Lotus japonicus SYMREM1 is a novel signalling component involved in symbiotic nitrogen fixation

Theses of PhD dissertation

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Introduction

Root nodule symbiosis (RNS) effectively contributes to biological nitrogen fixation with 200 million tons of nitrogen per year[1]. The symbiosis has been used for crop rotations in many agricultural traditions to enrich soils with organic nitrogen, and today it represents a significant part of sustainable agriculture. An intensively studied form of this mutualistic interaction is a symbiosis established between plants of family *Fabaceae* (legumes) and nitrogen-fixing soil bacteria commonly named as Rhizobia. Upon interaction with rhizobia, legume plants develop a new organ on their root where the bacteria are hosted and biological nitrogen fixation takes place. In these nodules rhizobia are differentiated into bacteroids, as which they convert atmospheric N_2 into ammonia (symbiotic nitrogen fixation, SNF) that can be utilized by the host plants. Nitrogen is an essential macronutrient for all living organisms since they represent one of the main components of the key building molecules such as amino acids and proteins. Legumes like *Medicago truncatula* that belong to the inverted repeat–lacking clade (IRLC)[2] form so-called indeterminate nodules that are elongated and are characterized by four different zones whereas other legumes like *Lotus japonicus* exhibit nodules of spherical shape, without zonation and with a certain lifespan, called determinate nodules[3]. To establish the symbiotic relationship, plants secrete flavonoid molecules to attract their compatible bacteria. As response to that, rhizobia produce and release lipochitooligosaccharide molecules, called Nod factors (NFs) that determine host specificity. Perception of rhizobial NFs is mediated by the plant symbiotic receptor-like kinases (RLKs) NFR5 (Nod Factor Receptor 5) and NFR1 (Nod Factor Receptor 1) in *L. japonicus,* and NFP (Nod Factor Perception) and LYK3 (LysM domain containing receptor-like kinase) in *M. truncatula* that trigger a signalling cascade resulting in bacterial infection and nodule development[3]. Recognition of the rhizobial symbiont is followed by molecular, cellular and morphological changes leading to nodule formation.

Recently a novel component of this symbiotic signalling pathway, the remorin protein MtSYMREM1 from *M. truncatula* was described to regulate rhizobial infections[4]. While members of six defined clusters of the remorin protein family can be found in all land plants[5], MtSYMREM1 is a member of the group 2 that has only been found in legumes, Poplar and winegrape[4,5]. Expression of *MtSYMREM1* is highly induced upon Nod factor treatment, during nodule development and in mature nodules.

Knock-out mutants of *MtSYMREM1* exhibit an impaired nodulation phenotype with significantly less and stunted nodules and are affected in bacterial release[3]. The protein localizes to nodular infection threads in zone II and at the symbiosome membrane (in the zone III of the indeterminate *Medicago* nodules[4]) that separates the bacteroids (differentiated form of nitrogen-fixing rhizobia in nodules) from the plant cytoplasm within the plant cell. Furthermore, MtSYMREM1 was shown to interact with NFP and LYK3, a further downstream receptor-like kinase DMI2 (Does not Make Infections 2) and to form homo-oligomer with itself[4].

The aim of this study was to identify and characterize the functional homolog of MtSYMREM1 in the second model legume *Lotus japonicus* that forms determinate nodules. LjSYMREM1 was identified as putative ortholog of MtSYMREM1. The remorin protein family is characterized by its conserved C-terminal domain, while the family was divided based on the variable N-terminal domain into six different groups[6]. Interestingly the variability between the N-terminal domains of the proteins in group 2 (that involves SYMREM1s) is significantly higher than in other groups. A further interesting fact is, that the two proteins (LjSYMREM1 and MtSYMREM1) exhibit a lower percentage of sequence identity than other orthologous symbiotic signalling proteins of the legumes *M. truncatula* and *L. japonicus*, forced us to further investigate LjSYMREM1 and its putative role in root nodule symbiosis.

Genetic analysis revealed a nodulation phenotype when altering expression by *LjSYMREM1*. Spatio-temporal expression pattern of *LjSYMREM1* was examined using its promoter region fused to a reporter protein and localization of the protein was determined under native expression conditions and constitutive expression in *L. japonicus*, respectively. Furthermore, interactions of LjSYMREM1 with three symbiotic receptor-like kinases (NFR1, NFR5 and SYMRK – Symbiosis Receptor-like Kinase) were analyzed and novel interaction partners were identified in large-scale split-ubiquitin yeast screen.

Materials and methods

In this study, lines of an EMS (ethyl methane-sulfonate) mutagenized *L. japonicus* (ecotype Gifu B-129) population were screened for mutations in *LjSYMREM1* gene. Three different lines were selected and phenotypically examined. One of the lines exhibits a mutation causing an amino acid change, one has a point mutation causing a

premature stop codon and one carrying a point mutation on the first exon-intron junction.

For over-expression studies, a LjSYMREM1:mOrange fluorophore fusion protein was expressed under control of a constitutive polyubiquitin promoter (pUb:LjSYMREM1:mOrange). The same experiments were performed with its N-terminal ($LjSYMREM1_N$) and C-terminal region ($LjSYMREM1_C$). For nodulation of composite plants carrying the respective constructs *Mesorhizobium loti* MAFF 303099 (expressing DsRed fluorophore) strain was used. Roots were subjected to analysis via Confocal Laser Scanning Microscopy (CLSM).

GUS (β-glucuronidase) histochemical assays were performed in order to analyse the spatio-temporal expression of *LjSYMREM1* using its promoter region. To define a functional promoter region a 2kb and 975bp sequence upstream of ATG start codon were fused to the *GUS* reporter gene in a binary construct. For the spatio-temporal experiments the 975bp *pLjSYMREM1:GUS* construct was chosen. Transgenic roots carrying the reporter constructs as well as the empty binary vector (as negative control) were generated via *Agrobacterium rhizogenes* mediated hairy root transformation. Composite plants were inoculated with 10^{-8} M NF and *Mesorhizobium loti* as well. Transgenic roots were harvested over a period of three weeks post inoculation upon *M. loti* treatment and subjected to GUS histochemical staining. Roots were observed using light stereomicroscopy. To study the GUS staining pattern on cellular level roots and nodules were embedded in 5% low-melt agarose and 100 µm sections were inspected via microscopy.

For localization of the LjSYMREM1 protein expressed under its endogenous promoter, transgenic *L. japonicus* (ecotype MG-20) plants were generated via *Agrobacterium tumefaciens* mediated gene transfer using *pLjSYMREM1: gLjSYMREM1:YFP* construct. By the help of PCR and antibiotic selection, plants carrying the transgene were selected. To reveal the protein expression in nodules, they were embedded in 5% low-melt agarose and 150µm sections were analyzed using CLSM.

For protein-protein interaction studies Bimolecular Fluorescence Complementation (BiFC) and split-ubiquitin yeast assay were applied. LjSYMREM1 and the symbiotic RLKs as well were fused C-terminally to N-terminal and C-terminal moieties of the yellow fluorescent protein (YFP; YFP_N; YFP_C). Leaf epidermal cells (of

Nicotiana benthamiana) expressing the respective constructs were inspected 2 dpi using epifluorescence microscopy or CLSM.

In the split-ubiquitin yeast system the constructs Alg5:NubI and Alg5:Cub/Alg5:NubG were used as positive and negative controls, respectively. Correct localizations and specificities of the proteins studied were tested before examining the interactions of the particular proteins. Interactions of LjSYMREM1 with the symbiotic RLKs NFR1, NFR% and SYMRK were investigated.

Large-scale split-ubiquitin yeast screen was performed using a NubG:library created from *L. japonicus* nodulated and mycorrhized roots to identify further putative interaction partners of LjSYMREM1. Several of the identified putative interactors were re-cloned and interactions were verified. In order to test for positive interactions, yeast co-transformants were subjected to drop-tests, where dilution series were plated onto selective yeast media (SD) lacking leucine (L) and tryptophan (W) (as a positive control for yeast growth and to test for the presence of both, bait and prey construct, respectively) and onto SD medium lacking L, W and histidine (serves as a readout of the system) supplemented with 15mM and 30mM 3-AT (3-amino-1,2,4-triazole, a competitive inhibitor of the *HIS3* gene product).

Results and Discussion

Based on sequence alignment and transcriptome profile analysis[7], a group 2 remorin, named LjSYMREM1, was identified as putative ortholog of MtSYMREM1 protein. Monophyletic clustering of LjSYMREM1 and MtSYMREM1 proteins in phylogenetic analysis strongly implies orthology of the two proteins. However, functional homology of the two remorins remains to be proven experimentally. Three different EMS mutagenized *L. japonicus* lines carrying point mutations in the *LjSYMREM1* locus were analyzed to identify a knock-down or knock-out mutant caused by point mutation in *LjSYMREM1*. One of the lines that carries a point mutation on the first exon-intron junction exhibited a significantly increased nodulation phenotype. However the expected impaired nodulation phenotype caused by knock-down or knockout of the LjSYMREM1 protein could not be identified.

Furthermore, when *L. japonicus* composite plants carrying an *LjMYCREM*-RNAi construct that potentially also silences expression of *LjSYMREM1*, the transgenic roots exhibited significantly less and prematurely aborted nodules (J. Bittencourt-Silvestre

and T. Ott, unpublished data). LjMYCREM is another member of the group 2 remorins that is induced in arbuscular mycorrhiza symbiosis[8]. The reduced nodulation phenotype might be caused by knock-down of *LjSYMREM1* since the C-terminal region is highly conserved among the two proteins. A further genetic approach in order to ascertain a role of LjSYMREM1 in the root nodule symbiosis was over-expression of the protein and its N-terminal and C-terminal regions as well. Not only the full-length protein but also solely over-expressed LjSYMREM1 C- and N-terminal regions led to increased nodulation, suggesting that both domains are involved in the biological function of LjSYMREM1.

Spatio-temporal expression analysis was done using a 975bp long *LjSYMREM1* promoter sequence fused to *GUS* reporter gene. These experiment series revealed that *LjSYMREM1* is active 24 hours after NF treatment in epidermal and cortical cells above the root tip, a susceptible zone for bacterial invasion. The same expression pattern was observed 2 dpi with *M. loti* while the expression could not be observed in epidermal cells from the fourth day onward post inoculation with *M. loti* but it was detected in dividing cortical cells (nodule primordia) underneath rhizobial infection foci. Promoter activity was also observed in young nodules (6 dpi) and mature nodules (3 wpi) in the inner cells but not in outer cortex of the nodules. This pattern was confirmed when 3 wpi old nodules were subjected to sectioning and microscopy. Additionally promoter activity was not only detected in the infected cells but also in nodule parenchyma where the protein could not be detected examining the localization of the LjSYMREM1:YFP fusion protein. At this stage no conclusions can be drawn on whether these differences are due to limitations in the sensitivity of the detection or to post-transcriptional regulation of *LjSYMREM1*.

Using stable transgenic plants carrying a *pLjSYMREM1:gLjSYMREM1:YFP* construct, localization of the protein was observed at the symbiosome membrane and nodular infection threads. Furthermore, plasma membrane localization of the protein in epidermal cells was observed when LjSYMREM1 was expressed under control of the *Lotus* polyubiquitin promoter. The N-terminal region of the protein localizes in the cytoplasm while the C-terminal region to the PM suggesting that this region is responsible the PM localization of the protein.

LjSYMREM1 exhibits the same interaction pattern as MtSYMREM1, as it physically interacts with the symbiotic RLKs NFR1, NFR5 and SYMRK. As for MtSYMREM1, homo-oligomerization of LjSYMREM1 was shown as well.

Furthermore, we raised the question whether the two proteins could hetero-oligomerize with each other because of the high sequence identity of the conserved coiled coil containing C-terminal domain or the highly variable N-terminal region would have an impact on interaction. Interestingly, oligomerization of the two remorins from different species, (LjSYMREM1 and MtSYMREM1) could be observed. Interaction occurred when proteins were fused C-terminally to the split YFP halves and also when MtSYMREM1 fused N-terminally (YFP_N:MtSYMREM1) but LjSYMREM1 fused C-terminally (LjSYMREM1:YFP_C) with the YFP moieties. These data indicate that they probably interact via coiled coils of their conserved C-terminal region.

In order to broaden our view on the biological function of LjSYMREM1, a large-scale split-ubiquitin yeast screen was performed to identify novel interaction partners of LjSYMREM1. Among the putative interaction partners we identified an acid phophatase and an AAA+ATPase core domain containing protein. Both proteins belong to large protein families that are involved in a broad range of cellular processes such as signalling, trafficking or refolding. Since LjSYMREM1 was found to be phosphorylated by NFR1 and SYMRK kinase domains[8] it can be hypothesised that the acid phosphatase might dephosphorylate LjSYMREM1. Despite the fact that no evidence has been described so far for LjSYMREM1 that it also would be located in membrane microdomains (like MtSYMREM1[3]), it might be hypothesized that it can recruit cytosolic proteins like the AAA+ATPase core domain containing protein to PM or its microdomains.

Summary

In this study, identification and characterization of the LjSYMREM1 protein, an ortholog of MtSYMREM1, is described. Both proteins are members of the plant-specific remorin family and belong to the almost legume-specific group 2 remorins. Despite their close phylogenetic relationship both proteins show surprisingly low degrees of sequence identities. This is in sharp contrast to other orthologous proteins involved in the symbiotic signal transduction pathway. Orthology between the two SYMREM1 proteins was proven via *in silico* phylogenetic analysis that clearly shows that the two proteins cluster together, however their functional homology could not be confirmed experimentally so far. Other data obtained during this project also indicate the conception of the functional homology of the two proteins.

Over-expression of LjSYMREM1 and its N-terminal and C-terminal regions resulted in increased nodule number that shows the importance of the protein and contribution of both LjSYMREM1 domains to the biological function of the protein involved in nodule formation.

Performing spatio-temporal expression analyses we found that *LjSYMREM1* is induced 24 hours upon NF treatment and 2 dpi with rhizobial treatment in epidermal and cortical cells. Furthermore, *LjSYMREM1* expression was observed in dividing cortical cells (nodule primorida) at 4 dpi and in young and mature nodules coinciding with bacterial infection, when epidermal expression could not be detected anymore. In nodules, promoter activity was observed not only in infected cells but also in nodule parenchyma. Whereas fluorophore tagged LjSYMREM1 was found to be localized to the symbiosome membrane and to the nodular infection threads in the infected zone of nodules and it could not be detected in nodule parenchyma.

Beside interactions of LjSYMREM1 with itself and the symbiotic RLKs NFR1, NFR5 and SYMRK, novel putative interaction partners were revealed in a split-ubiquitin yeast screen. An acid phosphatase that might dephosphorylate the protein caused by phosphorylation with NFR1 and SYMRK and an AAA+ATPase core domain containing protein were identified. The latter one belongs to the large family of AAA+ATPases with diverse cellular functions. It might be hypothesized that this cytosolic protein might be recruited to the plasma membrane via LjSYMREM1.

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List of publications related to this study

Lefebvre B, Timmers T, Mbengue M, Moreau S, Hervé C, Tóth K, Bittencourt-Silvestre J, Klaus D, Deslandes L, Godiard L, Murray JD, Udvardi MK, Raffaele S, Mongrand S, Cullimore J, Gamas P, Niebel A, and Ott T (2010): A remorin protein interacts with symbiotic receptors and regulates bacterial infection; Proc Natl Acad Sci USA, 107: 2343-2348.

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