The importance of sucrose synthase for AM symbiosis in maize, in pea and in Medicago

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ABBREVIATIONS

AGP: Arabinogalactane protein
AM: arbuscular mycorrhiza
ATP: adenosine triphosphate
BCIP: 5-bromo-4-chloro-3-indolyl phosphate
BSA (bovine serum albumine)
bp: base pair
cDNA: complementary deoxyribonucleic acid
CHI: chalcone isomerase
CHS: chalcone synthase
C/CON: control plant
DEPC: diethylpyrocarbonate
D: Dalton
DIG-AP: anti-digoxigenin alkaline phosphatase
DNA: deoxyribonucleic acid
DNTP: deoxyribonucleic acid
DTT: dithiothreitol
DW: Dry weigth
EDTA: ethylenediaminetetraacetic acid
FW: Fresh weight
GTP: guanosine triphosphate
HPLC: high-performance liquid chromatography
HR: hypersensitive response
HRGP: hydroxyproline rich glycoprotein
IFR: isoflavone reductase
IPTG: isopropyl β-D-thiogalactopyranoside
min: minute
mRNA: messenger RNA
MYC: plant treated with AM fungus
MYA: million years ago
NBT (nitroblue tetrazolium salt)
Nod factor: nodulation factor
OD: optical density
PAL: Phenylalanine ammonia lyase
PCR: polymerase chain reaction
PR: pathogenesis-related proteins
rDNA: ribosomal DNA
RNA: ribonucleic acid
rRNA: ribosomal RNA
RT-PCR: reverse transcription-PCR
rpm: revolutions per minute
Sh: shrunken
Sus: sucrose synthase

SS1: sucrose synthase 1
SS2: sucrose synthase 2
TAE: Tris/acetic acid acid/EDTA buffer
Tris: tris(hydroxymethyl)-aminomethane
TBS (100 mM Tris-HCl, pH 7.5, 400 mM NaCl)
Suc: sucrose
XET: xyloglucan endo-transglycosylase

ABSTRACT

The arbuscular mycorrhiza (AM) is probably the most widespread symbiotic associations on earth, occurring in the roots of about 90 % of the land plant species. The fungal partners are soil-borne fungi of the order Glomales in the phylum of Glomero. The first AM fossils were dated to the Ordovician (505-434 million years ago*)*, soon after the separation of the major groups of terrestrial fungi (around 600 million years ago) and at the time when the first land plants appeared. The existence of mycorrhizal fossils from very early epochs and its prevalence over all the different symbioses between plants and fungi indicate an important role of the AM symbiosis in the evolution of life on earth. They penetrate into the host cortical cells, invaginating the plasma membrane, and form highly branched structures, so-called arbuscules. This symbiosis is mutually beneficial since the fungus profits of the plant's photoassimilates and the plant of an increased supply of mineral nutrients provided by the fungus. Glucose is probably the main carbon compound taken up by the fungus at the plant-fungal interface but little is known about carbon metabolism in the AM symbiosis. In particular, the complex mechanisms of the carbon allocation from the host-plant to the fungus have not been elucidated so far.

In the last fifteen years, the AM symbiosis was more and more studied because of its widespread occurrence in the plant kingdom, including many agronomical interesting plants. The diversity of vesicular-arbuscular mycorrhizae in soils has been shown to be a determining factor for maintain of biodiversity and ecosystem functioning. The AM symbiosis shares similarities and common pathways with the *Rhizobium*-legume interaction, another symbiosis of great interest for grain and forage legumes used in agriculture.

In this thesis, after a thorough review on the current knowledge of the molecular aspects of AM symbiosis, an untargeted approach to find genes induced in this symbiosis is described. A reverse transcription-PCR differential display (RT-PCR-DD) was carried out in order to identify plant genes involved in an AM symbiosis, using as a model *Medicago truncatula* colonized by the AM fungus*, Glomus intraradices.* A clone

encoding a putative sucrose synthase (MtSucS2) was identified, and its regulation was studied with regard to mycorrhiza and nodulation. The expression of the newly discovered gene encoding MtSucS2 was compared to the expression of a nodule–enhanced sucrose synthase (MtSucS1) that had been isolated earlier from *Medicago.* Interestingly, the two sucrose synthases displayed an inverse expression pattern in mycorrhizal roots. Compared to control roots, MtSucs1 expression was lower in mycorrhizal roots, but MtSucS2 was 4 fold induced in 6 weeks old roots infected by *Glomus intraradices*. The expression of MtSucS2 was a higher in fruits, flowers and nodules than in leaves, roots and stems. The induction of MtSucS2 seems to be particularly important in "special sink tissues" while MtSucS1 is predominant in stems, flowers and nodulated roots. Based on an in-situ analysis of mRNA expression, it is likely that the induction of MtSucS2 is in fact much higher in the arbuscule-containing cells.

The role of sucrose synthase in AM symbiosis was investigated with the use of pea and maize mutants deficient in the sucrose synthase. Maize and pea lines affected in genes coding for sucrose synthase showed wild-type growth and development with regards to the plant phenotype but did not display a normal development of the symbiosis. In a time-course experiment, a reduction of AM colonization was observed in maize mutant plants compared to the wild-type plants. In particular, vesicle formation was strongly reduced. The results highlight the importance of sucrose synthase(s) for a normal development of the AM symbiosis. A model is presented to illustrate the role of sucrose synthase in AM symbiosis.

I. GENERAL INTRODUCTION

1.1. THE MYCORRHIZA: A KEY SYMBIOSIS OF PLANTS AND FUNGI

1.1.1. Definition and history

The term symbiosis, according to the dictionary of Science Technology (Academic Press) is defined as a relationship in which two dissimilar organisms live in close association with each other, and it is used to designate any close long-lasting association between two distinct living organisms regardless of the benefit for the individual partners. DeBary (1831-1888) first introduced this term in 1887, and he also coined the distinction between mutualistic and antagonistic symbiosis to describe, in the first case, a symbiosis in which two partners benefit from each other and in the second, an association where only one partner benefits from the other (De Bary, 1887).

Currently, the term "symbiosis" is often restricted to the mutualistic symbioses whereas the antagonistic symbioses are usually defined as cases of parasitism or pathogenic interactions.

"Symbiosis" in the narrower sense of mutualism is widespread in animal and plant kingdoms. Indeed, symbiotic interactions seem to exist in almost all ecosystems. The two symbioses of direct importance for the present study are the *Rhizobium*-legume symbiosis and the mycorrhiza.

In botany, mycorrhiza comes from the combination of the two greek words *mykes* (fungus) and *rhiza* (root). This term has been proposed by Franck in 1885 to define the association between a soil-borne fungus and plant roots (Frank, 1885). This relation consists in the colonization of plant roots by the microorganism and in the creation of a site of nutrient exchange. At this particular site, the plant will receive mineral nutrients and in turn will feed the fungus with carbohydrates. This mutualistic association is present in around 90% of all land plant species and is one of the most striking examples of compatibility between plants and microbes.

1.1.1.1. The different types of mycorrhiza

Several types of mycorrhiza association have been characterized according to morphological and histological criteria and have been classified into seven different groups. The endomycorrhiza, in particular the arbuscular mycorrhiza (AM), and the

ectomycorrhiza (Ecto) are the most widespread types, whereas the arbutoid, monotropoid, ectendo, ericoid and orchid mycorrhiza are only present within a few plants species as described in Smith and Read (1997).

The ectomycorrhiza is the association of fungi belonging primarily to the Basidiomycetes or Ascomycetes, with roots of woody Angiosperms and Gymnosperms. In this association, the fungus gets into contact with the young root forming a coat around it the so-called mantle. Then, from the inner part of the ectomycorrhizal mantle, hyphae develop a network of hyphae between the epidermal and cortical cells to form the so-called Hartig net (Tagu et al., 2000).

The AM symbiosis is the most widespread on earth, compared to the occurrence of the symbiosis types cited earlier. Indeed, it is present in most of the habitats and is defined as the association between the fungi of the phylum Glomeromycota and most of the terrestrial species ranging from thallophytes to angiosperms. The morphology of the fungus colonizing plant root tissues is highly elaborated in AM symbiosis. Among the different morphological patterns encountered, two main ones are the *Paris* and *Arum* types (Smith and Smith, 1997). In the *Arum* type, the fungus penetrates the root cortex and the fungal hypha grows intercellularly and intracellularly to differentiate into a complex intracellular branched haustorium, the arbuscule. The *Paris* type mycorrhiza is characterized by well-developed intracellular hyphal coils while arbuscules are absent.

In the last fifteen years, the AMs were more and more studied because of their widespread occurrence in the plant kingdom, including many agronomically interesting plants, as well as their importance in the function of ecosystems.

1.1.2. The Arbuscular Mycorrhiza (AM) symbiosis

1.1.2.1. Origin

The first report on fossil mycorrhizae came from Scottland; these AM fossils were dated to the Devonian (395-345 MYA*)* (Kindston and Lang, 1921). However, in many cases, it was still difficult to identify clearly the fungal structures inside the plant tissues (Harley, 1969). Some other cases of fossils from the carboniferous time (345-280 MYA) were discovered and provided clear evidence for colonization of rhizomes and fern roots (Pirozynski and Malloch, 1975; Remy et al., 1994). The colonization consisted of non-

septate hyphae and of arbuscules. More recently Redecker and collaborators combined estimates of fungal phylogeny derived from ribosomal small subunit sequences and fossil records to conclude that the major groups of terrestrial fungi originated around 600 million years ago (Redecker et al., 2000).

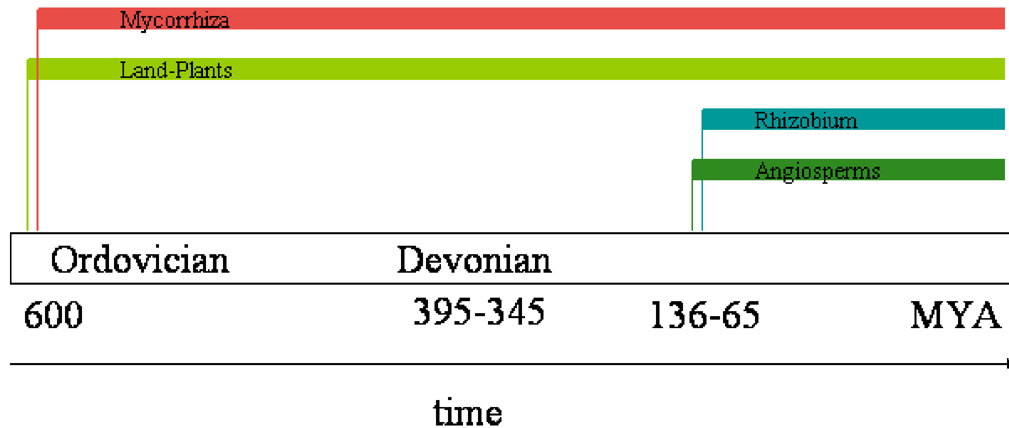

Figure 1.1: Appearance of AM and *Rhizobium* symbioses

The existence of mycorrhizal fossils from very early epochs and its prevalence over all the different symbioses between plants and fungi indicate an important role of the AM symbiosis in the evolution of terrestrial life on earth. The invasion of land by the ancestors of vascular plants may have been facilitated by the occurrence of a symbiotic association and the symbiosis is influencing the terrestrial ecosystems until present times (Pirozynski and Malloch, 1975; Pirozynski and Dalpe, 1989). How the colonization of land by the plants occurred is still largely unknown and the time when it happened remains somewhat speculative (Fig.1.1.).

1.1.2.2. Benefits of AM fungi for the host-plant

1.1.2.2.1. The mineral nutrition

AM fungi are obligate biotrophs, i.e. they cannot live in the absence of their association with plants. According to current knowledge, it is assumed that the AM symbiosis is

beneficial for the two organisms and is therefore a typical mutualistic interaction (Solaiman et al., 2001). The AM fungi colonize a host plant in order to be supplied by photosynthetically fixed carbon from the plant. In turn, the plants benefit from the fungus that contributes to their mineral nutrition, by the capture and the transport of mineral nutrients, essentially of phosphorus, from soil to plant (Koide and Schreiner, 1992). This chapter gives a brief overview of the present knowledge on the importance of AM fungi for mineral uptake. The carbon metabolism of the symbiosis will be summarized in another part of the manuscript.

1.1.2.2.2. Phosphate uptake and transport

Phosphorus is one of the most important plant macronutrient. It is required to build essential molecules such as nucleic acids and phospholipids, and it plays a central role in energy transfer (NADPH, ATP) and regulation of enzymatic and metabolic reactions (Bieleski and Ferguson, 1975; Theodorou and Plaxton, 1993). Free phosphate levels in the soils solution encountered by the plant are usually very low and range from less than 1 µM to 10 µM (Marschner, 1995). Phosphate is present in the soil in different forms, with an organic and a mineral pool (Holford, 1997). Because of different factors like adsorption, precipitation or conversion into organic forms, 80-99% of the phosphorus is barely available for plant uptake. For this reason, the mycorrhizal symbiosis is important for plant P supply since the fungal hyphae extend into the soil and allow roots to explore a larger soil volume (Smith and Read, 1997). The form of P most readily accessed by plants is Pi (in-organic Phosphate) (Bieleski, 1973). If the root is mycorrhized, it is primarily captured from the soil by external hyphae and transferred to the plant root cortex (Sanders and Tinker, 1971; Smith and Gianinazzi-Pearson, 1988). The phosphate flux has been estimated at 13 nmol $m^{-2}s^{-1}$ along the hyphae in the mycorrhiza (Cox and Tinker, 1976; Smith et al., 1994a). Despite numerous studies, the mechanisms underlying this P translocation in the hyphae are largely unknown (Smith et al., 1994b). A trans-membrane phosphate transporter has been cloned from the external hyphae of AM fungus *G.versiforme* and the function of the corresponding protein confirmed by complementation of a yeast phosphate transporter mutant. The localization of GvPT to the external hyphae strongly suggests it is responsible for phosphate uptake from the soil

(Harrison and van Buuren, 1995). In these external hyphae, phosphate may be condensed into polyphosphate (poly-P), in small vacuoles and the phosphate is probably translocated in this form via protoplasmic streaming into the intraradical hyphae (Cox et al., 1975; Cooper and Tinker, 1981). Enhanced polyphosphatase activity detected in colonized onion roots as well as enhanced alkaline phosphatases in vacuoles and intercellular hyphae suggest the hydrolysis of the poly-P and release of phosphate in the arbuscule where it is probably transferred to the plant (Gianinazzi et al. 1999). At this nutrient exchange site, the phosphate may be taken up by a plant phosphate transporter. It is indeed widely assumed that the phosphate translocation in the root occurs at the interface between cortical cells and the arbuscular fungal structures, i.e through the periarbuscular membrane. Among the first plant phosphate transporters cloned from different species, the tomato phosphate transporter gene LePT1 showed a specific localization of transcripts in the cells containing arbuscules as detected by in situ hybridization (Rosewarne et al., 1999) whereas MtPT1 and MtPT2, *Medicago truncatula* high affinity phosphate transporters, display decreased levels of transcripts during the AM symbiosis (Liu et al., 1998). Moreover, expression of the MtPT4 gene has been shown to be repressed upon both Pi fertilization and AM colonization (Burleigh and Harrison, 1997). This indicates that the trigger for the down-regulation of plant's own Pi uptake pathway is the enhanced P availability.

Recently, Rausch and collaborators (2001) have identified a mycorrhiza-induced phosphate transporter in potato, *StPT3*. The functionality of the corresponding protein was verified by complementation of a yeast mutant, and further analyses indicated that StPT3 is expressed in root cells colonized by the AM fungus (Rausch et al., 2001).

The uptake of nutrients and particularly phosphate by the plant cell may be linked to the high H^+-ATPase activity observed at the peri-arbuscular membrane (Gianinazzi-Pearson et al., 1991). This is supported by the observation of a differential activation of H^+-ATPase genes by the presence of *Glomus fasciculatum* in roots cells of tobacco (Gianinazzi-Pearson et al., 2000).

1.1.2.2.3. Uptake of other mineral nutrients

Nitrogen is the primary limiting factor besides water and light for plant growth in many ecosystems. The contribution of mycorrhizal symbiosis for N supply has been poorly defined until recently. In the soils, nitrogen is available for plants in the inorganic form (as NH_4^+ and NO_3^-) but most N is in-organic form, often occurring in complex molecules (Carling et al., 1978; Oliver et al., 1983). Initially, the common view was that the fungal hyphae were taking up NH_4^+ and were transporting it to the plant (Ames et al., 1983; Barea et al., 1987; Johansen et al., 1992). The idea that ammonium was the preferential nitrogen source for AM fungi has changed after several experiments with ^{15}N labeled nitrate had been conducted. Georges and collaborators (George et al., 1992) indicated that nitrate was taken up by *Glomus mosseae* hyphae while others (Tobar et al., 1994b; Tobar et al., 1994a) followed the nitrate transfer to the plant under water stress conditions. A more recent study demonstrated that the mRNA level of the maize nitrate reductase gene was lower in roots and shoots of mycorrhizal maize plants whereas it was the fungal nitrate reductase gene that was transcribed in the plant roots (Kaldorf et al., 1998).

The uptake of other nutrients, particularly Zn and Cu, seems to increase when the mycorrhizal fungi are present in the plants (Sieverding, 1991; Smith and Read, 1997). The AM symbiosis seems to be beneficial as well for the uptake of S, B and Mo (Sieverding, 1991) whereas the uptake of Mn is reduced in mycorrhizal plants. Detailed studies on the influence of AM on K nutrition have still to be done but the common view is that K accumulation is reduced in mycorrhizal tissues (Smith and Read, 1997).

1.1.2.2.4. Heavy metal tolerance

Many studies have been performed on the behaviour of mycorrhizal plants confronted with heavy metals (Gildon and Tinker, 1983). Some elements are necessary for plants but required at very low amounts. The excessive uptake and accumulation of Zn, Cu, Fe and Co as well as other non-essential elements and ions like Pb, Cd, Ni, Ti, Ba can lead to heavy metal toxicity, and a heavy metal tolerant AM fungus might improve the growth of plants and their tolerance to heavy metals. Indeed, plants from heavy-metal contaminated sites often show high levels of AM colonization (Smith and Read, 1997).

The mechanisms by which the AM symbiosis could improve tolerance to heavy metals in contaminated environments are still poorly understood. One hypothesis to explain this heavy metal tolerance proposes that a dilution of toxic elements occurs by improved absorption of other mineral nutrients (El-Kherbawy et al., 1989; Sieverding, 1991). Another hypothesis is that the sequestration of the metals by polyphosphates, accumulating due to improved phosphorus status in the fungus, might reduce the transfer of the toxic element to the plant, which could explain the diminished toxicity (Turnau et al., 1993). According to a third hypothesis, mycorrhizal plants can improve their growth due to AM symbiosis and thereby increase their heavy metal tolerance.

1.1.2.3. AM symbiosis and its relation to other microorganisms

Since the pioneering work of Barea et *al.*, (1975) followed by the studies of Meyer and Linderman and Secilia and Bagyaraj (1986 and 1987), the AM fungi have been viewed not only as plant symbionts, but as interactors with other microorganisms (Meyer and Linderman, 1986; Secilia and Bagyaraj, 1987).

One important aspect is the synergy that can develop between mycorrhizal fungi and rhizosphere bacteria and some attention has been drawn on the role of so-called plant growth-promoting rhizobacteria (PGPB), thought to be important in the plant and soil ecology (Linderman, 1992; Glick, 1995). These PGPB (*Pseudomonas* and *Bacillus*) as well as some fungi (*Trichoderma* and *Gliocladium*) could also influence the soil fertility and facilitate the plant growth by interacting with AM fungi (Azcón-Aguilar and Barea, 1992). Some of these bacteria would promote the germination and growth rate of fungal structures by releasing vitamins, amino acids, phytohormones and/or cell wall hydrolytic enzymes. Others could act directly on root development and on the susceptibility to AMF colonization (Garbaye, 1994). Finally, the stimulation of endomycorrhizal fungal development and increased root mycorrhization by several rhizosphere bacteria have been reported (Azcón-Aguilar and Barea, 1992; von Alten et al., 1993).

1.1.2.3.1. BLO's, a striking example

In addition to these interactions, the AM fungi also carry endosymbiotic bacteria in their own cytoplasm. The presence of bacteria from the genus *Burkholderia* has been noted in the cytoplasm of several representatives of the three families of Glomales. The biological

role of these endosymbionts is intruiguing, and many questions may be asked concerning this particular association. What is the significance of BLOs within the fungal cytoplasm? Do they represent a permanent endosymbiosis coupled with a strictly vertical transmission through generations or a more cyclical endosymbiosis in which the AM fungi take up and perhaps select their bacterial symbionts from the soil? What are the putative metabolic exchanges between the AM fungal cells and their endosymbionts? (Bianciotto et al., 2000).

1.1.2.3.2. AM and rhizosphere bacteria/microorganisms

Many potential symbionts live in the soil physically near the AM hyphae. In the layer of soil directly surrounding the root, there is a high density of microorganisms that can form a biofilm around the hyphae attached to the root with adhesive protein, flagella and extracellular polysaccharides (Perotto and Bonfante, 1997). The significance of bacterial attachment is still not clear but some hypotheses can be aired. The bacteria forming biofilms around the hyphae of mycorrhizal fungi may be an important mechanism to avoid dispersion by percolating soil water (Bianciotto et al., 1996; Sen and Chalk, 1996). In the study of Requena et al. in 1997, the authors displayed the efficiency of different microbial combinations including AM fungi, *Rhizobacteria* and PGPR, on the improvement either of plant development, nutrient uptake, N_2-fixation or root system quality. The authors further claimed that appropriate microbial combinations could be recommended to improve plant performance (Requena et al., 1997). More recently, the interaction between *Sinorhizobium meliloti* with two arbuscular mycorrhizal fungi has been investigated. The results of this study showed a growth promoting effect on lettuce plants (Galleguillos et al., 2000). In addition to these microbe-microbe interactions, the arbuscular mycorrhiza symbiosis may also increase significantly the plant's resistance or tolerance to diverse microbial pathogens by promoting the nutrionnal and development status of the plant (Barea and Jeffries, 1995). This is discussed below.

1.1.2.3.3. Bioprotection

A further most intriguing feature of AM symbiosis is that they interact with soil parasites and pathogens and that they minimize their development and impact (Harley and Smith, 1983). It has been reported that the AM colonization of root systems often reduces the

disease severity caused by nematodes and soil-borne pathogens like *Fusarium*, *Phytium*, *Rhizoctonia*, *Sclerotium*, *Aphanomyces* or *Phytophtora* (Rosendahl, 1985; Mulongoy et al., 1992; Azcón-Aguilar and Barea, 1996), a phenomenon called "bioprotection".

AM colonization has been reported to reduce damages caused by pathogens but the criteria necessary for efficient bioprotection were found to be different. Some claimed the necessity of a well-established AM infection (Rosendahl, 1985; Cordier et al., 1996a; Dassi et al., 1998; Slezack et al., 2000) whereas others found a co-inoculation or post-inoculation with an AM fungus sufficient to protect plants (Krishna and Bagyaraj, 1983; Caron et al., 1986).

In the AM symbiosis, plant defense responses increase transiently in the early phases of the symbiosis and then are suppressed (Harrison, 1999). This preactivation of plant defense response (Gianinazzi-Pearson et al., 1991) could perhaps explain why a precolonized root would exhibit a better reaction against pathogen attack than a non-colonized root. The improvement of plant nutrition and root biomass observed in some mycorrhized plants could contribute to increase plant tolerance and compensate for root damage caused by pathogens, change in root system morphology, modification of antagonistic microbial populations in the mycorrhizosphere and competition between AM and pathogenic fungi. The studies on bioprotective effects of AM colonization have to be followed since the conditions required for bioprotection by AM fungi and the mechanisms allowing its expression are still not well understood (Slezack et al., 2000).

1.1.2.3.4. Impact of AM in agriculture

In the future agriculture, new agricultural practices have to be created, on one hand to meet food production needs and on the other hand to shift managment towards greater resource efficiency and conservation while maintaining an environment favorable for the evolution of all species (Golley et al., 1992). In this context of sustainable agriculture, the AM will very probably play an important role.

As shown by Van der Heijden and collaborators (1998), the diversity of vesicular-arbuscular mycorrhizae in soils is a determining factor for the maintainance of biodiversity and ecosystem functioning (van der Heijden et al., 1998). In addition to its important protective effects on plant growing on polluted soils, the AM can increase

drought tolerance in response to water stress as it was shown in wheat (Ruiz-Lozano and Azcon, 1997; Al-Karaki, 1998; Goicoechea et al., 1998). Moreover, mycorrhizal plants are more and more used for revegetation purposes to halt desertification in arid regions (Bagyaraj and Varma, 1995; Mathur and Vyas, 1999; Varma, 1999) as well as to restore plant communities in disturbed areas (Smith et al., 1998).

1.2. THE AM FUNGI

1.2.1. Classification

1.2.1.1. Morphological criteria

AM fungi are members of the order Glomales, class Glomeromycetes in the new phylum of Glomeromycota. To differentiate between AM fungi species (Walker, 1983) and study the phylogeny of Glomales (Walker, 1992), morphological traits of spores are most frequently used together with other fungal structures as color, size, morphology, cell-wall structures. The order Glomales is classified into two suborders of *Glominae* and *Gigasporineae* (Morton and Benny, 1990).

In the latter suborder, the family *Gigasporaceae* exhibits abundant coiled, swollen hyphae and arbuscules and produces extraradical auxiliary cells.

The *Gigasporaceae* comprises the two genera *Gigaspora* and *Scutellospora* that are differentiated by absence and presence, respectively, of hyaline flexible inner spore walls (Morton and Benny, 1990).

The suborder of *Glomineae* has two families, the *Glomaceae* and the *Acaulasporaceae,* in which the fungal species produce arbuscules as well as vesicles but no auxiliary cells. Theses two families are themselves divided in two genera: *Glomus* and *Sclerocystis* for *Glomaceae* and *Acaulospora* and *Entrophosphora* for *Acaulasporaceae.* The *Glomus* species are thought to have appeared first whereas the families *Acaulasporaceae* and *Gigasporaceae* emerged later. These last ones were supposed to have diverged from each other around 250 MYA ago (Simon et al., 1993).

To summarize, AM fungi belong to one order, class, phylum comprising two suborders composed of three families with six genera of which around 150 species have been

described (Helgason et al., 1999). Their broad host spectrum combined with their capability to get spread easily in nature (Abbott and Robson, 1991) explain to some extent why such a small number of species is able to colonize up to 80 % of the land plant species (Walker and Trappe, 1993).

1.2.1.2. Molecular approaches

In addition to the morphological characteristics, molecular criteria provide reliable and useful information in order to identify and differentiate fungal isolates. Despite the inability to grow AM fungi in pure culture, the molecular investigations have been very successful tools to allow the genetic characterization of these microorganisms. The first DNA fungal regions that were sequenced and used as targets for phylogenetic analyses on the AM fungi were various parts of the genes for ribosomal RNA, including the large subunit (LSU), the small subunit (SSU) and the internal transcribed spacers (ITS) (Simon et al., 1993; Lloyd-MacGilp et al., 1996; Simon, 1996; Redecker et al., 1997).

Other molecular strategies based on PCR amplification have been carried out successfully to identify mycorrhizal fungi. Random amplified polymorphic DNA (RAPD)-markers were used to ascertain specifically isolates of *Glomus mossae* (Lanfranco et al., 1995) while using taxon specific primers, the PCR identification of different AM fungi co-existing in the same root fragment was possible (van Tuinen et al., 1998). The use of the SSU and the ITS sequences were coupled to the design of specific primers to identify AM fungi from both spores and fungal structures within plant roots by PCR amplification (Harrison, 1999). PCR systems using rDNA sequences also allow the detection and classification of as yet unknown AM fungal species, thereby extending the knowledge about various soil ecosystems.

A recent report presents the combination of morphological and molecular approaches to characterize isolates in the genus *Gigaspora* from different geographical areas (Lanfranco et al., 2001). The results show how these two complementary methods can clarify relationships among species of low morphological divergence.

1.2.2. Genetic diversity

The AM fungi have a coenocytic nature, meaning they enclose many nuclei within one cell. An estimation of DNA content of nuclei from spores of different glomalean fungi indicates that the genomes range from 0.13 to more than 1.00 pg DNA per nucleus, larger than the genome of other fungi (Bianciotto and Bonfante, 1992; Hosny et al., 1997). In nine glomalean species, the DNA has of a low GC content (at most 35%) with high levels of methylcytosine (Hosny et al., 1997) and the genomes are composed of extensively repeated DNA sequences (Zézé et al., 1996; Zézé et al., 1999). Genetic variations were revealed by this ribosomal DNA sequence analysis within a fungal species but as well between different AM fungal species (Sanders et al., 1995; Lloyd-MacGilp et al., 1996; Bago et al., 1998; Vandenkoornhuyse and Leyval, 1998; Clapp et al., 1999; Hosny et al., 1999; Lanfranco et al., 1999). This variability among spores of ITS sequences was confirmed for other loci by PCR amplification of satellite regions revealing intersporal genetic variation of *Gigaspora margarita* (Zézé et al., 1997). It was hypothesized that this genetical divergence of nuclei was not due to recombination events but due to several mutations (Sanders et al., 1996). In a recent report, in situ hybridization and statistical analysis of sequence variants in ITS regions of rDNA strongly suggest that most of the variant sequences are the results of accumulation of mutations in a clonal genome leading to the creation of a population of genetically different nuclei even if the authors do not exclude rare recombination events (Kuhn et al., 2001). Hence, it is likely that mycorrhizal fungi exhibit a multiple genome evolution**.** Until now, reports of within-individual sequence variation in AM fungi have been restricted to ITS regions of rDNA sequences and moreover mostly demonstrate genetic differences only among nuclei for one region of rDNA. Nevertheless, additional sequence comparisons between variants of a gene coding for a binding protein (*BiP* gene) from different nuclei show that silent mutations were found to be as frequent as non-silent mutations. This suggests that functional and non–functional variants of coding sequences coexist in one spore, another piece of evidence for a multiple genome evolution (Kuhn et al., 2001).

A further source of diversity among nuclei within a hyphal network may arise by fusion of hyphae anastomosis of different AM fungi although the study of Giovannetti suggests

that cross-incompatibility is more frequent than an anastomosis conductive behavior (Giovannetti et al., 2001).

1.2.3. Fungal genes

Molecular analyses on the AM fungus in the asymbiotic and symbiotic phases are necessary to better understand how the AM symbiosis functions. People are interested in finding genes important for AM symbiosis and use targeted and untargeted approaches.

1.2.3.1. Targeted approaches

Gene expression in AM fungi was studied by PCR approaches, and early results revealed the presence of mRNA from genes encoding glyceraldehyde-3-phosphate dehydrogenase, β–tubulin and further P-type ATPases in *Gigaspora rosea* (Franken et al., 1997). Butehorn and collaborators reported the use of β–tubulin to quantify *Glomus mosseae* fungal material in the asymbiotic versus symbiotic stages (Butehorn et al., 1999). In addition to these housekeeping genes, other fungal genes have also been isolated with targeted molecular approaches and can be classified according to their functional importance in nutrition and morphology (Harrier, 2001a). Many molecular studies have been oriented towards the major benefits associated to the AM symbiosis and more particularly towards the enhanced P status of mycorrhizal host plants that is allowed by the fungal partner (Smith and Smith, 1989). A phosphate-transporter has been cloned from *Glomus versiforme* and its transcripts have been found only in the extraradical hyphae (Harrison and van Buuren, 1995). This high affinity phosphate transporter was therefore suggested to be involved in the initial P uptake from the soil. A previous report of Lei et al. in 1991 have shown a positive correlation between the H^+-ATPase activity and ^{32}P uptake in hyphae from germinated spores of the AM fungus *Gigaspora margarita,* indicating a H^+–cotransport mechanism (Lei et al., 1991). More recently, a PCR cloning approach based on the use of highly degenerate primers allowed the isolation of five partial genomic clones encoding plasma membrane H^+-ATPases from the AM fungus *Glomus mosseae* (Ferrol et al., 2000). The corresponding ATPase function remains to be analyzed but the *Glomus mossae* H^+-ATPase (*GmHA*) genes found in this study are divergent and may even have different evolutionary origins. Indeed, e.g

the GmHA5 gene is similar to plant H^+-ATPases and could therefore have been transferred horizontally from the host plant (Ferrol et al., 2000).

A further gene that can be of importance in N uptake was isolated from spores of *Glomus* by a PCR based strategy (Kaldorf et al., 1994). This gene was an assimilatory nitrate reductase (NR) gene and was localized in arbuscules but not in vesicles, suggesting a differential function depending on the different symbiotic stages (Kaldorf et al., 1998). PCR with degenerate primers designed on highly conserved chitin synthase domains allowed the isolation of chitin synthase genes from *Gigaspora margarita* and *Glomus versiforme*; expression analysis of the latter by RT-PCR indicated an expression during the symbiotic stage but not during spore germination (Lanfranco et al., 1999). Chitin is a major component of the fungal cell wall, and the chitin synthases play therefore key roles in fungal morphogenetic events. Hence, during the symbiotic stage, the induction of fungal chitin synthases might be necessary to permit host colonization and the formation of the specialized fungal structures.

1.2.3.2. Untargeted approaches

Research on the genome of AM fungi is difficult. One of the first problems encountered for molecular analysis of fungi in the symbiotic stage is the presence of the plant genetic material. Indeed, even in highly colonized roots, only 1 % of the total mRNA is estimated to belong to the fungus.

The untargeted molecular approaches, including more particularly differential display (DD) and differential screening (DS), look for differentially expressed genes regardless of their identity (Harrier, 2001a). Several fungal genes were isolated by DD and here the results of some of these studies are summarized. Three partial cDNAs have been isolated from *Glomus intraradices* (Delp et al., 2000). The deduced amino acid sequence from GIMYC1 and GINMYC2 shows homology respectively to TRIP15, a human protein interacting with a thyroid receptor, and to *O*-linked *N*-acetylglucosamine transferases from vertebrates. GINBH1displays the same expression pattern as the gene GIMYC1 and contains a putative leucine zipper and a homeodomain indicating it may act as a transcriptional regulator (Harrier, 2001b). Another gene isolated by DD from *Glomus mosseae* coding for the phosphoglycerate kinase (*GPK*) exhibits the same expression pattern during the asymbiotic and symbiotic stages (Harrier et al., 1998). But, a

significant increase in the levels of the corresponding protein during the symbiotic phase compared to the presymbiotic development suggests a possible differential regulation of this gene during the symbiosis (Harrier, 2001b). Promoter analysis has revealed two sequence motifs with homology to carbon-source-controlled upstream activating elements from *Saccharomyces cerevisiae,* suggesting that *GPK* gene is probably regulated by sugar metabolism as in other organisms (Harrier, 2001b). A cDNA isolated from *Glomus versiforme* differentially expressed during the AM symbiosis was homologue to a cruciform DNA-binding protein from *Ustilago maydis* suggesting the involvement of common regulatory protein during the interaction with the plant (Burleigh and Harrison, 1998). Differential screening also allowed the detection of genes that show a down-regulation in response to any type of stimuli. A fungal cDNA fragment from *Glomus mosseae* corresponding to a gene encoding for the homologous of the fatty acid oxydase FOX2 from yeast and human was down regulated in the presence of the rhizobacterium *Bacillus subtillis* (Requena et al., 1999). The corresponding human gene encodes a 17-hydroxysteroid dehydrogenase IV protein that inactivates estradiol by converting it into estrone. The structural similarity between the estrogens and the plant flavonoids opens-up the possibility that the FOX2 homologue from *Glomus mosseae* is responsible for a structural change in a signal molecule (Franken and Requena, 2001).

Among the untargeted approaches, the construction of EST libraries and the screening of cDNA arrays are useful tools to analyse AM fungal gene expression. Some ESTs have been found after screening libraries constructed from activated spores of *Gigaspora rosea* or presymbiotic mycelium from *Gigaspora margarita* (Stommel et al., 2001). The sequence analyses showed similarities to genes coding for proteins having a role in multiple cell functions like translation and protein processing, replication and the cell cycle and cell signal transduction, primary metabolism and transport processes. Among the candidate genes, two encoded homologues of metallothioneins. These proteins have the capability to bind metal ions (Nordberg, 1998), and expression of these genes may explain the increased tolerance of mycorrhizal plants in heavy metal contaminated environments (Franken and Requena, 2001). Despite a substantial number of AM fungal genes found with the studies on the AM symbiosis, there is a lack of data on proteins corresponding to these genes. This can be explained in part by the difficulty

to differentiate fungal proteins from plant ones during the symbiotic phase. In early studies on AM enzymes activities, a phosphatase activity has been detected in vacuoles of hyphae and along the fungal tonoplast (Gianinazzi-Pearson and Gianinazzi, 1978; Gianinazzi et al., 1979) and measured in planta (Tisserant et al., 1993). In the last ten years, specific fungal enzymes have been identified, such as cell wall degrading enzymes such as pectinases, cellulase, endo- and exoglucanases, and xyloglucanases (García-Romera et al., 1991; García-Garrido et al., 1992a; García-Garrido et al., 1992b; García-Garrido et al., 1996). These enzymes may facilitate the penetration of the colonizing AM fungus into the root. Despite a limited use of antibodies against fungal proteins, some research has been recently done, mainly to identify fungal species and to detect AM fungi in plant root and soil systems (Harrier and Sawczak, 2000). The immunochemical methods are mainly focused on finding highly selective antibodies against fungal cell walls to determine modifications in fungal cell components related to infection and development (Harrier, 2001a). Recently, a polyclonal antibody was used to detect the presence of the protein encoded by the *Gmpgk* gene. The protein was not detected in non-mycorrhizal roots (Harrier and Sawczak, 2000). The recent development of techniques like proteomics and mass spectrometry opens new possibilities to identify new important AM fungal proteins and thereby explore the interaction between plants and fungi.

1.2.4. Transformation of AM fungi

For functional analysis of AM fungal genes a transformation protocol of AM fungi would be a great issue. The transformation of spores of *Gigaspora rosea* was performed by particle bombardment. For the first time in 1998, Forbes et al. managed to integrate the glucuronidase reporter gene under the control of the promoter of glyceraldehyde-phosphate dehydrogenase (GAPDH)-encoding gene from *Aspergillus nidulans* (Forbes et al., 1998). Despite a weak expression, possibly due to the use of the heterologous promoter, it was still possible to detect the presence of the GUS gene by PCR and of the protein by immunoblotting in the first generation of harvested spores (Forbes et al., 1998). Although these first results suggesting, a stable transformation, are promising, the mechanisms underlying transformation process have still to be understood. The presence of more that two thousand nuclei within one spore makes gene inactivation tedious and

the deletion of gene of interest not feasible. The antisense methodology therefore seems to be the most appropriate approach for inactivating target genes in fungi.

Coupled with traditional physiological experimentation, the developing molecular techniques will be essential to understand the mechanisms underlying the fungal development in its interaction with a host plant. In addition to the techniques cited above, microarray technology (DeRisi et al., 1997) allows the simultaneous comparisons of the different stages of the fungal cycle whereas strategies using ESTs offer the comparisons between different fungal development stages (Requena et al., 2000).

1.2.5. Cultures of AM fungi

A major challenge in the research on mycorrhiza is to understand the AM fungus-plant signaling mechanisms and the colonization procedure. This tight relation is reflected in the inability of fungi to grow in the absence of plant host. The obligate biotrophic nature of AM fungi is a handicap for the management of the symbiosis in agriculture and in laboratories. It was necessary to develop axenic, gnobiotic cultures of a single AM fungus with a single root system, if possible free of contaminants.

Usually, AM fungi are grown on roots of greenhouse plants and chopped mycorrhizal roots, often mixed with the growth media containing hyphae and spores, are used as source of inoculum. To avoid contamination, the spores collected from soil samples can be surface sterilized with antibiotics (Mertz et al., 1979). Several hydroponic culture systems were reported to produce external mycelium and spores of *G.mosseae*, *G. fasciculatum*, *G. caledonium*, and *Acaulospora laevis* with maize, beans, lettuce and white clover as host plants (Mosse and Thompson, 1979; Mosse and Thompson, 1984). A hydroponic system also allowed the obtention and growth maintainance of *Glomus intraradices* in symbiosis with linseed (*Linum usitatissimum*) where an extramatrical mycelium is produced in nutrient solution, free of plant tissue (Dugassa et al., 1995).

Other methods such as aeroponic culture (Sylvia and Hubbell, 1986; Hung and Sylvia, 1988; Jarstfer and Sylvia, 1999) and alginate entrapment of root fragments in alginate beads and their use as inoculum (Strullu and Plenchette, 1990) have been developed in order to increase the quantity, the quality and the infectivity of the inoculum.

Nevertheless, some problems remain in these systems, such as the need for special and large equipment, difficulty of management. A difficulty is the prevention of

contaminations, especially in the case of hydroponic systems that are readily contaminated by algae, bacteria, or other fungi.

For scientific purposes, particularly to observe the differential hyphal morphogenesis of the preinfection stages, the fungi and the roots were sandwiched between membranes and potted in sterile substrate (Giovannetti et al., 1993). This *in vivo* sandwich system was recently modified to visualize the extension of extraradical mycelium, anastomosis formation and structure of the mycorrhizal network (Giovannetti et al., 2001).

A system was recently adapted to allow the non-invasive microscopic observation of the symbiosis development between *Medicago truncatula* and *Glomus intraradices*. This "Mini-Mycorrhizotron" allowed the natural growth of the *Medicago* seedlings and permitted the observation of the fungal development in the first stage of the symbiosis (Bonanomi et al., 2001).

To circumvent the problem of the obligate biotrophic nature of the AM fungus and to study more particularly the pre-symbiotic stage, in vitro cultures showed considerable promise. Already, in the 1960s, Mosse developed for the first time AM fungal in-vitro colonization in the presence of Pseudomonas (Mosse, 1962). About ten years later, a bacteria-free symbiosis was reported (Phillips and Hayman, 1970). Subsequently, typical infections of *Glomus mosseae* in axenic culture were obtained by using root organ culture (Mosse and Hepper, 1975). This was followed by the work of Mugnier and Mosse (1987) who developed a method using Ri T-DNA transformed roots of *Convolvulus sepium* used as a root host for *Glomus mosseae* (Mugnier and Mosse, 1987).

Based on these studies, it was an important aim to improve the *in vitro* cultures under aseptic conditions. In order to study the early events of mycorrhiza formation, Bécard and Fortin (1988) inoculated carrot root slices with the A4 *Agrobacterium rhizogenes* strain. After confirmation of the transfer of the T-DNA from the *Agrobacterium rhizogenes* Ri plasmid in the carrot roots by detection of opines, the roots were left to grow on a minimal medium established for this purpose. A single non-germinated spore of *Gigaspora margarita* was used to initiate a primary mycorrhizal colonization. After this first step had taken place, a rapid development of extramatrical hyphae was observed whereas sporogenesis was regularly noticed between the first and the seventh month of dual culture (Bécard and Fortin, 1988). Following the set-up of this in vitro root organ

culture system with *Gigaspora margarita*, the use of dual culture is now useful in every research project needing aseptic conditions for a fungal life cycle completed in axenic conditions.

In vitro cultures of AM fungi have been established up-to now only for a limited number of species. This in vitro experimental system has been developed for culturing *Gigaspora margarita* in association with carrot roots (*Daucus carota*) (Bécard and Fortin, 1988), *Gigaspora gigantea* (Bécard and Piché, 1992), *Glomus etunicatum* (Schreiner and Koide, 1993), *Glomus intraradices* (Fig1.2.) (Chabot et al., 1992) and *Glomus versiforme* (Diop et al., 1994). These in vitro dual cultures have been improved concerning the growth requirements and the culture media as well as the pH conditions (Bécard and Piché, 1989b; Bécard and Piché, 1989a; Bécard and Piché, 1992; Chabot et al., 1992; St-Arnaud et al., 1996; Douds, 1997). The AMF culture in biofermentors has been successfully developed in producing commercially available inoculum in North America, in South-East Asia and in Europe. One of the most recent trials to scale up the root organ culture system was the work of Jolicoeur and collaborators who were using an airlift bioreactor (Jolicoeur et al., 1999). Despite the feasibility of the technique, the spore production was not optimal and proportionally ten times lower than the one of petri dish cultures. Nevertheless the in vitro system became a very efficient tool to produce sterile and axenic cultures of AM fungus for laboratory purposes.

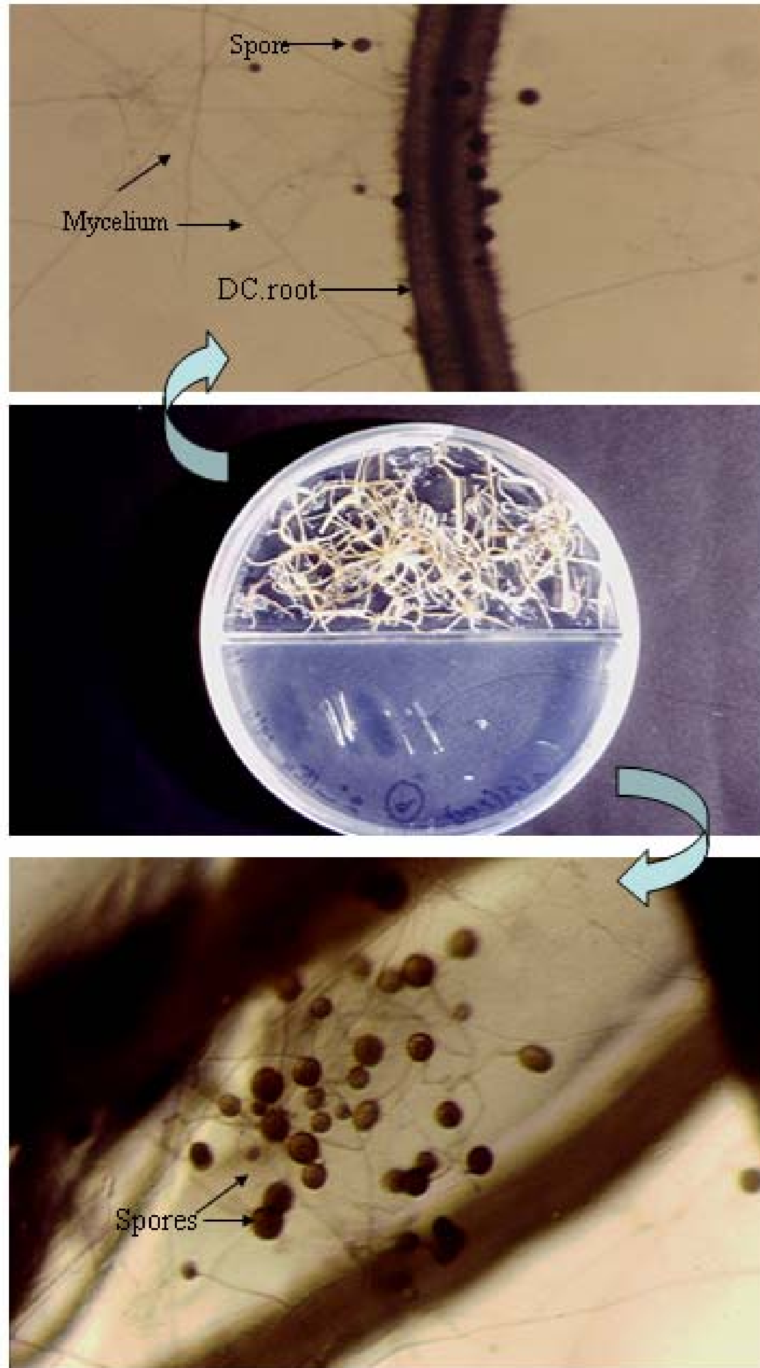

Figure 1.2: In vitro culture of *Daucus Carota* transformed roots and *Glomus intraradices*. **Center:** Photo of a divided agar plate (diameter: 9 cm) containing a culture of carrot roots (*Daucus carota*) transformed by *A. rhizogenes* in the upper half, and a root-free area colonized by AM fungi in the lower half.
Top: Detail of a transformed carrot root colonized by mycelium of *G. intraradices* growing in the upper part of the plate.
Bottom: Detail of the axenic culture of *G. intraradices* growing in the lower half of the agar plate.

1.3. PLANTS INVOLVED IN AM SYMBIOSIS

1.3.1. Hosts and non-hosts for AM fungi

As introduced in chapter 1.1.1, the AM symbiotic association predominates in the roots and soils of agricultural crops and weed plants. Among the 3% of the *Angiospermae* on which information of the mycorrhizal status was available, 18% were non-mycorrhizal, 15% of all species were forming another mycorrhizal association than AM type, and 67% were developing AM symbiosis (Trappe, 1987). Concerning tropical plant species, the ratio was as follows: 70.9% mycorrhizal with AM fungi, 15.7% mycorrhizal with other symbiotic fungi and 13.4% non mycorrhizal. Among the plants that are able to form AM symbiosis, the degree of infection may vary. Tropical crop plants, such as cassava, sweet potato, cowpea, soybean, maize, sorghum, barley, upland rice, sugarcane, tobacco, cotton, cacao, rubber, tea oil palms, tropical pasture grasses and legumes, are often heavily colonized by AM fungi under natural conditions. On the other hand, crops like wheat, beans, coffee, and tomato may only be infected to a moderate extent. Ecotypes, cultivars or clones of the same crop plant can exhibit large differences in colonization rates as has been reported for wheat, alfalfa, sugarcane, beans (Sieverding, 1991). The environmental conditions such as soil type or high N, K, P fertilization can also influence the AM formation and the root colonization extent.

Another distinction among mycorrhizal species has been made according to their dependence on the AM association. The degree of mycorrhizal dependence was defined by Gerdemann in 1975 as: "Degree to which a plant is dependent on the mycorrhizal condition to produce its maximum growth or yield, at a given soil fertility" (Gerdemann, 1975). The mycorrhizal dependence is often correlated to the morphology of root hairs as well as to the extension of the root system. For example, cassava (*Manihot esculenta*), *Citrus* spp., onions (*Allium* spp.) and many tropical legumes have a simple, weakly branched root system and therefore are heavily dependent on AM. Plant species with a high density of long root hairs depend less on AM than plants forming only a few, short root hairs. Plants may be obligately or facultatively dependent on AM, as defined according to their ability to grow with and without AM at different levels of soil fertility. Obligately mycorrhizal plants are unable to survive and grow without mycorrhiza, even

in the most fertile soil encountered naturally, whereas facultative mycorrhizal plants can survive and grow without mycorrhiza higher levels of soil fertility (Gerdemann, 1975). Another characteristic of the mycorrhizal plants is the host plant responsiveness to mycorrhiza (Janos, 1988). Responsiveness depends on soil fertility, is influenced by both the host plant and the AM fungus and is used to measure the fungal effectiveness. Therefore, obligately mycorrhizal plants may be highly responsive like cassava (*Stylosanthes guianensis*) or slightly responsive like *Genipa Americana*. Maize and sorghum, both facultatively mycotrophic plants, show high and low response, respectively, to mycorrhiza (Powell and Bagyaraj, 1984).

Most genera and species of the *Amaranthaceae*, *Brassicaceae*, *Caryophyllaceae*, *Cyperaceae*, *Chenopodiaceae*, *Commelinaceae*, *Lecythidaceae*, *Portulacaceae*, *Proteaceae, Restionaceae, Sapotaceae* and *Zygophyllaceae* do not form any mycorrhiza and are so-called non-mycorrhizal plants; however, some genera or species of these plant families are mycorrhizal. On the other hand, within the family of *Leguminosae* in which almost all genera or species are mycorrhizal, the genus *Lupinus* is non-mycotrophic (Sieverding, 1991). The investigations on the barriers to infection of non-mycorrhizal plants are important for the understanding the AM infection processes in general and for a discrimination between environmental and intrinsic factors affecting infection in particular. Current hypotheses were examined by Tester and collaborators concerning the lack of infection in non-host plants. The non-mycorrhizal plants could lack critical factors controlling mycorrhizal colonization, could produce root factors being either fungitoxic or produced in quantities insufficient for fungal nutrition (Tester et al., 1987). An alternative hypothesis was also proposed by the same authors who suggested that the control of mycorrhizal fungal penetration is exerted during later interaction between the organisms, at the level of the cell wall and (or) the middle lamella. An example of such a late control was observed in roots of *Salsola kali* L. (*Chenopodiaceae*) that, after being invaded by mycorrhizal fungi up to the stage of arbuscule formation, developed progressive, incompatible interactions with different AM fungi (Allen et al., 1989). Bécard and Piché used transformed roots of carrot and sugar beet as model for host and non-host plant respectively, for the AM fungus, *Gigaspora margarita.* Growth of fungal hyphae and formation of infection units were only observed in the presence of the carrot

roots. Hyphal growth was stimulated by root volatiles from both plants species, but only by root exudates from carrot and not by those of sugar beet. Hence, these results suggested that the non-mycorrhizal roots of sugar beet lack factors that promote AM infection rather than producing inhibitory factors (Bécard and Piché, 1992).

1.3.2. Plants that are hosts for both AM and Rhizobia

The *Leguminosae* (or *Fabaceae*) is one of the largest families of flowering plants, and they are important agricultural crops cultivated worldwide, providing a major source of protein and oil for human and animals, and contributing to the biological fixation of nitrogen for soil improvement. Legumes offer unique opportunities for the study of plant-microbe interactions such as symbiotic nitrogen fixation, mycorrhizal associations and legume-pathogen interactions, particularly at the level of genetics (Sprent, 2002). Among the legumes, pea is a significant crop being both a vegetable and an arable crop and it has been used as the first model legume for genetics even before Mendel's work (Sprent, 2002). Even if pea is not the best model for modern genetic analysis because the plants are large and the genome is complex, it has many interesting mutants available. To circumvent the problems of scale in the genome, the legumes *Medicago truncatula* and *Lotus japonicus*, with a smaller genome and taxonomically closely related to pea, can be useful (Ellis and Poyser, 2002).

1.3.2.1. Origin of *Rhizobium*

The members of the *Fabaceae* plant family have the ability to form a symbiosis with soil bacteria (*Rhizobiaceae*) of the genera *Rhizobium*, *Bradyrhizobium*, or *Azorhizobium*. This leads to symbiotic nitrogen fixation and provides the major source of nitrogen for the biosphere. In contrast to AM symbiosis, the legume-*Rhizobium* symbiosis is much younger, and its establishment is dated no more than 65 to 136 millions years ago (Fig.1.1.). *Rhizobium*-mediated nitrogen fixing symbioses are essentially restricted to legume species, unlike the AM symbiosis, which occurs in a majority of plant families. The process of root invasion by bacteria results in the formation of a completely new organ, the root nodule. The initial events in nodule formation are triggered by bacterial

signals, so-called nodulation (Nod) factors, the biosynthesis of which is catalyzed by the bacterial NodA, NodB and NodC proteins. Nod-factor-secreting rhizobia induce curling of root hairs creating thereby a microenvironment to establish an infection site. The mechanisms used by Nod factors secreting bacteria to redirect root hair tip growth and to induce curling are largely unknown. Morphological changes of the root hair tip involve a rapid influx of calcium into the root hair (Gehring et al., 1997; Felle et al., 1998) followed by an opposite-directed flux of chloride ions and a depolarization of the root-hair membrane (Felle et al., 1998). The bacteria locally degrade the plant cell wall and enter the root invaginating the plant plasma membrane (Turgeon and Bauer, 1985). The different root layers respond differently to Nod factors and for example in *Medicago* species, root hairs and outer cortical root cells form a tubular structure, "called inward tip growing", the infection thread. This infection thread then crosses several cortical cell layers before reaching the primordium and transports the bacteria towards the inner cortex. These cortical cells are induced to divide, leading to the formation of a nodule meristem (Mathesius et al., 2000). The bacteria are released into the cytoplasm of these cells, remaining enclosed by the host-cell membrane. This membrane is called the peribacteroid membrane (PMB). Within the PMB, the bacteria differentiate forming nitrogen-fixing bacteroids. The PMB and the bacteroids form the symbiosome that is the basic nitrogen-fixing unit of the nodule. Nodules of *Medicago* ssp. are indeterminate, i.e they have a persistent meristem where all stages of nodule differentiation remain present. The central region of the indeterminate nodules is divided in three major zones: zone I of the apical nodule meristem where cells proliferate; the zone II, the invasion zone where cells do not divide but undergo differentiation and become infected with rhizobia; and zone III, the nitrogen fixation zone where the bacteria are converted into nitrogen-fixing bacteroids (Long, 1996; Schultze and Kondorosi, 1998). The bacteria reduce N_2 to ammonia and in exchange for reduced carbon compounds from the plant. The reduced carbon is used as energy for bacteria to make its metabolism including nitrogen fixation (Udvardi and Day, 1997).

The mechanisms underlying symbiotic nitrogen fixation have been intensively studied, with particular emphasis on the attempt to understand the molecular basis of nodule development. Until now, a number of genes with enhanced expression in nodules

(nodulines) have been described and for some of them, their function in the nodule has been identified (e.g. leghemoglobin).

1.3.2.2. Genes involved in common events of AM and Rhizobia symbioses

The molecular mechanisms underlying the *Rhizobium*-legume symbiosis have been studied in much more detail than for the AM symbiosis. At first sight, the two root endosymbioses do not seem to involve related processes. They exhibit clear differences in terms of host specificity and developmental responses elicited in the host plant. However, molecular and genetic studies show that the *Rhizobium*-legume and AM symbioses share similarities, as demonstrated by legume mutants affected in both root nodule (Nod^-) and mycorrhiza development (Myc^-) (Schultze and Marsh, 2001). Furthermore, several legume genes have been identified that show transcriptional activation during both symbiotic interactions. The proteins encoded by genes that are specifically produced during formation and functioning of nodules are called nodulins, and they have been classified as early and late nodulins according to the timing of their expression (Brewin, 1991; Verma et al., 1992). Early nodulins (*ENOD*s) are associated with organogenesis and bacterial invasion of the root nodule. In general, late nodulin genes (*NOD*) are thought to play a role in nodule function; they include the oxygen transporter leghemoglobin. In *Medicago sativa*, two nodulation genes, *MsENOD40* and *MsENOD2* are induced also in mycorrhizal roots and have similar patterns of expression as in Rhizobium inoculated roots (van Rhijn et al., 1997). The authors propose that cytokinin is part of the mechanism of signal transduction mediating induction of these symbiotic genes. Indeed, the level of the cytokinin is elevated during both symbioses, and *MsENOD40* and *MsENOD2* are induced by the plant hormone in absence of AM colonization and nodulation. Recently, *MsENOD40* has been shown to be a regulator of mycorrhization (Staehelin et al., 2001). The *MsENOD2* gene product is a proline-rich protein on the basis of its nucleic acid sequence; in contrast, *MsENOD40* has no long Open Reading Frame (ORF) (Crespi et al., 1994) and is likely to function in part as an RNA and to encode small peptide(s) with hormone-like functions (Lindsey, 2001).

The hypothesis of a common signal transduction pathway in the AM symbiosis and *Rhizobium*-legume interaction is supported by the study of Albrecht and collaborators on *PsENOD5* and *PsENOD12A* genes (Albrecht et al., 1998). They found these genes to be induced in pea roots during the two symbioses. Transcription of both genes is rapidly activated in the epidermal root cells, which are in direct contact with rhizobial Nod factors (Heidstra et al., 1997). Their induction pattern helped to observe a possible common pathway for mycorrhizal fungi and Nod factors using a pea mutant (SYM8) blocked at a very early step of both symbiotic interactions (Markwei and LaRue, 1992). In this SYM8 mutant, the *PsENOD5* and *PsENOD12A* induction is blocked when inoculated with either mycorrhizal fungi or rhizobia suggesting that SYM8 is essential and could have a function in the signal transduction pathway for induction of these genes in the two symbioses (Albrecht et al., 1998). In situ hybridization experiments demonstrated that a gene encoding a lectine-like glycoprotein of pea (*PsNlec1*) was not only strongly expressed in root nodules (Kardailsky et al., 1996) but the transcripts were as well detected in host cells containing arbuscules (Balestrini et al., 1999). A difference appeared between the two symbioses at the protein level. The protein product present in AM corresponds to one isoform (PsNLEC-1C) present as well in nodule tissues whereas two different isoforms are apparently nodule specific. PsNLEC-1C might be correlated with development of host cells infected by AM or *Rhizobia* symbiontes.
Another plant gene, the expression of which is triggered by rhizobia and AM fungi is *VfLb29*. This gene, coding for a leghemoglobin, exhibits induction in root nodules as well as in broad bean roots colonized by the AM fungus, *Glomus fasciculatum* (Frühling et al., 1997). This leghemoglobin coding gene is atypical since it has a relatively low level of sequence identity compared to the other broad bean leghemoglobins and is thought to be a member of a new class of genes. Due to the localization of the *VfLb29* gene products in AM colonized root cells and to the role of leghemoglobins in root nodule, the authors suggested that protein encoded by *Vflb29* could assure an oxygen supply for respiration of the mycorrhizal fungi. Recently, the expression patterns of two *Medicago truncatula* genes coding for putative cell wall repetitive proline-rich proteins (RPRPs) have been studied in the context of symbiosis (Journet et al., 2001). One of them, the *MtENOD11* gene, has been reported to be a molecular marker for both early pre-infection and late

infection-related processes of the symbiosis with rhizobia. But it is also strongly expressed in the mycorrhizal symbiosis, in inner cortical cells containing arbuscules. A further gene, *MtENOD12* exhibits a similar transcriptional pattern in symbiotic contexts, and its expression is linked as well to pre- and infection events in epidermal, cortical and nodule cells. This indicates a possible role of the corresponding PRPRs in host cell wall modification induced by hyphal infection or in the elaboration of the new arbuscules matrix interface.

These different studies, and particularly the one on PsNLEC1, have lead their authors to propose that the *Rhizobium*-legume symbiosis, i.e the nodulation process, may have evolved by adaptation of components preexisting in the more ancient AM symbiosis (LaRue and Weeden, 1994; Gianinazzi-Pearson, 1997; Balestrini et al., 1999).

The advanced knowledge about *Rhizobium*-legume symbiotic interaction as well as about molecular processes showing similarities in the two root symbioses could be very useful for a better understanding of the AM association.

1.3.3. *Medicago truncatula*, a genetic model for symbiosis

Originated from the Mediterranean Basin, the so-called barrel medic, *Medicago truncatula* is a legume well adapted to grow under semi-arid conditions. Among the several hundred ecotypes reported, some are commonly used in rotation with cereal crops. *Medicago truncatula* is closely related to the important forage legumes, alfalfa and soybean. *Medicago truncatula* can be grown in greenhouse where it can perform several complete growth cycles within a year (Fig.1.3.).

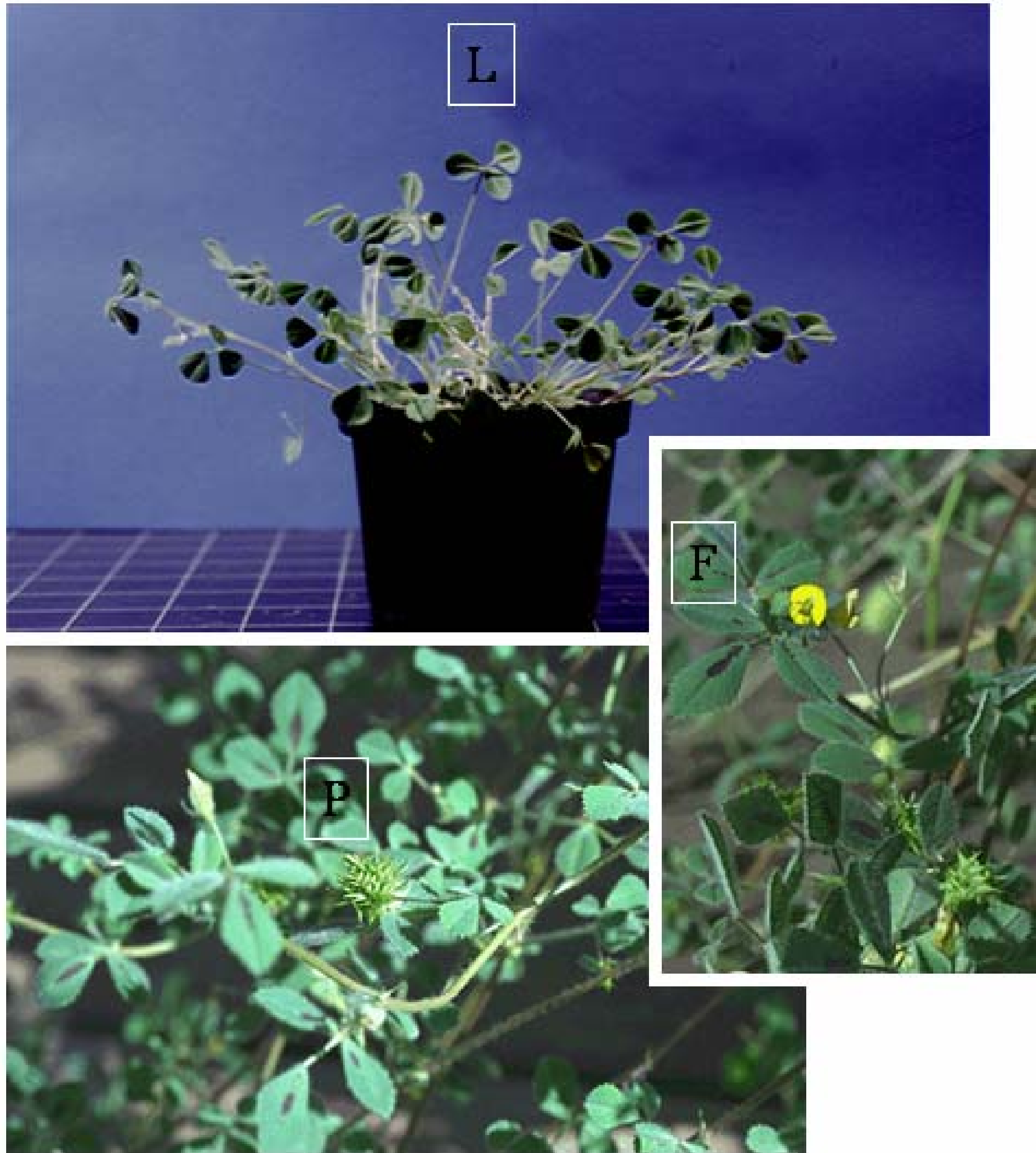

Figure 1.3: *Medicago truncatula* plant. Trifoliate leaves (L) and a flower (F) as well as an immature seed pod (P).

On the basis of a number of characteristics like self-fertility, prolific seed production, a small genome, and the possibility of regeneration via somatic embryogenesis, *Medicago truncatula* was proposed, at the beginning of the 90s, as a model system for molecular and genetic studies of legume biology (Barker et al., 1990). *Medigaco truncatula* is

diploid, and its DNA content represents about one fourth of the one of alfalfa, which is tetraploid. The small size of the MT genome is particularly advantageous for the construction and screening of gene libraries whereas diploidy is useful for genetic studies and the use of the mutants.

At least two ecotypes of *M. truncatula* can be efficiently transformed and regenerated with *Agrobacterium tumefaciens* (Chabaud et al., 1996; Trinh et al., 1998). Boisson-Dernier and collaborators recently proposed an *Agrobacterium rhizogenes* transformation of *Medicago truncatula*. In this protocol, the fast and efficient production of transgenic roots was coupled to the possibility of antibiotic selection for roots expressing a co-transferred transgene cloned in a binary vector (Boisson-Dernier et al., 2001).

In addition to its usefulness as a model legume with all the characteristics required for molecular, biological and genetical analysis of the nitrogen-fixing symbiosis with Rhizobium, *Medicago truncatula* is efficiently colonized also by AM fungi. A number of symbiosis-defective mutants have been identified after treatment of wild-type plants by ethylmethane sulphonate (EMS) or gamma irradiation. A considerable number of them are both affected in penetration of AM fungi in the root epidermis (Myc^-) and in nodules formation (Nod^-).

Medicago truncatula is currently the subject of major genomics initiatives. The *Medicago* Genome Initiative (MGI) is a database of EST sequences of this model plant. This database is part of the integrated *Medicago* functional genomics program at the Noble Foundation, which is taking a global approach in studying the genetic and biochemical events associated with the growth, development and environmental interactions of this model legume. The approach includes large-scale EST sequencing, gene-expression profiling, the generation of *M. truncatul*a activation-tagged and promoter trap insertion mutants, high-throughput metabolic profiling and proteome studies (Bell et al., 2001). The public interface to MGI database can be accessed at:

http://www.ncgr.org/research/mgi.

Another database where information on *Medicago truncatula* gene sequences can be accessible is the TIGR Gene Indices (http://www.tigr.org/tdb/tgi.html). This is a collection of species-specific databases that use a highly refined protocol to analyze EST sequences to identify the corresponding genes and to provide additional information on

them. Genes Indices are constructed by clustering and assembling EST and annotated gene sequences from GenBank for the targeted species. The result is the production of Tentative Consensus (TC) sequences that can be used to provide putative genes (Quackenbush et al., 2001).

1.4. DEVELOPMENT OF THE AM SYMBIOSIS

1.4.1. Description of the different steps of symbiotic association

To define the different stages of colonization, categories have been chosen and are mainly classified into 1) Pre-Penetration (Pre-Pen) (spore germination, hyphal elongation, hyphal branching, appressorium formation), 2) Penetration (Pen) (fungal penetration), 3) intracellular development and hyphal spread in the cortical tissue (Coi, Ici), 4) formation of arbuscule (Arb) 5) arbuscule collapse and senescence, 6) vesicle formation, 7) spore formation (Gadkar et al., 2001). Even if this classification is not exhaustive in reporting all stages observed so far, it is a quite useful to describe the development of the symbiosis.

1.4.1.1. Pre-penetration

1.4.1.1.1. Spore germination

A spore can germinate spontaneously despite the biotrophic nature of AM fungi, and this germination seems not to require the influence of a plant root. Quiescent spores will germinate under the appropriate environmental conditions of soil matric potential, temperature, and CO_2 level (Douds and Nagahashi, 2000). The germ tube will then elongate but in the absence of host root only for a short time and 20 times slower than in the presence of host roots. It ceases its development before total depletion of spore reserves. The hyphal growth has been improved by modifying the nutritional conditions but it has never been unlimited and extensive preinfection branching and hyphal growth cannot be obtained without host plant roots (Bécard and Piché, 1989a). The host induced differentiation starts before any contact between the AM fungus and its host plant, and the signaling events in pre-and post-infection stages, although not fully understood, are

on the way to be unraveled and can be described in quite some detail (Gadkar et al., 2001). In natural soils, infections do not necessarily originate from a germinating spore but predominantly from previously infected root segments surrounded by external hyphae (Schultze and Marsh, 2001).

1.4.1.1.2. Hyphal growth stimulation and hyphal branching

After it germinates, a spore has to find a host root in its immediate surrounding to proceed with the next colonization steps. Roots exudates are important in this early stage. Several authors have described growth stimulation of germinating AM fungi in the presence of host root exudates (Mosse and Hepper, 1975; Graham, 1982; Elias and Safir, 1987; Bécard and Piché, 1989b; Bécard and Piché, 1989a; Giovannetti et al., 1993) while exudates from non-mycotrophic species had no effect (Gianinazzi-Pearson and Gianinazzi, 1989; Bécard and Piché, 1990; Giovannetti et al., 1994) or appeared to be inhibitory (Koide and Schreiner, 1992; Vierheilig and Piché, 1996).

Among the important root exudates, oligosaccharides may be released from the plant cell wall (endogenous elicitors) (Fry et al., 1993). Observations that the initial infection occurs often next to emerging lateral roots, where some cell wall fragments are released lead to the hypothesis that those fragments serve to stimulate hyphal growth and act as attractants (Nagahashi et al., 1996). These possible signal compounds remain to be determined but roots also exude compounds that have been identified as flavonoids (Nair et al., 1991). During legume nodulation, they can induce nodulation genes and growth stimulants for *Rhizobium*. There are several parallels between the establishment of AM and *Rhizobium*-legume symbiosis as briefly outlined below. Flavonoids have been investigated and shown to indeed stimulate AM fungal growth (Bécard et al., 1992; Chabot et al., 1992). On the other hand, using maize mutant plants unable to produce flavonoids, Bécard and Piché found that they were equally mycorrhized as wild-type and that flavonoids are not essential for the establishment of the AM symbiosis (Bécard et al., 1995). On the other hand, the flavonoids coupled to nodulation factors effects promoted AM fungal colonization in soybean roots (Xie et al., 1995), suggesting they are involved

in a complementary mechanism in the fungus development next to the roots (Shaul et al., 2000).

The isolation of a potential "stimulating branching" compound has not yet been successful and its chemical structure is still unknown, but a hypothesis of Giovannetti and collaborators suggests that a factor eliciting branching of *Glomus mosseae* is a compound of <500 D (Giovannetti et al., 1996). Recently, Douds and Nagahashi measured the effects of carrot root exudates on *Gigaspora gigantea* hypha and reported a dose-dependent response in hyphal growth and branching. They conclude that roots of host plants of AM fungi constitutively exude signal molecule(s), which affect the growth of germ tube hyphae in the soil and that this branching response is a mechanism to increase the probability for the fungus to encounter with a site for colonization on the root surface (Nagahashi and Douds, 1999). With the use of the same system, a semi-purified root extract called "branching factor" has been shown to trigger a typical pre-symbiotic fungal response, i.e stimulation of a hyphal growth and branching (Buee et al., 2000). Even if this root signal is not yet chemically characterized, its presence with similar stimulating effect on three different fungi was detected in eight species of six families from mycotrophic plant and its absence correlated with the non-host character of four plant species (Buee et al., 2000). In agreement with the only other study on the subject by Bécard and collaborators (1995), this branching factor has been shown not to include flavonoids.

In this domain, many questions still need to be answered, concerning for example the nature of the signals involved, the existence of a unique or numerous signals acting at checkpoints during the colonization process, as well as the possible role of these signals in regulating fungal morphogenesis.

1.4.1.1.4. Formation of appressoria

Following the recognition between the two partners, the progress of fungal colonization is characterized by the formation of hyphal swellings preceding the differentiation of

structures of attachment at the root surface, the appressoria (Fig.1.4.A). This earliest defined recognition phenomenon on the root itself can occur as early as 36 hr after inoculation of the host plant (Giovannetti et al., 1994). Cells of the epidermis and hypodermis in contact with these first infection structures show no significant cytological modifications or response typical of plant defense (Gianinazzi-Pearson et al., 1996). Recently, Douds and Nagahashi produced carrot root cell wall "ghosts" 3-5 cells long with complete removal of protoplasts and incorporated them in a medium where pregerminated spores of *G. rosea* or *G. gigantea* were transferred. After 10 days, appressoria were formed on epidermal cell wall by either fungus but not on cortical or vascular cell walls. The authors conclude that this step is a contact recognition process not requiring a chemical signal (Nagahashi and Douds, 1997).

1.4.1.2. Penetration

The hypha can enter the root between two epidermal cells or invade an epidermal or root hair cell wall making its way through the cell (Bonfante-Fasolo, 1984). The fungus probably penetrates the root epidermis by exerting a hydrostatic pressure with the hyphal tip and by locally producing wall-degrading enzymes (Bonfante and Perotto, 1995). The study of Douds and Nagahashi described above indicates that the fungal penetration is only possible via a physiochemical signal intrinsic to the living epidermis cells or via the presence of a protoplast (Douds and Nagahashi, 1999).

1.4.1.3. Intercellular fungal growth

After the penetration of the epidermal cells, the fungus invades the root intercellularly (Fig.1.4.A and B). The plant's reaction to a hypha crossing an epidermal or hypodermal cell is apparently restricted to the synthesis of a host-derived membrane and the deposition of cell wall material continually around the fungus. Fungal development is extremely limited in outer root tissues and hyphae quickly progress toward the inner cortical parenchyma, where they proliferate intercellularly along the root (Gianinazzi-Pearson, 1996).

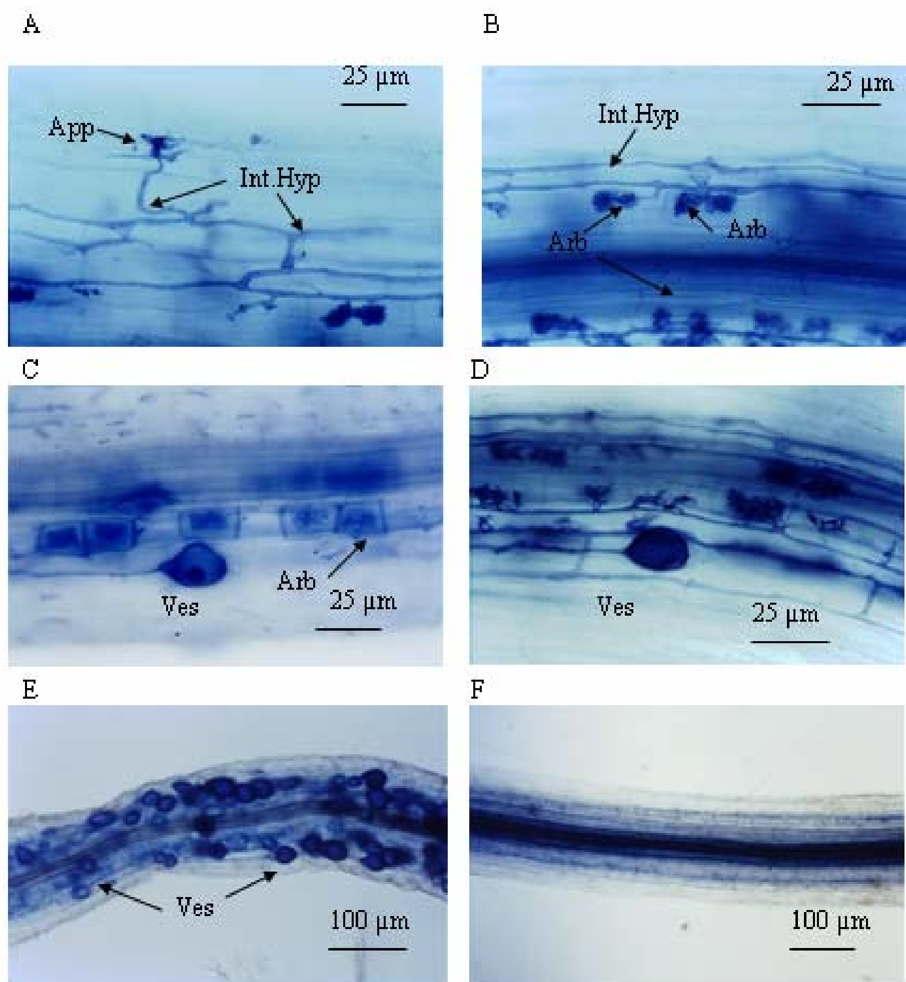

Figure 1.4: Examples of typical structures developed by *Glomus intraradices* within a plant root system (A, B, C, D, E). The different structures were stained with trypan blue, Appressorium (App.) at the surface of epidermal cells. Internal hyphae (Int.Hyp.) growing between root cells. Arbuscules (Arb.) invaginating cortical root cells. Vesicles: V. Control non-mycorrhized root (F).

1.4.1.4. Formation of arbuscules

After penetration and colonization of the inner cortex, the multi-step process of the mycorrhiza development proceeds with the formation of arbuscules within the plant cells (Fig.1.4.B and D). These highly branched haustorium-like structures create an invagination of the host plasma membrane, increasing the area of contact between fungus and cortical cells. The formation of arbuscules is accompanied by a vacuole fragmentation and with movement of the nucleus to the center of the cell (Bonfante and Perotto, 1995). The microtubules of the cytoskeleton are involved in these morphological changes (Genre and Bonfante, 1997). The transcriptional activation of a plant α-tubulin gene in colonized cells suggests a reorganization of the cytoskeleton itself (Bonfante et al., 1996). The branching progression of the fungus provokes de novo formation of a periarbuscular membrane. This surface of contact between the plant and the fungus creates a new subcellular symbiotic compartment. The arbuscules are thought to be the sites of carbon and phosphate transfer between the two partners (Smith and Smith, 1990). According to the results of experiments using acidotrophic fluorescent dyes, the periarbuscular matrix is seen as an acidic compartment probably due to the activity of the H^+-ATPases (Guttenberger, 2000). A cDNA corresponding to a gene coding a membrane intrinsic protein (MIP) has been obtained from mycorrhized parsley roots. The corresponding mRNA accumulates in roots during symbiotic and plant-fungal interactions (Roussel et al., 1997). This PcRB7 MIP belongs to the family of passive transporters, known to facilitate the movement of small molecules across the membrane, and thus, the PcRB7 protein might play a role in the transport of nutrient at the interface. Still at the level of membrane proteins, a cDNA, *psam1,* has been identified by RT-PCR differential display on pea roots. The function of the protein encoded by *psam1* is still unknown but the deduced amino acid sequence has similarities with phospholamban that regulates the activity of Ca^{2+}-ATPase in mammalian cells (Martin-Laurent et al., 1997). Three clones of *Medicago truncatula* corresponding to genes encoding an HRGP, a putative arabinogalactan protein (AGP) and a member of the xyloglucan endo-transglycosylase (XET) family are induced following colonization by *Glomus versiforme* (van Buuren et al., 1999). The enhanced activity of the corresponding enzymes would be

consistent with de novo synthesis of cell wall proteins responsible for the structural maintainance of the arbuscular interface matrix.

1.4.1.5. Arbuscule collapse and senescence

After the metabolic activity has taken place, the arbuscules collapse and degenerate within 7-12 days, leaving the cell undamaged and capable of hosting another arbuscule (Alexander et al., 1988; Alexander et al., 1989).

A recent interesting study reports the occurrence of cross walls in arbuscular trunk hyphae in relation to the development and senescence of arbuscules (Dickson and Smith, 2001). This material deposition in the trunk hyphae is similar to the ones observed by Bonfante in the intercellular hyphae (Bonfante et al., 1990). In many plant-pathogen interactions, these structures called neckbands are located in the interfacial apoplast and play a role in sealing the haustorium to probably facilitate the unidirectional transfer from plant to the fungus (Manners and Gay, 1983). In *Arum*-type colonization, the cross wall formation is supposed to occur after the loss of activity of at least the majority of the arbuscular branches. The authors hypothesize that the formation of the cross walls in intercellular hyphae would be useful for the translocation of nutrient from inactive arbuscule into more active structure.

A recent interesting report by David-Schwartz and collaborators ask different questions related to the hyphal growth and branching control (David-Schwartz et al., 2001). They describe a mutant from tomato (*Lycopersicum esculentum* cv. Microtom) that is unable to form AM when inoculated with fungal spores but yields a normal colonization of its roots when grown together with already colonized wild-type plants. These results suggest that one or several signals necessary to initiate spore germination, hyphal elongation or appressoria formation are missing in the mutant plants and that this signal is genetically controlled by the plant by a single locus in the key steps of the pre-infection process. Hence, this study gives more arguments in favor of a genetic control by the plant over the fungal development, not only within root tissue, as e.g reported in the publication of Gianinazzi et al., in 1996, but as well over recognition processes and early fungal differentiation necessary to establish the symbiosis at the pre-infection stage. A further

mutant in the Pre-Pen stage has been described in maize, another non-leguminous plant (Paszkowski et al., 2000).

1.4.1.6. Formation of vesicles

Following the formation of arbuscules, many AM fungal species form vesicles within the root tissue (Fig.1.4.D and E). These structures are not present in every AM symbiosis and some fungi even do not develop them at all. They are intracellular fungal storage structures that contain lipids and nuclei and are thought to act as propagules (Smith, 1988).

1.4.1.7. Spore formation in the extraradical mycelium, completion of life cycle

Root colonization is accompanied by the development of an extraradical mycelium. Under sterile conditions it includes so-called arbuscule-like structure (ALS) (Mosse and Hepper, 1975; Bécard and Fortin, 1988; Chabot et al., 1992; Bago et al., 1998) or branched absorbing structures (Bago et al., 1998). According to Bago et al. (1998), these ALS/BLS could be preferential sites for nutrient uptake by the extraradical mycelium of AM fungi. The formation of new spores in the rizosphere completes the life cycle of the fungus (Fig.1.2).

1.4.2. The use of plants mutants for a step by step dissection of AM development

In order to study the plants factors involved in successful colonization and to elucidate these processes at the molecular level, mutant plants have been searched exhibiting abnormal mycorrhizal phenotype, most frequently in legume species. These mutants can range from defective AM (Myc^-) to an enhanced mycorrhizal phenotype. It has been found that many legume mutants deficient in root nodule formation (Nod^-) were also deficient in mycorrhizal formation or function (Myc-). This demonstrates that the two symbioses are sharing common steps, but on the other hand, this does not allow the discovery of mycorrhiza specific genes. Nevertheless, a few $Nod^+(Fix^-)/Myc^-$ mutants, and recent reports of AM mutants in tomato (Barker et al., 1998) and in maize

(Paszkowski et al., 2001) two non-leguminous plants, suggests the existence of at least some mycorrhizal specific genes (Smith and Read, 1997).

Among the Nod- mutants deficient in mycorrhiza formation, the vast majority is defective in the earliest stages of AM symbiosis. They are called Pen$^-$ mutants in reference to the introduction of this chapter. Mutant plants affected in the penetration step exhibit the formation of complex and abnormal appressoria compared to wild-type (Marsh and Schultze, 2001). The first mutants isolated were from *Pisum sativum* populations and the mutations in six loci resulted in the Pen$^-$ phenotype as described above (Duc et al., 1989). In *Medicago truncatula*, three separate loci, dmi1, dmi2, dmi3 are associated with the same defective phenotype (Catoira et al., 2000). The mutants *dmi1*, *dmi2,* as well as *sym8* and *sym19* in pea are defective in the calcium spiking, one of the first steps of nodulation in the *Rhizobium*-legume symbiosis that occurs in root hairs (Wais et al., 2000; Walker et al., 2000). This suggests that calcium spiking may be part of the signaling events necessary also for mycorrhizal symbiosis. Further analysis of the *sym8* pea mutants E140, R19 and R25 revealed that SYM8 is essential for the induction of the early nodulation (*ENOD*) genes, *PsENOD5* and *PsENOD12A* in roots inoculated by *Gigaspora margarita* (Albrecht et al., 1998). A single Pen$^-$ mutant has been isolated from *Vicia faba* (Duc et al., 1989). Interestingly, this mutant is not Nod$^-$, as the mutants cited above, but is Nod$^+$/Fix$^-$ i.e. it makes nodules but those are not functional. Several other mutants are unable to develop the first step of the symbiosis. The mutant *mcbep* of *Lotus japonicus* (Senoo et al., 2000b) has a blocked colonization already at the epidermis level. The same is true for the *rmc* (reduced mycorrhized colonization) mutant of tomato (*Lycopersicon esculentum*) (Barker et al., 1998) and for the mutant MN-NN1008 of alfalfa (*Medicago sativa*) (Bradbury et al., 1991). The latter mutant also displays a lower transcript level of gene encoding a sugar transporter compared to wild-type AM roots where this level is normally increased during symbiosis (Albrecht et al., 1998). When the fungus tries unsuccessfully to enter the root epidermis, it develops a hyphal branching and a higher number of appressoria (Schultze and Marsh, 2001).

A battery of mutants unable to perform this step has recently been isolated in *Lotus japonicus*. The Coi- mutants allow normal appressoria development and penetration of the epidermis but they block fungal progress prior to cortex invasion (Wegel et al., 1998;

Bonfante and Perotto, 2000; Parniske, 2000). The epidermal cell penetration leads to death of root cells but as well to death of fungal hyphae. This can be considered somehow like a symptom of HR although no other typical defense response was detected in these mutants. Another characteristic of these Coi- plants is the occurrence of occasional inner cortex colonization and formation of normal arbuscules that points out that this mutation is both stage and cell specific (Schultze and Marsh, 2001). It is more difficult to categorize precisely the following mutants according to the stage. Again isolated from *Lotus japonicus*, the mutants *mcbex* and *mcbee* display a mycorrhizal colonization blocked in the cortex or blocked between epidermis and exodermis, respectively. These mutations affecting steps somewhere between Pen and Arb stages of development are responsible for the overproduction of deformed appressoria, absence of inner cortical invasion and occasional formation of abnormal arbuscules (Senoo et al., 2000a). These mutants have been called Ici- for inner cortex invasion defective. In the long list of mutants presented so far, there are only few mutants affected in the last stage of the colonization process. The MN-IN3811 mutant of *Medicago sativa* and R69 mutant of *Paseolus vulgaris* allow the inner cortex colonization but do not form arbuscules and they are called Arb- (Bradbury et al., 1991; Shirtliffe and Vessey, 1996). The last category of mutant is represented by the RisNod24 mutant of pea, which undergoes normal colonization within the cortex until the development of truncated arbuscules (Ard-) (Gianinazzi et al., 1996). Interestingly, these latter mutants described are Nod^+/Fix^- (i.e. they form nodules but those do not fix nitrogen) in contrast to the majority of the mutants being Nod^-/Fix^-.

1.4.3. Plant defense reactions and their suppression, systemic induced resistance

The penetration of a fungal pathogene in a plant tissue usually provokes the mobilization of various defense mechanisms to avoid the progress of the microorganism. These defense reactions include: the hypersensitive response, accumulation of phytoalexines and other secondary metabolites, formation of structural defense barriers, induction of genes encoding for plant defense processes and a set of proteins (pathogenesis-related or PR proteins) which often show antifungal activities. In many AM associations, there is a weak but transient increase of expression of plant defense responses in the early stage of

the fungal colonization followed by its suppression below constitutive levels at a later stage (Gianinazzi-Pearson et al., 1996; Kapulnik et al., 1996). To understand the cellular and molecular events underlying these induced defense-like responses in host plants and in particular their subsequent repression, cytochemical investigations as well as studies on regulation of plant genes encoding for different defense-related proteins have been carried out. At the cytological level, the host cell usually forms wall appositions or papillae at the site of a pathogen attack. These compounds like callose, phenolics, proteins or a silicon deposition are considered to reinforce the cell wall and hence to increase their resistance to pathogen ingress (Gianinazzi-Pearson et al., 1996). Although some thickenings can be observed occasionally in epidermal cell walls below points of contact of appressoria, neither callose (long chain of β-1,3-glucans) nor phenolics accumulate in mycorrhizal roots (Garriock et al., 1989). In addition, peroxidases that are usually involved in cell wall reinforcements and lignin synthesis occurring during plant-pathogen interactions are not activated in arbuscule containing cells (Gianinazzi-Pearson et al., 1996). In contrast, β-1,3-glucans and hydroxyproline rich glycoprotein (HRGP), two cell wall-localized defence-related molecules, have been detected by immunolocalization at the contact site between host cell and hyphae. The β-1,3-glucan synthesis takes place typically in mycorrhizas of pea, tobacco and leek but not in mycorrhizal maize roots. Thus, their presence cannot be considered as a general feature of the wall reaction to AM fungi invasion. When present, the β-1,3-glucans have been found around the point of penetration of hyphae but they are not detected anymore as the fungus differentiates into arbuscules (Gianinazzi-Pearson et al., 1996). In mycorrhizal parsley and maize roots, the mRNA coding for HRGP, a structural cell wall glycoprotein, accumulates to significant amounts. In maize roots, HRGPs have been immunolocalized around hyphae, both at the point of cell penetration and around arbuscules at the interface fungus-host (Balestrini et al., 1997). Another group of proteins -the PR proteins- are induced during a pathogenic infection, the pathogenesis-related proteins (PR). After their isolation in the 70ies, the PRs have been extensively characterized and later grouped into different families divided in PR-1a, PR3: acidic chitinases, PR2: acidic β-1,3-glucanase and PR11: peroxidases (Boller and Keen, 1999). Some PR proteins (PR-2, PR-3 and PR-11) can be potential inhibitors of fungal growth since they can degrade chitin and β-1,3-

glucans, major structural components of many fungal cell wall (Gianinazzi et al., 1994). As a consequence, it was expected to see an induction of these hydrolytic enzymes if a defense response occurs in a mycorrhizal plant; it was logical to orientate the research towards them. Indeed, increased activities of chitinase, cell wall-bound peroxidase have been measured in mycorrhizal roots during the early stages of colonization of *Allium porrum* (Spanù and Bonfante-Fasolo, 1988; Spanù et al., 1989). In bean and tobacco mycorrhized roots, a similar transient increase was found in both the transcripts and activities of β-1,3-endoglucanase and of chalcone synthase, an enzyme of the flavonoid pathway. The activity of these enzymes dropped even below the levels in control plants once mycorrhiza was established, following the transient induction pattern observed for the other defense responses mentioned above (Lambais and Mehdy, 1993; Vierheilig et al., 1994; Volpin et al., 1994; Kapulnik et al., 1996). In *Allium porrum*, further immunocytochemical analysis suggested a localization of a chitinase in the vacuole but not a direct contact with the hyphal cell wall (Spanu et al., 1989). Chitinases are widely distributed in plants, fungi and bacteria. They are hydrolytic enzymes that cleave the β-1,4-glycosidic bonds between N-acetyl glucosamines residues of chitin that is one of the main component of the wall of all true fungi. The detection of chitin in cell walls of AM an ectomycorrhizal fungi indicates that they are not only important in plant-pathogen interaction but as well in mutualistic symbiosis (Boller, 1987). Class I and V chitinases may contribute to plant defense, as their anti-fungal activity has been demonstrated in vitro (Schlumbaum et al., 1986; Mauch et al., 1988; Melchers et al., 1994), and class I chitinase can increase resistance of tobacco plants to *Rhizoctonia solani* when expressed constitutively (Broglie et al., 1991; Vierheilig et al., 1995). Their role in AM symbiosis has not yet been elucidated but a number of chitinases have been proposed to be specific symbiosis-related proteins showing induction in pea, tobacco and tomato (Dumas-Gaudot et al., 1992; Dassi et al., 1996; Pozo et al., 1999). On the other hand, a basic chitinase gene was suppressed in tobacco in response to colonization by *Glomus intraradices* (David et al., 1998). In *Medicago truncatula*, it was recently shown that mRNAs of a class III chitinase gene accumulate in AM colonized roots but not in Rhizobia or pathogen-infected roots or in myc- mutants nor in uninfected roots (Salzer et al., 2000).

In early studies on plant-pathogen interaction, it has been proposed that acidic apoplastic PR-1 defense-related proteins have an antifungal activity (Graham and Graham, 1991). In studies of PR-1 expression in tobacco roots colonized by *Glomus mosseae* or pathogen revealed a lower transcript level in the mycorrhizal roots than in the pathogen-infected ones (Gianinazzi-Pearson et al., 1992). A slight local increase of transcripts has been found around living arbuscules, and the localization of the corresponding PR-1 protein is limited to the interface between intracellular hyphae and the host protoplast. A promoter-fusion marker system confirmed an increased and local gene expression restricted to colonized cells (Gianinazzi-Pearson et al., 1996).

In response to pathogen infection, the resistant plant develops a hypersensitive response (HR) to limit the fungal spread. The common features of HR are deposition of lignin, polyphenol formation and production of antimicrobial phytoalexins followed by localized cell death (Knogge, 1996). The lack of HR after recognition of AM fungi indicates no "resistance" against this microorganism and denotes a non-activation, or low level of response or suppression of the defense mechanisms. The phytoalexins are produced via the isoflavonoid biosynthetic pathway, the genes of which are regulated at the transcriptional level by different stimuli (Allen et al., 1989). After the studies on the effect of flavonoids on fungal growth, a detailed analysis of flavonoid/isoflavonoid pathway at the enzyme and mRNA levels has been made in *Medicago truncatula* to determine whether the AM symbiosis modifies the flavonoid profile (Harrison and Dixon, 1993). *Medicago sativa* and *Medicago truncatula* respond to a pathogen attack with a rapid induction of the genes and enzymes of the isoflavonoid pathway and with the production of phytoalexins, mainly medicarpin. Colonization of *Medicago truncatula* roots by *Glomus versiforme* results in a change of transcript levels of five enzymes involved in the phenylpropanoid metabolism. A transient increase in free medicarpin in the early colonization coupled with accumulation of transcripts of phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) have been reported, consistent with general increased flavonoid biosynthesis (Harrison and Dixon, 1993). During the late stage of colonization, isoflavone reductase (IFR) transcripts, which encode an enzyme specific for medicarpin biosynthesis, decrease as well as the medicarpin levels. Further studies revealed the specific location of PAL and CHS transcripts in the cortical cells

containing arbuscules (Harrison and Dixon, 1994). The expression of chalcone isomerase (CHI) and isoliquiritigenin 2'-*0*-methyltransferase (ChaIOMT) was not affected by colonization (Harrison and Dixon, 1993).

The knowledge on regulation of expression defense-related genes needs to be developed albeit some possible functions have been proposed and will be presented in the following paragraphs in an attempt to answer the question: how does the fungus manage to circumvent the defense reaction triggered by the host-plant?

The deposition of cell-wall defense-related molecules (HRGPs and β-1,3-glucans) by the host cell around the point of penetration of hypha as well as the accumulation of HRGPs-encoding mRNA could be understood like a defense response to limit fungal development. The role of β-1,3-glucanases in AM symbiosis is not known. Further to their possible role in helping to degrade the fungal cell wall (a defense reaction), it is conceivable that the fungus would take advantage of the plant glucanases to destroy β-1,3-glucan depositions and thereby facilitate its penetration. The deposition of HRGPs could also be seen as a defense barrier to restrict the fungus growth. However, no significant cell wall alterations are observed during AM symbiosis. Therefore, HRGPs may be involved in a more general cell wall building activity as a result of the changes in the cellular environment (Gianinazzi-Pearson et al., 1996).

The induction of the two isoenzymes PAL and CHS around arbuscules during AM fungi invasion are unlikely to play a role in lignin biosynthesis, as lignin is absent in arbusculated cells. As reported for other plant-microbes interactions (*Agrobacterium*, *Rhizobium*), plant phenolics can regulate positively or negatively genes involved in the first steps of AM symbiosis. Hence, PAL and CHS may be induced for the synthesis of such signal molecules.

The fact that the fungal root colonization is not affected by the presence of these hydrolases suggests that the fungal cell wall components are not degraded either because they are not accessible to them or because their enhanced activities do not reflect a specific plant defense response (Gianinazzi-Pearson et al., 1996). This view is supported by the fact that the basic forms of β-1,3-glucanases and chitinases which have strong anti-fungal potential, are located in the vacuoles; thus as exemplified in colonized leek root cells (Spanu et al., 1989), there may be contact between the fungal hyphae and the

plant enzymes. From the combined results and observations collected out of experiments with EM (Ectomycorrhizal fungi) and AM fungi, a possible role for chitinases in AM symbiosis establishment has been proposed (Salzer and Boller, 2000). According to this speculative model, in the early colonization stage, chitin fungal elicitors would be partially destroyed by constitutively expressed chitinases. Some of them would be active enough to trigger low intensity defense response whereas in a later stage, a combined action of constitutive and mycorrhiza-induced chitinases would destroy all of them thereby, stopping further defense reactions. As an alternative model independent of chitinases, the authors propose that the fungi are able to suppress plant defense gene expression via plant hormone production. These latter, especially auxins, can be synthesized by AM and EM fungi and have been shown to down-regulate the expression of defense-related proteins, more particularly chitinases, peroxidases and basic glucanases (Felix and Meins, 1986; Sauter and Hager, 1989; Salzer and Hager, 1993; Mensen et al., 1998).

In arbuscule containing cells, the localization of the PR-1 protein that is limited to the fungal-plant interface contrasts with the generalized accumulation observed in pathogen infected tissue (Tahiri-Alaoui et al., 1993). This restricted localization of protein and transcripts associated with the low gene expression, speaks more in favor of a very localized induction rather than a general suppression of transcriptional activity (Gianinazzi-Pearson et al., 1996). Since, the PR-1 function is still unknown and its anti-fungal activity only tested against oomycetes (Niderman et al., 1995), the role of PR-1 in the early steps of AM symbiosis and restricted around the arbuscule needs to be clarified.

The suppression of plant defense response seems to be the rule in AM association. Indeed, the non-mycotrophic *Salsola kali* species triggers defense responses when AM fungi attempt to initiate the root colonization (Allen et al., 1989). This could be a typical example of a non-host response; however such a reaction has also been observed in a mycorrhizal species, alfalfa. Plants were eliciting HR in response to *G. margarita*, impeding further symbiosis (Douds et al., 1998).

It seems nevertheless that suppression of some of these defense-related genes is not necessary for the development of the symbiosis. As a matter of fact, tobacco plants

overexpressing chitinases, glucanases, PR, with the exception of PR-2 proteins, are not affected in their symbiotic capability (Vierheilig et al., 1994 and 1995).

Another aspect is a potential systemic induced resistance of mycorrhizal plants when challenged by pathogens. The usual symptoms of a systemic induced resistance, like up-regulation of PR protein in cells distant from the colonized ones, seem to be due to salicylic acid, and enhanced levels of salicylic acid do not exist in mycorrhizal roots. However, several reports indicate that a plant colonized by an AM fungus express an enhanced level of resistance to subsequent pathogen attack (Blee and Anderson, 2000). In a split-root experimental system where half of the roots of tomato plants were mycorrhized, a protective effect was observed against *Phytophtora parasitica* in both the AM colonized and the non-colonized compartment (Cordier et al., 1996b). The mechanism of this induced response seems to include the plant cell wall thickenings that can limit the pathogen progression in the root. In a pathogen attack, the plant responds with induction of the salicylic acid pathway subsequent of plant cell necrosis. In the absence of SA induction in colonized roots, the salicylic pathway, well defined for pathogen attack, cannot be considered as the only one involved in the systemic resistance (Métraux et al., 1990). Alternative SA-independent pathways (oxidase, jasmonate, ethylene) could mediate an induced systemic resistance in roots colonized by AM fungi (van Loon et al., 1998).

Plants that fail to form mycorrhizae are good systems to explore plant and fungal genes responsible for defense regulation. It is hypothesized that the fungus would be excluded through the abnormal expression of defense reaction in the mutant plants (Marsh and Schultze, 2001). The P2 (*sym 30*) pea mutant which is resistant to symbiont invasion, develops thick wall appositions, callose and phenolics on epidermal and hypodermal cell walls in contact with the appressoria, similar to those observed during an incompatible interaction (Gollotte et al., 1993). Another observation is the higher level of endogeneous salicylic acid as well as the steady levels of several plant defense genes in P2 compared to wild-type (Blilou et al., 1999; Ruiz-Lozano et al., 1999).

1.5. IMPORTANCE OF CARBOHYDRATE METABOLISM IN SYMBIOSIS

1.5.1. Carbon metabolism in the plant

1.5.1.1. Sucrose-cleaving enzymes

A typical feature of all the different pathogenic to mutualistic associations of higher plants with fungi is the occurrence of net assimilate fluxes from the plant to the micro-organism, established through a close contact between the two partners. In the leaves, the carbon is photosynthetically fixed and then supplied mainly as sucrose to the sink organs. In normal development, heterotrophic sinks are organs like developing fruits, seeds, roots, and tubers. In plant-microbe interactions, the heterotrophic microbes are similar sinks. The channeling of sucrose into the sinks often requires its cleavage by invertase or sucrose synthase.

These two sucrose-cleaving enzymes have entirely different properties. Invertase is a hydrolase cleaving sucrose irreversibly into glucose and fructose whereas sucrose synthase is a glycosyl transferase, which, in the presence of UDP, converts sucrose into UDP-glucose and fructose. Although sucrose synthase is able to synthetize sucrose under appropriate test-tube conditions, there is good evidence that sucrose synthase in vivo is primarily involved in its breakdown (Winter et al., 1997). Several isoforms of invertase and sucrose synthase are present in different subcellular compartments (Sturm, 1996). Plants usually have at least two isoforms of vacuolar acid invertases. These soluble proteins accumulate in the lumen of the vacuole and have pH optima for Suc cleavage between 4.5 and 5. In addition, plants usually have several isoforms of extracellular invertases, which are ionically bound to the cell wall. These acidic cell wall invertases have the same pH optima than the vacuolar invertases. Typical plant invertases are not Suc specific and also display a β-fructofuranosidase activity against other substrates. For example, they can hydrolyse raffinose and stachyose. At least two isoforms of neutral invertases are known with neutral or slightly alkaline pH optima for Suc cleavage. They are less well characterized. The activities of these hydrolases are thought to regulate the entry of sucrose into the different biochemical pathways. More specifically, cell wall invertases are probably responsible for the phloem unloading of Sucrose and participate

in this way to its partitioning between source and sink organs (Sturm, 1999). Even if the specific role and contribution of the different invertases have not been fully elucidated, several hypotheses have been proposed. In cleaving sucrose, invertases contribute to provide growing tissues with hexoses as a source of energy and carbon, to generate a sucrose gradient between source and sink tissues to aid sucrose transport (Sturm, 1999), to regulate cell turgor and to control sugar composition in storage organs (Tang et al., 1999). Lack of invertase activity in a natural mutant of maize (*miniature-1*) causes an early degeneration and elimination of maternal cells from the endosperm. The resulting interruption of the transport of photoassimilates into the developing kernel causes high decrease of seed weight (Miller and Chourey, 1992). In developing tomato fruits (Ohyama et al., 1995; Klann et al., 1996) and mature potato tubers (Zrenner et al., 1996), down-regulation of vacuolar invertase activity by gene suppression or an antisense mRNA approach altered the hexose-to-sucrose ratio without major effects on plant development. Different functions of cell wall invertases have been detected by studies on transgenic carrot plants in which the activities of the two enzymes are altered by anti-sense expression. The transgenic plants expressing antisense mRNA for cell wall invertase develop no tap roots, suggesting carrot cell wall invertase plays a role in sucrose partitioning to developing tap roots (Tang et al., 1999).

A study of Koch and collaborators showed a rapid repression of invertases under low oxygen and sharp changes in the invertase-sucrose synthase ratio in maize root tips. This could be a prompt control on sucrose use, possible energy savings and reduced signal transduction to genes for non-respiratory pathways for carbon utilization (Zeng et al., 1999).

The other sucrose-cleaving enzyme, sucrose synthase (Susy, EC 2.4.1.13) catalyses the reversible reaction that converts sucrose and UDP to fructose and UDP-glucose. It is a key enzyme of sugar metabolism, and like invertase, it plays a major role in sink/source relationships within the plant (Zrenner et al., 1995). The sucrose synthase polypeptides are located in the cytoplasm and are soluble or tightly attached to the cellulose synthase complex at the plasma membrane or the actin cytoskeleton (Amor et al., 1995; Winter et al., 1998). Despite some remaining uncertainty, it is strongly suggested that in association with this cellulose synthase complex, Sucrose synthase may provide UDP-Glc for

cellulose synthesis whereas a free form in the cytosol would supply products of sucrose metabolism into the general metabolism (Carlson and Chourey, 1996; Delmer et al., 2000). Mutants with reduced sucrose synthase activity have been isolated and studied in maize (Chourey and Nelson, 1976) and pea (Craig et al., 1996) as well as in transgenic potatoes, where the anti-sense inhibition of sucrose synthase results in a dramatic reduction in tuber starch content (Zrenner et al., 1995). An anti-sense inhibition of tomato fruit sucrose synthase was performed and resulted in a decrease of fruit setting and of the sucrose unloading capacity of young fruit. The authors took this as direct evidence that sucrose synthase activity was an important determinant of fruit growth rate and fruit set (D'Aoust et al., 1999). The regulation of sucrose synthase genes has also been studied in maize. In this plant, two genes encode sucrose synthase isozymes with very similar characteristics. *Sh1* encodes SS1 and *Sus1* encodes SS2 (Echt and Chourey, 1985). SS1 was initially considered an "endosperm form" of sucrose synthase, and the corresponding gene was called *Shrunken-1* (*Sh1*) referring to the shrunken phenotype of kernels (Chourey and Nelson, 1976). Expression of this sucrose synthase-encoding gene (*Sh1*) has been demonstrated in other maize tissues (Chourey et al., 1986; Springer et al., 1986; Heinlein and Starlinger, 1989). The second enzyme SS2 is more widely distributed among plant parts than SS1 and is present at higher levels in young leaves (Nguyen-Quoc et al., 1990). Initial work of Koch and McCarty indicated that carbohydrate depletion could up-regulate the *Sh1* gene in maize root tips and that sucrose addition would decrease levels of its message (Koch and McCarty, 1988). Later on, it was shown that the two genes encoding sucrose synthase were responding differently to changes in the carbohydrate content of tissues. In excised maize tips exposed for varying periods in different sugars and a range of concentrations, plentiful sugar supplies increased expression of *Sus1,* whereas reduced sugar availability enhanced *Sh1*. Although total enzyme activity did not show any net change, cellular localization of sucrose synthase protein was markedly altered. This differential response of the two sucrose synthase genes suggests that through an altered pattern of enzyme distribution, the products of sucrose synthase genes are implicated in many different metabolic pathways and processes like cell wall regeneration; respiratory pathways; low sugar tolerance/recovery; and phloem transport (Koch et al., 1992). Furthermore, the sucrose synthase genes

respond differentially to hypoxia and anoxia (Zeng et al., 1998). Sucrose synthase has been shown to be phosphorylated in maize leaves (Huber et al., 1996) and pea nodules (Zhang and Chollet, 1997). Phosphorylation was reported to stimulate the cleavage reaction and moreover to modify the subcellular localization of the sucrose synthase protein. While the dephosphorylated form was found to be associated with the membrane, the phosphorylated form was found in the cytoplasm (Winter et al., 1997). Most interestingly, a phosphorylated form was associated with the detergent-insoluble fraction of microsomal membrane preparations suggesting a possible cytoskeleton association; this hypothesis was supported by the affinity of phospho-sucrose synthase to G-and F-actin in vitro (Winter et al., 1998). This opens the possibility that sucrose synthase may have an additional function related to cytoskeleton and cellular architecture.

1.5.1.2. Mechanisms for C transfer and its significance for the AM symbiosis

As sucrose or as its components, glucose and fructose, the carbon becomes available to the root cells (Sonnewald and Willmitzer, 1992) to support root growth and metabolism as well as growth of a microsymbiont if present (Harley and Smith, 1983). Over 20 years ago, this transport from plant to fungus was demonstrated (Ho and Trappe, 1973; Bevege et al., 1975), and further measurements of carbon flux indicate that up to 20% of fixed carbon may be transported from leaves to roots during a mycorrhizal symbiosis (Douds et al., 1988). The source of carbon transferred from plants and utilized by the microorganism has been identified as sugars in the ectomycorrhizal symbiosis (Lewis and Harley, 1965), as well as in the interaction with endophytes like *Acremonium* (Lam et al., 1994), and in the infection by powdery mildew (Manners, 1989). It is worth noting that, in the *Rhizobium*-legume interaction, the bacteria use organic acids that have been produced from assimilates transported to the roots, rather that sucrose or hexoses (Day and Copeland, 1991). The molecular mechanisms of carbon transfer from plant root to the mycorrhizal fungus are still largely unknown. A cDNA clone encoding a hexose transporter (Mtst1) has been obtained from mycorrhized roots of *Medicago truncatula* (Harrison, 1996). Mtst1 is expressed in the phloem fibers and root tips, and its transcript levels increase in the cortical cells colonized by *Glomus versiforme*. Harrison (1996) proposes that the expression of Mtst1 in these particular cells could mean a higher

demand of plant cells to acquire more hexoses in order to support the increase metabolism during the symbiosis (Cox and Tinker, 1976). This does not exclude that the trigger is an increased demand for hexose supply to both partners. The arbuscule is traditionally proposed to be the key site of carbon exchange, due to its large contact area between the two partners. It has been suggested that the carbohydrate allocation would take place at the intercellular hyphae, the membranes of which show a high ATPase activity and thus a capacity for active transport (Gianinazzi-Pearson et al., 1991).

In the *Rhizobium*-legume symbiosis, the nodules themselves, as whole organs, are primarily dependent on the import and metabolism of sucrose. It is indeed the primary source of energy and carbon skeletons provided to both the plant tissues and the bacteroids. After being unloaded in the root cortex, sucrose is metabolized in the infected region of the nodule and the product of its catabolism (usually malic acid) is used by bacteroids to fuel N fixation (Gordon et al., 1999). The importance of sucrose hydrolysis in N fixation has been studied in part through investigation on the enzymes responsible for sucrose cleavage. The gene encoding sucrose synthase (SucS) belongs to the class of nodulins, highly or uniquely expressed in nodules (Thummler and Verma, 1987). Gordon and collaborators (1999) demonstrated that SucS is essential for nitrogen fixation in nodules and also suggested that the control of SucS gene expression may be as well of importance in the regulation of carbon metabolism and nitrogen fixation (Gordon et al., 1997). SucS has been characterized in nodules of soybean (Morell and Copeland, 1985) and broad bean (Ross and Davies, 1992) and SucS cDNAs have been also isolated from *Pisum sativum*, *Lotus japonicus*, *Glycine max* and *Medicago sativa* (Déjardin et al., 1997; Skot et al., 1997; Zhang and Chollet, 1997; Buchner et al., 1998). The gene corresponding to SucS cDNA from *Vicia faba* L. is reported to have a level of expression ten-times higher in nodules compared to uninfected roots (Küster et al., 1993).

In *G. max*, SucS was immunolocalized in both uninfected and infected nodule cells (Gordon et al., 1992), in accordance with the level of transcripts detected by in situ hybridization in *Pisum sativum* and *P. vulgaris* (van Ghelue et al., 1996).

The dominant role of sucrose cleaving enzymes has been reported by Wang and Hedley (Wang and Hedley, 1993b) and Gordon and collaborators (1998). They observed that pea and *Lotus* plants with reduced level of SucS enzymes were severely impaired in growth

on nitrogen-free medium. A further study reports the isolation of a sucrose synthase cDNA from *Medicago truncatula* that shows a ten-fold higher expression level in root nodules compared to control roots on a northern-blot (Hohnjec et al., 1999). This nodule-enhanced sucrose synthase encoding gene is also expressed in stems but transcripts are barely detectable in leaves, in seeds and flowers.

1.5.2. Carbon metabolism in AM fungi and assimilation

It is generally thought that in AM symbiosis, the plants benefit from the fungal presence with a better P supply and a better growth, to cite the most important examples. In turn, as an obligate biotroph, the mycorrhizal fungus has to get carbon from the plant, making the flux of nutrients bi-directionnal. The process of C allocation is allowed with important modifications on both parts and involves complex mechanisms that have not been elucidated so far. A step forward was done with the identification of glucose and to a lesser extent of fructose, as the major C compounds to be taken up by intraradical AM hyphae at the plant-fungal interface (Shachar-Hill et al., 1995). Further experiments revealed that glucose was transformed by the fungus into glycogen and trehalose, this latter being the most abundant carbohydrate within fungal spores (Bago et al., 1998). The studies of Pfeffer and collaborators (1999) revealed that the symbiotic and asymbiotic stages were quite different with respect to C metabolism. Carbohydrate analysis suggests that the intraradical hyphae can take up hexose and serves as major sites for lipids synthesis, providing the lipids to the extraradical mycelium and the newly-formed vesicles. No uptake of hexoses has been detected in the extraradical phase of the fungus. The developing germ tubes, representative of asymbiotic stage, use hexose but less effectively than the intraradical hyphae do (Bago et al., 1999; Pfeffer et al., 1999). In the germinating spores, gluconeogenesis based on degradation of storage lipids seems to be the major metabolic pathway.

On the fungal side, the process of carbon uptake has not been elucidated so far. Does the fungus require an active transport mechanism similar to the one of the plant? Or does it take the carbon by facilitated diffusion as in yeast? In the EM fungus *Amanita muscaria,* known to use both fructose and glucose that are released from sucrose cleavage by a plant

invertase (Chen and Hampp, 1993), a hexose transporter which seems to be responsible for sugar uptake (Nehls et al., 1998). Another example to illustrate the translocation of host metabolites to a fungus is the infection of plant cells by powdery mildew or rust. Their parasitic mycelium consists of intercellular hyphae that develop haustoria to penetrate host cells. These structures are supposed to play a special role in nutrition (Hahn and Mendgen, 2001). The expression of the protein encoded by a hexose transporter (HXT1) gene of the rust fungus, *Uromyces fabae* has been detected only in haustoria concomitantly with an increased H^+-ATPase activity (Hahn and Mendgen, 1997; Mendgen et al., 2000).

1.5.3. Hypothesis on source-sink relations in AM symbiosis

Because they are obligate symbionts, the AM fungi can exert a significant effect on the carbon economy of the plant (Smith and Read, 1997). Several studies have shown that support of the symbiosis requires the transfer of an additional 4-20% of the total net fixed by the plant (Koch and Johnson, 1984; Wang et al., 1989). This demand can be seen as a "cost" of the symbiosis; it can also be seen as an enhancement of sink strength. A stimulation of the process of C assimilation by mycorrhiza would reduce or eliminate the cost imposed on the plant's C economy and would contribute to the overall benefit derived from association with a symbiont (Fitter, 1991; Tinker et al., 1994). The mechanisms involved in this possible enhancement of sink strength have not been investigated in depth except in a few studies where several hypotheses were discussed. The study of Wright and collaborators (Wright et al., 1998) presented a manipulation of mycorrhizal and non-mycorrhizal clover plants, done with the aim of producing similar foliar N and P status, size, and growth rate in order to examine the effect of the fungal symbiont on the pattern of assimilation and allocation of C in the plant. An increased C allocation to roots of mycorrhized plants was observed, coupled to a stimulation of the activities of cell wall and cytoplasmic invertases and of sucrose synthase in roots colonized by AM fungi. The authors suggested that mycorrhizal colonization stimulates the activities of sucrose synthase and cytoplasmic and cell wall invertases in order to increase the sink activity of mycorrhized roots. This may result in the partitioning of the

additional C fixed to the mycorrhizal root system (Wright et al., 1998). Along the same line, the regulation of arbuscule formation by carbon in the plant has been proposed as a model by Blee and Anderson (1998). They followed the hypothesis that the arbuscule is the key site for nutrient exchange between the plant and its symbiotic partner. The location and function of arbuscules in cortical cells would be regulated by sink strength created by carbon availability. The authors address the question if altered expression of genes encoding sucrose-cleaving enzymes creates a sink for sucrose in arbusculated cells. Vacuolar invertase and cytoplasmic sucrose synthase in catalyzing the hydrolysis of sucrose would maintain a gradient for symplastic influx of sucrose in the cell. Then, a gradient for efflux of the product of sucrose cleavage would be created in direction of the apoplast where the fungus could by supplied by these hexoses (Blee and Anderson, 1998). Although being speculative and needing further experiments to be confirmed, this hypothesis is an interesting model for a better understanding of the importance of carbohydrates metabolism in the AM symbiosis.

1.6. OUTLINE OF THE THESIS

The AM symbiosis is of fundamental importance in nature, and so it is necessary to understand the molecular mechanisms underlying its development. This symbiosis is of great interest to agriculture with its effects on promoting plant growth, on bioprotection, on detoxification of polluted soils, but it is important as well in fundamental research to understand mechanisms of plant-microbe interactions, of signaling events and of processes in morphogenesis and cellular differentiation. Therefore, it has been studied more and more since 15 years in ecological, physiological and molecular projects. The AM symbiosis shares similarities and common pathways with the *Rhizobium*-legume interaction that are of great concern for grain and forage legumes used in agriculture. A better knowledge of both symbioses is particularly useful to improve practices in sustainable agriculture.

In this thesis, I did an untargeted approach of RT-PCR differential display in order to identify plant genes involved in an AM symbiosis. In Table 1.1., the genes already identified in a mycorrhizal context are summarized. The colonization was performed

between *Medicago truncatula* and the AM fungus, *Glomus intraradices* produced in vitro. The choice of *Medicago truncatula* to investigate molecular mechanisms particularly in an AM symbiotic context was first motivated by its capability to undergo both the mycorrhizal and the rhizobial symbioses, and secondly because it became in the last recent years a model plant for research purposes. A clone having homology with sucrose synthase have been found and its characterization has been done in *Medicago truncatula* with regards to mycorrhiza and nodulation in a time-course and in the different plant organs. The effects of the corresponding gene on symbiosis development were investigated with the use of pea and maize mutants deficient in the sucrose synthase encoding genes.

Table 1.1: Overview on mycorrhiza-regulated plant genes

GENE	Plant	Putative function	Expression during AM	Reference
LePT1	*Lycopersicum esculentum*	Phosphate transport	Up-regulated in the cells containing arbuscules	(Rosewarne *et al*., 1999)
MtPT1/MtPT2	*Medicago truncatula*	Phosphate transport	Down-regulated	(Liu *et al*., 1998)
MtPT4	*Medicago truncatula*	Phosphate transport	Repressed upon AM colonization	(Burleigh and Harrison, 1997).
StPT3	*Solanum tuberosum*	Phosphate transport	In mycorrhiza-colonized root sectors	(Rausch *et al*., 2001)
BMR78	*Hordeum vulgare*	H^+-ATPase	Up-regulated in the cells containing arbuscules	Murphy *et al*., 1997
Nitrate reductase	*Zeae mays*	Nitrate reduction	Down-regulated in mycorrhizal roots	(Kaldorf *et al.,* 1998)
MsENOD40/MsENOD2	*Medicago sativa*	Early nodulins	Induced in mycorrhizal roots	(van Rhijn *et al*., 1997).
PsENOD5/PsENOD12A	*Pisum sativum*	Early nodulins	Activated by AM fungi	(Albrecht *et al.,* 1998)
SYM8	*Pisum sativum*	Lectinelike glycoprotein, essential for induction of PsENOD5/PsENOD12A	Activated by AM fungi	(Albrecht *et al*., 1998)
PsNlec1	*Pisum sativum*	Lectine-like glycoprotein	In root nodules and cells containing arbuscules	(Kardailsky, 1996; Balestrini *et al*., 1999)

VfLb29	*Vicia faba*	Leghemoglobin	In root nodules & AM colonized cells	(Frühling *et al*., 1997)
MtENOD11	*Medicago truncatula*	Early nodulin	In inner cortical cells containing arbuscules.	(Journet *et al*., 2001)
MtENOD12	*Medicago truncatula*	Early nodulin	In epidermal, cortical cells upon mycorrhiza	(Journet *et al*., 2001)
α-tubulin	*Zea mays*	Reorganization of colonized cells	In mycorrhized cells	(Bonfante *et al*., 1996)
PcRB7	*Petroselinum crispum*	Membrane intrinsic protein, passive transporters	In mycorrhized roots	(Roussel *et al*., 1997)
psam1	*Pisum sativum*	May regulate the activity of Ca^{2+}-ATPase	In mycorrhized roots	(Martin-Laurent *et al.,* 1997)
Mt.AM1	*Medicago truncatula*	Shares homologies with AGPs	Induced exclusively in cells containing arbuscules	(van Buuren *et al*., 1998)
Mt.AM2	*Medicago truncatula*	XET protein	Detected under considerable colonization in roots	(van Buuren *et al*., 1998)
Mt.AM3	*Medicago truncatula*	Shares sequences similarity with a human translation initiation factor	Detected early in AM colonized roots	(van Buuren *et al*., 1998)
Acidic chitinases	*Nicotiana tabacum*	Symbiosis-related proteins	Induced by AM fung*i*	(Dumas-Gaudot *et al*., 1992)
Basic chitinases	*Nicotiana tabacum,*	Symbiosis-related proteins	Suppressed by *G. intraradices*	(David *et al*., 1998)
Class III-2,3,4 chitinases	*Medicago truncatula*	Symbiosis-related proteins	Induced in AM colonized roots	(Salzer *et al*., 2000)
PR-1	*Nicotiana tabacum*	Unknown	In colonized cells	(Gianinazzi-Pearson *et al*., 1996b).
Mtst1	*Medicago truncatula*	Hexose transporter	In the cortical cells under mycorhhiza	(Harrison, 1996)
PAL/CHS	*Medicago truncatula*	Phenylalanine ammonia lyase and chalcone synthase	Accumulation of transcripts around arbuscules	(Harrison and Dixon, 1994)
IFR	*Medicago truncatula*	Isoflavone reductase	Decrease level of transcripts around arbuscules	(Harrison and Dixon, 1994)

II. MATERIAL AND METHODS

2.1. BIOLOGICAL MATERIAL

Medicago truncatula Gaertn. cv. Jemalong, strain A17, was provided by T. Huguet (INRA-CNRS, Castanet-Tolosan, France). The seed was surface sterilized with 95-97% of H_2SO_4 for 5 min, washed several times with sterile water and pre-germinated for 5 days on 1% water agar plates at 20°C in the dark. The seedlings were transferred in single plastic pot and grown on Terra Green (Maagtechnic, Zürich, Switzerland) in a climate chamber (22°C, 16 h light, 100 µmoles photons $m^{-2}s^{-1}$). The plants were fertilized with B&D medium (Broughton and John, 1979) containing 0.5 mM K_3PO_4 and 2mM KNO_3 at every watering and harvested at the time-points indicated.

Pisum sativum L, isogenic lines, one carrying the wild-type and the other carrying the mutant allele at the *rug4-b* locus (Wang and Hedley, 1993a) originated from the laboratory of T. Wang (John Innes Centre, Norwich NR4 7UH, United-Kingdom). The seeds were soaked 8 hours in water and surface sterilized for 5 min in 0.125% NaOCl.
After intense rinsing with sterile water, the pea seeds were pre-geminated in the dark at 26 °C. The 5-days-old seedlings were planted in individual plastic pots on Terra Green and grown for 8 weeks in a climate chamber with the same conditions as described for *Medicago* plants and fertilized with B&D as described.

The four near isogenic lines of maize (***Zeae mays*** L.), Sh Sus, Sh sus, sh Sus, sh sus were kindly provided by P.Chourey (Department of Plant Pathology, University of Florida, Gainsville, FL 32611-0680,USA). Isolation of the double recessive, sh1 sus1-1, genetic stock was produced from an F2 ear obtained by selfing the dihybrid, Sh1sh1 Sus1sus1-1 (Chourey and Taliercio, 1994). All the different maize lines were in the W22 inbred background. The kernels were surface-sterilized in 3.5% NaOCl, rinsed 7-8 times with sterile water. They were then disposed on two layers of filter paper in petri dishes. Before putting the plates at 26 °C for 4 days, 2 ml of sterile water were added to each of them. After pre-germination, maize seedlings were grown in individual pots containing soil and

placed in a climate chamber with the following conditions (16 hours light at 100 µE $m^{-2}s^{-1}$ 20°C, 8 hours dark at 18°C) During the 12 weeks experiment, the maize plants were fertilized weekly with low phosphate fertilizer (32% total N, 6% P 20% K_2O, 2% Mg, 0.1% Fe, 0.05% Mn, 0.01% Mo, 0.02% B, 0,04% Cu, 0.01% Zn) and harvested at the time-points indicated.

The arbuscular mycorrhizal fungus, ***Glomus intraradices***, Schenck and Smith, was kindly offered by G. Bécard (Université Paul Sabatier, Castanet-Tolosan, France). This in-vitro inoculum was originally obtained by inoculation of Ri transformed *Daucus carota* roots by *Glomus intraradices* spores (Bécard and Piché, 1992). The *Glomus intraradices* inoculated roots were subcultured every two months onto M medium (Bécard and Fortin 1988) containing 0.3% Phytagel (Sigma, St Louis, MO, U.S.A) and kept at 26 °C.

The ***Rhizobium***-legume symbiosis was established between *Sinorhizobium meliloti* (strain 1021) and *Medicago truncatula.* The bacterial strain, provided by P. Curioni (ETH, Zürich, Switzerland) was cultured for 3 days in liquid succinate minimal medium (Schmidt et al., 1992) at 140 rpm at 27°C.

Escherichia coli strains DH5α were grown either on LB agar plates or in LB liquid medium at 37 °C under rotary shaking at 180 rpm. LB medium consisted of 10 g NaCl, 10 g tryptone and 5 g yeast per liter. According to the protocol of Sambrook (Sambrook et al., 1989), 2.5 % agar was added to LB liquid medium before autoclaving and bacteria were stored on LB agar plates at 4 °C and as glycerol stock at –80°C. Competent cells were as well prepared according to the protocol cited previously.

2.2. PLANT TREATMENTS

2.2.1. Inoculation with *Glomus intraradices*

Fungal material was separated from its culture medium by dissolving the Phytagel in 10 mM citric acid, 10 mM Na-citrate, pH 6 at 37°C. Spores were collected in water by sieving the suspension through a 33 µm mesh (Doner and Bécard, 1991). This inoculum was then adjusted to 600 spores/ml and used for all in vivo experiments. For every model plant used in this work, the seedlings were transferred to pots and the spore suspension was applied directly on the roots while planting. As control, water was poured on the roots of each control seedling. At indicated time-points, the plants were harvested, the root system was gently washed in water and dried between two layers of paper. Roots and shoots were weighted separately and sampled. The different samples obtained were either immediately frozen and kept at –80°C or used directly to estimate the degree of colonization.

2.2.2. Inoculation with *Sinorhizobium meliloti*

The overnight bacterial liquid culture of *Sinorhizobium meliloti* was diluted with one volume of culture medium and 3 ml were used to inoculate every seedling transferred into an individual pot. At the times indicated, the plants were collected and sampled as described in previous chapter.

2.3. DETERMINATION OF ROOT COLONIZATION

Root samples were cleared in 10% KOH at 95°C for 5 to 15 min and stained in 0.1% trypan blue (Philips and Hayman, 1970) for 20 min. The fungal structures were visualized under a Zeiss Axioplan microscope and the root colonization by *Glomus intraradices* was estimated by the gridline intersection method (Giovanetti and Mosse, 1980).

2.4. RNA EXTRACTION

2.4.1. Method A

From *Medicago truncatula* plants, total RNA was extracted according to the method described in Mohr (Mohr et al., 1998). This hot phenol method started with grinding 1g of frozen plant material with a pestle in a pre-cooled mortar under liquid nitrogen. The ground powder was transferred to 10 ml of 80°C preheated extraction buffer, 1:1 water-saturated phenol: 2x NETS (200 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5 and 1% SDS). After incubation on ice for 10 min, the preparations were centrifuged at 500*g* for 10 min at room temperature. The upper phase was transferred to a 5 ml tube containing water-saturated phenol and vortex for 30 sec. From a new centrifugation for 10 min at 500*g*, the supernatant was collected in a 30 ml Corex tube and the nucleic acids precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of ice cold 100% ethanol. The mixture was stored at –20°C for 1h to overnight and the nucleic acids were then collected as a pellet by centrifugation at 10’ 000*g* for 15 min at 4°C. The RNA was then resuspended in 6 ml water and transferred to a 15 ml Corex tube, 6 ml of 5 M LiCl were added and the mixture incubated at –20°C for 2h. The precipitated RNA was collected by a 10’000*g* centrifugation for 20 min at 4°C and the pellet was re-dissolved in 600 µl DEPC treated water. In order to repeat the precipitation step, 600 µl of LiCl were added to the re-suspended RNA and the mixture was incubated at –20°C for 2 h. The RNA pellet was finally obtained by centrifugation at 10’000 rpm for 20 min at 4°C and re-suspended in 100µl water. The OD_{260} and the ratio of OD_{260}/OD_{280} were measured to determine the concentration of RNA preparation and its purity.

2.4.2. Method B

Another method for RNA extraction was used particularly to extract RNA from maize roots. This procedure was used routinely also for extraction of RNA from *Medicago*

truncatula tissues. Two g of frozen root material were ground in pre-cooled mortar and pestle under liquid nitrogen and the powder obtained was extracted in 20 ml of TRIzol Reagent (Life Technologies, Cincinnati, U.S.A), a mono-phasic solution containing phenol and guanidine thiocyanate. The homogenized samples were incubated for 5 min at room temperature. After addition of 0.2 ml of chloroform per 1 ml of TRIzol Reagent, the mixture was shaked vigorously for 15 sec and incubated at room temperature for 2-3 min. The two phases were separated by a centrifugation at 12'000*g* for 15 min at 2 to 8°C in 15 ml Corex tubes and the RNA was collected from the upper aqueous phase. RNA precipitation from this aqueous phase was done in a new Corex tube, by mixing with 0.5 ml of isopropyl alcohol per 1 ml of TRIzol followed by a centrifugation at 12'000*g* for 10 min at 2 to 4°C. RNA pellet was then washed once with 20 ml of 75% ethanol and dissolved, after brief drying, in 100 µl of RNase free water. The OD_{260} and the ratio of OD_{260}/OD_{280} were measured to determine the concentration of RNA preparation and its purity. The RNA integrity was checked on a 0.8% agarose gel.

2.5. DIFFERENTIAL DISPLAY OF MRNA (DDRT-PCR)

Total RNA extracted as described in Method A was treated with DNaseI according to the Message Clean kit (GenHunter, Brooklin, USA). According to the protocols of Liang and Callard (Callard et al., 1994; Liang et al., 1994), mRNAs were reverse transcribed for 60 min at 37°C. The reverse transcription (RT) mix consisted of 0.2 µg of DNase I treated RNA, 1 µM final concentration of H-T11-A, H-T11C or H-T11G anchor primer. One x RT buffer (25 mM Tris-HCl, pH 8.3, 38 mM KCl, 1.25 mM MgCl2, 5 mM DTT) and 20 mM of each deoxynucleotide triphosphate in 20 µl final reaction volume. After 5 min at 65°C and 10 min at 37°C, 1 µl MMLV reverse transcriptase (100 U/ul) was added; after 50 more minutes at 37°C, the reaction was stopped incubating 5 min at 75°C. The first strand cDNAs obtained were then PCR amplified with the same anchored primer as for reverse transcription and with one of 18 different arbitrary primers (0.25 µM final concentration) and 1 µCi of ^{33}P(dATP).

The PCR was run for 30 cycles with a denaturation step at 94° for 30 sec, annealing step at 40°C for 2 min, and polymerization at 72 °C for 30 sec and was followed by a final elongation step at 72°C for 5 min.

PCR products aliquots of 3.5 µl then separated by electrophoresis on a 7 M urea/6% acrylamide sequencing gel (Sambrook et al. 1989). The gel was fixed onto 3 MM Whatman paper, dried, and exposed overnight to a Kodak X-RAY-OMAT film. Bands of interest were cut out of the gel and the cDNA fragments were eluted from the gel according to the protocol of Johnson et al., (1995). In Eppendorf tubes, gel slices were mixed with 100 µl TE and incubated for 2 h at room temperature, 1 h at 37°C and several hours up to overnight at 4°C. After centrifugation at 13'000g to pellet the debris, the supernatant was collected into a new tube and the cDNA precipitated with 10 µl 3M sodium acetate, pH 5.2, 5 µl glycogen (10 mg/ml) and 450 µl of 100 % ethanol. The mixture was incubated at –80°C for 40 min. The cDNA was pelleted by 10' centrifugation at 4°C, washed with 200 µl ice-cold 85% ethanol and re-suspended in 10 µl water. These cDNA fragments, called differentials, were re-amplified by PCR with the same arbitrary primer used to obtain the differential of interest (Vögeli-Lange et al., 1997).

2.6. CLONING PROCEDURE

The re-amplified fragments were cloned into the pCR-TRAP vector (GenHunter). According to the manufacturer's instructions, 5 µl of PCR products were ligated in the insert-ready pCR-TRAP vector with T4 DNA ligase (200 units/µl). Competent *E. coli* cells were transformed with the ligation products by heat shock and plated on LB agar plates containing 20 µg/ml of Tetracycline as selective marker. To identify colonies containing ligated inserts, colony PCR was performed according to GenHunter procedure and the primers provided by the kit were used for the amplification steps. The PCR products were then analyzed by electrophoresis according to standard procedures Sambrook et al. (1989) and size fractionated through 0.8-1.5% agarose gels (Seakem LE, FMC, Rockland, ME, USA) containing 0.5 µg/ml of Ethidium Bromide. The bands of interest were compared to a 100 bp ladder (Pharmacia) on the agarose gel.

2.7. REVERSE NORTHERN-BLOT ANALYSIS

The screening of cDNA fragments derived from differentially expressed genes was performed by using Reverse Northern method as described by Vögeli-Lange (Vögeli-Lange et al., 1996). For this procedure, a NY 13 N membrane, Schleicher and Schuell, Dassel, Germany) was floated in water and in 6x SSC and placed into a convertible filtration manifold equipped with a 48 well slot top plate (BRL, Gaithersburg, MD, USA). The differentials obtained were cloned and re-amplified by colony PCR, 19 µl of these PCR products were heat to 95°C for 5 min and then supplemented with 475 µl of 10x SSC (1X SSC is 0.15 M NaCl, 15 mM Na-citrate). This mixture was applied in aliquots of 156 µl to two slots of the slot-blot machine. Each blot was washed twice with 0.5 ml 10x SSC. After being removed from the slot blot apparatus, the membrane was floated for 7 min on a solution of 0.5 M NaOH, 1.5 M NaCl and twice for 3 min on a solution of 0.5 M Tris-HCl pH 7.5. 1.5 M NaCl. The membrane was washed briefly in 2x SSC and cut into two identical sets and baked for 1-2 h at 80°C in a vacuum oven. The membranes were incubated for 1-4 h at 60°C in 5 ml of pre-hybridization solution (5x SSC, 5x Denhardt's solution, 0.5% SDS and 100 µg/ml of denatured salmon sperm DNA). This solution was discarded and replaced by 4 ml of fresh hybridization solution. After boiling for 5 min, the original (^{33}P)-PCR reaction from the control sample was added to one membrane and the original (^{33}P)-PCR reaction of the inoculated sample was added to the second membrane. The hybridization was taking place overnight at 60°C under rotation. Different washings steps were then performed: 10 min at 54°C in 2X SSC, 0.1% SDS, 10 min at 65°C in 2x SSC, 0.1% SDS and finally, 10 min at 65°C in 0.5x SSC, 0.1% SDS. The membranes were at the end exposed for 4 to 8 h to BioRad Phosphor Imager high sensitivity screen.

2.8. DNA SEQUENCING AND ANALYSIS

To determine the sequence of differentials obtained by DD RT-PCR, the ABI protocol was followed (Applied Biosystems, Inc., Foster, CA, USA). The procedure consisted in amplifying by PCR 0.5 µg of DNA in 4 µl of sequencing reagent (ABI), 1 µl of sequencing primer (0.1 µM final concentration of either specific plasmid M13 primer forward or specific M 13 primer reverse) The final volume of the reaction was 10 µl. The PCR conditions were the followings: 94°C for 30 sec, 50°C for 15 sec, 60°C for 4 min for 30 cycles and 72°C for 5 min. The PCR products were then precipitated with 2.5 volumes of 100% ethanol, 1/10 volume of Na-acetate 3 M pH 5 at –20°C. After centrifugation the DNA pellets were washed and resuspended in 35 µl of template suppressing reagent (ABI) and the samples were boiled for 5 min. The sequencing analysis was automatically done in an ABI Genetic Analyzer 310. The DNA sequences were then analyzed with Genscan 2.1 for ABI Prism 310 and Sequencing analysis 3.0 or EditView 1. Sequence comparisons and alignment were performed using the GCG program (Genetics Computer Group, Madison, Madison WI, USA) on a UNIX server and Database searches were allowed with the help of BLAST network services of the National Center for Biotechnology Information.

2.9. RT-PCR PROCEDURE

This procedure was carried out according to the protocol of the Ready-To-Go RT-PCR beads of Amersham Pharmacia Biotech Inc, USA.

The RT-PCR was performed with Ready-To-Go RT-PCR beads that are stable at room temperature. Each bead contained all of necessary reagents except primer and template. The two-step protocol consisted in dissolving each bead with 44 µl of room temperature DEPC-treated water. The dissolved bead content was transferred to a PCR tube with 1 µg of the RNA template and the oligo dT primer (10 ng/µl final concentration) for first-strand cDNA synthesis. The reaction was put in a thermal cycler and incubated for 30 min at 42°C. The reverse transcriptase was inactivated and the DNA template completely denatured for 5 min at 95°C. The second step consisted in adding the two gene-specific

primers (0.1 µM final concentration) for PCR. The reaction was then cycled 32 times with denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min and polymerization at 72°C for 1 min. 10 µl of PCR products were then loaded on an agarose gel and photographed under UV light.

2.10. IN-SITU HYBRIDIZATION

The RNA analysis was performed according to the introduction and advices of Andy Fleming (ETH, Zürich, Switzerland) and following the protocol of Coen (Coen et al., 1990). All the manipulations described in this sub-chapter were done with DEPC-treated water and with all precautions necessary to avoid RNase contamination. This procedure was consisting in a tissue fixation of root tissues in 4% (w/v) formaldehyde (Polysciences 16% stock, EM grade) in final 1xPBS (phosphate buffered saline: 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4 pH 7.4 (Sigma P3813). Thereafter, the fixative solution was removed and the tissues were dehydrated step-wise in Histoclear/EtOH gradient-25%, 50%, 75%, 100% Histoclear. Finally the samples were embedded at 60°C in paraffin wax (Paraplast Plus, Sigma, St Louis, MO). The embedded tissues were cut with a microtome in 7 µm sections and mounted onto the center of a poly-L lysine coated slide (Polysine slides, BDH 406/0178/00), after drying overnight at 42°C, the slides were used immediately for in-situ hybridization or stored at room temperature. The sections were deparaffinized in Histoclear, rehydrated through an ethanol series and incubated with 14 µl of proteinase K (Pharmacia, 1 µg/ml) at 37°C for 30 min. The sections on slides were then treated with 0.25% acetic anhydride and dehydrated in an ethanol series from 30 to 100%. In the meantime, the appropriate hybridization buffer was prepared and consisted in 200 µl of 10x salts, 800 µl of Formamide, 400 µl of 50% Dextran sulphate, 40 µl of 50x Denhardts, 20 µl of 100 mg/ml tRNA, and 140 µl of water. The mounted samples were hybridized with appropriate denaturated, DIG-labeled RNA probes overnight at 50°C and then washed with 0.2x SSC at 55°C. After treatment with RnaseA for 30 min at 37°C, the slides were washed again at 55°C with 0.2x SSC. The RNA hybridized probe was detected by enzyme-linked immunoassay using an antibody conjugate, anti-DIG-AP (anti-digoxigenin alkaline phosphatase conjugate, Boehringer Manheim). A subsequent

enzyme-catalysed color reaction with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium salt) produced an insoluble blue precipitate, which visualized hybrid molecules. The DIG-antigen revelation consisted in washes in 0.5% blocking reagent in TBS (100 mM Tris-HCl, pH 7.5, 400 mM NaCl) for 5 min, followed by an incubation in the anti-DIG alkaline phosphatase conjugate diluted in BSA (bovine serum albumine) and a revelation color by incubating in NBT and X-phosphate for periods of 4 h to 2 days in the dark. The reactions were stopped with washing twice in 10 mM Tris-HCl pH 7 and mounted in DePeX with coverslip.
The DIG-labeled riboprobes (antisense and sense probes) used for in-situ hybridization were synthetized according to the manufacturer's protocol (DIG RNA labeling Kit, Boehringer Manheim). The probes utilized in this experiment were kindly provided ready to use by Andy Fleming (ETH, Zürich, Switzerland).

2.11. SUGAR EXTRACTION

200 mg of maize roots were incubated in 1 ml of 80% ethanol for 5 min at 80°C. The samples were then dried and ground in eppendorf tubes. Polyvinylpyrolidone was added to the ground samples as well as 750 µl of 80% methanol and 75 µl of 1 mg/ml of mannoheptulose as internal standart. After 10 min at 60°C, the supernatant was transferred to a new eppendorf and the extraction step was repeated. After drying in speed-vac, the pellet was resuspended in 600 µl water and supplemented with ion exchange beads. After several centrifugation steps, 100 µl of the extract were put in vials. The samples were analyzed by high-performance liquid chromatography (HPLC) using anion exchange column and pulsed amperometric detection as described in Lüscher et al., 2000.

III. MtSucS2 - A sucrose synthase differentially expressed in Medicago truncatula during AM symbiosis

3.1. INTRODUCTION

The arbuscular mycorrhiza (AM) is a symbiotic association that occurs between roots of the majority of terrestrial plants and the fungi from the order Glomales (Harley and Smith, 1983). The AM symbiosis is thought to be beneficial for the two partners through a mutual exchange where the plant supplies the fungus with carbon and in turn receives phosphate and other nutrients from the soil (Smith and Smith, 1997). AM associations have been shown to influence plant diversity within an ecosystem (van der Heijden et al., 1998) and the plant community structure (Bever et al., 2001). Moreover, AM associations are of great importance for sustainable agriculture practices and enhance to some extent the plant's resistance to root pathogens (Newsham et al., 1995).

Early investigations on gene expression during this tight relation between the plant and the obligate symbiont revealed that the colonization of root tissue is accompanied by a transient induction of defense-related genes of the plants (for review see (Gianinazzi-Pearson et al., 1996). To find other genes induced in plants during AM symbiotic events, the approaches making use of differential display or substractive library screens have been particularly fruitful (for review see (van Buuren et al., 2000). For example, in *Medicago truncatula*, a sugar transporter (Harrison, 1996), three clones encoding an HRGP (hydroxyproline rich glycoprotein), a putative arabinogalactan protein (AGP), and a member of the xyloglucan endo-transglycosylase (XET) family (van Buuren et al., 1999) all showed increased levels of expression upon AM colonization. Studies on differentially induced genes during AM colonization also lead to the isolation of genes in other species such as a gene encoding a membrane intrinsic protein (MIP) from parsley (Roussel et al., 1997), an ATPase from barley (*Hordeum vulgare*) (Murphy et al., 1997) and recently a gene encoding a phosphate transporter in potato (Rausch et al., 2001). The existence of legume plant mutants deficient in the AM and legume-rhizobia symbiosis indicates that these two interactions share some common pathways and common genes (Hirsch and Kapulnik, 1998; Schultze and Marsh, 2001).

We used a non-targeted approach by differential display reverse trancription PCR (DDRT-PCR) (Liang and Pardee, 1992) in order to isolate plant genes, differentially expressed during the AM symbiosis between *Medicago truncatula* and *Glomus intraradices. Medicago truncatula* L. (barrel medic) is closely related to alfalfa, an important forage legume and has been used as a model species for genomic studies (Cook, 1999). Indeed it posses a small, diploid genome, a short generation time, is self-pollinating and can be genetically transformed. *Medicago truncatula* can serve as a model for economically important legumes, such as soybean since it establishes symbiotic relationships with arbuscular mycorrhizal fungi as well as with nitrogen fixing *Rhizobia*. *Medicago truncatula* is currently the subject of major genomic initiatives. We report here the expression analysis of a new gene coding for a sucrose synthase, MtSucS2 that is specifically induced in AM symbiosis and in typical sink organs. We show that MtSucS2 has a different regulation pattern than the sucrose synthase, MtSucS1, isolated earlier in the context of nodulation (Hohnjec et al., 1999). It appears that similar genes are required in both AM and in nodulation but that the regulatory mechanisms involved in inducing these genes may be at least in part distinct.

3.2. RESULTS

3.2.1. AM symbiosis in roots of *Medicago truncatula* is established between 4 and 6 weeks after inoculation

Pre-germinated five day-old seedlings of *Medicago truncatula* were planted in individual pots and grown in a sterilized substrate. Concomitantly, the plantlets were inoculated with approx. 600 spores of *Glomus intraradices* obtained from *in vitro* culture of carrot roots and harvested at different time-points, after 2, 4, 6, 8 weeks of co-cultivation. The colonization rate was determined using the grid-line intersection method and the abundance and the occurrence of the different fungal structures within the root systems was scored (Table 3.1).

Table 3.1: Development of AM structures in roots of *Medicago truncatula* inoculated with *Glomus intraradices*

	Fraction of the roots found containing AM structures in % after			
	2 weeks	4 weeks	6 weeks	8 weeks
internal hyphae	5	13	18	20
arbuscules	0	18	20	25
vesicles	0	5	40	40
total colonization	5	36	78	85

Two weeks after inoculation, only 5% of the root length showed the presence of internal hyphae. Nevertheless, the presence of some appressoria and growth of fungal hyphae was apparent at the root surface, referred to as external hyphae. Arbuscules were detected after 3 weeks of colonization but intraradical vesicles could be seen only after 6 weeks. According to the different structures present within the roots, it was assumed that the symbiosis was established in all its structures between 4 and 6 weeks.

3.2.2. Differential RNA display and identification of a partial cDNA homologous to sucrose synthase

The DDRT-PCR experiments were performed on total RNA extracted from non-inoculated or *Glomus intraradices* colonized root systems from 6 weeks-old *Medicago truncatula* plants. We used 30 primer combinations of oligo (dT) and random primers and we obtained 35 bands revealing a differential pattern in colonized root systems. The 35 bands representing potentially up-regulated candidates in mycorrhizal roots were cut from the polyacrylamide gels and eluted, cloned. A reverse Northern approach (Vogeli-Lange et al., 1996) was used to eliminate false positive candidates and 3 clones were finally sequenced (Fig.3.1).

Mt-70

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  1 AGCTTCAAGA CCAACTATAT TAGAGCCTGT TTGCTTGTTG AGCTAAAAGT
    H-AP65
 51 CCAAATGAAT TTCAGGGAGC TGTTGCTGGT GACCTCAACA AGAGAAAGGG
101 TATGATTGTT GGCAATGATC AGGATGGAGA TGACTCTGTC ATTATAGCTC
151 ATGTCCCTCT TAACAATATG TTTGGGTACT CTACAGCTCT TCGTTCGATG
201 ACACAGGGAA AGGGTGAATT TACAATGGAA TATAAGGAAC ATTCACCTGT
251 TTCTCATGAT GTACAGACCC AATTGGTAAA CGCATACAAG GGCAATAAAG
301 GCACCTGAAT GATGACTTTG TTGAAGNATA CGGTCCTATA TTTTTTGGGA
351 GTAATTTATC TAGGAAAAAA AAAAAGCTT
                           oligo(dT)11
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Mt-28

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  1 AAGCTTTTTT TTTTTCGAAA AATACGACAA TCCTATTGAG GATGTTGCGT
    oligo(dT)11
 51 TGTAAGAAAA AAGATAATAG GTGAAGAAGA ATAATTTTTT TGAGCCGTGG
101 TTGTTTATTT ATTTTGATAA CTTTATCATA TGAATTCTAT TTTATCATAC
151 AAATCTCTTT TATTCTACCA CCTTCCCGGA GTTGAGTCTC CGGGGAATTT
201 TTATTAAGTT TATGATCTCC TTGGCAATCA AGCTT
                                  H-AP1
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Mt-19.2

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  1 AAGCTTGATT GCCGTGAGAG CAAACGTTAC CTTGAGATGT TCTATGCACT
          H-AP1
 51 TAAGTACAGC AAATTGGCTG AATTTGTGCC TCTTGCTGTT GAAGAGTAAG
101 ATCAATGATG AAGTATGAAG AGATTGCGGG ATTGGCTTTT TGTTGTTTGG
151 AGTTGATGGA GCATTTATAA ATAAAATATT AGTACCATTG ATTTGATATT
201 GAGATTATAT TTTCAATTGG AAAAAAAAAA AGCTT
                             oligo(dT)11
```

Figure 3.1: DNA sequences of 3 cDNAs clones obtained by differential display. The primers used for DDRT-PCR reactions are underlined Mt-70: homologous to mitochondrial elongation factor; Mt 28: homologous to chloroplast ribosomal protein; Mt-19.2: homologous to sucrose synthase.

Sequence similarity searches, revealed that clone Mt-70 had homologies with mitochondrial elongation factor G, clone Mt-28 had homologies with a chloroplast ribosomal protein encoding gene and a partial cDNA clone, and the clone called “19.2” was homologous to a sucrose synthase gene (Table 3.2).

<table>
<tr><th>clone</th><th>BLAST highest score</th><th>sequence identity</th><th>best alignment</th><th>Reference Accession #</th></tr>
<tr><td>Mt70</td><td>EMBL

Oryza sativa mEF-G mRNA for mitochondrial elongation factor G</td><td>88 – 91 %</td><td>Mt28 : 79 ggtgacctcaacaagagaaagggtatgattgttggcaatgatcagga 125
|||||| | ||||||||||||||||| |||||||| |||||||||||
OsmEF-G: 2147 ggtgacatgaacaagagaaagggtatcattgttggaaatgatcagga 2193

Mt28 : 163 aacaatatgtttgggtactctacagctcttcgttcgatgacacagggaaagggtgaattt 222
|||||||||||||| || || ||||||||||| || ||||||||||| || |||||||||
OsmEF-G: 2231 aacaatatgtttggatattcaacagctcttcggtcaatgacacaggggaaaggtgaattt 2290</td><td>Kato et al., 2000

AB040051</td></tr>
<tr><td>Mt28</td><td>EMBL

Medicago truncatula chloroplast, complete genome, ribosomal protein</td><td>96 %</td><td>HC7 : 131 tgattgccaaggagatcataaactaataaaaattccccggagactcaactccgggaaggt 190
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Mtchondrome : 58033 tgattgccaaggagatcataaactaataaaaattccccggagactcaactccgggaaggt 58092

HC70 : 191 ggtagaataaangagatttgtatgataaaatagantttntatgataaagttatnaantta 250
||||||||||| |||||||||||||||||||| || ||||||||||||| || ||
Mtchondrome : 58093 ggtagaataaaagagatttgtatgataaaatagaattcatatgataaagttatcaaaata 58152

HC70 : 251 aataaacaaccacgctcaaaaaaattattcttcttnacctattatntttnttcttacaac 310
||||||||||||||||||||||||||||||||||| ||||||||| ||| ||||||||||
Mtchondrome : 58153 aataaacaaccacgctcaaaaaaattattcttcttcacctattatctttttcttacaac 58212

HC70 : 311 gcaacatcctcaataggattgtc 333
|||||||||||||||||||||||
Mtchondrome : 58213 gcaacatcctcaataggattgtc 58235</td><td>Lin et al., unpublished

AC093544</td></tr>
<tr><td>Mt 19.2</td><td>EMBL

Medicago sativa cold acclimation-specific mRNA sequence pMcig12</td><td>92 %</td><td>19.2 : 84 tgaagagtaagatcaatgatgaagtatgaagagattgc-gggattggctttttgttgttt 142
||||||||||||||||||| |||||| ||||||||||||| |||| |||||||||| ||||||
PMcig12 : 8 tgaagagtaagatcaatgctgaagtgtgaagagattgccgggactggcttttattgttt 67

19.2 : 143 ggagttgatggagcatttataaataaaatattagtaccattga-tttgatattgagatta 201
|||||||||||||||||||||||| ||||| ||||||||||||| ||||||||||||||||
PMcig12 : 68 ggagttgatggagcatttataaat-aaataatagtaccattgattttgatattgagatta 126

19.2 : 202 tatttt 207
| ||||
PMcig12 : 127 tgtttt 132</td><td>Ivashuta et al., unpublished

AF411549</td></tr>
<tr><td></td><td>EMBL

Pisum sativum mRNA for sucrose synthase isoform 3 (sus3 gene)</td><td>92 %</td><td>19.2 : 5 gccgtgagagcaaacgttaccttgagatgttctatgcacttaagtacagcaaattggctg 64
|||||||||||||||| ||||| |||||||| |||||||| || ||||||||||||||||
Ps sus3 : 2403 gccgtgagagcaaacgctacctcgagatgttttatgcactcaaatacagcaaattggctg 2462

19.2 : 65 aatttgtgcctcttgctgttgaagagtaa 93
||| |||||||||||||| |||||||||
Ps sus3 : 2463 aatctgtgcctcttgctgtggaagagtaa 2491</td><td>Barratt et al., 2001

AJ311496</td></tr>
<tr><td>Mt 19.2</td><td>TIGR *Medicago trucatula* ESTs

tentative consensus (TC)
sucrose synthase, closely related to *Pisum sativum* sus3</td><td>99 %</td><td>19.2 : 207 AAAATATAATCTCAATATCAAATCAATGGTACTAATATTTTATTTATAAATGCTCCATCA 148
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TC31898 : 176 AAAATATAATCTCAATATCAAATCAATGGTACTAATATTTTATTTATAAATGCTCCATCA 235

19.2 : 147 ACTCCAAACAACAAAAAGCCAATCCCGCAATCTCTTCATACTTCATCATTGATCTTACTC 88
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TC31898 : 236 ACTCCAAACAACAAAAAGCCAATCCCGCAATCTCTTCATACTTCATCATTGATCTTACTC 295

19.2 : 87 TTCAACAGCAAGAGGCACAAATTCAGCCAATTTGCTGTACTTAAGTGCATAGAACATCTC 28
|||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
TC31898 : 296 TTCAACAGCAAGAGGCACAGATTCAGCCAATTTGCTGTACTTAAGTGCATAGAACATCTC 355

19.2 : 27 AAGGTAACGTTTGCTCTCACGGCAATC 1
|||||||||||||||||||||||| |||
TC31898 : 356 AAGGTAACGTTTGCTCTCACGGCGATC 382</td><td>TIGR *Medicago truncatula* Gene Index (MtGI)

TC31898</td></tr>
<tr><td>Mt 19.2 peptide</td><td>SwissProt / EMBL

Pisum sativum sucrose synthase isoform 3 (Sus3)</td><td>90 %</td><td>19.2 pep : 2 LDCRESKRYLEMFYALKYSKLAEFVPLAVEE 32
L+ RESKRYLEMFYALKYSKLAE VPLAVEE
Ps Sus3 : 774 LERRESKRYLEMFYALKYSKLAESVPLAVEE 804</td><td>Barratt et al., 2001
Q9AVR8</td></tr>
</table>

Table 3.2: Best sequence alignment search results with the "differential clones" obtained.

Based on these findings, clone 19.2 was chosen for further analysis. Fig. 3.2 presents the original finding of the induction of the 19.2 band in DDRT-PCR.

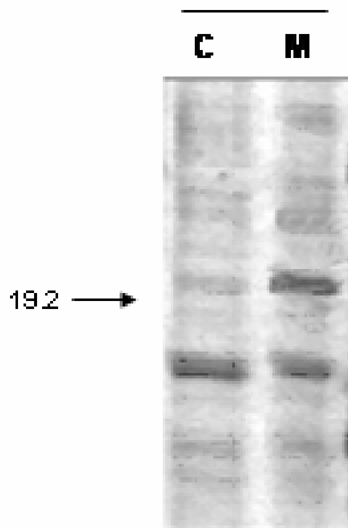

Figure 3.2: RT-PCR Differential Display analysis of mRNAs from *Medicago truncatula* roots. The banding pattern represents mRNAs expressed in control and mycorrhizal roots. (19.2) represents the differentially expressed clones in mycorrhizal roots.

Fig. 3.3 shows that clone 19.2 is indeed induced in mycorrhizal roots, based on a reverse Northern-blot experiment.

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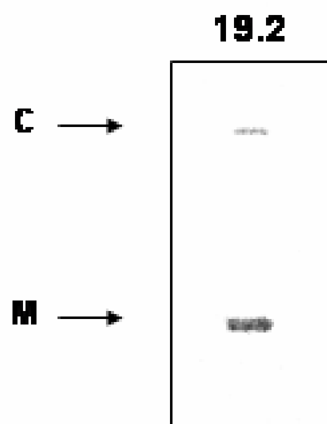


Figure 3.3: Reverse Northern-blot of the differential clone 19.2.
PCR amplified inserts of two individual *E.coli* obtained by transformation with the differential 19.2 were loaded onto two membranes. One membrane was hybridized with the ^{33}P –PCR reaction from the control roots (Con) and the other one with the ^{33}P-PCR reaction from the *Glomus intraradices* infected roots (Myc).

3.2.3. Clone 19.2 is a partial cDNA of a sucrose synthase

The mycorrhiza up-regulated 207 bp clone "19.2" was subjected to several searches using the BLAST network services (BLAST: basic local alignment search tool) (Altschul et al., 1990). Clone "19.2" mapped to the 3' end of more than 25 sucrose synthase mRNAs from several plant species in the EMBL gene bank including *Medicago truncatula* SucS1 (Hohnjec et al., 1999). The highest homology was found with a sequence encoding a putative sucrose synthase from *Medicago sativa* (92% identity over 117 bp at the 3'end; EMBL accession # AF411549). At the 5' end of clone "19.2" the identity was 92% over 80 bp with the *Pisum sativum* mRNA for the sus3 gene (AJ311496) and 88% over 85 bp with a *Lotus japonicus* sucrose synthase mRNA (LJA133726).

Using the BLAST service of the TIGR Gene Indices (Quackenbush et al., 2000; Quackenbush et al., 2001), clone "19.2" was found to share 99% identity over its 207 bp with sequence TC31898/9. TC31898/9 is a tentative consensus of the TIGR *Medicago truncatula* Gene Index (MtGI) library, assembled from a series of independently isolated ESTs. This sequence represents a putative second sucrose synthase of *M. truncatula,* closely related to the *Pisum sativum* sus3 gene (Barratt et al., 2001).

The deduced amino acid sequences of clone "19.2" were subjected to a BLAST search at the protein level. Only frames 2 and 3 contained putative ORFs of significant lengths. The deduced amino acid sequence of frame 2 had between 63 and 87% amino acid sequence identity with the C-terminal end of various sucrose synthases, whereas the one of frame 3 had no hits in the BLAST service. Therefore, it appears that clone "19.2" is a partial cDNA of a mycorrhiza up-regulated sucrose synthase in *Medicago truncatula* containing part of the ORF, encoding the 32 most C-terminal amino acids, and ca. 100 bp of the 3' untranslated region. As judged from sequence comparisons in the BLAST results this sucrose synthase is a new form, different from the one described as nodule enhanced (Hohnjec et al., 1999) and most likely it is represented by the TIGR MtGI sequence TC31898/9. Clone "19.2" will hereafter be referred to as MtSucS2.

In order to determine whether MtSucS2 and TC31898/9 are identical, a PCR analysis was performed. Four pairs of primers (P1.1, P2.1, P3.1, P4.2) were designed for amplification of DNA fragments covering most of the putative MtSucS2 gene given by TC31898/9 (Table 3.3). The size of the amplified fragments is shown on the Figure 3.4.

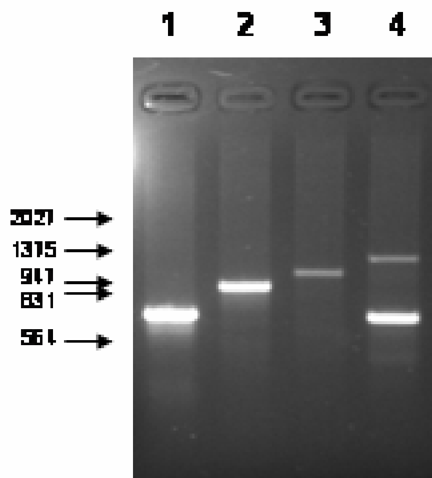


Figure 3.4: Electrophoresis gel of overlapping fragments of the gene TC31898/9 obtained by PCR on genomic DNA. Lane 1, amplicon A1; lane 2, amplicon A2; lane 3, amplicon A3; lane 4, amplicon A4. The positions of the molecular weight marker lambda (Hind III/EcoRI) in kb are indicated on the left.

The identity of the targeted amplicons obtained by PCR was verified by restriction digestion (data not shown), which allowed determining as well an approximate location of introns on the TC31898/9 (Table 3.3).

Primers 5'-3'	**Digestion**	Location (position on TC31898/9)	Expected size from cDNA after PCR / digestion (bp)	Amplicon targeted Name: length (bp)	Conclusion
P1.1 CACAGCCTTGTTGTCGATGA GCGAAGTAACCATGTGGAGA	 *Nco*I *Pst*I	434-993 634 756	559 200 + 359 326 + 233	**A1**: 850 ~ 360 + 500 ~ 550 + 300	Intron located between pos. 452 and 634
P2.1 ATCTTCTCGAGGCACCTGAT CTCCAGTGCATGAGCAATAG	 *Eco*RV	894-1444 1106	550 214 + 336	**A2**: 1100 ~ 450 + 650	Intron ~ 200 bp between pos. 912 and 1106. Intron ~ 300 bp between pos. 1106 and 1426
P3.1 TTGCTCATGCACTGGAGAAG TCAGCAGGACCACCATTACA	 *Cla*I	1428-2123 1661	695 234 + 461	**A3**: 1200 ~ 570 + 250 + 300	Intron of 500 bp after pos. 695 containing a *Cla*I site
P4.2 ATGTACGGCCTAATCGAGAC ACAGCAAGAGGCACAGATTC	 *Hind*III *Eco*RI	2006-2538 2145 2073, 2297	532 139 + 393 67 + 224 + 241	**A4**: 700 ~ 150 + 625 ~ 250 + 450	Contains two ~ 150 bp introns between pos. 2145 and 2520 and between pos. 2485 and 2582 *or* one intron between 2485 and 2520
19.2F/R GATTGCCGTGAGAGCAAAC GAGGTAGTTGAGGTTTGTTG		2467-2600	133	**19.2**: 300	

Table 3.3: Amplicons of the putative MtSucS2 gene defined by the TIGR MtGI sequence TC31898/9.

On Figure 3.5, the assembled genetic and restriction map of TC31898/9 is shown together with the positions of the five amplicons and the approximate position of the introns.

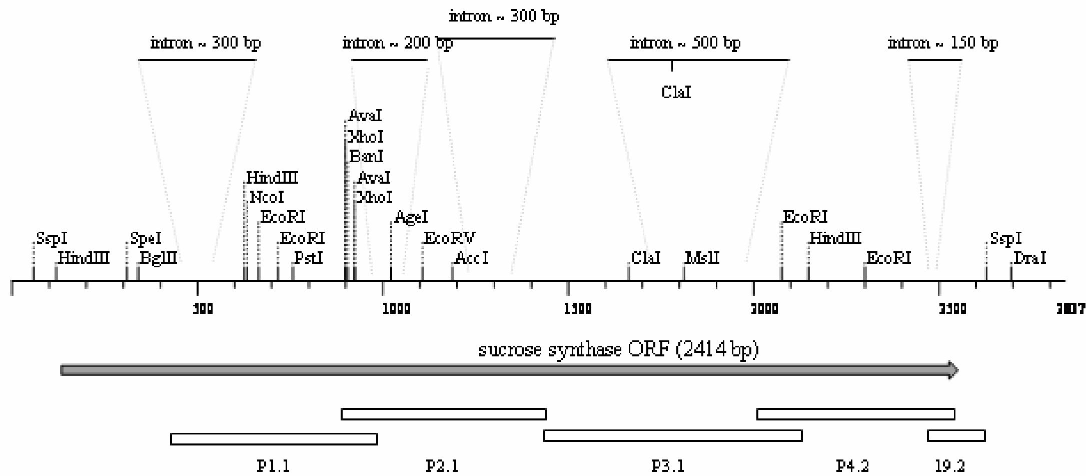
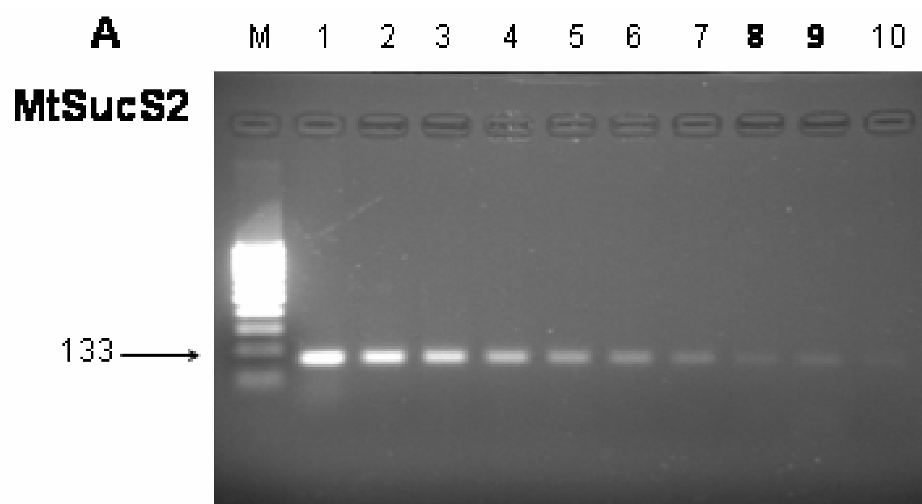
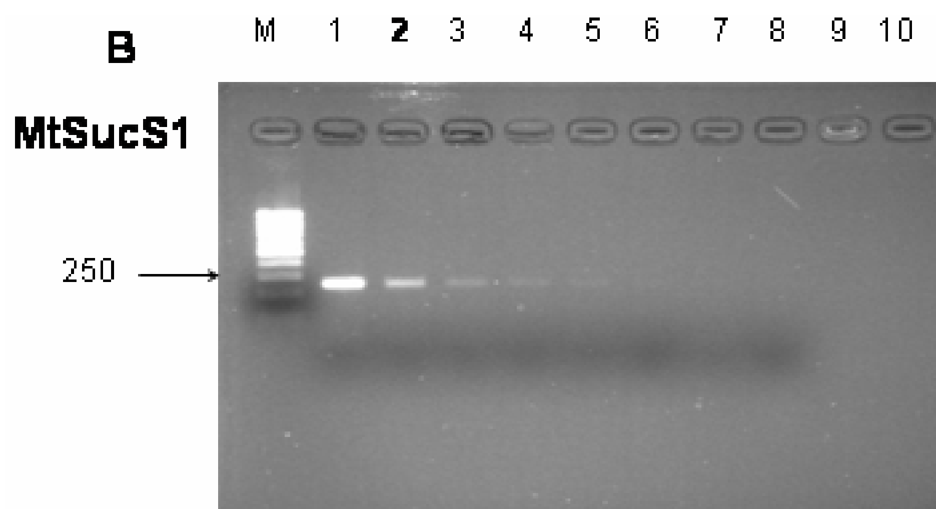


Figure 3.5: Genetic map and restriction map of TC31898/9, showing the positions of the five amplicons.

3.2.4. MtSucS2 is at least 4 fold induced during AM symbiosis

The differentially expressed clone 19.2 corresponding to MtSucS2 was detected by differential display in roots 6 weeks after inoculation with *Glomus intraradices*. So far, only one gene coding for sucrose synthase in *Medicago truncatula* had been isolated and characterized (Hohnjec et al., 1999). This gene, MtSucs1, codes for a nodule enhanced sucrose synthase. To study the induction of MtSucS2 during AM formation and to compare the regulation of MtSucS1 and MtSucs2 during the establishment of the two types of symbiosis, sets of *Medicago* plants were inoculated with *Glomus intraradices* and *Sinorhizobium meliloti*. Semi-quantitative RT-PCR was carried out with total RNA extracted from *Medicago truncatula* at different stages of symbiosis development. In Figure 3.6., an example of the analysis is shown. Starting from 1 µg RNA, dilution steps were performed. The sucrose synthase expression, based on the last dilution step giving a detectable amplification, was then expressed in arbitrary units relative to the last visible ubiquitin signal (Salzer et al., 2000).


A
MtSucS2
M 1 2 3 4 5 6 7 8 9 10
133


B
MtSucS1
M 1 2 3 4 5 6 7 8 9 10
250

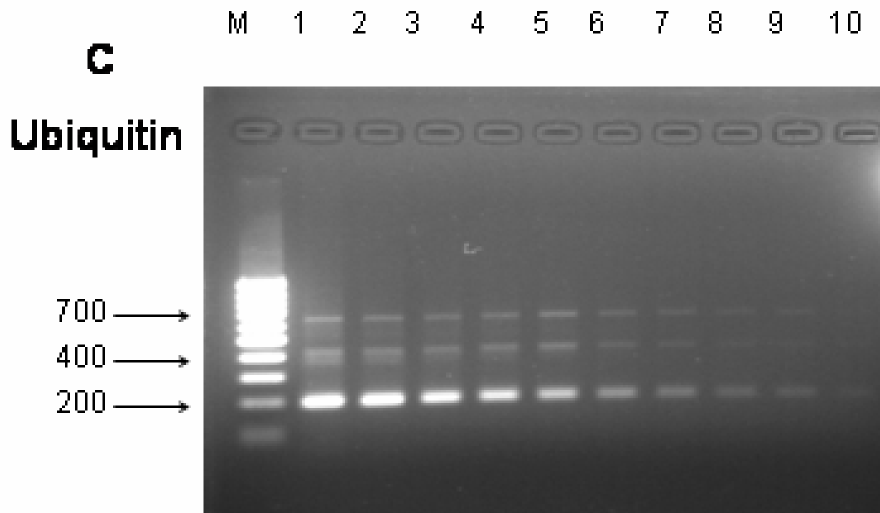


Figure 3.6: Semi-quantitative analysis of expression of MtSucS2, MtSucS1 and ubiquitin genes in *Medicago truncatula* roots. The ethidium bromide-stained gels show expression of the A) MtSucS2, B) MtSucS1 and C) ubiquitin mRNA levels. The PCR products were obtained with a dilution series of RNA using the primer combination specific for the three genes. The dilution factors 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 correspond to 50 ng, 3 ng, 0.75 ng, 0.375 ng, 187.5 pg, 93 pg, 46 pg, 23 pg, 11.5 pg and 5.75 pg of RNA. Samples were from roots harvested 6 weeks after colonization by *Glomus intraradices.*

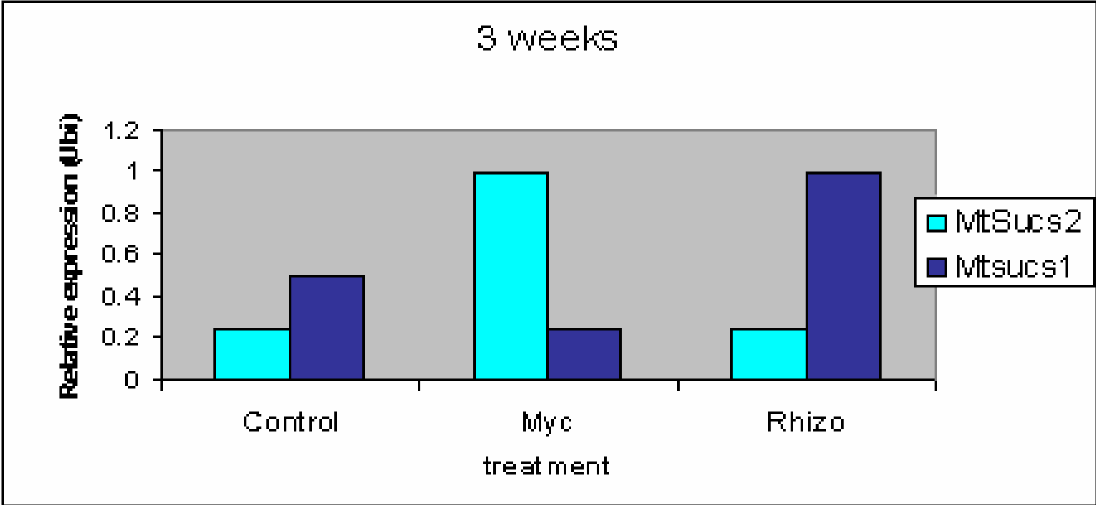
The expression patterns of MtSucS1 and MtSucS2 genes were compared in this way in roots 3, 6, and 8 weeks after treatment.

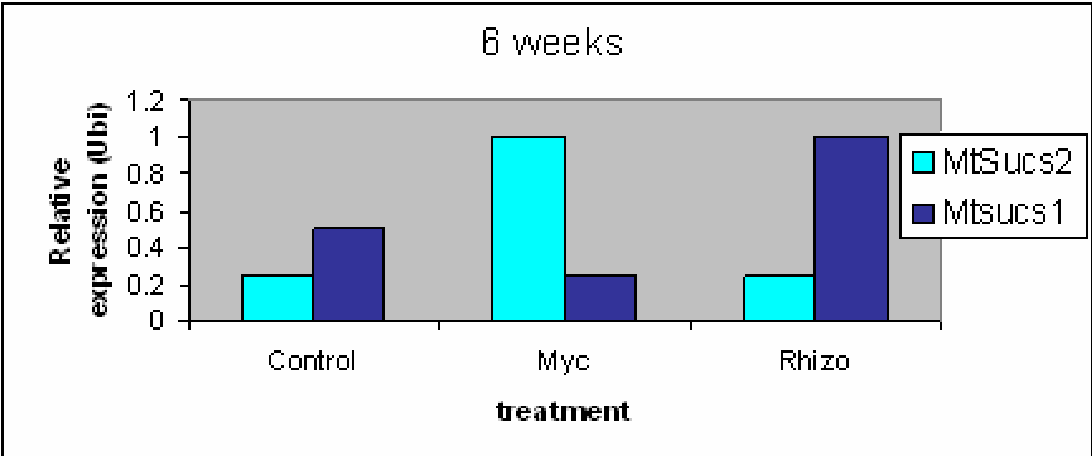
In the roots of control, non-inoculated plants, the level of MtSucS2 expression remained unchanged at 0.25 Ubi during the whole experiment. MtSucS1 had an increase in transcript levels from 0.5 Ubi at 3 and 6 weeks (Fig. 3.7.)

In contrast, in roots inoculated with *Glomus intraradices*, MtSucS2 steady state level was found at 1 Ubi already after 3 weeks, which corresponds to a 4-fold induction compared to the controls. The same level of expression was also found in root RNA after 6 and 8 weeks. Thus, the steady-state level of MtSucS2 transcripts measured by semi-quantitative RT-PCR confirmed the results obtained by differential display and reverse northern analysis and showed that the induction of this sucrose synthase occurs already earlier than 6 weeks after inoculation. Interestingly, the level of MtSucs1 expression was lower in mycorrhizal roots than in the controls. It remained at 0.25 Ubi at all time-points analyzed. This corresponds to a 2-fold reduction at the time point of 3 weeks and to a 4-fold reduction at 6 and 8 weeks.

The level of MtSucS2 expression was unaffected in roots inoculated with *Sinorhizobium meliloti*. It remained at the same level as in the control roots at 0.25 Ubi over the whole duration of the experiment. MtSuc1

expression on the other hand was increased 2-fold as compared to the control roots at 3 weeks (1 Ubi) and remained as high at 6 and 8 weeks.





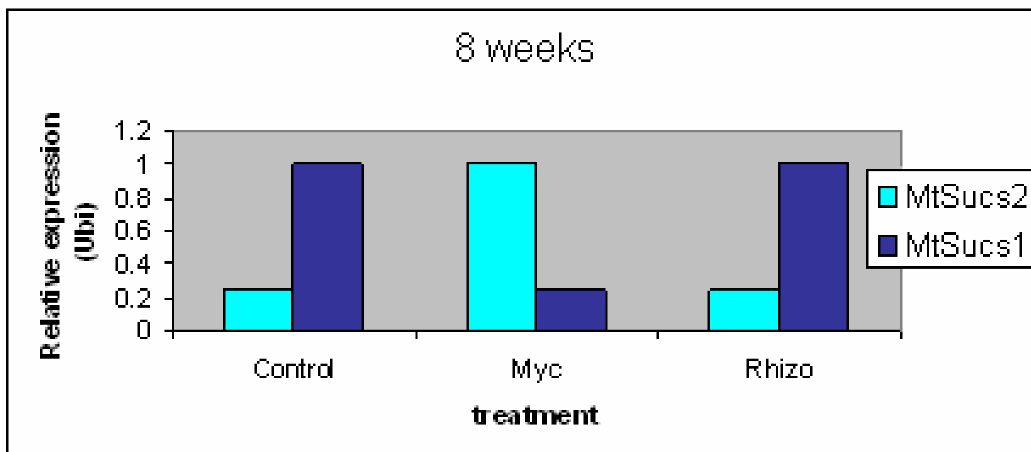


Figure 3.7: Expression of MtSucS2 and MtSucS1 relative to Ubi units in *Medicago* roots, non-inoculated (Control), mycorrhizal (Myc) and rhizobia infected (Rhizo) roots at 3, 6 and 8 weeks.

3.2.5. Expression in different plant tissues:

As already shown by Northern analysis by other scientists (Hohnjec et al., 1999), MtSucS1 is also expressed at a low level in control roots. This was confirmed by our analysis. In addition, MtSucS1 showed slight expression in stem RNA. To investigate the expression of MtSucS2 outside the context of mycorrhiza, RNA preparations from different plant organs (roots, stems and leaves) of 6 weeks old plants, were analyzed by semi-quantitative RT-PCR. Flowers and fruits were harvested at occurrence. Again, MtSucs1 and MtSucs2 were analyzed and their steady state level expressed in arbitrary Ubi units. In addition to the plant organs from untreated greenhouse plants, RNA from isolated nodules (i.e. nodules without surrounding roots tissue) harvested 6 weeks after inoculation with *Sinorhizobium* was analyzed (Fig. 3.8.).

In leaves, MtSucS1 and MtSucS2 displayed 0.125 Ubi and 0.25 Ubi respectively. The low steady-state level of MtSucS1 transcripts detected in this experiment by RT-PCR confirmed the results obtained by northern-blot analysis (Hohnjec et al., 1999). In the stems, MtSucS2 transcripts level was low (0.125 Ubi) whereas the MtSucS1 level was higher (0.50 Ubi). Interestingly, in fruits, the two genes displayed inverse patterns compared to stems. Indeed, the MtSucS1 expression level was very low (0.065 Ubi) and the MtSucS2 expression level higher, at 0.5 Ubi. Finally, the expression of MtSucS2 was highest in flowers (2 Ubi), whereas the one of MtSucS1 was at 0.50 Ubi. This means that in flowers, MtSucS2 messages were detected in as little $3 \cdot 10^{-6}$ µg total RNA.

In nodules, MtSucS2 steady-state level of transcripts was at 2 Ubi whereas the one of MtSucS1 represented only 1 Ubi. Taken together, these results showed that the MtSucS2 had a higher level in fruits, flowers and nodules than in leaves, roots and stems. On the other hand, the MtSucS1 had a higher level in stems, flowers and nodules.

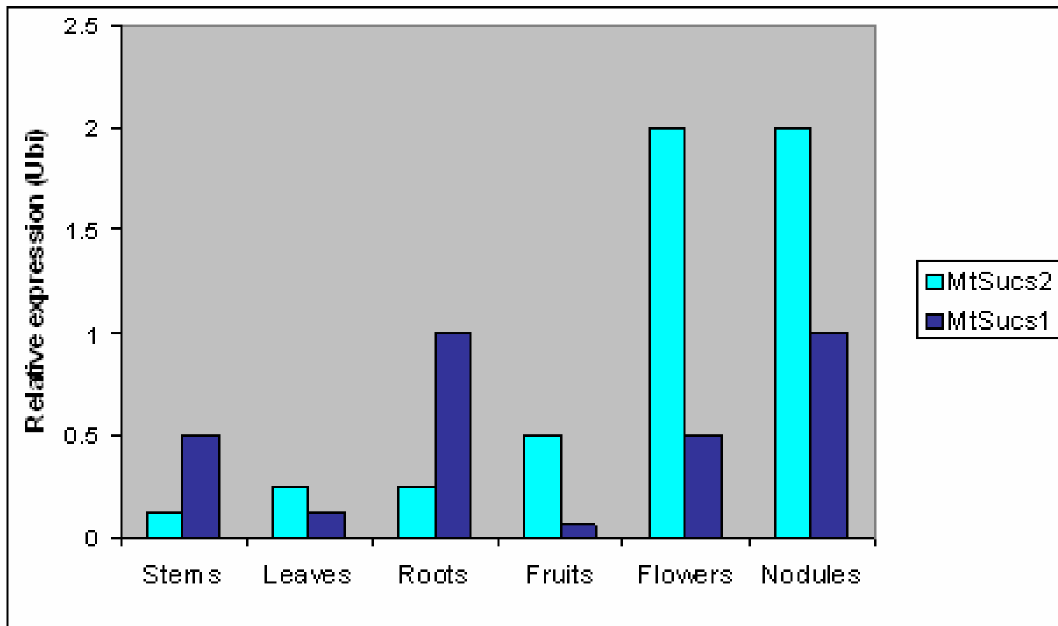


Figure 3.8: Expression of MtSucS2 and MtSucS1 relative to Ubi units in different organs of *Medicago truncatula.*

3.3. DISCUSSION

In this study, we report the identification and characterization of a gene encoding a mycorrhiza-regulated gene for sucrose synthase, MtSucS2, in *Medicago truncatula*. As judged from sequence comparisons with the deduced amino acid sequence, the MtSucS2 protein is closely related to the product of the *Pisum sativum sus3* gene. Hence, MtSucS2 is different from the *Medicago truncatula* sucrose synthase gene (MtSucS1) isolated earlier in the context of nodule formation with *Sinorhizobium meliloti* (Hohnjec et al., 1999). MtSucS2 is represented by the TIGR MtGI sequence TC31898/9. The deduced amino acid sequence of TC31898/9 shows the presence of the conserved phosphorylation site (Fig. 3.9) that is likely to have importance in regulation of enzyme activity (Huber et al., 1996; Zhang and Chollet, 1997; Chikano et al., 2001).

MtSucS2	MASLTRST**S**L**R**ERFD
MtSucS1	MATERLTRVH**S**L**K**ERLD
VfSucS	MATERLTRVH**S**L**R**ERLD
PsSucS	MATDRLTRVH**S**L**R**ERLD
VrSucS	MATDRLTRVH**S**L**R**ERLD
Gm-100	MATERLTRVH**S**L**R**ERLD
Asus1	MANAERMITRVH**S**Q**R**ERLN
ZmSus1	MAAK-LTRLH**S**L**R**ERLG
shrunken-1	MGEGAGDRVLSRLH**S**V**R**ERIG

Figure 3.9: Sequence alignment of the N-terminal amino acids of SucS proteins from different plants showing the phosphorylation sites for calcium-dependent protein kinase C. Adapted from Hohnjec et al. 1999. The MtSucS2 sequence is the deduced amino acid sequence TC31898/9 of the TIGR Mt GI.

Semi-quantitative measurements using RT-PCR and ubiquitin as internal reference for the relative level of expression indicate that MtSucS2 mRNA accumulation is induced at least 4 fold relative to the control, once the AM symbiosis is established, i.e. 6 weeks after inoculation. This corresponds to the time point at which the clone was first found by DDRT-PCR, indicative of the remarkable sensitivity of the technique in this case. Further analysis shows that MtSucS2 is already induced 3 weeks after inoculation. In untreated greenhouse plants, MtSucS2 shows as well relatively high expression in flowers and fruits but is detectable by RT-PCR in all organs tested.

Both sucrose synthases have been identified in the context of symbiosis. When RNA from mycorrhized, nodulated and control roots was analyzed (Fig. 3.7.), both the mycorrhiza-specific induction of MtSucS2 as well as the nodulation-specific induction of MtSucS1 was apparent. Interestingly, MtSucS2 appears repressed in RNA from nodulated roots and MtSucS1 repressed in RNA from mycorrhized roots relative to the control. However, an important induction of MtSucS2 mRNA accumulation was noted in RNA from isolated nodules and this induction seemed to be more important than the induction of MtSucS1. This indicates that the relatively low level of MtSucS2 mRNA detected in nodulated roots was due to a dilution effect by the RNA from root tissue surrounding the nodules. In analogy to the situation observed in nodules, it is well conceivable that during AM, MtSucS2 is induced locally only in specialized root tissues or cells, possibly in or around cells containing arbuscules. If this is the case, induction of MtSucS2 within these specialized cells is probably far more important than revealed by bulk analyses. When different tissues were analyzed, the comparison of MtSucS2 and MtSucS1 expression showed interesting differences. In stem tissues on one hand and in flowers and fruits on the other hand, the two sucrose synthases showed inverse relative expression patterns, MtSucS2 being abundant in flower and fruit RNA but poorly represented in stems, while the opposite was the case for MtSucS1.

Sucrose synthases catalyze the reversible reaction that converts sucrose and UDP to fructose and UDP-glucose. They play a central role in source-sink relationships within the plant and in phloem loading/unloading and provide hexoses for starch and cell-wall synthesis as well as for other metabolic purposes (Zrenner et al., 1995; Sturm and Tang, 1999). Several studies have indicated a complex regulation pattern of sucrose synthases at the transcriptional and post-transcriptional level by sugar, hypoxia and anoxia (Koch et al., 1992; Zeng et al., 1998; Zeng et al., 1999). In maize roots, the accumulation of *Sh1* and *Sus1* mRNA is differentially and dramatically modulated by variations in carbon supply while the total sucrose synthase activity remains constant (Koch et al., 1992).

In this study, we found that MtSucS2 mRNA accumulation relative to ubiquitin was highest in flowers and fruits – typical sink organs with respect to carbon. Moreover MtSucS2 appears to be specific to symbiosomes, strong carbon sinks in both AM and nodulation. In the AM symbiosis, the fungus is probably supplied with hexoses, and the arbuscule is believed to be the main site of nutrient exchange (Bago et al., 1999; Pfeffer et al., 1999). Therefore, sucrose synthases, and MtSucS2 in particular, could be directly involved in providing hexoses for the fungus at the site of exchange by cleaving the imported sucrose. Alternatively, the role of MtSucS2 might be most important for the early formation of AM structures. In tomato, the fruit-specific sucrose synthase (TOMSSF) has been shown by antisense inhibition to be required

for early sucrose import into the developing fruit. This reduced import had no influence on starch synthesis capacity and total sugar content of the fruits at maturity but antisense plants developed fewer fruits (D'Aoust et al., 1999). Therefore, TOMSSF is thought to enhance sucrose import into the young fruit as long as the import capacity rather than the sucrose availability is limiting. Once the fruits increase in number and size the import capacity into fruits is related to acid invertase and additional sucrose synthase activities cannot add to the import as the source is limiting. Interestingly, maize mutants with defective sucrose synthases develop fewer mycorrhizal structures and, in particular, a lower relative number of vesicles among these structures (Corbière et al., to be published elsewhere). Therefore, in analogy to TOMSSF, the role of MtSucS2 could be to enhance the sink strength for the developing arbuscule.

3.4. MATERIAL AND METHODS

3.4.1. Materials

Medicago truncatula

Medicago truncatula Gaertn. cv. Jemalong, strain A 17, was provided by T. Huguet (INRA-CNRS, Castanet-Tolosan, France). The seed was surface sterilized with 95-97% of H_2SO_4 for 5 min, washed several times with sterile water and pre-germinated for 5 days on 1% water agar plates at 20°C in the dark. The seedlings were transferred in single plastic pot and grown on Terra Green (Maagtechnic, Zürich, Switzerland) in a climate chamber (22°C, 16 h light, 100 µmoles photons $m^{-2}s^{-1}$). The plants were fertilized with B&D medium (Broughton and John, 1979) containing 0.5 mM K_3PO_4 and 2mM KNO_3 at every watering and harvested at the time-points indicated.

Glomus intraradices

The arbuscular mycorrhizal fungus, *Glomus intraradices*, Schenck and Smith was kindly offered by G. Bécard (Université Paul Sabatier, Castanet-Tolosan, France). This *in vitro* inoculum was originally obtained by inoculation of Ri transformed *Daucus carota* roots by *Glomus intraradices* spores (Bécard and Piché, 1992). The *Glomus intraradices* inoculated roots were subcultured every two months onto M medium (Bécard and Fortin, 1988) containing 0.3% Phytagel (Sigma, St Louis, MO, U.S.A) and kept at 26 °C.

Sinorhizobium meliloti

The *Rhizobium*-legume symbiosis was established between *Sinorhizobium meliloti* (strain 1021) and Medicago truncatula. The bacterial strain, provided by P. Curioni (ETH, Zürich, Switzerland) was cultured for 3 days in liquid succinate minimal medium (Schmidt et al., 1992) at 140 rpm at 27°C.

3.4.2. Plant treatments

-Inoculation with *Glomus intraradices*

Fungal material was separated from its culture medium by dissolving the Phytagel in 10 mM citric acid, 10 mM Na-citrate, pH 6 at 37°C. Spores were collected in water by sieving the suspension through a 33 µm mesh (Doner and Bécard, 1991). This inoculum was then adjusted to 600 spores/ml and used for all in vivo experiments. For every model plant used in this work, the seedlings were transferred to pots and the spore suspension was applied directly on the roots while planting. As control, water was poured on the roots of each control seedling. At indicated time-points, the plants were harvested, the root system was gently washed in water and dried between two layers of paper. Roots and shoots were weighted separately and sampled. The different samples obtained were either immediately frozen at –80°C or used directly to estimate the degree of colonization.

-Inoculation with *Sinorhizobium meliloti*

The overnight bacterial liquid culture of *Sinorhizobium meliloti* was diluted with one volume of culture medium and 3 ml were used to inoculate every seedling transferred into an individual pot. At the times indicated, the plants were collected and sampled as described previously.

3.4.3. Determination of root colonization

Root samples were cleared in 10% KOH at 95°C for 5 to 15 min and stained in 0.1% trypan blue (Phillips and Hayman, 1970) for 20 min. The fungal structures were visualized under a Zeiss Axioplan microscope and the root colonization by *Glomus intraradices* was estimated by the gridline intersection method (Giovannetti and Mosse, 1980).

3.4.4. RNA extraction

From *Medicago truncatula* plants, total RNA was extracted according to the method described in Mohr et al., 1998 (Mohr et al., 1998). This "hot phenol method" started with grinding 1g of frozen plant material with a pestle in a pre-cooled mortar under liquid nitrogen. The ground powder was transferred to 10 ml of 80°C preheated extraction buffer, 1:1 water-saturated phenol: 2x NETS (200 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5 and 1% SDS). After incubation on ice for 10 min, the preparations were centrifuged at 500g for 10 min at room temperature. The upper phase was transferred to a 5 ml tube containing water-saturated phenol and vortex for 30 sec. From a new centrifugation for 10 min at 500g, the supernatant was collected in a 30 ml Corex tube and the nucleic acids precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of ice cold 100% ethanol. The mixture was stored at –20°C for 1h to overnight and the nucleic acids were then collected as a pellet by centrifugation at 10' 000g for 15 min at 4°C. The RNA was then resuspended in 6 ml water and transferred to a 15 ml Corex tube, 6 ml of 5 M LiCl were added and the mixture incubated at –20°C for 2h. The precipitated RNA was collected by a 10'000g centrifugation for 20 min at 4°C and the pellet was re-dissolved in 600 µl DEPC treated water. In order to repeat the precipitation step, 600 µl of LiCl were added to the re-suspended RNA and the mixture was incubated at –20°C for 2 h. The RNA pellet was finally obtained by centrifugation at 10'000 rpm for 20 min at 4°C and re-suspended in 100 µl water. The OD_{260} and the ratio of OD_{260}/OD_{280} were measured to determine the concentration of RNA preparation and its purity.

3.4.5. Differential display of mRNA (DDRT-PCR)

Total RNA extracted as described in Method A was treated with DNaseI according to the Message Clean kit (GenHunter, Brooklin, USA). According to the protocol of Liang et al. (1993) (Liang et al., 1993) and Callard et al (1994) (Callard et al., 1994), mRNA were reverse transcribed for 60 min at 37°C. The reverse transcription (RT) mix consisted of 0.2 µg of DNase I treated RNA, 1 µM final concentration of H-T11-A, H-T11C or H-T11G anchor primer. One x RT buffer (25 mM Tris-HCl, pH 8.3, 38 mM KCl, 1.25 mM

MgCl2, 5 mM DTT) and 20 mM of each deoxynucleotide triphosphate in 20 µl final reaction volume. After 5 min at 65°C and 10 min at 37°C, 1 µl MMLV reverse transcriptase (100 U/ul) was added; after 50 more minutes at 37°C, the reaction was stopped incubating 5 min at 75°C. The first strand cDNAs obtained were then PCR amplified with the same anchored primer as for reverse transcription and with one of 18 different arbitrary primers (0.25 µM final concentration) and 1 µCi of α^{33}P) dATP.

The PCR was run for 30 cycles with a denaturation step at 94° for 30 sec, annealing step at 40°C for 2 min, and polymerization at 72 °C for 30 sec and was followed by a final elongation step at 72°C for 5 min.

PCR products aliquots of 3.5 µl were separated by electrophoresis on a 7 M urea/6% acrylamide sequencing gel (Sambrook et al., 1989). The gel was fixed onto 3 MM Whatman paper, dried, and exposed overnight to a Kodak X-RAY-OMAT film. Bands of interest were cut out of the gel and the cDNA fragments were eluted from the gel according to the protocol of Johnson et al., (1995) (Johnson et al., 1995). In Eppendorf tubes, gel slices were mixed with 100 µl TE and incubated for 2 h at room temperature, 1 h at 37°C and several hours up to overnight at 4°C. After centrifugation at 13'000g to pellet the debris, the supernatant was collected into a new tube and the cDNA precipitated with 10 µl 3M sodium acetate, pH 5.2, 5 µl glycogen (10 mg/ml) and 450 µl of 100 % ethanol. The mixture was incubated at –80°C for 40 min. The cDNA was pelleted by 10' centrifugation at 4°C, washed with 200 µl ice-cold 85% ethanol and re-suspended in 10 µl water. These cDNA fragments, called differentials, were re-amplified by PCR with the same arbitrary primer used to obtain the differential of interest (Vogeli-Lange et al., 1997).

3.4.6. Cloning procedure

The re-amplified fragments were cloned into the pCR-TRAP vector (GenHunter).

According to the manufacturer's instructions, 5 µl of PCR products were ligated in the insert-ready pCR-TRAP vector with T4 DNA ligase (200 units/µl). Competent E. coli cells were transformed with the ligation products by heat shock and plated on LB agar plates containing 20 µg/ml of Tetracycline as selective marker. To identify colonies containing ligated inserts, colony PCR was performed according to GenHunter procedure and the primers Lgh and Rgh provided by the kit were used for the amplification steps. The PCR products were then analyzed by electrophoresis according to standard procedures (Sambrook et al., 1989) and sizefractionated through 0.8-1.5% agarose gels (Seakem LE, FMC, Rockland, ME, USA) containing 0.5 µg/ml of Ethidium Bromide. The bands of interest were compared to a 100 bp ladder (Pharmacia) on the agarose gel.

3.4.7. Reverse Northern-blot analysis

The screening of cDNA fragments derived from differentially expressed genes was performed by using Reverse Northern method as described by Vögeli-Lange et al. (1996) (1996). For this procedure, a NY 13 N membrane, Schleicher and Schuell, Dassel, Germany) was floated in water and in 6x SSC and placed into a convertible filtration manifold equipped with a 48 well slot top plate (BRL, Gaithersburg, MD, USA). The differentials obtained were cloned and re-amplified by colony PCR, 19 µl of these PCR products were heat to 95°C for 5 min and then supplemented with 475 µl of 10x SSC (1x SSC is 0.15M NaCl, 15 mM Na-citrate). This mixture was applied in aliquots of 156 µl to two slots of the slot-blot machine. Each blot was washed twice with 0.5 ml 10x SSC. After being removed from the slot blot apparatus, the membrane was floated for 7 min on a solution of 0.5 M NaOH, 1.5 M NaCl and twice for 3 min on a solution of 0.5 M Tris-HCl pH 7.5. 1.5 M NaCl. The membrane was washed briefly in 2x SSC and cut into two identical sets and baked for 1-2 h at 80°C in a vacuum oven. The membranes were incubated for 1-4 h at 60°C in 5 ml of pre-hybridization solution (5x SSC, 5x Denhardt's solution, 0.5% SDS and 100 µg/ml of denatured salmon sperm DNA). This solution was discarded and replaced by 4 ml of fresh hybridization solution. After boiling for 5 min, the original (^{33}P)-PCR reaction from the control sample was added to one membrane and the original (^{33}P)-PCR reaction of the inoculated sample was added to the second membrane. The hybridization was taking place overnight at 60°C under rotation. Different washings steps were then performed: 10 min at 54°C in 2X SSC, 0.1% SDS, 10 min at 65°C in 2x SSC, 0.1% SDS and finally, 10 min at 65°C in 0.5x SSC, 0.1% SDS. The membranes were at the end exposed for 4 to 8 h to BioRad Phosphor Imager high sensitivity screen.

3.4.8. Semi-quantitative RT-PCR procedure

This procedure was carried out according to the protocol of the Ready-To-Go RT-PCR beads of Amersham Pharmacia Biotech Inc, USA.

RT-PCR was performed with Ready-To-Go RT-PCR beads that are stable at room temperature. Each bead contained all of necessary reagents except primer and template. The two-step protocol consisted in dissolving each bead with 44µl of room temperature DEPC-treated water. The dissolved bead content was transferred to a PCR tube with different dilut of the RNA template and the oligo dT primer (10 ng/µl final concentration) for first-strand cDNA synthesis. The dilution factors 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 corresponded to 50 ng, 3 ng,

0.75 ng, 0.375 ng, 187.5 pg, 93 pg, 46 pg, 23 pg, 11.5 pg and 5.75 pg of RNA. The reaction was put in a thermal cycler and incubated for 30 min at 42°C. The reverse transcriptase was inactivated and the DNA template completely denatured for 5 min at 95°C. The second step consisted in adding the two gene-specific primers (0.1 µM final concentration) for PCR. The specific primers used for MtSucS2 and MtSucS1 were: 5'-GATTGCCGTGAGAGCAAAC-3' (forward) and 5'-GATTGCCGTGAGAGCAAAC-3' (reverse) and 5'-AATCTGAGGCTTTGAAGAGT-3' (forward) and 5'-AAAATTGAATTGATGGGGGC-3' (reverse). For the gene coding for ubiquitin, the primers were: 5'-GTGAAGACCTTGACCGGCAAAAC-3' (forward) and 5'-GGTGAAGCGTGGACTCTTTCTGC-3'(reverse). The reaction was then cycled 32 times with denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min and polymerization at 72°C for 1 min. 10 µl of PCR products were then loaded on an agarose gel and photographed under UV light.

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IV. Normal development of arbuscular mycorrhizal symbiosis in plants requires sucrose synthase(s)

Abstract

Symbiotic interactions between plant roots and arbuscular mycorrhizal fungi (AM symbiosis) are the most prevalent associations dating from Ordovician times and concern the roots of land plant species and fungi from the order *Glomales*. It is generally accepted that in AM symbiosis, the plants benefit from the fungal presence, among others, with a better phosphorus supply and better growth. In turn, as an obligate biotroph, the mycorrhizal fungus has to obtain carbon assimilates from the plant, making the flux of nutrients bi-directional. Maize mutants in the genes Sh and Sus coding for the sucrose synthases, SS1 and SS2, were selected to investigate the involvement of this enzyme in AM symbiosis. The Sh and Sus genes show different development and tissue specificity and differential modulation in response to sugar availability. To assess the mycorrhizal phenotype in the mutant maize lines, we observed the colonization of the different root systems inoculated with the AM fungus *Glomus intraradices*. While there were no phenotypic differences between the wild-type and mutant plants, the mutations at the known loci encoding sucrose synthases caused a delay in colonization and poor development of mycorrhizal structures. Therefore, the sucrose synthase encoding genes appear to be important in the development of a normal symbiosis. Experiments with pea mutants confirm this conclusion.

4.1. INTRODUCTION

Among the different types of symbiosis between plants and microorganisms, the arbuscular mycorrhizal (AM) association is the most widespread. Indeed, this very ancient symbiosis dating from Ordovician times concerns the majority of terrestrial plant species (Smith and Read, 1997). It is generally thought that in AM symbiosis, the mycorrhizal fungus supplies phosphate to the plant in exchange for assimilates. The molecular mechanisms underlying this phosphate uptake has recently received much attention by the cloning of a potato phosphate transporter specifically expressed in arbuscule containing root cells (Rausch et al., 2001). Advances in the elucidation of the process of the carbon transfer from plant root to the mycorrhizal fungus have also been made. A cDNA clone encoding a hexose transporter (Mtst1) has been obtained from mycorrhizal roots of *Medicago truncatula* (Harrison, 1996). Mtst1 shows increased transcript levels in the phloem tissues and root tips and in the cortical cells colonized by *Glomus versiforme*. This expression pattern of Mtst1 has been interpreted as a consequence of the higher demand of plant cells for hexoses in order to

support the increased metabolism during symbiosis (Cox and Tinker, 1976; Harrison, 1996). This does not exclude that the trigger is an increased demand for hexose supply to both the plant and the fungal partners. Therefore, enhanced Mtst1 expression in the colonized cell would be the response to a sink signal from a tissue or directly the response to fungal colonization (Harrison, 1996). The finding that hexoses are the major carbon compound taken up by intraradical AM hyphae at the plant-fungal interface (Shachar-Hill et al., 1995) and the identification of a sucrose synthase enhanced in colonized roots in *Medicago* (Corbière et al., to be published elsewhere) underline the importance of this sucrose cleaving enzyme in the establishment of a functional symbiosis. Sucrose synthase catalyses the reversible reaction that converts sucrose and UDP to fructose and UDP-glucose. It is a key enzyme of carbon metabolism and it plays a major role in sink/source relationships within the plant (Zrenner et al., 1995). Despite some remaining uncertainties, it is strongly suggested that in association with a cellulose synthase complex, sucrose synthase may provide UDP-glucose for cellulose synthesis whereas a free form in the cytosol would supply products of sucrose cleavage into the general metabolism (Carlson and Chourey, 1996; Delmer et al., 2000).

In the context of symbiosis, sucrose synthase has mainly been studied in the *Rhizobium*-legume association. Sucrose synthase has been characterized as a "nodulin" in nodules of soybean (Morell and Copeland, 1985) and broad bean (Ross and Davies, 1992), and SucS cDNAs have been also isolated from *Pisum sativum*, *Lotus japonicus*, *Glycine max* and *Medicago sativa* (Déjardin et al., 1997; Skot et al., 1997; Zhang and Chollet, 1997; Buchner et al., 1998).

In pea, a gene encoding an isoform of sucrose synthase expressed in the embryo co-segregates with the rug4 locus where three mutants alleles, rug4-a, rug4-b and rug4-c were identified (Wang et al., 1990). With the use of rug4 mutants, responsible for the wrinkled-seeded phenotype, Wang and Hedley (Wang and Hedley, 1993a) observed that these mutations reduced the activity of sucrose synthase in the testa and in the leaves by 50%, but activity in *Rhizobium*-infected root nodules was reduced by 85%. In common with other wrinkled-seeded genotypes, seeds of the rug4 mutants have lower starch content than seeds of the round seeded wild-type (Wang and Hedley, 1993a).

In maize (*Zea mays*), two sucrose synthases, SS1 and SS2, have been identified. They have very similar characteristics and are encoded by the Sh and Sus loci respectively (Echt and Chourey, 1988). SS1 protein, encoded by the Sh gene, has been found mainly in developing endosperm, and the loss of this isozyme by mutation is the basis for the "shrunken seed" phenotype. The Sh-encoded protein is detected in other plant parts, including roots and shoots (Chourey et al., 1986; Springer et al., 1986). The SS2 protein, encoded by the Sus gene, is more widely distributed among plant parts than is SS1 (Chen and Chourey, 1989). It is

present in developing embryo and metabolic sinks and at high levels in young leaves (Chen and Chourey, 1989; Rowland et al., 1989; Nguyen-Quoc et al., 1990). The Sh gene product is thought to play a critical role in providing the substrates for cellulose biosynthesis and the production of cell wall material. The Sh-encoded SS1 could also take part in sugar tolerance with its capability to use or import low level of sugars. Indeed, under limited carbohydrate supply, Sh mRNA is maximally expressed and Sh gene is considered like a typical “famine” gene (Koch et al., 1992).

The Sus gene product is required to generate precursors for storage processes such as starch accumulation, and it has a different sugar regulation. Sus mRNA shows increased expression under plentiful sugar supplies and is a so-called “feast” gene (Koch et al., 1992). Three maize stocks corresponding to two single recessive mutants and a double recessive mutant were isolated. The double recessive genotype sh sus was not distinguishable by the seed phenotype from the two single genotypes, Sh sus and sh Sus, but could be distinguished by Southern-blot analyses (Chourey et al., 1988; Chourey and Taliercio, 1994).

In order to investigate the role of sucrose synthases in the establishment of the AM symbiosis, we used maize and pea lines deficient in sucrose synthase to assess the mycorrhizal phenotype. The results show that mutations at loci encoding sucrose synthases cause a delay in mycorrhiza formation and poor development of mycorrhizal fungus. Hence, sucrose synthase(s) is important for a normal development of the AM symbiosis.

4.2. RESULTS

The three near isogenic mutant maize lines, two single recessive mutants and one double recessive mutant, referred to as Sh sus, sh Sus and sh sus, were compared to the W22 wild-type (Sh Sus) (Chourey et al., 1988). Pregerminated maize plantlets were inoculated with approx. 600 spores of *Glomus intraradices*. The degree of colonization was determined after 3, 6 and 9 weeks of co-cultivation using the grid-line intersection method. The fungal structures indicative of AM colonization and scored for colonization were internal hyphae, arbuscules and vesicles (Fig.4.1).

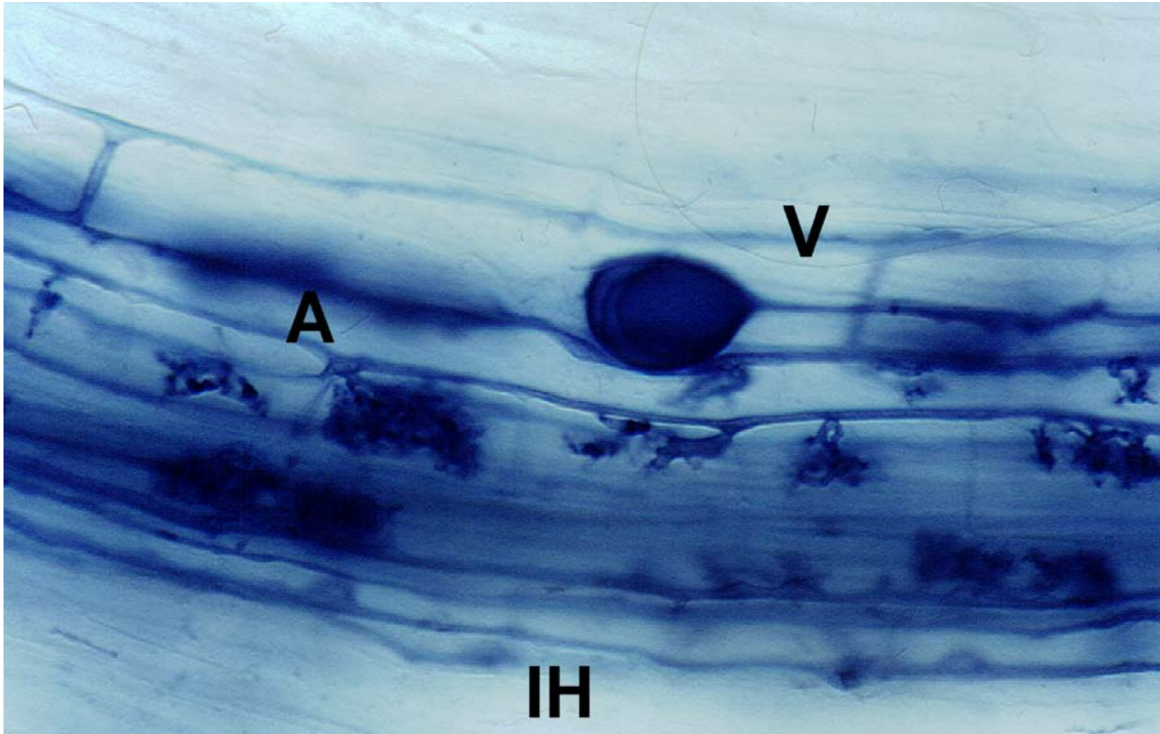

Figure 4.1: Typical structures of AM association. Maize wild-type roots were stained with trypan blue 9 weeks after inoculation. IH: internal hyphae; A: arbuscules; V: vesicles.

4.2.1. The maize sh and sus mutations do not affect the overall morphology

Plants from all four genotypes grew normally, and no phenotypic differences were visible, neither in the shoots nor in the roots. This was true for both non-mycorrhizal control plants (not shown) and mycorrhizal plants (Fig.4.2).

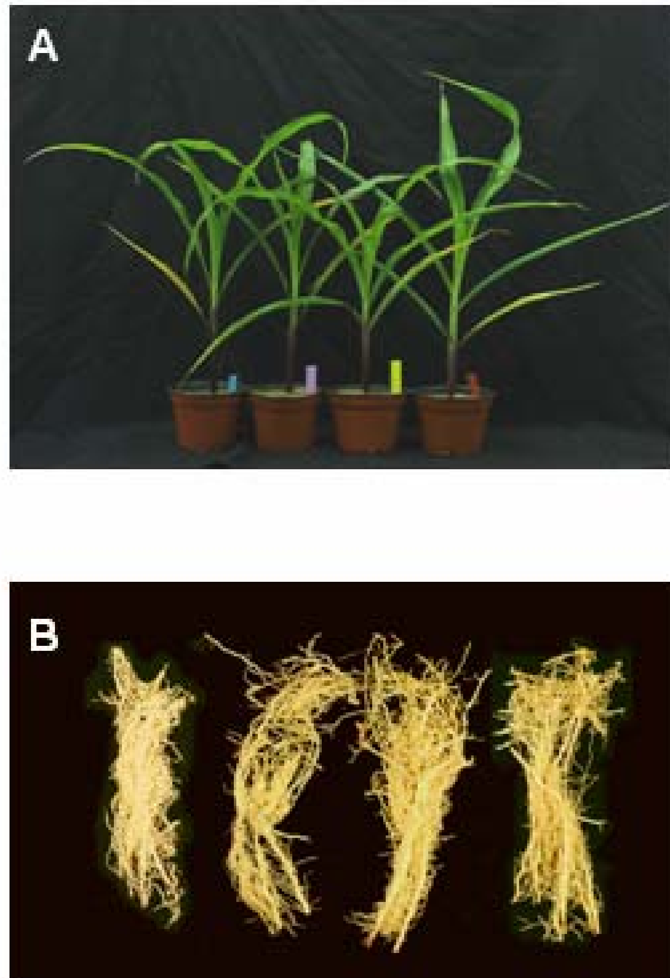

Figure 4.2: (A) Maize plants and (B) root systems of four genotypes, 6 weeks after inoculation with *Glomus intraradices*. Left to right: ShSus, Shsus, shSus, shsus.

The ratio between fresh weight and dry weight remained constant and comparable in all lines. Shoot dry weight (DW) was between 15 and 18% of the fresh weight (FW) and root DW between 8 to 12% of the FW in both control and mycorrhizal plants (data not shown). Six weeks after inoculation, FW and DW of root and shoot material were not significantly different between the four lines without treatment (Table 4.1). Similarly, at 9 weeks, the DW of roots and the DW of shoots in control plants were not different between the four lines (Table 4.1).

Table 4.1: Dry weight (DW) of four different maize genotypes at two time-points in control plants without mycorrhiza and mycorrhiza inoculated plants.

		DW after 6 weeks[1]			DW after 9 weeks[1]		
treatment	genotype	roots	shoots	total	roots	shoots	total
control	Sh/Sus	2.7 ± 0.4[1)]	8.2 ± 0.7	10.9 ± 0.4	2.9 ± 0.3	9.3 ± 1.4	12.2 ± 1.6
	Sh/sus	2.7 ± 0.4	7.6 ± 0.9	10.3 ± 0.7	2.6 ± 0.3	6.9 ± 0.4	9.5 ± 0.6
	sh/Sus	2.8 ± 0.4	9.9 ± 1.1	12.7 ± 1.3	3.0 ± 0.3	9.0 ± 1.0	12.1 ± 1.1
	sh/sus	5.7 ± 0.7	8.4 ± 0.7	14.1 ± 1.1	4.5 ± 0.8	9.5 ± 1.3	14.0 ± 1.8
mycorrhiza inoculated	Sh/Sus	2.9 ± 0.6	7.9 ± 1.6	10.8 ± 2.1	5.1 ± 1.1	9.2 ± 1.3	14.2 ± 2.2
	Sh/sus	3.2 ± 0.3	9.0 ± 0.5	12.2 ± 0.5	4.0 ± 0.5	8.9 ± 0.5	12.9 ± 0.6
	sh/Sus	2.3 ± 0.4	10.7 ± 0.7	13.1 ± 0.9	3.3 ± 0.3	10.2 ± 1.4	13.5 ± 1.3
	sh/sus	1.9 ± 0.1	10.2 ± 0.7	12.1 ± 0.8	2.7 ± 0.4	11.7 ± 1.6	14.4 ± 1.9

[1)] dry weight in grams plus/minus standard deviation for 5 replicates.

4.2.2. Mutations in Sh and Sus cause a decrease in colonization by the AM fungus

Colonization was determined in wild-type, Sh sus, sh Sus, and sh sus plants 6 and 9 weeks after inoculation using the grid-line intersection method, and the abundance and occurrence of the different fungal structures within the root systems was scored. To quantify the fungal presence within the inner cortical tissue, our criteria for mycorrhization were the presence of internal fungal hyphae, of arbuscules and finally of vesicles (Fig.4.1). Visible external fungal hyphae were not scored as mycorrhizal structures. After 3 weeks, the lines Sh sus, sh Sus, sh sus and wild-type had only 10% of their root system colonized by the AM fungus (data not shown). This low percentage of colonized sectors was routinely observed in the development of the AM symbiosis in maize plants and corresponds to the early ingress of the fungus.

After 6 weeks of co-cultivation, the wild-type roots were intensely colonized, with values in the range of 90% (88%) routinely obtained. The Sh sus, sh Sus and sh sus displayed 28, 58, and 37% of colonization, respectively (Fig.4.3).

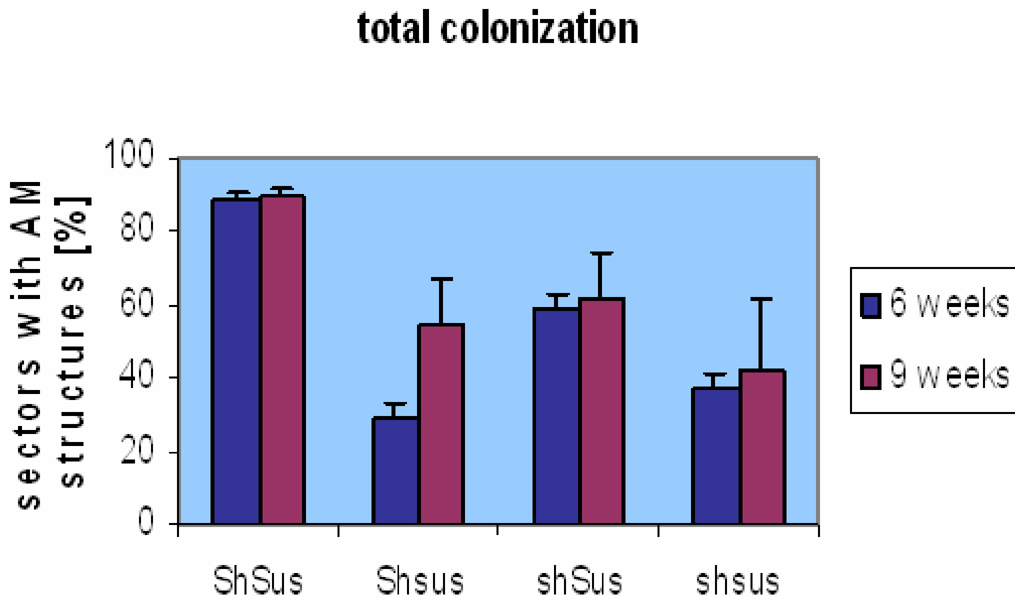

Figure 4.3: Total arbuscular mycorrhizal structures in the roots of four maize genotypes 6 and 9 weeks after inoculation with *Glomus intraradices*. Error bars: standard deviation. Five individual root systems were analyzed per treatment. The experiment was done twice with similar results.

Thus, the Sh sus mutant plants had the poorest colonization with fewest AM structures, even lower than the double mutant (sh sus), whereas the colonization of sh Sus plants was intermediate (58%). After 9 weeks, 90% of the root sectors in wild-type plants had mycorrhizal structures; 53% of the Sh sus, 62% of sh Sus and 42.4% of the double mutant (sh sus) roots were colonized. In conclusion, the colonization in the Sh sus and the sh sus mutants, which both lack the Sus encoded SS2, was initially delayed. Later, between 6 and 9 weeks, the Sh gene seems to take over an important role, as the colonization increased from 28 to 54% in the Sh sus roots. The sh Sus developed the symbiosis first until 58%, then, presumably due to the lack of Sh, could hardly develop the symbiosis any further (increase to 62%). The double mutant was colonized in 37% of the root sectors after 6 weeks and remained at a similar colonization level also after 9 weeks (42%).

In Figure 4.4, the percentage of internal hyphae, arbuscules and vesicles found in the sectors of the different root systems is shown.

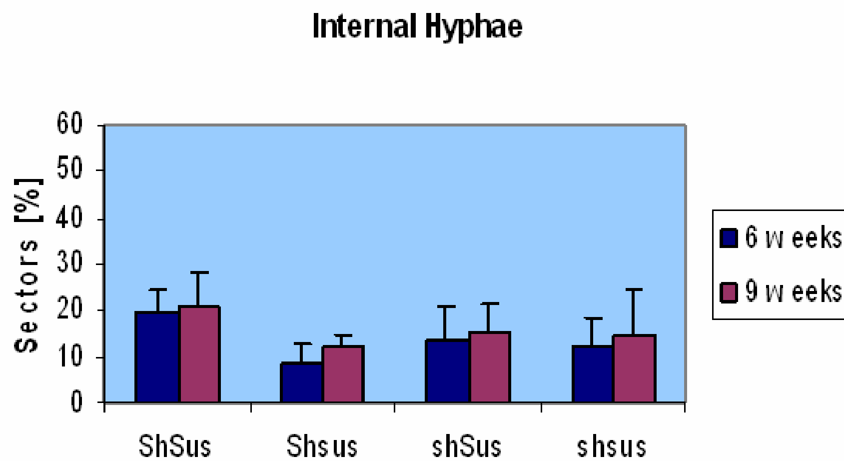

Figure 4.4 A: Development of internal hyphae in the roots of four maize genotypes 6 and 9 weeks after inoculation with *Glomus intraradices*. Errors bars: standard deviation. Five individual root systems were analyzed per treatment.

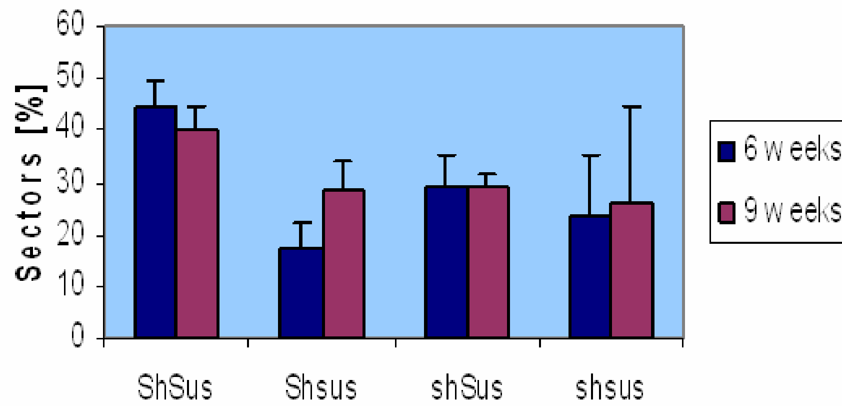

Figure 4.4 B: Development of arbuscules in the roots of four maize genotypes 6 and 9 weeks after inoculation with *Glomus intraradices*. Errors bars: standard deviation. Five individual root systems were analyzed per treatment.

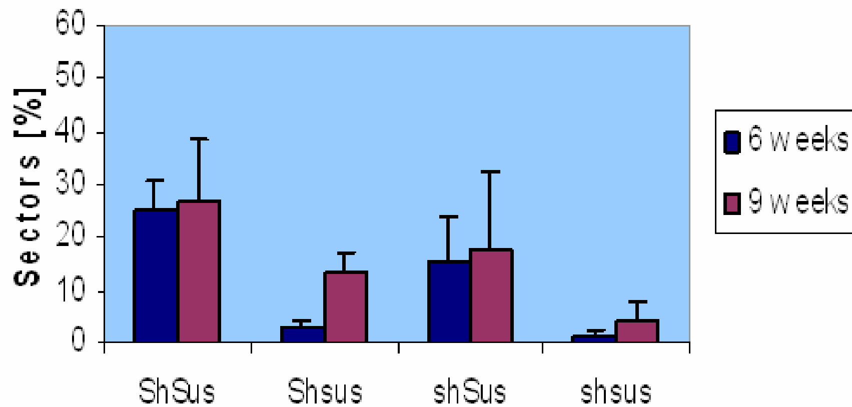

Figure 4.4 C: Development of vesicles in the roots of four maize genotypes 6 and 9 weeks after inoculation with *Glomus intraradices*. Errors bars: standard deviation. Five individual root systems were analyzed per treatment.

At 6 weeks, in wild-type, 20% of the root sectors showed internal hyphae, 43% arbuscules and 25% vesicles. In the Sh sus mutant, 8.5% of the root sectors had hyphae, 17% arbuscules and only 3% vesicles. Less than 14% of the sh Sus root sectors contained vesicles, almost 30% arbuscules, and around 15% vesicles.
Nine weeks after inoculation the percentage of root sectors containing particular structures were similar to those at 6 weeks in wild-type with more than 20% hyphae, 40% arbuscules and 27% vesicles and in the sh Sus mutant (15% hyphae, 29% arbuscules, less than 18% vesicles). All structures were more frequent in Sh sus mutant roots at 9 weeks, 12% hyphae, more than 28% arbuscules, and particularly, over 13% vesicles. The double mutant also had slightly more root sectors with AM structures at 9 than at 6 weeks (close to 15% hyphae, 26% arbuscules, and 4.5% vesicles). Taken together, the reduced total colonization in the mutant lines was correlated with a reduction of the incidence of every individual structure. Most striking was the small percentage of vesicles in the single Sh sus and double mutant sh sus lines (Fig.4.4C).
How abundant the different mycorrhizal structures were, within a given colonization, is shown in Table 4.2.

	Internal Hyphae		Arbuscules		Vesicles	
	6 weeks	9 weeks	6 weeks	9 weeks	6 weeks	9 weeks
Sh Sus	21.8	20.2	49.8	46.0	28.4	33.6
Sh sus	29.4	22.3	59.3	50.9	11.3	23.2
sh Sus	25.5	22.6	50.1	47.7	26.4	29.7
sh sus	33.5	34.0	63.7	54.7	2.8	9.0

Table 4.2: Distribution of mycorrhizal structures expressed in percent of all the mycorrhizal structures (100%) in maize plants 6 and 9 weeks after inoculation with Glomus intraradices. Red color emphasized comparable values of colonization in Sh Sus and sh Sus plants. Blue color emphasizes comparable values of colonization in Sh sus and sh sus plants. Values are in dark color when the values of colonization of Sh Sus and sh Sus plants and of Sh sus and sh sus plants are not comparable.

These data are independent of the total colonization and give better information about the development of the fungus inside the roots. Obviously, there was a similarity in the relative abundance of different fungal structures between the Sh Sus and sh Sus lines (highlighted in red) on one hand and in Sh sus and sh sus (highlighted in blue) on the other hand. The Sh Sus and sh Sus lines displayed 21.8% and 25.5% within the total of internal fungal structures (100%) at 6 weeks and the Sh sus and sh sus lines 29.4 and 33.5%, respectively. At 9 weeks, the percentage of internal hyphae was around 20% in the roots of Sh Sus, Sh sus and sh sus lines whereas it reached 34% in the double mutant. In Sh Sus and sh Sus plants, the arbuscules that made up for 50% of the total colonization at 6 weeks remained at ca. 50% after 9 weeks. In the lines lacking the Sus gene, the percentage of arbuscules was higher than in the other lines (59.3% and 63.7% at 6

weeks). At 9 weeks after inoculation, the percentage of arbuscules represented a smaller percentage of the total colonization but remained either close to the two other lines (50%) or still higher (54.7%). The percentage represented by arbuscules decreased for the four lines between 6 and 9 weeks. Compared to wild-type and sh Sus lines, in which the vesicles represented 28.4 % of the total of internal fungal structures, their incidence was only 11.3 % in the Sh sus and less than 3 % in the double mutant in which many whole root segments were free of any fungal structures over their entire length (2 cm.) In the later time-point, the percentage of root sectors with vesicles remained around 30% in the Sh Sus and sh Sus lines, increased to 23.2% in the single Sh sus mutant and stayed below 10% of the total colonization in the double mutant. In that case, the fungal structures inside colonized roots were mainly arbuscules (54.7 %) and internal hyphae (34 %). In conclusion, it appeared that the ratio between the fungal structures displayed small variations between 6 and 9 weeks in the lines where the Sus gene was still present. On the other hand, in the lines lacking Sus gene and having a delay of colonization, the variation between the two time-points was more pronounced.

4.2.3. Sugar content

Root samples of the different maize lines were collected at 6 and 9 weeks and soluble carbohydrates were extracted according to procedures reported earlier (Roth et al., 1997) and analyzed by high-performance liquid chromatography (HPLC) using anion exchange column and pulsed amperometric detection as described (Lüscher et al., 2000). Typically, roots contained between 6 and 16 µg glucose per mg dry weight, between 5 and 9 µg fructose per mg dry weight and 16 and 31 µg sucrose per mg dry weight (Table 4.3.A). To allow an optimal comparison, the data are expressed in ratios that have been calculated for mutant and wild-type lines. A high (F+G)/S ratio might represent a high sucrose-degrading activity related to invertases and a high F/G ratio might be related to a sucrose synthase activity. However, no notable differences in sugar ratios were noticed between the control of the four lines neither at 6 weeks nor at 9 weeks. Similarly, in mycorrhized plants, no major differences were noticed (Table 4.3.B).

A

Genotype	Glucose (µg/mgDW)	Fructose (µg/mg DW)	Sucrose (µg/mg DW)	G+F/S	F/S	F/G
ShSus	12.60 ± 6.12	8.50 ± 4.34	30.50 ± 14.07	0.73 ± 0.18	0.29 ± 0.07	0.73 ± 0.42
shSus	15.80 ± 6.56	6.99 ± 3.38	20.21 ± 7.55	1.12 ± 0.26	0.34 ± 0.10	0.43 ± 0.09
Shsus	11.82 ± 7.00	6.30 ± 5.03	31.04 ± 12.42	0.64 ± 0.40	0.23 ± 0.16	0.51 ± 0.13
shsus	6.19 ± 2.41	5.76 ± 2.05	16.30± 4.70	0.80 ± 0.43	0.38 ± 0.20	0.94 ± 0.15

B

	Genotype	(G+F)/S	F/S	F/G
6 weeks	**ShSus con**	0.88 ± 0.16	0.34 ± 0.09	0.62 ± 0.12
	ShSus myc	0.82 ± 0.08	0.32 ± 0.05	0.65 ± 0.13
	Shsus con	0.82 ± 0.09	0.23 ± 0.04	0. 39 ± 0.02
	Shsus myc	0.82 ± 0.17	0.27 ± 0.04	0.50 ± 0.05
	shSus con	1.32 ± 0.08	0.48 ± 0.08	0.58 ± 0.08
	shSus myc	0.95 ± 0.05	0.39 ± 0.05	0.70 ± 0.08
	shsus con	2.08 ± 0.24	1.31 ± 0.24	0.85 ± 0.24
	shsus myc	0.50 ± 0.07	0.23 ± 0.08	0.87 ± 0.14
9 weeks	**ShSus con**	0.56 ± 0.20	0.22 ± 0.07	0.77 ± 0.40
	ShSus myc	0.64 ± 0.03	0.25 ± 0.02	0.65 ± 0.05
	Shsus con	1.26 ± 0.17	0.18 ± 0.14	0.46 ± 0.09
	Shsus myc	1.09 ± 0.29	0.31 ± 0.12	0.39 ± 0.09
	shSus con	0.49 ± 0.17	0.15 ± 0.07	0.44 ± 0.09
	shSus myc	0.37 ± 0.13	0.11 ± 0.03	0.47 ± 0.09
	shsus con	0.68 ± 0.11	0.31 ± 0.06	0.87 ± 0.15
	shsus myc	0.65 ± 0.24	0.33 ± 0.11	1.05 ± 0.13

Table 4.3: **A.** Sugar content in µg per mg DW and sugar ratios of four different maize genotypes, measured in extracts from the root systems (mean and standard deviation for three to five replicates).
B. Sugar ratios of four different maize genotypes measured in extracts from the root systems, at two time-points in control and mycorrhiza-inoculated treatment (mean and standard deviation for three to five replicates).

4.2.4. In situ hybridization

To examine the localization of sucrose synthase expression, an in situ hybridization experiment was performed on roots of mycorrhizal versus control wild-type maize plants, using sense and anti-sense RNA corresponding to the cDNA of tomato Sh as a probe. As seen in Figure 4.5, the presence of sucrose synthase transcripts was detected only in a subset of cells of mycorrhizal samples and particularly around small arbuscules. In different preparations, an average of three cells was stained for ten arbuscules-containing cells.

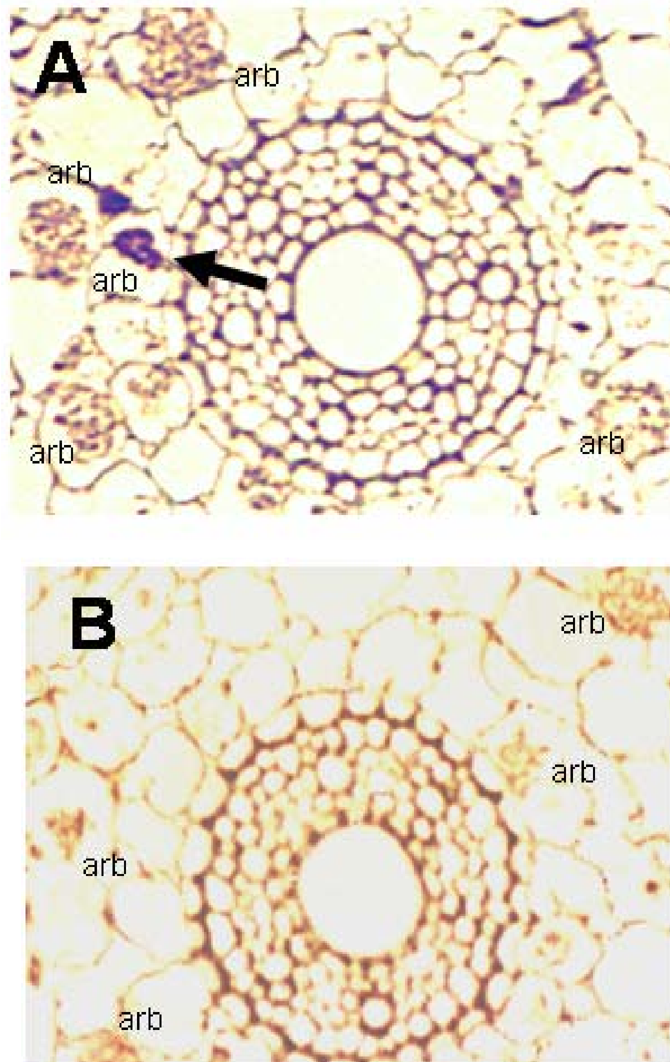

Figure 4.5: Localization of sucrose synthase mRNA by *in situ* hybridization. Root cross-sections of wild-type (ShSus) maize plants 6 weeks after inoculation with *Glomus intraradices* were hybridized with a DIG-labelled (A) antisense tomato sucrose synthase RNA probe and (B) a sense tomato sucrose synthase probe. Arbuscule-containing cells are indicated (arb). Arrow: DIG staining of an arbuscule-containing cell. No staining was observed in the controls.

4.2.5. The importance of sucrose synthase for AM formation in pea

Knowing the minimal pleiotropic effects of *rug4-b* mutation on the activity of other enzymes of primary metabolism (Craig et al., 1999) we used *rug4-b* mutants of pea to see the effect of the lack of sucrose synthase on the development of the symbiosis in a leguminous model plant. The colonization of pea plants by *Glomus intraradices* was estimated after 6 weeks of co-cultivation. The results presented in the Figure 4.6 show that 74% of the wild-type root systems were colonized. The rug*4-b* mutant plants were only colonized at 42.5%. The fresh weight measurements reflected only negligible differences between the mutant and wild-type plants (data not shown). The low fungal presence in the mutants could thus not be due to an overall growth delay of the plants. The decreased colonization in the pea mutants confirmed the results obtained with the maize mutants.

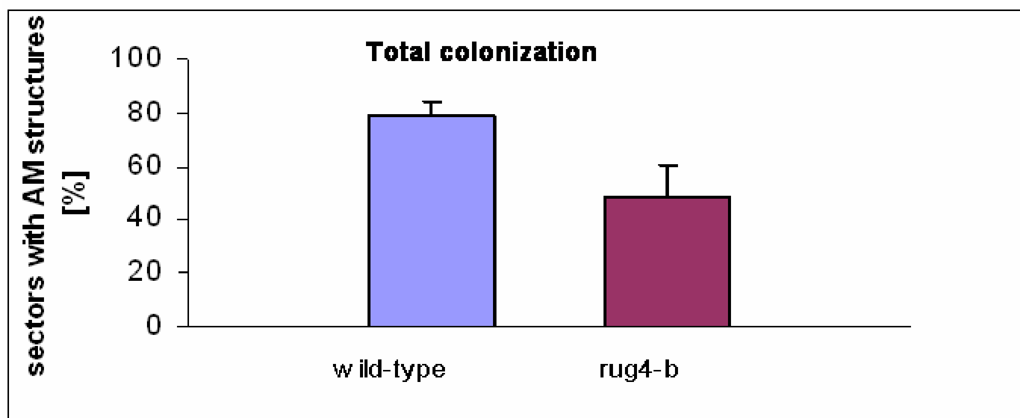

Figure 4.6: Total arbuscular mycorrhizal structures in the roots of two pea genotypes 6 weeks after inoculation by Glomus intraradices. Error bars: standard deviation. Five individual root systems were analyzed per treatment.

The distribution of the fungal structures was different from the one observed in maize plants (Table 4.4) and could not be directly compared to any of the maize mutants. The results of this experiment gave the same tendency of a decreased colonization in the pea mutant and provided further evidence that sucrose synthase encoding genes presence is required for a normal development of AM symbiosis.

	Internal Hyphae	Arbuscules	Vesicles
wild-type	13.51	83.78	2.70
***rug4*-b**	7.06	83.53	9.41

Table 4.4: Distribution of mycorrhizal structures expressed in percent of all the mycorrhizal structures (100%) in pea plants 6 weeks after inoculation with *Glomus intraradices*.The root system of wild-type had a degree of colonization of 74% whereas the *rug4-b* mutant was colonized by 42.5%.

4.3. DISCUSSION

The work presented in this paper consisted in studying the development of AM symbiosis in maize plants deficient in sucrose synthase compared to wild-type plants. The maize lines affected in genes coding for sucrose cleaving enzymes showed normal growth and development, but a decrease of AM colonization was observed in maize mutants plants whereas the wild-type line was heavily colonized. We can therefore conclude that normal expression of the sucrose synthase encoding genes is important in the development of a normal symbiosis. This conclusion is supported by the results obtained in pea where the *rug4-b* mutant showed a reduced colonization by *Glomus intraradices* when compared to wild-type pea plants.

Given that the sucrose synthases are key enzymes of carbon metabolism and partitioning, their absence is expected to cause changes in source-sink relationships. This is most apparent in the seed phenotypes which appear “shrunken” because of their reduced sink activity and ability to accumulate starch. A symbiosis in a wild-type context consists in a repartition of resources between the plant and its symbiont in order for the two partners to develop a tight association that is mutually beneficial. The plants with sucrose synthase deficiency appeared to be impaired in their response to respond to increased demands of sugars provoked by the symbiont, such that the fungal growth could not proceed and the colonization in mutants was delayed compared to wild-type. In addition to the decrease colonization in the mutant lines, the small number of vesicles present in the roots of the double mutant indicates that the fungus was not receiving sufficient carbon to invest into its storage structures. This can be compared to the “shrunken seeds” of the plant. This higher demand provoked by the fungus that cannot be satisfied by the mutant plants can also be seen as a diminished stimulation of the sink strength. Indeed, in addition to be a cost for the plant, mycorrhiza can enhance the process of carbon assimilation, equilibrating to reach an overall benefit (Fitter, 1991; Tinker et al.,

1994). Wright and collaborators (Wright et al., 1998) reported that the mycorrhiza in clover plants increases carbon allocation to roots and stimulates the activities of sucrose cleaving enzymes, above all sucrose synthase but also invertases. The activity of these sucrose-cleaving enzymes is expected to increase the sink strength of colonized roots (Wright et al., 1998). In a further report, it was hypothesized that sink strength could regulate the location and function of arbuscules in cortical cells (Blee and Anderson, 1998). In this case, the fungus would send out signals that stimulate enhanced sink strength in certain cells. These cells would, then, be preferentially targeted by the fungus. These two views on the consequences of AM colonization for carbon allocation are not mutually exclusive and the results of our study do not allow the distinction between them. In any case, the altered expression of genes coding for sucrose synthase in the mutant lines is likely to reduce the sink strength so that the symbiosis develops less extensively compared to wild-type.

Nevertheless, the fungus could develop to a considerable extent in all mutant root systems. The fungal development in the mutant lines can be explained by the presence of alternative ways to degrade sucrose. For example, invertase or other sucrose-cleaving enzymes could explain this remaining colonization in mutants. One cannot exclude a possible role of the third sucrose synthase that has been recently identified in maize (Carlson et al., 2002).

Our observations indicate that both sucrose synthases present in maize are involved in the symbiosis. However, they seem to have a different temporal contribution to the establishment of the symbiosis. Comparing the results given by the Sh sus and sh Sus lines, one can speculate that the product of the Sus gene would be needed first in the development of the symbiosis (until 6 weeks) and that Sh gene would be needed later (between 6 and 9 weeks). The presence of sucrose synthase encoded by the Sus gene appeared of primary importance for a full colonization. Apparently, the fungal development in the mutants lacking Sus gene was not only delayed but also rarely completed. Indeed, considering the strongly reduced number of vesicles in Sh sus and sh sus and the similarly high abundance of vesicles in wild-type and in sh Sus, the mutation of the Sus gene appears to be responsible for the reduction of vesicle development. The in situ hybridization experiments show only signals for sucrose synthase mRNA in a subset of cells containing small arbuscules and the measurements of sugars in whole root systems are not indicative of dramatic changes in sucrose cleaving activities due to colonization. Therefore, it is possible that the product of the Sus gene induces enhanced sink strength very locally to ensure sufficient sucrose supply for the nascent arbuscule. This view is in agreement with the observations made with sucrose synthase anti-sense plants in tomato, where sucrose import into the

fruit was only altered very early in development but where the consequence of the loss of sucrose synthase expression was a reduced number of fruits (D'Aoust et al., 1999).
In maize, sucrose synthase is composed of a tetramer that can be composed of different subunits encoded by Sh and Sus (Koch et al., 1992). The ratio between the subunits is possibly responsible for different activities because of different affinities (Km) for sucrose (Koch et al., 1992). Between 3 and 6 weeks of colonization process, in wild-type, both the Sus and Sh gene products may be present and may be important for the formation of particular types of tetrameric holoenzymes. This would implicate a larger spectrum of particular affinities (Km) and preferred localization of the functional proteins. In any case, the results obtained in the sugars measurement and particularly the negligible difference obtained in the different sugars ratios between WT and mutants as well as between control and mycorrhizal plants did not allow precise distinction of the relative implication of the two enzymes. Immunolocalization and sucrose synthase antisense experiments in the context of AM colonization will help to answer these questions.

Acknowledgements
Thanks to Trevor Wang for the pea seeds, to Guillaume Bécard and Nathalie Séjalon-Delmas, for in vitro system, to Prem Chourey for maize seeds, to Andrew Fleming for in situ probe, to Fred Meins for facilities for in situ, to Markus Briker for substrates of maize.

4.5. MATERIAL AND METHODS

4.5.1. Plants, fungal material and experimental conditions

Seed of four near isogenic lines of maize (*Zea mays* L.), Sh Sus, Sh sus, sh Sus, sh sus in W22 inbred background were kindly provided by P. Chourey (Chourey 1988). The kernels were surface-sterilized, pregerminated in Petri dishes for 4 days and the maize seedlings were grown in individual pots containing a mixture of commercial soil and sand and placed in a climate chamber (16 hours light at 100 µE $m^{-2}s^{-1}$ 20°C, 8 hours dark at 18°C). Seed of pea (*Pisum sativum* L.), wild-type and mutant at the rug4-b locus (Wang and Hedley, 1993a) were originated from the laboratory of T. Wang. The seeds were surface sterilized, germinated in the dark and after 5 days, the seedlings were planted in individual plastic pots in Terra Green and grown in a climate chamber under the same conditions as

above. Carrot roots, inoculated with spores of the arbuscular mycorrhizal fungus, *Glomus intraradices* (Schenck and Smith) (Bécard and Piché, 1992) were kindly offered by G. Bécard. They were subcultured every two months onto M medium (Bécard and Fortin, 1988) containing 0.3% Phytagel and kept at 26 °C in the dark. Spores were separated from the culture medium as described (Doner and Bécard, 1991). This inoculum adjusted to 600 spores/ml was applied directly onto the roots while transferring the plantlets to individual pots. During the time of the experiment, the maize plants were fertilized weekly with low phosphate fertilizer (32% total N, 6% P 20% K_2O, 2% Mg, 0.1% Fe, 0.05% Mn, 0.01% Mo, 0.02% B, 0,04% Cu, 0.01% Zn) and the pea plants with B&D solution (Broughton and John, 1979). At the time-points indicated, the plants were harvested, separated into roots and shoots and weighted. An aliquot was used directly to determine the degree of colonization and the remaining aliquots were stored at –80°C until further analysis.

4.5.2. Determination of root colonization

Root samples were cleared in 10% KOH at 95°C for 5 to 15 min and stained in 0.1% trypan blue (Phillips and Hayman, 1970) for 20 min. The fungal structures were visualized under a Zeiss Axioplan microscope and the root colonization by was determined by the gridline intersection method (Giovannetti and Mosse, 1980).

4.5.3. In situ hybridization was performed following the protocol of Coen et al. (Coen et al., 1990). In brief, the root tissue was fixed in formaldehyde, dehydrated and embedded in paraffin wax (Paraplast Plus, Sigma, St Louis, MO). The embedded tissues were cut with a microtome in 7 µm sections, mounted onto the center of a poly-L lysine coated slide (Polysine slides), and dried. After deparaffinization and proteinase K treatment the sections were hybridized with appropriate denaturated, DIG-labeled RNA probes overnight at 50°C and then washed with 0.2x SSC at 55°C. After treatment with RnaseA for 30 min at 37°C, the slides were washed again at 55°C with 0.2x SSC. The RNA hybridized probe was detected by enzyme-linked immunoassay using an antibody conjugate, anti-DIG-AP (anti-digoxigenin alkaline phosphatase conjugate, Boehringer Manheim). A subsequent enzyme-catalyzed color reaction with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium salt) produced an insoluble blue precipitate, which visualized hybrid molecules. The DIG-antigen revelation consisted in washes in 0.5% blocking reagent in TBS (100 mM Tris-HCl, pH 7.5, 400 mM NaCl) for 5 min, followed by an incubation in the anti-DIG alkaline

phosphatase conjugate diluted in BSA (bovine serum albumine) and a revelation color by incubating in NBT and X-phosphate for periods of 4 h to 2 days in the dark. The reactions were stopped with washing twice in 10 mM Tris-HCl pH 7 and mounted in DePeX with coverslip.
Preparation of the probes and hybridization were made following the instructions of the DIG RNA labeling kit (Boehringer, Manheim). The probes utilized in this experiment, sense and anti-sense RNA corresponding to the cDNA of tomato Sh were kindly provided by Andrew Fleming (Hänggi and Fleming, 2001). Tomato Sh has 75% sequence identity with the maize sus1 gene and 68% with the maize Sh gene.

4.5.4. Sugar extraction

According to Roth et al, (1997) 200 mg of maize roots were incubated in 1 ml of 80% ethanol for 5 min at 80°C. The samples were then dried and ground in Eppendorf tubes. Polyvinylpyrolidone was added to the ground samples as well as 750 µl of 80% methanol and 75 µl of 1 mg/ml of mannoheptulose as internal standard. After 10 min at 60°C, the supernatant was transferred to a new Eppendorf and the extraction step was repeated. After drying in speed-vac, the pellet was resuspended in 600 µl water and supplemented with ion exchange beads. After several centrifugation steps, 100 µl of the extract were put in vials for HPLC analysis. The samples were analyzed with the program analysed by high-performance liquid chromatography (HPLC) using anion exchange column and pulsed amperometric detection as described in Lüscher et al., 2000.

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V. GENERAL DISCUSSION

5.1. IMPORTANCE OF SUCROSE SYNTHASES IN SYMBIOSES

The data reported in the two chapters of this thesis provide strong evidence that sucrose synthases are important in the development of the AM symbiosis. In *Medicago truncatula*, we discovered a sucrose synthase represented by a newly isolated cDNA clone (MtSucS2). This gene is most closely related to the pea 3 gene, in contrast to MtSucS1 which is most closely related to the pea 1 gene (Fig.5.1). We found that MtSucS2 was strongly up-regulated upon colonization by the AM fungus *Glomus intraradices*. This up-regulation, seen in a differential display screening, was confirmed by semi-quantitative RT-PCR. We followed the expression of the gene corresponding to the differential clone in a time-course experiment, and we compared it to the expression of the nodule-enhanced sucrose synthase-encoding gene (MtSucS1) characterized by Hohnjec and collaborators (Hohnjec et al., 1999). This gene displays a ten-fold higher expression level in nodules compared to control roots but it is not induced in mycorrhizal roots. Using the same technique of semi-quantitative RT-PCR, we studied the expression of the two sucrose synthases in the main organs of *Medicago truncatula*. Compared to normalized ubiquitin expression, MtSucS2 expression was highest in fruits (8x higher than ubiquitin) and flowers (2x higher than ubiquitin). In AM colonized roots and in nodules, MtSucS2 expression was induced compared to non-colonized roots. In the present study, MtSucS2 was induced 4 fold by mycorrhiza. A study using cDNA chips confirmed for mycorrhizal maize that sucrose synthase was induced (Uta Paszkowski, personal communication).

Experiments in maize and pea deficient in sucrose synthases revealed the importance of functional sucrose synthase in mycorrhiza development. Mutations at loci encoding sucrose synthases cause a delay of mycorrhiza formation and poor development of mycorrhizal fungus. The RT-PCR revealed slightly different patterns of Medicago truncatula Sucs1 expression than the northern-analysis reported earlier (Hohnjec et al., 1999). While the authors detected expression of MtSucS1 only in nodules, roots and stems, RT-PCR shows expression in the roots containing nodules as well as in flowers. One can explain the difference in the two studies concerning the expression in flowers by a higher sensitivity of the RT-PCR compared to the northern-blot analysis or by the age of the plant at harvest. The results show that the MtSucS2 expression is enhanced most in mycorrhized roots, in isolated nodules and fruits.

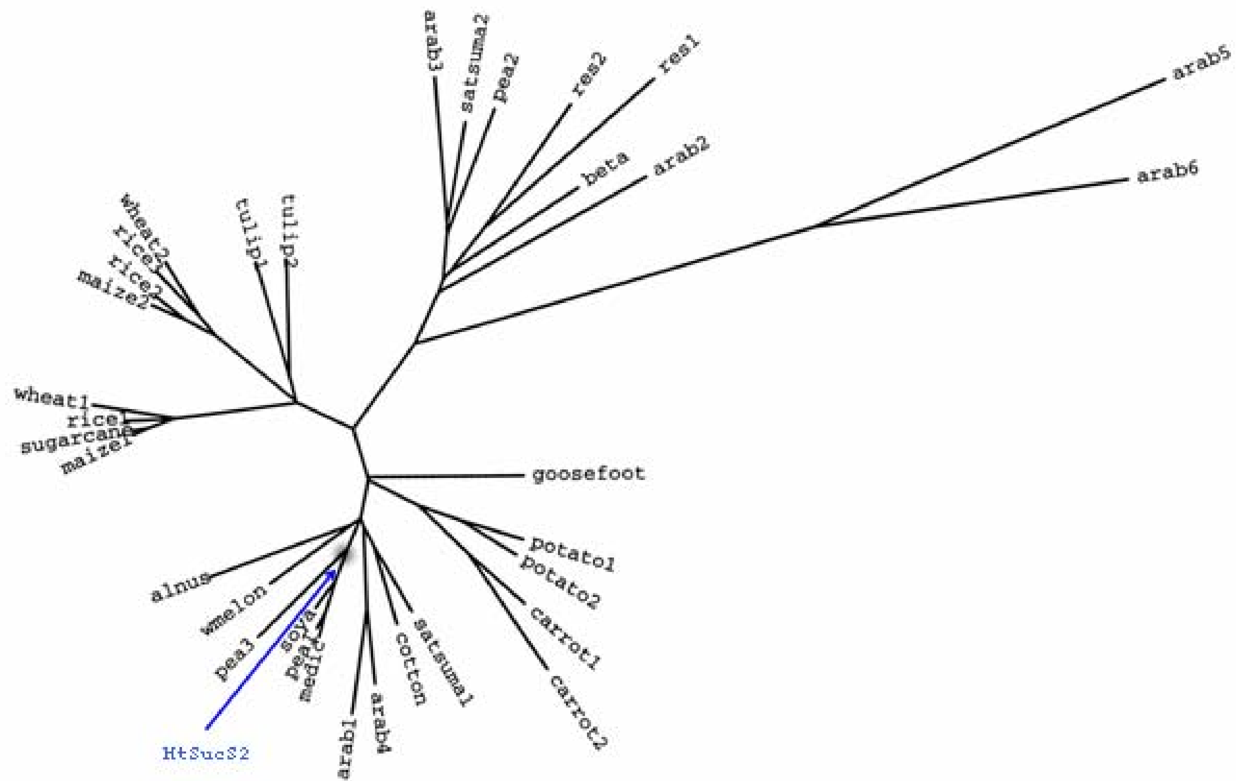


Figure 5.1: Unrooted phylogenetic tree of predicted amino acid sequences of Sus proteins. From Barrat et al., 2001.The position of MtSucS2 is indicated with the blue arrow.

Interestingly MtSucS2 level appears unaffected in nodulated roots compared to control roots whereas it is highly increased in isolated nodules. This can be explained by the dilution of the nodule-enhanced message of MtSucS2 by the total RNA of the surrounding root tissue. MtSucS2 expression is clearly higher in mycorrhizal roots than in control roots. Because it appears to be mainly expressed in arbuscule-containing cells, it is likely induced by a very large factor in these specialized cells. The results of in situ-hybrization on maize root colonized by AM fungi reveal that only some particular colonized cells were showing the presence of sucrose synthase transcripts and support the idea of a localized induction of the sucrose synthase.

MtSucS2 seems to be important in particular sink organs. Roots are sink organs by definition; they are supplied by sucrose from the classical source tissues, the mature leaves. In a growing plant, there are additional sinks, which are likely to perturb the flux of sucrose as described by a simplified leaf-root model. Flowers, fruits, nodules and mycorrhizal cells would obviously fall in this category. This model would therefore weaken the concept of sucrose synthase specifically induced during symbioses. The apparent diverse regulation patterns of the two sucrose synthases rather reflect specific functions required in certain cells or tissues. In pea, the analysis of expression patterns of the three isoforms of sucrose synthase shows that the contribution of each isoform to sucrose synthase activity differs between organs and through development (Barratt et al., 2001). The mutations at the rug4 locus cause a large and specific reduction in the activity of sucrose synthase in the developing embryo and in nodules on the roots and smaller reductions in sucrose synthase activity in testas and the leaf. The data obtained in Medicago are consistent with the ones obtained in maize and illustrate the idea derived from the maize sh and sus mutants (Chourey et al., 1998) that different isoforms of sucrose synthase may channel carbon from sucrose toward different metabolic fates within cells. In Sh sus maize plants, the delay of colonization was more obvious at 6 weeks than at 9 weeks therefore the lack of Sus protein would cause a delay in the early phase of AM symbiosis. Similarly, after 6 weeks the mutants sh Sus display a high delay of colonization, but after 9 weeks, the colonization increases. Hence, the lack of Sh protein inhibits the development of the symbiosis in a later phase.

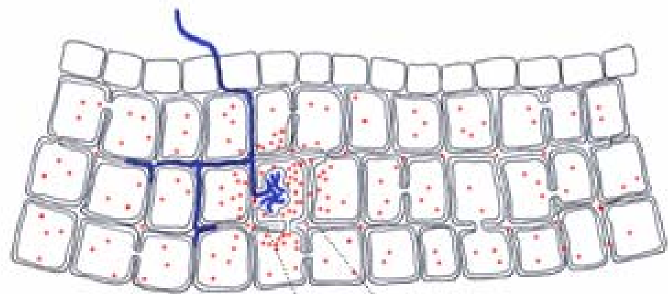
In *Medicago*, the expression of MtSucS2 encoding gene is already higher at 3 weeks in mycorrhized roots compared to control. Thus, MtSucS2 encoding gene appears to be functionally related to SS2 encoding gene (Sus) in maize in terms of the symbiosis development. The expression analysis of MtSucS1 gene showed the presence of the gene in 20 days old uninfected roots by northern analysis (Hohnjec et al., 1999) as it was found by the RT-PCR. During development, the expression of

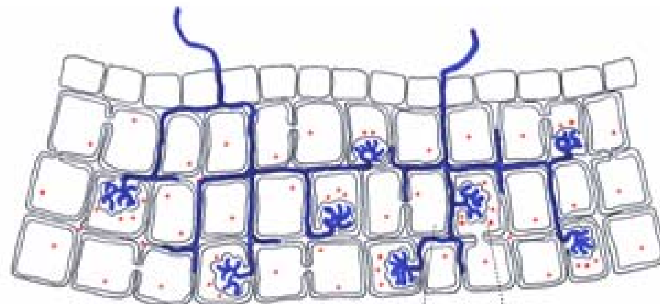
MtSucS1 increases both in control and nodulated roots. However, in mycorrhizal roots when the MtSucS2 expression increases, the MtSucS1 expression is reduced.

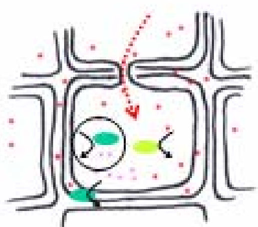
5.2. PROPOSED ROLES FOR SUCROSE SYNTHASES IN SYMBIOSES

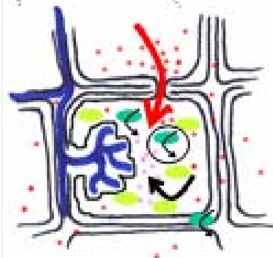
In the light of the results obtained, possible roles for sucrose synthase in the AM symbiosis can be proposed. In the *Rhizobium*-legume symbiosis, the nodules are primarily dependent on the import and metabolism of sucrose. With regard to its enhanced expression in nodules, the MtSucS2 may metabolize sucrose mainly in the cytoplasm of the infected zone of the nodules. According to different studies on the absence of transporters for most sugars in bacteroids (for review, see (Streeter, 1995)), the plant does not supply them with significant amounts of sugars. Instead, the organic acids (usually malic acid) are the main assimilates supporting respiration of bacteroids and indirectly nitrogen fixation (Udvardi and Day, 1997). These organic acids are ultimately produced from sucrose supplied through the phloem. Concerning the importance of carbon metabolism in AM symbiosis development, it has been reported that mycorrhiza formation changes the carbon balance (Wright et al., 1998) and that the glucose is the major carbon compound to be taken up by intraradical AM hyphae, particularly at the arbuscular interface (Shachar-Hill et al., 1995). The nodules and the cells containing arbuscules, would be similar with respect to an enhanced sink for sucrose and root cell colonized by arbuscules could be considered as a “special sink” tissue. Additional sink activity arising through mycorrhizal colonization or bacteria infection has been shown to enhance normally the activities of both sucrose synthases and invertases (Wright et al., 1998). It has also been proposed that the carbon availability would control the location and function of arbuscules in cortical cells. In the cell, the hydrolysis of sucrose by sucrose-cleaving enzymes would create a gradient for symplastic influx of sucrose and a gradient for efflux of cleavage products toward the apoplast. In the apoplastic space, the fungus could be supplied by the hexoses (Blee and Anderson, 1998).

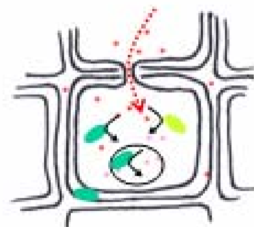
According to this model, we can propose a hypothesis (Fig. 5.2) to answer the following questions: What is/are the function(s) of sucrose synthase in AM symbiosis? What is the significance of a loss of sucrose synthases for mycorrhiza?











invertase
sucrose synthase
sucrose cleaving activity
sink strength
sucrose
hexoses

Figure 5.2: Scheme to illustrate the proposed role for sucrose synthase in AM formation. A wild-type root during an early phase (left) and a later phase (right) of AM development is symbolized. In the early phase, sucrose (symbolized by read dots) is not limiting and there are only few special sinks. The induction of sucrose synthase in arbuscule-containing cells enhances locally the sink strength, which results in a more important sucrose uptake than in non-colonized cells where the total sucrose cleaving activity (invertase plus sucrose synthase) is lower. Later during AM development, the number of arbuscule-containing cells (special sinks) has increased and these sinks compete for sucrose. Under these conditions, because the available sucrose becomes limiting, an enhanced sucrose synthase activity cannot lead to a higher sucrose uptake. Hence, lack of inducible sucrose synthase e.g. as in the maize and pea mutants causes delay and poor development of AM during the early phase because arbuscule containing cells cannot enhance the sucrose uptake while sucrose is non-limiting. Later, under conditions where sucrose is limiting, arbuscule-containing cells in wild-type and mutant roots are equivalent and the development proceeds similarly in both cases.

In a WT root cell, the sucrose provided by the leaves is available and non-limiting. The AM fungus starts to develop within the root system where there are two types of root cells. Among them, there are non-colonized cells where the cleavage products of sucrose are provided by both acidic invertases and sucrose synthases. The enhanced hexose consumption by the fungus is compensated by the induction of sucrose synthases and invertases activities. Next to this cell, the fungus develops and forms an arbuscule. The hexoses are provided to the fungus by the combination of invertases and of induced sucrose synthase. The sucrose in this case is not limiting and there is a stimulation of the sink strength in the colonized cells and consequently an increased sucrose import into these cells. The next step is characterized by an increased number of arbuscule-containing cells as well as by the formation of vesicles. Because the arbuscules containing cells compete among each other for hexoses supply, sucrose itself becomes now limiting rather than the sucrose cleaving activities of the different cells.

The mutant plants are not able to increase the sink strength in the root cells because they are deficient in sucrose synthase, therefore the development of colonization in maize mutant lines is delayed. Nevertheless, the fact that the double mutants are still colonized means the invertases or a third sucrose synthase present in the plant are sufficient for C supply. It is likely that the invertases and sucrose synthases act synergistically in the AM symbiosis. In many instances, the sucrose synthase pathway for metabolic utilization of sucrose in plant cells is thought to be favored because it requires only half the net energy of the alternate pathway through invertases (Black et al., 1987). This model is in agreement with the results obtained with sucrose synthase anti-sense tomato plants (D'Aoust et al., 1999). These plants produce fewer fruits than wild-type plants. The import of sugars is reduced during the early development of the fruit. Later, there is no difference in sugar uptake between wild-

type and anti-sense plants and there is no difference in the sugar content of fruits at the end. In maize mutants, the mycorrhizal colonization is reduced, but all the fungal structures are present and they are normal. Like in anti-sense plants of tomato where there are fewer fruits, in the maize double mutant, the number of vesicles is highly reduced.

Colonized cells have in common with other special sink organs the increased demand for carbon. This is in line with the observation that MtSucS2 expression is enhanced in the fruits, flowers and nodules in *Medicago* as well. MtSucS2 expression may well support a higher carbon demand for the organ development and in the case of nodules and arbuscule-containing cells, it may be responsible for the hexose supply for both the plant and the symbiont.

In higher plants, sucrose synthases are generally globular cytosolic proteins. However, it was found that part of the protein can be associated with the plasma membrane (Winter et al., 1997). The membrane-associated form of the sucrose synthase may be involved in a complex with β-glucan synthases to channel UDP-glucose into cellulose synthesis (Amor et al., 1995). The membrane association of sucrose synthase is dynamic and it was strongly suggested that a reversible protein phosphorylation of sucrose synthase may control this process in both maize leaves and soybean nodules. In maize, a single serine residue, namely the serine-15 of the maize SS2 protein is phosphorylated (Huber et al., 1996). Phosphorylation of the membrane-associated sucrose synthase causes release from the membrane while dephosphorylation of the soluble form promotes membrane association (Winter et al., 1997). Interestingly, it was also shown that at least a portion of the phosphorylated sucrose synthase is not simply soluble but bound to the actin cytoskeleton in vivo (Winter et al., 1998).

The insights on the importance of sucrose synthases in AM symbiosis reported in this thesis have to be considered with these additional elements of post-translational regulation and localization of the sucrose synthase. As reported in several publications, the mycorrhizal events lead to extensive reorganization of the host cell, like nucleus migration, plasma membrane invagination, vacuole fragmentation and deposition of new wall components (Bonfante and Perotto, 1995; Balestrini et al., 1996; Balestrini et al., 1997). Upon colonization, a α-tubulin gene is activated exclusively in the colonized cells and the microtubular cytoskeleton of the host cell is reorganized, the microfilament pattern showing particularly important changes (Bonfante et al., 1996; Genre and Bonfante, 1997; Genre and Bonfante, 1998). One can speculate that, alternatively or in addition to its role in the regulation of sink strength, a phosphorylated form of sucrose synthase binding to both G and F actin

could control the symbiosis development participating to the rearrangement of cytoskeleton for the formation of arbuscules and for a new repartition of the cell organelles.

5.3. OUTLOOK

In our study on a better understanding of the complex events underlying the AM symbiosis, initial insights were obtained concerning the determinant involvement of sucrose synthases in a symbiotic context.

1) In maize and in pea, sucrose synthases are necessary for an optimal development of the AM symbiosis.

2) The newly isolated cDNA clone corresponding to a second sucrose synthase in *Medicago truncatula* (MtSucS2) has an enhanced expression in mycorrhizal roots. Furthermore, MtSucS2 shows a strong induction in special sink organs like nodules, flowers and fruits.

Therefore, the most important questions to address now aim at elucidating the function(s) and the biological relevance of the sucrose synthase in AM symbiosis on one hand and in organs where the sink activity is increased on the other hand.

Different experiments may be performed to investigate the importance of MtSucS2 in AM symbiosis. First of all, the obtention of the full-length clone corresponding to MtSucS2 is required. A Southern analysis is important to determine if MtSucS2 is a single copy gene in the Medicago genome as it was shown for MtSucS1. The transformation of *Medicago truncatula* roots by *Agrobacterium* allows the possibility of performing anti-sense and overexpression studies. Using the in vitro system where *Glomus intraradices* is the fungal partner, it will be interesting to assess the mycorrhizal phenotype of the transformed *Medicago* roots. Does the knock-out of MtSucS2 decrease the development of the symbiosis? Does its overexpression increase the colonization rate or does it allow a faster expansion of the fungus in the root system?

In maize, studies on the expression of the sucrose synthase at the RNA level and the measurements of corresponding enzymatic activities can be envisaged as well. Experiments of immunogold localization conducted within root cell colonized by AM fungus may confirm the presence of the sucrose synthase protein around arbuscules and can give an idea on the subcellular localization of the corresponding protein at different time-points of the colonization. To investigate the possible attachment of sucrose synthase(s) to the cytoskeleton, with the use of antibodies against actin and sucrose synthase, a co-localization as well as a co-purification of the two proteins can be envisaged.

Concerning the expression of sucrose synthase in “special sink organs” described in this thesis, one would predict that MtSucS2 is induced also in response to the attack by pathogens or by parasites such as cuscuta. It will be very exciting to see if a maize pathogen, such as *Sporisorium reilianum zeae* responsible of the smut disease by an infection starting in the root, drives the induction of the sucrose synthases expression.
In conclusion, the insights presented in this thesis are exciting starting points for further experiments that will contribute to the elucidation of the elaborated mechanisms of the AM symbiosis.

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