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Project Report

Symbiosis and Defence in the Interaction of Plants with Microorganisms

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Abstract

In a TMR-network (Training and Mobility of Researchers) of the European Union about 30 young postdoctoral scientists were involved in the theme of this

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report, working in the 9 laboratories. The work covered the following aspects: 1. Molecular basis of competition, specificity and signalling in symbiosis; 2. The rhizobia infection process and triggering of defence reactions; 3. Membrane targeting, defence reactions and symbiosis specific gene expressions; 4. Biological control with rhizosphere bacteria and arbuscular mycorrhiza fungi. The following review reports on major achievements of these broad topics.

Keywords: Competition, infection, defence reactions, membranes, biological control

1. Molecular Basis of Competition, Specificity and Signalling in Symbiosis

The objectives in this subtopic were concentrated on the biosynthesis and functions of low and high molecular weight components as specific amino acids, lipopolysaccharides, new nodulins and the cytoskeleton for symbiosis development in legume nodules. The study of saprophytic rhizobia in nodules was a connecting point to the second subtopic.

A plasmid-borne locus in Rhizobium etli KIM5s is involved in lipopolysaccharide O-chain biosynthesis and nodulation of Phaseolus vulgaris

Derivatives of *Rhizobium etli* KIM5s that were mutagenized with mTn5SS*gusA*30 resulted in the identification of strain KIM-G1. Its rough colony appearance, flocculation in liquid culture, and Ndv^- Fix^- phenotype were indicative of a lipopolysaccharide (LPS) defect. Electrophoretic analysis of cell-associated polysaccharides showed that KIM-G1 produces only rough LPS. Composition analysis of purified LPS oligosaccharides from KIM-G1 indicated that it produces an intact LPS core trisaccharide (α-D-GalA-1→4[α-D-GalA-1→5]-Kdo) and tetrasaccharide (α-D-Gal-1→6[α-D-GalA-1→4]- α-D-Man-1→5Kdo), strongly suggesting that the transposon insertion disrupted a locus involved in O-antigen biosynthesis. Five monosaccharides (Glc, Man, GalA, 3-*O*-Me-6-deoxytalose, and Kdo) were identified as the components of the repeating O unit of the smooth parent strain, KIM5s. Strain KIM-G1 was complemented with a 7.2-kb DNA fragment from KIM5s that, when provided in trans on a broad-host-range vector, restored the smooth LPS and the full capacity of nodulation and fixation on its host *Phaseolus vulgaris*. The mTn5 insertion in KIM-G1 was located at the N terminus of a putative alpha-glycosyltransferase, which most likely had a polar effect on a putative beta-glycosyltransferase located downstream. A third open reading frame with strong homology to sugar epimerases and dehydratases was located upstream of the insertion site. The two glycosyltransferases are strain specific, as suggested

by Southern hybridization analysis, and are involved in the synthesis of the variable portion of the LPS, i.e., the O antigen. This newly identified LPS locus was mapped to a 680-kb plasmid and is linked to the lpsbeta2 gene reported for *R. etli* CFN42 (Vinuesa et al., 1998).

Rhizobium tropici CIAT899 leuA mutants altered in their symbiotic interaction with the hosts Phaseolus vulgaris and Leucaena leucocephala

There is a link between amino acid biosynthesis and nodulation ability in rhizobia with tryptophan (*trpB*) and methionine (*metz*) biosynthesis mutants of *Rhizobium etli* being altered in their Nod factor profiles and unable to colonize *Phaseolus vulgaris* nodules.

The creation of leucine biosynthesis (*leuA*) mutants from *R. tropici* CIAT899 and their characterization in terms of leucine auxotrophy, and their inability to form determinate and indeterminate nodules on *P. vulgaris* cv. Saxa and *L. leucocephala* cv. Cunningham, respectively was studied.

An internal fragment of the *R. tropici leuA* gene was PCR amplified using degenerate primers and cloned into an integrative plasmid for mutagenesis of the locus. Sequence and homology analysis confirmed the identity of the 550 bp gene fragment, with highest similarity (86% identity) to *leuA* from *Sinorhizobium meliloti* (Sanjuan, unpublished). The resultant mutants were tested on minimal medium at pH 6.8 and pH 5.5 with and without supplemented leucine (50 mg/l). All the mutants were strict leucine auxotrophs displaying no growth in medium without leucine and growth comparable with the wild-type in medium with leucine. The mutants could be divided into 2 groups dependent on their sensitivity to pH. Some were repairable with leucine and growth was not dependent on the pH of the medium. Others could only grow in medium at pH 5.5 in the presence of leucine, and could not grow in the same medium at pH 6.8.

The mutants were tested for their ability to nodulate *Phaseolus* and *Leuceana* plants. In both cases only the wild-type induced nodule formation. On mutant inoculated plants there were pseudonodules on the roots, at junctions of side roots, which were much smaller than the wild-type nodules, and no rhizobia could be recovered from them (Peick et al., 1999; Steele et al., 1999a, 2001).

Role of cytoskeleton

The plant cytoskeleton plays a major role during nodule ontogeny. Using *in situ* immunolocalisation methods, microtubular cytoskeleton (MtC) architectural changes were found during early developmental stages, both in

the inner plant tissues where the nodule forms (pericycle, inner cortex), and in the outer tissues where infection takes place (epidermis, outer cortex). Based on these changes a model was proposed, which aims to better define the timing of the early symbiotic stages. This model (i) suggests the existence of two opposing gradients of differentiation controlling respectively the formation of division centers in the inner cortex and plant preparation for infection and (ii) implies that the infection process proceeds prior to the formation of the nodule primordium-derived meristem. Major MtC changes also occur during nodule differentiation: a transient disorganization of MtC in zone II where bacteria are released, is followed by a newly-patterned reorganization in zone III, the nitrogen-fixing zone. These cytoskeletal changes take place concomitantly with cellular differentiation and are correlated with Nod factor internalization, suggesting that Nod factors are possibly involved in the control of cytoskeleton-directed cell differentiation. Cytoskeletal architecture in planta in transgenic *Medicago truncatula* carrying cytoskeleton-binding protein/GFP constructs are currently being studied.

MtN1 and MtN13 nodulin genes

MtN1 and *MtN13* are two nodulation-specific nodulin genes structurally related to proteins associated with plant defense reactions. *MtN1* is associated with the infection process, while *MtN13* is expressed in the nodule cortex. These nodulins may play (i) a protective role to prevent pathogenic infections of the root nodule and/or (ii) a developmental role. The possibility that *MtN1* represents a defense protein turned on by the infection process should not be excluded. To find out more about their respective roles plants were transformed with appropriate sense and anti-sense gene constructs. The analysis of these lines is in progress (Gamas et al., 1998; Timmers, 2000; Timmers et al., 1998, 1999).

Saprophytic rhizobia in nodules

Prior to this study, proximal zone IV of indeterminate nodules where symbionts undergo simultaneous degeneration was considered to be the end point of the symbiotic interaction. However, it was found that a novel zone proximal to zone IV, namely zone V, develops in nodules more than six weeks old. In zone V, a new round of bacterial release occurs leading to the reinvasion of senescent central cells. Such intracellular bacteria multiply and do not differentiate into nitrogen-fixing bacteroids. Oxygen is available in zone V at a concentration compatible with both the development of rhizobia and the expression of nitrogen fixation genes. However, this gene expression is not associated with

nitrogen fixation. Zone V appears to be an ecological niche where intracellular rhizobia take advantage of the interaction for their exclusive benefit as saprophytic partners, thereby providing support for the notion that rhizobia/legume interactions are indeed symbiotic, with benefits to the two partners occurring at different developmental stages (Timmers et al., 2000).

2. The Rhizobia Infection Process and Triggering of Defence Reactions

This subtopic had as major objectives the analysis of less known interactions such as *Rhizobium galegae*, *Sinorhizobium arboris* and *Sinorhizobium kostiense* with their respective host plants and also less studied reactions from the plant side such as new defence genes and avirulence genes in transgenic plants. Signalling between the microsymbionts and the host plants by flavonoids was a connecting aspect.

The first part of the project concerned the origin and evolution of the molecular interactions between rhizobia and their legume counterparts, which can be surmised from studies on extant organisms. During the early stages of the interaction, a molecular dialogue develops by the partners that leads to specific mutual recognition, induction of nodule organogenesis and an infection process. Tracing the molecular evolutionary process of the *Rhizobium*-legume association, with particular emphasis on the specificity of the cross talking of this interaction is difficult, if not impossible. This is because varying degrees of specificity that range from very narrow associations, for instance between *Azorhizobium* and *Rhizobium galegae* with their respective hosts, to highly promiscuous *Sinorhizobium* sp. NGR234 infecting several hosts from different tribes complicate the molecular approach. In addition, studies have shown that factors such as gene duplication, lateral transfer and recombination, among others, have shaped the molecular machinery.

This work started from the report of the organization of the two symbiotic regions of *R. galegae*. The first one, pRg30 contains the common *nod* genes with an organization similar to the *S. meliloti* and *R. leguminosarum* operons. The second one, pRg10 contains different genes mostly related to nodulation or nitrogen fixation. Further sequence analysis revealed that in addition to symbiotic genes, both regions contain sequences with high degree of similarity (over 85%) to insertion elements identified in *Agrobacterium* or *Rhizobium* species. Their localization at the ends of the symbiotic regions suggests a possible lateral transfer to or from *R. galegae*. The GC% of the pRg10, however, is not significantly different outside or inside the region delimited by the two insertion sequences. About 50 kb of DNA from the symbiotic regions of *R.*

galegae has been sequenced in collaboration with the Institute of Biotechnology, University of Helsinki.

Comparative phylogenies of ribosomal and nodulation genes were performed from these sequences to help to explain if this factor has contributed to the symbiosis evolution. Phylogenies inferred from 16S rRNA sequences show a great diversity among the rhizobia, and non-symbionts from other genera are found mixed with the various lineages of rhizobia. In general, this phylogeny is very similar to the one inferred from the large subunit ribosomal sequences (23S rRNA). However, comparison of phylogenies from 16S sequences and the *nodA* gene does not show significant correlation. Thus, the phylogeny of common genes in a lineage does not usually reflect organism phylogeny, and they can tell very different stories. Studies have shown that the nodulation genes may have been recruited from diverse ancestral functions.

To gain information on plant-bacterial coevolution, the phylogeny of *nodA* and the ITS2 (Internal Transcribed Spacer) sequences from the major legume families were compared. Two major groups are apparent from the phylogeny of *nodA*, with *A. caulinodans* as their distant relative. One of the groups clusters all the rhizobia producing Nod factors with polyunsaturated fatty acid, i.e. *S. meliloti*, *R. leguminosarum* bv. *viciae* and *trifolii* and *R. galegae*. Interestingly, the phylogeny of major legumes from the ITS2 sequences generates a tree where all the host plants to the rhizobia producing Nod factors with these specific fatty acids also appear to have a common ancestor.

From an evolutionary point of view, rhizobia species producing polyunsaturated Nod factors seem to follow a specific nodulation strategy, where the main origin of the diversity that allows adaptation to the host plant relies on the nature of the fatty acid. The specific acylation of the Nod factors by fatty acids, polyunsaturated or not, is dependent on the NodA protein. Thus, the identical phylogenetic trees obtained with the *nodA* rhizobial gene and the host plant ITS sequences strongly suggest that this adaptation is governed by functional constraint of host plant. From this result, it was developed a model of a pair wise "coevolution" of the *nodA* gene and the host plant was developed that can be an example of gene-organism association. Study of the function of putative proteins encoded by the open reading frames in the symbiotic region was also attempted. Antiserum against a putative ABC transporter was produced by immunizing with a synthetic peptide the structure of which was deduced from the DNA sequence. When tested against induced and uninduced bacterial and also nodule extract, the antiserum unfortunately turned out to be insufficiently, when used in western blots of 2D gels.

In the second part of the project, the effect of abiotic stresses on the production of Nod factors by tropical strains was investigated using *Sinorhizobium arboris* and *S. kostiense* isolated from leguminous trees. Strains

overproducing Nod factors were constructed by introduction of a multicopy plasmid harboring an exogenous *nodD* gene. Thin Layer and High Performance Liquid Chromatography were used to estimate the level of production of the different molecules that were detected by autoradiography and UV spectroscopy respectively. The relation of Nod factor metabolism to the general metabolism was investigated by estimating the number of total and culturable bacterial cells and by analyzing the cell shape by epifluorescence microscopy.

S. arboris and *S. kostiense* have a very similar host range, and, under non-stress conditions, their Nod factor profiles are very similar either by TLC or by HPLC, with two major peaks. It is therefore probable that the two strains produce a family of molecules with similar structural features. Osmotic, salt or heat stress alters the Nod factor profiles, and the responses of the two strains are globally similar, whichever the stress is involved. Both TLC and HPLC analyses showed a decrease in the proportion of the most hydrophobic peak compared to the most hydrophilic one. *S. arboris* nevertheless seemed to tolerate harsher conditions better than *S. kostiense*. In addition to the alteration of the Nod factor profiles, the stress conditions lead to a decrease in the cell viability associated with non-typical shapes.

The relevance of the alteration of the Nod factor profile under stress conditions will now be further studied with the purification of the different molecules by HPLC. Both structural characterization and biological tests should give more precise information about the symbiotic behaviour of these strains under abiotic stress conditions (Nowak, 2000; Radeva et al., 2001; Suominen et al., 1999, 2001; Tas and Lindström, 2000, 2001; Terefework et al., 1998, 2000; Yang et al., 1999)

New defence genes and early nodulins in Trifolium repens

A new defence peroxidase cDNA, *Trprx2*, was isolated from *Trifolium repens* roots via differential display reverse transcription PCR. This gene encodesis a putative extracellular or membrane-bound cationic class III peroxidase and yields with a full length cDNA sequence of 1.31 Kb. Extensive quantitative RT-PCR temporal expression assays in whole roots subjected to a variety of inoculation treatments revealed low levels of constitutive expression and no discernable up or down regulation in symbiotically challenged roots. When roots were inoculated with a compatible pathogen, *Pseudomonas syringae*, there was a dramatic reduction in peroxidase expression after only 1 hour and a complete absence of expression in whole root tissue up to seven days post-inoculation (Crockard et al., 1999). It is possible that localised down regulation of *Trprx2*, detectable by *in situ* hybridization, is also a feature of a successful interaction of this host plant with its rhizobial microsymbiont.

Using degenerate primers designed from regions of homology in previously published sequences, two new class III chitinases were identified in white clover roots. Full length cDNAs of each product were ca. 1.1 Kb. Both products contain conserved amino acid motifs characteristic of regions I and II of class III chitinases. RT-PCR expression assays of one product, *Chit1*, indicated temporal patterns in whole root tissue very similar to *Trprx2*, once again with complete elimination of expression in roots inoculated with pathogenic *Pseudomonas syringae*. Chitinases are thought to play an important role in the regulation of root infection by rizobia, either through degradation of their Nod factors or lysis of bacterial cells.

Two new early nodulins, *dd11* and *dd23b*, were also isolated from white clover by DDRT-PCR. Neither displayed significant homology with any published sequences and both demonstrated typical early nodulin gene expression patterns during RT-PCR assays: an absence of expression in sterile roots, expression within hours of inoculation with *Rhizobium leguminosarum* biovar *trifolii* and strong expression in root nodules. White clover homologues of several other early nodulin genes (eg. *Enod5*, *Enod8*, *Enod40*) have also been isolated as full length cDNAs and their temporal and spatial expression patterns in roots and root nodules have been obtained (Crockard et al., 2001).

Flavonoids in Lotus pedunculatus

In a collaboration between the TMR partners J.E. Cooper (Belfast) and D. Werner (Marburg) the first characterization of flavonoids in the epidermal exudates of any *Lotus* species was obtained. Emphasis was placed on diode array detection for collecting UV spectra that could be compared with spectra from authentic compounds in a user-generated reference library. Reconstituted seed and root exudate samples were first subjected to pre-separation by HPTLC-UV, followed by capillary zone electrophoresis-spectral array analysis (CZE-UV) and GC-MS. The principal compounds detected in seed exudates were: catechin, quercetin, several quercetin glycosides, naringenin and kaempferol. Root exudates contained most of these compounds plus rhamnetin, isorhoifolin and hesperidin. Whether some of the identified compounds act as *nod* gene inducers in *Lotus* rhizobia remains to be determined. The inability of most of them to induce *nod* genes of *Mesorhizobium loti* has been established but bacteria from the genera *Bradyrhizobium* and *Rhizobium* also nodulate this host and they have not yet been tested in *nod* gene induction assays.

This work has also identified several other candidate compounds for this function (Steele et al., 1999b, 2000). The feedback effect of the microsymbionts on flavonoid excretion by the host plants has been studied with beans nodulating rhizobia (Bolaños-Vasquez and Werner, 1997).

Avirulence genes and transgenic plants

In the first part of the project the work has been focussed on the characterisation of an avirulence locus in *R. leguminosarum* biovar *trifolii* bacteria. When the Sym plasmid of the test strain (LPR5020) was replaced by that from a *R. leguminosarum* biovar *viciae* strain, the resulting strain (called RBL5523) was not able to induce nitrogen fixing root nodules on pea plants (Roest et al., 1997). A locus not localized on the Sym plasmid was previously identified by the Leiden group by screening for mutants which enabled the ineffective bacteria to induce nitrogen fixing root nodules on pea plants. The locus was termed IMP (for impaired pea nodulation). A large cosmid library fragment (20 kb) was sequenced so that it was now possible to identify all other genes in the locus. Homologies of the open reading frames in this region indicate that the IMP genes are part of a type III secretion system. It was shown that the IMP locus is involved in negatively influencing infection thread development. Furthermore, an experimental test system was set up using various colour variants of the green fluorescent protein. This system was shown to be of general usefulness for studying the role of factors involved in symbiosis and defence. The results show that the CFP and YFP proteins can be efficiently used to monitor infection of the host plants. For following infection new bacterial vectors were constructed which are maintained very stable during all stages of symbiosis. Using this system it was shown that the avirulent strain RBL5523 can not be rescued by the IMP mutant during pea nodulation. This result was corroborated by showing that the growth medium of strain RBL5523 was able to negatively influence nodulation efficiency on pea plants also indicating that this strain produces a secreted avirulence factor. The second part of the project was focused on obtaining transgenic plant lines which contain reporter constructs for detecting symbiotic responses. *Lotus japonicus* was used as a model system for these studies. These lines were needed to further analyze, at the molecular level, the response to invading bacteria and to distinguish proper symbiotic responses from aberrant responses induced by avirulence factors. In collaboration with the group of Katinakis, the role of ENOD40 in symbiosis was studied (Flemetakis et al., 2000). The results show that this model of *L. japonicus* contains two distinct *ENOD40* genes that are expressed in symbiotic, nonsymbiotic and embryonic tissues. The *ENOD40* promoter was fused to a reporter gene construct consisting of the *gfp* and *gus* gene (Quaedvlieg et al., 1998) which have been developed recently as a sensitive and vital bifunctional reporter in plants. Transgenic *L. japonicus* lines containing this construct showed a clear response to Nod factors and rhizobia within 8 hours of inoculation. Interestingly, plant lines which contained more than one integration of the reporter construct showed no background expression in the absence of Nod factors which is otherwise present in the vascular system in the single integration

lines. Since vascular expression is also detected with the *in situ* experiments (Flemetakis et al., 2000) it was concluded that the double integration disturbs normal expression of this promoter yielding a reporter line which is more useful for studying effects of Nod factors than the more natural expression lines, in particular for detecting the differences in response with rhizobial strains containing different Nod factor modifications (Pacios Bras et al., 2000). The results show that the NolL protein, which is involved in the acetylation of a fucosyl residue on the Nod factors of *M. loti*, is an important determinant of host specificity. Apparently the NolL protein determines a difference between a symbiotic response, with normal infection threads, and a response characterized by poor infection leading to non-functional nodules, in which ENOD40 is aberrantly expressed.

In addition to reporter lines obtained by choosing known promoters, reporter lines as a result of a random promoter trapping approach were obtained. In general, promoter trapping provides a versatile tool to identify and clone genes that exhibit differential spatial and/or temporal expression during development. In addition to the cloning utility that a sequence tag provides, promoter-trapping offers the additional advantage that it does not necessarily rely on the creation of mutant phenotypes to successfully tag tissue-specific or signal-elicited genes; functional gene fusions can be generated which allow the detection of tagged cis-acting regulatory elements in a natural context and without disrupting gene function. Analysis for GUS expression of the transformants from a pilot experiment in which 130 independent transformants were generated showed that approximately 10% confer nodule specific or nodule enhanced expression. Of particular interest was line Lj2184.145 which showed low levels of reporter gene expression in the roots and a characteristic strong expression pattern in the nodule. These results make it attractive to undertake large scale promoter-trap approaches in this model legume aimed at further understanding the differences in symbiotic and defence responses (Diaz et al., 2000; Geiger et al., 1998; Olsthoorn et al., 1998, 2000; Ovtsyna et al., 1998, 1999, 2000; Spaink, 1999, 2000).

3. Membrane Targeting, Defence Reactions and Symbiosis Specific Gene Expressions

The objectives in this subtopic concentrated on later stages of symbiosis development. New symbiotic functions of signal peptidases in *Bradyrhizobium japonicum*, lipoxygenase and oxalate oxidases in pea nodules and another set of plant nodulins were studied, connecting this subtopic to the previous one.

Identification of putative signal peptidase substates in Bradyrhizobium japonicum

Two strains, 132 and 184, have been isolated from *Bradyrhizobium japonicum* 110*spc*4 after Tn*phoA* insertion. The gene disruptions occurred in two distinct genes encoding signal peptidases. Their functions were demonstrated by complementing the temperature sensitive lap of *E. coli* strain IT41 (Bairl and Müller, 1998). In symbiotic association with *Glycine max,* these mutant strains of *B. japonicum* present an altered symbiotic phenotype: acetylene reduction is decreased and bacteroids are collapsed in differentiated nodules. Moreover in soybean nodules infected by the strain 184, the pattern of symbiosome proteins is altered: the concentration of a 53 kDa protein is reduced in mutant nodules. The corresponding soybean cDNA sequence was cloned and exhibits no significant similarity to other sequences. However, the gene expression shows that it is also reduced in mutant nodules and therefore represents a nodulin gene (Winzer et al., 1999). These different phenotypes are probably caused by deficiencies in protein maturation of the microsymbiont during nodule organogenesis. Based on this assumption, several experiments were started in order to identify and isolate such signal peptidase substrates (or preproteins). Methodologies have been developed, using bacterial liquid cultures for isolating proteins contained in cytosol, membranes, periplasm and cell-free medium. These different cell fractions have been analysed by gel electrophoresis.

Symbiosome differentiation

A 1.6 Kb upstream sequence for *PsNlect1*, the lectin-like glycoprotein from pea nodules which is targeted into the symbiosome compartment, was characterized. The gene was localised to the pea RFLP map, demonstrating very close linkage to bud lectin (<10 Kb) and close linkage to pea seed lectin. A promoter-fusion was constructed with glucuronidase (*gus*) in order to monitor gene expression in transgenic *Vicia* and *Medicago* together with sense and antisense modules in order to knock out gene expression in transgenic peas. Expression of the glycoprotein is associated with nodule transfer cells (Dahiya et al., 1998; Dahiya and Brewin, 2000) and mycorrhizal symbiosis (Balestrini et al., 1999). High turnover of this glycoprotein in the symbiosome compartment is probably associated with a cysteine protease recently characterised in this laboratory (Vincent and Brewin, 2000; Vincent et al., 2000a, b). Also a novel bacterial inositol P-3 kinase was discovered (Drobak et al., 2000).

Lipoxygenase /oxalate oxidase

A study of pea nodule lipoxygenases was published (Wisniewski et al., 1998). The work was extended to include an oxalate oxidase activity recently identified in pea root extracts. This is the first well characterised report of an OxOx activity in dicotyledonous plants. In cereals, OxOx proteins are "germins" and it was demonstrated that antiserum raised against barley OxOx cross-reacts with a 30 Kd monomer from pea root extracts. A cDNA corresponding to a germin-like protein from peas was isolated and sequenced. It was hypothesised that this enzyme could serve as a local source of peroxide for cross-linking of plant extensins in the extracellular matrix. An extensin-like glycoprotein was identified as a major component of the infection thread matrix in all legumes (Brewin et al., 2000). A peroxide-driven hardening of extensin (by cross-linking of tyrosine residues) could be an important factor governing the biophysics of infection thread growth (Wisniewski et al., 1999, 2000).

ENOD40 nodulin gene

ENOD40, an early nodulin gene, has been postulated to play a significant role in legume root nodule ontogenesis. Two distinct *ENOD40* genes from *Lotus japonicus* were isolated. The transcribed regions of the two *ENOD40* genes share 65% sequence identify, while the two promoters showed no significant similarity. Both transcripts encode a putative dodecapeptide similar to that identified in other legumes forming determinate nodules. Both *ENOD40* genes are co-ordinately expressed following inoculation of roots with *Mesorhizobium loti* or treatment with purified Nod factors. In the former case, mRNA accumulation could be detected up to 10 days following inoculation while in the latter case the accumulation was transient. High levels of both *ENOD40* gene transcripts were found in non-symbiotic tissues such as stems, fully developed flowers, green seed pods and hypocotyls. A relatively lower level of both transcripts was observed in leaves, roots and cotyledons. *In situ* hybridization studies revealed that in mature nodules transcripts of both *ENOD40* genes accumulate in the nodule vascular system; additionally, in young seed pods strong signal was observed in the ovule, particularly in the phloem and epithelium, as well as in globular stage embryos (Flemetakis et al., 2000).

Carbonic anhydrase

A full length cDNA clone encoding carbonic anhydrase (CA) was isolated from a soybean nodule cDNA library. *In situ* hybridization and

immunolocalization were performed in order to assess the location of CA transcripts and protein in developing soybean nodules. CA transcripts and protein were present at high levels in all cell types of young nodules, whereas in mature nodules they were absent from the central tissue and were concentrated in cortical cells. In parallel, sucrose metabolism was investigated by examining the temporal and spatial transcript accumulation of SS and PEPC genes using *in situ* hybridization. In young nodules, high levels of SS gene transcripts were found in the central tissue as well as in the parenchymateous cells and the vascular bundles, while in mature nodules, the levels of SS gene transcripts were much lower, with the majority of the transcripts being located in the parenchyma and the pericycle cells of the vascular bundle. High levels of expression of PEPC gene transcripts were found in mature nodules, in almost all cell types, while in young nodules lower levels of transcripts were detected with the majority of them being located in parenchymateous cells as well as the vascular bundle. Immunogold cytochemistry indicated that, in mature nodules, CA is localized in the cytoplasm of the inner cortical cells and the cell wall of the endodermal cells (Kavroulakis et al., 2000).

A novel nodulin gene encodes a homologue to the mammalian selenium binding protein (SBP)

A novel *Lotus japonicus* nodulin *Lj*SBP and the corresponding gene (*Ljsbp*) were isolated and characterized, whose predicted amino acid sequences show striking homology to the mammalian 56 kDa selenium binding protein (SBP). Homologous cDNA clones were also isolated from soybean, *Medicago sativa* spp. *Falcata* and *Arabidopsis thaliana*. RT-PCR and Northern blot analysis indicated that *sbp* gene transcripts are present in differing abundance in various tissues of *Lotus japonicus* and *A. thaliana*. However, in *L. japonicus* nodules and seedpods and *A. thaliana* siliques the transcript levels appear to be dependent on the developmental stage. *In situ* hybridization studies showed that, in young nodules, *sbp* gene transcripts are located in infected cells and vascular bundles, while in mature ones, low levels of *Lj*SBP mRNAs were detected only in the parenchymatous cells. Expression of *sbp* gene transcripts in young seedpods and siliques, was clearly visible in vascular tissues and embryos, while in mature ones low levels of expression were detected in root epidermis and vascular bundles. A polyclonal antibody raised against a truncated *Lj*SBP recombinant protein recognizes a polypeptide of about 60 kDa. Immunohistochemical experiments showed that accumulation of *Lj*SBP protein was largely restricted to infected cells of young nodules, while lower levels were observed in the infected cells of mature ones (Flemetakis et al., 2001).

4. Biological Control with Rhizosphere Bacteria and Arbuscular Mycorrhiza Fungi

Research in the direction of potential applications of symbiosis and defence was a major objective of this subtopic, using rhizosphere bacteria such as *Pseudomonas* strains and arbuscular mycorrhiza fungi. This use of mutualistic and pathogenic fungi should also lead to a better understanding of the molecular basis of the differences between pathogens and symbionts, between "foes" and "friends".

The impact of Pseudomonas strains used as inoculants for biocontrol of soil-borne fungal pathogens on arbuscular mycorrhiza formation

The arbuscular mycorrhizal (AM) symbiosis, a key component in agroecosystems (Barea, 2000), has been assayed as rhizosphere biosensor for the evaluation of the impact of certain antifungal *Pseudomonas* inoculants used to control soil-borne plant pathogens. Two *Pseudomonas* strains were tested: a wild type (F113) producing the antifungal compounds 2,4-diacetylphloroglucinol (DAPG) and F113 (pCU203), a DAPG-overproducer. These biocontrol agents did not interrupt spore germination of the AM fungus *Glomus mosseae* nor mycorrhiza formation, by either *G. mosseae* or natural endophytes, in soil microcosms. In a field experiment, none of these *Pseudomonas* strains affected ($P<0.05$): (i) number and diversity of AM fungal populations; (ii) the percentage of root length that became mycorrhizal, (iii) AM performance. Furthermore, the antifungal *Pseudomonas* improved plant growth and nutrient (N and P) acquisition by mycorrhiza (Barea et al., 1998).

Local vs systemic effect of AM (arbuscular mycorrhiza) symbiosis on defence responses to fungal plant pathogens

Research was then focused on the comparison between the plant defence response against mutualistic (*Glomus* spp., AM fungi) and pathogenic (*Phytophthora parasitica*) fungi, looking for the underlying mechanisms of bioprotection exerted by the AMF (Pozo et al., 1998). A greater resistance/tolerance to *Phytophthora* attack induced in tomato plants by root colonisation with AMF was confirmed. The protective effect is exerted at a systemic level. Both local and systemic defence reactions at cellular level were evidenced (Cordier et al., 1998). Research was then focused on the pathogenesis-related proteins (PRs) chitinases, chitosanases and α-1,3-glucanases (Pozo et al., 1999). Enzymatic analysis after PAGE revealed that new isoforms of these enzymes were induced during the symbiotic interaction showing a possible role in the protective effect promoted by AM colonization.

New isoforms are locally induced in roots colonised by the AM fungi, and some quantitative changes in the activity level of some isoforms occur systemically. Thus, bioprotection by AM fungi seems to occur by a combination of local and systemic effects. The results obtained in these studies point to α-1,3-glucanases as the best candidates to be key in the local reduction of *Phytophthora* inside the roots by direct lysis of the pathogen cell wall. Similar results have been obtained in olive tree plants with regard to *Verticillum* attack.

To further understand the biological role of the differentially induced isoforms of these enzymes molecular studies are carried out on the induction of α-1,3-glucanases in tomato plants, either wild-type (mycotrophic) or mutants unable to establish the AM symbiosis. Up to now, using a RT-PCR approach, a new α-1,3-glucanase gene (EMBL accession number AJ278743) from mycorrhizal tomato roots has been cloned and the gene expression of all identified tomato α-1,3-glucanases during the symbiotic and/or pathogenic interaction was studied.

Additional results support a suppressive effect against the nematode population by pre-mycorrhization of tomato. Not only interactions between the two micro-organisms within roots but also an antagonistic activity of *G. mosseae* on nematode life cycle in soil were demonstrated.

Molecular approaches have been applied to study interface ATPases in AM symbioses (Ferrol et al., 2000).

Interactions of *Rhizobium* and AM fungi in non-legumes showed interesting PGPR effects of *Rhizobium* on mycorrhizal plants (Galleguillos et al., 2000).

Genetic characterisation and regulation of phloroglucinol genes of Pseudomonas fluorescens F113 strain involved in biocontrol interactions

The rhizosphere competent *Pseudomonas fluorescens* strain F113 produces a secondary metabolite, 2,4-diacetylphloroglucinol (PHL), known to be responsible for biocontrol activity towards the pathogenic fungus *Pythium ultimum*, the causal agent of damping off in sugarbeet. Research within the TMR project has led to the identification of the genetic locus responsible for the biosynthesis of PHL and its repressor gene. The involvement of GacA/GacS two component regulatory system and *prrB* (formerly *lgcA*) in the regulation of PHL production was also established.

A 6 Kb DNA fragment contained in pCU203 was able to restore PHL production to F113G22, the PHL non-producing mutant as well as to many *Pseudomonas* strains which normally do not produce PHL. This DNA fragment was fully sequenced on both strands. Sequence analysis revealed six open reading frames (ORFs): PhlA, PhlC, PhlB, PhlD, PhlE and PhlF. PhlA-E transcribed as a single operon encoding the biosynthetic genes, whereas phlF, transcribed in the other direction encodes a transcriptional repressor of the

phlA-E operon. It is believed that PhlD acts to condense acetyl-CoA and malonyl-CoA precursors to form monoacetylphloroglucinol (MAPG), whereas PhlACB are responsible for MAPG acetyltransferase activity for the acetylation of MAPG to PHL and *phlE* encodes for permease. It is known that carbon source influences PHL biosynthesis. It was found that sucrose and glucose induce the biosynthesis of PHL, whereas succinate represses this biosynthesis. Carbon source modulation of the PHL biosynthesis has been analysed at the transcriptional level by the construction of several *lacZ* transcriptional fusions. Using *phlF-lacZ* and *phlA-lacZ* fusions it was demonstrated that succinate represses PHL biosynthesis by increasing the expression of *phlF*, and conversely sucrose enhances PHL by repressing *phlF*. Thus carbon source regulates PHL production at the transcriptional level via *phlF* which acts as a repressor. It was also shown in this study that the nitrogen sources has a clear effect on PHL biosynthesis. The repressor function of PhlF has been confirmed by disruption of the *phlF* gene. The resulting mutant (F113IS) displays an increased phlA expression and PHL production during both the lag phase and logarithmic phase. Further analysis of PhlF function using a gelshift approach showed that the PhlF physically binds to a DNA region between *phlA* and *phlF*. These findings are in good agreement with the presence a helix-turn-helix at the N-terminal region of the PhlF protein.

Primer extension analysis revealed that the *phlA* transcription start is located at exactly -365 in the *phlA-phlF* intergenic region. This location is far beyond the PhlF binding region. It was not possible to show any primer extension product when primers specific to *phlD* were used showing that there is no transcription from the *phlB-phlD* intergenic region. These results and those obtained by complementation experiments confirmed that *phlACBD* is an operon and not a gene cluster.

PHL biosynthesis and *phlF* are under positive control of the GacS/GacA two component regulatory system. *gacS*$^-$ and *gacA*$^-$ mutants produce no PHL. Transcription of *phlA* and *phlF* is greatly reduced in *gacS*$^-$ and *gacA*$^-$ mutants background. This clearly shows that the GacS/GacA system regulates PHL biosynthesis and the *phlF* repressor gene mainly at the transcriptional level.

A locus complementing the regulatory mutants *gacS*$^-$ and *gacA*$^-$ was isolated. Sequence analysis of this locus, *prrB*, does not show an obvious open reading frame. However, the transcribed sequence, PrrB, folds in a similar manner to *CsrB* (*E. coli*) and *RsmB* (*Erwinia carotovora*), which are known regulatory RNAs. These observations strongly suggest that PrrB has a regulatory role in F113. The *prrB* gene is positively regulated by GacS/GacA as shown by Northern analysis.

In conclusion the locus responsible for PHL biosynthesis was characterised and its regulation by PhlF, the pathway specific repressor, and by the two component regulatory system was analysed. A new gene coding for regulatory

RNA was also characterised and its regulation was analysed. During the course of this project, results were also obtained to show that the biocontrol strain F113 can control many different pathogens like *P. ultimum, E. carotovora* and *G. rostochiensis* (Aarons et al., 2000; Cronin et al., 1997a, b; Delany et al., 2000; Dunne et al., 1998; Moenne-Loccoz et al., 2001). Two specific DNA probes have recently been identified. These probes are used to discriminate between antagonistic and non antagonistic strains (Redecker et al., 1999).

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