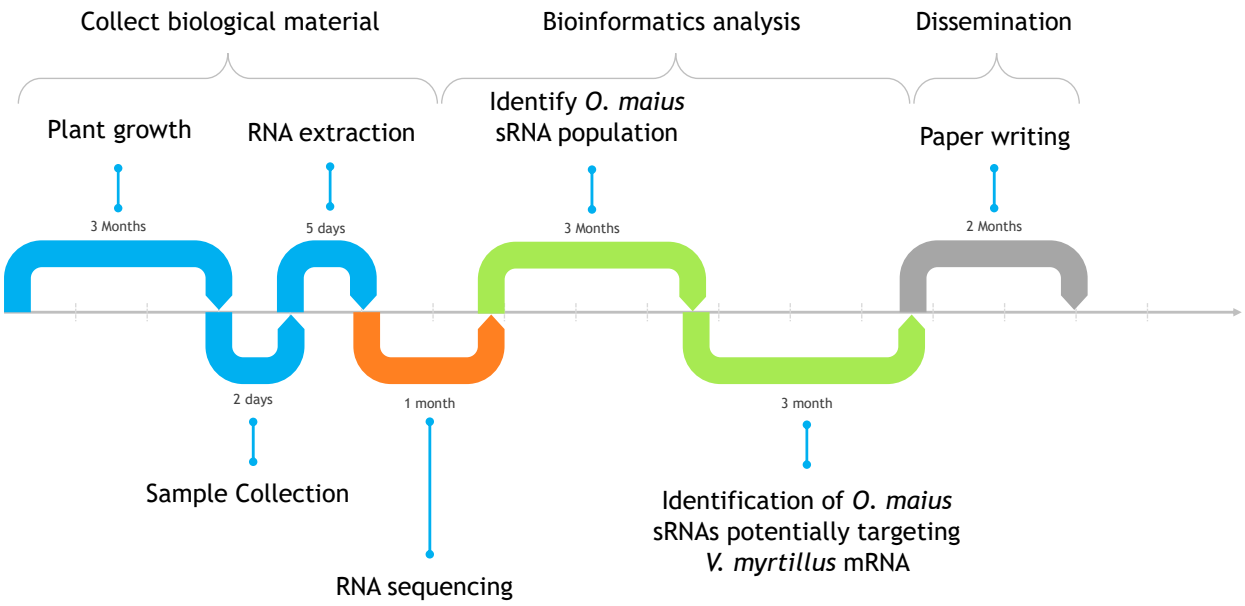
The production of RNA libraries from above-mentioned conditions will allow me to shed light on additional biological aspects that are not the direct goal of the project. The most interesting ones are:

1. Identification of plant mRNAs - targetted by the symbiotic fungus sRNAs - important for plant-fungus symbiosis
2. Identification of fungus mRNAs - targetted by the plant sRNAs - important for plant-fungus interaction

Possible future project development can be:

1. Among the identified plant mRNA targets, select few of them to silence or overexpress in order to study the effect on plant-Cd tolerance.
2. An interesting future development of the project could be the identification of a possible core set of plant mRNA targeted by fungus sRNA under different HMs growth conditions.

Time schedule



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