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FINAL REPORT

Small RNA regulation of plant NB-LRR defense gene family

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Abbreviations:

AGO: Argonaute protein
AMV: alfalfa mosaic virus
A. thaliana: Arabidopsis thaliana
DCL: DICER-LIKE
Exo Y: The key Sinorhizobium meliloti succinoglycan biosynthesis gene
Flg22: 22 amino acids peptide, corresponding to the most conserved domain of bacterial flagellin
N. benthamiana: Nicotiana benthamiana
miRNA: microRNA
mRNA: messenger RNA
M. truncatula: Medicago truncatula
pha-siRNA: phased siRNA
PolIV: RNA polymerase IV
PolV: RNA polymerase V
RDR: RNA Dependent RNA Polymerase
RITS complex: RNA Induced Transcriptional Silencing complex
ROS1: REPRESSOR OF SILENCING
S. meliloti: Sinorhizobium meliloti
siRNA: short interfering RNA
S. lycopersicum: Solanum lycopersicum
sRNA: small RNA
ta-siRNA: trans-acting siRNA
WT: wild type

Summary

Plant genomes contain NB-LRR innate immune receptors to recognize different pathogens. However, we have very little information about the expressional regulation of NB-LRR resistance genes. The widely accepted view is that they are constitutively expressed; however this has not been vigorously tested and also raises the problem of high fitness cost. Understanding the regulation of NB-LRR resistance genes are important, because it may result new type of pathogen resistant plants, which provide better protection of crop plants than the currently available methods. This is particularly urging, because actual yield losses to major crops due to pathogens are estimated at 15 % which represents a significant threat to agricultural production. Therefore, crop protection is a very important player securing food in the foreseeable future to meet the food and feed demands of a rising human population and increasing livestock production.

In recent years, miRNAs and tasiRNAs emerged as an important post-transcriptional gene expression regulators of various cellular processes including both biotic and abiotic stress. In this project we tried to understand the role of the tomato miRNA482 family and the *Medicago truncatula* miRNA2118 family and ta-siRNAs derived from TAS loci homologous to R genes during biotic stress in plants. We identified six TAS loci that are related to immune receptor genes of the nucleotide binding and leucine-rich repeat (NB-LRR) class and produce ta-

siRNAs from the conserved P-loop region. The expression level of the phase initiating miRNA482 family and the produced ta-siRNAs are changed during pathogen infection suggesting that a large number of immune receptor genes are potentially regulated by these miRNAs and ta-siRNAs. We determined the tissue and developmental stage specific expression pattern of the NB-LRR regulating tomato miRNA482 family and the tasiRNAs derived from the TAS loci showing homology to NB-LRR genes. We found that the tomato miRNA482 family regulates innate immunity during plant growth and development and keeps the level of NB-LRR genes at different level at the different developmental stages. We believe that miRNA mediated repression of NB-LRR gene expression provides an elegant and general mechanism for plants to reduce fitness cost, yet maintaining a large repertoire of NB-LRR receptors.

Legumes enter symbiotic interactions with microbes, however it is poorly understood how they differentiate between symbiotic or pathogenic microbial interactions. Based on the recent literature symbiotic interaction and nodulation may require the suppression of host defences to prevent immune responses. The *M. truncatula* genome encodes apr. 540 NB-LRR genes and more than 60 % of these genes can be targeted by the NB-LRR targeting miRNAs (miRNA2118, miRNA2109 and miRNA 1507) or by the phasiRNAs that are produced by the action of the NB-LRR targeting miRNAs. We found that upon pathogen infection these NB-LRR regulating miRNAs expression level is decreasing, similarly as miRNA482 in tomato. However, the expressions of these miRNAs are induced at the early phases of symbiotic interaction and it is also maintained in the symbiotic nodule. We also found that the target mRNAs of these miRNAs are downregulated in the symbiotic nodules. Furthermore, the inhibition of the expression of these miRNAs reduced the number of the symbiotic nodules, while their overexpression resulted increased number of nodules. These result shows that the three NB-LRR regulating miRNAs indeed contribute to the regulation of the NB-LRR genes in *M. truncatula* and this regulation could be very important in the symbiotic nodule development, because the downregulation of NB-LRR resistance genes in the developing nodule could produce a suitable niche that makes possible the bacterial colonization and the development of N-fixing nodule tissue.

The results of this research were continuously presented on international and national conferences, with 7 presentations (2 posters and 5 lectures) and it also resulted a MSc. thesis. Preparation of a manuscript based on the results on “small RNA regulation of plant NB-LRR defense gene family during pathogen and symbiotic interactions” is already in progress. The results and figures shown in this report outline the high quality of this study, which is anticipated to be published in a top academic peer reviewed journal with high impact factor. The results of this work are expected to be a highly cited reference for future studies.

Content:

1. Brief Introduction

Pathogens represent a major threat to agricultural production and it is estimated that they contribute to 15 % yield losses in major crops (Oerke and Dehne, 2004). Since, plants have evolved together with pathogens they developed several defence pathways to overcome pathogen infection. Plant genomes contain two major classes of innate immune receptors to recognize different pathogens (Chisholm et al., 2006; Jones and Dangle 2006). The first line of defence comprises the **p**attern **r**ecognition **r**eceptors (PRRs) that recognize conserved **p**athogen-**a**ssociated **m**olecular **p**atterns (PAMPs) and activate **P**AMP-**t**riggered **i**mmunity (PTI). However, PTI can be suppressed by the pathogen, usually through secreted proteins called effectors. Plant genomes also contain large numbers of resistance (R) genes with nucleotide binding (NB) and leucine-rich repeat (LRR) domains (Meyers et al., 2005), which function as an a second line of defense that recognize specific pathogen effectors and initiate **e**ffector **t**riggered **i**mmunity (ETI). In addition to innate immune receptors, in plants and other eukaryots small RNA (sRNA) systems mediate gene silencing which is thought to have evolved as a defence system against viruses and other molecular pathogens (Ding 2010; Ding and Voinnet,.2007). sRNAs are also an important gene expression regulators involved in many developmental processes and stress responses. These molecules are generally 21-24 nt long and based on their biogenesis we distinguish two main classes: microRNAs (miRNAs) and small interfering RNAs (siRNAs). miRNAs function in post-transcriptional manner by down regulating target mRNAs in a variety of cellular prosesses (Bartel 2004; Jones-Rhoades et al., 2006; Mallory and Vaucheret 2006). The large majority of the plant sRNAs are siRNAs and one class of siRNAs regulate protein coding genes through annealing to complementary sequence stretches on mRNAs and are called trans-acting siRNAs (ta-siRNAs) or phased siRNAs (phasiRNAs). These ta-siRNAs are derived from long single stranded RNAs transcribed from TAS loci. The primary transcript is cleaved by a miRNA, the cleavage product is turned into a double stranded RNA by RDR6, which is then processed into 21-nucleotide ta-siRNAs in a phased manner. The tasiRNAs are loaded into an AGO containing effector complex (RISC) and inactivate target mRNAs by cleavage in a similar fashion as miRNAs (Allen et al., 2005; Chen 2009). Other important difference between miRNAs and ta-siRNAs is that the latter ones can move out of the site of synthesis to a much greater range than miRNAs (Parizotto et al., 2004; deFelippes et al., 2011). This feature of ta-siRNAs allows the creation of a gradient that patterns the target gene (Chitwood et al., 2009).

Plant genomes contain large numbers of NB-LRR type resistance genes that recognize specific pathogen effectors and trigger resistance responses (Meyers et al., 2003) and as a consequence protecting plants from diseases caused by devastating pathogens. However, the unregulated expression of NB-LRR genes can inhibit growth and may result in autoimmunity in the absence of pathogen infection (Weaver et al., 2006). It was shown recently that a subset of plant miRNAs (miR482 family) can target NB-LRR genes and regulate plant immunity (Li et al., 2012; Shivaprasad et al., 2012; Zhai et al., 2011). Furthermore, some of the NB-LRR targeting miRNAs are capable of triggering the production of phasiRNAs from their cleaved target mRNAs and target a large number of NB-LRR mRNAs by phasiRNAs (Zhai et al., 2011). Furthermore, miRNAs and phasiRNAs are proposed as a mechanism for reducing

fitness costs associated with NB-LRR genes by targeting them in the absence of pathogens. It was proposed that in the absence of pathogens the NB-LRR resistance genes are transcribed but cleaved by miRNAs/phasiRNAs keeping their translation at a very low level. During pathogen attack the level of miRNA and tasiRNA are reduced and this should result in increased level of resistance gene transcript (Shivaprasad et al., 2012).

In our research grant we proposed to test the hypothesis that the NB-LRR targeting miRNA482 family and/or ta-siRNAs derived from TAS loci homologous to R genes play an important role in R gene regulation during infection by pathogens. Furthermore, we also proposed to investigate the miRNA and tasiRNA based regulation of NB-LRRs during symbiotic interactions in legumes. Since, the results of the funded project written here are not published yet, therefore we give a detailed description of our results here.

2. Identification of small RNAs and their target genes in a Solanaceae specie

As a preliminary research of this OTKA project, we prepared next generation sequencing data from *N. benthamiana* mRNAs, sRNAs and miRNA cleaved mRNA libraries (degradome library) to identify novel miRNAs in Solanaceae species. *N. benthamiana* is a widely used model plant from the Solanaceae family, for research on plant-pathogen interactions as well as other areas of plant science. It can be easily transformed or agro-infiltrated; therefore it is commonly used in studies requiring protein localization, interaction, or plant-based systems for protein expression and purification. Furthermore, it is very sensitive to pathogen infections and it is a widely used model plant to study plant-microbe interactions. We used a comprehensive molecular approach to detect and to experimentally validate *N. benthamiana* miRNAs and their target mRNAs from various tissues. We identified 40 previously described conserved miRNA families and 18 novel microRNAs and validated their target mRNAs with a genomic scale approach. We confirmed the expression pattern and the accumulation of thirteen novel miRNAs by Northern blot analysis. The conserved and novel miRNA targets were found to be involved in various biological processes including transcription, RNA binding, DNA modification, signal transduction, stress response and metabolic process. Among the novel miRNA's targets we found disease resistance proteins, polyphenol-oxydase and the most interesting is the mRNA of a DNA demethylase, REPRESSOR OF SILENCING (ROS1). Regulation of ROS1 by a miRNA provides a new regulatory layer to reinforce transcriptional gene silencing by a post-transcriptional repression of ROS1 activity. The identified conserved and novel miRNAs along with their target mRNAs also provides a tissue specific atlas of known and new miRNA expression and their cleaved target mRNAs of *N. benthamiana*. Thus this study will serve as a valuable resource to the plant research community that will be beneficial well into the future. The above summarised results were published in BMC Genomics (Baksa et al., 2015). The method of the high throughput identification of miRNAs target mRNAs was published in Methods in Molecular Biology (Baksa and Szittya., 2017).

3. Small RNA regulation of plant NB-LRR defense gene family

We and independently from us other research groups (Shivaprasad et al., 2012) also identified the miRNA482 microRNA family in *Solanum lycopersicum* that based on target complementary can target and regulate the expression of a large number of NB-LRR genes post-transcriptionally because they target the conserved P-loop domain coding region of these mRNAs. Furthermore, we also identified six tomato TAS non-coding transcripts which are cleaved by three miRNA482 family members (Figure 1.). Sequence similarity searches revealed that four of these transcripts show sequence similarity to tomato NB-LRR genes (TAS5, TAS6, TAS7 and TAS9). The cleavage of these TAS loci by miRNA482 results the biogenesis of phased siRNAs which in turn can target other NB-LRR genes which could not been cleaved and regulated by the miRNA482 family members. The productions of the phasiRNAs from these TAS loci contribute to the amplification of the original signal and help to down regulate a large number of NB-LRR genes. We think that the function of this miRNA 482 based regulatory layer is to reduce the fitness cost of producing NB-LRR proteins.

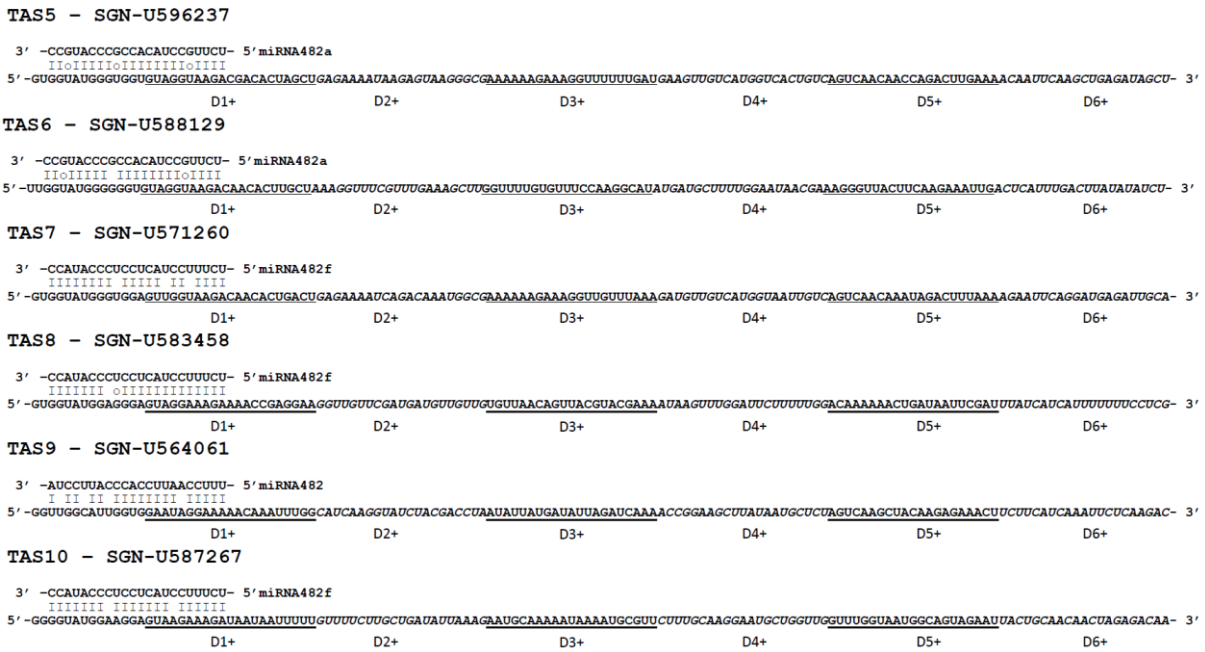

Figure 1. Targeting of TAS loci by miRNA482 family members
The figure shows the sequence of each validated TAS locus aligned with the phase initiating miRNA482. The tasiRNAs produced from each loci is also shown with the phase register (D1-D6).

3.1. The role of the miRNA482 family in biotic stress response

One locus (TAS6) showed high sequence similarity to tomato genes that are homologous to the potato (Solanum demissum) R3a gene, which is an NB-LRR immune receptor gene against the oomycete plant pathogen *Phytophthora infestans*. This raised the possibility that ta-siRNAs from TAS6 may regulate these immune receptor genes. First we tested whether the level of miRNA482a, which cleaves TAS6, and ta-siRNAs from TAS6 change upon *P. infestans* infection. Tomato leaves were inoculated with *P. infestans* (P17777 strain) vs. a

mock treatment and the level of sRNAs was analysed by Northern blot. miRNA482 family members,TAS5 and TAS6 derived ta-siRNAs were initially up-regulated and then down-regulated by the pathogen infection at 5 days after inoculation (Figure 2A). Next we tested whether the tomato homologs of the potato R3a gene are targeted by TAS6 derived ta-siRNAs. 5'RACE results confirmed that the mRNA from the Solyc08g007630gene (which is also an NB-LRR gene) was cleaved between the nucleotides that are complementary to the 10^{th} and 11^{th} nucleotides of TAS6-D5(-) (Figure 2B). Since miR482a also targets TAS5 we analysed the accumulation of TAS5-derived ta-siRNAs and found that TAS5-D1(+) was also down-regulated during *P. infestans* infection (Figure 2A). We confirmed trans-activity of TAS5-D13(-) by validating the cleavage of a predicted target mRNA (Solyc04g05550) by 5'RACE (Figure 2B). It is worth pointing out that Solyc04g05550 is also annotated as an NB-LRR resistance gene. If the immune receptor genes are only regulated by miRNAs and ta-siRNAs, the level of mRNAs is expected to show a mirror image to these sRNAs' accumulation level. However the mRNA level of Solyc08g007630 and Solyc04g05550 did not show a straightforward negative correlation to the level of miRNA482 or TAS5/TAS6 derived tasiRNAs (Figure 2C). This suggests that the expression of these genes is also regulated by other mechanisms. One possible explanation is that the pathogen interferes with the expression of this resistance gene through one of its effector molecules but other mechanisms may also affect the expression of these genes.

To test whether the RDR6 dependent ta-siRNAs play any role in disease development, we inoculated wild type and rdr6 mutant (Yifhar et al., 2012) tomato plants (cv. M82) with a *P. infestans* strain that expresses a fluorescent protein. The size of the lesions was measured 5 days after inoculation and we found that the lesions were bigger on the rdr6 mutant plants than on the wild type plants (Figure 3). These results indicate that potentially many NB-LRR resistance genes are regulated by ta-siRNAs and/or the miRNA482 family members. In the absence of pathogens the resistance genes are transcribed but cleaved by ta-siRNAs/miRNA482 keeping their translation at a low level. However, miRNA482 and therefore the ta-siRNAs are downregulated upon infection, allowing the resistance genes to be translated unless the pathogen developed another mechanism to suppress specific resistance genes. It was shown previously that plant viruses and also P. infestans encode proteins that can suppress RNA silencing (Csorba et al., 2015). RNA silencing is an ancient evolutionary conserved pathway that has a role in genome defence against pathogens and also to control gene expression transcriptionally and post-transcriptonally. During RNA silencing small RNAs (miRNAs and siRNAs) are produced that give sequence specificity to the effector complexes. The silencing suppressor protein of tombusviruses is very well characterized and we showed that it is functioning by binding and sequestering small RNAs (Vargason et al., 2003). This small RNA binding ability of the silencing suppressors raise the possibility that they can interfere with the miRNA482 mediated NB-LRR regulation.

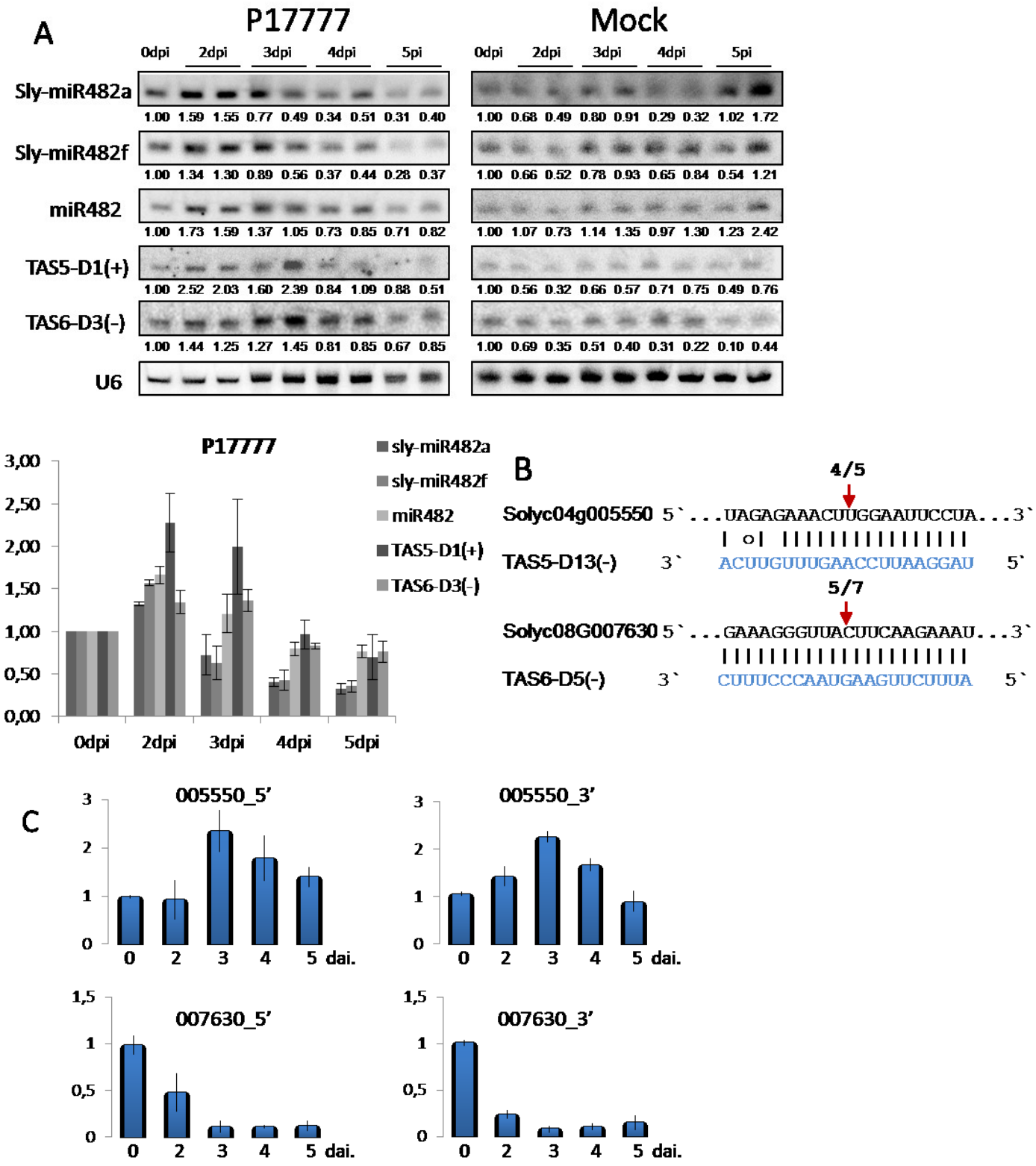

Figure 2. Regulation and function of NB-LRR resistance genes-related TAS genes.
Northern blot analysis of mock and *Phytophtora infestans* (P17777 strain) inoculated plants shows that miR482 family members and TAS5 and TAS6 derived ta-siRNAs are down-regulated during infection after a transient increase. Membranes were re-hybridised with a U6 probe to demonstrate equal loading. (B) Two tomato NB-LRR genes are cleaved by TAS5 and TAS6 derived ta-siRNAs. Red arrows indicate the most abundant cleavage sites found by 5' RACE. (C) RT-qPCR quantification of relative changes in the expression of NB-LLR genes targeted by TAS5-D1(-) and TAS6-D3(-) (Sly04g005550 and Sly08g007630, respectively). 5' and 3' regions were measured independently in mock and *Phytophtora infestans* infected plants 0,2,3,4 and 5 days after inoculation (dai.)(normalized to SKP1).

	Infected_Area (mm2)	Std error
M82	65,09	4,14
RD6	79,46	4,27

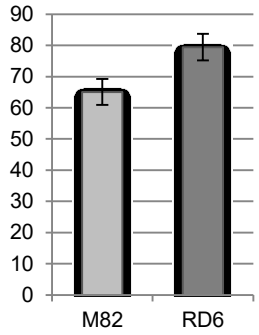

Figure 3. *P. infestans* causes larger lesions on *rdr6* (RD6) mutant tomato plants than on wild type

To test this possibility we infected tomato plants with wt TBSV and also a silencing suppressor mutant version of the virus called TBSV-p19Stop. Then we monitored the level of miR482 in the virus infected tissues by northern blot (Figure 4). We found that both wt and silencing suppressor mutant virus infected leaves showed reduction of miRNA482 level which suggest that the regulation of miRNA482 is not influenced by the pathogen silencing suppressor protein.

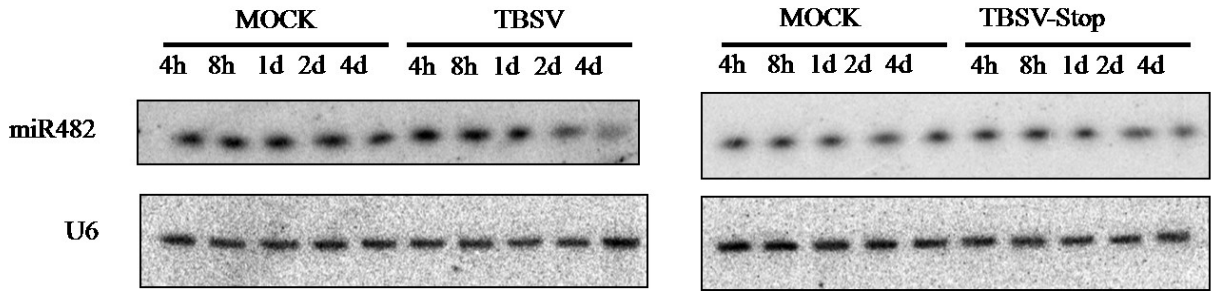

Figure 4. Viral silencing suppressor does not influence the regulation of miRNA482 expression. RNA gel blot analysis of miRNA482 in TBSV-P and TBSV-P19stop infected tomato leaves. TBSV-P and TBSV-P19stop infected and noninfected leaves of N. benthamiana were inoculated to M82 tomato (TBSV, TBSV-Stop and Mock). RNAs isolated at the indicated times after inoculation were separated on a 12% polyacrylamide gel. The RNA was transferred to a membrane and probed with radiolabelled DNA oligonucleotides for miRNA482 and U6 probes as loading controls.

3.2. Expression pattern analysis of the miRNA482 family

To determine the tissue and developmental stage specific expression pattern of the NB-LRR regulating miRNA482 and the tasiRNAs derived from the TAS loci showing homology to NB-LRR genes we conducted northern blot analysis from different M82 tomato tissues and fruits at different developmental stage. We analysed root, stem and leaf tissue from seedlings. We also analysed root, stem, petiole, leaf, flower bud and open flower from adult tomato plants. Furthermore, we tested fruits at different developmental stage, such as 2 mm diameter green fruit, 5 mm diameter green fruit, 10 mm green fruit, fully developed green fruit, fruits in breaker stage (fruit starts turning red) and red tomato fruits. miRNA482 showed clear developmental stage specific expression pattern and mainly expressed in during flower and fruit development. It showed the highest expression level in flower bud and its expression

level was decreasing during fruit growth and expansion. When the fruit reached its maximum size and did not grow any further the expression of miRNA482 has diminished (Figure 5).

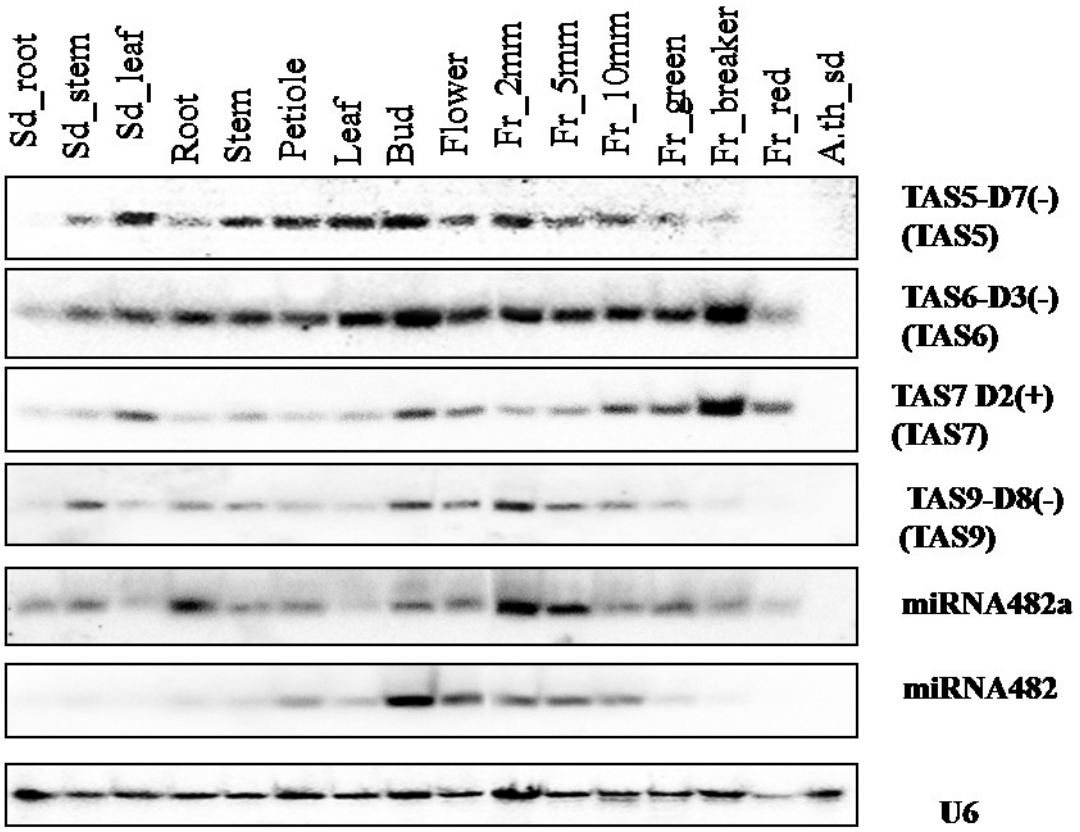

Figure 5. Tissue and developmental stage specific expression pattern of the NB-LRR targeting sRNAs. RNA gel blot analysis of miRNA482, miR482a and representative phasiRNAs from four NB-LRR homolog TAS loci (TAS5, TAS6, TAS7 and TAS9). RNAs isolated from different tissues and development stages of M82 tomato plants were separated on a 12% polyacrylamide gel. The RNA was transferred to a membrane and probed with radiolabelled DNA oligonucleotides for miRNA482, miRNA482a, TAS5-D7(-), TAS6-D3(-), TAS7-D2(+), TAS9-D8(-) and U6 probes as loading controls. Sd_root – seedling root; Sd_stem – seedling stem; Sd_leaf – seedling true leaf; root – adult plant root tissue; stem – adult plant stem tissue; petiole – adult plant petiole; leaf – adult plant leaflet blade; bud – unopened flower; flower – opened flower; Fr_2mm -2 mm diameter green fruit;Fr_5mm - 5 mm diameter green fruit; Fr_10mm - 10 mm green fruit; Fr_green - fully developed green fruit; Fr_breaker -fruits in breaker stage; Fr_red - red tomato fruits.

Another miRNA482 locus (miRNA482a) expressed throughout every tissue and developmental stage of the plant with the highest level in roots and small developing fruits (2-5 mm diameter green fruits). We also analysed the expression pattern of one individual phasiRNA from each NB-LRR homologue TAS loci. They expressed in almost every tissue and developmental stage, the only exception was the red fruit stage where TAS6-D3(-) and TAS7-D2(+) expression were detected only. Interestingly both of these phasiRNAs exhibited the highest expression level during the breaker stage of tomato fruit development (Figure 5).These results suggest that miRNA482 family regulates innate immunity during plant growth and development and keeps the level of NB-LRR genes at different level at the different developmental stages. NB-LRR genes recognize pathogen effectors and mediate effective immune responses to protect plants from disease caused by pathogens. However, NB-LRR expression in the absence of pathogen attack is accompanied by a high fitness cost. Furthermore, activation of immune responses often results in growth inhibition or promotes abnormal development which results a trade-off between defence and growth. Therefore,

miRNA mediated repression of NB-LRR gene expression provides an elegant and general mechanism for plants to solve this problem yet maintain a large repertoire of NB-LRR receptors. High levels of miRNAs and phasiRNAs that target NB-LRRs during key developmental stages can maintain optimal levels of NB-LRR expression and in turn minimize the possibility of developmental defects caused by unintended activationof NB-LRR mediated immunity.

To further investigate the expression pattern of the miRNA482 family we designed miRNA482 reporter constructs. First we identified the genomic loci of three miRNA482 family member (mir482b, mir482c and mir482e). Then 2,5 kb fragment of the miRNA482 promoter region were amplified by PCR. The PCR fragments were cloned into pGEM-T Easy and they were sequenced to ensure that the right sequences were amplified. After that we transferred the promoter sequences to binary vectors in front of GFP reporter gene sequence (Figure 6). The plasmid constructs containing the miRNA482 promoters and the reporter genes were transformed into C58C1 *Agrobacterium tumefaciens* strain. Next we checked the constructs in a transient reporter assay by infiltrating them into *Nicotiana benthamiana* leaves. The expression of the reporter genes were confirmed by checking the florescence of GFP with UV lamp. After the positive transient assay we concluded that the miR482b, miR482c and miR482e promoter construct can be used for tomato transformation.

To the transformation of tomato plants we transferred the promoter construct plasmids to the LB4404 *Agrobacterium tumefaciens* strain, since according to the literature it is the most suitable Agrobacterium strain to tomato transformation. We transformed 200 tomato (Moneymaker) cotyledons with each promoter construct. In all of the cases we were able to observe callus tissue formation and we transferred of the formed shoots on rooting medium.

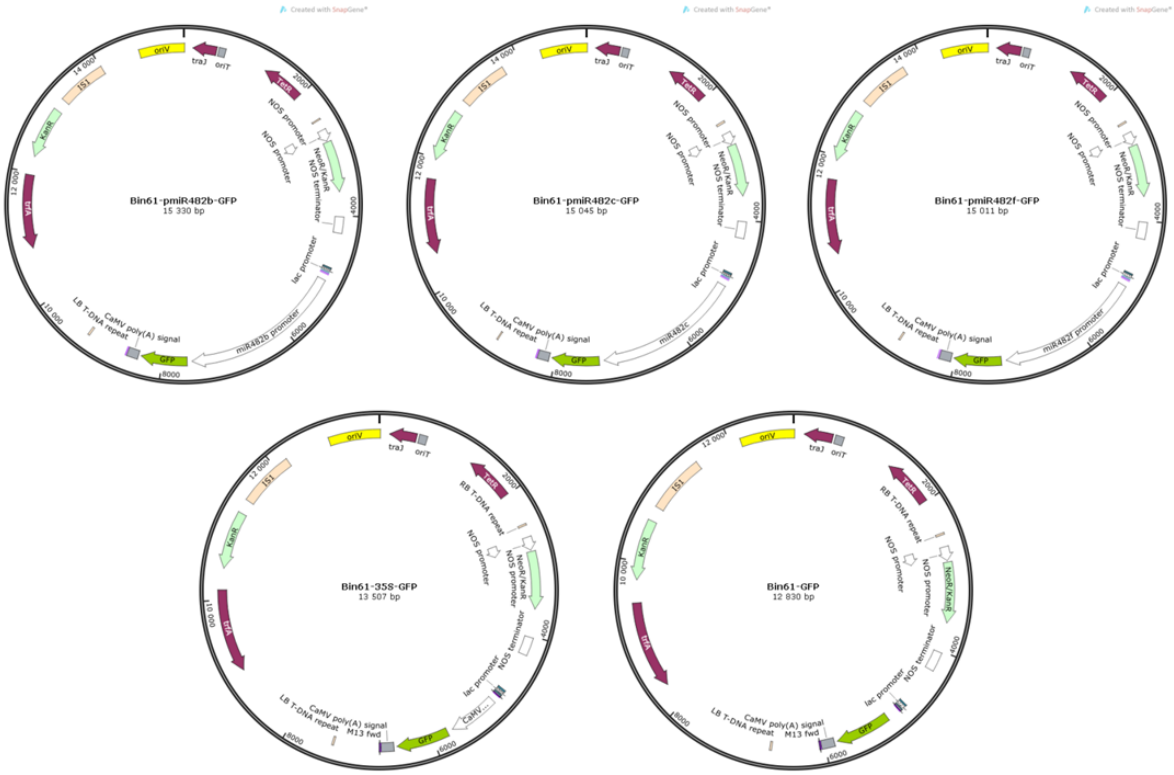

Figure 6. GFP expression vectors developed to investigate the expression pattern of three miR482 promoter sequence.

We were able to regenerate 15-20 tomato transformants (T0) with roots from each constructs and collected seeds from these plants. We used the T1 plants (pmiR482b:GFP - 9 individual line; pmiR482c:GFP - individual line; pmiR482f:GFP – 3 individual line) to monitor the spatial and temporal expression profile of the miR482 family in healthy plants. One of the pmiR482b:GFP line and 2 of the pmiR482f lines expressed the GFP mRNAs (they gave positive signal on the northern blot with GFP probe at the expected position). However, when we checked the GFP expression under UV light none of them gave strong enough signal that are detectable by UV lamp or UV microscope. Since we were not able to detect the GFP signal from these lines we did not continue this line of experiments any further.

3.3. Analysis of the miRNA482 family mediated regulation of NB-LRRs during symbiotic interactions

Legumes enter symbiotic interactions with microbes, however it is poorly understood how they differentiate between symbiotic or pathogenic microbial interactions. Based on the recent literature symbiotic interaction and nodulation may require the suppression of host defences to prevent immune responses (Yang et al., 2010). It was also shown recently that the model legume *Medicago truncatula* encodes at least 114 phased siRNA producing loci and the majority of these loci were located on defence related NB-LRR encoding genes (Zhai et al., 2011). Similarly to the tomato, the majority of phased siRNA producing loci initially were cleaved by the *Medicago truncatula* homologues of the miRNA482 family (miRNA2118) which set up the phase for the phasiRNA production. These data suggest that miRNAs and resulting phased phasiRNAs that target NB-LRR genes may play a role in the regulation of plant-microbe interactions in *M. truncatula* as well.
We demonstrated previously that in tomato the NB-LRR targeting miRNA482 family and the associated pasiRNAs expression is decreasing during pathogen infection and the down regulation of these miRNAs and phasiRNAs contribute to the upregulation of the NB-LRR genes and also to the activation of plant defence. In the case of *Medicago truncatula* three miRNAs (miR1507, miR2109 and miR2118) are known that targets NB-LRR type resistance genes. To test the effect of pathogen infection on the expression level of these miRNAs we infected *M. truncatula* plants with alfalfa mosaic virus (AMV) and monitored the level of the NB-LRR targeting miRNAs in the inoculated leaves 3 days post inoculation by northern blots (Figure 7). We found that similarly to tomato all three NB-LRR regulating miRNAs expression level were decreased during AMV infection and the largest decrease was in the expression level of miRNA2118 (miRNA2118 is the *Medicago* homolog of the tomato miRNA482).
The first line of plant defence comprises the **p**attern **r**ecognition **r**eceptors (PRRs) that recognize conserved **p**athogen-**a**ssociated **m**olecular **p**atterns (PAMPs) and activate **P**AMP-**t**riggered **i**mmunity (PTI). One of the most studied PAMP in plant biology is the bacterial flagellin. Flagellin is a globular protein that arranges itself in a hollow cylinder to form the principal component of bacterial flagellum, and is present in large amounts on nearly all flagellated bacteria. In plants a 22-amino acid sequence (flg22) of the conserved N-terminal part of flagellin is known to activate plant PTI defence mechanisms. Pre-stimulation of plants with a synthetic flg22-peptide led to activation of PTI and enhanced resistance against bacterial invaders (Zipfel et al., 2004). Therefore, next we tested the effect of the synthetic flg22-peptide on the level of NB-LRR regulating miRNAs (miR1507, miR2109 and miR2118). We immersed *Medicago* seedlings into media containing 1μM flg22-peptide and after 6 hours incubation transferred the seedlings into new media that did not contain flg22-peptide and incubated them for further 48 hours. After the incubation, harvested the seedlings, extracted the RNA and subjected it to northern blot to test the expression level of the NB-LRR regulating miRNAs. We found that all three miRNAs expression level were decreased as

a consequence of flg22-peptide treatment (Figure 7). This result shows that the activation of PTI with a synthetic peptide is able to trigger the plant defence signalling to downregulate NB-LRR regulating miRNAs and as a consequence upregulate NB-LRR mRNA level.

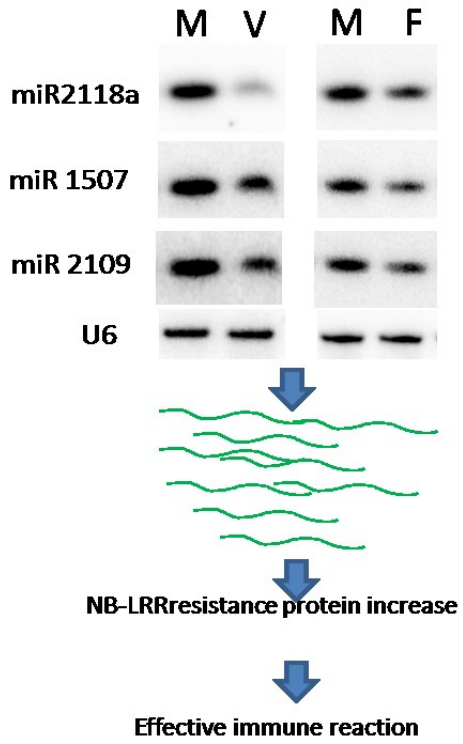

Figure 7. The NB-LRR regulating miRNA genes expression level is decreasing in *Medicago truncatula* during pathogen attack. RNA gel blot analysis of miRNA2118a, miRNA1507 and miRNA2109 in alfalfa mosaic virus (V) infected *Medicago* leaves and synthetic flg22-peptide treated *Medicago* seedlings (F). RNAs isolated from *M. truncatula* were separated on a 12% polyacrylamide gel. The RNA was transferred to a membrane and probed with radiolabelled DNA oligonucleotides for miRNA2118a, miRNA1507, miRNA2109 and U6 probes as loading controls. Mock – non-infected or non-treated leaves of *M. truncatula*.
Model: Pathogen infection leads to decreased NB-LRR targeting miRNAs expressions that in turn lead to the increasing level of NB-LRR level and effective immune reaction against pathogens.

The quick and precise regulation of plant immunity is not only paramount during pathogen attack it could also be very important during the recognition of beneficial microbes at the establishment of symbiotic interaction and nodulation. To test this hypothesis we used *Medicago truncatula* - *Shinorhizobium meliloti* symbiotic interactions as a model system to investigate the role of miRNAs in the regulation of NB-LRR genes during symbiosis. In our experiments we monitored the expression level of the three NB-LRR regulating miRNAs during the early phases of symbiotic interactions. Eight days old *Medicago truncatula* plants were inoculated with *Shinorhizobium meliloti* and we collected RNA samples from control non inoculated roots (mock) and inoculated roots one day post inoculation (dpi). The collected RNA samples were subjected to small RNA northern blot and all three NB-LRR regulating miRNAs expression level were tested (Figure 8). We found that the basal expression of all three miRNAs increased one day post inoculation. During this experiment we also used a mutant version of *S. meliloti* (ExoY mutant - do not produce succinoglycan) which is not able to induce symbiotic nodule formation upon infection. The exoY minus bacteria can enter the root, however it is not able to exit the infection thread and therefore it is not able to produce later N-fixing nodules. We used these bacteria to inoculate eight days old *Medicago truncatula* plants as well. We collected RNA samples from control non inoculated roots (mock) and inoculated roots (wt and mutant bacteria) one days post inoculation. The

collected RNA samples were subjected to small RNA northern blot and all three NB-LRR regulating miRNAs expression pattern were tested in every stages. We found that the expression level of miRNA2118a, miRNA1507 and miRNA2109 were also increased in exoY minus bacteria infected roots.
These results might indicate that the miRNAs that regulate NB-LRR genes may play a role during the establishment of symbiotic interactions.

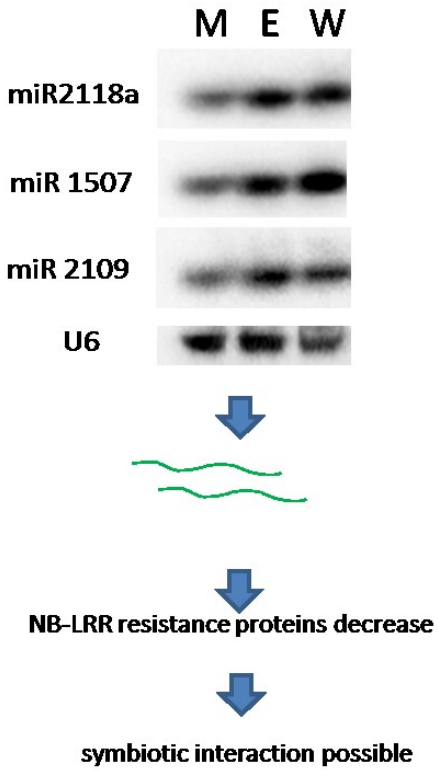

Figure 8. The NB-LRR regulating miRNA genes expression level is increasing in *Medicago truncatula* during symbiotic interactions. RNA gel blot analysis of miRNA2118a, miRNA1507 and miRNA2109 in *Shinorhizobium meliloti* treated *Medicago truncatula* roots one day post inoculation. RNAs isolated from *M. truncatula* roots were separated on a 12% polyacrylamide gel. The RNA was transferred to a membrane and probed with radiolabelled DNA oligonucleotides for miRNA2118a, miRNA1507, miRNA2109 and U6 probes as loading controls. Mock (M) –non-treated *M. truncatula* roots. exo Y mutant *Shinorhizobium meliloti* (E) treated *M. truncatula* roots. *Shinorhizobium meliloti* (W) treated *M. truncatula* roots.
Model: Symbiotic interaction leads to increased NB-LRR targeting miRNAs expression levels that in turn lead to the decreased level of NB-LRR proteins and helps the establishment of the symbiotic interaction.

The suppression of plant immunity could be important during two phases of symbiosis. The first one is the early phase, when the communication starts between the plant and the symbiotic bacteria. During this phase, plants can recognize their symbiotic partner very early and by suppressing NB-LRR genes expression help their colonization. The second important phase is when the symbiotic bacteria exit the infection thread, enter the cytosol and the symbiosome is formed. To monitor this step and also the nodule formation we carried out *in situ* hybridization of the NB-LRR targeting miRNAs in roots and developing nodules.
First we inoculated *M. truncatula* roots with wild type and exoY minus *S. meliloti* and investigated the distribution of miR2118 in 4, 7 and 14 days old nodules by *in situ* hybridization (Figure 9). In the 4 days old nodule the miR2118 expressed highly, it was distributed uniformly and we did not detected expression of the miRNA outside of the nodule. In the 7 and 14 days old nodule the miR2118 expression localized to the apical and middle part of the nodule, however we did not see miR2118 expression at the basal part of the

nodule. This indicates that higher NB-LRR gene expression level at the basal region of the nodule would arrest the spread of symbiotic bacteria outside the nodule into healthy root tissue. Furthermore we tested the expressional pattern of miR2118 in *M. truncatula* plants which roots were inoculated with exoY mutant *S. meliloti*. The nodules produced upon these infection processes are not able to fix nitrogen, because the exoY mutant *S. meliloti* bacteria are not able to exit the infection thread. In accordance with this we were not able to detect miR2118 expression in these nodules by *in situ* hybridization (Figure 9).

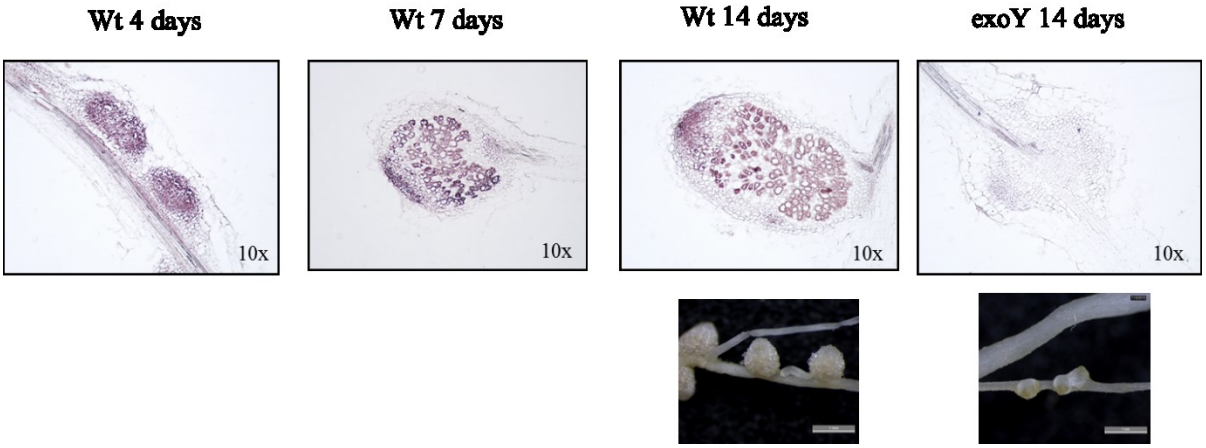

Figure 9. Spatial distribution of miRNA2118 in *M. truncatula* nodules. *In situ* hybridization of longitudinal section of *M. truncatula* nodules with 5′ DIG-labelled LNA-modified miRNA2118 oligonucleotide probe. Nodules were harvested 4, 7 and 14 days post-inoculation (dpi) with wt *Sinorhizobium meliloti*. Nodules were harvested 14 days post-inoculation (dpi) with exoY minus *Sinorhizobium meliloti*. Under the *in situ* hybridizations the image of 14 days old wt and exoY nodules are shown under light microscopy.

Next, we investigated the spatial distribution of all of the other NB-LRR targeting miRNAs during different stages of nodule development by *in situ* hybridization. In the 4 days old nodule (four days post *S. meliloti* inoculation – this is the earliest time point where we can detect the developing nodule primordia in a light microscope) all three miRNAs (miRNA2118, miRNA2109 an miRNA1507) were expressed, they were distributed uniformly in the developing nodule and we did not detect expression of the miRNAs outside of the nodule (Figure 10). In the 7 days old nodule (the N-fixation has not started yet, however the developing nodule can be seen by naked eye) we were able to detect the expression of all three NB-LRR targeting miRNAs (Figure 11). In the 14 days old N-fixing nodule all three NB-LRR targeting miRNAs were expressed, however they showed different spatial distribution. The miRNA2118 expression was localized to the apical (expression in the meristem zone) and middle part of the nodule (expression was detected in the infection zone and in the nitrogen – fixation zone), however we did not see miRNA2118 expression at the basal part of the nodule. The miRNA1507 expression was localized to the apical (expression in the meristem zone) and middle part of the nodule (the expression was mainly located to the infection zone, however not exclusively). The spatial expression pattern of miRNA2109 was the most restricted it mainly localized to the apical part of the nodule (meristem zone) (Figure 12). Next, we investigated the spatial distribution of all of the other NB-LRR targeting miRNAs in 14 days old nodules during *M. truncatula* – exoY mutant *S. meliloti* interaction. However, we did not detect the expression of any of the NB-LRR targeting miRNAs in this case (Figure 12).

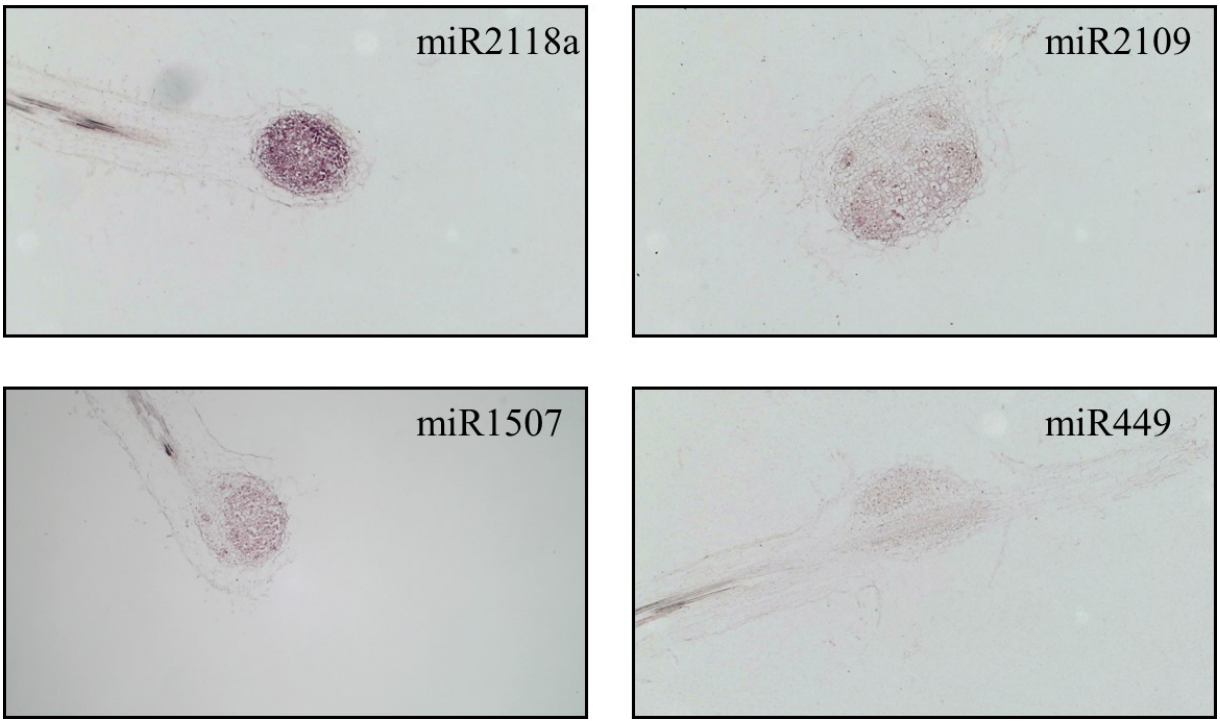

Figure 10. Spatial distribution of NB-LRR regulating miRNAs in 4 days old *M. truncatula* nodules with wt *Sinorhizobium meliloti*. *In situ* hybridization of longitudinal section of *M. truncatula* nodules with 5′ DIG-labelled LNA-modified miRNA2118, miRNA2109 and miRNA1507 oligonucleotide probes. The LNA probe for mouse miR449 was used as a negative control.

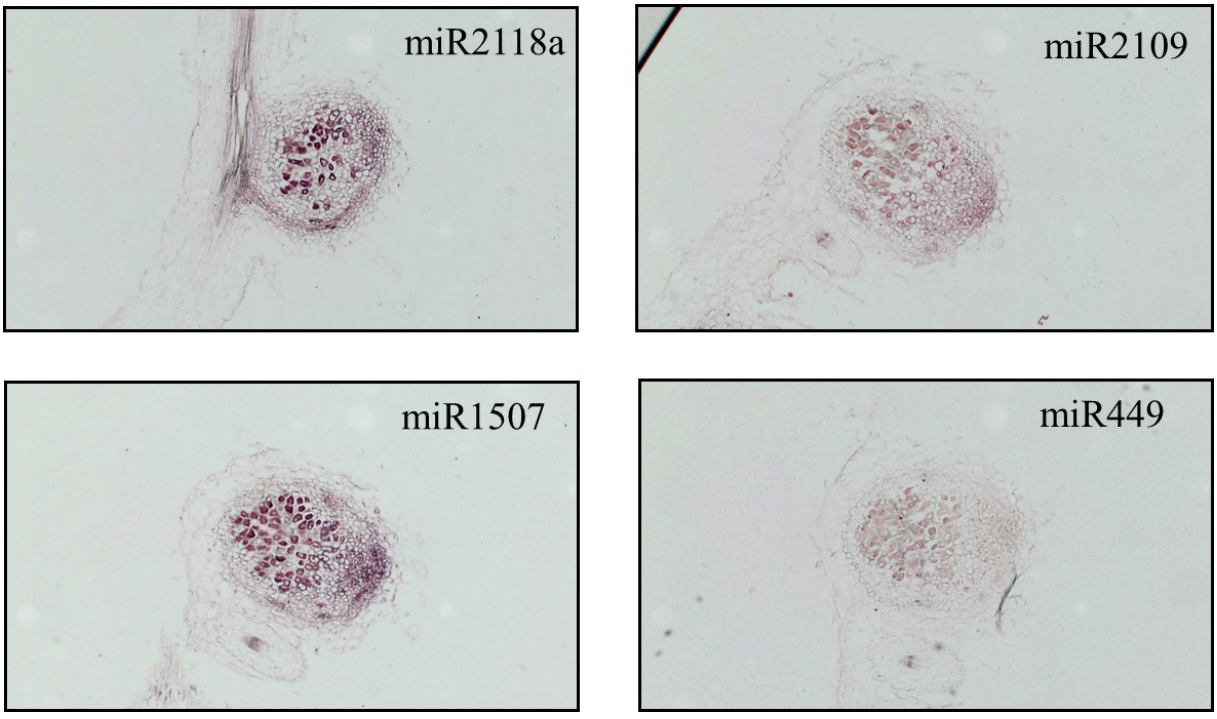

Figure 11. Spatial distribution of NB-LRR regulating miRNAs in 7 days old *M. truncatula* nodules with wt *Sinorhizobium meliloti*. *In situ* hybridization of longitudinal section of *M. truncatula* nodules with 5′ DIG-labelled LNA-modified miRNA2118, miRNA2109 and miRNA1507 oligonucleotide probes. The LNA probe for mouse miR449 was used as a negative control.

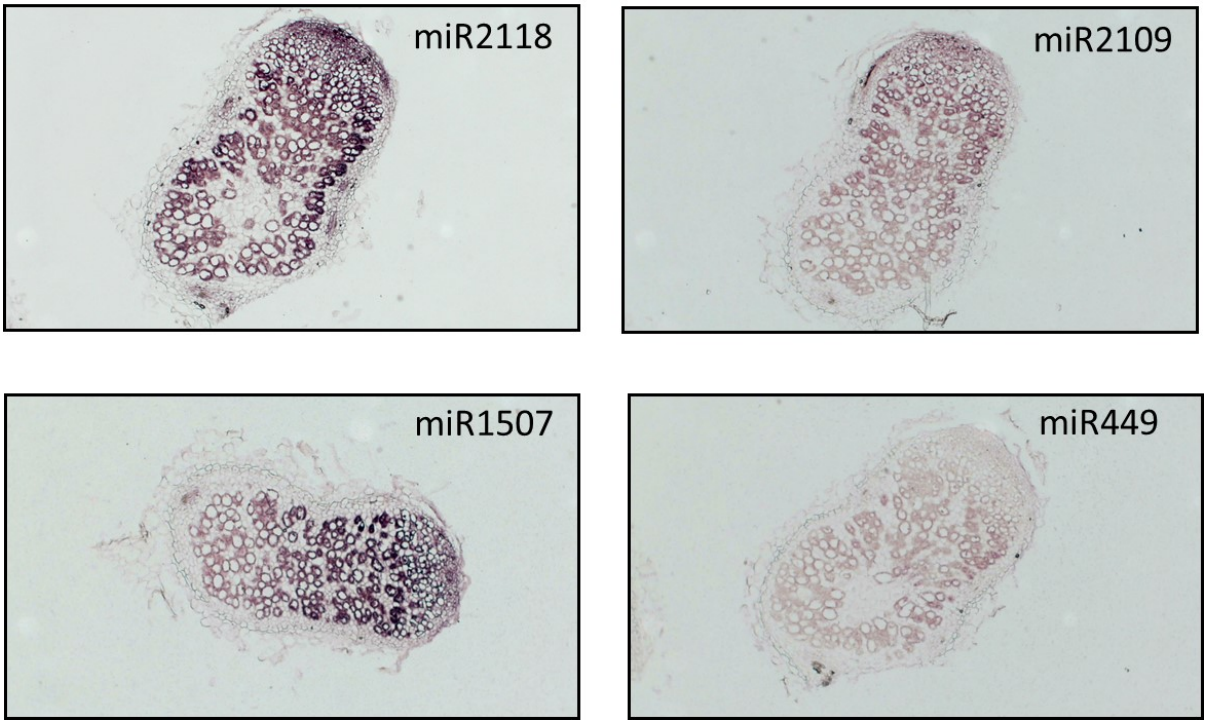

Figure 12. Spatial distribution of NB-LRR regulating miRNAs in 14 days old *M. truncatula* nodules with wt *Sinorhizobium meliloti*. *In situ* hybridization of longitudinal section of *M. truncatula* nodules with 5′ DIG-labelled LNA-modified miRNA2118, miRNA2109 and miRNA1507 oligonucleotide probes. The LNA probe for mouse miR449 was used as a negative control.

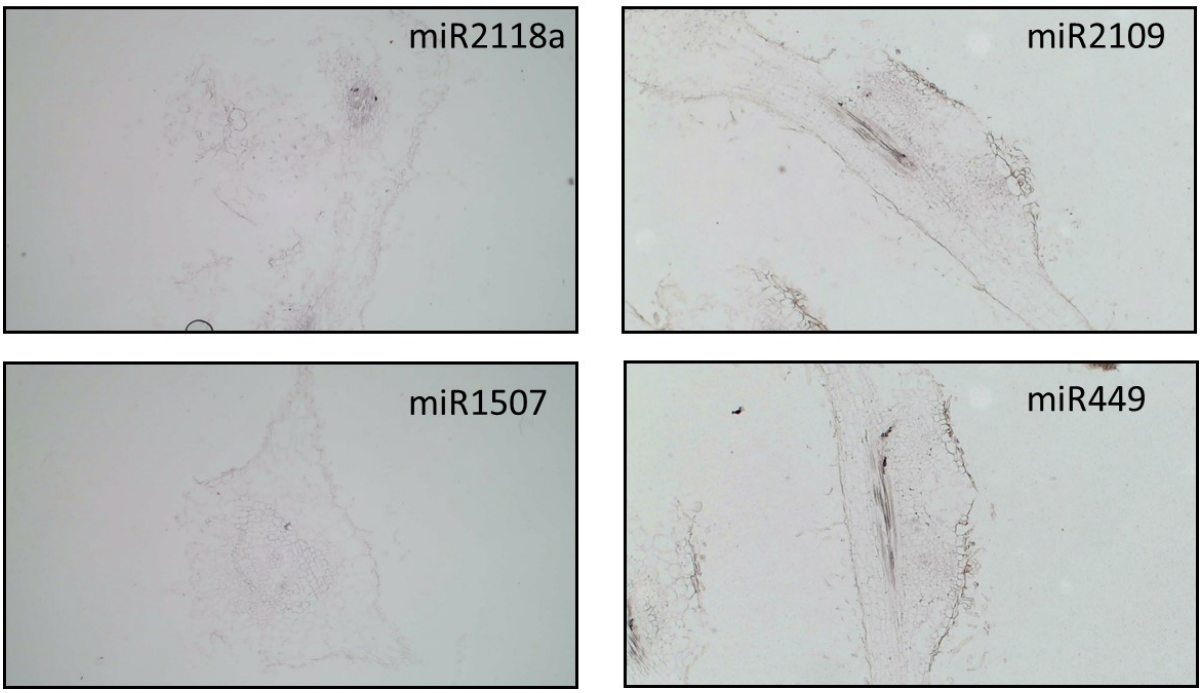

Figure 13. Spatial distribution of NB-LRR regulating miRNAs in 14 days old *M. truncatula* nodules with exo Y minus *Sinorhizobium meliloti*. *In situ* hybridization of longitudinal section of *M. truncatula* nodules with 5′ DIG-labelled LNA-modified miRNA2118, miRNA2109 and miRNA1507 oligonucleotide probes. The LNA probe for mouse miR449 was used as a negative control.

The nodules produced upon these infection processes are not able to fix nitrogen, because the exoY mutant *S. meliloti* bacteria are not able to exit the infection thread. Since the NB-LRR

targeting miRNAs are not expressed in these nodules the level of NB-LRR proteins could be elevated to a level that would contribute to the restriction of exoY mutant *S. meloliti* spread in these nodules.

The *M. truncatula* genome encodes apr. 540 NB-LRR genes and more than 60 % of these genes can be targeted by the NB-LRR targeting miRNAs or by the phasiRNAs that are produced by the action of the NB-LRR targeting miRNAs (Zhai et al., 2011). The upregulation of all three NB-LRR regulating miRNAs during symbiotic interactions in the nodules predicts that it could down regulate the level of the mRNAs of those NB-LRR genes that can be targeted by these miRNAs. To check that the targeted NB-LRR genes expressions are indeed downregulated we selected two NB-LRR genes targeted by each miRNA (miRNA2118, miRNA2109 and miRNA 1507) and measured their expression level in symbiotic nodule by qRT-PCR. As it was expected, all of the investigated miRNAs targeted NB-LRR gene expression level was downregulated in the symbiotic nodule. However there were differences in the level and timing of the downregulated NB-LRR gene expression level (Figure 14). These result shows that the three NB-LRR regulating miRNAs indeed contribute to the regulation of the NB-LRR genes and this regulation could be very important in the symbiotic nodule development, because the downregulation of NB-LRR resistance genes in the developing nodule could produce a suitable niche that makes possible the bacterial colonization and the development of N-fixing nodule tissue.

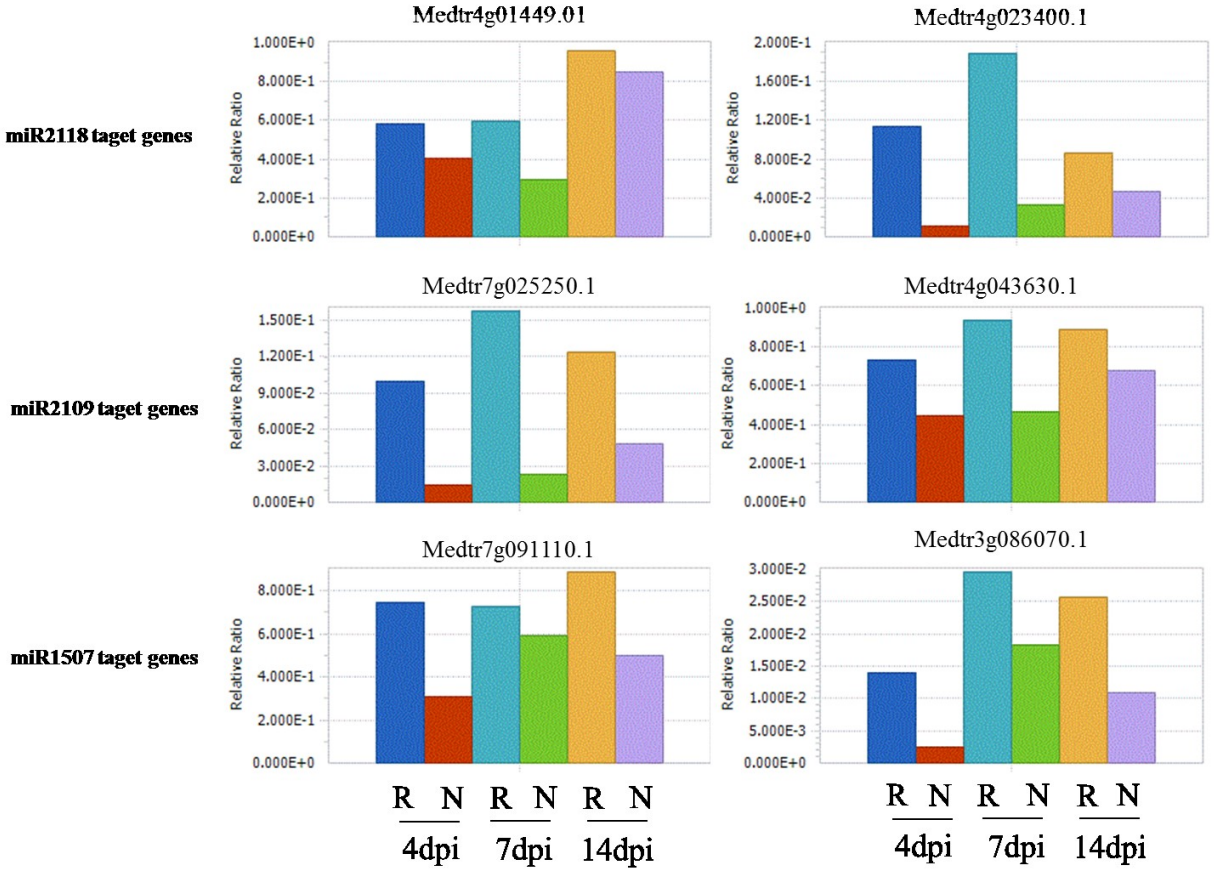

Figure 14. qRT-PCR analysis of miRNAs regulated NB-LRR genes in 4, 7 and 14 days old *M. truncatula* nodules infected with *Sinorhizobium meliloti*. Relative transcript levels at different time points were calculated in relation to PTB (polypyrimidine tract-binding-like protein Medtr3g090960.1.) R – samples were taken from roots that do not contain nodules. N – samples were taken from nodules.

Our model predicts that if the reduced level of NB-LRR gene expression is important to the formation and proper development of symbiotic nodules than the induction of NB-LRR gene expression would interfere with this process and it would result in the reduction of nodule number during symbiosis. We have shown previously that infection of *M. truncatula* plants with alfalfa mosaic virus (AMV) down regulates the expression level of the NB-LRR regulating miRNAs. Furthermore, tt was also shown that the inhibition of the function of these miRNAs leeds to the upregulation of their target mRNAs (Fei et al., 2015). Based on this, we speculated that AMV infection will result the downregulation of the NB-LRR regulating miRNAs which in turn results in the upregulation of NB-LRR genes. It is also important to mention that AMV infection, beside the downregulation of the expression of the NB-LRR regulating miRNAs, do not induce any visible phenotypes of *M. truncatula* plants. Therefore, the mock and the AMV infected plants are indistinguishable from each other. So we infected 5 days old *M. truncatula* seedlings with AMV or mock inoculum and 14 days after the virus infection we inoculated the plants with *S. meliloti*. We counted the nodules seven and ten days later and we found that the virus infected plants developed significantly reduced number of nodules than the mock inoculated control plants.

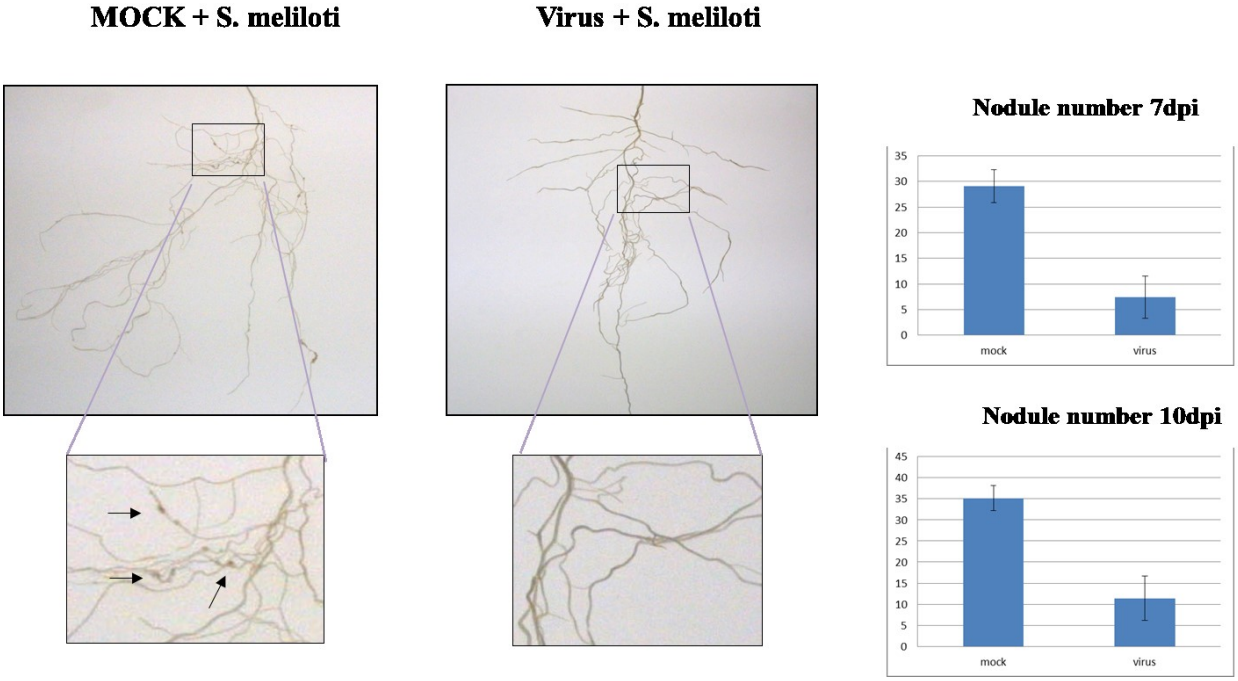

Figure 15. Down regulation of the expression of NB-LRR targeting miRNAs lead to the repression of nodule number of *M. truncatula* infected with *Sinorhizobium meliloti*. The pictures show the root architecture and the symbiotic nodules of representative plants from each group. The arrows on the magnified boxes point to the developing nodules. The bar chart shows the average nodule numbers after 7 and 10 days of *S. meliloti* inoculation. In each diagram we counted the nodules of 12 mock and 12 AMV infected plants.

Furthermore, we also prepared miRNA overexpressing constructs (miRNA2118oex, miRNA2109oex and miRNA1507oex) to test their effect on nodulation. We generated hairy root composite *M. truncatula* plants using *Agrobacterium rhizogenes*. The vector has a DsRed cassette for the identification of transgenic roots. The promoter that controlled miRNA expression was a constitutive promoter from *Arabidopsis thaliana* EF-1 alpha A1 gene. We used the empty vector transformed roots as a control. Hairy root composite plants were examined for root growth characteristics and nodulation by *S. meliloti*. First, we verified overexpression of miRNAs in transgenic roots by northern blot analysis. Total RNAs were

isolated from DsRed positive roots three weeks after *Agrobacterium* inoculation. The results confirmed that all three lines had significantly higher miRNA expression (Figure 16). Under our growing conditions, overexpression of these miRNAs did not cause any significant changes in root growth characteristics examined. Compared with empty vector transformed controls, the three transgenic lines showed no obvious differences in root length or lateral root density. Next we examined the nodule numbers in the transgenic hairy roots 3 weeks after *S. meliloti* inoculation. We found that nodule number per transgenic root varied significantly. Transgenic roots overexpressing miRNA2118 exhibited a large increase in the formation of mature nodules compared with control empty vector transformed roots. Transgenic roots overexpressing miRNA2109 and miRNA1507 showed a mild increase in the formation of mature nodules compared with vector controls (Figure 16). According to these results we concluded that the expression level of NB-LRR regulating miRNAs show a very good correlation with nodule number.

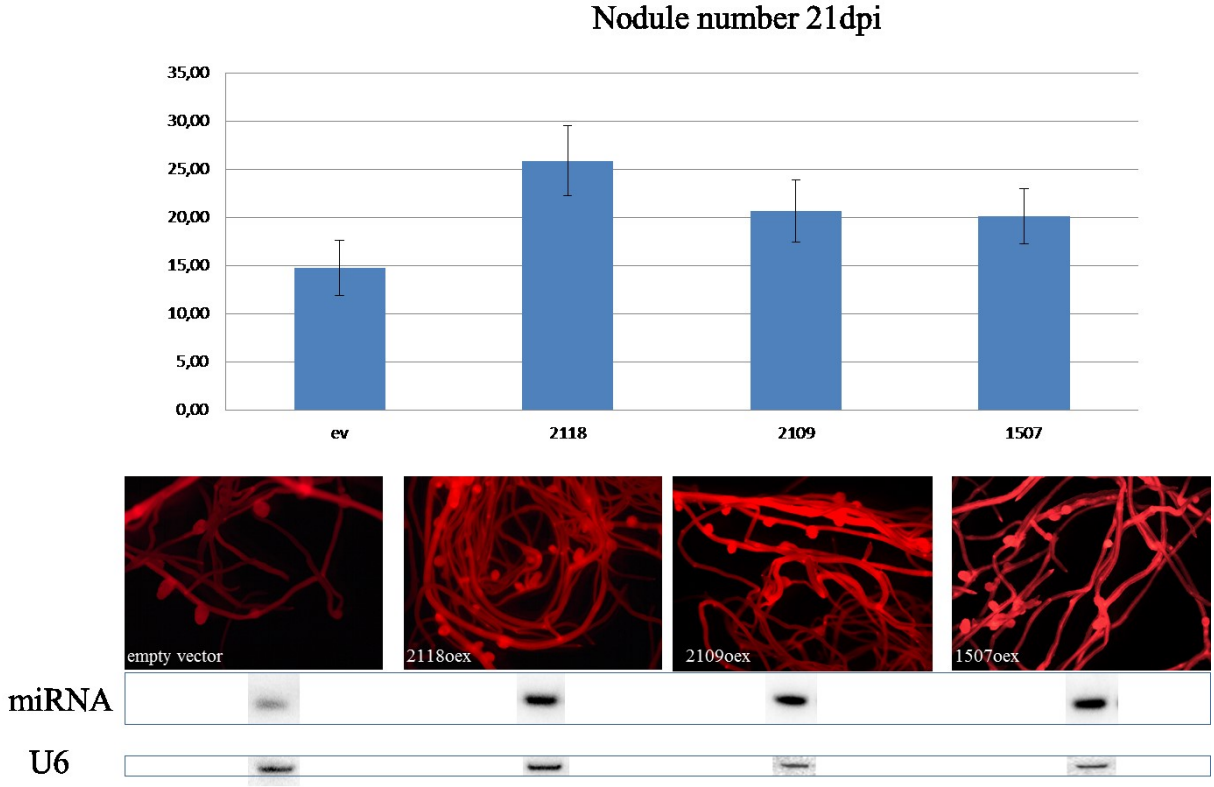

Figure 16. Constitutive overexpression of miRNAs in *M. truncatula* hairy root composite plants led to increased nodule number. NB-LRR regulating miRNAs (miRNA2118, miRNA2109 and miRNA1507) were driven by a constitutive *Arabidopsis thaliana* EF-1 alpha A1 gene promoter (At1g07940 - elongation factor 1-alpha) in *M. truncatula* composite plant roots. The top panel show a bar chart with the average nodule numbers three weeks after of S. meliloti inoculation (15 transgenic roots were counted with each construct). The middle panels show DsRed-expressing root nodules three weeks after inoculation with *S. meliloti*, including empty vector-transformed controls and roots overexpressing miRNA2118, miRNA2109 and miRNA1507. The bottom panels show northern-blot analyses of the same roots three weeks after inoculation, showing the increased abundance of mature miRNAs in transgenic lines. RNAs isolated from *M. truncatula* roots were separated on a 12% polyacrylamide gel. The RNA was transferred to a membrane and probed with radiolabelled DNA oligonucleotides for miRNA2118, miRNA2109, miRNA1507 and U6 probes as loading controls.

Based on our results we propose that NB-LRR regulating miRNAs are important regulators of nodulation by regulating the expression level of NB-LRR genes and therefore providing a suitable environment to the establishment and development of N-fixing symbiotic nodules.

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