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ALL-RUSSIAN RESEARCH INSTITUTE FOR AGRICULTURAL MICROBIOLOGY

BURGUNDY UNIVERSITY

ECOLE DOCTORALE ENVIRONNEMENT – SANTE-STIC

Laboratory 'Genetics of Plant-Microbe Interactions'
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THESIS

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presented by

KUZNETSOVA Elena Vladislavovna

CHARACTERIZATION OF PEA (*PISUM SATIVUM* L.) GENES IMPLICATED IN ARBUSCULAR MYCORRHIZA FORMATION AND FUNCTION

Defended publicly, 21 October 2010

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Abbreviations

AFLP	amplified fragment lenght polymorphism
ALP	alkaline phosphatase
AM	arbuscular mycorrhiza
App	appressoria formation
Arb	arbuscule formation
bp	base pair
CAPs	cleaved amplified polymorphic sequences
cDNA	complementary deoxyribonucleic acid
Coi	hyphae cortex invasion
cv.	cultivar
dCAPs	derived cleaved amplified polymorphic sequences
DNA	deoxyribonucleic acid
DEPC	diethyl pyrocarbonate
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetracetate
ER	endoplasmic reticulum
EST	expressed sequence tag
Gi	*Glomus intraradices*
Hac	root hair curling
Ici	inner cortex invasion
Idd	infection droplet differentiation
Ith	infection thread growth inside root hair
Iti	initiation of the infection thread growth
Itn	infection thread growth inside nodule tissue
Myc	mycorrhiza
LCM	laser capture microdissection
NFS	nitrogen-fixing symbiosis

Nod	nodule formation
PAM	periarbuscular membrane
PCR	polymerase chain reaction
Pen	penetration into root
Pi	inorganic phosphate
polyP	polyphosphate
PPA	prepenetration apparatus
RAPD	random amplified polymorpic DNA
RFLP	restriction fragment lenght polymorphism
Rmd	rate of mycorrhiza development
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SSH	suppressive subtractive hybridization
STS	sequence target site
SYM	symbiotic
TAC	transformation-competent artificial chromosome
TAGs	triacylglycerols
Taq	*Thermophilus aquaticus*
Tris	tris-(hydroxymethyl)-aminomethane

SUMMARY

The arbuscular mycorrhizal (AM) association results from a successful interaction between the genomes of the two symbiotic partners. In this context, the aim of my research was to better characterize the role of the late stage symbiosis-related pea genes *PsSym36*, *PsSym33* and *PsSym40* in the functional AM (i) by investigating the effect of mutations in the three genes on fungal and plant gene responses and (ii) by creating conditions for the localization of two of the genes, *PsSym36* and *PsSym40*, on the pea genetic map for future map-based cloning. The expression of a subset of ten fungal and eight plant genes, previously reported to be activated during mycorrhiza development, was compared in *Glomus intraradices*-inoculated roots of wild type and *Pssym36, Pssym33* and *Pssym40* mutant pea plants. Most of the fungal genes were down-regulated in roots of the *Pssym36* mutant where arbuscule formation is defective, and several were upregulated with more rapid fungal development in roots of the *Pssym40* mutant. Microdissection of mycorrhizal *PsSym40* roots corroborated preferential expression of the three *G. intraradices* genes *SOD*, *DESAT* and *PEPISOM* in arbuscule-containing cells. Inactivation of *PsSym36* also resulted in down regulation of plant genes whilst mutation of the *PsSym33* and *PsSym40* genes affected plant gene responses in a more time-dependent way.

Results thus indicate an implication of the investigated pea *SYM* genes in the modulation of plant and fungal molecular interactions linked to signaling, nutrient exchange or stress response regulation during AM symbiosis formation and functioning. Conditions for localization of the *PsSym36* and *PsSym40* genes on the pea genetic map were developed for their future map-based cloning. Based on the molecular markers obtained, it was possible to conclude that localization of the *PsSym40* gene most likely resides outside the linkage groups I, II, III or V of the genetic map of pea.

RESUME

L'association mycorhizienne à arbuscules (AM) est le résultat d'une interaction compatible entre les génomes des deux partenaires symbiotiques. Dans ce contexte, le but de mes recherches a été de mieux caractériser le rôle des gènes de pois liés aux stades tardifs de la symbiose, *PsSym36*, *PsSym33* and *PsSym40*, dans le fonctionnement de la symbiose MA (i) en étudiant l'effet des mutations de ces trois gènes sur l'expression des gènes de la plante et du champignon, et (ii) en créant les conditions pour positionner deux de ces gènes, *PsSym36* and *PsSym40*, sur la carte génétique afin d'envisager leur clonage futur. L'expression d'un groupe de dix gènes fongiques et de huit gènes de plante, déjà décrits pour être activés durant le développement de la mycorhize, a été comparée dans les racines de pois inoculées avec *G. intraradices* chez les plantes de génotypes sauvages, ou les mutants *Pssym36, Pssym33* et *Pssym40*. L'expression de la plupart des gènes fongiques a été inhibée dans les racines du mutant *Pssym36* où la formation des arbuscules est avortée, tandis que l'expression de plusieurs d'entre eux a été activée lorsqu'il existe un développement plus rapide du champignon dans les racines du mutant *Pssym40*. Des microdisséquats obtenus à partir de racines mycorhizées du mutant *PsSym40* confirment l'expression préférentielle de trois gènes de *G. intraradices* (*SOD*, *DESAT* et *PEPISOM*) dans les cellules contenant les arbuscules. L'inactivation du gène *PsSym36* provoque également une inhibition des gènes de plante alors que la mutation des gènes *PsSym33* and *PsSym40* affecte l'expression des gènes de plante plutôt de façon temporelle.

Les résultats indiquent ainsi une implication des gènes *SYM* de pois dans la modulation des interactions moléculaires entre la plante et le champignon impliquées au niveau de la signalisation, des échanges nutritifs ou de la régulation des réponses au stress durant la formation et/ou le fonctionnement de la symbiose AM. Les conditions pour la localisation des gènes *PsSym36* and *PsSym40* sur la carte génétique du pois ont été développées pour leur clonage basé sur la cartographie. En utilisant les marqueurs moléculaires obtenus, il a été possible de conclure que la localisation du gène *PsSym40* réside vraisemblablement à l'extérieur des groupes de liaison I, II, III ou V de la carte génétique du pois.

РЕЗЮМЕ

Формирование арбускулярной микоризы (АМ) является результатом успешного взаимодействия между геномами двух симбиотических партнёров. Целью моего исследования являлось изучение роли поздних симбиотических генов гороха *PsSym36*, *PsSym33* и *PsSym40* в формировании функционального АМ симбиоза. Для этого было проведено исследование эффекта мутаций в генах *PsSym36*, *PsSym33* и *PsSym40* на экспрессию грибных и растительных генов, предположительно (по литературным данным) вовлечённых в процессы формирования АМ, а так же проведена работа по локализации генов *PsSym36* и *PsSym40* на генетической карте гороха для последующего более точного картирования и позиционного клонирования данных генов. Экспрессия десяти грибных и восьми растительных генов была определена в корнях растений дикого типа и *PsSym36*, *PsSym33* и *PsSym40* мутантов, инокулированных *G. intraradices*. В корнях *PsSym36* мутанта, имеющего дефект развития арбускул, большая часть грибных генов была супрессирована, в то время как в корнях *PsSym40* мутанта, для которого характерна более быстрая по сравнению с диким типом микоризация, был отмечен более высокий уровень экспрессии грибных генов. Использование метода микродиссекций позволило выделить клетки, содержащие арбускулы, из микоризованных корней мутанта *PsSym40* и подтвердить, что гены *G. intraradices SOD*, *DESAT* и *PEPISOM* преимущественно экспрессируются в клетках, содержащих арбускулы. Мутация в гене *PsSym36* также привела к подавлению экспрессии большинства вовлечённых в анализ растительных генов, тогда как мутации в генах *PsSym33* и *PsSym40* оказали влияние на эксперессию растительных генов в меньшей степени. Полученные результаты свидетельствуют о роли исследуемых *SYM* генов гороха в контролировании растительно-грибных молекулярных взаимодействий, связанных с сигналингом, обменом питательными веществами и стрессовыми реакциями в процессе формирования и функционирования АМ симбиоза. Проведённое генетическое картирование не привело к локализации генов *PsSym36* и *PsSym40* на генетической карте гороха. Однако разработка и использование молекулярных маркеров для картирования позволили исключить локализацию гена *PsSym40* в I, II, III и V группах сцепления с высокой долей вероятности.

CHAPTER I

INTRODUCTION

I. Introduction

I.1. Arbuscular mycorrhiza, a ubiquitous symbiosis

The arbuscule mycorrhiza (AM) symbiosis is probably the most widespread beneficial interaction between plants and microorganisms. This symbiosis between roots and the fungal phylum of *Glomeromycota* originated around 450 million years ago and may have been a key event that enabled plants to colonize emerging landscapes (Remy et al., 1994). Since then, AM associations have become an integral part of most terrestrial plants in different ecosystems. More than 80% of land plants form AM which play a major role in improving plant nutrient (mainly phosphorus) and water uptake (Parniske, 2004). Colonization of the root system by AM fungi provides an additional pathway for the uptake of nutrients by formation of an extended network of extraradical hyphae and of a system for transfer to the plant by means of specialized intracellular structures, arbuscules (Harrison et al., 2005). More generally, the AM symbiosis may enhance resistance of plants to biotic (root pathogens) and abiotic (heavy metals, salinity, drought) stress (Dumas-Gaudot et al., 2000; Gianinazzi et al., 2005). In return, the plant provides photosynthetic carbon to the fungi which are unable to obtain carbon saprophytically. It has been estimated that worldwide, about 5 billion tons of carbon are transferred from plants to AM fungi each year (Parniske et al., 2004). The potential agronomic and ecological importance of this unique beneficial fungal/plant association is largely recognized (Koide and Dickie, 2002; Ryan and Graham, 2002; Johansson et al., 2004).

I.2. Formation of the AM symbiosis

I.2.1. Stages of AM development

AM fungi are obligate biotrophs, their life-cycle depending on the formation of a symbiotic relationship with plant roots. The asymbiotic phase of their life cycle is restricted to spore

germination and production of a limited amount of mycelium (Belestrini and Lanfranco, 2006). In the presence of root exudates of a host plant, spores of AM fungi usually show higher germination frequencies, and increased growth and branching of fungal hyphae (Gianinazzi-Pearson et al. 1989; Tamasloukht et al., 2003). After a series of recognition events between the symbiotic partners involving plant and fungal signals (Gianinazzi-Pearson et al. 2007), the fungi colonize the root surface, form appressoria, and enter the rhizodermis intercellularly or intracellularly depending on the host plant and fungus involved (Dickson, 2004). Fungal hyphae then grow through the outer cell layers to invade cells of the inner cortical parenchyma, where the fungi form highly branched intracellular structures, named arbuscules. Arbuscules, which can occupy the major part of the inner cortical root tissues, are key structures of the AM symbiosis as they are responsible for reciprocal nutrient and signal exchange between partner cells. They are completely surrounded by a periarbuscular membrane (PAM) which is formed de novo from the plant plasma membrane, giving rise to a symbiotic interface (Gianinazzi-Pearson, 1996).

Arbuscules senesce and degrade after 4-10 days of symbiotic activity (Alexander *et al.* 1988), whereupon the original organization of the plant cells is restored and they can become colonized again by an AM fungus (Jacquelinet-Jeanmougin et al., 1987; Gianinazzi-Pearson, 1996). The life cycle of AM fungi is completed by the formation of extraradical hyphae and new spores (Sanders, 1977; Jacquelinet-Jeanmougin, 1987; Marsh and Schultze, 2001; Hause and Fester, 2005).

I.2.2. Cytological reorganization of plant cells during AM formation

Upon first contact with the appressoria of AM fungi, the contents of a root epidermal cell undergo an important reorganization to form a specialized membrane-bound cytoplasmic assembly, the prepenetration apparatus (PPA), which directs the penetrating AM hypha through the plant cell. The PPA is a novel structure comprising microtubules, microfilaments and endoplasmic reticulum (ER) assembled within a thin column of cytoplasm, created by repositioning of the plant nucleus directly below the site of appressorium contact, and

subsequent migration of the nucleus across the epidermal cell (see figure I.1 A, B; Genre et al., 2005). PPA plays an important role in the creation of the membrane/matrix interface surrounding hyphae during AM colonization of the root (Genre et al., 2008).

A similar penetration mechanism operates during the next steps of AM fungal colonization of outer and inner cortical cells (Genre et al., 2008; Smith et al., 2006). Once the fungus has entered and fully traversed the epidermal cell, a second PPA forms across the adjacent outer cortical cell in advance of the progressing hyphal tip (see figure I.1 A, B). The subsequent pattern of AM fungal colonization of the inner cortical tissues is dependent on the identity of the host plant. In the case of the *Medicago truncatula*/*Gigaspora* spp association, intercellular hyphae grow through the apoplastic space of the cortical parenchyma before penetrating individual cells and initiating arbuscule development (*Arum*-type colonization; Smith and Smith, 1997). Intercellular hyphal growth triggers enlargement and repositioning of the plant nucleus, associated with an accumulation of cytoplasm which aggregates on the inner cell side in contact with the hypha. The subsequent colonization of the individual plant cell is preceded by nuclear movement to the center of the cell and assembly of a cortical PPA. Formation of prebranching cytoplasmic aggregations along the intracellular hypha determines arbuscule branching (see figure I.1 A; Genre et al., 2008).

A different pattern of AM development occurs in the inner cortex of *Daucus carota* roots (see figure I.1 B) which is characterized by an intracellular cell-to-cell colonization (*Paris*-type of colonization; Smith and Smith, 1997). In this case, there is a linear long-distance coordinated PPA development which is defined by specific nuclear dynamics and the formation of a cytoplasmic bridge in adjacent cortical cells in advance of the approaching hypha (Genre et al., 2008).

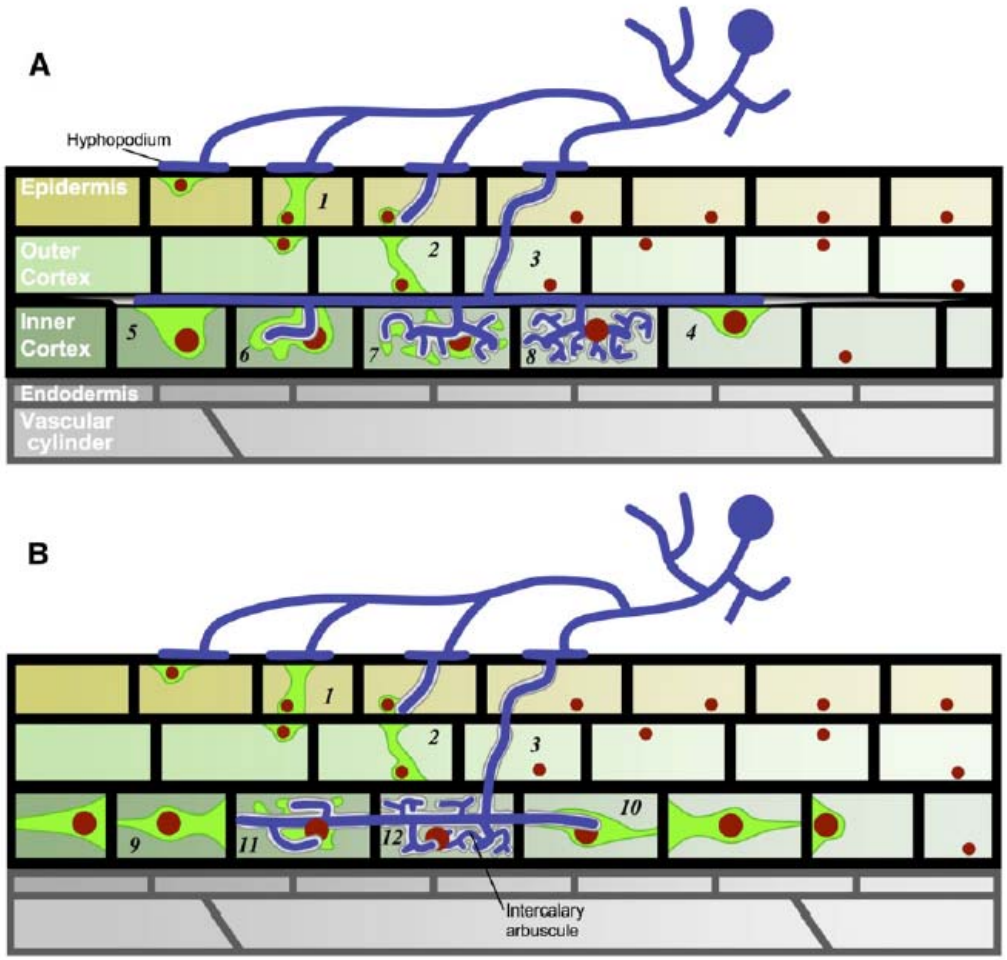


Figure I.1. Schematic summarizing the different AM colonization patterns and associated cellular dynamics for *M. truncatula* and *D. carota* (Genre et al., 2008).

1-3 (A, B) – common steps of AM colonization: 1 – the adhesion of a fungal hyphopodium to the root surface triggers the assembly of a PPA in the contacted epidermal cell, 2 – PPA formation in the underlying outer cortical cell, 3 – fungal crossing of the outer root layers;

4-8 (A) – colonization of inner cortical cells of *M. truncatula* (intercellular hyphae grows along and between the cells): 4 – nuclear reposition upon direct hyphal contact, 5,6 – assembly of large PPAs in inner cortical cells, 7 – assembly of prebranching cytoplasmic aggregation preceding the arbuscular branches development, 8 – completed arbuscule;

9-12 (B) - colonization of inner cortical cells of *D. carota* (intracellular hypha grows from one cell to the next): 9, 10 – organization of the polarized funnel-shaped PPAs in adjacent cells, 11 – assembly of prebranching cytoplasmic aggregation, 12 - completed arbuscule.

The presence of the fungus in cells of the inner root cortex induces a decrease in size and fragmentation of the vacuole, disappearance of amyloplasts, nuclear hypertrophy and proliferation of organelles (plastids, mitochondria, dictyosomes) (Jacquelinet-Jeanmougin et al., 1987; Bonfante-Fasolo and Perotto, 1992; Balestrini et al., 1992). Mitochondria aggregate in the vicinity of arbuscules and plastids form stromules (stroma filled tubules) leading to extensive network-like structures, both of which may be necessary to support the general increase in metabolic activity of the colonized host cells (Lohse et al., 2005). The nuclear hypertrophy of arbuscule-containing cells (Balestrini et al, 1992) is characterized by enhanced fluorochrome accessibility, increased nuclease sensitivity and chromatin dispersion which reflect an increase in chromatin decondensation (Gianinazzi-Pearson, 1996). These modifications indicate a strong activation of host cell transcription in response to fungal colonization.

Formation of the arbuscule results in the genesis of a new interfacial compartment between the fungal wall and PAM which provides the structural basis of the biotrophic interaction between plant and fungus. The presence in this interface of molecules typical of the primary plant cell wall (i.e., cellulose, pectins, hemicellulose, HRGPs) suggests that the PAM retains the enzymatic machinery involved in both synthesis and secretion of cell wall material (Bonfante, 2001; Balestrini and Bonfante, 2005). Specific activation of genes encoding H^+-ATPase and a low-affinity phosphate transporter in arbuscule-containing cells, and location of the corresponding protein at the PAM suggest transmembrane transport activities at the symbiotic interface (Gianinazzi-Pearson et al., 2000; Harrison et al., 2002).

When the arbuscule begins to senesce, fibrillar material encapsulates the collapsed fungal structures that are subsequently degraded by the plant cell. Host cell contents regain their original morphology and recolonization by another arbuscule can occur (Jacquelinet-Jeanmougin et al., 1987; Strack et al., 2003).

I.2.3. Mycorriza-defective pea mutants

Plant mutants are key tools for defining genes involved in distinct morphological stages of AM symbiosis development and for the analysis of specific plant or fungal gene responses during

symbiotic interactions. Mutants with a mycorrhiza-defective phenotype were first designated as Myc$^-$ by Duc et al. (1989). The majority of such mutants are affected in the earliest stages of symbiosis development, after appressoria formation and preventing penetration of fungi into the root (Myc^{-1} phenotype). More infrequent are mutants which do not fully block the symbiotic process but are altered for arbuscule formation (Myc^{-2} phenotype) (Gianinazzi-Pearson et al., 1991) or in the rate of AM symbiosis development (Rmd$^-$ and Rmd^{++} phenotypes) (Jacobi et al., 2003a, b).

Mutants having alterations at the appressoria formation stage (App) have been identified for maize and tomato. The maize *nope 1* (no perception 1) mutant does not support appressoria formation, suggesting the absence of a plant-encoding function necessary for early recognition prior contact (Paszkowski et al., 2006). In another maize mutant *taci 1* (taciturn 1), appressoria form at a reduced frequency but their morphology is normal and leads to penetration of the rhizodermis. However, the majority of hyphae are septate, resulting in limited fungal spread (Paszkowski et al., 2006). Appressorium formation is significantly reduced in roots of tomato *pmi* (premycorrhizal infection) mutants, M161 and M20 (David-Schwartz et al., 2001, 2003). Enhanced and early fungal invasion was detected in roots of maize *pram 1* (Precocious arbuscular mycorrhiza 1) mutant (Paszkowski et al., 2006).

The majority of plant mutants block fungal penetration (Pen$^-$) into the root epidermis. These mutants prevent penetration of the root epidermis resulting in the formation of complex hyphal branching, and multiple and usually swollen appressoria (Marsh and Schultze, 2001). A number of Pen$^-$ mutants have been identified for *Pisum sativum* (Duc et al., 1989), *Vicia faba* (Duc et al., 1989), *Medicago truncatula* (Sagan et al., 1995; Catoira et al., 2000), *Medicago sativa* (Bradbury et al., 1991), *Lotus japonicus* (Senoo et al., 2000a), *Lycopersicon esculentum* (Barker et al., 1998) and *Petunia hybrida* (Reddy et al., 2007). In pea, mutants having a Pen$^-$ phenotype are *Pssym8*, *Pssym9* and *Pssym19* (see table I.1). In some Myc$^-$ mutants, appressorium formation and epidermal cell penetration can develop normally, but further development into the cortex does not occur (Coi$^-$) (Marsh and Schultze, 2001). Mutations in at least six distinct loci of *L. japonicus* (*SYMRK, CASTOR, POLLUX, LjSym3*, *LjSym5* and *LjSym30*) result in the Coi$^-$ phenotype (Wegel et al., 1998; Bonfante et al., 2000; Kistner et al., 2005).

In addition, mutants in which inner cortex invasion (Ici^-) does not occur were identified by Senoo et al. (2000b). They are the *L. japonicus* mutants *mcbee* (mycorrhizal colonization blocked between epidermis and exodermis) and *mcbex* (mycorrhizal colonization blocked in exodermis). Mutants which allow colonization of the inner cortex but where the fungus cannot form arbuscules are designated Ard^- (arbuscule defective). This is the case of the *Pssym36* mutant in pea (see table I.1). In contrast, the mutation in the *mcbco* (mycorrhizal colonization blocked in cortex) gene of *L. japonicus* results in the formation of normal arbuscules but leads to premature arbuscule senescence (Senoo et al., 2000b). Other plant mutants affected in the rate of AM symbiosis development are found in pea: *Pssym33* and *Pssym40* (Jacobi et al., 2003a, b). The mutation in the *PsSym33* gene decreased mycorrhizal colonization of roots (Rmd^- phenotype), whereas the mutation of the *PsSym40* gene results in faster arbuscule development and turn-over in comparison with wild-type plants (Rmd^{++} phenotype).

P. sativum L. is one of the most developed model plants for genetic studies of the nitrogen-fixing symbiosis (NFS) and the AM symbiosis. More than 40 NFS genes have been identified in pea, a part of which are also determinant for the AM symbiosis (Table I.1; Borisov et al., 2004).

Duc et al. (1989) were the first to demonstrate that some pea mutants impaired in symbiosis with nitrogen-fixing root nodule bacteria are also defective for AM formation. Gianinazzi-Pearson (1998) and Gherbi et al. (2008) have hypothesized that at least a part of the genetic machinery necessary for the *Rhizobia* nodulation symbiosis may have been recruited by legumes from functions in the more ancient plant/AM fungal symbiosis. Since their discovery, the exploitation of mutants defective in both bacterial and fungal symbioses has made an important contribution to investigations of the evolution and fundamental processes involved in plant - microbe interactions.

Table I.1. NFS and AM symbiosis-related pea genes

Gene symbol	AM phenotype	NFS phenotype	References
PsSym8/MtDMI1/ LjCastor, LjPollux	Pen^{-}	Hac^{-}	Engvild, 1987; Borisov et al., 1994; Kneen et al., 1994; Balaji et al., 1994 ; Gianinazzi-Pearson, 1996; Albrecht et al., 1998; Catoira et al., 2000; Ané et al., 2004 ; Imaizumi-Anraku et al., 2005
PsSym9=PsSym30/ MtDMI3/LjCCaMK	Pen^{-}	Hac^{-}	Duc, Messager, 1989; Duc et al., 1989; Kneen et al., 1994; Balaji et al., 1994; Sagan et al., 1994 ; Gianinazzi-Pearson, 1996; Catoira et al., 2000; Levy et al., 2004
PsSym14	Rmd^{-}	Iti^{-}	Kneen et al., 1990; Weeden et al., 1990; Tsyganov et al., 1999, 2002
PsSym19/MtDMI2/ LjSYMRK	Pen^{-}	Hac^{-}	Kneen & LaRue, 1988; Duc, Messager, 1989; Duc et al., 1989; Weeden et al., 1990; Schneider et al., 1999; Catoira et al., 2000; Hogg et al., 2006
PsSym33	Rmd^{-}	Itn^{-}	Engvild, 1987; Tsyganov et al., 1998; Rozov et al., 1999 ; Voroshilova et al., 2001; Jacobi et al., 2003 a, b
PsSym36	Arb^{-}	Ith^{-}	Engvild, 1987; Gianinazzi-Pearson et al., 1996;
PsSym40	Rmd^{++}	Idd^{-}	Tsyganov et al., 1998; Voroshilova et al., 2001; Jacobi et al., 2003 a, b
PsSym41	Rmd^{-}	Itn^{-}	Engvild, 1987; Tsyganov et al., 2001

Abbreviation of AM phenotypes: Pen – fungal penetration into root; Arb – arbuscule formation; Rmd – rate of mycorrhiza development. Abbreviation of NFS phenotypes: Hac- root hair curling; Iti – initiation of the infection thread growth; Ith – infection thread growth inside root hair; Itn – infection thread growth inside nodule tissue; Idd – infection droplet differentiation.

I.3. Molecular background of the AM symbiosis

I.3.1. Early signaling events

Successful root colonization and a functional interaction between host plant and AM fungi are based on exchange of signaling molecules at different stages of symbiosis development. Flavonoids and strigolactones, root secreted molecules, can activate the presymbiotic growth of AM fungi (Gianinazzi-Pearson et al. 1989; Akiyama et al., 2005). Flavonoids are considered signals in symbiotic plant-microbe interactions but a specific role during early stages of AM establishment seems unlikely (Steinkellner et al., 2007). Strigolactones are a novel class of plant hormones shown to be active at sub-picomolar concentrations and to stimulate fungal spore germination, branching of germinating hyphae and growth of AM fungi (Akiyama et al., 2005; Besserer et al., 2006). Previously, they were characterized as germination stimulants of parasitic weeds such as *Striga* and *Orobanche* (Bouwmeester et al., 2003; Matusova et al., 2005). Chemically, strigolactones are a group of sesquiterpene lactones derived from carotenoid biosynthesis (Délano-Frier and Tejeda-Sartorius, 2008). Treatment of maize seedlings with fluridone, an upstream inhibitor of the carotenoid metabolism, results in a significant reduction in root mycorrhization which can be restored by the addition of GR24, a strigolactone analogue (Gomez-Roldan et al., 2007). Similar results were obtained with the *y9* mutant of maize defective in an upstream step of carotenoid synthesis, underlining the role of strigolactones as symbiotic signals (Gomez-Roldan et al., 2007).

Strigolactones induce a rapid (within 1 hour) increase in O_2 consumption by AM fungi and strong changes in the shape, density and motility of mitochondria in hyphae (Besserer et al., 2006). Further investigations (Besserer et al., 2008) have shown that treatment of *Gigaspora rosea* with a strigolactone analog, GR24, also causes a rapid (within minutes) increase in the NADH concentration, the NADH dehydrogenase activity and the ATP content in the fungal cells, especially in the hyphal tips. A stimulation in the fungal mitotic activity takes place several days after this initial boost to the cellular energy of the fungus. However, the alterations in

metabolism do not require new gene expression. The striking stimulation of fungal metabolic activity by strigolactones has led to the elaboration of a model in which mitochondrial or cytoplasmic receptors could be possible strigolactone targets needed to stimulate mitochondrial biogenesis, lipid catabolism and respiration (Besserer et al., 2008; Délano-Frier and Tejeda-Sartorius, 2008). Other models propose the interaction of strigolactones with a hypothetical fungal receptor, followed by the quick inactivation of the lactone ligand through the removal of its D ring (Akiyama et al., 2005; Yoneyama et al., 2009), or the response of fungi to strigolactones via an enhancement of endogenous ethylene synthesis which could then stimulate mitochondrial metabolism (Besserer et al., 2008). In spite of the discovery of strigolactones, only scarce data are available about interactions between strigolactones and fungi other than AM fungi. The mechanism(s) by which AM fungi are able to detect host plant roots therefore still remains a source of speculation (Délano-Frier and Tejeda-Sartorius, 2008). Purification and characterization of active root-exuded molecules are in progress (Buée et al., 2000; Vierheilig and Piché, 2002; Vierheilig, 2004; Naqahashi and Douds, 2007).

I.3.2. Plant genes known to be activated at the first steps of AM formation

Further establishment of a functional symbiosis is known to require activation of a host plant signaling pathway involving the *NORK/SYMRK/DMI2/SYM19* Leu-rich-repeat receptor kinase, the *POLLUX/DMI1/SYM8* putative nuclear channel protein and the *CCaMK/DMI3/SYM9* calcium and calmodulin-dependent protein kinase (*CCaMK*) (Endre et al., 2002; Stracke et al., 2002; Ane et al., 2004; Levy et al., 2004; Mitra et al., 2004; Kistner et al., 2005). Mutations in the genes encoding these proteins either prevent or reduce entry of the fungus into the root (Oldroyd et al., 2005). However, whilst *dmi1* mutants are tight and always have a myc^{-1} phenotype, *dmi2* and *dmi3* mutants are leaky and can allow fungal colonization under certain conditions (Morandi et al., 2005). Hypothetically, *DMI2* is involved in the direct or indirect perception of a fungal signal which is then transduced through the intracellular kinase to activate the ion channel *DMI1*. Both genes are responsible for induction of oscillations in calcium concentrations to which the *DMI3* kinase potentially responds directly (Parniske, 2004;

Navazio and Mariani, 2008). The implication of Ca^{2+} as an intracellular messenger has been predicted by several authors based on similarities with the nitrogen-fixing root nodule symbiosis, and it was recently confirmed that AM fungi can induce Ca^{2+} oscillations in plant cell cultures and root epidermal cells (Navazio et al., 2007a, b; Kosuta et al., 2008).

Several genes are strongly induced in *M. truncatula* roots in response to unknown signaling molecules exuded by AM fungi prior to and after contact with the host root (Kosuta et al., 2003; Weidmann et al., 2004). An essential part of the reported genes are related to signal transduction or to synthesis of the early nodulin MtENOD11. In the case of the signal transduction-related genes, Weidmann et al. (2004) showed that their activation is dependent on the *DMI3* gene both before contact and with appressorium formation. The *MtENOD11* gene, which encodes a repetitive Pro-rich protein, is believed to be a functional component of the plant extracellular matrix. *Gigaspora*-targeting experiments in root organ cultures systematically revealed P_{ENOD11}:GFP-HDEL activation in epidermal cells in contact with fungal appressoria as well as in a very limited number of adjacent cells. Observations suggest that the *ENOD11* gene is activated in epidermal cells after appressorium differentiation both before and during PPA formation and probably also during hyphal penetration of the root (Genre et al., 2005). However, the issue about the place of the *ENOD11* gene in the signaling pathway between AM symbionts remains open. Kosuta et al. (2003) reported that activation of the *ENOD11* gene by diffusible fungal signals is not dependent on *DMI* gene function in *M. truncatula*, because P_{ENOD11}:GUS activation by AM fungal exudates was unaltered in mutants of all three *DMI* genes. In contrast, *ENOD11* gene expression associated with appressorium formation, described by Chabaud et al. (2002) using a P_{ENOD11}:GUS reporter construct and by Genre et al. (2005) using a P_{ENOD11}:GFP-HDEL reporter construct, requires activation of the *DMI* genes. In experiments by Chabaud et al. (2002), for example, *MtENOD11* gene expression was not detectible in a *dmi2* mutant background despite the formation of appressoria at the root surface.

Exploiting the Myc^- pea mutant P2 (now known to be *Pssym9*), Roussel et al. (2001) by RNA display analysis identified a PsAM5 pea gene to be transiently activated at early stages of fungal-root interactions. Deduced PsAM5 protein shares significant similarity with a ClpP serine protease. The authors proposed that this protein may have multiple functions related to the

cross-talk between symbiotic partners during early interactions and that in addition to housekeeping functions, it may be involved in controlling levels of key regulatory proteins within a signal-transduction pathway in the AM symbiosis.

I.3.3. Plant and fungal gene expression related to AM establishment and function

Genes associated with plant cell wall and cell matrix modifications

The colonization of a root by AM fungi is accompanied by the reorganization of host cell walls and cell matrix. Most genes associated with these modifications have been described in *M. truncatula*. A set of genes associated with cell wall degradation and modification during AM symbiosis was identified by Hohnjec et al. (2005) through a microarray analysis. Activation of genes coding for cell wall-degrading enzymes ((endo)-glucanases, pectinesterase, polygalacturonase, pectate lyase, α-D-xylosidase) was detected in the AM symbiosis. α-D-xylosidase is involved in the degradation of complex carbohydrates and a suggested function is to modify the extracellular matrix during fungal spread into the root and the formation of the PAM. Up-regulation of genes encoding proline-rich proteins, extensins or arabinogalactans points to cell wall modifications by the incorporation of structural or glycosylated proteins (Honhject et al., 2005).

The *M. truncatula* gene *MtCel1,* which is predicted to be involved in cell wall modifications, was reported to be specifically induced in mycorrhizal roots by Liu et al. (2003). In AM roots, expression of the *MtCel1* gene was associated with cells containing arbuscules. It was suggested that *MtCel1* is located in the periarbuscular membrane and involved in the assembly of the cellulose/hemicellulose matrix of the interface compartment (Liu et al., 2003).

A cDNA of *M. truncatula* gene coding a xyloglucan endotransglucosylase/hydrolase (*Mt-XTH*) that catalyzes the hydrolysis and transglycosylation of xyloglucan polymers in plant cell walls has been isolated by van Buuren et al. (1999). Later Maldonado-Mendoza et al. (2005) identified the corresponding *XTH* genes, *Mt-XTH1* and *Mt-XTH2*. Analysis of transgenic roots expressing an *Mt-XTH1* fusion promoter revealed that *Mt-XTH1* expression is elevated in cells throughout the root system with significant higher levels of activity in mycorrhizal roots,

especially in the mycorrhizal regions. This expression pattern is considered to be consistent with gene activation in response to a systemic signal which may enable fungal penetration of roots (Maldonado-Mendoza et al., 2005).

Expression of *M. truncatula* and pea genes encoding α-expansin and extensin, respectively, that contribute to plant cell-wall extensibility, has also been detected in mycorrhizal roots (Journet et al., 2001; Liu et al., 2003; Grunwald et al., 2004). Such proteins may be involved in the accommodation of the AM fungus by the colonized cortical cells (Balestrini et al., 2005). Genes encoding putative *AGP* (arabinogalactan protein) and *HRGP* (hydroxyproline-rich glycoprotein) are also induced in mycorrhizal roots of *M. truncatula* and maize, respectively, and transcripts are specifically localized in the cells containing arbuscules (Balestrini and Lanfranco, 2006).

Of the genes associated with cell matrix functions, alterations in the microtubular network in cells of AM colonized roots are reflected in AM-specific up-regulation of α- and β-tubulin genes, especially in cells containing arbuscules (Bonfante et al., 1996; Grunwald et al., 2004; Hause and Fester, 2005).

The proliferation and reorganization of root plastids which take place in cortical cells of AM-colonized roots are correlated with the increased transcript levels of the first two enzymes of the plastid-located methylerythritol phosphate (MEP) pathway, 1-deoxy-d-xylulose 5-phosphate synthase (DXS) and 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXR), and of the carotenoid pathway, phytoene desaturase, ζ-carotene desaturase, carotenoid-cleaving dioxygenase (Strack and Fester, 2006; Walter et al., 2007; Floss et al., 2008; Sun et al., 2008).

Genes related to defense and/or stress responses

The colonization of host roots by AM fungi elicits a cascade of general and specific plant defense/stress responses (Dumas-Gaudot et al., 2000). AM-related expression has been reported in different plants for genes encoding proteins of the phenylpropanoid biosynthetic pathway, pathogenesis-related (PR) proteins and enzymes involved in responses against oxidative stress (Garcia-Garrido and Ocampo, 2002; Grunwald et al., 2004; Hause and Fester, 2005; Balestrini and Lanfranco, 2006). Increased expression of defense and stress-related

genes is detected during the early stage interactions between roots and AM fungi, with a subsequent decrease for several gene families as the AM symbiosis develops (Gianinazzi-Pearson, 1996; Liu et al., 2003; Deguchi et al., 2007). However, cytological localization of defense and stress-related gene expression in mycorrhizal roots has shown that in nearly all cases transcripts of the genes accumulate specifically in arbuscule-containing cells (Gianinazzi-Pearson, 1996; Garcia-Garrido and Ocampo, 2002; Liu et al., 2003; Wulf et al., 2003; Grunwald et al., 2004).

Phenylpropanoid metabolism is activated in AM interactions but to a much lower extent than in plant-pathogen interactions (Hause and Fester, 2005). Also, the expression of genes encoding enzyme components of the phenylpropanoid pathway is often uncoordinated and can vary during AM establishment. For example, Deguchi et al. (2007) reported that phenylalanine ammonia-lyase (*PAL*) and chalcone synthase (*CHS*) genes are moderately up-regulated on the initial association of *L. japonicus* with an AM fungus and repressed in the later stages, whilst Harrison and Dixon (1994) described the specific induction of the isoflavone reductase-encoding gene in *M. truncatula* cells containing arbuscules. The induction of phenylpropanoid biosynthesis during the AM symbiosis has been discussed either in relation to the biosynthesis of antimicrobial phytoalexins and bioprotection against root pathogens or the biosynthesis of flavonoid compounds stimulating the growth of AM fungi during interactions with host roots (Dumas-Gaudot et al., 2000).

PR protein-encoding genes are also more weakly expressed in mycorrhizal roots when compared to plant-pathogen interactions (Hause and Fester, 2005). PR1 protein (pathogenesis-related protein 1) was first detected in mycorrhizal tobacco roots in the symbiotic interface between the PAM and the fungal wall (Gianinazzi-Pearson et al., 1992). Since then, PR genes and proteins have been studied in roots of a range of mycorrhizal plant species (Dumas-Gaudot et al. 2000). For example in pea, a PR10 gene (pathogenesis-related protein 10) is over-expressed during early interactions with *G. mosseae* (Ruiz-Lozano et al., 1999) whilst genes encoding members of the protein families PR1 and PR6 (proteinase inhibitor) are up-regulated in colonized roots (Grunwald et al., 2004). The activity and transcript accumulation patterns of PR proteins that are hydrolytic enzymes (chitinase, β-1,3-glucanase) suggest that these

enzymes could also form part of the plant defense response against the invading AM fungus (Dumas-Gaudot et al. 2000; Garcia-Garrido and Ocampo, 2002). It has also been speculated that chitinases may also have a role in the degradation/turnover or formation of arbuscules (Dumas-Gaudot et al. 2000; Hause and Fester, 2005). In fact, a class III chitinase gene is expressed specifically in cells of roots *M. truncatula* containing functionally active arbuscules, suggesting a role of this enzyme in arbuscule turnover (Salzer et al., 2000).

One of the most rapid reactions in plants to stress situations, including pathogen attack, is the so-called oxidative burst which is marked by the production of reactive oxygen species (ROS) such as superoxide, hydroxyl radicals, and hydrogen peroxide (Apel and Hirt, 2004; Garcia-Garrido and Ocampo, 2002). Increased production of ROS may result in the activation of various signaling cascades involved in direct toxic effects on a pathogen, in modification of apoplastic structures directed against the pathogen, or in cell senescence (Apel and Hirt, 2004). It was shown that in AM colonized roots, ROS are accumulated close to fungal structures and that H_2O_2 accumulation is correlated with arbuscule senescence (Fester and Hause, 2005). Increased ROS production induces the activity of various antioxidative enzymes such as superoxide dismutase (SOD), catalases and peroxidases which play a role in detoxification processes and H_2O_2 release (Apel and Hirt, 2004). There are several reports of a general stimulation of SODs by the AM symbiosis in different plants (Dumas-Gaudot et al., 2000), and mycorrhizal protection against oxidative stress caused by drought is associated with increased expression of a *Mn-SODII* gene in lettuce (Ruiz-Lozano et al., 2001). Gene expression for a germin-like protein, which can posses oxalate oxidase or superoxide dismutase activities, has been repeatedly found to be enhanced in AM roots of *M. truncatula* (Wulf et al., 2003; Küster et al., 2004; Brechenmacher et al., 2004; Güimil et al., 2005). On the fungal side, expression of the superoxide dismutase genes of *Gig. margarita* (Lanfranco et al., 2005), *G. intraradices* (Seddas et al., 2008) and *G. mosseae* (Brechenmacher et al., 2004), and the thioredoxin peroxidase-encoding gene of *G. intraradices* (Seddas et al., 2008), have been shown to be up-regulated when the fungi colonize host tissues. Synthesis and activity of corresponding enzymes may help the fungus to contend with ROS production by host roots and deal with plant defense responses during AM symbiosis development.

Genes coding for proteins involved in the enzyme synthesis of nitric oxide (NO), a key signaling molecule crucial to plant defense against pathogens (Wendehenne et al., 2004), are up-regulated in mycorrhizal *M. truncatula* roots (Gianinazzi-Pearson et al., 2007). Plant leghemoglobins are thought to bind NO and enhanced expression of the plant leghemoglobin gene *VfLb29* detected in arbuscule-containing cells of *V. faba* may reflect a role in regulating such a defense response (Vieweg et al., 2004).

Arbuscule-related expression has also been shown for plant glutathione S-transferase (*GST*) genes (Strittmatter et al., 1996; Wulf et al., 2003; Brechenmacher et al., 2004) which are known to be activated under various stress conditions, such as pathogen attack, chemical treatments, or changes in environmental conditions (Wulf et al., 2003). In AM roots of potato, expression of a *Gst1* gene was limited to arbuscule-containing cells (Strittmatter et al., 1996) whilst in *M. truncatula*, the gene *MtGst1* was shown to be transcribed in mycorrhizal root tissues, not only in arbuscule-containing cells but also in the direct vicinity of cells containing fungal hyphae (Wulf et al., 2003). Since glutathione S-transferase is considered to have protective functions and it is induced during programmed cell death, the corresponding gene could be involved in defense responses to the symbiotic fungus but it may also be related to arbuscule senescence (Brechenmacher et al., 2004).

Pea metallothionein gene expression was detected during the late stage of AM symbiosis development (Grunwald et al., 2004; Rivera-Becerril et al. 2005). In both publications, the gene is not significantly affected by AM. Other genes of *M. truncatula* known be implicated in plant defense to pests or pathogens and up-regulated in mycorrhizal roots include those encoding a serine protease, a cysteine-rich antifungal protein (defensin), a Kunitz-type trypsin inhibitor and a subtilisin inhibitor (Massoumou et al., 2007; Wulf et al., 2003). Several defense or stress-related genes are down-regulated with establishment of the functional AM symbiosis. In addition to the phenylpropanoid pathway and genes, for example, plant genes encoding WRKY transcription factors, which are mainly involved in tolerance to pathogen-related stress, and BURP domain-like proteins, one of which is a stress-related transcriptional factor, are repressed in AM roots of *L. japonicus* (Deguchi et al., 2007).

In conclusion, a large number of defense- and stress-related genes are known to be expressed during AM symbiosis development. However, the underlying mechanisms of their regulation and the implication for the symbiosis are still mostly unclear.

Genes involved in nutrient exchange between symbiotic partners

Bidirectional nutrient transfer between the plant and the fungus is a key feature of the AM symbiosis. Nutrient exchange takes place across the intraradicular symbiotic interfaces which are bordered by the plant and fungal cells. The major nutrients exchanged are reduced carbon, assimilated through plant photosynthesis and transferred to the fungus, and phosphate, taken up from the soil by the fungal hyphae and transported to the plant cells.

Carbon. Carbon is transferred from the plant to the AM fungus as hexose which is then rapidly converted within hyphae to trehalose and glycogen, the main transient storage carbohydrates (see figure I.2; Bago et al., 2003). Significant modifications in the host plant's C metabolism are seen at the level of the expression of corresponding genes. In AM colonized roots, up-regulation of gene expression and/or activity of sucrose hydrolyzing enzymes (invertases, sucrose synthase) (Ravnskov et al., 2003; Hohnject et al., 2003; García-Rodríguez et al., 2005) and sugar transporters (Harrison, 1996; Hohnjec et al., 2005) has been observed. Enhanced expression of a sucrose synthase gene in AM-colonized roots leads to the hypothesis that the enzyme is involved in generating a sink strength (Balestrini and Lanfranco, 2006). Upregulation of a cell wall invertase gene in mycorrhizal roots supports the general assumption that the C transfer across the symbiotic interface requires host sucrose hydrolysis. Transcriptional upregulation of sucrose-splitting enzymes during early root colonization events agrees with the decreased levels of sucrose detected in these roots (García-Rodríguez et al., 2006). It is generally assumed that plant invertase and sucrose synthase are crucial for the delivery of hexoses to the fungal partner (Délano-Frier and Tejeda-Sartorius, 2008). Moreover, on the fungal side, a monosaccharide transporter gene GpMST1 was isolated from *Geosiphon pyriformis* and characterized (Schüßler et al., 2006). It was shown that GpMST1 has the highest affinities for glucose then mannose, galactose and fructose. Authors hypothesized that GpMST1 is active at the symbiotic interface and not able to take up glucose through the non-symbiotic

plasma membranes. Nevertheless, the question about the site of carbon transfer in the AM symbiosis remains open.

A substantial amount of the hexose C transferred from plant to AM fungal cells can be transformed to storage lipids, mostly in the form of triacylglyceroles (TAGs). Most of the carbohydrates are known to be metabolized in the intraradical mycelium, and transport of C to the extraradical mycelium is performed in the form of TAGs and glycogen (see figure I.2; Bago et al., 2003; Délano-Frier and Tejeda-Sartorius, 2008).

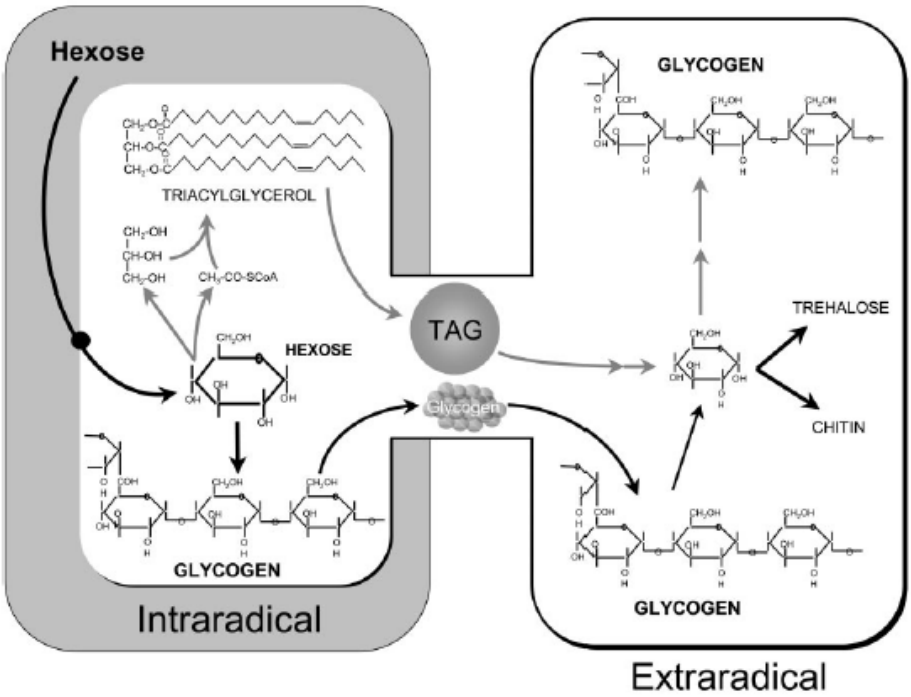


Figure I.2. Biochemical pathways of carbon metabolism active in symbiotic intraradical and extraradical mycelium of AM fungi (Bago et al., 2003). Carbon taken up from the host in the form of hexose is converted to carbohydrates and storage lipids in the intraradical mycelium. Lipid and glycogen are translocated from intraradical to extraradical mycelium. Storage (glycogen and trehalose) and structural (chitin) carbohydrates are synthesized in the extraradical mycelium from hexose that is derived from exported carbohydrate as well as from lipid.

Bago et al. (2003) reported expression of fungal genes encoding glycogen synthase, glycogen branching enzymes, chitin synthase and glutamine fructose-6-phosphate aminotransferase (enzyme in chitin synthesis) in AM colonized roots. The quantified glycogen synthase gene

expression of *G. intraradices* within mycorrhizal roots, extraradical mycelium and germinatimg spores is consistent with C-labeling assays, which indicate that glycogen is synthesized by the fungus in germinating spores and within the symbiosis (Bago et al., 2003). It has been speculated that a putative acyl-coenzyme A dehydrogenase gene may function in the anabolic flux of C from lipid to carbohydrate (Bago et al., 2002). These expression data confirm that TAGs and glycogen are synthesized by the fungus inside the root and then transferred to the extraradical mycelium. In contrast, genes coding for enzymes of the glycoxylate cycle (isocitrate lyase, malate synthase) are actively expressed in the extraradical mycelium where the fungus, unable to take up hexose, convert lipids into carbohydrates via this metabolic pathway (Balestrini and Lanfranco, 2006; Lammers et al., 2001).

Phosphorus. Phosphate is a key nutrient contributing to the symbiotic functioning of arbuscular mycorrhiza. Once the symbiosis is established, AM fungal hyphae take up inorganic phosphate (Pi) from the soil, accumulate it in the form of vacuolar polyphosphate (polyP) and translocate it along hyphae by means of a motile tubular vacuolar network to the intraradical compartment. Here, Pi ions resulting from polyP by hydrolysis are assumed to be released by membrane-passive carriers into the periarbuscular space and actively taken up into the plant cell via transporters at the PMA (Cox et al., 1980; Rasmussen et al., 2000; Uetake et al. 2002; Ezawa et al., 2002).

The AM fungal-mediated translocation of phosphate from the soil to plant roots requires specific transporters that function in at least two fungal structures: the external hyphae which mediate uptake from the soil and arbuscules within root cortical cells where an efflux transporter or channel is expected (Ferrol et al., 2002). AM fungal phosphate transporter genes have already been isolated from *G. versiforme* (*GvPT*), *G. intraradices* (*GiPT*) and *G. mosseae* (*GmosPT*) (Harrison and van Buuren 1995; Maldonado-Mendoza et al. 2001; Benedetto et al., 2005). Expression of the *GiPT* and *GmosPT* genes in extraradical mycelium is induced in response to phosphate concentrations typical of those found in the soil solution, and modulated by the overall phosphate status of the mycorrhiza (Maldonado-Mendoza et al., 2001; Benedetto et al., 2005). The intraradical mycelium of *G. mosseae* also showed a significant level of *GmosPT* expression that nevertheless was independent of external Pi concentrations (Benedetto et al.,

2005). These data suggest that inside the root the fungus may exert a control over the amount of phosphate delivered to the plant (Balestrini and Lanfranco, 2006).

It has been proposed that fungal alkaline phosphatases (ALP) may have a role in the transfer of phosphate by hyphae from the soil to the host plant (Tisserant et al., 1993). AM-dependent expression of ALP-encoding genes has been described in *G. intraradices* and *G. mosseae*: the levels of *GiALP* and *GmALP* transcripts were higher in mycorrhizal roots than in germinating spores and extraradical mycelium, and gene expression in AM roots was irrespective of colonization rates and environmental Pi concentration (Aono et al., 2004). These results agree with ALP enzyme location and activity data (Gianinazzi et al., 1979; Gianinazzi-Pearson et al., 1995). Moreover, Ezawa et al. (1999) revealed that ALP in the extraradical mycelium has an increased affinity for sugar phosphate. Detailed information on the dynamics of polyP turnover in AM fungi and corresponding gene activity is lacking.

Active uptake into the plant cell of phosphate released by the fungus across the arbuscule interface requires transporters at the PMA. Plant phosphate transporter genes identified from potato (*StPT3*), maize (*ZEAma*) and L. japonicus (*LjPT3*) show an increased expression in symbiosis (Rausch et al., 2001; Karandashov et al., 2004; Nagy et al., 2006; Maeda et al., 2006), whilst phosphate transporter genes from rice (*OsPT11*), tomato (*LePT4*) and *M. truncatula* (*MtPT4*) have been identified to be expressed exclusively within the AM symbiosis (Paszkowski et al., 2002; Nagy et al., 2005; Javot et al., 2007). The *MtPT4* gene was shown to be located in the periarbuscular membrane, at the symbiotic interface of arbuscules which is generally assumed to be the preferential site of phosphate transfer from the AM fungus to the host plant (Balestrini and Lanfranco, 2006). Javot et al. (2007) observed that loss of *MtPT4* function leads to premature death of the arbuscules, inability of the fungus to proliferate within the root and arrest of the symbiosis.

Other known key enzymes for the AM symbiosis are H^+-ATPases. They play a role in establishing the electrochemical gradient required for the transfer of nutrients (either phosphate or carbon) across the plasma membrane in both fungi and plants (Ferrol et al., 2002). Few genes encoding membrane H^+-ATPase have been identified in AM fungi. Five were isolated from *G. mosseae* (Ferrol et al., 2000; Requena et al., 2003) and expression of two of them,

GmPMA1 and *GmHA5*, was investigated during AM formation. It was shown that the *GmPMA1* gene is highly expressed during asymbiotic fungal development and that its expression is not affected either by the presence of different nutrients in the growth medium, or by the presence of the host plant. In contrast, the *GmHA5* gene is strongly induced once appressoria begin to form, and it remains highly expressed during the in planta phase. Moreover, expression of *GmHA5* is induced during symbiotic fungal growth in roots by micromolar amounts of phosphate in the medium (Requena et al., 2003). A set of membrane H^+-ATPase genes has been identified on the plant side (Gianinazzi-Pearson et al., 2000; Ferrol et al., 2002; Krajinski et al., 2002; Manthey et al., 2004). Up-regulation within the established AM symbiosis and arbuscule-specific induction of expression were detected for the tobacco H^+-ATPase genes *pma2* and *pma4*, and for *Mtha1* from *M. truncatula* (Gianinazzi-Pearson et al., 2000; Krajinski et al., 2002). These data indicate that different genes coding for plant and fungal membrane H^+-ATPases are differentially regulated during the AM symbiosis. This together with the specific localization of H^+-ATPase and phosphate transporter proteins in the PMA underline the complexity of plant-fungal interactions and of the mechanisms leading to a functional AM.

Nitrogen. In addition to phosphorus, AM fungi can take up and transfer significant amounts of nitrogen to their host plants (Bago et al., 1996; Johansen et al., 1993; He et al., 2002). Stable isotope labeling experiments on *G. intraradices*/carrot root mycorrhiza (Jin et al., 2005) showed that inorganic nitrogen taken up by the fungus outside the roots is incorporated into amino acids and translocated from the extraradical to the intraradical mycelium as arginine. N is then probably transferred from the fungus to the plant as ammonium without any loss in the C skeleton (see figure I.3). Data from quantitative real-time PCR are consistent with the described mechanisms. The AM fungal gene of primary nitrogen assimilation (glutamine synthase) is preferentially expressed in the extraradical tissues, whereas genes associated with arginine breakdown (urease accessory protein, ornithine amino transferase) and NH^+_4 transfer (ammonium transporter) are more highly expressed in the intraradical mycelium (Govindarajulu et al., 2005; López-Pedrosa et al., 2006).

AM fungi may also be involved in the acquisition of organic N. The activity of an amino acid permease gene (*GmosAAP1*) from *G. mosseae* and of a glutamine synthase gene from *G.*

mosseae and *G. intraradices* appear to be modulated in AM roots by different N sources (Cappellazzo et al., 2008; Breuninger et al., 2004). *GmosAAP1*, which encodes a functional amino acid transporter, binding nonpolar and hydrophobic amino acids, is expressed in the external mycelium but not in the intraradical fungal structures. It was suggested that *GmosAAP1* plays a role in the first steps of amino acid acquisition, allowing direct amino acid uptake by the fungus from the soil (Cappellazzo et al., 2008).

On the plant side, induction of genes encoding AM-related nitrate transporters has been reported in *M. truncatula* and in tomato (Hohnjec et al., 2005; Burleigh, 2001; Hildebrandt et al., 2002). These findings suggest the existence of mechanisms not only supporting increased ammonium uptake but also nitrate acquisition through the AM symbiosis (Balestrini and Lanfranco, 2006).

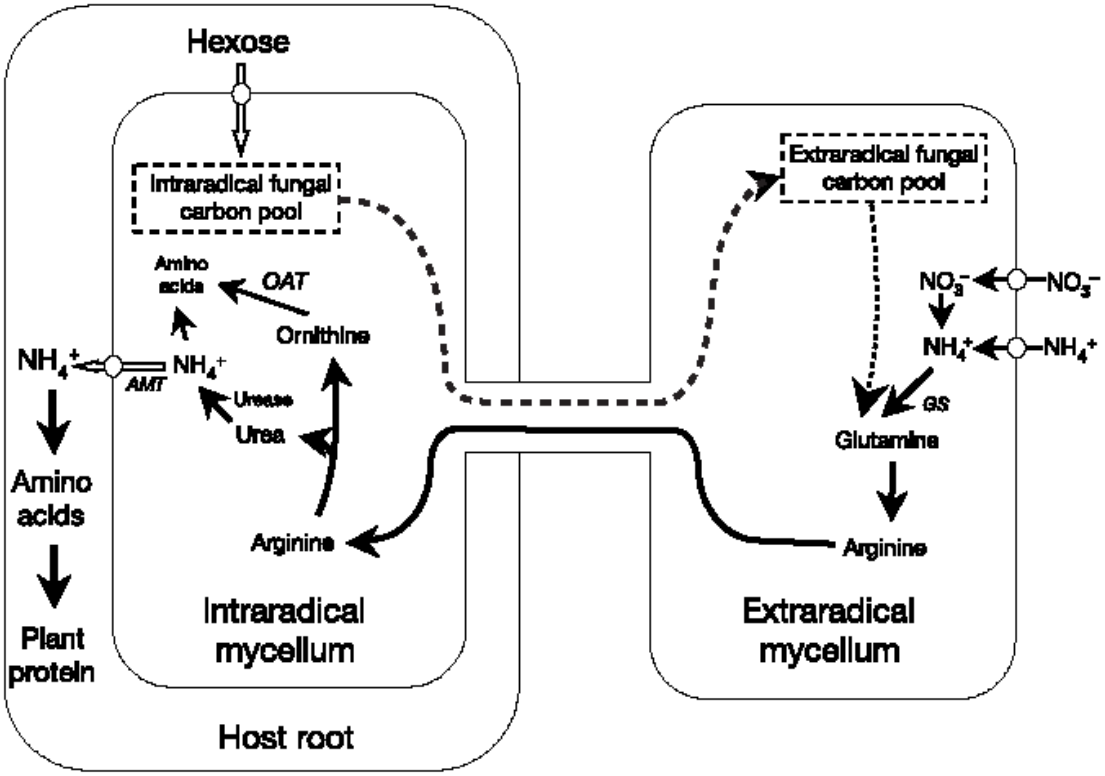


Figure I.3. Model of nitrogen movement in the arbuscular mycorrhizal symbiosis (Govindarajulu et al., 2005). Inorganic nitrogen is taken up by the fungal extraradical mycelium, assimilated via nitrate reductase and the glutamine synthetase-glutamate synthase cycle and converted into arginine, which is translocated along the fungal hyphae to the intraradical mycelium. Arginine is

broken down in the intraradical mycelium, releasing urea and ornithine, which are further broken down by the actions of urease and ornithine aminotransferase (OAT). Ammonia released from arginine breakdown passes to the host via ammonia channels (AMT). Amino acids from ornithine breakdown and/or NH_4^+ assimilation in the intraradical mycelium may be catabolized within the intraradical mycelium or translocated to the extraradical mycelium.

I.3.4. High throughput analyses of symbiosis-related transcriptional changes in plant and fungal genes

Targeted approaches based on EST screening by homology with known genes are not sufficient to enable identification of novel genes which may be specific to the AM symbiosis. In order to obtain a comprehensive picture of transcriptional changes during AM symbiosis development, transcriptome-wide studies principally based on large-scale EST sequencing, suppressive subtractive hybridization (SSH) and DNA microarray analysis have been performed.

In the earliest approaches using differential RNA display, only a limited number of novel AM-related genes were isolated by comparing RNA accumulation patterns in different samples (i.e. mycorrhizal and non-mycorrhizal) to isolate differentially transcribed mRNAs (Martin-Laurent et al., 1997; Lapopin et al., 1999). A greater number of genes were identified exploiting suppressive subtractive hybridization (SSH) (Wulf et al., 2003; Brechenmacher et al., 2004; Weidmann et al., 2004; Breuninger and Requena, 2004; Grunwald et al., 2004) which consists in a PCR-based cDNA subtraction method allowing selective amplification of target cDNA fragments (differentially expressed) and simultaneous suppression of non target amplified cDNA (Diatchenko et al., 1996). SSH analysis of interactions at the appressorium stage and in fully developed mycorrhiza of *M. truncatula* colonized by *G. intraradices* or *G. mosseae* has led to the identification of AM-induced plant genes which are mostly involved in signaling processes, defence/cell-rescue, membrane transport or cell recognition (Wulf et al., 2003; Brechenmacher et al., 2004; Weidmann et al., 2004). ESTs from the SSH library obtained by Brechenmacher et al. (2004) were also used as templates to identify plant genes commonly or differentially

activated during *M. truncatula* root colonization by AM fungi (*G. mosseae*), rhizobacteria (*Pseudomonas fluorescens*) and nodule bacteria *Sinorhizobium meliloti* (Sanchez et al., 2004). Application of SSH to specifically target events associated with arbuscule development, by comparing mycorrhizal wild type pea and the *Pssym36* mutant, led to the identification of over 20 genes which seem to be induced during this functional stage of AM (Grunwald et al., 2004).

G. mosseae genes up-regulated during appressoria formation have also been identified using this technique (Breuninger and Requena, 2004; Brechenmacher et al., 2004). Several genes related to Ca^{2+}-dependent signaling were found amongst the up-regulated fungal genes, suggesting a role for fungal Ca^{2+} in the early stages of mycorrhiza formation (Breuninger and Requena, 2004).

A significant advance in knowledge about molecular responses in the plant partner during AM interactions has been made by large-scale EST sequencing of the legume model species, *M. truncatula* and *L. japonicus*. A number of research groups around the world have participated in sequencing projects and numerous EST sequences have been generated by random sampling of cDNA libraries constructed from different tissues of comparable plant material grown under a variety of physiological conditions (Gianinazzi-Pearson and Brechenmacher, 2004). Resulting EST sequences are deposited in public databases: TIGR *Medicago truncatula* Gene Index (MtGI)(http://combio.dfci.harvard.edu/tgi/), *Medicago truncatula* Data Base (MtDB) (http://medicago.org/MtDB), European Medicago EST Navigation System (MENS) (http://medicago.toulouse.inra.fr/Mt/EST), TIGR *Lotus japonicus* Gene Index (LjGI) (http://combio.dfci.harvard.edu/tgi/ljgi/index.shtml.old), and Kazusa *Lotus japonicus* EST-database (http://www.kazusa.or.jp/lotus/). Such databases allow *in silico* gene expression analysis from different tissues (electronic Northerns) as well as macro- and microarray construction.

In silico analysis of EST data by Journet et al. (2002) from three cDNA libraries (MENS database), corresponding to nonmycorrhizal, mycorrhizal and nodulated roots, led to the identification of a large number of novel plant genes predicted to be up- or down-regulated during either symbiotic root interaction. Using a similar strategy based on *in silico* analysis of *M. truncatula* random cDNA (MtAmp) and SSH (MtGim) libraries, Frenzel et al. (2005) identified

additional AM-induced genes with a putative function in transport and signaling processes, as well as a novel family of AM-specific lectin genes. In situ arbuscule-specific expression of two members of this lectin family indicates a role for AM-specific lectins during arbuscule formation or function (Frenzel et al., 2005).

In silico analysis also formed a useful starting point for cDNA array construction for transcriptome analyses (Liu et al., 2003, 2007; Küster et al., 2004, 2007; Gianinazzi-Pearson et al., 2004; Manthey et al., 2004; Hohnjec et al., 2005; Deguchi et al., 2007). The construction of *M. truncatula* cDNA macroarrays for AM studies was first reported by Liu et al. (2003) to examine transcript profiles of *M. truncatula* roots during the development of AM with *G. versiforme* and during growth under different phosphorus nutrient regimes. Using a set of 2268 cDNAs, 67 genes were shown to be differentially regulated during AM symbiosis development and transcript profiling revealed two distinct temporal expression patterns. One group of genes (mostly defense and stress-response related) showed an increase in transcripts during the initial period of contact between symbionts and a subsequent decrease as the symbiosis developed. A second group (genes linked to signaling transduction pathways) showed a sustained increase in transcript levels that correlated with colonization of the root system (Liu et al., 2003). The first symbiosis-targeted microarray, designated Mt6k-RIT, was constructed using 6359 MtC EST clusters identified by Journet et al. (2002) (Küster et al., 2004). Expression profiling revealed additional marker genes for nodule and arbuscular mycorrhiza development. An extended *M. truncatula* Mt16kOLI1 microarray, containing 16086 probes, was used to highlight the overlapping genetic program activated by two different AM fungi, *G. mosseae* and *G. intraradices* (201 genes co-induced) and identify plant genes uniquely present in mycorrhizal root cDNA libraries (Hohnjec et al., 2005).

A contribution to the detection of tissue- or cell-specific gene expression during AM symbiosis has been made by exploiting microdissection techniques. Recently, a number of novel plant and fungal genes specifically induced in colonized cortical cells and in arbuscules were identified using laser microdissection of arbuscule-containing cells of *M. truncatula* and the Affymetrix Gene Chip®Medicago Genome Array containing 32167 probes designed to sequences from the MtGI database. Within the arbuscule, expression of genes associated with the urea cycle,

amino acid biosynthesis and cellular autophagy was detected. Analysis of gene expression in the colonized cortical cell revealed up-regulation of a lysine motif (LysM)-receptor kinase, members of the GRAS transcription factor family and a symbiosis-specific ammonium transporter that is a likely candidate for mediating ammonium transport in the AM symbiosis (Gomez et al., 2009). Laser microdissection offers a new way to obtain RNA from subsets of cells, and coupled with transcript profiling approaches, it provides an opportunity for monitoring gene expression in an individual cell type.

In conclusion, the large numbers of mycorrhiza-related plant genes identified during different large-scale analyses cover a substantial part of the root endosymbiotic transcriptome. However, further investigations are still necessary to confirm their significance in the fungal-plant interactions and to map their involvement in the molecular pathways driving or regulated by the AM symbiosis.

I.3.5. Influence of *SYM* gene mutations on AM-related molecular responses

Plant mutants defective for symbiotic AM have been used to obtain information about the role of *SYM* genes in plant and fungal gene regulation associated with the different stages of symbiosis development. Responses of plant genes associated with signaling pathways at the appressorium stage were compared by Weidman et al. (2004) and Sanchez et al. (2005) in wild type and Myc$^-$ *dmi3* roots of *M. truncatula* roots inoculated with *G. mosseae*. Weidman et al. (2004) identified 11 plant genes activated from the appressorium stage up to the fully established AM symbiosis. Expression of these genes in roots of the *dmi3* mutant was not activated by appressorium formation or diffusible fungal molecules, indicating lack of recognition of AM fungus by the *dmi3* mutant. Likewise, none of the plant genes identified by Sanchez et al (2005), coding for proteins associated with a putative signal transduction pathway and induced during early stages of AM colonization, were responded to inoculation of roots of the *dmi3* mutant.

To identify genes specifically regulated during PPA formation in *M. truncatula*, Siciliano et al., (2007) built a PPA-targeted cDNA SSH library and monitored expression profiles of differentially

expressed genes in the wild type and *dmi3* mutant plant genotypes. This led to the identification of 107 genes potentially involved in early AM interaction. 15 of them were shown to be specifically up-regulated at the time points of appressorium and PPA development, including genes encoding signal responses (receptor-like protein kinase RK20-1), defense responses (resistance protein KR1, PR10 protein, *Avr9/Cf-9*-rapidly elicited protein 26A ACRE264) and cell wall modifications (cellulase synthase, expansin-like protein 1 precursor, pectin-glucuronyltransferase-like, cationic peroxidase). Two novel genes specifically up-regulated during the formation of PPA were identified. One of them encodes an expansin-like protein preferentially expressed in epidermal cells in contact with an appressorium. Another gene encodes a putative *Avr9/Cf-9*-rapidly elicited protein 26A (ACRE264) found to be up-regulated in the *dmi3* mutant, suggesting that it is suppressed by *DMI3* to possibly facilitate fungal entry into the host plant. The authors suggest that *DMI3* may play a pivotal role in interpreting and modulating plant responses to early AM colonization by boosting the up-regulation of plant genes required for the accommodation of the AM fungus and suppression of the basal defense-related genes (Siciliano et al., 2007).

On the fungal side, mutations of the *DMI1*, *DMI2* and *DMI3* genes not only block root penetration but also affect fungal gene expression (Seddas et al., 2009). Transcript accumulation of 14 *G.intraradices* genes, with predicted functions in transcription, protein synthesis, primary/secondary metabolism or of unknown function, were monitored by real-time RT-PCR in quiescent spores, germination spores and roots of wild type or mutant plants at appressoria formation stage. Tested fungal genes were mostly down-regulated or not expressed in contact with roots of the *DMI* mutants. Authors suggest that plant *SYM* genes may regulate AM fungal activity through stimulatory pathways and/or control of inhibitory factors.

In non-targeted approaches, microarray hybridization experiments comparing *M. truncatula* roots colonized by *G. mosseae* (Myc^+ genotype) and its inoculated mycorrhiza defective (Myc^-) mutant ($dmi3^-$) gave consistent and significant differential expression for 72 genes (57 up-regulated, 15 down-regulated) from the mycorrhizal wild type genotype compared with 47 genes (37 up-regulated, 10 down-regulated) for the inoculated *dmi3* mutant (Gianinazzi-Pearson et al., 2004). The main classes of AM up-regulated genes in the wild type genotype related to protein

synthesis/processing (~ 18%), and about 47% were genes with unknown function or homology. Only two genes of unknown function showed a similar expression pattern in wild type and mutant genotypes, which concords with their very different phenotypes in interactions with AM fungi.

To study arbuscule-related gene expression, differential RNA display (Lapopin et al., 1999), SSH and macroarray hybridization (Grunwald et al., 2004) were performed exploiting the *Pssym36* mutant. Comparison of RNA accumulation patterns between controls, *Glomus mosseae*-colonized *Pssym36* mutant and wild type pea (Lapopin et al., 1999) resulted in the identification of four differentially occurring cDNA fragments, one from the fungus and three of plant origin. More detailed analysis of the pea *Psam4* (*Pisum sativum* arbuscular mycorrhiza-regulated) gene showed that this gene encodes a proline-rich protein and its RNA accumulation is higher in the mutant compared with the wild type. Authors proposed that *Psam4* might be itself mutated or belong to a regulon of genes altered in their expression and involved in the greater resistance of the *Pssym36* tissues and cortical cells to invasion by the symbiotic microorganisms (Lapopin et al., 1999). In the investigation performed by Grunwald et al. (2004), 67 unique fragments presumably up-regulated during the late stage of AM development were identified. Arbuscule-related expression was confirmed by macroarray analysis for 22 of them. Identified genes belong to different functional groups, part of them being reported to be AM-induced for the first time.

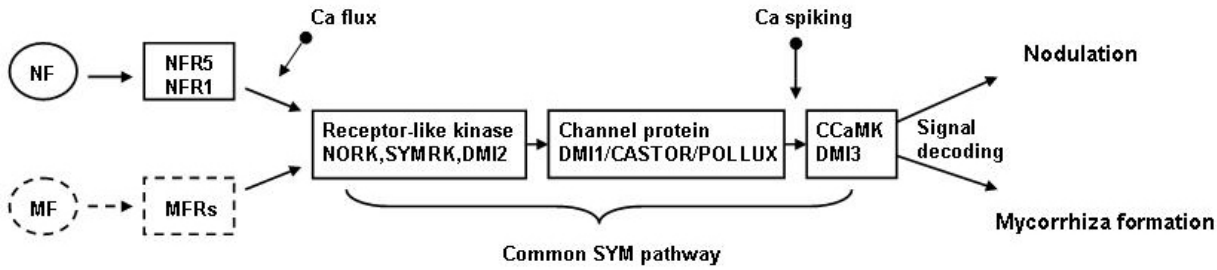
I.3.6. Molecular similarities between legume/AM fungi and legume/*Rhizobia* symbioses

Although AM fungal and rhizobial symbioses are morphologically distinct, the genetic programs underling them partially overlap in legumes. One of the first evidences for a genetic overlap was the discovery of pea mutants affected in both root symbioses (Duc et al., 1989) indicating the existence of some shared symbiotic steps. Common symbiosis mutants have since been identified in *M. truncatula*, *M. sativa*, *L. japonicus*, *Phaseolus vulgaris*, *Vicia faba* and *Melilotus alba* (Bradbury et al., 1991; Sagan et al., 1995; Shirtliffe and Vessey, 1996; Schauser et al., 1998; Szczyglowski et al., 1998; Wegel et al., 1998; Catoira et al., 2000; Senoo et al., 2000;

Kawaguchi et al., 2002; Lum et al., 2002). In pea, *SYM* genes common to both symbioses are essential for comparable stages in partner interactions: fungal and bacterial root penetration (*PsSYM8*, *PsSYM9/PsSYM30*, *PsSYM19*), arbuscule development and infection thread growth inside root hairs (*PsSYM36*), rate of mycorhization/arbuscule turnover and infection thread growth or infection droplet differentiation (*PsSYM14*, *PsSYM33*, *PsSYM40, PsSym41*) (see table I.1).

Researchers have paid special attention to early steps of symbioses establishment. Analysis of signaling pathways taking place during root colonization by AM fungi and rhizobial bacteria have revealed that a legume host plant utilizes a partially common Ca^{2+}-dependent pathway for both symbioses (see figure I.4).

Figure I.4. Signaling pathways of arbuscular mycorrhizal and nitrogen fixing symbioses (Smith and Read, 2008)



NF- Nod factor; MF – putative 'myc factors'; MFR- myc factor receptor.

The common *SYM* genes encoding a Leu-rich repeat receptor kinase, a nuclear cation channel and a calcium/calmodulin-dependent protein kinase (*DMI2*, *DMI1, DMI3*, respectively, in *M. truncatula* and their orthologs in other plant species) operate downstream of nod factor receptor genes (*NFR1*, *NFR5*) in the nitrogen-fixing symbiosis pathway. These *SYM* genes are known to be required for the Ca^{2+}-dependent transduction of both bacterial and fungal signals. *DMI2*/*PsSYM19*/*LjSYMRK* is involved in the perception of a fungal or bacterial signal and the activation of *DMI1*/*PsSYM8*//*LjPOLLUX* that could function as a calcium channel (Oldroyd et al., 2005). *dmi1* and *dmi2* mutants lack the calcium spiking response. *DMI3*/*PsSYM9*/*LjCCaMK* has been placed downstream of the generated calcium response. The role of *DMI3* is considered to

be the interpretation of the calcium signals and conversion of them to phosphorylation events (Kistner et al., 2005).

However, in spite of the involvement of the same *DMI1* and *DMI2* proteins, the symbiosis signaling pathway in legumes differentially transduces bacterial and fungal signals to generate distinct calcium responses. Mathematical analyses of calcium oscillations indicate that both the fungal and the bacterial-induced calcium responses are chaotic in nature (Kosuta et al., 2008). Chaotic systems require minimal energy to produce a wide spectrum of outputs in response to marginally different inputs. Consequently, chaos provides a possible mechanism for flexibility that must be an inherent component of this common *SYM* pathway (Kosuta et al., 2008).

The common activation of a number of nodulin genes during early nodulation and mycorrhization (*MsENOD2*, *MtENOD11*, *MtENOD12, MsENOD40, PsENOD5*, *PsENOD12A, VfLb29*) lends support to the existence of common molecular events in the two symbiosis (Van Rhijn et al., 1997; Albrecht et al., 1998; Journet et al., 2001; Frühling et al., 1997). Existence of plant genes common to both symbioses led to the hypothesis that the evolutionary younger legume root nodule symbiosis might have evolved from the more ancient AM symbiosis (Gianinazzi-Pearson, 1997; Gherbi et al., 2008).

Global comparisons of the complex root symbiotic programs activated during host plant interactions with AM fungi and nitrogen-fixing bacteria have been performed using *in silico* comparisons in the MENS database, targeted expression analyses and the microarray tools described above (Journet et al., 2002; Manthey et al., 2004; Sanchez et al., 2004, 2005; Küster et al., 2004; Kistner et al., 2005; Deguchi et al., 2007). A considerable number of *M. truncatula* and *L. japonicus* genes are reported as being modulated in root nodules as well as in mycorrhizal roots, and a significant proportion is involved in build-up of cell walls, metabolism, transport, protein processing or signal transduction. Deguchi et al. (2007) reported that many of the plant genes repressed during both nodule and AM symbioses are involved in host plant defense and stress responses, suggesting that host plants accept AM fungi and compatible rhizobia in similar manners, their defense mechanisms being suppressed. However if all the published data are taken together, the overall overlapping of induced or repressed genes is

limited in nodules and AM tissues, suggesting that co-regulated genetic networks will not be such a common feature of the established symbioses as so far supposed.

I.4. Genetic mapping of symbiosis-related plant genes for map-based cloning

I.4.1. Cloning of pea symbiotic genes

Gene cloning is an essential step for the determination of the molecular basis of mutant phenotypes. The first cloned *SYM* gene of legumes was the *Nin* gene from *L. japonicus* (Schauser et al., 1999). The *LjNin* mutant was identified in screening of symbiotic mutants carrying the autonomous maize transposon Ac and isolated using a transposon tag. It is one of the possible ways for gene cloning. Briefly, the transposon insertion knocks out the gene and is used for the gene isolation at the same time. A few years later, the comparison of the phenotype of *Ljnin* with known pea mutants facilitated the identification of a possible *Nin* orthologue in pea, the *Pssym35* gene (Borisov et al., 2003). Synteny between genomes of legume species allows isolation of orthologous genes based on the nucleotide sequence of the identified gene in other legume species (Table I.4.1). Primer design and PCR amplification of the pea *Nin* orthologue were performed as well as subsequent co-segregation analysis and sequencing of the three independent *Pssym35* mutant alleles (Borisov et al., 2003). The same approach was later applied to isolate the *M.truncatula Nin* orthologue, *MtNin* (Marsh et al. 2007).

A modification of this approach was applied to clone the *PsSym19* gene (Stracke et al., 2002; Endre et al., 2002), which differed by the initial isolation from *L. japonicus* of the orthologous gene *SYMRK* by map-based cloning. The *LjSYMRK* gene was positioned on the bottom arm of chromosome 2 between the molecular markers *AUX* and *SHMT*. A contig of transformation-competent artificial chromosome (TAC) clones, comprising the SYMRK region and flanking markers, was assembled and then sequenced in the context of the *L. japonicus* genome sequencing project. Eighteen putative genes were predicted within the targeted region. Among them, a receptor-like kinase-encoding gene (*RLK*) was suggested as an obvious candidate for a

signal transduction component, a function compatible with the *SYMRK* mutant phenotype and which was confirmed by subsequent sequence analysis (Stracke et al., 2002).

Such a strategy based on the positional cloning of a gene of interest in model legume species and subsequent homology-based cloning of the orthologous gene in pea has been applied quite often. Up to day, artificial chromosomes available in the model legume species *L. japonicus* and *M. truncatula* significantly simplify the process of map-based (or positional-based) gene cloning.

Table I.4.1. Cloned symbiotic genes of pea and their orthologues

Gene name			Mutant phenotype	Encoding protein	References
P. sativum	*M. truncatula*	*L. japonicus*			
PsSym7	*MtNsp2*	*LjNsp2*	Myc^{+} Nod^{-}	transcriptional regulator GRAS family	Kaló et al., 2005 Murakami et al., 2006
PsSym8	*MtDMI1*	*LjCastor, LjPollux*	Myc^{-} Nod^{-}	multimeric ion channel	Ané et al., 2004 Imaizumi-Anraku et al., 2005
PsSym9	*MtDMI3*	*LjCCaMK*	Myc^{-} Nod^{-}	Ca^{2+}/calmodulin-dependent protein kinase	Mitra et al., 2004 Lévy et al., 2004 Tirichine et al., 2006
PsSym10	*MtNfp*	*LjNfr5*	Myc^{+} Nod^{-}	receptor-like kinase	Radutoiu et al., 2003 Madsen et al., 2003 Arrighi et al., 2006
PsSym19	*MtDMI2*	*LjSYMRK*	Myc^{-} Nod^{-}	receptor-like kinase	Stracke et al., 2002 Endre et al., 2002
PsSym29	*MtSunn*	*LjHar1*	Myc^{++} Nod^{++}	receptor-like kinase	Krusell et al., 2002 Nishimura et al., 2002 Schnabel et al., 2005
PsSym35	*MtNin*	*LjNin*	Myc^{+} Nod^{-}	transcriptional regulator	Schauser et al., 1999 Borisov et al., 2003 Marsh et al., 2007

PsSym37	*MtHcl* *(MtLyk3)*	*LjNfr1*	Myc^+ Nod^-	receptor-like kinase	Radutoiu et al., 2003 Limpens et al., 2003 Smit et al., 2007 Zhukov et al., 2008

Myc^+ - wild type AM formation, Myc^- - blocked AM formation, Myc^{++} - increased AM formation, Nod^+ - wild type root nodule formation, Nod^- - blocked root nodule formation, Nod^{++} - increased root nodule formation.

I.4.2. Expressed sequence tag-based molecular markers for gene mapping

The genetic map of pea has been developed gradually, starting with the first version by Lamprecht in 1948 up to the significant advance in the last years resulting from the development of DNA techniques for molecular marker creation.

A number of methods allowing detection of random nucleotide sequence polymorphism, such as RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism) and RFLP (Restriction Fragment Length Polymorphism), have led to the creation of moderate density pea linkage maps (Vos et al., 1995; Lacou et al., 1998; Flavell et al., 1998). The subsequent integration of generated molecular markers into previous classical genetic and cytogenetic maps has resulted in the development of consensus genetic maps containing different types of markers (Weeden et al., 1998; Ellis and Poyser, 2002). However, random DNA markers are not universal tools for genetic mapping because the majority of them cannot be transferred from one cross to another or from one legume species to another.

In this context, expressed sequence tag (EST)-based DNA markers appear more promising. This approach is based on the exploitation of information about conserved DNA sequences like those found in exons of genes. Such information is available in electronic databases (NCBI, different EST databases). For EST polymorphism detection, PCR-based methods using Cleaved Amplified Polymorphic Sequences (CAPS), Derived Cleaved Amplified Polymorphic Sequences (dCAPS) or size markers are usually used. These methods reveal differences in DNA sequence between individuals (polymorphism) by cutting of amplified PCR products with a

restriction endonuclease (CAPS and dCAPS) or by differences in the size of amplified PCR products. The first pea map containing 18 EST-based DNA markers was created by Gilpin et al (1997). They converted sequenced-characterized pea cDNAs into CAPS markers. This linkage map consists of 209 markers, covers 1330 cM and also includes RFLP, RAPD and AFLP markers. A number of pea genes were afterwards successfully located on the pea linkage map as EST (mostly as CAPS) markers using nucleotide sequence polymorphisms (Brauner et al., 2002; Irzykowska and Wolko, 2004; Konovalon et al., 2005; Aubert et al., 2006). In fine, the EST approach has proved to be most effective for genetic mapping and for comparative genome studies.

I.4.3. Exploitation of synteny between legume genomes

The development of genetic, cytogenetic and physical maps of legume species combined with data from large-scale *M. truncatula* and *L. japonicus* genome sequencing has facilitated the understanding of conservation (synteny) of genome structure among legumes. Macrosynteny generally refers to conserved gene order between species (an example is loci found on corresponding chromosomes), whilst microsynteny applies to conserved gene content and order over a short, physically defined DNA contig (an example is loci found on a corresponding bacterial artificial chromosome). The degree of synteny is correlated with the phylogenetic distance of the investigated species.

Several comparative genome studies were performed recently to evaluate synteny between legume species based on mapping of common DNA markers or *in silico* mapping of homologous sequences (Brauner et al., 2002; Kaló et al., 2004; Choi et al., 2004; Zhu et al., 2003, 2005; Moffet et al., 2006; Hougaard et al., 2008).

Zhu et al. (2005) demonstrated macrosyntenic relationships between legume species in a simplified consensus map (Figure I.5).

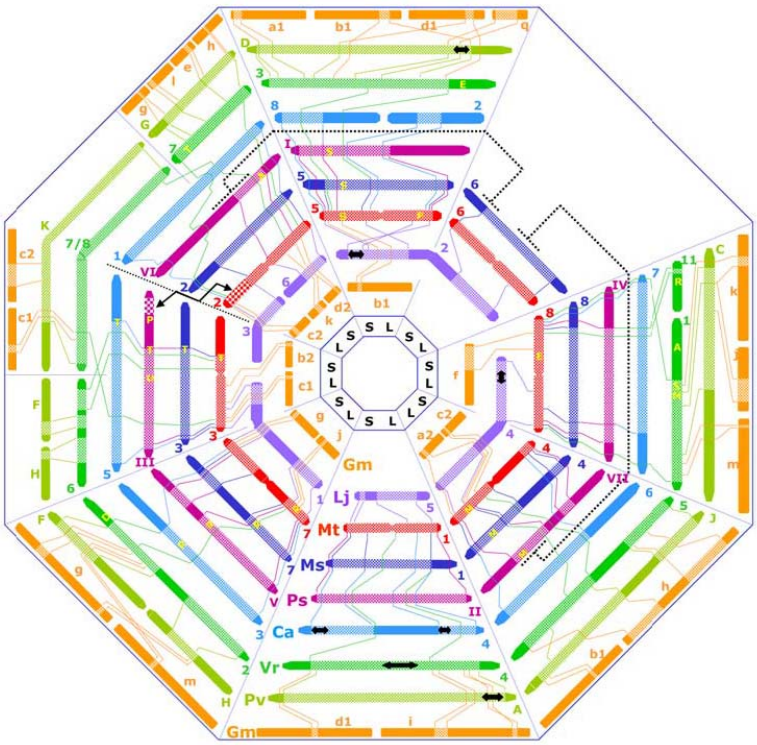


Figure I.5. A simplified consensus comparative map of legume species (Zhu et al., 2005). Mt- *Medicago truncatula*, Ms- *Medicago sativa*, Lj- *Lotus japonicus*, Ps- *Pisum sativum*, Ca- *Cicer arientinum*, Vr- *Vigna radiata*, Pv- *Phaseolus vulgaris*, Gm- *Glycine max*. S and L denote the short and long arms of each chromosome in *M. truncatula*. Syntenic blocks are drawn to scale based on genetic distance.

The central point of the map with which genomics of other legumes are compared belongs to *M. truncatula*, the model legume species for which the genome has been investigated in most detail. Developed pseudochromosomes of *M. truncatula,* containing information about the precise position of most of the genes in this species, are freely accessible (http://www.medicago.org/).

The comparative mapping of *M. truncatula* and *P. sativum* genomes shows that, despite some major differences (the pea genome is approximately 10 times larger than that of *M. truncatula* and has one less chromosome), the co-linearity of genes is remarkably conserved between the two species, with main evident differences being inferred from inter-chromosomal rearrangements (Choi et al., 2004; Zhu et al., 2005). Much of the expansion in the genome size of *P. sativum* may be due to retro-elements (Choi et al. 2004). The difference in chromosomes

number between pea and *Medicago* has been suggested to result from rearrangements involving the *Medicago* chromosome 6 (Zhu et al., 2005).

The conserved genome structure between *M. truncatula* and *P. sativum* has allowed for map-based cloning of genes (homology-based gene cloning; see I.4.1.) and creation of EST-based DNA markers, using *M. truncatula* as a surrogate genome. For example, Aubert et al. (2006) used syntenic relationships between pea and *M. truncatula* to infer gene position in the pea genome from *M. truncatula* gene position, and provided a functional pea map containing 111 genes and mutations. EST-based cross-species genetic markers have also been used for comparative mapping between *P. sativum* and *M. sativa* (Kaló et al., 2004) and between *P. sativum* and *M. truncatula* (Choi et al., 2004).

The location of each new gene identified using synteny between genomes provides an additional tool for future studies. Moreover, the idea that conserved genome structure can facilitate transfer of knowledge among related plant species may also be applicable to the map-based gene cloning for the creation of molecular tools for the fine mapping of genes of interest.

I.5. Thesis objectives

My thesis work has been performed within the framework of a co-operation programme between the Laboratory of Genetics of Plant-Microbe Interactions (ARRIAM, St. Petersburg, Russia) and the Plant-Microbe-Environment Research Unit (UMR INRA 1088/CNRS 5184/Université de Bourgogne PME, France). The research focuses on the characterization of the late symbiotic pea (*Pisum sativum* L.) genes *PsSym36*, *PsSym33* and *PsSym40* in arbuscular mycorrhiza formation and functioning.

In this context, the objectives of my thesis were:

- to monitor effects of *PsSym36, PsSym33* and *PsSym40* gene mutation on *G.intraradices* gene expression,
- to monitor effects of *PsSym36, PsSym33* and *PsSym40* gene mutation on plant gene expression in order to identify those involved in late stage of symbiotic interactions,

- to determine *G.intraradices* genes expression related to arbuscule formation stage of AM symbiosis,
- to localize the symbiotic pea genes *PsSym36* and *PsSym40* on the pea genetic map in order to create conditions for future map-based cloning of theses genes.

The research focuses on the characterization of fungal and plant gene responses during AM formation and functioning was performed in 'Plant-Microbe-Environment' Mixed Research Unit, INRA Dijon. In this part of my study, the pea symbiotic mutants *Pssym36*, defective for arbuscule formation (Engvild, 1987), *Pssym40*, and *Pssym33* having alterations in rate of AM development (Jacobi et al., 2003 a, b) and their corresponding wild type genotypes were used as genetic background for investigations of plant and fungal (*Glomus intraradices*) gene expression. The aim of exploiting the symbiotic pea mutants was to gain insight into the possible symbiotic function of the investigated genes in the AM symbiosis. *G. intraradices* genes were chosen amongst those that have been previously shown to be specifically up-regulated during AM symbiosis establishment by exploitation of early symbiotic mutants of *M. truncatula* (Seddas et al., 2008, 2009). Plant genes were selected among genes reported as being modulated during plant-fungal interactions in the established AM symbiosis (Balestrini and Lanfranco, 2006; Massoumou et al., 2007).

The second part of my research focused on the creation of conditions for localization of pea symbiotic genes *PsSym36* and *PsSym40* on the pea genetic map was performed in the Laboratory of Genetics of Plant-Microbe Interactions, ARRIAM, St. Petersburg. For this aim, a set of morphological and molecular markers was used for *PsSym3*6 and *PsSym40* gene mapping.

CHAPTER II

MATERIAL AND METHODS

II. Material and methods

II.1. Biological material

Pea genotypes: Throughout this study the following genotypes of *Pisum sativum* L. were used:

- cv Finale and its isogenic mutant RisNod24 (*Pssym36*) (provided by Gerard Duc, UMR Légumineuses à Graines, INRA, Dijon, France)
- line SGE and its corresponding mutant lines SGEFix$^-$2 (*Pssym33*), SGEFix$^-$1 (*Pssym40*) (provided by Alex Y. Borisov, Laboratory of Plant-Microbial Interactions, ARRIAM, Saint-Petersburg, Russia)
- laboratory lines NGB1238, NGB851 and NGB2715 (provided by Stig Blixt, Nordic Gene Bank, Alnarp, Sweden)
- lines K3034, K1693, K7128 (from the N. I. Vavilov Institute of Plant Industry (VIR) collection)

AM fungal isolate: The arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith (BEG 141) was supplied by the International Bank for Glomeromycota (IBG, http://www.ukc.ac.uk/bio/beg/) (INRA/Dijon) where it was produced on *Allium porrum* L. in pot cultures in neutral γ-irradiated Epoisses clay-loam soil.

Root nodule bacteria: *Rhizobium leguminosarum bv. vicia* strain CIAM1026 supplied by All-Russia Collection of Nonpathogenic Microorganism for Agriculture (ARRIAM, Saint-Petersburg, Russia).

II.2. Growth conditions and sampling of plant material

For gene expression analysis

Seeds were surface-disinfected by 10-min treatment with a hypochlorite solution (4%) at room temperature for the genotypes cv Finale and RisNod24, and by a 25-min treatment with 98 % sulphuric acid at room temperature for lines SGE, SGEFix-1, SGEFix-2, and washed with

distilled water. The method of surface-disinfection was chosen based on seed coat properties. Seeds were germinated in Petri dishes (12-cm diameter) on humid filter paper at room temperature in the dark for 3 days.

Seedlings were planted into pots containing 200 ml of a 1:1 mixture of quartz sand and mycorrhizal soil-based inoculum produced in pot cultures, or quartz sand and gamma-irradiated and heat-sterilized Epoisses soil for controls. Non-inoculated controls received a filtered (Whatman n°2) water suspension of inoculum to introduce the associated microflora. Plants were raised in a growth cabinet under control conditions (24/19^{0}C, 16 h light, 420 μmol m^{-2} s^{-1}, 70 % relative humidity), fertilized with 10 ml/pot of a modified Long Ashton solution (Table II.1, Hewitt, 1966) without phosphorus three times a week, or 10 ml of distilled water on other days.

Tab.II.1. Modified Long Ashton solution

Component	Concentration, mg/l	Component	Concentration, mg/l
KNO_3	808	$ZnSO_4 \times 7H_2O$	0.29
$Ca(NO_3)_2 \times 4H_2O$	1890	H_3BO_3	3.10
$MgSO_4 \times 7H_2O$	368	NaCl	5.90
$MnSO_4 \times H_2O$	2.23	$H_{24}Mo_7N_5O_{24} \times 4H_2O$	0.088
$CuSO_4 \times 5H_2O$	0.25	FeNa-EDTA	22

Plants were harvested at 7, 14, 21 or 28 after inoculation with *G. intraradices* and weighed fresh, after washing the root systems in ice-cold water. Root samples were taken for RNA extraction and stored in liquid nitrogen.

For genetic crossing experiments

Seeds were surface-disinfected by a 25-min treatment with 98% sulphuric acid, washed with distilled water and directly soaked in distilled water for 2 h before sowing. Plants were grown in 4l pots in a sod-podzol sandy soil (pH_{KCl} 5.5, P_2O_5 18.5 mg/100g soil, K_2O 4.3 mg/100g soil) (parental lines for crossing, F1 population of plants) or sterile quartz send (F2 population plants), 4 plants per pot, in greenhouse conditions in ARRIAM.

Plants of F2 populations were inoculated by *Rhizobium leguminasarum bv. vicia* strain CIAM1026 known to have high symbiotic capacity (Safronova, Novikova, 1996). Bacteria were cultured on solid nutrient medium №79 (Sambrook et al., 1989) with streptomycin (600 µg/l) at 28°C for 3 days. Plants were inoculated with an aqueous suspension of bacterial cells (10^7-10^8 cells per plant) at sowing, and fertilized with 250ml per pot of a nutrient solution (tab. II.2.) every 2 weeks (Borisov et al., 1997).

Tab.II.2. Plant nutrient solution

Component	Concentration, mg/l	Component	Concentration, mg/l
$MgSO_4 \times 7H_2O$	1000	$CuSO_4$	0.4
K_2HPO_4	1000	$Al_2(SO_4)_3 \times 18H_2O$	0.3
$Ca_3(PO_4)_2$	200	$ZnSO_4 \times 7H_2O$	0.2
NH_4NO_3*	600	$CoCl_2 \times 6H_2O$	0.2
NaFe-EDTA	10	NaCl	0.2
H_3BO_3	5	$NiSO_4$	0.2
$(NH_4)_2MoO_4$	5	$MnSO_4$	0.2
KBr	0.6	Li_2SO_4	0.2
KI	0.5		

* - not added in the nutrient solution for plants inoculated by *Rh. leguminasarum*.

Plant material (leaves) was collected for DNA extraction and root systems examined for nodule formation in F2 populations at 4 weeks after inoculation by *Rh. leguminosarum*. Samples were stored at -80°C.

II.3. Estimation of mycorrhizal root colonization

Mycorrhization parameters were determined at 3 and 4 weeks after inoculation with *G. intraradices*. To estimate total root colonization, staining with black ink (Noir de Jais of Shaeffer, Sheaffer Manufacturing Co., USA) was performed. Roots were digested in 10% KOH

at 90°C for 80 min, rinsed several times with distilled water, stained for 8 min in 5% ink solution in 8% acetic acid and destained in 0.8% acetic acid for 25 min (Vierheilig et al., 1998).

Detection of fungal alkaline phosphatase activity in colonized roots was used to evaluate presence of a functionally active symbiosis (Tisserant et al., 1993). A 0.5 g root sample was incubated in 20 ml of 0.05 M Tris/citric acid, pH 9.2, containing sorbitol (50 mg/ml), cellulose (15 units/ml) and pectinase (15 units/ml) for 2 h at room temperature, rinsed with distilled water several times and left overnight at room temperature in 20 ml of the staining solution (0.05 mol/l Tris/citric acid pH 9.2; 1 mg/ml α-naphthyl acid phosphate; 1mg/ml Fast Blue RR salt; 0.5 mg/ml $MgCl_2$; 0.8 mg/ml $MnCl_2 \times 4H_2O$). The next day, root samples were washed with distilled water, destained in hypochlorite solution (containing 1% active chlorine) for 5 min at room temperature, and rinsed with water again.

For light microscopy, 30 1cm-long root pieces were mounted on glass slides, observed according to Trouvelot et al. (1986), and colonization estimated using the Mycocalc programm (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). The parameters used were F% (frequency of mycorrhizal colonization in the root system), M% (intensity of mycorrhizal colonization in root cortex), m% (intensity of mycorrhizal colonization in mycorrhizal root fragments), A% (arbuscule abundance in the root system), a% (arbuscule abundance in mycorrhizal root fragments).

II.4. Laser Capture Microdissection (LCM) of arbuscule-containing cortical root cells

II.4.1. Tissue preparation

Mycorrhizal roots were cut into 1-cm pieces and fixed in freshly prepared Farmer's fixative solution (acetic acid/absolute ethanol [1v/3v]) overnight at 4°C. The next day, the liquid was discarded and root tissues were rinsed twice with 96% ethanol, incubated for 15 min with 96% ethanol at 4°C, then incubated for 15 min with ethanol/eosin (SIGMA ALDRICH 861006 solution (0.1% in DEPC (diethyl pyrocarbonate) water) at room temperature, rinsed with 100% ethanol,

and incubated with 100% ethanol for 1 hour. After the last ethanol step, root tissues were treated at room temperature with a 100% ethanol (VWR ref: 370720601)/histoclear II solution series (3v/1v, 1v/1v and 1v/3v) for 30 min each. They were then embedded in paraffin (paraplast plus SIGMA réf P 3683) through a series of histoclear II /paraffin solutions, in which the histoclear II was gradually replaced by paraffin (15 pastilles of paraffin/25 ml of histoclear II, 30 pastilles of paraffin/25 ml of histoclear II, 45 pastilles of paraffin/25 ml of histoclear II and pure paraffin). Roots were incubated at 55°C for 1h in each paraffin-containing solution, and subsequently overnight in pure paraffin. Embedded samples were stored in pure paraffin at 4°C in small Petri dishes until use.

Sectioning (10-µm) was performed at room temperature using a Jung RM 2065 (Leica) microtome and RNAse-free tools. Sections were placed on a slide, stretched with few drops of distilled RNase-DNase-free water and kept in a slide warmer at 42°C overnight. Just before use, sections were deparaffinized by treating slides twice with histoclear II for 5 min,

histoclear II/100%ethanol solution (1v/1v) for 2 min, 100 % ethanol for 2 min, and fresh 100% ethanol for 1 min. Deparaffinized slides were placed in a desiccator for 1 hour before dissection.

II.4.2. Dissection of arbuscule-containing cells

Dissection was performed using an Arcturus™Microdissection instrument (ARCTURUS NIKON inverse XT100). Slides with sections were visualized on a computer monitor through a video camera. Selected cells were marked using graphical software, cut twice with an IR laser (810 nm, laser spot size of 10 µm, laser Power of 69 mw, laser pulse duration of 25 ms), and captured automatically into CapSure HS LCM Caps (Arcturus). Harvested cells were transferred into RNA extraction buffer (Picopure Kit Arcturus) within a maximum of 30 min after dissection had begun. RNA was frozen at -80°C and stored until extraction (see II.7.4).

II.5. Plant hybridization analysis

Parental pea lines were crossed when plants were budding or started flowering. Immature stamens in maternal flowers were cut out using sterile tweezers. Mature pollen from paternal flowers was transferred to pistil stigma of maternal plants with another set of sterile tweezers. Plants of F2 populations were obtained from self-fertilization of plants of F1 population. Goodness-of-fit for the co-dominant 1:2:1 or dominant 1:3 ratios in F2 populations were tested by χ^2 analysis using the computer program MapL98.

II.6. Nucleic acid preparation

II.6.1. RNA extraction from plant roots

Total RNA was isolated from roots stored in liquid nitrogen following the method of Logemann et al. (1987), modified by Franken and Gnadinger (1994). Samples were ground into a fine powder in ceramic mortars in liquid nitrogen and transferred into plastic 50 ml tubes containing NTES extraction buffer (50 mM Tris/HCl, pH 9; 150 mM NaCl; 5 mM EDTA; 5% SDS; 1% β-mercaptoethanol; 1.6 buffer volume (ml) per mg sample). An equal volume of phenol:chloroform:isoamyl alcohol solution (mixed by volume as 25:24:1, correspondingly) was added to remove proteins. Tubes were inverted several times to mix, then centrifuged 15 minutes at 14500 g at room temperature, and the upper aqueous phase transferred into new 1.5 ml eppendorf tubes. This procedure was repeated twice with the phenol:chloroform:isoamyl alcohol solution and once with pure chloroform. To precipitate RNA and eliminate polysaccharides, 0.7 vol of 96% ethanol (from -20°C) and 0.05 vol of 1 M acetic acid were added to the aqueous phase and left overnight at - 20°C. The next day, tubes were centrifuged for 35 min at 10000 g at 4°C and the liquid phase was discarded. The pellet was resuspended in DEPC water (vol=vol NTES/4) and RNA was specifically precipitated from the solution by 4 M LiCl (1 vol) treatment for 4 h at 4°C. Tubes were centrifuged for 35 min at 10000 g at 4°C, the

aqueous phase was discarded, the pellet was resuspended in DEPC water and another RNA precipitation was performed overnight with 3 M NaAc (0.1 vol) and 96 % ethanol (3 vol) at -20°C. The final pellet was air-dried at room temperature and dissolved in 40 µl RNase-free water. Quantity and quality of RNA were checked in an Eppendorf BioPhotometer and RNA integrity was checked by electrophoresis in a denaturing agarose gel (see II.8.2). The DNase-containing columns of the Promega kit for total RNA isolation (SV Total RNA Isolation System) were used to remove any DNA contamination. RNA quantity, quality and integrity were checked again.

II.6.2. RNA extraction from laser-dissected arbuscule-containing cells

RNA was extracted from dissected cells using the PicoPure RNA Isolation kit (Arcturus) according to the manufacturer's protocol. CapSure HS Caps (Arcturus) containing dissected cells were incubated with extraction buffer for 30 min at 42°C and then cells were collected in microcentrifuge tubes by centrifugation for 2 min at 800 g. Cell extract containing RNA was stored at -80°C and RNA isolation was continued after grouping together extracts from a series of cell sections. Cell extract was loaded onto a preconditioned purification column (600-700 sections/column) to bind RNA to the column membrane and washed with washing buffer. After removing DNA by DNase treatment (RNase-Free DNase Set, Qiagen), the membrane was washed twice with washing buffer and the RNA was eluted into a fresh centrifuge tube using a low ionic strength buffer. RNA was stored at -80°C. Quality and quantity of the RNA were estimated using a Bio-Rad Experion System and Bio-Rad Experion HighSens Analysis kit following the manufacturer's protocol.

II.6.3. DNA extraction from plant shoots

DNA was extracted using CTAB (Cetyl trimethyl ammonium bromide) buffer using the modified protocol of Rogers and Bendich (1985). Plant tissue (approximately 70 mg) was ground in

1.5 ml tubes containing 700 µl of 2×CTAB buffer and approximately 30 µg of Al_2O_3 powder, and incubated for 1 h at 65°C.

After incubation, an equal volume of phenol/chloroform solution (1/1, vol/vol) was added, tubes were inverted several times to mix, and centrifuged for 15 min at 14000 rpm. The upper phase was transferred into a new 1.5 ml eppendorf tube, and the procedure was repeated twice with phenol/chloroform solution and once with pure chloroform. DNA was precipitated from the final solution by adding an equal volume of 96% ethanol, transfering into new 1.5 ml eppendorf tubes containing 1 ml of 75 % ethanol, and centrifuging 5 min at 14000 rpm. After centrifugation, the liquid phase was removed, and the pellet was air-dried then diluted in distilled DNase free water.

Composition of 2×CTAB buffer: 2% CTAB (m/V), 1.4 M/l NaCl, 0.1 M/l Tris-HCl pH 8.0, 20 mM/l EDTA, H_2O.

DNA quantity and quality were checked using Beckman Coulter DU800 Spectrophotometer.

II.6.4. Plasmid DNA preparation

Amplified parts of genes were cloned into a plasmid vector using the TOPO TA Cloning kit (Invitrogen) (see II.9). After plasmids were recuperated by white-blue selection and PCR (see II.9). Recombinants (white colonies) were multiplied in liquid LB culture containing ampicillin (100 µg/ml), and plasmid DNA was isolated using the NucleoSpin®Plasmid kit (Machrey-Nagel) following the manufacturer's instructions.

2 ml of liquid culture of recombinant colony was centrifuged 30 s at 11000 g at room temperature. The supernatant was discarded and the cell pellet was resuspended by vigorous vortexing in buffer A1. Plasmid DNA was liberated from the *E. coli* host cells by SDS/alkaline lysis (Buffer A2) and Buffer A3 was used to neutralize the resulting lysate and create appropriate conditions for binding of plasmid DNA to the silica membrane of the NucleoSpin®Plasmid column.

Precipitated protein, genomic DNA and cell debris were removed by a centrifugation step. Contaminations like salts, metabolites and soluble macromolecular components were removed

by washing with Buffer AW and ethanolic Buffer A4. At the final step, pure plasmid DNA was eluted under low ionic strength conditions with slightly alkaline Buffer AE (5 mM Tris/HCl, pH 8.5). Plasmid DNA yield and purity was estimated using an Eppendorf BioPhotometer.

II.7. Nucleic acid separation by gel electrophoresis

II.7.1. DNA electrophoresis in native agarose gel

Agarose gel (1% to 2% depending on the size of DNA fragments) was prepared in 0.5×TAE buffer (0.02 M Tris-acetate, 0.5 mM EDTA). DNA samples were mixed with 0.1 vol loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) (Sambrook et al., 1989), loaded into agarose gel and separated at 100V in 0.5×TAE buffer for 15-40 minutes (depending on agarose gel concentration and DNA fragment size). DNA was visualized under UV light after gel staining with ethidium bromide (0.1 µg/µl).

II.7.2. RNA separation in denaturing agarose gel

1 µg RNA diluted in 5 µl RNase-free water was denatured in 5 µl denaturing buffer (50% deionized formamide, 20% formaldehyde [37%], 1×MOPS buffer [10×MOPS buffer: 200 mM MOPS pH 7.0, 50 mM sodium acetate, 10 mM EDTA]) and 1 µl loading buffer (50% sucrose, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF, 1% ethidium bromide [10 µg/µl]) (Sambrook et al., 1989) for 10 minutes at 70°C. RNA was separated for 60 min at 50V in a 1.2% denaturing agarose gel prepared with 20% formaldehyde (37%) and 1×MOPS buffer. RNA integrity was checked by gel observation under UV light.

II.8. Polymerase chain reaction (PCR)

II.8.1. PCR

The polymerase chain reaction was performed in either a T3 Thermocycler (Biometra), a Personal Cycler (Biometra) or an iCycler™ (Bio-Rad) in 20 µl final volume mix using DiaTaq polymerase (DiaTaq) or aTaq DNA polymerase (Promega).

Tab.II.3. Composition of PCR mix for DNA (or cDNA) amplification

Product	Final concentration
Reaction buffer	×1
dNTPs	125 µM
Specific primer 1 (for)	0.25 µM
Specific primer 2 (rev)	0.25 µM
Taq DNA polymerase	1unit
DNA	app. 100 ng
Sterile water	qps 20 µl

PCR protocol parameters were: initial denaturation at 95°C for 4 min, 35 cycles at 95°C for 45 s, primers annealing temperature 55-62°C (see tables II.4 and II.5) for 45 s, elongation at 72°C for 1 min, followed by a final elongation at 72°C for 5 min (15 min if PCR product cloning was planned).

PCR of cDNA was carried out in 20 µl of PCR mix (Invitrogen) supplemented with 1.5 mM $MgCl_2$, containing 0.5 U of *Taq* polymerase (Invitrogen), 125 µM dNTP, and 0.5 µM of each primer using 1 µl of diluted 1:5 cDNA for both fungal and plant genes. PCR was performed in a Biometra thermocycler (Göttingen, Germany) at 94°C for 1 min, followed by 30 cycles at 94°C for 45 s, the appropriate primer annealing temperature for 45 s, 72°C for 45 s, and a final extension at 72°C for 1 min.

Amplification products were analyzed by 1.4 % (wt/vol) agarose gel electrophoresis in 0.1 Tris-acetate-EDTA (4 mM Tris, 0.1 mM EDTA, 2 mM acetic acid) (Fluka, St Gallen, Switzerland),

stained with ethidium bromide for 10 min and observed under UV light using GelDoc EQ apparatus (Weidmann et al. 2004).

II.8.2. Primer design

For trancriptome studies

Primers for real-time RT-PCR were designed to amplify PCR product with a size of 120-350 bp. The protein-encoding genes from *G. intraradices* genes chosen for this study have predicted functions in transcription, protein turnover, primary or secondary metabolism (table II.4), and are known to be active in *G. intraradices* at the appressoria stage of root colonization (Seddas et al. 2008, 2009). The primers used had previously been designed by Seddas et al. (2009). Primer sequences, melting temperature and expected amplicon size are presented in Table II.4.

Table II.4. Specific primers for *G. intraradices* gene expression analysis

Gene	Primer sequences	Expected size, bp	Tm, °C
H+-ATPase (*H-ATP*)	TTGAGGATTGGCAAATGAGTGC GTCCAAGACGCCATTTATCAGG	126	55
Alkaline phosphatase (*ALP*)	GCTCGTCAAGTTTCCGATCTAC CTCCGATTCCTAATCCGCTAC	163	55
Translation elongation factor 1, α-subunit (*TEF*)	AGC CGA ACG TGA ACG TGG GCA CAA TCG GCC TGA GAA GTAC	247	55 to 60
RHO/GDP dissociation inhibitor (*RHO*)	GCT GAC GAT GAA TCT CT CCC TGA TTC CCT TTC CTC TG	214	55
Peptidylprolyl isomerase 2 (*PEPISOM*)	GAT GTT CAT GCC GGT AAA AG ACT GGA TGA ACC CAA TGT CT	235	55
26S proteasome subunit 2 (*26SPROT*)	CAC GTG TTT TGC CAA CG GCT TCT TCT TCT TCA GTA AC	183	55
26S proteasome regulatory subunit (*26SREG*)	CCT ACT TTT GAC CGA CGT CA CGA CTT TCT TAA CAT TGG CT	231	55
Stearoyl-CoA desaturase	TCG TGT TCC TGA AAA TG	269	60

(*DESAT*)	GCT TTA GTG GAG TCT TTA CC		
Thioredoxin peroxidase (*THIO*)	GGG AAG CTA AGG CAG T CGC AAT ATG AGC GTG ATG T	214	60
Superoxidase dismutase (*SOD*)	CTG GAC CTC ATT TTA AC CCG ATA ACA CCA CAA GCA A	273	55
Hypothetical protein 1 (*VACU*)	CGC TCC GAA CTT TTA TG CCG AGG ACT ATC CTG AGT T	345	55

Protein-encoding plant genes were selected amongst those reported in the literature to be specifically up-regulated during AM interactions or in arbuscule-containing cells of *P. sativum* or *M. truncatula* (table II.5). A part of the chosen *P. sativum* genes had been studied before (Grunwald et al., 2004); these encode a *disease resistance protein pI230*, a *trypsin inhibitor* and a *MAP kinase*. Since nucleotide sequences were not known for the other genes in pea, primers were designed for specific amplification of those genes in pea based on nucleotide sequences of conserved regions of homologous genes of *M. truncatula* and some other plant species (annex 1). Amplicons from RT-PCR were cloned and sequenced to confirm the identity of the amplicon and to verify primer specificity. Using the obtained sequences, primers were then designed for amplification of 130-300 bp size PCR products in real-time RT-PCR experiment (table II.5.).

Table II.5. Specific primers for *P. sativum* gene expression analysis

Gene	Primer sequences	Expected size, bp	Tm, °C
Plasma membrane ATPase (H^+*ATPase*)	TACAGGTGAGTCCCTGCCG CCCAATTGCAGTCAAGACCT	252	60
Inorganic phosphate transporter (*PT4*)	CTTCACGTGCCATGTTCATC GCGTCGGAAACAGCTCC	273	58
Blue copper binding protein (*BCOP*)	GTTGGGTGATTGGTGGTGA AAGAGGAATGGTTGTCGCAC	187	55
Disease resistance protein pI230 (*DRP*)	GCGGCCGAGGTCAAGAAATAG CCGGGCAGGTACAAATACTGC	278	58

Trypsin inhibitor (*TI*)	ACCTTACAGCGTGAGCCTATAAGA GCGGCCGAGGTACGAAAGGTG	302	60
Glutathione-S-transferase (*GST*)	ACCAAAAGGTGCCTGTGTTC CTAGCTTGTGCGCGATCATA	133	58
MAP kinase (*MAPK*)	GCACTGGCACACCCTTACTTG GGGGTTAAATGCTAGAGCTTCTCTG	154	60
Serine protease (*SERPROT*)	AGAGTAGCCAAAGGTCAAGCAGT GACCTCTTGATGAAAATGAAGCA	253	58
Glyceraldehyde phosphate dehydrogenase (*GAPDH*)	AAGAACGACGAACTCACCG TTGGCACCACCCTTCAAATG	187	55 to 60

For molecular marker creation

Primers were designed based on information about EST nucleotide sequences of genes for *P. sativum*. For some genes, primer sequence information was taken from the literature (Aubert et al., 2006). The list of ESTs and primers used is presented in table VII.1 and annex 2, respectively.

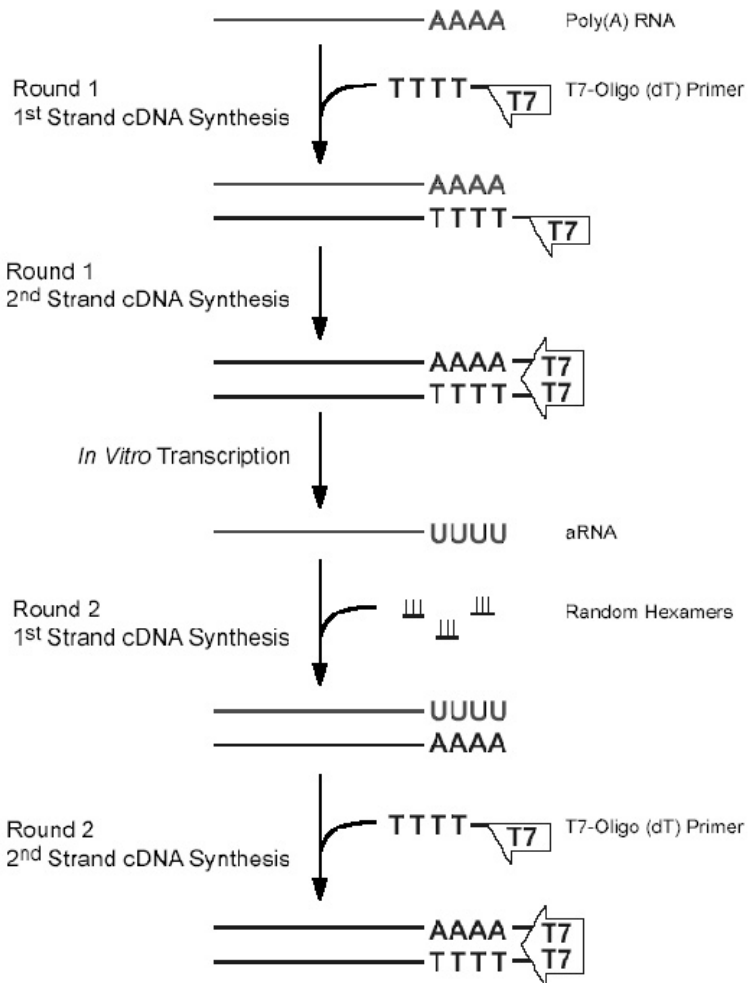
II.8.3. cDNA synthesis

Reverse transcription-PCR (RT-PCR). cDNA was synthesized from 1 μg DNA-free RNA using 300 U M-MLV Reverse transcriptase RNase H Minus (Promega), 1 μg oligo$(dT)_{15}$ primer (Promega), 40 U RNasine RNA inhibitor (Promega) and 7.5 mM dNTPs. RNA, oligo$(dT)_{15}$ primer and dNTPs were preheated at 70^0C in a thermocycler for 5 min and cooled on ice for 3 min. Reverse transcriptase, reaction buffer, RNasine and distilled water were then added to obtain a final mix volume of 25 μl and the reaction was performed under conditions recommended by Weidmann et al. (2004) (25^0C for 15 min, 42^0C for 1 h and 96^0C for 2 min). cDNA was stored at -20°C.

aRNA amplification. RNA extracted from laser microdissected arbuscular-containing cells (see II.6.4) was amplified using TargetAmp™2-Round aRNA amplification kit (Epicentre Biotechnologies, Madison,WI) and 40 pg - 200 pg of total RNA in the following protocol.

The first reverse transcription reaction was primed with a synthetic T7-oligo(dT) containing a phage T7 RNA Polymerase promoter sequence at its 5'-end. The primer was annealed to the RNA template at 65°C for 5 min then chilled on ice for 1 min. First-strand cDNA synthesis was performed at 50°C for 30 min using a premix containing primer and RNA, 50 U of SuperScript III reverse transcriptase (Invitrogen), reverse transcription premix (supplied with the amplification kit) and DTT (dithiothreitol). The RNA component of the resulting cDNA:RNA hybrid was digested by RNase H into small RNA fragments that were primed for second-strand cDNA synthesis. For this purpose DNA polymerase and DNA polymerase mix from the amplification kit was added to the reaction mix and incubated for 10 min at 65°C and for 3 min at 80°C. The reaction was stopped by adding finishing solution from the amplification kit. *In vitro* production of anti-sense RNA (aRNA) was obtained by adding T7 RNA polymerase, T7 transcriptional buffer, *In Vitro* transcriptional premix and DTT. The reaction was performed at 42°C for 4 hours followed by a DNase I treated at 37°C for 15 min. Qiagen RNeasy MinElute Cleanup kit was used to purify aRNA according to the manufacturer's protocol. A second round of reverse transcription was catalyzed by SuperScript II reverse transcriptase (Invitrogen). After adding random sequence hexamer primers, the volume mix was adjusted to 3 µl by speed vacuum centrifugation without heating and the reaction was incubated at 65°C for 5 min**.** The first-strand cDNA synthesis mix was combined with the RNA-primer mix, 50U of SuperScript II reverse transcriptase (Invitrogen), reverse transcription premix and DTT. Reaction was incubated for 10 min at room temperature and for 1 h at 37°C. The RNA component of the cDNA:RNA hybrid was digested with RNase H, as previously described. Double-stranded cDNA was produced using a T7-Oligo(dT) primer, DNA polymerase and DNA polymerase premix supplied by the amplification kit. The reaction was incubated for 10 min at 37°C, for 3 min at 80°C, chilled on ice and stored at -20°C. The obtained cDNA was used for semi-quantitative and real-time PCR.

Figure II.1. Target Amp™2-Round aRNA Amplification principle



II.8.4. Absolute quantitative real-time PCR

Absolute real-time RT-PCR was carried out using the Absolute QPCR SYBR Green kit (ABgene, UK) and fluorescence was quantified using an ABI PRISM 7900 real-time cycler (Applied BioSystems, Foster City, CA, USA). Reactions were performed in 20 µl full mix volume containing tested cDNA, 10 µl of reaction mix (from Absolute QPCR SYBR Green kit), 2 pM of each primer and sterile water. After 10 min denaturation at 95°C, 40 amplification cycles (95°C for 15 s, primer annealing temperature for 30 s and 72°C for 30 s) were followed by a melting curve program included at the end of each PCR run according to the thermal profile suggested by the manufacturer (95°C for 15 s, primer annealing temperature for 15 s, 95°C for 15 s) to verify amplification of each specific target cDNA. The expression of each gene was assayed in

three technical replicates and analyzed by the SDS 2.2 program (Applied Biosystems, Foster City, USA). Expression analyses for each gene were performed in replicate RNA batches (three biologically independent experiments) from *G. intraradices*-inoculated or non-inoculated roots of each wild type pea genotype and the corresponding mutants.

To calculate the absolute amount of transcripts present in original samples, plasmid DNA containing each amplicon was prepared (see II.7.3.), quantified by UV absorbance spectroscopy, and linearized by restriction enzyme digestion (see II.11). Standard amplification curves were determined from duplicate samples of plasmid DNA at dilutions of 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 copies for each assay. Relative amounts of transcripts for each gene were calculated as a ratio to the amount of *translation elongation factor 1,* α-*subunit* (*TEF*) transcripts as the fungal reference gene (Seddas et al. 2009) or of *glyceraldehyde phosphate dehydrogenase* (*GAPDH*) transcripts as the plant reference gene (Weidmann et al. 2004; Sanchez et al. 2005).

II.9. DNA fragment sequencing

Sequencing was performed using CEQ™8000 Genetic Analysis System (Beckman Coulter) and GenomeLab™ DTCS Quick Start Kit (Beckman Coulter). To purify PCR products for DNA sequencing, 40 µl of PCR reaction was loaded into 1% agarose gel. PCR products were cut from the gel and dissolved in solution A (1 µl of solution A to 1 µg of gel) for 5 min at 65°C. 50 µl of solution B containing silica was added to each reaction, tubes were vortex well and centrifuged for 1 min at 3000 rpm. The liquid phase was discarded then the pellet was rinsed sequentially with 100 µl of solution A, 100 µl of solution C, 100 µl of 70% ethanol and dried for 10 min at 37°C. DNA was washed from the silica particle using 10 µl of 10 mM Tris-HCl pH 8.0 and stored at -20°C.

<u>Composition of solution A</u>: 3 M guanidine(iso)tiocianat, 20 mM Na_2EDTA, 10 mM Tris-HCl pH=6.8, 40 mg/ml Triton X-100, 20 mg/ml β-mercaptoethanol

<u>Composition of solution B</u>: 40 mg/ml Silica in solution A

Composition of solution C: 25% ethanol, 25% isopropanol, 100 mM NaCl, 10 mM Tris-HCl pH=8.0

Reaction was performed in 5 µl full mix containing 25 fmol PCR product, 1 µl of DTCS mix (supplied by the kit), 0.25 µl of reaction buffer (supplied by the kit) and 1.6 pmol of original primer following the manufacturer's protocol.

II.10. Cloning and sequencing of PCR products

To prepare standard plasmid dilutions for real-time RT-PCR, PCR fragments of cDNA were cloned into a plasmid vector using the TOPO TA Cloning kit for sequencing (Invitrogen). 1 µl of salt solution (supplied by the kit) and 1 µl of Vector (supplied by the kit) were added to 4 µl of PCR product; the mix was incubated for 30 min at room temperature and chilled on ice. To transform 1 aliquot of *Escherichia coli* competent cells (supplied by the kit), 2 µl of the previously prepared mix was added and transformation was performed by a heat shock at 42°C for 30 sec followed by cooling on ice.

To increase transformation efficiency, bacteria were cultured with 250 µl of SOC medium (Super Optimal broth with Catabolite repression: 2% [m/V] bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM Mg [$MgCl_2 \times 6H_20/MgSO_4 \times 7H_2O$], 20 mM glucose) for 1 hour at 37°C. 200 µl of the cell culture were transferred into Petri dishes containing LB medium (Luria Broth: 1% [m/vol] bacto-tryptone, 0.5% [m/vol] yeast extract, 5% [m/vol] NaCl pH 7.0, 1.5% [m/vol] bacto-agar) containing ampicillin (50 µg/ml), IPTG (20 µg/ml), X-Gal (20 µg/ml) and incubated overnight at 37°C. Recombination was checked by white-blue selection (recombinant colonies are white) and by PCR. Recombinant colonies were transferred into liquid LB medium containing ampicillin (50 µg/ml) and grown overnight at 37°C with shaking. Plasmid DNA was extracted as described in chapter II.7.3.

Cloned PCR products were sent for sequencing to NWG Biotech (Ebersberg, Germany).

II.11. Digestion of DNA fragments by restriction enzymes

Plasmid DNA digestion

Reactions were performed in 50 µl final volume mix containing 300 ng of plasmide DNA, 1 unit of restriction enzyme NotI, ×1 reaction buffer (corresponding to this restriction enzyme) and sterile water (qsp 50 µl) and incubated for 3 hours at 37°C. Effectiveness of digestion was checked by electrophoresis in 1% agarose gel (see II.7.1.).

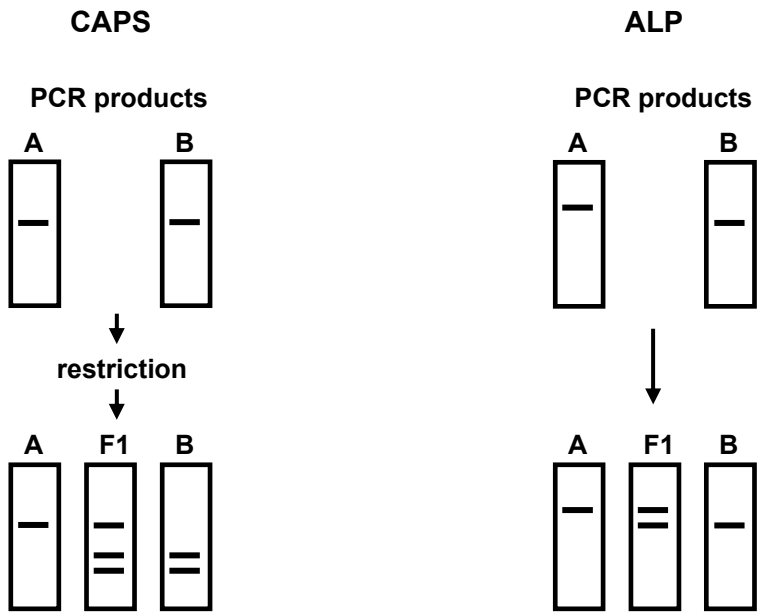
PCR fragment digestion

PCR fragment digestion was used for CAPS (Cleavage Amplified Polymorphic Sequences) marker analysis. The reaction mix contained 10 µl of PCR product, 1 unit of restriction enzyme, ×1 reaction buffer (recommended to be used with restriction enzyme) and sterile water (qps 20 µl). A set of restriction enzymes provided by Fermentas (Lithuania) and СибЭнзим (Russia) were used for the analysis. Reactions were performed according to conditions recommended by producer for each enzyme and analyzed by electrophoresis in agarose gel (see II.8.1.).

II.12. Molecular marker creation

In this study two types of EST (Expressed Sequence Targ) molecular markers were used. They are CAPS (Cleavage Amplified Polymorphic Sequences) and ALP (Amplified Length Polymorphic sequences). CAPS marker creation was done in three steps: specific amplification of the part of the gene, search for polymorphic restriction sites and checking of the size of PCR products after restriction by electrophoresis in agarose gel. To check specific amplification, PCR products were sequenced (see II.10.). Analysis of obtained nucleotide sequences was done using online computer programme SDSC Biology Workbench (http://workbench.sdsc.edu/). In process of sequences analysis for some genes, polymorphism was detected by the size of PCR products which can be visualized by electrophoresis in agarose gel. It became a base for ALP marker creation.

Figure II.2. Molecular marker development



A – parent 1, B – parent 2, F1 – heterozygote

II.13. Statistical analysis

- All data from real-time RT-PCR analyses were statistically compared between treatments at each time point using one-way ANOVA for each fungal gene and two-way ANOVA for mycorrhiza-mutant interactions for each plant gene (SPSS 16.0 package, SPSS Inc., Chicago, Illinois); significant differences between means were established by Tukey at $P \leq 0.05$. Where ANOVA gave marginally significant ($P \leq 0.1$) differences across treatments, data between *G. intraradices*-inoculated and noninoculated roots were compared separately for each genotype using the Student's t-test at $P \leq 0.05$.
- Gene mapping and construction of genetic maps were done using computer program MapL98 (Prof. Yasuo Ukai, Biometrics Laboratory, Graduate School of Agricultural Life Science, University of Tokyo).

CHAPTER III

EFFECT OF MUTATIONS IN THE GENES *PSSYM36*, *PSSYM33* AND *PSSYM40* ON ARBUSCLAR MYCORRHIZA FORMATION AND FUNCTION

III. Effect of mutations in the genes *PsSym36*, *PsSym33* and *PsSym40* on arbuscular mycorrhiza formation and function

III.1. Introduction

Pssym36 (line RisNod24) was first reported as a nitrogen fixation symbiosis (NFS) mutant by Engvild (1987). Later, more detailed investigations of the process of NFS development showed that the mutation in the *PsSym36* gene blocks infection thread growth in the root hair and nodule primordium development (Sagan et al., 1994). *Pssym36* is also an AM-defective mutant where the stages of appressorium formation, root penetration and cortex colonization by intercellular hyphae are completed, but arbuscule formation is reduced to a few stumpy branches (Gianinazzi-Pearson et al., 1991; Gianinazzi-Pearson, 1996), leading to a lower level of root colonization than in the wild type genotype (Lapopin et al., 1999; Grunwald et al., 2004). The pea symbiotic mutants *Pssym40* and *Pssym33* were identified after a complementation analysis in SGEFix$^-$-1 and SGEFix$^-$-2 lines, respectively, and were shown to control stages in symbiotic root nodule development by Tsyganov et al. (1998). SGEFix$^-$-1 (*Pssym40*) is blocked at the stage of infection droplet formation during nodule organogenesis, whilst SGEFix$^-$-2 (*Pssym33*) is impaired in the process of endocytosis and rhizobia are not properly released into the nodule cells. These mutants were later shown to affect AM formation (Jacobi et al., 2003 a,b). Mycorrhizal colonization of the *Pssym40* roots is significantly increased but only at the early time-points of development. The mutation in the gene *PsSym33* results in decreased mycorrhizal colonization of roots but does not affect arbuscule abundance in the mycorrhizal root fragments. Transmission electron microscopy of arbuscule-containing cells revealed differences in the dynamics of arbuscule development between mutant and wild type plants in fully-developed mycorrhiza at an early flowering stage (Jacobi et al, 2003 b). The majority of cells of the *Pssym40* mutant contained arbuscules in an almost fully-degraded state. In contrast, the large majority of cells in roots of the *Pssym33* contained arbuscules without signs of degradation. In comparison, roots of the wild type contained cells belonging to all defined groups: cells with arbuscules without signs of degradation, cells with degrading and degraded

arbuscules (see figure III.1.). Based on these data, Jacobi et al. (2003 b) concluded that the *Pssym40* mutant accelerated mycorrhiza development and increased rate of arbuscule turn-over, whilst *Pssym33* has a slower rate of AM development than wild type plants.

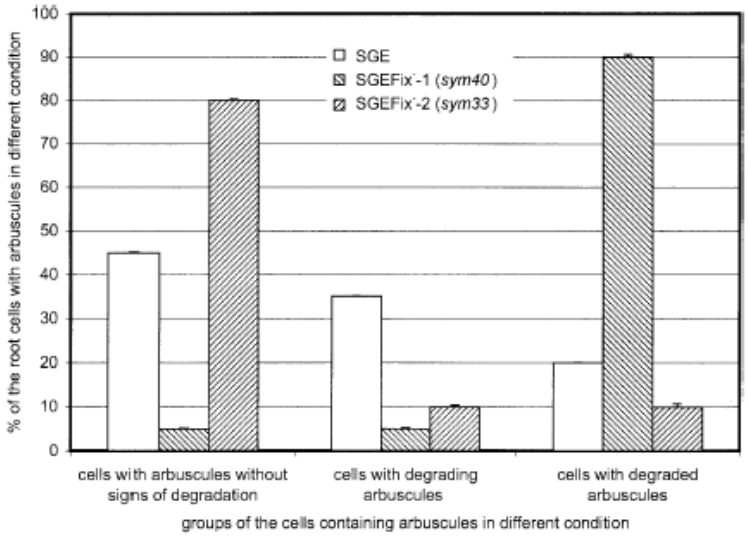


Figure III.1. Proportion of cells containing arbuscules in different developmental stages in roots of wild type, *Pssym40* and *Pssym33* plants (Jacobi et al., 2003 b) inoculated by *G. intraradices*.

Both *Pssym40* and *Pssym33* mutants showed differences in plant development compared with the wild type plants (Jacobi et al., 2003 a). Analysis of rate of biomass accumulation and P accumulation was performed under bioavailable P fertilization, full NPK nutrition, AM inoculation with *G. intraradices* and control conditions without inoculation and without additional mineral nutrition. *Pssym40* mutant had improved root biomass accumulation and P accumulation in roots in the presence of AM fungi. Mutation in *PsSym33* gene resulted in increased biomass accumulation in both shoots and roots and P accumulation in roots for all experimental treatments (addition of bioavailable P fertilization, full NPK nutrition and inoculation with *G. intraradices*). An increased rate of P accumulation in shoots was observed with the addition of biovailable P and NPK. The mutant plants were characterized by a delayed timing of flowering and pod ripening. These observations suggest that mutations in the *PsSym40* and *PsSym33* genes have pleiotropic effects and induce slight systemic changes in whole plant development (Jacobi et al, 2003 a).

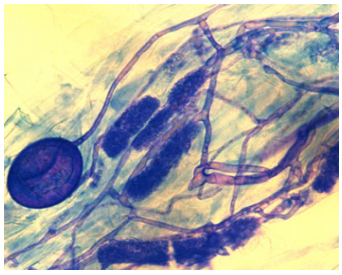
III.2. Results

III.2.1. Mycorrhiza development

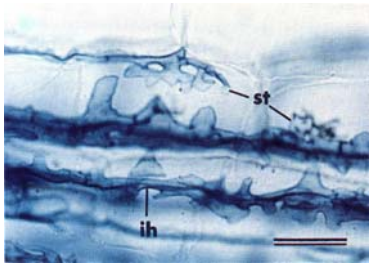
Mycorrhization parameters were estimated by ink staining and alkaline phosphatase-active fungal structures at 21 and 28 days after inoculation (dai) with *G. intraradices* (see Material and Methods II.3.). The high frequency of mycorrhiza development in colonized roots of the wild type, *Pssym33* and *Pssym40* mutant genotypes (parameter F) indicated high inoculum infectivity (table III.1 - 4).

<u>*Pssym36* mutant versus wild type cv Finale</u>

The mycorrhization of *Pssym36* mutant roots was much lower than wild type cv Finale, as indicated by all mycorrhization parameters (tables III.1 and III.2). Only aborted arbuscules with stumpy branches were observed within root cortical cells (see figure III.2).



wild type cv Finale



Pssym36 phenotype

Figure III.2. Light microscopy of mycorrhizal roots of wild type and *Pssym36* mutant plants (Gianinazzi-Pearson et al., 1996)

No vesicles were observed within roots. The proportion of overall alkaline phosphatase-active compared to total (ink-stained) mycelium in roots remained low in either cv Finale (6%) or *Pssym36* (7%) roots at 21 dai but increased to 19% in cv Finale and 51% in *Pssym36* at 28 dai.

Table III.1. Mycorrhization parameters of the wild type (wt) cv Finale line and mutant RisNod24 (*Pssym36*) line at 21 days after inoculation with *G. intraradices*. F – frequency of mycorrhiza in the root system; M - intensity of mycorrhizal colonization in the root system; m – intensity of mycorrhizal colonization in mycorrhizal root fragments; A - arbuscule abundance in the root system; a - arbuscule abundance in mycorrhizal root fragments.

Plant line	F %	M %	m%	a %	A %
Ink staining					
Finale (wt)	78.3 ± 5.5	19.7 ± 3.6	24.7 ± 3.1	19.5 ± 2.3	3.9 ± 0.9
RisNod24 (*Pssym36*)	48.9 ± 3.9	5.6 ± 1.8	11.3 ± 3.7	1.6 ± 1.0	0.2 ± 0.1
Alkaline phosphatase-active fungal structures					
Finale (wt)	51.0 ± 5.9	1.2 ± 0.4	2.2 ± 0.5	9.4 ± 1.6	0.1 ± 0.1
RisNod24 (*Pssym36*)	24.8 ± 2.2	0.4 ± 0.1	1.4 ± 0.2	4.1 ± 1.2	0.01 ± 0.01

Table III.2. Mycorrhization parameters of the wild type (wt) Finale line and mutant RisNod24 (*Pssym36*) line at 28 days after inoculation with *G. intraradices*. Mycorrhiza parameter abbreviations are those defined in table III.1.

Plant line	F %	M %	m %	a %	A %
Ink staining					
Finale (wt)	83.8 ± 5.3	47.6 ± 7.0	55.6 ± 5.4	19.9 ± 3.1	9.8 ± 2.1
RisNod24 (*Pssym36*)	61.2 ± 6.8	11.6 ± 3.0	18.4 ± 3.6	4.6 ± 0.7	0.6 ± 0.2
Alkaline phosphatase-active fungal structures					
Finale (wt)	78.5 ± 3.7	9.1 ± 2.7	11.1 ± 2.8	19.0 ± 3.8	2.0 ± 1.0
RisNod24 (*Pssym36*)	69.8 ± 6.1	6.0 ± 0.9	8.7 ± 1.1	13.8 ± 2.0	0.8 ± 0.1

Pssym33 and *Pssym40* mutants versus the wild type SGE line

Mycorrhizal colonization (M, m) and arbuscule abundance (A, a) of *Pssym33* mutant roots were significantly lower in comparison with wild type roots at 21 dai whereas mycorrhizal levels in roots of *Pssym40* mutant were higher than wild type ones (see table III.3.). At 28 dai,

differences between mutants and wild type became less significant (see table III.4). The rate of root colonization (M, m) of *Pssym33* was still significantly less but arbuscule abundance rose to wild type levels. Colonization of *Pssym40* roots slowed down. No significant differences in mycorrhizal colonization and arbuscule abundance between wild type and *Pssym40* roots were detected at 28 dai.

Table III.3. Mycorrhization parameters of the wild type (wt) SGE line and mutant SGEFix$^-$-2 (*Pssym33*) and SGEFix$^-$-1 (*Pssym40*) lines at 21 days after inoculation with *G. intraradices*. Mycorrhiza parameter abbreviations are those defined in table III.1.

Plant line	F %	M %	m %	a %	A %
Ink staining					
SGE (wt)	94.1 ± 3.9	34.5 ± 4.8	36.2 ± 3.9	30.9 ± 5.7	10.8 ± 2.9
SGEFix$^-$-2 (*Pssym33*)	99.2 ± 0.8	24.1 ± 1.1	24.3 ± 1.1	21.3 ± 2.2	5.1 ± 0.5
SGEFix$^-$-1 (*Pssym40*)	100 ± 0	44.2 ± 9.9	42.7 ± 16.9	33.1 ± 8.1	16.2 ± 6.5
Alkaline phosphatase-active fungal structures					
SGE (wt)	96.5 ± 0.1	16.8 ± 2.1	17.4 ± 2.2	30.6 ± 5.0	5.3 ± 1.3
SGEFix$^-$-2 (*Pssym33*)	94.9 ± 3.2	10.9 ± 1.6	11.4 ± 1.4	27.5 ± 4.3	3.1 ± 0.7
SGEFix$^-$-1 (*Pssym40*)	93.3 ± 2.7	24.5 ± 5.7	26.1 ± 5.9	30.8 ± 3.9	8.0 ± 2.9

Table III.4. Mycorrhization parameters of the wild type (wt) SGE line and mutant SGEFix⁻-2 (*Pssym33*) and SGEFix⁻-1 (*Pssym40*) lines at 28 days after inoculation with *G. intraradices*. Mycorrhiza parameter abbreviations are those defined in table III.1.

Plant line	F %	M %	m %	a %	A %
Ink staining					
SGE (wt)	99.1 ± 0.9	36.3 ± 5.6	36.5 ± 5.5	21.5 ± 3.6	9.1 ± 3.9
SGEFix⁻-2 (*Pssym33*)	99.1 ± 0.9	27.7 ± 3.0	27.9 ± 3.0	24.8 ± 1.9	7.0 ± 1.1
SGEFix⁻-1 (*Pssym40*)	99.2 ± 0.8	29.3 ± 1.9	29.6 ± 2.1	25.1 ± 3.5	7.5 ± 1.4
Alkaline phosphatase-active fungal structures					
SGE (wt)	93. ± 3.71	15.0 ± 3.2	15.9 ± 3.0	22.5 ± 4.8	3.8 ± 1.3
SGEFix⁻-2 (*Pssym33*)	94.1 ± 2.8	11.6 ± 1.5	11.8 ± 1.6	23.2 ± 5.1	2.7 ± 0.8
SGEFix⁻-1 (*Pssym40*)	95.7 ± 2.3	29.5 ± 4.2	30.5 ± 3.9	24.5 ± 3.7	7.3 ± 1.6

The AM fungus developed rapidly in the root systems of the SGE line and corresponding mutants *Pssym33* and *Pssym40*, which all showed consistently higher values for overall root colonization (M%) and arbuscule abundance (A%) at 21 dai than in either cv Finale or *Pssym36*. The proportion of overall alkaline phosphatase-active compared to total (ink-stained) mycelium in roots at 21 dai was also considerably higher in parent and mutant SGE lines (45-55% of ink-stained structures) than in either cv Finale or *Pssym36* roots. The proportion of alkaline phosphatase-active/total (ink-stained) mycelium remained fairly constant up to 28 dai for SGE and *Pssym33*, and increased in *Pssym40*.

III.2.2. Plant growth

Pssym36 mutant versus the wild type cv Finale line

Mutation in the *Pssym36* gene did not affect the shoot biomass accumulation but root biomass of non-inoculated roots of *Pssym36* mutant was marginally decreased as compared with wild type cv Finale plants under the same conditions (see figures III.3, III.4). *Pssym36* root colonization by *G. intraradices* slightly increased the shoot and root biomass accumulation at 21

dai but at 28 dai both parameters were significantly lower in inoculated roots than in non-mycorrhizal ones. Wild type cv Finale shoot and root biomass accumulation was not modified by root colonization by *G. intraradices* at 21 dai but at 28 dai, similar to *Pssym36* mutant, shoot and root biomass of wild type plants was significantly decreased in comparison to non-mycorrhizal plants (see figures III.3, III.4). At 28 dai, the root/shoot biomass ratio for wild type cv Finale was equal to 2.02 in mycorrhizal plants and 2.12 in non-mycorrhizal ones. For the *Pssym36* mutant it was equal to 1.29 and 1.55 in *G. intraradices*-inoculated and non-inoculated plants, respectively.

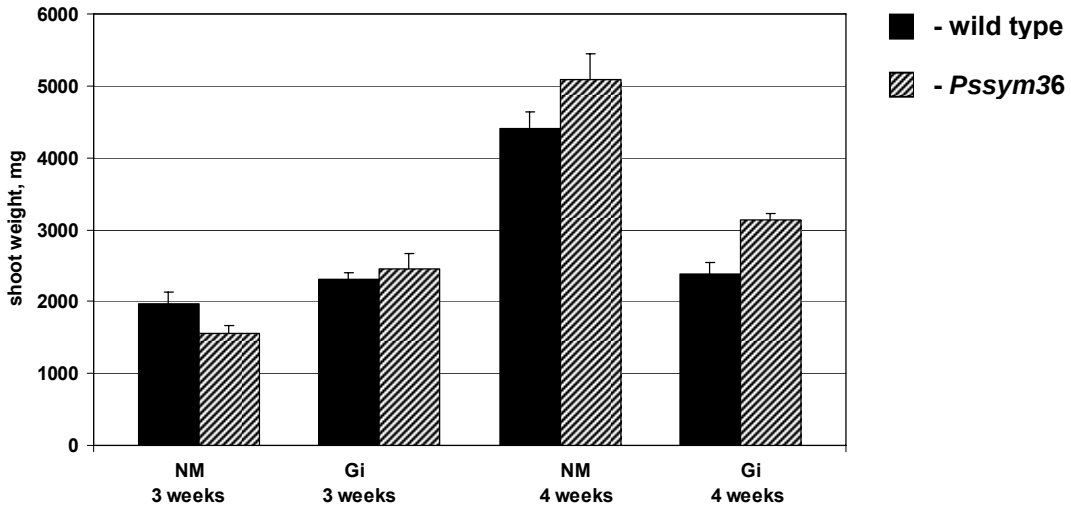


Figure III.3. Shoot biomass accumulation of wild type (wt) cv Finale and the RisNod24 (*Pssym36*) mutant lines under non-inoculated (NM) conditions and in the presence of *G. intraradices* (Gi) at 21 and 28 days after inoculation.

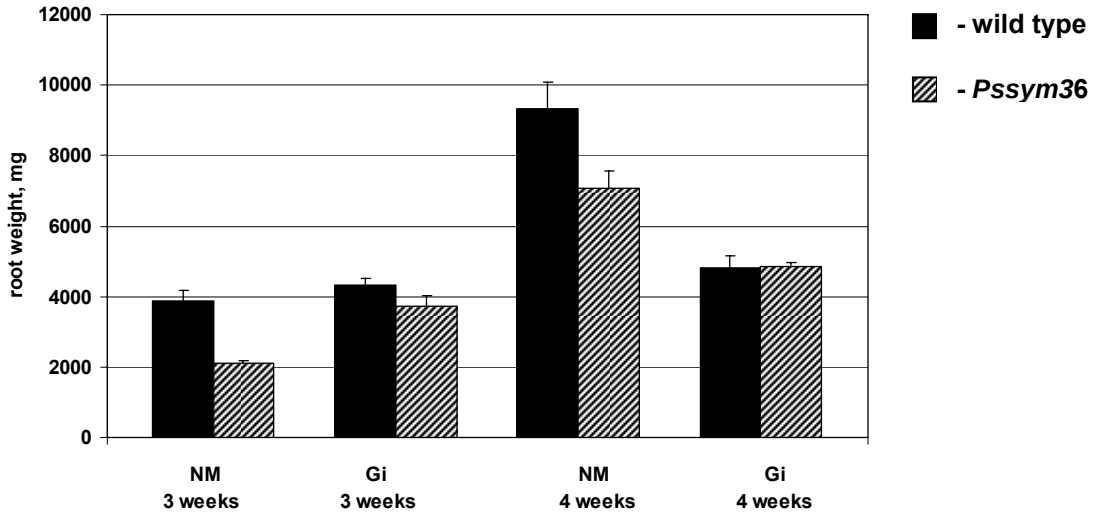


Figure III.4. Root biomass accumulation of wild type (wt) cv Finale and RisNod24 (*Pssym36*) mutant lines under non-inoculated (NM) conditions and in the presence of *G. intraradices* (Gi) at 21 and 28 days after inoculation.

<u>*Pssym33* and *Pssym40* mutants versus the wild type SGE line</u>

Both the *Pssym33* and *Pssym40* mutants had significantly higher root biomass than the wild type plants at 28 dai for all treatments but only *Pssym33* presented higher root biomass than wild type SGE at 21 dai (see figure III.6.). Inoculation with *G. intraradices* did not affect shoot and root biomass accumulation of the pea genotypes SGE (wt), SGEFix$^-$-2 (*Pssym33*) and SGEFix$^-$-2 (*Pssym40*) but some differences were detected between mutants and wild type (see figures III.5, III.6). Shoot biomass of the *Pssym40* mutant was significantly reduced in comparison with wild type under non-mycorrhizal control conditions and at 28 dai with the AM fungus (see figure III.5.). There were no significant differences in shoot biomass accumulation between the wild type and the *Pssym33* mutant for all treatments.

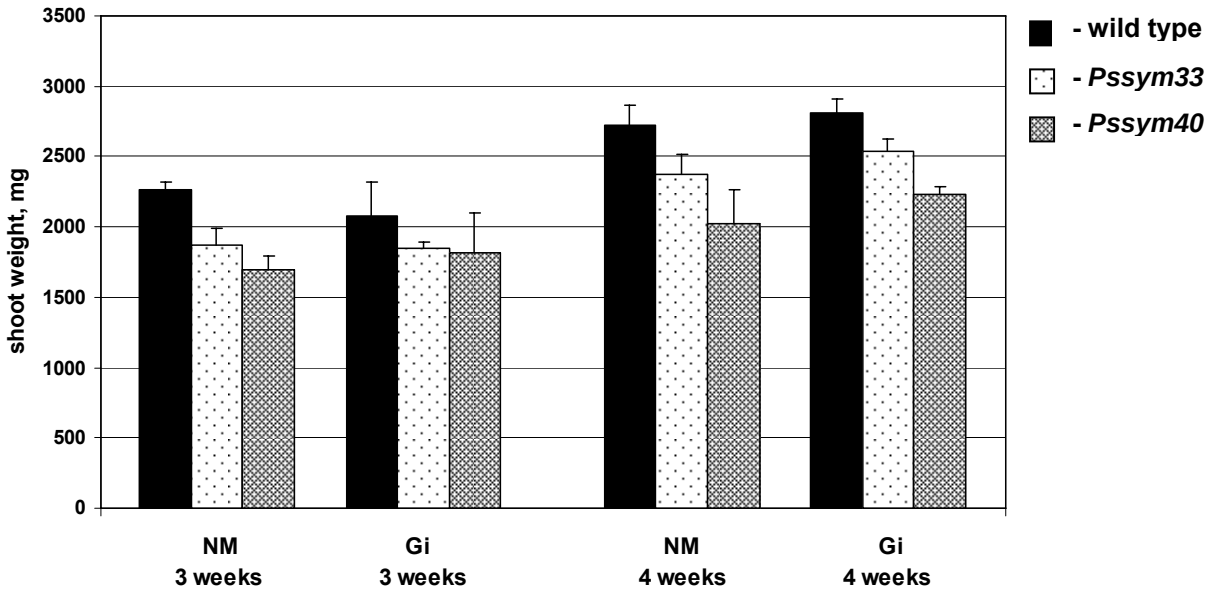


Figure III.5. Shoot biomass accumulation of the wild type (wt) SGE line and SGEFix⁻-2 (*Pssym33*) and SGEFix⁻-2 (*Pssym40*) mutant lines under non-mycorrhizal (NM) conditions and in the presence of *G. intraradices* (Gi) at 21 and 28 days after inoculation.

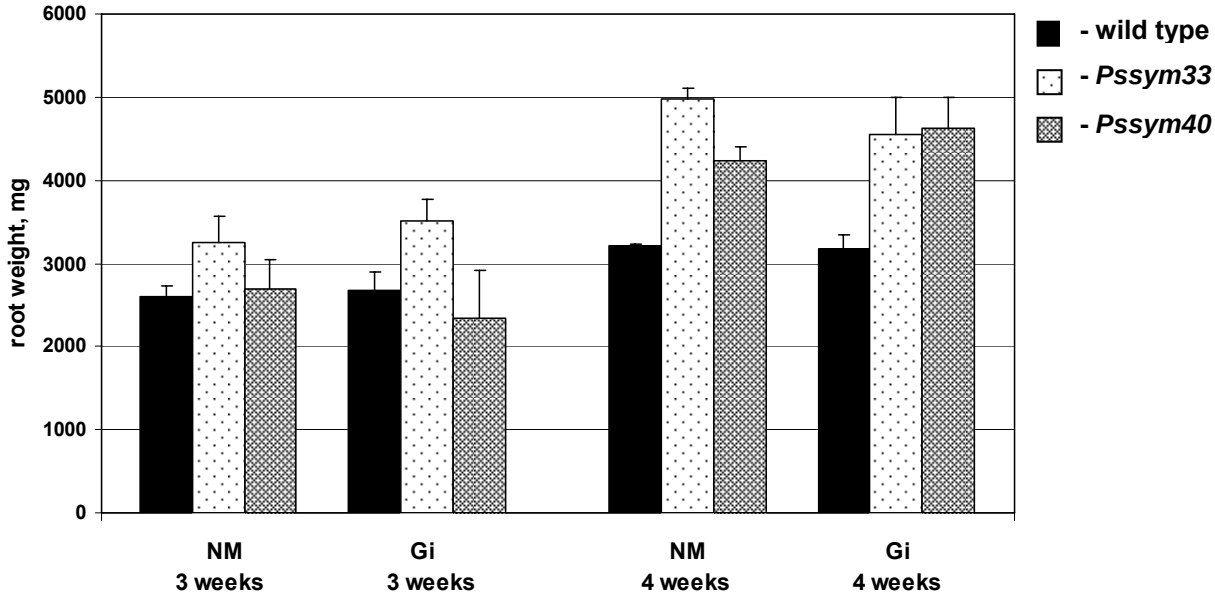


Figure III.6. Rate of root biomass accumulation of the wild type (wt) SGE line and SGEFix⁻-2 (*Pssym33*) and SGEFix⁻-2 (*Pssym40*) mutant lines under non-mycorrhizal (NM) conditions and in the presence of *G. intraradices* (Gi) at 21 and 28 day after inoculation.

III.3. Conclusions

Estimation of AM development confirmed previous observations (Gianinazzi-Pearson et al., 1992; Jacobi et al., 2003 a, b) that the *Pssym36*, *Pssym33* and *Pssym40* mutants have modified AM phenotypes as compared to the wild type parent genotypes:

- *Pssym36* is blocked at the arbuscule developmental stage. Mutation in the *PsSym36* gene also leads to a reduction in mycorrhizal colonization of roots.
- Mutation in the *PsSym33* gene decreases the rate of mycorrhizal colonization but does not influence development of an effective AM symbiosis.
- Mutation in the *Pssym40* results in an early increased rate of AM development.

Mutations in both of the *PsSym33* and *PsSym40* genes influence plant development (shoot and root biomass accumulation), suggesting a pleiotropic effect of these mutations. Mutation in the *PsSym36* gene affected plant development in the absence of an AM fungus and *G. intraradices* colonization of the *Pssym36* roots had a negative influence on both wild type and mutant plant growth at 28 dai.

An increased rate of AM development and a higher proportion of alkaline phosphatase-active mycelium in roots of the SGE line and the corresponding mutants *Pssym33* and *Pssym40*, as compared to the cv Finale genotype, suggests an influence of the genetic background of these genotypes on the ability of pea to form a successful AM symbiosis.

CHAPTER IV

MODIFICATION IN *GLOMUS INTRARADICES* GENE EXPRESSION PATTERNS IN INTERACTIONS WITH ROOTS OF MUTANT PEA PLANTS

IV. Modification in *Glomus intraradices* gene expression patterns in interactions with roots of mutant pea plants

IV.1. Introduction

Identification of AM symbiosis-related plant and fungal genes has progressed rapidly over the last decade due to the realization of sequencing projects and transcriptome-wide studies (Journet et al., 2002; Liu et al., 2003, 2007; Wulf et al., 2003; Brechenmacher et al., 2004; Gianinazzi-Pearson et al., 2004; Küster et al., 2004, 2007; Sanchez et al., 2005; Weidmann et al., 2004; Frenzel et al., 2005; Deguchi et al., 2007; Massoumou et al., 2007; Seddas et al., 2009). However, despite the extensive list of plant and fungal genes identified, information about molecular events related to defined steps of AM symbiosis development is still poor. In this context, previous research of AM fungal behavior has been limited to the use of plant mutants affected in the early steps of appressoria formation and root penetration (Seddas et al., 2009). For this reason, we chose to use the pea mutants *Pssym36*, *Pssym33* and *Pssym40* to study how corresponding *SYM* genes affect fungal gene expression in the late stage of AM formation or functioning. *G. intraradices* genes were selected from a recent investigation showing their expression during early and late stages of AM symbiosis development in *M. truncatula* (Seddas et al., 2009). These genes are predicted to have functions in transcription, protein synthesis, primary/secondary metabolism or have unknown function. They encode for a plasma membrane H^+-ATPase (*H^+-ATPase*), an alkaline phosphatase (*ALP*), a putative Rho/GDP dissociation inhibitor (*RHO*), a peptidylprolyl isomerase (*PEPISOM*), a 26S proteasome subunit 2 (*26S*), a 26S proteasome regulatory subunit (*26SREG*), a stearoyl-CoA desaturase (*DESAT*), a thioredoxin peroxidase (*THIO*), a superoxide dismutase (*SOD*) and a hypothetical vacuolar import protein (*VACU*). Expression of these fungal genes was compared at 21 and 28 dai in *G. intraradices*-colonized roots of *Pssym36*, *Pssym33* and *Pssym40* mutants and the corresponding wild type pea genotypes cv. Finale and SGE, by real-time RT-PCR. Expression was quantified as transcript levels and normalized against the expression of the house-keeping *TEF* gene of *G. intraradices*.

IV.2. Results

To amplify the *G. intraradices* genes *RHO*, *PEPISOM*, *26S*, *26SREG*, *DESAT*, *THIO*, *SOD* and *VACU*, the primers previously designed by Seddas et al. (2009) were used. Nucleotide sequences of amplified products were identical to those present in the MENS database. For amplification of *H^+ATPase* and *ALP* transcripts, primers were newly designed; the sequences of the amplified products showed high similarity with those of *GiHA5* (AF420481, 1e-48) and *GiALP* (AB114298.1, 6e-70) genes, respectively.

IV.2.1. Interactions with the *Pssym36* mutant

Mutation in the *PsSym36* gene affected expression of all tested *G. intaradices* genes. At 21 dai, *RHO*, *PEPISOM*, *DESAT*, *THIO*, *SOD* and *VACU* genes were significantly down-regulated in colonized roots of the *Pssym36* mutant in comparison with wild type cv Finale roots (see figure IV.1). Differences in fungal gene expression during colonization of cv Finale and *Pssym36* roots were greatest for the *PEPISOM* (4.7 fold), *DESAT* (39 fold), *THIO* (5.6 fold) and *SOD* (8.3 fold). *26SREG* tended to be also down-regulated in *Pssym36* roots (P=0.1) and *26S* and *ALP* tended to be up-regulated (P=0.1) as compared to cv Finale roots. No difference was observed in expression of the fungal *H^+ATPase*, *26S*, *26SREG* and *ALP* genes. The fungal genes generally had lower expression levels in roots of both pea genotypes at 28 dai than at 21 dai, except for the *DESAT* gene. The *DESAT* gene continued to be significantly down-regulated in roots of the *Pssym36* mutant at 28 dai. Transcripts of the *26S* gene were not detected at 28 dai whilst transcript accumulation of fungal *H^+ATPase* and *26SREG* was significantly decreased in *Pssym36* roots by 3.2 and 10 fold, respectively. ALP was the only gene of *G. intraradices* to show significantly enhanced expression (4.5 fold) in mutant as compared to wild type colonized roots at 28 dai. None of the other fungal genes showed significant differences in expression levels between mutant and wild type-colonized roots at 28 dai.

IV.2.2 Interactions with the *Pssym33* mutant

The *PsSym33* gene mutation had less pronounced effects on *G. intraradices* gene expression than mutations in the *PsSym36* and *PsSym40* genes (see figure IV.2). At 21 dai, only the *ALP* and *26S* genes were significantly up-regulated in colonized roots of the *Pssym33* mutant as compared with mycorrhizal wild type SGE plants. No other significant modifications in *G. intraradices* gene expression were detected between mutant and wild type at both, 21 and 28 dai, time points. Transcript levels of *RHO*, *PEPISOM*, *26SREG*, *THIO*, *SOD* and VACU fungal genes increased from 21 to 28 dai in mycorrhizal roots of the *Pssym33* mutant.

IV.2.3 Interactions with the *Pssym40* mutant

At 21 dai, expression of the *RHO*, *26S*, *26SREG* and *DESAT* genes was significantly up-regulated in mycorrhizal roots of the *Pssym40* mutant as compared with wild type-colonized roots (see figure IV.3). No other *G. intraradices* gene expression was significantly modified by mutation in the *PsSym40* gene at this time point. At 28 dai, transcript accumulation of H^+*ATPase*, *26SREG* and *THIO* genes was significantly higher in mycorrhizal *Pssym40* mutant roots than in mycorrhizal wild type SGE. Expression of the other fungal genes did not significantly differ in mycorrhizal *Pssym40* and wild type roots. As for *Pssym33,* expression of *RHO*, *PEPISOM*, *26SREG*, *THIO*, *SOD* and *VACU* fungal genes was increased from 21 to 28 dai in colonized roots of wild type and/or mutant plants.

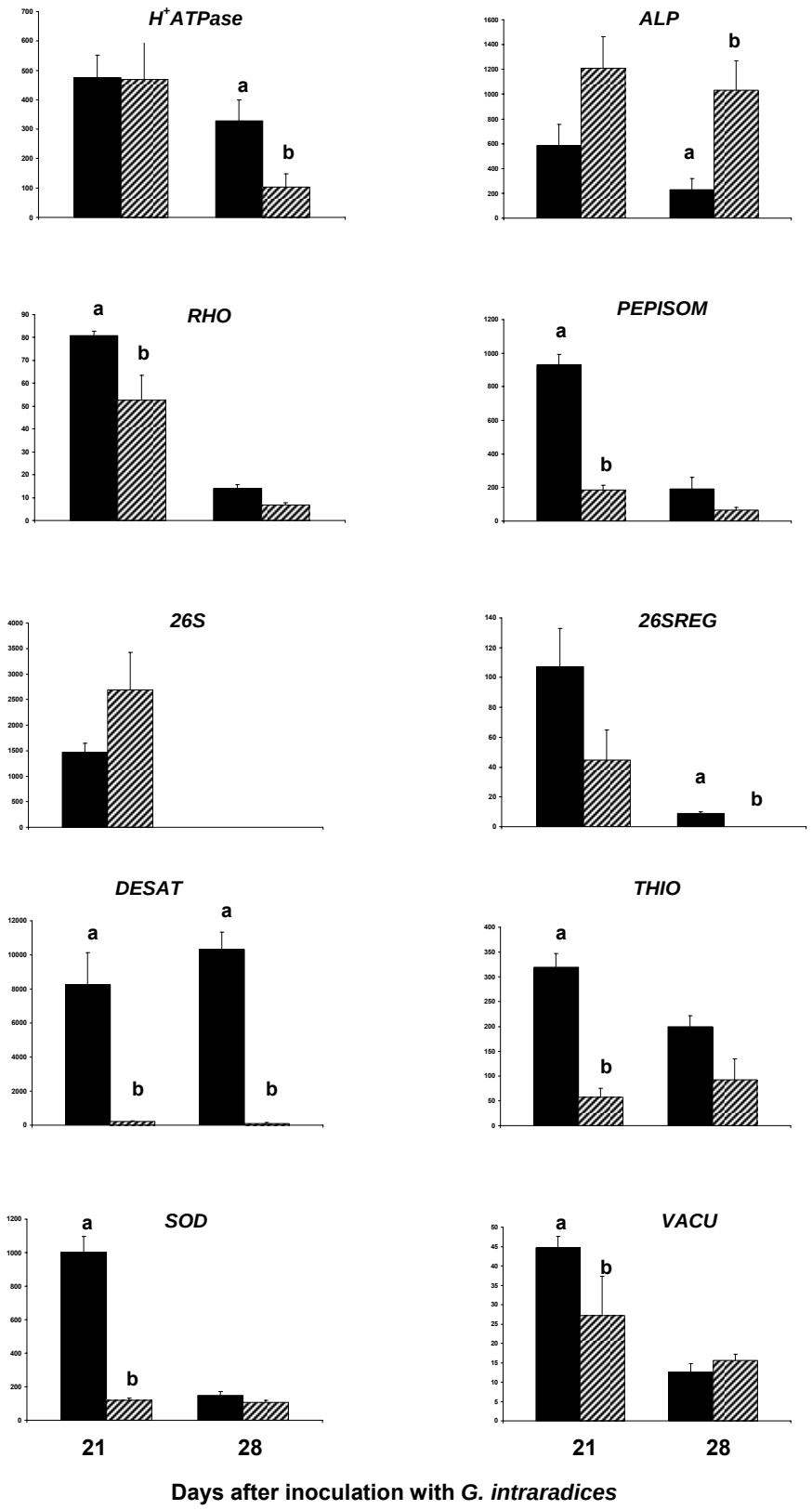


Figure IV.1. *G. intraradices* gene expression in colonized roots of wild type cv Finale (■) and mutant *Pssym36* (▨). Transcript abundance of each gene, measured by real-time RT-PCR, is expressed relative to that of the house-keeping fungal *TEF* gene. Letters indicate significant differences between treatments (P≤0.05, n=3).

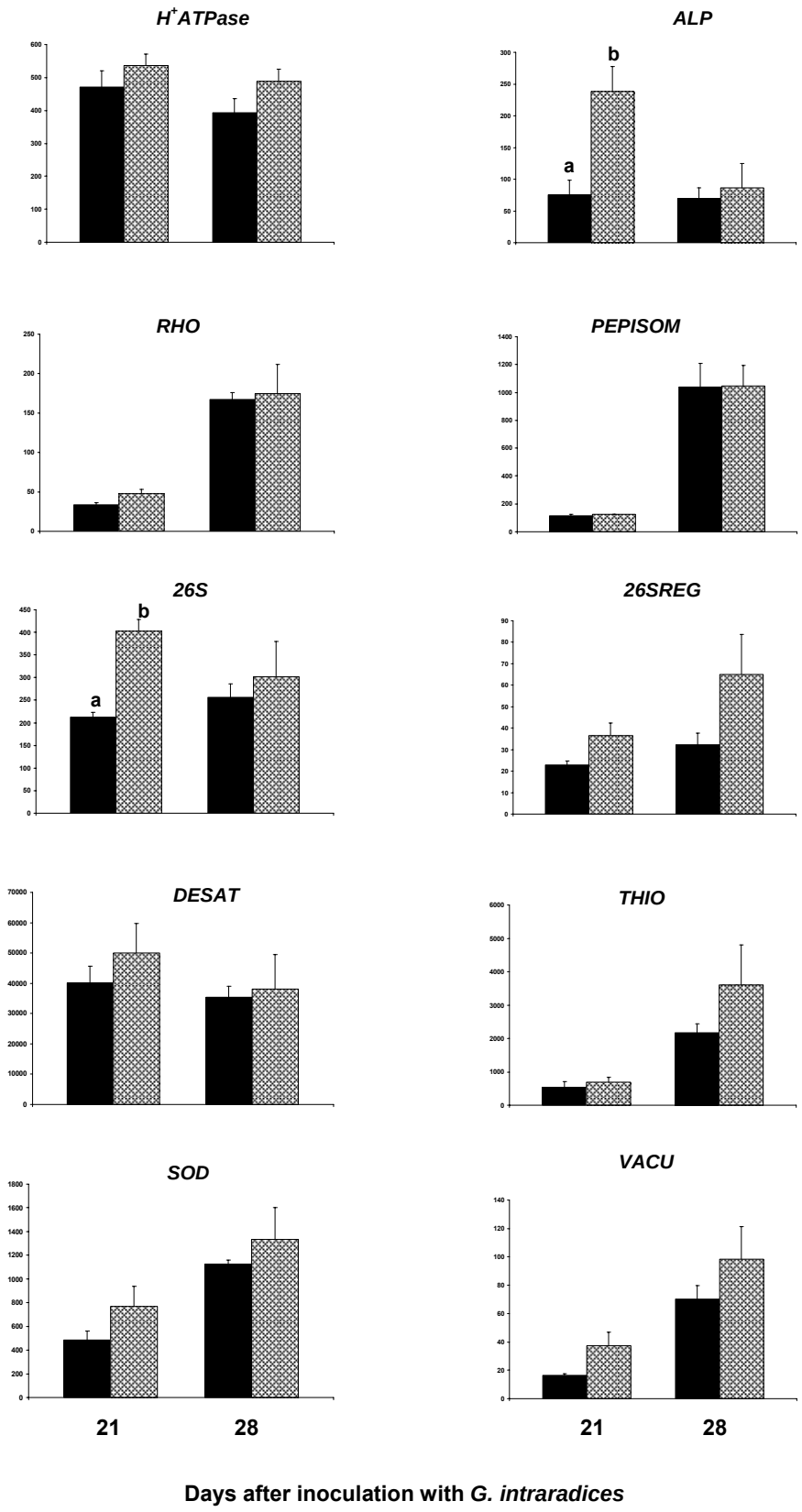


Figure IV.2. *G. intraradices* gene expression in colonized roots of wild type SGE (■) and mutant *Pssym33* (▩). Transcript abundance of each gene, measured by real-time RT-PCR, is expressed relative to that of the house-keeping fungal *TEF* gene. Letters indicate significant differences between treatments (P≤0.05, n=3).

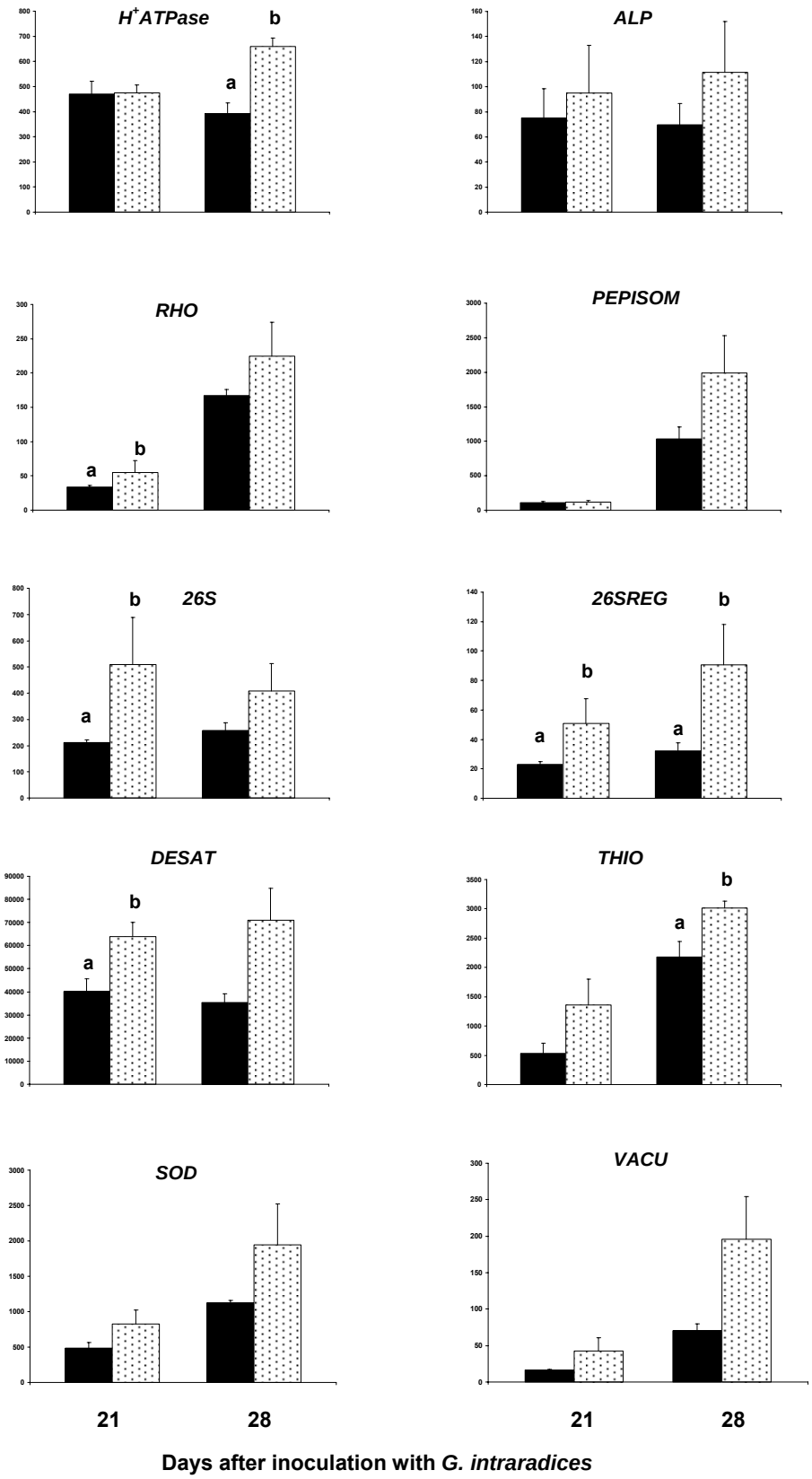


Figure IV.3. *G. intraradices* gene expression in colonized roots of wild type SGE (■) and mutant *Pssym40* (▒). Transcript abundance of each gene, measured by real-time RT-PCR, is expressed relative to that of the house-keeping fungal *TEF* gene. Letters indicate significant differences between treatments (P≤0.05, n=3).

IV.3. Discussion and conclusions

In this study, we used the late stage symbiotic mutants *Pssym36*, *Pssym33* and *Pssym40* of pea to gain insight into AM fungal molecular responses linked to AM formation and functioning. Modifications in transcript accumulation of *G. intraradices* genes resulting from mutations in the *Pssym36*, *Pssym33* and *Pssym40* genes gave an opportunity to better define mutant phenotypes and the role of the selected fungal genes in the symbiosis. The mutation in the *PsSym36* gene not only led to a lack of arbuscule as was shown before (Gianinazzi-Pearson et al., 1991) but also to a considerably lower expression of most of the monitored *G. intraradices* genes, suggesting that sub-optimal conditions are created in the *Pssym36* mutant for fungal development and activity inside the root. Lower levels of expression were detected for genes involved in fungal membrane transport, transcription, protein synthesis, primary/secondary metabolism.

The decreased expression of the fungal membrane *H^+-ATPase* gene when arbuscule formation is defective in *Pssym36* roots correlates with the lack of enzyme activity on the fungal plasma membrane bordering aborted arbuscules branches, shown before in the same pea genotype (Gianinazzi-Pearson et al., 1995). H^+-ATPase enzymes are known to generate the electrochemical gradient of protons that drives active secondary transport system across membrane. It can therefore be hypothesized that in the AM symbiosis the fungal H^+-ATPase plays an important role in the active transport of nutrients across the plasma membrane, and that absence of the enzyme activity will limit fungal uptake of photosynthetic carbon at the plant-fungal interface in *Pssym36* roots. This could explain the strong down-regulation of the *DESAT* gene of *G. intraradices* which reflects modifications in fungal lipid metabolism, particularly reduction in biosynthesis of fatty acids due to carbon limitation. Down-regulation of this gene was also recently shown when *G. intraradices* develops on the roots of the *M. truncatula* mutants *dmi1*, *dmi2/Mtsym2* and *dmi3/Mtsym13* which block AM symbiosis development at early stages after appressoria formation (Seddas et al., 2009). In contrast, expression of fungal *H^+-ATPase* and *DESAT* genes in mycorrhizal roots of the *Pssym33* and *Pssym40* mutants was maintained at a similar level or increased as compared mycorrhizal wild type roots. Increased

expression of these *G. intraradices* genes in *Pssym40* mutant may be connected with greater carbon acquisition by the fungus linked to the more rapid root colonization, arbuscule formation and turn-over typical for this mutant (Jacobi et al. 2003 b). Transcript analysis of microdissected arbuscule-containing cells from *Pssym40* mutant performed in this study (see chapter VI) confirmed arbuscule-related expression of the *DESAT* gene.

The weaker expression of the fungal *RHO* gene, encoding a putative Rho/GDP dissociation inhibitor, in *G. intraradices*-colonized *Pssym36* roots could indicate effects on cell signaling events at the fungal-plant interface, as was suggested for mutations in the *DMI2* and *DMI3/MtSym13* genes of *M. truncatula* which similarly repressed *RHO* expression when appressoria are formed (Seddas et al. 2009). Such inhibitors regulate GTP-GDP conversion on Rho/GTPase proteins which are essential to basic cell functions including metabolism, proliferation and differentiation (Groysman et al. 2002). Lower transcript levels in colonized *Pssym36* roots of the *PEPISOM* and *26SREG* genes may contribute to, or result from, defective arbuscule development in the pea mutant. *PEPISOM* plays a role in cellular regulation as a chaperon protein that activates or inhibits regulatory proteins by catalyzing proline isomerization (Miele et al. 2003). The *26SREG*-regulated proteasome is a proteasome complex that is responsible for selective degradation of abnormal proteins and naturally short-lived proteins related to metabolic regulation and cell cycle progression (Murray 1995). Inactivation of the *Pssym33* gene was not detrimental to the expression of these three genes whilst mutation of *Pssym40* significantly up-regulated the *RHO* and *26SREG* genes, which may be explained by quicker arbuscule turn-over in roots of this mutant (Jacobi et al., 2003 b).

Expression of the *ALP* gene, considered to have a role in phosphate transfer within AM fungi to the host plant, unexpectedly increased in the *G. intraradices*-colonized roots of *Pssym36* and *Pssym33*, as compared to mycorrhizal roots of the corresponding wild type pea genotypes. This correlated with an increased proportion of *ALP*-active mycelium in the two mutants although intraradical development of *G. intraradices* was lowest in their root systems. Mutation in the *Pssym40* gene did not affect *ALP* transcript accumulation. Because purification of the *ALP* enzyme from AM fungi has so far been unsuccessful, little is known about its enzymatic characteristics or its function in the AM symbiosis (Kojima et al., 2001). Microorganisms produce

alkaline phosphatases in response to various environmental stresses (Lim et al., 1996) and the up-regulation of *G. intraradices ALP* expression in *Pssym36* and *Pssym33* roots may be indicative of the perception of a stress by the fungus in these genotypes.

Transcript accumulation of the fungal genes of anti-oxidative stress metabolism, *THIO* and *SOD*, in the mycorrhizal roots of wild type, *Pssym33* and *Pssym40* pea genotypes is typical for AM symbiosis (Balestrini and Lanfranco, 2006). Such gene activation may enable the AM fungus to contend with plant defense/stress responses which are usually activated in mycorrhizal roots, especially within arbuscule-containing cells (Gianinazzi-Pearson et al., 1996). Activation of these two genes has been reported previously for mycorrhiza of other plant/fungal combinations (Brechenmacher et al. 2004; Seddas et al. 2009). In this context, expression of *THIO* and *SOD* genes was significantly decreased in *G. intraradices*-colonized roots of the *Pssym36* mutant in the absence of full arbuscule development.

In conclusions, mutation of the *PsSym36* gene had a stronger effect on *G. intraradces* gene expression, as compared with the *Pssym40* and *Pssym33* mutants. Expression of most of the tested fungal genes was considerably repressed in *G. intraradices*-colonized roots of the *Pssym36* mutant as compared with those of mycorrhizal wild type cv Finale. This could be the result of incomplete arbuscule development and the impossibility for nutrient exchanges between the fungal and plant cells in the absence of a functional symbiotic interface. Mutation in the *PsSym40* gene resulted in an increase in the expression of most of the investigated *G. intraradices* genes, which could be associated with the more rapid AM symbiosis development in roots of the *Pssym40* mutant. Of the three tested pea mutants, *Pssym33* had less pronounced effects on *G. intraradices* gene expression as well as on AM formation and function (see chapter III).

CHAPTER V

DIFFERENTIAL EXPRESSION OF SYMBIOSIS-RELATED *PISUM SATIVUM* L. GENES IN ROOTS OF *PSSYM36*, *PSSYM33* AND *PSSYM40* MUTANTS

Chapter V. Differential expression of symbiosis-related *Pisum sativum* L. genes in roots of *Pssym36*, *Pssym33* and *Pssym40* mutants

V.1. Introduction

Successful AM symbiosis development requires tight coordination between the symbiotic partners at the molecular level. Any alteration in one symbiotic partner will lead to modifications in the other. Most of studies of the effect of mutations in *SYM* genes on plant gene responses to AM fungi have focused on early steps of arbuscular mycorrhiza formation (Kistner et al., 2005; Sanchez et al., 2005; Seddas et al., 2009). Only Lapopin et al. (1999) and Grunwald et al. (2004) have reported plant gene expression in a mutant affected in the late stages of mycorrhiza development (*Pssym36*). In order to further investigate a role of *SYM* genes in late stages of the AM symbiosis, we studied the effect of mutations in the pea *PsSym36*, *PsSym33* and *PsSym40* genes on symbiosis-associated plant molecular responses. Eight protein-encoding plant genes were selected amongst those reported in the literature to be up-regulated during AM interactions or in arbuscule-containing cells of *P. sativum* or *M. truncatula* (Krajinski et al., 2002; Wulf et al., 2003; Liu et al., 2003; Brechenmacher et al., 2004; Grunwald et al., 2004; Massoumou et al., 2007). The chosen plant genes are putatively involved in processes of signaling and gene regulation (MAP kinase (*MAPK*)), membrane transport (plasma membrane H^+-ATPase (*H^+-ATPase*), inorganic phosphate transport (*PT4*), blue copper protein (*BCOP*), protein synthesis and processing (serine protease (*SERPROT*)), defense and cell rescue (pathogenesis-related protein pI230 (*DRP*), trypsin inhibitor (*TI*), glutathione-S-transferase (*GST*)). Expression of pea genes was analyzed at 21 and 28 dai with the *G. intraradices* in mycorrhizal and non-inoculated roots of *Pssym36*, *Pssym33* and *Pssym40* mutants and their corresponding wild type genotypes cv. Finale and SGE by real-time RT-PCR. Expression was quantified as transcript levels related to those of the pea house-keeping *GAPDH* gene.

V.2 Results

Nucleotide sequences were known for two of the eight analyzed pea genes (kunitz-like trypsin inhibitor (*TI*) and disease resistance response protein pI230 (*Drp*)) and primers published by Grunwald et al. (2004) were used to amplify them. For the other genes, primer design was performed based on conserved regions of amino acid and nucleotide sequences of homologous genes of different plant species. Obtained pea nucleotide sequences showed high homology to *Vicia faba* H^+-ATPase *vha4* (AJ310523.1, 1e-79), *M. truncatula* phosphate transporter *MtPt4* (QY116211, 9e-35), *P. sativum* blue copper protein (Z25471, 1e-37), *M. truncatula* glutathione-S-transferase (AY134608, 2e-15), *P. sativum* MAP kinase homologue (X70703, 8e-28) and a putative *M. truncatula* serine protease (AL382601, 7e-40). The new pea sequences have been deposited in EMBL database under the accession numbers FN554871-76.

V.2.1. *Pssym36* mutant

At 21 dai, significant enhancement was observed in the expression of *TI* and *GST* pea genes in wild type mycorrhizal roots compared to non-mycorrhizal ones; the same tendency persisted in roots of the *Pssym36* mutant but only the expression of *GST*-encoding gene was increased significantly. Expression of the *DRP*, *MAPK* and *SERPROT* genes was, on the contrary, down-regulated in colonized roots of wild type plants (see table V.1, figure V.1.). No significant differences were detected in expression levels of these genes in mycorrhizal and non-mycorrhizal roots of the *Pssym36* mutant. Neither *G. intraradices* root colonization nor mutation in the *PsSym36* gene significantly affected the expression of *H^+-ATPase*, *PT4* and *BCOP*-encoding genes at the 3 weeks time point, although significant mycorrhiza-mutant interactions were observed (see table V.1).

At 28 dai, expression of genes encoding *PT4*, *DRP* and *GST* was significantly up-regulated in AM-colonized wild type roots. No significant responses to *G. intraradices* root colonization were observed in roots of the *Pssym36* mutant. Mycorrhiza development strongly repressed expression of the *BCOP* gene in both wild type and mutant plants but differences were only

marginally significant for the mutant genotype (P=0.1). Expression of the pea genes *H^+-ATPase*, *TI*, and *SERPROT*, was not significantly modified by *G. intraradices* development at 28 dai in wild type or in *Pssym36* mutant roots, whilst *MAPK* gene expression followed the same reduced trend (P=0.1) in cv Finale mycorrhizal roots. No significant mycorrhiza-mutant interactions were observed at this time point (see table V.1).

V.2.2 *Pssym33* mutant

Expression of *PT4*, *TI*, *GST* and *SERPROT* genes was significantly up-regulated in response to *G. intraradices* colonization of wild type and *Pssym33* mutant roots at 21 dai (see table V.1, figure V.2). Transcript abundance of the *MAPK* gene was significantly lower in mycorrhizal roots of wild type SGE plants than in non-mycorrhizal ones, but mutation of the *PsSym33* gene reduced this effect of *G. intraradices* colonization on *MAPK* gene expression so that no significant differences were detected between mycorrhizal and non-mycorrhizal mutant roots. No significant differences in *H^+-ATPase* gene expression were detected in all roots whatever the treatment at 21 and 28 dai, although it tended to be lower in mycorrhizal roots at 28 dai.

At 28 dai, only expression of the *GST* gene was significantly up-regulated in mycorrhizal roots of wild type in comparison with non-mycorrhizal roots. Increases in *PT4* and *TI* gene expression also persisted in mycorrhizal SGE roots (P=0.1, P=0.08). Expression of all other genes was not affected by *G.intraradices* colonization either of wild type or *Pssym33* mutant roots. Expression of the *DRP* gene was not detected in any roots of either genotypes 21 and 28 dai. Significant mycorrhiza-mutant interactions were observed for *GST* gene at both time points (see table V.1).

V.2.3 *Pssym40* mutant

It was observed that, at 21 dai, *G. intraradices* colonization results in significant up-regulation of the expression of the genes *PT4*, *TI*, *GST* and *SERPROT* in wild type and *Pssym40* mutant plants and down-regulation of the *MAPK* gene in mycorrhizal roots of the SGE line (see table V.1, figure V.3). Mutation in the *PsSym40* gene did not affect transcript accumulation of these

genes in mutant compared to wild type roots. Transcript accumulation of the *DRP* gene was only detected at 28 dai when expression was reduced in response to AM colonization although differences were only significant for the wild type SGE line. No significant modifications in expression of *H^+-ATPase* or *BCOP* genes were observed in mycorrhizal wild type and *PsSym40* mutant roots at 21 dai. At 28 dai, significant increases in gene expression in response to AM formation were detected for *PT4* and *GST* genes only in mutant roots, although marginally significant (P=0.06, P=0.1) differences in the wild type genotype were observed. Expression of the *H^+-ATPase, BCOP*, *TI*, *MAPK* and *SERPROT* genes was not significantly modified by *G. intraradices* colonization of roots in either wild type SGE or *Pssym40* mutant roots 28 dai, although *MAPK* expression tended to remain higher in mycorrhizal wild type plants.

Table V.1. Two-way ANOVA for influence of the factors mycorrhiza (Myc), pea genotype (mutant) and an interaction between both factors (Myc^- mutant) on plant gene expression 21 and 28 dai by *G. intraradices* in roots of cv Finale vs *Pssym36*, line SGE vs *Pssym33* and line SGE vs *Pssym40*. Values in bold indicate significant P-values (P<0.05)

Plant gene:	*H+ATPase*	*PT4*	*BCOP*	*Drp*	*TI*	*GST*	*MAPK*	*SERPROT*
Finale/*sym36*								
21 dai								
Myc	0.678	0.098	0.815	0.092	**0.001**	**<0.001**	**0.001**	**0.049**
Mutant	0.348	0.077	0.114	0.770	**0.002**	0.502	0.063	0.849
Myc*mutant	**0.027**	0.317	**0.036**	0.206	**0.006**	0.175	**0.014**	0.120
28 dai								
Myc	0.594	**0.050**	**0.003**	**0.027**	0.348	0.100	0.110	0.302
Mutant	0.115	0.184	0.266	0.577	0.086	0.096	0.903	0.497
Myc*mutant	0.988	0.098	0.373	0.205	0.240	0.063	0.311	0.299
SGE/*sym33*								
21 dai								
Myc	0.402	**0.003**	0.270	-	**0.010**	**<0.001**	**0.025**	**0.031**
Mutant	0.260	0.467	**0.010**	-	**0.219**	0.059	0.542	0.339
Myc*mutant	0.667	0.435	0.987	-	0.208	**0.046**	0.254	0.399
28 dai								
Myc	0.081	0.060	0.139	-	0.065	**0.021**	0.182	0.251
Mutant	0.491	0.606	0.128	-	0.377	0.140	0.215	0.495
Myc*mutant	0.660	0.231	0.751	-	0.096	**0.026**	0.220	0.221
SGE/*sym40*								
21 dai								
Myc	0.114	**0.007**	0.873	-	**0.002**	**0.031**	**0.049**	**0.033**
Mutant	0.593	0.411	**0.001**	-	0.994	0.308	0.158	0.562
Myc*mutant	0.787	0.387	0.431	-	0.954	0.821	**0.034**	0.824
28 dai								
Myc	0.326	**0.028**	0.422	**0.027**	0.165	**0.012**	0.238	0.384
Mutant	0.669	0.410	0.048	0.509	0.597	0.074	0.324	0.309
Myc*mutant	0.708	0.496	0.660	0.663	0.233	0.361	0.317	0.125

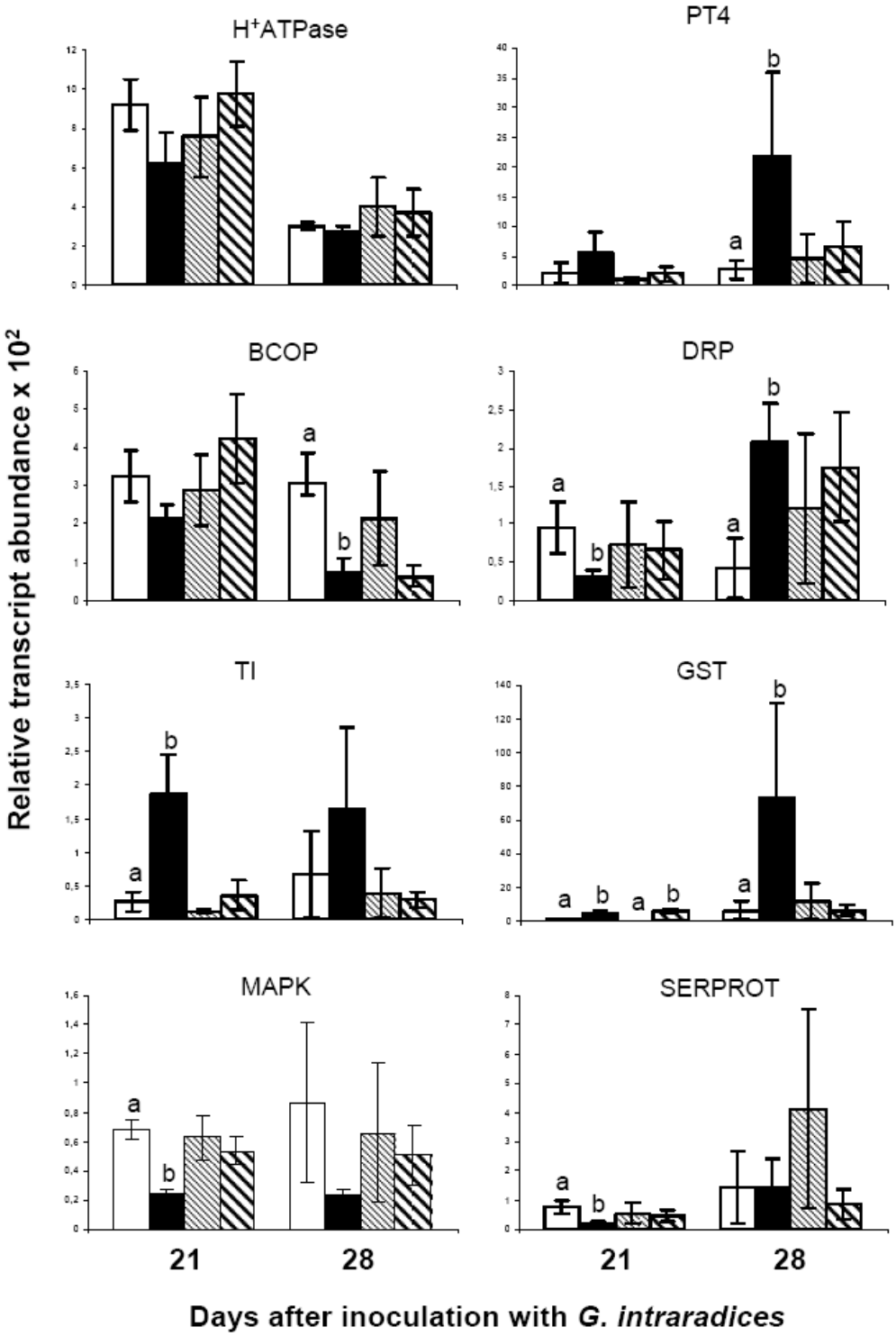


Figure V.1. Expression of pea genes in mycorrhizal (■) and non-mycorrhizal (□) roots of wild type cv Finale and in colonized (▧) and non-colonized (▨) roots of the mutant *Pssym36*, 21 and 28 days after inoculation with *G. intraradices*. Transcript abundance of each gene, measured by real-time RT-PCR, is expressed relative to that of the house-keeping pea *GAPDH* gene. Different letters indicate significant differences between inoculated and non-inoculated plants for each pea genotype ($P \leq 0.05$, n=3).

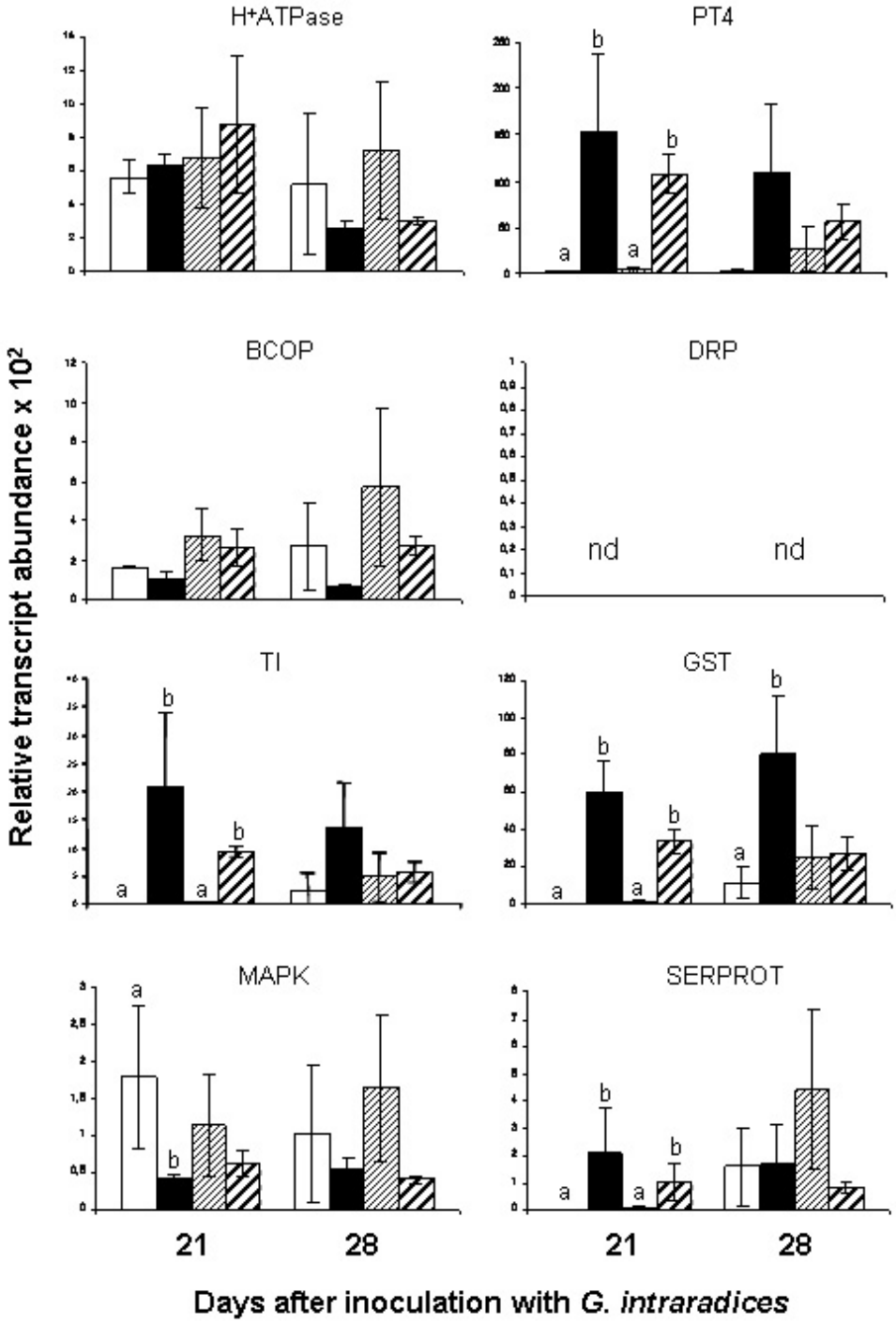


Figure V.2. Expression of pea genes in mycorrhizal and non-mycorrhizal roots of the wild type SGE genotype (■, □) and of the *Pssym33* mutant (▨, ▨), 21 and 28 days after inoculation with *G. intraradices*. Transcript abundance of each gene, measured by real-time RT-PCR, is expressed relative to that of the house-keeping pea *GAPDH* gene. Different letters indicate significant differences between inoculated and non-inoculated plants for each pea genotype ($P \leq 0.05$, n=3).

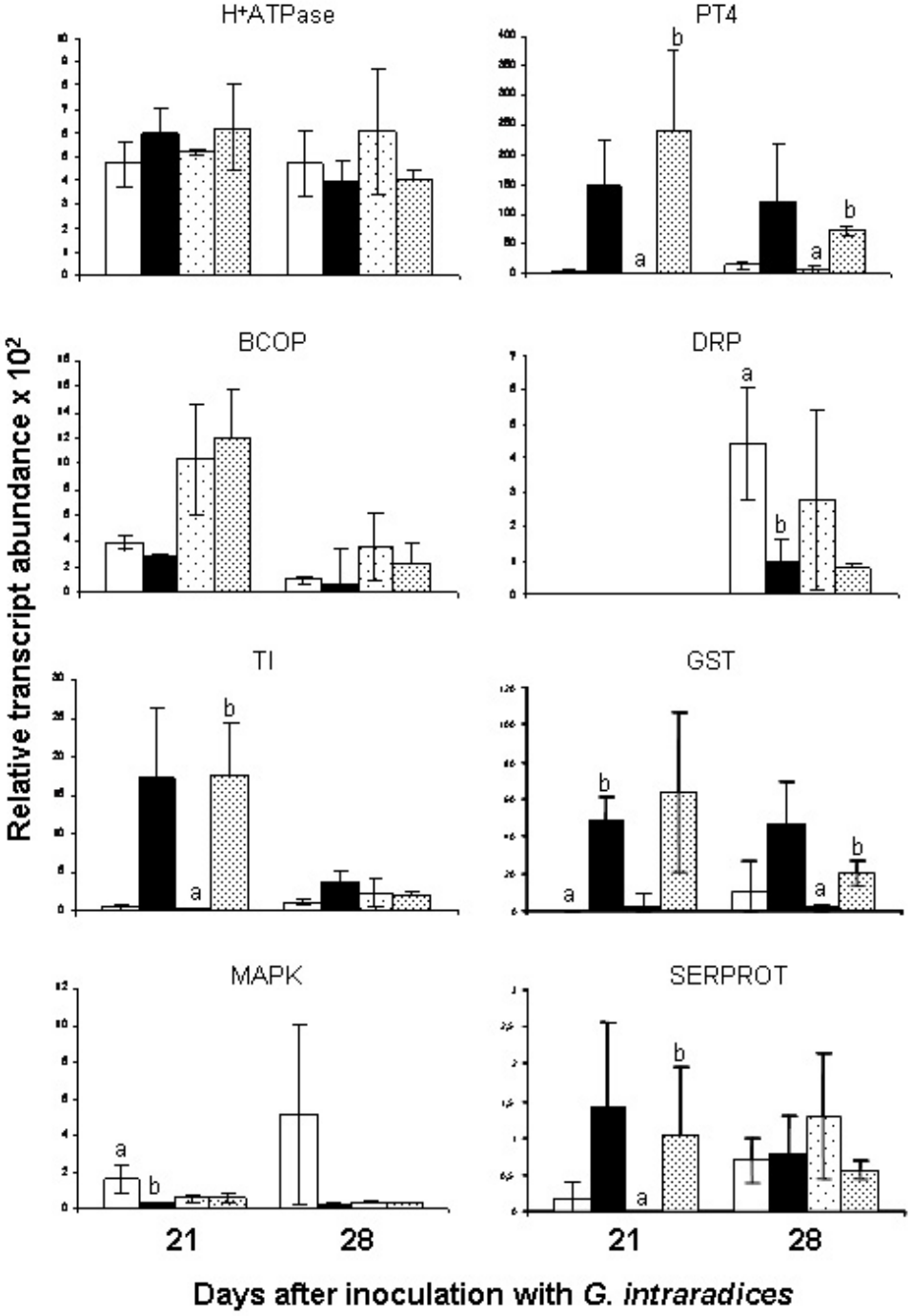


Figure V.3. Expression of pea genes in mycorrhizal and non-mycorrhizal roots of the wild type SGE genotype (■, □) and of the *Pssym40* mutant (▨, ⊡), 21 and 28 days after inoculation with *G. intraradices*. Transcript abundance of each gene, measured by real-time RT-PCR, is expressed relative to that of the house-keeping pea *GAPDH* gene. Different letters indicate significant differences between inoculated and non-inoculated plants for each pea genotype ($P \leq 0.05$, n=3).

V.3. Discussion and conclusions

Mutations in the *PsSym36*, *PsSym33* and *PsSym40* genes had a less pronounced effect on expression of the pea genes monitored in this study to reflect AM symbiosis development and functioning than on *G. intraradices* genes reported in the previous chapter.

The pea *H^+-ATPase* gene identified here was active in non-mycorrhizal roots of wild type pea genotypes and of all three *Pssym36*, *Pssym33* and *Pssym40* mutant plants, but its expression was not affected by *G. intraradices* colonization of root in any of the genotypes. This contrasts with reported up-regulation of an *H^+-ATPase*-encoding gene in mycorrhizal roots of tobacco and *M. truncatula* (Strittmater et al., 1996; Krajinski et al., 2002). Likewise, expression of the pea *BCOP* gene selected for this study was also not influenced by AM formation, contrary to a *M. truncatula* gene (Kuster et al., 2004). Both *H^+-ATPase* and blue copper proteins belong to large gene families and members may behave differently in response to signals or external stimuli in different plant species.

The pea phosphate transporter gene (*PsPT4*) which was identified in this study shows a high homology with the *MtPT4* reported to be induced during the AM symbiosis in *M. truncatula*. There is recent evidence that *MtPT4* transporter activity is essential for the maintenance of arbuscules (Javot et al. 2007), and lyso-phosphatidylcholine has been proposed as a possible signal molecule at the symbiotic interface to maintain this activity (Drissner et al. 2007). Expression of the *PsPT4* gene was strongly up-regulated in fully mycorrhizal roots of wild type pea genotypes, as well as in those of the *Pssym33* and *Pssym40* mutants but it did not respond to *G. intraradices* colonization of roots in the *Pssym36* mutant, which concords with a role of *PsPT4* in P_i transport at the symbiotic interface of arbuscules (Harrison et al., 2002; Javot et al., 2007).

Up-regulation of the expression of genes encoding for the pI230 disease resistance response protein (*DRP*), a trypsin inhibitor (*TI*) and glutathione-S-transferase (*GST*) in mycorrhizal roots of wild type, *Pssym33* and *Pssym40* genotypes may reflect activation of pea defence/stress responses to the AM fungal development. Glutathione-S-transferases are enzymes that catalyze the conjugation of the tri-peptide glutathione which is known to play an important role in

plant defence mechanisms (Anderson and Davis, 2004). Glutathione-S-transferase transcripts have been found to accumulate in the arbuscule stage of mycorrhiza (Wulf et al., 2003; Brechenmacher et al. 2004). Activation of the *DRP* and *TI* genes may be involved in the control of fungal development within host plant roots. The disease resistance response protein pI230 and the trypsin inhibitor have been described as having an antifungal activity (Chiang et al., 1991; Blee, 1998; Griudici et al., 2000). Plant *DRP* and *TI* genes were not activated by *G. intraradices* colonization of roots of the arbuscule-defective *Pssym36* mutant, suggesting a role of these genes at the arbuscule development stage. These observations on *DRP* and *TI* gene expression in roots of the *Pssym36* mutant are in agreement with data reported previously by Grunwald et al. (2004). It is interesting that lack of activation of these two plant defence-related genes in *Pssym36* roots coincides with a lack of up-regulation of the *G. intraradices THIO* and *SOD* genes implemented in anti-oxidative stress metabolism, which may reflect arbuscule formation as a stress situation for the fungus.

The MAP kinase-encoding pea gene was consistently down-regulated in mycorrhizal roots of the wild type pea genotypes but expression was not significantly affected in the three mutants. Liu et al. (2003) reported down regulation of two *M. truncatula* MAP kinase genes as *G. versiforme* developed within roots, although the genes were transiently up-regulated during the initial stages of symbiosis establishment, whilst Grunwald et al. (2004) found that a MAP kinase gene was up-regulated in mycorrhizal cv Finale roots and unaffected in *Pssym36*. MAP kinases belong to a gene family which interacts with a large number of receptors making it difficult to hypothesize as to a specific function in mycorrhizal interactions since distinct MAP kinase pathways are involved in a wide variety of biological processes including cell growth and death, differentiation, the cell cycle and stress responses (Jonak et al. 1999).

The pea serine protease (*SERPROT*) gene identified here shows a high homology with a previously reported serine protease gene activated in mycorrhizal roots of *M. truncatula* (Gianinazzi-Pearson et al., 1996; Garcia-Garrido and Ocampo, 2002; Massoumou et al., 2004). Likewise, expression of pea *SERPROT* was also up-regulated by AM development.

In summary, expression of seven of the eight selected plant genes (except the H^+*-ATPase* gene) was affected by mycorrhiza formation and functioning in wild type cv Finale and SGE

roots, compared to non inoculated roots. At 21 dai in cv Finale roots colonized by *G. intraradices*, *TI* and *GST* genes were up-regulated, whilst the *DRP*, *MAPK* and *SERPROT* genes were all down-regulated. Similar trends persisted for the *TI*, *GST* and *MAPK* genes at 28 dai, whilst *DRP* became up-regulated in mycorrhizal roots. At this time point, development of *G. intraradices* within cv Finale roots significantly repressed expression of the *BCOP* gene, and activated the *PT4* gene. In roots of the mutant *Pssym36*, only *GST* expression was significantly modified (up-regulated) by *G. intraradices* colonization at 21 dai; no significant responses were observed for any of the other seven plant genes at either time point which could reflect absence of signalling responses from the plant side to AM fungal colonization in roots of the *Pssym36* mutant. When plant gene responses to *G. intraradices* in *Pssym33* and *Pssym40* were compared to those in the wild type SGE line, modifications in gene expression were of the same tendency as for the wild type genotype in the two analyses: *PT4*, *TI*, *GST* and *SERPROT* were up-regulated, and *BCOP* and *MAPK* down-regulated in mycorrhizal roots.

In conclusions, mutations of pea *PsSym36, PsSym33* and *PsSym40* genes modify both *G. intraradices* and pea gene expression during AM interactions. Mutation of the *PsSym36* pea gene results in strong alterations in the process of AM symbiosis development that are reflected not only in defects in fungal morphogenesis but also in reduced expression of plant genes which may be connected with arbuscule formation and/or functioning. Inactivation of the *PsSym40* and *PsSym33* genes has less pronounced effects on the AM symbiosis. Modifications in pea gene expression in roots of the *Pssym33* and *Pssym40* mutants were less significant and more time dependent.

CHAPTER VI

TRANSCRIPTOME PROFILING OF *G. INTRARADICES* GENES IN ARBUSCULE- CONTAINING CORTICAL CELLS OF THE *PSSYM40* MUTANT

Chapter VI. Transcriptome profiling of *G. intraradices* genes in arbuscule-containing cortical cells of the *Pssym40* mutant

VI.1. Introduction

One of the objectives of this study was to verify expression of some investigated genes during arbuscule development in the pea AM symbiosis. For this, three *G. intraradices* genes *SOD*, *PEPISOM* and *DESAT* were chosen as candidate genes expressed in arbuscules. These fungal genes were active in mycorrhizal roots of both cv Finale and SGE wild type genotypes and in *Pssym33* and *Pssym40* mutants. At the same time, their expression was significantly lower in the arbuscule-defective *Pssym36* mutant, suggesting a role in arbuscule development and/or functioning. To localize their expression, arbuscule-containing cells from mycorrhizal roots of the *Pssym40* mutant were dissected using a laser capture microdissection system (see Material and Methods II.5.). This approach has previously been applied to monitor plant and fungal gene expression within arbuscule-containing cells of mycorrhizal roots of *Lycopersicon esculentum* (Balestrini et al., 2007) and *M. truncatula* (Gomez et al., 2009). Transcript levels of the three selected *G. intraradices* genes were quantified by absolute quantitative real-time RT-PCR and normalized against the expression of the house-keeping *TEF* gene, as reported in chapter IV.

VI.2. Results

RNA for fungal gene expression analysis was isolated from dissected arbuscule-containing cells of the *Pssym40* mutant at 28 dai by *G. intraradices* using an Arcturus™Microdissection instrument (ARCTURUS NIKON inverse XT100). Samples were visualized on a computer monitor (figure VI.1. A); each arbuscule-containing cell was marked using graphical software (figure VI.1. B) and sampled with IR laser. Dissected cells (figure VI.1. D) were automatically captured into CapSure HS LCM Caps (Arcturus) (figure VI.1. C) and bulked in 4 samples. RNA was extracted from each of these (see details in Material and Methods II.7.4.). The number of dissected arbuscule-containing cells in each bulked sample and the yield of total RNA are

presented in table VI.1. As a control, RNA was extracted from 40 whole mycorrhizal root sections (~240 arbuscule-containing cells) before microdissection.

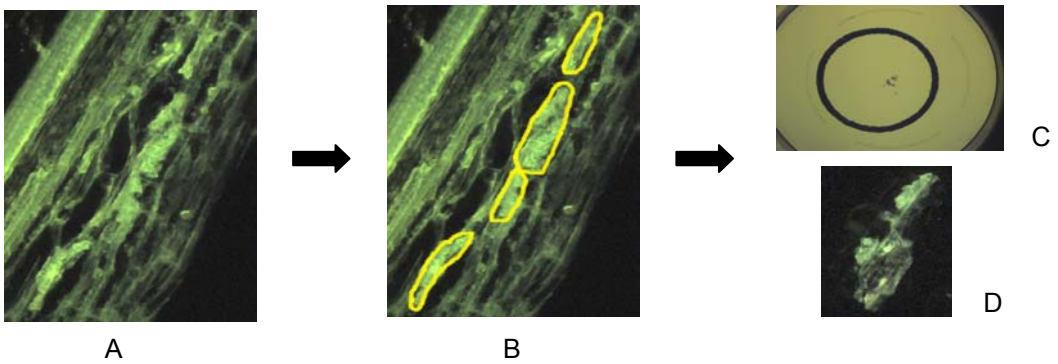


Figure VI.1. Laser microdissection of cortical cells containing arbuscules in mycorrhizal *Pssym40* roots. Explanations are in the text.

Table VI.1. Number of arbuscule-containing cells dissected from mycorrhizal roots of the *Pssym40* mutant at 28 dai and yield of extracted RNA evaluated with a BIO-RAD Experion system.

№ of sample	Number of dissected cells	RNA yield, pg/µl
1	600	20
2	724	36
3	586	100
4	662	40

Fungal gene expression was monitored after cDNA synthesis and multiplication using TargetAmp™2-Round aRNA amplification kit (Epicentre Biotechnologies, Madison,WI; see Material and Methods II.9.6.). Expression of the three selected *G. intraradices* genes was significantly higher in dissected cells containing arbuscules in comparison to that detected from whole slide extracts of mycorrhizal root sections (figure VI.2.).

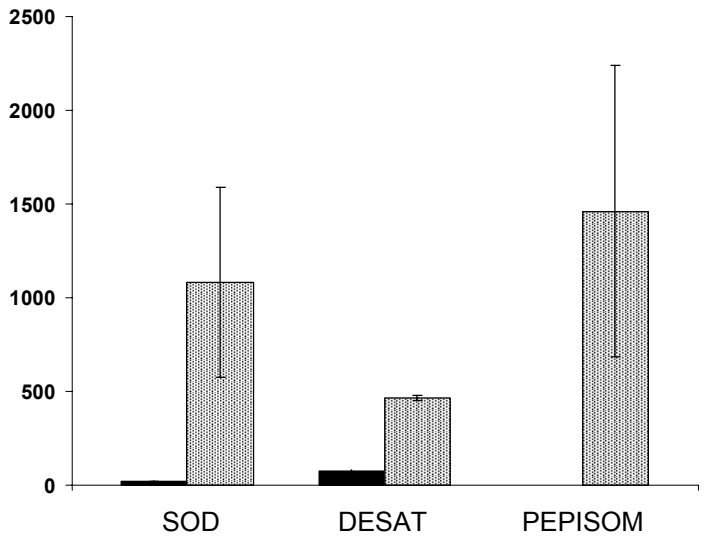


Figure VI.2. *G. intraradices* gene expression in whole sections of colonized *Pssym40* roots (■) and in microdissected arbuscule-containing cells of mycorrhizal *Pssym40* mutant roots (▩).

VI.3. Discussion and conclusions

It was previously shown that expression of *G. intraradices SOD*, *PEPISOM* and *DESAT* genes is strongly related with the arbuscule stage of AM symbiosis development (see chapter IV). These genes had an important level of transcripts in dissected arbuscule-containing cells of the *Pssym40* mutant which was chosen for this study because it has a more rapid arbuscule development. Transcript levels of *SOD*, *DESAT* and *PEPISOM* genes were higher by 60, 6 and 2117 times, respectively, in dissected arbuscule-containing cells than in the pool of all cells of a mycorrhizal root. The significant up-regulation of these genes observed in mycorrhizal roots of wild type SGE, *Pssym33* and *Pssym40* mutants, but not in roots of the arbuscule-defective *Pssym36* mutant, supports the hypothesis of their arbuscule-related expression.

As was discussed previously (chapter IV), activation of the anti-oxidative *SOD* gene may result from a reaction of *G. intraradices* to stress/defense responses generated in roots by the AM fungus. These responses, especially localized in arbuscule-containing cells (Gianinazzi-Pearson et al., 1996), probably explain up-regulation of the *SOD* gene in that the fungus has to

overcome the oxidative defense system of the plant for its establishment and functioning in the root.

The *DESAT* gene which is also significantly activated in dissected arbuscule-containing cells, has a function in lipid metabolism. This gene may be involved in *G. intraradices* lipid metabolism necessary for fungal plasma membrane modifications during symbiosis development, especially during symbiotic interface formation. Its activity may also be related to photosynthetic carbon uptake at the arbuscule interface and subsequent storage as lipid globules in mycelium because we observed low expression level of the *DESAT* gene in *G. intraradices*-colonized roots of the arbuscule-defective *Pssym36* mutant.

PEPISOM is also a fungal gene highly expressed in dissected arbuscule-containing cells. *PEPISOM* encodes a chaperon protein that activates or inhibits regulatory proteins by catalyzing proline isomerization (Miele et al., 2003). This gene may play a role in protein turnover during cellular regulation of arbuscule formation and/or functioning, which would explain the lack of expression in *G. intraradices*-colonized roots of the *Pssym36* mutant.

In conclusion, the expression analysis of *SOD*, *DESAT* and *PEPISOM* genes in dissected arbuscule-containing cells provides strong evidence that the up-regulation observed by qRT-PCR monitoring of transcripts in mycorrhizal roots preferentially occurs in the fungal arbuscules.

CHAPTER VII

GENOME LOCALIZATION OF THE *PSSYM36* AND *PSSYM40* GENES

VII. Genome localization of the *Pssym36* and *Pssym40* genes

VII.1. Introduction

Genetic mapping, as a tool for gene cloning, is a necessary step in studies of the functioning and maintenance of different living processes, including interactions between plants and microbes during beneficial symbioses. One way to define the molecular basis of a mutant phenotype is by map-based cloning which first necessitates localization of the corresponding gene on the genetic map. In this context, the objective of this study was to create conditions for genetic mapping of the mutated *PsSym36* and *PsSym40* genes.

Neither *PsSym36* nor *PsSym40* genes have been localized on the pea genetic map so far. Previous attempts to localize the *PsSym40* gene using a set of 10 morphological markers (*A, b, d, Fs, gp, k, r, s,* tl^{W}*,wb*) were not successful (Dr. V.Tsyganov, personal communication). For *PsSym36*, weak linkage to the morphological marker *wb* of linkage group (LG) II was observed in a small mapping population of 50 F2 plants (Dr. V. Zhukov, personal communication). This makes it necessary to resort to the use of DNA-based molecular markers for mapping *PsSym36* and *PsSym40* in the pea genome.

Recent efforts to construct pea genetic maps have produced an extensive list of molecular markers based on genes of known function and known position in pea genome (so called anchor markers) (Gilpin et al., 1997; Konovalov et al., 2005; Aubert et al., 2006). This information provides a tool for mapping new pea mutations in relation to the anchor markers. Most of the protein encoding genes used in the present study to create molecular markers for mapping of the *PsSym40* gene were localized on the pea genetic map by Aubert et al. (2006). In the present study, molecular markers (mostly CAPS – Cleaved Amplified Polymorphic Sequences (Konieczny and Ausubel, 1993)) were firstly developed on the basis of gene nucleotide sequence polymorphisms which were identified between parental lines (one of which was the *Pssym40* mutant) by sequencing of PCR products, and afterwards they were localized on the pea genetic map. The position of the *PsSym36* gene in the linkage group II close to the morphological marker *Wb* was also investigated.

VII.2. Results

VII.2.1. Creation and screening of F2 populations

For mapping of the *PsSym36* and *PsSym40* genes, F1 and F2 populations were produced (see Material and Methods II.2, II.5) by the following crosses:

RisNod24 (*Pssym36*) mutant pea line with:

- morphologically mapped laboratory lines: NGB 1238, NGB 851, NGB 2715,
- lines from the N.I. Vavilov Institute of Plant Industry (VIR) collection: K 3034, K 1693, K 7128.

SGEFix$^-$-1 (*Pssym40*) mutant pea line with:

- laboratory line NGB 1238
- lines from VIR collection: K 3034, K 1693, K 7128, K 8274.

A total of 217 individual F2 plants from the RisNod24(*Pssym36*)×NGB1238 cross and 158 individual F2 plants from the SGEFix$^-$-1(*Pssym40*)×NGB1238 cross were used for *Pssym36* and *Pssym40* gene localization, respectively. *Pssym36* and *Pssym40* mutant phenotypes in corresponding F2 plant populations were observed by alterations in root nodule formation in quartz sand after inoculation with *Rhizobium leguminosarum bv. viciae* (strain CIAM 1026; see Material and Methods II.2). *Pssym36* mutant plants do not form root nodules. *Pssym40* mutant plants form small white root nodules whilst wild type nodules are larger and pink.

VII.2.2. EST-based molecular marker development

For *PsSym40* gene localization, 34 protein encoding genes known to be localized in the pea linkage groups LGI, LGII, LGIII, LGIV and LGV were selected (see table VII.1). 18 molecular markers were identified between parental lines, based on nucleotide sequence differences of amplified parts of the chosen genes. The origin of primers, primer sequences, melting temperature of primers and restriction enzymes used for CAPs (Cleaved Amplified Polymorphic sequences) polymorphisms detection are presented in annex 2. Computational analysis of

segregation of the constructed markers was performed using the shareware program MapL98 (Prof. Yasuo Ukai, Biometrics Laboratory, Graduate School of Agricultural Life Science, University of Tokyo).

Table VII.1. ESTs selected for molecular marker creation for segregation analysis in F2 progeny of the SGEFix$^-$-1(*Pssym40*)×NGB1238 cross

Gene name	Gene symbol	Linkage group	Marker nature
Nodule inception protein (PsSym35)	*NIN*	I	allele-specific amplification
Receptor-like kinase (PsSym37)	*PsSym37*	I	CAPs
1-aminocyclopropane-1-carboxylic acid oxidase	*ACCox*	I	CAPs
Receptor-like kinase (PsSym10)	*PsSym10*	I	CAPs
Thiol protease	*ThiolP*	II	size
Putative peptide/amino-acid	*PepTrans*	II	CAPs
Granule-bound starch synthase I	*Gbsts1*	II	no polymorphism
P54 protein, putative sucrose binding	*P54*	II	no polymorphism
Cell-wall invertase I	*Cwi1*	II	no polymorphism
Cytosolic malate dehydrogenase	*MDHc*	II	no polymorphism
Putative peptidyl-prolyl cis-trans-isomerase	*PPIlike*	II	CAPs
Gigantea-like protein	*Gigantea*	II	no polymorphism
Caffeic acid 0-methyltransferase	*cOMT*	II	no polymorphism
Plastidial phosphoglucomutase	*pPgm*	II	CAPs

Sugar transporter 1	*Sut1*	II	no polymorphism
ADP-glucose pyrophosphorylase	*Agpl*	III	CAPs
Aspartate amino transferase	*PsAAT1*	III	size
NIP protein	*NIP*	III	allele-specific amplification
Amino-acid permease 2	*PsAAP2*	III	no polymorphism
Dipeptidyl peptidase IV-like protein	*DiPept IV*	III	CAPs
Plasma membrane intrinsic protein 1	*Pip 1*	III	no polymorphism
Ribosomal protein S13	*TE002L09*	III	no polymorphism
Asparagine synthase-1	*PsAS1*	III	no polymorphism
Glucose-6-phosphate/phosphate-translocator	*Gpt*	III	no polymorphism
Early nodulin 12B	*Eno12B*	III	CAPs
Phosphoenolpyruvate carboxylase	*PepC*	III	CAPs
Phosphoglycerate kinase 1	*PGK1*	III	no polymorphism
Beta-fructofuranosidase	*bfruct*	III	no polymorphism
Xyloglucan fucosyltransferase	*Xyft*	IV	no polymorphism
Cell-wall invertase II	*Cwi2*	IV	no polymorphism
Ineffective greenish nodules 1	*IGN1*	V	CAPs
Enolase (2-phospho-D-glycerate hydrolase)	*Enol*	V	CAPs
Metallothionein	*MET2*	V	CAPs

UDP-glucose dehydrogenase	*UDPgd*	V	CAPs

VII.2.3. Genetic mapping

PsSym36

The F2 population obtained from the RisNod24(*Pssym36*)×NGB1238 cross was analyzed (see VII.1) in order to verify whether *PsSym36* is linked to morphological marker *Wb* of linkage group II.

Table VII.2. Segregation data in the F2 progeny of the RisNod24(*Pssym36*)×NGB1238 cross

Gene pare	Number of progeny							Rec.	S.E.	LOD	Joint χ^2
	PP	PH	PQ	QP	QH	QQ	Total				
Sym36 - Wb	34	0	10	135	0	38	217	0.513	0.056	0.01	0.04

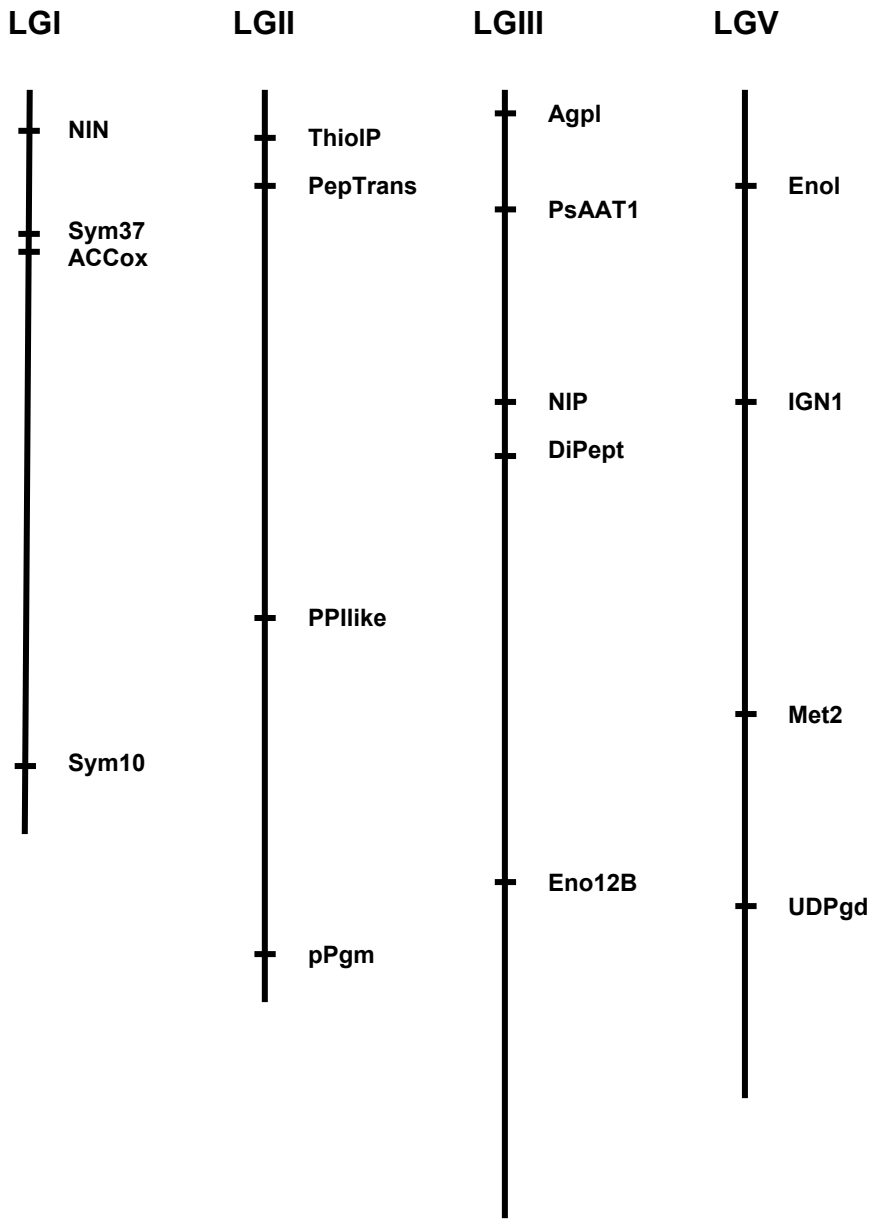
P-allele 1, Q – allele 2, H- heterozygote; Rec. – recombination; S.E. – standard error

No significant linkage between the two genes was detected. The plant material obtained will be used in the future for *PsSym36* gene localization in relation to gene-based molecular markers.

PsSym40

A joint segregation analysis was performed using the F2 population from the SGEFix$^-$-1 (*Pssym40*) × NGB1238 cross. 17 molecular gene-based markers positioned in LGI, LGII, LGIII and LGV were adopted for *PsSym40* gene localization (see figure VII.1).

Figure VII.1. Location of gene-anchored markers on the pea genetic map



Linkage group I

Three CAPs (*Sym37, ACCox and Sym10*) and one allele-specific (*NIN*) molecular marker are known to be positioned in LGI. Significant linkage was detected between the *NIN, PsSym37* and *ACCox* markers (see table VII.3). No linkage of the *PsSym10* gene with *PsSym37, ACCox* or *NIN* was observed, probably because *PsSym10* lies quite far from these. No significant linkage of the *PsSym40* gene with the tested molecular markers was detected.

Table VII.3. Segregation analysis data (linkage group I)

Gene pair	Number of progeny										Rec.	S.E.	LOD	Joint χ^2
	PP	PH	PQ	HP	HH	HQ	QP	QH	QQ	Total				
Sym40 - NIN	10	15	7	0	0	0	6	12	4	54	0.519	0.069	0.02	0,51
Sym40- Sym37	10	19	7	0	0	0	2	6	0	44	0.545	0.104	0.04	0.89
Sym40-ACCox	10	18	9	0	0	0	6	12	5	60	0.512	0.067	0.01	0.13
Sym40-Sym10	6	15	9	0	0	0	0	0	0	30	0.637	0.162	0.13	0.20
NIN-Sym37	5	6	0	6	15	1	0	2	5	40	0.213	0.054	3.19	14.55
NIN-ACCox	7	7	2	7	17	3	0	4	7	54	0.269	0.052	2.78	14.70
Sym37-ACCox	12	0	0	0	24	1	0	0	7	44	0.011	0.012	16.89	75.68
ACCox-Sym10	3	4	2	3	7	5	0	4	2	30	0.409	0.084	0.24	2.00

Abbreviations are those defined in table VII.2.

Linkage group II

11 protein encoding genes with known location in LG II (see table VII.1) and 4 molecular markers *ThiolP, PepTrans, PPIlike* and *pPgm*, developed on basis of nucleotide sequences polymorphism, were selected for segregation analysis. Significant linkage was detected between the pairs of molecular markers *ThiolP-PepTrans* (7.3 cM; LOD=13.38) and *PPIlike-pPgm* (25.5 cM; LOD=3.11). No linkage was observed between *PepTrans* and *PPIlike* markers, probably due to the long distance between them. Again, no significant linkage of the *PsSym40* gene with the tested molecular markers was detected.

Table VII.4. Segregation analysis data (linkage group II)

Gene pair	Number of progeny										Rec.	S.E.	LOD	Joint χ^2
	PP	PH	PQ	HP	HH	HQ	QP	QH	QQ	Total				
Sym40 -ThiolP	11	17	7	0	0	0	5	14	3	57	0.512	0.069	0.01	2.16
Sym40-PepTrans	12	19	6	0	0	0	5	16	3	61	0.500	0.067	0.00	2.89
Sym40-PPIlike	6	21	10	0	0	0	3	14	6	60	0.467	0.071	0.05	0.30
Sym40-pPgm	9	21	7	0	0	0	5	13	6	61	0.525	0.068	0.03	0.22
ThiolP-PepTrans	12	4	0	3	28	0	0	1	9	57	0.073	0.026	13.38	60.44
PepTrans-PPIlike	2	10	5	7	17	10	0	8	1	60	0.538	0.068	0.07	2.50
PPIlike-pPgm	5	4	0	8	22	5	1	7	8	60	0.255	0.050	3.11	12.87

Abbreviations are those defined in table VII.2.

Linkage group III

Molecular markers were developed for 5 of the 13 selected ESTs positioned in LGIII. Significant linkage was detected in the *Agpl-PsAAT1* pair of molecular markers. Linkage in pairs of markers *PsAAT1-DiPept* and *DiPepet-Eno12B* were marginally significant and no significant linkage of the *PsSym40* gene with the tested molecular markers was detected.

Table VII.5. Segregation analysis data (linkage group III)

Gene pair	Number of progeny										Rec.	S.E.	LOD	Joint χ^2
	PP	PH	PQ	HP	HH	HQ	QP	QH	QQ	Total				
Sym40 -Agpl	7	24	6	0	0	0	8	10	6	61	0.536	0.073	0.05	3.16
Sym40-PsAAT1	5	20	12	0	0	0	5	13	6	61	0.525	0.068	0.03	0.22
Sym40-NIP	6	0	31	0	0	0	5	0	19	61	0.503	0.088	0.00	0.00
Sym40-DiPept	5	20	12	0	0	0	1	15	6	59	0.447	0.070	0.12	3.21
Sym40-Eno12B	8	16	12	0	0	0	6	10	8	60	0.489	0.065	0.01	0.40
Agpl-PsAAT1	6	8	1	4	23	7	0	2	10	61	0.215	0.044	4.76	23.46
PsAAT1-NIP4	4	0	6	6	0	27	1	0	17	61	0.290	0.074	1.31	4.38
PsAAT1-DiPept	3	7	0	3	18	10	0	10	8	59	0.302	0.054	2.10	8.49
DiPept-Eno12B	3	3	0	8	15	11	3	8	7	58	0.382	0.060	0.75	3.72

Abbreviations are those defined in table VII.2.

Linkage group V

Four CAPs markers *Enol, IGN1, Met2* and *UDPgd* were developed based on sequences of genes localized in LGV. Significant linkage was detected between *Met2* and *UDPgd* molecular markers only. Markers *Enol, IGN1* and *Met2* are positioned too far from each other to detect any linkage (see figure VII.1). No significant linkage of the *PsSym40* gene with the tested molecular markers was detected.

Table VII.6. Segregation analysis data (linkage group V)

Gene pair	Number of progeny										Rec.	S.E.	LOD	Joint χ^2
	PP	PH	PQ	HP	HH	HQ	QP	QH	QQ	Total				
Sym40 -Enol	4	15	16	0	0	0	4	10	5	54	0.560	0.070	0.16	1.40
Sym40-IGN1	1	3	1	0	0	0	3	11	5	24	0.443	0.145	0.03	0.11
Sym40-Met2	9	24	4	0	0	0	8	10	5	60	0.530	0.075	0.03	2.40
Sym40-UDPgd	11	20	6	0	0	0	10	12	2	61	0.658	0.106	0.51	2.23
Enol-IGN1	1	1	2	2	7	2	1	4	2	22	0.500	0.125	0.00	1.91
IGN1-Met2	2	1	1	5	8	1	1	3	2	24	0.386	0.107	0.21	3.00
Met2-UDPgd	14	2	1	6	25	3	0	5	4	60	0.207	0.081	2.05	52.83

Abbreviations are those defined in table VII.2.

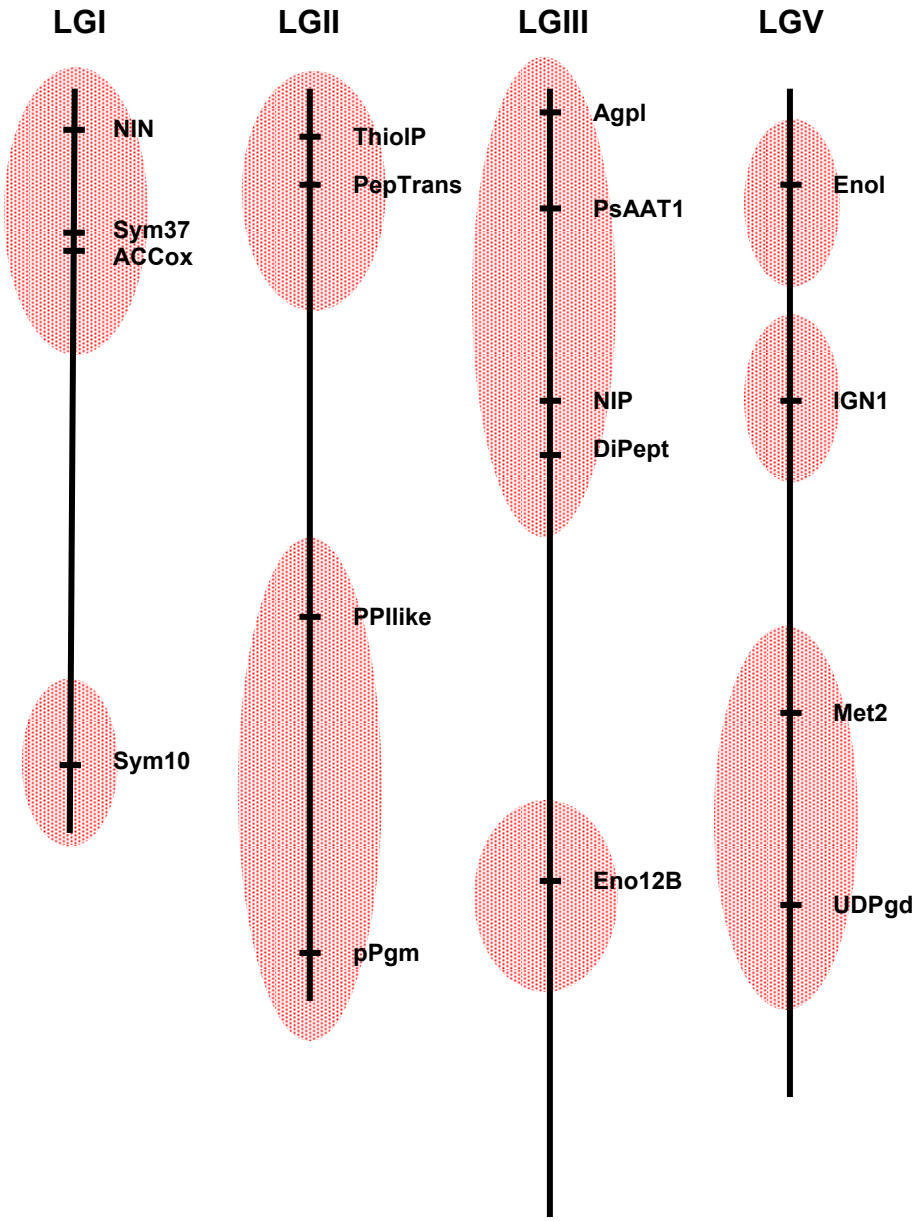
VII.3. Discussion and conclusions

The main aim of the present study was to develop conditions for the localization of the *PsSym36* and *PsSym40* genes without creating a highly saturated pea genetic map. After generating the necessary plant material, the possible linkage between the *PsSym36* gene and the morphological marker *Wb* shown in a previous study was examined. No significant linkage between these two genes was detected in the F2 population. However, plant material developed for this analysis will be useful for future experiments based on molecular markers to localize the position of the *PsSym36* gene on the pea genetic map.

A total of 34 protein encoding genes with known function and position on the pea genetic map were selected for molecular marker creation to determine the position of the *PsSym40* gene,

and 17 molecular markers were developed on the basis of the nucleotide sequence polymorphism identified between parental lines in amplified parts of the chosen genes. The segregation data obtained did not reveal any significant linkage of the developed molecular markers with the *PsSym40* gene. Thus, the three molecular markers *NIN, PsSym37* and *ACCox* localized in linkage group I are significantly linked to each other but do not link with *PsSym40* which excludes localization of this gene in this part of the pea genome. Another molecular marker *PsSym10*, which is positioned lower in LGI, does not link with the *NIN*, *PsSym37*, *ACCox* markers or the *PsSym40* gene, which suggests that the *PsSym40* gene is also not positioned in the region around *PsSym10* marker. Similar conclusions can be drawn about the possible absence of *PsSym40* from LGII, LGIII and LGV. Overall, the performed analyses make it improbable that the *PsSym40* gene lies in one of these four linkage groups, although this can not be absolutely confirmed at present. However, it does appear likely that *PsSym40* lies outside regions close to the molecular marker locations (see figure VII.2) and continuation of the investigations is necessary. In conclusion, the performed study has permitted some advance towards defining the localization of the *PsSym40* gene; the plant material and data obtained will facilitate further investigations.

Figure VII.2. Location of gene-anchored markers on the pea genetic map with regions of the pea genetic map where location of the *PsSym40* gene can be excluded on the base of obtained segregation data



CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSIONS

VIII. General discussion and conclusions

Pea (*Pisum sativum* L.) is an important legume crop in French and in Russian agriculture and it is one of the most developed model plants for genetic studies of symbiotic root interactions with beneficial soil microorganisms (Zhukov et al., 2009). Phenotypic characterization of pea mutants has led to the identification of plant genes that are implicated in the establishment or in the functioning of nodulation and AM symbioses (Borisov et al., 2004). Such mutants provide tools for genomic and molecular studies to identify plant *SYM* genes and to gain insight into their control of the metabolic circuits driving symbiotic interactions. In AM associations, the symbiotic fungi facilitate uptake of mineral nutrients (especially Pi) from the soil to plant roots and, in return, they receive photosynthetically fixed carbon (Smith and Read, 1997). The principal site of Pi and carbon exchange between plant and fungus occurs across a symbiotic interface formed between specific intracellular fungal structures, arbuscules, and cortical root cells. Whilst plant genes involved in the early steps of AM formation are a focus of research (Parniske, 2008), little is known about the genetic programme controlling the formation and function of arbuscules which are the key symbiotic structures in the established mycorrhiza.

The aim of my research was therefore to better characterize the late stage AM symbiosis-related pea genes *PsSym36*, *PsSym33* and *PsSym40* by investigating the effect of mutations in the three genes on fungal and plant gene responses and by creating conditions for the localization of two of the genes, *PsSym36* and *PsSym40*, on the pea genetic map for future map-based cloning. Mutation in the *PsSym36* gene results in incomplete, aborted arbuscule development (Gianinazzi-Pearson et al., 1992) whilst mutations in the *PsSym33* and *PsSym40* genes induce alterations in the rate of AM development (Jacobi et al., 2003 a, b). The study focusing on the genetic localization of *PsSym36* and *PsSym40* was performed in the Laboratory of Genetics of Plant-Microbe Interactions, ARRIAM. During this research period, production of necessary plant material was achieved and 34 protein encoding genes with known location in the linkage groups LGI, LGII, LGIII and LGV of the pea genetic map were selected. 17 molecular markers were developed from these and segregation analysis in the F2 population from a SGEFix$^-$-1(*Pssym40*)×NGB1238 cross was performed. Whilst the position of some of the

molecular markers was located on the pea genetic map, neither the *PsSym36* nor the *PsSym40* genes could be localized. There is a high probability of the *PsSym40* gene being located outside the molecular marker regions of LGI, LGII, LGIII or LGV.

Symbiosis-associated fungal and plant molecular responses were monitored to study the effect of inactivation of late stage symbiosis-related pea genes *PsSym36*, *PsSym33* and *PsSym40* on AM functioning. This part of my research work was performed in the Plant-Microbe-Environment Research Unit, Dijon. Expression of fungal and plant genes was analyzed in roots of the *Pssym36*, *Pssym33* and *Pssym40* mutants and corresponding wild type cv Finale and SGE plants at 21 and 28 day after inoculation with *G. intraradices* by real-time RT-PCR. It was shown that mutations in the *PsSym36*, *PsSym33* and *PsSym40* genes modify both fungal and plant responses during AM establishment but mutation of the *PsSym36* gene had a greater impact than inactivation of the *PsSym33* and *PsSym40* genes.

All the studied *G. intraradices* genes were expressed in roots of the mycorrhizal wild type and mutant pea genotypes. Most of them were strongly down-regulated in roots of the *Pssym36* mutant and up-regulated in roots of the *Pssym40* mutant, which could be associated with the absence of full arbuscule development in roots of the *Pssym36* mutant and the more rapid AM symbiosis development in roots of the *Pssym40* mutant. Of the three tested pea mutants, *Pssym33* had less pronounced effects on *G. intraradices* gene expression as well as on AM formation and function. Expression of selected pea genes for this study was less affected by *Pssym36*, *Pssym33* and *Pssym40* mutations than fungal gene responses.

Observed down-regulation of the *G. intraradices* H^+*-ATPase* gene when arbuscule formation is defective in *Pssym36* roots correlates with the lack of enzyme activity on the fungal plasma membrane bordering aborted arbuscules branches, shown before in the same pea genotype (Gianinazzi-Pearson et al., 1995). It can be hypothesized that this H^+-ATPase enzyme plays a role in the active fungal uptake of photosynthetic carbon at the plant-fungal interface in wild type roots. If so, limited carbon uptake of plant-derived carbon could be expected in *Pssym36* roots and this could explain the strong down-regulation of the fungal *DESAT* gene which suggests a reduced biosynthesis of fatty acids as a result of reduced carbon acquisition. In the *Pssym33* and *Pssym40* pea genotypes, where arbuscules are fully developed, expression of fungal

DESAT and *H^+-ATPase* was maintained or increased. Furthermore, the pea phosphate transporter (*PsPT4*) gene was not activated in *G. intraradices*-colonized roots of the *Pssym36* mutant, in contrast to its up regulation in mycorrhizal roots of the wild type, *Pssym33* and *Pssym40* pea genotypes. Observed expression of the *PsPT4* gene is in agreement with a role of *PT4* in P_i transport only at the symbiotic interface of fully developed, functional arbuscules in mycorrhizal pea roots, as was reported for *M. truncatula* mycorrhiza (Harrison et al., 2002; Javot et al., 2007).

The lack of activation of the *DRP* and *TI* plant defence-related genes in *Pssym36* roots coincides with a lack of up-regulation of the *G. intraradices THIO* and *SOD* genes implemented in anti-oxidative stress metabolism. This may reflect absence of a stress situation for the fungus when arbuscule formation is inhibited. In fact, fungal development and arbuscule formation appears to result in the activation of defence/stress responses, with up-regulation of the expression of pea genes encoding for the pI230 disease resistance response protein (*DRP*), a trypsin inhibitor (*TI*) and glutathione-S-transferase (*GST*) in mycorrhizal roots of wild type, *Pssym33* and *Pssym40* genotypes. In this context, the activation of the fungal *THIO* and *SOD* genes in the mycorrhizal roots of wild type, *Pssym33* and *Pssym40* pea genotypes may enable the AM fungus to contend with plant defense/stress responses which are usually activated in mycorrhizal roots, especially within arbuscule-containing cells (Gianinazzi-Pearson et al., 1996).

The weaker expression of the *G. intraradices RHO* gene, encoding a putative Rho/GDP dissociation inhibitor, in *Pssym36* roots could indicate effects on cell signaling events at the fungal-plant interface, as was suggested for mutations in the *DMI2* and *DMI3/MtSym13* genes of *M. truncatula* (Seddas et al. 2009). The lower transcript levels in colonized *Pssym36* roots of the *PEPISOM* and *26SREG* genes which are implicated in cellular recognition (Miele et al. 2003) and metabolic regulation/cell cycle progression (Murray 1995), respectively, may also contribute to or result from defective arbuscule development in the pea mutant. In this context, inactivation of either *PsSym33* or *PsSym40* was not detrimental to the expression of these three pea genes or to arbuscule development.

Exploitation of the microdissection technique confirmed that expression of the three *G. intraradices* genes *SOD*, *DESAT* and *PEPISOM* does occur preferentially in arbuscule

containing cells, supporting the above conclusions as to their eventual function in fungal interactions with the plant cells.

In conclusion, the present study contributes to a better understanding of the role of the *PsSym36*, *PsSym33* and *PsSym40* genes in the AM symbiosis and of the implication of molecular responses in the two partners during mycorrhiza formation and functioning, as well as progressing knowledge about *PsSym36* and *PsSym40* gene positioning on the pea genetic map. The most significant results obtained can be resumed as follows:

- the *Pssym36*, *Pssym33* and *Pssym40* mutants have modified AM phenotypes which is in agreement with previous observations (Gianinazzi-Pearson et al., 1992, Jacobi et al., 2003 a, b): mutation in the *PsSym36* gene affects arbuscule formation whilst the *Pssym33* and *Pssym40* mutants have alterations in rate of AM development,
- the pea genes *PsSym36*, *PsSym33* and *PsSym40* not only control root colonization and morphological differentiation of an AM fungus but they also influence plant and fungal gene responses linked to symbiotic interactions between the partners,
- mutation in the *PsSym36* gene results in strong alterations in *G. intraradices* and *P. sativum* gene expression, mostly down-regulation, which is coherent with a role of the *PsSym36* gene in the coordinated cellular development in plant and fungus to elaborate a functional interface in arbuscule-containing cells,
- mutation in the *PsSym33* gene has less pronounced effects on plant and AM fungal gene expression as well as on AM formation and functioning than mutations in *PsSym36* or *PsSym40*,
- mutation in the *PsSym40* gene results in an increased expression of most of the investigated *G. intraradices* genes and some of the plant genes which may be associated with the more rapid mycorrhiza development in *Pssym40* roots,
- observed up-regulation of *G. intraradices SOD*, *DESAT* and *PEPISON* genes in microdissected arbuscule-containing cells provides strong evidence of the arbuscule-related expression of these fungal genes,

- localization of the *PsSym40* gene in linkage group I, II, III or V of the genetic map of pea can be almost excluded.

The methodology and molecular tools developed in the present study will be useful for further research activities with respect to other plant genes controlling development and functioning of the AM symbiosis, including those yet to be discovered. The plant material (mapping populations), molecular markers and data obtained will facilitate future experiments to localize the *PsSym36* and *PsSym40* genes on the pea genetic map and create conditions for map-based cloning of these genes. The gene expression data obtained in the present study contribute to the accumulating knowledge about plant, fungal and bacterial molecular responses to interactions between symbiotic partners in legumes. They provide a basis for further research to discover the role of the pea genes *PsSym36*, *PsSym33* and *PsSym40* (and their orthologs in other legume species) in the development of both the fungal and bacterial symbioses of legume plants, to decipher interrelated signal transduction pathways during development of such complex plant-microbe systems and to facilitate creation of approaches to manage such systems in sustainable agriculture.

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ANNEXES

Annex 1. Genes of *M. truncatula* and other plant species used for design of primers for corresponding pea gene amplification.

Name of pea gene	Name of corresponding genes used for primer design.
Plasma membrane ATPase	*Medicago truncatula Plasma membrane ATPase Mtha1* (AJ132892) *Vicia faba Plasma membrane ATPase VHA4* (AJ310523.1)
Inorganic phosphate transporter	*Medicago truncatula Inorganic Phosphate transporter PT4* (AY116210) *Lupinus albus phosphate transporter 1 PT1* (AF305623)
Blue copper binding protein	*Pisum sativum Blue copper binding protein* (Z25471)
Glutathione-S-transferase	*Medicago truncatula Glutathione-S-transferse-like protein* (AY134608) *Arabidopsis thaliana putative glutathione transferase* (AY062941)
Serine protease	*Medicago truncatula Serine protease* (AL382601) *Arabidopsis thaliana subtilisin-type serine endopeptidase XSP1* (AF190794)

Annex 2. ESTs selected for molecular marker development. Technical parameters.

Gene symbol	GenBank accession №	Primer sequences	Tm, °C	Restriction enzyme	Reference
NIN	Ps-AJ493066	GAAAGAGGAAGCGGACTTGT TGGTCTTCTCCGCCTTGG GTGTCATTGATGTTGTTATCGCA GATGATGACCTGCGTCCACCA	60	-	Borisov et al., 2003
PsSym37	EU564102	ATGCAAGCTTAACAACTGTTGAG GAATGCCCGACCATTTGTGCC	60	RsaI	Zhukov, personal communication
ACCox	Ps-CD859427	GAATCATGGCATACCTCATGACC GAGAAGGATGATCCCGCCAG	58	HinfI	Aubert et al., 2006
PsSym10	AJ575252	CAAGGCTAATATAGATGGTAGAG GCTAATACCAATTATAAAAGCAGG	58	PstI	Zhukov, personal communication
ThiolP	Ps-X66061	CCGAAGAGGATTACCCCTAYCGTGC GCTTCTCCCCAGCTACCACCCC	58	-	Aubert et al., 2006
PepTrans	Ps-CD860432	GCCGTGATTCGGATCTGATGG CGGTCGTATAAAGGAATGACTAC	58	NlaIII	Aubert et al., 2006
Gbsts1	Ps-X88789	GTCAACTGGCAACCCAATCT CACTGTAAGTCCACATTATGCC GACATGGAACATAGAAACTGA AAAATGACAGTTTTGATGAACAC	55	-	Aubert et al., 2006
P54	Ps-Y11207	CTATGCTCAAACTTAGCCGTAGGC CAGAACCAGAAGAAGGTGGC	55	-	Aubert et al., 2006

Cwi1	Ps-Z83747	TCCCAAAGGAGCTGTTTGGGG GAATCGGGTGCTTGGCCCG	60	-	Aubert et al., 2006
MDHc	Ps-CD858827	mATGCTGCTTTCCCTCTTTTG TCTAGGGAATCCACCAACCA	58	-	Aubert et al., 2006
PPIlike	Ps-CD860684	hTTTTGAGCATGGCGAATG GCTTGCTTGTCTCCTGTGATT	58	HhaI	Aubert et al., 2006
Gigantea	AY826734	TCCTCGTGGAAGCCCGT CCGATTCTAGACCATTCTGC	60	-	Aubert et al., 2006
cOMT	Ps-CD858528	AGGTGTCATGGTCATGAACA ATGGTCGTCGCTCCAGTC	58	-	Aubert et al., 2006
pPgm	Ps-AJ250770	AACCTGCACCAGAATCCATC CACCATCACCATCACTAGCG	58	RsaI	Aubert et al., 2006
Sut1	Ps-AF109922	GTCTATCTTTTTTGTCCCCG GTCTTCTACACAACGGCTTC	55	-	Aubert et al., 2006
AgpII	Ps-X96766	CGTCGAGAATGTTATTGATCTTGG CAATCCATAATCAGATGCGCGGC	58	AvaII	Aubert et al., 2006
PsAAT1	Ms-L25334	CAGTTTCACAAGGAGTGTCGC CATAGCCTATGAGTTCTTAACCAG	60	-	Aubert et al., 2006
NIP	Ps-U15036	AGTTGAGTTTTTCTGTTCTAGGCCATAGTAC CTGCATGTCAGAGGTCCATAGG	64	-	Aubert et al., 2006
PsAAP2	-	TTTGGACCATATATGGCATATGC CAATAAAATGCAGCAATCACAGCC	55	-	Aubert et al., 2006
DiPept IV	Ps-CD859478	CATACAGCTGTGGATGGTTACCC	58	NlaIII	Aubert et al., 2006

		CTTATCACTCTGCTTGGTTCTGTGG			
Pip1	Ps-AJ548795	CATTCGGTTTGTTCTTGGCGAGG GAGTCTCTGGCACTACGTTTGGC	60	-	Aubert et al., 2006
TE002L09	Ps-CD860523	hAACGGCCACTTCTCCAATC TCAACACCACCAACACGAAC	60	-	Aubert et al., 2006
PsAS1	Ps-Y13321	GCATCCATCACGTCCGTGTGACG CGATGTCACTACGATAAGGGCTGC CATCAAAGGCCTTTCTTAGAATCC	55	-	Aubert et al., 2006
Gpt	Ps-AF020814	TGCACACACAATTGGACTTGTCG CTTACCTCAAGCTTCTACCTTGCC	62	-	Aubert et al., 2006
Eno12B	Ps-X57232	GAGGATCCTTACTAGTATAAAACC CCTGAAACACATATACCAAAAGAC	55	NlaIII	Aubert et al., 2006
PepC	Ps-D64037	CCGTACCGATGAAATCAAGAGG GCATCCATCACGTCCGTGTGACG	62	BstBI	Aubert et al., 2006
PGK1	Ps-CD858627	mCCATGGGAGTGTTCGAGTTT CGACGGAATCTCCTCCTACA	60	-	Aubert et al., 2006
Bfruct	Ps-X85327	GGACCAATGAGATATGGAGG CTTGGTTGGCCAATTTACCGG	55	-	Aubert et al., 2006
Xyft	Ps-AF223643	GTGTTCCCAAACGATAAACCAATT CTTAAATGGCCAGAATTGAGATCC	55	-	Aubert et al., 2006
Cwi2	Ps-Z8339	CTGATCTTGTTAACTGGACCCC TAATCCATTCCCTTAGAAAAGGGCC	58	-	Aubert et al., 2006
IGN1	Lj-AB251640	WGTRGTGAARTTGCTTCTTGMTGC	58	AluI	Kumagai et al., 2007

		GTMACMAGRATTGCMGCCCAC			
Enol	At-AY150418	AGGATGACTGGGAGCACTATG CCAAGCTCCTCCTCAATTC	60	HpaII	Kuznetsova et al., 2005
MET2	AB176565	AACTGTGGTTGCGGTACTAGC TTATTCTATAACTCCAAAAGGGCG	58	RsaI	Zhukov et al., 2007
UDPgd	At-NM123294	TGGTGAAGATTTGCTGCATTGGTGC TCATGGATAGATCCCTCTGG	58	HinfI	Zhukov et al., 2007

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