

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

Summary of Ph.D. Thesis

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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 (N₂) 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 scavenge 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 ($F1_E$). One possible step is to self-pollinate these $F1_E$ 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 possible explanation for the absence of the embryogenic individuals (I_{ME}) in the F2 population is that one of the genes required for embryogenicity 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 endogenous 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 β -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.

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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 DMI2 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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