Regulation of plant carbon transport mechanisms during arbuscular mycorrhizal symbiosis

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“It is a natural human impulse to think of evolution as a long chain of improvements, of a never-ending advance towards largeness and complexity – in a word, towards us. We flatter ourselves. Most of the real diversity in evolution has been small-scale. We large things are just flukes – an interesting side branch. Of the twenty-three main divisions of life, only three – plants, animals and fungi – are large enough to be seen by the human eye, and even they contain species that are microscopic. Indeed, according to Woese, if you totaled up all the biomass, microbes would account for at least 80 per cent. The world belongs to the very small – and it has done for a very long time.”

— Bill Bryson, A Short History of Nearly Everything

Abstract

The symbiotic interaction between plants and fungi is present in 70 to 90% of all terrestrial plant species. The main benefit of this symbiotic interaction is the supply of the fungi with photosynthetically fixed carbon in exchange for mainly phosphate (P), nitrogen and water for the plant. The regulatory mechanisms of carbon transport and partitioning between plant and fungi have not yet been fully discovered. Hence, it is still a matter of debate whether the mycorrhizal fungi can become parasitic in case of less beneficial conditions for the symbiotic interaction. In this study, plants in an established mycorrhizal interaction with AM fungi were shaded or optimally supplied with P to create a situation in which the symbiotic interaction is less beneficial for the plant. Additionally, a super less beneficial situation was created, in which mycorrhizal plants were both, optimally supplied with P and shaded.

Mycorrhization of plants supplied with limited P (AM-P plants) resulted in an increased growth, independent of the light treatment. Mycorrhization of plants grown under optimal P supply (AM+P) had no effect on biomass gain during growth, independent of the light treatment. The carbon transport from roots to the AM fungi (traced by ^{13}C labeling) was reduced by shading. Furthermore, the concentration as well as the ^{13}C content in neutral lipid fatty acids and phospholipid fatty acids were reduced under shading. Additionally, optimal P supply further reduced the neutral lipid fatty acid concentration. Plant biomass, fungal fatty acid contents and analysis of carbon flux from the plant to the fungi did not indicate a shift to a parasitic interaction between the mycorrhizal fungi and the plant in case of less beneficial situations. A comparative transcriptomic analysis between beneficial and less beneficial situations identified two sucrose synthases, and a hexokinase as possible candidates to be involved in sugar transport and partitioning between plant and fungi. The pathways of lipid synthesis, glycolysis, and pyruvate dehydrogenase of the tricarboxylic acid cycle were found to be partly regulated during mycorrhizal symbiosis. This study provides new insights into the regulation of carbon partitioning between plant and fungi when an established mycorrhizal symbiotic interaction becomes less beneficial for the plant. Our study demonstrates, that intensive shading for 21 days did not turn the symbiont into a parasite as the plant is able to reduce the carbon supply of the fungi.

Keywords: plant-mycorrhiza interaction, carbon limitation, phosphate supply

Zusammenfassung

Die symbiotische Interaktion zwischen Pflanzen und Pilzen bildet sich in 70 bis 90% aller Landpflanzen. Ein wesentlicher Teil der Symbiose zwischen Pflanze und Pilzen ist der Austausch von Kohlenstoff (C) aus der Photosynthese gegen Phosphat (P), Stickstoff und Wasser aus dem Boden. Gegenwärtig sind die regulatorischen Mechanismen von Kohlenstofftransport und -verteilung zwischen Pflanze und Pilzen unbekannt und es wird kontrovers diskutiert ob arbuskuläre Mykorrhizapilze in Situationen, in denen die Pflanze den Pilz nicht benötigt, parasitär werden können. Um dies zu untersuchen, wurden Pflanzen in einer etablierten Mykorrhiza-Symbiose schattiert oder optimal mit P versorgt (AM+P). Zusätzlich wurde durch Schattieren von optimal mit P versorgten Pflanzen eine besonders unvorteilhafte Situation für die Symbiose erzeugt. Die Mykorrhizierung von P-limitierten Pflanzen (AM-P) führte unabhängig von der Lichtintensität, zu einem gesteigerten Wachstum. Mykorrhizierung von AM+P Pflanzen hatte keinen Einfluss auf den Biomassezuwachs der Pflanzen während des Wachstums. Der Transport von C aus den Wurzeln zum Mykorrhizapilz (quantifiziert durch ^{13}C labeling) sowie die Konzentration der Pilzfettsäuren der Neutrallipide und Phospholipide wurde durch Schattierung reduziert. Eine optimale P-Versorgung der Pflanze reduzierte ebenfalls den C Transport in die Wurzeln, sowie die Fettsäuren der Phospholipide und der Neutrallipide. In für die Symbiose weniger günstigen Situationen, konnte bezüglich der Pflanzenbiomasse, des Pilzfettsäuregehalt und anhand der Analysen des Kohlenstoffflusses von der Pflanze zu den Pilzen keinerlei Verschiebung in Richtung einer parasitären Wechselwirkung ausgehend von den Mykorrhizapilzen detektiert werden. Eine vorteilhafte und weniger vorteilhafte Situation vergleichende Transkriptomanalyse identifizierte zwei Saccharose-Synthasen und eine Hexokinase als mögliche Kandidaten, die in den Zuckertransport und die Partitionierung zwischen Pflanzen und Pilzen involviert waren. Zusätzlich zeigten Lipidsynthese, Glykolyse und die Pyruvatdehydrogenase eine erhöhte Regulation. Diese Studie bietet neue Einblicke in die Regulation der Kohlenstoffverteilung zwischen Pflanze und Pilzen, wenn eine etablierte Symbiose weniger vorteilhaft für die Pflanze wird und zeigt dass die Pflanze in der Lage ist den Kohlenstofftransport zum Pilz zu reduzieren. Es konnte gezeigt werden, dass auch eine intensive Schattierung für 21 Tage keine Transformation des Symbionten in einen Parasiten verursacht.

Schlagwörter: Pflanze-Mykorrhiza Interaktion, Kohlenstoff Limitierung, Phosphat

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List of abbreviations

AM	Arbuscular Mycorrhiza
AM-P	Mycorrhiza infected and supplied with 20 µM P
AM+P	Mycorrhiza infected and supplied with 1 mM P
dpi	Days past infection
dpl	Days past label
ERM	Extraradical hyphae
FAME	Fatty acid methyl ester
GC-MS	Gas chromatographie with mass spectrometry
IRM	Intraradical hyphae
MGR	Mycorrhizal growth response
MST	Monosaccharide transporter
Mt	*Medicago truncatula*
NLFA	Neutral lipid fatty acids
NMR	Nuclear magnetic resonance spectroscopy
PAM	Periarbuscular membrane
PAS	Periarbuscular space
PLFA	Phospholipid fatty acids
PPA	Prepenetration apparatus
SUT	Sucrose transporter
TC	Total carbohydrates
wpi	Weeks past infection

1. Introduction

Crop production is highly dependent on fertilizers to promote plant growth and to increase crop productivity. The three major nutrients which are required by plants and provided by fertilizers are nitrogen, phosphorus and potassium (Roy-Bolduc and Mohamed 2011). In this composition, phosphorus represents the most critical compound, as it is a limiting factor for crop yield and in addition, a non-renewable resource (Cordell et al. 2009; Plenchette et al. 2005; Vance et al. 2003). During the next 50 to 125 years, the global resources of phosphate could be critical depleted, due to the predicted need for food and current phosphorus extraction rates (Cordell et al. 2009; Vance et al. 2003; Gilbert 2009). Today, agriculture is highly dependent on external phosphate supply and the input of phosphate-based fertilizers has increased from 9 million to 40 million tons per year between the years 1960 and 2000 (Vance et al. 2003). This potential crisis of agriculture phosphate availability, as it was pointed out by Gilbert (2009), needs to be addressed with several approaches against the issue of phosphate scarcity. One of these approaches is the use of mycorrhizal fungi which can supply plants with additional phosphate (Smith and Gianinazzi-Pearson 1990). Today, the application of mycorrhizal fungi is not integrated in agricultural systems in Europe and North America. In countries such as Cuba, India and Mexico, striking results for mycorrhizal application in agriculture have already been made (reviewed in Zimmerman et al. 2009). Up to now, it is not known in detail, how the symbiotic interaction between mycorrhizal fungi and host plants is regulated. Plants adapt to phosphate starvation with several mechanisms, which could possibly influence the symbiosis. Additionally, the consequences of less beneficial situations on the symbiosis is poorly understood. The less beneficial situation for the symbiosis could be an optimal P supply for the plant, due to fertilization. Such a situation could occur if crops are not homogenously inoculated with mycorrhizal fungi and phosphate fertilization becomes necessary. Another less beneficial situation could be a reduction of carbon availability for the plant, due to shading. These shading situations can occur due to cloudy weather, shading by neighboring plants or the development of microbial biofilms on leaves. To apply mycorrhizal fungi in agriculture systems it is important to understand how the symbiotic interaction is regulated. This knowledge can be used to increase yield and avoid yield losses.

1.1 The symbiotic interaction between plants and mycorrhizal fungi

The symbiotic interaction between plants and fungi occurred early in evolution (Redecker 2000) and can be found in nearly all ecosystems. Basically, there are two types of mycorrhizal interactions. The ectomycorrhiza remains, as its name indicates, outside of the plant cells and builds a network around the plant root (Frank 1885). Ectomycorrhiza, which are mainly represented by basidiomycetes and to a lower percentage by ascomycetes, can be found predominantly at trees that are growing in temperate forests. In contrast, endomycorrhiza grow inside the plant cell. Endomycorrhiza can be divided into three groups: orchid, ericoid and arbuscular mycorrhiza (AM). About 70 to 90% of all terrestrial plant species are associated with fungi belonging to the monophyletic phylum of *Glomeromycota* (Schüβler et al. 2001; Hibbett et al. 2007; Smith and Read 2008). About 18% of vascular plants are estimated to not interact with arbuscular mycorrhizal fungi and 6% do not interact with mycorrhizal fungi at all (reviewed in Brundrett 2009). These “non-host” plants can be divided in two groups: one group of plants with highly specialized nutrition like carnivores, parasites, species with cluster roots, and species which mainly grow in wet, arid, saline, very cold and disturbed habitats (reviewed in Lambers et al. 2010 and Brundrett 2009). The other group consist of species of the families Brassicaceae, Polyganaceae, Amaranthaceae and Caryophyllaceae (reviewed in Wang and Qiu 2006). The high number of angiosperm species which can be colonized by mycorrhizal fungi may suggest that these plants are harboring a special genetic program to perform symbiotic interactions with mycorrhizal fungi (Oldroyd et al. 2009; Parniske 2008). AM fungi are considered to be ancient asexuals with an aseptate, coenocytic hyphal network. The spores contain hundreds of nuclei and up to now there are no confirmed reports of a sexual stage in the life cycle, but there is a possibility of exchange and recombination of genetic material via the fusion of hyphae (Giovannetti et al. 2004).

The main benefit of the symbiotic interaction is the exchange of carbon, photosynthetically fixed by the plant, for mainly phosphate, nitrogen and water, supplied by the fungus (Smith and Smith 1990; Smith and Smith 1997). The extensive hyphal network of the fungi is able to acquire nutrients behind the root depletion zones (Smith and Smith 1990). Furthermore, plants can benefit in many ways from the symbiosis with the fungus. The plant growth and reproduction is supported, and the

resistance to pathogens as well as the tolerance to abiotic stress is increased (Valentine et al. 2001; Wu and Xia 2006; van Wees et al. 2008; Liu et al. 2007).

In exchange for nutrients, 20 to 30% of photosynthetically fixed carbon is transported to the obligate biotrophic fungi (Bago et al. 2003; Douds, 2000; Jakobsen and Rosendahl 1990). This means that approximately 5 billion tons of carbon are estimated to be consumed by the fungi, per year (Parniske 2008).The symbiotic interaction between plants and fungi contributes significantly to global phosphate and carbon cycling and influences primary productivity in terrestrial ecosystems (Fitter, 2005). Up to now, it is not well understood which regulatory mechanisms are underlying this symbiosis, but it was shown, that the amount of colonization typically decreases with increasing nutrient availability (Olsson et al. 2010).

When plant cells are infected by a mycorrhizal fungus, they actively prepare the intracellular environment for the mycorrhizal fungal hyphae (Genre et al. 2005; Genre et al. 2008). A prepenetration apparatus (PPA) is built in the plant cell, defining the intracellular passage for the fungus. A PPA is a subcellular structure, build by a cytoplasmic bridge across the vacuole of the host cell. This cytoplasmic bridge contains microtubes and microfilaments. Together with cisternae from the endoplasmic reticulum a hollow tube in the PPA is formed, which connects the nucleus with the site of the apressorium (Genre et al. 2005; Siciliano et al. 2007).

1.2 Arbuscules: The main exchange site between plant and arbuscular mycorrhizal fungi

During arbuscular mycorrhizal symbiosis, tree-shaped structures are formed within the plants cells. This so called arbuscules are important sites for the uptake of nutrients like phosphate and they are a result of a coordinated development of plant and fungus (Harrison 2002). The fungal hyphae enter root cortical cells and form the arbuscules generated by repeated hyphal branching. A characteristic of arbuscules is the large membrane surface, which represents the interface between plant and fungi (Parniske 2008; Bapaume and Reinhardt 2012). This so called periarbuscular membrane (PAM) contains a special protein composition delivered from the plasma membrane and, in addition, transporters which play a keyrole in the nutrient exchange between fungi and host (Wright et al. 1998a; Boldt et al. 2011; Graham and Abbott 2000). The structure can vary depending on the fungal and host genotype (Smith and Read, 2008), but is

not dependent on the environment they are growing in (Smith and Smith, 1997). Two different forms of arbuscules are known (Smith and Smith, 1997). The arnum type, in which hyphae penetrate the cell walls at the inner cortex, then branch in the apoplast and finally form the above described tree-like structure. The other arbuscule type is called paris type. Here, the hyphae grow from cell to cell and build an intracellular structure which is coil shaped. These structures are growing in the entire root cortex, and several arbuscules can be found in one cell. Nutrients and perhaps signals are exchanged via this plant-fungal interface. The periarbuscular space exists between the two periarbuscular membranes, the fungal plasmamembrane and the periarbuscular membrane (Harrison 2005). The mycorrhiza specific phosphate transporter PT4 for example, is located in the PAM (Harrison 2002). Additionally, a H+-ATPase is localized in arbusculated cells and provides the proton gradient for active transport processes across the PAM (Krajinski et al. 2002). Arbuscules have a lifetime of around 8.5 days, which is shorter than the lifetime of the host cells (Alexander et al. 1989). Arbuscules grow until their maximum size is reached. After that, degradation and senescence are induced and the arbuscular hyphae are separated by septation from the of remaining cytoplasm (Javot et al. 2007). Subsequently, arbuscules collapse and disappear (Fig. I.1)

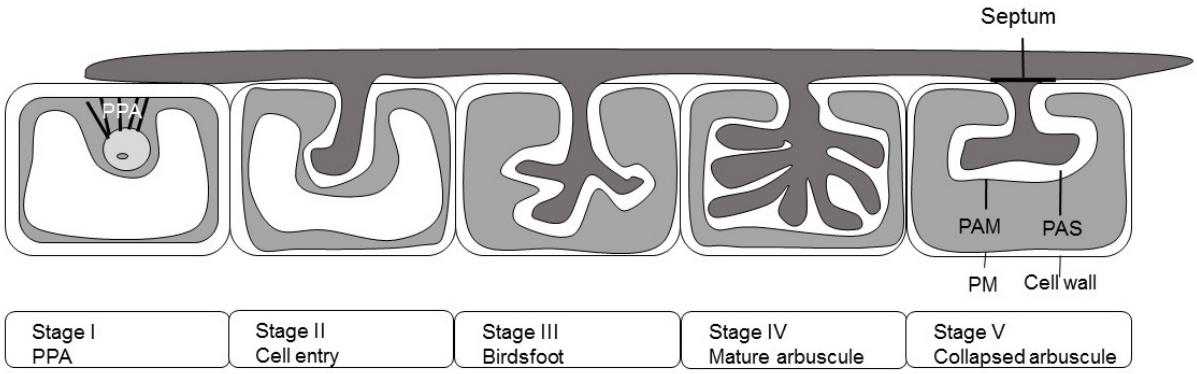

Fig. I.1 **Stages of arbuscule development**. In Stage I the prepenetration apparatus (PPA) is formed, displayed by a conically arranged microtubule array (Genre et al. 2005; 2008). In Stage II the fungus enters the cell and the arbuscule trunk is formed. Stage III is characterized by rough and low branching with a pattern similar to a bird´s foot. In Stage IV mature arbuscules with a tree-like structure are built. In Stage V arbuscules collapse and are disconnected from the hyphal network via a septum. PM: peripheral plasma membrane; PAS: periarbuscular space. Figure adapted from Gutjahr and Parniske (2013).

1.3 The role of carbohydrates in the mycorrhizal symbiotic interaction

1.3.1 Sucrose as the main transport form of carbohydrates in plants

Mycorrhizal fungi are acting as an additional sink for the host plant, which results in an increase of sucrose export from the source organs (Wright et al. 1998a; Boldt et al. 2011). Sucrose is mainly synthesized in the cytosol of photosynthetically active cells. To a minor part, sucrose can result from a breakdown of starch and lipid reserves. It has been hypothesized that disaccharides are translocated in the phloem by pressure flow (Münch 1930). This high concentration chases the sieve sap towards the sites of low turgor which is caused by the escape of photosynthates at the sink ends (VanBel, 1995; Knoblauch and Peters 2010). While sucrose is transported in the phloem and can be unloaded for the supply of flanking tissues (Thompson 2006). When plants are colonized by heterotrophic organisms like arbuscular mycorrhiza, sugars can be transported via the phloem to non-plant sinks which have an increased sugar demand (Doidy et al. 2012).

1.3.2 Carbohydrate uptake by mycorrhizal fungi

During symbiotic mycorrhizal interaction respiration is increased by the fungi resulting in a higher substrate demand. Caused by the interaction with the mycorrhizal fungi, the plant metabolism is reorganized and photosynthesis, carbon metabolism and transport processes are adapted to the symbiosis (Wright et al. 1998a; Wright et al. 1998b; Black et al. 2000). Arbuscules are located in the cortical cells near the epidermis which results in the direct access to the carbon in the phloem (Blee and Anderson 1998). NMR spectrometry experiments demonstrated that intraradical hyphae of mycorrhizal fungi are not able to take up sucrose, but hexoses mainly represented by glucose and to a smaller extent fructose (Shachar-Hill et al. 1995; Solaiman and Saito 1997; Pfeffer et al. 1999). Up to now, no sucrose cleaving activities have been identified in mycorrhizal fungi (Schubert et al. 2004). Therefore, sucrose has to be cleaved by apoplastic and symplastic invertases (Sturm 1999) and/or sucrose synthases (Huber and Akazawa, 1986) before the arbuscular mycorrhizal fungus is able to take up the carbohydrates. Inverse carbon transport from the fungus to the plant was not detected

(Pfeffer et al. 2004). Additionally, the expression profiles of genes which are involved in sucrose breakdown (Blee and Anderson 2002; Hohnjec et al. 2003) and sugar transport are upregulated during mycorrhizal interaction (Harrison 1996; Garcia-Rodriguez et al. 2005).

In a model for sucrose transport from plant to fungi adapted from Manck-Götzenberger and Requena (2016) the SWEET transporters play an important role (Fig.I.2). Sucrose reaches the cortex symplastically and can be directly exported into the periarbuscular space (PAS) and to the apoplast of cells which are directly in contact with the fungus by SWEET transporters such as SWEET12a. The sucrose in the PAS can then be cleaved with the help of cell wall invertases (CWIN) and glucose would be taken up into the fungal cell by the fungal monosaccharide transporter MST2 (Helber et al. 2011). Sucrose can also be cleaved in the cytoplasm by sucrose synthase and/or cytoplasmic invertases, which could increase the glucose concentration, so that glucose is further exported into the PAS.

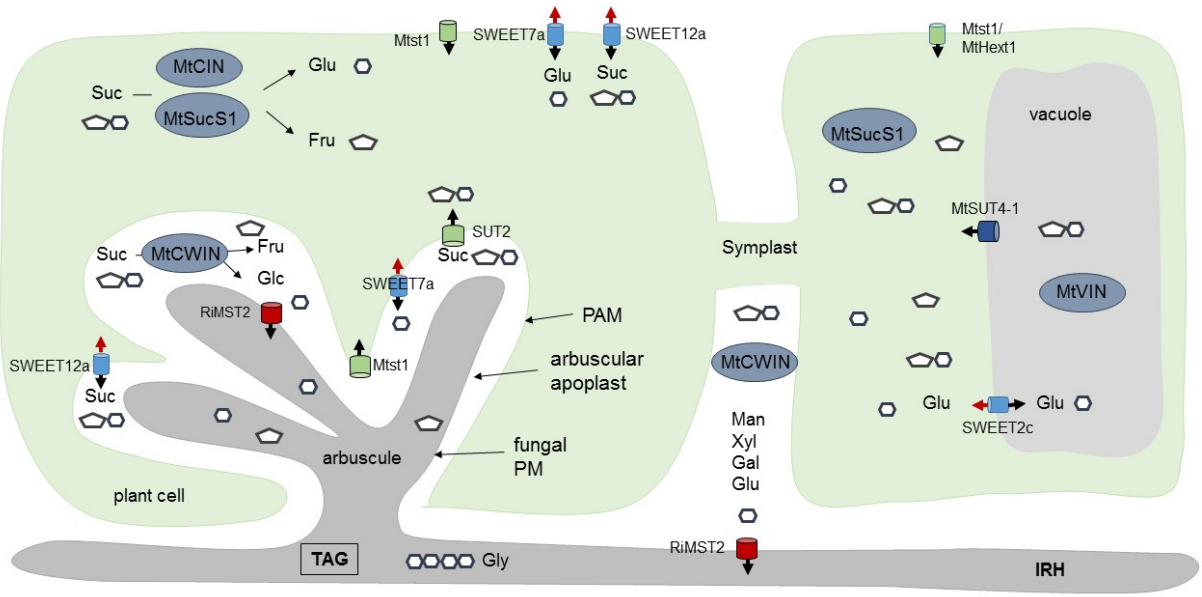

Fig. I.2 **Model of sugar transport and partitioning during arbuscular mycorrhiza symbiosis.** Sucrose (Suc) and glucose (Gluc) are imported into arbusculated cells symplastically or by transporter (e.g. Mtst1, SWEET7a and 12a). In the cytoplasm, sucrose can be cleaved into glucose and fructose (Fru) by invertases (MtCIN) or sucrose synthases (MtSucS1). The hexoses are then transported via e.g. SWEET7a or 12a through the periarbuscular membrane (PAM) into the arbuscular apoplast. In the arbuscular apoplast, sucrose could also be cleaved by a cell wall invertase (MtCWIN). Sucrose which is in the vacuole can also be cleaved into hexoses via a vacuolar invertase (MtVIN) and transported in the cytoplasm via the SWEET2c. Once cleaved, hexoses can be taken up via the RiMST2 transporter by the fungi. The transporter can also take up mannose (Man), xylose (Xyl), galactose (Gal) and glucose from the apoplast. Hexoses are further transformed into triacylglycerade (TAG) and glycogen (Gly). The figure is adapted from Manck-Götzenberger and Requena (2016) and (Doidy 2012).

When hexoses reach the intraradical mycelium (IRM), there are two routes for the transfer into the extraradical mycelium (ERM) (Bago et al. 2003). Hexoses can be converted into glycogen or triacylglycerol. ^{13}C labeling experiments showed, that the first carbon pools from hexose which are found to be labeled in the intraradical mycelium are trehalose and glycogen (Shachar-Hill et al. 1995).

The regulation of carbon partitioning in the interaction between plant and fungi is not well understood. Several candidates like carbohydrate transporters and sucrose cleaving enzymes which are specifically induced during symbiosis, came into focus during the last years (Hohnjec et al. 2003; Schaarschmidt et al. 2006) .

The restructuring of cells due to arbuscule development induces changes in transcript levels of hundreds of genes (Gaude et al. 2012; Gomez et al. 2009; Hogekamp et al. 2011). The most prominent functional groups which showed an induction of transcripts were related to transport processes, transcriptional regulation and lipid metabolism (Gaude et al. 2012). Furthermore, genes which are involved in sucrose breakdown (Blee and Anderson 2002; Hohnjec et al. 2003; García-Rodríguez et al. 2007) and sugar transport are optimized for mycorrhizal energy supply (Harrison 1996; Garcia-Rodriguez et al. 2005). The highest number of induced genes was found in cortical cells next to arbusculated cells (Gaude et al. 2012).

1.3.3 Carbohydrate transporter exclusively influenced by mycorrhization

In *G. mossae* colonized tomato plants, SUT1 and SUT4 (sucrose transporter) family members were found to be upregulated in leaves and roots (Boldt et al. 2011), which could show a possible role in sugar allocation towards the mycorrhizal fungi. The role of SUT1 may be specific for the individual to the AM species. A downregulation for this transporter was shown in *Rhizophagus intraradices* colonized plants Ge et al. (2008). Additionally, the expression of the SUT1 transporter correlates with the level of phosphate supply. An overexpression of this transporter doubled the colonization of mycorrhiza when phosphate was highly concentrated (Gabriel-Neumann et al. 2011). Some of the transporters which are of importance during mycorrhizal symbiosis are exclusively expressed in arbusculated cells and hyphae. One of these transporters is the MST2 high affinity monosaccharide transporter, which can be found in arbusculated cells and intercellular hyphae (Helber et al. 2011). MST2 is able to transport several monosaccharides, including pentoses, but the highest affinity is found

for glucose. The knockdown of MST2 interferes with the formation of arbuscules (Helber et al. 2011). Some of the SWEET transporters are also induced by the fungus in arbuscule containing cells (Manck-Götzenberger and Requena 2016).

Not only transporters are upregulated during mycorrhization. Mycorrhizal roots show a significant increase in cell wall bound invertase activity (Schaarschmidt et al. 2006). However, increasing invertase activity did not increase colonization rates (Schaarschmidt et al. 2007). The importance of sucrose and sucrose cleaving in mycorrhizal roots is demonstrated by the finding that a higher sucrose level can be observed in mycorrhizal roots, but glucose and fructose levels are lower, compared to non-mycorrhizal roots (Campos-Soriano and Segundo 2011). Transcript amounts of MtSUT1 increased in cells of mycorrhizal roots which were not directly infected (Gaude et al. 2012). MtSUT1 has a function in export of sucrose from the vacuole and mobilization of carbohydrates which are close to mycorrhizal structures in the root. The uptake of glucose by the fungi is carried out by the GiMST2. This transporter can also be induced in the ERM by Xylose (Helber et al. 2011). The localization of GiMST2 in arbuscules and hyphae suggests a sugar transfer between host and fungi in both structures. Starch granules are typically absent from arbusculated cells, indicating the sink strength of the mycorrhizal fungi (Bonfante 2001; Kovács et al. 2003).

1.3.4 Mycorrhizal impact on invertases and sucrose synthases

Sucrose synthase and invertase activities have been found to be high in meristems of sink tissues (Koch 1996; Chin and Weston 1973). Vacuolar invertases and sucrose synthases of cortical cells are responsible for a gradient allowing the diffusion of sucrose through plasmodesmata. Hohnjec et al. (2003) showed the promoter activity of a *Medicago* sucrose synthase gene (MtSucS1) in colonized cells and cells next to arbuscules and hyphae. Schaarschmidt et al. (2006) found an increased expression of a cell wall bound invertase in colonized cells and in the central cylinder. Several experiments showed an upregulation for all type of sucrose cleaving enzymes when arbuscular mycorrhiza were present (Wright et al. 1998a; Ravnskov et al. 2003; García-Rodríguez et al. 2007; Tejeda-Sartorius et al. 2008). MtSucS1 antisense lines showed a downregulation of carbon related genes and that arbuscules could not develop (Baier et al. 2010). Plants which have a decrease in acid invertase activity

showed a reduction in mycorrhization, whereas an overexpression did not increase the fungal growth (Schaarschmidt et al. 2007).

1.4 Influence of mycorrhization on lipid metabolism

1.4.1 Synthesis of lipids in plants

Lipids play a major role in plants. For example, structural lipids are synthesized to function as membrane constituents. Furthermore, carbohydrates can be stored in the form of lipids. Membrane and storage lipids consist of a trivalent alcohol with three acyl residues. Storage lipids are triacylglycerine or triacylglycerides, whereas phospholipids contain two esterified acyl residues and a polar substituent like phosphate (phospholipids) or sugar moieties (glycolipids). During lipid metabolism, plastids, cytoplasm and endoplasmic reticulum (ER) are involved. In plants, the synthesis of fatty acids takes place in plastids, whereas fungi synthesize their fatty acids in the cytoplasm (Los and Murata 1978). In plastids, fatty acids are synthesized from acetyl-CoA by pyruvate dehydrogenase or from acetate (Harwood 1996). The synthesis is interrupted as soon as C16 or C18 chains are synthesized, which are namely palmitoyl-ACP (16:0-ACP) and stearoyl-ACP (18:0-ACP). The products of fatty acid synthesis in plastids are used for membrane lipid synthesis or they are exported into the cytoplasm (Browse et al. 1993). Acyl-CoA-synthetase generates acyl-CoA from the fatty acids under consumption of ATP (Kornberg and Pricer 1953). The acyl-CoAs (palmitoyl-CoA, stearoyl-CoA and oleoyl-CoA) can then be connected in several ways. Firstly chain elongation at the ER can result in producing storage lipids. A second option is the integration into membrane lipids. And finally, the acyl-CoAs can participate in the synthesis of the polyunsaturated fatty acids linoleic acids and linolenic acids (Browse and Somerville 1991). The plastidic glycolysis plays a major role in converting sugars for *de novo* fatty acid biosynthesis. Pyruvate dehydrogenase acts as a primary source of acetyl-CoA for lipid synthesis (Oliver et al. 2009). Based on this finding, Daher et al. (2017) suggested depending on the mycorrhizal development, the fatty acid biosynthesis pathway may be rapidly starved for plastidic carbon sources necessary to assemble lipids. Saturated and monosaturated fatty acids are mostly synthesized in the plastid stroma to be further used for synthesis of phospholipids at the ER.

1.4.2 Synthesis of lipids in arbuscular mycorrhizal fungi

During root colonization, plant genes for lipid biosynthesis are induced to produce lipids for the establishment of the periarbuscular membrane (Gaude et al. 2012). The intraradical growth, development of fungal storage organs and membrane proliferation requires large amounts of fatty acids. Studies of Pfeffer et al. (1999) and Trepanier et al. (2005) suggested that the *de novo* synthesis of fatty acids is absent from the extraradical mycelium (ERM) and germinating spores, but the intraradical mycelium (IRM) is able to synthesize 16:0 fatty acids.

AM fungi receive hexoses directly from the host and transform them into trehalose and glycogen, which are the characteristic fungal carbohydrates (Shachar-Hill et al. 1995). In the IRM, carbohydrates are mainly stored in the form of triacylglyceride (TAG) (Beilby and Kidby 1980; Jabaji-Hare 1988; Pfeffer et al. 1999). After building vesicles as storage organs in the IRM, some of the storage lipids are transported into the ERM (Pfeffer et al. 1999). When TAG reach the ERM, they can be transformed into carbohydrates via the glyoxylate cycle (Fig. I.3).

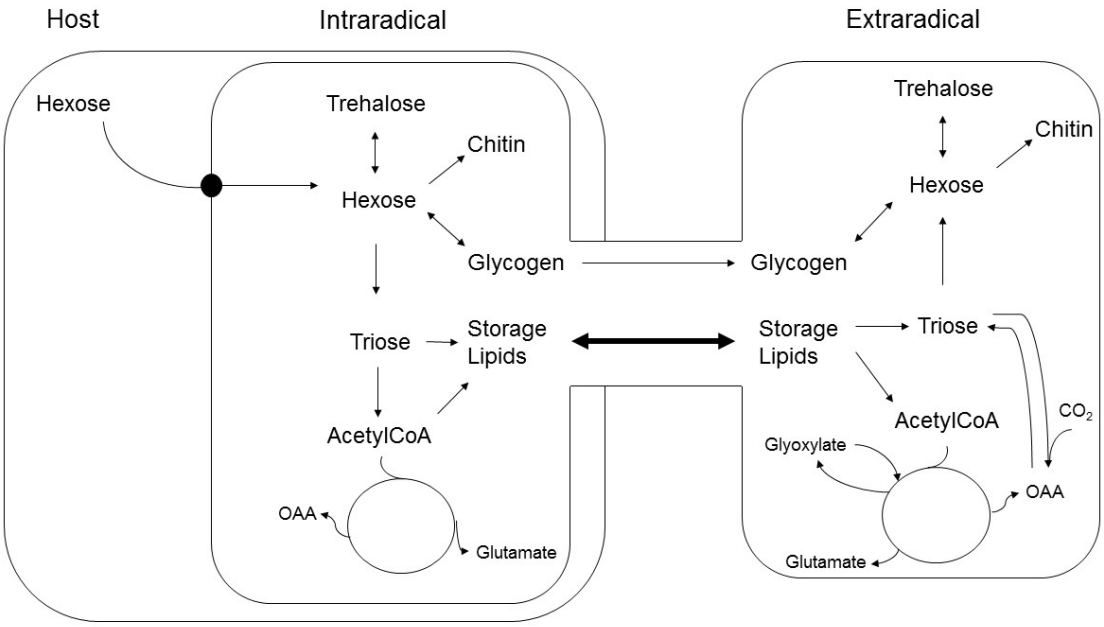

Fig.I.3 **Scheme of proposed routes by which carbon can move from the IRM to the ERM during mycorrhizal symbiosis.** Carbon, represented by hexoses can be taken up from host and is then converted to storage lipids in the IRM. The lipids and glycogen are translocated from IRM to ERM. The storage carbohydrates, represented by glycogen and trehalose and the structural carbohydrates, namely chitin, are synthesized in the ERM. The figure is adapted from Bago et al. (2002a; 2002b; 2003)

The lipid bodies, which are built by the fungi, were found in arbuscular trunks, intercellular hyphae, extraradical spores and germ tubes (Bonfante et al. 1994). Most of the lipid bodies were observed to move with the cytoplasmic stream, others are stationary (Bago et al. 2002a). These so called oleosomes consist of insoluble lipids with a surrounding phospholipid monolayer, often with inserted proteins of the TAG metabolism (Kamisaka and Noda 2001). The large amounts of lipids which are present in the AM fungal hyphae of the *Glomales* reveal that they are “oleogenic” fungi. Oleogenic fungi are able to accumulate about 25% of their dry weight as lipids (Jabaji-Hare 1988). It was postulated that the regulation of lipid utilization would be at the level of transcription and/or posttranscriptional activation of the proteins responsible for directing carbon from lipid bodies into storage, catabolism or anabolism (Bago et al. 2002a).

The fatty acids typically found in AM fungi are 16:1ω5 and 18:1ω7 (Nakano et al. 2001; Pearson and Jakobsen 1993). Additionally polyunsaturated 20-carbon fatty acids can be found. To estimate the biomass of AM mycelium in soil and roots the fatty acids in PLFA and NLFA can be used (Olsson et al. 1995). *Rhizophagus intraradices* contains 50 to 70% of 16:1ω5 fatty acids in their NLFA (Olsson and Johansen 2000) which is uncommon for other fungi (Müller et al. 1994; Olsson 1999). The specific fatty acids in PLFA and NLFA show the relation between biomass of mycelium and storage structures, which can be used to determine the amount of carbon allocation to the fungi (Peng et al. 1993; Olsson et al. 1997). Olsson and Johansen (2000) hypothesized that energy stores in the mycelium can mainly be found in roots and that successful root colonization is a prerequisite for accumulation of energy storage products in external mycelium.

The use of PLFA analysis was first applied to soil by Frostegård (Frostegård et al. 1993). PLFA can be used as biomarker for the living hyphae because they are rapidly hydrolyzed after cell death.

1.5 Adaptions of plants to phosphate deficiency

The interactions between plants and mycorrhizal fungi are mostly analyzed under phosphate limited conditions. The phosphate limitation itself influences the plant among other factors in growth and carbohydrate metabolism.

1.5.1 Plants adapt to phosphate starvation with changes in root morphology

Plants can increase their ability to acquire P from soil and improve their ability to reutilize P internally via transcriptional, biochemical and physiological responses (Vance et al. 2003; Franco-Zorrilla et al. 2004). There are some adaptions to P starvation like a reduced primary root growth, increased lateral root number, increased root hair number and length as well as anthocyanin accumulation (Miura et al. 2005). Concentrations as well as the transport from the shoots to the roots of the phytohormones auxin, ethylene and cytokinin have to be balanced. Hormone regulation and distribution is maybe also involved in systemic responses to P starvation (Abel et al. 2002; Franco-Zorrilla et al. 2005; Hammond et al. 2004; Vance et al. 2003). A low rhizosphere P concentration, combined with an increased ethylene production results in the initiation and elongation of root hairs (Zhang et al. 2003). This increases the root surface and as a result, an increased amount of soil can be reached for P acquisition (Zhang et al. 2003). Many plant species respond with the formation of root clusters to P starvation (Lambers et al. 2006). In these clusters, root exudates can be concentrated and insoluble organic and inorganic sources of P can be exploited (Shen et al. 2005). Cytokinin concentrations were shown to decrease in roots when plants were starving due to a lack of P (Kuiper et al. 1988).

1.5.2 Plants adapt to P starvation by modifying the rhizosphere

Phosphate starving plants are able to modify their rhizosphere via secretion of organic acids (e.g. citrate, malate or oxalacetate) to release inorganic phosphate which is bound to clay particles (Lopez-Bucio et al. 2000a; López-Bucio et al. 2000b). Additionally to the release of organic acids, plants increase the amount of phosphate transporters to optimize the phosphate uptake (Versaw and Harrison, 2002). Three Pi transporter subfamilies were identified, namely Pht1, Pht2 and Pht3 (Daram 1999; Rausch and Bucher 2002; Mudge et al. 2002; Takabatake et al. 1999).

1.5.3 Phosphate starvation influences the carbohydrate metabolism

Several studies indicate that shoot-derived carbohydrate signals, like sucrose, are involved in the systemic control of plant P starvation responses (Muller et al. 2007). The main problem of studies of phloem-sucrose signaling is that sucrose participates in many mechanisms. Sucrose participates in signaling, drives the phloem transport, delivers C skeletons to sink tissues, influences the osmotic status of tissues and is further rapidly broken down into glucose and fructose (Franco-Zorrilla et al. 2005; Gibson 2005).

Phosphate starvation has direct consequences for photosynthesis, glycolysis and respiration (Plaxton and Carswell, 1999). When inorganic phosphate reserves in the vacuole decrease, a lack of Pi occurs in the cytoplasm which inhibits photosynthesis and results in an inhibition of Calvin Cycle enzymes. In turn, this results in a feedback inhibition based on pH changes across the thylakoid membrane or redox state of electron carriers (Plaxton and Carswell, 1999). Another effect of decreasing cellular Pi contents, is a reduced activity of ATP synthases in the thylakoid membranes and RuBisCo resulting in a reduced but not terminated carbon assimilation (Cakmak et al. 1994).

1.5.4 Phosphate starvation influences transcript amounts of genes of the carbohydrate metabolism

Transcriptional studies of phosphate starving plants showed a rapid change in shoot carbohydrate metabolism (Muller et al. 2007). Transcripts encoding invertases, sucrose synthase, sucrose phosphate synthase and sucrose-phosphate phosphatase were found to be differentially expressed under phosphate starvation (Hammond et al. 2005; Uhde-Stone et al. 2003). Additionally, carbohydrate transporters of shoots and roots responded to P starvation (Muller et al. 2007). Hexokinase acts as a sensor for glucose and glucose signaling pathways (Moore et al. 2003). During phosphate starvation, hexokinase activity decreases (Rychter and Randall 1994).

1.5.5 Carbon availability can influence plant responses to phosphate starvation

When sucrose is reduced by decreasing photosynthetic capacity via shading, phosphate starving plants show a reduction in transcripts which are otherwise known to be upregulated during phosphate starvation (for example PT1, Liu et al. 2005). This means, that carbon limited plants with a reduced sucrose phloem transport are not able to react to phosphate starvation fast enough (Hammond and White 2008).

1.6 Mycorrhizal symbiosis in less beneficial situations

It is unclear what happens in less beneficial situations when plants are already in an established symbiotic interaction with arbuscular mycorrhizal fungi. A less beneficial situation could be an optimal phosphate supply. Due to this the plant would not need the fungi anymore for a better phosphate supply, but the fungi would still gain carbon from the host.

Plants colonized by AM fungi change their phosphate uptake from the direct rhizodermal uptake pathway to the symbiotic-uptake pathway, which also includes a switch of host phosphate transporters (Smith 2003; Yang et al. 2012). In contrast, the symbiotic pathway of phosphate uptake is suppressed during high phosphate availability (Breuillin et al. 2010). High phosphate availability can have several effects on AM development. These effects encompass the quantitative suppression of colonization and abnormal arbuscule formation in petunia (Breuillin et al. 2010) to a decrease of hyphopodium induction and nearly no intraradical colonization in pea (Balzergue et al. 2011). But the particular mechanisms inhibiting the growth of AM fungi in host roots under sufficient phosphate conditions are unknown.

As it is unknown whether the plant or the fungi controls the amount of arbuscules in the roots, the investment of energy in new arbuscules is used as a sign for mutualism, whereas an investment in fungal storage organs like vesicles is an indicator for a shift to parasitism of the fungus (Johnson 1993). Another less beneficial situation could be carbon limitation of mycorrhizal plants. When plants would not have enough carbon to supply their own metabolism, the fungi could possibly turn useless. However, responses of mycorrhizal plants to carbon limitation caused by shading are variable (reviewed in Konvalinkova and Jansa 2016). It was reported that the limitation of plant

photosynthesis reduced mycorrhizal growth response and inorganic phosphate uptake (Tester et al. 1985). Up to now it is not even clear whether the decrease of arbuscular mycorrhizal fungal colonization of roots under low light is actively driven by the host plant or just a consequence of the lack of assimilates within the roots (Konvalinkova and Jansa 2016). Another theory for the regulation of the symbiotic interaction between plant and fungi in a less beneficial situation assumes that carbohydrate and phosphate distribution are controlled by the actual needs of the symbionts (Landis and Fraser 2008). Accordingly, plants should not supply AM fungi with carbohydrates in situations where growth and/or reproduction are not primarily limited by nutrients, but by energy availability such as occurring light deprivation (Konvalinkova and Jansa 2016). The extent to which plants can reduce the carbon flux to arbuscular mycorrhizal fungi is unknown (Konvalinkova and Jansa 2016).

1.7 Aim

As it is unknown how light limitation and/or optimal phosphate supply by fertilization can influence yields in agricultural systems which are influenced by mycorrhizal fungi, it was analyzed whether plants are able to control the carbon partitioning to fungi when a less beneficial situation occurs. To this end, *Medicago truncatula* plants under phosphate limitation were inoculated with the arbuscular mycorrhizal fungi *Rhizophagus intraradices*. After establishing the symbiotic interaction, mycorrhizal plants were transferred in less beneficial situations. The less beneficial situations were induced via shading to limit the carbon availability, or by providing optimal phosphate supply, or both. Mycorrhizal plants in less beneficial situations were compared with plants in conditions optimal for mycorrhiza (low phosphate availability, fully illuminated) and nonmycorrhizal control plants. Influence on physiological parameters was determined. Furthermore, the analyses of ^{13}C transport from plants to fungi under less beneficial conditions and the changes in amounts of fungal lipids were used to gain insights on the influence of less beneficial situations on the carbon partitioning from the host plant to the fungal partner. Finally, transcriptomic analyses based on Microarray analyses were performed to study the influence of less beneficial situations on plants genes in a mycorrhizal interaction, which control the major and minor carbohydrate pathway. Additionally, enrichment analyses of pathways in plant metabolism were done. In summary, the following hypothesis was drafted:

Plants are able to reduce the carbon transport to the fungi under less beneficial conditions.

The hypothesis was grouped in three parts:

1. How does a less beneficial situation caused by shading and/or optimal phosphate supply influence the colonization status, fresh weight gain, root carbohydrate and root phosphate content of mycorrhizal plants?

2. Does the mycorrhizal fatty acid content and the carbon transport to the fungi change under less beneficial conditions?

3. Are specifically regulated genes or pathways for carbon partitioning between plant and fungi detectable when a less beneficial situation occurs?

2. Material and Methods

2.1 Material

2.1.1 Chemicals and consumables

The commonly used chemicals had at least a purity of *p.a.* if not further specified in the method description. Chemicals were ordered at the following companies:
AppliChem (Darmstadt), Carl Roth GmbH (Karlsruhe), Duchefa Biochemistry (Harlem, Netherlands), Larodan Ltd. (Sweden), Merck KGaA (Darmstadt), Sigma Aldrich (St. Louis, USA)

The commonly used consumables were ordered from the following companies:
BioRad Laboratories GmbH (München), Eppendorf (Hamburg), Greiner (Solingen), Sarstedt (Nümbrecht), Serva (Heidelberg), Sigma Aldrich (St. Louis, USA), VWR International GmbH (Darmstadt)

2.1.2 Special chemicals and consumables

Table II.1 **Special chemicals and consumables**

Chemical/Consumable	Company	
Rhizophagus intraradices research grade inoculum 10 000 spores/g	Symplanta (München)	DAOM197198, DAOM181602 and MUCL43194
Carrier Material for inoculation of control plants	Symplanta (München)	Attapulgit-clay based carrier in mineral powder
Phosphate standard solution	Merck KGaA (München)	
Germ Agglutinin, Alexa Fluor® 488 Conjugate (catalogue W11261)	Thermo Fisher Scientific (Waltham, USA)	Staining of mycorrhizal roots for magnified intersection method
Sodium carbonate- ^{13}C 99 atom % ^{13}C	Sigma Aldrich (St. Louis, USA)	^{13}C label of plants
cOmplete™ Protease Inhibitor Cocktail	Sigma Aldrich (St. Louis, USA)	

2.1.3 Reaction kits

Table II.2 **Reaction kits used for experiments**

Reaction kit	Company	
NucleoSpin® RNA Plant	Macherey-Nagel (Düren)	
RNeasy MinElute Cleanup Kit	Qiagen (Hilden)	Clean up of RNA prior Microarray analyses
Total Carbohydrate Assay Kit	Sigma Aldrich (St. Louis, USA)	

2.1.4 Frequently used Buffer and Solutions

Table II.3 **Frequent used buffer and solutions**

Buffer	Component	Final concentration
PBS Buffer	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO_4	8 mM
	KH_2PO_4	2 mM
	pH 7.3 with 10% KOH	
0.5 x Hoagland Solution	$CA(NO_3)_2$ x 4 H_2O	2.5 mM
	KNO_3	2.5 mM
	$MgSO_4$ x $7H_2O$	1 mM
	KH_2PO_4+K_2HPO_4	20 µM/1 mM
	NaFeEDTA	20 µM
	$Na_2MoO_4x2H_2O$	0.2 µM
	H_3BO_3	10 µM
	$NiSO_4x6H_2O$	0.2 µM
	$ZnSO_4x7H_2O$	1 µM
	$MnCl_2x4H_2O$	2 µM
	$CuSO_4x5H_2O$	0.5 µM
	$CoCl_2x6H_2O$	0.2 µM

2.1.5 Software and databases

Table II.4 **Software and databases**

Software/Tool	
PageMan for Enrichment analyses	http://mapman.gabipd.org/web/guest/robin
ionOS 3.0 for Stable Isotope processing	http://www.isoprime.co.uk/products/software/ionos.html
R for multiple variance testing	https://www.r-project.org/ Version 3.2.2
Databases	
NCBI for Sequence analyses	http://ncbi.nlm.nih.gov/
Medicago truncatula Genome Project v4.0	http://jcvi.org/medicago/search.php?pageName=Search&section=Locus
Mtgea noble database	http://mtgea.noble.org/v3/probeset.php?id=Mtr.46023.1.S1_at&submit=Go

2.2 Methods

2.2.1 Plant growth

2.2.1.1 Seed sterilization

Medicago truncatula Gaertn. Jemalong line A17 seeds (provided by Prof. Dr. Helge Küster, Leibniz Universität Hannover, Institute of plant genetics) were surface sterilized. At first *Medicago truncatula* seeds were put into 98% sulfuric acid. After 10 minutes the seeds were washed three times with sterilized ultrapure water, to avoid sulfuric acid leftovers. After that the surface was sterilized with 20% hypochlorite for 15 minutes. After this the seeds were again washed three times with sterile ultrapure water and then left in water for 30 minutes for swelling. For germination seeds were put onto water agar (phyto agar 7.5 g/l) and wrapped in foil to ensure complete darkness. Seeds were transferred to 4°C for two days to overcome seed dormancy, and following that they stayed for two days in the dark at 24°C. The seeds were then transferred into 23°C temperature and 350 µmol*$m^{-2}s^{-1}$ light intensity for 10 days.

2.2.1.2 Inoculation

Plants were potted in 13 cm diameter pots filled with sterile foamed clay. Mycorrhizal plants were inoculated with 5g of *Rhizophagus intraradices* spores, which were in an attalpugit-clay based mineral powder mixture, directly into the potting hole. Control plants were potted in the same substrate but inoculum was replaced with sterile carrier material.

2.2.1.3 Growth conditions

Plants were grown in phytochambers (Johnson Controls, Ireland). Plants were watered with demineralized water when needed until three weeks after potting. After three weeks, plants were supplied with 50 mL 0,5x Hoagland solution (see Table II.3) once a week. Depending on treatment, the 0.5x Hoagland solution contained either 20 µM or 1 mM Phosphate. Additionally plants were watered with demineralized water whenever needed. Temperature and light intensity followed a program published by

Sulieman et al. (2013). The temperature was 23°C during the day which lasted for 16 hours and 18°C at night. The light intensity was 350 µmol*$m^{-2}s^{-1}$ during the day. The humidity was around 70 %.

2.2.1.4 Harvest

Plants were harvested six, eight and nine weeks after inoculation. Leaves were counted and shoot length was measured. After that the plants were removed from the pots and roots were washed with demineralized water. After that root and shoot fresh weight was measured, root samples for Alexa Fluor® staining (see 2.2.3) were taken and shoots and roots were directly frozen in liquid nitrogen.

2.2.2 Induction of less beneficial situations

Less beneficial defines a situation in which the fungi are not beneficial to the plant, due to the limitation of carbon availability or optimal nutrient availability. The experiments are analyzed under the assumption that mycorrhizal plants are able to control and therefore limit carbon transport to the fungi under less beneficial conditions. These less beneficial situations were created in two different ways. Firstly, plant carbon availability was limited by reducing photosynthetic performance through light limitation. Secondly, high phosphate fertilization was used to create a situation, in which the plant does not need the fungi for phosphate supply and hence, symbiotic interaction with the mycorrhizal fungi can also be considered as less beneficial.
After establishing the symbiotic interaction between plant and fungi for six weeks, twelve plants per replicate were shaded for the following three weeks and twelve plants per replicate remained in full light. All plants were grown in the same phytochamber, independent of light or phosphate treatment. For shading treatments light intensity was reduced to ~9 µmol $m^{-2}s^{-1}$ light intensity with a shading mesh.
Additionally to the light treatments, plants were supplied with 20 µM P (-P) or 1 mM P (+P) in 0.5x Hoagland´s solution to investigate the influence of P on carbon transport from the plant to the fungi. Furthermore, the shading of AM+P plants was used to create a super less beneficial situation.

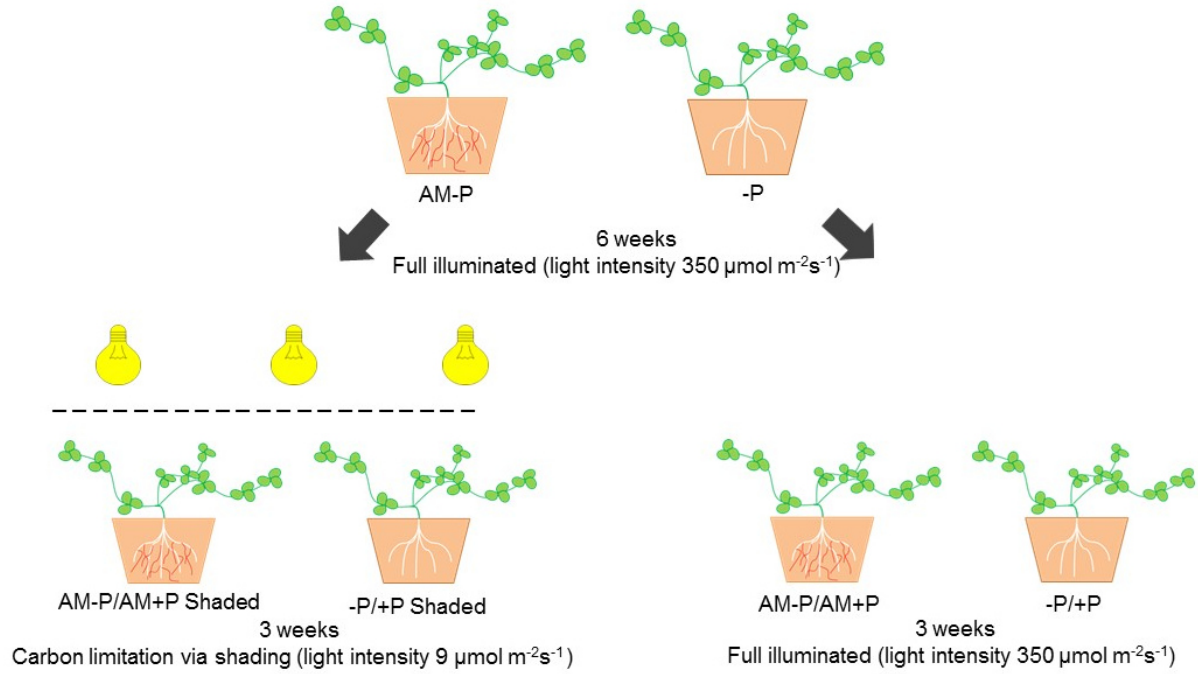

Fig. II.1 **Overview over the experimental setup to create less beneficial situations with mycorrhizal and nonmycorrhizal plants.**

2.2.3 Alexa Fluor® staining of roots and determination of mycorrhizal colonization

Root pieces were incubated for 10 min in 10% KOH (w/v) at 95°C. After that, roots were washed three times with sterile water and covered with staining solution. Staining stock solution contained 20 µg/ml Alexa Fluor® in PBS buffer. For staining, the stock solution was diluted 1:50 with PBS buffer. After staining for 12 hours in darkness, root samples were washed with sterile water and stored in sterile PBS buffer in the dark.
The Alexa Fluor® stained roots were used to determine mycorrhizal colonization with the magnified intersection method of McGonigle et al. (1990) with the additional parameter of stunted arbuscules. Fluorescence microscopy was performed using a Nikon Eclipse Ti fluorescence microscope (Nikon, Japan). Images and counting were carried out through a Nikon Plan Apo, 10x/0.45 objective. The fluorescence of Alexa Fluor® was analyzed by excitation at 480/20 nm. Image processing was performed using the NIS-Elements AR 4.40.00 software (Nikon, Japan).

2.2.4 Physiological measurements

2.2.4.1 Determination of inorganic phosphate

Inorganic phosphate of root material was measured following an adapted protocol of Taussky and Shorr (1953). 100 mg root material was ground in liquid nitrogen and transferred into 800 µL of 3% perchloric acid. After 20 minutes of shaking and centrifugation for 5 minutes at 14 000xg, 120 µl of supernatant was transferred to a 96 well plate for photometric purposes. 80 µl reaction solution (10 ml 10 % (w/v) $(NH_4)_6Mo_7O_{24}$ in 10 N H_2SO_4, 0.18 M $FeSO_4$, in H_2O) were added, and a KH_2PO_4 solution was used as standard. The measurement was done with a BioTek Synergy MX (BioTek, USA) at 750 nm absorption.

2.2.4.2 Total soluble carbohydrate assay

The total soluble carbohydrate assays was performed with the Total Carbohydrate Assay Kit (Sigma-Aldrich, USA). The procedure followed the description in the manual. 50 mg of root material ground in liquid nitrogen and then homogenized in 200 µl of cold assay buffer. To get a clear supernatant, homogenized root material was centrifuged at 13 000 xg for 5 minutes. 30 µl of the supernatant was used for the assay. The absorbance was measured at 490 nm with the BioTek Synergy MX (BioTek, USA).

2.2.4.3 RNA isolation

Root RNA was isolated with the NucleoSpin® RNA Plant kit. 50 mg of ground root material was used for RNA isolation. The procedure was followed as described in the manual. 350 µl of buffer RA1 with 3.5 µl β-mercaptoethanol was used. RNA was eluated with 60 µl H_2O. After eluating the RNA, an additional DNase digestion was done, as described in the manual.

2.2.5 Transcriptomic analyses

The root RNA of mycorrhizal plants was isolated with the NucleoSpin® RNA Plant kit and additionally cleaned up with the RNeasy MinElute Cleanup Kit. The microarray analysis was done by IMGM Laboratories GmbH (Planegg) and was based on the GeneChip® Medicago Genome Array (Medicago Genome (IVT) Array) from Affymetrix (Santa Clara, USA).
A total number of 12 Gene Chips was analyzed. The resulting relative expression numbers were assigned to identifiers of the *Medicago truncatula* genome.

2.2.5.1 Analyses of regulated candidates in the sucrose pathway

To analyze if candidates for the regulation of carbon transport from plant to fungi can be found in carbohydrate pathways, AM-P Light to Shading and AM+P Light to Shading was compared. The analyses was restricted to identifier which were assigned to the pathways of major and minor CHO metabolism and sugar transport.
The pattern for the identification of candidate identifiers is exemplified in Fig. II.2. Firstly, the ratios of AM-P (AM-P Light/AM-P Shaded) and AM+P (AM+P Light/AM+P Shaded) roots were calculated. Ratios between 0.5 and 1.5 were assigned as not relevant. Secondly, the other ratios <0.5 and >1.5 were analyzed for significantly different regulated identifiers, using Student´s t-test. Thirdly, significantly differing ratios (p-values AM-P compared to AM+P <0.05) with a difference (ratio AM-P-ratio AM+P) of more than 0.5 or less than -0.5 were then assigned as candidates.

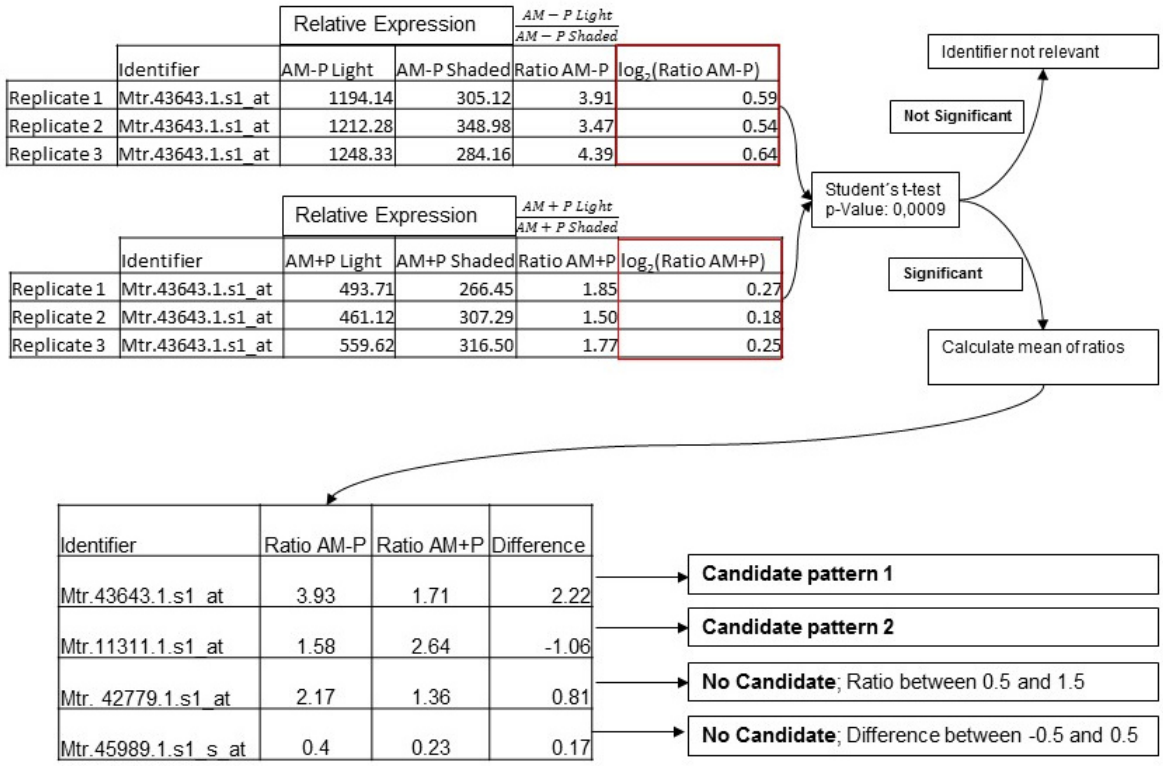

Fig. II.2 **Example calculation for the identification of candidates in carbohydrate metabolism**. Ratios from AM-P Light to AM-P Shaded and AM+P Light and AM+P Light were calculated and statistically compared. Not significantly different ratios were assigned as not relevant. Significant differences were used for further calculations. The differences between the ratio of AM-P (AM-P Light/AM-P Shaded) and AM+P (AM+P Light/AM+P Shaded) were calculated. Based on the difference the identifiers were assigned as candidate matching pattern 1, candidate matching pattern 2 or as a not candidate. The criteria for the assignment of a not candidate can be based on the calculated ratio or on the calculated differences. Calculated ratios (AM-P Light/AM-P Shaded; AM+P Light/AM+P Shaded) between 0.5 and 1.5 were defined as not relevant. Likewise, calculated differences between ratios (Ratio AM-P-Ratio AM+P) between -0.5 and 0.5 were defined as not relevant.

Additionally, candidate genes were analyzed for mycorrhiza induction. For this the microarray dataset of control plants and mycorrhizal plants of Hogekamp et al. 2011 was used. The datasets were derived from the mtgea homepage (https://mtgea.noble.org/v3/slides.php#a_treatment_type_3). Candidates which did not show an increase in relative expression due to mycorrhization were excluded. Exceptions were made for candidates with mycorrhizal induction and known role in fungal carbohydrate supply.

2.2.5.2 Enrichment analyses with the PageMan tool

To analyze if mycorrhiza dependent pathways were influenced by shading, an enrichment analysis was performed. For the enrichment analysis the PageMan tool of

the MapMan software was used. Here, the identifier of the *Medicago truncatula* Gene Chip with the associated expression data were sorted into the pathways. With this informations the PageMan tool analyzed, if identifiers of a metabolic pathway are over- or underrepresented in regulation, compared to the data from the *Medicago truncatula* Affymetrix GeneChip. The analyses of the influence of shading on mycorrhiza dependent metabolic pathways were split into two parts. Firstly it was analyzed which pathways in AM-P and AM+P plants were influenced by carbon limitation. These analyses were based on the ratios of AM-P Light/AM-P Shaded and AM+P Light/AM+P Shaded. Second, we tested the effect of mycorrhization on the identified pathways. The ratios of AM-P Light/AM-P Shaded and AM+P Light/AM+P Shaded were compared to nonmycorrhizal *Medicago truncatula* Gene Chip data from Hogekamp et al. (2011). These nonmycorrhizal data are based on 6 weeks old –P (20 µM) and +P (2 mM) *Medicago truncatula* roots.

To investigate which pathways in AM-P and AM+P roots were more regulated by carbon limitation, many informations of the performed experiments needed to be summarized. For this purpose, two tables were built and analyzed with PageMan (Usadel et al. 2006). One table contained information about differentially regulated identifiers from AM-P Light to AM-P Shaded plants. The other table contained information about differently regulated identifiers from AM+P Light to AM+P Shaded plants. At first the fold changes of the relative expression of the identifiers from Light to Shaded were calculated (AM-P Light/AM-P Shaded and AM+P Light/AM+P Shaded). This was done for each identifier in every biological replicate. The three ratios of the identifier of AM-P (AM-P Light/AM-P Shaded) were compared to the three ratios of the identifier of AM+P (AM+P Light/AM+P Shaded; Fig. II.3).

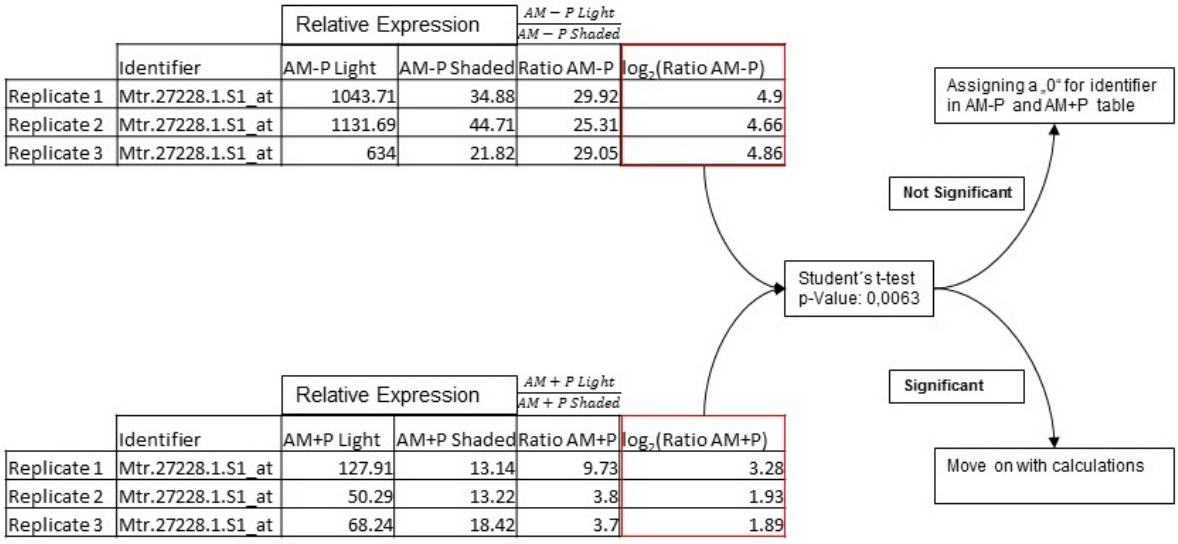

Fig. II.3 **Example calculation for the first step of 1 and 0 decision.** Regulatory ratios from AM-P Light to AM-P Shaded and AM+P Light and AM+P Shaded were calculated and significance was calculated with Student´s t-test. Only significantly different regulated identifier were used for further calculations.

The information about regulated identifiers were represented by yes and no decisions, indicated by “zero” (no) or “one” (yes). On the one hand, zero can represent no significant differences in regulation between AM-P (AM-P Light/AM-P Shaded) and AM+P (AM+P Light/AM+P Shaded) ratios. On the other hand the zero was assigned for example in the AM+P table when the regulation of an identifier was found to be higher in AM-P plants from Light to Shaded (Fig. II.4).

When the differences from light to shading were found to be significant, the differences between the AM-P ratio (AM-P Light/AM-P Shaded) and the AM+P ratio (AM+P Light/AM+P Shaded) were calculated. All differences between -0.5 and 0.5 were also assigned with zero, because the difference was defined as no different regulation. Differences higher than 0.5 were defined as higher regulated in AM-P from Light to Shaded compared to AM+P. This resulted in the assignment of a 1 for AM-P and a 0 for AM+P. Differences lower than 0.5 were defined as higher regulated in AM+P from Light to Shaded compared to AM-P (AM-P Light/AM-P Shaded). This resulted in the assignment of a 1 for AM+P and a 0 for AM-P.

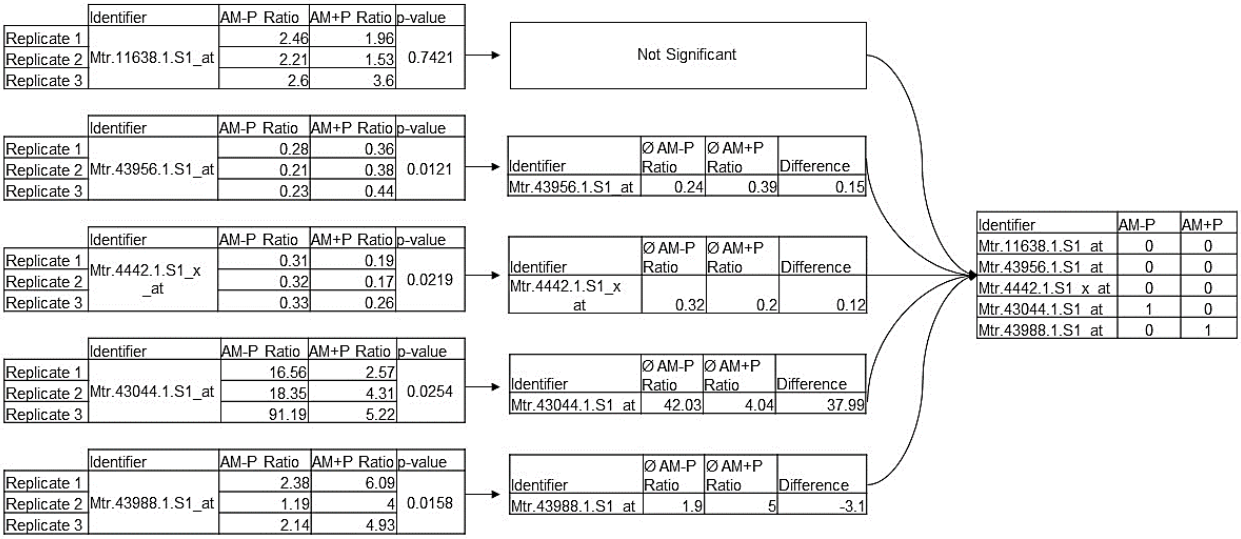

Fig. II.4 **Example calculation of regulated identifier.** Firstly it was analysed the difference between AM-P and AM+P ratios for each identifier using the Student´s t-test. Significantly different identifier were substracted to get an overview in which treatment identifier were higher regulated.

Both lists were analyzed using the Medicago_Affy_09 Map, summary statistics type ORA-Fisher, and Benjamini-Hochberg correction. The statistics and corrections were done with the tool of the RobinA software (Lohse et al. 2010).

2.2.6 ^{13}C-Tracer experiment

2.2.6.1 $^{13}CO_2$ pulse labelling

After the plants were shifted in light limitation for three weeks, a $^{13}CO_2$ pulse labeling experiment was conducted. To ensure that shaded plants were able to take up enough ^{13}C, labelling was performed in full light (light intensity of 350 µmol $m^{-2}s^{-1}$) for 2 hours. Plants were put into an airtight foilbox (45 cm x 60 cm x 130 cm). 1 g of ^{13}C sodium carbonate (99 atom% ^{13}C, Sigma-Aldrich, USA) was mixed with 10 ml 1M Sulfuric acid. To ensure a proper distribution of $^{13}CO_2$ in the foilbox, three fans were used. The labeling period was restricted to two hours. After labelling plants were shifted into light limitation and full illumination again, and harvested three and seven days later.

2.2.6.2 Measurement of ^{13}C isotopy in plant material

The frozen and ground root and shoot samples were dried in a freeze dryer (Christ alpha 1-4 LD plus, Christ, Osterode am Harz, Germany) and ground into a fine powder with a ball mill (MM200 ReTCh GmbH, Haan, Germany). Five to ten mg of homogenized root and shoot material was filled in tin capsules. The measurement of carbon content and stable carbon isotope composition ($\delta^{13}C$) was done with an elementar analyzer (vario ISOTOPE cube, elementar Analysensysteme GmbH, Hanau, Germany) coupled with an isotope ratio mass spectrometer (GC-5 System, elementar Analysensysteme GmbH, Hanau). For calibration, a number of standard substances was used (see appendix Tab. V.3). The ^{13}C concentrations were referenced with a linear calibration of five IAEA standards (see appendix Tab. V.3), with an additional $CaCO_3$ “in-house” standard.

The ^{13}C values were corrected with the ^{13}C values of plants which were not labeled, but grown in the same phytochamber like the labeled plants. Additionally, the isotopic composition was calculated with respect to the Vienna Pee Dee Belemnite (VPDB) standard:

$$^{13}C\ (\text{atom }\%) = \frac{(100*0.0111803*(\text{corrected } ^{13}C/1000+1))}{(1+0.0111803*(\text{corrected } ^{13}C/1000+1))}$$

2.2.7 Analyses of fungal fatty acids

2.2.7.1 Extraction of fungal fatty acids

Root samples were ground in liquid nitrogen. 110 mg of root material was transferred into centrifugation beakers with 18 ml of Bligh and Dyer Solution (Bligh and Dyer 1959). Additionally 25 µl of Internal Standard 1 and Internal Standard 2 were added. The samples were put on a horizontal shaker for 15 min at 325 rpm, than sonificated for 15 minutes and again put on a horizontal shaker at 325 rpm for 15 minutes. Samples were centrifuged at 7 °C and 3000 xg for 12 minutes. Supernatant was transferred into a

separating funnel. Additional 20 ml of Bligh and Dyer solution was added onto the root samples, and the beakers were put again on a horizontal shaker for 15 minutes at 325 rpm. Again beakers were centrifuged at 7°C and 3000 xg for 12 minutes. The supernatant was removed and combined with the supernatant from the first extraction step. 20 ml of chloroform and 20 ml citrate buffer (0.15 M citric acid monohydrate, pH 4 with NaOH) were put into the separating funnel, to establish a two phase solution. The separating funnel were transferred onto a horizontal shaker for 15 minutes at 300 rpm. After establishment of a phase boundary, the lower phase was transferred into a copped bottom flask. An additional 20 ml of chloroform was add into the separating funnel and they were shook on a horizontal shaker for 15 minutes at 300 rpm. The separating funnels were left over night to establish two phases. Lower phases were drained into the copped bottom flasks containing the lower phase from the day before. To reduce the lower chloroform phase copped bottom flasks were fixed on a rotation evaporator. At 45°C and maximum rotation speed, the pressure was reduced to 400 mbar. Evaporation was stopped when the liquid reached a volume of 500 µl. Custom made glass columns were packed with silica and the liquid remaining in the copped bottom flasks was transferred onto the silica columns, including 3 time of rinsing with chloroform. Neutral lipids were eluted with 5 ml chloroform into a copped bottom flask. The column was washed with 20 ml of acetone to remove glycolipids. Finally phospholipids were eluted with 40 ml of methanol into another copped bottom flask. The volume of the samples was reduced down to 500 µl with a rotation evaporator (400 mbar for chloroform and 150 mbar for methanol). The samples were transferred into reactivials and dried under a nitrogen stream. 25 µl of internal standard 1 and 3 were added to the reactivials. To hydrolyze the samples, 500 µl of 0.5 M NaOH in methanol were added into the reactivials, ultrasonificated for 10 minutes and vortexed for 5 minutes. The vials were heated for 5 minutes at 100 °C. 750 µl of 12.5 M BF_3 in methanol were added to each sample and heated at 80 °C for 15 minutes. 500 µl of a saturated NaCl solution and 1 ml of hexane were added to each vial. After 30 seconds of intensive shaking the upper apolar phase was transferred into a new reactivial. The hexane extraction was repeated two more times. The samples were dried under a nitrogen stream. 15 µl of internal standard 2 was added to each vial, as well as 185 µl of toluol. Vials were sonificated for 10 minutes, and vortexed for 5 minutes. All liquid was transferred into GC vials, containing an inlet and then sealed tightly. GC vials were stored at -20°C until measurement.

All chemicals used for the extraction and measurement of fungal fatty acids had a suitability for GC. Glasware was incubated at 300°C overnight, or washed with acetone.

2.2.7.2 Content and stable carbon isotope composition of fatty acids

The samples were measured with a gas chromatograph (Agilent Technologies 7890A GC-System) combined with an isotope ratio mass spectrometer (IRMS, GC5 System; elementar analyzer, vario ISOTOPE cube, elementar Analysensysteme GmbH, Hanau) for analyzing isotopy. The samples were analyzed with additional 17 substances as multistandards and three internal standards (see appendix table V.1 and table V.2). The settings of GC-IRMS are given in table II.6.

Table II.6 **Settings of the GC-IRMS**

Parameter	Setting
Oven temperature	80°C 1 min, heat rate of 7°C/min up to 180°C 180°C 0 min, heat rate of 0.7°C/min up to 190°C, 190°C for 3.5 min, heat rate of 0.7°C/min up to 209°C, 209°C for 0 min, heat rate of 50°C/min up to 300°C, 300°C for 5 min
Injector temperatur	250°C
Septum purge	3 ml/min
Split modus	Splitless
Splitless time	0.75 min
Column flow	1 ml/min for 61 min, afterwards with 1 ml/min up to 1.5 ml for 6 min
Detector	IRMS
Interface temperature	350°C
Oven temperature (filled with CuO_2)	850°C
H_2O removal	With Nafion tube
Online calibration	With reference gas CO_2 (Linde, purity: 99.99995%)
Referencing	International standards: Pure substances were measured with EA-IRMS and referenced to international standards

2.2.8 Statistical methods

2.2.8.1 Multi-factor analysis of variance (ANOVA)

Analyses of variance was performed with the acknowledgement of Philipp Bohnhorst (Institute for Botany, Leibniz Universität Hannover).

The statistical analyses of the magnified intersection method to calculate the mycorrhizal colonization was performed with a multi-factor ANOVA in R (version 3.2.2). For this the following packages were used:

- “lsmeans” , Lenth 2016
- “lme4” , Bates et al. 2015
- “lmerTest” , Kuznetsova et al. 2015

Confidence level: 0.95
P value adjustment: tukey method for comparing a family of 4 estimates
Significance level used: alpha= 0.05

2.2.8.2 Student´s t-test

P values were calculated according to Students t-test, two-tailed with homogeneity of variance.
Significance level used: $p< 0.05$

3. Results

To investigate how carbon transport in an arbuscular mycorrhizal symbiotic interaction is influenced under carbon limitation and optimal phosphate supply, plants in an established symbiotic interaction were shaded and analyzed (2.2.2).
The scheme in figure 3.1 displays the analyses performed with the different treatments. The results in chapter one are based on mycorrhizal and nonmycorrhizal plants. In chapter two and three only mycorrhizal plants were analyzed.

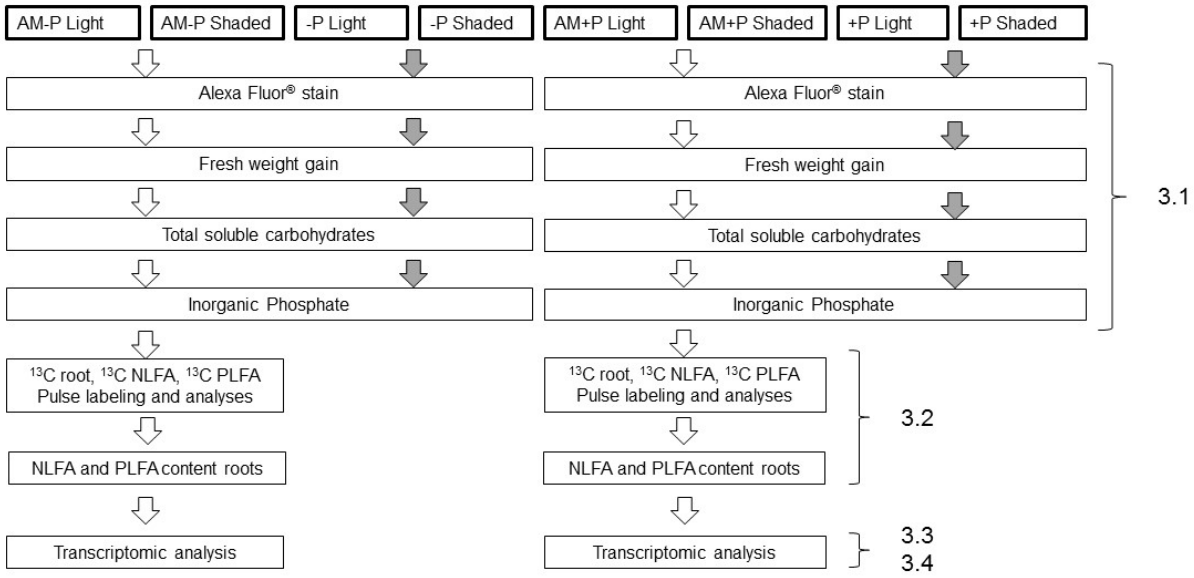

Fig. III.1 **Scheme of experiments, methods and analyses which were done with different treated plants.** Numbers show the chapters in which the assigned experiments are shown.

The results of the experiments are divided into four chapters. In chapter one, the influence of mycorrhization and phosphate supply on growth, root carbohydrate content and root phosphate content are shown. The second chapter shows the results of ^{13}C pulse labeling experiments. The ^{13}C pulse labeling was used to investigate changes in ^{13}C contents in roots and fungal fatty acids and to analyze how the carbon transport from plant to fungi is directly influenced by shading and phosphate supply. In the third and fourth chapter, results of transcriptomic analysis are shown. These analyses were used to identify possible candidates which are involved in the regulation of carbon transport mechanisms during the interaction between plant and fungi.

3.1 Mycorrhizal infections influence plant growth under different light and phosphate treatments

The following experiments were performed with the aim to test, if mycorrhizal plants are able to control carbon transport to the fungi in a less beneficial situation. If mycorrhizal plants are able to control the carbon transport mechanism to the fungi when a less beneficial situation occurs, weight gain of mycorrhizal plants would not be negatively affected by shading or phosphate overabundance.

3.1.1 Plant phenotypes were influenced by mycorrhiza, shading and phosphate supply

AM-P plants showed an increased shoot growth compared to -P plants, when light was not limited (Fig. III.2a, b). Furthermore, the minimal P supply (-P) was not too low to survive for nonmycorrhizal plants in full light treatments. AM+P plants showed an increased shoot growth compared to +P control plants in full light conditions (Fig. III.2c, d). All fully illuminated plants showed a black spot on their leaves, which was found in pre-experiments to occur under high light conditions. AM-P plants showed an increased shoot growth compared to -P control plants under shading conditions (Fig. III.2e, f). The growth of AM+P plants and +P control plants did not show differences under shading conditions (Fig. III.2g, h). Shading generally resulted in the loss of the black spot on the leaves. All plants survived the shading treatment.
In summary, mycorrhization had no visible negative effect on plant growth during the less beneficial and super less beneficial situations.

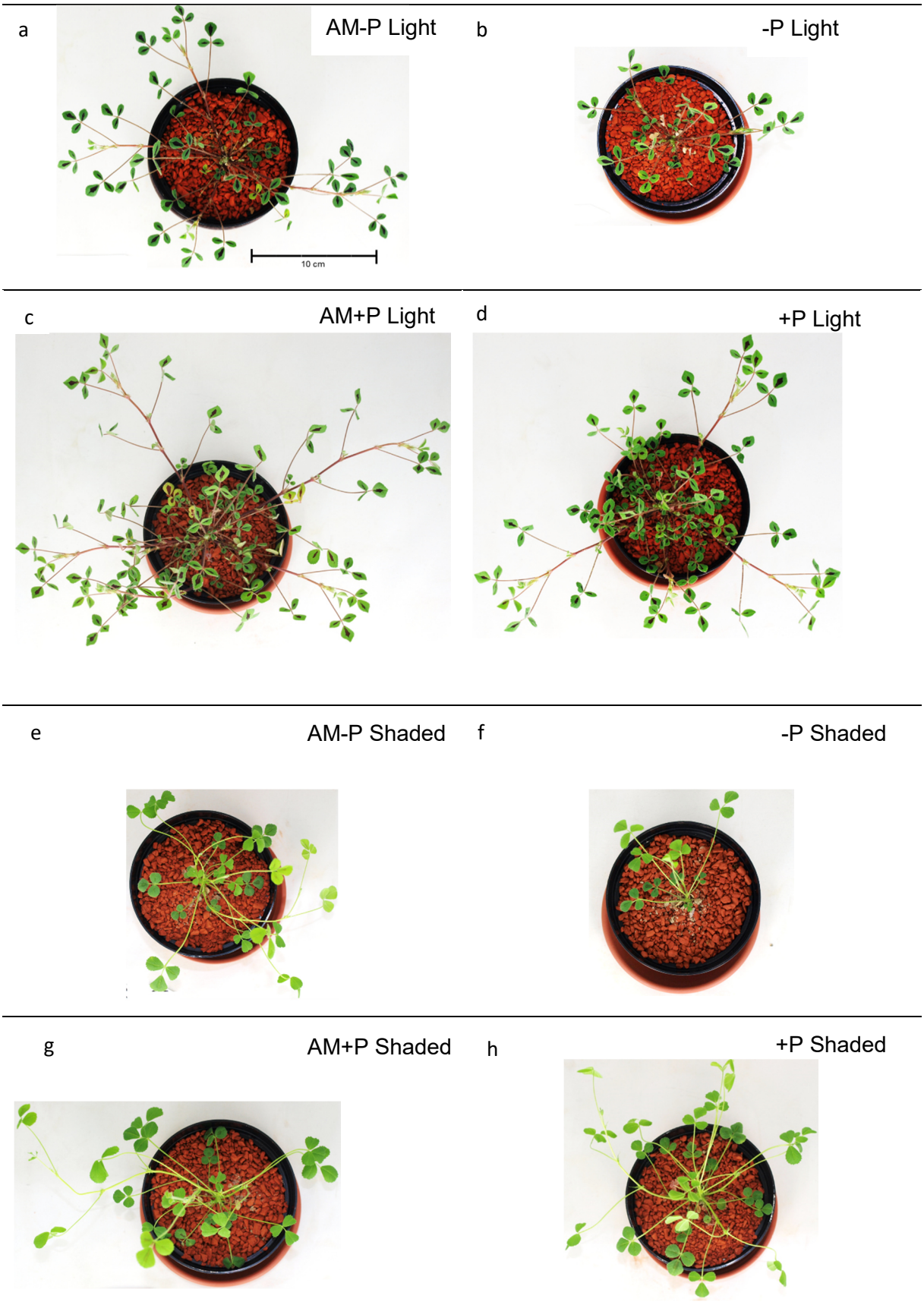

Fig. III.2: **Pictures of mycorrhizal (a, c, e, g) and nonmycorrhizal control plants (b, d, f, h)**, 9 weeks past infection and 21 days past shift; Scale bar = 10 cm, -P plants are supplied with 20 µM P, +P plants are supplied with 1 mM P. The pictures are representative for the six replicates which were used in the different experiments. C and e are less beneficial situations, g shows the super less beneficial situation.

3.1.2 Influence of optimal phosphate supply and light limitation on mycorrhizal structures

To investigate the influence of phosphate supply and light limitation on mycorrhizal structures, the colonization status was determined using the magnified intersection method (McGonigle et al. 1990). After staining the roots with Alexa Fluor® (2.2.3), different mycorrhizal structures like hyphae, arbuscules, vesicles and stunted arbuscules (Fig. III.3) were quantified.

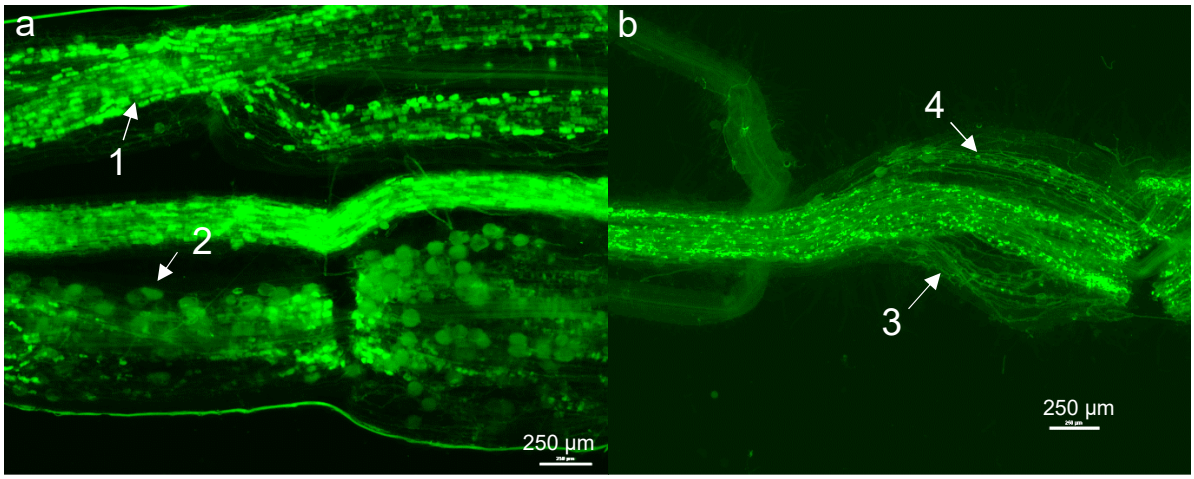

Fig. III.3 **Alexa Fluor® staining of mycorrhizal roots.** In panel a, the fungal structures arbuscules (1) and vesicles (2) are visible. In part b, hyphae (3) and stunted arbuscules (4) are marked visible.

AM-P plants showed a high amount of vesicle and hyphae, independent of light limitation (Fig. III.4a, b). The amount of arbuscules was significantly reduced by about 45% when AM-P Shaded plants were compared to AM-P Light treated ones. Consistent with this observation, the amount of stunted arbuscules increased by about 13%. Nonmycorrhizal controls did not show any stained fungal structures.

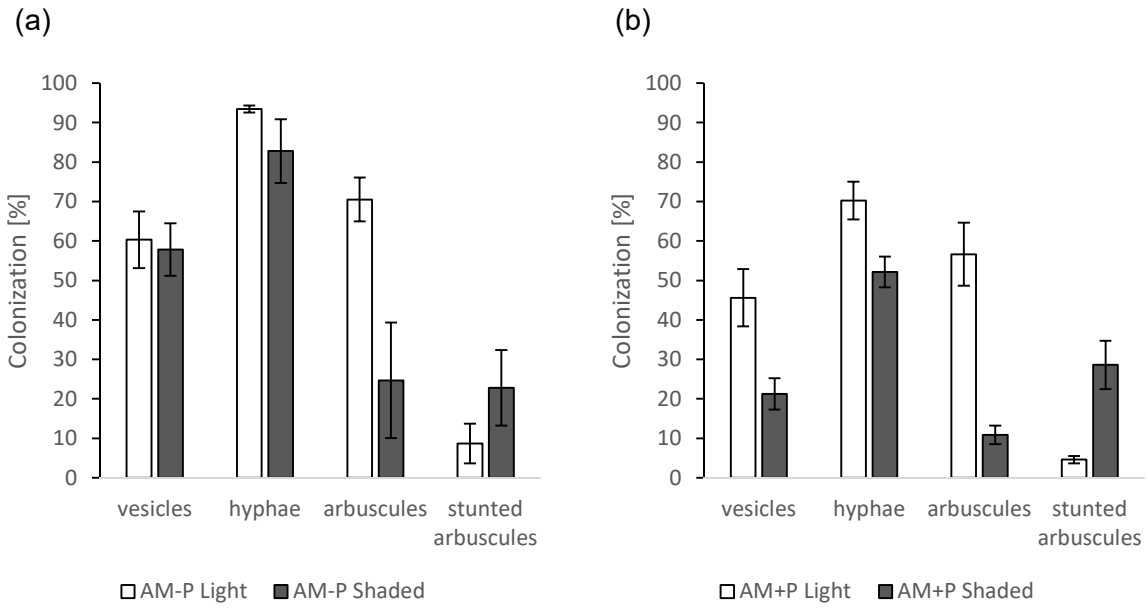

Fig. III.4 **Mycorrhizal colonization under P limitation (a) and optimal P supply (b) under full illumination and light limitation using the magnified intersection method** (± SE; n=4). Each experiment is based on 4 biological replicates with 12 individual plants in each replicate.

The counting data were further statistical analyzed (Table III.1, 2.2.8.1, for detailed results see appendix table V.4-7). The comparison of counting data from AM-P Light and AM-P Shaded plants, which represented a less beneficial situation, showed that shading resulted in a significant reduction of arbuscules, with a significant increase of stunted arbuscules at the same time. Due to phosphate supply a less beneficial situation was induced, AM+P Light plants showed a significant reduction of hyphae, compared to AM-P Light plants. The comparison from AM-P Light to AM+P Shaded, which presented the super less beneficial situation, resulted in a significant reduction of hyphae, vesicles and arbuscules. The same result was observed for the comparison of AM+P Light and AM+P Shaded plants. The comparison of AM-P Shaded and AM+P Shaded plants showed a significant reduction of hyphae and vesicles due to phosphate supply.

Table III.1 **Statistical analyses for significant variances between the treatments.** Data were tested for variance with a multivariate ANOVA. NS abbreviates no significant variance, S abbreviates significant variance.

comparison			Hyphe	Vesicle	Arbuscule	st. Arbuscule
AM-P Light	→	AM-P Shaded	NS	NS	S	S
AM-P Light	→	AM+P Light	S	NS	NS	NS
AM-P Light	→	AM+P Shaded	S	S	S	NS
AM+P Light	→	AM+P Shaded	S	S	S	NS
AM-P Shaded	→	AM+P Shaded	S	S	NS	NS

Fungal structures were found in AM+P plants under full illumination (Fig.III.3b). Due to shading, the amount of fungal structures was reduced (Fig. III.4b). The amount of vesicles (45%), hyphae (70%) and arbuscules (58%) was quite high. When AM+P plants were shaded, a super less beneficial situation was induced. It was expected that the amount of fungal structures in AM+P Shaded plants would be highly reduced compared to AM+P Light plants. Shading reduced the amount of vesicles significantly by about 50%. The amount of hyphae was significantly reduced by about 20%. The amount of arbuscules showed the highest reduction by about 48%, which was also significant. Accordingly, the amount of stunted arbuscules increased by about 25%.

In summary, all treatments showed fungal structures. Shading of AM-P plants reduced only the amount of arbuscules, whereas shading of AM+P plants resulted in a reduction of all mycorrhizal structures.

Nonmycorrhizal controls did not show any stained fungal structures.

When AM-P Light plants were compared to AM+P Light plants, the amount of vesicles was reduced by about 15%. The amount of hyphae was significantly reduced by about 22% due to phosphate supply. The amount of arbuscules was reduced by about 12%. The amount of vesicles was significantly reduced by about 38% in AM+P Shaded compared to AM-P Shaded plants. The amount of hyphae was significantly reduced to about 30%, whereas the arbuscule abundance did not show a difference. This result indicates, that the influence of optimal phosphate supply is more important, when carbon availability is limited.

To summarize the results, the induction of less beneficial situations reduced the amount of fungal structures in roots. In AM-P plants, shading and the resulting reduction of carbon availability reduced the arbuscule amount. Optimal phosphate supply of mycorrhized plants resulted in the reduction of all fungal structures. In this super less beneficial situation, in which AM+P plants were shaded, the reduction of fungal structures was found to be the highest.

3.1.3 Influence of mycorrhization on plant weight under light limitation and optimal phosphate supply

To investigate if the induction of less beneficial situations or a super less beneficial situation via shading, optimal phosphate supply or shading of optimal phosphate supplied plants results in a negative effect of mycorrhizal fungi on the host plant, the root and shoot weight of mycorrhizal and nonmycorrhizal plants was determined (Fig. III.5a, b). When AM-P plants were carbon limited, no negative effect of the mycorrhizal fungi was detectable (Fig. III5a). AM-P Shaded plants were bigger in shoot, root and total fresh weight. The total fresh weight of AM-P plants was significantly higher compared to -P plants, in shaded (p= 0,013, according to Student´s t-test, two-sided, homogeneity of variance, 2.2.8.2) and fully illuminated (p= 0,035 according to Student´s t-test, two-sided, homogeneity of variance, 2.2.8.2) treatments. Compared to fully illuminated plants, fresh weight of shaded plants was always much smaller in both, mycorrhizal and nonmycorrhizal plants. This indicates, that shading indeed resulted in carbon limitation.

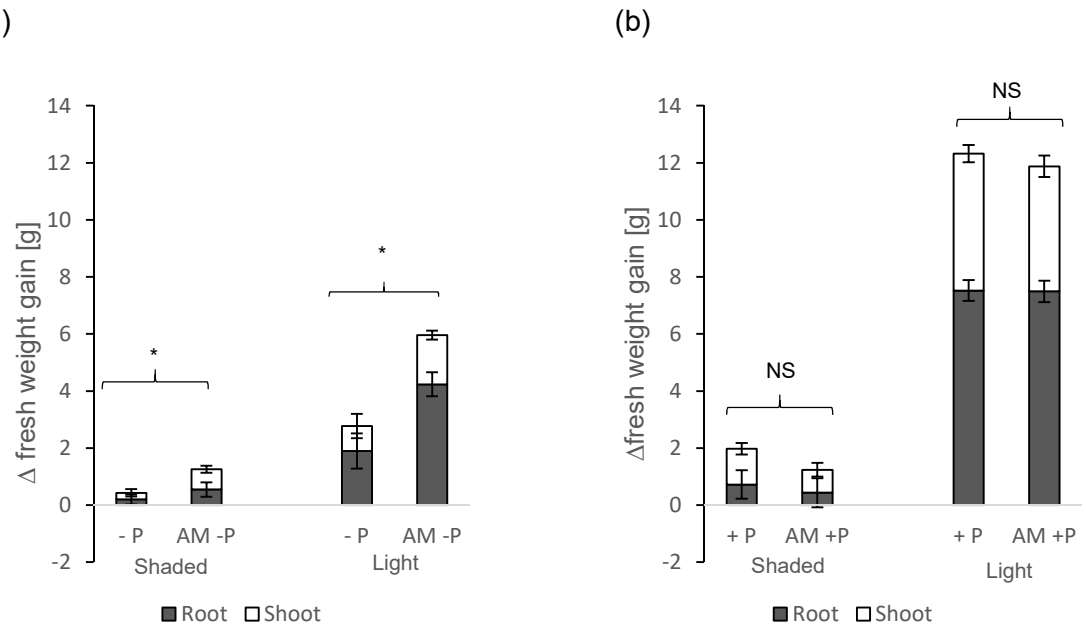

Fig. III.5 **Fresh weight gain of shoots, roots and whole plants under phosphate limitation (a) and optimal phosphate supply (b) within 21 days of carbon limitation.** (± SE; n=4). Each experiment is based on 4 biological replicates with 12 individual plants in each replicate. Asterisks show significant differences according to Students t-tests (*p<0.05, n.s. not significant)

The super less beneficial situation of optimal phosphate supply and shading of mycorrhizal plants, was also investigated. Therefore, the fresh weight gain in optimal P supplied mycorrhizal plants (AM+P Shaded) compared to nonmycorrhizal plants (+P Shaded) was additionally tested (Fig. III.5b). No significant differences were observed. This indicates, that likewise the combination of the two parameters shading and optimal P supply did not result in a negative growth effect for mycorrhizal plants compared to nonmycorrhizal plants. Notably, the positive effect of mycorrhization for plants under phosphate limitation and shading (AM-P Shaded) was not detected for AM+P plants. A positive effect of mycorrhization was neither detected for +P plants when they were fully illuminated.
In summary, both, the light limitation and optimal P supply experiments gave no indication for a parasitic behavior since plants fresh weight was not negatively influenced compared to the nonmycorrhizal control.

3.1.4 Influence of mycorrhization on total soluble carbohydrate content in roots under light limitation

The previous experiments indicated that plants might be able to regulate carbon allocation to the fungi in less beneficial situations. Therefore, the carbon content in roots, again under light limitation and full illumination, as well as under phosphate limitation and optimal P supply was measured (2.2.4.2).
Based on the hypothesis that plants are able to control carbon transport to the fungi in less beneficial situations, reduction of total soluble carbohydrates (TC) caused by mycorrhization was not expected. The amount of TC in AM-P Shaded roots was not differing from the amount determined in –P Shaded roots (Fig. III.6). In fully illuminated plants, the TC content of AM-P roots was significantly ($p= 0,014$ according to Student´s t-test, two-sided, homogeneity of variance, 2.2.8.2) higher compared to -P roots. Notably, the TC content in -P Light roots was as high as in -P Shaded and AM-P Shaded roots.

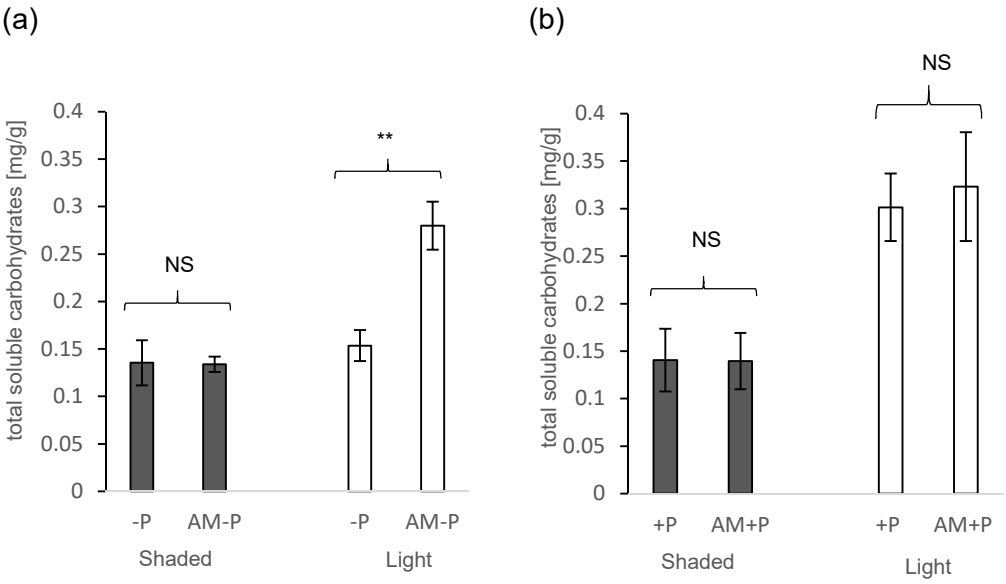

Fig. III.6 **Amount of total soluble carbohydrates in roots under P limitation (a) and optimal P supply (b).** Total soluble carbohydrates were measured in roots after 21 days of shading. (± SE; n=4). Each experiment is based on 4 biological replicates with 12 individual plants in each replicate. Asterisks show significant differences according to Student´s t-tests (*p<0.05, **p<0.001, n.s. not significant)

Under optimal P supply, mycorrhization did not influence the content of TC in roots, independent of light treatments (Fig. III.6b).
Under shading conditions, the TC content in roots was found to be the same for all treatments. In full light, AM-P, AM+P and +P roots showed the same TC.

3.1.5 Influence of mycorrhization on inorganic phosphate content in roots under light limitation and optimal phosphate supply

The previous data indicated, that plants are able to control carbon transport to the fungi when a less beneficial situation occurs. The P content of roots was measured to investigate the influence of shading and optimal P supply on mycorrhizal plants (2.2.4.1). It was hypothesized, that plants are able to control carbon transport to the fungi in a less beneficial situation. Thus, we analyzed, whether in such situations a positive effect of the mycorrhizal fungi on root phosphate content was measurable.
Only at the starting point of different light treatments (0 dps) AM-P plants showed a significantly higher root phosphate content than -P plants (p= 0.04 according to Student´s t-test, two-sided, homogeneity of variance; Fig. III.7a).

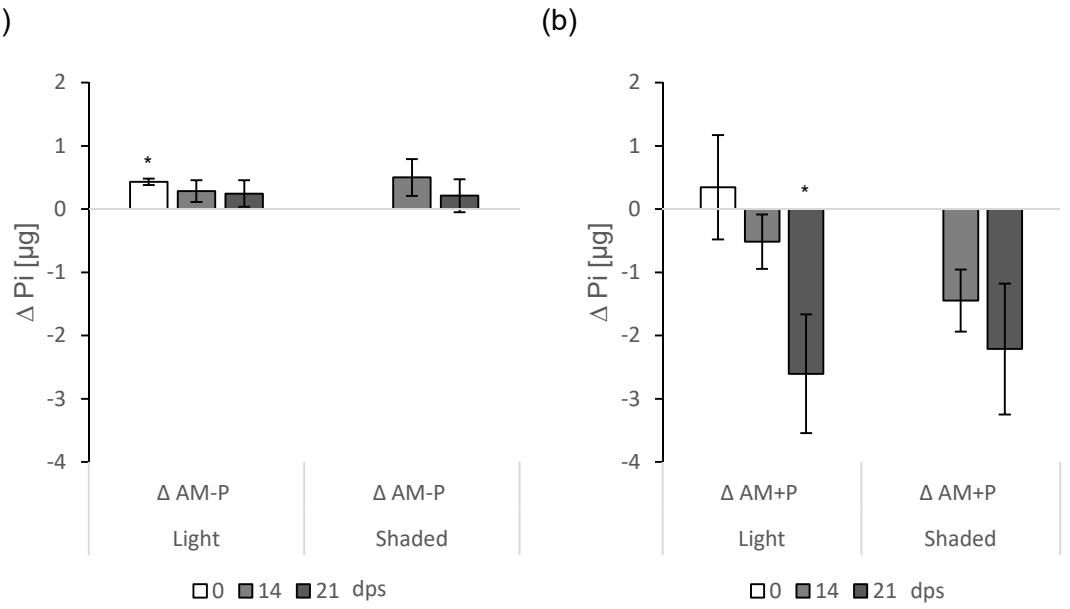

Fig. III.7 **Changes of inorganic phosphate (Pi) content in roots under P limitation (a) and optimal P supply (b) during light treatments.** The changes in Pi content were calculated between mycorrhizal roots and the nonmycorrhizal control roots (± SE; n=3). Data in each column is based on 3 biological replicates with 9 plants in each biological replicate. Asterisks show significant differences according to Students t-tests (*p<0.05)

In AM+P plants, mycorrhization did not have a positive effect on root phosphate content (Fig. III.7b). Fully illuminated AM+P plants showed a significantly reduced root phosphate content, after 21 days of light treatments (p= 0.03 according to Students t-test, two-sided, homogeneity of variance, 2.2.8.2).
In summary, less beneficial situations and the super less beneficial situation did not increase the root phosphate content, but a negative trend for AM+P Shaded plants was visible

3.2 Analyses of fungal fatty acids and carbon transport from plant to fungi with ^{13}C labeling

To see how the carbon transport from the plant to the fungus is influenced by light limitation, plants were labeled with $^{13}CO_2$, 21 days after carbon limitation (2.2.6). After another three and seven days, the plants were harvested. Mycorrhization was determined by Alexa Fluor® (2.2.3) staining. Fresh weight was determined and incorporation of ^{13}C into shoots, roots and mycorrhizal fatty acids was measured. The following figures are based on two replicates. Therefore, only descriptive statistics were performed and the standard error was calculated.

3.2.1 Validation of mycorrhization during light limitation and optimal phosphate supply using the magnified intersection method

To validate how mycorrhization of AM-P plants was influenced by light limitation, colonization was counted with the magnified intersection method (Fig. III.8, McGonigle et al. 1990), which was described previously (3.1.2). The abundance of stunted arbuscules was not determined. It was expected that shading would reduce the amount of fungal structures. In AM-P roots, shading reduced the amount of vesicles by about 15 % at 3 dpl and by about 40 % at 7 dpl. The amount of hyphae in AM-P roots was reduced by about 13 % at 3 dpl and by about 24 % at 7 dpl. The reduction of arbuscules in AM-P plants was reduced to about 5 % at 3 dpl and to about 46 % at 7 dpl, due to shading.

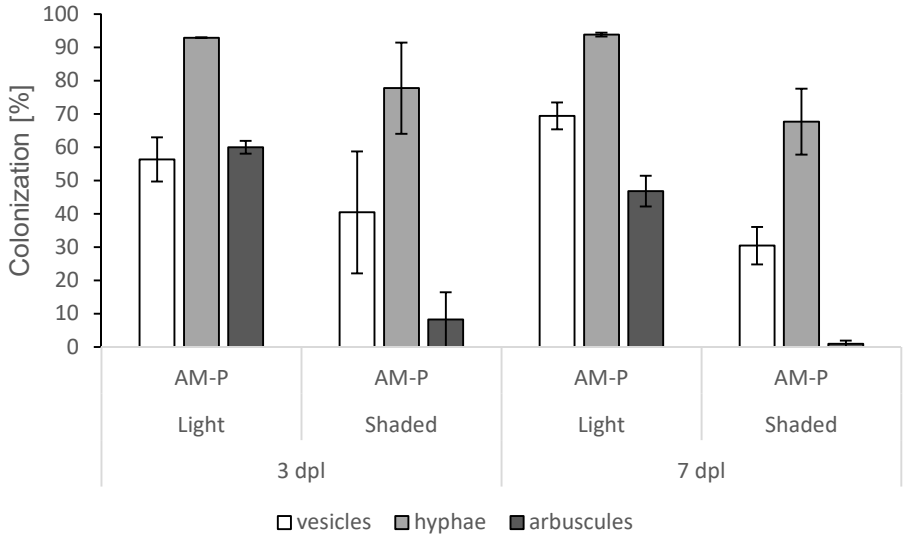

Fig. III.8 **Mycorrhizal colonization under P limitation under full illumination and light limitation using the magnified intersection method** (± SE **;** n=2**).** Plants were labeled 9 weeks past infection, and harvested three and seven days after labeling.

AM-P Light roots showed about 10% more vesicles compared to AM-P roots in Fig. III.4. The amount of hyphae did not differ between the experiments. The amount of arbuscules in AM-P Light plants was reduced by about 10 to 22% compared to the former experiment. AM-P Shaded plants, the amount of vesicles was reduced by about 20 to 30% compared to the experiment explained in 3.1.2. Only the amount of hyphae did not differ between the two experiments.

In summary, shading reduced the amount of fungal structures in the roots of AM-P plants.

The magnified intersection method was also used to evaluate the influence of shading on mycorrhizal plants which were supplied with an optimal amount of phosphate (Fig. III.9). Due to shading of AM+P plants a reduction of all fungal structures was expected. This expectation was based on the hypothesis and the results displayed in Fig. III.4b, which showed a significant reduction of hyphae, vesicles and arbuscules for AM+P Shaded plants (3.1.2). Shading of AM+P plants reduced the amount of vesicles by about 10% at three dpl and by about six% at seven dpl. The amount of hyphae did not change at three dpl. At seven dpl the amount of hyphae was reduced by about 50% due to shading. The abundance of arbuscules was reduced by about 35% at three dpl in AM+P Shaded plants compared to AM+P plants. At seven dpl, the amount of arbuscules was reduced by about 58% in AM+P Shaded plants.

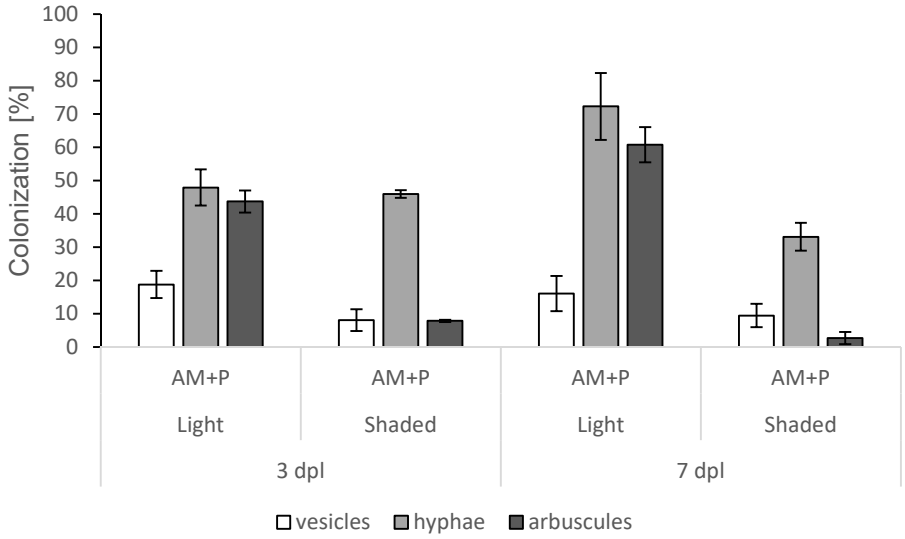

Fig. III.9 **Mycorrhizal colonization under optimal P supply in full illumination and light limitation using magnified intersection** (± SE **;** n=2)**.** Columns and bars are based on two independent biological replicates. Plants were labeled 9 weeks past inoculation, after 21 days of different light treatments and harvested three and seven days past label (dpl).

In comparison to the results described in 3.1.2, the amount of fungal structures in AM+P Light and AM+P Shaded plants was different. In AM+P Light roots, the amount of vesicles was reduced by about 26 to 29% compared to the results in 3.1.2. The amount of hyphae was reduced by 22% at 3 dpl and no difference was detectable at 7 dpl. The amount of arbuscules was reduced by about 4 to 14% in the AM+P roots (Fig. III.9). In AM+P Shaded roots, the amount of vesicles was reduced by about 10 to

11%, compared with the data in 3.1.2. The amount of hyphae was reduced by 10 to 20% and 2 to 9% reduction of arbuscules was detected.
In summary, shading of AM+P roots resulted in a reduction of fungal structures, compared to AM+P roots.

3.2.2 Influence of carbon limitation on carbon transport from plants to mycorrhizal fungi under phosphate limitation and optimal phosphate supply

To investigate how much carbon is transported from plants to the mycorrhizal fungi when a less beneficial situation occurs, plants were pulse labeled with $^{13}CO_2$ as a tracer (2.2.6). The ^{13}C content in roots and in the mycorrhizal fatty acid was measured. The fatty acids which are commonly used for the identification are represented by 16:1ω5 fatty acids (2.2.7). The ^{13}C in 16:1ω5 phospholipid fatty acid (PLFA) is indicative for the amount of plant derived carbon, integrated into growing fungal structures like hyphae, whereas the ^{13}C in 16:1ω5 neutral lipid fatty acids (NLFA) indicates the amount of carbon integrated into the vesicles of the fungi. For the analyses of carbon transport the ^{13}C content of the whole root was compared to the ^{13}C content in PLFA and NLFA. The ratio of root ^{13}C divided by PLFA ^{13}C or NLFA ^{13}C displays how much carbon from the root is transported to the fungi and how this ratio is influenced in less beneficial situations. The ^{13}C incorporation in NLFA over time at three and seven days past label was investigated. It was hypothesized, that less beneficial situations would result in a reduction of carbon transported from plant to fungi under shading, optimal phosphate supply and the super less beneficial situation in which AM+P plants are shaded.
The ratios of ^{13}C in roots compared to ^{13}C in fatty acids in AM-P plants showed, that the amount of ^{13}C which was incorporated into the fungal fatty acids was reduced by shading (Fig. III.10a). In the fully illuminated plants, the amount of ^{13}C in NLFA and PLFA was found to be twice as high as in roots at three dpl. This allocation of ^{13}C was reduced to a distribution of the same amount of ^{13}C in roots and NLFA or PLFA, respectively, at 7 dpl. When AM-P plants were shaded, the amount of ^{13}C found in fatty acids was lower than the amount of ^{13}C measured in AM-P Shaded roots. At three dpl, the ^{13}C content in PLFA was not detectable. The ^{13}C atom% in NLFA was low at three dpl and showed an increase at seven dpl, but the standard error was quite high. These

findings show that shading reduces the carbon transport from plants to fungi under phosphate limitation.

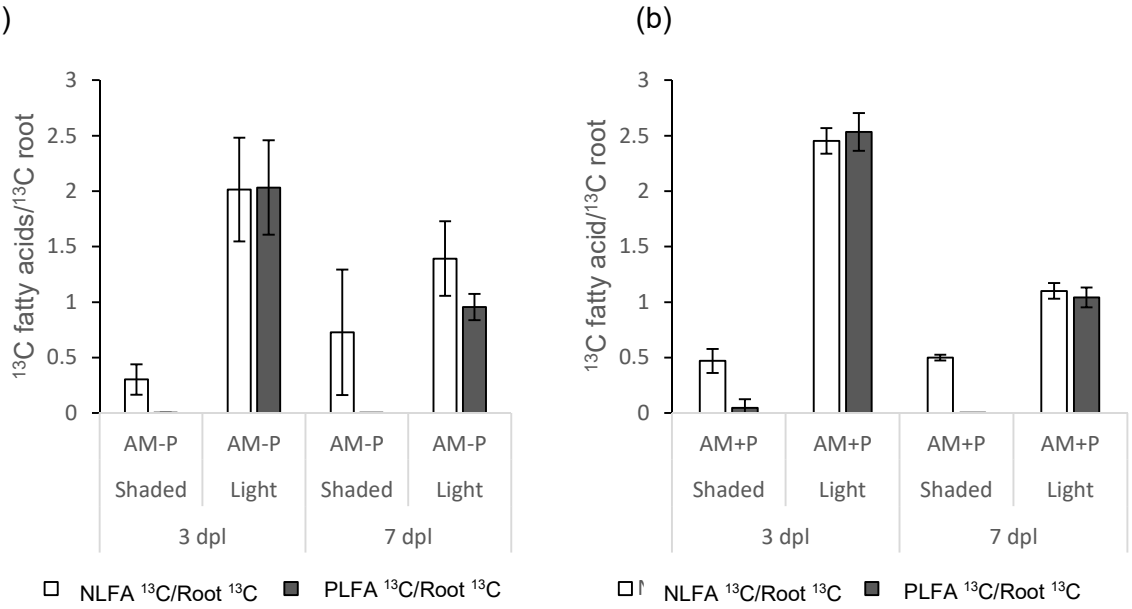

Fig. III.10 **Influence of shading on ^{13}C content in mycorrhizal fatty acids divided by ^{13}C content in roots under phosphate limitation (a) and optimal phosphate supply (b)** (± SE **;** n=2). Columns and bars are based on two independent biological replicates. Plants were labeled 9 wpi, after 21 days of different light treatments and harvested 3 and 7 days past label.

In AM+P Shaded roots the ^{13}C amount was found to be more abundant compared to ^{13}C in NLFA and PLFA (Fig. III.10b). The ^{13}C in NLFA did not differ between three and seven dpl. The ^{13}C in PLFA was close to the detection threshold at seven dpl. In AM+P Light roots, the ^{13}C was more abundant in PLFA and NLFA compared to root ^{13}C. The ratio of ^{13}C in PLFA and NLFA to ^{13}C in roots was higher at three dpl compared to seven dpl. In fully illuminated plants the ratios for ^{13}C NLFA/^{13}C root and ^{13}C PLFA/^{13}C roots were nearly the same, independent of phosphate treatment.

In summary, shading reduced the ^{13}C in NLFA and PLFA compared to root ^{13}C. This was independent of phosphate treatment. It is suggested that plants are able to control carbon transport to the fungi when a less beneficial situation occurs.

The ratios of ^{13}C NLFA/^{13}C root and ^{13}C PLFA/^{13}C root were nearly the same for AM-P Light and AM+P Light plants.

3.2.3 Influence of shading and optimal phosphate supply on neutral- and phospholipid fatty acids in roots

In the previous experiment, the carbon transport dynamics were investigated. The ^{13}C labeling illustrated how much carbon was incorporated in NLFA and PLFA three and seven dpl. The labeling experiment did not allow any conclusions about the total amount of NLFA and PLFA in the mycorrhizal roots.

Based on the previous results and the hypothesis that plants are able to control carbon transport to the mycorrhizal fungi when a less beneficial occurs, it was analyzed if the amount of NLFA and PLFA was reduced when mycorrhizal plants were shaded, under optimal P supply or both (2.2.7).

In AM-P Shaded plants, the amount of NLFA was considerably reduced to 1000 µg/g root fresh weight (Fig. III.11a). PLFA were not detectable at both points in time. These data indicating, that AM-P plants are able to control carbon transport to the fungi when plants are shaded.

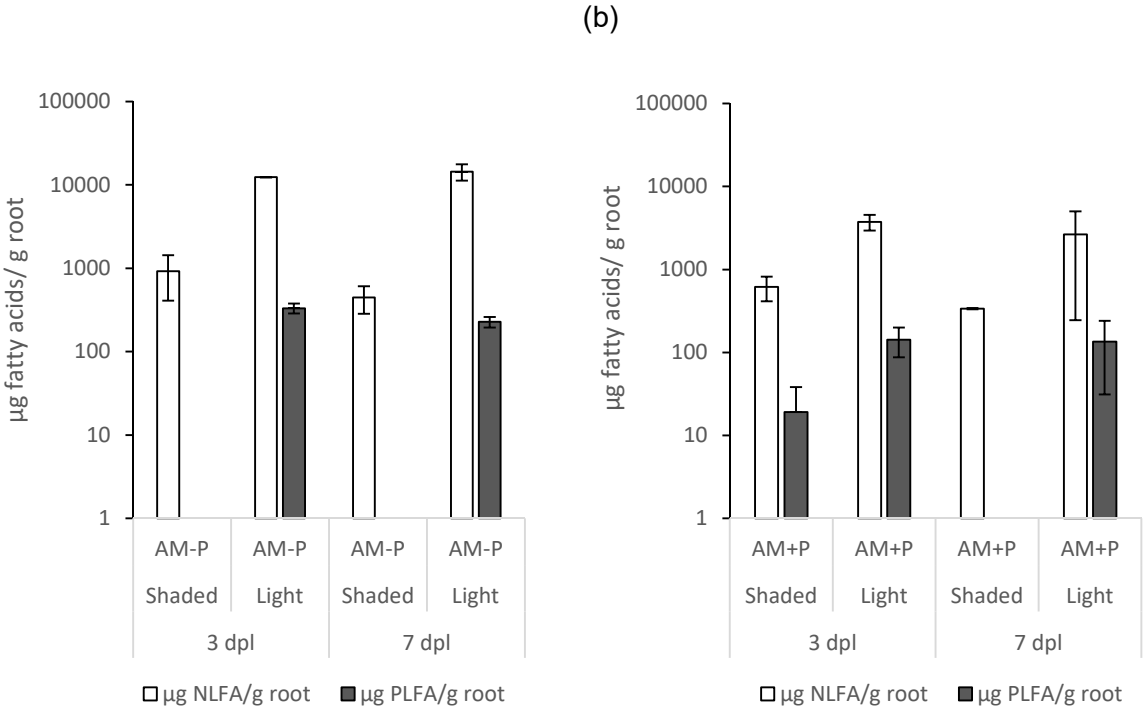

Fig. III.11 **Concentrations of neutral lipid fatty acids (NLFA) and phospholipid fatty acids (PLFA) in mycorrhizal roots under phosphate limitation (a) and optimal phosphate supply (b) in full illumination and shading treatments.** (± SE ; n=2). Columns and bars are based on two independent biological replicates. Plants were labeled 9 wpi, after 21 days of different light treatments and harvested 3 and 7 days past label.

In AM+P Shaded plants, the amount of NLFA in AM+P Shaded roots was reduced compared to AM+P Light (Fig. III.11b). PLFA were detected in a low amount with 20 µg/g root at 3 dpl. At 7 dpl PLFA were not detectable. The results are indicating that plants under optimal P supply might control the carbon transport to the fungi when shading reduces the carbon availability.
Additionally, the amount of NLFA and PLFA in AM-P Light roots was compared to the amount in AM+P Light roots. Plants under optimal phosphate supply and full illumination showed a reduction of NLFA and PLFA compared to AM-P Light plants. Under shading conditions, the amount of NLFA and PLFA was similar in AM-P Shaded and AM+P Shaded plants.
In summary, these results indicate that shading as well as optimal phosphate supply, reduced the amount of NLFA and PLFA in mycorrhizal roots.

3.3 Influence of carbon limitation via shading on relative expression patterns of genes involved in the carbohydrate pathway in mycorrhizal roots

Previously analyzed physiological data (see 3.1) and analyses of mycorrhizal fatty acids (see 3.2) indicated, that plants are able to control carbon transport mechanisms to the mycorrhizal fungi under shading conditions. To investigate possible mechanisms of regulation, transcriptomic analyses with Microarrays were conducted (2.2.5). Therefore, roots of the following treatments were used: AM-P Light, AM-P Shaded, AM+P Light, AM+P Shaded in three independent biological replicates. At first, identifier which are known to be involved in the mycorrhizal interaction were analyzed (Fig. III.12). The relative expression of the mycorrhiza induced phosphate transporter MtPT4 was reduced due to shading and optimal phosphate supply (Fig. III.12a). In contrast, the relative expression of ENOD40 was found to be induced in shading treatments (Fig. III.12b). The optimal phosphate supply did not influence the relative expression of ENOD40.

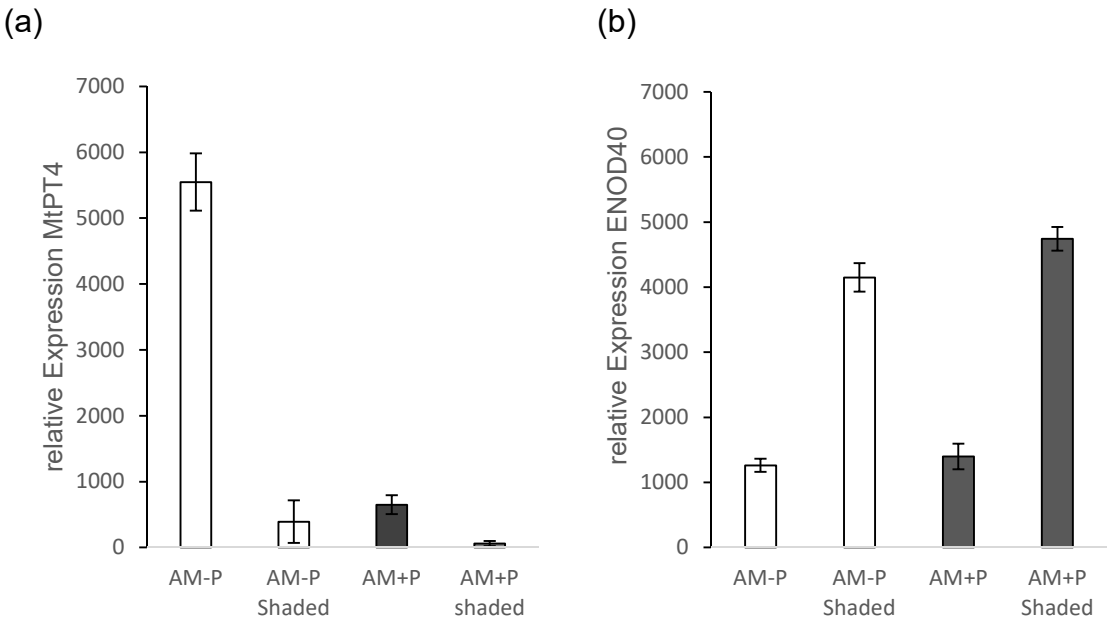

Fig. III.12 **Relative expression of the identifier of MtPT4 (a) and ENOD40 (b) known to be influenced during symbiotic interaction** (± SE; n=3).

3.3.1 Identification of candidate genes for regulation of carbon transport mechanisms in carbohydrate pathways in mycorrhizal roots

Sucrose plays a major role in carbon transport in plants. Due to the fact that mycorrhizal fungi are not able to take up sucrose but hexoses, all mechanisms which are involved in sucrose transport, sucrose degradation and transport of hexoses are of further interest. Furthermore, the synthesis of sucrose could be interesting, because it could be involved in mechanisms for the reuptake of carbon. The metabolism of sucrose degradation is one of the most important parts in mycorrhizal C supply.

3.3.1.1 Identification of candidates differentially regulated in AM-P and AM+P roots

To analyze if candidates for the regulation of carbon transport from plant to fungi can be found in carbohydrate pathways, the changes of expression of identifiers from AM-P Light to Shading and AM+P Light to Shading was compared. The analyses were restricted to identifier which were assigned to the pathways of major and minor CHO metabolism and sugar transport (2.2.5.1).
Changes in relative expression from light to shading of AM-P and AM+P roots were compared. Two different regulatory patterns were accepted as candidate identifier for

the regulation of carbohydrate transport mechanisms between plant and fungi (Fig.II.2).

The first pattern showed a high regulation from AM-P Light to AM-P Shaded. AM+P plants showed a regulation from AM+P Light to AM+P Shaded to a lower extent. This pattern is based on the theory, that AM-P plants show a higher downregulation of candidate genes, because they are more colonized by the AM fungi than AM+P plants. The second pattern showed a higher reduction from AM+P Light to AM+P Shaded plants compared to the regulation from AM-P Light to AM-P Shaded. Mycorrhizal fungi could represent an unnecessary additional carbon sink under shading conditions, when phosphate is available in an optimal amount. At this point plants would have the highest interest to quit the carbon transport to the fungi.

Candidate identifiers of genes which were higher regulated in AM-P roots compared to AM+P roots (pattern 1) were found in several pathways of the major and minor CHO metabolism (Table III.2).

Table III.2 **Candidates for regulation of carbon transport mechanisms between plant and fungi in less beneficial situations, following pattern 1.** Candidates are higher regulated in AM-P roots compared to AM+P roots. p-values according to Student´s t-test, two-sided, homogeneity of variance (2.2.8.1).

	Pathway	Identifier	Ratio AM-P	Ratio AM+P	p-value
	major CHO metabolism				
1	synthesis.sucrose.SPP	mtr.43643.1.s1_at	3.93	1.71	0.00
2	synthesis.starch.AGPase	mtr.40327.1.s1_at	1.71	1.21	0.01
3	synthesis.starch.AGPase	mtr.51589.1.s1_at	2.61	1.62	0.00
4	synthesis.starch.starch synthase	mtr.37384.1.s1_at	9.09	1.96	0.01
5	synthesis.starch.transporter	mtr.37984.1.s1_at	10.22	3.91	0.00
6	degradation.sucrose.fructokinase	mtr.26527.1.s1_at	2.97	1.74	0.03
7	degradation.sucrose.hexokinase	mtr.11173.1.s1_at	1.84	1.35	0.03
8	degradation.sucrose.hexokinase	mtr.42779.1.s1_at	2.17	1.36	0.03
	minor CHO metabolism				
9	myo-inositol.InsP-Kinases	mtr.22065.1.s1_at	6.55	1.95	0.00
10	myo-inositol.inositol phosphatase	mtr.14192.1.s1_at	4.85	2.00	0.00
11	galactose.alpha-galactosidases	mtr.10236.1.s1_at	9.31	2.51	0.00
12	others	mtr.45308.1.s1_at	7.02	3.72	0.00
13	others	mtr.12414.1.s1_at	6.04	2.60	0.00
14	others	mtr.31917.1.s1_at	8.39	4.27	0.01
15	transport.sugars	mtr.8579.1.s1_at	2.12	1.66	0.01

Only one candidate was found in the pathway of sucrose synthesis (Table III.2). The candidate represents a sucrose-phosphatase (pathway 1). Four candidates were found in the pathway of starch synthesis. Two identifier represented AGPases (pathway 2, 3). The other identifier represented a starch synthase (pathway 4) and a transporter which is part of starch synthesis (pathway 5). Both were highly downregulated when AM-P plants were shaded. The downregulation was also found when AM+P plants were shaded, but to a lower extent. Three candidates were found in the pathway of sucrose degradation. The candidates were represented by one fructokinase (pathway 6) and two hexokinases (pathway 7, 8). Fructokinases are involved in the degradation of sucrose. Hexokinases phosphorylating hexoses and convert them into hexosephosphates. Two hexokinases were found to be higher regulated in AM-P plants during shading (higher ratio in AM-P Light/AM-P Shaded) compared to AM+P (AM+P Light/AM+P Shaded) plants. In the minor CHO metabolism, six candidates were found. Two of them were part of the myo-inositol pathway (pathway 9, 10) and the other represented a galactosidase (pathway 11). The other three identifier were not assigned in a special pathway (pathway 12, 13, 14). For sugar transporters, only one identifier (pathway 15) was found to be a possible candidate for the regulation of carbon transport mechanisms from plant to fungi when a less beneficial situation occurs.

Based on regulatory pattern 2, shading of AM+P plants would create a super less beneficial situation. The major and minor pathways were analyzed for candidates which are downregulated from AM+P Light to AM+P Shaded to a higher extend compared to AM-P Light to AM-P Shaded. One candidate in the minor CHO metabolism and three candidates in sugar transport followed regulatory pattern 2 (Table III.3).

Table III.3 **Candidates for regulation of carbon transport mechanisms between plant and fungi in less beneficial situations, following pattern 2**. Candidates are higher regulated in AM+P roots compared to AM-P roots. p-values according to Student´s t-test, two-sided, homogeneity of variance (2.2.8.1).

	Pathway	Identifier	Ratio AM-P	Ratio AM+P	p-value
	minor CHO metabolism				
16	raffinosefamily. galactinolsynthases.putative	mtr.25494.1.s1_at	6.25	9.30	0.01
17	transport.sugars	mtr.11311.1.s1_at	1.58	2.64	0.01
18	transport.sugars.sucrose	mtr.12339.1.s1_at	1.56	2.34	0.03
19	transport.sugars.sucrose	mtr.43055.1.s1_s_at	1.59	3.00	0.03

The candidate which was identified in the minor CHO metabolism, represented a putative galactinolsynthase (pathway 16). Two of the three sugar transporters (pathway 17, 18, 19) were identified as sucrose transporter (pathway 18, 19).
In summary, 15 identifier were found to be significantly higher downregulated from AM-P Light to AM-P Shading compared to AM+P Light to AM+P Shading. One identifier was part of sucrose synthesis, four were found in the pathway of starch synthesis and three identifiers represented enzymes of the sucrose degradation pathway. In the minor CHO metabolism two candidates were found in the myo-inositol metabolism, one was a galactosidase and three candidates have not been specified. The last candidate represented a sugar transporter. For the hypothesis of the super less beneficial situation, in which the regulation of candidates would be higher from AM+P Light to AM+P Shading compared to AM-P Light to AM-P Shading, four candidates have been identified. One candidate was a galactinolsynthase, the three other candidates were identified as sugar transporters.

3.3.1.2 Identification of candidates with similar regulatory patterns in AM-P and AM+P roots

The analyses of candidates shown in 3.3.1.1 were based on the hypothesis that mycorrhizal plants under low and optimal phosphate supply would react differently to carbon limitation. Hence, identifiers were expected to show significant differences in regulation. Possible candidates for the regulation of carbon transport mechanisms in mycorrhizal symbiosis have been published before and some of them have already been shown to be involved in carbon supply for the fungi. In the analyses which identified candidates following regulatory pattern 1 and 2, none of these candidates were found. Plants could react to less available carbon with a general downregulation of transcripts of carbohydrate metabolism. This would result in a downregulation of identifiers of carbon metabolism which would not be correlated with the amount of colonization. To analyze the data for such candidates, the ratios of AM-P and AM+P plants were compared to each other. Candidates were those identifiers, which were downregulated due to shading, but to the same extent in AM-P ratios and AM+P ratios. These candidates were specifically analyzed in the “major CHO pathway. degradation.sucrose” and in sugar transport (Table III.4).

Table III.4 **Candidates which are generally regulated in the major CHO metabolism when mycorrhizal plants are in less beneficial situations.** Candidates are regulated in the same way in AM-P and AM+P roots due to shading. p-values according to student´s t-test, two-sided, homogeneity of variance (2.2.8.1).

Pathway	Identifier	Ratio AM-P	Ratio AM+P	p-value
major CHO metabolism				
major CHO metabolism.degradation.sucrose				
20 fructokinase	mtr.43020.1.s1_at	2.17	2.20	0.99
21 fructokinase	mtr.39221.1.s1_s_at	2.49	1.75	0.09
22 fructokinase	mtr.43528.1.s1_at	2.51	1.91	0.12
23 invertases.cell wall	mtr.24871.1.s1_s_at	2.35	1.90	0.41
24 invertases.cell wall	mtr.24817.1.s1_at	2.68	3.75	0.22
25 invertases.cell wall	mtr.45666.1.s1_at	3.87	2.58	0.58
26 invertases.vacuolar	mtr.43881.1.s1_at	5.97	7.26	0.95
27 Susy	mtr.45190.1.s1_at	1.58	1.80	0.80
28 Susy	mtr.32293.1.s1_at	1.76	1.69	0.84
29 Susy	mtr.22018.1.s1_s_at	2.21	2.00	0.54
30 Susy	mtr.43059.1.s1_at	5.77	5.69	0.83

Based on the hypothesis that candidates for regulation of carbon transport mechanisms from plant to fungi can be regulated generally as a reaction to carbon limitation, 11 candidates were identified. Three of the candidates represented fructokinases (pathway 20, 21, 22), three represented cell wall invertases (pathway 23, 24, 25) and one identifier was a vacuolar invertase. The other four candidates represented sucrose synthases (Susy: 27, 28, 29, 30). Especially, mtr.22018.1.s1_s_at is of further interest, because this is the identifier for MtSucS1. The sucrose synthase MtSucS1 is known to be a keyplayer during mycorrhizal carbon supply (Baier et al., 2010).

In the cluster of sugar transporters, 16 candidates were found to be downregulated in the same amount in AM-P roots as well as in AM+P roots due to shading (pathway 31-46; Table III.5).

Table III.5 Candidates for regulation which are involved in carbon transport between plant and fungi in less beneficial situations. Candidates are regulated in the same way in AM-P and AM+P roots due to shading. p-values according to Student´s t-test, two-sided, homogeneity of variance (2.2.8.1).

Pathway	Identifier	Ratio AM-P	Ratio AM+P	p-value
31 transport.sugars	mtr.35093.1.s1_at	1.59	2.12	0.37
32 transport.sugars	mtr.39507.1.s1_at	1.63	2.40	0.10
33 transport.sugars	mtr.17856.1.s1_at	1.67	1.69	0.92
34 transport.sugars	mtr.48631.1.s1_at	1.69	2.42	0.19
35 transport.sugars	mtr.21035.1.s1_at	1.97	2.22	0.75
36 transport.sugars	mtr.50247.1.s1_at	2.25	2.49	0.78
37 transport.sugars	mtr.43120.1.s1_at	2.33	2.85	0.54
38 transport.sugars	mtr.37475.1.s1_at	2.63	2.73	0.85
39 transport.sugars	mtr.9967.1.s1_at	2.81	2.12	0.24
40 transport.sugars	mtr.38695.1.s1_at	2.82	4.02	0.55
41 transport.sugars	mtr.48836.1.s1_at	2.83	2.50	0.33
42 transport.sugars	mtr.433.1.s1_at	3.60	2.84	0.65
43 transport.sugars	mtr.31164.1.s1_at	3.60	3.17	0.85
44 transport.sugars	mtr.38001.1.s1_at	3.85	3.28	0.95
45 transport.sugars	mtr.39031.1.s1_at	4.06	6.49	0.28
46 transport.sugars	mtr.10221.1.s1_at	18.81	19.10	0.78

In summary, 27 candidates were found to react to shading with the same pattern of downregulation. Eleven candidates represented enzymes of sucrose degradation, and 16 represented sugar transporters. We hypothesized that plants are able to control carbon transport to the fungi when a less beneficial situation occurs. The candidates which were identified in this study, were all regulated in the less beneficial situation of carbon limitation caused by shading, but not under optimal phosphate supply. These candidates could indicate that plants might react with different regulatory mechanisms to different less beneficial situations. Alternatively, regulation of carbon transport from plants to fungi could be driven by carbon availability.

3.4 Enrichment analyses of mycorrhizal plants under carbon limitation

After focusing on the identifiers of sucrose synthesis, an enrichment analyses as an additional method to study metabolic pathways was performed (2.2.5.2, Fig. II.3, Fig. II.4). Small changes, with a ratio (AM Light/AM Shaded) of less than 0.5 in the relative expression have not been recognized as candidates, but can have an influence.

3.4.1 Comparison of transcriptomic regulations in shaded AM-P and AM+P plants

We found an enrichment in the “major CHO metabolism.synthesis.starch.AGPase” for the AM-P (AM-P Light/AM-P Shaded) treatment. Additionally, parts of glycolysis and the TCA cycle showed an enrichment. The most prominent part was an enrichment in lipid metabolism. This was split into the metabolic pathways of “FA synthesis and FA elongation” and “lipid degradation”. Furthermore, the regulations in AM-P (AM-P Light/AM-P Shaded) plants showed more enriched pathways than AM+P (AM+P Light/AM+P Shaded) plants. In AM+P plants starch synthesis was the only carbohydrate involved metabolism which showed an enrichment in regulation from Light to Shaded. Based on an enrichment analysis, no difference in gene regulation in carbon transport mechanisms or sugar converting enzymes was found. To ensure that this finding did not result from a phosphate effect, the identifiers were compared to -P (20 µM) and a +P (2 mM) treated *Medicago truncatula* plant dataset from Hogekamp et al. (2011). This comparison showed, that most of the enriched pathways were not based on a phosphate effect (Table III.6).

Table III.6 **Results from PageMan enrichment analyses for AM-P (AM-P Light/AM-P Shaded) and AM+P (AM+P Light/AM+P Shaded) treatments compared to enrichment analyses of nonmycorrhizal –P and +P datasets.** Blue color indicates a higher abundance of regulated identifier than it would be expected. The nonmycorrhizal –P and +P data are based on the work of Hogekamp et al., 2011.

Pathway	AM-P	AM+P	-P	+P	
major CHO metabolism					major CHO metabolism
					major CHO metabolism.synthesis
					major CHO metabolism.synthesis.starch
					major CHO metabolism.synthesis.starch.AGPase
glycolysis					glycolysis
					glycolysis.enolase
					glycolysis.PK
					glycolysis.PEPCase
TCA/org. transformation					TCA/org. transformation
					TCA/org. transformation.TCA
					TCA/org. transformation.TCA pyruvate. DH
					TCA/org. transformation.TCA pyruvate. DH.E3
lipid metabolism					lipid metabolism
					lipid metabolism. FA synthesis and FA elongation
					lipid metabolism. FA synthesis and FA elongation. Acetyl CoA Carboxylation
					lipid metabolism. FA synthesis and FA elongation. ketoacyl ACP synthase
					lipid metabolism. FA synthesis and FA elongation. ACP oxoacyl reductase
					lipid metabolism. FA synthesis and FA elongation. beta hydroxyacyl ACP dehydratase
					lipid metabolism. FA synthesis and FA elongation. enoyl ACP reductase
					lipid metabolism. FA synthesis and FA elongation. pyruvate kinase
					lipid metabolism. FA synthesis and FA elongation. pyruvate DH
					lipid metabolism. 'exotics' (steroids, squalene etc) sphingolipids. ceramide.glucosyltransferase
					lipid.metabolism.lipid degradation. lysophospholipases
					lipid.metabolism.lipid degradation.lysophospholipases.carboxylesterase
					lipid.metabolism.lipid degradation.lysophospholipases.glycerophosphodiester phosphodiesterase
					lipid.metabolism.glycolipid synthesis
					lipid.metabolism.glycolipid synthesis.MGDG synthase
					lipid.metabolism.glycolipid synthesis.sulfolipid synthase

In summary starch synthesis AGPase, enolase and pyruvate kinase in glycolysis, pyruvate dehydrogenase E3 in TCA/org transformation and the FA synthesis and FA elongation pathway in lipid metabolism could be involved in the regulation of plant carbon transport mechanisms between plant and fungi in a less beneficial situation. Candidates for the regulation of carbon transport mechanisms between plant and fungi which were published by other groups, did not meet the requirements for mycorrhizal dependent regulation.

Additionally, we found pathways which were higher regulated from AM-P Light to AM-P Shaded, compared to AM+P Light to AM-P Shaded.

4. Discussion

It was hypothesized that plants are able to control carbon transport mechanisms to the fungi when a less beneficial situation occurs. The symbiotic interaction between plant and fungi has been observed to potentially depress plant growth and is expected to play an important role for plant growth when carbon is limited (Hayman 1974; Peng et al. 1993). It has been shown before that light limitation or phosphate supply could result in a lower root colonization (Tester et al. 1986; Bruce et al. 1994; Olsson et al. 1997). Especially under light limited conditions, it could be a consequence of a lack of assimilates in the roots (Konvalinkova and Jansa 2016). Plants which were in an established symbiotic interaction were supplied with low phosphate (AM-P) to create a control situation and with an optimal phosphate supply (AM+P) to create a less beneficial situation (2.2.2). Additionally both treatments were shaded to create a carbon limited situation (AM-P Shaded and AM+P Shaded), to investigate how the regulation of carbon transport mechanisms is done.

4.1 Plants regulate the carbon transport to mycorrhizal fungi when a less beneficial situation occurs

To answer the question, how the induction of less beneficial situations influence physiological parameter of the plant, several situations where the fungi could be less beneficial for the plant were induced. We expected that mycorrhizal plants in such less beneficial situations would not show any negative responses if they are able to control carbon allocation to the fungi. The fresh weight measurement showed that shading led to a reduction in plant weight, demonstrating that the artificial carbon limitation, which was one of the most important aims of the experimental setup, was successful (3.1.3). In comparison to -P plants, AM-P plants showed a higher fresh weight gain which was surprisingly independent of light treatment (3.1.3). Shading experiments of mycorrhizal plants were conducted before, and the studies observed no or even negative reactions of mycorrhizal growth response (MGR) on shading treatments (Bereau et al. 2000; Zheng et al. 2015; Son and Smith 1988; Konvalinkova et al. 2015; Marschner and Timonen 2005; Gehring 2003; Smith and Gianinazzi-Pearson 1990; reviewed in Konvalinkova and Jansa 2016). Studies with mycorrhizal *Medicago truncatula* and

Trifolium sp. in treatments comparable to the shown experimental setup, did not show a benefit from the fungi under long-term shading (Olsson et al. 2010; Konvalinkova et al. 2015).

AM-P Light and AM-P Shaded plants showed a significantly higher fresh weight gain compared to -P Light and -P Shaded plants. The increase in plant weight showed that there must be a regulatory mechanism that prevents a parasitic situation.

The novel observation of positive influence on fresh weight gain of AM-P Shaded plants fits in the model of (Tuomi et al. 2001) which states that nutrient limited plants profit most from the symbiosis with AM fungi. Under shading conditions the positive effect could still occur if the increase of P-acquisition by AM symbiosis allows an increase of C assimilation, which is high enough to compensate the carbon demand of the fungi. Additionally, this observation confirms the suggestion that C costs of AM fungi are lower than the photosynthetic capacity of the host (Johnson et al. 2015). If plants can regulate the amount of transported carbon to the fungi, both partners can stay in a symbiotic interaction. As soon as the photosynthetic capacity is upregulated again, the fungi can again receive more carbon from the plant.

The surprising observation of the positive influence of mycorrhizal fungi on the fresh weight gain of AM-P Shaded plants could also be explained with the establishment of the symbiotic interaction in full light. The mycorrhizal fungi had the chance to store a lot of energy in the form of vesicles during phases of light treatment and can use this energy store to keep on growing if the plant does not supply the fungus with carbon anymore. This hypothesis was confirmed by the observation, that shading reduced the fungal neutral lipid fatty acids. The analysis of NLFA and PLFA allowed an inside view on nutritional and growing status of the fungus, as their synthesis is based on host carbon supply. NLFA are found in mycorrhizal vesicles, which are used for carbon storage in mycorrhizal fungi. The reduction of NLFA has been compared with the status of mycorrhizal colonization before (Olsson et al. 2010). The hypothesis of a regulation of carbon partitioning from plant to fungi was also promoted by the finding, that the amount of NLFA was massively reduced due to shading (3.2.3). PLFA, as membrane constituents, can be used as biomass indicators for the amount of mycorrhizal fungi in roots and soil. PLFA were found to be reduced in AM-P Shaded plants (3.2.3).

Mycorrhizal colonization, evaluated with the magnified intersection method, showed no clear reduction of mycorrhizal fungi (3.1.2, 3.2.1). The shading of AM-P plants resulted in a significant reduction of arbuscules, but no significant changes for the amount of

vesicles and hyphae were observed. In comparison to other studies, the reduction of arbuscules confirmed the results of Son and Smith (1988) and Vierheilig et al. (2002). Using the mycorrhizal marker MtPT4 (Fig. III.12), a clear reduction of interaction due to shading was detected. This observation confirms the above described reduction of arbuscules, as MtPT4 is located in the periarbuscular membrane (Harrison 2002). It was shown before that shading of mycorrhizal plants resulted in a reduction of mycorrhizal structures in plant roots (Konvalinkova et al. 2015), but the fungi are not completely eliminated (Schubert et al. 1992). The decrease of fungal colonization due to shading has also been described in different experimental setup in pots (Olsson et al. 2010; Shi et al. 2014) and in field studies (Heinemeyer et al. 2004). A significant reduction of arbuscules under shaded conditions point towards a reduced amount of carbon allocated from the plant to the fungi. As arbuscules are important exchange sites in the mycorrhizal symbiosis, with a lifetime of about 8.5 days (Alexander et al. 1989), the observed reduction is of further interest. A reduction of carbon transport from plant to fungi should result in a reduction of arbuscules, if a reduction of carbon allocation from the plant to the fungi affects living and growing structures first. Following the reduction of arbuscules, a reduced growth of vesicles and hyphae should follow. It was hypothesized by others before, that a reduction of exchange structures like arbuscules, with an additional increase of storage organs like vesicles, indicates a shift towards parasitism by the fungi (Johnson 1993).

Another indication for the control of fungal carbon availability by the plant was the result of total carbohydrate determination. We observed an increase of total carbohydrates in AM-P Light roots compared to -P roots (3.1.4). Shading decreased the amount of total soluble carbohydrates to the same amount found in nonmycorrhizal -P plants. The amount of total soluble carbohydrates in roots is known to be increased by mycorrhization (Hampp and Schaeffer 1999). The reduction could be a reaction of the plant to reduce the available carbohydrates for the fungi. As the fungi depend on plant hexoses, a reduction of hexoses could be crucial for a continuing interaction with the fungi. The mycorrhizal fungus is an obligate biotroph and is not able to take up sucrose. The fungus needs the plant to cleave sucrose in hexoses, these hexoses are taken up by the fungi.

A reduction of carbohydrates in the plant roots reduces the amount of hexoses available for the fungi. This is an indication for the reduction of carbon transferred from

the plant to the fungi. To confirm this findings, a specific measurement of sucrose and hexose concentrations needs to be performed.

The influence of optimal phosphate supply on mycorrhizal plants was investigated with the comparison of AM-P Light to AM+P Light plants. Plants under optimal phosphate supply showed a lower abundance of hyphae (3.1.1). Furthermore, the total amount of PLFA and NLFA was reduced in AM+P Light roots compared to AM-P Light (3.2.3). The total amount of NLFA was reduced by a factor three.

AM+P Light plants were not able to benefit from the fungi, but also no reduction of fresh weight was observed (3.1.3). Shading likewise did not result in a fresh weight reduction for AM+P Shaded plants (Olsson et al. 2010). The counting data of mycorrhization were again differing from transcriptomic and NLFA data. Furthermore, vesicles were found in AM+P plants (3.1.1). To build these storage organs, fungi need to be supplied with carbon by the plant. NLFAs which serve as carbon storage to the fungi showed a clear reduction from AM+P Light to AM+P Shaded plants. A high phosphorus availability in soil usually reduces the mycorrhizal formation in plants (Jasper et al. 1979) and as a result reduces the carbon allocation to roots and mycorrhizal components (Eissenstat 1993; Graham et al. 1997). Olsson et al. (2010) suggested an influence of phosphate supply on mycorrhization rates, independent of the fact that *Rhizophagus intraradices* is highly tolerant to phosphate supply (Douds and Schenck 1990). Former studies showed that phosphate supplied mycorrhizal plants decreased in mycorrhization rates when they were shaded (Tester et al. 1986; Bruce et al. 1994; Olsson et al. 1997). The reduction of colonization following the phosphate supply which is often observed (Bruce et al. 1994) has been explained with a reduction in AM germ tube formation of spores, reduced exudation of branching factors and finally a faster growth of roots (Smith and Read 2008). The argument of faster growing roots is valid for the AM+P treatment, because the gain in root fresh weight for AM+P plants was higher compared to AM-P plants. During full illumination, vesicles were detected in AM+P Light plants, but not as much as in AM-P Light plants. It is still questionable if the mycorrhizal structures are actively reduced by the plants, as fungal structures usually do not disappear, but growing roots are not newly colonized by the mycorrhizal fungi (Olsson et al. 2010).

This explanation would be also valid for our observation during the shading treatments. It has been shown that the C allocation to fungal partners is coupled with the exchange of phosphate or nitrogen (Hammer et al. 2011; Kiers et al. 2011; Fellbaum et al. 2014).

But the +P plants may supply the fungi with carbohydrates in advance of environmental conditions in which the plant would need an arbuscular mycorrhizal fungi. It was hypothesized, that the additional carbon sink can be easily compensated in full light, but can also turn out to be problematic during light limitation. We did not observe a shift into a parasitic situation. Furthermore, there may exist other benefits for the plants from the fungi, like increased resistance against pathogens, which are currently not known.
A super less beneficial situation was created by shading of mycorrhizal plants under optimal phosphate supply when AM-P Shaded plants were compared to AM+P Shaded plants, the amounts of arbuscules, vesicles and hyphae were found to be significantly reduced in AM+P Shaded plants (3.1.1). The comparison of fungal fatty acids in the super less beneficial situation showed no outstanding effect (3.2.3). PLFA amounts were mainly influenced by light intensity. The amount of NLFA was only reduced by optimal phosphate supply, when light was not limited.
It has been shown in a comparable experimental setup with *Trifolium sp.* that NLFA are reduced by phosphate supply and not by shading (Olsson et al. 2010). In our experiments, whereas the total amount of PLFA was found to be mainly influenced by light intensity, the amount of NLFA was found to react to phosphate availability as well as to light intensity.
The ^{13}C labeling was used to follow the carbon transported from plant to fungi and to determine the amount of carbon that was transported from the plant to the fungi under less beneficial situations (3.2.2). When AM-P plants were shaded, the ^{13}C transport from plant to fungi was reduced. Olsson et al. (2010) found a reduction of carbon transport to *Rhizophagus intraradices* when clover plants in an established symbiotic interaction were supplied with phosphate, whereas shading did not have the same effect. With regards to the fact that shading influences the total amount of ^{13}C in the roots, the fraction of root ^{13}C to fungal ^{13}C was calculated. This calculation showed a clear reduction of ^{13}C in fungal fatty acids, due to shading. This reduction was independent of the optimal phosphate supply of the plant. Furthermore, the ^{13}C amount in NLFA and PLFA was also reduced due to shading. The ^{13}C in PLFA was nearly not detectable in shading treatments. When the ^{13}C transport from AM+P plants to the fungal fatty acids was analyzed, the influence of light was found to be more important than the amount of available phosphate.
The evaluation of the physiological data showed, that mycorrhizal plants are able to control the carbon partitioning in less beneficial situations. AM-P plants can benefit

from the symbiotic interaction with *Rhizophagus intraradices*, independent of light treatments. For AM+P plants, no effect of the mycorrhizal fungi on fresh weight gain was observed and this was independent of light treatment. Several studies were carried out to investigate the influence of less beneficial situations on the mycorrhizal symbiosis (reviewed in Kovalinkova and Jansa, 2016). The studies were based on different plants species and fungi and in addition, the experimental setup were highly variable. The plants were shaded at differing times after inoculation, with different light intensities for different shading durations. Even the results of shading are displayed in various ways. The mostly used mycorrhizal growth response (MGR) is not always calculated in the same way, but always displays the differences between mycorrhizal and nonmycorrhizal plants. Due to this, published data are hardly comparable. A detailed review of the effects of light limitation on mycorrhizal fungi has been published by Konvalinkova and Jansa (2016). Their comparison showed, that the effects of light limitation on mycorrhizal fungi were highly variable. It was hypothesized, that the amount of vesicles are an indicator for the influence of shading on the fresh weight gain of mycorrhizal plants. To review this hypothesis, an experimental setup focused on vesicle number and NLFA concentration would be of further interest. If mycorrhizal plants with nearly no vesicles become shaded, the fungi should turn parasitic immediately if vesicles are keyplayer in the interaction and if carbon transport is not regulated. In a study with a similar experimental setup in which mycorrhized *Trifolium subterraneum* were shaded under an optimal P supply, it was shown that the plants were able to control the carbon transport to the fungi under optimal P supply, but not under shading conditions (Olsson et al. 2010). This result was not reproduced in this experiment with *Medicago truncatula.* Instead it was shown that light intensity was more important than phosphate supply. Another study with mycorrhized *Medicago truncatula* showed that host plants could not benefit from the *Rhizophagus intraradices* under long-term shading depending on plant weight and phosphate uptake (Konvalinkova et al. 2015). Therefor both of these studies differ from this one. One reason could be the different inocula used. Whereas in this study only mycorrhizal spores were used, the inocula of Konvalinkova and Olsson also contained bacteria which represent an additional sink of unknown intensity. This was not the case for the plants which were used in this study. Furthermore, Olsson et al. (2010) could not show that *Trifolium* benefit from the symbiotic interaction in full light. Shading massively reduced the amount of vesicles and living fungal structures indicating that the fungi

had to use their energy storage to keep on growing when light was limited. The low colonization rate in shaded plants observed by other groups was hypothesized to be the reason for a reduced C supply of the fungi, and not an active regulation of carbon flux by the plant (Olsson et al. 2010). At this point it is obvious, that plants can control the carbon transport and partitioning to the fungi under our experimental conditions. If this is due to an active or a passive mechanism cannot be differentiated.

PLFA and NLFA amounts showed a clear reduction due to shading which supported the results of the transcriptomic MtPT4 measurements. The growth data revealed the symbiotic interaction between plant and fungi must underlie a regulatory mechanism when plants were shaded and phosphate was limited. The total amount of PLFA and NLFA in AM-P Shaded and AM+P Shaded roots was reduced. A reduction of NLFA shows a decrease in storage organs of the fungi, an indicator for fungal starvation. In summary, the mycorrhizal infection of *Medicago truncatula* was reduced by additional P supply and/or shading. The amount of ^{13}C was reduced in NLFA and PLFA due to shading. It was hypothesized that the C drain of mycorrhizal fungi would not be high enough to decrease mycorrhizal growth response under low light (Hayman et al. 1974). This hypothesis was confirmed in our experimental setup. When AM-P and/or AM+P plants were shaded no negative effects on plant fresh weight gain were observed and the ^{13}C amount transported from the host to the fungi were found to be reduced. These data lead to the assumption that plants are able to control the carbon partitioning to the fungi, and the mycorrhizal interaction did not get parasitic.

4.2 Transcriptomic analyses

With the knowledge of a regulatory mechanism, transcriptomic analyses were performed to identify possible candidates involved in the regulation of carbon transport and partitioning mechanisms. We hypothesized, that candidates can be found in the carbohydrate metabolism, and that the regulation of carbon transport and partitioning is an active process.

4.2.1 Candidates for regulation of carbon partitioning in carbohydrate metabolism

The analyses of transcriptomic data identified potential candidates which could be involved in the regulatory processes. The candidates were identified as an aldehyde

dehydrogenase homolog, two sucrose synthases, a hexokinase, a porin/voltage dependent anion selective channel protein and a sugar porter /SP) family Major Facilitator Superfamily (MFS) transporter (3.3). The aldehyde dehydrogenase could not be further specified. The sucrose synthases were the Mtr.22018.1.s1_at and Mtr.43059.1.s1_at. It was hypothesized before that sucrose synthases might partly support the hexose demand of symbionts (Shachar-Hill et al. 1995). Studies revealed that sucrose synthases play important roles in symbiosis, for example during the interaction with rhizobia (Gordon et al. 1999; Xie et al. 2003) and mycorrhiza (Hohnjec et al. 2003). The sucrose synthase which is assigned to Mtr.22018.1.s1_at represent MtSucS1. MtSucS1 was first shown to be nodule enhanced and specifically upregulated in endosymbiotic tissues (Hohnjec et al. 1999), and later also during mycorrhizal symbiosis (Hohnjec et al. 2003). Promoter studies showed a predominant activity in colonized cells (Hohnjec et al. 2003). A knockdown of MtSucS1 affected arbuscule maturation and conservation (Baier et al. 2010). Considering the importance of sucrose synthases for symbiotic interactions, these synthases are suitable candidates to act as keyplayer in the regulation of carbon transport from plant to fungi when a less beneficial situation occurs. However, it is not clear if a reduction of transcripts results from an active regulation of the plant or from a previous reduction of mycorrhizal colonization, as the candidate was identified to be regulated by light intensity (3.3.1.2).

This hexokinase was identified by Mtr.11173.1.s1_at and Mtr.42779.1.s1_at. Hexokinases are key enzymes for glucose utilization and sensing (Jang et al. 1997). These enzymes are catalyzing the phosphorylation of glucose to glucose-6-phosphate. Furthermore, hexokinases can act as important signaling molecules. Saito (1995) found a high activity of hexokinase in intraradical hyphae of *Gigaspora margarita*, which showed that there is also an active glycolysis in fungal hyphae (Solaiman and Saito 1997). To ensure that the identified hexokinase was not only expressed in intraradical hyphae, expression was compared to expression data in the mtgea.noble database. As hexokinases play a major role in the degradation of sucrose they are suitable candidates for the regulation of carbon transport from plant to fungi in a less beneficial situation.

Additionally, to the enzymes which were identified as candidates for the regulation of carbon transport from plant to fungi when a less beneficial situation occurs, two sugar transporters were found. The transporter Mtr.37475.1.s1_st was assigned to a

porin/voltage-dependent anion selective channel protein (VDAC) and Mtr.10221.1.s1_at was identified as a sugar porter (SP) family MFS transporter. Both transporters are not specified in more detail, and their role in mycorrhiza symbiotic interactions is unknown.

4.2.1.1 Sucrose synthase as a keyplayer in symbiotic interactions

One of the candidates which remained from the analysis described above was the sucrose synthase Mtr.22018.1.S1_s_at and Mtr.43059.1.S1_at. Sucrose synthases are known to play important roles in symbiosis, e.g. during the interaction with rhizobia (Gordon et al. 1999; Xie et al. 2003) and mycorrhiza (Hohnjec et al. 2003). Thus, sucrose synthases are suitable candidates as keyplayers in the regulation of carbon transport from plant to fungi when a less beneficial situation occurs. The regulation of sucrose synthase is carried out in many ways. Sucrose synthases respond, among others, to sugar availability and some of them are upregulated by sugar depletion, whereas others are upregulated by sugar abundance (Koch et al. 1992; Koch 1996). Additionally, the localization of sucrose synthase can be affected by sugars and other signals (Hardin et al. 2004; Winter and Huber 2000; Winter et al. 1998). Under some conditions the sucrose synthase protein shows an extreme stability and several mechanisms control its turnover (Halford et al. 2004; Koch 2004). This means that an increase in transcript amount does not necessarily have an influence on carbon flux from plant to fungi. The enzyme is activated via phosphorylation (Huber et al. 1996; Zhang et al. 1999) and the phosphorylation at the first site marks the sucrose synthase to be phosphorylated at the second site (Hardin et al. 2003). The second phosphorylation site plays an important role for the degradation of the protein. This site targets the sucrose synthase for ubiquitin-mediated degradation via the proteosom (Hardin et al. 2003). An important point when sucrose synthase stability is discussed in context with symbiotic interaction is the influence of ENOD40. The breakdown of sucrose synthase can be inhibited by blocking the second phosphorylation site, for example by ENOD40 proteins. The ENOD40 protein plays an important role in early nodule development, many other functions of this protein in plants were detected (Hardin et al. 2003; Rohrig et al. 2002; Kouchi et al. 1999). The association of ENOD40 with sucrose synthase potentially indicates involvement of ENOD40 in the control of vascular function, phloem loading/unloading and assimilate import (Hardin et al. 2003;

Varkonyi-Gasic and White 2002; Kouchi et al. 1999) Therefore, symbionts can possibly have an active influence on the stability of proteins in carbon transport pathways in the plant. The ENOD40 is known to be induced by mycorrhizal interactions (van Rhijn et al. 1997). Due to the lower mycorrhization in shaded plants, a reduction of ENOD40 in the shading would be expected, but an increase of ENOD40 due to shading was observed. This could mean, that a symbiotic interaction could influence the activity of sucrose synthase by increasing the stability and as a result the lifetime of the enzyme. Due to the fact that the mycorrhizal fungi depend on the supply of hexoses by the plant, an increased stability of the sucrose converting enzyme is of high interest because it could increase the carbon supply for the fungi. As a consequence, the fungi would be able to control its situation and this would be a hind for a parasitic tendency of the fungi.

It was hypothesized by Shachar-Hill et al. (1995) that the encoded sucrose synthase might partly support the hexose demand of symbionts. Due to MtSucS1 expression patterns it is likely that not only arbuscule containing cells (Blee and Anderson 1998) but also intraradical hyphae are a predominant interface for hexose supply from plant to fungi (Bago et al. 2002b). Due to the known role of MtSucS1 in the carbon supply of mycorrhizal fungi and the influence of stabilizing symbiotic plant factors, it is likely that the reduction in transcript amounts plays an important role in carbon flux regulation. It cannot clearly be differentiated if the MtSucS1 transcripts are downregulated due to the reduction of mycorrhization, or if the downregulation results from a reduction of mycorrhization. In summary, MtSucS1 plays an important role in mycorrhizal carbon supply and a downregulation of transcripts is an important part of regulation of the transport mechanism.

4.2.2 Shading of mycorrhizal plants results in an enrichment of regulation in different pathways

It was hypothesized that metabolic pathways or components would be influenced by shading of mycorrhizal plants. Glycolytic enolase, pyruvate kinase, the pyruvate dehydrogenase E3 of the TCA/org. transformation pathway and the pathway of fatty acid synthesis were overrepresented in regulation (3.4).

4.2.2.1 Shading of mycorrhizal plants results in a regulation of the fatty acid synthesis pathway

The most prominent pathway regulated in AM-P plants which were shifted from light to shading was the fatty acid synthesis. When the parts of the pathway were compared to the synthesis of fatty acids in plants, it was obvious that nearly every step of the pathway showed a higher regulation and transcript amount in AM-P plants compared to the AM+P treatment (Fig. IV.1). 3-ketoacyl-ACP-synthase is crucial for the synthesis of fatty acids. The reaction which is catalyzed by this enzyme cannot be reversed.

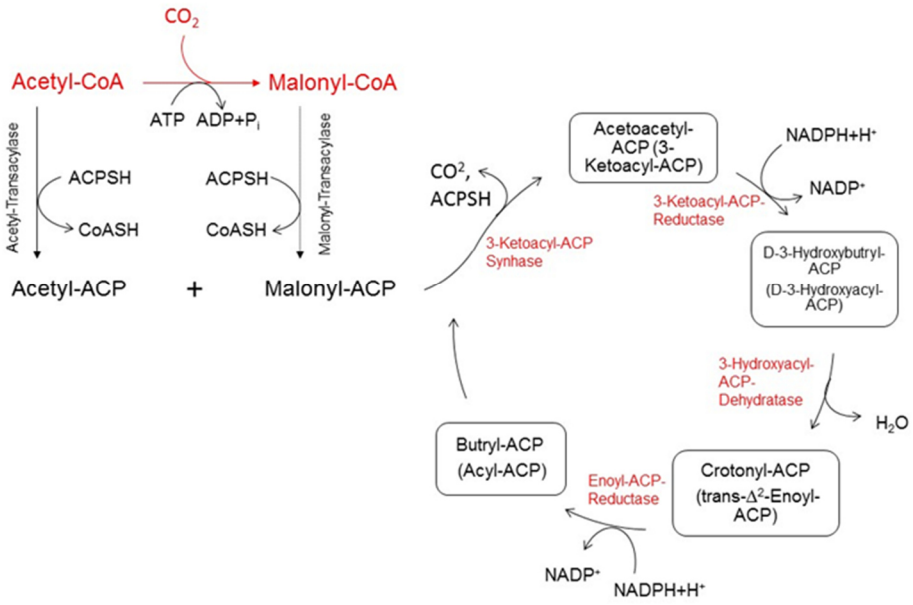

Fig. IV.1 Synthesis of fatty acids in plants. Enzymes and reactions enriched in regulation are written in red.

It is known, that mycorrhizal interactions increase the plant's need for fatty acids. During mycorrhizal infection, membranes and cell morphology needs to be massively reorganized. Therefore large amounts of plant cell membranes are required (Gaude et al. 2012). The periarbuscular membrane (PAM) represents an area between fungus and plant. These lipids can be delivered by the synthesis of new fatty acids, or via the breakdown of storage lipids. Gaude et al. (2012) showed that the remodeling of cellular membrane systems leads to an induction of expression of genes which are involved in lipid metabolism. The colonization of host cells resulted in an upregulation of 27 transcripts involved in lipid metabolism. Only two transcripts were downregulated. In adjacent cells the enhanced expression was comparable to colonized ones.

Furthermore, Gaude et al. (2012) showed an upregulation of transcripts involved in lipid breakdown in arbusculated cells. High expression levels were found for example for genes encoding key enzymes of fatty acid synthase system.

This could explain our observation that fatty acid synthesis is downregulated when the plants are shaded, since mycorrhization is also reduced under shading. However, phospholipid synthesis which is mainly needed to build up new membranes, was not upregulated. If these fatty acids are used for synthesis of membranes, the plant could stop the symbiotic interaction by a downregulation of the transcript. Another explanation could be the supply of the fungi with lipids from the plant. Up to now it has only been described that plants support the fungi with carbohydrates. In GUS staining experiments of hyphae it was shown that hyphae of *Rhizophagus intraradices* showed a blue coloration, even when the GUS construct was under control of the plant MtSucS1 promoter (Hohnjec et al. 2003). It was hypothesized that the hydrophobic GUS complex might be stored in lipid bodies and would visualize the proposed flow of lipids in vicinity of fungal carbon uptake (Hohnjec et al. 1999; Bago et al. 2002b; Bago et al. 2002a). Journet et al. (2001) observed the same but hypothesized that this was an artifact resulting from local diffusion of soluble monomeric products of the GUS reaction. In fungi, the fatty acid synthase complex is necessary to catalyze the fatty acid synthesis (Wakil et al. 1983). It was found, that *Glomus intraradices* and the phylogenetically distant fungus *Gigaspora rosae* are lacking the fatty acid synthase, and it was hypothesized that fungi acquire palmitic acid from their plant host (Trepanier et al. 2005). Furthermore, it was proposed that the large amounts of fatty acids in AM fungi could be a result of a very efficient lipid transfer mechanism between two organisms (Trepanier et al. 2005). It has been shown by Pfeffer et al. (1999) that the fungal lipid synthesis occurs in root compartments. In plant defense mechanisms, lipid transfer proteins are active (Kader 1996), and they could be produced during hyphal penetration (Blilou 2000). These proteins could be involved in this transport mechanisms (Trepanier et al. 2005). Labeling experiments which used ^{14}C sucrose and acetate showed that synthesized 16-carbon fatty acids occur exclusively in intraradical hyphae, but not in extraradical hyphae or germinating spores (Trepanier et al. 2005). The major problem of analyzing fatty acids is the fact, that plants and fungi are synthesizing the same fatty acids. Therefore, it is hard to distinguish between their origins. The downregulation of the metabolic pathways which are used to synthesize the lipids could be a hint for a regulation of fungal carbon support. One way of the plant

to control the transfer of carbon or lipids to the fungus could be the inhibition of the transport of 16-C carbon fatty acids. Without these substructures the fungus is not able to synthesize its own fatty acids and would not be able to grow. If the fatty acids are used to prepare the membrane structures for the symbiotic interaction, a downregulation of the synthesis of these fatty acids would result in a downregulation of mycorrhization. Both mechanisms would mean that the plant controls the situation and could quit the interaction when it turns less beneficial.

4.2.2.2 Shading of mycorrhizal plants results in a regulation of glycolysis enolase and pyruvate kinase

We found glycolytic enolase and pyruvate kinase to be enriched amongst the regulated genes. Enolases are ubiquitous enzymes which are catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate. They are also called 2-phospho-D-glycerate hydrolases, and the reaction catalyzed by these enzymes is the only dehydration step in the glycolytic pathway (van der Straeten et al. 1991). Enolases play an important role in non-green tissues, which depend on glycolysis and oxidative phosphorylation for their energy supply (Goodwin and Mercer 1983). The regulation of enolases is based on complex mechanisms, which involves the control at the level of transcription and also at translational and post-translational levels (van der Straeten et al. 1991). Other studies found that in nodules enolases were expressed at high levels in infected cortical cells, as well as in the pericycle of the central vascular bundle of a nodule lobe (van der Straeten et al. 1991).
Pyruvate kinases catalyze the reaction of Phosphoenolpyruvate to pyruvate (Plaxton 1996). The generated pyruvate can participate in different pathways. Pyruvate can be respired, or it can participate in the biosynthesis of fatty acids, for example. It was hypothesized that the decrease in transcript amounts of enolase and pyruvate kinase results from a downregulation of root respiration or the downregulation of fatty acid biosynthesis due to shading of mycorrhizal plants. The other option is the usage of pyruvate in mitochondrial respiration (Plaxton et al. 1996). Due to the reduction of mycorrhization, it is likely that the transport of pyruvate to mitochondria is likewise reduced. It is unclear if glycolysis is downregulated because of the reduction of mycorrhization, or if the mycorrhization is reduced due to a downregulation of glycolysis. Plant glycolysis is known to be regulated from the bottom up. Primarily, the

regulation is based on the usage of PEP by pyruvate kinase (Paul and Pellny 2003). An explanation could be that the major reason for the downregulation of the transcripts of enolases and pyruvate kinases is the reduction of pyruvate usage in the above described mechanisms and not *vice versa*.

4.2.2.3 Downregulation of pyruvate dehydrogenase as a part of TCA/organic transformation

A possible explanation for the downregulation of pyruvate dehydrogenase transcripts could be the downregulation of pyruvate kinase (4.2.2) which could result in a reduction of pyruvate. Pyruvate dehydrogenase produces acetyl-CoA from mitochondrial pyruvate for the TCA cycle (Sweetlove et al. 2010). As already mentioned above (4.2.1, 4.2.2), enzymes of glycolysis showed up as candidates for regulation of carbon partitioning between plant and mycorrhizal fungi (Fig. IV.2). A possible reason for the enrichment in downregulation of pyruvate dehydrogenase could be a reduction of pyruvate delivered from glycolysis.

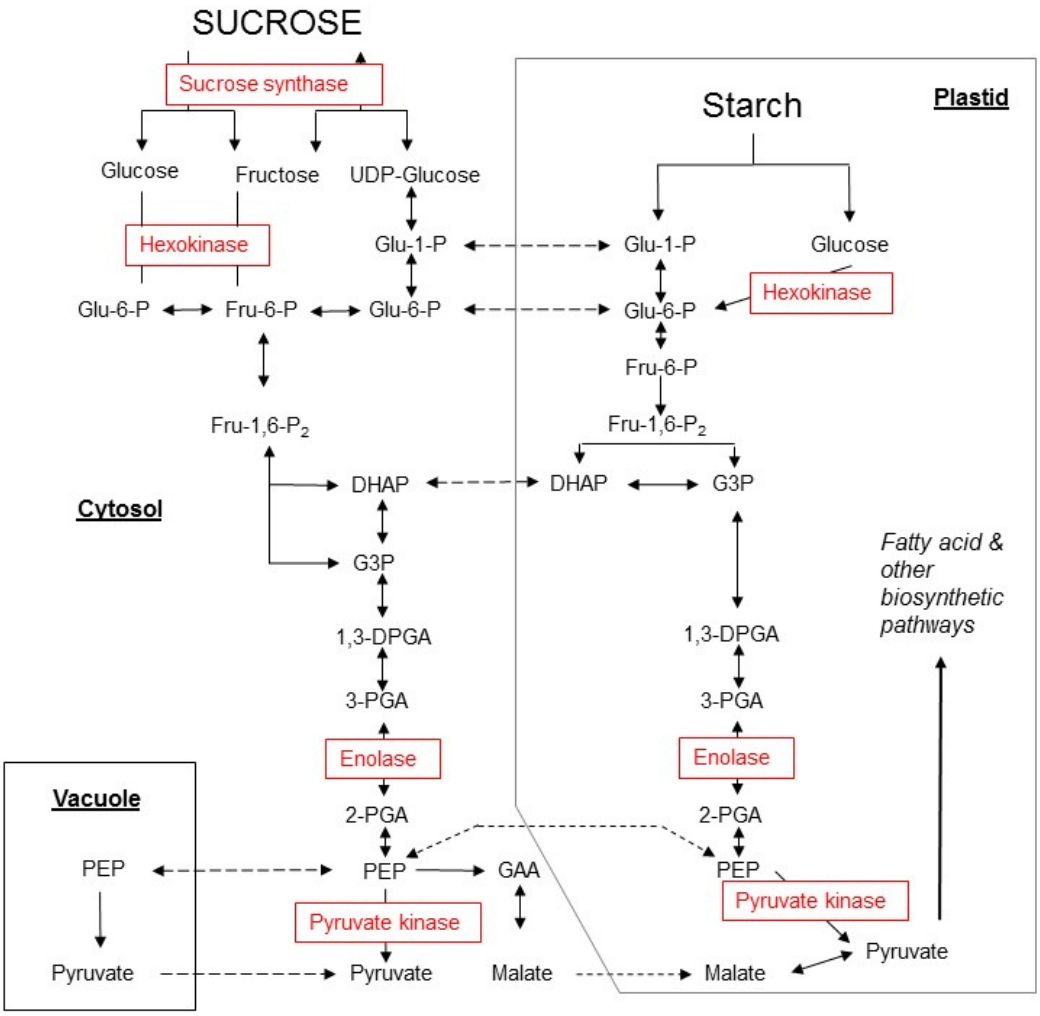

Fig.IV.2 **Scheme of the glycolytic pathway with candidates for regulation of carbon partitioning between plant and fungi.** Candidates are marked in red.

Overall, no negative influence of arbuscular mycorrhizal fungi on fresh weight gain of AM-P and AM+P plants, independent of light treatment was found. Analyses of fungal fatty acids and carbon partitioning via ^{13}C label showed a reduction due to shading. AM-P Shaded plants showed a reduction of soluble carbohydrates in roots, compared to AM-P Light plant roots. These results allowed the assumption that plants were able to control the carbon partitioning in less beneficial situations and the arbuscular mycorrhizal fungi did not become parasitic. Transcriptomic analyses identified three candidates in the carbohydrate metabolism. One candidate represented a hexokinase, the other two sucrose synthases. It cannot be differentiated if these candidates were passively regulated by light intensity or actively by the plant. In glycolysis, enolase, pyruvate kinase and pyruvate dehydrogenase were found to be enriched in regulation from light to shaded, in AM-P plants. Furthermore, the “TCA/ org. transformation

pathway” and “Fatty acid synthesis pathway” showed up to be enriched in regulation from light shaded in AM-P plants. These pathways were identified to be candidates for the regulation of carbon partitioning between plant and fungi. To review the candidate genes from the carbohydrate metabolisms and the enriched pathway a detailed validation would be needed. Up to now, the candidates can only be seen as hinds for keyplayers in regulatory mechanisms of the symbiotic interaction between plant and fungi.

With the novel observation that the plant is in charge to control the transport of carbon to the fungi under long-term shading and optimal phosphate supply, we were able to confirm the hypothesis that carbon transport to arbuscular mycorrhizal fungi is reduced under less beneficial conditions.

4.3 Counting data did not show the same results as transcriptomic and fatty acid data

There are some reasons for the discrepancy between counting data, transcript (MtPT4) and NLFA/PLFA amounts (3.1.1, 3.2.3, 3.2.1, 3.3). During the first three weeks of establishment of the symbiotic interaction, plants were only supplied with water. In this time, the plant needed the fungus and an intensive hyphae growth was expected. Living and dead hyphae were still stained if no vital stain was used. Fungal structures were grown during the three weeks without additional P supply. For hyphae this could be the case, for arbuscules it is not a probable reason as they are only active for around 8.5 days and arbuscules degenerate at the end of their life cycle (Alexander et al. 1989). This is visible after staining and microscopy. Furthermore, only the presence or absence but not the amount of arbuscules, hyphae or vesicles could be determined, which is a weakness of the microscopy technique we used. Another problem with the counting data of vesicles is that the formation of the lipid storage organs is not correlated with lipid accumulation. The results of van Aarle and Olsson (2003) indicated that vesicles are formed first and are filled with lipids only later.
In summary, NLFA and PLFA analyses are the best method to get an overview over the mycorrhizal status in plant roots. The methods of fatty acid analyses are time consuming and special equipment is needed. Depending on the experimental setup, the magnified intersection method can be sufficient. The use of vitality stains increases the usability.

4.4 The content of inorganic phosphate in roots was not positively influenced by mycorrhizal interaction

After 21 days of shift AM+P Light plants showed a significantly lower Pi content compared to their +P Light control (3.1.5). When plants were mycorrhized, the formation of symbiosis changed the expression of plant epidermal Pht1 transporters. For *Medicago truncatula* this was shown for Pht1;1 and 2 by Liu et al. (1998). This downregulation of phosphate transport by the plant is usually equalized by the phosphate transport of the fungi and may underlie the Pi reduction in AM+P Light plants. When the plant phosphate transport is not induced again after the reduction of mycorrhization, a lack in phosphate would occur, which could result in a reduced Pi

content in the roots. Alternatively, due to a reduced carbon supply of the plant by the fungi, the phosphate transfer of the fungi to the plant may be reduced. Without reactivating the plant phosphate transport mechanisms, the plant suffers from reduced phosphate uptake compared to control plants. The analyses of the mycorrhiza induced phosphate transporter MtPT4, showed a massive reduction due to optimal phosphate supply and shading of mycorrhizal plants in our experiment.

The inorganic phosphate content in roots was found to be significantly higher in AM-P plants compared to -P plants, six weeks after inoculation. However, at 14 and 21 days past shift this difference was not detectable, independent of light treatment. The reason for this can be explained on the above described mechanisms.

For AM+P Light plants, a significant reduction of inorganic phosphate content in roots was observed at 21 days past shift. To investigate the reasons for this, transcripts of above named genes should be analyzed. Based on the agricultural view, phosphate fertilization of mycorrhizal plants could be less beneficial for the plant.

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V. Appendix

Tab. V.1 **Composition of multistandards for the determination of fatty acid concentrations**

Single standard	Concentration in Multistandard [µg/µl]
Straight chain saturated fatty acids	
14:0	0.0300
15:0	0.0299
17:0	0.0299
18:0	0.0299
Cyclopropyl branched fatty acids	
Cy19:0	0.0300
10-Methyl branched saturated fatty acids	
10Me16:0	0.0300
Anteiso branched saturated fatty acids	
a15:0	0.0300
a17:0	0.0302
Iso branched saturated fatty acids	
i15:0	0.0284
i16:0	0.0300
i17:0	0.0300
Monosaturated straight chain fatty acids	
16:1w5c	0.0300
16:1w7c	0.0300
18:1w7c	0.0300
18:1w9c	0.0291
Polyunsaturated straight chain fatty acids	
18:2w6,9c	0.0300
18:3w3,6,9c	0.0300
20:4w6,9,12,15c	0.0300

Tab. V.2 **Internal standards for the analyses of fatty acid concentrations**

Analysed fatty acid	Designation	Standard	Conc.
PLFA	Internal Standard 1	19:0	1 µg/µl
NLFA	Internal Standard 2	12:0	1 µg/µl
FAME	Internal Standard 3	13:0	1 µg/µl

Tab. V.3 **Standard substances for ^{13}C measurements**

Substance	Weight [mg]	Company
Quartz sand * (blank)	20	In-house standard
High organic sediment (HOS)	20	IVA Analysetechnik, Meerbusch, Germany (In-house)
Cellulose	4.8	IAEA**
Caffeine	4.2	IAEA**
$CaCO_3$	16	In-house standard
Needle litter (Nadel)	5-10	In-house standard (Waldstein)
Acetanilide	2-2.5	Merck KGaA, Darmstadt, Germany (In-house)

*washed with HCl and glowed at 1040 °C

** International Atomic Energy Agency, Seibersdorf Laboratory, Vienna, Austria

Tab. V.4 **Detailed results of analysis of variance (ANOVA) for hyphae**

```
Myc1H <- lmer(Hyphae ~ phosphate*Illumination + (1|Replicate), data = Myc1)

 # summary(Myc1)

 anova(Myc1H, test = "F")

 # Analysis of Variance Table of type III  with  Satterthwaite
 # approximation for degrees of freedom
 #                         Sum Sq   Mean Sq  NumDF DenDF   F.value    Pr(>F)
 #   phosphate             8469.8   8469.8      1    43     40.889    9.75e-08 ***
 #   Illumination          2422.0   2422.0      1    43     11.693    0.001385 **
 #   phosphate:Illumination  160.7    160.7      1    43     0.776     0.383332
 #   ---
 #  Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Appendix

Tab. V.5 **Detailed results of analysis of variance (ANOVA) for vesicle**

```
Myc1V <- lmer(Vesicle ~ phosphate*Illumination + (1|Replicate), data = Myc1)
```

```
anova(Myc1V)

# Analysis of Variance Table of type III  with  Satterthwaite
# approximation for degrees of freedom
#                          Sum Sq  Mean Sq    NumDF  DenDF    F.value   Pr(>F)
#   phosphate              7581.9  7581.9     1      40.105   28.1088   4.476e-06 ***
#   Illumination           2037.0  2037.0     1      40.105   7.5518    0.008941  **
#   phosphate:Illumination 1337.6  1337.6     1      40.105   4.9590    0.031634  *
```

Tab. V.6 **Detailed results of analysis of variance (ANOVA) for arbuscules**

```
Myc1A1 <- lmer(Arbuscule ~ phosphate*Illumination + (1|Replicate), data = Myc1)
```

```
anova(Myc1A1)

# Analysis of Variance Table of type III  with  Satterthwaite
# approximation for degrees of freedom
#                           Sum Sq   Mean Sq   NumDF  DenDF   F.value   Pr(>F)
# phosphate                 2323.4   2323.4     1     40.04   7.699     0.008354 **
#  Illumination             24792.3  24792.3    1     40.04   82.156    3.008e-11 ***
#  phosphate:Illumination   0.7      0.7        1     40.04   0.002     0.961280
```

Tab. V.7 **Detailed results of analysis of variance (ANOVA) for stunted arbuscules**

```
Myc1A2 <- lmer(standed.Arbuscule ~ phosphate*Illumination + (1|Replicate), data = Myc1)
```

```
anova(Myc1A2)

# Analysis of Variance Table of type III  with  Satterthwaite
# approximation for degrees of freedom
#                           Sum Sq  Mean Sq   NumDF  DenDF    F.value   Pr(>F)
# phosphate                 5.1     5.1        1    40.149   0.0185    0.8926286
# Illumination           4191.9     4191.9     1    40.149   15.2561   0.0003512 ***
# phosphate:Illumination  263.5     263.5      1    40.149   0.9592    0.3332623
```

Appendix

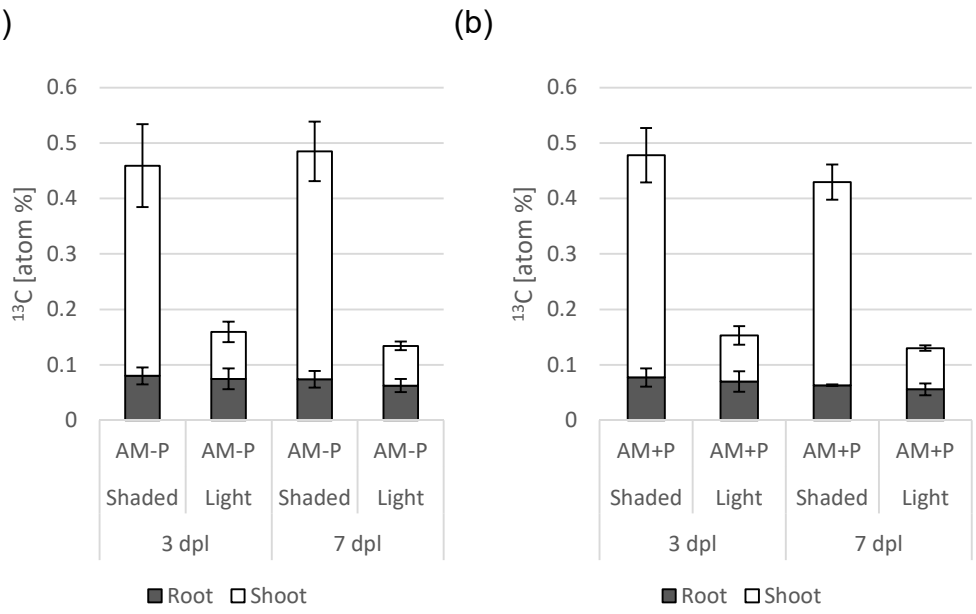

Fig. V.1 **^{13}C content in shoots and roots under P limitation (a) and phosphate overabundance (b) in full illumination and under shading treatments.** (± SE; n=2), Plants were labeled 9 wpi, after 21 days of different light treatments and harvested 3 and 7 days past label.

Conference contributions

Oral presentation

3rd Doctoral Researchers Conference 2016, Bad Salzdetfurth (09/2016)

“Regulation of plant carbon transport mechanisms during AM symbiosis”

2nd Doctoral Researchers Conference 2015, Goslar (10/2015)

“Regulation of plant carbon transport mechanisms during AM symbiosis”

Poster presentation

3rd Doctoral Researchers Conference 2016, Bad Salzdetfurth (09/2016)

"Influence of shading and P supply on arbuscule formation"

29th Conference Molecular Biology of Plants, Dabringhausen (02/2016)

"Influence of carbon limitation on nutrient exchange and arbuscule development"

36th New Phytologist Symposium Cell Biology at the plant-microbe interface, München (11/2015)

"Can plants still benefit from mycorrhizal fungi when carbon is less available"

2nd International Molecular Mycorrhiza Meeting, Cambridge UK (09/2015)

"Do plants regulate the carbohydrate flux to AM fungi under carbon limiting conditions"

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