Laboratory of Plant Molecular Biology
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The roles of *Medicago truncatula* NADPH oxidases during legume-*Rhizobium* symbiosis

Thesis Molecular Biology - Plant Biotechnology

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Premature nodule senescence as the Consequence of *Medicago truncatula* *MtNADPHoxF* Open Reading Frame-mediated Silencing

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Abstract

The nitrogen-fixing endosymbiosis between legume plants and *Rhizobium* is controlled by a plant genetic program that governs two processes in parallel, including nodule primordia formation and a bacterial infection and development process. In this study, we focus on the second process of bacterial development activities during symbiosis with the participation of NADPH oxidases using RNAi approach. We show that at least one of experimented NADPH oxidases, for instance *MtNADPHoxF* which regulates reactive oxygen species (ROS) production in plant cells, attends in symbiosome development process, since their open reading frame (ORF)-mediated RNAi (*orf_F)* nodules in *Medicago truncatula* A17 gives rise to many dead cells inside nodule with a very unstable symbiosis and improper bacteroid development.

Keywords: symbiosis, legume, Rhizobium, bacterial infection and development, NADPH oxidases, silencing, cell death.

INTRODUCTION

The symbiosis between legumes plants and *Rhizobia* bacteria, which converses atmosphere nitrogen into ammonium for plants, is fascinating for evolutional reason. As a result of this association, a new organ is formed on the root of the plant: the nodule.

In order to develop in N_2-fixing symbiosomes, *Rhizobia* have to infect the plant root and subsequently the nodule. This infection process takes place through three main steps: growth of infection thread, bacteria release, and bacteria development. Ultimately, differentiated bacteria fix atmospheric nitrogen into ammonia and receive, in turn from the plant, carbohydrates and other nutrients [Oldroyd and Downie, 2008].

Although some plant defense response genes are differently modulated during the infection process [Samac and Graham, 2007], still, *Rhizobia* successfully invade the plant roots. Most of the single genes essential in legume-*Rhizobia* symbiosis have been identified in the last 10 years, for instance, *MtLYK3* and *MtNFP* [Limpens *et al.*, 2003; Arrighi *et al.*, 2006; Mulder *et al.*, 2006], *DMI1/DMI2/DMI3* [Catoira *et al.*, 2000; Hogg

et al., 2006]. However, the identification of redundant genes is still in progress since this redundancy makes it difficult to separate and assign the specific role of these plant genes.

Our approach to discover the gene network behind the bacterial infection and the later processes during symbiosis is based on an evolutional perspective with a whole genome duplication (WGD). Legumes evolved about 60 million years ago (Ma), and nodulation evolved 58 Ma ago [Sprent, 2007]. It is assumed that a WGD predated the evolution of *Rhizobia*-legumes symbiosis [Guerts *et al.*, 2008, unpublished]. Most of the genes duplicated after WGD are discarded under a very less strong selective pressure; few of these genes under a higher selective pressure will be maintained over time to, possibly, sub- and/or neo-functionalize.

We hypothesize that some of the duplicated genes that have been maintained in nodulating legumes, after the WGD, have evolved a new function. We postulate that the new function might be essential during legumes-*Rhizobia* symbiosis.

More than 250 duplicated gene pairs have been maintained in nodulating legumes up to date [De Mita, 2009, unpublished]. From the expression profile retrieved from Atlas Gene Expression Database [Benedito *et al.*, 2008], only 18 gene pairs among those 250 in *Medicago truncatula* are specifically induced during nodulation. Most of them have relative low expression patterns in non-symbiotic plant tissues. Among these conserved legume-specific gene duplicates there are two NAPDH oxidases, in plant also known as Respiratory Burst Oxidase Homologs (Rboh).

NADPH oxidases of Nox family, present in various supergroups of eukaryotes (but not in prokaryote), are membrane-bound proteins that can produce reactive oxygen species (ROS). NADPH oxidases activation reduces molecular oxygen to superoxide anion (O^{2-}). Rboh in plants consists of three main regions (Fig. 1). In particular, the trans-membrane part contains six α-helices, two of which contain histidine amino acids which can bind the oxygen species; the N-terminus part contains several regulatory sites, among which are two EF-hands to which for instance calcium can bind; and the C-terminus part contains two binding sites, one for the co-factor FAD and one for NADPH. NADPH is the reducing agent in this reaction and donates electrons to oxygen compounds to produce ROS [Sumimoto, 2008].
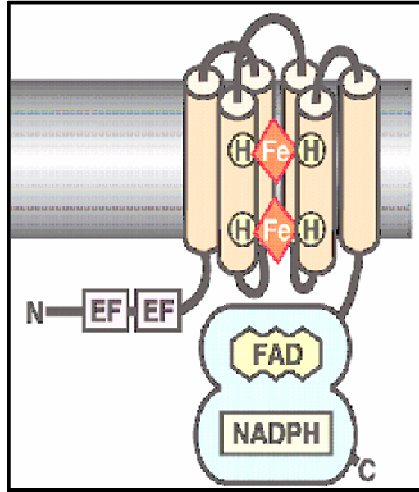
Figure 1. General structure of a candidate plant NADPH oxidase. There are three regions: (1) Six trans-membrane α-helices; (2) N-terminus regulatory region and (3) C-terminus binding sites for (cofactor) FAD and (reducing agent) NAPDH [Sumimoto, 2008].

NADPH oxidases are well-studied *Arabidopsis thaliana*. They are known to produce ROS and to be involved in a wide range of biological process including defense responses against pathogens (i.e. *AtRbohD*) [Torres *et al.*, 2005]; abiotic factors (i.e. *AtRbohA*) [Torres *et al.*, 2002], and developmental processes such as: systematic signaling (i.e. *AtRbohD*) [Kwak *et al.*, 2003; Miller *et al.*, 2009], cell death (i.e. *AtRbohD*) [Levine *et al.*, 1994; Torres *et al.*, 2005], stomata closure [Kwak *et al.*, 2003], and polar root hair growth (*ROOT HAIR DEFICIENT 2* or *AtRHD2* or *AtRbohC*) [Foreman *et al.*, 2003; Monshausen *et al.*, 2007; Takeda *et al.*, 2008]. Recently Tsukagoshi *et al.* [2010] have shown that NADPH oxidase-mediated ROS regulation plays an important role in transition from cell proliferation to cell differentiation.

Although in *A. thaliana* NADPH oxidases have been extensively studied, in *M. truncatula* little is known about their biological roles. Moreover, phylogenetic studies do not shine a light on the putative function of the legume-specific duplicated *MtNADPH E/F* since no clear *A. thaliana* orthologues of *MtNADPHoxE/F* are present (Fig. 2).
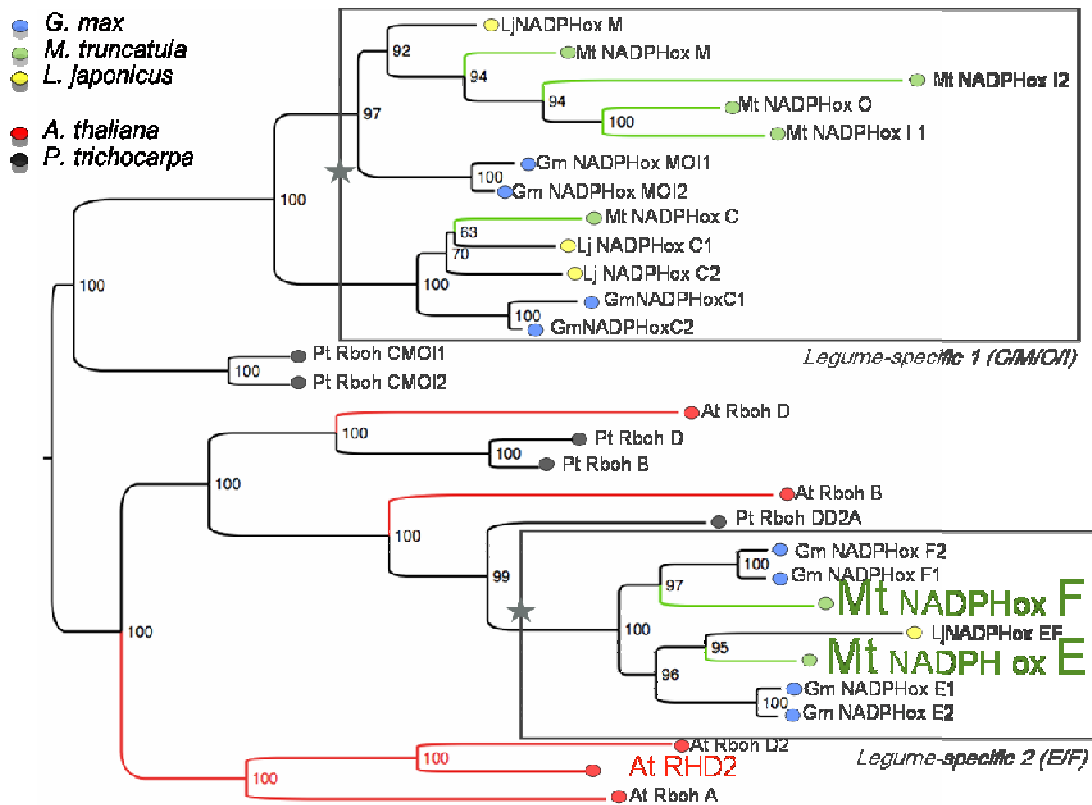
Figure 2. Phylogenetic tree of NADPHox gene family among non-leguminous and leguminous plants [Mita, 2009, unpublished]. Five plant species of three legumes (*Glycine max* – "Gm"; *Medicago truncatula* – "Mt" and *Lotus japonicus* – "Lj") and non-legumes (*Arabidopsis thaliana*– "At" and P*arasponia trichocarpa* – "Pt") are embedded in the phylogenetic tree. Two legume-specific duplications are represented in black square boxes, namely (i) *MtNADPHox C/I/M/O* and (ii) *MtNADPHoxE/F*.

Despite their poorly understood roles during symbiosis, these legume-specific NAPDH oxidases, *MtNADPHoxE/F,* are of our strong interest not only because of their phylogenesis, but also because of their expression in nodules. According to Atlas microarray data [Benedito *et al.*, 2008], on one hand, *MtNADPHoxF* is highly induced from 10 to 14 day old nodules and during fungal responses of *Phymatotrichum*. On the other hand, its duplicate, so-called *MtNADPHoxE*, is mostly constitutively expressed ubiquitously in plant tissues, except for seed, and highly induced 96 h.p.i with *Phymatotrichum* (Fig. 3).
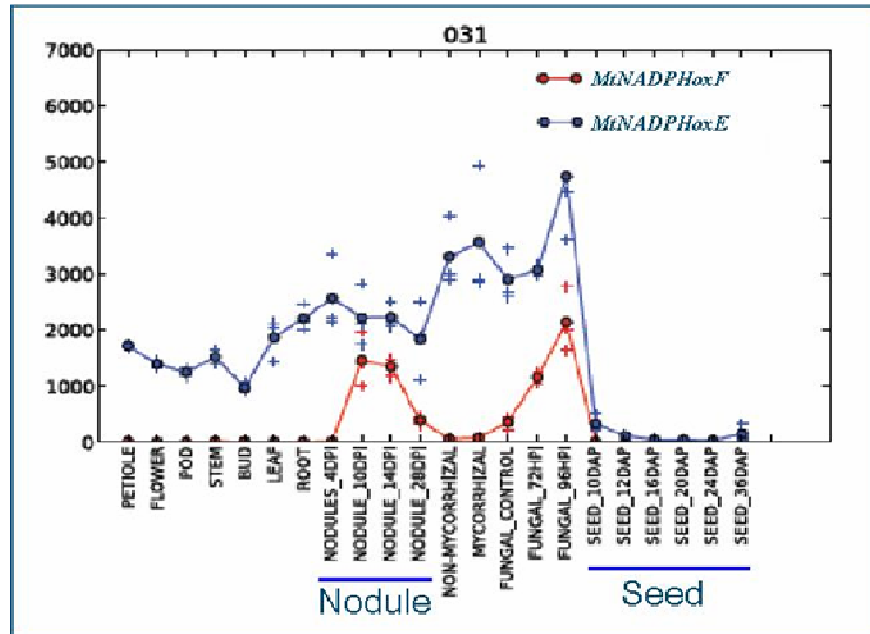
Figure 3. Expression profile of *MtNADPHoxE* (blue line) and *MtNADPHoxF* (red line) in *M. truncatula A17*. Values in vertical and horizontal axes are levels of Affymetrix probe singal based on microarry data from the *M. truncatula* Gene Expression Atlas Database [Benedito *et al.*, 2008]. DPI: days post inoculation; HPI: hour post inoculation.

Furthermore, detailed promoters studies have shown that both NADPH oxidases are expressed in the same tissues but with different level of expression. *MtNADPHoxF* is highly expressed in the apical part of the nodule, while its expression is lower in the root tips and vasculatures [unpublished]. In the nodule apical part the promoter is active in the meristem, in the infection zone, and also in one or two cell layers in the fixation zone where symbisomes start differentiating (Fig. 4, right). On the other hand, *MtNADPHoxE* is highly express in the root tip and vasculature, and lower in the apical part of nodule: meristem and infection zone (Fig. 4, left).

The specific expression patterns of these two duplicated genes with opposite expression levels suggest that at least one of them, most likely the highest induced, *MtNADPHoxF,* might play a role during nodulation.
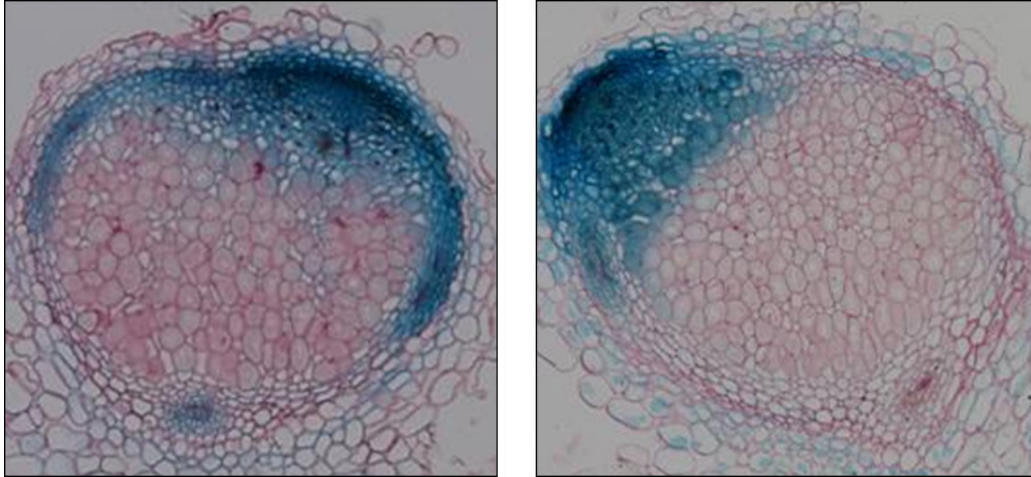
Figure 4. GUS staining of micro-sectioned 14 d.p.i *M. truncatula A17* nodules transformed with *NADPHoxidaseE* (left) or *NADPHoxidaseF* (right) promoter driving *GUS*.

This hypothesis is supported by the sub-cellular localization of both NADPH oxidases *E* and *F*. Both proteins are shown to localize mainly in the infection zone of the nodule (Fig. 4, left) and, more precisely, in the releasing zone, where they are possibly associated with bacterial release (Fig. 4, right) [Lillo *et al.*, 2010, unpublished].
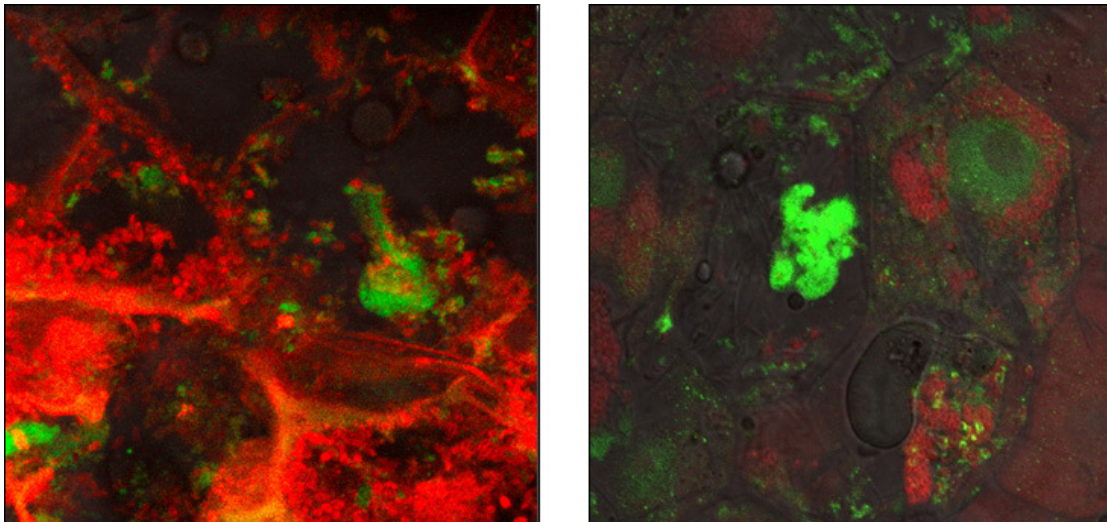

Figure 5. Confocal images of *MtNADPHox E* **and** *F*. **Left: infection zone; right: releasing zone.**

Since the precise sub-cellular localization cannot be fully resolute, it is still unclear whether these two trans-membrane proteins are localized in ITs plasma membranes or in endoplasmic reticulum, or in vesicles around the place of the release.

Considering all data from the previous studies mentioned above, during my Msc. Thesis I tried to investigate the following research question:

"What is the biological function of MtNADPHoxE and/or MtNADPHoxF during nodule formation?"

To address it, we hypothesized that at least one of the two possibly redundant gene duplicates is involved in a biological role during symbiosis. In addition, based on the tissue specificity and the localization studies, I hypothesized a role in: infection threads growth, and/or bacteria release associated, and/or in the early steps of symbiosomes development. To determine which of these are indeed the role of *MtNADPHoxE* and/or *MtNADPHoxF* during nodule formation, I used a RNAi silencing approach for functional analyses of these genes.

APPROACH

With RNAi silencing experiment, I aim to investigate the effect of reduction in expression of each NADPH oxidases, *E* and *F*, individually and simultaneously during symbiosis. I designed specific and cross silencing constructs to investigate the functions of each single gene, *MtNADPHoxE* or *MtNADPHoxF,* but also of both of them.

Two strategies have been adopted to knock-down the mRNA levels of *MtNADPHoxE* and or *MtNADPHoxF*:

(1) Specific silencing of single genes using short RNAi fragments with low identity ($\leq 15\%$) between *MtNADPHoxE* or *MtNADPHoxF* (about 500 bp each);

(2) Cross silencing of both *MtNADPHox E* and *MtNADPHoxF*. I used short RNAi fragments (under 300 bp) highly identical (approximately 80%) between *NADPH oxidases E* and *F* (2a); and the open reading frame of each gene (2b).

Since I hypothesize a role of *MtNADPHoxE* and/or *MtNADPHoxF* during symbiosis in infection zone, and or in place of release, and or during the first step of bacteria development, a specific approach is required to direct the silencing in each zone of nodule. To knock down *MtNADPHoxE* and/or *MtNADPHoxF* in different zone of the nodule I use two promoters to drive the silencing constructs: *p35S and pENOD12*. The first is used to reduce the target gene(s) mRNA levels constitutively in the root and in the apical zone of nodule: meristematic and infection zone; while the second is utilized to point towards the infection zone only [Nap and Bisselling, 1990].

RESULTS

Based on the approach described above, the following results were obtained:

(1) Specific silencing of *MtNADPHoxE* and *MtNADPHoxF* gives no symbiotic phenotype.

To elucidate the function of NADPH oxidase *E* and NADPH oxidase *F*, RNAi studies have been performed in *M. truncatula* roots and nodules transformed with specific short silencing constructs (269 and 413 bp, respectively). Furthermore, to better identify in which zone of the nodule these genes could have a symbiotic function, two different promoters have been used to drive the silencing constructs: *pr35S* and *prENOD12*.

Nodules and roots transformed with the six different silencing constructs (*pr35S*:*spec_E*, *pr35S*:*spec_F*, *pr35S*:*cross_EF*; *prENOD12*:*spec_E*, *prENOD12*:*spec_F*, *prENOD12*:*cross_EF*) have been analyzed after 14 days of inoculation (d.p.i). In all cases transformed nodules did not show a phenotype different from the control.

Cytologically, the 14 d.p.i. RNAi nodules showed a proper zonation: the apical part with small meristematic cells reach of cytoplasm, the infection zone with several ITs and the fixation zone with fully infected cells. Thus, bacteria were released properly into nodule cells and were capable of differentiating into elongated bacteroids. These observations indicate a normal nitrogen-fixing activity and a functional symbiosome development in RNAi nodules (Fig. 5). In brief, the exclusive short RNAi constructs of *MtNADPHoxE* and/or *MtNADPHoxF* introduced into the *Medicago* roots do not interfere with the normal nodule organgenesis and development.

(2) Cross silencing of MtNADPHoxE and MtNADPHoxF triggers premature nodule senescence.

(2a) Short RNAi fragment mediated silencing of MtNADPHoxE and MtNADPHoxF gives no significant difference in the total numbers of forming nodules.

To elucidate the putative redundant function of NADPH oxidase *E* and NADPH oxidase *F*, RNAi studies have been performed in *M. truncatula* roots and nodules transformed with short cross silencing constructs (263 bp). Fourteen d.p.i nodules transformed with short cross-silencing fragment showed neither significant difference in total number or in cytology compared with the control's (see the example in Fig. 6), independently from the promoter used: *35S* or *ENOD12*.
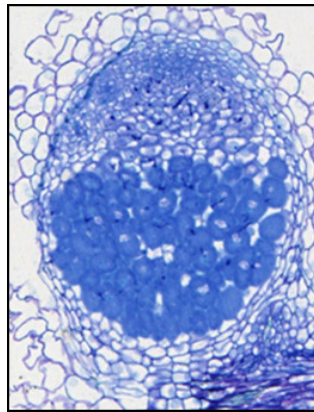

Figure 5. Toluidine blue-stained section through a 14-day-old root nodule of *prENOD12*:*cross_EF* RNAi.
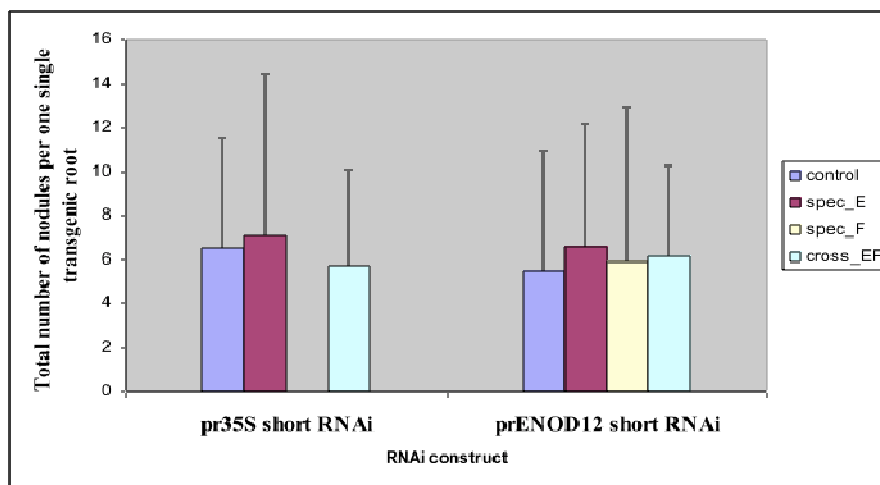

Figure 6. Effect of *pr35S* or *prENOD12*: short RNAi fragment silencing on the total number of nodules per transgenic root of *M. truncatula* A17 compared with the control. Black arrows represent the standard

deviation of the values. Values were scored on 20-30 single transgenic roots. The *LSD* test was performed based on the *ln* values of the original data, which gave a relatively normal distribution. The significant difference among the means was statistically considered at $p < 0.05$.

(2b) ORF-mediated silencing of MtNADPHoxF triggers premature nodule senescence and a reduction in nodule numbers.

To elucidate the putative redundant function of *NADPHoxE* and *NADPHoxF* during symbiosis, RNAi studies have been performed in *M. truncatula* roots and nodules transformed with full ORFs of *NADPHoxE* (2749 bp) or *NADPHoxF* (3063 bp). The combination of each ORF, *NADPHoxE* or *NADPHoxF,* and each promoter, *pr35S* or *prENOD12,* eventually resulted in four RNAi constucts: *pr35S:orfE*, *pr35S:orfF*, *prENOD12:orfE*, *prENOD12:orfF*.

Only 14 d.p.i nodules transformed with *pr35S:orfF* silencing construct showed significant difference in total number and cytology compared with the control's. In details, *pr35S:orfF* total nodule number was nearly two-fold decreased compared with control plants (Fig. 8). The *LSD* of means of the total nodule number between *pr35S:orfF* vs. the control showed a significant difference (*p*-value 0.004). From these data it is likely that the gene silencing of *MtNADPHoxF* resulted in the abnormality in capacity to form nodules.
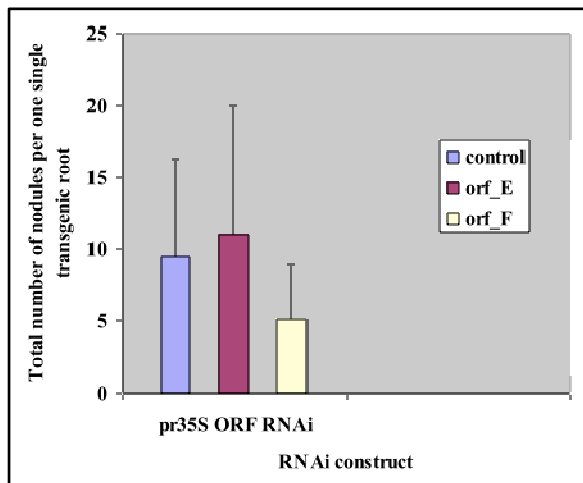

Figure 8. Effect of *pr35* ORF RNAi knock-down on the total number of nodules per one single transgenic root of *M. truncatula* A17 compared with the controls that were transformed with orf-free *pr35S* destination vectors. The black arrows represent the standard deviation of the values. The RNAi treatment group mean was scored on a sample of 20-30 single transgenic roots. The *LSD* test was performed based on the *ln* values of the original data, which gave a relatively normal distribution. The significant difference among the means was statistically considered at $p < 0.05$.

For the *pr35S:orfF* transformed nodules, no proper zonation but early senescence was observed in the fixation zone. Cytologically, the nodules showed a proper apical zonation: small meristematic cells reach of cytoplasm in the meristemaic zone, and

several ITs the infection zone where bacteria release appeared to be successful. Only after bacteria release, plant cells showed early senescence (Fig. 7, B, C, light-green staining). It is likely that these cells have lost the ability to maintain the symbiosomes during development soon after the bacteria. Moreover, the phenotype is statistically significant as the percentage of aberrant *pr35S:orfF* nodules is seven-fold higher (23%) than the control (3%) (Table 1). In brief, 14 d.p.i *pr35S:orfF* transformed nodules gave rise to an unstable symbiosis, in which many nodule cells in the fixation zone became dead without fixing N_2, and restricted the ability to form functional nodules.
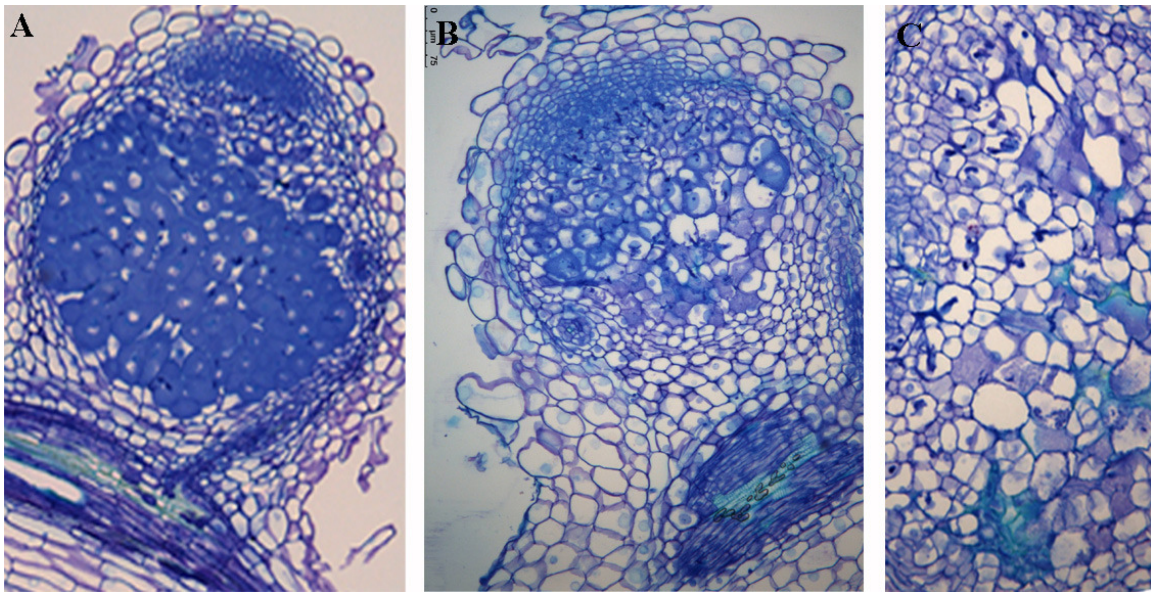

Figure 7. Toluidine blue-stained section through a 14-day-old developing root nodule. (A) The control nodule transformed with an empty *pr35S* destination vector. (B) RNAi nodule transformed with *pr35S*:*orfF*. (C) Magnified views of infection zone and fixation zone of nodule section in (B).

Table 1. The percentage of aberrant nodules scored in *orfF* RNAi *M. truncatula* plants driven by promoter 35S. The control plant was transformed with *Agrobacterium rhizogenes* carrying an empty promoter *35S* destination vector.

35S:orf RNAi	Total number of nodules	Number of aberrant nodules	Percentage (%)
Control	42	1	3
Orf F	44	10	23

CONCLUSIONS AND DISCUSSIONS

In the present study, I aim to investigate the function of *M. truncatula* NADPH oxidase *E* and *F* during symbiosis. Using RNAi approach is known to be one way possibly to cause symbiotic mutants, a strong evidence to address the function of these genes. Identified plant symbiotic mutants are either perturbed in their capacity to form nodules (absent or very few nodules per plant) or show increased numbers of nodules (supernodulators) [Crespi and Galvez, 2000]. Silencing experiments support the hypothesis that at least one of the NADPH oxidases is essential in symbiosis. It has been shown that RNAi effect, for example with *35S:orfF*, not only induce the nodule senescence, but also decrease the nodule number of the roots. The nodule early senescence and reduction of nodule can be explained by two scenarios:

    (1) Effective cross silencing of multiple and redundant NADPH oxidases

    (2) Effective specific silencing of NADPH oxidase *F* only.

Since NADPH oxidases *E* and *F* are both localized at the place of bacterial release but the phenotype is evident only in few cell layers, NADPH oxidase *F* (and *E*) should have a function after bacteria release. To clarify whether NADPH oxidase *F* only or more NADPH oxidases have the same function after bacteria release, qPCR analysis is still needed.

Reduction in ROS production associated with the place of release seems to impair cell longevity. ROS burst could be important for degradation of components released in association with bacteria release. Since bacteria differentiation and development does not seem to be directly impaired in silenced nodule, a more general process associated with symbiosis might be impaired. Transmission Electron Microscopy (TEM) images of silenced nodules might be informative on their sub-cellular phenotype. These further studies might in turn pave the way for unraveling the biological roles of *MtNADPHoxE/F*.

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MATERIALS AND METHODS

Plant materials and growth conditions

Plants from line *M. truncatula* accession A17 were the materials for all transformations. Surface-sterilized seeds were germinated in Petri dishes on Farhaeus media and grown vertically during 10 days in a growth chamber at 23°C under 16h light/8h dark photoperiod. Plant transformation was performed by a cut through the hypocotyls, using a blade covered in transgenic *Agrobacterium*. After one week of transformation, plants were transferred to Emergence media and grow for two weeks or until the root systems were fully developed. Transformed plants continued growing in perlite substrates watered with nitrate-free Farhaeus media, and after two days inoculated with a rhizobial suspension in water ($OD_{600} = 0.1$). Plants were left to grow under the same photoperiod of 16h light/8h dark. For all RNAi silencing experiments nodules were harvested and investigated at 14 d.p.i..

Bacterial strains

Agrobacterium rhizogenes strain MSU440 was used for *M. truncatula A17* hairy root transformations according to the protocol of Boisson-Dernier *et al.* [2001]. Inoculated *Rhizobium* was *Sinorhizobium meliloti* strain 2011 pHC60 expressing GFP [Limpens *et al.*, 2003]. Rhizobia were grown on YEM or TY media supplemented with the appropriate antibiotics at 28°C until the OD reaches 0.6.

RNAi constructs and plant transformation

Five different *attB*-tagged RNAi fragments of *MtNADPHox E* and *F* including three short (*spec_E* and *spec_F* for specific and *cross_EF* for cross silencing) and two long open reading frame (ORF) (*orfE* and *orfF* for cross silencing) were amplified from the *M. truncatula* cDNAs with Phusion High Fidelity DNA Polymerase (provided by New England Biolabs). The primer sequences and PCR product lengths were described in Table 2.

The PCR products of target RNAi fragments from the cDNA templates were constructed using Gateway Technology (Invitrogen) and first cloned into the entry pENTR1,2 vector (BP recombination) before individually sub-cloned into each pDEST-277 (pDEST-277 *pr35S* and pDEST-277 *prENOD12*) (LR recombination) according to the manufacturer's instructions (Directional TOPO Cloning Kit, Invitrogen[R]). The control is the corresponding empty pDEST-277 without cloning any RNAi fragment. BP recombinant

plasmids were check by digestion with relevant restriction enzymes and double checked with sequencing using reverse and forward M13 primers. LR recombinant plasmids with two directional RNAi fragments were subsequently verified by restriction enzyme digestion and/or colony PCR. DNASTAR software and Plasmid Editor ApE program (version 1.17) were used to analyze the DNA sequences.

Table 2. RNAi fragments, corresponding primers and PCR lengths.

Name	Forward and reverse primer sequences	PCR length (bp)
	ATCTATACATATTCATTTCTCCTCCAAA]	
Spec_E	CGTAATCCTGATCCACCTCA	413
	AAAGTAGTAATTGGCTTGGAATTG	
Spec_F	CCATTTCACCATCGTCCTTG	269
	AGCAAGCGGTGACCAAAGT	
Cross_EF	TTTCATGTTGTTTAGTACATCTTTGA	263
	AGATGGAGATTAGTCAAGAGAGAGAA	
OrfE	TCTAAAAATTCTCTTTATGAAACTCAAATT	2749
	GAATGGAAATTGATCAAGAGAACAAA	
OrfF	TCTAAAAATTCTCTTTATGAAAATCAAACT	3063

Table 3. The scheme of transformation with different constructs made and their corresponding controls. Each of three destination vectors (pKG277-pr35S and -prENOD12) recombined with each of five different RNAi fragments (spec_E, spec_F, cross_EF, orfE anf orfF), eventually 10 RNAi constructs were transformed into Medicago truncatula WT A17. The control was transformed with an empty destination vector contained the corresponding promoter carried by Agrobacterium rhizogenes without the target RNAi fragment.

Promoter	RNAi fragment	Construct name	Control
pr35S	*Spec_E*	*pr35S*:*spec_E*	*pr35S*:control
	Spec_F	*pr35S*:*spec_F*	
	Cross_EF	*pr35S*:*cross_EF*	
	OrfE	*pr35S*:*orfE*	
	OrfF	*pr35S*:*orfF*	
prENOD12	*Spec_E*	*prENOD12*:*spec_E*	*prENOD12*:control
	Spec_F	*prENOD12*:*spec_F*	
	Cross_EF	*prENOD12*:*cross_EF*	
	OrfE	*prENOD12*:*orfE*	
	OrfF	*prENOD12*:*orfF*	

Plant transformant selection

Successfully transformed roots and nodules expressing the marker gene DsRed were selected using Leica MZIII fluorescence stereomicroscope with DsRed filter setting.

Micro-sectioning and staining

RNAi and WT nodules were fixed in 4% paraformaldehyde with 3% glutaraldehyde in 50 mM phosphate buffer (pH7.4) and embedded using Technovit 7100 (Heraeus Kulzer). 5-10 μm sections were cut using a Reichert-Jung 2035 microtome. Sections were counterstained using 0.05% toluidine blue and analyzed under a Nikon Optiphot-2 microscope.

Statistics analysis

Total number of nodules per one single transgenic root was scored on RNAi transgenic root system. RNAi treatment group mean was obtained from a sample of 20-30 transgenic root individuals and Least Significant Difference (*LSD*) test was performed on the *ln* values of the original data, which gave a relatively normal distribution. SPSS software (version 16.0) was employed to analyze the data. The significant difference among the means was statistically considered at $p < 0.05$.