Development and application of transgenic approaches to study the *NORK* gene implicated in symbiotic interactions

Ph.D. Thesis

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Table of contents

List of Original Publications

List of original publications directly related to the thesis:

1. Perhald A, Endre G, Kevei Z, Kiss GB, Kereszt A. *Strategies to obtain stable transgenic plants from non-embryogenic lines: complementation of the nn1 mutation of the NORK gene in Medicago sativa MN1008*. Plant Cell Rep. 2006 Aug;(8):799-806.
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2. Bersoult A, Camut S, **Perhald A**, Kereszt A, Kiss GB, Cullimore JV. *Expression of the Medicago truncatula DM12 gene suggests roles of the symbiotic nodulation receptor kinase in nodules and during early nodule development.* Mol Plant Microbe Interact. 2005 Aug;(8):869-76
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1. Introduction

Almost every animal on the planet requires plants in order to sustain life. Plants have the ability to convert solar energy into chemical energy to provide us with food, fuel, and fiber, but their growth is often limited by the availability of nitrogen (Gresshoff, 2003) which is an essential component of many important structural, genetic and metabolic processes. Although nitrogen is a highly abundant element on this planet and commonly it exists in the form of an inert gas (N_2) in the atmosphere. Plants require nitrogen to be in the form of ammonia (NH_3), urea, or nitrate (NO_3) for uptake. There are three main natural nitrogen sources available for plants to utilize: (1) nitrogen acquired slowly from decomposed minerals and organic material, (2) nitrogen fixed by lightning strikes, and (3) nitrogen acquired from the atmosphere as a result of biological nitrogen fixation. The latter contributes to most of the nitrogen found in the soil (Igarashi and Seefeldt, 2003; Jensen and Hauggaard-Nielsen, 2003).

1.1. Symbiotic Nitrogen Fixation

During biological nitrogen fixation, the nitrogen gas (N_2) from the atmosphere is converted to ammonia (NH_3) by bacteria and becomes available for plants use. There are three types of nitrogen fixation: free living, associative and symbiotic. These three types of nitrogen fixation processes differ in the source of energy used and the level of fixing ability (Burris and Roberts, 1993):

The free living process is carried out by bacterium species such as *Azotobacter* or *Klebsiella* which have the photosynthetic ability to produce their own energy source but only small amounts of nitrogen is converted in this process as these species fix nitrogen for their own use.

Associative nitrogen fixation is a process during which bacteria reside close to the plants' roots or leaves. Bacterial species like *Acetobacter* or *Azospirillum* rely on plant exudates and secretions for their energy source used for nitrogen fixation, however, 90% of the nitrogen fixed is only available when the bacterium dies.

The third type of nitrogen fixation is a symbiotic process in which each partner benefits from the interaction with the other partner. Studies have shown that this process occurs predominantly between a specific type of soil-dwelling bacteria broadly called rhizobia and leguminous plant species. *Frankia* and non-legumes (such as *Casuarina*) also enter nitrogen fixing symbioses. Symbiosis between leguminous plants and rhizobia, under conditions of nitrogen limitation, leads to the development of new plant organs, the N_2–fixing nodules that are usually formed on roots but also on stems in a few plants. Inside the nodule the differentiated form of rhizobia, the bacteroids fix molecular nitrogen which is then assimilated by the plant partner. This mutualistic symbiosis is important not only because of its tremendous agricultural and ecological value but also because of the interest for studies of plant organogenesis, signaling and plant-microbe interactions (for reviews see Albrecht et al, 1999; Kistner and Parniske, 2002; Limpens and Bisseling, 2003).

1.1.1. Development of the symbiosis

The legume-rhizobia (LR) symbiosis is characterized by the production of nodules on the roots of the plants in which the bacteria reduce dinitrogen in exchange for nutrients derived essentially from carbon dioxide fixed during photosynthesis which uses solar energy as an energy source. The LR symbiosis is generally initiated in a region, called the susceptible zone (Caetano-Anollés and Gresshoff, 1991) close to the root tips where root hairs are developing. Based on the origin and persistence of the nodule meristem nodules fall into two different types: indeterminate or determinate (Hirsch 1992). The primordia of indeterminate nodules, usually formed on roots of temperate legumes (pea, alfalfa, vetch), starts in the root inner cortex. Cells of the outer cortex are also activated: microtubules reorientate and these outer cortical cells form a preinfection structure, named the preinfection thread (PIT), which later allows for passage of the infection thread (Timmers et al 1999; van Brussel et al 1992). Mature indeterminate nodules are characterized by the presence of a persistent apical meristem responsible for nodule growth. Such nodules are divided into several regions along a differentiation gradient (Vasse et al 1990): zone I or the meristem; zone II or the invasion zone, where bacteria are released into the plant cytosol; interzone II–III, characterized by an accumulation of amyloplasts, a zone where the bacteria differentiate into bacteroids; zone III or the nitrogen-fixing zone, composed of both bacteroid-containing plant cells and small bacteroid-free plant cells where the fixed nitrogen is assimilated; and zone IV or the senescing zone, where both the bacteroids and plant cells degenerate. These zones are enveloped by peripheral cell layers, known as the outer and the inner cortex. Indeterminate nodules export fixed nitrogen predominantly as asparagine and glutamine, which are the most abundant nitrogen species found in the phloem tissues of these legumes.

Determinate nodules are usually formed on tropical and subtropical legume plants (for example, soybean, bean). Nodule primordia are originated from the root outer cortex, and their meristematic activity disappears very early after nodule initiation. Accordingly, nodule growth takes place by cell expansion rather than by cell division and shows only a temporal differentiation pattern. Like indeterminate nodules, determinate nodules are also surrounded by outer and inner cortex but unlike indeterminate nodules, they export mainly ureides (Vance and Gantt 1992).

As mentioned before, legumes form symbiotic relationships with soil bacteria commonly known as rhizobia. These include genera such as *Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium*, and *Allorhizobium*. Many of these species are extremely host specific while others have shown a wide host range (Gresshoff, 2004). For instance, it is known that *Rhizobium leguminosarum* biovar *viciae* almost only nodulates *Pisum* and *Vicia* species while *Rhizobium* sp. NGR234 establishes symbiosis with over 80 species. Extensive genome sequencing of these species have indicated that these genera of bacteria share many highly similar gene sequences such as the genes controlling nodulation (*nod, nol, noe*) or the genes (*nif, fix*) responsible for nitrogen fixation (Gresshoff, 2004). Gene sequences highly similar to the latter group have also been found within free-living and associative nitrogen-fixing bacteria.

To initiate the symbiotic process, the plant roots exude a wide range of sugars, carboxylic acids and flavonic substances which essentially feeds the rhizosphere (Denarie, 1996). This mixture of substances contributes to a certain level to the specificity observed in a symbiotic relationship because different rhizobia respond to specific flavonic signals (Györgypál et al 1991). In response to these chemicals, the nodulation (*nod*, *nol, noe*) genes of bacteria are activated and the microsymbiont synthesizes lipo-chito-oligosaccharides (LCOs)

or otherwise known as Nod Factors (NF). Nod Factors consist of an oligosaccharide backbone of *N*-acetyl-D-glucosamine units and a fatty acyl group which is always attached to the non-reducing saccharide. Nod factors differ between rhizobial species with regards to the saturation, hydroxylation and length of the acyl chain, the number of *N*-acetyl-D-glucosamine units and the decoration of the sugar moieties with different substituents such as fucosyl, sulphate, carbamoyl, carboxyl, etc. groups. This variation in the decoration of the Nod Factors contributes to the host specificity. Nod factors are among the most potent developmental regulators: their effect is expressed at concentrations of $10^{-8} – 10^{-12}$ M only.

Effective nodulation requires two interlinked processes: nodule organogenesis and infection. Nodule organogenesis is initiated in the cortex and pericycle by cell divisions that lead to the formation of the nodule primordium. This process is initiated earlier than infection, which generally occurs through curled root hairs followed by the production of infection threads which route the bacteria towards the developing primordium. Infection of the primordium leads to formation of a meristem from which the nodule develops.

Application of purified Nod Factors or inoculation with rhizobia leads to (electro)physiological, morphological and gene expression changes in the plant cells. Using ion-specific micro-electrodes, Felle et al. 1996 observed a rapid (within 1 minute) Nod-factor-induced Ca^{2+} influx followed by the efflux of Cl^{-}, then K^{+} which result in membrane depolarization and an alkalinization of the cytoplasm. Oscillations in cytosolic Ca^{2+} (Ca^{2+} spiking) have been observed with a lag of approximately 10 minutes in legume root-hair cells following the addition of Nod factor or rhizobia (Ehrhard et al. 1996). The major morphological responses of host plants to Nod signals are deformation (in the presence of rhizobia, curling) of root hairs, development of preinfection threads, and division of cortical cells in front of the xylem poles. These morphological changes are accompanied by the induction of genes expressed early in nodule development (early nodulin or *ENOD* genes).

Further nodule morphogenesis requires the rhizobial infection through root hairs which are strongly deformed and curled. Within curls, the hair wall is weakened and the plasmalemma invaginates. These processes mainly involve the plant lytic enzymes and biogenesis of intracellular membranes (ER and Golgi vesicles) ensuring active uptake of bacteria by the host (Gualtieri and Bisseling, 2000). The bacteria are encapsulated in a special tunnel, the infection thread (IT) which grows within the root hair towards and then within the developing nodule tissue. The walls of ITs are built up from the material of plant cells, while the internal space contains a matrix synthesized by both partners. Although ITs develop either between or within the plant cells, rhizobia inside the IT always have a topologically intercellular location. Therefore, ITs should be considered as intercellular symbiotic compartments (Brewin, 1998). The initiation and development of ITs are regulated by the presence of bacteria as well as factors produced by the microsymbiont. Bacteria that are unable to adapt to osmotic or ionic or pH changes because of a defect in cyclic β-glucan or K+/H+ antiporter (Putnoky et al. 1998) production or defective in EPS/LPS synthesis usually do not infect the plant roots but do elicit the formation of non-infected "pseudonodules" which resemble the normal nodules in tissue structure (Becker and Puhler, 1998; Kannenberg et al., 1998).

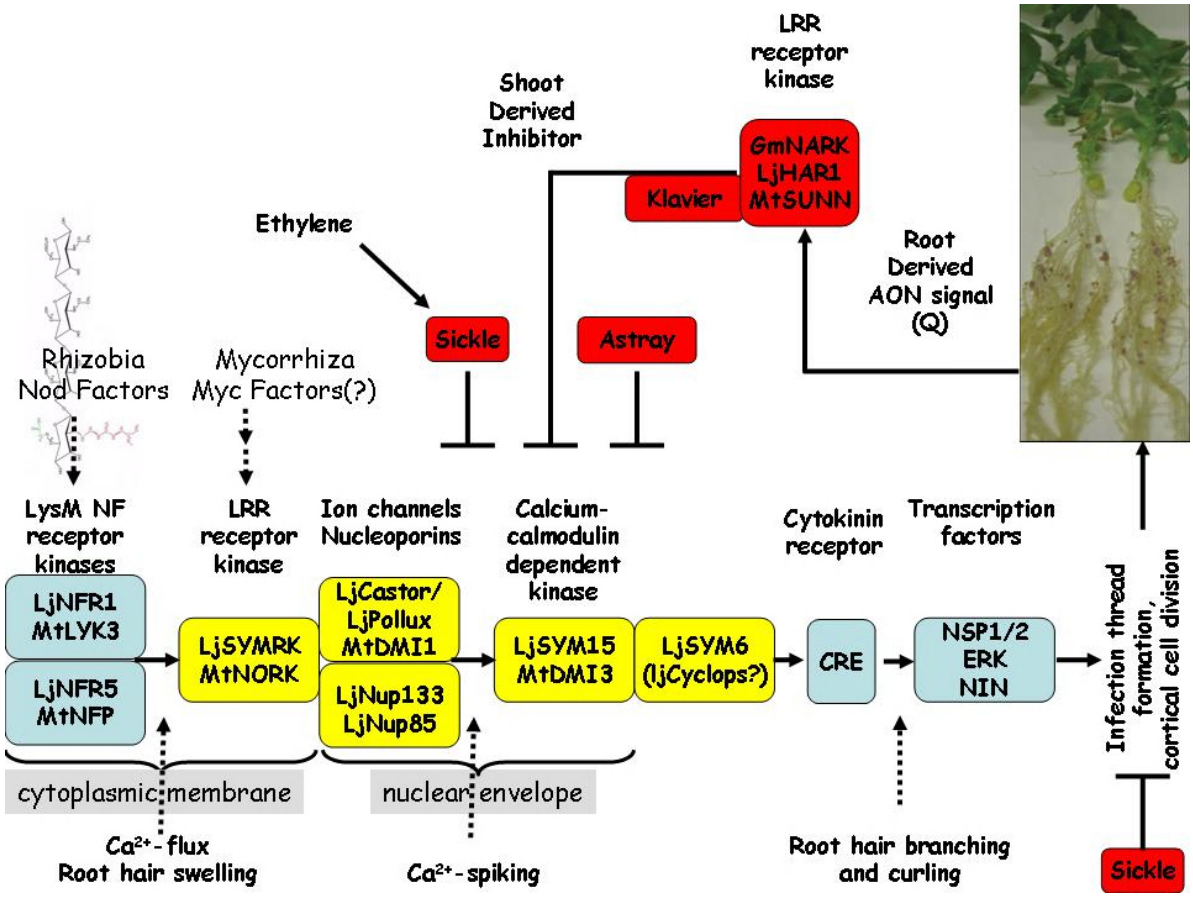

Figure 1. The steps of nodule morphogenesis, the changes determined by the presence of the bacteria at the cytoplasmic membrane and at the nuclear envelope of the plant cells. The plant genes implicated in the process of the nodule formation. (Figure modified from Kinkema et al., 2006)

A key stage of endosymbiosis is represented by the bacterial "release" from infection droplets to the plant cytoplasm via an endocytosis-like process. Infection droplets are unwalled regions of the ITs (they usually arise at the growing tips of ITs) at which the rhizobial cells come into close contact with the plant cell plasma membrane. Inside the plant cytoplasm, the bacteria are surrounded by special peribacteroid membranes (PBM) that initiates from the plasmalemma of infection droplets and then are formed mainly from ER and Golgi vesicles. The bacterial cell(s) surrounded by PBM represents a major intracellular

symbiotic compartment, the symbiosome (Roth and Stacey, 1989). Within symbiosomes the bacteria differentiate into nitrogen-fixing bacteroids which are several times larger than free-living bacteria and have an altered shape (e.g. Y-like).

1.1.2. Genes involved in the development and functioning of the symbiosis

To identify genes involved in the development two basic approaches have been used: (1) biochemical isolation, when RNAs/proteins synthetized specifically in nodules are identified, and (2) genetic analysis, when mutants defective in the establishment, maintainance and regulation of the nitrogen-fixing symbiosis are used to pinpoint genes required for the process.

1.1.2.1 Identification of nodule-specific genes by biochemical methods

The legume symbiotic genes identifed using their molecular products are called nodulin genes if their activity is expressed *de novo* or Nst (Nodule stimulated) genes if their activity is enhanced greatly in nodules compared to uninoculated roots (Franssen et al., 1992). Usually, the term "nodulin" represents an operational definition designed to identify nodule up-regulated gene products (they can comprise more than a half of the total protein pool in nodules). However, in many cases, *nodulin* genes are also expressed in nonsymbiotic tissues, and homologues of these genes may exist in non-legumes (Hirsch AM, 1997). This suggests that symbiosis-specific functions have been recruited from genes of general function.

To identify nodulin genes, spectra of proteins (RNAs) were compared via different methods either for nodules and uninoculated roots or for different time-points after inoculation/Nod factor application or for Fix^+ and Fix^- nodules. This approach provided a

general differentiation of nodulin genes into early (activated before the induction of N_2 fixation) and late (activated during or after N_2 fixation onset). Pre-genomics studies to isolate nodulin genes were based on the differential screening of nodule- and root specific RNA samples or cDNA libraries using methods like differential hybridization, differential display reverse transcription (DDRT-) PCR or cDNA-AFLP. Structural information – being obtained via sequencing high number of genomic and cDNA clones – on the (expressed) genome of several legumes has established a base for new approaches to study gene expression patterns in these plants. Microarray technology allows rapid gene expression analysis on a whole genome scale. Thousands of DNA fragments can be spotted at high density on a solid substrate (such as a glass microscope slide) and analysed simultaneously in a single experiment. Gene expression profiles can then be determined over a range of experimental conditions and organised into patterns that reflect the state of the tissue, or the plant as a whole.

Studies of nodulin genes have shown that they can be used as markers both of early stages of nodulation and of different developmental stages in the nodule (see Albrecht et al, 1999). For many nodule-specific proteins, the subcellular location (cytoplasm, PBM, IT wall) or enzymatic activity was determined. Some nodulins is believed to be involved in the formation of the symbiotic structures, e.g. early nodulin ENOD2 is actively synthesized in the nodule parenchyma, while proline-rich proteins ENOD5, ENOD10, ENOD11, ENOD12, PRP4 (Hirsch and LaRue, 1997; Munoz et al,. 1996; Mylona et al, 1995), extensins (2), glycine-rich proteins or GRPs (Kevei, 2002), and the Rip1 peroxidase (Cook et al., 1995, Peng et al. 1996) are accumulated in or modifying the IT and cell walls (Mylona et al., 1995). Nodulin ENOD40 is thought to be involved in balancing the hormonal status of the developing nodule. This factor may control the auxin/cytokinin ratio that is altered greatly after inoculation and may play an important role in nodule histogenesis (Hirsch and LaRue, 1997;

Mathesius et al., 1998). Nodulin N-26 synthesized during endocytosis is a component of PBM which may be required for the transport of signals or nutrients between partners.

Although sequence information and global gene expression analyses enable the researchers to speculate about gene function, definitive allocation of function requires the introduction of genetic mutations and analyses of their phenotypic repercussions. To achieve this goal a number of reverse genetics tools has been developed which are used also in legumes to study symbiotic nitrogen fixation.

One group of approaches is based on the identification of individuals in a mutagenized (by chemicals like EMS, by ionizing radiation like fast neutrons, γ- or X-rays, or by T-DNA or (retro)transposon insertions) population carrying mutations in the gene of interest. Targeting-induced local lesions in genomes (TILLING) is a PCR-based strategy that can identify single nucleotide changes in a known sequence in ethyl methane sulfonate (EMS)-mutagenized populations (Henikoff and Comai, 2003). This technique relies on the formation of heteroduplexes between PCR products of the wild-type and mutated fragments, and mismatch cleavage by the endonuclease CEL I. TILLING generates a series of alleles and enables us to carry out targeted studies on particular protein domains. It can also be rapid once the set up is in place. Ionizing radiation is another effective mutagen but, unlike EMS, it causes DNA deletions and other chromosomal rearrangements (Li and Zhang, 2002). In a typical fast neutron bombardment, deletions ranging in size from a few base pairs to more than 30 kb can be obtained, thus, fast neutrons or γ-rays readily generate knockout mutants. Fast neutron or γ-ray mutated lines are excellent for high-throughput reverse genetic screens. Such screens require optimization of PCR conditions to amplify the deleted sequence preferentially in the presence of >1000-fold excess of the wild-type sequence in DNA preparations from pooled samples. Current high-throughput PCR methods, however, might not readily detect deletions smaller than 500 base pairs. Insertional mutagenesis is a tractable genetic system in which a

DNA sequence (T-DNA, transposon or retrotransposon) is used to mutate and tag the genome, with the convenience of looking for the mutated site using the tag as an identifier. All insertional mutagenesis approaches rely on genome size and gene size. The larger the genome, the more insertions are required for saturation; the smaller the gene, the lower the chance that it will be a target site for the mutagen. Reverse genetic screening of DNA pools can also be done rapidly and efficiently using a combination of mutagen-specific and gene-specific primers.

Another approach is based on the down-regulation of gene expression. RNA-induced gene silencing, commonly called RNA interference in animals and post-transcriptional gene silencing (PTGS) in plants, is a powerful reverse genetics tool based on small double-stranded RNAs (dsRNAs) that guide sequence-specific mRNA degradation (Waterhouse and Helliwell, 2003). This technique requires that constructs are made for each gene of interest and introduced into the plant by transformation. RNA-induced gene silencing usually results in a range of variable phenotypes from wild-type to knockouts, which necessitates the analysis of sufficiently large numbers of transformants for each gene being silenced.

Despite the availability of the methods the function of very few legume genes identified with a biochemical method was studied in the course of symbiotic interaction. The most abundant late nodulin, leghemoglobin (Lb) accumulates to millimolar concentrations in the cytoplasm of infected plant cells prior to nitrogen fixation and are thought to buffer free oxygen in the nanomolar range, avoiding inactivation of oxygen-labile nitrogenase while maintaining high oxygen flux for respiration (Appleby, 1984). To test this hypothesis, Ott et al (2005) abolished symbiotic leghemoglobin synthesis in nodules of the model legume *Lotus japonicus* using RNA interference (RNAi). This caused an increase in nodule free oxygen, a decrease in the ATP/ADP ratio, loss of bacterial nitrogenase protein, and absence of symbiotic nitrogen fixation. However, RNAi plants grew normally when fertilized with mineral nitrogen.

These data indicated roles for leghemoglobins in oxygen transport and buffering and proved for the first time that a nodulin family, i.e. leghemoglobins were crucial for symbiotic nitrogen fixation. In contrast, genes like *ENOD12* may be functionally substituted by other genes because the absence of the *ENOD12* genes in a *Medicago* line resulted in no visible defect in its nodulation capacity (Csanádi et al. 1994).

1.1.2.2. Identification of genes based on mutant phenotype

Until now the most effective way to identify genes that are indispensable for the development, functioning and regulation of the symbiotic interaction has been the classical or "forward" genetics approach. The classical or 'forward genetics' way of acquiring mutants with altered nodulation phenotypes involves mutagenesis of a large number of wild-type seeds/plants by treatment with chemical reagents, irradiation or by tagging with inserted foreign sequences (T-DNA, transposon/retrotransposon). The goal of forward genetics screens is to use mutants to identify all of the genes involved in this specific process. Through the detailed phenotypic analysis of these mutants and the characterization of the corresponding genes, it is possible to begin to outline the biochemical mechanisms that underlie this process.

The first step during the cloning of the mutated gene is the crossing of the mutant individual with a wild-type plant then the creation of an F2 population either to show that the insertion is linked to the mutation and thus causing the phenotype or to identify markers linked to the mutation and to determine the map-position of the mutated gene. After fine-mapping of the trait and identifying recombinations on both side of the mutation as close to the mutation as possible, clones have to be identified from a genomic library of large insert size with the help of closely linked markers and a contig of overlapping clones covering the mutation has to be built. Sequence analysis of the contig between the recombination points may identify one

or more candidate genes which is followed by the sequencing of the mutant allele(s) to identify which gene is affected. For the determination of the position of the T-DNA, transposon/retrotransposon insertions PCR based methods like thermal asymmetric interlaced (TAIL) PCR (Liu et al, 1995) or inverse PCR (Yephremov and Saedler, 2000) can be used. To prove that the mutation in a given gene was responsible for the mutant phenotype, the wild type sequence has to be introduced into the mutant individual to restore the phenotype.

Large-scale mutagenesis programs and phenotypic screens in legumes led to the identification of several nodulation mutants representing ~40 genetic loci both in pea and *Lotus japonicus* (Stougaard, 2001; Provorov et al, 2002). These mutants can be divided into five classes on the basis of their phenotypes: (1) Mutants that are non-nodulating (Nod^-) and are impaired in early rhizobial interaction, Nod-factor perception or downstream signaling. (2) Ineffective nodulating mutants (Fix^-) in which nodulation is arrested during nodule organogenesis or that are impaired in nodule function. (3) Mutants with increased nodule numbers (Nod^{++}). In these mutants, inactivation of the normal autoregulatory mechanism leads to an excess of root nodules. (4) Mutants with delayed nodulation or reduced nodule number. Reduced nodule numbers may be caused by a variety of mutations, including leaky mutations. (5) Mutants that form so-called spontaneous nodules in the absence of rhizobia.

Cloning of the genes responsible for the non-nodulating mutant phenotypes has revealed a molecular network that can be best described as a series of three distinct but linked processes. Initially, there is the perception of the rhizobia-derived signal(s) by membrane receptors, principally Nod factor perception by LysM type protein receptor kinases (Limpens *et al.* 2003; Madsen *et al*. 2003; Radutoiu *et al.* 2003), followed by a LRR-receptor kinase called NORK or SYMRK (Endre *et al.* 2002; Stracke *et al.* 2002). Next, the reception of the rhizobia-derived signal is processed via the action of channels in the nuclear, cytoplasmic and plastid membranes (Ané *et al*. 2004; Imaizumi-Anraku. *et al* 2005; Kanamori *et al.* 2006),

which is seen most readily by depolarisation of the host cell membrane and through an initial spiking and subsequent fluctuations in cytosolic Ca^{2+} concentration (Wais *et al*. 2000; Oldroyd 2001) sensed by a calcium-calmodulin dependent protein kinase (CcaMK: Lévy *et al*. 2004; Mitra *et al*. 2004a). Interestingly, generating constitutively active forms of this CcaMK by either removing its autoinhibitory domain or changing an autophosphorilation site lead to the formation of spontaneous nodules on the roots of *Medicago truncatula* and *Lotus japonicus* (Gleason et al, 2006; Tirichine et al, 2006). Finally, there is execution of the response to rhizobial infection, primarily through the action of transcription factors, which are proposed to activate as yet uncharacterized target genes (Schauser *et al*. 1999; Borisov *et al*. 2003; Kalo *et al*. 2005; Smit *et al*. 2005).

Several mutants have been identified that develop nodules with metabolic defects that cannot fix nitrogen efficiently and exhibit retarded growth under symbiotic conditions but only one gene affected by a mutation has been identified with the help of map-based cloning. The *sym13/sym81* mutants of the model legume *L. japonicus* carry mutation in a gene which is expressed in a nodule-specific manner and encodes a protein homologous with eukaryotic sulfate transporters (Krussel et al, 2005). Full-length cDNA of the gene complemented a yeast mutant defective in sulfate transport. Hence, the gene was named *Sst1* (for Symbiotic Sulfate Transporter 1).

It was also shown by multiple groups that the regulation of nodule number required the activity of a putative transmembrane, leucine-rich repeat (LRR) receptor-like kinase in soybean (*GmNARK*, *G. max* Nodule Autoregulation Receptor Kinase; Searle *et al.* 2003), *L. japonicus* (*HAR1*, Hypernodulation and Aberrant Root; Krusell *et al.* 2002; Nishimura *et al.* 2002*a*), pea (*SYM29*, SYMbiosis; Krusell *et al.* 2002), and *M. truncatula* (*SUNN*, SUper Numeric Nodules; Schnabel *et al*. 2005).

1.2. Transformation of legumes

The importance of nodulation and nitrogen fixation to agriculture, natural ecosystems and the global nitrogen cycle are indisputable (Graham and Vance 2003). Legumes - domesticated for the production of food, feed, forage, fiber, industrial and medicinal compounds, flowers, and other end uses - are second only in importance to the *Graminiae* with respect to agricultural production and human and animal consumption. They are cultivated on 12 to 15% of available arable land and constitute more than 25% of the world's primary crop production. They reduce and provide roughly 200 million tons of nitrogen per year. Since 30 – 50 % of applied chemical nitrogen fertilizers (containing 16-35% nitrogen) on agricultural land is lost to run-off or leaching (consequently causing environmental problems) legumes replace approximately 800 million tons of chemicals worth of 200 billion US $. Thus, understanding the molecular basis of nitrogen fixation and the unique metabolic pathways that result in the myriad of end uses of legumes is both a matter of scientific curiosity and of economic necessity because of their importance in the biosphere and to the sustainability of the human race. In accordance, model legumes are being rapidly developed as experimental systems to pursue a number of important biological questions unique to these plants using molecular tools including genomics. A key component of most functional genomics approaches is a high-throughput transformation system useful for developing various gene identification strategies mentioned before. In the past decades, considerable success has been achieved in transformation of forage and pasture, grain and pulse, and tree legumes (Atkins and Smith, 1997; Babaoglu et al., 2000; Somers et al., 2003) facilitating studies in different species. Transformation also is emerging as an important crop improvement tool. This is particularly evident in soybean (*Glycine max*), in which Roundup Ready soybean cultivars have captured a major stake in market share of soybeans planted in the U.S. and Argentina. Transformation

theoretically expands the sources of genes for plant improvement to all organisms, far beyond the gene pool accessible via sexual hybridization.

In a scientific point of view, transgenic organisms play an important role in functional studies of plant genes and can be used in different experiments:

The spatial and temporal regulation of the genes can be followed by promoter-reporter gene fusions in different backgrounds or developmental stages, under different environmental conditions (Wood, 1995).

To test whether a gene is required for or associated with a given biological process, its expression level can be modified by overexpression or silencing (Vaucheret et al., 2001). Thus, introducing new genes or manipulating endogenous gene expression via transformation generates new phenotypic variation useful for investigating gene function and for crop improvement.

Transgenic organisms also have important role in genetic approaches that are based on mutant phenotype (map-based cloning, Tanksley, 1995; insertion mutagenesis, Azpiroz-Leehan and Feldman, 1997), where the mutants can be produced by the introduction of a foreign DNA and/or the role of the isolated gene can be proven by functional complementation. In the latter case the wild type allele of the identified gene is transformed into the mutant plant and the restoration of the normal function is tested in the transgenic plants.

1.2.1. Plant transformation methods

There are different methods to introduce foreign genes into plants in order to obtain transgenic individuals: e.g. protoplasts can be forced to take up DNA with the help of high concentrations of polyethylene glycol (Zhang et al., 1988), DNA can be injected into the nuclei of embryogenic single cells (Neuhaus et al., 1987) or can be shot into the plant tissue when coated onto small particles of heavy metals (Klein et al., 1987), but the most commonly used methods (also for legumes) are the *Agrobacterium* mediated plant transformation systems (Hooykaas, 1989).

1.2.1.1. Agrobacteria as tools for engineering plant genomes

The plant pathogenic agent of crown gall tumor, *Agrobacterium tumefaciens* and its close relative, the hairy root agent *Agrobacterium rhizogenes*, cause hyperplasia on plant hosts. This can be considered a true disease syndrome, as it is certainly debilitating to the plant, but it is not lethal.

Years before scientists elucidated the molecular mechanism of *Agrobacterium*-mediated transformation of plants (shown in Figure 2), Armin Braun (1947) proposed the concept of a "tumor-inducing principle" that was stably transferred to and propagated in the plant genome. He was also the first who demonstrated that cells from the crown gall tumors are transformed, they can be freed from *Agrobacteria* and grown *in vitro* without the supplemental auxin and cytokinin required by normal plant cells (Braun, 1958). Research in the 1970s resulted in the identification of large plasmids in virulent Agrobacterium strains (Zaenen et al. 1974), although we now know that many strains contain plasmids unrelated to virulence. Genetic experiments indicated that a particular class of plasmids, the Ti (for Tumor induction) and later Ri (for hairy Root induction) plasmids, were responsible for tumor genesis (Van Larebeke et al, 1974). It was discovered that large amounts of new metabolites -

octopine and nopaline - were present in cultured crown gall tumor cells that were free from agrobacteria (Petit et al., 1970). They also showed that the *Agrobacterium* strain, not the plant, determines the opine made by the tumor. Ti (and Ri) plasmids are classified according to the opines, which are produced and excreted by the tumors (roots) they induce. An important milestone was the discovery that a mobile portion of these Ti (and Ri) plasmids, the T-DNA, was transferred to plant cells and incorporated into the plant genome (Chilton et al. 1977). T-DNA was shown to contain the genes for inducing tumor formation and opine biosynthesis, and it was revealed that these genes, even though they are bacterial in origin, have evolved to function only in plant cells. The T-DNA fragment is flanked by 25-bp direct repeats, which act as a *cis* element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (vir genes) and in the bacterial chromosome. The Ti plasmid also contains the genes for opine catabolism produced by the crown gall cells, and regions for conjugative transfer and for its own integrity and stability. It was also shown that the removal of all the genes within the T-DNA does not impede the ability of *Agrobacterium* to transfer this DNA but does prevent the formation of tumors (De Framond et al 1983, Hoekema et al, 1983). Moreover, any foreign DNA placed between the T-DNA borders can be transferred to plant cells, no matter where it comes from.

It was thus obvious to propose that Ti plasmids be used as a vector to introduce foreign genes into plant cells. However, Ti plasmids are very large (2-800 kbp) and T-DNA regions do not generally contain unique restriction endonuclease sites not found elsewhere on the Ti plasmid. Therefore, one cannot simply clone a gene of interest into the T-region. Scientists therefore developed a number of strategies to introduce foreign genes into the T-DNA. These strategies involved two different approaches: cloning the gene, by indirect means, into the Ti plasmid such that the new gene was in *cis* with the virulence genes on the

same plasmid, or cloning the gene into a T-region that was on a separate replicon from the *vir* genes (T-DNA binary vectors). Because of the complexity of introducing foreign genes directly into the T-region of a Ti plasmid, several laboratories developed an alternative strategy to use Agrobacterium to deliver foreign genes to plants. This strategy was based on seminal findings of Hoekema et al. (1983) and de Frammond et al. (1983) mentioned above: These authors determined that the T-region and the *vir* genes could be separated into two different replicons. When these replicons were within the same *Agrobacterium* cell, products of the *vir* genes could act in *trans* on the T-region to effect T-DNA processing and transfer to a plant cell. Hoekema et al. (1983) called this a binary-vector system; the replicon harboring the T-region constituted the binary vector, whereas the replicon containing the *vir* genes became known as the *vir* helper. The *vir* helper plasmid generally contained a complete or partial deletion of the T-region, rendering strains containing this plasmid unable to incite tumors. These Ti plasmids and their host *Agrobacterium* strains that are no longer oncogenic are termed 'disarmed'. The binary plasmids are small and easy to manipulate in both *E. coli* and *Agrobacterium* and generally contain multiple unique restriction endonuclease sites within the T-region into which genes of interest could be cloned. Many vectors were designed for specialized purposes, containing different plant selectable markers, promoters, and poly(A) addition signals between which genes of interest could be inserted, translational enhancers to boost the expression of transgenes, and protein-targeting signals to direct the transgene-encoded protein to particular locations within the plant cell.

With the help of *A. rhizogenes*, the causative agent of hairy root disease, composite plants with transgenic roots can be obtained. Mutant phenotypes determined by the root genotype can be rescued by this transformation system while the complementation of the shoot-determined defects is possible only if regeneration of whole plants from the hairy root can be achieved. In contrast, the *A. tumefaciens* mediated transformation system results in the

generation plant lines with all tissues being transgenic. The drawback of the method is that it requires somatic embryo formation and regeneration capability, therefore its application is restricted to certain species or certain genotypes/ecotypes of a given species. For example, in tetraploid alfalfa (*M. sativa*), only few genotypes have the ability of somatic embryogenesis, and genetic studies suggested this trait to be under the control of dominant alleles of at least two genes that are usually in simplex/simplex (Aaaa/Bbbb) configuration in the embryogenic lines tested (Wan et al. 1988; Hernandez-Fernandez et al. 1989; Kielly and Bowly 1992; Crea et al. 1995). Using either of the *Agrobacterium* mediated transformation an important criterion is that the T-DNA encoded functions should not interfere with the mutant phenotype or the wild type function.

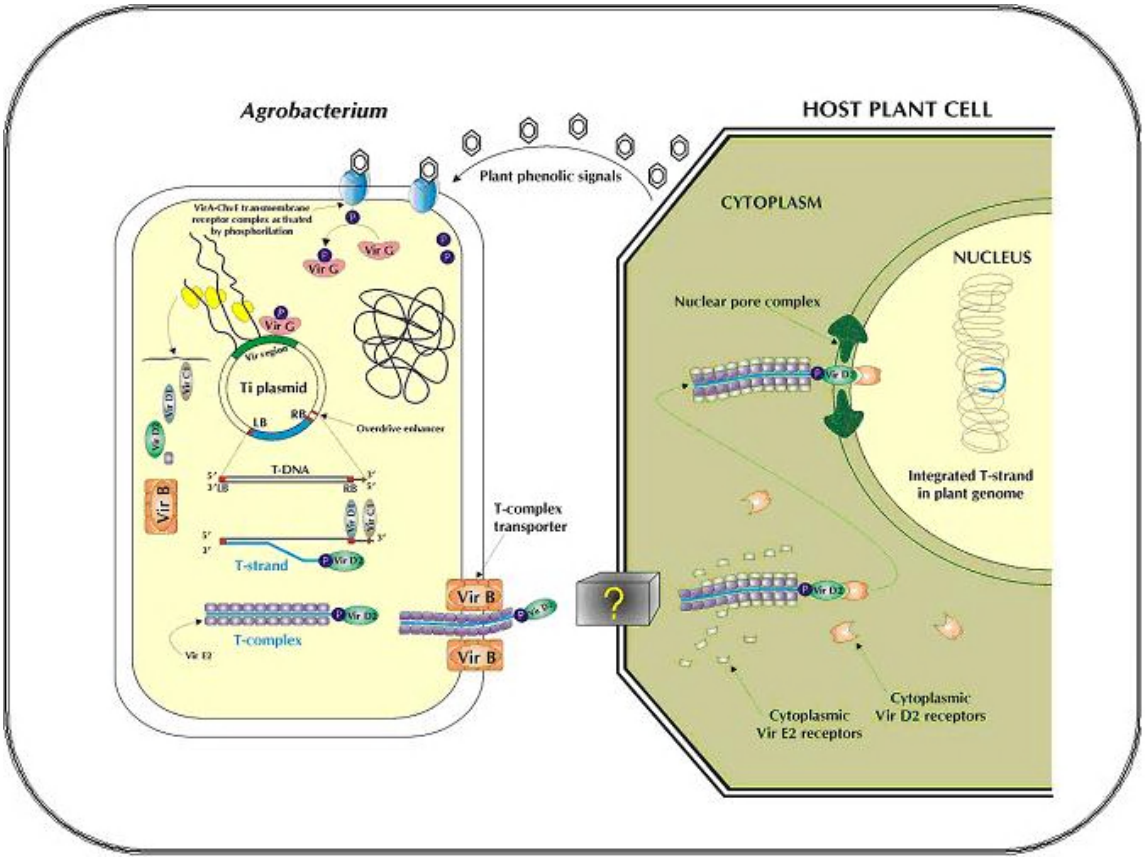

Figure 2. The mechanism of DNA transfer from *Agrobacterium* to the plant cell. Picture from research.cip.cgiar.org

1.2.2. Reporter genes

The central concept of a reporter gene is simple: it is a defined nucleotide sequence, which when introduced into a biological system, yields a readily measurable phenotype upon expression. This provides a convenient parameter that is correlated to the molecular events associated with genetic expression. The most widely accepted reporter genes encode chloramphenicol acetyltransferase (CAT), β-galactosidase, firefly luciferase, and β-glucuronidase (GUS), the latter two have been extensively used in gene-expression studies in plants. Luciferin-luciferase imaging has been used, for example, to isolate and characterize circadian-clock mutants, phenotypes, or genes regulated by (Millar et al., 1992; 1995; Hall et al., 2002; Kevei et al., 2006), or tagging stress responsive genes (Alvarado et al, 2004) in *Arabidopsis*. Substrates for *in vivo* labeling are available, but they suffer from a high non-specific background. Furthermore, low levels of light emission require the use sensitive photon counting cameras, and the overall signal strength is not sufficient for high resolution.

1.2.2.1.The GUS reporter gene

The *Escherichia coli uidA* gene encoding β-glucuronidase (GUS) is widely used as a reporter gene in plant transformation studies (Jefferson, 1997) because the gene expression patterns can be quantified by fluorometric and spectrophotometric analysis. Additional advantages of the GUS assay are that it is very straightforward and requires no expensive equipment. The major disadvantage of the GUS assay is that the chemicals necessary for this assay are expensive. Also the GUS assay is lethal for the plant tissues. With these limitations, however, the GUS assay is still one of the most effective reporter gene systems used by the scientists in plant gene expression studies.

The key advantage of GUS is the absence of GUS activity in many organisms other than vertebrates and their attendant microflora. Lower and higher plants and most bacteria, fungi

and many insects that exist in the phylo and rhizosphere are largely, if not completely lacking in GUS activity (Jefferson, 1987). Minute quantities of GUS activity can therefore be accurately measured, even in single cells (Harkins et al., 1990) when *uidA* is used as a reporter gene in these systems.

Since the development of the GUS system, many thousands of transgenic plants have been generated expressing GUS. In such plants, the spatial distribution of gene activity can be visualized in the absence of any background signal.

This has been instrumental in the development of new metohods of plant transformation such as particle bombardment (Klein et al.1988, Christou et al 1989) and in the succesful transformation of many crop plants including soybean and monocots such as maize (Songstaad et al, 1996, Toriyama et al, 1988). The power and simplicity of GUS histochemical methods is also demonstrated in assays for transposon excision (Masson et al.1989), lineage analysis (Klein et al, 1988, Finnegan et al,1989), developmental analyses (Bevan et al,1989) and discrimination between family members (Forde et al,1989).

GUS fusions are now widely used to study plant-pathogen and plant-symbiont interactions. They can be used both to study expression of particular genes (in different genetic backgrounds, developmental states etc.) and to mark and monitor populations of microorganisms in soil or in association with plants.

2. Background and aim of the present work

The tetraploid non-nodulating (Nod$^-$) alfalfa (*Medicago sativa*) mutant MnNC-1008(NN) (Peterson and Barnes 1981; Barnes et al. 1988) identified in the progeny from crosses between different cultivars and referred here as MN-1008, was among the first symbiotic mutants reported (Caetano-Anolles and Gresshoff, 1991). Studies on MN-1008 revealed the lack of the early physiological and morphological changes like membrane depolarization (Felle et al. 1996), calcium spiking (Ehrhardt et al. 1996), root hair deformation and curling, cortical cell division (Dudley and Long 1989; Endre et al. 1996) that are characteristic responses to rhizobia and Nod factors. In addition, the mutant also fails to establish the other symbiotic interaction, the vesicular-arbuscular mycorrhizal symbiosis (Bradbury et al. 1991). To identify the gene required for the development of these symbioses a map-based cloning strategy was conducted (Endre et al. 2002a; 2002b). In this way, we have shown that in the MN-1008 line, a gene coding for a receptor-type protein kinase designated as NORK (Nodulation Receptor Kinase) carried a non-sense mutation resulting in translational termination in the kinase domain (Endre et al. 2002b). Complementation of the MN-1008 mutant with the wild type *NORK* could not be accomplished by the rapid *A. rhizogenes* mediated transformation since symbiotic nodules are not formed on the hairy roots induced on alfalfa (Beach and Gresshoff 1988). Furthermore, the traditional transformation method using *A. tumefaciens* was also not feasible for MN-1008 being a non-embryogenic alfalfa line. Therefore, the orthologous *Medicago truncatula dmi2* mutant was used in complementation experiment at first to confirm that the *NORK* gene is indispensable for the development of the symbiotic interactions (Endre et al. 2002b).

The second part of the work was performed in order to find out what is the function of *NORK*. Different approaches can be followed for this purpose, like fusions between reporter genes and the gene of interest. GUS reporter gene was succesflully used in early nodulin genes

including *ENOD12* and *ENOD40*. That is why we built a construct between GUS reporter gene and *NORK* and we introduced it in *M. truncatula* Jemalong using *Agrobacterium rhizogenes*. We chose *Agrobacterium rhizogenes* plant transformation because it is quite a rapid method resulting only in transgenic roots. This was not a problem in the case of *NORK* due to the fact that in the case of *DMI* genes, the phenotype is controlled in all the cases by the roots.

Following this route, we could localize the *NORK* expression in the root tissue of *M. truncatula*.

3. Materials and methods

3.1. Plant material, nodulation assay and growth conditions

The non-nodulating tetraploid alfalfa line designated as MnNc-1008 (NN) (Peterson and Barnes, 1981; Barnes et al., 1988) is referred as MN-1008 throughout the paper.
M. sativa Regen S (Brown and Atanassov, 1985) was used as highly embryogenic line. Maintenance of the plants, crossings and the determination of the nodulation ability were carried out as described earlier (Endre et al., 2002a).

3.2. Bacterial strains and plasmids

An 8.5 kb *Nhe*I fragment carrying the *M. truncatula NORK* gene (Endre et al., 2002b) was cloned into the plant transformation vector pCAMBIA 2201 (AF234314) resulting in the clone pBRC1667.
The same 8.5 kb *Nhe*I fragment of the *M. truncatula* NORK gene was cloned into the modified pPR97 (Szabados et al., 1995) plant transformation vector carrying a constitutively expressed *uid* A gene (clone pBRC 1720). *A. rhizogenes* carrying the pBRC 1720 clone or empty pPR97 vector (negative control) were used to transform seedlings of the *M. truncatula* TR 25 and R 38 Nod $^-$ mutants.
For the plant transformation *A. tumefaciens* strain LBA 4404 (Hoekema et al., 1983) carrying clone pBRC1667 were used.
A *Bam*HI fragment of 3.1 kb upstream the ATG start codon of the *NORK* gene were cloned into pPR97 binary vector resulting in the clone pBRC1772. For the transformation A. rhizogenes ARqua carrying clone pBRC1772 was used.

3.3. Embryogenic test

To test the embryogenic capacity of the *M. sativa* lines MN-1008 and Regen S, young trifoliate leaves were sterilized with 0.1% $HgCl_2$ + 0.1% Tween, followed by five rinses in sterile distilled water. To initiate callus formation the sterilized explants were wounded by scalpel and placed onto solidified B5H medium (Brown and Atanassov, 1985) containing

auxin (2.4D) in different concentrations (0.5 mg/l, 1 mg/l, 1.5 mg/l and 2 mg/l) and a constant level (0.2 mg/l) of citokinin (BAP). After a 3 week culture period in a growth cabinet at 24°C, 16h light/ 8h dark, calli were transferred to B5H medium without hormones. The calli were subcultured every three weeks on the same medium for six months to observe embryo formation. The embryogenic capacity of the F1 hybrids and the Nod^- F2 plants was tested similarly using B5H medium containing 0.2 mg/l BAP and 1.5 mg/l 2.4D on which the highest number of embryos (25 per explant) was formed for *M. sativa* Regen S.

3.4. *Agrobacterium tumefaciens* mediated plant transformation

For the transformation of alfalfa variation of the explant method of Horsch et al. (1985) was used.

Edges of sterilized leaves were cut on moist filter paper and the tissue were dropped into liquid B5H medium containing *A. tumefaciens* with cell density adjusted to 0.6-0.8 at OD_{600}. After 30 minutes inoculation, the explants were gently blotted on filter paper and placed onto B5H medium for three days. After rinsing twice in sterile distilled water they were cultured on B5H medium containing hormones, 50 mg/l kanamycin and 200 mg/l carbenicillin. Plates were maintained at 24° C, with 16 h photoperiod and light intensity of 60-80 μE $m^{-2}s^{-1}$. Calli which formed within 3 weeks were moved to B5H with antibiotics but without hormones to allow embryo production and development. After 3-4 weeks, embryos were transferred to MMS medium (Murashige & Skoog salts (Murashige and Skoog, 1962), Nitsch & Nitsch vitamin stock (Nitsch and Nitsch, 1969), 0.1 mg/l myo-inositol and 30 g/l sucrose) containing the antibiotics for plant regeneration. The embryos developed into plants within 12-14 weeks from the start of the experiment. The regenerated plantlets were moved first into perlit and then into soil.

3.5. *Agrobacterium rhizogenes* plant transformation

M. truncatula seeds were surface sterilized and germinated on inverted agar plates at 14°C. The *A. rhizogenes* strain ARqua1 (Quandt et al. 1993) containing the binary vector of interest was streaked on agar plate of LB medium with appropriate antibiotics and grown for approximately 48h.

After 30 hours germination, the radicle of the seedlings was cut approximately 3 mm from the root tip. After removing the radicle tip, the sectioned surface of the seedling was scraped on the surface of the *A. rhizogenes* plate. The seedling were then placed on a Petri dish containing agar with TM-1 medium and kanamycin 25 mg/l. The Petri dishes were placed in a 20 ^{o}C growth room. The first co-transformed roots (i.e. those having integrated both the Ri T-DNA and the binary vector T-DNA) should begin to appear approximately 7 days after inoculation.
2-3 weeks after inoculation, the transformed roots were sufficiently well developed for experimental studies.

3.6. DNA isolation and hybridization

Total DNA was isolated from young leaves according to Kiss et al. (1993). Aliquots (15 µg) of total DNA were digested with the restriction enzymes *Dra*I, *Eco*RI, *Eco*RV and *Alu*I (Amersham or Fermentas) according to the suppliers' instructions.
The DNA fragments were separated in 1.1% agarose gel and transferred by the capillary method (Southern, 1975) to nylon membranes (Hybond-N+, Amersham), in accordance with the supplier's protocol. For probe preparation, PCR-amplified DNA fragments were isolated from agarose gels using the QIAEX Gel Extraction Kit (Qiagen), and were labelled with [α-32P]dCTP by random priming (Feinberg and Vogelstein, 1983). Hybridization experiments were performed at 55–60ºC, and the washes were carried out as described by Kiss et al. (1993).
Using *npt*II as probe to *Eco*RI digested DNA the number of hybridizing bands should indicate the number of integration events because an *Eco*RI site separates the T-DNA border and the *npt*II gene from the insert in the T-DNA construct.
Hybridization with a *NORK* specific probe to *Dra*I digested DNA identified two fragments of 3.9 and 3 kb. size representing the wild type and mutant *M. sativa* alleles, respectively, and two shorter fragments (1.8 and 0.8 kb.) specific for *M. truncatula* Jemalong.

3.7. DNA amplification

The PCR reactions were carried out as it follows: 30 sec at 92°C, 1 min at the annealing temperature (55°C) and 1 min at 72°C for 35 cycles, with a denaturation step at 94° for four minutes at the start and final extension step at 72°C for 5 min. The primer pairs used

for the PCR amplification of the hybridization probes as well as for the genotyping of the F2 individuals are shown in Table 1.

To genotype the F2 individuals a primer pair (PF_U1-PF_D1) designed for a gene in ~15 kilobase pair distance from the *NORK* gene in *M. truncatula* (Endre et al., 2002b) was used in PCR reactions. The amplification products originating from the mutant and wild type alleles could be distinguished by a length polymorphism of 1.5 and 1.2 kb, respectively.

3.8. Reverse transcription (RT-) PCR analysis

Total RNA was isolated from alfalfa roots and leaves by the High Pure RNA Isolation Kit (ROCHE). For RT-PCR, first strand of cDNAs were synthesized with MuLV reverse transcriptase (Fermentas), by treating 1 μg of total RNA, in the presence of RNase inhibitor and oligo-dT primers. The *NORK* transcripts were amplified in 35 cycles (94°C 30 sec, 55°C 45 sec and 72°C 1 min) using the primers NORK_U4 and NORK_D4B (Table 1). The nucleotide sequence difference between the cDNAs corresponding to the positions 740 and 741 in the *M. truncatula* databank entry (AJ418369) made possible to distinguish the expressed *NORK* sequences originated from the endogenous *M. sativa* gene and the *M. truncatula* transgene. The amplified products were digested with *Pvu*I enzyme (Fermentas), which recognizes the *M. truncatula* sequence.

Table 1. Primers used for PCR amplification

Name of the primer	Sequence
npt_U1	ACCCAGCCGGCCACAGTCG
npt_D1	GGGCGCCCGGTTCTTTTTG
NORK_U1	TACAGGGGCACTCTAGACGATGGT
NORK_D1	GGCCGATATGTTGAGTAGGGTTCT
PF_U1	TCAAAACATGCACTTCAACCT
PF_D1	TCACCCTTCTCCCCCAATG
NORK_U4	TTCCAGGCCTAAAGTCAAACACCA
NORK_D4B	CCATGTAGGTATTCTTCAGGTA

3.9. Histochemical localization of GUS activity

For histochemical assays, tissues were fixed in 90% cold acetone, rinsed with 100 mM pH: 7.2 Na-phosphate buffer and incubated overnight in the presence of 2 mM chromogenic substrate, *X-Gluc* (5-bromo-4-chloro-3-indolyl-β-D-glucuronide). They were fixed in fresh glutaraldehyde, dehydrated in ethanol. Histochemical fixation of GUS stained roots was performed as described by Beeckam and Ronald Viane (1999).

The tissues were infiltrated with a hydroxyethylmetacrylate based resin, Technovit 7100 embedding kit (Heraeus Kulzer, Wehrheim, Germany). The infiltration solution, a mixture of the basic resin with hardener I (dibenzoylperoxide), was prepared as instructed by the manufacturer. Polymerization was induced using a mixture of the basic resin with hardener II and the infiltrated specimens were placed into the histoform S and positioned.

Mounting was performed using Technovit 3040 poured in the histoblocks.

Sections of 8-13 μm thick were cut on a rotary microtome using glass knives. The sections were collected and placed on glass slides.

Sections were observed by bright-field microscopy for roots and nodules. Digital pictures were taken with Leica camera using Leica DC Viewer software.

Tissues were examined under light microscope. Morphological and cellular localisation of GUS expression were performed in microtome sectioned material and analysed in transmitted light.

4. Results

4.1 Complementation of *NORK* mutations

4.1.1. *Agrobacterium rhizogenes* mediated transformation

The *NORK* gene, which is indispensable for the development of rhizobial and mycorrhizal symbioses, has been identified by map-based cloning with the help of the Nod^- alfalfa mutant MN-1008. *A. rhizogenes*-mediated transformation system was used to show functional complementation of the *M. truncatula dmi2* mutants carrying mutation in the orthologous sequence by the wild type *NORK* gene (Endre, 2002b), since neither *A. rhizogenes* (hairy root transformation) nor *A. tumefaciens* (stable transformation) could be used to complement MN-1008:

168 Nod^- plants (MN-1008) formed roots after treatment with *A. rhizogenes* and only on four plants roots showed GUS expression, indicating transformation event. However, after rhizobia infection, no nodule formation could be observed on the transgenic roots. The roots of Nod^+ alfalfa plants (Regen S) transformed with the same *A. rhizogenes* strain also did not form nodules after rhizobia treatment (data not shown; Beach and Gresshoff, 1988).

The wild type sequence of the *NORK* gene was then introduced into *M. truncatula* TR25 with the help of *A. rhizogenes* in order to complement the *dmi2* mutation. In a typical experiment 20 plants were transformed. After their transfer to perlit, 10 plants survived and were analysed. 37 roots appeared after transformation on these 10 plants. Four roots on four different plants carried nodules. In order to prove that the appearance of the nodules was due to the *Agrobacterium* transformation process, the roots were stained for GUS activity since *uid* A gene was present in the T-DNA. Three plants were found to carry 15 nodules on the roots that in the same time were GUS positive, indicating that the transformation took place (Fig. 3). One root was GUS negative and still carrying one nodule. It may be due to the silencing of the reporter gene or the incomplete transfer of the hairy roots on TR25 plants carrying the T-DNA of the empty vector (PCAMBIA 2201) did not form nodules.

The experiments were repeated several times with similar results.

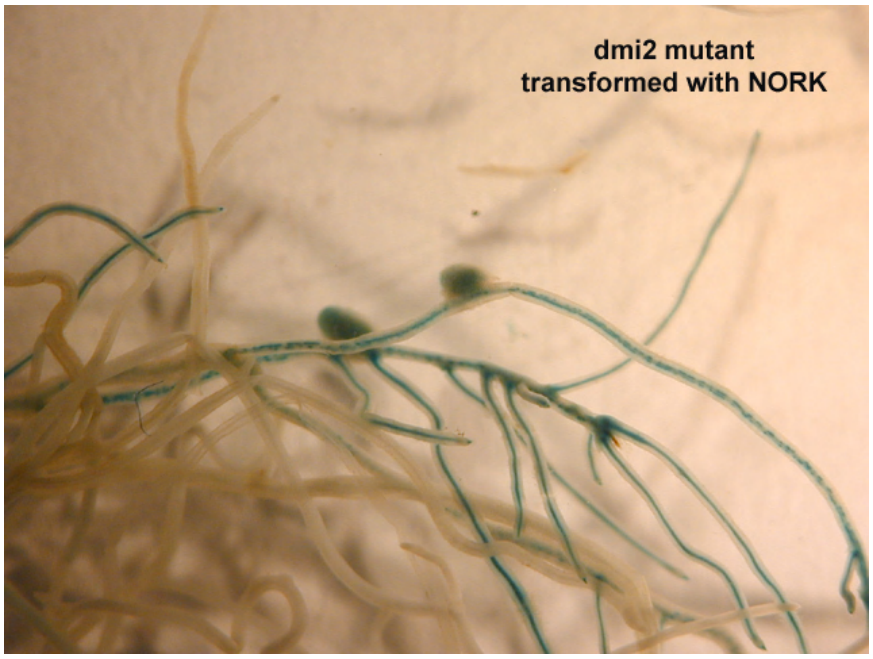

Figure 3. *Dmi2* mutant transformed with *NORK*

We could conclude that the nodulation phenotype was restored by using this construct.

4.1.2 Alternative strategies to complement the Nod- mutation

On the other hand, *A. tumefaciens* based complementation seemed to be unfeasible for MN-1008 being non-embryogenic. As it is shown in Table 2, there was callus formation on the MN-1008 explants, but no embryo development could be seen in a six months period, while on embryogenic alfalfa Regen S embryos appeared in about six weeks.

Nevertheless, for the complementation of the original Nod^- mutation of the MN-1008 plant new alternative strategies were looked for.

Three general strategies could be utilized to complement a mutation residing in a non-embryogenic plant genotype: the DNA construct to be used for complementation could be introduced into an embryogenic line followed by the crossing of the transgenic and the mutant plants or the mutant allele(s) could be transferred into an embryogenic line which is followed by the transformation experiment either in the hybrid F1 progeny or in selected F2 plants.

These three complementation approaches are illustrated in Fig. 4 with the designated names referring to the generation in which the transformation is performed (i.e. P, F1 and F2).

In the course of the first alternative way (Fig. 4a: Route P) the gene of interest is introduced into the embryogenic parent (P_{WE}). Transgenic regenerants are selected (P_{WET}) and then crossed with the non-embryogenic mutant line (P_M) to combine the mutation with the transgene in one plant. Transgenic F1 individuals are selected (F1T) and a segregating population is produced by self-pollinating or back-crossing these F1 plants with the mutant parent. As a final step, individuals (I_{MT}) homozygous for the mutant gene and also carrying the transgene are identified from this second generation.

Routes F1 and F2 are based on the findings that the embryogenic capacity in alfalfa is genetically determined by dominant alleles of two genes (Wan et al. 1988; Hernandez-Fernandez and Christie 1989; Kielly and Bowley 1992; Crea et al. 1995). Therefore, it is feasible to combine embryogenic capability with other traits via genetic crossing. Both approaches start with a cross between the nonembryogenic mutant (P_M) and the embryogenic parent (P_{WE}) followed by the selection of embryogenic plants from the F1 population that are also heterozygous for the mutation (F_{1E}). One possible step is to self-pollinate these F_{1E} hybrids and subsequently to identify embryogenic individuals that are also homozygous for the mutation (I_{ME}) in the F2 population (Fig. 4a: Route F2). The desired construct(s) can later be introduced into these plants using the *A. tumefaciens*-mediated transformation. In this case, since the embryogenic mutant plant is already available, several transformation experiments can be designed to test different gene constructs. If a candidate gene is available it is possible to transform the embryogenic F1 individual(s) (Fig. 4a: Route F1) with the foreign DNA. After selecting the transgenic hybrid plants (F_{1T}), further steps to get I_{MT} plants are just the same as followed in the Route P approach.

Individual	No. of explants	No. of explants producing callus	No. of calli producing embryos	Time required for embryo appearance (weeks)
MN-1008	20	14	0	NA
Regen S	12	9	7	6
RN/28	13	9	7	6
RN/41	12	8	6	6
RN/50	12	8	5	6

Table 2 Testing the parental and hybrid plants for their embryogenic ability

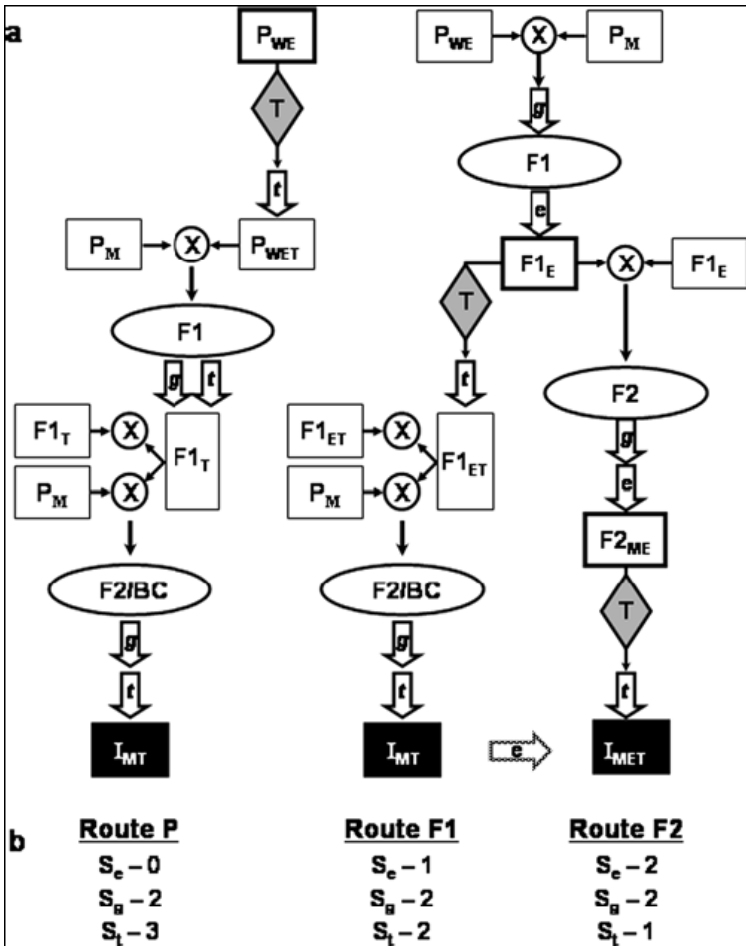

Fig. 4 a and **b** Summary of the complementation strategies for a mutation existing in a non-embryogenic genetic background. **A** Flowchart of the three possible routes. The different routes are named according to the generation in which the transformation experiment (*T in diamond highlighted in grey*) is carried out. *Circles* with X indicate crossing to obtain populations (*ovals*) from which the desired individuals are selected. The selection steps are indicated by *block arrows*, the type of the selecion is labelled by *letters in the arrow* like (e) embryogenic test, (g) genotyping, or (t) demonstrating the presence of the transgene. *Rectangles* denote individuals that have been selected for either crossing or transformation steps from the parental (P) as well as the first (F1) and second (F2 or back-cross, BC) generations. Their most important features are indicated in subscript (W: wild-type, M: mutant, E: embryogenic, T: transformed). The desired transgenic individuals with homozygous mutant bacground ($I_{MT(E)}$) are shown with *white letters in black rectangles*. **b** The number of different selection steps (S) required in the course of the three routes

4.1.2.1 Generating embryogenic F1 plants carrying mutant alleles of the *NORK* gene

To achieve the complementation of the mutation identified originally in the non-embryogenic MN-1008 *M. sativa* plant and test the method in practice, we followed the common initial steps of the F1 and F2 approaches parallel to the (physical) mapping and sequencing efforts of the positional cloning work. At that time, in the absence of a candidate gene the Route P approach was set aside. As a first step, we generated embryogenic hybrid plants (F_{1E}) carrying the Nod^+ and Nod^- alleles in heterozygous configuration. To complete this goal, crosses were carried out between the non-embryogenic, Nod^- MN-1008 mutant (P_M) and the highly embryogenic, Nod^+ Regen S parent (P_{WE}) plants in both directions. F1 seeds obtained from the crosses were collected, vernalized, germinated and grown in pots. The hybrid nature of the progeny was determined by either the nodulation phenotype or the genotype depending on the direction of the cross. Because of the recessive nature of the mutant allele the appearance of nodules on the progeny plants was an indication of the hybrid nature in those cases where the maternal plant was the Nod^- (MN-1008), and the pollinator was the Nod^+ (Regen S) parent. In the reciprocal cross since cross-pollination is favored in alfalfa but self-pollination can occur as well the nodulation phenotype was not informative to disclose the offspring of self-pollination; therefore, an RFLP marker (U492, Kiss et al. 1993) was used to check the heterozygous genotype of the descendents in a DNA–DNA hybridization experiment (data not shown). All 56 progeny tested turned out to be hybrid F1 plants, none of them originated from the self-pollination of the parents. These 56 F1 individuals were subjected to embryogenic test to select those plants ($F1_E$) that had the ability to form somatic embryos. Three out of the 56 individuals (RN/28, RN/41, RN/50) were found to develop embryos with an efficiency comparable to that of the embryogenic parental line, Regen S (Table 2). Under our conditions, the rest of the individuals did not produce any embryos on the calli induced on their explants. One of these $F1_E$ plants, RN/28 was selected for use in further experiments because it had high efficiency seed production after self-pollination that was advantageous for progeny generation. This highly embryogenic F1 hybrid plants offered two possibilities to produce stable transgenic lines in order to investigate the complementation by the transgene. One approach was to self-pollinate the embryogenic hybrid to identify embryogenic Nod^- (homozygous for the mutant *NORK* allele) plants (I_{ME}) that would be adequate for *A. tumefaciens* mediated transformation (Route F2 in Fig. 4a). The other possibility was to introduce the wild-type

NORK gene into the F1 embryogenic plant followed by self-pollination of a stable transgenic line ($F1_{ET}$) to select F2 plants (I_{MT}) homozygous for the mutant allele (Route F1 in Fig. 4a).

4.1.2.2. Generating F2 population segregating the embryogenic capacity and the nodulation ability

Following the Route F2 strategy (Fig. 4a) F2 plants were generated by self-pollination of the RN/28 hybrid. To accelerate the identification of the plants carrying the mutation in homozygous configuration the F2 individuals were genotyped for the PF marker closely linked to the *NORK* gene (Endre et al. 2002b) as described in Materials and methods section. In the meantime, a nodulation test was also performed to identify Nod^- plants. In more than 800 plants, 15 individuals were identified based on their phenotypic and genotypic characteristics to be homozygous for the genomic region carrying the Nod^- trait. These plants were tested for embryogenic capacity (see Materials and methods section), but none of them was able to form embryos seven after 6 months of culturing. Hence, the Route F2 strategy was determined to be impractical in our experimental system and was abandoned. If the trait is determined by dominant alleles in simplex configuration of two unlinked genes (AaaaBbbb) the theoretical ratio of embryogenic and non-embryogenic plants in a self-mated population is 9:7. One possible explanation for the unexpected failure to identify an embryogenic mutant (I_{ME}) is that one of the two genes proposed to determine embryogenicity in alfalfa might be linked to the *NORK* gene and none of the 15 Nod− individuals carried a recombinant chromosome with both the *nn1* mutation and the dominant allele required for embryo development.

4.1.2.3 Transformation of the selected embryogenic F1 plant

After the identification of the candidate gene in the course of map-based cloning we followed the Route F1 strategy (Fig. 4a) to complement the *nn1* mutation. First, the wild type *NORK* gene from *M. truncatula* was introduced into the embryogenic $F1_E$ plant RN/28 as described in Materials and methods section. The *A. tumefaciens*-mediated transformation protocol resulted in 52 regenerated, potentially transgenic plants. The most rapidly regenerated 10 plants were tested for the presence of the transgene(s) and their copy number in the genome by DNA–DNA hybridization (see Materials and methods section). According to the detected hybridization signals all plants tested (Fig. 5) were transgenic ($F1_{ET}$). Three (TRN 1, TRN 2 and TRN 4) and seven (TRN 3 and TRN 5–10) plants carried six and three copies of the transgene, respectively. Based on the hybridization pattern it was concluded that these plants originated from two independent transformation events, and the plants displaying similar hybridization patterns were clones (Fig. 5).

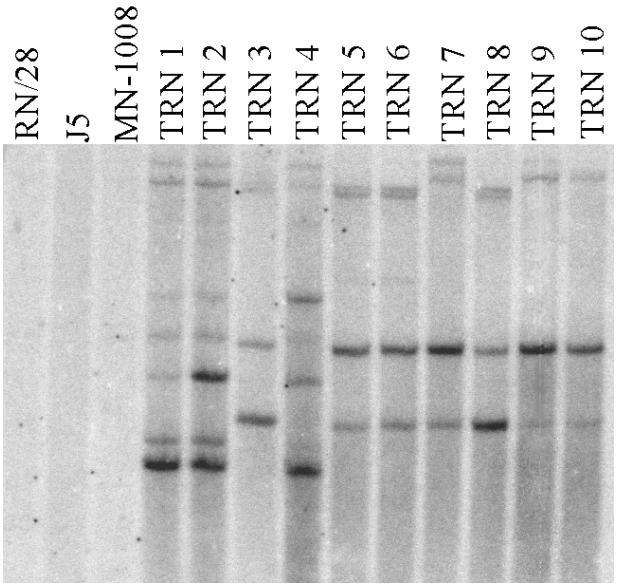

Figure 5. DNA-DNA hybridization with the *nptII* probe to the *Eco*RI digested genomic DNA of the regenerated F1 plants. J5: *M. truncatula* cv Jemalong, MN-1008: Nod^- parent, RN/28: embryogenic F1 plant, TRN 1-10: transformed-regenerated F1 plants.

The presence of the *M. truncatula NORK* gene in the transformed F1 plants was also demonstrated. Using a *NORK* specific probe (see Fig. 6) hybridized to *Dra*I digested genomic DNA the *M. truncatula* and *M. sativa* specific *NORK* alleles could be distinguished (Figure 7a). The NORK probe covers the genomic sequence from exon 10 till exon 15. The enzyme *Dra*I cleaves the *M. truncatula* genomic sequence in the intron 9, intron 13 and exon 15 resulting in two hybridizing bands of 0.8 and 1.8 kb (Fig. 6 and 7a). The difference detected between the hybridizing fragments of the Nod^+ and Nod^- *M. sativa* alleles was used later to identify the plants homozygous for the mutant allele in the F2 population.

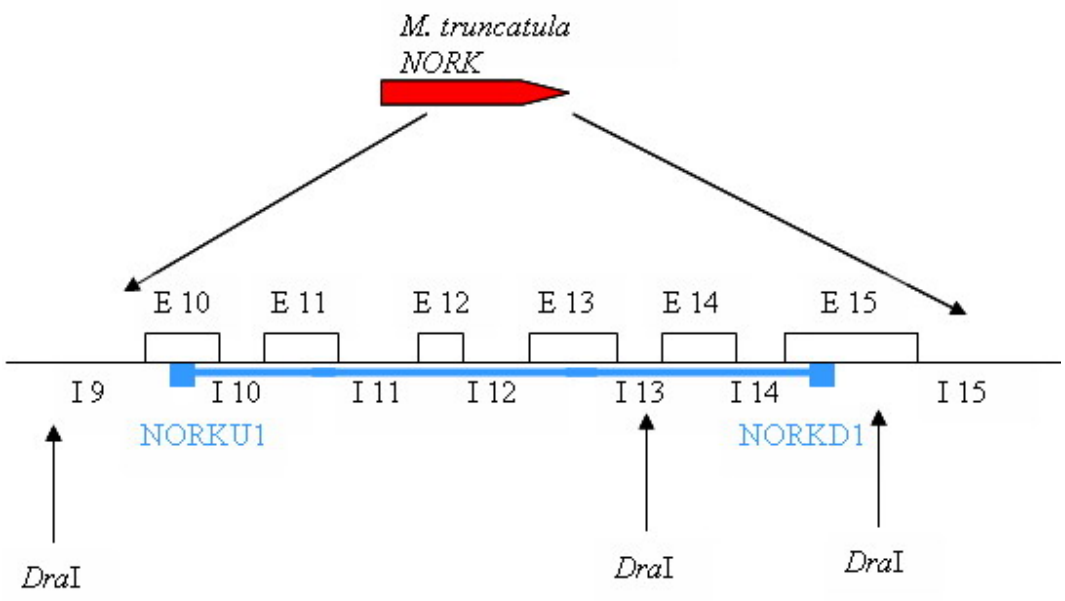

Figure 6. Sites of *Dra*I digestion (indicated by arrows) in the *M. truncatula NORK* gene between intron 9 and exon 15. E10-E15 exons of *M. truncatula NORK*, I9-I15 introns of *M. truncatula NORK*, RKU1-RKD1- primers (blue block) used for the amplification of the *NORK* specific probe (blue bar)

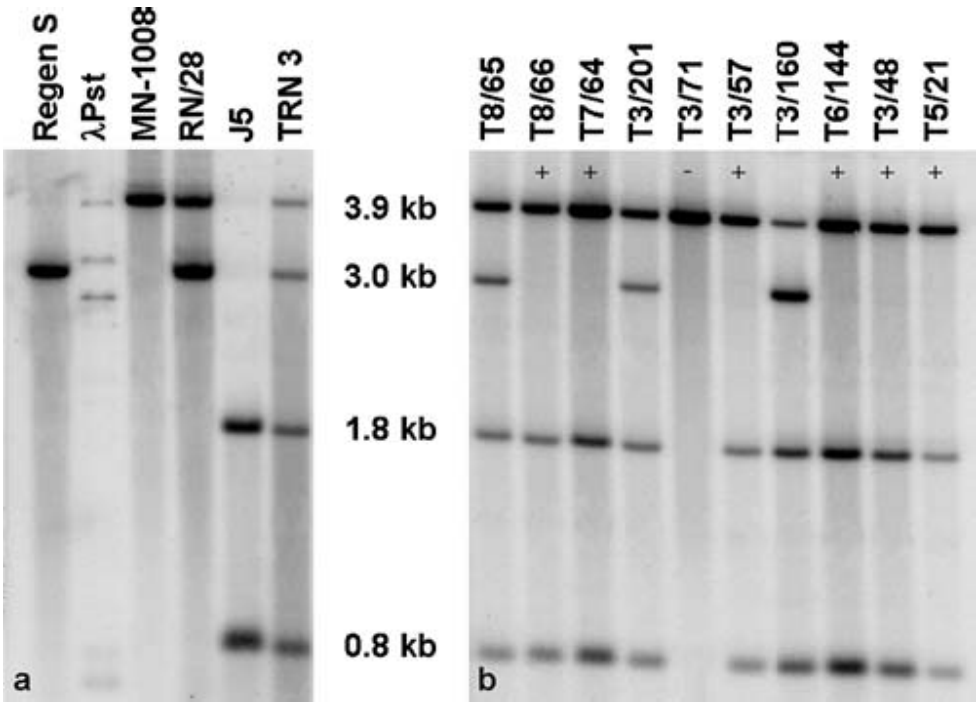

Fig. 7 a and **b** DNA–DNA hybridization with the *NORK* probe to *Dra*I-digested DNA of selected plants. **a** Parental, hybrid F1 and transgenic F1 plants. Regen S: Nod+ parent,MN-1008: Nod− parent, RN/28: embryogenic F1 hybrid, J5: *M. truncatula* cv. Jemalong,TRN 3: transformed F1 plant. **b** F2 plants selected from the population obtained after the self-pollination of TRN 3 and its clones. The phenotype of the individuals homozygous for the Nod− allele are indicated (+: Nod^+, −: Nod^-)

In order to show that the integrated wild type *M. truncatula NORK* (*MtNORK*) gene was expressed in the transgenic *M. sativa* plants reverse transcription (RT-) PCR amplification coupled to restriction enzyme digestion was carried out. After the isolation and reverse transcription of the RNA from the roots and leaves of the transgenic plants, *NORK* specific transcripts were amplified and subjected to *Pvu*I digestion. In this way, we have shown that in all four F1 plants tested (TRN 1, TRN 2, TRN 3 and TRN 4) the transgene was transcribed in the root tissue (Figure 8).

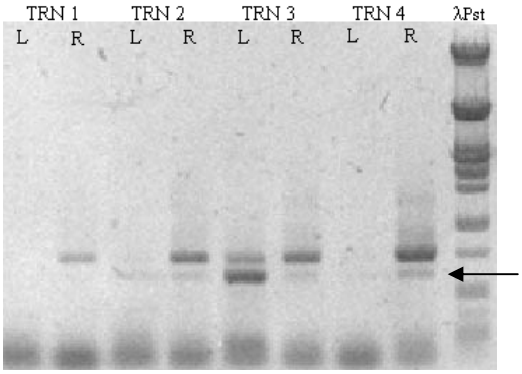

Figure 8. Expression of the *M. truncatula NORK* gene in the transgenic *M. sativa* F1 plants (TRN 1-4). RNA was isolated from leaves (L) and roots (R) and analyzed by RT-PCR coupled with *Pvu*I digestion. Arrow shows the cleavage products characteristic for the transgene.

4.1.2.4 Identification of the homozygous mutant plants carrying the wild-type transgene

The transformed $F1_{ET}$ individual TRN 3 and its clones (TRN 5–10) with the highest seed producing capacity were chosen to generate the F2 population. After self-pollination, 727 seeds were collected and germinated, of which 622 F2 plants grew up and were analyzed. The genetic marker closely linked to the mutation was amplified with the PF U1-PF D1 primer pair (see Materials and methods section) and was used to identify F2 individuals with the proper homozygous genotypes in the Nod region. Seven F2 plants were found to be homozygous for the chromosomal region in the vicinity of the *NORK* mutation. The appearance of the homozygotes (I_M) exhibited lower proportion in the F2 progeny (1:88) than the theoretical ratio (1:35). This type of distorted segregation is a well-known phenomenon in different alfalfa populations (Kaló et al. 2000; Endre et al. 2002a). In a nodulation assay, six out of these seven plants were able to form symbiotic nodules on the roots after inoculation with *S. meliloti*, indicating complementation events.

In order to confirm the homozygous configuration of the mutant alleles and the presence or absence of the wild-type *NORK* transgene in these seven F2 individuals, hybridization experiments with the *NORK* probe were carried out (Figure 7b). The hybridization to *Dra*I digested DNA of selected F2 individuals revealed the presence of one band representing the *M. sativa* Nod^- alleles in seven plants (T8/66, T7/64, T3/71, T3/57,

T6/144, T3/48, T5/21) that confirmed the homozygous genotype. The genomes of the six Nod^+ plants T8/66, T7/64, T3/57, T6/144, T3/48, T5/21, also carried the introduced *M. truncatula NORK* gene as indicated by the two additional hybridizing fragments (Fig. 7b), while the transgene could not be detected in the genome of the Nod^- individual, T3/71. This hybridization pattern demonstrates that the absence of the endogeneous wild-type *NORK* allele from Regen S results in a non-nodulation phenotype, and, on the other hand, the presence of the *M. truncatula* wild-type *NORK* transgene is responsible for the complementation of the Nod^- mutant phenotype originating from the MN-1008 genetic background.

Using the *npt*II probe in hybridization to *Eco*RI-digested genomic DNA the number of transmitted T-DNAinsertions could be determined in the segregating F2 plants (Fig. 9).

T3/71 did not carry any copies, as we expected, T8/66, T3/48, and T8/65 inherited one, T7/64, T3/201, and T3/57 inherited two, and T3/160, T6/144, and T5/21 inherited all three copies of the transgene from TRN 3 and its clones, respectively. This result, i.e. nine out of 10 tested individuals carried one or more transgene(s), is in accordance with the expectations that the transgene (a dominant trait) had to be inherited in high ratio by the F2 individuals. It is worth noting that the two smaller hybridization fragments did not segregate in the individuals tested indicating their possible tandem integration (Fig. 9).

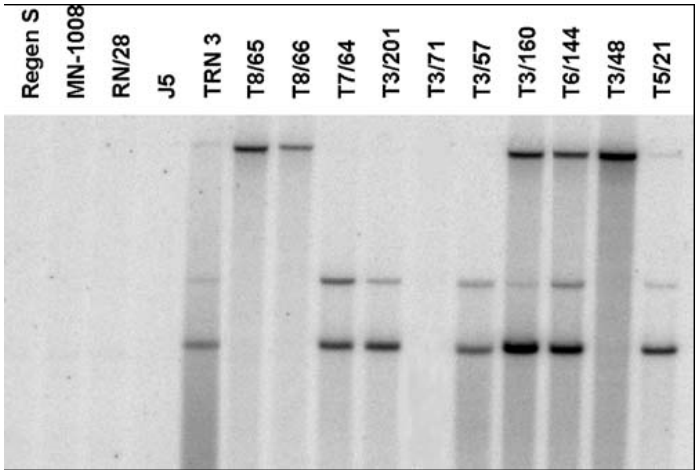

Figure 9 Segregation of the transgenes in the F2 population. DNA–DNA hybridization with the *nptII* probe to the *Eco*RI-digested DNA of the F2 plants selected from the population obtained after the self-pollination of TRN 3 and its clones. Further abbreviations are as indicated in Fig. 7.

4.2. Investigating the possible function of the *NORK* gene with gene expression studies

For mutants in the three *DMI* genes (*DMI1*, *NORK*= *DMI2*, *CCaMK*= *DMI3*) the grafting experiments (Ane et al, 2002) clearly showed that the phenotype is only controlled in each case by the roots. No diminution of the nodule number was observed when a mutant shoot was grafted on a wild-type stock. The root control of the mutant phenotypes shows that it should be possible to use *A. rhizogenes* transformation to speed up the following of the *NORK* expression.

We also made homological searches in the TIGR data bank against the *M. truncatula* ESTs and identified 13 *NORK* sequences of which 11 originated from different root and nodule cDNA libraries. That is why, *Agrobacterium rhizogenes* plant transformation was used to follow the spatial regulation of the *NORK* gene expression in *Medicago truncatula* plants.

To analyze the cell-specific expression of the *NORK* gene in roots and nodules, one construct was made of the promoter and upstream regions of the gene fused to the ß-glucuronidase (GUS) marker gene.

The validity of such a fusion between the promoter of a gene and the GUS reporter gene has already been demonstrated for several other early nodulin genes including *ENOD12* (Pichon et al.1992; Bauer et al. 1996), *ENOD40* (Fang and Hirsch 1998), and *Mtlec1&3* (Bauchrowitz et al. 1996), and is particularly appropriate when gene expression is limited to discrete subpopulations of cells within a given organ.

One fusion was made: *p3.1 NORK-GUS* corresponding to *NORK* promoter and upstream region of 3.1 kbp. This region is functional because of its direct complementation of *dmi2* mutants by the *DMI2* gene (Endre et al. 2002).

This fusion was introduced into *Agrobacterium rhizogenes* Arqua strain and the strain was used to produce *M. truncatula* Jemalong composite plants in which the roots, but not the shoots, were transgenic (Boisson-Dernier et al. 2001). As each transgenic root results from a different transformation event and analysis of many plants eliminates the possibility that the observed GUS expression is due to the position of insertion of the transgene.

In 20 day old transgenic roots GUS activity was observed over most of the root systems but noticeably no activity was detected in the root apices (Fig. 10A and B). It was not possible to define where expression started in the roots, as the GUS activity was very low in the developing root hair zone (relative, for example, to expression from the *MtENOD11* or *12*

promoters – Pichon et al, 1992; Journet et al, 2001). However GUS activity increased progressively in this zone and was highest in the region of lateral roots where the root hairs had just attained their maximal length. The older regions of the roots and particularly the primary roots showed lower GUS activity than the lateral roots (Fig 10A).

In order to point which are the elements of the root where *NORK* is active, we performed binocular analysis and root sectioning. This revealed that *NORK* didn't have expression in the root hairs of the primary roots (Fig. 10B). This result might be due to the fact that in hairy root transformation experiments we always investigated elder roots.

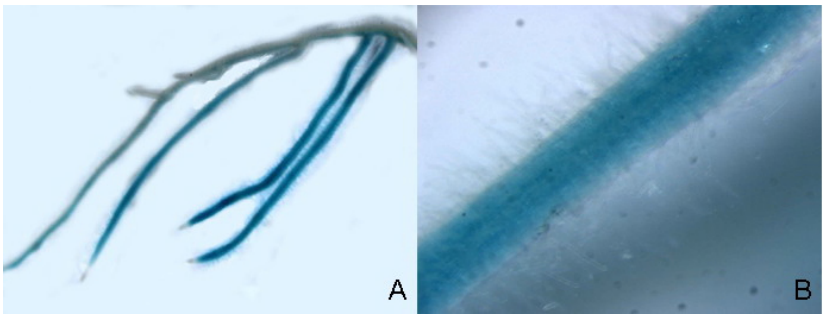

Figure 10. A- Whole segments of primary and secondary roots, showing GUS expression. B- primary root showing lack of GUS expression in the root hairs

To go further, we produced 14 root sections from the secondary roots and these showed *NORK* expression in all tissues including the epidermis, the root hairs as well as the cortex.

4 transversal sections of two different plants were performed and the GUS activity could be detected in the root hairs, cortical cells and in the central cylinder (Fig. 11 A, B, C, D, E, F).

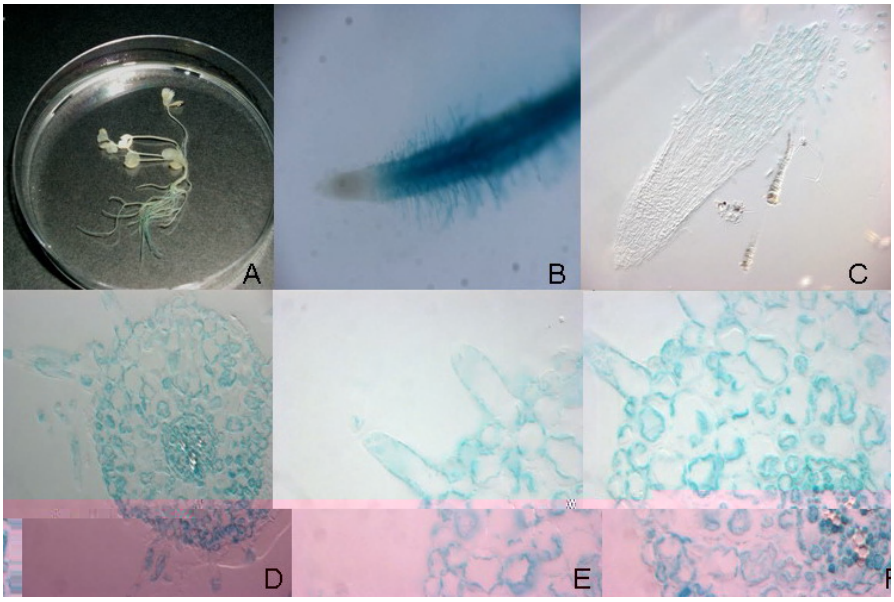

Figure 11. 20 day old transgenic plant carrying the *NORK* promoter fused to GUS. A- The whole root system of a stained plant. B- Secondary roots showing lack of GUS expression in the root apices and increasing expression in the developing root hair zone. C- longitudinal section through the root tip of a secondary root. D- Section, 10 µm thick, of a secondary root cut in the region behind the root apex, showing *NORK* expression in the root hairs and epidermal and cortical cells. E- root hairs, F- cortex and central cilinder of a secondary root

To examine expression of *NORK* promoter during nodulation the transgenic composite plants were transferred to growth pots. 26 day old plants were inoculated with *Sinorhizobium meliloti* 2011 strain in order to produce nodules. The transgenic roots and nodules were analyzed histologically for GUS activity.

The GUS staining of six plants in the first two days after rhizobia addition reveals weaker intensity of the GUS expression than the uninoculated roots of 20 day old plants (Fig. 12 A).

Still the pattern of the *pNORK- GUS* is similar to the uninoculated plants. *NORK* activity was very weak in the primary roots and stronger in the secondary roots but no GUS activity was detected in the root apices. In the primary roots the pattern of GUS remained the same, the root hairs with no GUS expression, the cortex with *NORK* expression but weaker than the uninoculated plants.

At three days following rhizobial inoculation, two plants (out of three analyzed) showed a clear increase in NORK expression in the susceptible zone. The increased cortical expression clearly was associated with the developing nodule primordia, whereas lateral root primordia showed basal expression (Fig. 12 C).

In some cases there *NORK* activity could be observed in the root hairs of the secondary root, also carrying nodules, but in general *NORK* activity in the root hairs of the nodulating roots was much weaker than the uninoculated ones (Fig. 11 C).

At four days after inoculation the nodulation centers were more visible in two plants out of three analysed (Fig. 12 B). *NORK* expression was strong in all of the roots of the three plants analysed and the conclusion is it was not dependent on the nodulation.

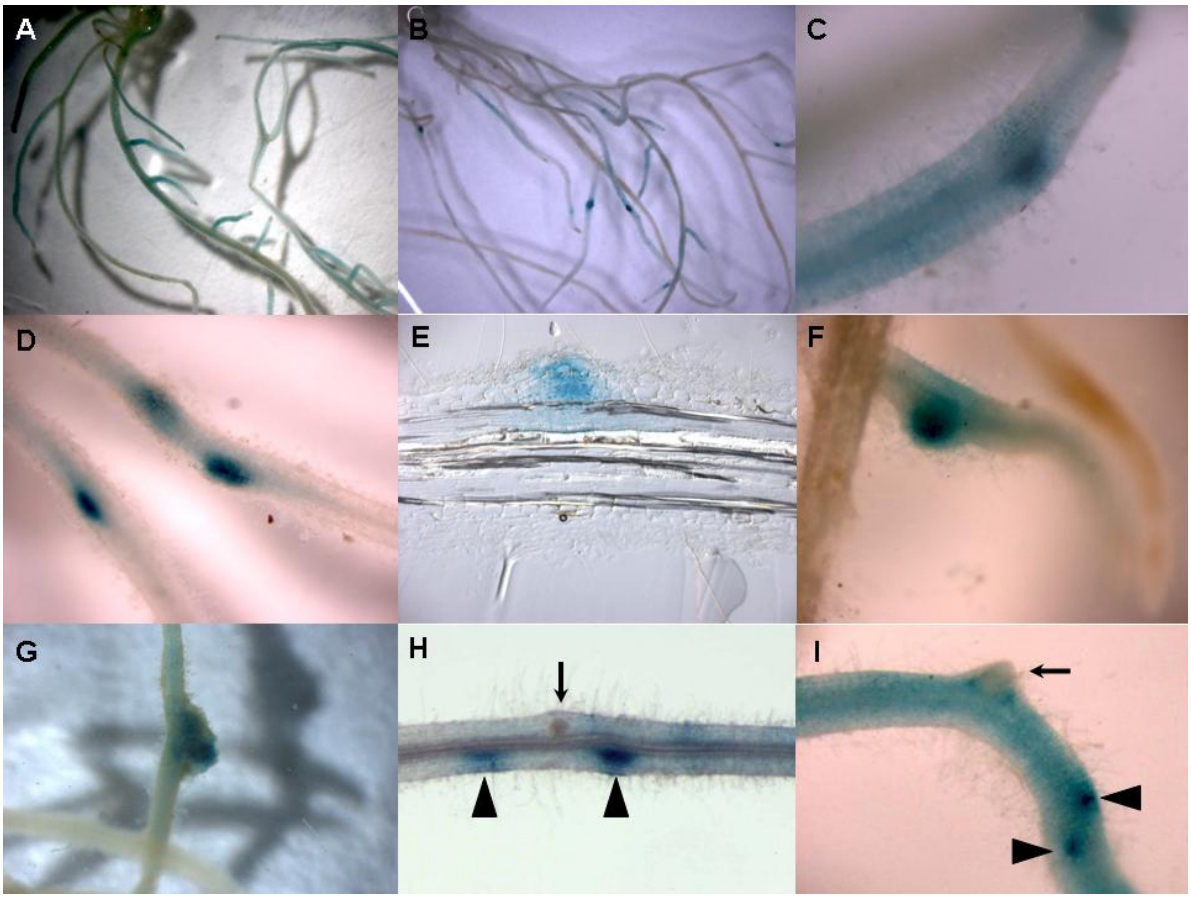

Figure 12. A-Secondary root showing expression of *pNORK-GUS*. A-Whole root segments of secondary roots showing expression of *pNORK-GUS* in the developing nodule primordia, in the third day after inoculation, B- In the fourth day from inoculation, C- In the third day, nodulation centers appear in the susceptible zone, and *NORK* is strongly expressed, D- Root segments of secondary roots showing expression of *pNORK-GUS* in the developing nodule primordia at 5 days after inoculation, E- Section of developing nodule at 6 days after inoculation showing *pNORK-GUS* expression in the central, undifferentiated tissues, G- nodule in the eighth day after inoculation, H, I- difference between nodule primordia and secondary root primordial (thin arrow shows secondary root primordia and triangle arrow indicates nodule primordia).

In the fifth day after the inoculation three plants were analyzed. One plant developed nodules (Fig 12 D). It is noteworthy that this plant had a very weak *NORK* expression in the primary and the secondary roots. The other two plants that did not develop nodules had a stronger *NORK* expression both in the primary and secondary roots.

On the longitudinal sections of 6 and 7 day old nodules we could observe that the cortex dividing cells have a strong *NORK* activity.

Sectioning of some of the primordia showed that the increased expression occurred throughout the internal tissues of the developing nodule, whereas the surrounding root tissues showed relatively little activity. At this stage, the internal tissues did not show differentiation into zones (Fig. 12 E, 13 A).

Expression of *pNORK-GUS* was observed in sections of older nodules of 10 days following inoculation. 15 nodules from 4 different plants were sectioned longitudinally. Strong expression in all GUS-positive plants was localized in a small region at the distal part of the nodule (Fig. 13B).

The typical zonation of indeterminate nodules was apparent: a distal, apical and persistent meristem (zone I) followed by zones of increasing cell age, comprising an infection zone (zone II), an interzone (II-III), and a nitrogen-fixation zone (zone III) (Vasse et al. 1990) (Fig. 13B).

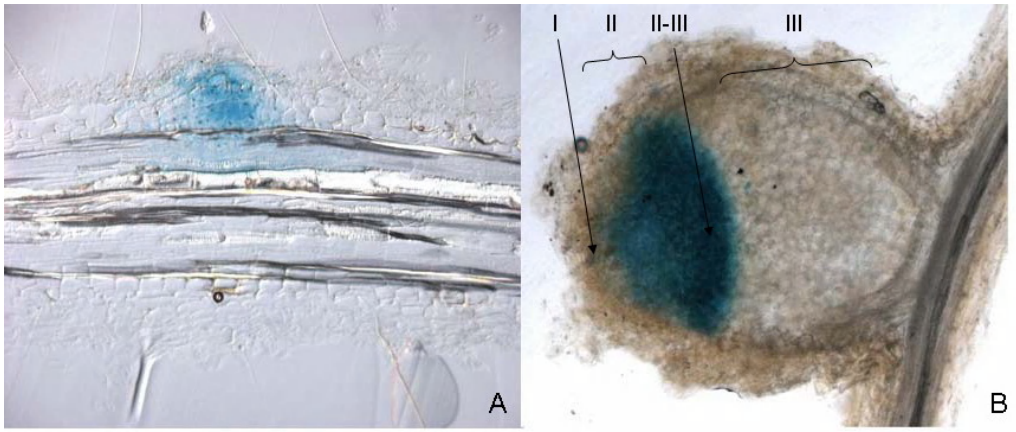

Figure 13. A- Longitudinal section through a seven day old nodule, B- Longitudinal section of 10-day-old nodule showing strong GUS expression in the pre-infection zone.

In order to show that nodulation did not affect the basal level of *NORK* expression we analyzed the number of plants carrying nodules and their GUS intensity in roots. (table 3)

There were five cases. Plants having a very weak (w) *NORK* expression in the root system, and not carrying nodules Nod^-) (4 individuals), plants with a stronger *NORK* expression (s), not carrying nodules (Nod^-) (7 individuals), plants having a stronger *NORK* expression (s), carrying nodules (Nod^+) (5 individuals), plants with weak *NORK* expression (w) , carrying nodules (Nod^+) (4 individuals) and one plant with very strong (ss) GUS expression , carrying nodules (Nod^+) (1 individual).

Intensity of GUS and presence of the nodulation	w Nod^-	s Nod^-	s Nod^+	w Nod^+	ss Nod^+
Number of individuals	4	7	5	4	1

Table 3. Intensity of GUS expression coupled with the nodulation phenotype

From this table, we can conclude that when there was a strong *NORK* expression in the roots, 7 individuals were not nodulating and 6 individuals were having nodules. On the other hand, when *NORK* expression was weak 4 individuals did not carry nodules and 4 individuals were nodulating. This indicates that the GUS intensity was not dependent on the nodulation process rather on the possible positional effect.

5. Discussion

5.1 Comparison of the three complementation strategies

Comparison of the three complementation strategies reveals five selection steps in each route (Fig. 4b), but their components are diverse. All three strategies need the genotyping of two populations (the identification of the F1 hybrids and the selection of the homozygous mutants in the segregating population), while the number of selection steps to determine the embryogenic capacity and to show the presence of the transgene is different. This also means substantial variations in the time course of the experiments. In order to decide which strategy to use the following questions should be answered: (1) is the gene of interest already in hand or is there sufficient time to produce one or two appropriate generations prior to transformation (e.g. during map-based cloning work)? and (2) what are the future plans with the homozygous mutant or the resulting transgenic plants (i.e. they are subjects for further transformation experiments or not)

Route P method is less arduous and takes less time as it does not require any selection step for embryogenic ability. On the other hand, from the aspect of a complementation experiment, it needs longer time (two generations) after having the candidate gene in hand to produce the transgenic plant. Thus, it is rather suggested for other purposes, e.g. to check the function (by overexpression or silencing) or expression (by promoter–reporter constructs) of certain genes in different genetic (mutant) backgrounds. Once the desired construct is introduced into the embryogenic plant and transgenic individuals selected (P_{WET}), they can be used in crosses with several different mutant lines (P_{M1}, P_{M2}. etc.). After obtaining transgenic F1 plant(s), the next generation segregating the mutation and the transgene can be produced either by self-pollination or to facilitate the emergence of homozygous mutants by back-crossing (BC) with the mutant parent. The inheritance of the transgene into the second generation is relatively frequent (75% in the F2, 50% in the BC population) since it behaves as a dominant trait. In an experimental approach similar to this strategy the *ENOD11* promoter-GUS construct was introduced into *M. truncatula* individuals with different mutant genetic backgrounds (Catoira et al. 2000).

In the course of Route F1 experiments, the desired gene construct has to be in hand for transformation after the first embryogenic selection step at the F1 level (Fig. 5a). The next generation (i.e. the offspring of the transformed plants) can be produced by either self-

pollination or backcrossing as was mentioned above for Route P approach. The last selection steps identify the individuals carrying the mutation in homozygous configuration and the transgene (I_{MT}) to check for complementation. During this approach, there is no need for a second selection for embryogenic capacity, however, if additional transformation experiments were needed (as mentioned later for Route F2), there is the opportunity to select for embryogenic competence among the F2 (I_{MT}) plants.

The two selection steps for embryogenic ability in Route F2, both at F1 and F2 levels, make this strategy longer and more laborious than the other approaches. On the other hand, embryogenic individual(s) with the desired homozygous mutant genetic background (I_{ME}) will be generated at the end that can easily be transformed with several gene constructs afterwards. Accordingly, one can start the $P_M \times P_{WE}$ cross at the beginning of a positional cloning work- even use this cross to produce the mapping population if suitable- and the identified candidate genes can be introduced into the I_{ME} plants to test for complementation. Because of its embryogenic nature there is a potential to introduce further gene(s) into the complemented transformant plants (I_{MET}) to test possible interaction(s) or follow reporter gene expression. The UMN 3176 alfalfa germplasm (Samac and Lamb 2000), which has 98% ineffectively nodulated plants, (i.e. homozygous for the *in1* mutation; Peterson and Barnes 1981), and 55% regenerating plants was created similarly through Route F2, by crossing two F1 populations obtained from crosses of the embyogenic line Regen-SY with two ineffective populations, Ineffective Agate (Reg. no. GP-228, PI536529) and Ineffective Saranac (Reg. no. GP-229, PI536530), respectively.

5.2. Investigating the possible function of the *NORK* gene with gene expression studies

The *NORK* gene of *M. truncatula* was identified in screens for Nod$^-$ mutants; thus, all alleles reported to date are either completely or severely restricted to nodulation. Further analysis has revealed that *dmi2* mutants are blocked in infection at the root epidermis and that these cells are defective in most Nod factor responses, including induction of some early nodulin genes and calcium spiking (Catoira et al. 2000; Wais et al. 2000). Thus, the gene plays an essential role in the early steps of Nod factor signal transduction leading to infection of the epidermal cells and of the root. By studying the expression of the fusions of the *NORK* promoter with the *GUS* gene, data presented here suggest that *NORK* plays also a role in the root cortex during early nodule development and in the nodules.

Studies reported here using GUS fusions have shown that the *NORK* gene is highly expressed in a very specific region of the nodules, located between the meristem and the main part of the infection zone (Fig. 13B). A similar, narrow zone of *NORK* mRNA abundance recently has been shown by in situ hybridization (Mirabella 2004), thus validating the GUS fusion approach. This zone of expression has been termed the "preinfection zone" (Pichon et al. 1992) and is characterized by the induction of several nodulin genes of *M. truncatula,* including *MtENOD12* (Pichon et al. 1992), *MtENOD11* (Journet et al. 2001), *MtN1* (Gamas et al. 1998), *MtN6* (Mathis et al. 1999), and *MtAnn1* (de Carvalho-Niebel et al. 1998). By comparison of the depths of the zones in which the nodulin genes are expressed, *NORK* and *MtENOD11* appear to have very similar, narrow expression patterns in this apical region of the nodule. Indeterminate nodules show a gradation of cells of increasing age from the meristem to the base (Vasse et al. 1990); therefore, this localization of expression suggests that the *NORK* gene is expressed transitorily during growth and differentiation of the nodule. However, it is noteworthy that *NORK* also appears to be expressed, albeit at a lower level, in the nitrogen-fixing zone of the nodule.

In roots, our studies with GUS fusions have shown that *NORK* is expressed in the epidermis and cortex and throughout most of the root system but not in the root apices (Fig. 9A and B). Highest expression was observed in the younger part of lateral roots. During nodulation, the gene is strongly induced in the nodule primordia, forming particularly on the lateral roots, relative to the low expression in the surrounding tissues and newly developing roots. At 2 days following rhizobial inoculation, induction of *NORK* could be seen clearly in patches of cells in

the cortex and, at 3 to 5 days, this induction clearly was confined to the nodule primordia and the central, undifferentiated tissues of the young emerging nodules. At these stages of nodulation, the expression of *NORK* resembles more closely the expression pattern of *MtENOD20* (Vernoud et al. 1999) and *MsENOD40-1* and *–2* (Fang and Hirsch 1998), rather than *MtENOD11* and *MtENOD12,* which are not induced in the primordia. However, the *ENOD* genes also are induced in the epidermal and cortical cells through which infection threads are initiated or passing (Journet et al. 2001; Pichon et al. 1992; Vernoud et al. 1999).

5.2.1. Role of *NORK*

Because *nork* mutants develop normally when grown with a combined nitrogen source, it appears that *NORK* is essential only for establishing root endosymbioses. The much higher levels of mRNA in roots and nodules compared with leaves and stems suggest a role in nodules in addition to roots and do not exclude functions in the aerial organs.

Recently, roots of *nork* mutants have been found to be more sensitive to touch than those of wild-type plants (Esseling et al. 2004), suggesting that the gene may have a subtle, nonsymbiotic role which should be investigated further. Because *nork* mutants are blocked in infection of the epidermal cells by both rhizobia and AM fungi, a common symbiotic role of the gene has been suggested in the very early steps of infection. In the LR symbiosis, *NORK* clearly plays a role in Nod factor-mediated signal transduction in roots prior to infection and, moreover, also is involved in establishing the nodule primordia (Catoira et al. 2000). The expression of *NORK* in epidermal and cortical cells before inoculation is consistent with a role in perceiving the symbionts before allowing infection to proceed. In accord with an infection role in roots, the simplest explanation for the induction of the *NORK* gene in the nodule primordium and in the preinfection zone of the nodule is that the NORK receptor is required to prepare for infection during nodule organogenesis. Bacterial *nod* genes required for Nod factor synthesis seem to be induced not only in a molecular dialogue before root infection but also in the apical zone of nodules (Schlaman et al. 1998); therefore, it appears that NORK is mediating Nod factor signal transduction in both roots and nodules. Moreover, the *NORK* gene appears to be expressed, albeit at a lower level, in the fixation zone of the nodule, thus suggesting that *NORK* may play a role in continual interactions between the plant and its

functioning bacterial symbiont. From the expression pattern of the gene in proximity to infecting or nitrogen-fixing rhizobia, we cannot state whether the signal that binds to and activates the NORK receptor is more likely to be derived from the bacterium or the plant (or, for the AM symbiosis, from the fungus); at present, the ligands for NORK and related proteins remain unknown.

In conclusion, our studies on the localization of *NORK* expression suggest that the gene plays a specific role in the root cortex and in nodules preceding or leading to rhizobial infection.

In concurrence with its role in root epidermal cells, these results support the idea that Nod factor perception and transduction leading to rhizobial infection occur continuously during development of indeterminate nodules.

Parallel to our studies and in accordance with our results, it was shown by *in situ* hybridization that the *NORK* expression level was elevated in two-three cell layers of the infection zone adjacent to the meristem (Limpens et al. 2005). In addition, other genes (*DMI*1, *DMI*3, *LYK*3) playing also an essential role in nodule initiation and development are expressed in the same zone. These are the cells where the bacteria are released from the infection thread. In the existing *dmi2* mutants, the function of the *NORK* gene is totally lost. That is why in additional experiments the authors tried to reduce the level of *NORK* expression in order to allow nodule initiation and investigate the role of the gene in the later steps of nodule development. For this purpose, silencing of *NORK* expression using RNAi and also expression of *NORK* at lower level in the mutant background, introducing a 35S derived promoter driven gene were performed. Decreased *NORK* expression resulted in the extensive growth of infection threads and block of bacteria release from these infection threads. These observations suggest that a threshold level of *NORK* expression should be achieved in order to switch from infection thread growth to release bacteria.

Also it is possible that *NORK* is implicated in other processes that symbiosome formation, but they are not affected by this partial reduction.

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8. Summary of the Thesis

Introduction

Legumes form symbiotic relationships with soil bacteria commonly known as rhizobia. The legume-rhizobia (LR) symbiosis is characterized by the development of nodules on the roots of the plants in which the bacteria reduce atmospheric nitrogen (N_2) in exchange for nutrients derived essentially from carbon dioxide fixed during photosynthesis which uses solar energy as an energy source.

Until now the most effective way to identify all of the genes that are indispensable for the development, functioning and regulation of the symbiotic interaction has been the classical or "forward" genetics approach by using mutants affected in the biological process.

The tetraploid non-nodulating (Nod^-) alfalfa (*Medicago sativa*) mutant MnNC-1008(NN) (Peterson and Barnes 1981; Barnes et al. 1988) identified in the progeny from crosses between different cultivars and referred here as MN-1008, was among the first symbiotic mutants reported (Caetano-Anolles and Gresshoff, 1991). This mutant fails to produce the characteristic responses to rhizobia and Nod factors. In addition, the mutant is also unable to establish the other symbiotic interaction, the vesicular-arbuscular mycorrhizal symbiosis to scavange phosphorus (Bradbury et al. 1991). To identify the gene required for the development of these symbioses a map-based cloning strategy was conducted and a receptor-like protein kinase designated as NORK (Nodulation Receptor Kinase) was identified (Endre et al. 2002a; 2002b). The final step of the map based cloning was the complementation of the MN-1008 mutant with the wild type *NORK* gene. It could not be accomplished by the rapid *Agrobacterium rhizogenes* mediated transformation since symbiotic nodules are not formed on the hairy roots induced on alfalfa (Beach and Gresshoff 1988). Furthermore, the traditional transformation method using *A. tumefaciens* was also not feasible for MN-1008 being a non-embryogenic alfalfa line.

Aims of the study

1. To confirm that the *NORK* gene is indispensable for the development of the symbiotic interactions using new alternative complementation strategies.

2. The analysis of the *NORK* gene expression and its regulation in the root tissues of *M. truncatula* to reveal its potential roles during nodulation.

Methods

- *Agrobacterium rhizogenes* mediated plant transformation
- *Agrobacterium tumefaciens* mediated plant transformation
- DNA isolation and hybridization
- PCR analysis
- Reverse transcription (RT-) PCR analysis
- Histochemical localization of β-glucuronidase (GUS) activity

Results and discussion

Complementation of the *NORK* mutations in another species

Since neither *A. rhizogenes* nor *A. tumefaciens* mediated transformation was feasible to complement the MN-1008 line, *A. rhizogenes*-mediated transformation system was used to show functional complementation of the *M. truncatula dmi2* mutants carrying mutation in the orthologous sequence by introducing the wild type *NORK* gene (Endre, 2002b).

Alternative strategies to complement the Nod$^-$ mutation of MN-1008

A procedure has been developed and successfully applied to obtain stable transformants and to complement the Nod$^-$ mutation that originated from the non-embryogenic mutant alfalfa line MN-1008. A few genotypes of tetraploid *M. sativa* have the ability of somatic embryogenesis, and genetic studies suggested this trait to be under the control of dominant alleles of at least two genes that are usually in simplex/simplex (Aaaa/Bbbb) configuration in the embryogenic lines tested (Wan et al. 1988; Hernandez-Fernandez et al. 1989; Kielly and Bowly 1992; Crea et al. 1995). The basis of our approach is that the dominant alleles of the genes conditioning the embryogenic potential, the mutant phenotype, as well as the wild-type sequence to complement the mutation can be combined using several crossing and selection steps. The strategies described here can generally be used in similar circumstances where the plant to be transformed is not embryogenic, but other embryogenic lines are available in the plant species studied.

Three general strategies could be utilized to complement a mutation residing in a non-embryogenic plant genotype: the DNA construct to be used for complementation could be introduced into an embryogenic line followed by the crossing of the transgenic and the mutant plants (Route P) or the mutant allele(s) could be transferred into an embryogenic line which is

followed by the transformation experiment either in the hybrid F1 progeny or in selected F2 plants (Routes F1 and F2).

In the course of the first alternative way (Route P) the gene of interest is introduced into the embryogenic parent (P_E). Transgenic regenerants are selected (P_{ET}) and then crossed with the non-embryogenic mutant line (P_M) to combine the mutation with the transgene in one plant. Transgenic F1 individuals are selected ($F1_T$) and a segregating population is produced by self-pollinating or back-crossing these F1 plants with the mutant parent. As a final step, individuals (I_{MT}) homozygous for the mutant gene and also carrying the transgene are identified from this second generation. Routes F1 and F2 start with a cross between the nonembryogenic mutant (P_M) and the embryogenic parent (P_{WE}) followed by the selection of embryogenic plants from the F1 population that are also heterozygous for the mutation (F_{1E}). One possible step is to self-pollinate these F_{1E} hybrids and subsequently to identify embryogenic individuals that are also homozygous for the mutation (I_{ME}) in the F2 population (route F2). The desired construct(s) can later be introduced into these plants using the *A. tumefaciens*-mediated transformation. In this case, since the embryogenic mutant plant is already available several transformation experiments can be designed to test different gene constructs. If a candidate gene is available it is possible to transform the embryogenic F1 individual(s) with the foreign DNA (route F1). After selecting the transgenic hybrid plants (F_{1T}), further steps to get I_{MT} plants are just the same as followed in the Route P approach.

Generating embryogenic F1 plants carrying mutant alleles of the *NORK* gene

To achieve the complementation of the mutation identified originally in the non-embryogenic MN-1008 *M. sativa* plant and test the method in practice, we followed the common initial steps of the F1 and F2 approaches parallel to the (physical) mapping and sequencing efforts of the positional cloning work. At that time, in the absence of a candidate gene the Route P approach was set aside. As a first step, we generated embryogenic hybrid plants (F_{1E}) carrying the Nod^+ and Nod^- alleles in heterozygous configuration. To complete this goal, crosses were carried out between the non-embryogenic, Nod^- MN-1008 mutant (P_M) and the highly embryogenic, Nod^+ Regen S parent (P_{WE}) plants in both directions. The hybrid nature of the progeny was determined by either the nodulation phenotype or the genotype depending on the direction of the cross.

The hybrid F1 individuals were subjected to embryogenic test to select those plants ($F1_E$) that had the ability to form somatic embryos. One of the embryogenic plants ($F1_E$), was

selected for use in further experiments because it had high efficiency of seed production after self-pollination that was advantageous for progeny generation. This highly embryogenic F1 hybrid plants offered two possibilities to produce stable transgenic lines in order to investigate the complementation by the transgene.

Following the Route F2 strategy F2 plants were generated by self-pollination of the F1 hybrid. The F2 individuals homozygous for the genomic region carrying the Nod^- trait were identified. These plants were tested for embryogenic capacity but none of them was able to form embryos. One posssible explanation for the absence of the embryogenic individuals (I_{ME}) in the F2 population is that one of the genes required for emryogenicity is genetically linked to the *NORK* gene and we have not obtained a mutant with a recombinant chromosome carrying the proper alleles (i.e. the dominant allele for embryogenicity and the mutation in *NORK*).

Transformation of the selected embryogenic F1 plant

After the identification of the candidate gene in the course of map-based cloning we followed the Route F1 strategy to complement the *nn1* mutation. First, the wild type *NORK* gene from *M. truncatula* was introduced into the embryogenic $F1_E$ plant.

The most rapidly regenerated plants were tested for the presence of the transgene(s) and their copy number in the genome by DNA–DNA hybridization. According to the detected hybridization signals all plants tested were transgenic.

In order to show that the integrated wild type *M. truncatula NORK* (*MtNORK*) gene was expressed in the transgenic *M. sativa* plants reverse transcription (RT-) PCR amplification coupled to restriction enzyme digestion was carried out. In this way, we have shown that in all the F1 plants tested the transgene was transcribed in the root tissue.

Identification of the homozygous mutant plants carrying the wild-type transgene

The transformed $F1_{ET}$ individual and its clones with the highest seed producing capacity were chosen to generate the F2 population. After self-pollination, 727 seeds were collected and germinated, of which 622 F2 plants grew up and were analyzed. The genetic marker closely linked to the mutation was amplified and was used to identify F2 individuals with the proper homozygous genotypes in the Nod region. Seven F2 plants were found to be homozygous for the chromosomal region in the vicinity of the *NORK* mutation.

In order to confirm the homozygous configuration of the mutant alleles and the presence or absence of the wild-type *NORK* transgene in these seven F2 individuals,

hybridization experiments with the *NORK* probe were carried out. The genomes of the six Nod^+ plants, also carried the introduced *M. truncatula NORK* gene while the transgene could not be detected in the genome of the Nod^- individual. This hybridization pattern demonstrates that the absence of the endogeneous wild-type *NORK* allele from Regen S results in a non-nodulation phenotype, and, on the other hand, the presence of the *M. truncatula* wild-type *NORK* transgene is responsible for the complementation of the Nod^- mutant phenotype originating from the MN-1008 genetic background.

Investigating the possible function of the *NORK* gene with gene expression studies

To analyze the cell-specific expression and the regulation of the *NORK* gene in roots and nodules, the 3.1 kb long promoter and 5'-untranslated regions of the gene were fused to the ß-glucuronidase (GUS) reporter gene. This fusion was introduced into *Agrobacterium rhizogenes* strain Arqua which was used to produce *M. truncatula* Jemalong composite plants in which the roots, but not the shoots, were transgenic.

We have shown that *NORK* is expressed in the epidermis and cortex and throughout most of the root system but not in the root apices. Highest expression was observed in the younger part of lateral roots. During nodulation, the gene is strongly induced in the nodule primordia, forming particularly on the lateral roots, relative to the low expression in the surrounding tissues and newly developing roots. At 2 days following rhizobial inoculation, induction of *NORK* could be seen clearly in patches of cells in the cortex and, at 3 to 5 days, this induction clearly was confined to the nodule primordia and the central, undifferentiated tissues of the young emerging nodules. At these stages of nodulation, the expression of *NORK* resembles more closely the expression pattern of *MtENOD20* (Vernoud et al. 1999) and *MsENOD40-1* and *–2* (Fang and Hirsch 1998), rather than *MtENOD11* and *MtENOD12*, which are not induced in the primordia. However, the *ENOD* genes also are induced in the epidermal and cortical cells through which infection threads are initiated or passing (Journet et al. 2001; Pichon et al. 1992; Vernoud et al. 1999).

NORK gene is highly expressed in a very specific region of the mature nodules, located between the meristem and the main part of the infection zone termed the “preinfection zone”. By comparison of the depths of the zones in which the nodulin genes are expressed, *NORK* and *MtENOD11* appear to have very similar, narrow expression patterns in this apical region of the nodule.

In conclusion, our studies on the localization of *NORK* expression suggest that the gene plays a specific role in the root cortex and in nodules preceding or leading to rhizobial infection. In concurrence with its role in root epidermal cells, these results support the idea that Nod factor perception and transduction leading to rhizobial infection occur continuously during development of indeterminate nodules.

9. ÖSSZEFOGLALÓ

Bevezetés

A pillangósvirágú növények szimbiotikus kapcsolatot alakítanak ki a *Rhizobiaceae* családba tartozó talajlakó baktériumokkal. A pillangós-rhizobium szimbiózisra jellemző egy új szerv, a gyökérgümő kialakulása a növények gyökerén, melyben a baktériumok végzik a légköri nitrogén (N_2) redukcióját cserében a Nap fényenergiáját használó fotoszintézis során beépített szén-dioxidból előállított tápanyagokért.

A szimbiotikus kapcsolat kialakulásához, működéséhez és regulációjához elengedhetelen gének megismerésének leghatékonyabb módja a klasszikus genetikai megközelítés, melynek során a folyamatban hibás mutánsok segítségével azonosítjuk az érintett géneket.

A különböző tetraploid lucernafajták (*Medicago sativa*) keresztezésével előállított utódpopuláció egyedei között azonosított, gümőképzésre képtelen (Nod^-) MnNC-1008(NN) (Peterson and Barnes 1981; Barnes et al. 1988) vonal volt az egyik elsőként azonosított szimbiotikus mutáns (Caetano-Anolles and Gresshoff, 1991). Ez a mutáns képtelen az összes jellegzetes, a rhizobiumok illetve a bakteriális jelmolekulák, a Nod faktorok által kiváltott válaszreakciók megjelenítésére. Mi több, ez a növény a foszformobilizáló mycorrzizás szimbiózis kialakítására is képtelen (Bradbury et al. 1991). A szimbiotikus kapcsolatok kialakításához elengedhetetlen gént az MTA Genetikai Intézetének Lucerna Genetika Csoportja térképezésen alapuló klónozással izolálta és egy NORK-nak (Nodulation Receptor Kinase) elnevezett receptor-szerű protein kinázt azonosított (Endre et al. 2002a; 2002b). A térképezésen alapuló klónozás utolsó lépése lett volna az MN-1008 mutáns komplementálása a vad típusú *NORK* génnel. Ezt azonban nem lehetett megvalósítani a gyors, *Agrobacterium rhizogenes* segítségével végrehajtott transzformáció segítségével, ugyanis a képződött transzformált gyökereken (hairy roots) nem képződnek szimbiotikus gümők (Beach and Gresshoff 1988). Mi több, a tradicionális, A. tumefaciens segítségével végzett transzformációs módszert sem lehetett alkalmazni, mert az MN-1008 vonal nem embriogén.

Célkitűzések

1. Új, alternatív komplementációs stratégiák segítségével a *NORK* génnek a szimbiotikus kapcsolatok kialakulásában betöltött nélkülözhetetlen szerepének igazolása.

2. A *NORK* gén kifejeződésének és regulációjának vizsgálata a célból, hogy feltárhassuk a gümőképzés során betöltött potenciális funkcióit.

Módszerek

- *Agrobacterium rhizogenes* segítségével megvalósított növénytranszformáció
- *Agrobacterium tumefaciens* egítségével megvalósított növénytranszformáció
- DNS isoláció és hibridizáció
- PCR analízis
- Reverz transzkripciós (RT-) PCR analízis
- β-glükuronidáz (GUS) aktivitás hisztokémiai lokalizálása

Eredmények és megvitatásuk

***NORK* mutációk komplementálása más fajokban**

Mivel sem az *A. rhizogenes* sem az *A. tumefaciens* segítségével végrehajtott növénytranszformációval sem volt megvalósítható az MN-1008 vonal komplementálása, a közeli rokon faj, a *M. truncatula* ortológ génjében mutációt hordozó *dmi2* mutánsainak funkcionális komplementálását értük el a vad típusú NORK szekvenciának az *A. rhizogenes* segítségével történő bejuttatásával.

Alternatív stratégiák kidolgozása az MN-1008 vonal Nod$^-$ fenotípust okozó mutációjának komplementálására

Kidolgoztunk egy eljárást, melyet sikeresen alkalmaztunk stabil transzformáns vonalak előállítására a nem embriogén MN-1008 lucerna vonal Nod$^-$ fenotípust okozó mutációjának komplementálása céljából. A tetraploid *M. sativa* néhány fajtája képes szomatikus embriogenezisre, mely tulajdonságot – több vonal genetikai analízise alapján – legalább két gén domináns allélja határoz meg, melyek általában szimplex/szimplex (Aaaa/Bbbb) konfigurációban vannak a tesztelt embriogén vonalakban (Wan et al. 1988; Hernandez-Fernandez et al. 1989; Kielly and Bowly 1992; Crea et al. 1995). Megközelítésünk alapja az, hogy az embriogenitást meghatározó gének domináns alléljei, a mutáns fenotípus, valamint a komplementációt biztosító vad típusú transzgént halmozni lehet egy egyedben több keresztezési és szelekciós lépés kombinálásával. Az itt leírt stratégiát általánosan alkalmazni lehet hasonló körülmények között, azaz amikor a transzformálni kívánt növény embriogenezisre képtelen, de a vizsgált fajból rendelkezésünkre áll(nak) emriogén vonal(ak).

Három stratégiát lehet használni egy nem embriogén növényi vonalban levő mutáció komplementálására: A komplementációra használandó DNS konstrukciót be lehet transzformálni egy embriogén vonalba, majd a transzgenikus és a mutáns növényt keresztezzük (route P= szülői út), vagy pedig a mutáns allél(eke)t keresztezéssel bejuttatjuk egy embriogén vonalba, melyet a hibrid F1 vagy az F2 (/BC) populáció embriogén egyedeinek transzformálása követ (F1 és F2 utak).

Az első alternatív út (Route P) során a vizsgálni kívánt gént az embriogén szülőbe (P_{WE}) transzformáljuk. A regenerált transzgenikus egyedeket kiválasztjuk (P_{ET}), majd keresztezzük a nem embriogén mutáns (P_M) vonallal, hogy egy növényben legyen jelen a mutáció és a transzgén. Azonosítjuk a transzgenikus F1 egyedeket ($F1_T$) és önbeporzással, vagy a mutáns növénnyel történő visszakeresztezéssel szegregáló populációt állítunk elő. Utolsó lépésként a második utódgenerációban azonosítjuk azokat a transzgenikus egyedeket (I_{MT}), melyek homozigóta formában hordozzák a gén mutáns alléljét. Az F1 és F2 utak (Routes F1 and F2) a nem embriogén mutáns (P_M) vonal és az embriogén szülő (P_{WE}) keresztezésével kezdődnek, mely után az embriogén heterozigóta egyedeknek (F_{1E}) a hibrid F1 populációból történő kiválasztása következik. Az egyik lehetséges következő lépés (Route F2) ezen F_{1E} hibrid növények önbeporzása, majd az embriogén, homozigóta mutáns egyedek (I_{ME}) kiválasztása az F2 populációból. A kívánt génkonstrukció(ka)t később *A. tumefaciens*-mediálta transzformációval be lehet juttatni ezen növényekbe. Ebben az esetben, mivel az embriogén mutáns növény a rendelkezésünkre áll, számos transzformációs kísérletet tervezhetünk különböző gének/génkonstukciók tesztelésére. Ha a jelölt gén már a kezünkben van, akkor mód van az embriogén F1 egyedek idegen DNS-sel történő transzformálására (Route F1). A transzgenikus hibrid növények (F_{1T}) azonosítása után a transzgenikus, homozigóta mutáns növények (I_{MT}) előállításának lépései megegyeznek a "szülői út" (Route P) során leírtakkal.

A *NORK* gén mutáns alléljait hordozó embriogén F1 növények előállítása

Az eredetileg a nem embriogén MN-1008 lucerna növény által hordozott mutáció komplementálása valamint a módszer gyakorlatban történő kipróbálása céljából a térképzésen alapuló génklónozás térképezési és szekvenálási munkáival párhuzamosan végrehajtottuk az F1 és F2 utak közös kezdeti lépéseit. Akkor, a jelölt gén hiánya miatt a "szülői út" (Route P) megvalósításának lehetőségét elvetettük. Első lépésként a Nod^+ és Nod^- allélokat heterozigóta konfigurációban hordozó, embriogén F1 növény (F_{1E}) előállítása céljából kereszteztük a nem

embriogén, Nod^- MN-1008 mutánst (P_M) a nagyon embriogén, Nod^+ Regen S szülővel (P_{WE}). Az utódok hibrid jellegét a keresztezés irányától függően gümőképzési teszttel illetve hibridizációval ellenőriztük.

Az F1 hibridek közül azonosítottuk azokat az egyedeket ($F1_E$), melyek képesek voltak szomatikus embriók létrehozására, s közülük kiválasztottunk egyet, melynek az volt az előnyös tulajdonsága, hogy önbeporzás után nagy mennyiségű magot, azaz nagy utódgenerációt tud létrehozni. Ez az embriogén F1 vonal kétféle lehetőséget kínált a stabil transzgenikus vonalak előállítására, a transzgén általi komplementáció vizsgálatára.

Az F2 stratégiát követve az F1 növény önbeporzásával előállítottuk a második utódgenerációt, melynek egyedei közül kiválasztottuk azokat a növényeket, melyek homozigóták voltak a Nod^- tulajdonságot hordozó genomi régióra nézve. Meglepetésünkre, ezen mutáns növények közül egyetlen egy sem volt képes szomatikus embriogenezisre. Egy lehetséges magyarázata lehet az embriogén mutáns egyedek (I_{ME}) hiányának, hogy az embriogenitásért felelős gének egyike genetikailag szorosan kapcsolt a *NORK* génhez, s a populációban nem találtunk olyan mutáns egyedet, mely a megfelelő (azaz az embriogenitásért felelős domináns és mutáns *NORK*) alléleket hordozó rekombins kromoszómával rendelkezett volna.

Az embriogén F1 növények transzformációja

A jelölt gén azonosítása után az *nn1* mutáció komplementálására az F1 stratégiát (Route F1) követtük, s a *M. truncatula* vad típusú *NORK* génjét transzformáltuk az embriogén $F1_E$ növénybe.

Az elsőként regenerált növényeket DNS–DNS hibridizációval teszteltük, hogy genomjukban a transzgén jelenlétét kimutassuk, kópiaszámát meghatározzuk. Restrikciós enzim emésztéssel kapcsolt reverz transzkripciós (RT-) PCR amplifikációval kimutattuk, hogy a vad típusú *M. truncatula NORK* (*MtNORK*) gén minden vizsgált $F1_{ET}$ növény gyökérszövetében kifejeződött.

A vad típusú transzgént hordozó homozigóta mutáns növények azonosítása

A legnagyobb magtermelő képességgel rendelkező transzformált $F1_{ET}$ egyedet és klónjait választottuk ki az F2 populáció létrehozására. Önbeporzás után 727 magot gyűjtöttünk össze és vetettünk el, közülük 622 F2 növény nőtt fel, melyeket analizáltunk. Egy a mutációval szorosan kapcsolt, PCR alapú genetikai markert használtunk azon hét F2 egyed

kiválasztására, melyek a Nod régióban, azaz a *NORK* gén szomszédságában megfelelő (mutáns) homozigóta genotípussal rendelkeznek.

A mutáns allélek homozigóta konfigurációjának igazolására, valamint a vad típusú *NORK* transzgén jelenlétének illetve távollétének kimutatására hibridizációs kísérleteket végeztünk a hét növény genomiális DNS-ével egy *NORK* próba felhasználásával. A hat Nod^+ fenotípusú, de a mutációra homozigóta növény genomja hordozta a *M. truncatula NORK* génjét, míg a hetedik homozigóta, s Nod^- fenotípusú egyed genomjában nem lehetett kimutatni a transzgén jelenlétét. Ez a hibridizációs mintázat egyrészt azt bizonyítja, hogy a Regen S vonalból származó endogén, vad típusú *NORK* allél hiánya Nod^- fenotípust eredményez, másrészt a vad típusú *M. truncatula NORK* (*MtNORK*) transzgéngén felelős az MN-1008 genetikai háttér által meghatározott mutáns fenotípus komplementciójáért.

A *NORK* gén lehetséges szerepének vizsgálata génexpressziós vizsgálatokkal

A *NORK* gén sejt-specifikus expressziójának és regulációjának gyökérben és gyökérgümőben történő vizsgálata céljából a gén 3.1 kb hosszúságú promóter és 5' nem transzlálódó régióját a ß-glükuronidáz (GUS) riporter gén elé klónoztuk. Ezt a hibrid gént bejuttattuk az *A. rhizogenes* Arqua törzsbe, melynek segítségével olyan kiméra *M. truncatula* növényeket állítottunk elő, melyekben a vad típusú hajtások transzgenikus gyökerekkel rendelkeztek.

Kimutattuk, hogy a *NORK* gén a gyökércsúcsot kivéve a gyökér teljes hosszában kifejeződik az epidermiszben és a kéregben, s a legmagasabb expressziót az oldalgyökerek fiatalabb részében figyeltük meg. A gümőképzés során a gén erősen indukálódott a gümő-primordiumokban, magas kifejeződési szintet mutatott összehasonlítva a környező szövetekkel illetve az újonnan fejlődő oldalgyökerekkel. A *NORK* gén indukcióját már két nappal a baktérium inokulációt követően meg lehetett figyelni a kéreg sejtcsoportjaiban, ami a 3. és az 5. nap között tisztán a gümő-primordiumokra korlátozódott, majd fiatal kiemelkedő gümők központi, differenciálatlan sejtjeiben volt látható. A gümőfejlődés ezen szakaszában a *NORK* gén aktivitása inkább az *MtENOD20* (Vernoud et al. 1999), valamint az *MsENOD40-1* és *–2* (Fang and Hirsch 1998), mint az *MtENOD11* és *MtENOD12* gének expressziós mintázatára emlékeztet, melyek nem indukálódnak a primordiumokban. Azonban az *ENOD* gének azokban az epidermális és kéregsejtekben is indukálódnak, melyekből az infekciós fonalak kiindulnak, illetve amelyeken keresztül haladnak (Journet et al. 2001; Pichon et al. 1992; Vernoud et al. 1999).

A NORK gén kifejeződésének lokalizációjára irányuló vizsgálataink arra utalnak, hogy a gén terméke speciális feladatot lát el a gyökér-kéregben és a gümőben is, mely megelőzi és melyre szükség van a baktériumok inváziójához. Egyetértésben az epidermiszben betöltött szerepével, ezek az eredmények alátámasztják azt az elgondolást, hogy bakteriális infekcióhoz vezető Nod faktor felismerésre és jeltovábbításra folyamatosan szükség van az indeterminált gümők fejlődése során.