Expression of the *Sinorhizobium meliloti* C_4-dicarboxylate transport gene during symbiosis with the *Medicago* host plant.

Bert Boesten

Promotor: dr. Ab van Kammen, em. hoogleraar in de moleculaire biologie

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The investigations described in this thesis were carried out between juli 1995 and juli 1997 in the laboratory of Prof. Pierre Boistard at the laboratoire de Biologie Moléculaire des Rélations Plantes-Microorganisms INRA-CNRS, Toulouse, France. The work was financially supported by a grant from the Institute Nationale des Récherches Agronomiques (INRA), France.

Stellingen:

1) The alternative mechanism of symbiotic activation (ASA) was postulated for *Rhizobium leguminosarum* despite earlier results, which gave no evidence of any symbiosis-specific regulation of the *dct*A gene
(Ronson, C.W. and Astwood, P.M. 1985. Nitrogen fixation research progress. Bottomley, P.J. and Newton, W.E. eds. Martinus Nijhoff, Dordrecht. 201-207.
Ronson, C.W., Astwood, P.M., Nixon, T.B. and Ausubel, F.M. 1987. Nucl. Acids Res. 15, 7921-7934)

2) It is unlikely that the alternative mechanism of symbiotic activation (ASA) in *Sinorhizobium meliloti* has a significant influence on the crop yield of *Medicago sativa*.
(Rastogi *et al.*, 1992. Can. J. Microbiol. 38, 555-562.)

3) The use of *Medicago truncatula* as a model plant in studying the *Rhizobium*-legume interactions may leave the functioning of the bacteria in the symbiosis underexposed.

4) The conclusion that NifA is not involved in the expression of the *dct*A gene during the symbiosis of *Sinorhizobium meliloti* and *Medicago sativa*, is not contradictory to the finding that NifA is essential for the alternative mechanism of symbiotic activation (ASA).
(Jording *et al.*,1992, J. Plant Physiol. Vol. 141:18-27)

5) The hypothesis that the endodermis of indeterminate nodules functions as an oxygen barrier should not be accepted as an established fact without support of solid experimental data
(Hunt and Layzell, 1993, Ann. Rev. Plant Physiol. Plant Mol. Biol., 44:483-511).

6) Research on the potential risks associated with the release of genetically engineered microorganisms (GMO's) should be more concerned with the conditions that provide a positive selective advantage to potential harmful microorganisms, than with lateral transfer of genes in the environment.

7) Conclusions in a scientific paper should always be treated with reservation. Additional information may shed a different light on earlier results.

8) Applying age limits in grant applications is a form of discrimination and therefore illegal.

9) The creative thinking of a scientist may be inhibited by reading scientific publications.

Bert Boesten:

Expression of the *Sinorhizobium meliloti* C_4-dicarboxylate transport gene during symbiosis with the *Medicago* host plant.

Wednesday September 8 1999,
Agricultural university Wageningen.

Contents

Rhizobium spp. are gram⁻ soil bacteria that are able to induce the formation of specialised organs on their leguminous host plants which they can invade and where they can fix atmospheric nitrogen to ammonia. This fixed nitrogen is made available to the host plant, rendering it independent from the availability of nitrogen in the soil. The plant in turn provides an unique ecological niche to the microsymbiont. Symbiotic N_2-fixation is a high energy requiring process and this energy is derived from the host plant photosynthate. The bacteria remain at all times separated from the plant cell cytoplasm by a plant derived peribacteroid membrane. This membrane is the interface between the host plant and the endosymbiont. Not every available carbon source can pass the peribacteroid membrane. Early work on this subject has demonstrated that C_4-dicarboxylic acids (dCA) rather than succrose which is present in abundance in the nodule cytosol, are partitioned into the peribacteroid space. Consequently the ability of the microsymbiont to take up these dCA compounds is essential for the establishment of an effective symbiosis.

The uptake of dCA by *Sinorhizobiom melliloti* and some other *Rhizobium spp.* under free-living conditions is controlled by three genes: *dct*A coding for a high affinity dCA uptake permease, and a two-component regulatory system encoded by *dct*B and *dct*D. A mutation in the structural gene *dct*A , renders the mutants unable to transport dCA and therefore such mutants cannot use dCA as a carbon source. Such *dct*A mutants can invade their host-plants and induce the formation of nodules, but are unable to fix nitrogen.

A mutation in either of the regulatory *dct*BD genes also renders the mutants unable to take up dCA under free-living conditions, but generally such mutants establish an effective symbiosis when inoculated on their host plant. This latter phenotype suggests that the *dct*A gene is expressed efficiently during symbiosis even in the absence of the cognate regulatory *dct*BD genes. This observation led to the postulation of an alternative system of symbiotic activation of the *dct*A gene. This ASA, which stands for Alternative Symbiotic Activator has been the subject of much research by several groups including our own at the Microbiology Dept. University College Cork. Despite this considerable effort, which I will summarise in **Chapter 2**, the ASA has not been identified to date. The problem in pinpointing the ASA probably lies in the strict symbiotic nature of the phenomenon. The mechanism can not be induced under free-living conditions. Therefore it has to be studied *in situ* during symbiosis with the host plant. Using gene fusions of the *dct*A expression signals to the *lacZ* and *uid*A reporter genes, in combination with histochemical staining of plant tissue, I have studied the regulation of the *dct*A gene *in situ* during the symbiosis with the *Medicago* host plants.

The construction of various gene fusions is described in **Chapter 3** and their behaviour under free-living conditions is analysed in **Chapter 4**. In **Chapter 5** I describe the use of some of the gene fusions to study the *in situ* patterns of *dct*A expression during symbiosis. First of all it is established that in a wild-type background, the *dct*A gene is expressed during the early and late stages of symbiosis. In contrast in nodules induced by a regulatory *dct*B or *dct*D mutant, a distinct late symbiotic pattern of *dct*A expression was observed. This meant that the ASA is an exclusive late symbiotic mechanism. In addition we also found that certain gene fusions lacking the extreme N-terminal domain of the *dct*A coding region did not respond to the ASA. The use of these gene fusions allowed us to monitor the

activity of the DctBD system without the interference of the ASA and conclude that the DctBD system alone is sufficient to express the *dct*A promoter during all stages of symbiosis.

Having identified a gene-fusion construct that responded to the DctBD mechanism, but not the ASA, we wished to obtain a complementary gene fusion that would respond to the ASA, but not the DctBD mechanism. In **Chapter 6** we describe such gene fusion construct which was obtained by deleting the Upstream Activator Sequences (UAS) from the *dct*A promoter. These sequences are essential for the activation of the *dct*A promoter by DctBD, under free-living conditions and during symbiosis. This gene fusion was found to be activated equally well during symbiosis in nodules induced by wild-type as well as a *dct*D mutant strain. This *dct*D independent activity however was totally abolished in nodules induced by a strain mutated in the *nif*A gene. This is probably the best indication to date that a functional *nif*A gene is required for the ASA activity.

Besides alfalfa (*Medicago sativa*) *Medicago truncatula* is also a host plant for *S.meliloti*. Because of its better amenability for genetic manipulation, the latter has been proposed as a model-plant to study symbiotic N_2-fixation. In **Chapter 7** we evaluated the *in situ dct*A activity on *M.truncatula*. To our surprise we observed that *dct*D mutant strains induced ineffective nodules on this host plant. No *dct*A activity could be observed suggesting that the ASA does not operate. The *in situ* activity of *nif*A::*lacZ* and *nif*H::*lacZ* gene fusions demonstrated that the pattern of *nif* gene expression is similar as in the alfalfa background, but the level of gene expression is about a tenfold lower. These findings are in agreement with the previous results suggesting that NifA is required for ASA activity in the *S.meliloti* alfalfa symbiosis.

Finally in **Chapter 8** we discuss the major findings of this work and assess their implication in developing our understanding of gene regulation in *S.meliloti* during symbiosis.

Chapter 1)

Introduction

1.1) The *Rhizoblum* - legume symbiosis.

Rhizobia are gram-negative soil bacteria, capable of fixing atmospheric nitrogen in symbiosis with their leguminous host plants. This symbiosis leads to the development of nodules on the roots of the host plant. These nodules are specialised organs where the host-plant provides an ecological niche for bacteria, which can invade these nodules. Here they can differentiate to endosymbiotic bacteroids, which can fix atmospheric nitrogen in a microaerobic environment. The fixed nitrogen is made available to the host plant, which provides a competitive advantage in growth conditions where nitrogen is limited.

1.2) Exchange of nutrients during symbiosis.

During symbiosis between *Sinorhizoblum meliloti* and the *Medicago* host plant, the energy required for the bacteria to colonise the roots, to invade and multiply inside the host plant and to fix atmospheric nitrogen, is all ultimately derived from the plant photosynthate. At all times during infection and

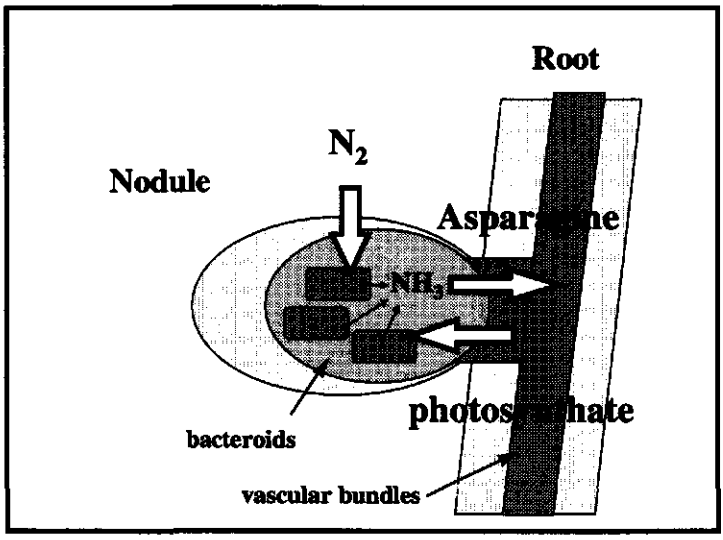

Fig. 1a: Exchange of nutrients during symbiosis.
During the *S.meliloti*-alfalfa symbiosis the bacteroids fix atmospheric nitrogen (N_2) and make it available to the plant cells in the form of ammonia (NH_3). There it is converted to asparagine and transported via the vascular system to the other parts of the host plant. The energy required for these processes is ultimately derived from the photosynthesis, which takes place in the leaves.

symbiotic N_2-fixation, the bacteria, or bacteroids, remain separated from the plant cell cytoplasm by plant derived membranes. During invasion and infection, the bacteria are enclosed collectively inside infection threads. When released from these infection threads into the plant cell cytoplasm, the bacteria are enveloped individually in a peribacteroid membrane. In general biological membranes are

impermeable to large hydrophilic molecules such as those that may serve as carbon and nitrogen sources for the growth of the microsymbionts. Specific transporters will be required to allow the selective passage of molecules from the host cell cytoplasm to reach the internalised bacteria. As such the host plant determines the direct environment of its microbial partner and may influence its behaviour. The bacteria are able to produce a wide range of more or less specific transport proteins in order to be able to take up and utilise molecules that it encounters in its environment. The production of these permeases is highly regulated. Synthesising these proteins only makes sense when the compounds that are to be transported are actually present in the environment, in sufficiently high amounts to merit the effort involved in synthesising the permease and adjusting the metabolism.

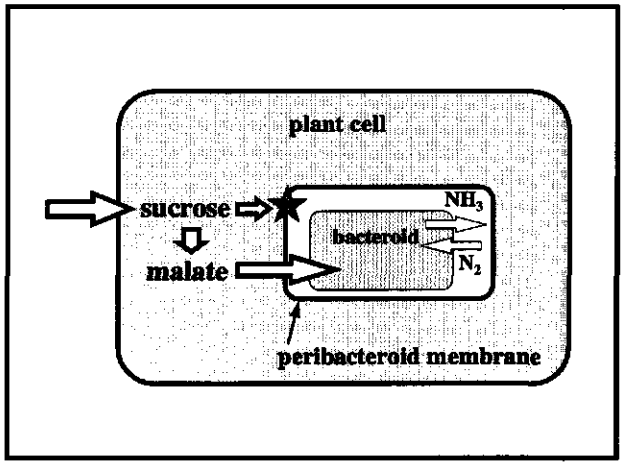

Fig. 1b: The peribacteroid membrane.
The nitrogen fixing bacteroids are enveloped in a plant derived peribacteroid membrane, which prevents the free exchange of large molecules. Selective passage of carbohydrates is obtained by means of specific transporter proteins, or permeases. Although sucrose is the most abundant form of photosynthate in the plant cells, it is not directly available to the bacteroids. Malate is probably the only carbon source provided to the bacteroids in sufficiently high amounts to support symbiotic N_2-fixation. Consequently ability of the bacteroids to take up this C_4-dicarboxylate is essential for an effective symbiosis.

1.3) The capacity of the microsymbionts to take up C_4-dicarboxylates is essential for symbiotic N_2-fixation.

Current evidence indicates that C_4-dicarboxylates (dCA = fumarate, succinate, malate) are the major and probably only source of carbon provided to the bacteroids in sufficiently high amounts to support

symbiotic N_2-fixation. They are translocated through the peribacteroid membrane and were found to support nitrogenase activity of isolated *S.meliloti* bacteroids (McRea *et al.*, 1989). Consequently, efficient expression of the *S.meliloti* C_4-dicarboxylate transport gene (*dct*A), coding for a high affinity uptake system for dCA, is essential for an effective symbiosis (Ronson *et al.*, 1981; Glenn and Dilworth, 1981; Finan *et al.*, 1983; Glenn *et al.*, 1984; Arwas *et al.*, 1985; Driscoll and Finan, 1993).

1.4) The regulation of the *dct*A gene.

The genes of *S.meliloti* involved in the uptake of C_4-dicarboxylic acids have been studied extensively. The *dct* gene cluster consists of the structural gene *dct*A and the divergently transcribed regulatory genes *dct*BD. The *dct*A gene codes for a high affinity permease (DctA), which is required for the

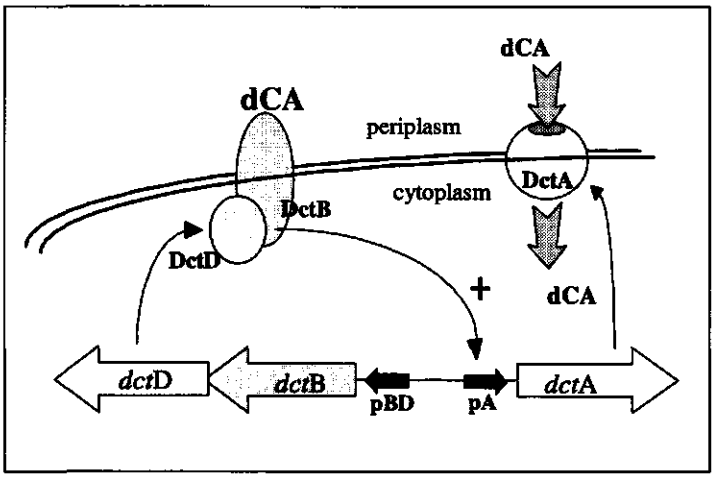

Fig. 1c: The *dct*ABD gene cluster.
The structural gene (*dct*A) codes for a high affinity permease (DctA), which is required for the efficient uptake of dicarboxylic acids (dCA = fumarate, succinate, malate). The regulatory *dct*BD genes are transcribed from a low level constitutive promoter (pBD) and code for a two component regulatory system (DctB and DctD). DctB is a sensor protein that is located in the cytoplasmic membrane and detects the presence of dCA in the periplasmic space. DctB interacts with the transcriptional activator DctD. In the presence of dicarboxylic acids, or aspartate in the environment DctD is activated by DctB and in turn activates transcription from the *dct*A promoter (pA).

efficient uptake of dicarboxylic acids. The *dct*BD genes are transcribed constitutively at a low level and were found to code for a two-component regulatory system (DctB and DctD). DctB is a sensor protein that is located in the cytoplasmic membrane and detects the presence of dCA in the periplasmic space. DctB interacts with the transcriptional activator DctD. In the presence of dicarboxylic acids, or

aspartate in the environment DctD is activated by DctB. DctD in turn activates transcription from the *dct*A promoter (Fig. 1c). Under free-living conditions, the regulatory *dct*BD genes are essential for activation of the *dct*A promoter (Ronson *et al*, 1984, 1987; Ronson and Astwood, 1985; Engelke *et al.*, 1989; Jiang *et al.*, 1989; Wang *et al.*, 1989

1.5) The symbiotic phenotype of the *dct* mutants.

S.meliloti strains mutated in the structural *dct*A gene without exception display a fix⁻ phenotype during symbiosis. This is in line with the notion that the ability to take up dCA is essential during symbiotic N_2-fixation.

Strain	Growth on dCA	Symbiotic phenotype
wild-type	+	+
*dct*A⁻	-	-
*dct*B⁻	-	+
*dct*D⁻	-	+

Table 1a: Phenotypic behaviour of the *dct* mutant strains.
S.meliloti strains mutated in any of the *dct* genes are unable to grow on media containing a dicarboxylic acid (dCA) as a sole carbon source. Mutations in the structural *dct*A gene affect the permease directly, whereas the regulatory *dct*BD mutants are unable to activate the *dct*A promoter under free-living conditions. Regulatory mutants however do often allow an efficient symbiosis. This indicates that during symbiosis the *dct*A gene is still expressed in these strains.

Although certain regulatory *dct* mutants are unable to fix nitrogen during symbiosis, most were hardly affected in their symbiotic efficiency. Bacteroids isolated from nodules induced by such mutants were found to efficiently transport dCA (Engelke *et al.*, 1987). It became clear that the *dct*A gene is expressed during symbiosis, even in the absence of the regulatory *dct*BD genes. Accordingly, nodules induced by these *S.meliloti* mutants, still fix nitrogen although sometimes at an reduced rate (Yarosh *et al.*, 1989). This demonstrated that in the specific environment of the nodule, regulatory molecules other than DctBD, are involved in the expression of the *dct*A promoter. Despite the extensive study of the dicarboxylate transport (Dct), this alternative system of symbiotic activation (ASA) has not been identified to date.

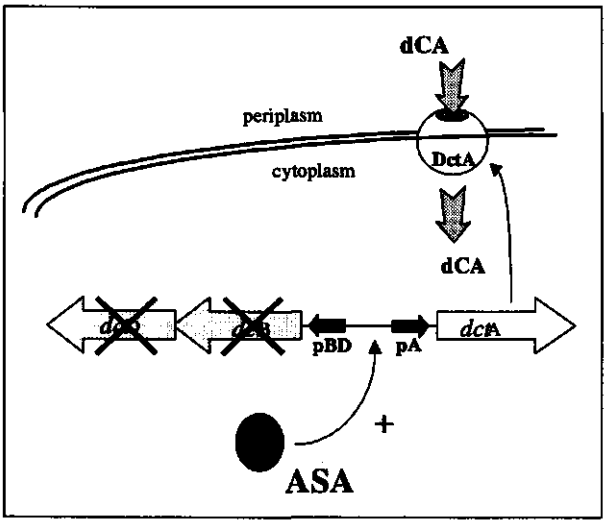

Fig. 1d: The alternative mechanism of symbiotic *dctA* activation.

The presence of an alternative mechanism of activation of the *dctA* promoter (ASA) is only revealed during symbiosis. When the regulatory *dctBD* genes are mutated, the *dctA* gene is still expressed at significant levels in a DctBD-independent manner. Despite extensive studies the ASA has not been characterised to date.

Abbreviations are as in Fig. 1c.

1.6) The alternative symbiotic activator (ASA).

The presence of a second mechanism of symbiotic activation of *dctA* during symbiosis, poses a question with respect to the symbiotic regulation of this gene. It is not clear what role the ASA may play in the context of overall gene regulation in a mature efficient nodule. It is conceivable that the cognate DctBD system is primarily responsible for symbiotic *dctA* expression and that the ASA is a regulatory artefact that only comes to light when the DctBD system is mutated. On the other hand the ASA may represent a specific regulatory mechanism, characteristic of the nitrogen fixation process, which takes over from DctBD when the bacteroids have fully differentiated and symbiotic N_2-fixation begins. Simple cross-talk between regulatory mechanisms, or a novel regulatory mechanism specific to symbiotic N_2-fixation? The relatively high efficiency at which N_2-fixation takes place in nodules induced by some *dctD* mutant strains, suggests that the *dctA* gene is expressed at nearly wild-type levels. Such efficient expression argues for the latter possibility.

1.7) Aim of this work.

The work presented in this thesis is aimed at the study of the regulation of the *S.meliloti dct*A gene *in situ* during symbiosis with the *Medicago* host plants. There is a big discrepancy between the knowledge that has been obtained in recent years of DctBD dependent regulation of the *dct*A gene in free-living conditions and the unknown nature of the alternative mechanism of symbiotic activation, or ASA. Consequently, it is still not known if either the ASA, or the regular DctBD system is primarily responsible for the expression of *dct*A during symbiosis.

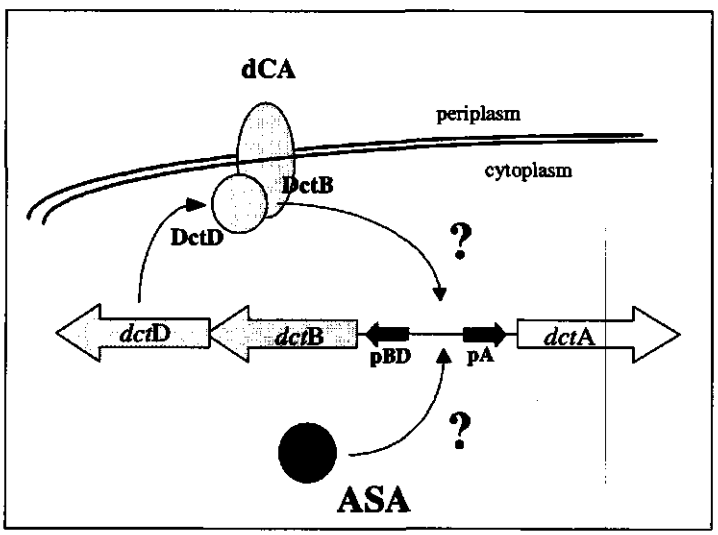

Fig 1e: Two possible mechanisms of *dct*A activation during symbiosis.
Since there are two possible ways of activating the *dct*A gene, it is not clear which mechanism (DctBD or ASA) is primarily responsible for the expression of the gene during symbiosis. Abbreviations are as in Figs. 1d

This lack of knowledge concerning the ASA originates probably from its strict symbiotic nature. Attempts to obtain DctBD independent expression of the *dct*A promoter under free-living conditions, for example by microaerobiosis (Wang *et al.*, 1989), have failed so far. Others have been looking for secondary mutations that allow a *dct*D mutant to grow on minimal medium with dCA as sole carbon source. One such mutation has been characterised (Labes *et al.*, 1993). This worked out to be an altered *ntr*C allele, which resulted in a NtrC molecule that activates NtrA dependent promoters constitutively. Such a mechanism is unlikely to be responsible for the activation of *dct*A during symbiosis. Although such approaches are relevant and the results obtained instructive, they have to date not lead to the characterisation of the ASA. In order to elucidate the role of the ASA during symbiosis, we considered it imperative to study the ASA *in situ* during symbiosis with the *Medicago* host plants. In this work we have undertaken such a study of the regulation of expression of the *S.meliloti dct*A gene *in planta*.

Chapter 2)

The state of the art

2.1) Organisation of the *dct* gene cluster.

The *dct* genes of *S.meliloti* and a number of related *Rhizobium* species have been extensively studied. The first complementation studies, which were done with *dct* genes from a *R.leguminosarum* gene bank, identified three loci (Ronson *et al.*, 1984). These loci were designated *dct*A, *dct*B and *dct*C. Furthermore, the results suggested that *dct*A encoded a structural component necessary for dCA transport. The *dct*B and *dct*C genes, which are transcribed opposite to the direction of the *dct*A gene, were thought to encode positive regulatory elements. Further transposon insertions indicated the presence of a fourth locus between *dct*B and *dct*C. This fourth locus was designated *dct*D. Eventually it turned out that *dct*C was in reality a truncated version of *dct*D, which led to a constitutive activation of the *dct*A promoter (Ronson *et al.*, 1988). This left only two regulatory *dct* genes: *dct*B and *dct*D. Sequence analysis of the *dct*BD genes revealed a striking homology to the nitrogen regulatory gene products NtrBC (Ronson *et. al.*, 1987a & c; For a review see: Parkinson and Kofoid, 1992). Conserved sequences in the C- terminal part of the *dct*B gene and the amino terminus of the *dct*D gene were also found in an ever growing family of regulatory proteins. Many of these regulatory proteins act in pairs to relay environmental signals to an appropriate response at the molecular level (Fig. 2a). Genetic analysis and nucleotide sequencing of the *S.meliloti dct* genes revealed a high degree of homology with the *dct* gene cluster of *R.leguminosarum* (Engelke *et al.*, 1989; Jiang *et al.*, 1989; Wang *et al.*, 1989). In summary, the *S.meliloti dct* region consists of three genes, required for the uptake of dCA in free-living conditions. The structural gene *dct*A, which codes for a high affinity uptake permease and the regulatory genes *dct*BD, that code for a two-component sensor-regulator system (Fig. 1c).

2.2) Role and function of the *dct*A gene.

2.2.1) DctA is a high affinity permease located in the periplasmic membrane.

C_4-dicarboxylic acids: fumarate, succinate and malate (dCA) do not freely pass through the periplasmic membrane. Therefore, to be able to use dCA as a carbon source, bacteria require a specialised uptake system. The *S.meliloti dct*A gene codes for a high affinity permease for the uptake of dCA. This permease also transports the amino acid aspartate. Aspartate is an efficient inducer of the *dct*A gene, but the affinity of DctA for the uptake of this amino acid is lower than that for dCA (McRea *et al.*, 1989). In practice this means that if dCA are present as well as aspartate, the latter is probably not taken up by DctA.

Because DctA is a permease, it was expected to be located in the periplasmic membrane. This was confirmed by gene fusions to the *E.coli* alkaline phosphatase (*pho*A). This alkaline phosphatase is normally located in the periplasm. To study protein secretion, Hoffman and Wright (1985) constructed a modified form of the gene from which the promoter and signal sequence encoding region of the gene have been removed. Fusion proteins with this alkaline phosphatase are therefore only active

when the *pho*A part of the chimeric protein is located in the periplasmic space. Manoil and Beckwith (1985) exploited this feature to construct a Tn5 derivative (Tn5 IS50$_L$::*pho*A) which can be used to identify protein transport signals. This transposon has been exploited by several groups to obtain random gene fusions of the *pho*A gene to genes coding for proteins which are located in the periplasmic membrane (Long *et al.*, 1988). The *dct*A gene proved to be a suitable target to obtain active *dct*A::*pho*A gene fusions (Long *et al.*, 1988; Yarosh *et al.*, 1989; Jording *et al.*, 1993). Although DctB also is thought to contain an extracellular domain, no active *dct*B::*pho*A gene fusions were obtained (Yarosh *et al.*, 1989).

The topology of the DctA protein has been determined using the complementary features of alkaline phosphatase (*pho*A) and β-galactosidase (*lac*Z) gene fusions. Contrary to the *pho*A gene fusions, which are only active when the PhoA part of the fusion protein is exposed into the periplasmic space, gene fusions to *lac*Z are only effective when the *lac*Z portion remains in the cytoplasm (Manoil, 1990). From the deduced protein sequence of the DctA protein, 12 membrane spanning regions were predicted. The topology of the DctA comprised twelve transmembrane α-helices with the amino- and carboxyl- terminus located in the cytoplasm. This topology is confirmed by the activity of a range of *dct*A::*pho*A and *dct*A::*lac*Z gene fusions (Jording *et al.*, 1993). All translational gene fusions to the *lac*Z genes, that have been constructed to date (Wang *et. al.*, 1993; This work) also confirm this proposed topology of the DctA protein.

2.2.2) DctA is involved in the regulation of its own expression.

The absence of a functional DctA protein generally results in a constitutive high level of expression of the *dct*A gene. Gene fusions of the *dct*A promoter sequences to various reporter genes are highly expressed in a *dct*A$^-$ background (Yarosh *et al.*, 1989; Jording *et al.*, 1992; This thesis). This is true under free-living conditions as well as during the symbiosis (Jording et al., 1992). This suggests that DctA is somehow involved in the regulation of its own expression. To date, it is not known which part of the DctA protein is involved in this autoregulation. Yarosh and coworkers (1989) demonstrated that the regulatory *dct*BD genes are required for the elevated expression of the *dct*A gene under these conditions. Being membrane located, it seems unlikely that DctA interacts directly with its own promoter region. The DctA protein may interact with the regulatory DctBD system. This may involve a direct protein-protein interaction with for example the DctB protein, which is also located in the periplasmic membrane (See Chapter 2.3). The existence of an additional regulator possibly sensing the internal levels of dCA and acting on the *dct*A promoter cannot be excluded. In this context it is interesting to observe that in the heterologous *E.coli* background, the resident cAMP receptor protein CRP has been demonstrated to negatively regulate the activity of the *dct*A promoter (Wang et al., 1993; Chapter 2.7).

2.3) Role and function of the regulatory *dct*BD genes.

Under free-living conditions, the expression of the *dct*A gene is regulated by the *dct*B and *dct*D genes. These regulatory genes are transcribed in the opposite direction from the *dct*A gene (Ronson *et al*, 1984, 1987; Ronson and Astwood, 1985; Engelke *et al.*, 1989; Jiang *et al.*, 1989; Wang *et al.*, 1989). The *dct*BD genes code for a two-component regulatory system belonging to a family of NtrBC sensor-regulator molecules (Ronson *et al.*, 1987; Parkinson and Kofoid, 1992). Members of this family of regulators are characterised by conserved domains in the C-terminus of the sensor proteins and the N-terminus of the regulators.

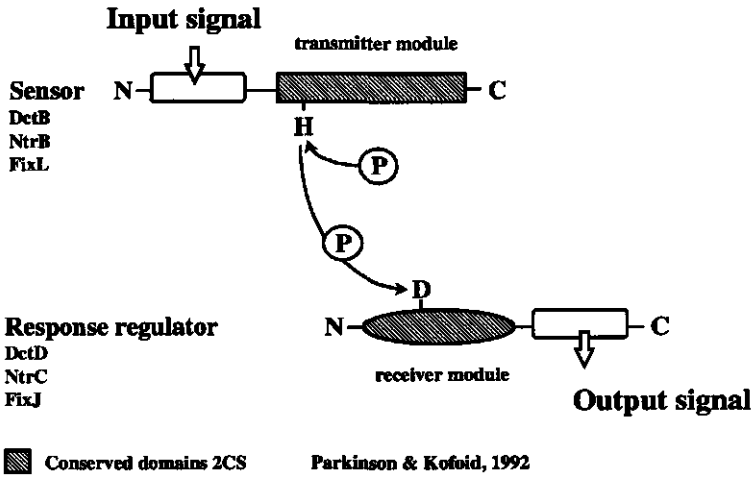

Fig. 2a The two-component regulatory system:
Signal transduction within a NtrBC type regulatory system takes place by means of auto-phosphorylation of a conserved domain in the sensor and subsequent specific phosphor transfer to a conserved domain in the response regulator. The input- and output signals are specific for each system.

Based on these homologies, Ronson *et al.* (1987a), proposed a model for the regulation of *dct*A expression in response to the presence of dCA in the environment of the bacteria. In this model, DctB was proposed to be located in the cytoplasmic membrane and capable of sensing the presence of dCA in the periplasmic space. In the presence of dCA, DctB would activate DctD by means of a protein-protein interaction. By homology to the NtrBC system, this was proposed to involve a phospho-transfer reaction (Fig. 2a). The phosphorylated DctD, which is free to move in the cytoplasm would activate the *dct*A promoter in turn. The *ntr*A gene product (NtrA, or σ^{54}), was also shown to be required for *dct*A expression (Ronson *et al.*, 1987b). To date, this model largely still holds true. Signal transduction between the DctB and DctD has been studied *in vitro* with purified proteins (Giblin *et al.* 1994; Chapter 2.8). Conform to the consensus, it has been shown to involve a mechanism of

phospho-transfer between the two proteins. The protein sequence of DctB reveals two potential transmembrane domains in the N-terminal part of the protein and suggests that a sizeable portion of DctB protrudes into the periplasmic space. This portion of the molecule presumably is involved in the sensing of the appropriate signal leading to the activation of the Dct system. The C-terminal portion of DctB contains a highly conserved Histidine residue (H_{410}). This is the site of autophosphorylation of the sensor molecules. In the presence of an inducer compound (dCA, or the amino acid aspartate) in the environment, the phosphor group is transferred to the conserved aspartate residue in the amino terminus of DctD (D_{55}). The C-terminus of the DctD molecule contains a helix-turn-helix motif, which is presumably involved in binding of DctD to the *dct*A promoter region. The phosphorylated DctD has an increased affinity for the *dct*A promoter DNA (Giblin *et al.* 1994; Chapter 2.8). The *dct*A promoter depends on the NtrA sigma factor (σ^{54}) for expression. The *S.meliloti ntr*A gene product (σ^{54}) is required for diverse metabolic functions. Apart from Dct, the *ntr*A gene is required for nitrate assimilation and symbiotic nitrogen fixation (Ronson et al., 1987b).

DctD belongs to a family of transcriptional regulators which interact with σ^{54}, to activate their target

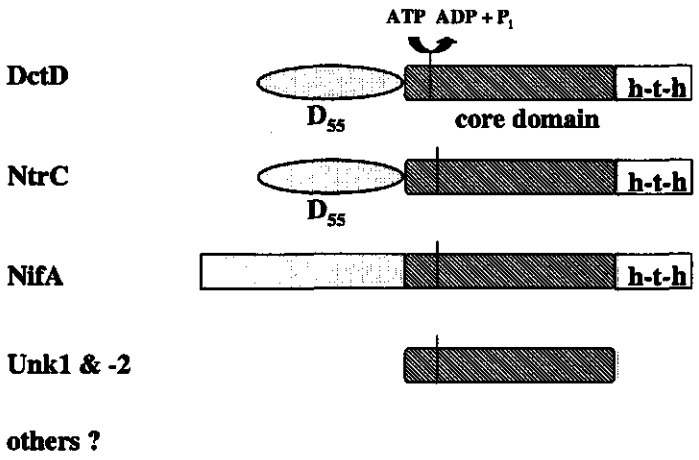

Fig. 2b: Modular structure of DctD and related enhancer binding proteins:
These transcriptional activators all have a highly conserved core domain which interacts with the Eσ^{54} polymerase complex. They also feature a typical helix-turn-helix (h-t-h) domain for DNA binding. DctD and NtrC both belong to a two component system and have a similar N-terminal domain which can be phosphorylated by their respective sensor molecule.

promoters (Kustu et al., 1989). This family of regulators are characterised by a highly conserved central domain in the proteins (Fig. 2b, Morett and Segovia, 1993). Hybridisation of the *S.meliloti* genome with DNA probes representing this conserved central portion of *dct*D potentially identified more than 20 homologous regulatory genes (Jiang et al., 1989). The best known examples of these

regulators are NifA (*nif* gene regulation) and NtrC (nitrate assimilation). Two more genes have been cloned from *S.meliloti*, using a PCR approach to amplify the central portion of homologous regulatory genes. The function of these genes is not yet known, although it has been suggested that they may be involved in the expression of the *dct*A gene during symbiosis (Kaufman and Nixon, 1996).

2.4) Primary structure of the *dct*A promoter region.

When the closely related *S.meliloti* (Wang *et al.*, 1989; Chapter 2.6) and *R.leguminosarum* (Jiang *et al.*, 1989) *dct*A promoter regions were compared, an overall homology of 58% at the nucleotide level was observed. Moreover a number of domains were found to be conserved at a much higher degree (Wang *et. al.*, 1989; Chapter 2.6). It seems likely that these conserved domains are essential for *dct*A (and *dct*BD) expression. The region between the *dct*A and *dct*B genes, where the promoters are located, measures 240 bp or 209 bp (depending on which of the two possible ATG start codons is taken as the translational start point of *dct*A). Each of these two ATG's is preceded by a good consensus ribosome binding site. Only the second ATG however is conserved between the *S.meliloti* and *R.leguminosarum dct*A coding regions (Fig. 2e) and therefore represents the more likely start site. In either case, the promoter and the start site for transcription are positioned upstream of the *Sma*I site as transcriptional gene fusions at this point (pCU22 and pCU32, Wang *et al.*, 1989) are fully functional. A consensus NtrA binding site (GG-N_{10}-GC) has been identified 47 bp upstream of this *Sma*I site. Transcription has been demonstrated to start around 10-11 bp downstream from this promoter site (Ledebur *et. al.*, 1990). NtrA dependent transcriptional activators, such as NtrC and NifA bind to sequences located 80 bp or more upstream from the promoter site to activate transcription. These upstream activator sequences, or UAS, are essential for efficient transcription from the target promoters. Based on their similarity with consensus NifA binding sites (TGT-N_{10}-AGA, Buck *et al.*, 1986), two UAS sites have been identified in the *dct*A promoter region (Ronson *et al.*, 1987). These sites were later confirmed by *in vitro* methylation footprints and gel shift assays as being target sites for binding of the DctD transcriptional activator (Ledebur *et al.*, 1990 and 1992). Both UAS are required for efficient transcriptional activation of the *dct*A promoter, despite the fact that the affinity of DctD for the downstream site is much higher than for the upstream sequence (Ledebur *et. al.*, 1992). It seems likely that DctD binds co-operatively to both sites, to activate the *dct*A promoter.
An additional conserved region is apparent about 30 bp upstream of the NtrA binding site. The function of this region has not been identified to date. Apart from the *dct*A promoter, there must also be a start site for transcription of the *dct*BD genes. The *dct*BD promoter has not been identified. The *dct*BD genes are expressed constitutively at a low level (Wang *et al.*, 1989; Jording *et al.*, 1992). This seems plausible in view of the fact that in order to detect the presence of the inducer and quickly activate the *dct*A promoter, the regulatory DctBD system must be already in place. The housekeeping σ factor component of the *S.meliloti* RNA polymerase, encoded by the *sig*A gene, is homologous to the *E.coli* RpoD σ^{70} subunit (Rushing and Long, 1995). Not much is known about the consensus DNA

sequence recognised by this polymerase holoenzyme. It seems likely that both the *E.coli* $E\sigma^{70}$ and the *S.meliloti* holoenzyme bind to similar -35 sequences (Boesten *et al.*, 1987; Bae and Stauffer, 1991). However, despite the fact that the start site of the *dctB* transcript has never been identified, it is clear that it must be located in the same region as the UAS sites of the *dctA* promoter. There are a number of TTG sequences, representing possible -35 recognition sites of the *dctBD* promoter. One of these corresponds with the conserved sequence about 50 bp upstream of the *dctA* promoter. This may well represent the *dctBD* promoter site. Binding of DctD to its UAS sequences is very likely to interfere with the initiation of transcription of the *dctBD* genes. This suggests a possibility of autoregulation of the *dctBD* operon. However, no significant difference has been observed in the level of transcription of *dctB* and *dctD* in the presence, or absence of inducer (Jording *et al.*, 1992). These authors have also demonstrated that *dctD* probably has its own promoter and gene fusions to *dctD* are expressed at a higher level than those to *dctB*. This holds true for free-living cultures as well as bacteroids. Why this should be so, is not clear. Possibly more DctD molecules are required for an effective Dct system than DctB molecules. A DctB molecule located in the cytoplasmic membrane may phosphorylate and dephosphorylate many DctD molecules.

2.5) Additional regulatory features of *dctA*.

2.5.1) Expression of *dctA* during symbiosis.

Under free-living conditions, the regulatory *dctBD* genes are essential for activation of the *dctA* promoter. However at an early stage it became clear that certain regulatory *dct* mutants unable to use dCA for growth under free-living conditions, were fully effective for symbiotic N_2-fixation (Ronson and Astwood, 1985). Similarly nodules induced by many *S.meliloti* strains bearing mutations in the *dctBD* genes efficiently fix nitrogen during symbiosis (Watson *et al.*, 1988; Engelke *et al.*, 1989; Jiang *et al.*, 1989; Wang *et al.*, 1989, Yarosh *et al.*, 1989). This demonstrates that in this specific environment regulatory molecules, other than DctBD, are involved in the expression of the *dctA* promoter. Symbiotic expression of a *dctA*::*lacZ* gene fusion demonstrated that the *dctA* gene is indeed expressed during symbiosis in a *dctD* mutant background (Wang *et al.*, 1989; Birkenhead *et al.*, 1990). Despite extensive study of Dct, this alternative system of symbiotic activation (ASA) has not been identified to date.

The existence of a specific symbiotic mechanism of *dctA* regulation led to the search of alternative mechanisms of *dctA* regulation *ex planta*. This search has concentrated on the one hand on attempts to induce secondary mutations that affect *dctA* expression in a *dctD* mutant background and on the other hand on attempts to induce related regulatory systems that may have a cross-talk effect on *dctA* expression. Both approaches are based on the presumption that there exists a gene that is capable of activating the *dctA* promoter, which is not expressed under free-living conditions. Further it seems a foregone conclusion that the activation of the *dctA* promoter by the ASA is also σ^{54} dependent. This

assumption is mainly based on the fact that a strain mutated in *ntr*A induces fully infected nodules, but *dct*A is not expressed in such nodules (This work and personal communication with other workers). However the transcription start point in a DctBD mutant background, during symbiosis has not been determined. Therefore the possibility remains that a second promoter, exclusively active under symbiotic conditions is responsible for the expression of *dct*A in the absence of the regulatory *dct*BD genes.

By forcing a regulatory *dct* mutant to grow on a minimal medium with a dicarboxylate as sole carbon source one may select mutations that lead to the expression of the alternative activator. A second approach represents a systematic search for environmental conditions that will lead to the induction of the alternative activator under free-living conditions. These may be conditions that closely resemble the conditions that prevail inside a mature nodule during symbiotic N_2-fixation. To date, none of these experimental approaches has been successful in identifying unequivocally the alternative symbiotic activator.

2.5.2) The σ^{54} dependent transcriptional activators.

The transcriptional activator DctD has a modular structure. The N-terminal domain has homology with response regulators belonging to a family of two-component regulatory systems. The remainder of the

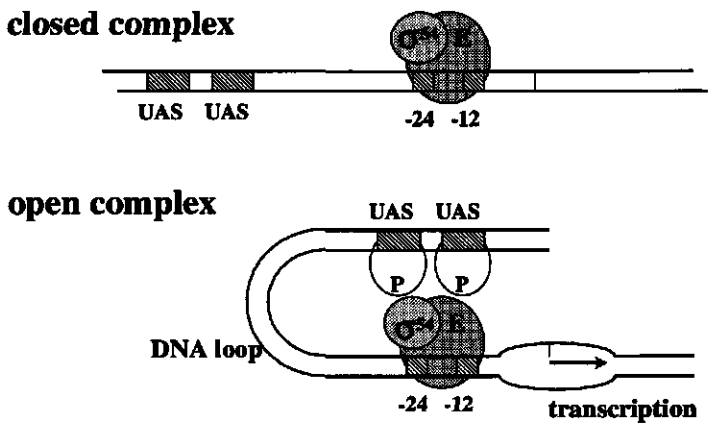

Fig. 2c: Positive activation of a σ^{54} dependent promoter.

The polymerase-sigma^{54} complex (Eσ^{54}) binds to the promoter site (-12,-24), but is unable to denature the DNA and form an open complex. A positive activator protein (P) is required that binds to an enhancer site (UAS), located upstream of the promoter. Simultaneous interaction with the polymerase-sigma^{54} complex results in looping of the DNA. This results in the formation of an open complex and efficient initiation of transcription.

molecule belongs to a family of proteins that work with the $E\sigma^{54}$ holoenzyme to initiate transcription of their target genes (Kustu *et al.*, 1989). The σ^{54} subunit is peculiar among prokaryotic sigma factors in that the polymerase-sigma factor complex ($E\sigma^{54}$) can bind to the promoter boxes, which are centred around -12 and -24 bp from the transcriptional start site. It forms a stable closed promoter complex, but is unable to isomerise into an active open complex (Fig. 2c). This step is mediated by the transcriptional activator, which binds to recognition sites typically about 80-100 bp upstream of the promoter site and interacts with the promoter complex. Initiation of transcription requires energy generated by ATP hydrolysis, catalysed by the transcriptional activator. For a review see Morett and Segovia (1993) and references therein. This mechanism of initiation of transcription is reminiscent of the activation of transcription by eukaryotic RNA polymerase II (Wang et al., 1992). The upstream activator sequences (UAS) are also referred to as enhancer like elements (ELE) and the transcriptional activator proteins as enhancer binding proteins (EBP). This family of transcriptional activators is characterised by a highly conserved domain of about 240 amino acids which contains the site for ATP hydrolysis and is shown to interact with σ^{54} and the β-subunit of the polymerase (Lee *et al.*, 1995). This core domain has been shown to be sufficient to initiate transcription by the $E\sigma^{54}$ complex (Huala and Ausubel, 1989; Huala *et al.*, 1992). All these activators contain a domain, which features a classical helix-turn-helix motif and interacts with the UAS elements. This domain is usually located at the C-terminal end of the molecule. An exception forms the LevR regulator, involved in the regulation of the levanase operon from *Bacillus subtillis*, in which this domain is located upstream of the core domain in the N-terminal part of the molecule (Martin-Verstraete *et al.*, 1991).
Binding of the activators to the UAS sites confers specificity to the promoters (Ledebur *et al.*, 1990). This may be because specific binding of the cognate activator to the UAS is a prerequisite for transcription. It has been shown that binding to the DNA stimulates the ATP hydrolysis by the activator and thereby the ability to activate transcription from the promoter (Lee *et al.*, 1994). The simultaneous interaction with the UAS and the $E\sigma^{54}$ complex involves the bending or looping of the intervening DNA (Su *et. al.*, 1990). However, the interaction with the UAS is not an absolute requirement for transcription activation (Huala and Ausubel, 1989; Huala *et. al.*, 1992). In such cases, it can often be argued that the experiments do not reflect the natural circumstances because they are either performed *in vitro*, or in a heterologous background. One notable exception is the symbiotic expression of the NifA regulated promoters P1 (*nif*HDK) and P2 (*fix*ABC). Under free-living microaerobic conditions the UAS are absolutely required for transcription from these promoters activated by NifA. On the other hand, these promoters are highly expressed during symbiosis, even when their UAS sequences are removed (Better et al., 1985; Wang et al., 1991). It is not known if other sequences do compensate for the absence of the UAS in that case. An additional deletion of DNA sequences downstream from the transcriptional start site further reduced the levels of symbiotic expression of P1 (Wang *et. al.*, 1991). In the context of symbiotic expression of the *dct*A promoter, these observations are interesting. NifA is highly active during symbiosis and its target promoters are activated to very high levels of transcription. The interaction with the UAS of the target promoters is

not strictly required for transcriptional activation. However, the UAS are thought to be major determinants in conveying specificity of the transcriptional activators for their target promoters. Considering that NifA can efficiently activate transcription from its target promoters under symbiotic conditions, even when these are lacking their UAS sequences, one can also envisage NifA activating other σ^{54}-dependent promoters such as the promoter of the *dct*A gene.

2.5.3) Activation by NifA.

The most obvious candidate for symbiotic activation of *dct*A seems to be the transcriptional activator of the *nif* genes: NifA. Like DctD, NifA is a transcriptional activator of NtrA (σ^{54}) dependent promoters. They have a similar central "core" domain which has been shown to be able to activate heterologous NtrA (σ^{54}) dependent promoters (Huala & Ausubel, 1989, Huala et al., 1992). Unlike DctD, or NtrC, NifA itself is not a member of a two component regulatory system, but rather its transcription is regulated by the FixLJ system (David *et al.*, 1988). FixLJ constitutes a two component regulatory system (Fig. 2a) which under microaerobic conditions turns on the transcription of the transcriptional activators NifA and FixK (Fig. 2d). These molecules in turn activate a substantial number of *nif* and *fix*

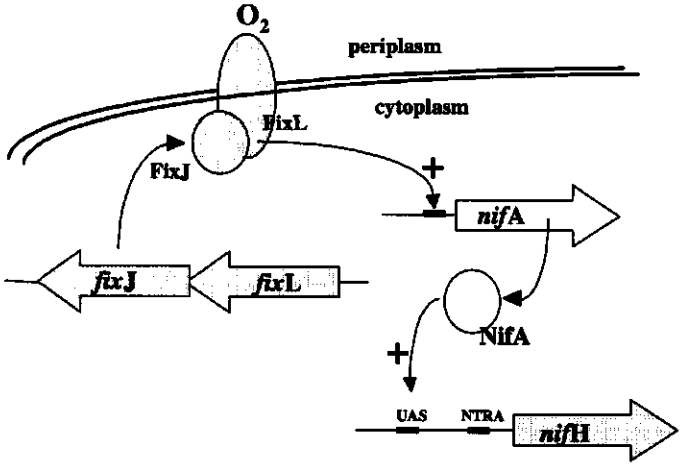

Fig. 2d: Regulatory cascade leading to the expression of the *nif*H gene.
Under microaerobic conditions the two component regulatory system FixLJ activates among others the transcription of the *nif*A gene. The NifA gene product in turn activates a number of genes required for symbiotic nitrogen fixation.

genes. FixK is an Fnr/Crp type protein and therefore an unlikely candidate for the alternative symbiotic activator of *dct*A. NifA on the other hand belongs to the family of σ^{54} dependent transcriptional activators and is highly active during symbiosis. The primary target of NifA are the P1 and P2 promoters which direct the transcription of the *nif*HDK- and the *fix*ABC operons respectively (Better *et al.*, 1985). During symbiosis, these genes are highly expressed to levels about 20 to 30 times higher than the *dct*A gene (Birkenhead *et al.*, 1990). The overall structure of the P1 and P2 promoters and the *dct*A promoter are very similar. For instance, the UAS sites upstream of the *dct*A promoter were first identified because of their similarity to the NifA target sites and were then proposed to be involved in the symbiotic activation of *dct*A (Ronson, 1988).

When the symbiotic expression of a *dct*A::*lac*Z gene fusion was measured in nodules induced by a *S.meliloti* strain defective in *nif*A, only background levels were obtained (Wang et al, 1989; Chapter 2.6). This suggests that *nif*A does indeed play a role in the symbiotic expression of *dct*A. It also suggests that the cognate DctBD regulatory system is unable to activate *dct*A in such nodules. This may be explained by the assumption that the fix⁻ phenotype may lead to an inability of the host plant to provide dCA to the bacteroids. This result is in contrast with the results presented in another report (Jording *et al.*, 1992). These authors showed symbiotic levels of *dct*A expression from nodules induced by a *nif*A and also *fix*L and *fix*J mutant strains, which did not differ from those obtained in a wild-type background. Therefore, these authors concluded that NifA does not play a role in the symbiotic expression of the *dct*A gene

The *nif* and *fix* genes are controlled by a regulatory cascade which lead to the activation of these genes under microaerobic conditions (David *et al.*, 1988). It has been reported that the expression of *nif*A is regulated by oxygen (Ditta *et al.*, 1987). Under conditions of low oxygen pressure (1% O_2, or less) the *nif*A gene is induced. This induction of *nif*A is followed by high levels of expression of its target gene *nif*H. When such microaerobic conditions were imposed on a *S.meliloti* wild-type strain in the absence of dCA, no increase in the levels of *dct*A expression were observed. In the wild-type background it is possible that DctD, in an inactive state is bound to the *dct*A promoter DNA and as such prevents NifA from interacting with the *dct*A UAS. However, a *dct*D mutant strain also did not allow expression of a *dct*A::*lac*Z fusion under these conditions, which led to high levels of expression from both the P1 and P2 promoters (Wang *et al.*, 1989). . These results also seem to exclude NifA as a possible candidate for the ASA.

2.6) Genetic analysis and regulation of the *Rhizobium meliloti* genes controlling C_4-dicarboxylic acid transport.

Authors: Wang, Y-P., Birkenhead, K., Boesten, B., Manian, S. and O'Gara, F.

Published in: Gene 85 (1989) 135-144.

Summary.

In this work we cloned and analysed the *dct*ABD genes from *S.meliloti* CM2. The DNA region between the *dct*A and *dct*B genes was sequenced and compared with the sequence of the *R.leguminosarum dct* genes. A high degree of overall homology was found. A number of highly conserved regions were identified (Fig 2e). These were proposed to be involved in regulatory features such as a NtrA binding site, two upstream binding sites for NifA, a region we proposed to be a possible DctD binding site and the most likely start of translation of the *dct*A gene. As discussed previously (Chapter 2.4) the proposed NifA binding sites were later demonstrated to be the UAS recognised by DctD and required for DctD dependent activation of the *dct*A promoter under free-living conditions (Ledebur *et al.*, 1990 and 1992). As discussed in Chapter 2.4, the proposed DctD binding site is in hindsight more likely the promoter region of the *dct*BD genes. Transcriptional gene fusions of the *dct*A and *dct*BD promoters to the enteric *lac*Z gene were constructed and the regulation studied under free-living and symbiotic conditions. During symbiosis the *dct*A promoter was shown to be active in nodules induced by the wild-type strain and the *dct*D mutant, but not the *dct*B mutant strain. Although C.W. Ronson (1988) already made mention of wild-type levels of activity from a *dct*A::*lac*Z gene fusion during symbiosis in nodules induced by *dct*B and *dct*D mutant strains, this work was the first to actually show such data.
We also investigated the possible role of NifA in the regulation of the *dct*A promoter. The finding that the *dct*A::*lac*Z gene fusions were not expressed in nodules induced by a *nif*A mutant strain, suggested that there may be indeed an involvement. On the other hand we were unable to activate the *dct*A::*lac*Z gene fusions under free-living microaerobic conditions, although a *nif*H::*lac*Z gene fusion was highly expressed under the same conditions, demonstrating that NifA was active.

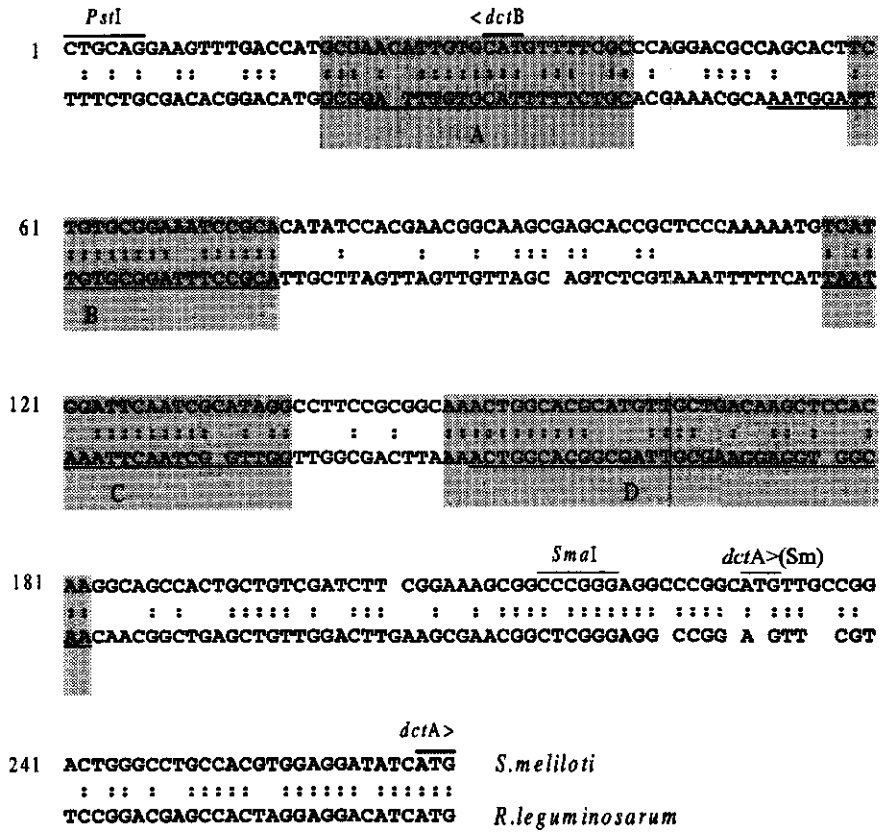

Fig.2e: Sequence alignment of the *S.meliloti* (upper strand) and *R.leguminosarum* (lower strand) *dct* promoter regions.

Conserved sequences in the *dct*A promoter region are shaded. According to Wang *et al.*, 1989 these correspond to: A & B, NifA binding sites; C, possible DctD binding site; D, NtrA binding site. The two possible translation start sites for *dct*A and the one for *dct*B are overlined. Also marked are the *Pst*I and *Sma*I restriction sites.

2.7) The *Escherichia coli* cAMP receptor protein (CRP) represses the *Rhizobium meliloti dct*A promoter in a cAMP dependent fashion.

Authors: Wang, Y-P., Giblin, L., Boesten, B. and O'Gara, F.

Published in: Mol. Microbiol. 8 (1993) 253-259.

Summary.

In this work we studied the regulation of the *S.meliloti dct*A promoter in *E.coli*. In this heterologous background the *dct*A promoter was found to require the presence of the *dct*BD genes for activity. High levels of activity of the *dct*A::*lac*Z gene fusion was observed in the presence of dCA in the growth medium. When other carbon sources were used in the growth medium, different levels of activity were observed. These differences were related to the activity of the CRP molecule, which is modulated by the level of cAMP in the cell. On glucose the level of cAMP is low and the CRP molecule is inactive. Under these conditions, high level of activity was observed from the *dct*A promoter even in the absence of inducer. On non-PTS sugars such as maltose, the level of cAMP is high and the CRP-cAMP complex is active. Under these conditions, in the absence of inducer, the activity of the *dct*A promoter was low. We could demonstrate that the CRP-cAMP complex binds to the *dct*A promoter. We also found that the most likely binding sites for the CRP-cAMP complex are overlapping with the DctD binding sequences. We proposed that the CRP-cAMP complex competes with DctD for binding to the *dct*A promoter and prevents activation by the unphosphorylated DctD. In the presence of inducer, DctD will be phosphorylated and have an increased affinity for binding. In that case the CRP-cAMP complex cannot compete and high levels of *dct*A::*lac*Z activity were observed. This mechanism assured a low background level of promoter activity in the absence of inducer. As such, it appeared to be essential for proper regulation of the *dct*A promoter in the *E.coli* background. We reasoned that a similar mechanism may also operate in *S.meliloti*.

2.8) Signal transduction in the *Rhizobium meliloti* dicarboxylic acid transport system.

Authors: Giblin, L., Boesten, B., Turk, S., Hooykaas, P. and O'Gara, F.

Published in: FEMS Microboil. Lett. 126 (1995) 25-30.

Summary.

In this work we investigated the signal transduction between DctB and DctD *in vitro*. The regulatory proteins were overproduced in *E.coli* and purified. DctB was shown to have autophosphorylation activity in the presence of γP^{32}-ATP and was able to transfer the radioactive phosphate to DctD. This supports the hypothesis that signal transduction between these regulatory molecules occurs by means of phospho-transfer. The phospho-transfer was specific in as far as no cross-talk could be observed with the purified components of the *Agrobacterium tumefaciens* VirA/VirG system. We could also demonstrate that the phosphorylated DctD had an increased affinity for binding to a DNA fragment containing the *dct*A promoter sequences. This supported our theory that phosphorylation of DctD is an important factor in its ability to compete with other regulatory molecules for binding to the DNA of its target promoter (as we discussed in 2.7).

2.9) NtrBC-dependent expression from the *Rhizobium meliloti dct*A promoter in *Escherichia coli*.

Authors: Allaway, D., Boesten, B. and O'Gara, F.

Published in: FEMS Microboil. Lett. 128 (1995) 241-245.

Summary.

In this work we investigated the possibility of 'cross-talk' between the DctBD system and the NtrBC regulatory system. In other words: the effect of the NtrBC system on the expression of a *S.meliloti dct*A::*pho*A gene fusion was studied in *E.coli*, either in the presence, or absence of the *S.meliloti dct*BD genes. Under nitrogen limiting conditions, a significant induction of the *dct*A promoter was observed in *E.coli*. A mutation of the *ntr*C gene abolished this induction. This demonstrated that the activated NtrC could efficiently activate the *S.meliloti* promoter. We also could demonstrate that NtrB had a significant effect on the activity of DctD, suggesting that 'cross-talk' also existed on the level of sensor-regulator interaction. No significant effect of nitrogen limitation on *dct*A activity could be observed in *S.meliloti*. This suggested that unlike its *E.coli* counterpart, the *S.meliloti* NtrC could not

efficiently activate the *dct*A promoter. A comparison of the amino acid sequences revealed that DctD is more similar to NtrC from *E.coli*, than NtrC from *S.meliloti*. This was especially true for a region including the aspartate residue, which is the specific acceptor for phosphor transfer by the sensor protein. We reasoned that NtrC and DctD in *S.meliloti* may have diverged evolutionary from a consensus activator sequence in order to minimise interference between the Dct and Ntr two component systems.

2.10) The state of the art.

From the data presented in the previous chapters, it can be seen that a lot of knowledge has been gathered on the regulation of expression of the *S.meliloti dct*A gene. We have discussed very little about the Dct system in other Rhizobia. In particular the *dct*A gene of *R.leguminosarum* also has been studied extensively and to date it appears that the two systems are very similar. In fact, information gathered from both systems has contributed to the current knowledge of the Dct system. Signal transduction by phospho-transfer within the DctBD two component regulatory system and the activation of the *dct*A promoter by DctD and the E-σ^{54} holoenzyme are now well understood. The study of other 2-component systems, especially the NtrBC system and other σ^{54} dependent transcriptional regulators such as NifA also have contributed enormously to the current level of understanding. On the other hand, several aspects of the Dct system are less well understood. For example the signal perception by DctB, or the role of DctA on its own expression. These items are more particular to the Dct system. It has probably been more attractive to study the general aspects of the Dct system because this research contributes to and benefits from the larger body of understanding of similar systems.

However, this argument does not apply to the study of the ASA. The question of the alternative mechanism of symbiotic activation of the *dct*A gene was raised very early on by Ronson and Astwood (1985). It was subsequently addressed by several more research groups (Watson *et al.*, 1988; Engelke *et al.*, 1989; Jiang *et al.*, 1989; Wang *et al.*, 1989, Yarosh *et al.*, 1989). Significant efforts were made to try and identify the genes involved. It is therefore the more surprising that no real progress has been made on this subject. The reason for this may be the strict symbiotic nature of the ASA. The majority of efforts to study the ASA have concentrated on trying to induce the *dct*A promoter under free-living conditions in absence of a functional DctBD system. So far, no one has succeeded in finding the right conditions to induce the ASA under free-living conditions. Obviously, our understanding of the process of bacteroid development and what constitutes symbiotic conditions, is still very limited. A different approach would be to study the ASA *in situ* during symbiosis. The use of gene fusions in combination with histochemical staining techniques is a powerful tool to study the temporal and spatial patterns of gene expression *in situ*. In this thesis we will use these techniques to study *S.meliloti dct*A expression *in situ* during symbiosis with the *Medicago* host plants.

Chapter 3

Methodology

3.1 Introduction.

In order to study the *in situ* expression of the *S.meliloti dct*A gene, we will use various reporter genes fused to the *dct*A expression signals (transcriptional and translational initiation signals). We will describe the construction of the various gene fusions that have been used in these studies. Many factors may influence the levels and the patterns of expression obtained from different gene fusions. The level of expression depends for example on the promoter strength and level of induction. Other factors such as the copy number of the carrier plasmid; the stability of the RNA, the efficiency of initiation of translation and the stability of the fusion protein all may have an effect. No doubt there are many more factors. Therefore a careful examination of how the gene-fusions are constructed and comparative examination of expression patterns under controlled conditions are necessary for each gene fusion plasmid that is to be used for further studies.

Gene fusions may be located on free replicating plasmids, or may be integrated into the chromosome by a single or double cross-over event. Free replicating plasmids may be lost in the absence of a selective pressure. An antibiotic resistance gene on the plasmid and the corresponding antibiotic in the medium is normally used for this purpose. Gene fusions located on free replicating plasmids were mostly used in this study because of the relative ease at which they can be manipulated and introduced into a range of wild-type and mutant strains. Because no selective pressure by means of antibiotics can be applied during symbiosis, there may be some loss of plasmids during the *in situ* experiments. However in the two to three week time span of the experiments this was not found to be a problem. More sophisticated solutions such as the use of plasmids containing a stabilising locus to ensure correct partitioning of the plasmids during division of the bacteria (Weinstein *et al.*, 1992) were considered, but found to be unnecessary.

3.2) The reporter genes.

To study the regulation of genes of interest, genes coding for readily assayable products such as the classical β-galactosidase (coded for by the enteric *lac*Z gene), may be exploited as reporter genes. Chimearic gene-fusions are constructed whereby the expression signals of the gene of interest are fused at the DNA level, to a gene fragment coding for the reporter protein. In this way, expression of the gene of interest can be monitored, assaying for the activity of the reporter gene product. In case of the β-galactosidase, the production of the enzyme in culture can be assayed by the hydrolysis of the ONPG substrate (*o*-nitrophenyl-β-D-galactopyranoside). This substrate is hydrolysed by the enzyme and the release of o-nitrophenol results in a vivid yellow colour which can be measured spectrophotometrically. The presence of the enzyme can be demonstrated *in situ* non-destructively by a chromogenic substrate: X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, or X-β-D-gal). This substrate is taken up by the living bacteria and hydrolysed by the β-galactosidase resulting in a dark blue precipitate. For example colonies formed by *lac*$^+$ bacteria can be readily distinguished from *lac*$^-$ bacteria (unable to produce the β-galactosidase) by virtue of their blue colour when grown in presence of X-gal. Over the years many variations of this theme have been developed. Many substrates have

been developed for different reporter genes. There are now substrates for various enzymes such as the alkaline phosphatase (*pho*A) or β-glucuronidase (*uid*A), resulting in precipitates of different colours. This allows the simultaneous study of several gene-fusions to different reporter genes in a single bacterial strain.

Gene fusions have been proven to be invaluable in the study of gene regulation in general and no less so for the study of *dct*A regulation. To study free-living expression of the *dct*A gene many *Lac*Z (Jiang *et al.*, 1989; Wang *et al.*, 1989; Jording and Pühler, 1993;) and *pho*A (Long *et al.*, 1988; Yarosh *et al.*, 1989; Ledebur *et al.*, 1990; Jording and Pühler, 1993) fusions have been constructed.

When reporter genes are to be used to study gene regulation, a number of things must be considered. First of all, which reporter gene is to be used? In these studies we have considered three of the most commonly used reporter genes: *lac*Z encoding β-galactosidase; *pho*A encoding alkaline phosphatase and *uid*A encoding β-glucuronidase (GUS). Each of these genes originates from *Escherichia coli.* Second, what is the background activity in the *Rhizobium* host strain? If the constructs are to be used for *in situ* studies, the background activity in the plant tissue must also be considered. Third, which vector should be used? Free replicating plasmids are easy to handle and quickly introduced in a wide range of host strains. However in absence of a selective pressure, the free-replicating plasmids may be lost. Also the copy number of the carrier plasmid may influence the levels of expression obtained. Narrow hostrange plasmids integrated in the genome of the host are more stable than free replicating plasmids and the gene fusion is present in a single copy. Furthermore the upstream DNA is continuous with the chromosome, which ensures that promoter activity is always registered, even if the promoter is located further upstream than the DNA fragment cloned on the plasmid. On the negative side, integration may disrupt certain genes and influence the regulation of the gene of interest. The way the gene-fusions are constructed can have an important effect on the results obtained. This means that the more the studies become sophisticated, the more important it becomes to carefully design the gene fusions.

3.2.1) The *lac*Z gene.

The *lac*Z gene is by far the best known and most widely used reporter gene to date. Casadaban *et al.* (1980) constructed a range of plasmid vectors for the detection and cloning of translational initiation signals. Several of these constructs are at the basis of *dct*A::*lac*Z gene fusions used in this work. In particular, they introduced a *Bam*HI restriction site preceding the eight amino acid of the *lac*Z gene, which can be used for the construction of translational gene fusions. Ditta et al. (1985) adapted these reporter genes to the use in a wider range of gram⁻ bacteria by cloning them onto a broad host range replicon. *S.meliloti* has several genes coding for a β-galactosidase (Niel *et al.*, 1977, Fanning *et al.*, 1988; Jelesko and Leigh, 1994). However the presence of these endogenous *S.meliloti lac*Z genes generally does not interfere with the gene fusions. This is probably because these *lac*Z genes either have to be induced, or are only expressed constitutively at a low level (Niel *et al.*, 1977). *S.meliloti lac*Z mutant strains are available, but the use of such strains means that any additional mutation that may be of interest must be introduced into the *lac*Z⁻ parent strain. This limits the range of possible host

strains that may be used. Also it must be verified that the *lacZ* parent strain has no mutant phenotype that may interfere with the studies. For example in case of the GMI 5600 *lacZ* mutant strain, we have observed a significant effect on nodule morphology and symbiotic efficiency on alfalfa (See also Chapter 5.3.).

3.2.2) The *phoA* gene.

The enteric alkaline phosphatase (orthophosphoric-monoester phosphohydrolase EC 3.1.3.1; *phoA*) is an easily assayable enzyme, which is located in the periplasm of *E.coli* K12. It is synthesised with an N-terminal signal sequence, which is required for its secretion. The enzyme is not active in the cytoplasm but only after it has been transported to the periplasm. In order to develop a tool for the study of protein secretion, Hoffman and Wright (1985) removed the promoter and N-terminal region coding for the signal sequence from the *phoA* gene. Gene fusions between the N-terminal part of a gene of interest, with this modified form of the *phoA* reporter gene, only show phosphatase activity when the *phoA* part of the protein is located in the periplasmic space. Manoil and Beckwith (1985) exploited this feature to construct a Tn5 derivative (Tn5 $IS50_L$::*phoA*), that carried this truncated *phoA* reporter gene and which can be used to identify protein transport signals. This transposon has been exploited by several groups to randomly obtain gene fusions to the *phoA* gene (Long *et al.*, 1988). The *dctA* gene, coding for a permease protein that is located in the periplasmic membrane, proved to be a suitable target to obtain *dctA*::*phoA* gene fusions that show phosphatase activity (Long *et al.*, 1988; Yarosh *et al.*, 1989; Jording *et al.*, 1993). Although DctB also is thought to contain an extracellular domain, no active *dctB*::*phoA* gene fusions were obtained (Yarosh *et al.*, 1989).
The use of the *phoA* gene as a reporter gene in *S.meliloti* requires a mutant strain, which has a low background activity of alkaline phosphatase (Rm8002, Long *et al.*, 1988). This significantly limits the range of *S.meliloti* wild-type and mutant strains that can be readily used for expression studies. Also its use is limited to proteins which are located in the cytoplasmic membrane, or are exported to the periplasm. DctA is located in the periplasmic membrane, but only certain regions of the protein are exposed to the periplasm. Therefore only a fraction of the possible *dctA*::*phoA* gene fusions resulted in chimearic proteins that also have phosphatase activity (Jording *et al.*, 1993).

3.2.3) The *uidA* gene.

The *Escherichia coli* β-D-glucuronidase gene (*uidA*, GUS), coding for a β-D-glucuronoside glucuronosohydrolase (EC 3.2.1.31). This enzyme is an acid hydrolase that catalyses the hydrolysis of a wide range of β-D-glucuronides. Many substrates for spectrophotometric, fluorometric and histochemical analysis are available. The gene is widely used as a sensitive and versatile gene fusion marker, both in prokaryotes as well as eukaryotic organisms (Jefferson *et al.*, 1986; 1987; Gallagher, 1992). There is no GUS activity detectable in *S.meliloti* under free-living or symbiotic conditions.

3.3) Background enzymatic activities in plant tissue.

In-situ studies with reporter gene fusions are hampered by the endogenous enzymatic activity of the plant tissues. Effective ways of avoiding background β-galactosidase activity in plant tissues has been reported by Teeri *et al.* (1989) and phosphatase activity by Reuber *et al.* (1991). The latter actually used a *dct*A::*pho*A gene fusion as a positive control to study *S.meliloti exo* gene expression in alfalfa nodules. This study is to our knowledge the only report on *in-situ dct*A expression to date. The *Escherichia coli* β-D-glucuronidase gene (GUS) is widely used as a sensitive and versatile gene fusion marker in higher plants (Jefferson *et. al.*, 1987). There is virtually no background GUS activity in the roots of alfalfa and other *Medicago* species, which makes it a very reliable marker in this symbiotic system. It has been successfully used to study the temporal and spatial regulation of symbiotic genes of *S.meliloti in planta* (Sharma and Signer, 1990). The major drawback of the GUS marker is its sensitivity to fixation agents such as glutaraldehyde.
For maximum reliability of the *in situ* expression patterns it is advisable to use more than one fusion construct, if possible with different reporter genes.

3.4) Construction details of the gene fusion plasmids.

3.4.1) Basic strategies.

The *lac*Z gene used in this work, is lacking both transcriptional and translational start signals and has been constructed by M.Casadaban *et al* (1980). It was originally located on a plasmid called pMC874. A close relative of this plasmid, which only differed in the available useful restriction sites downstream of the *lac*Z gene, is pMC931. The *lac*Z gene was transferred from pMC931 to a derivative of the broad host-range, low copy-number plasmid pRK290, which resulted in pGD926 (Ditta *et. al.*, 1985). The transfer of the *lac*Z gene from pMC874 to the narrow host-range, high copy number pBR322 resulted in pMC1403 (Casadaban *et. al.*, 1980).
In this work we have used three basic strategies to obtain *dct*A::*lac*Z gene fusions located on broad host-range mobilisable derivatives of pRK290. The 700bp *dct*A gene fragment from pCU700 was recovered and cloned into pBluescript SK⁻. This is a small, high copy-number plasmid, ultimately derived from pBR322. After manipulation the *dct*A fragment may be cloned into pGD926 to obtain the desired construct. It is also possible to liniarise both the pSK derivative and pGD926 and combine the two plasmids. Obviously, these cointegrates are constructed in such a way that the desired *dct*A::*lac*Z gene fusion is created through fusion of the two plasmids. An advantage of this procedure is that the cointegrated plasmid can be selected for, after transformation, by combining the antibiotics selective for each plasmid. The third strategy is to construct the *dct*A::*lac*Z gene fusion in a pMC1403 derivative and then form cointegrates of these plasmids with the broad host range plasmid pRK290. All three strategies have been used to construct the various plasmids used in this work.

3.4.2) The parent plasmid pCU700.

Plasmid pCU700 used in these studies was constructed by Wang *et al.* (1993; Chapter 2.7). This gene fusion was obtained by cloning a 700 bp *Pst*I fragment, with suitable linkers into pGD926 (Fig. 3a B).

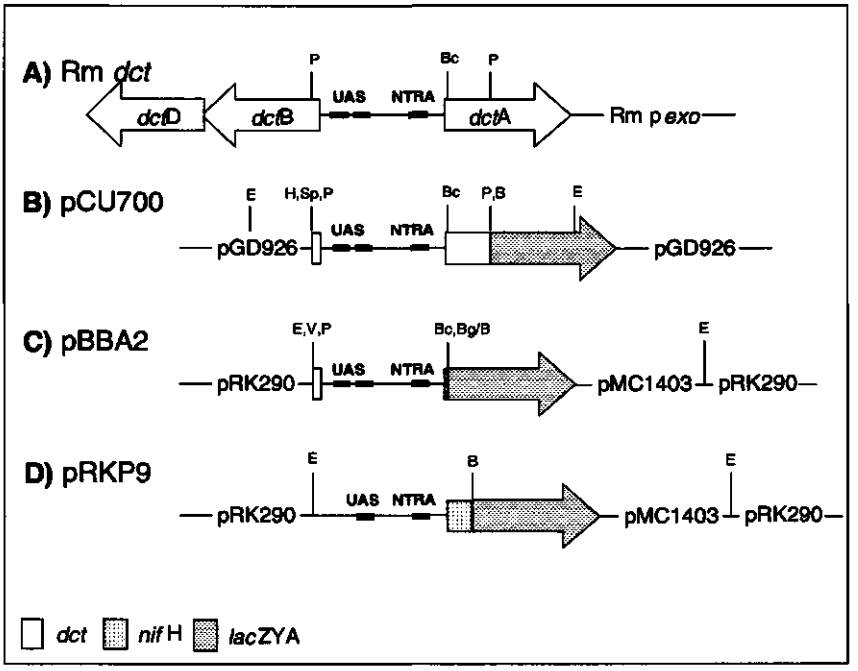

Fig. 3a: The *S.meliloti dct* genes and gene fusions to the *lacZ* reporter gene.
A) The *S.meliloti dct* genes are located on the p*exo* megaplasmid. B) Plasmid pCU700 contains a 700 bp *Pst*I *dct* fragment cloned, with a suitable linker into pGD926. C) Plasmid pBBA2, is a derivative from pRKP9 in which a 260 bp *Pst*I-*Bcl*I *dct*A fragment replaced the *nif*H promoter. D) The *nif*H::*lacZ* fusion plasmid pRKP9. B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; V, *Eco*RV; H, *Hind*III; Sp, *Sph*I; P, *Pst*I; NtrA, NtrA binding site (promoter); UAS (upstream activator sites), binding sites for transcriptional activator (DctD; NifA).

Plasmid pGD926 is a low copy number, broad host range, translational *lacZ* fusion plasmid. The upstream *Pst*I restriction site is located just inside the coding region of the divergently transcribed *dct*B gene. The downstream fusion site is located about 500 bp inside the *dct*A gene. The 700 bp *Pst*I fragment contains the complete intergenic region between the *dct*A and *dct*B genes and therefore presumably all necessary elements for expression of the *dct*A gene. The *S.meliloti dct*A gene contains two possible ATG start codons, only 33 bp apart from each other. Each ATG is preceded by a good consensus ribosome binding site (Engelke *et al.*, 1989; Wang *et al.*, 1989; Chapter 2.6; Fig. 2e). Only the second ATG is conserved between the *S.meliloti* and *R.leguminosarum dct*A genes (Jiang *et. al.*, 1989). Assuming this second ATG is the start codon of the *dct*A gene, the *Pst*I site is located at amino acids 161-162 (Fig. 4a). This N-terminal region of the DctA protein contains 4 of the 12

transmembrane domains (Jording *et al.*, 1993). The resulting DctA::LacZ fusion protein therefore is likely to be inserted into the periplasmic membrane.

3.4.3) The "Short" *dctA*::*lacZ* gene fusions.

The location of the pCU700 encoded *dctA*::*lacZ* gene fusion product in the membrane led to some concern about the "fitness" of the bacteria expressing this gene fusion. Although under free-living conditions strains bearing this plasmid generally behave normal, we noticed that inducing the gene-fusion led to smaller colony size, suggesting that expression of the fusion protein slows down the

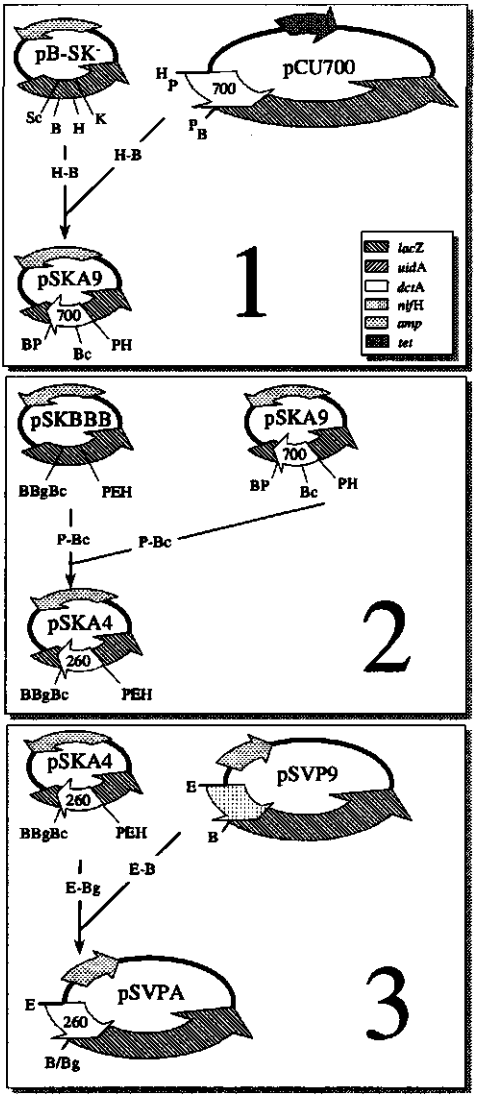

Fig.3b: Construction of a *dctA*::*lacZ* gene fusion lacking the *dctA* coding region.

1) The 700 bp *PstI dctA* fragment from pCU700 was cloned into a high copy number plasmid (pBluescript SK⁻) to facilitate genetic manipulation. **2)** The 260 bp *PstI*-*BclI* fragment from pSKA9 was cloned into pSKBBB. This removed most of the *dctA* coding region. The *BamHI BglII* and *BclI* restriction sites allowed to manipulate the reading frame. **3)** The 267bp *PstI*-*BglII dctA* fragment from pSKA4 was used to replace the *nifH* fragment from pSVP9.

B = *BamHI*; Bc = *BclI*; Bg = *BglII*; E = *EcoRI*; H = *HindIII*; K = *KpnI*; P = *PstI*; B/Bg = *BamHI*- *BglII* hybrid site. A colour code for the various is given in panel 1

growth of the bacteria. Also we noticed that a *dct*A mutant strain (F642) with pCU700 died during storage; even when stored at -80°C. Therefore, novel "short" *dct*A::*lac*Z gene fusions were constructed. Unlike the gene fusion on plasmid pCU700 that was taken as a starting point of these studies, the reporter genes were now directly fused to the putative ATG translational start codon of the DctA protein. A *Bcl* restriction site conveniently overlapping this ATG was used to construct these gene fusions.

The first step was to clone the ca. 700 bp. *Pst*I *dct*A promoter fragment from pCU700 into pBluescript SK⁻. This resulted in a plasmid designated pSKA9 (Fig. 3b 1). Next, the 260 bp *Pst*I-*Bcl*I from pSKA9 was cloned into a custom made pBluescript SK⁻ derivative (pSKBBB) containing the compatible *Bcl*I; *Bgl*II and *Bam*HI restriction sites in three different reading frames. This resulted in plasmid pSKA4 (Fig. 3b 2) Plasmid pSVP9 was obtained by restriction of the *nif*H::*lac*Z fusion plasmid pRKP9 (Fig. 3a D) with *Eco*RI and resolving the pSVP9 and the pRK290 moieties. The promoter fragment from pSKA4 was then excised as a *Eco*RI-*Bgl*II fragment and used to replace the *nif*H promoter in pSVP9 (Fig. 3b 3). This resulted in an in-frame gene fusion of the second amino acid of *dct*A to the *lac*Z gene. Finally this plasmid, designated pSVPA, was linearised using the unique *Eco*RI restriction site and cloned into the *Eco*RI site of the broad host-range plasmid pRK290. Three plasmids conferring resistance to ampicillin as well as tetracycline were obtained and conjugated into a wild-type *S.meliloti* strain. All three plasmids (pBBA2, -4, & -6) gave similar patterns of expression during symbiosis with alfalfa. One such plasmid (pBBA2, Fig.3a C) was used for more detailed studies. The orientation of pSVPA in pBBA2 with respect to pRK290 has not been determined. The "short" promoter fragment of pSKA4 has also been cloned as a *Hind*III-*Bgl*II fragment into pGD926, the parent plasmid of pCU700. This resulted in plasmids pBBBg1 & -2 (Fig.3d panel C). The gene fusions on these plasmids are identical to that on to pBBA2.

3.4.4) The GUS fusions.

The same *Bcl*I restriction site overlapping the ATG start codon was used to construct a *dct*A::*uid*A gene fusion (Fig.3c). Plasmid pSKA9 was used to construct the GUS fusion. The 975 bp *Sca*1-*Bcl*I fragment was fused with a 3,75 Kb *Sca*1-*Bam*H1 fragment from pCOOGUS, bearing the *uid*A reporter gene. The resultant fusion of the two plasmid halves was designated pBBGA. The gene *dct*A::*uid*A gene fusion on pBBGA can be excised as a *Pst*I or *Hind*III cartridge. The *dct*A::*uid*A gene fusion was cloned into the *Pst*I restriction site in the β-lactamase (amp) gene of pRKP9. This resulted in a plasmid pRKP4, containing the *dct*A::*uid*A gene fusion as well as a *nif*H::*lac*Z fusion. Similarly the fusion cartridge was also cloned into the amp gene of the narrow host range plasmid pSUP202. This plasmid pSUPGA1, may be mobilised into *S.meliloti* and is expected to integrate into the genome by a single crossover event in the *dct*A promoter region. Both plasmids have the gene fusion cloned in an orientation opposite to the direction of transcription of the β-lactamase gene.

Despite the fact that the ATG of *dct*A is not in frame with the ATG of *uid*A, this gene fusion is expressed efficiently. Translation may initiate at the ATG of the *dct*A gene, resulting in a peptide of 12 amino acids before running into an TGA stop codon. The *uid*A start codon is located only 7 bp.

downstream of the *dct*A ATG. It seems likely that this ATG is utilised to express the β-glucuronidase. The fact that translation of the *dct*A::*uid*A gene fusion is efficiently initiated at the *uid*A-ATG may

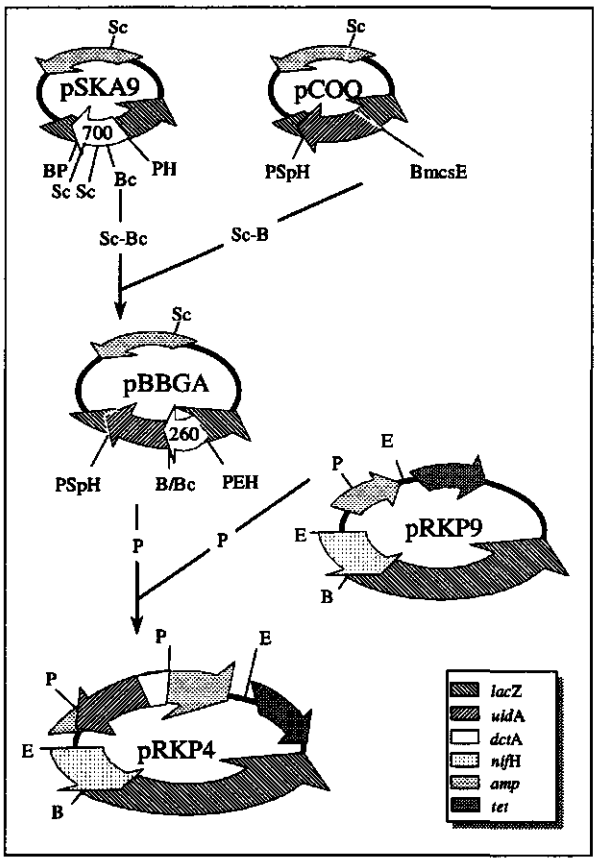

Fig.3c: Construction broad host range plasmid bearing a *dct*A::*uid*A gene fusion.
The moieties of pSKA9, containing the *dct*A promoter and pCOO, containig a promoterless *uid*A gene, were fused together to produce a plasmid pBBGA bearing a *dct*A::*uid*A gene fusion. The *dct*A::*uid*A cartridge was excised from pBBGA as a *Pst*I fragment and cloned into the ampicillin resistance gene (amp) of pRKP9. This resulted in pRKP4 bearing both, a *nif*H::*lacZ* and a *dct*A::*uid*A gene fusion.
B = *Bam*HI; Bc = *Bcl*I; Bg = *Bgl*II; E = *Eco*RI; H = *Hind*III; K = *Kpn*I; P = *Pst*I; Sc = *Sca*1; Sp = *Sph*I; B/Bc = *Bam*HI - *Bcl*I hybrid site; mcs = multiple cloning sites. A colour code for the various genes is given in the bottom righthand corner.

indicate that translation of the *dct*A gene is indeed initiated from the second ribosome binding site, rather than the one 33 bp further upstream. As the *dct*A::*uid*A gene fusion gave satisfying results, no further attempts were made to correct the reading frame. The possibility remains that such corrections will result in more efficient expression of the fusion protein.

Even though the GUS activities in terms of nmoles o-nitrophenol released per minute, are low (when compared with the rates obtained from of the *lac*Z gene fusions), background levels in *S.meliloti* and *Medicago* are undetectable. This means that this particular GUS fusion can be used reliably as a reporter gene of *dct*A activity.

A second *dct*A::*uid*A gene fusion cartridge has also been constructed. The design of this plasmid was based on the fact that a *Sac*I restriction site, located at amino acids 43-44 and just downstream of the first transmembrane domain of the *dct*A gene, is in-frame with the *Sac*I cloning site upstream of the *uid*A gene in pCOOGUS. A method of construction, similar as for pBBGA, was used to yield a plasmid designated pBBGA23. In theory this cartridge contains an in-frame *dct*A::*uid*A gene fusion. However,

when cloned into pRKP9 (resulting in a plasmid designated pRKP23) and transferred into *S.meliloti*, no expression was ever obtained from this GUS fusion. Probably, the periplasmic location of the GUS moiety prevents this fusion protein from being active. If that is the case, than it would confirm the presence and functionality of the first transmembrane domain of the DctA protein.

3.4.5) Construction of *dct*A::*lac*Z gene fusions using PCR.

During these studies, it became clear that sequences downstream of the translational start site of the *dct*A gene are involved in the regulation of expression of the gene. Also, just like the *nif*H gene, which

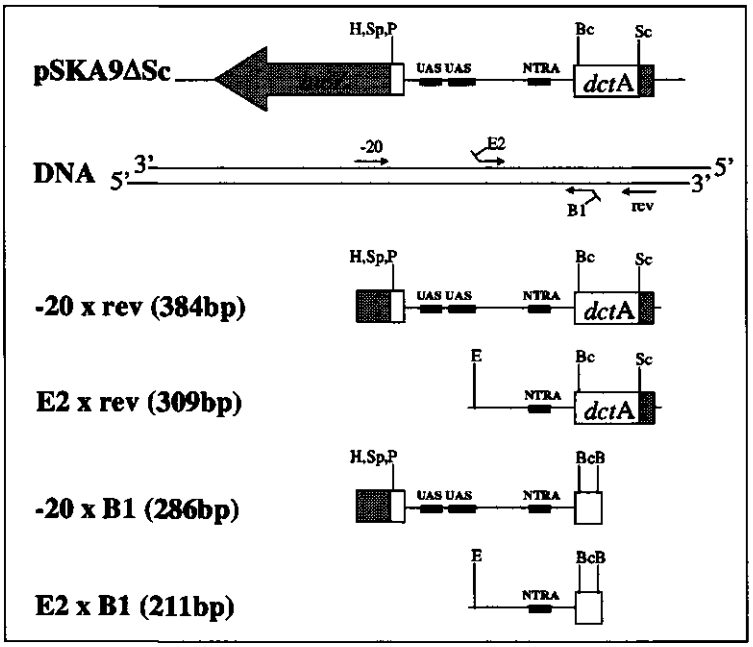

Fig. 3d: The PCR products.

A pSKA9 derivative from which the *Sac*I fragment has been deleted (pSKA9ΔSc) was used to PCR amplify DNA fragments. Various combinations of primers were used to obtain the required fragments. The –20 and rev primers are standard M13 primers. The B1 and E2 primers were purpose designed to introduce suitable restriction sites for cloning purposes. The sizes given in brackets are those of the *dct* sequences contained within the amplified fragments. B = *Bam*HI; Bc = *Bcl*I; E = *Eco*RI; H = *Hind*III; P = *Pst*I; Sc = *Sac*1; Sp = *Sph*I; UAS = upstream activator sites; NTRA = *dct*A promoter.

is efficiently expressed during symbiosis even without the UAS (Better et al., 1985), the possibility existed that the upstream activator sequences (UAS) might be dispensable for symbiotic expression of the *dct*A gene. To investigate to what extent promoter sequences are required for expression we decided to construct a number of gene fusions with predefined limits on the *dct*A fragments. To

achieve this, a number of oligonucleotide primers were designed for PCR amplification of *dct*A DNA fragments with suitable restriction sites in their extremities for fusion with the *lac*Z reporter genes. The B1 primer (CTCGGATCCGCGGAATGTTCGAT) introduced a *Bam*HI restriction site at the eight codon

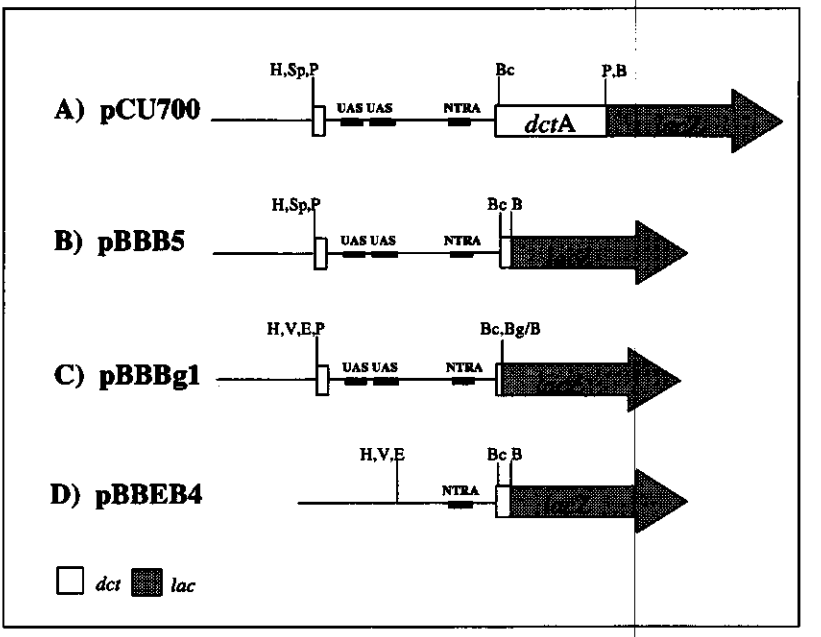

Fig.3e: The pGD926 *dct*A::*lac*Z fusion plasmids.

A) Plasmid pCU700 contains a 700 bp *Pst*I *dct* fragment cloned into pGD926. **B)** The cloning of a 286 bp PCR amplified fragment, directly into pGD926 resulted in pBBB5. This DNA fragment codes for only the first 8 amino acids of the DctA protein. **C)** For the construction of pBBBg1, a 266 bp *Pst*I-*Bcl*I fragment was cloned, with a suitable linker, into pGD926. This DNA fragment does not contain any part of the *dct*A coding region. **D).** The cloning of a 211 bp PCR amplified fragment with suitable linkers into pGD926 resulted into BBEB4. This plasmid carries a *dct*A::*lac*Z gene fusion , identical to pBBB5, but the fragment is lacking the UAS sequences. . B = *Bam*HI; Bc = *Bcl*I; E = *Eco*RI; H = *Hind*III; P = *Pst*I; Sc = *Sac*1; Sp = *Sph*I; UAS = upstream activator sites; NTRA = *dct*A promoter.

of the *dct*A coding region. This site can be used for cloning in-frame with the *lac*Z genes, into the *Bam*HI restriction sites in pGD926, or pMC1403. The E2 primer (ACGAATTCCACGAACGGCAAGCGAG) was designed to introduce an *Eco*RI restriction site just downstream of the UAS. A version of pSKA9, restricted with *Sac*I and religated (pSKA9ΔSc) was used as a DNA source to PCR amplify the various *dct*A gene fragments using different combinations of the B1, E2 and the standard M13 forward (-20) and reverse primers. Four different size fragments were obtained whose identity could be confirmed by restriction digests (Fig. 3d).The DNA fragments amplified with the B1 and -20 primers were restricted with *Bam*HI and *Hind*III and cloned directly into pGD926. This resulted in a trio of gene fusion plasmids pBBB5, -6 & -7 (Fig. 3e panel B) which

displayed the correct restriction pattern. All three plasmids were conjugated into *S.meliloti* and found to be efficient *dct*A::*lacZ* gene fusions.

To obtain a *dct*A::*lacZ* gene fusion comparable to the pBBB plasmids, but lacking the UAS, the 211 bp PCR fragment obtained from the B1 and E2 primers was used. The *Eco*RI restriction site incorporated in the upstream primer did not allow the direct cloning of this PCR DNA into pGD926. Therefore this DNA was restricted with *Bam*HI and *Eco*RI and first cloned into the pBluescript SK$^-$ plasmid. A number of plasmids featuring the correct restriction pattern were obtained. The DNA from ten such pSKEB plasmids was pooled and liniarised with the *Bam*HI restriction enzyme to cointegrate these plasmids with pGD926, also liniarised with *Bam*HI. Nine cointegrates were obtained on medium containing antibiotics selective for both plasmids. Interestingly all nine contained the pSKEB plasmids in the correct orientation resulting in a *dct*A::*lacZ* gene fusion. In order to obtain the pGD926 plasmids containing the *dct*A::*lacZ* gene fusions, the cointegrates were restricted with *Hind*III and religated. This resulted in the loss of the pBluescript replicon. Transformation of DH5 and selection on tetracycline produced the required pBBEB fusion plasmids. Similarly, corresponding plasmids containing the *dct*A promoter fragment in the high copy number pBluescript vector (pSKEB), were obtained by restriction of the cointegrates with *Bam*HI and selection on ampicillin. In this manner the nucleotide sequence of the *dct*A fragment of each pBBEB plasmid could be verified by sequencing the corresponding fragments in the pBluescript replicons. Three pBBEB plasmids (pBBEB1, -2 & -4; Fig. 4e D) were used for further studies. Sequence analysis of the pSKEB1, -2 & -4 was carried out and each one contained the correct *dct*A fragment without any mutations, or alterations.

3.5) Microbiological techniques and plant growth conditions.

3.5.1) Microbiological techniques.

Complex medium for *E. coli* and *S. meliloti* was Luria broth (Gibco). Minimal medium for *Rhizobium* was as described by Vincent (1970), supplemented with 6 mM nitrate as a source of nitrogen and 1 nM biotin. Carbon sources were added at 0.2 % w/v.

Plasmids were transferred from the *E. coli* donors into *S. meliloti* by tri-parental matings using pRK2013 as helper plasmid as described by Ditta *et al.* (1980). Exconjugants were selected on minimal medium supplemented with suitable antibiotics and purified on complex medium.

Transduction experiments with the N3 transducing phage were carried out as described by Martin and Long (1984).

3.5.2) Plant growth conditions.

Medicago sativa cv. Europe and *Medicago truncatula* cv. Jemalong plants were used in this work. Seeds were sterilised and germinated and plants were grown on agar slants in glass tubes, under controlled conditions as described by Ardourel *et al.* (1994). Two plants were grown in each tube. Three days old seedlings were infected with appropriate *S.meliloti* strains. The strains were pregrown on solid complex medium in the presence of the appropriate antibiotics to ensure the presence of the

plasmids. A loopfull of bacteria was resuspended in 3 ml. sterile water. Inoculation from a liquid culture was avoided because of possible transfer of nitrogen containing compounds. 0.3 ml of the bacterium suspension was used to infect the 2 seedlings/tube. In our experimental setup, mature N_2-fixing nodules were obtained from the wild-type strains within two weeks after infection.

3.6) Enzymatic assays and histochemical staining.

3.6.1) The enzymatic assays of fusion activity.

β-D-galactosidase and β-D-glucuronidase assays were modified in order to have a standard assay for both types of enzyme, suitable for free-living cultures as well as bacteroid suspensions. In a typical assay, 250 µl of a cell suspension (in growth medium, or diluted in 0.1M phosphate buffer pH 7) was mixed with 250µl assay buffer (0.1 M $NaHPO_4$ pH 7; 50 mM β-mercaptoethanol; 0.2 % Sodium lauryl sarkosine; 0.2 % Triton X-100; 10 mM KCl and 1mM $MgSO_4$). These were incubated at 37°C for 5 min, before adding 50 µl substrate (4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside or *p*-nitrophenyl-β-D-glucuropyranoside). Incubation proceeded at 37°C until a yellow colour developed (Usually 15 mins. for LacZ and 60 mins. for the GUS fusions). The reaction was stopped by the addition of 200µl 1M $NaCO_3$. The release of *o*-, or *p*-nitrophenol was measured at 420 nm, the enzyme activities calculated and expressed in terms of nmoles nitrophenol released / min. x OD600. Because the β-D-galactosidase assay according to Miller (1972) is done at 28°C, the effect of the increased temperature on the assay was evaluated. The levels measured at 37°C were on average about 50% higher than those measured at 28°C. This is more than would be expected from the increased temperature alone and may be due to a better lysis of the cells at the elevated temperature.

3.6.2) Preparation of bacteroid suspensions.

Bacteroid suspensions for the measurement of the activity of the gene fusions during symbiosis were prepared as follows. For the measurement of the β-D-galactosidase activity the nodules were first fixed in glutaraldehyde as described below. The fixation step specifically reduces the background β-galactosidase activity of plant origin. For the β-D-glucuronidase assays freshly picked nodules were taken. The nodules were sampled in 1 ml of 0.1 M sodium-phosphate buffer pH = 7,0; 0.1% Triton. The nodules were crushed with a appropriately shaped glass rod. The use of glass powder was avoided as it influences the OD_{600} readings. After vortexing for 30 seconds the samples were left standing for 15 mins. to allow the plant debris to settle. A 0,5 ml sample of the supernatant was carefully taken off without disturbing the pellet, and used for immediate assays as described in 3.6.1.

3.6.3) Histochemical techniques.

Plant root material infected with *S.meliloti* strains carrying *lacZ* gene fusions was sampled in petri dishes containing 20 ml 0.1 M Sodium-phosphate buffer pH = 7; 0.1% Triton. The free air was expelled under vacuum for 30 min. After the vacuum step, the samples were fixed in 1.25 % glutaraldehyde in 0.1 M phosphate buffer pH = 7, for 30 mins. This fixation step specifically inactivates the β-galactosidase activity of plant origin (Teeri *et al.*, 1989). Root systems and young nodules (less than 7 dpi), were stained directly in staining buffer (0.1 M Sodium-phosphate buffer pH = 7; 0.1% Triton, 5 mM $K_3[Fe(CN)_6]$ and 5 mM $K_4[Fe(CN)_6]$), containing 0.02% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Undisected samples were stained overnight for 20 hrs. The penetration of mature nodules by the dyes is hampered, especially into the late symbiotic zone III. Therefore the mature nodules were fixed in glutaraldehyde and mounted in a H1250 Microcut vibrating blade microtome (Energy Beam Sciences) under 0.1 M Sodium-phosphate buffer pH = 7 and sliced into 80 µm sections. The sections were incubated in staining buffer with X-gal for various amounts of time, depending on the strength of the fusions and the intensity of staining required. Typical staining times for nodule sections are less then 1 hr for the highly expressed *nif*H::*lacZ* gene fusions and about 2,5 hrs. for most *dct*A::*lacZ* gene fusions. The staining reaction was stopped by washing the sections in acetone. The sections could be stored under clean water for several weeks at 4°C.
In case of the GUS fusions the fixation step is omitted. Entire root systems and young nodules were stained in petri dishes containing 20 ml staining buffer containing 0.02% 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-glc). The free air was expelled under vacuum for 30 min. and the samples incubated overnight at 37°C. Mature nodules were sliced in the vibratome and the sections stained for several hours. The staining reaction was terminated by fixation in 2% glutaraldehyde.
Double staining involved an initial staining of the unfixed sample for GUS activity, followed by a fixation step and staining for β-galactosidase activity with the Magenta™-β-D-gal substrate. The chromogenic substrates were obtained from Biosynth AG, Staad, Switzerland.
The specimens were observed by brightfield microscopy at low magnification with a Wild Leitz M3Z binocular equipped with a MPS 48 photoautomat and at higher magnifications with a Zeiss Axiophot photomicroscope.

3.7) Bacterial Strains, Plasmids and Bacteriophage.

Table 3a: Bacterial Strains, Plasmids and Bacteriophage used in this study.

Designation	Relevant Characteristics	Reference / Source
***S.meliloti*:**		
RCR2011	SU47, wild-type, nod+, fix+ on *Medicago* spp	Rosenberg *et al.*, 1981
1021	SU47, Str^R	Meade *et al.*, 1982
102F34	commercial inoculant strain, Nitragin Co., Milwaukee, Wisconsin.	Better *et al.*, 1983

1531	1021 pSym20::Tn5, Nm^R, Str^R	Ruvkun *et al.*, 1982
GMI 211	2011 L$^-$; a *lac* negative derivative of 2011 Str_3, Str^R	Niel *et al.*, 1977
GMI 5600	GMI211 pSym20::Tn5, Nm^R, Str^R (Transduced from 1531)	David *et al.*, 1988
F121	Rm 1021, *dct*D16::Tn5, Nm^R, Str^R	Yarosh, 1989
F332	Rm 1021, *dct*B18::Tn5, Nm^R, Str^R	Yarosh, 1989
F642	Rm 1021, *dct*A14::Tn5, Nm^R, Str^R	Yarosh, 1989
1354	Rm 1021, *nif*A::Tn5, Nm^R, Str^R	Zimmerman *et al.*, 1983
20D1	*dct*D$^-$, Nm^R , Tn5 insertion of F121 transduced into 2011	This work
D2R	F121/pBBA2 transduced back to wild-type phenotype, *dct*D$^+$, Nm^S.	This work
E.coli:		
DH5	*rec*A1, *end*A1, *hsd*R17, *sup*E44, λ$^-$, *thi*-1, *gyr*A96, *rel*A1, *deo*R	Hanahan, 1983
GM48	F$^-$, *thr*, *leu*, *thi*, *lac*Y, *gal*K, *gal*T, *ara*, *fnu*A, *tsx*, *dam*, *dcm*, *sup*E44	Marinus *et al.*, 1973
Plasmids:		
pBluescript SK$^-$	Phagemid, fl(-) origin of replication, ColE1 replicon, Ap^R	Stratagene
pSKBBB	pSK$^-$ derivative with *Bam*HI; *Bgl*II and *Bcl*I restriction sites in three different reading frames, Ap^R.	This study
pSKA9	700bp *Bam*HI-*Hin*DIII *dct*A promoter fragment of pCU700 cloned into pSK$^-$, Ap^R	This study
pSKA9ΔSc	Derived from pSKA9 by deleting the *Sac*I fragments from the *dct*A sequence.	This study
pSK4	260bp *Hin*DIII-*Bcl*I *dct*A promoter fragment cloned into pSKBBB, Ap^R	This study
pRKP9	pSVP9 & PRK290 joined at their *Eco*RI sites, Ap^R, Tc^R	Szeto *et al.*, 1987
pMB1156	200 bp *nif*HDK promoter fragment lacking UAS cloned into pGD926, Tc^R, mob	Better *et al.*, 1985
pML330	cointegrate of pMB210 (*nif*H::*lac*Z; Better *et al.*, 1985) and pDK330 (truncated *fix*J; Kahn & Ditta, 1991) , Ap^R, Tc^R, mob.	Soupène, 1996
pCHK57	295 bp *nif*A promoter fragment cloned into pGD926, Tc^R, mob	Ditta *et al.*, 1987
pSVP9	pMC1403 derivative bearing the *nif*H expression signals fused to the *lac*Z reporter gene, Ap^R	Sundaresan et al., 1983
pRK290	Broad host range replicon, Tc^R, mobilisable (mob)	Ditta *et al.*, 1985
pSVPA	*nif*H promoter of pSVP9 replaced by the 267 *Hin*DIII-*Bgl*II *dct*A promoter fragment of pSK4, Ap^R	This study
pBBA2, -4 & -6	pSVPA & PRK290 joined at their *Eco*RI sites, Ap^R, Tc^R	This study

pGD926	Probe plasmid for translational gene fusions to the enteric *lacZ* gene, Tc^R, mob	Ditta *et al.*, 1985
pCU700	translational *dct*A::*lacZ* gene fusion. 700bp *Pst*I *dct*A promoter fragment cloned into pGD926, Tc^R, mob	Wang *et al.*, 1993
pBBBg1 & -2	translational *dct*A::*lacZ* gene fusion. 260bp *Pst*I-*Bcl*I *dct*A promoter fragment cloned into pGD926, Tc^R, mob	This study
pBBB5,-6 & -7	translational *dct*A::*lacZ* gene fusion. 279bp *Pst*I-*Bam*HI PCR -*dct*A promoter fragment cloned into pGD926, Tc^R, mob	This study
pBBEB1,-2 & -4	translational *dct*A::*lacZ* gene fusion. 200bp *Eco*RI-*Bam*HI PCR *dct*A promoter fragment (ΔUAS) cloned into pGD926, Tc^R, mob	This study
pSUP202	narrow host range, Ap^R, Tc^R,Cm^R, mob.	Simon *et al.*, 1983
pCCOGUS	pUC19 derivative with a promoterless GUS cartridge, Ap^R.	Axelos *et al.*, 1989
pBBGA	PSK - pUC19 derivative with *dct*A expression signals fused at the *Bcl*I site to the *uid*A (GUS) gene, Ap^R	This study
PBBGA23	*dct*A expression signals fused at the *Sac*I site to the *uid*A (GUS) gene, Ap^R	This study
pSUPGA1	*dct*A::*uid*A gene fusion cartridge from pBBGA cloned as a *Pst*I fragment into β-lactamase gene of pSUP202, Ap^S, Tc^R,Cm^R	This study
pRKP4	*dct*A::*uid*A gene fusion cartridge from pBBGA cloned as a *Pst*I fragment into β-lactamase gene of pRKP9, Ap^S, Tc^R	This study
PRKP23	*dct*A::*uid*A gene fusion cartridge from pBBGA23 cloned as a *Pst*I fragment into β-lactamase gene of pRKP9, Ap^S, Tc^R	This study
pRK2013	tra^+ helper plasmid for conjugations, narrow host range, Nm^R	Ditta *et al.*, 1980
Bacteriophage:		
N3	Transducing phage of *S.meliloti*	Martin & Long, 1984

Ap, ampicillin; Cm, chloramphenicol; Nm, neomycin; Tc, tetracycline; ^{R}, resistant; ^{S}, sensitive.

Chapter 4)

Regulation of the *S.meliloti dct*A gene under free-living conditions

4.1) Introduction.

In Chapter 3.4 we have described the construction of a number of *dctA*::*lacZ* gene fusions. The main difference between the various constructs lies in the location of the fusion point with respect to the nucleotide sequence of the *dctA* coding region. The parent plasmid pCU700 contains a 700 bp *dctA* fragment, that contains the coding sequence for the N-terminal 161 amino acids of the DctA protein. This gene fusion will be referred to as the "long" gene fusion. We have constructed a number of "short" fusion plasmids (pBBA2; pBBBg1 & -2) that carry a 260 bp *dctA* fragment. In these plasmids, the *lacZ* gene is fused to the ATG start codon of the *dctA* gene. The third group contains a number of plasmids (pBBB5 to –7) where the *lacZ* gene is fused to the 8th amino acid of the *dctA* gene. The upstream boundary of the *dctA* fragments is the same for all these plasmids. The pBBEB1, -2 & -4 plasmids also belong to this third group, but carry a shorter *dctA* fragment which is lacking some regulatory sequences upstream from the *dctA* promoter. The fusion points for the three groups of plasmids,

BclI *PstI*

ATGATCATCGAACATTCCGCGGAGGTCCGCGGCAAGACACCC - - - CTGCAG

Sequence	CAGATCC	TCC	GAGGATCC
Linker	*BclI-BglII* linker	B1 primer	pUC18-1 linker
Plasmids	pBBA2 pBBBg1 pBBBg2	pBBB5 pBBEB1 pBBB6 pBBEB2 pBBB7 pBBEB4	pCU700
Class	"short"	"third group"	"long"

Fig. 4a: The fusion points between the *dctA* and the *lacZ* genes.

The top line represents the *dctA* coding sequence commencing with the ATG start codon. The *Bcl* restriction site (underlined) partially overlaps this start codon. The second line represents the linker sequence fusing the two genes together. The GAT codon within the *Bam*HI restriction site at the N-terminal end of the *lacZ* gene and in frame with *lacZ* is underlined. The *Pst* restriction site (underlined) is located about 500 bp downstream from the ATG. This site was used to construct the "long" gene fusion on pCU700. The "short" gene fusions have the *lacZ* gene fused to the ATG start codon of the *dctA* gene. This group includes pBBA2 and the pBBBg plasmids. The third group plasmids have the *lacZ* gene fused to the 7th amino acid of the *dctA* gene. The third group includes the pBBB and pBBEB plasmids.

between the *dctA* sequence and *lacZ*, including the transition sequences are given in Fig. 4a.
In this chapter we evaluate the regulation of the different gene fusions of the *S.meliloti dctA* gene to the *lacZ* reporter gene. The aim of our work is to study the regulation of the *dctA* gene *in situ* on the *Medicago* host plant. However, before the gene-fusions can be used for the *in situ* work, the expression under free-living conditions must be evaluated. Several factors can influence the levels and the patterns of expression obtained from different gene fusions. The level of expression depends

for example on the promoter strength and level of induction, but also the copy number of the carrier plasmid, the stability of the mRNA, the efficiency of initiation of translation and the stability of the fusion protein, can have an important effect. Therefore a careful examination of the construction details of the gene-fusions and a comparison of the expression patterns under controlled conditions are necessary for each gene fusion plasmid that is to be used for further studies. Several groups have studied the regulation of the *dct*A promoter under free-living conditions (Yarosh *et. al.*, 1989; Wang *et al.*, 1989; Jording *et al.*, 1992). Some fusions were also used to study the regulation in the well characterised *E.coli* background (Ledebur and Nixon, 1992; Wang *et al.*, 1993; Chapter 2.7; Allaway *et al.*, 1995; Chapter 2.9). Plasmid pCU700 which was used as the parent for the construction of the different fusions in this work, has also been used to study the regulation of the *dct*A gene in the heterologous *E.coli* background (Wang *et al.*, 1993). The methods used in the various laboratories to induce the *dct*A gene differ somewhat in detail, especially in the timing of the assay after induction. Induction of the gene fusions can be obtained in several different ways. The cultures can be grown on complex medium, or minimal medium including an inducer and ever increasing levels of β-galactosidase can be obtained until well into the stationary growth phase. Another method is to grow the cultures on minimal medium without inducer into the mid-log phase and then add the inducer. In this case a rapid induction of the gene fusions can be observed within a few hours. In any case the levels obtained are highly dependent on the growth phase of the cultures and the time point that the data is taken. Often data are published where cultures are harvested and the fusion activity measured at a fixed period after addition of the inducer. Such data however do not consistently give a good indication of the activity of the gene fusions. It is advisable to use growth curves and multiple measurements to evaluate the activity of a gene fusion.

Conform to the model of DctBD dependent regulation of the *dct*A promoter, a typical *dct*A gene fusion displays the following pattern of expression under free-living conditions. A low level of expression is observed in a wild-type strain in the absence of specific inducers in the growth medium. In a strain mutated in either, the *dct*B or the *dct*D gene, no expression is observed. The *dct*A promoter is induced by the presence of C_4-dicarboxylic acids (dCA: malate, succinate and fumarate) in the growth medium. It is also strongly induced by the amino-acid aspartate (Engelke *et al.*, 1989; Watson *et al.*, 1990). The latter is generally thought responsible for induction of the *dct*A promoter on complex media such as TY and LB. In the absence of a functional *dct*A gene the *dct*A promoter is expressed "constitutively" at a high level (Ronson *et. al.*, 1985, 1987a; Yarosh *et al.*, 1989; Jording *et al.*, 1992). It has been demonstrated that the *dct*BD genes are required for *dct*A expression under these conditions (Yarosh et al., 1989).

4.2) Expression of the "short" gene fusion under free-living conditions.

Plasmid pBBA2 (Fig. 3a C) was the first plasmid constructed for these studies and belongs to the group of "short" gene fusions, in which the ATG start codon of the *dct*A gene is directly fused to the *lac*Z gene. It was simultaneously conjugated from the *E.coli* donor into the *S.meliloti* 2011 wild-type strain and the *dct* mutant strains: F642 (*dct*A$^-$), F332 (*dct*B$^-$) and F121 (*dct*D$^-$).

4.2.1) pBBA2 activity on complex medium.

The pBBA2 containing *S.meliloti* strains were grown in liquid TY medium for 23 hrs. The β-galactosidase activity of these overnight cultures was measured (Fig. 4b, time = 0). In the wild-type background the fusion was highly expressed. This high level of activity was attributed to the assumed presence of aspartate in this medium which is known to be an effective inducer of the *dct*A gene. As expected the *dct*B and *dct*D mutant strains (F332/pBBA2 and F121/pBBA2) did not show any expression of the gene fusion under these conditions. A strain derived from F121/pBBA2, transduced

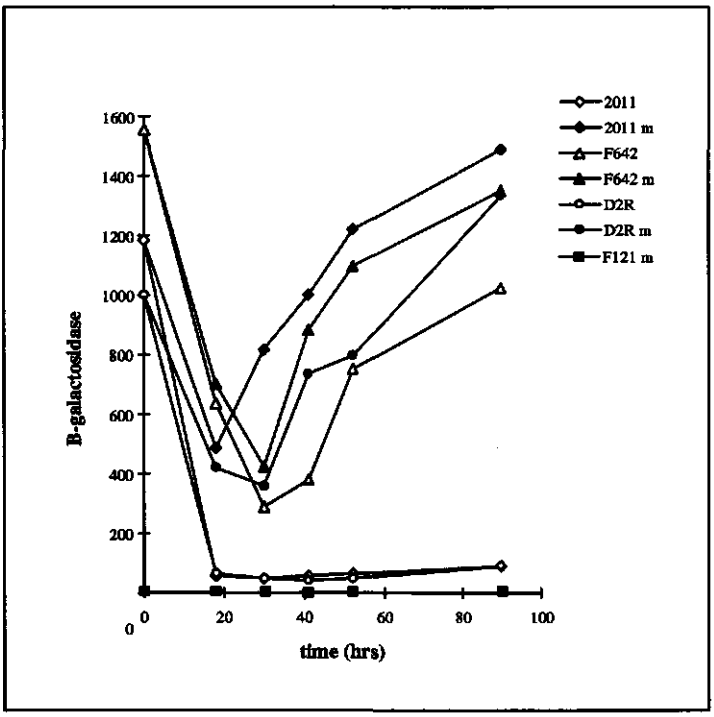

Fig. 4b: Expression of the pBBA2 gene fusion in wild-type and *dct* mutant strains.
The various *S.meliloti* strains containing the pBBA2 plasmid were pre-grown overnight in rich medium (time = 0 hrs). These cultures were used to inoculate minimal medium containing mannitol as sole carbon source (open symbols), or the same medium with malate added (m; closed symbols). One unit of β-D-galactosidase represents 1 nmole nitrophenol released at 37°C / min. x OD_{600}. 2011 = wild-type; F642 = *dct*A mutant; F121 = *dct*D mutant; D2R = F121/pBBA2 transduced back to wild-type phenotype. The curves for the *dct*B mutant (F332) and F121 without malate are not given as they coincide with the curve for F121 + malate.

back to a wild-type phenotype (D2R *dct*D^+, Nm^S) was included in this experiment. This strain behaved like the wild-type and demonstrated that the lack of expression from the gene fusion in the *dct*D

mutant background is not due to a possible mutation in the plasmid itself. The *dct*A mutant strain F642/pBBA2 showed levels of expression, about 50% higher than the wild-type.

4.2.2) Induction of the pBBA2 gene fusion on minimal medium.

For induction of the *dct*A gene, a small volume of the overnight cultures grown on TY was used to inoculate (5% v/v) mannitol minimal medium with and without malate (Fig.4b). All cultures grew equally well on either medium and reached the stationary phase in less than 40 hours (Data not shown). The final OD_{600} obtained on minimal medium was only about half of that usually obtained on complex medium. The final OD_{600} obtained on minimal mannitol medium, supplemented with malate, was similar for *dct*$^+$ and *dct*$^-$ strains. This indicates that the availability of a carbon source was not the growth limiting factor in the minimal medium used. The levels of β-galactosidase from the *dct*D and *dct*B mutant strains remained very low either with, or without malate in the growth medium. The β-galactosidase activity from the wild-type strain, which had been induced on the complex medium, dropped back in the absence of malate within the first 24 hrs of incubation. The activity from then on remained at a level about tenfold higher than that of the *dct*D and *dct*B mutants for the rest of the experiment. The wild-type strain in the presence of malate initially also dropped back substantially over the first 24 hrs, but then recovered and reached high levels of expression towards the end of the logarithmic growth phase. A similar pattern was observed for the *dct*A mutant strain, no matter if malate was present in the medium or not. It is not clear why the activity of the fusions drops, but it may be related to the lag period after the transition from complex to minimal medium. The D2R strain, essentially behaved like the wild-type. Apart from malate, the gene fusion in 2011/pBBA2 was found to be equally induceable by fumarate, succinate and aspartate (Data not shown).
In summary, three distinct levels of expression can be observed: No expression of the gene fusion in the *dct*B and *dct*D mutant strains (less than 10 U.). A basal level of uninduced expression in the wild-type strains in absence of dCA (less than 100 U.). And finally a high level of expression in the induced wild-type and the *dct*A mutant strains (more than 1000 U.). The *dct*A mutant also shows high level of expression even in the absence of inducer. Plasmids pBBBg1 and –2 which contain the same *dct*A::*lac*Z gene fusion, cloned directly into pGD926 (Fig. 3e C), gave the same results as pBBA2. These experiments demonstrate that pBBA2 and the pBBBg plasmids carry a correctly regulated *dct*A::*lac*Z gene fusion suitable for *in situ* studies during symbiosis with the *Medicago* host plants.

4.3) Activity of pBBA2 relative to pCU700.

The regulation of the "short" gene fusion on pBBA2 was as expected for a properly regulated *dct*A::*lac*Z gene fusion. The expression pattern was similar to that obtained with the pCU700 gene fusion. However, the levels of expression of the pBBA2 gene fusion appeared to be consistently several times higher than those usually obtained with pCU700. On complex medium, maximum levels of β-galactosidase activity from 2011/pBBA2 reached close to 2000 U. at the late logarithmic growth phase. For example, maximum expression from 2011/pCU700 under identical conditions only reached

to about 600 U. The pBBA2 and pCU700 gene fusions differ in the extent of DNA from the N-terminal coding region of the *dct*A gene (Figs. 3a & 4a). The fact that pCU700 is derived from pGD926 and pBBA2 is a cointegrate of pMC1403 and pRK290, seems unimportant as the pBBBg plasmids behave exactly as pBBA2. We wanted to evaluate if the difference in expression was due to differences in

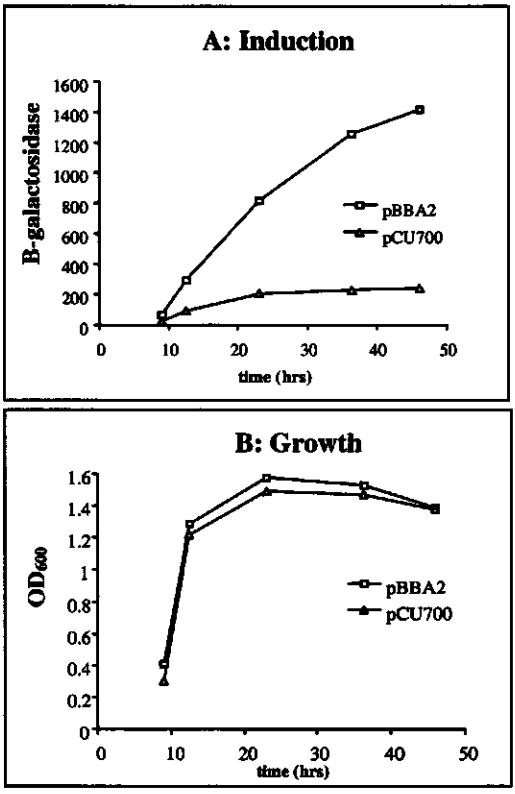

Fig. 4c: Direct comparison of *dct*A::*lacZ* gene fusions pCU700 and pBBA2 in the 2011 wild-type background.
A: Induction of the gene fusions. The difference in activity obtained from the two plasmids measured a factor of 2.9 at 9 hrs(uninduced); 3.3 at 12.5 hrs; 3.9 at 23 hrs and 5.5 after 36.5 hrs of incubation. **B:** Growth. Both cultures grew equally well. Malate was added at time = 9 hrs. One unit of β-D-galactosidase represents 1 nmole nitrophenol released at 37°C / min. x OD_{600}. Each point is the average of three independent cultures.

efficiency of the two gene fusions (in terms of for example RNA stability or copy number effects), or could be contributed to some regulatory effect linked to the N-terminal sequence of the *dct*A coding region. Therefore the two gene fusions were compared directly in an induction experiment. Overnight, early logarithmic pre-cultures on complex medium were used to inoculate minimal mannitol medium. The cultures were incubated for 9 hrs. to allow the fusion activities to drop back to uninduced levels. At this point malate was added to induce the fusions (Fig. 4c). The fusion activity, as judged by the rate of accumulation of β-galactosidase activity from 2011/pBBA2 was found to be about three times higher than that from 2011/pCU700. No difference was observed in the growth rate of the two strains. When the stationary growth phase was reached after about 24 hrs. No further increase in β-galactosidase levels was observed with pCU700. However the β-galactosidase levels with pBBA2 continued to increase for the duration of the experiment. The difference in β-galactosidase levels between the two fusions approached a factor 6 in the late stationary phase.

The initial difference in activity suggested that the "short" gene fusion is more efficient than the "long" one. However, the difference in behaviour in the stationary phase suggests that the long fusion may be more tightly regulated and suggests the presence of a cis-acting regulatory element in the N-terminal part of the coding region of the *dct*A gene.

4.4) Gene fusion expression in a *dct*A mutant background.

In a *dct*A mutant background, gene fusions of *dct*A to various reporter genes generally results in high levels of expression, no matter if dCA are added to the medium or not (Ronson *et al.*, 1985, 1987a; Yarosh *et al.*, 1989; Jording *et al.*, 1992). From this constitutive expression in the absence of a functional DctA protein, various researchers have concluded that there must exist some kind of feed-

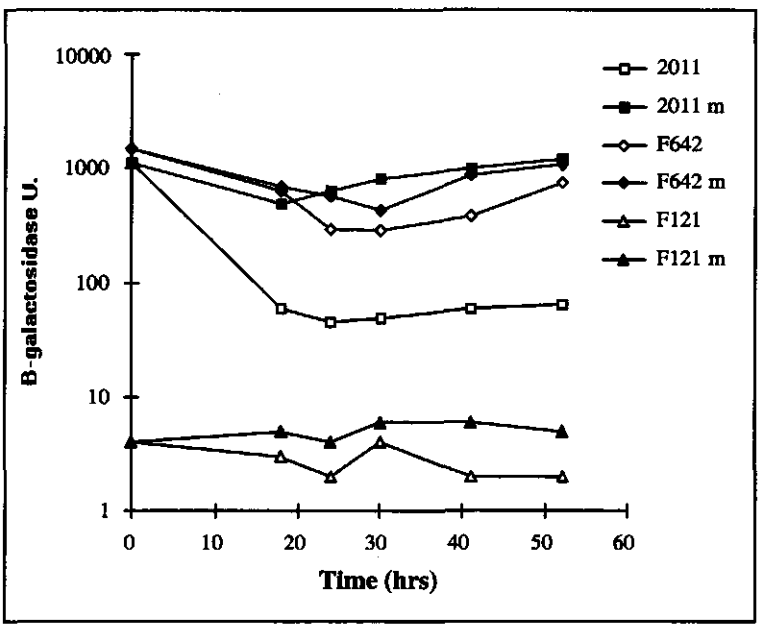

Fig. 4d: Constitutive expression in of pBBA2 a *dct*A background.
Some of the data presented in Fig. 4b is plotted against a logarithmic scale. This clearly demonstrates the three different levels of *dct*A activity. **1)** Low level of activity in the absence of the transcriptional regulator DctD. **2)** Basal level of uninduced *dct*A expression. **3)** High level of activity either in the induced wild-type or *dct*A mutant backgrounds. The cultures were grown in minimal medium containing mannitol as sole carbon source (open symbols), or with malate added (m; closed symbols). One unit of β-D-galactosidase represents 1 nmole nitrophenol released at 37°C / min. x OD_{600}. 2011 = wild-type; F642 = *dct*A mutant; F121 = *dct*D mutant

back regulation of the *dct*A promoter by the DctA gene product. Often the actual levels of expression obtained in the *dct*A⁻ strain were found to be several times higher than the maximum levels obtained in

a wild-type strain (Yarosh *et al.*, 1989; Jording *et al.*, 1992). Yarosh and co-workers also demonstrated that the *dct*BD system is required for the elevated *dct*A expression in the *dct*A mutant background. This led to the hypothesis that the DctA gene product may interact with the DctB product, also located in the cytoplasmic membrane, to exert this regulatory effect.

Although in general this high level of *dct*A expression in the in the absence of inducer is referred to as "constitutive", such levels may still be increased even further by the addition of dCA to the growth medium (Y-P Wang, personal communication). On the other hand, it has been shown that prolonged culturing in minimal medium, in the absence of dCA reduces the levels of expression in a *dct*A mutant background (Batista *et al.*, 1992). Under those conditions, several stimuli not related to Dct, such as osmotic stress, can trigger high levels of expression of the *dct*A gene.

With the pBBA2 gene fusion high levels of expression were obtained in the F642 strain. However, these levels of expression were not significantly higher than the induced levels of expression obtained in the wild-type. This was in contrast with earlier observations made with the pCU700 gene fusion and the above mentioned reports from other researchers (Jording *et al.*, 1992; Yarosh *et al.*, 1989). In most cases fusion activity in a *dct*A$^-$ background is much higher than can be obtained in a wild-type. In the F642 *dct*A$^-$ background, the pCU700 and pBBA2 gene fusions are expressed to about 1500 U with or without the addition of dCA to the growth medium. In a wild-type background levels of expression of pCU700 rarely exceed 500 U. The fact that equal levels of expression are obtained from both fusions in a *dct*A mutant background, suggests that the difference in levels of expression observed in the wild-type background, is probably not due to a difference in efficiency between the two fusions. Rather this would indicate that the pCU700 gene fusion is repressed in the wild-type background, whereas the pBBA2 gene fusion is not. This in turn would indicate that some *cis*-acting elements, located in the amino terminus of the *dct*A coding region are involved in this repression. If some kind of feed-back regulation is involved, than one may predict that overexpression of the *dct*A gene should result in low expression of *dct*A gene fusions when introduced *in trans*. Although strains with constitutive expression of the *dct*A gene have been constructed (Rastogi *et al.*, 1992; D. Jording, personal communication), to my knowledge such a predicted negative regulatory effect on gene fusions has not been reported.

From these results we may conclude that the "short" *dct*A::*lac*Z gene fusions are efficiently induced by the DctBD system and are suitable for *in situ* studies during symbiosis with the *Medicago* host plant. The higher level of expression may be a positive advantage over pCU700, in order to detect low levels of *dct*A activity. However, a certain amount of caution should be taken, as some differences between the "long" and the "short" gene fusions have been observed that imply differences in regulation between the two fusions.

4.5) The PCR generated gene fusions.

The pBBB and pBBEB plasmids all carry gene fusions in which the 8th amino acid of the *dct*A gene is fused to the *lac*Z reporter gene (Fig. 3e). They belong to the third group of fusions used in this work (Fig. 4a). The pBBB plasmids are highly active gene fusions. On complex medium 3000 Units of β-

galactosidase were readily obtained. This is nearly twice as much as for the "short" gene fusions and 5-6 times more than pCU700. In induction experiments on minimal medium these gene fusions behaved like correctly regulated *dct*A::*lac*Z gene fusions except that both uninduced levels and induced levels of expression were higher than that observed for any of the other constructs.
The three types of gene fusions were compared in an induction experiment (Fig. 4e). The experiment differs from those presented before (Fig. 4b & d) in that the inducer was added at a late point in the growth phase, when the cells were already starved for carbon. Induction of the fusions followed immediately. It can be clearly seen that the pBBB gene fusions are the most highly expressed.

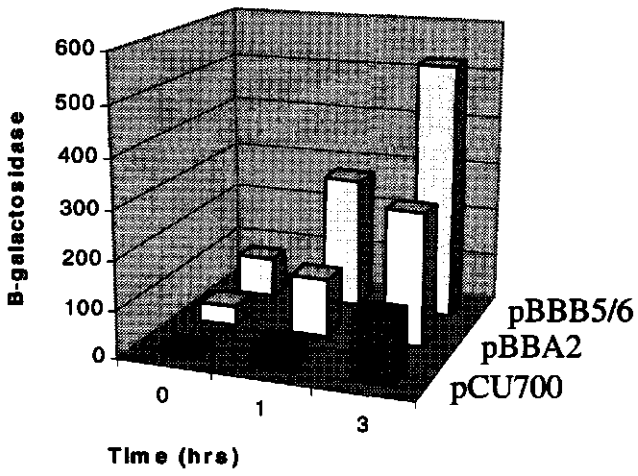

Fig. 4e: Induction of the *dct*A::*lac*Z gene fusions at the late logarithmic growth phase. The 2011 wild-type strains containing representatives of the three types of gene fusions were grown on minimal medium until the mannitol carbon source became limited. At this point (t = 0) malate was added to the cultures. Samples were taken 1 hr and 3 hrs after malate addition. Induction of the fusions was immediate.

However the induction rate (i.e. induced levels of expression divided by the uninduced levels) is not all that different for the three types of gene fusions and in fact is highest for pCU700. This indicates that all three types of fusions are equally good indicators of *dct*A promoter activity. Generally, the more active gene fusions would be preferred for the *in situ* work, but this may also result in higher background noise.
For the construction of the PBBEB gene fusions the same downstream fusion site was chosen as in the pBBB gene fusions, because these were the most active gene fusions. The upstream DNA was truncated to remove the UAS sites required for binding of the DctD transcriptional regulator. The prediction was that DctD would be no longer able to interact with this promoter and indeed no expression at all could be obtained from these gene fusions under free-living conditions (Fig. 6b). The effect of removing the UAS was essentially the same as deleting the *dct*D gene from the strain, in that the basal level of expression seen in the uninduced wild-type disappeared. Sequence analysis of the

*dct*A region demonstrated that no mutations had taken place. However we cannot exclude the possibility that some mutations elsewhere in the reporter gene may be responsible for the lack of activity from these gene fusions.

4.6) Summary.

Under free-living conditions, the different *dct*A::*lac*Z gene fusions generally behaved as one might expect. In the wild-type background, a low basal level of expression was observed in the absence of an inducer compound. When dCA, or aspartate were added to the growth medium an up to tenfold increase of activity could be obtained. In the absence of either, or both the *dct*B and the *dct*D genes, no induction was obtained and the uninduced basal levels of *dct*A expression dropped even further. A distinctly reduced basal level was also observed in a wild-type background for the pBBEB gene fusions where the UAS sites have been deleted from the *dct*A promoter fragment. Either way, this demonstrates that the DctBD system maintains a certain basal level of *dct*A expression under uninduced conditions. This basal level of DctBD dependent expression varied depending on the type of gene fusions used. In general the gene fusions that displayed the highest basal level of expression were also expressed highest after induction.

In the absence of a functional *dct*A gene the gene fusions are expressed "constitutively" at a high level. This has been verified for the pCU700 and pBBA and the pBBBg gene fusions, but not the pBBB and pBBEB gene fusions. In the wild-type background we observed a much higher activity of the "short" gene fusions, when compared to pCU700. In the *dct*A mutant background however, the levels of gene fusion activity were found to be similar. As it seems unlikely that differences in RNA or protein stability would manifest themselves in various *dct* backgrounds, this may indicate that the reduced activity of pCU700 in the wild-type background is more likely a regulatory effect (as discussed in Chapter 4.4).

The strongest expression was obtained with the pBBB gene fusions. The induced levels of activity are about twice as high as for the pBBA and pBBBg gene fusions. Even though the *dct*A fragments used to construct the two type of fusions only differ by merely 16 bp of the *dct*A coding region. Although for the *in situ* studies one tends to use the strongest gene fusions for easy detection of gene activity, the undoubted variations in regulatory effects on different gene fusions dictates to exploit a number of different fusions simultaneously. As we will see in the following chapters this is certainly not a case of being over-cautious. Moreover, the fact that a gene fusion behaves as expected under free-living conditions, is still no guarantee that it will also register all possible regulatory effects during symbiosis.

Chapter 5)

In situ expression of the *Sinorhizobium meliloti dctA* gene in mature nodules induced on the alfalfa (*Medicago sativa*) host plant

5.1) Introduction.

In the previous chapter we have analysed a number of *dct*A::*lac*Z gene fusions under free-living conditions. The activity of each of these fusions was found to be fully dependent on the presence of a functional DctBD system. Unlike *S.meliloti* strains mutated in the structural *dct*A gene, many strains mutated in either, or both the regulatory *dct*B and *dct*D genes are still capable of establishing an effective symbiosis. This indicates that the *dct*A gene is efficiently expressed during symbiosis, even in absence of a functional DctBD system. This observation led several researchers to postulate an alternative mechanism of *dct*A activation, which apparently is only active during symbiosis (ASA, see Chapter 2.5.1.). The existence of ASA poses a number of questions. Apart from the obvious question of the identity of ASA, which has not been solved to date, one must ask what role it plays in the establishment of an effective symbiosis. Is it an essential mechanism and is ASA also operating in a wild-type background? And if so, than which mechanism, ASA or the regular DctBD system, is primarily responsible for the activity of the *dct*A gene during symbiosis?
During symbiosis the microsymbionts differentiate from free-living bacteria, through various stages, into N_2-fixing bacteroids. The different stages of bacteroid development correspond with distinct zones in a mature nodule (See 5.1.1). Using histochemical staining techniques for β-galactosidase activity, we have tried to establish where, and by inference when, ASA becomes active. By making a link between ASA and certain stages of bacteroid development, we have tried to establish what regulatory pathways are involved in its activity.

5.1.1) The spatial organisation of a mature N_2-fixing nodule.

A mature indeterminate nodule is organised into different zones (Vasse *et al*, 1990), which correspond to a temporal and spatial pattern of bacterial and plant gene expression (Fig. 5a). The most distal and youngest part of a mature nodule contains the apical meristematic zone, which is free from bacteria. Zone II designates a region immediately behind the meristem containing the proliferating infection threads filled with dividing bacteria, followed by a region where the bacteria are released into the plant cells. These are the type 1 bacteroids. Type 1 bacteroids are rod shaped and still dividing and are contained in a plant derived peribacteroid membrane. This peribacteroid membrane separates the microsymbionts from the plant cell cytoplasm and forms a selective barrier against the free exchange of nutrients between the symbiotic partners (Mylona et al., 1995; Verma, 1992). Type 2 bacteroids are elongated, not longer dividing and are found in the most proximal part of zone II. Type 3 bacteroids are found exclusively in the interzone II/III, which is characterised by a high level of starch deposition in the plastids of the invaded cells. Type 3 bacteroids have stopped elongating and unlike type 2 bacteroids, fill most of the host cells. The plant cell organelles are lined against the cell wall. The interzone is of special interest as expression of plant and bacterial genes needed for symbiotic nitrogen fixation are sharply induced at this zone (De Billy *et al.*, 1991; Soupène *et al.*, 1995). Other genes such as the *rop*A gene coding for an outer membrane protein of *R.leguminosarum* are abruptly down regulated at this zone (de Maagd et al., 1994). The interzone II/III marks the transition from early

to late symbiosis. Symbiotic N_2-fixation takes place only in the fully differentiated type 4 bacteroids in the distal part of the late symbiotic zone III. Type 4 bacteroids are the same size as the non-fixing type 3 bacteroids, but show a more distinct heterogeneity at the ultrastructural level, with marked electron dense zones enriched with ribosomes. Type 5 bacteroids in the proximal part of zone III are not fixing nitrogen anymore. They are gradually losing the ribosome enriched areas and decrease in numbers. Finally after about 5 weeks a zone of senescence develops in the most proximal region of the nodule (Zone IV).

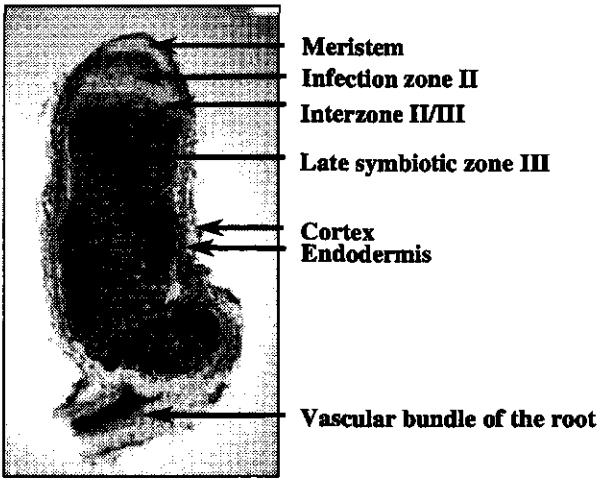

Fig. 5a: The spatial organisation of a two weeks old effective nodule on alfalfa. The various zones of bacterial and plant cell differentiation can be readily distinguished. The meristem at the apex of the nodule is the most distal from the root. This is followed by the infection zone II; the interzone II/III and the late symbiotic zone III, where the nitrogen fixation takes place. This nodule does not yet contain a senescence zone IV, which in a healthy N_2-fixing nodule develops after about 5 weeks in the oldest part of the nodule, most proximal to the root.

Cytological studies can provide valuable information about regulatory pathways operating in such intricate biological processes as nitrogen fixing symbiosis. The microsymbiont inside a mature indeterminate nodule exists in different physiological forms (Vasse et al. 1990; McRae et al. 1989a), which match the extent of differentiation starting with free-living bacteria up to N_2-fixing bacteroids. Having verified the correct regulation of the gene fusion under free-living conditions, we have evaluated *in-situ dct*A expression during symbiosis with alfalfa (*Medicago sativa* cv Europe).

5.2) *In situ* expression of the *dct*A::*lac*Z gene fusions during symbiosis.

To evaluate the *in-situ dct*A expression during symbiosis of *S.meliloti* with the alfalfa (*Medicago sativa* cv Europe) hostplant, nodules were collected between 2 and 3 weeks after inoculation. At this time wild-type nodules are well established and pink in colour. Plants inoculated with efficient strains are vigorous and green, whereas plants inoculated with non-fixing strains begin to show the first signs of stress at this age. Having established the efficiency under free-living conditions of both, the "short" gene fusion on pBBA2 and the "long" gene fusion on pCU700, these plasmids were used to study the spatial pattern of *dct*A activity in mature N_2-fixing nodules induced by the 2011 wild-type strain.

The spatial distribution of *dct*A activity was similar for both gene fusions. Expression of the *dct*A::*lacZ* gene fusions could be observed at all stages of bacteroid development. No induction of *dct*A activity was obvious in the infection threads immediately behind the apical meristem. Activity was induced as

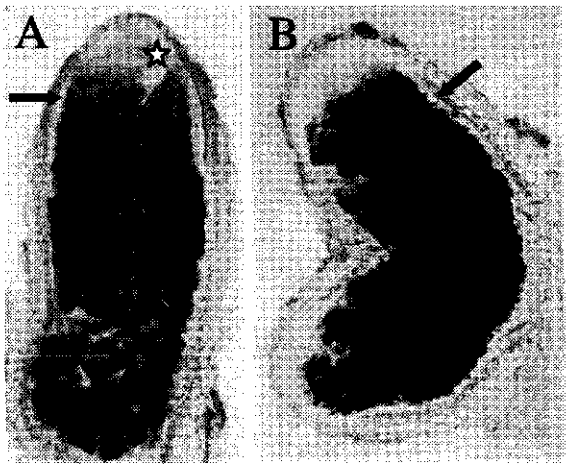

Fig 5b: *In situ dct*A::*lacZ* fusion activity in mature N_2-fixing nodules.
A) *In situ* expression of a "short" gene fusion in a wild-type background. Strain: 2011(pBBA2); 16dpi; coloration: X-gal for 2.5 hrs. The white star indicates an area of plasmid loss.
B) *In situ* expression of the "long" gene fusion in a wild-type background. Strain: 2011(pCU700); 16dpi; coloration: X-gal for 2.5 hrs. The arrows indicate the position of the interzone II/III. The spatial distribution of *lacZ* activity is similar for both types of gene fusions.

soon as the bacteria were released into the host cell cytoplasm. The genes remained strongly induced throughout the infection zone II, the interzone II/III and into the late symbiotic zone (Fig. 5b). No senescence zone could yet be distinguished. The intensity of staining did not vary significantly throughout the nodules. An apparent increase in intensity at the interzone being due to a higher density of bacteroids and the presence of amyloplasts in this zone. Although under free-living conditions, the "short" gene fusion was consistently more active than the "long" gene fusion, this difference was not observed *in situ*. On the contrary, when processed simultaneously with 2011/pBBA2 induced nodules and stained for an equal period of time, the coloration obtained with the pCU700 fusion was consistently more intense.

The activity of the *dct*A gene fusions in nodules induced by other wild-type strains such as 1021 and 102F34 was also monitored. In all cases similar patterns of *in situ dct*A activity were observed. Strain Rm 1021 and its derivatives, such as for example Rm 1531, or the *dct* mutant strains F332 and F121 transduced back to wild-type phenotype (Fig. 5f, panel B), seemed somewhat delayed in nodule development when compared to 2011. They also gave rise to somewhat smaller "shorter" nodules. Otherwise, nodule morphology and spatial organisation is similar to those induced by 2011. Strain 102-F34 is probably the nicest one to work with, in that nodule development is fast and efficient like 2011, but plasmid stability during symbiosis appeared to be superior. This allowed prolongation of the *in situ* experiments for up to four weeks without plasmid loss becoming a concern. In these older nodules, *dct*A activity in the infection zone II all but disappeared and fusion activity was confined to the symbiotic zone III (Fig. 5c).

Fig. 5c: *Dct*A::*lacZ* activity in a 3 weeks old nodule.
Increased plasmid stability in nodules induced by 102F34 allowed experiments to continue beyond two weeks after infection. In this nodule, which is more than three weeks old, there is no sign of plasmid loss (Compare to Fig. 5b A). Strain: 102F34(pCU700); 23dpi; coloration: X-gal for 2.5 hrs.

5.3) The *lacZ* mutant strains are affected in their symbiotic efficiency.

In absence of an active *lacZ* gene fusion, no *in situ* staining could be observed. This indicates that the resident *S.meliloti lacZ* gene(s) are not induced during symbiosis. Even so, the use of a lac⁻ strain designated GMI 5600, was considered in order to avoid the possibility of the *lacZ* gene interfering with

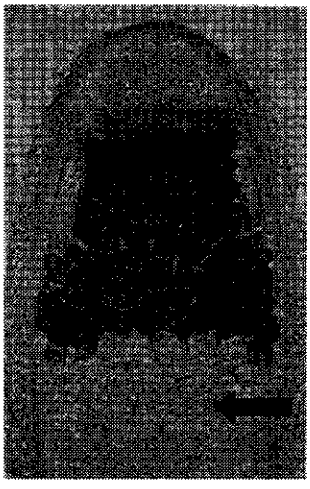

Fig. 5d: Nodule induced by a *lacZ* mutant strain.
An extensive zone of senescence is obvious in the proximal part of this two weeks old nodule induced on *M.truncatula* by a *lacZ* mutant strain (arrow). The strain carries a *nifA*::*lacZ* gene fusion which is induced by the microaerobic conditions that prevail in the interzone II/III and the late symbiotic zone III. Strain: 211/pCHK57; 15 dpi.

the results. No differences with the 2011 strain were observed during the early stages of infection up to the development of a mature nodule. The temporal and spatial patterns of *dctA* expression were also similar, but the senescence zone V developed at an early stage and was already distinct in two

weeks old nodules. In contrast, a senescence zone only appears after about 5 weeks in a normal healthy nodule. In time the nodules became elongated, developing only in the longitudinal direction without the lateral increase in volume that produces the usual pear shaped nodules. The symbiotic zone remained small and a long zone of "empty" cells developed which eventually turned green when exposed to the light. This phenotype was observed both on *Medicago sativa* and - *truncatula* host plants. The dry weight of *Medicago sativa* plants infected with GMI 5600 amounted to only about 60 % of that of plants inoculated with 1021, or 2011.

GMI 5600 is a Tn5 derivative of GMI 211 (David *et. al.*, 1988). As this defective phenotype was also observed for the GMI 211 strain (Fig. 5d), it is not due to the Tn5 insertion. Also Rm 1531, a derivative of 1021 and the donor strain of the Tn5 insertion in GMI 5600 (pSym20Tn5, Ruvkun *et. al.*, 1982), gave rise to healthy nodules. GMI 211 is another name for 2011 L$^-$, which is a lac$^-$ mutant selected after chemical N-methyl-N-nitro-N-nitrosoguanidine (NTG) mutagenisis from 2011 str^R (Niel et al., 1977). It is not know if this symbiotic deficiency is directly related to the lac$^-$ phenotype. However the presence of the active *dct*A::*lacZ* gene fusions did not suppress this phenotype. Therefore the defective phenotype is likely due to some other mutation possibly caused by the NTG treatment. Even though the *in situ* patterns of *dct*A activity were found to be similar to that observed for the other strains, the use of the lac$^-$ strain and its derivatives was avoided as much as possible in this work.

5.4) DctBD-independent *dct*A expression is strictly late symbiotic.

Nodules induced by F332 (*dct*B$^-$) and F121 (*dct*D$^-$) developed at the same rate and were externally indistinguishable from those induced by the wild-type strains. After sectioning, a higher density of amyloplasts throughout the symbiotic zone III was evident in the nodules induced by the *dct*B$^-$ and

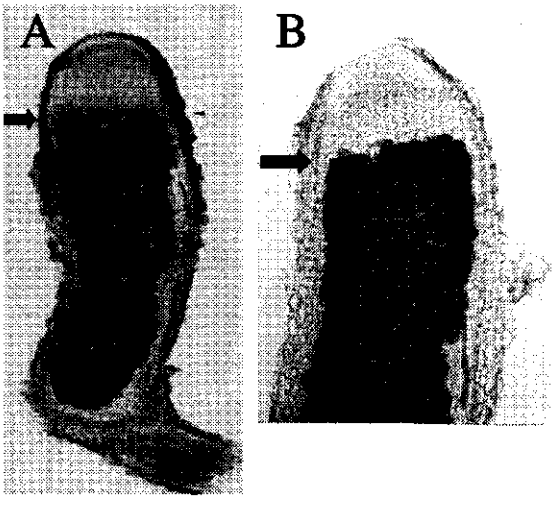

Fig 5e: *In situ* activity of *dct*A and *nif*H in a *dct*D mutant background.
A) DctBD-independent expression of the "long" *dct*A::*lacZ* gene fusion. Strain: F121/pCU700; 26 dpi; coloration: X-β-D-gal for 1 hrs.
B) Typical late symbiotic expression of the *nif*H::*lacZ* gene. Strain: F121/pRKP4; 15 dpi; coloration: X-β-D-gal for 1 hr.

*dct*D$^-$ mutant strains. Strong expression of the "long" fusion could be observed in the late symbiotic zone III of mature nodules induced by F332/pCU700 or F121/pCU700 (Fig. 5e, A). Unlike *dct*A activity

in a wild-type nodule, which could be observed in all infected zones, this DctBD-independent expression was strictly confined to the nitrogen fixing zone III of the mature nodules. It was induced sharply at, or one cell layer behind, the first important starch depositions of the interzone II/III and remained strong all the way to the most proximal part of the nodules. This pattern of *dct*A activity is very similar to that observed for *nif*H (Fig. 5e, B). The temporal and spatial pattern of *nif*H::*lac*Z expression in nodules induced by the *dct*D mutant strains was similar to that observed in a wild-type background (Fig. 6a, A).

5.5) The ASA does not act on the "short" gene fusion.

No DctBD-independent activity of the *dct*A gene could be observed with the "short" fusion on pBBA2. Neither in F121/pBBA2 (Fig. 5f, A) induced nodules, nor in nodules induced by F332/pBBA2. This result was rather unexpected as symbiotic activity of this "short" fusion was readily observed in nodules induced by the wild-type (2011) exconjugants, which were obtained from a simultaneous conjugation experiment using the same donor strain. We considered the possibility that the failure to

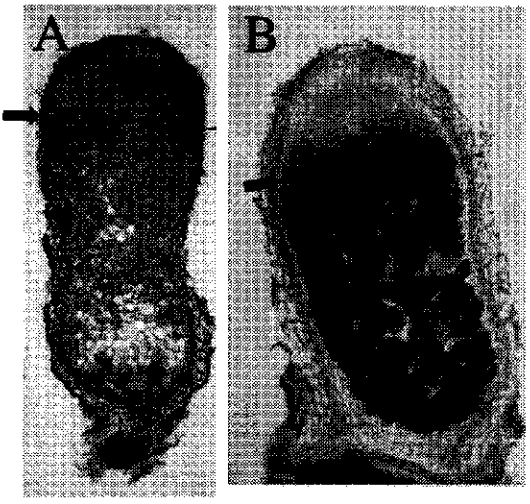

Fig 5f: ASA does not act on the "short" *dct*A::*lac*Z gene fusions.
A) No *in situ* activty was observed from the "short" *dct*A::*lac*Z gene fusion in a *dct*D⁻ background. Strain: F121/pBBA2; 26 dpi; coloration: X-β-D-gal for 1 hr.
B) When the strain was reverted back to wild-type phenotype by phage transduction, fusion activity *in situ* was also restored. Strain D2R; 19 dpi; coloration: X-β-D-gal for 1 hr. The arrows indicate the position of the interzone II/III

express the "short" gene fusion might be due to secondary mutations in the *dct*B⁻ and *dct*D⁻ mutant strains, or alterations of the plasmids, during or after the conjugation. A transduction experiment with the phage N3 was carried out to introduce the wild-type *dct* genes from *S.meliloti* 2011 into the *dct*B and *dct*D mutant strains. These transductions were carried out with the pBBA2 *dct*A::*lac*Z gene fusions in place. Transductants from F332/pBBA2 and F121/pBBA2 were selected on minimal medium with malate as the sole carbon source and tetracycline to maintain the fusion plasmids. In each case several transductants were obtained and the loss of the Tn5 insertion was checked by replica-plating on complex medium containing 25 µg/ml neomycin. These strains all behaved like wild-type. The *dct*A::*lac*Z fusions could now be induced under free-living conditions and were expressed normally during symbiosis with alfalfa (D2R, Fig. 5f, B). The pBBBg1 & -2 plasmids, which behaved exactly like

pBBA2 under free-living conditions, also behaved similarly during symbiosis. This confirmed the *in situ* results obtained with pBBA2. Furthermore, sequence analysis of the "short" *dct*A fragment from pSK4 did not reveal any alterations in the *dct*A promoter region. Therefore the lack of DctBD-independent expression from the "short" gene fusions is not caused by some mutation, neither in the host strains, nor in the gene fusion plasmids.

5.6) The 20D1 *dct*D mutant derivative of 2011.

5.6.1) *In situ* expression in 20D1 induced nodules.

F121 is a *dct*D$^-$ derivative from *S.meliloti* 1021 rather than 2011. Although in theory these wild-type strains are very close (Meade *et al.*, 1982), an isogenic *dct*D$^-$ derivative of 2011 was constructed. Such a strain was obtained by transduction of the Tn5 insertion from F121 into 2011. Transductants were selected on complex medium with 25 µg/ml neomycin. A small number of colonies were taken after 5 days from the selective plates and purified. None of the transductants were able to grow on minimal medium with malate as sole carbon source. Nodules induced by these transductants were efficient in

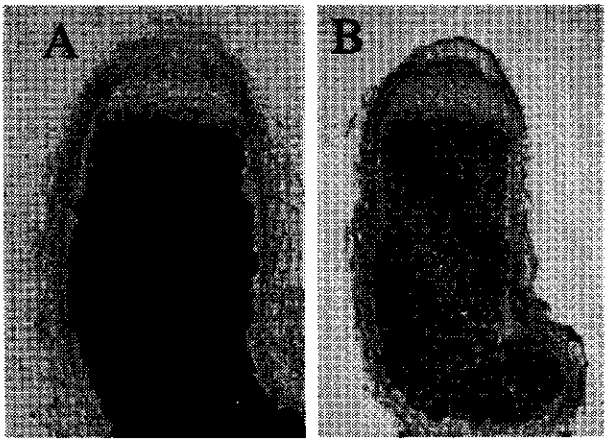

Fig 5g: *In situ dct*A::*lac*Z activity in the 20D1 *dct*D mutant background.
A) DctBD-independent expression of the "long" gene fusion. Strain: 20D1/pCU700; 15 dpi; coloration: X-β-D-gal for 2.5 hrs.
B) No DctBD-independent expression can be observed from the "short" gene fusion. Strain: 20D1/pBBBg1; 15 dpi; coloration: X-gal for 1 hr.

dinitrogen fixation and only distinguishable from their parent strain, by a higher level of starch depositions in the late symbiotic zone. One such strain (20D1) was taken for further studies. Plasmid pBBBg1 was used in these experiments because this construct is more similar to pCU700 than pBBA2. The plasmids pBBBg1, pCU700 and the *nif*H::*lac*Z gene fusion plasmid pRKP4 were introduced into 20D1 by conjugation. As expected for a *dct*D mutant strain, no expression of the *dct*A gene fusions could be obtained in 20D1 under free-living conditions.
The spatial pattern of expression from the pBBBg1 gene fusion in the wild-type background was similar to that of 2011/pBBA2 and has been described above. The pattern of temporal and spatial expression of the *nif*H gene fusion in nodules induced by 20D1 was similar to that observed before in the wild-type and also to that observed in F121 induced nodules (Fig. 5e, B). In 20D1 induced

nodules, no expression of the *nif*H fusion could ever be observed at the early stages of the symbiosis, such as during infection and in zone II of the mature nodules. The *nif*H fusion was induced sharply at the interzone II/III and remained highly expressed throughout the late symbiotic zone III. The temporal and spatial pattern of expression from pCU700 in the 20D1 background (Fig. 5g, panel A) closely resembled that observed for the *nif*H gene fusion. Strong expression of the "long" *dct*A::*lac*Z gene fusion was observed in mature nodules. This DctD-independent expression was strictly confined to the nitrogen fixing zone III of the nodules. No expression was ever observed at the early stages of colonisation and infection, or in zone II of the mature nodules. It was induced sharply at the interzone II/III and remained strong all the way to the most proximal part of the nodules. In contrast to the "long" *dct*A::*lac*Z gene fusion, no DctD-independent expression could be observed in the 20D1-alfalfa symbiosis at any stage with the "short" fusion on pBBBg1 (Fig. 5g, B).
These results confirm once more that unlike the "long" gene fusion in pCU700 which is strongly expressed in a DctBD independent manner during symbiosis, the ASA does not act on the "short" *dct*A::*lac*Z gene fusions. Since all other parameters have been excluded, the inability of the ASA to act on the short gene fusions must be related to some cis acting elements located in the 5' coding region of the *dct*A gene, present on pCU700, but missing from the "short" gene fusions.

5.6.2) Measuring gene fusion activity during symbiosis.

The *in situ* staining for β-galactosidase activity is a qualitative technology, which shows the spatial distribution of gene fusion activity. An approximation of the level of activity can be made from the time required to obtain a certain intensity of colour. In order to obtain more quantitative data we decided to measure the level of β-galactosidase activity from bacteroid suspensions. The *S.meliloti* 2011 wild-type and the isogenic 20D1 *dct*D mutant strain containing either pCU700 (The "long" *dct*A::*lac*Z gene fusion), one of the "short" *dct*A::*lac*Z fusion plasmids (Either pBBBg1, or pBBA2), or the *nif*H::*lac*Z gene fusion (pRKP4), were inoculated onto alfalfa plants. At 15 days after infection all sizeable, healthy looking, nodules were harvested (between 50 and 100 nodules/ batch of 10 plants). After fixation in glutaraldehyde, the nodules were examined visually and 5-8 average size nodules of each batch were put aside for histochemical staining. The remainder of the nodules were crushed and the levels of β-galactosidase activity from the bacteroid suspensions was measured as described in Chapter 3. The compiled data of a number of identical experiments is presented in Table 5a. The *nif*H promoter was highly expressed in both the wild-type and the *dct*D mutant strain. The *nif*H activity varied a lot between the batches. Possibly the activity of the *nif*H gene is very much dependent on the condition of the plants. Nevertheless it appears that the *nif*H gene is equally well expressed in both the wild-type and the 20D1 induced nodules. The β-galactosidase levels obtained from the *dct*A::*lac*Z fusions were much more consistent. In contrast with the activity under free-living conditions where the "short" gene fusions were found to be about twice as active than the "long" fusion, β-galactosidase activity from 2011/pCU700 induced nodules was consistently higher than those from 2011 containing the "short" gene fusions. A very low level of activity was obtained from nodules induced with 20D1 bearing either one of the "short" gene fusions (pBBA2 or pBBBg1). Although lower than in the wild-

type background, a significant level of activity was obtained from the "long" gene fusion in nodules induced by the 20D1 *dct*D mutant strain.

The intensity of the *in situ* staining of the nodules, put aside for this purpose, were always in good agreement with the β-galactosidase measurements. The homogeneous staining also indicated that there was no significant loss of plasmids evident in the nodules harvested at this age.

S.meliloti strain	β-galactosidase activity	repeats
2011(pBBBg1)	153 ± 27	5*
2011(pCU700)	305 ± 53	3
2011(pRKP4)	2570 ± 1178	15
20D1(pBBBg1)	14 ± 4	2*
20D1(pCU700)	187 ± 29	2
20D1(pRKP4)	2287 ± 1219	4

Table 5a: Symbiotic expression of the *dct*A- and *nif*H::*lacZ* gene fusions.

Nodules were crushed in a phosphate buffer and the β-galactosidase activity of the bacteroid suspension was measured as described in Chapter 3.6. Values are expressed in terms of nmoles nitrophenol released / min. x OD_{600}. pBBBg1 = a "short" *dct*A::*lacZ* gene fusion plasmid; * = pBBA2 is also included in these repeats; pCU700 = "long" *dct*A::*lacZ* gene fusion plasmid; pRKP4 = *nif*H::*lacZ* gene fusion plasmid.

The β-galactosidase measurements confirmed that the ASA does not act on the "short" *dct*A::*lacZ* gene fusions. They also demonstrated that the "long" gene fusion is more active in both, nodules induced by the 20D1 *dct*D mutant strain as well as in the wild-type background.

5.7) Discussion.

In planta regulatory determinants of *dct*A expression were studied using two types of *dct*A::*lacZ* gene fusions. Plasmid pCU700 contains the first 162 amino acids of the *dct*A coding region and is referred to as the "long" gene fusion. The gene fusions on pBBA2 and pBBBg1 & -2 only conserved the ATG translational start codon and the second amino acid of the DctA protein. These fusions are referred to as "short" *dct*A::*lacZ* gene fusions. Under free-living conditions, both types of gene fusion behaved exactly as would be expected from correctly regulated *dct*A::*lacZ* gene fusion. The only difference was that the "short" fusions were expressed at a level about twice as high as found for the "long" fusion (See Chapter 4). The aim of these studies was to confirm the existence and of the alternative mechanism of symbiotic *dct*A activation (ASA) and to approach its nature by using the *dct*A::*lacZ* gene

fusions and histochemical staining techniques for *in situ* β-galactosidase activity. We intended to establish where and when, the *dct*A gene is activated in nodules induced by *dct*B or *dct*D mutant strains. By making a link between *dct*A activity and the different stages of bacteroid development, we may be able to establish what regulatory pathways are involved in its activity. We further posed the question what the role of the ASA could be in the establishment of an effective symbiosis. Whether it is an essential mechanism and whether the ASA is operating in a wild-type background. And if so, which mechanism, the ASA or the regular DctBD system, is primarily responsible for the activity of the *dct*A gene during symbiosis?
The results presented in this chapter do not enable us to answer all these questions. However an amount of new information has been obtained and several significant conclusions can be made with respect to *dct*A activity in wild-type as well as in nodules induced by the *dct*BD mutant strains.

5.7.1) The efficient uptake of dCA is not required for bacteroid development.

Both types of *dct*A::*lac*Z gene fusions were used to monitor *dct*A expression during symbiosis. The fusions were efficiently expressed in mature nodules induced by the wild-type *S.meliloti* strains. Fusion activity could be detected as soon as the bacteria were released from the infection threads into the host cell cytoplasm. The fact that the fusions were not strongly induced inside the proliferating infection threads immediately behind the meristem suggests that carbon sources other than dCA or aspartate are used by the dividing bacteria at the early stages of the symbiosis. This agrees with the observation that *dct*A mutant strains lead to the formation of nodules, which are fully infected but fail to fix nitrogen. We have also observed that a *nif*H::*lac*Z gene fusion is expressed in nodules induced by a *dct*A mutant strain (Data not shown). Since *nif*H is usually only expressed in the fully differentiated bacteroids in the nitrogen fixation zone III (Soupène et al., 1995), this indicates that bacteroid differentiation in the absence of DctA reaches a stage normally associated with the late symbiosis.
In contrast with strains mutated in the structural *dct*A gene, deletion mutants in the *dct*D, or *dct*B genes generally are effective for symbiotic N_2-fixation and have been shown to transport dCA. This implies that the *dct*A gene is expressed in such strains during symbiosis. We were able to confirm this DctBD-independent *dct*A expression using the "long" *dct*A::*lac*Z gene fusion on pCU700. We also observed that this *dct*BD-independent expression is confined to zone III of mature nodules. Therefore the alternative mechanism of symbiotic expression of the *dct*A gene (ASA) has a typical late symbiotic phenotype. As a consequence *dct*BD mutant strains are effectively *dct*A negative at the early stages of the symbiosis. The difference between the regulatory *dct*BD mutants and the structural *dct*A mutant strains is that once the bacteroids are fully differentiated, the activity of ASA suppresses the *dct*A negative phenotype of the *dct*BD mutants. As this allows efficient symbiotic N_2-fixation it indicates that, although dCA are present at the early stages of bacteroid development, their efficient uptake is not essential for bacteroid differentiation.

5.7.2) The ASA requires cis-acting elements downstream of the promoter for activity.

No DctBD-independent symbiotic induction of the *dctA* gene at the late symbiotic stage was observed with the "short" gene fusions on pBBA2 (or its equivalent pBBBg1). It thus appears that the "short" fusion does not respond to the ASA, even though under free-living conditions it behaved as would be expected from correctly regulated *dctA*::*lacZ* gene fusion. Probably, efficient activation of the *dct*A gene by ASA requires some cis-acting regulatory elements located in the 5' one third of the coding region of the *dct*A gene. Such sequences may have been deleted partially, or entirely in the construction of the "short" gene fusion. A gene fusion containing this truncated *dctA* regulatory region appears to monitor correctly the activity of the DctBD system but displays a mutant phenotype when tested for its response to ASA. It is worth noting that the exclusive use of the "short" fusion would have led to conclusions about symbiotic expression of *dctA* in the *dct*BD mutants in contradiction with the data, which led to the ASA hypothesis.

5.7.3) The DctBD system is sufficient for symbiotic expression of *dct*A.

The "long" fusion on pCU700 can be activated by either the cognate DctBD system, or the ASA. Therefore, with this *dct*A::*lacZ* gene fusion, we could not distinguish which mechanism was primarily responsible for the late symbiotic expression of *dct*A. By contrast, the "short" gene fusion allowed the monitoring of the DctBD-driven expression of the *dctA* gene during symbiosis, without interference of the ASA. Therefore the use of this "short" fusion made it possible to show that DctBD activation of *dctA* expression was operating in the whole nodule and notably in the nitrogen fixation zone. This indicates that the highly differentiated bacteroids retain the ability to respond to the presence of C_4-dicarboxylic acids in the peribacteroid space, in agreement with the fact that C_4-dicarboxylic acids are the preferred energy source for the nitrogen-fixing bacteroids. Although ASA-driven expression of *dctA* could be demonstrated only in the absence of a functional DctBD system, we speculate that it may also play a role in a wild-type background. Possibly augmenting the level of *dctA* activity in nitrogen fixing bacteroids, in which there is a high demand for energy.

Chapter 6)

NifA is required for DctBD-independent expression of the *S.meliloti* C_4-dicarboxylate transport gene (*dct*A) during symbiosis

6.1) Introduction.

The symbiosis between *Sinorhizobium meliloti* and the *Medicago* host plants leads to the formation of nodules on the roots which are invaded by the microsymbiont and in which symbiotic N_2-fixation takes place in a microaerobic environment. The regulation of gene expression in mature N_2-fixing *S.meliloti* bacteroids differs substantially from that in free-living bacteria. It is still not well understood which factors determine the differentiation of the bacteria into N_2-fixing bacteroids and to date it is not possible to induce N_2-fixation by *S.meliloti* under free-living conditions.

The *S.meliloti ntr*A gene is required for diverse metabolic functions and is essential for symbiotic N_2-fixation (Ronson *et. al.*, 1987b). The gene codes for an alternative sigma factor (σ^{54}), which is among others required for the transcription of the *S.meliloti nif*HDK and *fix*ABC operons and is also required for transcription of the *dct*A gene. The σ^{54} dependent promoters also require the activity of a transcriptional activator to initiate transcription. In case of the P1 (*nif*HDK) & P2 (*fix*ABC) promoters this activator is NifA. The transcription of the *nif*A gene is induced under free-living microaerobic conditions (Ditta *et. al.*, 1987; David *et al.*, 1988). Furthermore the NifA protein itself is sensitive to oxygen. To initiate transcription, the NifA protein interacts with enhancer like sequences upstream of the P1 and P2 promoters. These upstream activator sequences (UAS) are absolutely essential for transcription of these genes under free-living microaerobic conditions. In contrast, it was found that gene fusions of the P1 and P2 promoter regions to the *lac*Z gene were expressed efficiently during late symbiosis, even when the UAS sequences were deleted from the promoter fragments. Apparently, the UAS are not essential for expression of the genes in bacteroids under symbiotic conditions (Better *et. al.*, 1985; Wang *et. al.*, 1991). It is not known if other sequences do compensate for the absence of the UAS in this case. We have however observed that besides deleting the UAS, a deletion of DNA sequences downstream from the transcriptional start site, further reduced the levels of symbiotic expression of the P1 promoter (Wang et al., 1991).

In the context of our work, the symbiotic regulation of the *nif*H gene is of particular interest because of the similarity of the P1 promoter region with the *dct*A promoter. The upstream activator sequences (UAS) of the *nif*H promoter are also similar to those of the *dct*A promoter. In Chapter 5 we have demonstrated that the DctBD-independent expression of *dct*A::*lac*Z gene fusions displays a typical late symbiotic phenotype. We also showed that sequences located downstream of the promoter in the coding region of the *dct*A gene are required for DctBD-independent expression. In this chapter we will demonstrate that the first 23 bases of the *dct*A coding region are sufficient for DctBD independent activity of the gene fusions during symbiosis. Furthermore we will show that the UAS sequences of the *dct*A promoter are not essential for the late symbiotic expression of the gene and we provide data indicating that a functional *nif*A gene is required for the DctBD independent activity of *dct*A during symbiosis.

6.2) *In-situ* expression of the *nif*H promoter without UAS.

As mentioned in the introduction, the *nif*H promoter is of special interest because of its homology with the *dct*A promoter. Particularly interesting is the fact that this promoter is efficiently expressed during symbiosis, even when the UAS are missing from the promoter sequence. We wanted to verify this symbiotic expression of a *nif*H promoter lacking the UAS and in particular to examine if the spatial distribution of the *in situ* expression had changed. As an example of a *nif*H::*lac*Z gene fusion, we used the plasmid pRKP4. Plasmid pRKP4 is essentially the same as pRKP9 (Fig. 3a, D), but additionally contains a *dct*A::*uid*A gene fusion cloned into the β-lactamase gene. The presence of the *dct*A::*uid*A gene fusion has no influence on the regulation of the *nif*H promoter. For a *nif*H promoter lacking the UAS, we used the

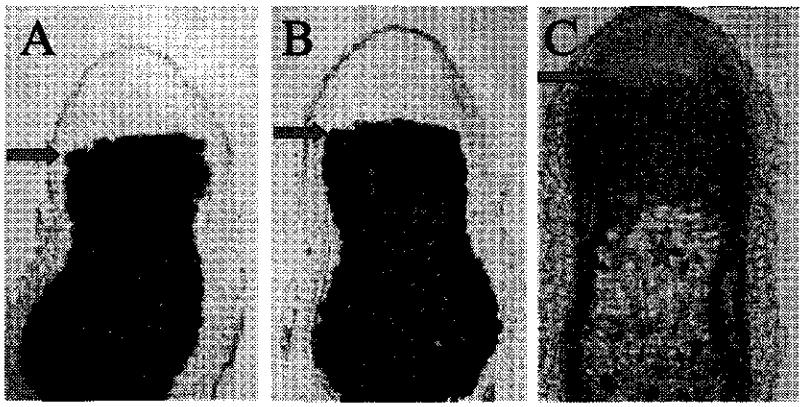

Fig. 6a: *In situ* expression of the *nif*H gene.
A) The pRKP4 *nif*H::*lac*Z fusion is expressed in a typical late symbiotic pattern. No activity is seen in the infection zone and the gene is induced sharply at the interzone II/III. Strain: 2011/pRKP4, 17 dpi, staining X-β-D-gal for 1 hr.
B) The *in situ* pattern of activity of the pMB1156 gene fusion, lacking the UAS, is indistinguishable from that of pRKP4. Strain: 2011/pMB1156, 17 dpi, staining X-β-D-gal for 1 hr.
C) No expression of the pMB1156 gene fusion could be observed in the *nif*A mutant background. A senesce zone IV has already developed at this early stage. This is typical for fix⁻ nodules. Strain: 1354/pMB1156, 16 dpi, staining X-β-D-gal for 1 hr. The arrows indicate the position of the interzone II/III. The star indicates the senescence zone.

pMB1156 gene fusion constructed by Better *et al.* (1985). This plasmid carries a *nif*H::*lac*Z gene fusion similar to that on pRKP4, but the UAS sequences are deleted from the *nif*H promoter fragment. Alfalfa seedlings were inoculated with the 2011 wild-type strain containing either pMB1156, or pRKP4. The nodules were harvested at 18 dpi and the β-galactosidase activity of the bacteroid suspensions measured. The *nif*H promoter lacking the UAS was expressed at a

level about half that of the complete *nif*H promoter (Data not shown). This is in agreement with previously reported values (Better *et al.*, 1985; Wang *et al.*, 1991).
A number of nodules were cut and stained to observe the spatial distribution of the activity of the *nif*H::*lac*Z gene fusions. The fusions were found to be expressed in a typical late symbiotic pattern in that expression was observed exclusively in zone III of the nodules containing the fully differentiated bacteroids (Fig. 6a, A & B). The pattern of expression of the pMB1156 *nif*H::*lac*Z gene fusion was indistinguishable from that observed with the pRKP4 gene fusion. The expression of the P1 promoter during symbiosis is fully dependent on a functional *nif*A gene (Wang *et al.*, 1991). Therefore it might be expected that the symbiotic expression of the pMB1156 gene fusion also required the presence of a functional *nif*A gene. Indeed, when introduced into the Rm1354 *nif*A mutant strain, no symbiotic expression of the pMB1156 gene fusion could be observed (Fig. 6a, C).

6.3) The first 23 bases of the *dct*A coding region are sufficient for ASA activity.

In the previous chapters (Chapter 4 & 5) we have evaluated the activity of a number of *dct*A::*lac*Z gene fusions under free-living conditions and also *in situ* during symbiosis with the alfalfa host plant. We evaluated the activity of a "long" *dct*A::*lac*Z gene fusion located on pCU700 and several "short" gene fusions located on plasmids pBBA2 and pBBBg1 & -2. Unlike pCU700, which contains a substantial part of the *dct*A coding region, the "short" gene fusions have the *lac*Z gene fused directly to the ATG start codon of the *dct*A gene. Under free-living conditions both types of gene fusions were fully dependent on a functional DctBD system and were regulated as expected for a *dct*A gene fusion. The "short" gene fusions were expressed at a higher level than the "long" gene fusion on pCU700. During symbiosis both types of fusions were active at the early, as well as the late stages of bacteroid development in nodules induced by a wild-type strain (Fig. 5b). In nodules induced by a *dct*D mutant strain however, only the "long" gene fusion was expressed in a typical late symbiotic pattern (Fig. 5g). No DctBD independent activity could be observed from the "short" gene fusions. These results indicated that sequences in the N-terminal part of the *dct*A coding region which are not required for DctBD dependent expression of the gene fusions under free-living and symbiotic conditions, are essential for DctBD independent activity during symbiosis.
In order to investigate how much of the 5-prime region of the *dct*A coding region is required for ASA activity on the gene fusions, a number of oligonucleotide primers were designed to introduce restriction sites at various distances from the ATG start codon. Starting off with the first primer introducing a *Bam*HI restriction site at the 8th amino acid of the *dct*A gene, we have constructed a highly effective *dct*A::*lac*Z gene fusion (See Chapter 4.5). Under free-living conditions these pBBB gene fusions were found to be correctly regulated as expected from a *dct*A::*lac*Z gene fusion. However, both uninduced and induced levels of gene fusion activity were found to be higher than both the "long", and the "short" gene fusions. During symbiosis the pBBB gene fusions were found to be expressed efficiently in wild-type induced nodules (Fig. 6c, A). The *in situ* pattern of *dct*A activity was found to be similar to that observed with the other *dct*A::*lac*Z gene fusions. Especially the higher level

of fusion activity resulted in a strong staining in zone II of the nodules. The fusion remained active throughout all subsequent stages of bacteroid differentiation. No senescence zone was yet visible in these 2 weeks old nodules. In a *dct*D mutant strain (20D1), no fusion activity could be obtained under free-living conditions. This was as expected and demonstrated that although a higher level of uninduced activity was observed in comparison with the "long" and "short" gene fusions, this activity is still fully DctBD dependent. During symbiosis, the pBBB gene fusions were found to be expressed efficiently in nodules induced by the 20D1 *dct*D mutant strain. The *in situ* pattern of expression was confined to the late symbiotic zone III only (Fig. 6c, B). This pattern of DctBD independent fusion activity was similar to that obtained with the "long" gene fusion located on pCU700 and was in strong contrast with the "short" gene fusions, which are not expressed in a DctBD independent manner. The only difference between the pBBB gene fusions and the "short" gene fusions on the pBBBg plasmids is that the pBBB gene fusions contain an extra 16 bases of the *dct*A coding region. These results demonstrate that all determinants required for ASA activity are present on the pBBB gene fusions, which contain the first 23 nucleotides of the *dct*A coding region.

6.4) The UAS are essential for free-living expression of the *dct*A promoter.

Having determined the extent of downstream DNA required for DctBD independent expression of the *dct*A promoter, we turned our attention to the upstream sequences. In particular we wanted to investigate if the UAS sequences would be required for ASA activity. An oligonucleotide primer was designed to introduce an *Eco*RI restriction site, just downstream of the second biding site for the DctD protein. The construction of the pBBEB gene fusions has been described in Chapter 3.4.5. The gene fusion on the pBBEB plasmids is identical to that on the pBBB plasmids, but in the pBBEB constructs the upstream activator sites (UAS) are deleted from the *dct*A promoter (See Fig. 3e).
Since we were particularly interested in the role of NifA in the symbiotic expression the *dct*A gene, the various *dct*A::*lacZ* fusion plasmids were introduced into the RM1354 *nif*A mutant strain. A mutation in the *nif*A gene was not expected to have an influence on the free-living expression of the different gene fusions. An induction experiment was carried out similar to those described in Chapter 4.5. The gene fusions were expressed to levels similar to those seen previously in the wild-type strain (Fig.6b). This confirmed that NifA does not play a role in the free-living expression of the *dct*A gene. As expected, no activity at all could be obtained from the pBBEB4 gene fusion which is lacking the UAS. This indicated that the UAS are essential for DctBD dependent expression of the *dct*A gene promoter under free-living conditions. However it must be born in mind that at this stage we can not exclude the possibility that the lack of activity of the pBBEB gene fusions under free-living conditions is not due to another unforeseen artefact of the construction of the plasmids.

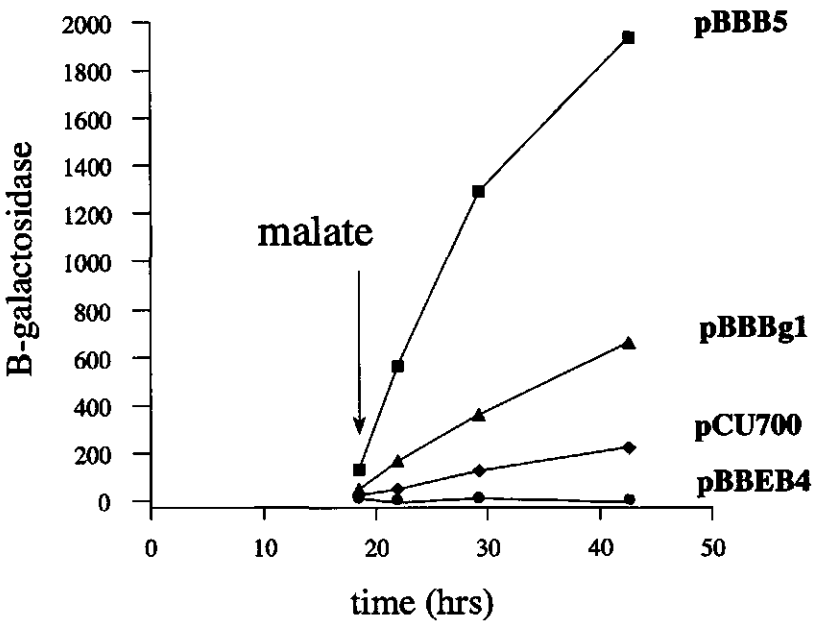

Fig. 6b: Free-living dctA activity in the RM1354 *nifA* mutant strain.
The gene fusions showed levels of activity similar to those observed in a wild-type background. Plasmid pCU700 carries the "long" dctA::lacZ gene fusion. pBBBg1 carries a "short" gene fusion. pBBB5 contains the first 8 amino acids of the *dctA* coding region and therefore belongs to the "third group" of gene fusions (See Fig. 4a) The pBBB5 gene fusion shows the highest levels of activity. The pBBEB gene fusion is similar to the pBBB5 gene fusion except that the UAS are lacking from the promoter region. The pBBEB4 gene fusion is not expressed under free-living conditions. The X-axis has been lowered slightly to reveal this curve. Cultures were pre-grown overnight in rich medium and inoculated into minimal medium containing mannitol as sole carbon source (time = 0 hrs). Malate was added after 18 hrs. An unit of β-D-galactosidase represents 1 nmole nitrophenol released at 37°C / min. x OD_{600}.

6.5) The UAS are not essential for *dctA* activity during symbiosis.

We have shown that the pBBB5 *dctA::lacZ* gene fusion is efficiently expressed under free-living conditions and during symbiosis. The temporal and spatial pattern of *in planta* activity in wild-type nodules is similar to that observed for both, the "long" as well as the "short" gene fusions (Fig. 6c, Panel A). Unlike the "short" gene fusions, which are not activated in a DctBD independent manner, the slightly longer pBBB5 gene fusion is also efficiently expressed in a *dctD* mutant background (Fig. 6c,

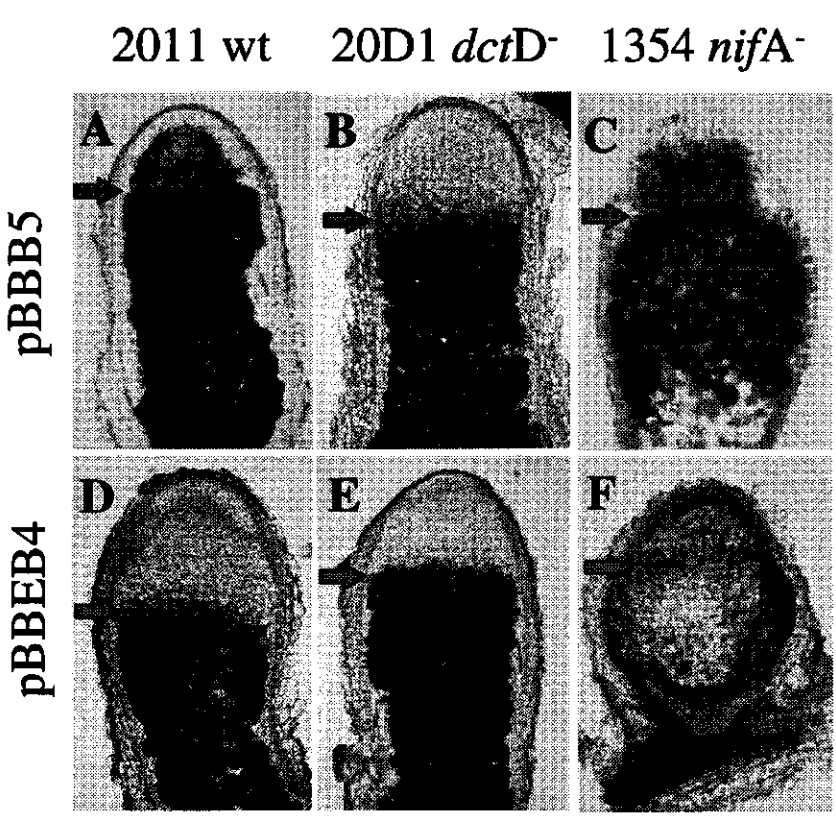

Fig 6c: *In situ dctA::lacZ* gene fusion expression in 16 day old nodules.

A) Activity of the pBBB5 gene fusion in a wild-type nodule. The gene fusion is expressed in zone II as soon as the bacteroids are released from the infection threads and remain expressed throughout the late symbiotic zone III. Strain: 2011/pBBB5; 16 dpi; coloration: X-β-D-gal for 1 hr.. **B)** DctD-independent activation of the pBBB5 gene fusion in a mature nodule induced by a *dctD*⁻ strain. The fusion is sharply induced at the interzone II/III and remains strongly expressed throughout the late symbiotic zone III. Strain: 20D1/pBBB5; 16 dpi; coloration: Xgal for 2.5 hr. **C)** Activity of the pBBB5 gene fusion in a *nifA*⁻ nodule. The *in situ* pattern is similar to a wild-type nodule. The early development of a senescence zone and the large amount of starch deposition are a typical feature of nodules inefficient for N_2-fixation. Strain: 1354/pBBB5; 16 dpi; coloration: X-β-D-gal for 1 hr. **D)** The *in situ* expression of the pBBEB gene fusion lacking the UAS displays a typical late symbiotic pattern in a wild-type nodule. Strain: 2011/pBBEB; 16 dpi; coloration: Xgal for 1 hr. **E)** DctBD-independent expression of the pBBEB gene fusion does not differ from that observed in the wild-type nodules. Strain: 20D1/pBBEB; 16 dpi; coloration: Xgal for 1 hr. **F)** A mutation in the *nifA* gene completely abolishes the symbiotic activation of the pBBEB gene fusion. Strain: 1354/pBBEB; 16 dpi; coloration: Xgal for 1 hr. The arrows point at position of the interzone II/III.

Panel B). As previously observed with the "long" gene fusion, this DctBD independent activation of the pBBB5 gene fusion displays a typical late symbiotic pattern of *in situ* expression. As such, the pBBB

gene fusions behave similar to the pCU700 "long" gene fusion and bear all the elements required for activation by the DctBD system, as well as ASA.
A mutation in the *nif*A gene had no influence on the free-living activity of the gene fusions (Fig. 6b). During symbiosis the pBBB5 gene fusion displays an early to late pattern of gene expression in a *nif*A mutant background, similar to that observed in wild-type induced nodules (Fig. 6c, Panel C). Incidentally the pBBBg1 "short" gene fusion, which is not expressed in nodules induced by a *dct*D mutant strain, is expressed efficiently in nodules induced by the *nif*A mutant strain (Data not shown). The pBBEB gene fusions lacking the UAS, could not be induced under free-living conditions, indicating that the UAS are essential for DctBD dependent activation of the *dct*A gene. However, during symbiosis in nodules induced by the wild-type strain, a significant level of activity could be observed from the pBBEB4 gene fusion. In contrast with the other gene fusions, which all displayed an early to late pattern of symbiotic expression in a wild-type background, the temporal and spatial pattern of *in situ* expression of the pBBEB gene fusion in wild-type 2011 induced nodules was a typical late symbiotic one (Fig. 6c, D). Therefore the pattern of *in situ* expression of the pBBEB gene fusion in wild-type induced nodules, is similar to the pattern observed with the "long" gene fusions in a *dct*D mutant background. Moreover, the intensity and the pattern of the *in situ* expression of the pBBEB gene fusion did not change in the 20D1 *dct*D mutant background (Fig. 6c, E). The observed *in situ* activity of the pBBEB4 gene fusion in wild-type induced nodules appears to be DctD independent. This DctD independent activation of the pBBEB gene fusion, was found to be completely abolished in nodules induced by the Rm1354 *nif*A mutant strain (Fig. 6c, F).

6.6) Discussion.

During symbiosis the *dct*A gene can be activated by either the DctBD mechanism, or independently of DctBD by an alternative mechanism of symbiotic activation (ASA). *In planta* regulatory determinants of *dct*A expression were studied using a range of pGD926 derived *dct*A::*lac*Z gene fusions. The "long" gene fusion on pCU700 and the gene fusion located on pBBB5 were found to be efficiently expressed during symbiosis in nodules induced by wild-type and *dct*D mutant strains. This indicates that these fusions contain all necessary determinants to be efficiently activated by DctBD and the ASA. The "short" gene fusion on pBBBg1 is efficiently expressed by the DctBD system, but not during symbiosis in a *dct*BD mutant background. This is in contrast with the pBBEB gene fusion, which can not be activated by DctBD, but is still expressed during symbiosis independently of DctBD. With the latter two plasmids we were able to monitor the two mechanisms of *dct*A activation (DctBD or ASA) independently of each other in nodules induced by a wild-type *S.meliloti* strain.

6.6.1) Elements downstream of the *dct*A promoter are required for ASA activity.

In contrast with the "long" gene fusion on pCU700, the "short" gene fusion on pBBBg1 is not activated by the ASA (chapter 5.4). Like the "long" gene fusion, the pBBB5 gene fusion was also found to be expressed efficiently by DctBD mechanism, as well as the ASA. Therefore the *dct*A fragment of the

pBBB5 gene fusion, which conserved only the first 8 amino acids of the *dct*A coding region, contains all elements required for activation by both mechanisms. The difference between the pBBB gene fusions and the "short" pBBBg gene fusions is limited to 16 bp in the extreme N-terminal part of the *dct*A coding region. Therefore a cis-acting element essential for ASA activation during symbiosis, is entirely or in part located in this region downstream of the *dct*A promoter. No further attempts have been made to characterise this element. A systematic approach involving a range of point mutations in this region of the DNA would probably be a logical way to pursue the analysis of this transcriptional element.

6.6.2) The UAS are not essential for ASA activity.

The pBBEB gene fusion could not be induced by dCA under free-living conditions, confirming that the UAS sequences are essential for DctBD dependent activation of *dct*A. Under free-living conditions, deletion of the UAS from the *dct*A promoter has the same effect as deleting the *dct*D gene. In both cases activity of the *dct*A promoter is diminished. During symbiosis, although much reduced in comparison with the highly efficient pBBB5 gene fusion, a significant level of gene fusion activity was retained. In nodules induced by a wild-type *S.meliloti* strain the *in situ* pattern of expression of pBBEB4 was found to have changed with respect to the other *dct*A::*lac*Z gene fusions retaining the UAS region. No activity could be observed at the early stages of symbiosis and the *in situ* pattern had changed to an exclusively late symbiotic one. The level of expression was similar to that of the pCU700 gene fusion in 20D1 induced nodules, or the pBBBg1 gene fusion in the wild-type background (Data not shown). Therefore the change in pattern was not a simple reflection of the lower level of activity of this gene fusion in comparison to pBBB5. The level of expression and the *in situ* pattern did not change when a *dct*D mutant strain (20D1) was used. This demonstrated that the symbiotic activation of the pBBEB gene fusion is truly DctD independent. The pBBEB4 gene fusion behaves in a nodule induced by a wild-type strain, just like the pBBB gene fusions in a *dct*D mutant background and allows us to study the effect of mutations in other regulatory genes without having to construct double mutants.

6.6.3) A functional *nif*A gene is required for ASA activity.

The pBBB5 and the pBBBg1 gene fusions were efficiently expressed in nodules induced by a *nif*A mutant strain. Both displayed an early to late pattern of *in situ* expression similar to those observed in the wild-type background. No activity was obtained in a *nif*A mutant background from the pBBEB4 gene fusion lacking the UAS sequences. This latter observation strongly suggests that NifA is involved in the DctBD independent activation of the *dct*A gene during symbiosis. It is not clear from these results if NifA itself interacts with the *dct*A promoter, or if another gene is activated by NifA, whose product acts on the *dct*A promoter. This latter scenario has been suggested by Kaufman & Nixon (1996). These authors identified a possible DctD homologue designated gene 19, which according to the authors, might be involved in the symbiotic activation of *dct*A.

When it became clear that during symbiosis a mechanism, other than the DctBD system, was capable of efficiently activating the *dct*A promoter, NifA was immediately suggested as a likely candidate for the ASA (Ronson *et al.*, 1985, 1987a, 1988). This was based on the fact that the UAS sequences for DctD resembled a consensus sequence for NifA binding. To date no concrete evidence has been provided to support this hypothesis. Certain experimental data even contradicts that NifA plays a role in the regulation of the *dct*A promoter (Wang *et al.*, 1989; Jording *et al.*, 1992). Here we have shown that NifA does indeed play a role in the symbiotic activation of the *dct*A promoter. It is also clear that, although the UAS sequences contribute to the expression of the *dct*A promoter, they are not absolutely essential in this process. The ASA appears to require certain cis-acting sequences downstream of the -12 -24 promoter site for its activity. Therefore the mechanism by which the ASA activates the *dct*A promoter during symbiosis must differ in several aspects from the classical DNA looping model involving an enhancer site located upstream from the promoter. It will be interesting to determine the actual mechanism of activation and, in particular, the nature of the cis-acting elements located in the extreme N-terminal part of the *dct*A coding region. Furthermore it will be interesting to examine if a similar mechanism is also involved in the symbiotic activation of the P1 and P2 promoters.

Chapter 7)

In situ dctA expression during symbiosis of *Sinorhizobium meliloti* with *Medicago truncatula*

7.1) Introduction.

The *S.meliloti* – alfalfa symbiosis is one of the most extensively studied system of symbiotic N_2-fixation, especially the bacterial side of the symbiosis. Progress in molecular studies on the plant side of this system is hampered by the complexity of the *Medicago sativa* (Alfalfa) genome. *Medicago sativa* is a tetraploid and cross-fertilising plant. The so-called barrel medic, *M. truncatula* is a diploid and autogamous plant with a relatively small genome. Therefore *M. truncatula* is more amenable to genetic analysis and has been proposed as a model plant to study the molecular genetics of the *Rhizobium*-legume symbiosis (Barker *et. al.*, 1990).
In order to test its suitability for the study of the symbiotic regulation of the *dct*A gene we checked the *in situ* expression of the *S.meliloti dct*A gene in nodules induced on *M. truncatula*.

7.2) A *S.melliloti dct*D mutant strain induces inefficient nodules on *M. truncatula*.

Nodules induced by *S.meliloti* strains mutated in the regulatory *dct*BD genes, on the alfalfa (*Medicago sativa*) host plant, are still effectively fixing nitrogen. As described in chapter 5, the *dct*A negative phenotype of these strains is overcome at the late symbiotic stage by an alternative mechanism of symbiotic activation (ASA). The *dct*D mutant strain 20D1 is a typical example and this strain, together with the 2011 wild-type strain, was used to inoculate *M.truncatula* (cv Jemalong 15-16) seedlings. Nodules induced by 20D1 developed at the same rate as those induced by the 2011wild-type strain and for the first two weeks looked perfectly normal. Between two and three weeks the plants inoculated with 20D1 started to lag behind in growth, in comparison to those inoculated with the wild-type strain. At 16 days after infection, the N_2-fixation activity was measured using the acetylene reduction assay (ARA). The activity of *M.truncatula* plants infected with the wild-type strain was found to be 48 ± 9 nanomoles of ethylene produced per plant, per minute. This is comparable to the activity of this wild-type strain with *M.sativa* (alfalfa). For example in another experiment, the 2011 wild-type strain (with the pRKP4 gene fusion) gave rise to 37 ± 13 nm/plant*min when inoculated on alfalfa and 40 ± 3 nm/plant*min when inoculated on *M.truncatula*. The N_2-fixation activity of plants inoculated with 20D1 *dct*D mutant strain was found to be 11 ± 6 nm/plant*min. This amounted to only 23% of the activity obtained with plants inoculated with the wild-type strain. This is also much less than the activity obtained with alfalfa when inoculated with the 20D1 strain. Therefore, it appeared that in contrast to the situation on alfalfa where the 20D1 strain is efficient for symbiotic N_2-fixation, the N_2-fixation activity is very much reduced when this strain is inoculated onto *M. truncatula*.

7.3) *In situ dct*A and *nif*H gene expression in nodules induced on *M.truncatula*.

The low N_2-fixing activity of the 20D1 *dct*D mutant strain on *M.truncatula* raised the question if the *dct*A gene was efficiently expressed in these nodules. This might indicate that unlike the *S.meliloti* –alfalfa symbiosis, the ASA does not operate in nodules induced on *M.truncatula*. In order to investigate this possibility, *M.truncatula* seedlings were infected with the isogenic 2011wild-type and 20D1 *dct*D mutant strains each bearing the pBBB5 *dct*A::*lac*Z gene fusions. As shown in chapter 6, this gene fusion is efficiently activated by the ASA in nodules induced by the 20D1 strain on alfalfa. To check the possibility that the lack of nitrogen fixation activity in the 20D1 induced nodules could be attributed to a reduced level of the *nif* gene expression, the activity of the pRKP4 (*nif*H::*lacZ*) gene fusion was also monitored.

The *in situ* pattern of early and late expression of the pBBB5 *dct*A::*lac*Z gene fusion in nodules induced by the 2011 wild-type strain on *M.truncatula* was similar to the pattern observed in nodules induced on alfalfa (Fig7a A). The typical late symbiotic pattern of *in situ* expression of the *nif*H gene was also similar to that observed in wild-type nodules induced on the alfalfa host plants (Fig7a C). The nodules induced by the 20D1 *dct*D mutant strain were completely infected and the various zones of temporal and spatial development could be clearly distinguished. Only a higher level of starch deposition and a premature development of a central zone of senescence, typical for fix$^-$ nodules, distinguished these nodules from those induced by the wild-type strain. The *nif*H gene was expressed in a typical late symbiotic pattern similar to the wild-type nodules (Fig7a D). In contrast the typical late symbiotic pattern of *in situ dct*A activity which can be observed in nodules induced by 20D1/pBBB5 on alfalfa, was absent from nodules induced by this strain on *M. truncatula* (Fig7a B).

In order to evaluate the efficiency of *dct*A and *nif*H gene expression, bacteroid suspensions were prepared and the symbiotic activity of the gene fusions measured. The level of *nif*H activity obtained from 20D1 mutant bacteroids amounted to about 70 % of that obtained from the wild-type (252 units of β-galactosidase activity versus 371 U. respectively). This ratio is similar to what was previously observed in nodules induced on alfalfa (See Table 5a). Therefore it seems unlikely that a reduction in *nif*H activity is responsible for the much reduced N_2-fixing activity of *M.truncatula* plants nodulated with the 20D1 strain. The pBBB5 *dct*A::*lac*Z gene fusion was found to be expressed efficiently in nodules induced by the 2011 wild-type strain. The β-galactosidase activity of 489 U. was comparable to that obtained from the same strain-plasmid combinations on alfalfa. In contrast to 20D1/pBBB5 bacteroids from nodules induced on alfalfa, bacteroids purified from nodules induced by this strain on *M.truncatula* did not show β-galactosidase activity of any significance (25 U.). These results indicate that the reduced symbiotic N2-fixing activity of the 20D1 *dct*D mutant strain on *M.truncatula* is due to the absence of efficient expression of the *dct*A gene.

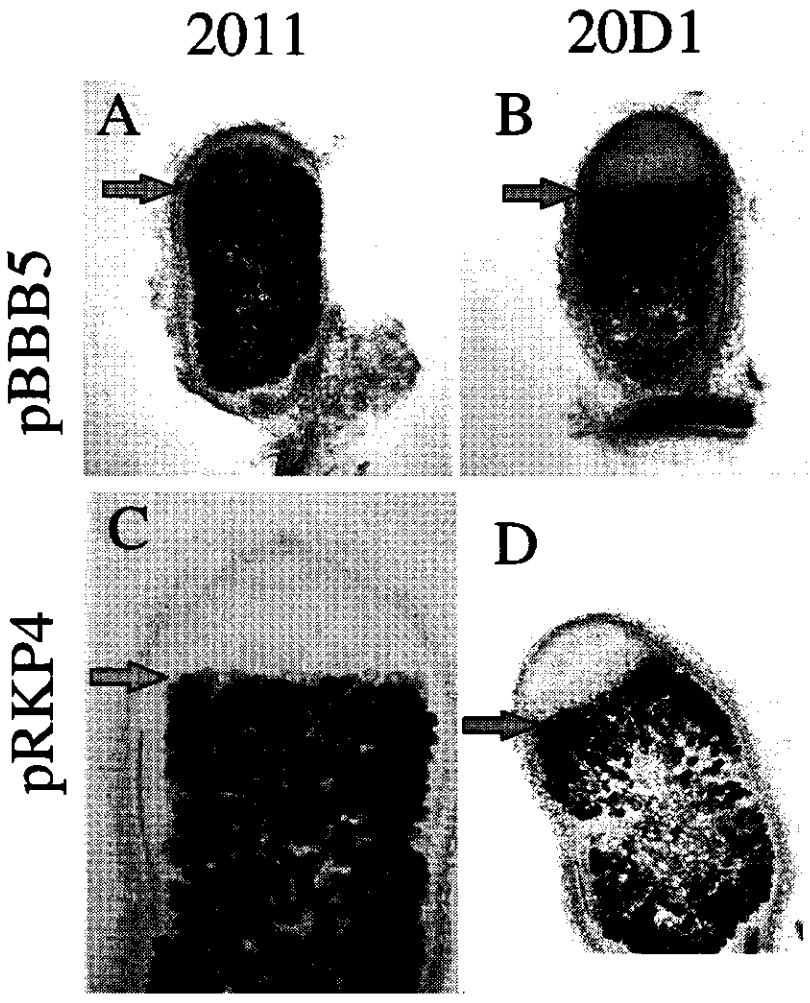

Fig. 7a: *In situ* expression of the *dct*A::*lac*Z and the *nif*H::*lac*Z gene fusions on *M.truncatula*.

The first column shows nodules induced by the 2011 wild-type strain. The second column shows nodules induced by the 20D1 *dct*D mutant strain. The top row shows the *in situ* expression of the pBBB5 *dct*A::*lac*Z gene fusion. The bottom row shows the activity of the pRKP4 *nif*H::*lac*Z gene fusions. The arrows indicate the position of the interzone II/III. **A)** Strain 2011/pBBB5; 15 dpi; coloration Xgal for 2.5 hrs. Strong fusion activity is observed in the infection zone II and continues throughout the interzone II/III and the late symbiotic zone III. **B)** Strain 20D1/pBBB5; 15 dpi; coloration Xgal for 2.5 hrs. Virtually no DctBD independent expression of the gene fusion can be observed. **C)** Strain 2011/pRKP4; 16 dpi; coloration Xgal for 2.5 hrs. Typical late symbiotic activity of the *nif*H gene is observed. **D)** Strain 20D1/pRKP4; 16 dpi; coloration Xgal for 2.5 hrs. The *nif*H gene is efficiently expressed in these nodules. Some early senescence of the cells in the central zone of the inefficient 20D1 nodules can be seen.

Considering the levels of β-galactosidase activity, a striking difference between alfalfa and *M.truncatula* was that bacteroid suspensions from *M.truncatula* nodules induced by the 2011 wild-type strain, had comparable levels of β-galactosidase activity for the pBBB5 and pRKP4 gene fusions. Previously with alfalfa, the *nif*H gene fusion was always found to be expressed at a level several times higher than the *dct*A fusions. Since the *dct*A::*lac*Z gene fusions gave comparable levels of β-galactosidase activity on alfalfa and *M.truncatula*, this indicated a real difference in *nif*H gene activity. This was the more surprising since wild-type nodules from *M.truncatula* were as efficient in N_2-fixation as those from *M.sativa*

7.4) NifA activity on *M.truncatula*.

The expression of the *nif*H gene is regulated by the NifA protein (Better *et al.*, 1985). Therefore the low level of activity of the *nif*H gene fusions on *M.truncatula* (in comparison with *nif*H activity in nodules induced on *M.sativa*) suggested a difference in NifA activity. This could be either a reduced level of expression of the *nif*A gene, or a lower activity of the NifA protein. To investigate these possibilities, we set out to compare the symbiotic activity of a *nif*A::*lac*Z gene fusion (pCHK57, Ditta *et al.*, 1987) in nodules induced by the 2011 wild-type *S.meliloti* strain on *M.truncatula* and *M.sativa* respectively.

	M.truncatula	*M.sativa*
pBBBg1	193 +/- 9	280 +/- 43
pCHK57	59 +/- 8	530 +/- 52
pPRKP4	138 +/- 53	2504 +/- 959

Table 7a: Symbiotic activity of the *nif* genes.
The β-galactosidase activity from bacteroid suspensions is presented. The values are the average of at least three samples of at least 5 plants each. It can be clearly seen that the expression of the "short" *dct*A gene fusion (pBBBg1) is comparable between the two hosts. However the *nif*A (pCHK57) and *nif*H (pRKP4) gene fusions are much less active on the *M.truncatula* host plant than on alfalfa.

Seedlings were also inoculated with the 2011 strain containing the pBBBg1 *dct*A::*lac*Z gene fusion and the pRKP4 *nif*H::*lac*Z gene fusions. The ASA apparently operates on alfalfa, but not on *M.truncatula*. Therefore the "short" *dct*A::*lac*Z fusion was used because its activity is not influenced by the ASA. We expected to observe similar levels of β-galactosidase activity with this gene fusion in nodules induced on either of the two host plants. The *nif*H gene fusion pRKP4 was used to consolidate the previous results.

The results of this experiment are summarised in Table 7a. As expected, comparable levels of β-galactosidase activity were obtained from the *dct*A gene fusion from nodules induced on either host. In contrast a very large difference in *nif* gene expression was observed. The expression of the *nif*H gene from nodules induced on *M.truncatula* was only a fraction of the activity obtained from alfalfa nodules. This corresponded with a similar difference in *nif*A gene expression between the two hosts. These results clearly suggest that a low level of *nif*A gene expression, rather than a reduction in NifA activity, is responsible for the low level of *nif*H gene expression in nodules induced by *S.meliloti* on *M.truncatula*.

7.5) Discussion.

In this chapter, we studied the expression of the *dct*A gene fusions during symbiosis with *Medicago truncatula* in order to assess the suitability of this model legume for further genetic studies on the symbiotic regulation of the *dct*A gene. The *M.truncatula* cv. Jemalong seedlings are efficiently nodulated by the 2011 strain and this results in an effective symbiosis. This incidentally is apparently not the case for all *S.meliloti* strains, as several wild-type strains that are effective on alfalfa lead to fix$^-$ nodules on certain cultivars of *M.truncatula* (L.Trichine Laboratoire de Biologie Moléculaire des Rélations Plantes - Microorganismes, INRA-CNRS Toulouse, France. personal communication). No significant differences were observed in the temporal and spatial pattern of *in situ dct*A expression in nodules induced by the 2011 wild-type strain on either one of the two host plants. In contrast with alfalfa however, nodules induced by the 20D1 *dct*D mutant strain on *M.truncatula*, were found to be ineffective for symbiotic N_2-fixation. This fix$^-$ phenotype was correlated with an inability to express the *dct*A gene during symbiosis. Therefore, unlike the situation on alfalfa, the *dct*A$^-$ phenotype of the 20D1 *dct*D mutant strain is not overcome during symbiosis by an alternative mechanism of symbiotic activation (ASA).

At the same time, in nodules induced on *M.truncatula* by either the wild-type strain or the *dct*D mutant, we observed a much lower activity of the *nif*H gene, than with alfalfa. This corresponded with a significantly reduced level of expression of the *nif*A gene. Surprisingly, this reduced level of *nif* gene expression was not reflected in a lower symbiotic N_2-fixation activity of the *S.meliloti-M.truncatula* symbiosis. Apparently the level of *nif* expression in nodules induced on *M.truncatula*, although much reduced in comparison to alfalfa, is still sufficient to establish high levels of nitrogenase activity. In any case the temporal and spatial patterns of expression of the *nif* gene fusions, also suggest a correct regulation. On the other hand, one may wonder if the very high level of *nif*H activity in nodules induced on alfalfa might suggest some deregulation of *nif* gene expression.

However, these findings may help to understand the observed differences in ASA activity in nodules induced on alfalfa and *M.truncatula*. If the *nif*A gene is over-expressed in the alfalfa nodules, than one can understand how this would allow NifA to cross activate the *dct*A promoter. Similarly the much reduced level of NifA activity on *M.truncatula*, not only explains the reduced level of *nif*H activity, but also the absence of ASA activity on the *dct*A gene. On the other hand, since the 2011 wild-type strain is efficient for symbiotic N_2-fixation with *M.truncatula*, this indicates that the ASA is dispensable in the *Sinorhizobium-Medicago* symbiosis. Consequently we conclude that the expression of the *dct*A gene by the DctBD mechanism alone is sufficient to provide the bacteroids with C_4-dicarboxylic acids and meet their energy requirement for symbiotic N_2-fixation.

Chapter 8)

Conclusions and General Discussion

8.1) Conclusions.

The *S.meliloti dct*A gene codes for a permease that is required for the uptake of C_4-dicarboxylates, which are the major carbon source to fuel symbiotic N_2-fixation. The aim of this work was to study the regulation of the *dct*A gene during symbiosis of *S.meliloti* with the *Medicago* host plants. In particular we wanted to characterise the alternative mechanism of symbiotic activation (ASA). In the absence of the regular mechanism of *dct*A activation (DctBD), the ASA leads to an efficient activation of the *dct*A gene during symbiosis. We set out to determine the role of the ASA in the overall regulation of *dct*A gene expression in nodules induced by *S.meliloti* and to examine whether the ASA, or the regular DctBD mechanism, is primarily responsible for the activation of the *dct*A gene during symbiosis. The work presented in this thesis has resulted in a number of observations that contributed significantly to our understanding of the regulation of the *dct*A gene during symbiosis. The major findings are summarised here.

8.1.1) The ASA mechanism is only active at the late stages of the symbiosis.

The first significant observation was that the ASA only manifests itself at the late stages of the symbiosis, after the bacteria have differentiated into N_2-fixing bacteroids. The temporal and spatial pattern of DctBD-independent *dct*A expression in nodules induced on the alfalfa host plant was always very similar to that of the *nif*H gene fusion. This late symbiotic manifestation of the ASA means that the early expression of *dct*A observed in wild-type nodules is necessarily DctBD dependent. This also means that *dct*BD mutant strains remain unable to express the *dct*A gene at the early stages of the symbiosis. The only significant difference between *dct*BD and *dct*A mutant strains, is that once the bacteroids are fully differentiated, the ASA suppresses the *dct*A negative phenotype of the *dct*BD mutants, whereas the *dct*A mutants remain Dct negative. It has been suggested that the metabolism of C_4-dicarboxylates may be required for the differentiation of the bacteria into N_2-fixing bacteroids (Gardiol *et al.*,1987). As indicated by the DctBD dependent activation of the *dct*A::*lac*Z gene fusions, dCA indeed appear to be present at the early stages of symbiosis. However, *dct*BD mutant strains, which are unable to utilise this dCA at the early stages of the symbiosis, still differentiate into N_2-fixing bacteroids. This indicates that efficient uptake of dCA at the early stages of the symbiosis is not essential for bacteroid differentiation.

8.1.2) The ASA requires sequences downstream of the *dct*A promoter for activity.

The second interesting observation was that the ASA did not act on the "short" *dct*A::*lac*Z gene fusions. This was remarkable since these "short" gene fusions appeared to be correctly regulated by the DctBD system under free-living conditions. In these gene fusions, the *lac*Z gene was fused directly to the ATG start-codon of the *dct*A gene. In contrast, gene fusions containing the first 8 amino-acids of the *dct*A coding region were efficiently activated by the ASA. The only difference between these fusions and the ones that were not activated by the ASA is a short stretch of 16 bp of DNA. We

therefore concluded that some cis-acting nucleotide sequences required for ASA activity, are located immediately downstream of the *dct*A promoter in the beginning of the coding region. Close examination of this region of the *dct*A gene and comparison with other *dct*A promoter sequences did not reveal the presence of any obvious regulatory sequences.
Some of our earlier work has indicated that the *nif*H promoter also contains sequences downstream of the transcriptional start site, which are involved in the regulation of this promoter (Wang *et. al.*, 1991). A direct comparison of the 5' amino acid sequences of the *nif*H gene with the *dct*A gene revealed some striking similarities, especially in the sequences coding for AA8-12. However these homologies are located downstream of the region required for activation of the *dct*A promoter by the ASA. Possibly, the *lac*Z sequences immediately downstream of the fusion site in the pBBB and pBBEB plasmids are sufficiently similar to compensate for the absence of these sequences. A systematic study of this region, involving site directed mutagenesis, will be necessary to unambiguously identify the regulatory elements.
The fact that the "short" *dct*A::*lac*Z gene fusions are efficiently expressed at the late symbiotic stages, in nodules induced by a wild-type *S.meliloti* strain, demonstrates that the DctBD mechanism also operates in the N_2-fixing bacteroids.

8.1.3) The UAS sites of the *dct*A promoter are not essential for ASA activity.

The third observation was that the upstream activator sites (UAS) were not essential for activation of the *dct*A promoter during symbiosis. The UAS are absolutely required for activation by the DctBD system, which was also demonstrated by the fact that gene fusions lacking these UAS sites could not be induced by dCA under free-living conditions. Yet, during symbiosis these fusions were expressed in bacteroids, at a level of about 30% of that of an equivalent fusion containing the UAS. Moreover these gene fusions, lacking the UAS, were expressed at a similar level in a wild-type nodule as they were in the 20D1 *dct*D mutant background. The pattern of temporal and spatial expression in a wild-type nodule was similar to that observed for DctBD independent activation of other gene fusions containing the UAS. We therefore concluded that the UAS sites, which are indispensable for the activation by the DctBD system, are not essential for the activation of the *dct*A promoter by the ASA. The fact that these fusions were activated in nodules induced by a wild-type strain indicated that (beside the DctBD system) the ASA contributes to the level of expression of the *dct*A gene during symbiosis.
The *dct*A::*lac*Z gene fusions, lacking the UAS, facilitated the search for the ASA because they circumvented the requirement for a strain mutated in the *dct*BD genes to monitor ASA activity. The effect of a mutation of interest on ASA activity could be evaluated directly, without the need for a double mutant strain mutated in *dct*BD as well.

8.1.4) The alternative symbiotic activator requires NifA.

From this work it has become clear that an efficient expression of the *nif*A gene is required for ASA activity. This conclusion was drawn from two separate lines of investigation:
We observed that the *dct*D mutant strain 20D1 induces nodules that are virtually fix$^-$ when inoculated on the *Medicago truncatula* host plant. This result was correlated with the fact that the *dct*A gene is not expressed efficiently in these nodules. We also observed that the *nif*A gene is expressed in these nodules at a level, which is strongly reduced in comparison to the level of *nif*A expression in nodules induced on *M.sativa*. These results suggested that a high level of *nif*A gene expression is required for ASA activity.
The second line of evidence came from the fact that the pBBEB gene fusions, lacking the UAS sequences, are not activated in nodules formed by the 1354 *nif*A::Tn5 mutant strain. These gene fusions are efficiently expressed in the wild-type and *dct*D mutant backgrounds. This also suggests a role for NifA in the DctBD independent activation of the *dct*A promoter.

8.1.5) The ASA is not essential for symbiotic N_2-fixation.

Monitoring *dct*A activity with the "short" *dct*A::*lac*Z gene fusions that are not activated by the ASA, demonstrated that the DctBD system alone is sufficient to express the *dct*A promoter during symbiosis. Furthermore we observed that the ASA does not operate in nodules induced on the *Medicago truncatula* host plant. Despite the absence of ASA activity, these nodules were as efficient for symbiotic N_2-fixation as those induced on alfalfa. This indicates that activation of the *dct*A promoter by the ASA is not required for symbiotic N_2-fixation.

8.2) Discussion.

In this work we have studied the *in planta* expression of a number *dct*A::*lac*Z gene fusions. The constructs differed only in the extent of DNA upstream, or downstream of the *dct*A promoter, that was retained. During symbiosis the *dct*A promoter can be activated by two independent regulatory mechanisms. The upstream activator sequences (UAS) are known to be required for the activation of the *dct*A promoter by the DctBD mechanism. Deleting these sequences resulted in gene fusions that no longer could be activated by DctBD, but still responded to the ASA. Conversely, deleting sequences immediately downstream of the promoter resulted in gene fusions that no longer could be activated by the ASA, but under free-living conditions were still correctly regulated by the DctBD system. It is curious to think that a gene fusion, which is apparently properly regulated by its cognate two-component regulatory system, does not necessarily reflect the regulation of the gene in question under all conditions. For example, the exclusive use of the "short" *dct*A::*lac*Z gene fusions might have led us to erroneously believe that there was no ASA activity. This demonstrates that great caution should be taken when interpreting results obtained with this technology. Using the various gene fusions we were able to distinguish between DctBD, or ASA driven *dct*A expression *in planta*.

8.2.1) The mechanism of *dct*A activation by the ASA.

The major finding of this work is that a functional NifA is required for ASA activity. NifA and DctD both belong to the same family of NtrC type of transcriptional activators and NifA is highly active during symbiosis. Therefore NifA was proposed as being the most likely candidate for the ASA, as soon as the mechanism was discovered. However, no conclusive evidence could be generated to support this hypothesis. Other results, such as the inability to express *dct*A under microaerobic conditions seemed to exclude NifA from the list of possible candidates (Wang *et al.*, 1989).
Here then, NifA is back as the most likely alternative symbiotic activator of the *dct*A promoter. Considering the fact that NifA and DctD are closely related, it seems likely that NifA interacts directly with the *dct*A promoter to activate its transcription. However, the possibility can not be excluded that NifA is required for the activation of another gene, which in turn is responsible for activation of the *dct*A promoter. A third possibility is that in addition to nifA, another gene product is required to allow NifA to act on the *dct*A promoter.
The most interesting aspect of the ASA is probably going to be the actual mechanism by which it performs its function. Two novel features are revealed in this work: First, the upstream activator sites (UAS) contribute significantly to the level of DctBD independent expression of the *dct*A promoter, but are not essential. Second, there seem to be cis-acting sequences downstream of the *dct*A promoter which are indispensable for ASA activity. Possibly another gene product which interacts with these downstream sequences is required in addition to NifA. A similar mechanism may also be involved in the symbiotic expression of the *nif*HDK- and *fix*ABC promoters. Both these promoters require NifA for activation and their UAS's were also found to be dispensable for their activity during symbiosis (Better *et al.*, 1985; Wang *et al.*, 1991; This work).

8.2.2) The role of the ASA in the Rhizobium-legume symbiosis.

We have concluded that the ASA contributes significantly to, but is not essential for, the symbiotic activation of the *dct*A promoter in the *Sinorhizobium-Medicago* symbiosis. On the other hand, NifA is required for the activation of various *nif* and *fix* genes and as such is a prerequisite for symbiotic N_2-fixation. Because of the high levels of NifA activity the *dct*A promoter is activated efficiently *in planta* by the ASA and this makes the regulatory *dct*BD genes redundant for an efficient symbiosis. At least this is true for alfalfa, but not for *Medicago truncatula*. It is interesting to observe that in *Rhizobium* sp. NGR234 a homologue of the *dct*A gene has been identified. This bacterium nodulates a wide range of host plants, among which also certain *Medicago* species. The NGR234 *dct*A gene was found to be essential for an effective symbiosis, although not for growth on dCA under free-living conditions (van Slooten *et al.*, 1992). No equivalent of the regulatory *dct*BD genes were found in the vicinity of this *dct*A gene. One can imagine that in absence of the DctBD mechanism, this bacterium relies solely on the ASA for the expression of this *dct*A gene during symbiosis. The symbiosis between *S.meliloti* and *M.truncatula* demonstrates that high levels of NifA activity, as observed on *M.sativa*, are not required

for efficient expression of the *nif* and *fix* genes. On the other hand, a high level of NifA activity does ensure expression of other σ^{54}-dependent genes such as the *dct*A gene, even in the absence of their own regulatory systems. The NGR234 system may be one step further in this development in that the *dct*A gene in this bacterium has become an exclusively late symbiotic gene, solely activated by the ASA.

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Samenvatting

Voor een optimale opbrengst van landbouwgewassen moeten de planten over voldoende stikstof in gebonden vorm kunnen beschikken. Een tekort aan stikstof in de bodem kan verholpen worden door toedienen van kunstmest. Sterker nog, zonder kunstmest zouden de opbrengsten van de hedendaagse landbouw niet mogelijk zijn. De lucht die wij inademen bestaat voor 80 % uit stikstof. Deze atmosferische *stikstof kan echter niet als zodanig gebruikt worden door de planten, maar moet eerst omgezet worden in* in ammonia om door de planten voor hun groei gebruikt te kunnen worden. Het "binden" van deze stikstof, dat wil zeggen het reduceren van N_2 tot NH_3, kan gebeuren via een chemisch proces. Door deze vorm van kunstmest produktie zijn in de jaren 50 sommige chemie-concerns groot geworden. Chemische stikstof binding verbruikt veel energie en is dan ook een kostbaar proces wat zich uit in de prijs die voor de kunstmest betaald moet worden. Kunstmest is dan ook een van de grootste kostenposten voor de boeren. Er zijn daarnaast ook planten die goed gedijen op stikstofarme gronden. Daartoe behoren vlinderbloemigen, die een symbiose kunnen aangaan met *Rhizobium* bacterien. Deze Rhizobia zijn in staat om de atmosferische stikstof te reduceren tot ammonia en deze ten goede laten komen aan de plant. Deze zogenaamde biologische stikstofbinding vindt plaats in knollen die zich onder invloed van de Rhizobia vormen op de wortels van de vlinderbloemige plant en gevuld zijn met bacterien. De wisselwerking tussen de vlinderbloemige plant en de Rhizobia, die leidt tot deze stikstofbindende knollen is complex en al vele jaren een belangrijk onderwerp van wetenscappelijk onderzoek. Het onderwerp dat wordt beschreven in dit proefschrift is slechts een klein stukje van de interactie van Rhizobia met hun gastheerplanten.

De gastheerplant reageert op de aanwezigheid van de Rhizobia met de vorming van knollen op de wortels. In een vroeg stadium worden de planten geinfecteerd door Rhizobia die de plant binnendringen via de wortelharen. *Aanvankelijk bevinden de bacterien zich in infectiedraden waar ze zich nog kunnen* vermeerderen door deling. Vervolgens worden de bacterien gedeponeerd in de cellen van de zich ontwikkelende wortelknol. Vanaf dat moment spreken we niet meer van bacterien, maar van bacteroiden. De bacteroiden worden bij hun opname in de plant cel omgeven door een membraan van plantaardige afkomst. Dit membraan scheidt de bacteroiden van het cytoplasma van de plantencel. Na de opname in de plantencel ondergaan de bacteroiden een ontwikkeling waarbij zij wel nog groeien, maar stoppen met delen en zich differentieren tot een vorm die in staat is tot symbiontische stikstof binding. Deze stikstof binding vind plaats in de centrale zone van de knollen. De bacteroiden maken het nitrogenase enzym dat verantwoordelijk is voor de stikstof binding. Dit enzym is zeer gevoelig voor zuurstof en de zuurstofdruk in de centrale zone van de knollen is dan ook zeer laag. Biologische stikstofbinding kost veel energie en de voeding voor de bacteroiden nodig voor de enrgieproductie, wordt geleverd door de plant. In de bladeren van de plant wordt door de fotosynthese sucrose gemaakt dat via de vaten wordt getransporteerd naar de andere delen van de plant. In de centrale zone van de knollen bevinden zich de stikstofbindende bacteroiden. Het membraan dat de bacteroiden omgeeft is niet doorlaatbaar voor sucrose. In de plant cell wordt de sucrose omgezet in dikarboonzuren zoals bijvoorbeeld malaat dat wel via de membraan tot de bacteroiden kan doordringen. Om van deze energiebron gebruik te kunnen maken, moeten de bacteroiden beschikken over een systeem om dikarboonzuren op te nemen. Dit opnamesysteem voor dicarboonzuren

en in het bijzonder de vraag hoe de expressie van de genen die betrokken zijn in de opname van dicarboonzuren wordt gereguleerd tijdens de symbiose, is het onderwerp van deze studie.
Sinorhizobium meliloti behoord tot de meest onderzochte Rhizobia. Deze stam kan knollen induceren op planten van de *Medicago* familie. Een cultuur van *S.meliloti* bacterien kan uitstekend groeien in een medium dat dikarboonzuren bevat als enige bron van koolstof. De dikarboonzuren worden dan opgenomen via een transporteiwit, een zogenaamd permease, dat aangemaakt wordt door het *dct*A gen. "Dct" staat voor: dicarboxylate transport. Het Dct systeem omvat behalve het DctA permease ook nog twee regulerende eiwitten die aangemaakt worden door de *dct*B en *dct*D genen. DctB is een "sensor" eiwit dat in het membraan gelokaliseerd is en gedeeltelijk naar buiten steekt. DctB is in staat de aanwezigheid van dikarboonzuren in het milieu te detekteren. DctD is een "regulator" eiwit welk in staat is het *dct*A gen te activeren. Als het wordt aangemaakt door het *dct*D gen, is het DctD eiwit inactief. Als de aanwezigheid van dikarboonzuren in het milieu wordt gedetecteerd door het DctB eiwit, activeerd DctB het DctD eiwit. DctD op zijn beurt activeert vervolgens het aflezen van het *dct*A gen. Via het DctA transport eiwit worden de dikarboonzuren dan opgenomen en verbruikt in het metabolisme van de bacterien. Het mag duidelijk zijn dat zonder *dct*A de opname van dikarboonzuren niet mogelijk is. Het ontbreken van de regulerende *dct*BD genen heeft hetzelfde effect omdat het *dct*A gen dan niet geactiveerd kan worden.
Een *S.meliloti* stam waarvan het *dct*A gen gemuteerd is, kan de gastheerplant wel infecteren en dit leidt ook tot de vorming van volledig geinfecteerde knollen. De bacteroiden in die knol zijn echter niet in staat om stikstof te binden omdat geen dikarboonzuren kunnen worden opgenomen. Men zou nu verwachten dat dit ook het geval zou zijn voor *dct*BD mutanten. In vrijlevende vorm zijn deze stammen immers niet in staat om het *dct*A gen te activeren. Het is echter zeer opmerkelijk dat bacterie stammen die gemuteerd zijn in *dct*B en/of *dct*D, doorgaans wel een effectieve symbiose aangaan met de gastheerplant en actief stikstof bindende knollen vormen. Nader onderzoek heeft aangetoond dat in die gevallen het DctA transport eiwit wordt aangemaakt tijdens de symbiose, ook al ontbreken de regulerende DctBD eiwitten. Dit heeft geleid tot de veronderstelling dat er een alternatief mechanisme moet zijn om de expressie van het *dct*A gen te activeren tijdens de symbiose. Dit mechanisme wordt in het engels "The alternative symbiotic activator", of kortweg de "ASA" genoemd.
De vraag wat het ASA precies inhoudt is het centrale onderwerp van dit proefschrift. Allereerst de vraag: "Wat is precies dit alternatieve mechanisme dat in staat is om *dct*A te activeren tijdens de symbiose?" Daarnaast rijst de vraag wat het belang is van dit mechanisme voor de symbiose en of er in aanwezigheid van een functioneel DctBD systeem ook een rol is voor de ASA. Men kan zich ook afvragen of ASA, of DctBD in eerste instantie verantwoordelijk is voor de expressie van het *dct*A gen tijdens de symbiose.
Om expressie van het *dct*A gen te kunnen detecteren werd het gen gefuseerd met het *Escherichia coli* lactose gen (*lac*Z). Het *lac*Z gen codeert voor het β-galactosidase. Xgal is een substraat voor het β-galactosidase dat door dit enzym wordt omgezet in een intens blauwe kleurstof. Met behulp van Xgal kan de activiteit van de *dct*A::*lac*Z gen fusies gevisualiseerd worden. De activiteit van de fusie eiwitten kan quantitatief worden bepaald met behulp van het ONPG substraat. Dit substraat wordt door de β-galactosidase activiteit in extracten van de bacterien of bacteroiden omgezet in een gele kleurstof die fotometrisch gemeten kan worden.

De β-galactosidase activiteit van de *dct*A::*lacZ* gen fusies in vrij levende bacterien is doorgaans laag als er geen dikarboonzuren aanwezig zijn in het medium. Door het toevoegen van dikarboonzuren wordt de activiteit van de fusies sterk geinduceerd. In de afwezigheid van een functioneel DctBD systeem worden de fusie eiwitten niet geinduceerd. Omgekeerd betekend een hoge activiteit van de gen-fusies dat het DctBD systeem actief is en dikarboonzuren aanwezig zijn in het milieu. Tijdens de symbiose is dat niet altijd waar omdat dan het *dct*A gen ook geactiveerd kan worden door de ASA.

De *in planta* expressie van de *dct*A::*lacZ* gen fusies werd bestudeerd door *Medicago sativa* (alfalfa) te infecteren met een stam van het wild-type *S.meliloti* die een *dct*A::*lacZ* gen fusie bevatte. Volwassen knollen werden in de lengte in plakjes gesneden en deze microscopisch dunne "coupes" werden geincubeerd in een oplossing met Xgal. Na ongeveer een uur kan dan onder het microscoop de blauwe kleur gedetecteerd worden die aanduid waar het *dct*A gen tot expressie komt (Fig.10). In wild-type knollen

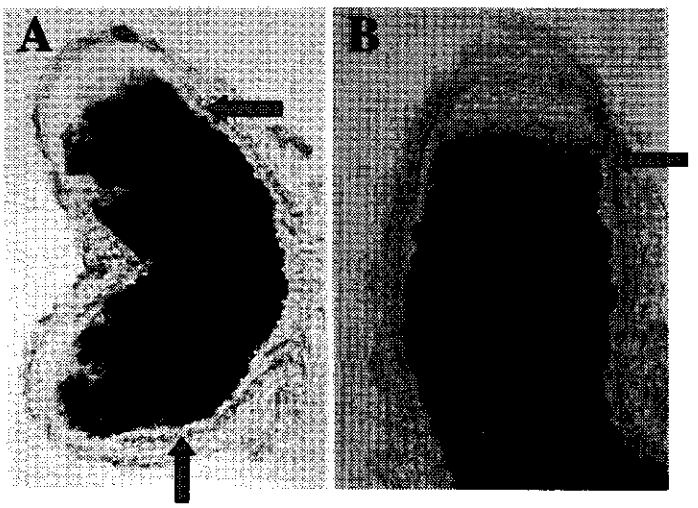

Fig.10: *In planta* expressie van een *dct*A::*lacZ* gen fusie.
De blauwe kleur wordt veroorzaakt door de omzetting van het Xgal door het β-galactosidase eiwit wat aangemaakt wordt door het geactiveerde *lacZ* gen. De pijlen wijzen naar de overgangszone van de vroege stadia van de symbiose in de top van de knollen, naar de centrale zone van de knollen waar de stikstofbinding plaatsvind. A) Deze foto toont een dubbele knol die geinduceerd werd door een wild-type *S.meliloti* stam. De expressie van *dct*A vind reeds plaats in een vroeg stadium van de symbiose. B) Deze foto toont de activiteit van dezelfde *dct*A::*lacZ* gen fusie, maar nu in een knol die geinduceerd werd door een *dct*D mutant. De expressie van *dct*A door de ASA vind uitsluitend plaats in de centrale zone van de knollen

blijkt het *dct*A gen actief in de zones die het verst van de wortels verwijderd zijn en overeen komen met de vroege stadia van de symbiose, en ook in de centrale zone van de knollen, waar zich de volledig gedifferentieerde bacteroiden bevinden en stikstofbinding plaatsvind. Vervolgens werd gekeken in welk stadium van de symbiose de ASA actief is. Daartoe werden alfalfa planten geinfecteerd met *S.meliloti*

stammen die gemuteerd waren in het *dctD* gen of in het *dctB* gen. De *in planta* expressie van het *dctA* gen was in deze knollen beperkt tot de centrale zone. Dit betekent dat de ASA uitsluitend actief is gedurende de latere stadia van de symbiose. Omgekeerd betekent dit dat gedurende de vroege stadia van de symbiose alle *dctA* activiteit van het DctBD systeem afhankelijk is.

Niet alle *dctA*::*lacZ* gen fusies werden echter door de ASA goed geactiveerd. Met name constructen waarin het *lacZ* onmiddelijk met het ATG start codon van het *dctA* gen gefusioneerd is, werden niet geactiveerd door de ASA. Dit was temeer opmerkelijk omdat deze "korte" fusies normaal geactiveerd werden door een functioneel DctBD systeem. Constructen die een wat groter deel van het *dctA* gen bevatten, met name de 24 nucleotiden die voor de eerste 8 aminozuren coderen, konden wel weer door de ASA geactiveerd worden. Deze resultaten toonden aan dat in het DNA dat vor het aminoterminale uiteinde van het DctA eiwit codeert, zich sequencies bevinden die noodzakelijk zijn voor de activiteit van het ASA systeem. In knollen geinduceerd door wild-type *S.meliloti* stammen met "korte" fusies wordt het dctA gen in vroege als latere stadia van de symbiose geactiveerd. Dit toont aan dat het DctBD systeem ook in de late stadia van de symbiose actief is. Om de activiteit van de ASA te kunnen detecteren in wild-type knollen, werd er vervolgens gezocht naar gen fusies die wel door de ASA geactiveerd worden, maar niet door het DctBD systeem. Hiervoor werd uitgegaan van de de *dctA*::*lacZ* gen fusies die de eerste acht aminozuren van het *dctA* gen bevatten en effectief door de ASA geactiveerd kunnen worden. Van deze fusies werden vervolgens de DNA sequenties verwijderd die de "upstream activator sites, of UAS" bevatten. Deze UAS zijn de sequenties waar het geactiveerde DctD eiwit moet binden om de *dctA* promotor te activeren. De resulterende fusies konden inderdaad niet meer geactiveerd worden in vrijlevende culturen. Tijdens de symbiose werden deze fusies echter wel geactiveerd, maar alleen in de centrale zone van de knollen. Bovendien was het patroon van de *in planta* expressie in knollen geinduceerd door de wild-type bacterien identiek aan die in knollen geinduceerd door de *dctD* mutanten. Dit toont aan dat de UAS niet essentieel zijn voor ASA activiteit en ASA ook actief is in de late stadia van de symbiose in knollen geinduceerd door een wild-type stam.

Deze fusies openden ook de mogelijkheid om het effect van mutaties op de ASA te bestuderen, zonder dat de mogelijke effecten overschaduwd worden door het DctBD systeem. Zo bleek in knollen die geinduceerd werden door een *nifA* mutant, de "korte" fusies (die uitsluitend door DctBD geactiveerd worden) normaal tot expressie te komen, maar dat de fusies zonder de UAS (die uitsluitend nog door de ASA geactiveerd worden) niet tot expressie komen. Dit is een sterke aanduiding dat een functioneel NifA nodig is voor ASA activiteit.

In zekere zin was dit niet verbazingwekkend want NifA is een activator eiwit voor de transcriptie van *nif* genen en zeer actief gedurende de late fase van de symbiose. Bovendien is NifA functioneel nauw verwant aan DctD daar beide behoren tot de familie van activeerders van NtrA afhankelijke promoters. Vreemd is echter dat voor het mechanisme waarbij NifA de expressie van de *dctA* promoter bewerkstelligt, NifA geen gebruik maakt van de UAS sequencies, maar sequencies nodig zijn die stroomafwaards van de promoter liggen. Anderzijds is bekend dat de UAS sequencies van de *nifHDK* en *fixABC* promoters, die door NifA geactiveerd worden, ook niet essentieel zijn voor efficiente expressie van deze genen tijdens de symbiose.

Het mechanisme waardoor ook deze promoters tijdens de symbiose geactiveerd worden is echter nog niet nader onderzocht.

Een tweede aanwijzing dat efficiente expressie van *nif*A nodig is voor ASA activiteit werd verkregen uit experimenten met de *Medicago truncatula* als gastheer. Vanwege het feit dat deze plant meer geschikt is voor geneties onderzoek en manipulatie dan alfalfa, is voorgesteld toekomstig onderzoek naar plant-Rhizobium interacties op *M.truncatula* te concentreren. Om te zien of deze plant ook geschikt is voor het bestuderen van de *in planta dct*A expressie, werd een aantal planten geinfecteerd met de wild-type *S.meliloti* stam en een aantal anderen met een *dct*D mutant. Het eerste wat daarbij opviel was dat *M.truncatula* planten met knollen geinduceerd doorde *dct*D mutant een sterk gereduceerde stikstofbinding vertoonden. Vervogens bleek dat de *dct*A::*lac*Z fusies niet geactiveerd werden in de knollen die geinduceerd waren door de *dct*D mutant. Dit in tegenstelling tot alfalfa knollen, waarin deze fusies wel tot expressie komen in afwezigheid van DctD. Dit wijst erop dat de ASA niet actief is in knollen geinduceerd op *M.truncatula*. De expressie van de *dct*A::*lac*Z fusies en de stikstofbinding in knollen geinduceerd door de wild-type waren wel vergelijkbaar op beide gastheerplanten. Vervolgens werd gekeken naar de expressie van de *nif*HDK promoter in knollen geinducerd op *M.truncatula*. Hoewel het patroon van de *in situ* expressie identiek was met dat in knollen geinduceerd op alfalfa, bleek dat het niveau van de expressie in knollen op *M.truncatula* vele malen lager was. Omdat NifA direct verantwoordelijk wordt geacht voor de expressie van *nif*HDK, werd vervolgens de expressie van een *nif*A::*lac*Z gen fusie bestudeerd. Hieruit bleek dat ook de expressie van *nif*A in knollen geinduceerd op *M.truncatula* veel lager was dan het niveau wat gemeten wordt in knollen op alfalfa.

Uit twee verschillende lijnen van onderzoek kan dus worden geconcludeerd dat efficiente expressie van het *nif*A gen noodzakelijk is voor ASA activiteit. Het is echter niet duidelijk of NifA zelf de *dct*A promoter activeert, of dat NifA een ander, nog niet geindentificeerd gen tot expressie brengt. NifA is onmisbaar voor de expressie van *nif* en *fix* genen, maar de bijdrage van NifA aan de expressie van het *dct*A gen is blijkbaar niet essentieel voor een effective symbiose en goede stikstofbinding. Met een functioneel DctBD mechanisme alleen kan voldoende expressie van *dct*A worden verkregen voor een efficiente stikstofbinding.

Curriculum Vitae:

Personal:

Surname:	Boesten
Christian names:	Hubertus Joseph Anna Maria
First name:	Bert
Sex:	male
Date of birth:	3rd January 1957
Place of birth:	Geleen (NL)
Family Status:	Married since 23rd September 1982, two children aged 11 and 13.
Nationality:	Dutch
Address:	Industrielaan 52, 3630 Maasmechelen, Belgium.
Telephone:	00 32 89 775097

Education:

1970-1975:	**Albert Schweitzer Atheneum, Geleen (NL).**
	June 1975: Atheneum B (Natural Sciences)
1975-1982:	**Agricultural University Wageningen (NL).**
	June 1979: Kandidaatsexam Phytopathology
	October 1981 - March 1982: Practical stage at the Biochemistry
	Department of the Animal Virus Research Institute in Pirbright, Surrey, U.K.
	September 1982: Doctoraalexam Phytopathology:
	Subjects: Virology; Molecular Biology; Cell Biology

Employment:

February 1983-March 1994:	Sr. Research Scientist
	Food Microbiology Department,
	University College Cork (UCC)
	Ireland.
September 1994-June 1995	Self employed in classic car restoration business
July 1995-July 1997:	Research Scientist
	Laboratoire de Biologie Moleculaire des Rélations Plantes-Microorganismes,
	INRA-CNRS, Toulouse,
	France.
September 1997- to date:	Reseach Scientist
	Ökologie des Bodens,
	Bio-1, RWTH Aachen,
	Germany

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