The genetic mapping and functional analysis of *IPD3* gene required for microbial symbiotic relationships in *Medicago truncatula*

PhD thesis

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Introduction

Leguminous plants (Fabaceae) compose the third largest family of flowering plants including several important crops used for human nutrition and animal feeding. The high level of protein content of legumes owes their capacity to form symbiotic interaction with nitrogen fixing soil bacteria, termed rhizobia. In addition they are able to establish symbiosis either arbuscular mycorrhizal (AM) that facilitate the uptake of phosphorus, water and microelements. These symbiotic capacities confer selective advantages on leguminous plants to grow in habitats of low nutrient. In order to describe the rhizobial and AM symbiotic associations at molecular level in more details, two model legume systems have been developed. The intensive study of the symbiotic interactions established either by *Medicago truncatula* or *Lotus japonicus* (1) helped us to better understand the developmental and differential events of these symbioses.

The most effective form of biological nitrogen fixation is the endosymbiotic interaction between legumes and rhizobia. The interaction between the two partners culminates in the formation a new organ, the root nodule, wherein the conversion of the nitrogen gas and the assimilation of fixed nitrogen takes place. The legumes are able to develop two types of nodules depending on the host plant. Determinate nodule is typical for tropical legumes such as *Lotus japonicus* and soybean. In this type of nodules, the cell division activity is terminated rapidly at the early stage of nodule organogenesis and nodule growth occurs through enlargement of nodule cells. Temperate legumes (e.g. *Medicago truncatula*, pea, alfalfa, etc.) develop indeterminate nodules, wherein the activity of apical meristem is maintained during the nodule lifetime. The nodule meristem continuously produces new cells to generate the tissues of the root nodule and the cells develop through the different of the nodule (2).

The nitrogen-fixing symbiotic relationship is the result of consecutive signal exchanges between the symbiotic partners (3). The perception of plant released host specific flavonoid compounds by the bacteria induces the synthesis of the nodulation factor (Nod factor; NF) in the rhizobium (4). In *M. truncatula* the NF is perceived by NF receptors (NFP and LYK3 proteins) located on the plant plasmamembrane. The receptors with the same function are NFR1 and NFR5 proteins in *L. japonicus*. The binding of NF to the plant receptors initiate the NF signalling pathway (5), which induces the development of nodule primordia and expression of different nodule specific genes and in addition allows the bacteria to enter into the nodule cells.
Several components of the NF signalling pathway are also required to establish the AM symbiotic association (6). Many key components of the symbiosis signalling (Sym) pathway have been already identified and one of them, the CCaMK (DMI3) protein has the presumed role to decode the NF-induced calcium spiking in the epidermal cells of the nodules. Former

studies identified the IPD3 protein as an interacting partner of CCaMK but no information was available about the function of the IPD3 protein (7).

In order to dissect a signalling pathway and identify the genes essential for a biological process, the forward genetic approach has been often applied successfully in many laboratories all over the world. The genetic analyses of symbiotic mutants have been identified more than 30 plant genes up to now which are required for the rhizobial and/or AM symbiotic interactions (8). These genes function in the SYM pathway and they are involved in the infection and invasion of the symbiotic nodule (8).

The objectives of the research project

The aim of our research is to identify plant genes involved in the bacterial colonization of the symbiotic nodule, differentiation of rhizobia and the effective function of the root nodule in the *Medicago truncatula – Sinorhizobium meliloti* symbiotic interaction. In order to identify genes required for the later stages of the nitrogen fixing symbiotic interaction we carry out the morphological characterization and genetic analysis of the mutant plants and finally we clone the genes by map based cloning approach. Previously we analysed the nodulation phenotype of eight mutants which develop nodules but they are impaired in efficient nitrogen fixation (Fix- mutant). In this PhD dissertation, the results of one (*ipd3-1*) of these Fix- mutants are presented. To identify the *IPD3* gene we planned to carry out the following experiments:

1. Characterize the symbiotic nitrogen fixing phenotype of *ipd3-1* mutant line by microscopic analysis to determine what stages of the infection process is blocked in *ipd3-1*.
2. Identify the gene in impaired in *ipd3-1* line by map-based cloning.
3. In order to prove the gene identity of the cloned gene, carry out genetic complementation experiment.
4. Characterization of the *IPD3* gene to study its function in the infection process.

Materials and Methods

Plant material

M. truncatula ssp. *truncatula* (Jemalong and A20 genotype) and *M. truncatula* ssp. *tricycla* (R108 genotype) were used as a wild-type plants in the experiments. The *ipd3-1* mutant line was isolated in the John Innes Centre, Norwich UK in mutant screen of fast neutron mutagenized *M. truncatula* Jemalong plants The *ipd3-2* line was identified from a insertion

mutant collection of *M. truncatula* R108 in the S. Noble Foundation (Ardmore OK, USA). To carry out genetic mapping of the *ipd3-1* mutant locus, an F2 segregating population was generated by the self pollination of an F1 plant of the cross of *M. truncula ipd3-1* and A20 plants. The backcrossed population was created with the cross of *ipd3-1* and Jemalong plants to investigate the inheritance of the mutant locus. The plants were grown under long day light condition (16 h daytime 24 ºC/8 h night, 20 ºC) in a SANYO MLR-350 growth chambers and were inoculated with different rhizobium strains depending on the experiments.

Microscopic analysis of *ipd3-1* and *ipd3-2* nodules

The kinetics of nodule development, rhizobium infection process and the structure of the nodule were analysed in wild-type and *ipd3* mutant plants following the inoculation with *S. meliloti* 1021 *hemA::LacZ* (pXLGD4) bacteria which constitutively express the *LacZ* marker gene (9). 70 µm thick nodules sectioned were prepared and bacteria were visualized with histochemical staining with solution containing X-Gal substrate and assessed by Olympus BX41M light microscope. The presence of bacteria in the different nodule zones was also analysed with Olympus Fluoview FV1000 confocal laser scanning microscopy after green fluorescent nucleic acid-binding SYTO13 staining (10).

Identification of *IPD3* gene mutation by map-based cloning

We analysed the symbiotic phenotype of F2 segregating population individuals 3 weeks post inoculation with B1 *S. meliloti* strain. To determine the genotype of the individuals, we isolated the genomic DNA from one leaf of *M. truncatula* plants using ZenoGene DNS purification Kit. The rough map position of the *IPD3* locus was determined by analysing the genotype of F2 plants with microsatellit markers. For fine mapping, we used intron targeted markers, which were designed for the coding sequences of identified BAC clones. The polymorphic bands were visualized by agarose gel electrophoresis and SSCP. The genotype data were organized and evaluated by the colormap method (11). In order to analyse the sequence of seven candidate genes in the IPD3 region, exon-specific primers were designed using the Primer Premier 5.5. The sequences of the candidate genes from the mutant background were amplified and the PCR products were sequenced and searched for mutations.

Complementation experiments

The genetic complementation of *IPD3* mutant alleles was carried out by generating transgenic hairy roots using the *Agrobacterium rhizogenes* mediated transformation system (12). The constructs of the complementation experiments were created with Gateway cloning system

(Invitrogen). We created the constructs that express the *IPD3* gene driven by constitutive (*35S::IPD3*cDNA-3'UTR in pK7WG2D) and native promoters (*pIPD3::IPD3*cDNA-3'UTR in pK7WG2D). We transformed *ipd3-1* plants with the *IPD3* ortholog of *L. japonicus CYCLOPS* using the construct of *pCYCLOPS::CYCLOPS* in pCAMBIA 1301 provided by M. Hayashi (Osaka University, Japan) (13). The three weeks old transformed roots were inoculated with *S. meliloti* 1021 pXLGD4 (*hemA::LacZ*) and *S. meliloti* CSB357 (*nifH::uidA*) rhizobium strains. Transformed roots were detected by either the green fluorescent protein (GFP) or DsRed fluorescent markers using Olympus SZX12 stereo microscope. We assessed the nodulation phenotype on the transformed root and structure of the nodules following X-Gal and GUS staining.

Spontaneous nodule experiment

In order to analyze the position of *IPD3* gene in the symbiotic signal pathway we carried out spontaneous nodule test in *A. rhizogenes* transformation system. The autoactive deletion version of the *CCaMK* gene was introduced into mutant plants to activate nodule morphogenesis without the presence of rhizobium in nitrogen-free medium. The truncated form of CCaMK containing only the kinase domain of CCaMK (14) was provided by Erik Limpens (Wageningen University, Netherlands). We examined the structure of the induced nodules following toluidine blue staining.

Gene expression analysis

In order to carry out gene expression analyses, the total RNA was extracted from roots. complementary DNA was prepared from 1 µg of total RNA with Superscript III Reverse Transcriptase Kit (Invitrogen) using oligo-dT primers. To monitor double-stranded DNA synthesis in quantitative real-time PCR, we used SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) and Maxima SYBR Green Master Mix (Fermentas) kits. The reactions were carried out with 40x diluted template DNA and the final primer concentration of each gene-specific primer was 100 nM. The primers were designed by Primer premier 5.5 Program. Semi-quantitative PCR experiments were performed on cDNA templates and the final primer concentration was 400 nM. To monitor the expression of symbiotic genes, five-day-old plants were inoculated with *S. meliloti* 1021 strain. For NF treatment, five-day-old seedlings were transferred into beakers and incubated for 16 h in liquid BNM medium supplemented with 10 nM NF. The *M. truncatula* roots were assayed for expression of the early nodulin gene *ENOD11* by histochemical staining for GUS activity. Wild-type plants carrying the *pMtENOD11::GUS* construct (16) were crossed to *ipd3-1* line to introduce the reporter gene into the mutant

background. F2 plants carrying the *pMtENOD11::GUS* construct and containing the *ipd3-1* deletion allele were identified by PCR amplification using allele specific primers of *IPD3* and the GUS gene. The seven-day-old plants were transferred into liquid BNM media containing 1 nM NF and treated for 24 h. As a next step the roots were staining GUS solution and incubated overnight in the dark and analysed with using Olympus SZX12 stereo microscope.

Results

In the John Innes Centre (Norwich, UK) research institute fast neutron mutagenized *Medicago truncatula* Jemalong plants were screened for mutant plants to identify mutant plants showing defects in symbiotic nitrogen fixation. Eight Fix^- mutant plants were identified and selected from this screen. We carried out morphological analysis one of the Fix- plants (*ipd3-1*) to determine what stages of the symbiotic process is blocked.

1. The *ipd3-1* mutant showed the symptoms of nitrogen starvation, yellowish leaves, stunted growth habit and developed small white nodules in symbiotic conditions. We detected a significant increase in nodule number in the mutant plants compared with wild type plants. The kinetics of nodule development was also analysed in wild type and *ipd3-1* plants. The mutant plants showed delayed nodule development and delayed and defective bacterial infection. No evident difference was observed in root length, but an extended infected region of the root and significantly more nodule primordia were detected. After four or more weeks post rhizobium inoculation, two types of small nodules developed on the mutant plants; (i) in most of nodules the infection threads (IT) were limited to the nodule apex, (ii) 15-20% of the nodule totally completed with ITs. After six weeks post inoculation a small number of the nodule (5-10%) developed into white elongated cylindrical structures. These nodules contained an extended invasion zone hypertrophied with ITs. Based on confocal laser scanning microscopy study most IT displayed blister-like formations and the bacteria retained inside of the ITs. From these analyses, we conclude that the mutated gene has role in release of bacteria from the ITs.
2. Two research institutes (Noble Foundation and University of Wisconsin, USA) identified another *ipd3* allele (*ipd3-2*) from a *M. truncatula Tnt1*-insertional mutant population. The nodule development and the infection was blocked at a slightly earlier stage in the *ipd3-2* mutant compared to *ipd3-1*; while the cortical cell division was induced, no IT growth was observed indicating the defects of IT development.
3. In order to confirm the results of infection process and microscopy experiment for nodule development, we carried out quantitative and semi-quantitative RT-PCR experiments. The expression of the monitored late-early nodulin marker genes were reduced and the most of

the late nodulin genes were not expressed suggesting that the expression profile of symbiotic marker genes correlates with the microscopy studies. Based on these data we concluded that the *ipd3-1* mutant have defects in the middle stages of symbiotic nitrogen fixing process.

4. The allelism test F1 plants of the cross of *ipd3-1* and *ipd3-2* plants indicated that they belong to the same complementation group.
5. We determined the inheritance of the mutant phenotype. The segregation ratio of the F2 mapping populations (328 Fix^+ / 130 Fix^-) indicated that the mutant phenotype was inherited as a single recessive locus.
6. Using a map-based cloning approach the mutant locus was positioned on the upper arm of chromosome 5 between genetic markers ENOD40 and MtB93. Fine mapping positioned the locus in the genomic region covered by BAC clones (mth4-3n15 and mth4-28c15) using an extended F2 mapping populations. We analysed the gene content in the genomic region between two flanking markers (BRL and GTP). Oligonucleotide primers were synthesized for the seven predicted genes in the region and PCR amplifications were carried out to amplify the genes from genomic DNA and cDNA samples prepared from mutant plant. The amplification of a part of the *IPD3* gene detected a deletion in the *IPD3* transcript. Sequence analysis of the *IPD3* cDNA revealed a single base-pair change at the end of the fourth exon and the deletion of the next six base-pairs at the start of the fourth intron. These mutations caused imperfect splicing mechanism and the *IPD3* transcript lacking exon four.
7. In order to prove that the mutation in *IPD3* caused the mutant phenotype, we carried out successful genetic complementation experiments with both mutant alleles. In addition the ortholog of *MtIPD3, LjCYCLOPS* also restored the symbiotic phenotype in the *ipd3-1* mutant.
8. In contrast to wild-type plants, *ipd3-1* showed a very limited NF inducible activity of *pMtENOD11::GUS*; the activity was restricted to a smaller region than the wild-type and the intensity of GUS staining was seriously reduced.
9. Using quantitative (q)RT-PCR, we examined the expression of *ENOD11* and other early nodulin genes, *NIN* and *HAP2* in *ipd3-1, ipd-2 and ipd3-3* (identified by Ovchinnikova et al. 2011) plants following 16 h of NF treatment and 1, 8, 14 days post inoculation with *S. meliloti*. A significant reduction of NF-inducible *ENOD11*, *NIN* and *HAP2* expression were detected in all of *ipd3* alleles compare with wild-type. We observed reductions in the expression of these genes during rhizobium infection as well, but the nature of this reduction was not consistent across all genes.
10. In the spontaneous nodulation test carried out on *ipd3-1* and *ipd3-2* plants, we could not detect development of nodule-like structures. The spontaneous nodule formation was

confirmed by toluidine blue staining that revealed that spontaneous nodules did not contain any bacteria.

Conclusions

IPD3 was identified previously in a yeast two-hybrid screen as an interaction partner of DMI3 (CCaMK), a component of the NF signal transduction pathway (7). In this PhD dissertation I report the identification of *ipd3* mutant alleles and the characterization of their symbiotic defects. The microscopic analysis of *ipd3* lines indicated that *IPD3* functions in IT growth and the release of bacteria from ITs. Nodule development was severely retarded in *ipd3* mutants and the nodule invasion was blocked at an early developmental stage. We observed more developed nodules in *ipd3-1* mutant compared to *ipd3-2* but these more-developed nodules never showed bacterial release or symbiosome formation. The late-early- and late nodulin genes expression was reduced or not detectable in the mutant lines, and this correlates well with the defects we observed in nodule development. These results imply that the phenotypic differences between *ipd3-1* and *ipd3-2* alleles may depend on their different genetic background. We suppose that an additional genetic component in *M. truncatula ssp truncatula* Jemalong genotype, which can partially complement the defects of *IPD3* in nodule development and this component is absent or less active in *M. truncatula ssp. tricycla* R108 background causing the earlier block of nodule development. The lack of expression of early nodulin genes suggested that IPD3 functions upstream of these genes in the NF induced signal pathway. The spontaneous nodule test enables us to determine the position of the genes relative to *CCaMK* in the NF signal pathway. The genes act downstream of *CCaMK* or lie in a parallel signal pathway, result in spontaneous nodule development in their mutated alleles using the autoactivated form of *CCaMK* while the genes positioned upstream of *CCaMK* unable to initiate spontaneous nodule morphogenesis. We could not observed spontaneous nodule development in the *ipd3* alleles using the gain of function version of CCaMK protein.

Based on these results and several other collaborative experiments (data not show), we concluded that the complex of IPD3 and DMI3/CCaMK functions between calcium spiking and transcription activation of early nodulin genes *NSP1* and *NSP2* and have an essential role in the accommodation of the rhizobial and mycorrhizal partners in the host plant.

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1. **Beatrix Horváth**, Gábor Halász, Ágota Domonkos, Ferhan Ayaydin, Krisztina Miró, John Marsh, Giles E. D. Oldroyd, Péter Kaló
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