Uncovering the Gene Regulatory Mechanisms of Carbon Provision during the Arbuscular Mycorrhizal Symbiosis

Abstract

During AM symbiosis, plants obtain mineral nutrients from AM fungi in exchange for a significant amount of fixed carbon. Despite its ancient origins and agricultural significance, this symbiosis lacks optimisation in modern agriculture. This project focuses on understanding the transcriptional regulation of plant genes involved in carbon provision to AM fungi. Key objectives include identifying cis-regulatory elements (CREs) and transcription factors that regulate these genes during symbiosis and characterizing their roles within signalling pathways. The study focuses on *Medicago truncatula*, using techniques like ATAC-seq and promoter-reporter assays for CRE identification, transactivation assays for transcription factor identification, and transient and stable plant transformation for targeted functional analyses. Findings of this project will contribute to the optimisation of carbon provision during AM symbiosis in crops, improving their nutrient homeostasis and productivity under symbiosis, which is integral to a sustainable agriculture that leverages nutrient symbioses over heavy chemical fertilisation.

Background

Most land plants, including most staple crops, engage in an ancient, intimate, and mutualistic endosymbiosis with arbuscular mycorrhizal (AM) fungi [1]. During AM symbiosis, the two symbionts exchange nutrients, which takes place predominantly at the plant inner cortex cells containing highly branched AM fungal hyphae called arbuscules [1]. Plants receive mineral nutrients efficiently foraged from the soil by AM fungi, including up to 90% of its phosphate in some cases, while investing up to 30% of its photosynthetically fixed carbon in the form of sugars and lipids [2]. In particular, lipid provision is essential for sustaining AM symbiosis [3-7], as AM fungi are fatty acid auxotrophs lacking fatty acid synthase (FAS) genes [8].

There is an urgent need in agriculture to reduce the reliance on heavy fertilisation, and a promising yet underused strategy is to leverage nutrient symbioses to improve nutrient utilisation. AM symbiosis is as ancient as the first land plants [7], yet its benefits have not been subject to optimisation through domestication and modern agriculture. Indeed, the benefit a plant receives from AM symbiosis highly depends on the plant species and environmental conditions [2]. A key determinant of the mycorrhizal benefit for a plant is its resultant nutrient homeostasis [2]. A plant must provide enough carbon to support AM symbiosis to gain sufficient mineral nutrients from the fungus, while also fine-tuning carbon provision to prevent excess carbon cost.

A key layer of control over carbon provision is enforced by a precise spatio-temporal transcriptional regulation of plant enzymes, transporter, and transcription factor genes involved in this process (carbon provision genes). Many are upregulated in arbusculated cells only during AM symbiosis [3-7]. These include an acyl-ACP thioesterase *FatM [3-4]*, a glycerol-3-phosphate acyltransferase *RAM2 [3-4,9]*, two half-ABC transporters that potentially transport lipids to AMF, *STR* and *STR2 [10-12]*, and a AP2/EREBP transcription factor that positively regulate lipid biosynthesis,

WRI5a [6-7]. While their general expression profiles are clear, the mechanisms governing their specific expression in arbuscule-containing cells and the fine-tuning of their expression by various abiotic and biotic factors remain unresolved. I hypothesise that a combination of fungal signals, nutrient status, carbon availability, and light are involved in the regulation of carbon provision genes (Figure 1a).

Fungal signals can activate, via the common symbiosis signalling pathway, the transcription factor CYCLOPS [13], which can induce the expression of other transcription factors like RAM1 that upregulates carbon provision genes [14]. Nutrient starvation promotes AM symbiosis via the actions of the transcription factors PHR2, NSP1, and NSP2 [15-17]. The light-induced, shoot-to-root mobile transcription factor HY5 has been shown to promote AM symbiosis via increasing the biosynthesis of strigolactones [18-19], hormones used by plants to attract AM fungi [13], while its potential roles in regulating carbon provision remains unclear. Many other transcription factors induced or activated upon AM symbiosis probably play significant regulatory roles. However, for many of these transcription factors, the cis-regulatory elements (CREs) they bind to are unknown. Moreover, the interactions between these transcription factors that underpin the plant's ability to integrate various abiotic and biotic cues to fine-tune AM symbiosis and carbon provision are poorly understood.

Research Aim:

In this project, I aim to discover how carbon provision during AM symbiosis is regulated. To this end, I will first uncover the CREs that regulate the expression of carbon provision genes during AM symbiosis. I will then identify the transcription factors that act through these CREs during symbiosis. Finally, I will characterise these CREs and transcription factors within the signalling pathways they operate, aiming to precisely modulate carbon provision during AM symbiosis for enhanced crop productivity. In the long term, the findings of this project will contribute to engineering crop varieties that achieve optimal nutrient homeostasis and thus higher productivity under AM symbiosis.

Research Programme

Objective 1: Uncover the CREs that regulate the expression of carbon provision genes during AM symbiosis

The significant transcriptional reprogramming of plant metabolism, physiology, and development during AM symbiosis requires transcription factors activating or repressing gene expression through binding to or dissociating from the CREs. Since most transcription factors can only bind the exposed regions of the chromatin, changes in the chromatin accessibility landscape can gate differential gene expression during AM symbiosis. To test this hypothesis and to guide promoter CRE analysis, I will perform an Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) on bulk shoot and root tissues of *Medicago truncatula* (henceforth *Medicago*) grown under asymbiotic conditions as well as during AM symbiosis, following already-established protocols at the lab. *Medicago* is chosen as the model system due to its well-characterised mycorrhizal biology and its hairy root transient transformation allowing quick characterisation of transgenic lines. Despite not being a crop species, *Medicago* likely uses similar mechanisms to regulate carbon provision during AM symbiosis as many crops, as AM symbiosis is widely conserved across land plants [1]. ATAC-seq is chosen over in vitro methods like yeast one-hybrid (Y1H) assays because

the latter overlook the importance of CRE accessibility changes and transcription factor complexes during AM symbiosis.

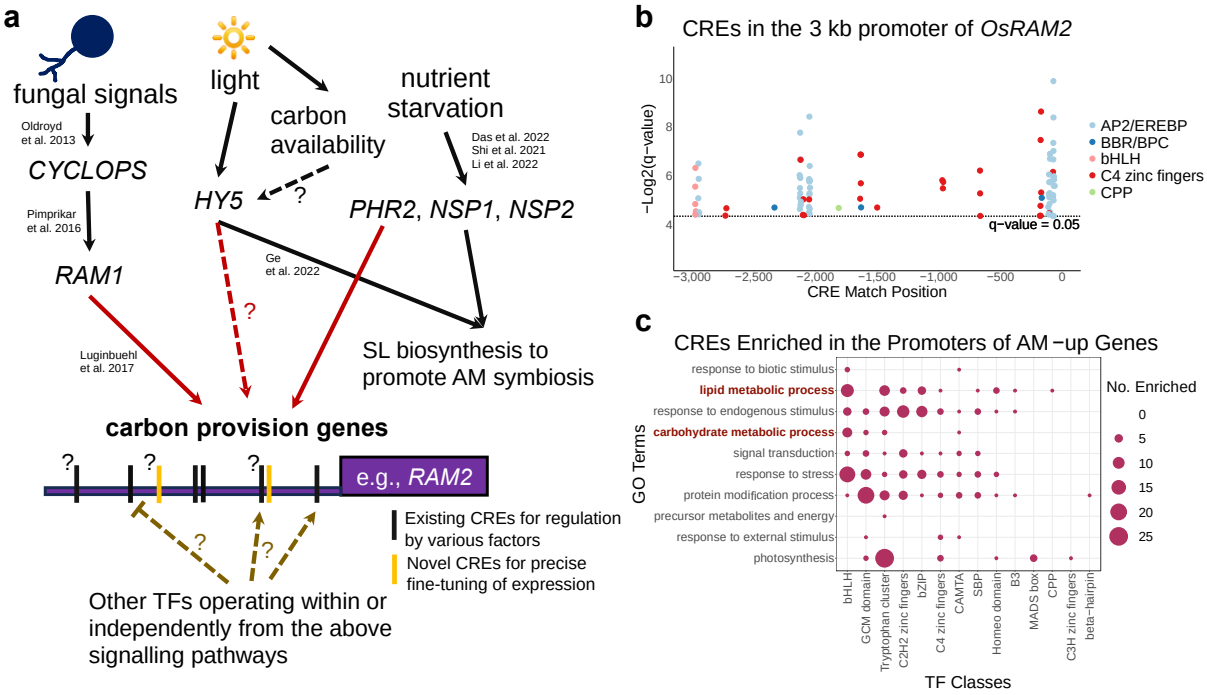

Figure 1| **Brief summary of key research questions. (a)** Overview of known and potential transcriptional regulators of carbon provision genes during AM symbiosis. **(b)** Significant CREs in the 3kb promoter region of rice *RAM2* gene found using Find Individual Motif Occurrence (FIMO). **(c)** CREs enriched in the promoters of genes belonging to the Gene Ontology (GO) terms that are overrepresented in the AM-upregulated genes in rice. This points to a myriad of transcription factors with potentially important roles in AM symbiosis that are yet to be studied. The RNAseq dataset used is by Das et al. 2022.

I will identify CREs in the promoters of carbon provision genes that exhibit increased accessibility during AM symbiosis and compare them to those found in other AM-upregulated genes. For accessible CREs particularly enriched in carbon provision gene promoters, I will examine their roles in modulating gene expression intensity and spatio-temporal patterns, using promoter:reporter assays in *Medicago* hairy roots with native and modified promoters lacking the CREs. For the CREs that activate or repress reporter expression, I will confirm their roles in AM symbiosis through complementation assays using promoters containing or lacking these CREs to drive carbon provision gene expression in their mutant backgrounds in the *Medicago* hairy root system. Mutants like *ram2* with published defects in AM symbiosis are available in the lab.

Following the hypothesis that the transcriptional regulation of carbon provision genes during AM symbiosis is not optimised, I will also test the ability of CREs uniquely found/enriched in other AM-regulated genes (Figure 1a, novel CREs) to modulate carbon provision gene expression when introduced to their promoters.

Objective 2: Identify the specific transcription factors governing the transcriptional regulation of carbon provision genes during AM symbiosis

It is important to establish which transcription factors modulate carbon provision gene expression during AM symbiosis through the identified CREs. I will first predict transcription factors bound to these CREs during AM symbiosis, based on the CRE

sequences and differential transcript accumulation of the transcription factors (using *Medicago* RNAseq datasets available at the lab). Promising candidates could include the bZIP transcription factor HY5 and members of the bHLH transcription factor family, whose CREs showed significant enrichment in AM-upregulated gene promoters (preliminary data, Figure 1c). bHLHs have well-established roles in regulating plant growth, which, like AM symbiosis, involves carbon metabolism reprogramming[20].

For the selected candidates, I will test whether they can activate carbon provision gene expression using transactivation assays in *Medicago* hairy roots and tobacco leaves, following already-established protocols available in the lab. I will combine the knowledge of spatial proximity of CREs within promoter sequences to predict the transcription factors that are likely to function in complexes. For those, I will multiplex their expression in the transactivation assay to determine their combined ability to modulate target gene expression.

I will characterise the most promising transcription factor candidates. This involves generating overexpression and CRISPR knockout mutants in *Medicago* and examining their carbon provision gene expression and colonisation by AM fungi during symbiosis. In addition, I will perform carbon tracing using stable isotopes in wild type and mutant plants grown with or without AM symbiosis. I will measure the abundance of labelled carbon in the form of soluble sugars, starch, and lipids in different plant tissues, including shoots, roots, and extraradical hyphae, using gas chromatography-mass spectrometry (GC-MS). These analyses will elucidate how carbon is differentially distributed and utilized throughout the plant-mycorrhizal network in the mutants. Lastly, to determine the signals that these transcription factors respond to, I will generate stable *Medicago* lines expressing fluorescently-tagged transcription factors. I will trace the spatio-temporal protein accumulation patterns of these transcription factors through a colonisation time course and test how these patterns change in response to altered light and nutrient conditions. This will pave ways to engineering enhanced responsiveness of carbon provision during AM symbiosis to changes the environment.

Objectives	1st year			2nd year			3rd year		
1.a. Perform ATAC-seq and select candidate CREs									
1.b. Identify CREs by promoter:reporter assays									
1.c. Confirm CRE roles by complementation assays									
2.a. Identify TFs by transactivation assays									
2.b. Generate overexpression and knockout lines of TFs									
2.c. Generate stable tagged TF lines									
2.d. Characterise mutant and tagged lines									

Reference:

[1] LH Luginbuehl and GED Oldroyd, Curr Biol **27** (17), R952 (2017).
[2] F Walder and MG van der Heijden, Nat Plants **1**, 15159 (2015).
[3] LH Luginbuehl et al., Science **356** (6343), 1175 (2017).
[4] A Bravo et al., New Phytol **214** (4), 1631 (2017).
[5] A Keymer et al., Elife **6** (2017).
[6] Y Jiang et al., Mol Plant **11** (11), 1344 (2018).
[7] MK Rich et al., Science **372** (6544), 864 (2021).
[8] N Tang et al., Front Microbiol **7**, 233 (2016).
[9] YN Liu et al., Mol Plant Microbe Interact **35** (3), 187 (2022).
[10] Q Zhang et al., Plant Cell **22** (5), 1483 (2010).
[11] C Gutjahr et al., Plant J **69** (5), 906 (2012).

[12] Q Zhang et al., Nat Commun **14** (1), 5743 (2023).
[13] GE Oldroyd, Nat Rev Microbiol **11** (4), 252 (2013).
[14] P Pimprikar et al., Curr Biol **26** (8), 987 (2016).
[15] D Das et al., Nat Commun **13** (1), 477 (2022).
[16] J Shi et al., Cell **184** (22), 5527 (2021).
[17] XR Li et al., Nat Commun **13** (1), 6421 (2022).
[18] X Chen et al., Curr Biol **26** (5), 640 (2016).
[19] S Ge et al., New Phytol **233** (4), 1900 (2022).
[20] ZF Zuo et al., Int J Mol Sci **24** (2) (2023).