FINAL REPORT

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Molecular factors changing the fate of plant-microbe interactions

The beneficial microbes promote plant growth by supplying the host with nutrient from the soil. Symbioses between mycorrhizal fungi and 80% of terrestrial plant species as well as between *Rhizobium* soil bacteria and legume plants are the most important and the best known examples of beneficial interactions. These symbiotic associations require mutual recognition and coordinated plant and microbial responses. Symbiotic nitrogen fixing nodules host only their cognate *Rhizobium* partners and any other microbes present in the rhizosphere are kept out. This raises the questions, how the plant can simultaneously accommodate mutualists and ward off pathogens or other microbes and how the rhizobial symbionts can evade plant defense.

In our project we have focused on the identification, molecular and functional characterization of bacterial and plant components that have critical functions in the bacterial invasion of the host plant and guiding the process either to the symbiotic or to the pathogenic direction. To dissect the components of these complex processes we have used genetic, genomic, association mapping and functional studies of the nitrogen fixing symbiosis and explored its interference with pathogenic interactions.

RECOGNITION OF *M. TRUNCATULA* FIX$^-$ MUTANTS SHOWING ALTERED DEFENSE RESPONSE AND IDENTIFICATION OF THE MUTATED GENES

In order to identify symbiotic mutants of *M. truncatula* that also exhibit induced defense responses during the symbiotic interaction with the compatible rhizobial partner, we analyzed a collection of ineffective symbiotic (Fix$^-$) mutants from the fast neutron and transposon-tagged mutant collection of *M. truncatula* for the symptoms of defense responses. Nodules were harvested 21 days post inoculation (dpi) with *Sinorhizobium medicae* WSM419, nodule sections were prepared and stained with toluidine-blue to detect the accumulation of phenolic compounds, which is a remarkable sign for enhanced defense response. Greenish blue color is generated when toluidine-blue binds to polyphenolic substances, such as lignin and tannins. The *M. truncatula dnf2* mutant identified by Bourcy and co-workers (New Phytologist 2012 197: 1250–1261.) showing strong activation of defense-like reactions (Fig. 1) was used as a control for toluidine-blue staining. We detected that the nodules of two Fix$^-$ mutants, *14S* and *7Y* showed strong greenish blue staining indicating accumulation of polyphenolics and hence probably an altered defense responses in these mutant lines (***Fig. 1***.). This greenish blue staining could not be detected in wild type plants.

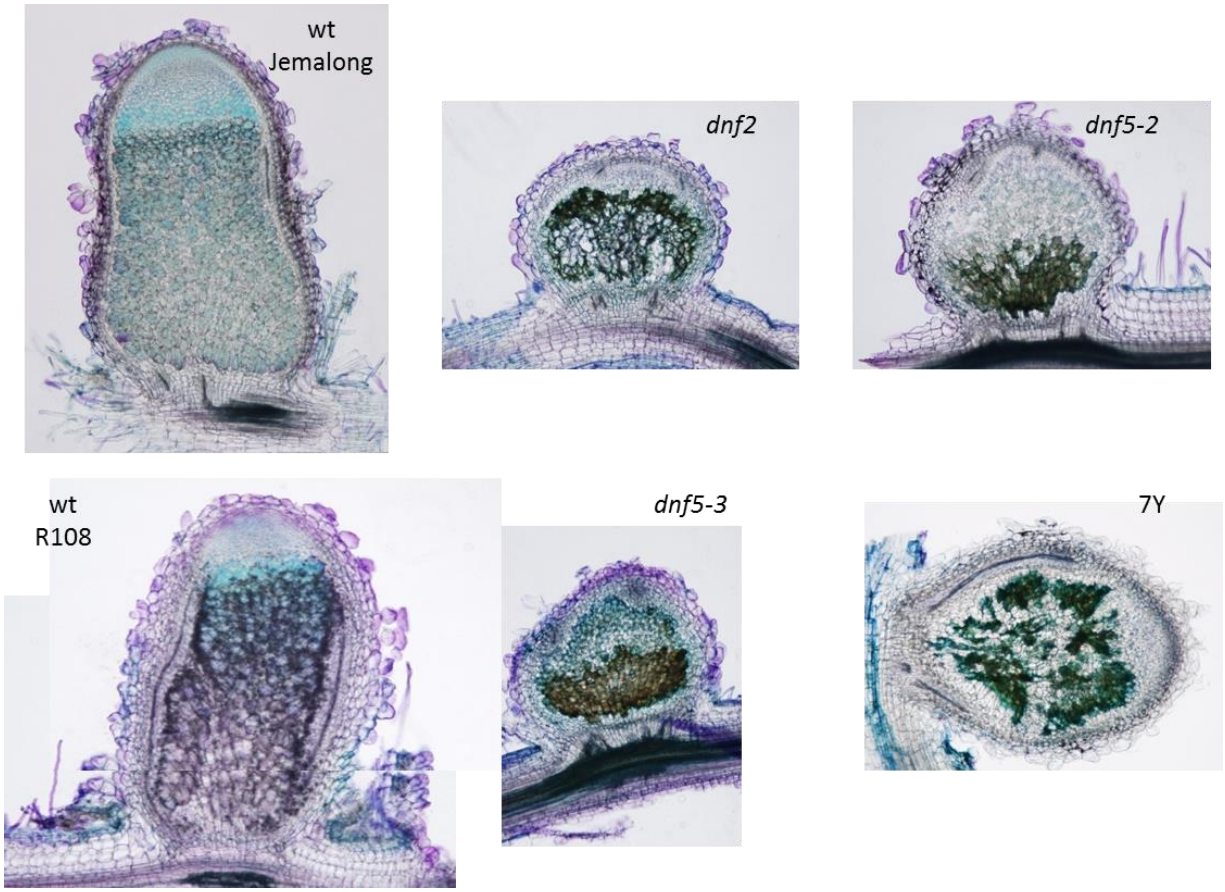


Figure 1. The toluidine blue staining of 50 μm thick sections of nodules harvested 21 dpi with *S. medicae* WSM419. Greenish blue coloration at the base or central part of the nodules indicates accumulation of phenolic compounds. wt = wild-type

To gain more information about the impaired gene in *14S*, a positional cloning project was initiated. We positioned the mutant locus on the lower arm of chromosome 3 between markers PRTS and EST400 within a distance of 1.2 cM. An extended segregating population of 2056 F2 individuals was used to map the mutant locus between genetic markers 75f6-SSR and 3g110480. This genomic region was covered by three BAC clones, mth2-75f6, mth2-33j9 and mth2-94b15, and their sequence analysis revealed 20 predicted coding sequences. Oligonucleotide primers were synthesized for the best candidate genes and PCR reactions were carried out to amplify genomic and cDNA samples of mutant *14S* and the wild type plants. In the meantime we have determined that mutant *14S* was allelic to a *dnf5* insertion mutant showing similar accumulation of polyphenolic compounds, therefore the mutant *14S* was referred to as *dnf5-2*. Thus we could extend the sequencing of the candidate genes also in the other alleles of *DNF5* gene. The sequence analysis of the candidate genes revealed a single base pair deletion in the second exon and an eight base pair deletion in the first exon of a gene encoding a cysteine-rich receptor-kinase-like protein in the *dnf5-2* and *dnf5-1* mutants, respectively. In order to prove the identity of the *DNF5* gene, we carried out genetic complementation experiments us ing the *Agrobacterium rhizogenes* transformation system.

The introduction of the wild-type or fusion version of *DNF5* with the gene of the green fluorescent protein (GFP) into the mutant *dnf5-2* restored the symbiotic phenotype indicating the gene identity of *DNF5*. We attempted to analyze the subcellular localization of the GFP-tagged version of DNF5 protein (DNF5-GFP) using confocal laser scanning microscopy. Although the DNF5-GFP was functional in complementing the symbiotic phenotype of the *dnf5* mutant, we could not detect the fluorescent signal of GFP indicating either the low production and/or the high turnover of the DNF5-GFP protein. We are preparing new constructs containing the DNF5-GFP gene fusion driven by highly active promoters of other symbiosis specific genes (leghemoglobin and NCR169) that might produce sufficient amount of gene product to detect the fluorescent signal. The manuscript describing the phenotype of *dnf5-2* and the cloning of *DNF5* is in progress.

The observed greenish blue coloration indicating the presence of polyphenolic compounds is often associated with defense reactions and/or senescence. In order to discriminate between the activated defense responses and the senescence induced by defects in the symbiotic interaction, we also monitored the transcriptional activation of genes annotated as senescence and defense related genes 14 dpi using by qRT-PCR. We detected that the senescence induced in the older nodules of the mutant *ncr169*, impaired in one of the nodule-specific cysteine-rich genes required for complete differentiation of rhizobia (Horváth et al. PNAS 2015 112:15232-15237) is associated with the up-regulation of cysteine proteinase genes, while genes associated with pathogenic responses, such as *NDR1* (a Non-race-specific Disease Resistance), *PR-10* (pathogen-related) and a *chitinase* gene, were not induced in the mutant *ncr169*.

We monitored the expression level of the genes associated with either senescence or defense responses in the several ineffective symbiotic (Fix^-) mutants using qRT-PCR (***Fig. 2.***). Both the *7Y* and the control *dnf2* mutants showed the up-regulation of the *NDR1* and *PR-10* genes while these genes were not induced in the other Fix^- mutants or wild-type plants (***Fig. 2***.). The two senescence associated *cysteine proteinase* genes exhibited increased transcriptional activation in all symbiotic mutants while none of these genes were induced in wild-type plants and the *6L* developmental mutant, which produced fewer but effective nitrogen fixing nodules. The transcriptional data indicated that the mutant *dnf5* showed similar expression pattern of defense response and senescence associated genes as the ineffective *5L, 11S, 13U* and *ncr* mutants but the polyphenol accumulation followed the inoculation with rhizobia relatively soon, which indicated the early induction of defense responses. Based on these data we concluded that the defect in the *dnf5* mutant probably induced a very early senescence process due to an enhanced defense response compared to the other Fix^- mutants.

The 7Y mutant showed a very similar defense response to mutant *dnf2* (***Fig. 1.***) and transcriptional activation (***Fig. 2***.), and it is likely that the impaired symbiotic gene in *7Y* controls the defense responses during the symbiotic interaction. We have recently also identified an insertional *M. truncatula* mutant being allelic with *7Y*. The cloning and the verification of the impaired gene in *7Y* are in progress.

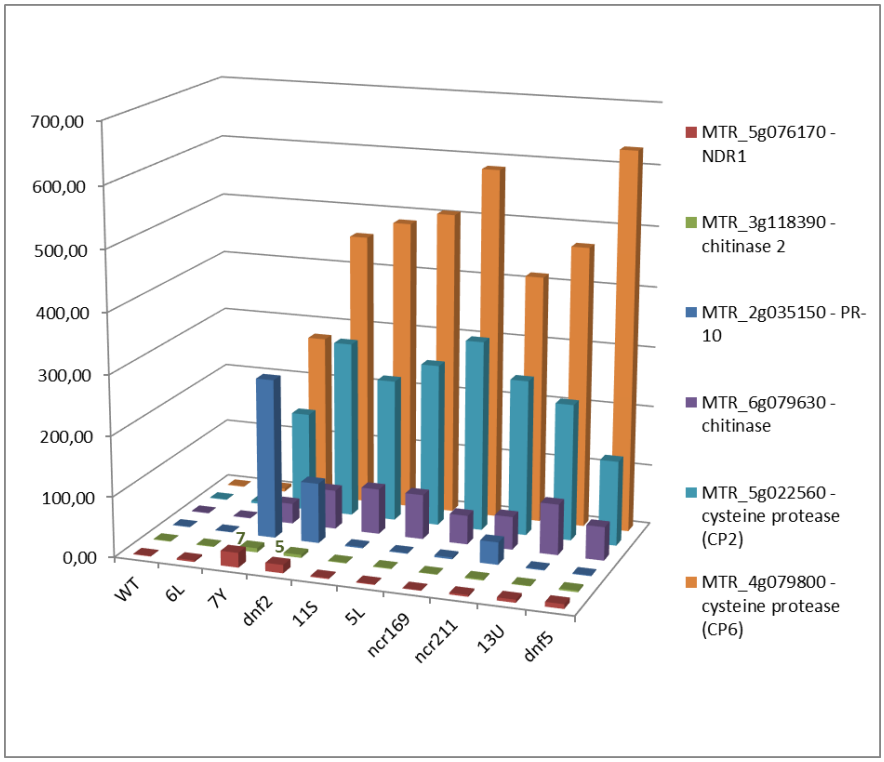


Figure 2. The expression of defense and/or senescence related genes was monitored at 14 dpi with *S. medicae* WSM419 using qRT-PCR in different Fix$^-$ mutants and wild-type and a developmental mutant 6L. Senescence associated genes were induced in all symbiotic mutants but enhanced pathogen related gene expression was detected only in *7Y* and *dnf2* plants. Relative transcript levels were calculated in relation to the wild-type plants.

INVESTIGATION OF THE INCOMPATIBLE *S. MELILOTI* - *M. TRUNCATULA* INTERACTION

Screening the *M. truncatula* HAPMAP population with different *S. meliloti* strains

To identify incompatible interactions between the different ecotypes of the model legume *M. truncatula* and *S. meliloti*, 356 and 136 lines from the *M. truncatula* HAPMAP collection were screened with the sequenced *S. meliloti* strains RM1021 and RM41, respectively, under sterile conditions. Three lines proved to be completely non-nodulating (Nod$^-$) with RM1021, 14 and 10 lines were Fix$^-$ with RM1021 and RM41, respectively. There were also 18 and 11 lines that showed the signs of less efficient nitrogen fixing symbiosis, which were designated as Fix+/- phenotype with RM1021 and RM41, respectively. Four of the Fix$^-$ lines showed incompatibility with both bacteria. We also tested a number of *Medicago* lines with *S. meliloti* strains GR4, AK83 and SM11 that proved to be Fix$^-$ with Jemalong. For further studies, we chose the *M. truncatula* ecotype Jemalong A17 and *S. meliloti* strain RM41 (see below).

Genetic analysis of the incompatible interactions between RM41 and ecotypes Jemalong and F83005 showed that the two traits were determined by different loci. Similarly, the

incompatibility of the four bacterial strains was mapped to two different loci. These results indicated that association mapping of the symbiotic trait is impossible without classical genetic mapping.

Phenotypic analysis of the interaction between strain RM41 and the *M. truncatula* ecotypes A17 and DZA315.16 by microscopy

To reveal at which stage of the nodule development in the RM41-A17 interaction is arrested, eight days old plants were inoculated with strain RM41 carrying the nifH::GUS reporter construct under aeroponic growths conditions (also used during sample collection for transcriptome analysis). For microscopic analysis nodules were harvested at 7, 10, 14, 17 and 21 dpi. (***Fig. 3.***)

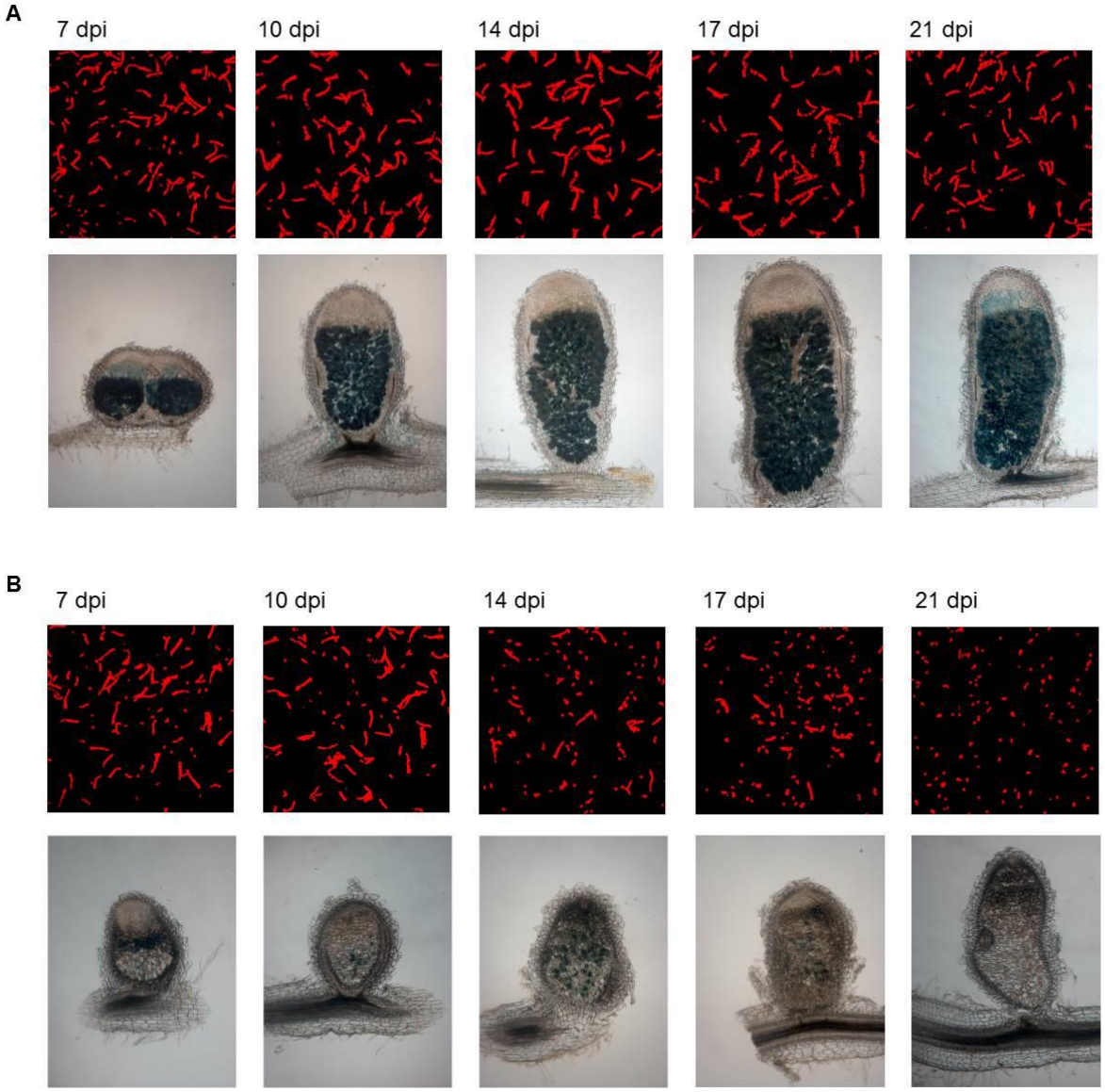


Figure 3.: Phenotypic analysis of the GUS stained nodules induced by RM41 (nifHpromoter-GUS) on *M. truncatula* ecotypes DZA315.16 (**A**) and A17 (**B**) as well as the propidium-iodide stained bacterial populations isolated from the nodules.

At 7 dpi, strain RM41 could invade nodule tissues where its *nif* genes were activated but at later time points, the plant cells quickly became empty indicating early senescence. The bacterial populations reflected this observation; the majority of bacteria isolated from the DZA nodules were in bacteroid form at all time-points, while bacteroids were observed mainly at early time points in the case of A17 but cells similar to the free-living form appeared already at 7 dpi and became the only form by 21 dpi. These free-living like cells most probably represent saprophytic forms that repopulate the senescent nodules.

Isolation of the bacterial gene(s) required for the efficient symbiosis with A17

The ineffective phenotype of RM41 can be caused by an activity that is either absent from or present in this strain. To resolve whether the absence of an activity determines the trait, first we constructed a library that contained all predicted ORFs of strain RM1021 that forms effective symbiosis with both *M. truncatula* ecotypes. The *S. meliloti* strain RM1021 ORFeome library was transferred into the broad host-range expression vector pHC60 and placed under the control of the bacA promoter which provides strong expression during nodule invasion then the library was introduced into strain RM41. *M. truncatula* A17 seedlings grown under aeroponic conditions were inoculated with RM41 bacteria carrying the 1021 ORFeome library but no confirmed complementing ORFs could be identified. The lack of complementation by the ORFeome library might be due to the lack of not a single gene but an operon. To overcome this limitation we obtained a cosmid library prepared from the genome of *S. medicae* strain WSM419 (that also forms effective symbiosis with both *M. truncatula* ecotypes), however, it proved to be not satisfactory because it contained mainly small inserts. We also initiated the construction of a novel library using transposons that carries replication origins.

To test whether the phenotype is caused by the presence of a gene in RM41 that causes the cessation of the interaction, we created over 16000 transposon insertion mutants and divided them into pools of 100 strains. The testing of the pools is in progress.

Mapping of the *M. truncatula* gene(s) determining the efficiency of symbiosis with RM41

To map the *M. truncatula* gene required for the efficient symbiosis with RM41 the symbiotic phenotype of the individuals of an F2 mapping population from a manual cross between A17 and A20 parental lines as well as of a recombinant inbred line population obtained after the cross of Jemalong and DZA315.16 ecotypes was determined. The genotype of a number of marker genes were determined and their possible linkage to the symbiotic trait were tested. Based on our first data, the Fix^- trait was recessive and monogenic and was mapped to linkage group eight between the molecular markers MtU10 and 8C24. The F2 mapping population was extended and the phenotype and genotype of the individuals were determined. In this way, novel recombinations have been identified, which were used to further

delimit the region to between genes 8g070760 and 8g073260 where the gene affecting the trait is located.

Transcriptome analysis of *M. truncatula* ecotypes A17 and DZA315.16 inoculated with *S. meliloti* strain RM41

To investigate the global bacterial and plant gene expression changes in the incompatible (*S. meliloti* RM41 - *M. truncatula* A17) and effective (*S. meliloti* RM41 - *M. truncatula* DZA315.16) interactions genome-wide transcriptome profiling was performed by the RNA-Seq method of nodules formed on the roots of the two ecotypes after inoculation with RM41. To collect the biological material the same growth conditions were applied as for microscopic analysis. Based on the results of the microscopic analysis two time points, 10 and 21 dpi, were chosen for further investigation where the less and the most differences could be observed in the nodulation of the two ecotypes.

In total, differential expression of 232 plant and 348 bacterial genes could be observed between the two ecotypes at 10 dpi, 220 plant and 204 bacterial genes at 21 dpi. There were only a few gene expression changes between the two time points of the interaction (10dpi vs. 21dpi) while there was a huge difference when we compared the transcriptome of the two interactions (A17 vs. DZA315.16) in both symbiotic partners (***Fig. 4.***).

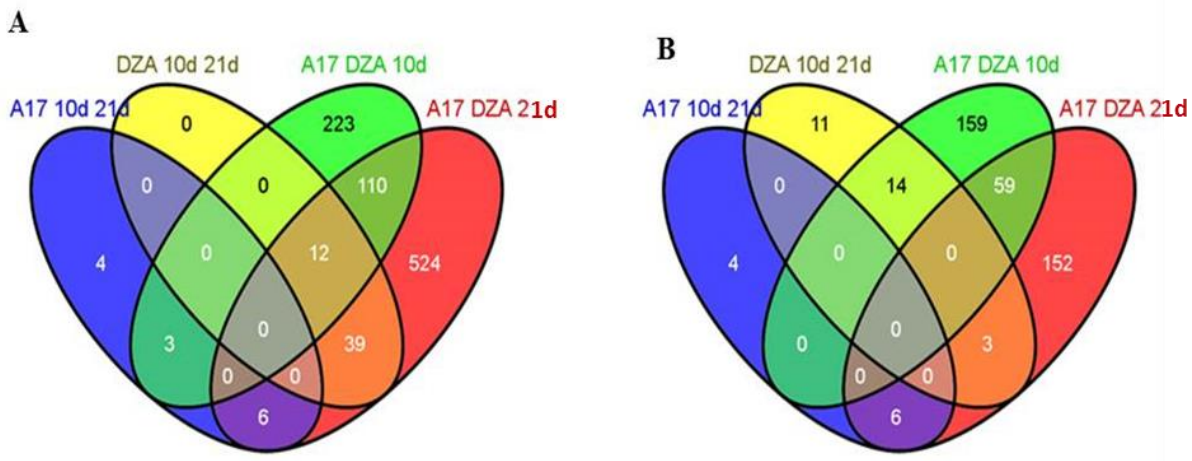


Figure 4.: Number of differentially regulated genes in RM41 (**A**) and in A17 (**B**).

Plant genes with altered expression profile

On the plant side we could observe high upregulation of several *cysteine protease* genes (Medtr4g079770, Medtr2g075830, Medtr4g080730, Medtr4g107930, Medtr4g047610, Medtr5g022560, Medtr4g079800, Medtr4g079470) in the 10 dpi A17 nodules. Cysteine

proteases play important role during the whole nodulation process, since it requires dramatic changes in metabolic activity. At the early steps of nodulation cysteine proteases play role in defense response and root invasion. They are involved in nodule formation, protein turnover, adaptation to physiological stress and also play important role in late phase of nodulation, controlling the senescence process. In senescing nodules the upregulation of cysteine proteases and pathogen related genes is a common process, since increase in proteolytic activity is one of the key features of senescence (Sheokand, 2003). A defense marker gene (Medtr6g079630, *chitinase*) and a *purple acid phosphatase* gene (Medtr7g104360) involved in nucleic acid degradation and nutrient remobilization also showed upregulation in 10 dpi A17 nodules. These genes and two of the *cysteine protease* genes (Medtr5g022560, Medtr4g079800) are early senescence and defense marker genes during nodule development. We tested the expression level of these senescence markers by qRT-PCR and found that they are highly upregulated not just in 10 dpi A17 nodules but also in later phase of nodulation. The *Leghemoglobin* gene (*LHG*, Medtr5g066070) showed more than three fold upregulation in the 21 dpi transcriptome of DZA315.16 nodules compared to the A17. *LHG* is crucial for maintaining low-oxygen environment inside the nodule and essential for the activity of the nitrogenase enzyme. The low *LHG* expression in the A17 nodules also proved the inefficiency of the symbiosis and verified the low level of *nifH* expression observed earlier.

The altered expression of defense response related proteins (most of them members of the NBS-LLR-type resistance protein family) was observed in 10 and 21 dpi A17 nodules as well.

Bacterial genes with altered expression profile

We could observe the upregulation of the *nifH* gene and also other members of the nitrogenase complex in both the 10 and 21 dpi DZA315.16 nodules confirming the qRT-PCR results described earlier.

47 transport mechanism related genes showed altered expression in 10 dpi A17 nodules compared to DZA315.16 and 25 genes in the 21 dpi nodules. Most of them were members of the ABC transporter gene family. More than 20 *dehydrogenase* genes were upregulated in the 10 dpi A17 nodules and 7 in the 21 dpi nodules. A *LysR family transcriptional regulator* gene was more than 20-fold upregulated in the 10 dpi A17 nodules. Another member of the *S. meliloti* LysR gene family, *lsrB* was identified previously to be essential for alfalfa nodulation in *S. meliloti*. This gene was shown to play key role in preventing premature nodule senescence and abortive bacteroid formation. A *glutathione S-transferase* gene showed about four-fold upregulation in RM41 infected A17 nodules. It was shown in soybean, that glutathione plays important role in the nodulation process and in nodule senescence. Plants infected with glutathione biosynthesis mutant *Rhizobium tropici* showed early senescent phenotype.

To verify our results we compared the expression of genes that showed different expression in 10 and 21 dpi nodules of the two ecotypes by qRT-PCR. The results of the qRT-PCR experiments confirmed the up- or downregulation of the selected genes (***Figs. 5, 6, 7.***).

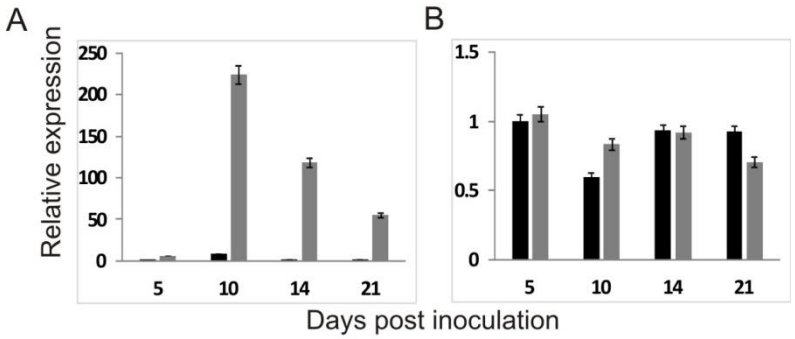


Figure 5.: Quantitative analysis of *nifH* (**A**) and *BacA* (**B**) gene expression in nodules. RNA samples from A17 and DZA315.16 nodules 5, 10, 21 dpi with *S. meliloti* RM41 were subjected to qRT-qPCR. Black bars represent A17 and gray bars represent DZA315.16. Three biological replicates each with two technical repeats were used. Shown are means ± standard deviation; n = 3.

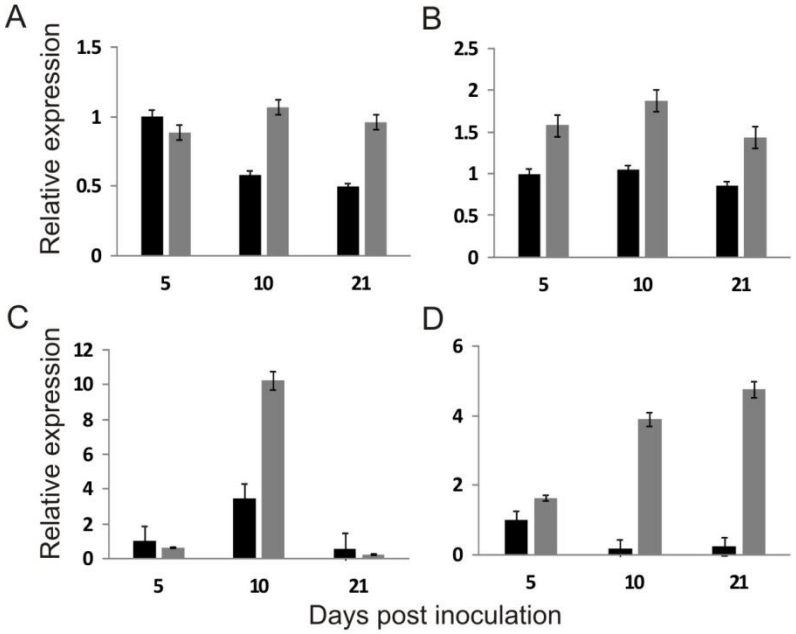


Figure 6.: Quantitative analysis of **A**, BN406_01695 (glycine betaine ABC transporter periplasmic protein), **B**, BN406_02064 (periplasmic binding ABC transporter protein), **C**, BN406_02041 (periplasmic binding ABC transporter protein) and **D**, BN406_06326 (RND family efflux transporter MFP subunit) gene expression in nodules. A17 and DZA315.16 nodules 5,10,21 dpi with *S. meliloti* RM41 were subjected to quantitative reverse-transcription polymerase chain reaction. Black bars represent A17 and gray bars represent DZA315.16. Three biological replicates each with three technical repeats were used. Shown are means ± standard deviation; n = 3.

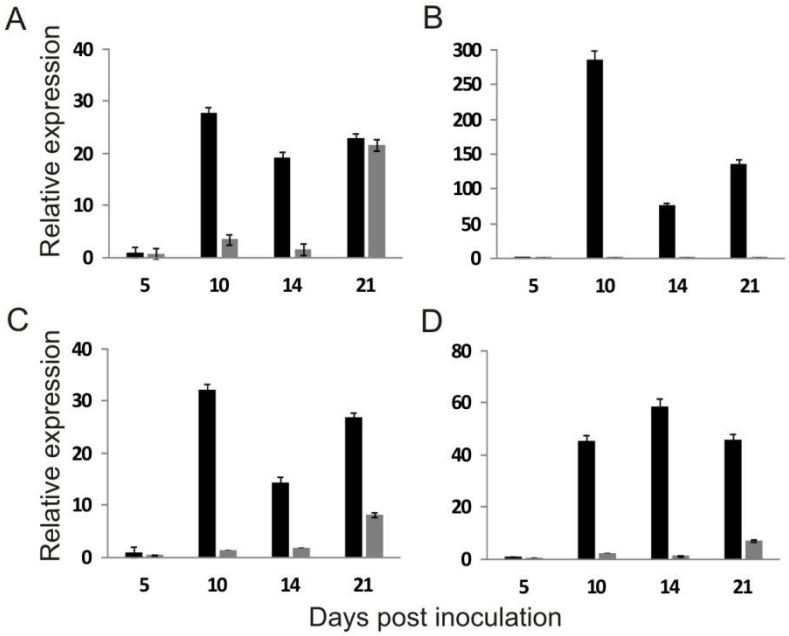


Figure 7.: Upregulation of early senescence and nutrient remobilization marker genes in A17 nodules. qRT-PCR of A, cysteine protease (Medtr4g079800); B, cysteine protease (Medtr5g022560); C, purple acid phosphatase (Medtr7g104360); D, chitinase (Medtr6g079630) was performed at the indicated time points. Black bars represent A17 and gray bars represent DZA315.16. Three biological replicates each with two technical repeats were used. Shown are means ± standard error; n = 3.

CHARACTERIZATION OF THE PLANT DEFENSE RESPONSES ON SYMBIOTIC *MEDICAGO* MUTANTS AND UPON PATHOGEN CHALLENGE WHILE PLANT ROOTS ARE AT SYMBIOSIS

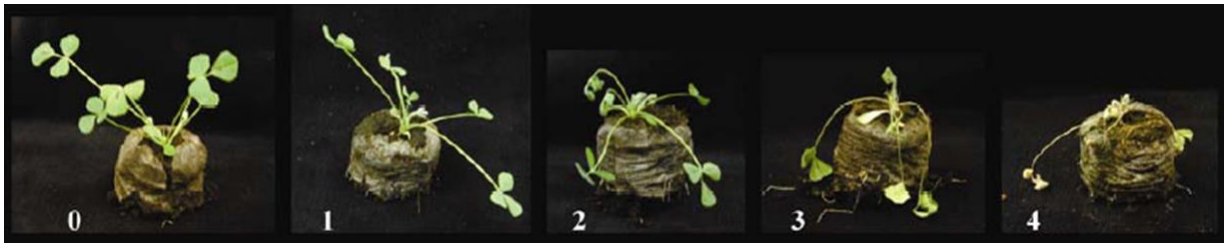
Wilt pathogen *Ralstonia* strains used to detect plant defense responses on symbiotic *Medicago* mutants

Beside the characterization of the plant pathogen responses during the impaired symbiotic processes we have also designed and carried out experiments to follow the molecular responses of concomitant symbiotic and pathogenic challenges in *Medicago* roots. For this we decided to use the root pathogen bacteria *Ralstonia solanacearum* since they have already been shown to be virulent on *M. truncatula* roots. First, we have tested two *Ralstonia solanacearum* strains (TUDK and R190) available in Hungary for their virulence capacity on *M. truncatula*, but they showed only random slight effects. Therefore, we obtained published strains from a French laboratory and got five different strains that show various virulence on different *Medicago* ecotypes. Two strains (Rd15 and GMI1000) that were reported having the strongest

virulence phenotype on *M. truncatula* were tested on A17 plants to adjust the optimal parameters for infections using the conditions published by the French laboratory.

The *R. solanacearum* Rd15 strain showed strongest virulence, it caused wilting symptoms on plants 4-5 dpi (strain GMI 1000 induced symptoms 3-4 days later). After setting up the conditions using the wild type plants we have compared two symbiotic mutants (*lin* and *7Y*) and the wild type *M. truncatula* sensitivity to Ralstonia infections. The mutant plants showed earlier wilting symptoms comparing to wild type plants (***Fig. 8.***). We have also provided individuals from the *lin* mutant line to another research group that developed an efficient pathogenic test on *M. truncatula* roots with *Phytophthora palmivora* and one of the *lin* mutants showed the most severe difference of the extent of symptoms compared to the wild type (Rey et al. New Phytol. 2014). These data suggest us that some genes that had been identified to be required in the nitrogen fixing symbiosis based on their mutant phenotype also have function in the plant responses to the pathogen attack. Thus we are in the process to explore in more details the possible role of our symbiotic genes *Lin* and 7Y in the plant immunity.

A



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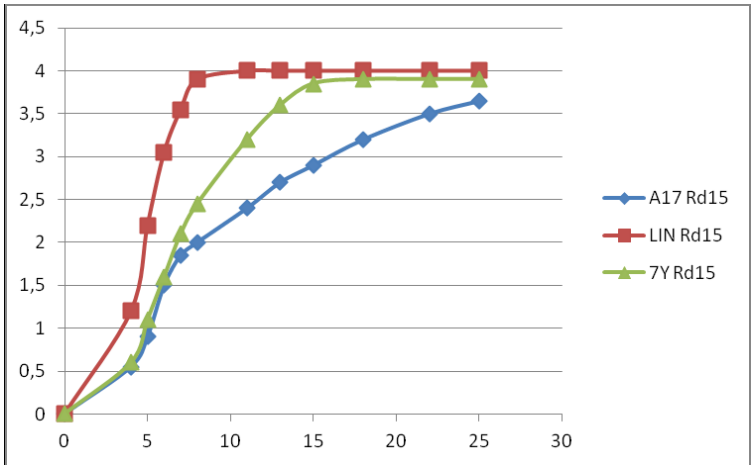

Fig. 8. Development of symptoms of the Ralstonia infection on wild type and mutant *M. truncatula* roots. **A**) Scoring of the phenotypes as published earlier by Vailleau et al. 2007. **B**) Development of the symptoms of the virulent *Ralstonia* on the studied roots using the scores shown in **A**) on the axis Y plotted against the days after inoculation of roots on the axis X.

Characterization of the plant response to the pathogen attack of the symbiotic root

The other set of experimentations aimed to investigate the effect of symbiotic interactions on disease development upon pathogenic challenge. After selecting the *Ralstonia* strains showing the strongest virulence phenotype on *M. truncatula* A17 plants, our next goal was to adjust the optimal parameters for double infections with symbiotic and pathogenic bacteria, as well as for proper root sample collection for transcriptome analysis. In these experiments we have applied microsymbiont *Rhizobium* bacteria together or before infecting the *M. truncatula* roots with the wilt pathogen *Ralstonia* strains. The first trials included several arrangements of conditions and time points of the inoculations and infections. The plant growing substance for the pathogenic test was the Jiffy pot, while for the symbiotic tests generally zeolite/perlite (frequently) or plant agar plates (more rarely with *M. truncatula*) are used. In several plant tests we have determined the best parameters for our experiments: germinated plants were grown on sterile plant agar plates covered by sterile blotting paper, inoculated by symbiotic rhizobia 3-4 days after germination. The results of the first trials, when applied the pathogenic *Ralstonia* at different time points after the symbiotic induction, showed us that cutting the root tips needed for the *Ralstonia* infection disturbed the onset of the symbiotic nodule development and therefore the *Ralstonia* infection was done following the appearance of pink nodules in the subsequent experiments. Control non-symbiotic roots were infected by the pathogenic bacteria at the same time. Root samples were collected at 1 and 3 dpi with *Ralstonia*, total RNA samples were prepared and qRT-PCR was carried out to check gene expression of five symbiotic genes and ten genes related to the plant responses to pathogenic attack (***Fig. 9.***). The induction of these marker genes confirmed that both the symbiotic and the pathogenic processes were well initiated in this system.

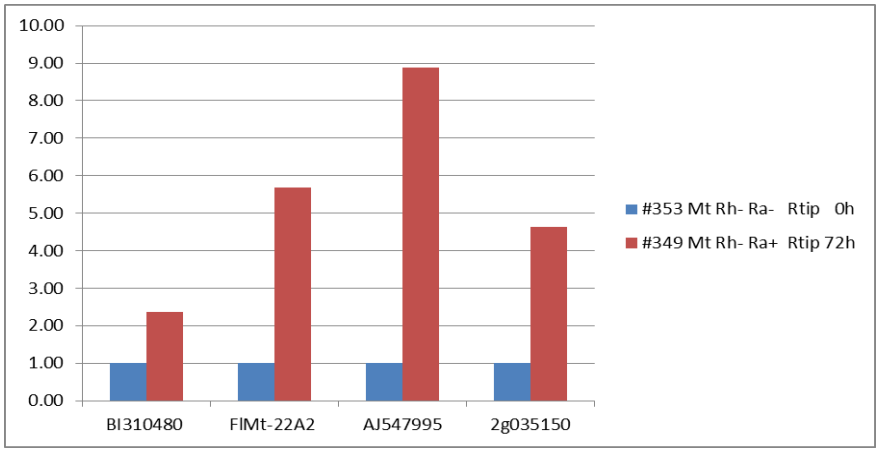

Fig. 9. qRT-PCR results of four genes related to the plant response to the pathogenic attack. 72 hours after infection of the roots by *Ralstonia* the expected induction of the genes was detected compared to the 0 hour controls.

In the meantime a new paper was published (Moreau et al., New Phytology, 2014) in which transcriptome analysis was done on *M. truncatula* root tips challenged with *Ralstonia*. Since they characterized the plant gene expression responses only to this pathogenic bacteria we have decided that the better way for us to do our experiments using the same experimental set, i.e. applying *Ralstonia* on the intact roots (without cutting the tip as before), then collecting samples at the same time point so our results can be directly compared later with the published data. Thus our controls are similar to the published experimental set - roots infected only with *Ralstonia* (0 hour, 24 hours, 72 hours), while our other samples were inoculated first with *Rhizobium* to induce the formation of the nitrogen fixing nodules on the *Medicago* roots, followed by the infection with *Ralstonia.* We have completed to repeat these carefully designed experiments three times, root samples were collected and RNA were isolated. The RNA-Seq method could be finished by the end of the grant, but analyses of the data is in progress yet.

As it is noticeable from this report some parts of the project have just finished or close to be finished, therefore the writing of several manuscripts from the above reported work is in progress. The current/running titles of these manuscripts are:

1) A symbiosis specific gene controls the defense responses during the bacterial colonization of the symbiotic nodule.
2) The deficiency in a cysteine-rich receptor-kinase-like protein prevents differentiation of rhizobia and induces early senescence of the symbiotic nodule.
3) The incompatible interaction between Medicago truncatula cv. Jemalong and Sinorhizobium meliloti strain RM41 induces early nodule senesce.
4) The symbiotic LIN gene has a role on the plant defense against pathogen attack in Medicago roots
5) Altered plant response to the wilt pathogen Ralstonia attack is determined by the symbiotic roots of Medicago