A plant chitinase controls cortical infection thread progression and nitrogen-fixing symbiosis

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Abstract:

Morphogens provide positional information and their concentration is key to the organized development of multicellular organisms. Nitrogen-fixing root nodules are unique organs induced by Nod factor-producing bacteria. Localized production of Nod factors establishes a developmental field within the root where plant cells are reprogrammed to form infection threads and primordia. We found that regulation of Nod factor levels by *Lotus japonicus* is required for the formation of nitrogen-fixing organs, determining the fate of this induced developmental program. Our analysis of plant and bacterial mutants shows that a host chitinase modulates Nod factor levels possibly in a structure-dependent manner. In *Lotus*, this is required for maintaining Nod factor signalling in parallel with the elongation of infection threads within the nodule cortex, while root hair infection and primordia formation are not influenced. Our study shows that infected nodules require balanced levels of Nod factors for completing their transition to functional, nitrogen-fixing organs.

Keywords: morphogen, symbiosis, Nod factor, chitinase, *Lotus japonicus*
Introduction

Molecules of microbial origin have the capacity to induce a morphogenetic response in symbiotic eukaryotic hosts. Nitrogen-fixing rhizobia produce species-specific decorated chitin-like molecules, Nod factors, that are recognized by LysM receptors in legume roots and initiate two different processes; nodule development and infection thread (IT) formation. In addition to their signalling capacity, Nod factors have been considered morphogens based on their effect on host developmental programs, but direct measurements of in planta-produced Nod factor levels proved intractable, and therefore limited our knowledge on their concentration-dependent effect on the host. ITs are tubular structures used by bacteria as conduits to advance from the root hair tip towards the root cortex. IT passage through cortical layers involves reprogramming of individual cells for microbial infection. Inside nodule primordia, branched IT arrays spread from the main shaft, guiding the confined rhizobia towards competent cortical cells where endocytosis takes place. Although a number of components are known, the mechanisms governing IT formation are not defined, and even less understood is the control of IT branching inside root nodules. Rhizobia grow and multiply during infection thread progression and consequently bacterial signals accumulate inside the limited space within ITs. Nevertheless, the host determines the compatibility of Nod factors and exopolysaccharide produced by bacteria during root hair infection, via NFR1/NFR5 and EPR3 receptors, respectively.

In parallel with the signalling events induced by Nod factor recognition, legumes express chitinases with Nod factor cleaving activity. Plant chitinases are primarily found as components of immune responses induced by chitin producing pathogens, but novel functions of these enzymes have evolved in plants and in symbiotic interactions between eukaryotes and microbes. The identification of Nod factor-cleaving chitinases induced during legume-rhizobia symbiosis led to a model where these enzymes are required for Nod...
factor hydrolysis to avoid activation of immune responses induced by these chitin-like molecules during symbiosis. However, unlike chitin, Nod factors do not trigger immune responses in *Lotus* and in this context the function of Nod factor cleaving chitinases remained unclear. This was further confirmed by the *Medicago truncatula*, Nod factor hydrolase (NFH1) mutants that display delayed infection thread formation and nodule hypertrophy.

**Results and discussion**

We identified *chit5-1, chit5-2* and *chit5-3* as Fix’ Gifu mutant alleles from screens aimed at discovering symbiotic defective mutations following inoculation with *Mesorhizobium loti*. The impaired symbiotic association was reflected in significantly reduced shoot growth (Fig. 1A). This defective phenotype was alleviated by the addition of nitrogen (10mM KNO₃) to the growth substrate. Detailed analysis of the symbiotic phenotype manifested by the three alleles revealed that nodule organogenesis was initiated in the presence of *M. loti* (Fig. 1 source data 1), but most nodules remained arrested at the primordia stage with only a few developing into large, white or pink-spotted nodules (Fig. 1B, C). Assessment of bacterial nitrogenase activity through acetylene reduction assays revealed that mutant nodules, despite their occasional functional appearance (pink-spotted), had significantly reduced nitrogenase activity compared to wild-type (Fig 1D). The limited nitrogen fixation capacity is reflected in the observed plant growth and nodule phenotypes. This was further supported by reduced transcriptional activation of leghemoglobin (*Lb3*) and sulfate transporter (*Sst1*) genes which are prominent molecular markers for mature nodules and successful nitrogen-fixing symbiosis. In contrast, transcripts associated with early rhizobial infection, early nodulin *N6* and pectate lyase *Npl1*.
and transcription factor Ern1, were induced to higher levels in the chit5 mutants compared to wild-type plants (Fig. 1 Figure supplement 3). No morphological signatures associated with activation of plant defense responses, such as the onset of early senescence, previously reported for some Fix’ mutants was observed in chit5 plants (Fig. 1B). Mpk3, Wrky29 and Wrky33, marker genes upregulated during plant immunity, were found to have a wild-type level of expression in both uninoculated and 21-dpi whole mutant roots (Fig. 1 Figure supplement 3 and Figure 1 source data 2). Recent transcriptome analyses of Lotus revealed that progression of symbiosis in wild-type roots and formation of the symbiotic nodules (21 dpi) is accompanied by downregulation of these immunity marker genes (Fig. 1 Figure supplement 4 A). We have analysed the expression of these marker genes in 21dpi nodules of wild-type and chit5 mutants. We found a higher level of the Npl1, Mpk3, Wrky29 and Wrky33 transcripts in chit5 mutant nodules compared to wild-type nodules (Fig. 1 Figure supplement 4). The differences in the expression levels of the early symbiotic and defense markers detected between wild-type and chit5 mutants indicate a deregulated symbiotic and immune signalling, seemingly arresting nodules at an early infection stage. We investigated whether rhizobial infection was affected and found that a normal number of ITs were formed in chit5 mutant root hairs (Fig. 2A), and these proceeded in a manner similar to wild-type plants (Fig. 2 Figure supplement 1). Together, these results demonstrate that the early programs initiated by Nod factor signalling, nodule organogenesis and formation of root hair ITs, operate normally in chit5 mutants.

In determinate nodules, like those formed by L. japonicus, ITs are present inside the cortex of mature nodules and can be quantified. We have performed detailed investigations of IT phenotypes in wild-type and chit5 mutant nodule sections (n=30) using light and electron microscopy. Counting of the cortical ITs formed inside wild-type and infected mutant nodules revealed a severe and significant reduction in their number in the mutant nodules.
Mutants displayed an impaired symbiotic occupancy of the nodule central tissue, with only scattered infected cells that formed inside the pink-spotted nodules (Fig. 2C). Bacteria accumulated in the intercellular spaces between nodule cells (Fig. 2C and D), giving rise to fewer, localized intracellular infections (Fig. 2D). Symbiosomes present in the infected mutant cells contained multiple bacteroids of irregular shape and of atypical appearance (accumulation of white matrix) (Fig. 2D). These phenotypes are likely a result of symbiotic and defense genes deregulation inside chit5 nodules. These results demonstrate that CHIT5 functions during cortical IT development and branching within nodules, and is required for the establishment of fully functional nitrogen-fixing symbiosis.

Map-based cloning and whole genome sequencing revealed that all three alleles are defective in one of the three chitinase-coding genes present at the identified genomic location (Fig. 3A, Figure 3 Figure supplement 1, and supplement 2), while the remaining two paralogs are pseudogenes with premature stop codons (Fig. 3 Figure supplement 2). The chit5-1 and chit5-3 mutants were found to have large genomic deletions encompassing the chitinase gene, while chit5-2 carries a point mutation (CCA to CTA), that results in a proline to leucine (P\textsubscript{168}-L) transition in the predicted protein sequence (Fig. 3A and Fig. 3 Figure supplement 2). The symbiotic defective phenotype of all three alleles was restored by genetic complementation with the wild-type Chit5 gene expressed from its native promoter (2072bp) and terminator (2108 bp) regions, or from the LjUbiquitin promoter and Nos terminator (Fig. 3 Figure supplement 3). Analyses of Chit5 expression using transcriptional reporters (Chit5-tYFPnls and Chit5-GUS) revealed promoter activity in all cells of uninoculated roots, in infected root hairs (Fig. 3B), and nodule primordia (Fig. 3 Figure supplement 4). Chit5 promoter activity decreased inside fully functional nodules, indicating a reduced requirement for Chit5 at later stages of a functional symbiosis (Fig. 3B, Fig. 3 Figure supplement 4).
The CHIT5 protein consists of a predicted signal peptide at the amino terminus followed by a class V (glycosyl hydrolase 18) domain with a conserved catalytic DxDxE motif (Fig. 3). The proline residue mutated in chit5-2 allele is located immediately after the catalytic site, and is conserved across GH18-type chitinases, indicating a crucial role for the function of this protein (Fig. 3 Figure supplement 5). CHIT5 shares a high level of similarity to Medicago truncatula NFH1, including the presence of A and B loops and the proline residue (P_{294}), previously reported as essential for Nod factor hydrolysis (Fig. S8). Furthermore, we found that a version of CHIT5 mutated in the DxDxE motif (E_{166} to K) was no longer able to complement the symbiotic defective phenotype, suggesting that enzymatic activity is required for CHIT5 function in planta (Fig. 3 Figure supplement 6). To investigate the enzymatic properties of CHIT5 in planta, we took advantage of its predicted secretion (Fig. 3 Figure supplement 5) and ubiquitous expression in Lotus roots (Fig. 3B) and analysed the hydrolytic capacities of wild-type and mutant root exudates on chitin hexamers (CO-VI) and on R7A Nod factor (LCO-V (18:1, Me, Cb, FucAc)).

For testing CHIT5-dependent hydrolysis of CO-VI by root exudates we made use of a physiological test based on the differential capacity of CO-VI, CO-III/CO-II and Nod factor elicitors to induce ROS production in Lotus. CO-VI elicitors present in the control samples (not exposed to plant root exudates) induced ROS production, while those exposed to wild-type or mutant roots lost this capacity (Fig. 3C). This shows that CO-VI hydrolysis took place during incubation with plant roots irrespective of the genotype and that CHIT5 is thus not solely required for CO-VI hydrolysis in the Lotus rhizosphere. Similar analyses performed with LCO-V showed no ROS production, confirming that intact R7A Nod factor molecules (present in the control samples) or their possible hydrolysis products resulting from exposure to plant roots, lack the ability to induce ROS in Lotus roots (Fig. 3D). Analyses of butanol-extracted fractions from LCO-V samples determined that hydrolysis of the R7A Nod factor...
into LCO-II (18:1, Me, Cb) was induced by exudates from roots (Fig. 3E). Exudates from all
chit5 mutants induced lower production of LCO-II than wild-type exudates, and there was a
corresponding reduction in LCO-V degradation by mutants compared with wild-type (Fig. 3E
and Figure 3 source data 1). Since LCO-II was produced after exposure to chit5 exudates,
additional plant chitinases expressed in Lotus roots (Fig. 3 Figure supplement 7) may
contribute to Nod factor hydrolysis in the Lotus rhizosphere. Our in planta assays
demonstrate that secreted CHIT5 contributes to hydrolysis of R7A Nod factor. Nevertheless,
CHIT5 and unknown hydrolase(s) induce only a limited hydrolysis of the fully compatible
Nod factor, most likely to ensure that symbiotic signalling occurs, as shown in wild-type
plants.

*M. loti* NodD1 and NodD2 transcriptional regulators control the expression of Nod factor
biosynthesis genes and are preferentially active at distinct stages of *M. loti*-Lotus symbiosis
34. NodD1 is primarily active inside root hair ITs, while NodD2 activates the transcription of
Nod factor biosynthesis genes within nodules. NodD1 and NodD2 were found to differ
significantly in their capacity to induce Nod factor biosynthesis genes transcription on Lotus
roots and coordinated activity between them ensures an intermediate gene expression in wild-
type R7A 34. Our analysis of chit5 mutants revealed a requirement of CHIT5 for cortical IT
extension leading to effective colonization of nitrogen-fixing nodules (Fig. 2). This provided
us with the opportunity to further investigate the necessity of CHIT5 for monitoring Nod
factor levels in planta, by assessing the phenotypes of nodD1 (D1+/D2+) and nodD2 (D1+/D2−
) bacterial mutants. chit5 mutants inoculated with nodD1, that induces a 3-fold higher level of
Nod factor biosynthesis gene transcription compared to wild-type R7A in response to Lotus
root exudates 34, displayed a severe nitrogen-deficient phenotype (Fig. 4A). Comparable root
hair IT formation was observed on wild-type and chit5 plants (Fig. 4 Figure supplement 1)
yet nodules induced by nodD1 on chit5 mutants were even more severely impaired than those
induced by R7A. Nodules were rarely or superficially infected and were found to be severely ineffective in acetylene reduction assays (Fig. 4 and Figure supplement 1). In contrast, inoculation with nodD2, that induces a 4-fold lower level of Nod factor biosynthesis gene transcription compared to wild-type R7A, resulted in full restoration of chit5 defective phenotypes (Fig. 4 and Figure supplement 1). A similar number of pink nodules were formed on wild-type and chit5 mutant roots and plants were no longer nitrogen-deficient. Fully colonized nodules with efficient nitrogen-fixing symbiosomes were formed (Fig. 4 Figure supplement 1). These contrasting phenotypes of chit5 mutants in response to M. loti strains affected in the regulation of Nod factor biosynthesis indicate that CHIT5-dependent modulation of Nod factor levels within nodules is critical for cortical IT development and complete transition to nitrogen-fixing symbiosis.

Nod factors are perceived by the NFR1/NFR5 receptors. The expression of Chit5 is similar to that of Nfr1/Nfr5 and follows the spatio-temporal development of symbiosis in the root and nodules (Fig. 3B and Fig. 3 Figure supplement 4). The observed requirement for tight modulation of Nod factor levels inside nodules (Fig. 4A and Figure supplement 1) prompted us to investigate whether the chit5 phenotype in the presence of wild-type M. loti could reflect a bottleneck in Nod factor signalling due to limited/tightly controlled NFR-dependent signalling. We tested this possibility by overexpressing Nfr1 and Nfr5 receptors in chit5 mutants, but observed no change in their symbiotic phenotype after inoculation with M. loti R7A (Fig. 4 Figure supplement 2). This indicates that increased and ectopic expression of Nod factor receptors is insufficient to restore the chit5 defective phenotype.

Bacterial genetics coupled with in planta phenotypic studies and chemical analyses of Nod factors produced by rhizobial mutants have determined that various Nod factor decorations are required for initiation and progression of symbiosis. We investigated the importance of Nod factor decorations for CHIT5 activity in Lotus by assessing the phenotypes induced
by R7A *nolL* and *nodZ* mutants that produce Nod factors lacking acetyl (LCO-V(18:1, Me, Cb, Fuc)) and acetylated-fucosyl decorations (LCO-V(18:1, Me, Cb)), respectively on the reducing-end of the Nod factor. Compared to wild-type *M. loti*, these bacterial mutants are delayed in initiating nodule organogenesis and form fewer infected nodules (Fig. 4 Figure supplement 3), a phenotype similar to the one induced by *M. loti* *nodD1* mutant.

Interestingly, we found that these bacterial mutants infected *chit5* mutants and wild-type similarly and that, compared to wild-type or *M. loti* *nodD1*, no significant differences in nodule number, infection rate and nitrogen fixing ability were observed between plant genotypes (Fig. 4 Figure supplement 3). This suggests that cortical infection and onset of nitrogen fixation in the symbiotic organ might require tight modulation of Nod factor levels in a structure-dependent manner. Decorations have been shown to be important for determining the rate of Nod factor degradation by different chitinases. Nod factors produced by *nodZ* and *nolL* mutants may become accessible to other chitinases present in the *chit5* mutants for degradation. Alternatively, Nod factor signalling induced by *nodZ* and *nolL* mutants may be reduced compared to wild-type bacteria and therefore not requiring CHIT5-dependent modulation. These findings based on analyses of plant and bacterial mutants in binary interactions questioned the functional relevance of CHIT5 in the context of natural environments where a diverse rhizobial population, presumably producing Nod factors with various structures at various levels, is present. We investigated this by analysing the phenotypes of *chit5* mutant plants grown in soil and exposed to the natural rhizobial population compared with wild-type and *nfr5*-2 plants. We found that both *chit5* and *nfr5* mutant plants were clearly distinctive from wild-type, as they were nitrogen starved in spite of numerous nodule primordia formed on *chit5* plants (Fig. 4 Figure supplement 4). This indicates, that at least in the presence of the tested soil rhizobial population, CHIT5 is a major
determinant for the onset of nitrogen-fixing symbiosis in the nodules whose formation is controlled by NFR5.

Our study revealed that in L. japonicus, CHIT5-dependent hydrolysis of the Nod factor morphogen plays a crucial role during primordia infection and for full-transition to symbiotic nitrogen fixation. Chit5 in L. japonicus and Nfh1 in M. truncatula are highly similar, however the mutant phenotypes in the two model-legumes are very different. Unlike in the nfh1 mutant, the number and appearance of root hair ITs and the size of nodule primordia appear not to be affected in chit5 mutants, indicating that in Lotus other mechanisms independent of CHIT5 control these early stages of symbiosis. Chit5 and Nfh1 are part of genomic clusters with closely-located paralogs (Fig. 3 Figure supplement 1, Fig. 4 Figures supplement 5 and 6). In Lotus, two of the three paralogs evolved as pseudogenes (Fig. 3 Figure supplement 2 and Fig. 4 Figure supplement 7), while in Medicago all are functional genes and expressed (Fig. 4 Figure supplement 5). This may contribute to the differential phenotypes observed in the two model legumes, and may explain the strong phenotype displayed by Chit5 mutants in Lotus. Alternatively, Chit5 and Nfh1 might be highly similar but non-orthologous genes. Based on our observations from Lotus we propose a likely scenario explaining CHIT5 function (Fig. 4D). Bacteria maintain a low level of Nod factors within root hair-traversing ITs by preferential activity of NodD1. Bacterial amplification inside primordia-elongating ITs, coupled with the switch for preferential activity of NodD2 leads to higher levels of fully decorated pentameric Nod factors (LCO-V) that unbalance the symbiotic and immune signalling in the cortical cells. CHIT5 hydrolytic activity on LCO-V is crucial for maintaining balanced symbiotic and defense signalling in the root developmental field and is required for cortical IT extension inside primordia, efficient bacterial endocytosis and ultimately, development of the nitrogen-fixing organ. In the absence of Chit5, IT elongation is impaired leading to bacteria accumulation in
the intercellular space and sparse intracellular infection (Fig. 4D). However, we cannot
exclude that a CHIT5-dependent product derived from Nod-factor hydrolysis plays a role
during nodule infection. Interestingly, similar but less frequent infections, as observed in
chit5 mutants inoculated with wild-type M. loti, was reported for spontaneous nodules
formed on nfr1snf1 plants when infected by an M. loti nodC mutant lacking the ability to
produce Nod factors. Surprisingly, the symbiosomes formed by the M. loti nodC mutant were
reported to have normal appearance and the plants to be nitrogen proficient. These results
suggest that Nod factors, or their hydrolytic products, are dispensable for nitrogen fixation
within infected cells. Based on these results and our findings from symbiotic gene expression
in chit5 mutants and their phenotype in the presence of mutant rhizobia, we consider the
scenario proposed in Fig. 4D as the most likely framework for CHIT5 function. Future
studies will likely reveal which molecular determinants are responsible for decoding the
CHIT5 output into nitrogen-fixing state inside infected cells.

Materials and Methods

Plant materials. Lotus japonicus ecotype Gifu B-129 was used as the wild-type
plant. Plants were grown at 21°C with 16 h day and 8 h night cycles. Agrobacterium strain
AR1193 was used for hairy-root transformation experiments, carried out as described
previously. Plants were inoculated with corresponding bacterial suspensions, OD_{600}=0.02.

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are
listed in Figure 1 source data. Mesorhizobium loti R7A and mutant strains were
cultured at 28°C in YMB or G/RDM medium. Strains expressing fluorescent reporters
were constructed by the introduction of pSKDSRED or pSKGFP. Antibiotics were added
to media as required at the following concentrations: tetracycline, 2 μg ml\(^{-1}\); rifampicin, 100 μg ml\(^{-1}\); spectinomycin, 100 μg ml\(^{-1}\); ampicillin, 100 μg ml\(^{-1}\).

**Chit5 gene cloning.** The \(\text{chit5-1 (sym43)}\), \(\text{chit5-2 (sym103-1)}\) and \(\text{chit5-3 (sym103-2)}\) mutants were isolated as fix\(^{-}\) mutants in the background of \(L.\ japonicus\) ecotype ‘Gifu’, and they were described by Sandal et al., 2006. An F\(_2\) mapping population was established by crossing \(\text{chit5-1}\) mutant to wild-type \(L.\ japonicus\) ecotype ‘MG-20’. In total, 1508 F\(_2\) offspring mutant plants allowed us to limit the region of interest to 483 kb on chromosome 5 (Fig. S4). Additional attempts to further limit the candidate region were not successful due to suppression of recombination in this chromosomal region. Next, we used a combination of BAC/TAC subcloning and whole genome sequencing of nuclear DNA \(^{46}\) from the three alleles by Illumina technology (NexTera Library Kit) to pinpoint the candidate mutated gene. SHOREmap strategy \(^{46}\) was used for SNP calling based on \(L.\ japonicus\) v.3.0 genome. We identified that \(\text{chit5-1}\) and \(\text{chit5-3}\) contain deletions of app. 13 and 9 kb, respectively in this region while \(\text{chit5-2}\) has a point mutation C/T in a predicted chitinase gene. Three chitinases (\(\text{Chit5}\), \(\text{Chit5a}\) and \(\text{Chit5b}\)) sharing high identity in the coding, 5’ and 3’ DNA flanking regions were identified to be present in this genomic region (Fig. S5). \(\text{Chit5a}\) and \(\text{Chit5b}\) have early stop codons and are therefore pseudogenes (Fig. S5). The \(\text{chit5-1}\) and \(\text{chit5-3}\) mutants lack \(\text{Chit5}\) gene, and one of the pseudogenes, while \(\text{chit5-2}\) has all three genes, but contains a point mutation (C/T) in \(\text{Chit5}\) (Fig. S4). The precise location of the three genes in the current version of \(L.\ japonicus\) genome is not possible to assign due to the high level of similarity and repetitive nature of the DNA sequences present in this region.

**Plant phenotypic assessment.** Seed sterilization and plant-growth setups for nodulation and infection thread assays were as previously described \(^{13}\). For nodulation assays plants were inoculated with R7A, \(\text{nodD1}, \text{nodD2}, \text{nolL}\) and \(\text{nodZ}\) and scored weekly. For IT counts, plants were inoculated with strains carrying the pSKDSRED reporter plasmid. Roots
of 10 dpi plants (n=20 per biological replica) were cut into 1 cm pieces and 20 pieces were
examined for infection thread counts. For phenotypic assessment of plants grown in soil,
sterile seedlings were planted in pods containing Cologne soil\(^47\) and the shoot biomass,
together with the number of pink and total nodules were counted at 9 weeks post-planting.
The soil was supplemented with 10mM KNO\(_3\) for phenotypic assessment in the presence of
nitrogen.

**Acetylene reduction assays.** Ethylene production was monitored in acetylene
reduction assays using a SensorSense (Nijmegen, NL) ETD-300 ethylene detector as
previously described\(^48\).

**RT-qPCR.** Root systems formed by chit5 mutants and wild type plants were
harvested 21 days after inoculation with *M. loti* R7A mock (diluted YMB medium). The
plants were grown on plates supplemented with 1/4 B&D medium in a 16/8 h day/night regime
of 21/16 °C. The mRNA was extracted using Dynabeads (Invitrogen) and the quality of the
purified mRNA was examined with a 2100 Bioanalyzer RNA pico chip (Agilent). cDNA was
synthesized by RevertAid Reverse Transcriptase (Fermentas) with an OligodT primer. For
quantitative real-time PCR analysis of symbiotic transcripts levels, three housekeeping genes
(ΑTP, UBC, and PP2A encoding L. japonicus ATP synthase, Ubiquitin Conjugating Enzyme
and Protein Phosphatase 2A) were used as references. Specificity of the primers was ensured
by melting curve analysis and sequencing of the amplification products. Quantitative real
time PCR using specific primers for the reference and target genes (Figure 1 source data 2)
was performed using the LightCycler 480 II (Roche) with the LightCycler SYBR Green I
Master kit. The relative quantification software by Roche was used to determine the
efficiency-corrected relative transcript levels, normalized to a calibrator sample. The
geometric mean of the relative expression ratios for the three biological and three technical
replicates has been calculated as well as the corresponding upper and lower 95% confidence intervals.

**Light microscopy (LM) and transmission electron microscopy (TEM) analyses**

including cortical infection thread (IT) counts. For cortical ITs white nodules inoculated with ML001 DsRed were scored at 4 wpi. Nodules were prepared for LM and TEM investigation of infection from the indicated genotypes. Nodules that were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.0) overnight at 4 °C were dehydrated in an ethanol series, and embedded in LR White acrylic resin (Agar Scientific, UK). Semithin sections (1 μm) of these were taken for light microscopy. Ultrathin (70 nm) sections were taken for TEM using a Leica UCT ultramicrotome from samples that had been post-fixed in 1% osmium tetroxide and embedded in Araldite epoxy resin. The semithin sections were collected on glass slides and stained with 0.1% toluidine blue, whereas the ultrathin sections were collected on pioloform-coated nickel grids. The number of ITs in semithin sections was counted in 2 representative sections of 10 different nodules for wild-type and chit5-1 genotypes.

**Chit5 expression analyses.** Chit5 promoter region of 2072 bp upstream of start codon was PCR-amplified from L. japonicus Gifu using primers caccCATACTTAACCAATGTGGTACTTCAATTC (PM-9331) and GTGTATATATGTGAAACCTTGCATCTC (PM-9332). The amplification product was cloned into pIV10 carrying GUS or tYFP-NLS reporters using a Gateway cloning strategy. Promoter activity was investigated in transformed roots expressing the reporter constructs at 1, 3, 7, 10, 14, 21 dpi after inoculation with M. loti. For analysis of Chit5 promoter-GUS activity, roots were stained as previously described. Chit5 promoter-tYFP-NLS activity was investigated using a Zeiss LSM780 meta confocal microscope.

**Genetic complementation.** For complementation analysis, a construct consisting of Chit5 promoter (2072 bp) followed by genomic Chit5 sequence (3206 bp) was used. The
clone was obtained by a sequential subcloning from BAC69G19 following: 1) *HindIII* digest and cloning in pGreen29, and 2) *SmaI* and *SalI* digest of pGreen29 construct, and cloning into pIV10. The construct was integrated into *Agrobacterium rhizogenes* AR1193 that was used for hairy root plant transformations. AR1193 with empty pIV10 vector was used as a control. To introduce the E to K mutation in CHIT5 sequence, the pIV10 *Chit5* construct was used as a template for introducing the point mutation using primers GATTGGaAGTGGCCAGGAGATG (PM-11341) and CCACTtCCAATCCAGTCAAGACC (PM-11342). The construct was then moved to *Agrobacterium rhizogenes* AR1193 for hairy root plant transformations. For *Chit5* overexpression the *Chit5* coding region from start to stop codon was amplified using pIV10-*Chit5* as template and primers caccATGATCATCAAGCTCTTGGTTGC (PM-9870) and TCAATCATTATAAAGAGGTGAAAACAAGTG (PM-9829), followed by cloning into pIV10 vector containing the *LjUbiquitin* promoter and *Nos* terminator using Gateway cloning strategy. For *Nfr1/Nfr5* over-expression the constructs described by Radutoiu et al.\(^\text{51}\) have been used. All constructs have been sequence-verified.

**Root exudate hydrolysis assays** were performed as described by Staehelin et al.\(^\text{52}\) with adaptation for *L. japonicus*. Surface sterilized seeds of wild-type and *chit5* mutants were germinated on upright plates with wet filter paper in a 16/8 h day/night regime of 21/16 °C for 3 days. The emerging plant roots were pre-treated with R7A Nod factor by transfer into 0.5 ml dark glass vials and grown O/N in ⅓ B&D medium supplemented with 0.1 μM LCO-V(C18:1, Me, Cb, FucAc). Pre-treated seedlings were transferred to new 0.5 ml dark glass vials filled with 300 μl of ⅓ B&D medium supplemented with 10 μM LCO-V(C18:1, Me, Cb, FucAc) or CO- VI for 18h incubation time in the dark. Seedlings were removed and the incubating solution was used for measurements of ROS induction as described by Bozsoki et al.\(^\text{22}\). Nod factor hydrolysis by root exudates was determined by extracting the Nod factors...
from the incubating solution with equal volume of distilled n-butanol for extraction. Freeze dried samples were analysed as described below.

**Analyses of Nod factor hydrolysis.** The n-Butanol-extracted samples from the root exudate hydrolysis assay were dissolved in acetonitrile-water mixture (1:1 (v/v), 50 μL) and analysed on a Dionex UltiMate 3000 UHPLC+ focused system. Separation of LCOs was performed using a C4 column (Phenomenex Aeris 3.6 μm widepore, 50 × 2.1 mm). Samples were eluted by applying a gradient of CH3CN in H2O containing 0.1 % formic acid with a flow rate of 1 mL/min for 12 minutes. Peak integration was performed using the wavelength range 190-440 nm to maximize signal intensity. HPLC data were complemented by high-resolution mass spectrometric identification of LCO-V, LCO-III, and LCO-II using a Bruker Impact HD UHR-QTOF mass spectrometer connected to the LC. HR-MS (ESI-TOF): calcd. for LCO-V(18:1, Me, Cb, FucAc), [M+H]+: m/z 1501.7394; found 1501.7414. HR-MS (ESI-TOF): calcd. for LCO-II(18:1, Me, Cb), [M+H]+: m/z 704.4328; found 704.4346.

**References:**


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We thank Clive Ronson and John Sullivan for providing the *M. loti* R7A mutants. We thank Keisuke Yokota for his contribution to fine mapping of *Chit5*. We thank Finn Pedersen for plant care. We thank Clive Ronson and Christian Staehelin for constructive discussions. We thank Jens Stougaard for critical read of the manuscript. This work was supported by the Danish Research Foundation, DNRF 79 grant.
Figure legends:

Fig. 1. *chit5* mutants are defective in nitrogen-fixing symbiosis.

(A) Three representative plants of wild-type Gifu and *chit5* mutant alleles 6 weeks post-inoculation with *M. loti* R7A. Scale bar is 1 cm. (B) Gifu plants form pink nodules whilst *chit5* mutants form mainly small white nodules, and occasionally pink-spotted (white arrows) nodules. Scale bars are 0.5 cm. (C) Average number of pink and white nodules formed by *M. loti* R7A over 4 weeks. (D) Nitrogenase activity measured as the amount of ethylene production from acetylene in nodules of Gifu and *chit5* mutants inoculated with *M. loti* R7A. (C) and (D) Error bars represent SEM and statistical comparisons of the number of pink nodules formed between genotypes at each time point are shown using ANOVA and Tukey post hoc testing with p values <0.01 as indicated by different letters.

Fig. 1 Figure supplement 1. Shoot lengths of Gifu and *chit5* mutants following inoculation with *M. loti* R7A. Average shoot lengths of the indicated genotypes were measured 4-weeks post-inoculation with *M. loti* R7A. Error bars represent SEM and statistical comparisons of shoot lengths between genotypes are shown using ANOVA and Tukey post hoc testing with p values <0.01 as indicated by different letters.

Fig. 1 Figure supplement 2. Wild-type and *chit5*-3 mutant show similar growth phenotype when grown in nitrogen-supplemented soil. Shoot weight (A) and root weight (B) of plants after 7 weeks growth in soil +10mM KNO₃. (C) Plant growth of representative plants as indicated. For (A) and (B) error bars represent SEM and statistical comparisons between the genotypes are shown using ANOVA and Tukey post hoc testing with p values <0.01. No significant difference was detected between wild-type and *chit5*-3 plants.
**Fig. 1** Figure supplement 3. RT-qPCR analysis of symbiotic and defense gene expression in whole roots. Expression of the indicated symbiotic and defense genes was analysed in Gifu and chit5 mutant whole roots 21 dpi with *M. loti* R7A or mock-inoculation. Results are from biological and technical triplicates. Error bars represent SEM.

**Fig. 1** Figure supplement 4. Expression of symbiotic and defense gene during symbiosis A) and in nodules wild-type and chit5 nodules B). A) Expression heatmap of the analysed genes as found in Kelly et al., 2018. B) Expression of the indicated symbiotic and defense genes was analysed by RT-qPCR in Gifu and chit5 mutant nodules at 21 dpi with *M. loti* R7A. Results are from biological and technical triplicates. Error bars represent SEM.

**Fig. 1** Source data 1. Bacterial strains used in this study

**Fig. 1** Source data 2. Primers used for RT-qPCR analyses

**Fig. 2.** chit5 mutants have a defective nodule infection phenotype.

(A) Root hair infection threads counted in Gifu and chit5 mutants 10 dpi induced by *M. loti* R7A+DsRed. Error bars represent SEM and statistical comparisons of the number of full length infection threads formed between genotypes at each time point are shown using ANOVA and Tukey post hoc testing with p values <0.01 as indicated by different letters. (B) Box plot of cortical infection thread counts performed on Gifu and chit5-1 nodule sections. $t$ test $p$ values are indicated by asterisks (** <0.01). (C) Light microscopy of nodule sections from the indicated plant genotypes 4 wpi stained with toluidine blue. Black arrows indicate bacteria between nodule cells. Scale bars are 200 µm (D) Transmission electron microscopy of nodule sections from the indicated plant genotypes 4 wpi. Red arrows point out bacteroids of stressed appearance with accumulated white spots, red dashed lines outline infection threads or invagination from the intercellular space. Note the difference between PHB bodies (large, white spots) characteristic for *Mesorhizobium* when present inside infection threads.
(red dashed outlines), and the small white spots present in the symbiosomes formed in the
*chit5* mutant nodules (red arrows). Scale bars are 2 µm. Corresponding images of *chit5*-2 and
*chit5*-3 are in Fig.2 Figure supplement 1.

**Fig. 2 Figure supplement 1.** Root hair infection thread formation in wild-type Gifu and *chit5*
mutants. Representative images of full length root hair infection threads formed by *M. loti*
R7A+DsRed 10 dpi on the indicated genotypes. Scale bars are 100 µm. (B) Light microscopy
of nodule sections from the indicated plant genotypes 4 wpi stained with toluidine blue.
Black arrows indicate bacteria between nodule cells. Scale bars are 200 µm (C) Transmission
electron microscopy of nodule sections from the indicated plant genotypes 4 wpi. Red arrows
point out bacteroids of stressed appearance with accumulated white spots, red dashed lines
outline infection threads or invagination from the intercellular space. Note the difference
between PHB bodies (large, white spots) characteristic for *Mesorhizobium* when present
inside infection threads (red dashed outlines), and the small white spots present in the
symbiosomes formed in the *chit5* mutant nodules (red arrows). Scale bars are 2 µm.

**Fig. 3.** *Chit5* encodes a root expressed class V chitinase with Nod factor hydrolase activity.

(A) *Chit5* gene structure. Mutation in *chit5*-2 allele is shown. *Chit5* gene is deleted in *chit5*-1
and *chit5*-3 alleles. (B) *Chit5* promoter activity was monitored in Gifu roots transformed with
a *Chit5* promoter-tYFP-NLS (green nuclei) in uninoculated and *M. loti* R7A+DsRed
inoculated roots. White arrows highlight examples of uninfected cells showing *Chit5*
promoter activity. Scale bars are 200 µm. (C) ROS induced by exudates of CO-VI-treated
plants. (D) Absence of ROS induction by exudates of *M. loti* R7A Nod factor-treated plant
exudates. (E) *M. loti* R7A Nod factor hydrolysis in the presence of the indicated plant
genotypes measured by HPLC-MS. The average relative percentage of LCO-V and LCO-II
fractions from two biological replicates (Figure 3 Source data 1) determined from peak-peak
integration is indicated in brackets. The peak eluting near 4.1 min is present in all plant

treated samples but was not detected in the control sample. It lacks the characteristic

fragmentation pattern of LCOs, and appears to be an aromatic, small molecule unrelated to

chitinase activity.

**Fig. 3 Figure supplement 1.** Map-based cloning of Chit5 gene. (A) Positions of bacterial

artificial chromosome (BAC) and transformation-competent artificial chromosome (TAC)

clones from *L. japonicus* MG20 and BAC clone 69G19 from *L. japonicus* Gifu are shown.

The closest markers with the number of informative recombinations delimiting the Chit5

locus are indicated. A schematic representation of the three Chit5 paralogs within the

delimited region is shown with PCR primer binding sites indicated. (B) Amplicon detection

in the indicated genotypes using primers specific for Chit5 (P1L/P1R) and common for the

three Chit5 paralogs (P2L/P2R).

**Fig. 3 Figure supplement 2.** Alignment of Chit5 paralogs. Nucleotide sequence alignment of

Chit5 and its two paralogs (start to stop codons). Exons are highlighted in green and

nucleotide polymorphisms are shown in red. The premature stop codons in the paralogs are

highlighted by red boxes and the position of the C to T point mutation in chit5-2 is indicated

with an asterisk.

**Fig. 3 Figure Supplement 3.** Complementation of chit5 mutant alleles with Chit5. Gifu (A)

and chit5 mutant plants (B-D) were transformed with an empty vector (EV) control or Chit5

expressed from its native or *Ljubiquitin* promoter. Plants were harvested 6 weeks post-

inoculation with *M. loti* R7A. Similar results were obtained using the two promoters and

images show three representative plants and examples of the nodules formed. Scale bars are 1

cm for plant images and 1 mm for nodules.

**Fig. 3 Figure supplement 4.** Chit5 promoter-GUS reporter analysis of promoter activity.
Gifu plants transformed with a Chit5 promoter-GUS reporter construct were analysed after inoculation with *M. loti* R7A+DsRed. (A) Chit5 promoter activity in transformed roots uninoculated or inoculated with *M. loti* R7A+DsRed at the indicated time points. Scale bars are 0.5 mm. (B) Upper panel shows light microscopy of GUS-reporter staining in nodules at different developmental stages and the lower panel shows *M. loti* R7A+DsRed colonization state of these same nodules. Note the increase in DsRED fluorescence in the nodules with reduced GUS activity. Scale bars are 0.5 mm. (C) Sections of mature nodules show that Chit5 promoter activity is restricted to non-colonized nodule cells. Scale bars are 200 μm.

**Fig. 3 Figure supplement 5.** Amino acid alignment of CHIT5. Amino acid sequence alignment of *Lotus japonicus* CHIT5, *Medicago truncatula* NFH1 (KC833515) and *Arabidopsis thaliana* CHIC (NP_193716). (*) indicates a conserved residue, (:) indicates strongly similar properties, (.) indicates weakly similar properties, (-) indicates gaps. A predicted signal peptide is indicated in bold for CHIT5. The conserved catalytic motif DxDxE is indicated as is the position of the E-K mutation (green) introduced to produce an inactive CHIT5 construct. The position of the P-L substitution in chit5-2 is indicated (magenta). The position of A and B loops (in red) and of the proline residue (red box) identified as important for Nod factor hydrolase activity of MtNFH1 are indicated.

**Fig. 3 Figure supplement 6.** Complementation of chit5 mutant alleles with *Chit5* (E-K). Gifu (A) and chit5 mutant plants (B-D) were transformed with an empty vector (EV) control or an active site mutant version of *Chit5* (E to K) expressed from its native promoter. Plants were harvested 6 weeks post-inoculation with *M. loti* R7A. Images show three representative plants for each transformation construct and examples of the nodules formed. Scale bars are 1 cm for plant images and 2 mm for nodules.

**Fig. 3 Figure supplement 7.** Several *Lotus japonicus* chitinase genes are expressed in roots.
Phylogenetic analysis of protein sequences of annotated chitinases or glycosyl hydrolases from *Lotus* genome v3.0. (B) Log10 total counts from RNA-seq analysis performed on Gifu roots and shoots 3 dpi with water or *M. loti* R7A from *Lotus* base (www.lotus.au.dk). *Chit5* (as assigned in v3.0) is highlighted in red.

**Fig. 3 Source data 1.** HPLC-MS analysis of Nod factor isolated after exposure to roots of wild-type Gifu or *chit5* mutants.

**Fig. 4.** The symbiotic impairment of *chit5* mutants can be overcome by an *M. loti* R7A mutant affected in the regulation of Nod factor biosynthesis. (A) Representative images of Gifu and *chit5*-1 plants inoculated with the indicated *M. loti* R7A strains 7 wpi. Scale bars are 1 cm. (B) Average number of pink and white nodules formed on Gifu and *chit5*-1 by the indicated *M. loti* R7A strains over 3-weeks. Error bars represent SEM and statistical comparisons of the number of pink nodules formed between strains on each genotype at each time point are shown using ANOVA and Tukey post hoc testing with p values <0.05 as indicated by different letters. (C) Light microscopy of whole nodules 3 wpi and confocal images of nodule sections from Gifu and *chit5*-1 plants inoculated with the indicated strains tagged with DsRed. Scale bars are 0.5 cm. (D) Proposed model of CHIT5 activity and representative nodule images of wild-type and *chit5* mutant illustrating the observed phenotype. (1) Bacteria maintain a low level of Nod factors within root hair-traversing ITs by preferential activity of NodD1. (2) and (3) Bacterial amplification inside ITs, coupled with the switch for preferential activity of NodD2 leads to higher levels of fully decorated pentameric Nod factors (LCO-V). *Chit5* expression (green filled cells in wild-type) is crucial for maintaining a balanced Nod factor level enabling IT extension inside primordia, efficient bacterial endocytosis and, ultimately, development of the nitrogen-fixing
organ (3). (4) In the chit5 mutants (light grey filled cells) higher levels of Nod factors (LCO-V) impede IT elongation and branching inside primordia leading to bacteria accumulation in between the cells (dotted brown line) and scattered infection.

Fig. 4 Figure supplement 1. chit5 mutant phenotypes with M. loti R7A mutants affected in the regulation of Nod factor biosynthesis. (A) Root hair infection thread formation on the indicated genotypes with M. loti R7A strains. 20 plant roots were cut into 1 cm pieces and 60 of these were analysed per condition. Error bars represent SEM and statistical comparisons of the number of full length infection threads formed across genotypes by the different M. loti R7A strains are shown using ANOVA and Tukey post hoc testing with p values <0.01 as indicated by different letters. (B) Light microscopy and transmission electron microscopy of nodule sections from Gifu and chit5-1 inoculated with the indicated strains. Scale bars are 200 μm for light microscopy images and 2 μm for TEM. (C) Nitrogenase activity measured as the amount of ethylene production from acetylene incubated nodules of Gifu and chit5 mutant alleles inoculated with the indicated M. loti R7A strains.

Fig. 4 Figure supplement 2. Overexpression of Nfr1 and Nfr5 does not rescue the symbiotic impairment of chit5-1. (A) Nodule counts on Gifu and chit5-1 plants transformed with an empty vector control or the Nfr1/Nfr5 over-expression construct 6-weeks post-inoculation with M. loti R7A. Error bars represent SEM and statistical comparisons of pink nodules formed are shown using ANOVA and Tukey post hoc testing with p values <0.01 as indicated by different letters. (B) Representative images of Gifu and chit5-1 plants transformed with an empty vector control or the Nfr1/Nfr5 over-expression construct 6-weeks post-inoculation with M. loti R7A. Scale bars are 1 cm.

Fig. 4 Figure supplement 3. chit5 mutant phenotypes with M. loti R7A mutants affected in the biosynthesis of fully decorated Nod factor. (A) Representative images of Gifu and chit5-1 plants inoculated with the indicated strains 7 wpi. Scale bars are 1 cm. (B) Average number
of pink and white nodules formed on Gifu and *chit5-1* by the indicated *M. loti* R7A strains over 3-weeks. Error bars represent SEM and statistical comparisons of the number of pink nodules formed between strains on each genotype at each time point are shown using ANOVA and Tukey post hoc testing with p values <0.05 as indicated by different letters. (C)

Light microscopy and transmission electron microscopy of nodule sections from Gifu and *chit5-1* inoculated with the indicated strains. Scale bars are 200 μm for light microscopy images and 2 μm for TEM. (D) Nitrogenase activity measured as the amount of ethylene production from acetylene incubated nodules of Gifu and *chit5* mutant alleles inoculated with the indicated *M. loti* R7A strains.

**Fig. 4 Figure supplement 4.** *chit5* mutants show a nitrogen-deficient phenotype when grown in soil. (A) Plant growth of the indicated genotypes when grown in soil containing its native microbiota for 9 weeks. (B) Shoot weight of plants after 7 weeks growth in soil. (C) Counts of total nodules formed on the soil grown plants. (D) Number of pink nodules formed on the soil-grown plants. For (B), (C) and (D) error bars represent SEM and statistical comparisons between the genotypes are shown using ANOVA and Tukey post hoc testing with p values <0.01 as indicated by different letters.

**Fig. 4 Figure supplement 5.** Genomic location and expression pattern of *MtNfh1* and the two close-located paralogs. The genomic information available on Phytozome 12 (https://phytozome.jgi.doe.gov) is given according to *Medicago truncatula* Mt4.0v1 version of the genome, and their expression level according to Gene Atlas expression tool. The three GH18 coding genes are marked (red circle) and their expression pattern is shown.

**Fig. 4 Figure supplement 6.** GH18 proteins from *M. truncatula* and *L. japonicus*. Alignment (A) and phylogenetic tree B) of *Lotus* and *Medicago* proteins along with CHIT5 from *Arabidopsis* (AT4G19810.1) and *Nicotiana tabaccum* (XP_016468204.1). Protein sequences were obtained from https://phytozome.jgi.doe.gov, and https://lotus.au.dk/.
**Fig. 4 Figure supplement 7.** Sequencing traces from PCR products using genomic DNA (gDNA), cDNA from water treated roots (R_M), *M. loti* inoculated roots-3dpi (R_3dpi),-10dpi (R_10dpi) and nodules-21 dpi (N_21dpi) as template. Calibrator template contains a mixture of *L. japonicus* cDNAs from various organs and *M. loti* infected roots and nodules. The PCR products were sequenced with forward (F) or reverse primer (R). Note that sequences from gDNA have double peaks in the regions with nucleotide polymorphisms between chitinase paralogs (X and line), while the cDNA sequences have only one variant corresponding to *Chit5*. 
A

Relative expression

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1

MPK3
WRKY33
WRKY29
NPL

Root_H2O_3dpi
Root_R7A_3dpi
Primordia_R7A_7dpi
Nodule_R7A_21dpi

B

MPK3

Absolute abundance

Gifu chit5-1 chit5-2

WRKY29

Absolute abundance

Gifu chit5-1 chit5-2

WRKY33

Absolute abundance

Gifu chit5-1 chit5-2

NPL

Absolute abundance

Gifu chit5-1 chit5-2
A

![Bar graph showing average nodules per plant for EV and OE Nfr1/Nfr5 genotypes in Gifu and chit5-1 backgrounds.](image)

<table>
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<th></th>
<th>Gifu</th>
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<td>n=30</td>
</tr>
<tr>
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<td>n=28</td>
<td>n=30</td>
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B

![Images comparing crop plants under different conditions.](image)