Role of mycorrhiza symbiosis and phosphorus nutrition in plant growth, photosynthesis and secondary metabolism

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Abstract

Inorganic phosphorus (P_i) is an important and often limiting nutrient for plants. Large amounts of P_i fertilizers derived from non-renewable rock phosphorus, are used in agriculture. These are applied in excess but crops take up only a small amount of P_i; the residual P_i ends up in water systems where it causes problems with eutrophication. Plants can increase their P_i uptake efficiency by forming a symbiotic association between their roots and arbuscular mycorrhizal (AM) fungi. During symbiosis, AM fungi provide the host with P_i in return for carbohydrates synthesized in the leaf chloroplast through photosynthetic assimilation of CO_2. For AM symbiosis to be a plausible tool in modern agriculture, the symbiotic interaction needs to be optimized for generating a positive growth response of the crop. To achieve this, knowledge about the signaling between the plant and the fungus is crucial. It is known that both P_i signalling and AM symbiosis are tightly connected to metabolic processes in the chloroplast. In response to P_i limitation, more sugars and starch accumulate in leaves, and transport of sucrose towards roots increases. AM symbiosis increases the flow of sucrose towards the root system and induces production of secondary metabolites, which is initiated in the chloroplast.

An *Arabidopsis thaliana* mutant lacking the chloroplast-localized P_i transporter PHT4;1, was studied in **Paper I** to get a deeper understanding about the role of P_i supply in the chloroplast. The mutant displayed a reduced activity of the chloroplast ATP synthase due to P_i limitation, which resulted in less CO_2 assimilation, decreased levels of sugars in the shoot, reduced leaf size and biomass. The influence of AM symbiosis and P_i fertilization on growth and chloroplast processes such as photosynthesis and secondary metabolism was studied in *Medicago truncatula*. In **Paper II,** it is shown that AM symbiosis and P_i fertilization stimulate the expansion of shoot branches and leaves, whereas AM symbiosis specifically increases the number of chloroplasts. The increased surface area of the shoot enables the plant to harvest more sunlight. These morphological alterations are attributed to an enhanced level of cytokinins in leaves of AM- and P_i-treated plants (**Paper III).** In **Paper III,** it is also shown that AM symbiosis and P_i fertilization induce largely different transcriptional and metabolic responses. AM-specific responses were increased expression of secondary metabolite genes, and enhanced production of flavonoids and the hormone abscisic acid (ABA).

In conclusion, a model is proposed where a long distance signal in mycorrhized roots, derived from the enhanced carbon demand of the fungus, affects production of secondary metabolites in leaf chloroplasts. Validating this model will help to better understand the signaling between the plant and the fungus during AM symbiosis. This will allow the development of systems where AM symbiosis is used in agriculture for more efficient Pi uptake by crop plants.

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List of publications

This thesis is based on the following papers, which are referred to by their Roman numerals in the text:

I Karlsson PM, Herdean A, **Adolfsson L**[*], Beebo A[*], Nziengui H, Irigoyen S, Ünnep R, Zsiros O, Nagy G, Garab G, Aronsson H, Versaw WK and Spetea C (2015) The *Arabidopsis* thylakoid transporter PHT4;1 influences phosphate availability for ATP synthesis and plant growth. *The Plant Journal* 84(1): 99–110**

II **Adolfsson L**, Solymosi K, Andersson MX, Keresztes Á, Uddling J, Schoefs B and Spetea C (2015) Mycorrhiza symbiosis increases the surface for sunlight capture in *Medicago truncatula* for better photosynthetic production. *PloS ONE* 10(1): e0115314.

III **Adolfsson L**[*], Nziengui H[*], Abreu IN, Šimura J, Beebo A, Aboalizadeh J, Široká J, Moritz T, Novák O, Ljung K, Schoefs B and Spetea C. Transcriptomic and metabolomic profiling reveal enhanced secondary- and hormone metabolism in leaves of arbuscular mycorrhizal *Medicago truncatula. Manuscript.*

Other papers not included in this thesis:

Andersson MX, Nilsson AK, Johansson ON, Boztas G, **Adolfsson LE**, Pinosa F, Garcia C, Aronsson H, Mackey D, Tor M, Hamberg M, Ellerström M (2015) Involvement of the electrophilic isothiocyanate sulforaphane in *Arabidopsis* local defense responses. *Plant Physiology* 167(1): 251-261

Dusenge M, Wallin G, Gårdesten J, Niyonzima F, **Adolfsson L**, Nsabimana D, Uddling J (2015) Photosynthetic capacity of tropical montane tree species in relation to leaf nutrients, successional strategy and growth temperature. *Oecologia* 177(4): 1183-1194.

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List of abbreviations

3-PGA	3-phosphoglycerate
ABA	Abscisic acid
AGPase	ADP-glucose phosphorylase
AM	Arbuscular mycorrhiza
ATP	Adenosine triphosphate
CKs	Cytokinins
Cyt b_6f	Cytochrome b_6f
DMAPP	Dimethylallyl pyrophosphate
ECM	Ectomycorrhiza
ECS	Electrochromic band-shift
ETR	Electron transport rate
FBPase	Fructose bisphosphatase
Fru	Fructose
Glc	Glucose
IPP	Isopenthenyl pyrophosphate
JA	Jasmonic acid
LHC	Light-harvesting complex
MFS	Major facilitator superfamily
NADPH	Nicotinamide adenine dinucleotide phosphate
NPQ	Non-photochemical quenching
PAM	Periarbuscular membrane
P	Phosphorus
P_i	Inorganic phosphorus
P_o	Organic phosphorus
PEP	Phosphoenolpyruvate
PHO1	Phosphate1
PHT	Phosphate transporter
PMF	Proton motive force
PS	Photosystem
RC	Reaction center
ROS	Reactive oxygen species
RuBP	Ribulose 1,5-bisphosphate
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SA	Salicylic acid
SL	Strigolactone
W*p*i	Weeks *post* inoculation
WT	Wild type

Table of contents

1. Introduction

1.1. Phosphate

Plant cells need phosphorus (P) as a constituent of nucleotides and phospholipids and for energy transfer. In soil, P is present in different pools of organic phosphorus (P_o) and inorganic phosphorus (P_i, *i.e.*, phosphate). Only a small amount is directly accessible to plants as solubilized P_i. Therefore, P is one of the major limiting nutrient elements for plant growth (reviewed by Elser (2012)). A large amount of P_i fertilizers derived from non-renewable rock P_i are used in agriculture giving rise to two opposing problems. First, global reserves of rock P_i are limited and restricted to a few countries, mainly Western Sahara, Marocko, China and USA, creating a sensitive political situation. The production of P_i from rock reserves is estimated to peak within a few decades, and to go down until the reserves are depleted (Cordell et al., 2009). Second, the P_i fertilizers applied to soil are not taken up efficiently by plants, and are therefore used in excess (MacDonald et al., 2011). As a consequence, much of the excess P_i will end up in oceans and lakes, where it causes problems with eutrification, *e.g.*, algal blooms and oxygen depletion (reviewed by Elser (2012)). Together these problems call for more efficient use of P_i fertilizers, *e.g.*, by recycling waste water or improving the uptake efficiency of plant through mycorrhizal interaction (reviewed by George et al. (2016)).

1.1.1. Plant adaptions to phosphate limitation

Plants take up P from soil as P_i in the form of $H_2PO_4^-$ or HPO_4^{2-} (reviewed by Shen et al. (2011)). Naturally occurring P_i in the soil is either tightly bound in rock minerals or forms soluble mineral particles together with calcium, iron and aluminum. The solubility of P_i depends on the pH of the soil; at lower pH the solubility of P_i bound to iron and aluminum decreases, whereas the solubility of P_i bound to calcium increases. In natural soils, the concentration of available P_i is about 1,000 fold lower compared to the cytosolic concentrations in the root (Schachtman et al., 1998). Usually a depletion zone arises around the roots because the diffusion rate of P_i in soil is very low. Plants are adapted to grow in P_i-limited conditions and have developed several strategies to deal with this, *e.g.*, most plants are able to form symbiosis with mycorrhizal fungi (see section 1.2.).

Plants respond to P_i limitation by sending out root exudates, which serve several purposes. Plants exudate organic acids and secrete phosphatases to mineralize P_o to P_i and enhance the accessibility of P in the soil. The organic acids will also feed soil microorganism, which mineralize P_o through decomposition (reviewed by Richardson and Simpson (2011)). In addition, the plant hormones and apocarotenoids (see section 1.3.3.) strigolactones (SLs), are released to attract arbuscular mycorrhizal (AM) fungi (Akiyama et al., 2005). SLs and auxins together modify the root architecture so that branching and root hair proliferation

are favored over primary root elongation (reviewed by Chang et al. (2013)). The response of shoot growth towards P_i fertilization and mycorrhiza symbiosis is described in **Paper II**. Usually P_i limitation results in altered biomass allocation towards roots due to increased transport of sucrose (reviewed by Hermans et al (2006)). In addition, more soluble sugars and starch are accumulated in leaves. Sucrose appears to work as a signaling molecule during P_i-limiting conditions (reviewed by (Hammond and White (2011)). Increased sucrose concentration in roots during P_i limitation leads to accumulation of anthocyanins and morphological alterations orchestrated by plant hormones. The correlation between P_i homeostasis and sugar- and starch metabolism is described in section 1.3.2.

Plants can also undergo metabolic adaptations to save P_i, *e.g.,* reallocate P_i from older to younger tissues, export P_i stored in vacuoles and recycle P_i inside the cell by converting P_o to P_i with acid phosphatases (reviewed by Baker et al. (2015)). The use of P_i can be restricted by using alternative pathways for glycolysis, and replacing membrane phospholipids with galactolipids and sulfolipids (Tjellström et al., 2008). How the cellular responses to P_i limitation in roots and shoots are coordinated is not fully elucidated. Members of the transcription factor families: NAC-, MYB-, ethylene response factor/APELATA2-, zinc-finger-, WRKY-, and CCAAT-binding, has been suggested as likely candidates (Nilsson et al., 2010). One of the main regulators of the signaling is the MYB transcription factor PHOSPHATE STARVATION RESPONSE 1 (PHR1, reviewed by Baker et al. (2015)), which activates components important for P_i signaling such as microRNA399 as well as root P_i-transporters PHOSPHATE TRANSPORTER (PHT) 1 and PHOSPHATE1 (PHO1), involved in P_i-uptake from soil and loading into xylem (see below).

1.1.2. Phosphate transporters in plants

Plant roots actively take up P_i from soil through members of the PHT1 family (reviewed by Baker et al. (2015)). All PHT transporters belong to the major facilitator superfamily (MFS), have 12 transmembrane helixes and represent the largest group of secondary transporters (reviewed by Yan (2015)). They facilitate transport of an ion or solute down their concentration gradient (uniporters) *or* use the electrochemical potential of an ion or solute that is either cotransported (symporters) or exchanged (antiporters). PHT1 proteins are $P_i H^+$ symporters, driven by energy-demanding H^+ pumps (H^+-ATPase, reviewed by (2015)). Not all PHT1 members are root-specific; some are expressed in shoots, leaves and flowers. In the root, they are localized in the plasma membrane and use H^+ gradients to transfer P_i between cortical cells and towards the xylem. There are high- and low-affinity PHT1s, which are differently expressed in roots depending on the P_i concentration in the soil. Some members are specifically expressed during AM symbiosis (*e.g.*, PT4, see section 1.2.2.)

Within the plant, P_i is transported from the root to the shoot through the xylem. Transport of P_i from cortical cells to xylem vessels is down the concentration gradient and does not require energy. PHO1 protein has been identified to facilitate this transport (Hamburger et

al., 2002). It is localized to the Golgi and trans-Golgi network, and appears to play a role in P_i signaling when P_i is limiting (Wege et al., 2016). Excess P_i is stored in vacuoles to maintain P_i homeostasis of the cell. AtPHT5;1, also named Vacuolar phosphate transporter 1 (VP1), was recently discovered in *Arabidopsis thaliana* to import P_i into the vacuole (Liu et al., 2015, Liu et al., 2016). In contrast, the *Oryza sativa* (rice) translocator OsSPX-MFS3 is proposed to export P_i from the vacuole (Wang et al., 2015). In addition to belonging to the MFS family, AtPHT5;1 and OsSPX-MFS3 proteins contain a SPX domain, which is conserved in proteins involved in P_i homeostasis (reviewed by Secco et al. (2012)). PHO1 also has a SPX domain but is not a MFS transporter.

Distribution of P_i to different compartments of the cell is facilitated by translocators. Members of the PHT2 and PHT3 families are $P_i H^+$ symporters, the former being localized to chloroplasts and the latter to mitochondria in *Arabidopsis* (reviewed by Poirier and Bucher, (2002)). Members of the PHT4 family in *Arabidopsis* are $P_i H^+(Na^+)$ symporters and are found in different organelles: PHT4;2 in root plastids, PHT4;3 and PHT4;5 in leaf phloem, PHT4;1 and PHT4;4 in chloroplasts, and PHT4;6 in Golgi (Guo et al., 2008a). PH4;4 expression is induced by light and it functions as an ascorbate transporter in the envelope (Miyaji et al., 2015). *Arabidopsis* mutants lacking the P_i transporter PHT4;1 are characterized in **Paper I**. PHT4;1 was initially annotated as ANION TRANSPORTER 1 (ANTR1) due to its homology to the mammalian Na^+-dependent P_i transporter (Roth et al., 2004). It was characterized as a Na^+-dependent P_i transporter in the bacteria *Escherichia coli* (Pavón et al., 2008), and as a H^+-dependent P_i transporter in the yeast *Saccharomyces cerevisiae* (Guo et al., 2008b). PHT4;1 was localized to either the stromal side of the thylakoid membranes (Pavón et al., 2008) or to the chloroplast envelope (Ferro et al., 2010). The expression pattern for PHT4;1 is both light induced and under circadian regulation, with the highest expression at the end of the light period (Guo et al., 2008a), a pattern resembling the one of the chloroplast ATP-synthase (Robertson McClung, 2000).

In addition, there are several sugar/P_i antiporters involved in supplying P_i to plastids, as follows: triose-P_i/P_i translocator (only expressed in the chloroplast), phosphoenolpyruvate (PEP)/P_i translocator and pentose/P_i translocator (reviewed by Flügge et al. (2011)). One additional plastidial translocator, namely glucose (Glc)-6P_i/ P_i translocator, is only expressed in non-photosynthetic plastids, where it serves to import carbon skeleton.

1.2. Arbuscular mycorrhiza symbiosis

Mycorrhiza symbiosis is an association between plants and fungi, where carbohydrates produced by plant photosynthesis are exchanged for minerals and water in specialized structures in roots. In addition the symbiosis can protect the plant against abiotic and biotic stresses, *e.g.,* heavy metals (*e.g.*, Aloui et al. (2009)), drought (reviewed by López-Ráez (2016)) and pathogen attacks (reviewed by Jung et al. (2012)). However, the outcome of the symbiosis, in the sense of growth response, is not always positive but varies with species and

environmental conditions (*e.g.,* Lendenmann (2011), Walder (2012)). There are different forms of mycorrhiza, the dominating types being ectomycorrhiza (ECM) and AM. ECM is formed by woody perennial plants and fungi belonging to the *Ascomycetes* or *Basidomycetes* (Smith and Read, 2008). The fungal hypha forms a mantle around the roots and a hartig net that penetrates the epidermal or cortical cell wall but does not enter the cell. About 80% of all land plants (including mosses and ferns) are estimated to form AM symbiosis together with fungi belonging to the monophyletic phylum *Glomeromycota* (reviewed by Parniske (2008)). It is a very ancient form of association; fossil records dates the age of AM symbiosis to *circa* 400 million years ago, and it is believed to have played a pivotal role when plants first colonized land.

1.2.1. Establishment of the symbiosis

AM spores can germinate and survive a short time in the soil whiteout a host, a stage called presymbiotic growth (reviewed by Smith and Read (2008)). But all AM fungi are obligate symbionts and need a plant host to sustain the mycelia network, which is formed both in the soil (extra radicle) and inside the host root (intra radicle). AM fungi have a simple morphology compared to the ECM-forming fungi. The hyphae are coenocytic, *i.e.,* not divided by septae, and no sexual structures have been identified. Instead, fungi reproduce with asexual spores that have multiple nuclei with high genetic variability. Like some *Ascomycetes* and *Basidiomycetes*, AM fungi form a mycelia network through fusing of hyphae in a process called anastomosis. Anastomosis is generally restricted to hyphae originated from the same spore, *i.e.,* the same clone. But cases have been reported where anastomosis between hyphae of different clones occur during the presymbiotic growth (*e.g.,* Purin and Morton (2013)).

Germinating spores sense the presence of roots through substances in root exudates *e.g.,* the plant hormone SL which initiates branching of fungal hyphae, to facilitate growth towards roots and formation of appressoria in root epidermal cells (Akiyama et al., 2005). Fungi in turn send out short-chain chitin oligomers called MYC factors (Maillet et al., 2011, Genre et al., 2013). Perception of these starts a signaling cascade in roots that involves Ca^{2+} spiking. This signaling pathway is shared with nodule-forming bacteria called *Rhizobia,* and is referred to as the common symbiosis pathway, and the genes involved are collectively called *SYM* genes (reviewed by Parniske (2008)). Expression of *SYM* genes leads to the formation of the pre-penetration apparatus underneath the appressoria. The pre-penetration apparatus is an apoplastic tunnel that allows the hypha passage through the epidermal cells (Genre et al., 2005). It is formed by the cytoskeleton and the endoplasmic reticulum, and directed by the nucleus.

AM fungi propagate in the cortical root cells, through different colonization patterns. Some fungi form intracellular hyphal coils (*Paris* type) within the cells; most grow with intercellular hyphae between the cells and form arbuscules inside the cells (*Arum* type), but all

intermediate forms exist (reviewed by Smith and Read (2008)). The arbuscules are invaginations in the plant plasma membrane, formed by repeated dichotomous branching of hyphae. In arbuscules the fungal cell wall is separated from the plant cytoplasm through the periarbuscular membrane (PAM) formed by the plant plasma membrane. The space between the PAM and the fungal cell wall is called apoplast (**Figure 1**). Fungi also form vesicles between or inside the cortical cells. Vesicles function as storage units and can work as propagules for new colonization events by root fragments.

1.2.2. Nutrient exchange – uptake, delivery and control

AM fungi mainly provide the host with P_i but can also deliver water, N, Zn, S and other mineral nutrients (reviewed by Behie and Bidochka (2014)). Mycorrhiza-facilitated uptake of P_i is referred to as the mycorrhizal pathway, in contrast to the direct pathway where P_i is taken up by the roots. Through the extra radicle mycelia, plants have access to nutrients and water outside the depletion zone of the root (see section 1.1.1.). Some reports also indicate that AM fungi can release P_i bound as P_o by excreting enzymes (*e.g.,* Koide and Kabir (2000)). H^+-ATPase-driven P_i transporters in hyphae take up P_i from the soil, similarly to the plant P_i transporter PHT1 (reviewed by Bucher (2007), section 1.1.2.). P_i taken up by the mycorrhizal pathway is transported through vacuoles inside the hyphae as poly-Pi though (**Figure 1**). The driving force for this transport might be the water flow from the fungus to the root, mediated by fungal aquaporins (Kikuchi et al., 2016). Poly-P_i is converted back to P_i before it is transferred across the apoplast to the plant (**Figure 1**). P_i is further taken up in the PAM through the PHT1-family P_i transporter PT4, that has been characterized in *Medicago truncatula* to be specifically expressed in roots during AM symbiosis (Javot et al. (2007).

On the plant side, sucrose produced through photosynthesis in the leaf mesophyll is transported to roots by the phloem (see section 1.3.2.). Sucrose is transported to the apoplast between the PAM and the fungal membrane by sucrose H^+ symporters (reviewed by Doidy et al. (2012a), **Figure 1**). Invertase excreted either from the plant of the fungi, cleaves sucrose to fructose and glucose in the apoplast. Monosaccharide transporters are used to import fructose and glucose to the fungus (Helber et al., 2011).

1.3. Different functions of plastids

Plastids are plant organelles derived from an endosymbiont event where a cyanobacterium was taken up by an eukaryotic organism (reviewed by Niyogi et al. (2015)). As a result, plastids are surrounded by inner and outer membranes, called envelopes. Plastids contain their own DNA, even though most proteins are coded in the nucleus. Inside the plastid are the stroma and an inner membrane system. There are several different types of plastids in the plant, all developing from proplastids, *e.g.,* amyloplasts that store starch in root cells,

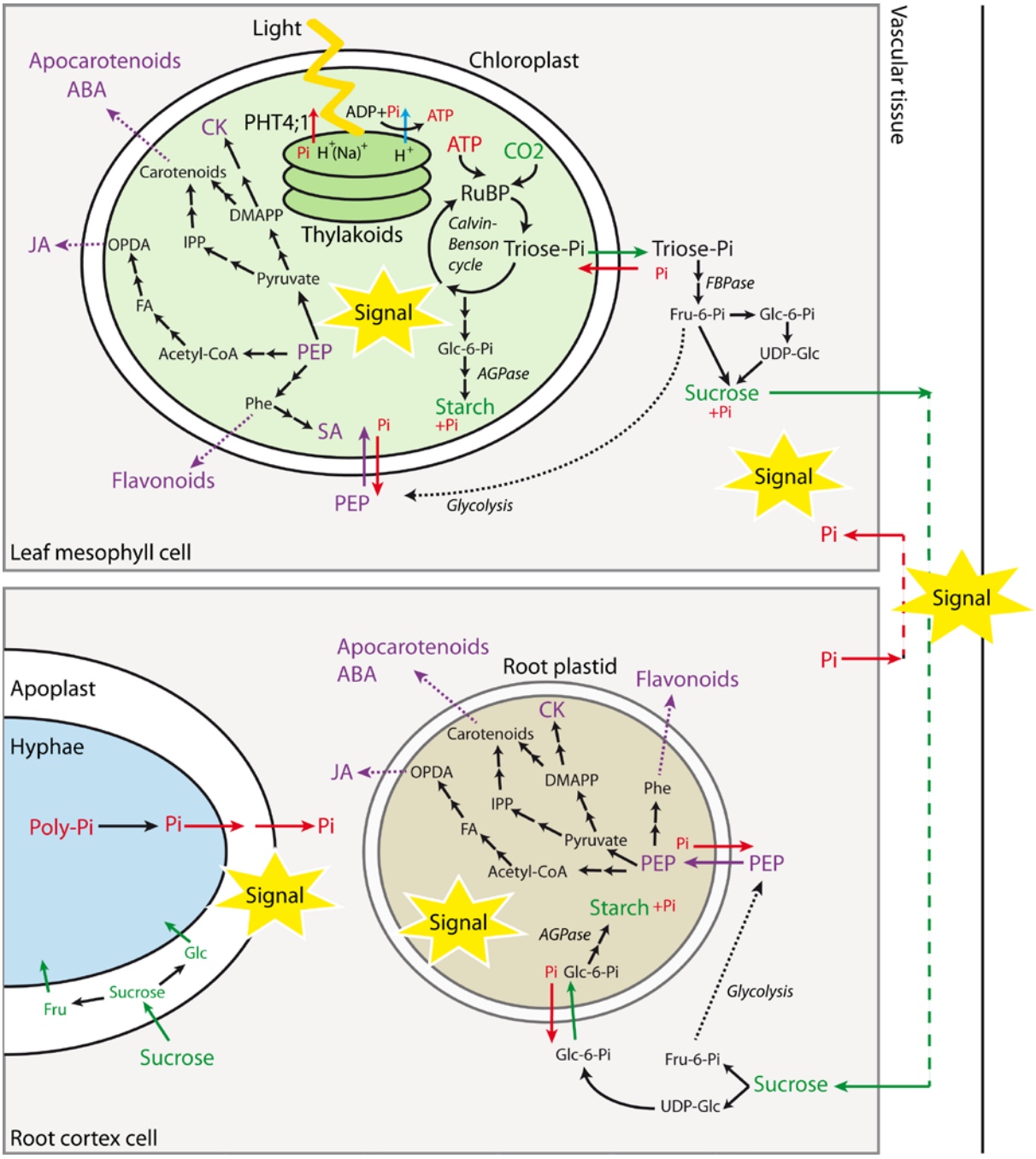

Figure 1. Model of nutrient exchange and signaling between an arbuscular mycorrhizal (AM) fungus and a plant. Black arrows indicate metabolic steps and colored arrows indicate metabolite transport. Red: Inorganic phosphorus (P_i) is transferred from fungal hyphae across the apoplast to the cortical root cell. P_i is further transported through the xylem to other parts of the plant (section 1.2.1-2.). Green: CO_2 is assimilated trough photosynthesis, and converted to starch and sucrose. Sucrose is transported through the phloem to root cortical cells, and further on across the apoplast to fungal hyphae (section 1.2.2. and 1.3.1-2). Purple: Phosphoenolpyruvate (PEP) is used as a precursor for secondary metabolism in plastids, possibly as a response to an AM-derived signal from the root.

and chromoplasts that contain carotenoids in various organs such as fruits, flowers and roots. Chloroplasts are found in photosynthetic tissues, primarily leaves. They are used for conversion of light energy to chemical energy, and for production of primary- and secondary metabolites. Chloroplasts need light to develop (Solymosi and Schoefs, 2010). If a plant is grown in darkness, proplastids will develop into etioplasts that will further develop into chloroplasts upon illumination. The inner membrane of chloroplasts is arranged in grana stacks called thylakoids. The inner space of thylakoids is called thylakoid lumen. Plastoglobules are outgrowths on the stromal side of thylakoids, and store lipids and carotenoids (reviewed by Niyogi et al. (2015)).

1.3.1. The photosynthetic light reactions

Photosynthesis is divided into the light-dependent (light) and light-independent (dark) reactions (reviewed by Niyogi et al. (2015)). The light reactions occur in the thylakoid membrane where light energy is converted into chemical energy in the form of adenosine triphosphate (ATP) and reducing power, nicotinamide adenine dinucleotide phosphate (NADPH). The light reactions take place in four macrocomplexes in the thylakoid membranes, *i.e.,* photosystems I and II (PSI, PSII), cytochrome b_6f (cyt b_6f) and ATP synthase. PSI and PSII are surrounded by antenna, called light-harvesting complexes I and II (LHCI, LHCII), consisting of proteins and the pigments chlorophyll and carotenoids. Photons are absorbed by LHCs and the energy is transferred to a special chlorophyll molecule in the reaction center (RC) of PSI and PSII. In RC of PSII the energy is strong enough to trigger chlorophyll ionization, resulting in the release of one electron (reviewed by Foyer et al. (2012)). The excited electron moves through a series of redox reactions from PSII via the electron carrier plastoquinones to cyt b_6f and PSI. More energy is received in the RC of PSI and the electron continues via ferredoxin to ferredoxin-$NADP^+$ reductase where $NADP^+$ is reduced to NADPH. Altogether, these reactions are known as the linear electron transport chain. The electron released from the excited chlorophyll in RCII is replaced by oxidizing water into H^+ and O_2 by the oxygen-evolving complex. Protons are released in the thylakoid lumen, where a proton motive force (PMF) is built up. The PMF is mainly used by the ATP-synthase to form ATP from P_i and ADP (**Figure 1**).

If more energy is absorbed by chlorophyll than can be utilized in the electron transport chain, excited chlorophyll molecules can react with oxygen and form reactive oxygen species (ROS, reviewed by Tripathy and Oelmüller (2012)). ROS can also be produced in other compartment (*e.g.*, mitochondria and peroxisomes) and through reaction with reducing agents other that exciteted chlorophyll (*e.g.*, Fe(II)), and is damaging for proteins, lipids and DNA. If not quenched in the thylakoids, ROS will cause damage to PSII and PSI, leading to photoinhibition. The chloroplast has several ways to handle this problem. State transition is a way for the plant to redistribute excitation energy between PSII and PSI, by phosphorylating and moving LHCII (reviewed by Minagawa (2011)). Excess energy can also

be dissipated by the xanthophyll cycle, where acidification of the lumen through buildup of H^+ triggers de-epoxidation of the carotenoid violaxanthin to zeaxanthin (reviewed by Moulin et al. (2010)). This causes a rearrangement of the chlorophyll and carotenoid molecules, which prevents transduction of energy to the RC in a process called non-photochemical quenching (NPQ). In addition zeaxanthin is an antioxidant that protects the chloroplast from ROS.

1.3.2. Photosynthetic carbon assimilation and partitioning

Photosynthetic assimilation of CO_2 takes place in the chloroplast stroma (**Figure 1**). CO_2 is incorporated into Ribulose 1,5-bisphosphate (RuBP) by Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) to 3-phosphoglycerate (3-PGA, Farquhar et al. (1980)). The name RuBisCO is due to the fact that the enzyme can use both O_2 and CO_2 as substrates. Oxygen will outcompete CO_2 when the CO_2 levels are low in the cell. ATP and NADPH produced in the light reactions (see above), are used to convert 3-PGA to triose-P_i and other compounds, in a series of reactions known as the Calvin-Benson cycle (**Figure 1**). Depending on the levels of triose-P_i and enzyme activity in the stroma and the cytosol, triose-P_i is either exported to the cytosol for sucrose- and amino acid synthesis or metabolized in the chloroplast for starch production or regeneration to RuBP (reviewed by Singh and Malthora (2000)).

Starch is a polymer consisting of ADP-glucose molecules linked together in linear or branched chains. It is produced during day-time in the chloroplast stroma from fructose(Fru)-6-P_i, derived in the Calvin-Benson cycle (Stitt et al. (2010), **Figure 1**). Fru-6-P_i is further converted to Glc-6-P_i and Glc-1-P_i. The enzyme ADP-glucose phosphorylase (AGPase) uses ATP to form ADP-glucose from Glc-1-P_i (Ballicora et al., 2004). Several branching enzymes and starch synthase work together and produce linear amylose and branched amylopectin. Starch is also produced in root plastids, from Glc-6-P_i imported from the cytosol by the Glc-6P_i/ P_i translocator (Kammer et al. (1998), **Figure 1**). During night-time starch is broken down to maltose and partly glucose (reviewed by Stitt et al(2010)). Maltose is exported by maltose exporter to the cytosol, where it is converted to sucrose, and used for growth. The amount of starch stored and the rate of breakdown are regulated diurnally.

Sucrose is synthesized in the cytosol of mesophyll cells. Triose-P_i is exported from the stoma to the cytosol by the triose-P_i/P_i translocator, where it is converted to Fru-1,6-bisphosphate (reviewed by Flügge et.al (2011), **Figure 1**). The enzyme Fru bisphosphatase (FBPase) further converts Fru-1,6-bisphosphate to Fru-6-P_i (reviewed by Sing and Malthora (2000)). Part of Fru-6-P_i is converted to Glc-6-P_i and UDP-glucose. Finally, the enzyme sucrose phosphate synthase forms sucrose from UDP-glucose and Fru-6-P_i. From the source cell, sucrose moves freely between mesophyll cells via plasmodesmata, until it reaches the phloem. Phloem consists of sieve element surrounded by companion cells. Phloem loading can either be passive through plasmodesmata or active through the apoplast, depending on the plant

species. In the latter case, sucrose is exported to the apoplast by Sweet proteins and exported to companion cells by sucrose H^+ symporters (SUT, reviewed by Doidy et al. (2012a)). Likewise, sucrose is unloaded from the phloem into the sink tissue either passively via plasmodesmata or actively but sucrose H^+ symporters (**Figure 1**).

The metabolic fate of triose-P_i is dependent on the relative levels of triose-P_i and P_i in the stroma and cytosol. High levels of P_i has an inhibitory effect on AGPase and FBPase, key enzymes in starch and sucrose metabolism respectively (reviewed by Sing and Malthora (2000)). Instead, AGPase is activated by 3-PGA, whose level increases when export of triose-P_i is prevented (Ballicora et al., 2004). Fructose-2,6-bisphosphate functions as a regulatory metabolite that inhibits sucrose synthesis by deactivating FBPase, and thereby promoting starch synthesis (reviewed by Nielsen et al. (2004)). In addition, the enzymatic activity of carbon metabolism is tightly connected to the photosynthetic light reactions through the ferredoxin-thioredoxin regulatory system (reviewed by Schürmann and Jacquot (2000)). Ferredoxin is reduced by the electron transport chain (see section 1.3.1) and in turn reduces thioredoxin via ferredoxin:thioredoxin reductase. By reducing cysteine residues and breaking sulfur bridges, reduced thioredoxin activates: several enzymes involved in the Calvin-Benson cycle, ATP-synthase, FBPase and AGPase (reviewed by Geigenberger et al. (2005)).

1.3.3. Production of secondary metabolites and hormones

Secondary metabolites are compounds that are not necessary for plant growth, development or reproduction, but serve other purposes *e.g.*, as defense molecules or for attracting pollinators (reviewed by Ncube and Van Staden (2015)). Alterations in secondary metabolite production in response to P_i fertilization and AM symbiosis are discussed in **Paper III**. Many secondary metabolites, and also hormones, are produced in plastids. An important precursor for many biosynthetic pathways is PEP, which is produced from Glc and Fru breakdown during glycolysis in the cytosol, and are imported into plastids by the PEP/P_i translocator (reviewed by Flügge et al. (2011), **Figure 1**). In the stroma, PEP can be metabolized to chorismate in the shikimate pathway to produces aromatic amino acids, namely phenylalanine, tyrosine, and tryptophan (Rippert et al. (2009), **Figure 1**). The plant hormone auxin is derived from tryptophan, whereas the hormone salicylic acid (SA) and the secondary metabolites flavonoids and lignin are derived from phenylalanine in the phenylpropanoid pathway (Vogt, 2010)). SA is produced in chloroplasts (Fragnière et al., 2011) whereas flavonoid biosynthesis takes place in the cytosol (reviewed by Gholami (2014)).

PEP can also be converted to pyruvate, which serves as a precursor for isopenthenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), used in isoprenoid biosynthesis (reviewed by Parisa et al. (2014)). Biosynthesis of IPP and DMAPP can take place either in the cytosol by the mevalonate pathway or in plastids by the mevalonate-independent- or MEP/DOXP pathway. Biosynthesis of cytokinins (CKs) takes place in plastids

where an isoprenoid side-chain derived from DMAPP is added to an adenine derivative (reviewed by Sakakibar (2006), **Figure 1**). Some CKs are further modified in the cytosol. DMAPP and IPP are also used in carotenoid biosynthesis in plastids (reviewed by Walter (2013), **Figure 1**). Carotenoids are pigments present in various plant tissues. They can be used for light harvesting and photoprotection (see section 1.3.1) or cleaved into apocarotenoids by carotenoid cleavage dioxygenases (CCD), and exported to the cytosol. Carotenoid cleavage products can be further converted to the plant hormones abscisic acid (ABA) and SLs. ABA is derived from 9-*cis* violaxanthin by the enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED).

Puryvate can be further metabolized to acetyl-CoA, which is a precursor for fatty acid biosynthesis, from which lipids are derived. The hormone jasmonic acid (JA) is derived from the fatty acid α-linolenic acid, which originates from galactolipids in the chloroplast membrane (reviewed by Wasternack and Hause (2013), **Figure 1**). Lipoxygenases (LOX) are used to produce oxygenated lipids, collectively called oxylipins, from α-linolenic acid and other fatty acids. The JA precursor and signaling molecule, OPDA (cis-12-oxo-phytodienic acid), is produced by the enzymes allene oxide cyclase (AOC) and allene oxide synthase (AOS). OPDA is exported to the peroxisome, where JA biosynthesis continues. JA can be further converted into many different forms, collectively called jasmonates. The amino acid isoleucine is conjugated to JA by the enzyme jasmonyl isoleucine conjugate synthase 1 (JAR1), to the active form JA-Ile.

2. Scientific Aims

The overall aim of this thesis is to understand how the shoot responds to AM symbiosis and P_i nutrition with emphasis on growth, development and various functions of the chloroplast such as photosynthesis, secondary- and primary metabolism.

The specific aim of each paper is:

Paper I To understand the role of P_i supply to chloroplast photosynthesis and shoot growth by studying *Arabidopsis thaliana* mutant plants lacking the chloroplast-localized P_i transporter PHT4;1.

Paper II To compare the alterations in shoot growth, development, nutrient content and photosynthesis in *Medicago truncatula* plants in response to AM symbiosis and P_i fertilization.

Paper III To understand and distinguish how root colonization by AM fungi and P_i fertilization are perceived by the shoot of *Medicago truncatula* plants, with respect to gene regulation, secondary metabolism and hormone levels.

3. Methodology

3.1 Available resources for model organisms

Arabidopsis and *Medicago* are well-studied plant species, for which genomic and metabolic resources are available. The webpage www.ncbi.nlm.nih.gov/ of the National Center for Biotechnology Information founded by the US government provides several tools that together contain a huge amount of genomic information for a vast number of species including *Arabidopsis* and *Medicago*. *Genebank* which provides an annotated collection of DNA sequences, *Unigene* which collects transcript belonging to the same locus and gives them an annotation, and *BLAST* (Basic Local Alignment Search Tool which compares the similarity of a nucleotide/protein sequence with those available in the database, were all used in **Paper III**. ATTED-II at http://atted.jp/ (used in **Paper I**) is a useful tool to study co-expression of genes in model plants, including *Arabidopsis* and *Medicago* (Aoki et al., 2016). For information on metabolic pathways, the webpage www.kegg.jp/kegg/ of Kyoto Encyclopedia of Genes and Genomes (KEGG, used in **Paper III**) is crucial. KEGG is founded by the Japanese government and contains a tool where you can map the function of different enzymes for different species.

3.1.1. *Arabidopsis thaliana*

Arabidopsis thaliana (*Arabidopsis*, **Figure 2A**) is a small, annual herb belonging to the family *Brassicaceae*. It was proposed as model plant already in the 1940s because it produces many seeds, has a fast generation time, is self-fertile, diploid and has few chromosomes (reviewed by Somerville and Koornneef (2002)). Already at that time there was a large collection of different accessions with morphological differences, which made *Arabidopsis* suitable for studying phenotypic traits. Still most scientists preferred to work with crops and *Arabidopsis* was not generally adopted as model plant until the 1980s. This happened simultaneously with the discovery of *Agrobacterium* transformation. *Arabidopsis* turned out to be suitable for making genetic manipulations. This was partly because of its very small genome (70 Mbp), which was sequenced in the beginning of 2000s by the *Arabidopsis* Genome Initiative (AGI, 2000).

Today the main node for *Arabidopsis* research is hosted at the webpage https://www.arabidopsis.org/, by The *Arabidopsis* Information Resource (TAIR). This webpage contains a large database with genomic information about *Arabidopsis*. It also provides the opportunity to order seeds from the seed stock the *Arabidopsis* Biological Resource Center at The Ohio State University, which contains a large amount of mutant lines in various ecotypes. Recently the *Arabidopsis* information portal (Araport) at https://www.araport.org/, has been brought forward as an alternative tool for *Arabidopsis* genomic information. Still, *Arabidopsis* has its limitations as a model plant. For example, the inability to produce secondary meristem or form symbiosis with neither nitrogen-fixing

bacteria nor mycorrhizal fungi makes it useless for studying wood formation or symbiotic interaction. This creates a need for additional model plants.

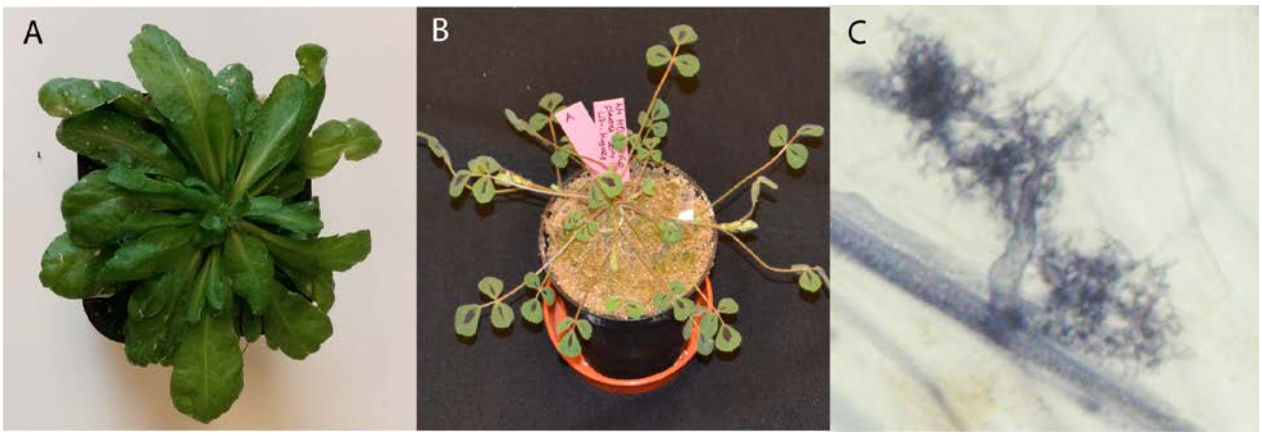

Figure 2. Species studied in this thesis: **A**. *Arabidopsis thaliana* ecotype Landsberg *erecta*, **B**. *Medicago truncatula var.* Jemalong 5 and **C**. *Rhizophagus irregularis* syn. *Glomus intraradices* BEG 141.

3.1.2. *Medicago truncatula*

Medicago truncatula (*Medicago,* **Figure 2B**) is an annual legume that originates from the Mediterranean basin and is used as a forage crop in Australia. In contrast to *Arabidopsis*, *Medicago* and other legumes have the ability to form symbiosis with both AM fungi and with nitrogen-fixing bacteria. *Medicago* was suggested as a model plant for studying those interactions in the 1990s because it is diploid, self-fertile, has a relatively small genome (500-600 Mbp), short generation time and many collections of ecotypes are available (Cook, 1999). *Medicago* has a high degree of genomic synteny with several important crops such as alfalfa (*Medicago sativa*), clover, pea, chickpea and soybean, making it useful in comparative studies (reviewed by Young et al. (2005)). In addition *Medicago* is fairly easy to genetically manipulate. The genome of *Medicago* cultivar Jemalong was completely sequenced in 2011 (Young et al., 2011). The latest annotations are available at the webpage http://plantgrn.noble.org/LegumeIP/index.jsp by LegumeIP (Li et al., 2012). Still a lot of work remains to be done in order to fully annotate the *Medicago* genome. Mutant populations are constantly generated using ethyl methane mutagenesis, fast neutron bombardment and transposon-insertion mutagenesis (reviewed by Young and Udvardi (2009)).

3.1.3. Rhizophagus *irregularis* syn. *Glomus intraradices*

Glomus intraradices can be considered a model fungus of the phylum *Glymeromycota*. It has been widely studied in mycorrhizal interactions, and was the first AM fungus to have its genome sequenced (Martin et al., 2008). Unfortunately, the phylogeny within the *Glomeromycota* is not completely resolved. The fungus reproduces with asexual spores, creating high genetic variability within a species (Rosendahl, 2008). The fungal strain used in

Paper II and **Paper III** was originally annotated as *Glomus intraradices* BEG 141 (**Figure 2C**). The inoculum was kindly donated to our lab by Dr. Vivienne Gianinazzi-Pearson from UMR Plante Microbe Interactions at INRA, Dijon, France. We now refer to it as *Rhizophagus irregularis* syn. *Glomus intraradices,* based on up-dated molecular evidence: Phylogenetic comparison of ribosomal DNA between the *Glomus intraradices* type strain and two other strains commonly used in publications, DAOM197198 and BEG 195, revealed that they rather belong to *Glomus irregulare* (Stockinger et al., 2009). Later on, both *Glomus intraradices* and *Glomus irregulare* were moved to the resurrected genus *Rhizophagus* and given the name *Rhizophagus intraradices* and *Rhizophagus irregularis,* respectively (Schüßler and Walker, 2010).

3.2. Experimental design

3.2.1. *Arabidopsis pht4;1* mutant

To investigate how plant growth and photosynthetic performance were affected when a chloroplast P_i transporter was impaired, the *Arabidopsis* mutant lines *pht4;1-2* and *pht4;1-3* (hereafter referred as *pht4;1*) in the ecotype background Landsberg *erecta* (L*er*) were characterized in **Paper I** (**Figure 2A**). Both *pht4;1-2* and *pht4;1-3* are loss-of-function mutation caused by transposon insertions (Wang et al., 2011).

3.2.2. Mycorrhizal interactions in *Medicago truncatula*

For studying the influence on *Medicago truncatula* cultivar Jemalong 5 (**Figure 2B**) from AM symbiosis and P_i nutrition with respect to growth, photosynthesis and secondary metabolism, the following treatments were compared in **Paper II** and **Paper III**:

Control	*Medicago* plants inoculated with non-colonized leek roots
AM	*Medicago* plants inoculated with leek roots colonized with *Rhizophagus irregularis syn. Glomus intraradices* (**Figure 2B**)
Pi	*Medicago* plants inoculated with non-colonized leek roots and watered weekly with a 5 mM P_i nutrient solution.

The outcome of the symbiosis is not constant over time. When studying plants grown during 8 weeks *post* inoculation (w*p*i), the highest degrees of mycorrhization and arbuscular abundance were found at 3-5 w*p*i, (**Paper II**). Hence, all other experiments described in section 4 were carried out between 3-5 w*p*i.

3.3. Approaches

3.3.1 Photosynthesis

The photosynthetic light reactions were studied by measuring changes in chlorophyll fluorescence in **Paper I** and **Paper II**. Chlorophyll fluorescence measurements are based on the principle that in a leaf the light energy absorbed is either used for photosynthesis, emitted as fluorescence or dissipated as heat (reviewed by Roháček et al. (2008)). By measuring the chlorophyll fluorescence emitted from a sample illuminated with light of a known wavelength, information about photosynthesis and heat dissipation can be calculated. Fluorescence is emitted when energy absorbed by the reaction center of PSII is not able to reduce the plastoquinone Q_A and continues the electron transport chain (see section 1.3.1.). A common parameter to look at is the F_v/F_m, which estimates the maximum efficiency of PSII (reviewed by Maxwell and Johnson (2000)). F_m is the maximum fluorescence, dervived when a strong pulse of light is applied and Q_A in all PSII centers is in reduced state. F_v is the difference between F_m and F_o, the fluorescence emitted after illumination with a light that is not strong enough to drive electron transport so Q_A in all PSII centers is oxidized. A healthy plant has an F_v/F_m value around 0.8. By measuring chlorophyll fluorescence several other parameters can be derived through mathematical calculation, such as electron transport rate (ETR), NPQ and PMF. ETR gives an estimate over how fast electrons move in the linear electron transport chain (reviewed by Ralph and Gademann (2005)). By studying ETR at different light intensities we can find out how well the plant adapt to increasing light. In contrast, NPQ is a measure of how much energy is dissipated as heat through the xanthophyll cycle, state transition and photoinhibition (see section 1.3.1., reviewed by Maxwell and Johnson (2000).

PMF was estimated in **Paper I** by recording electrochromic band-shift (ECS). PMF is a measure of the H^+ gradient across the thylakoid memrbane generated from release of H^+ in the lumen during electron transport (see section 1.3.1). PMF is composed of ΔpH, based on the difference in H^+ concentration on the lumenal and stromal side of thylakoids, and ΔΨ, based on the electric field across the thylakoid membrane (reviewed by Foyer et al.(2012)). The latter affects the light absorption by chlorophyll and carotenoids, and can be measured as a band-shift in absorption maxima during illumination (Bailleul et al., 2010). ATP-synthase conductivity (g_{H+}) can be measured by recording how long time the ECS signal will continue after illumination has stopped and H^+ are no longer supplied to the lumen by electron transport.

CO_2 assimilation was studied by recording gas exchange in ambient conditions (**Paper I**) and as A/C_i-curves, where assimilation is recorded at increasing CO_2 concentrations (**Paper II**). Based on the assumption that photosynthetic CO_2 assimilation is either limited by availability to CO_2 or ATP and NADPH for recycling of RuBP (see section 1.3.2), the parameters V_{cmax} (maximum rate of RuBisCO carboxylation) and J_{max} (maximum electron transport rate) can be derived from the A/C_i-curves (Farquhar et al., 1980).

3.3.2. Mycorrhizal interactions

Mycorrhizal status was monitored by root staining in all experiments described in **Paper II** and **Paper III**. Different techniques can be used both for staining the fungus and estimation of colonization (Vierheilig et al., 2005). In most methods, roots are first boiled in an alkaline solution to remove the cell content and cell wall pigments of the roots. This step is called clearing. Next, roots are boiled in different kinds of dyes that bind to fungal structures. In Paper II and Paper III a protocol was applied that use ink and vinegar, which is a non-toxic alternative to methods using *e.g.* trypane blue (Vierheilig et al., 1998). For estimation of root colonization, the gridline intersect and the slide method are most commonly used (Giovannetti and Mosse, 1980). In the first one, stained roots are placed in a Petri dish containing a gridline and examined with a stereo-microscope. The number of colonized vs. non-colonized root segments at the intersection points gives the percent colonized root length. In the second method, which was used in **Paper II** and **Paper III**, root fragments are mounted on a microscopy slide and examined in a compound microscope. The degree of mycorrhization and arbuscular abundance are calculated based on formulas described by Trouvelot (1986).

4. Shoot responses to mycorrhiza symbiosis and phosphate nutrition

4.1. Growth

AM symbiosis is known to facilitate the uptake of P_i from soil, a process which is often limiting for plant growth (Smith et al., 2011). On the other hand, high concentration of soil P_i is known to inhibit AM symbiosis (Breuillin et al., 2010). Hence the outcome of AM symbiosis, in the sense of growth- and P_i uptake-response of plants, is highly variable depending on environmental and experimental conditions. For example, in two independent studies using *Glomus intraradices/Rhizophagus irregularis* as fungal partner, positive growth response was found by Lendenmann et al. (2011), but not by Schweiger et al. (2014b). In our growth system, AM symbiosis increased the shoot biomass and the total shoot content of P, but not the tissue concentration of P (expressed as mg/DW, **Paper II** and **Paper III**). The apparent lack of P_i response in mycorrhized plants may be due to down-regulation of the direct P_i uptake pathway (Smith et al., 2011). Several studies have reported down-regulation of the root P_i transporters PHT1 in response to AM symbiosis (*e.g,* Christophersen (2009) and Grundwald et al. (2009)). This effect appears to be mainly local and not systemic, according to a study by Watts-William et al. (2015) using split root experiments and mutants lacking the mycorrhiza-specific P_i transporter PT4 (see section 1.2.2). The authors suggested that down-regulation of direct uptake P_i transporters during AM symbiosis is more an effect of increased P_i concentration in roots than the fungus *per se*. Taken together, this means that even when the P_i response is not positive, the P_i supply to the plant can be considered to be under control of the fungus. In this sense, AM symbiosis is crucial for modulating the P_i uptake in agricultural systems, regardless of the P_i- and biomass responses of crops.

In **Paper II,** AM- and P_i-fertilized plants had similar shoot biomass at 3 w*p*i, despite higher P levels in P_i-fertilized plants. For older plants, both shoot biomass and P levels were higher in P_i-fertilized than AM plants. This demonstrates that P levels are not always correlated with biomass. The same notion was demonstrated in a study by Grundwald et al (2009), comparing *Medicago* colonized with three different AM fungi. Only one of the fungi increased the level of P (measured as soluble P_i) in host leaves. This fungus, in contrast to the other two, did not increase the biomass of the shoot. Lack of correlation between growth- and P_i response could be explained by plant's ability to compensate for P_i deficiency up to a certain point (as described in section 1.1.1). Exchanging phospholipids for glycolipids is one of the mechanisms plants use to save P_i (Tjellström et al., 2008). Indeed, the levels of glycolipids were slightly higher in AM- and control plants as compared to P_i-fertilized plants (**Paper II**). However, the observed difference is small enough to conclude that in our experimental system, even though the growth of AM- and control plants were limited by the P_i supply, they did not suffer from severe P_i starvation. This provides a good base for studying mycorrhizal interactions in healthy plants.

The correlation between shoot P levels and biomass was also investigated in the *Arabidopsis pht4;1* mutant. Lack of the chloroplast P_i transporter did not alter either the P_i or P_o concentration in leaves of the *pht4;1* mutant as compared to leaves from wild type (WT) plants (**Paper I**). This is expected since most of the P_o is bound to nucleotides and plasma membrane lipids, and excess P_i is stored in vacuoles rather than chloroplasts. Despite this, the shoot biomass and also the leaf area of *pht4;1* mutant plants were lower than those of WT (**Paper I**). Smaller leaf area can be due to fewer cells or smaller cells. A closer examination of the leaves of the *pht4;1* mutants revealed that the reduced leaf size is due to both reduced epidermal cell number and cell area as measured by microscopy (**Paper I**). Supplementation with P_i in excess to mutant and WT plants resulted in similar biomass, leaf area, epidermal cell area, but not cell number. This implies that the reduced growth of *pht4;1* plants is mainly due to incomplete cell expansion. It is relevant here to compare these results with those obtained in previous studies on *pht2;1* and *pht4;4*, mutants lacking P_i transporters localized to the chloroplast envelope. In contrast to *pht4;1* mutants, the leaf biomass of p*ht2;1* mutants was reduced at high but not low P_i condition (Versaw and Harrison, 2002). P*ht4;4* mutants were found not different in rosette size from WT plants (Miyaji et al., 2015). The different growth phenotype of *pht4;1* mutants implies a distinct function in the chloroplast for PHT4;1 than for PHT4;4 and PHT2;1. As will be discussed in section 4.3, the function of PHT4;1 is most likely to maintain P_i homeostasis within the chloroplast rather than importing P_i into this organelle.

4.2 Development

AM symbiosis and P_i fertilization positively influenced the leaf area in *Medicago*. The leaves of both AM- and P_i-fertilized plants had more cells but of similar size compared to leaves of control plants (**Paper II**), despite the fact that the P concentration was similar in AM and control shoots but much higher in P_i-fertilized shoots (**Paper II** and **Paper III**). In contrast, *Arabidopsis pht4;1* mutants had similar shoot P levels as WT but less cells (**Paper I**). Together this suggests that for cell division, distribution of P_i within the chloroplast is more important than the actual P_i levels in the leaves. Cell division in the shoot is under control of CKs (reviewed by Schaller et al. (2014)), hence the increased leaf area of AM- and P_i-fertilized plants in **Paper II** may be a result of CK signaling. The levels of CKs were indeed enhanced in leaves from both AM- and P_i-fertilized plants (**Paper III**). CKs, auxin and SL are important for controlling shoot branching, where CKs have a stimulatory effect on axillary bud outgrowth (reviewed by Domalgalska and Leyser (2011)).

The shoot of *Medicago* plants has a branched architecture. Based on numbering systems proposed by Moreau *et al.* (2006) and Bucciarelli *et al.* (2006) the shoot development can be described as follows. Leaf, petiole and stalk, together referred to as metamer, first grow along a main axis and are numbered according to their order of appearance, *e.g.,* m1, m2, m*n* (**Figure 3**). The m1 leaf is one lobed; all other leaves have three leaflets. From axillary

buds new metamers are developed and grow out into primary branches. These are numbered after the metamer they grow out from, *e.g.,* B1, B2, B*n*. Later on, secondary and tertiary branches are also formed. AM symbiosis stimulated formation of the first primary branches in the sense that they appeared earlier in AM plants compared to control and P_i-fertilized plants (**Paper II**). Both AM- and P_i-fertilized plants also had more and longer primary branches as compared to control plants. This is likely due to the increased levels of CKs in leaves of both treatments (**Paper III**). CKs are mainly synthesized in root plastids and transported to the shoot via the xylem, but can also be synthesized in chloroplasts (reviewed by Sakakibara (2006)). There may be a connection between CKs and P_i signaling since CKs have been shown to repress several genes involved in P_i starvation response and P_i transport (Shen et al., 2014). In conclusion, it is likely that the developmental alterations detected in mycorrhized plants are due to CK signaling mediated by P_i delivered from the fungus.

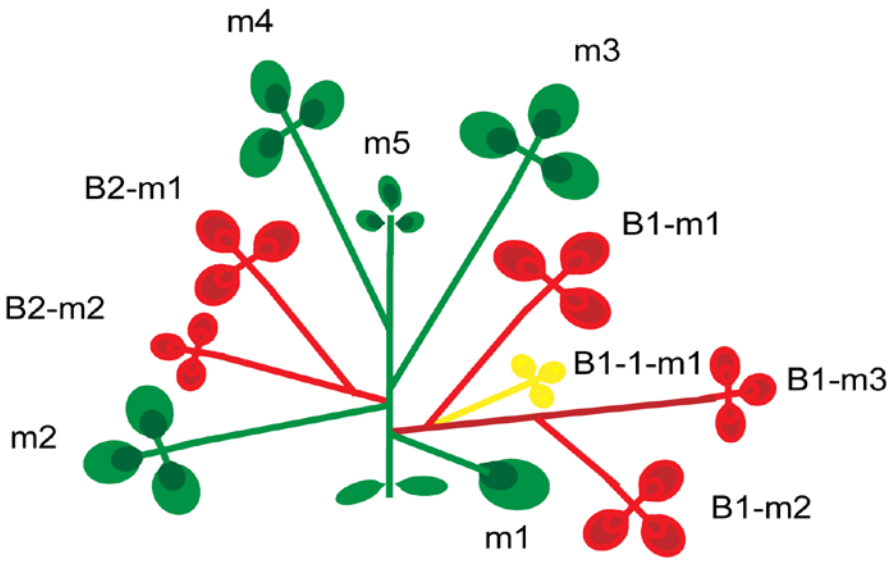

Figure 3. Shoot architecture of *Medicago truncatula*. New metamers (leaf, petiole and stalk) first develop along a main axis (green). Later metamers are developed from axillary buds and grow into primary- (red) and secondary (yellow) branches.

4.3. Photosynthesis

One of the functions of CKs is development of chloroplasts (Cortleven and Schmülling, 2015). Interestingly, AM leaves have more chloroplasts than both P_i-fertilized and control plants (**Paper II**), which could be due to AM-specific increase in the level of dihydrozeatin, a CK derivative (**Paper III**). Increased number of chloroplasts in leaves has also been observed in finger millet (Krishna et al., 1981). In addition, the shape of chloroplasts was altered towards being longer and narrower in AM-inoculated *Medicago* plants (**Paper II**). Despise this, AM symbiosis had no impact on photosynthetic activity of *Medicago* in our experimental conditions (**Paper II**). Neither concentrations of chlorophyll and carotenoids, the PSII

efficiency (measured as F_v/F_m, see section 3.3.1) nor CO_2 assimilation were different in AM plants compared to control. This is in in contrast with studies on *e.g.*, clover (Wright et al., 1998), alfalfa (Tsimilli-Michael et al., 2000) and tomato (Boldt et al., 2011), which reported an enhanced photosynthetic efficiency. In our growth system, the photosynthetic activity was neither increased by P_i fertilization (**Paper II**), indicating that photosynthesis in *Medicago* control plants is not limited by the P_i supply, which might have been the case in the above mentioned studies. This raises the question if the larger number of chloroplasts in AM plants serves any other purpose beside photosynthesis, *e.g.,* secondary metabolism. The observation of more and elongated plastids has been associated with mycorrhized roots and increased production of mycorrhiza-specific apocarotenoids (mycorracidicines and blumenol glucosides, reviewed by Strack and Fester (2006)). In our experimental conditions, the levels of different apocarotenoids were found altered in leaves of mycorrhized plants (**Paper III**, see further discussion in section 4.5.2). It can be speculated if the effects on chloroplast number in mycorrhized plants are due to apocarotenoid production in this organelle.

Chloroplast ultrastructure and photosynthetic function were also examined in *Arabidopsis pht4;1* mutants. Electron microscopy images of *pht4;1* and WT leaves revealed no difference in the ultrastructure of chloroplasts (**Paper I**). At first glance, the photosynthetic light reactions did not seem to be affected by lack of this P_i transporter. There was no difference in levels of photosynthetic proteins, pigments or in ETR (described in section 3.3.1.) between WT and mutant plants. However, NPQ measurements revealed a faster response in leaves of the *pht4;1* mutant compared to WT (**Paper I**). Increased concentration of H^+ in the lumen generated by electron transport is normally used to drive the chloroplast ATP-synthase to add P_i to ADP (as described in section 1.3.1.). NPQ is triggered as a mechanism to protect the photosynthetic machinery against oxidative stress (see section 3.3.1.), when acidification starts to buildup in the lumen. This notion was strengthen by ECS measurements (described in section 3.3.1.) showing a larger contribution of H^+-concentration gradient to PMF in the *pht4;1* mutant than in WT (**Paper I**). In addition, *pht4;1* mutants displayed decreased H^+ conductivity through ATP synthase. If the ATP synthesis is restricted in *pht4;1* mutants this would affect CO_2 assimilation, since the continuation of the Calvin-Benson cycle relies upon a steady supply of P_i in the form of ATP (see section 1.3.2 and **Figure 1**). Indeed, the *pht4;1* mutants grown in standard conditions assimilated less CO_2 than WT, but supplementation with excess P_i resulted in similar assimilation levels in *pht4;1* as in WT plants (**Paper I**), strengthening the assumption of restricted ATP synthesis in the mutants in standard conditions. Together with the observation that PHT4;1 has a similar expression pattern as the chloroplast ATP synthase (Robertson McClung (2000) and Guo et al. (2008a)), this implies that PHT4;1 is most likely localized to the thylakoid membrane where it serves to modulate the levels of P_i available for ATP synthesis (**Figure 1**).

4.4. Sugar metabolism

Plants have several ways to regulate the flow of carbon from source to sink tissue. One important aspect is the partitioning of carbon. Part of the carbon assimilated by the plant is stored as starch in plastids or as cellulose in cell wall, and part it is converted to sucrose in the cytosol for storage in vacuoles or for further transport to sink tissues (see section 1.3.2.). The remaining carbon is used for synthesis of lipids, amino acids and other metabolites. Starch is accumulated during day time, to be converted to sugars and utilized for energy during night time. Partitioning between sucrose and starch is tightly regulated and dependent on light, for supply of ATP and activation of key enzymes through the ferredoxin-thioredoxin regulatory system, and P_i levels in the stroma, since P_i is used as a counter ion in transport of sucrose and triose-P_i (**Figure 1**, see section 1.3.2.).

The observed decrease in CO_2 assimilation in the A*rabidopsis pht4;1* mutant (**Paper I,** see above) is expected to affect the levels of carbohydrates. The starch and soluble sugar contents were measured at the beginning, middle and end of the light cycle. There was no alteration in starch content in *pht4;1* at any time during the cycle (**Paper I**). However, at the beginning of the light cycle levels of soluble sugars were significantly decreased in leaves of the *pht4;1* mutant. The difference disappeared during the day. This suggests that less sugars are used for building biomass and utilizing energy in *pht4;1* mutants as compared to WT, which would explain the reduced biomass and epidermal cell size in the *pth4;1* mutant.

In the case of *Medicago*, leaves from mycorrhized and non-mycorrhized plants contained much more starch as compared to P_i-fertilized plants (**Figure 4A**), in line with the inhibitory role of P_i on starch synthesis (see section 1.3.2). Instead, the increased sink strength of mycorrhized plants was reflected as enhanced level of Fru and Glc, but not sucrose, in AM-inoculated plants compared to control- and P_i-fertilized plants (**Figure 4B**). In contrast, Doidy et al. (2012b) found decreased levels of sucrose, Fru and Glc in leaves of mycorrhized *Medicago* plants, compared to control but not compared to P_i-fertilized plants. One possible explanation for this discrepancy could be that mycorrhized plants in the Doidy study had higher leaf P_i concentration than control, which was not the case in our study (**Paper II** and **Paper III**). Regardless, up-regulated expression of leaf sucrose transporters has been reported in mycorrhized *Medicago* and tomato plants (Doidy et al., 2012b, Boldt et al., 2011); which is most likely due to the enhanced sink strength caused by the carbon demand of the AM fungus. Enhancement of photosynthetic CO_2 assimilation in mycorrhized plants has been suggested as a mechanism to compensate for the increased sink strength (Kaschuk et al., 2009). In our growth system, neither AM symbiosis nor did P_i-fertilization increase the CO_2 assimilation per leaf area in *Medicago* plants. However, if the larger leaf area of AM and P_i plants are taken into account, the overall assimilation of the leaf is larger in those treatments compared to control plants (**Paper II**).

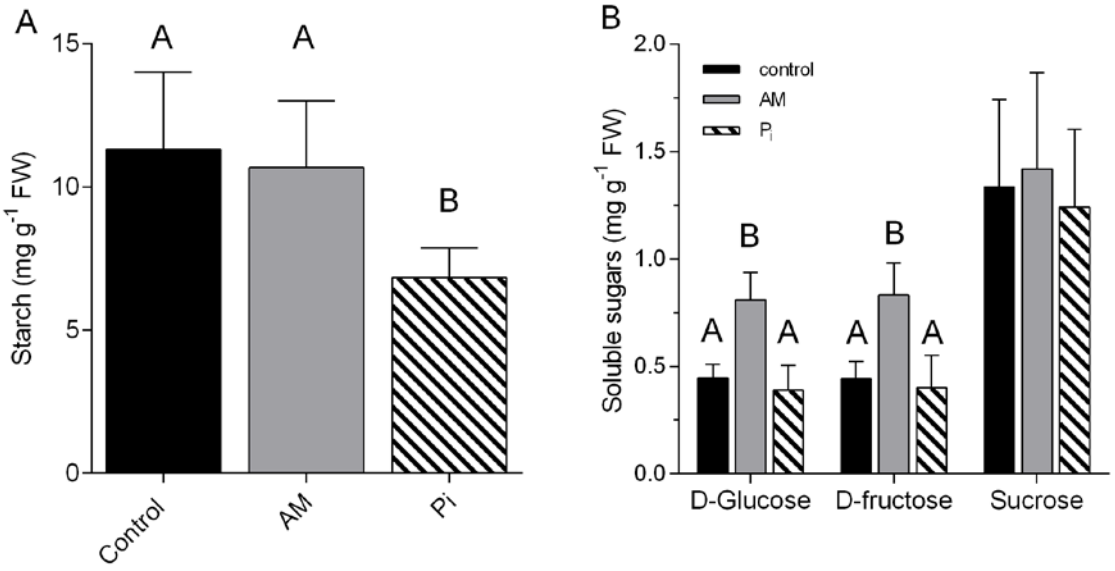

Figure 4. Content of starch (**A**) and soluble sugars (**B**) in leaves of control, mycorrhized (AM) and Pi-fertilized *Medicago truncatula* plants. The bars show mean of four replicate plants ±SD. Different letters represent significant difference at $P<0.05$ according to one-way ANOVA with Fisher LSD as posterior test.

It appears that both the plant and the fungal partner can regulate how much nutrients are transferred (Hammer et al., 2011, Kiers et al., 2011). In the study by Kiers et al., radiolabeled C and P were used to track the nutrient flow on plates with roots and hyphae in separate compartments and different amounts of available sucrose or P_i. It was shown that both the root and the hypha were able to give more C/P to the partner that provided most P/C in return. The mechanism behind this is not known, but the flow of C is likely regulated at the arbuscule level. The turnover of arbuscules is high and appears to be connected with the ability to deliver P_i. Without the mycorrhiza-specific P_i transporter PT4, arbuscules collapse and the symbiosis is aborted (Javot et al., 2007). Likewise, the ability to deliver carbon seems to be crucial for maintaining arbuscules in the root. Development of arbuscules are disrupted without the fungal monosaccharide transporter MST2T, which has a strong expression correlation with PT4 (Helber et al., 2011).

4.5. Secondary- and hormone metabolism

4.5.1. Alterations in the transcriptome

In addition to the benefits related to an improved uptake of P_i, AM symbiosis is known to enhance plant growth by improving the tolerance to biotic and abiotic stresses (see section 1.2). This demonstrates that AM fungi affect plants in more ways than the strict nutritional. To understand how the shoot responds to mycorrhization, changes in the transcriptome

were studied based on microarray data in *Medicago* leaves of AM-inoculated and P_i-fertilized plants (**Paper III**). Of the genes that were up- or downregulated by AM symbiosis compared to control, about 5 % were altered in the same- respective opposite direction as P_i treatment; demonstrating both shared and antagonizing responses from the two treatments. Genes found differently regulated compared to control plants, were divided into functional categories using a system previously proposed by Journet et al. (2002). This gave us the opportunity to compare if the same type of genes were affected in both *Medicago* shoots (**Paper III**) and roots (Journet et al., 2002, Hohnjec et al., 2005). The dominating category in both tissue types was *Unknown function/No homology* due to the incomplete annotation of the *Medicago* genome (see section 3.1.2). For genes with known function in shoots most fell into two categories: *Gene expression and RNA metabolism* and *Secondary- and hormone metabolism* (**Paper III**). Most genes in the former category are transcription factors, and more or less the same number was up- and downregulated in AM- and P_i-fertilized plants (**Paper III**). For example, *Gigantea* and *Sepallata* genes regulating flowering time (Tzeng et al., 2003, Park et al., 2013) were found upregulated in both treatments. This suggests that AM-inoculated and P_i-fertilized plants share some basic developmental responses, as was also observed in the developmental study made in **Paper II** (see section 4.2).

The results from the two studies in *Medicago* roots (Journet et al., 2002, Hohnjec et al., 2005) were not completely conclusive. Here it should be pointed out that the different categories are not strictly defined so one gene can potentially be placed in different categories. In the Hohnjec et al. (2005) study most of the AM-induced genes with known function fell into the categories of *Secondary- and hormone metabolism* and *Primary metabolism*; whereas most fell in the category *Protein synthesis and degradation* in the study by Journet et al. (2002). The altered expression of genes involved *primary-* and *protein metabolism* in roots are likely a reflection of the morphological rearrangements taking place in the cortical cells during symbiosis (described in section 1.2.1). A recent proteomic study on mycorrhized *Medicago* roots demonstrated high impact on primary metabolism and response to oxidative stress in the colonized cells (Daher et al., 2016). The fact that genes involved in *secondary- and hormone metabolism* were upregulated by AM symbiosis in both roots and shoots (Hohnjec et al. (2005); **Paper III**), indicate that responses to the fungus in roots have an impact on the signaling in shoots. Enhancement of defense genes in *Medicago* shoots in response to mycorrhization has indeed previously been observed by Liu et al. (2007). In our study, genes involved in biosynthesis of flavonoids and saponins, the two major groups of secondary metabolites in *Medicago* (reviewed by Gholamin et al. (2014)) as well as ABA and oxylipins were upregulated by AM symbiosis but not by P_i fertilization (**Paper III**), indicating that this response is not coupled to the P_i status of shoots. Interestingly, the transcription factor *MYC2* and the repressor *JAZ* involved in jasmonic acid (JA) signaling (Figueroa and Browse, 2012), as well as the enzyme 9s-LIPOXYGENASE involved in oxylipin biosynthesis (Griffiths, 2015) were upregulated by AM and downregulated by P_i fertilization (**Paper III**), suggesting an antagonizing role of P_i and mycorrhization in JA

signaling. Both JA and 9-oxylipins are believed to be important for regulating fungal proliferation in roots (León-Morcillo et al., 2012).

As expected, genes known to be involved in P_i transport and signaling (see section 1.1.1-2) such as purple acid phosphatase, *SPX2* and *SPX3* were downregulated by P_i fertilization (**Paper III**). Interestingly, genes involved in iron transport and homeostasis, including ferritin, were strongly downregulated in both AM-inoculated and P_i-fertilized plants. Ferritin protects the cell from oxidative stress by chelating iron into large complex stored in the cytosol or plastids (reviewed by Arosio et al. (2009)). As will be discussed below, the finding of reduced levels of ferritin indicates that AM- and P_i-fertilized plants have complementary mechanisms to deal with excess iron.

4.5.2. Flavonoids and apocarotenoids

Several attempts have been made to characterize metabolic responses in mycorrhized shoots of *e.g.,* willow, (Aliferis et al., 2015), rice (Gerlach et al., 2015) and *Plantago major* (Schweiger et al., 2014a). It has been proven difficult to find any universal metabolic response to AM symbiosis among different species. This is partly because of high variation in shoot metabolome between different species, even when they are not colonized (Schweiger et al., 2014b). In the case of *Medicago*, only one of the metabolites altered in leaves of AM plants compared to control (a ferulic acid derivative), were altered in the same direction by P_i treatment (**Paper III**). This demonstrates a very low overlap in secondary metabolite response between AM-inoculated and P_i-fertilized plants. Only AM treatment altered the composition of apocarotenoids (see discussion below) and saponins (triterpenoids produced in the cytosol, reviewed by Gholami et al. (2014)) and enhanced the levels of several flavonoids (see discussion below). For comparison, a study with mycorrhized *Medicago* roots revealed enhancement of fatty acids, isoflavonoids and apocarotenoid derivatives (Schliemann et al., 2008). Lipids and the precursors for flavonoids and apocarotenoids are all produced in plastids (see section 1.3.3), which points towards activation of plastidial metabolism in both roots and shoots of mycorrhized plants (**Figure 1**).

Flavonoids, including anthocyanins, are a large group of polyphenolic pigments, derived from phenylalanine produced in plastids, and further synthesized in the phenylpropanoid pathway in the cytosol (**Figure 1**, see section 1.3.3). AM symbiosis, but not P_i-fertilization, enhanced the levels of flavonoids; both for specific metabolites and when looking at the total levels of flavonoids and anthocyanins (**Paper III**). Flavonoids are important for various functions in the cell *e.g.,* protection against UV damage and oxidative stress and interaction with other organisms (reviewed by Gholami et al. (2014)). The latter includes their function as Nod factors in rhizobia symbiosis, allelopathic compounds that inhibit growth of other plants and phytoalexins that are toxic to pathogens. Flavonoids are also released as root exudates during P_i deficiency, where they help release bound P_i by chelating metals (reviewed by Cesco et al. (2010)) and have a stimulatory effect on presymbiotic growth of AM hyphae

(Scervino et al., 2005). In addition, some flavonoids have the ability to reduce and chelate iron in cells (Mira et al., 2002). This would explain the lower expression of ferritin observed in AM treatment in **Paper III**; but not the similar reduction of ferritin in P_i-fertilized plants, which did not accumulate flavonoids. P-containing compounds *e.g.,* phospholipids and phytic acid, can also chelate iron (Rasmussen and Toftlund, 1986). Because of the much higher P concentration in leaves of P_i-fertilized plants compared to AM leaves, it is likely that the iron-chelating role of flavonoids mainly applies to AM plants. Iron, in the form Fe(II), can cause oxidative damage to the cell by reacting with ROS (see section 1.3.1) in the Fenton reaction, which is prevented by flavonoid chelation (reviewed by Perron and Brumaghim (2009)). It is possible that flavonoids serve to modulate oxidative stress in AM leaves.

Apocarotenoids are another group of metabolites that were altered in *Medicago* leaves in response to AM symbiosis (**Paper III**). They are carotenoid cleavage products produced in plastids and include the plant hormones SL and ABA (**Figure 1**, see section 1.3.3.). The level of several apocarotenoids was altered only by AM symbiosis; ABA which were enhanced and dihydroxy blumenol glucoside which almost completely disappeared in AM leaves (**Paper III**). ABA is associated with abiotic stress responses and its signaling role during AM symbiosis is discussed below. Apocarotenoids have previously been associated with mycorrhized roots, especially the yellow linear C_{14} pigment mycorradicines (Klingner et al., 1995) and cyclic C_{13} cyclohexenon derivative (*e.g.,* blumenol C 9-O-(2'-O-β-glucuronosyl)-β-glucoside, Maier et al. (1995)), where the latter are believed to be important for arbuscule turnover (reviewed by Walter (2013)). Both ABA and blumenol are derived from all-trans-lycopene (reviewed by Hou et al. (2016)). The strong reduction of the dihydroxy blumenol glucoside in leaves of mycrrohized leaves, can be due to either competing biosynthesis with ABA or to transport towards mycorrhized roots.

4.5.3. Jasmonate and abscisic acid signaling

Secondary metabolite levels are usually under control of hormone signaling. Previous works indicate that AM symbiosis can inhibit defense responses in the shoot that are regulated by the hormone SA *e.g.,* resistance towards biotrophic pathogens and phloem-feeding insects, but enhance defense responses in the shoot that are regulated by the JA signaling pathway *e.g.,* resistance towards necrotic pathogens and chewing insects (Pozo and Azcón-Aguilar, 2007). JA is present in many forms in the plant and its precursor is synthesized in plastids (see section 1.3.3, **Figure 1**). Its biologically active form JA-Ile is formed in response to wounding. JA-Ile binds to the repressor JAZ, which is target for degradation, and allows transcription of JA-regulated genes (reviewed by Wasternack and Hause (2013)). Our large-scale hormone analysis of shoots revealed that both AM symbiosis and P_i fertilization decreased the levels of SA and JA-Ile in *Medicago* leaves (**Paper III**). However, in studies of mycorrhized willow shoots most of the JA was in the volatile form MeJA (Aliferis et al., 2015), used for long-distance transport. MeJA was not detectable by the method used in

Paper III. It is possible that AM symbiosis did indeed increase the levels of MeJA in *Medicago* shoots as well. This would be in line with the observed up-regulation of genes involved in JA- and oxylipin signaling and -biosynthesis in mycorrhized AM shoots (**Paper III**). Alternatively, JA produced in leaves might have been transported to the roots, where their levels are known to increase during AM symbiosis (reviewed by Wasternack and Hause (2013)).

Two antagonizing branches of JA signaling occur (reviewed by Kazan and Manners (2013) and Wasternack and Hause (2013)). One is regulated by the hormone ethylene and provides defense against necrotic pathogens. The other is regulated by the hormone ABA (produced in plastids, **Figure 1**) and the transcription factor MYC2, and is involved in defense towards herbivores. In *Medicago* shoots *MYC2* was up regulated, and ABA levels increased in AM-inoculated but not in P_i-fertilized plants (**Paper III)**. This suggests an enhancement of the ABA/*MYC2*-pathway in mycorrhized plants. Defense towards herbivores can be accommodated through production of secondary metabolites such as flavonoids. Several enzymes involved in flavonoid biosynthesis are regulated by MYC2 (Dombrecht et al., 2007). Hence it is tempting to speculate that the observed up-regulation of enzymes for flavonoid biosynthesis as well as increase in total flavonoid level in shoots of mycorrhized plants in **Paper III**, are due to activation of MYC2 through ABA and JA signaling. Indeed, application of ABA and MeJA on *Medicago* seedlings caused up-regulation of *MYC2* and flavonoid biosynthesis genes (**Paper III**).

5. Conclusions and Outlook

The current use of P_i fertilizer derived from rock P, where a large portions of the limiting resources ends up in rivers and oceans where it cause problems with eutrophication, are not sustainable in the long run (see section 1.1.). Alternative means for more efficient use of P_i-fertilizers are called for, and AM symbiosis has been proposed as an important tool (reviewed by Berruti et al. (2016)). However, the growth- and P_i response of the plant to AM fungi is not always positive (see discussion in section 4.1.). For AM symbiosis to function as a realistic approach to manage sustainable agriculture, it is important to establish a good interaction between plants and fungi. To achieve this, understanding of the signaling between the two symbionts is crucial. AM symbiosis is an interaction that physically takes place in roots (**Figure 1**). Still the maintenance of the symbiosis is dependent on a steady supply of sugars produced in leaves (see section 1.2.2). Furthermore, P_i supplied by the fungus has an intimate connection with carbohydrate production and partitioning in the plant (see section 1.3.2). AM symbiosis is also known to induce systemic defense responses that affect the resistance of the shoot (see section 4.5.3). Still, it is not clear exactly how the symbiosis is perceived by the shoot and to what extent this is dependent on the P_i supply. Two different experimental systems were used in this thesis (described in detail in section 3.2) to gain a deeper understanding of how AM symbiosis and P_i nutrition are perceived by the shoot.

In **Paper I,** *Arabidopsis* mutants lacking a chloroplast P_i transporter were studied to understand how the shoot is dependent on P_i supply within the chloroplast for development, photosynthesis and carbon partitioning. It was shown that disruption of P_i homeostasis within the chloroplast, caused by the loss of the P_i transporter, reduced the activity of chloroplast ATP synthase. This resulted in reduced CO_2 assimilation and less sucrose for building biomass, especially during night time, and was reflected in reduced shoot biomass and smaller leaf area. There was no difference in P levels in the shoot of the WT and mutant, but supplementation of P_i nutrition rescued the biomass and leaf area phenotype. This could be interpreted as: the level of P_i within the chloroplast is more important for carbon partitioning than the P_i level in the whole leaf. Of course more research is needed to support or disregard this hypothesis.

In **Paper II** and **Paper III,** the interaction between *Medicago* plants and an AM fungus was used to study how the symbiosis affects the development, photosynthesis and metabolism of the host shoot. In **Paper II,** it is demonstrated that, under the experimental conditions used, the total P content (mg P/total shoot) of shoots is higher in mycorrhized plants compared to control. But the concentration of P (mg P/DW shoot) is similar in both treatments. Mycorrhized plants also had larger shoot biomass, longer branches and larger leaves compared to control. However the symbiosis did not stimulate the photosynthetic activity of the host plant. The same features were observed in P_i-fertilized plants. The number of chloroplasts was increased and the shape altered in response to mycorrhiza but

not P_i fertilization. Taken together, AM symbiosis induce a growth phenotype with longer branches, larger leaves and more chloroplasts, that increase the surface area of the shoot and enables the plant to harvest more sunlight.

In **Paper III**, it was investigated how the alterations described in **Paper I** were reflected in gene expression and secondary metabolism of the shoot. Analysis of gene expression revealed that genes related to *secondary metabolism and hormones* (primarily flavonoids and saponin-type of terpenoids as well as ABA and oxylipins including JA) were strongly upregulated in response to AM symbiosis, but either not altered or downregulated by P_i treatment. The induction of secondary metabolite genes by AM symbiosis was reflected in an enhancement of flavonoids and an altered composition of saponins and apocarotenoids. An altered composition of some secondary metabolites was also observed in P_i-fertilized plants. Overall, AM-inoculated and P_i-fertilized plants had poor overlap in metabolic responses and in gene expression, most likely due to different P_i status resulting in different signaling pathways. The biosynthesis of flavonoids and apocarotenoids both start in plastids (**Figure 1**), which is an indication that chloroplast metabolism is affected in leaves of AM-inoculated plants. Hormone analysis showed enhancement of the apocarotenoid ABA in leaves of AM-inoculated plant and CKs (also produced in plastids, **Figure 1**) in AM-inoculated and P_i-fertilized plants. The increased level of CKs explains the developmental alterations, including increased number of chloroplasts observed in **Paper II**. Enhanced level of ABA is likely responsible for activation of flavonoid and oxylipin biosynthesis through an AM-specific signaling pathway (see discussion in section 4.5.3.).

The question still remains how the increased sugar demand of mycorrhized roots is communicated to the shoot. It is well established that massive signaling events take place in roots during AM symbiosis. Root plastids appear to be especially targeted, as reflected in increased number of plastids and enhanced plastidial activity producing fatty acids and secondary metabolites (Seddas et al., 2009). Secondary metabolites derived from plastids are enhanced both in the root and the shoot of mycorrhized plant (**Figure 1**). This demonstrates that the signaling events taking place in the root are also reflected in the shoot, but the identity of the signal going from root to shoot still remains to be elucidated. There are several potential actors; one is P_i, which has been proven to work as a signaling molecule in arbuscules (reviewed by Yang and Paszkowski (2011)) and during P_i limitation (see section 1.1.1). However, despite the alteration in secondary metabolism in AM plants (**Paper III**) the P concentration in shoots were the same for AM and control plants (**Paper II**), which makes P_i a less likely signal in this case.

Another potential signal is the altered levels of sugar (**Figure 4B**) caused by the increased sink strength of AM plants (see discussion in section 4.4.). Sugars have been shown to play an important role during P_i limitation (reviewed by Hammond and White (2011)), and can together with various hormones including JA and ABA, induce synthesis of anthocyanins (reviewed by Das et al. (2012)). In a recent proteome study (Daher et al., 2016), it is

proposed that arbuscules cause carbohydrate starvation which result in an oxidative stress signature in mycorrhized roots. The enhanced levels of flavonoids in leaves of mycorrhized plants (**Paper III**) could be an indication of ROS signaling (see section 1.3.1. and discussion in section 4.5.2). ROS is known to stimulate production of ABA (Mittler and Blumwald, 2015), whose levels were indeed found enhanced in shoots of mycorrhized plants (**Paper III**). It is possible that a ROS signature from the root activates ABA biosynthesis in shoot. Alternatively, ROS signaling in roots can stimulate production of ABA, which is further transported to the shoot. In either case; in the mesophyll cells ABA can stimulate production of PEP-derived flavonoids (and possibly apocarotenoids and oxylipins, **Figure 1**), through stmulation of *MYC2* expression as described above. Together this would create a link between the sugar demand of the root and the systemic signaling in the shoot during AM symbiosis.

6. Populärvetenskaplig sammanfattning

Växter är beroende av grundämnet fosfor som byggstenar i genetiskt material och cellmembran samt för att lagra energi. Större delen av fosforn i jorden är hårt bundet till mineraler och är inte tillgängligt för växter, som tar upp fosfor i form av fosfat. Därför är brist på fosfat oftast en begränsande faktor för växters tillväxt. Inom jordbruket används stora mänger av fosfatgödsel, som till större delen bryts ur fosforrika bergarter. Mineralfosfor är en begränsad resurs som beräknas vara förbrukad inom de närmaste 100 åren. Samtidigt går mycket av fosfaten som sprids på åkrarna till spillo då grödorna inte tar upp all fosfat som tillförs marken. En stor del av överskottet hamnar i olika vattendrag där det orsakar problem med övergödning. Därför finns ett stort behov av att effektivisera fosfatgödslingen inom jordbruket. Flera förslag har förts fram, varav ett av dem är att använda mykorrhizabildande svampar.

De flesta grödor, förutom vissa kålväxter, kan naturligt bilda symbios med s.k. arbuskulära mykorrhiza (AM) svampar. Dessa hjälper växterna att ta upp fosfat och andra näringsämnen ur jorden, samtidigt som växterna blir mer tåliga mot torka och sjukdomar. I gengäld förser växterna svampen med kolhydrater som bildats igenom fotosyntes i kloroplaster i växternas blad. Fotosyntes är en process där ljusenergi används för att binda in koldioxid i luften och göra om den till kolhydrater. I kloroplaster tillverkas även sekundära metaboliter, vilket är ämnen som fyller en annan funktion än tillväxt, utveckling och reproduktion, t.ex. försvar mot skadedjur. Eftersom växterna ger bort en stor del av kolhydraterna till svampen, leder inte alltid symbiosen till ökad tillväxt hos växterna. För att AM-symbios ska vara ett realistiskt alternativ inom jordbruket, krävs en ökad förståelse för hur växten och svampen kommunicerar och påverkar varandra. Man vet att tillverkning av kolhydrater påverkas av mängden fosfor i växten och att AM-symbiosen är beroende av ett ständigt tillflöde av socker. Däremot vet man inte om AM-symbiosen har någon direkt påverkan på kloroplasterna.

In min avhandling har jag velat ta reda på hur växters skott påverkas av fosfattillgång och AM-symbios, främst med fokus på tillväxt, utveckling och processer i kloroplasten som fotosyntes och produktion av kolhydrater och sekundära metaboliter. För att undersöka detta har jag använt mig av två modell-system: I **artikel I** jämförs muterade *Arabidopsis thaliana* (backtrav) växter som saknar proteinet PHT4;1 vars uppgift är att transportera fosfat inom kloroplasten, med omuterade (vildtyp) backtrav. Backtrav är en modellväxt som är tacksam att jobba med då det finns tillgängligt väldigt mycket information om den. Däremot kan den inte bilda AM-symbios. Därför har istället *Medicago truncatula* (lusern) som är en modellärtväxt undersökts i **artikel II** och **artikel III**. I dessa artiklar har plantor som antingen inokulerats med AM-svamp eller fosfat gödslats, jämförts med obehandlade kontroll-plantor.

I **artikel I** har vi tittat närmare på hur avsaknaden av fosfattransportören i kloroplasten påverkar fotosyntes, produktion av kolhydrater och tillväxt. Vi visar att

mutanter som saknar PHT4;1 har en försämrad förmåga att tillverka ATP, vilket är en fosfatinnehållande molekyl som används för att lagra energi i cellen. Energin som är lagrad i ATP behövs för att t.ex. fixera koldioxid till kolhydrater. Bristen på ATP visar sig i *pht4;1*-mutanterna i form av minskad koldioxidfixering, mindre mängd socker och minskad skottbiomassa. Däremot påverkades inte fosforinnehållet i skottet. Resultaten tyder på att tillgången på fosfat inom kloroplasten har väldigt stor betydelse för växtens tillväxt.

I **artikel II** jämförs tillväxt och fotosyntes hos växter som lever i symbios med AM-svampar och växter som fosfatgödslats med kontrollväxter. Resultaten visar att växter som har odlats med AM-svampar har fler kloroplaster. I övrigt påverkas inte effektiviteten på fotosyntesen, varken av AM-symbios eller av fosfatgödsling. Däremot ger båda behandlingarna längre grenar och större bladyta, vilket ökar växternas förmåga att ta upp solljus. Dessa morfologiska skillnader beror troligen på att både AM-inokulerade och fosfatgödslade växter innehåller större mängder av hormonet cytokinin, vilket beskrivs i **artikel III**. I denna artikel jämförs även mängden av andra hormoner och sekundära metaboliter, samt genuttryck hos de olika behandlingarna. Resultaten visar på en ökad mängd av hormonet abskisinsyra samt ökat uttryck av gener som kodar för proteiner som behövs vid tillverkning av sekundära metaboliter, i blad hos växter som inokulerats med AM-svamp men inte hos de som fosfatgödslats. AM symbios ökar även mängden av sk. flavonoider, som fungerar som antioxidanter och därför påverkar näringsinnehållet i växten.

Sammanfattningsvis presenterar jag en modell där det ökande suget efter kolhydrater i mykorrhizerade rötter alstrar en signal som leder till ökad produktion av sekundärmetaboliter i bladen. Genom dessa resultat bidrar min forskning till en ökad förståelse för hur kommunikationen med AM svampen i växters rötter påverkar tillväxten och näringsinnehållet i skottet. Denna förståelse är viktig för att skapa gynnsamma samspel mellan växter och AM-svampar. Kunskapen kan i förlängningen användas inom jordbruket för att skapa både ökad tillväxt och ett mer effektivt upptag av fosfat hos de odlade växterna.

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