



Figures and figure supplements

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

Anna Malolepszy *et al*

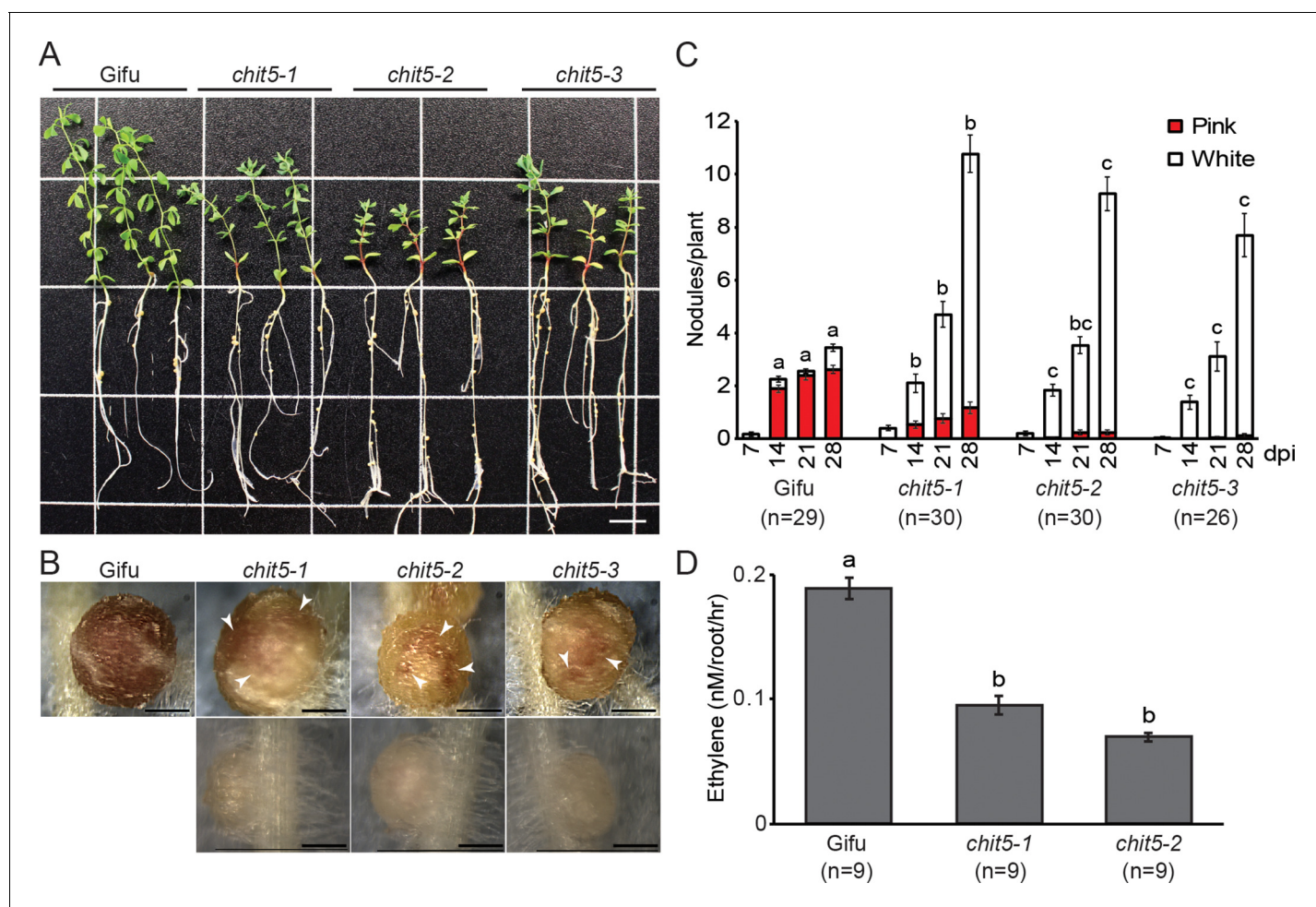


Figure 1. *chit5* mutants are defective in nitrogen-fixing symbiosis. (A) Three representative plants of wild-type Gifu and *chit5* mutant alleles 6 weeks post-inoculation with *M. loti* R7A. Scale bar is 1 cm. (B) Gifu plants form pink nodules whilst *chit5* mutants form mainly small white nodules, 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 produced 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.

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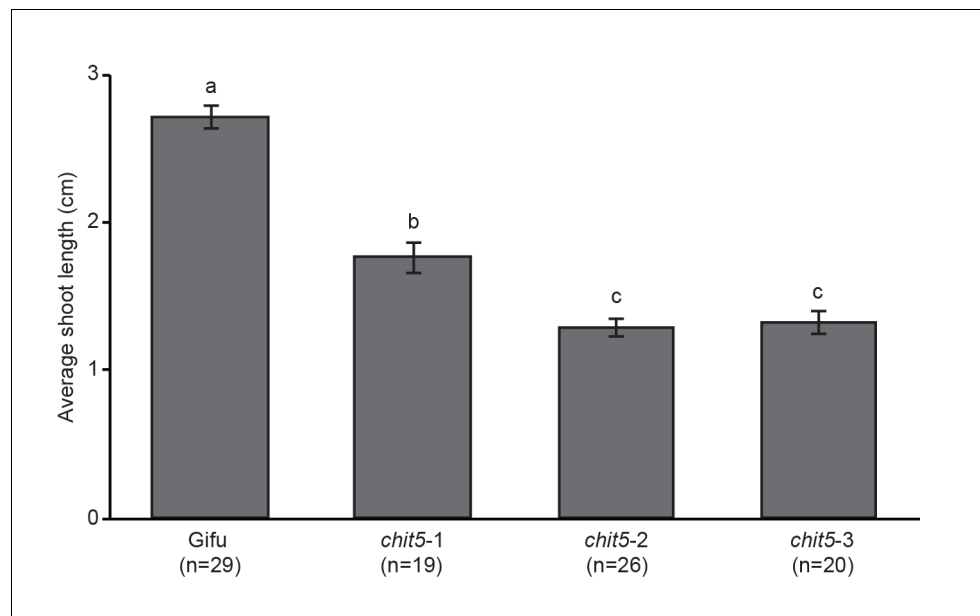


Figure 1—figure supplement 1. Shoot length of Gifu and *chit5* mutants following inoculation with *M. loti* R7A. Average shoot lengths of the indicated genotypes were measured 4 weeks post-inoculation with *M. loti* R7A. Error bars represent SEM and statistical comparisons of shoot lengths between genotypes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

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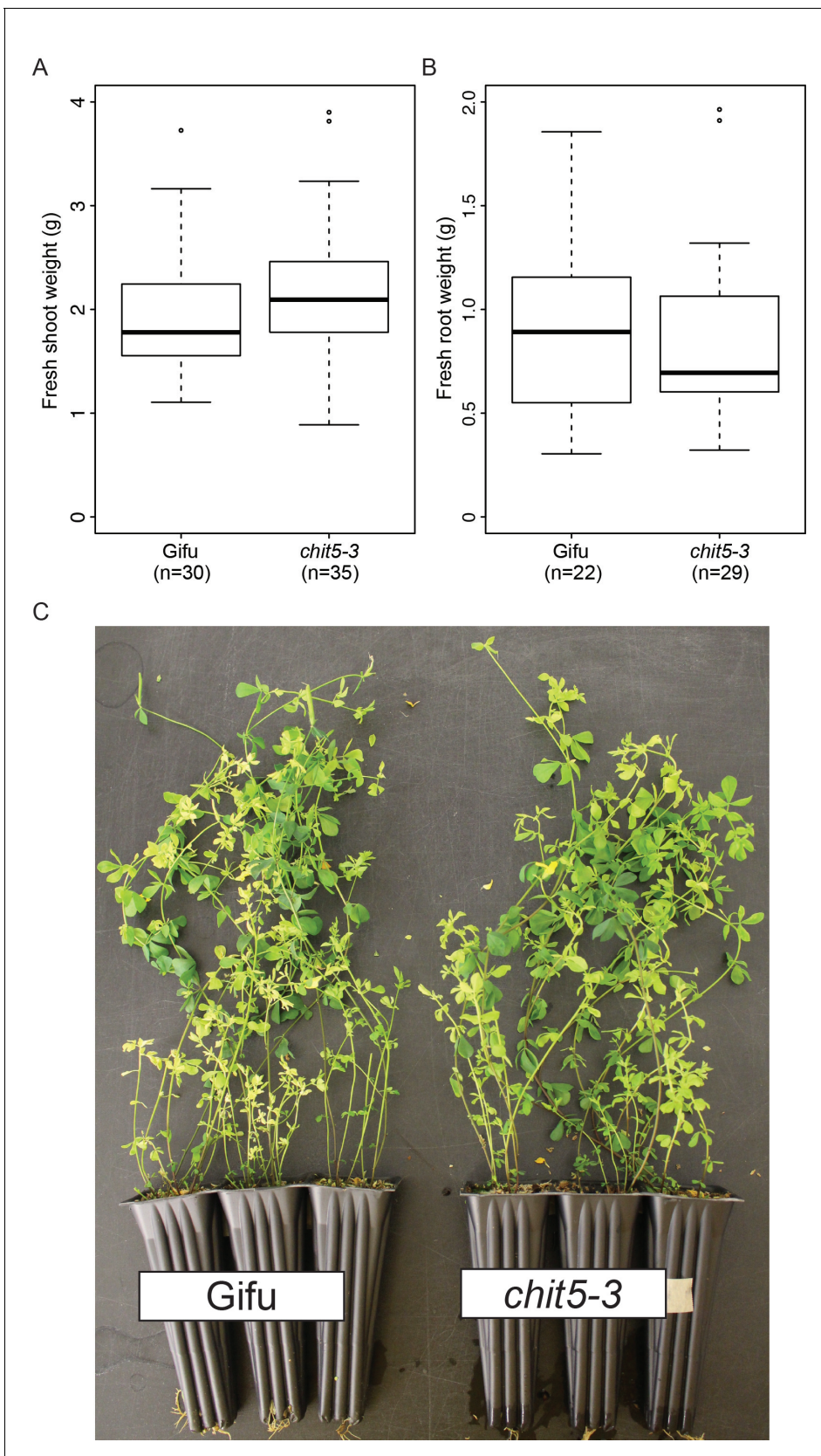


Figure 1—figure supplement 2. Wild-type and *chit5-3* mutant show similar phenotype when grown in nitrogen-supplemented soil. Shoot weight (A) and root weight (B) of plants after 7 weeks growth in soil +10 mM KNO₃. (C) Plant growth of representative plants as indicated. For (A) and (B) error Figure 1—figure supplement 2 continued on next page

Figure 1—figure supplement 2 continued

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.

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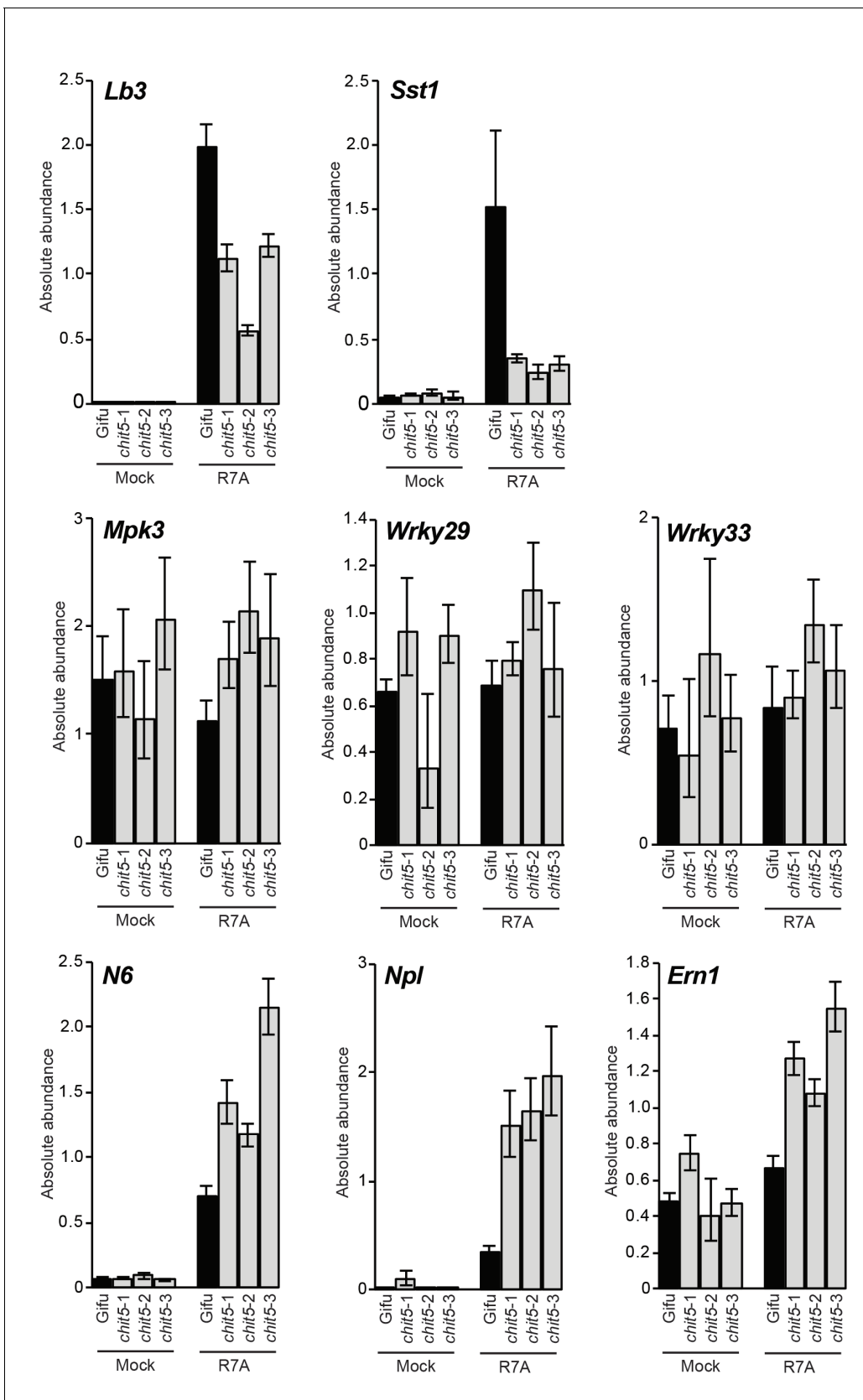


Figure 1—figure supplement 3. RT-qPCR analysis of symbiotic and defense gene expression in whole roots. Expression of the indicated symbiotic and defense genes was analysed in Gifu and *chit5* mutant whole roots 21 dpi with *M. loti* R7A or mock-inoculation. Results are from biological and technical triplicates. Error bars represent SEM.

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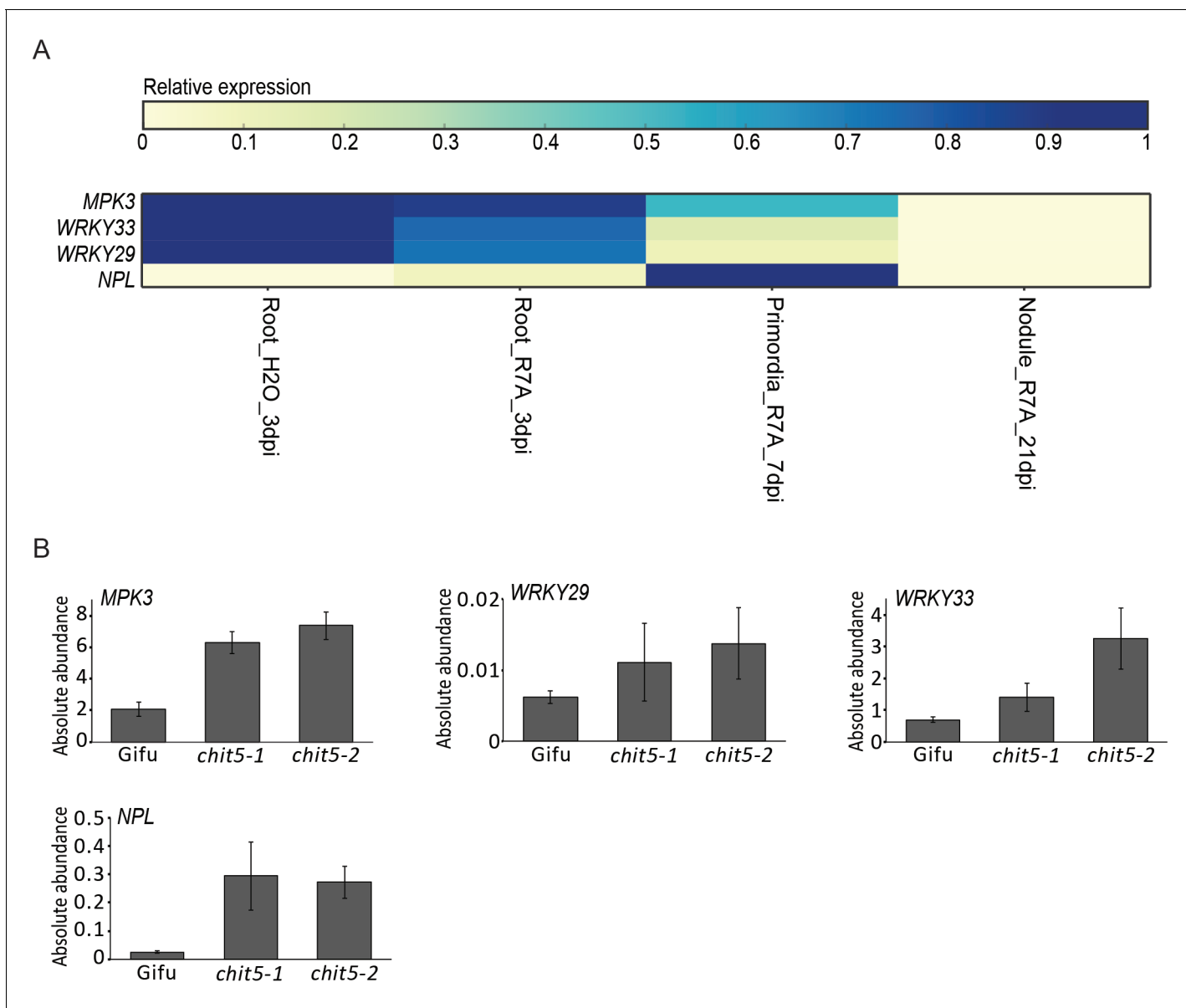


Figure 1—figure supplement 4. Expression of symbiotic and defense gene during symbiosis (A) and in wild-type and *chit5* nodules (B). (A) Expression heatmap of the analysed genes as found in Kelly et al., 2018a. (B) Expression of the indicated symbiotic and defense genes was analysed by RT-qPCR in Gifu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

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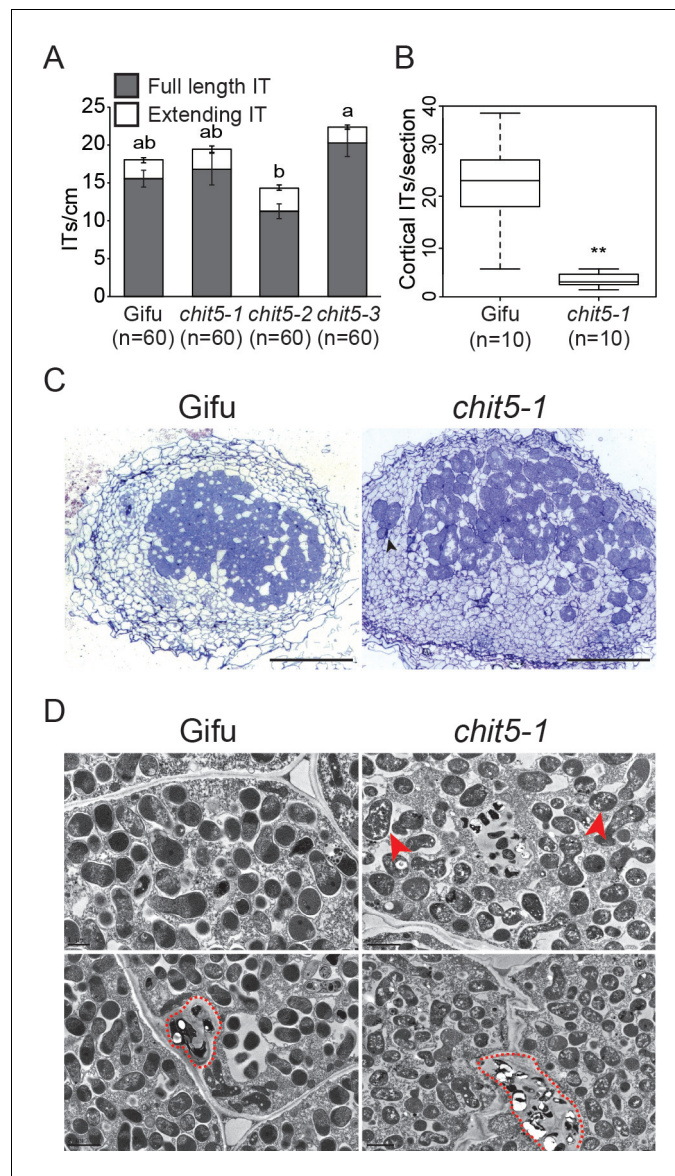


Figure 2. *chit5* mutants have a defective nodule infection phenotype. (A) Infection threads induced by *M. loti* + DsRed in Gifu and *chit5* mutant root hair at 10 dpi. Error bars represent SEM and statistical comparisons of the number of full length infection threads formed between genotypes at each 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 μ m (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 μ m. Corresponding images of *chit5-2* and *chit5-3* are in **Figure 2—figure supplement 1**.

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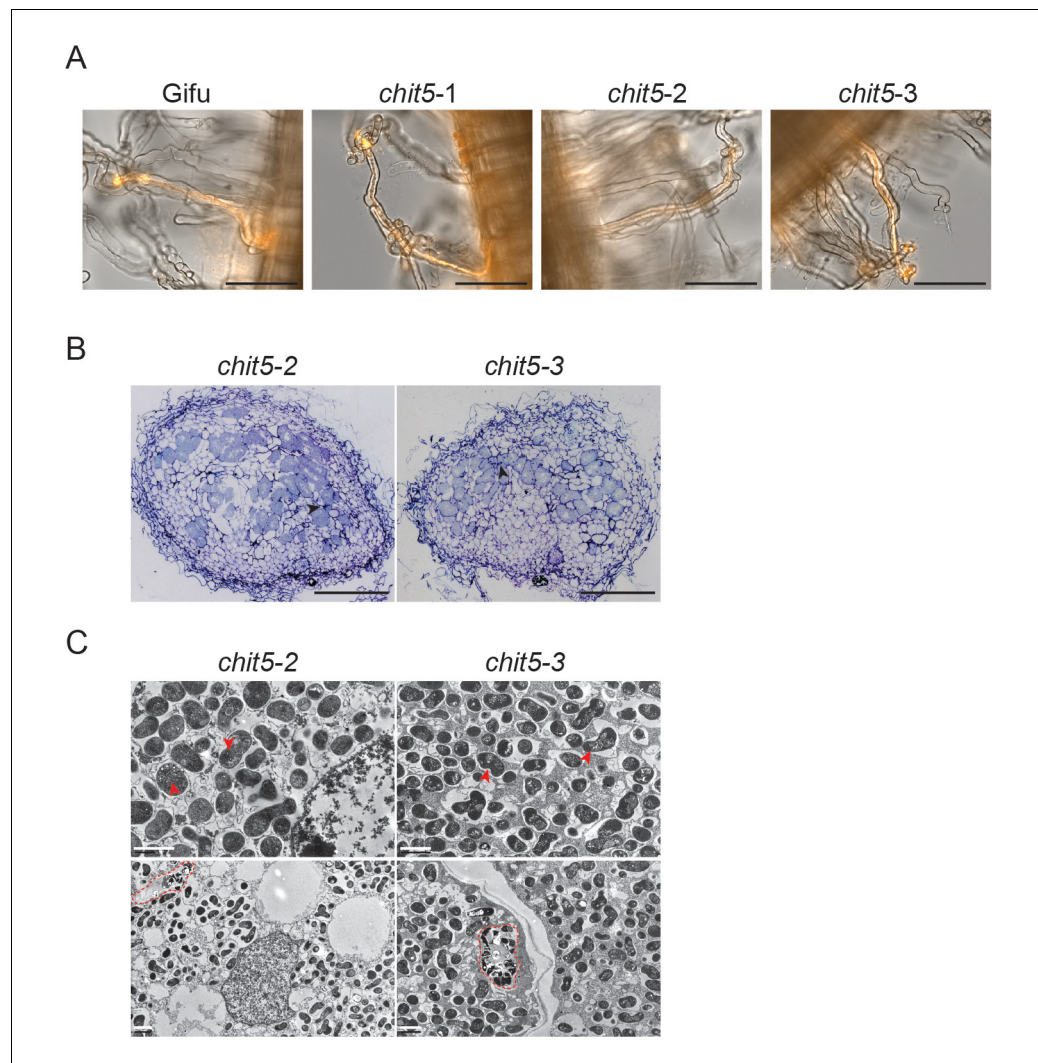


Figure 2—figure supplement 1. Infection phenotype of wild-type and *chit5* mutants. Representative images of full length root hair infection threads formed by *M. loti* R7A + DsRed 10 dpi on the indicated genotypes. Scale bars are 100 μ m. (B) Light microscopy of nodule sections from the indicated plant genotypes 4 wpi stained with toluidine blue. Black arrows indicate bacteria between nodule cells. Scale bars are 200 μ m (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 μ m.

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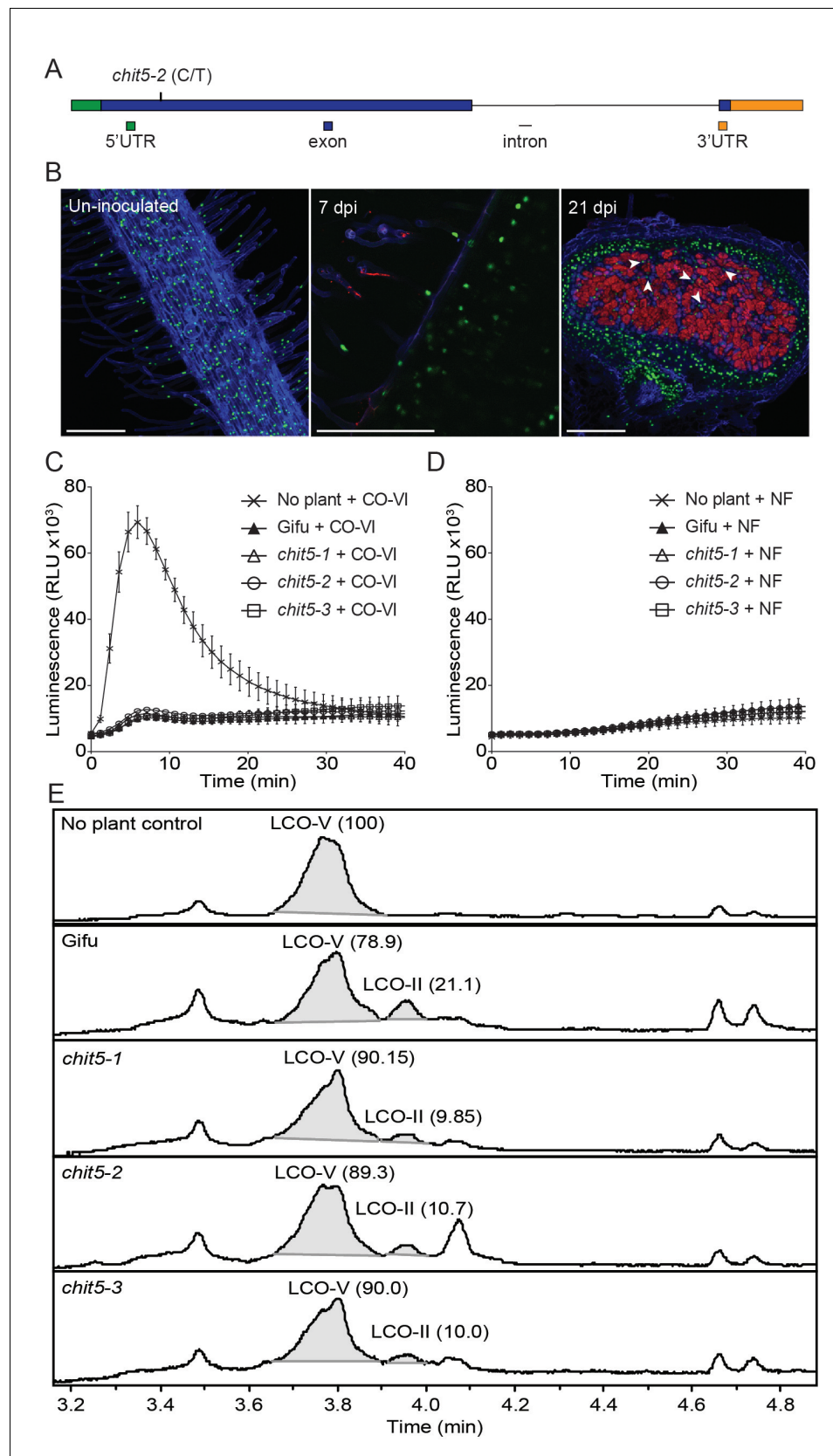


Figure 3. *Chit5* encodes a root expressed class V chitinase with Nod factor hydrolase activity. (A) *Chit5* gene structure. Mutation in *chit5-2* allele is shown. *Chit5* gene is deleted in *chit5-1* and *chit5-3* alleles. (B) *Chit5*

Figure 3 continued on next page

Figure 3 continued

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.

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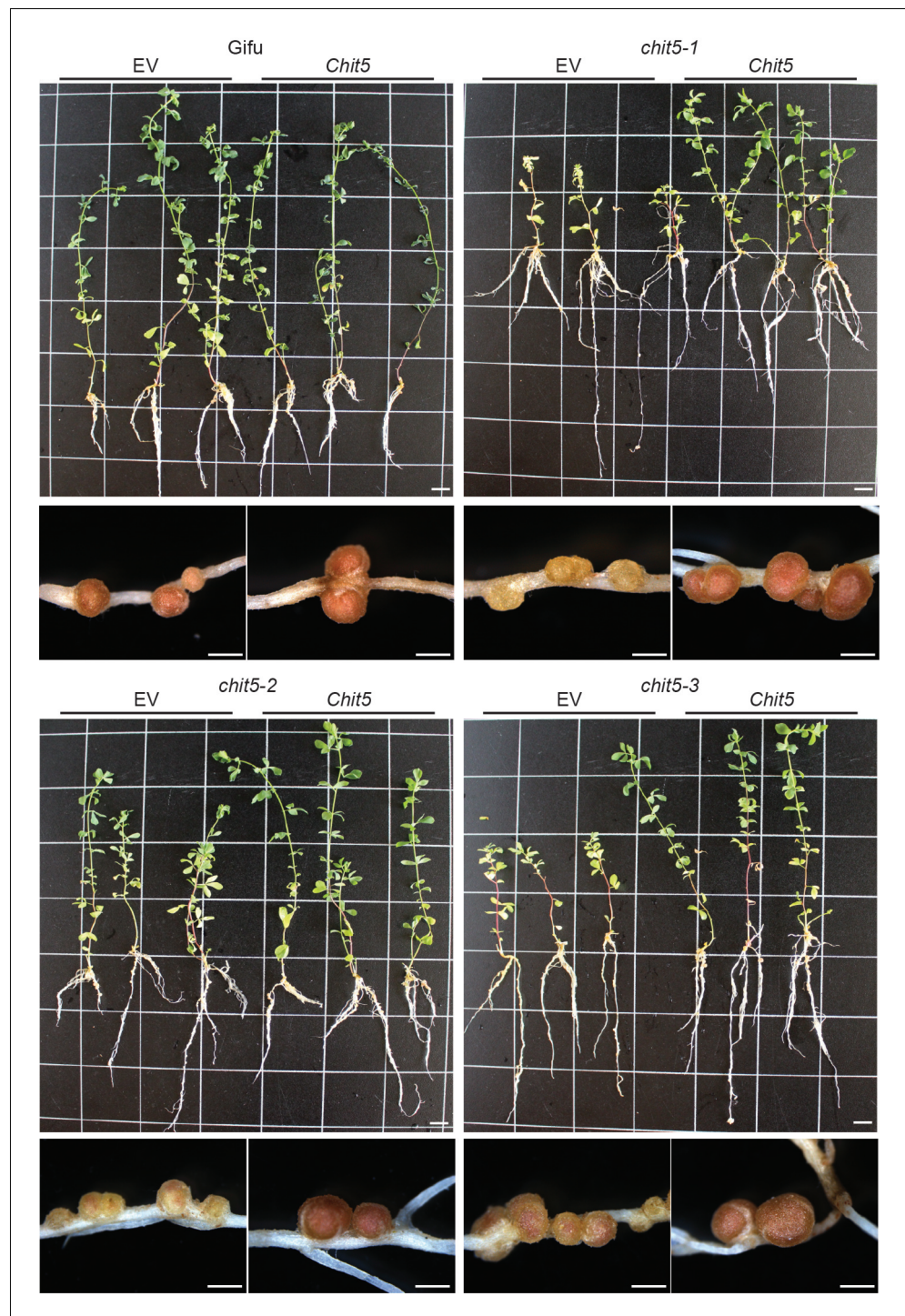


Figure 3—figure supplement 3. Complementation of *chit5* mutant alleles with *Chit5*. Gifu (A) and *chit5* mutant plants (B–D) were transformed with an empty vector (EV) control or *Chit5* expressed from its native or *Ljubiquitin* promoter. Plants were harvested 6 weeks post-inoculation with *M.loti* 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.

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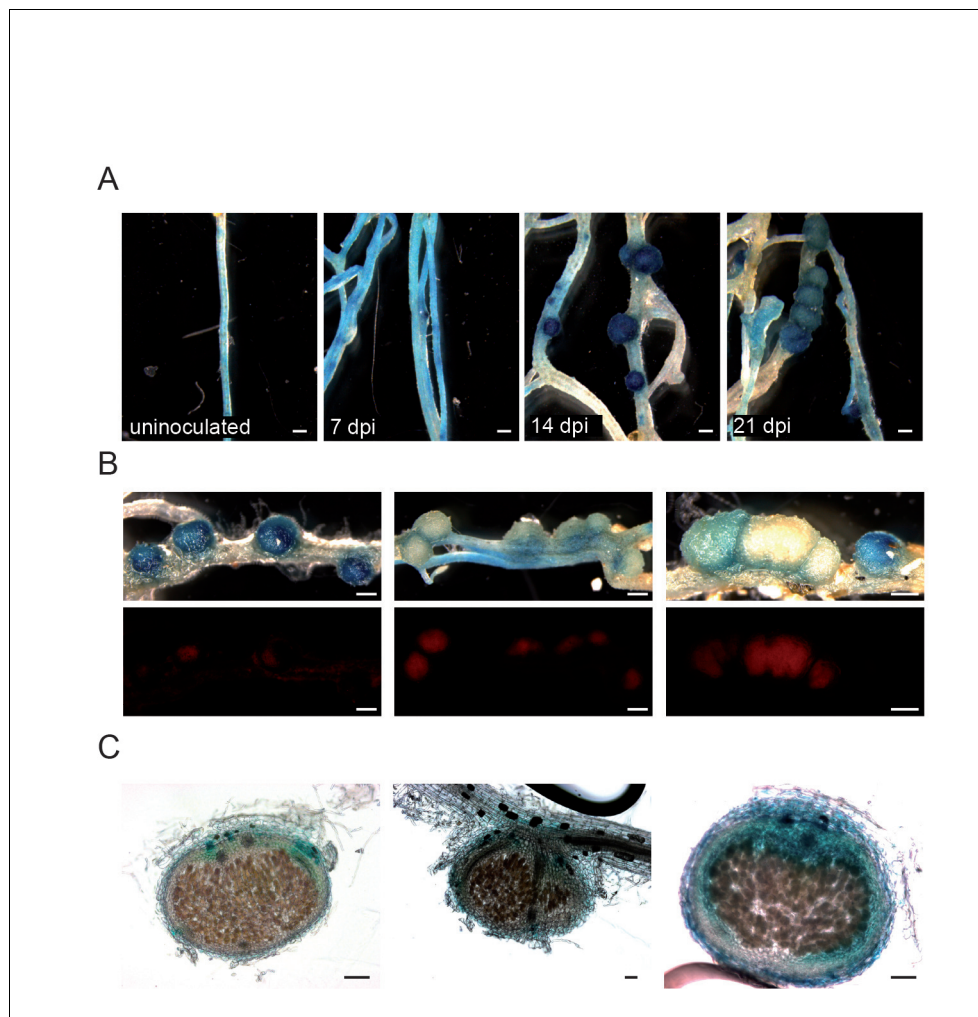


Figure 3—figure supplement 4. *Chit5* promoter-GUS reporter analysis . *Gifu* plants transformed with a *Chit5* promoter-GUS reporter construct were analysed after inoculation with *M. loti* R7A + DsRed. (A) *Chit5* promoter activity in transformed roots uninoculated or inoculated with *M. loti* R7A + 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 μ m.

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AtCHIC	-----MSS-----TKLISLIVSITFF-----LTLQCSMAQTVVKASYW	33
CHIT5	MIIKLLVALIHYLHETMA VQSIITTPLLVILMSLRSYAFTEPSLHRQQPPSKGGVRAAYW	60
MtNFH1	-----MANFLKCLKQFLTL--VLILLALA----AKSSTSTTSPSSTTRVKGIYW	42
	: : : : : : : : *	
AtCHIC	FPASEFPVTDIDSSLFTHLFCADFADLNSQTNQVTV---SSANQPKFSTFTQTVQRRNPSV	90
CHIT5	PAWSDFTSSIDTNYFTHIYYAFVQPAPESEFNLEITESYKKWAPKY----DGIHNIIRPV	116
MtNFH1	IENPLFPPASIDTSLFTHIFYAFVSPNKFTYKLEEEEDSTTVATSLTFTNTFKTKTPPI	102
	* :.***. ***: : *. . : : : . : : * :	
	Catalytic motif (E->K mutant)	
AtCHIC	KTLMSIGGGIADKTAYASMASNPTSRSKSFIDSSIRVARSYGFHGLDLWEYPSATEMNN	150
CHIT5	TLLSIGGGGNNATLTFSEMASSKQNRASFINSTIHVARKHEFNGLDLDEWPGDEKDMSN	176
MtNFH1	PTLLSIGGATSNSTLFAFIASDPTARATFINSTIQVARTFGFDGIDFDWEFPTTKEMND	162
	**:* : * : : ** . * : * : * : * : * : * . * : * : * : * : * : * : *	
	<i>chit5-2</i> (P->L)	
AtCHIC	FGTLLREWRSAAVVAEASSGKPRLLLLAAAVFYSNYYSS----VLYPVSAVASSLDWVNL	205
CHIT5	LALLLKEWYKALVVEANTSRSKSRLLLLSAVYFNSTISLIGNGPRSPVRAIRKYLDWASP	236
MtNFH1	LGELLFQWRRAISDEAAASTSRPPLLLTAAVYFAVNFLLSG-ERRMYPVDSINKNLDWVNV	221
	:. * * : * * : * * : : * * : * * : : . * * * : : . * * * .	
	Loop A	
AtCHIC	MAYDFYGPWSRVTPGPAALFDPSNAGPSGDAGARSWIQAGLPAKKAVLGFPPYGYAWRL	265
CHIT5	MCFDYNG-AWANETGFNAALYDPNS-NISTKYGIGSWGIVGSGVPAEKLVMGLPLYGRRAWEL	294
MtNFH1	MSYDLRG-SGSNVTGAPSGMFDSSK-NVSVVSGLFSWIRGGVAPEKIVMGMPLYGKSWKL	279
	*. : * * . : . * * : : : * . . * * * * . * : * * : * : * * * : * . *	
	Loop B	
AtCHIC	TNANSHSYAPTTGAAISPDGSIYGQIRKFIVDNGATTVYNSTVVGDYCYAGTTWIGYD	325
CHIT5	KDPNDHGVGAKAVGPAVDTDGSMYDEILVFNKDTGAKVVYDEVAVSFYSYSGTTWIGYD	354
MtNFH1	QDPNVHGIGAPNVG EGPGVDGG MAYFQVVDFNKQMGAKVVYDKETGSVYSYSGSTWIGYD	339
	: * * . * . * . . * * : * : * : * * . * * . . . * . * : * : * * * * *	
AtCHIC	DNQSIIVTKVRYAKQRGLLGYFSWHVGADDN-SGLSRAASQAWDATATTTRTIQKV	379
CHIT5	DGPSITKKVQFARSMGLKGYFFWAIGKDK-DWTISKQASNAWGY*-----	397
MtNFH1	DPFTVSVKVGFAQALKLGGYFFWAAGYDTSDWKVSTQGKINTLS-----	383
	* : : * * : * : * * * * * * * : * . .	

Figure 3—figure supplement 5. Amino acid alignment of CHIT5, AtCHIC, MtNFH1. 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 DxExE is indicated as is the position of the E-K mutation (green). 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.

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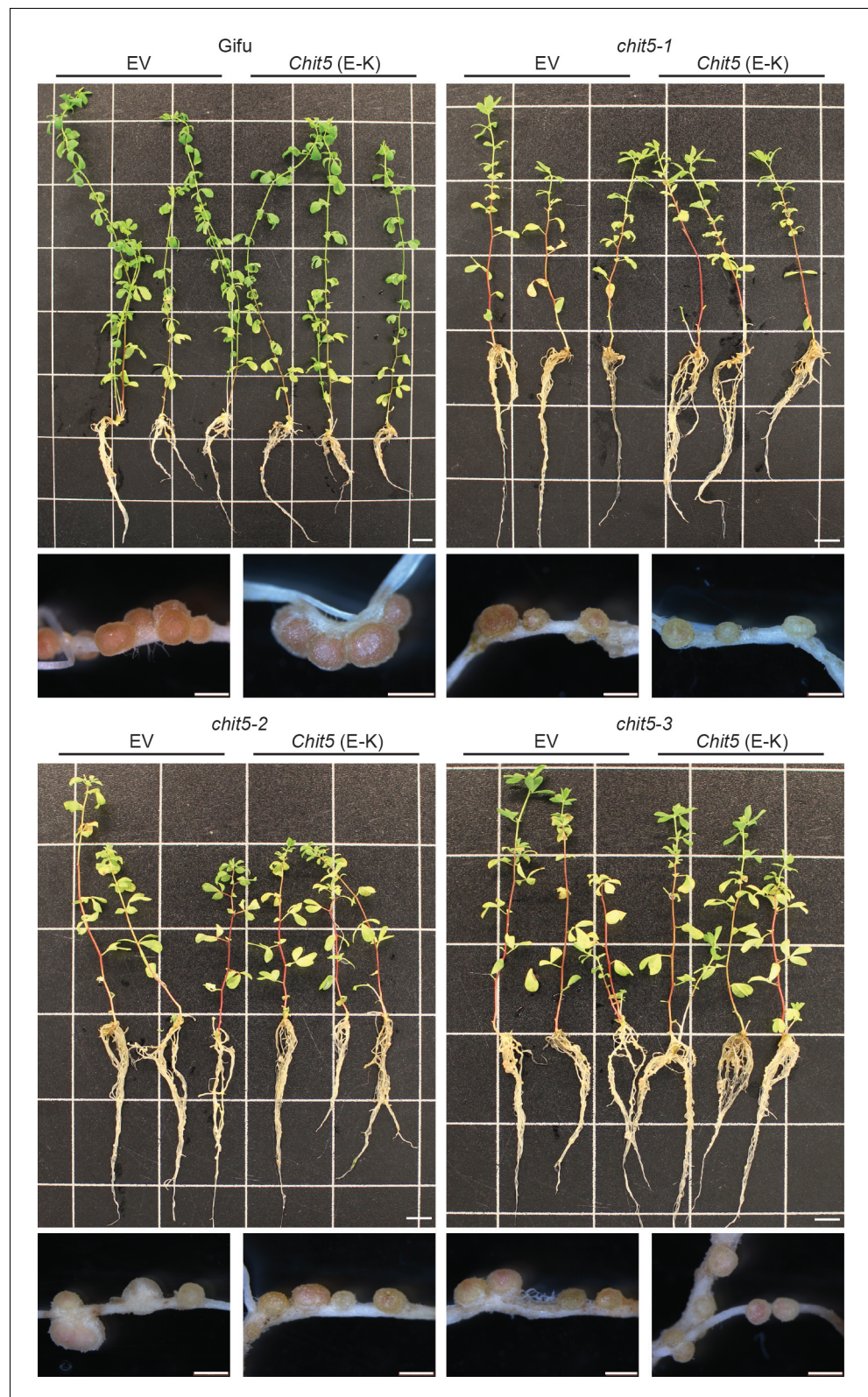


Figure 3—figure supplement 6. Complementation of *chit5* mutant alleles with *Chit5* (E166-K). Gifu (A) and *chit5* mutant plants (B–D) were transformed with an empty vector (EV) control or an active site mutant version of *Chit5* (E to K) expressed from its native promoter. Plants were harvested 6 weeks post-inoculation with *M. loti* R7A. Images Figure 3—figure supplement 6 continued on next page

Figure 3—figure supplement 6 continued

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.

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Chit5 ATGGCCGTCCAAAGCATCATAACAAACACCCCTTTGGTTATCTCATGAGCCTTCGTCTTATGCATTCACTGAACCTTC 80
Chit5b ATGGCCGTCCAAAGCATCATAACAAACACCCCTTTGGTTATCTCATGAGCCTTCACCTTTATGCATTCACTAAACCTTC 74
Chit5a ATGGCCGTCCAAAGCATCATAACAAACACCCCTTTGGTTATCTCATGAGCCTTCACCTTTATGCATTCACTGAACCTTC 80

Chit5 TTTACATAGGCAGCAGCCACCGTCCAAGGGTGGTGTAGAGCAGCATATTGGCCTGCA GGTCTGATTTCTCAACCTCTT 160
Chit5b TTTACATAGGCAGCAGCCACCGTCCAAGGATGGTGTAGAGCGGCATATTGGCCTGCAGGGTCTGATTTCTCAACCTCTT 154
Chit5a TTTACATAGGCAGCAACCCACCGTCCAAGGGTGGTGTAGAGCAACATATTGGCGTGCAGGGTCTGATTTCTCAACCTACT 160

Chit5 CCATTGACACAAACTACTTCACTCACATATACTACGCCTTTGTCCAACCTGCACCAGAATCCTTCAACCTCGAAATCAC 240
Chit5b CCATTGACACAAACTACTTCACTCACATATACTACGCCTTTGTCCAACCTGCACCAGAATCCTTCAACCTCGAAATCAC 234
Chit5a CCATTGACACAAACTACTTCACTCACATATACTACGCCTTTGTCCAACCTGCACCAGAATCCTTCAACCTCGAAATCAC 240

Chit5 GAATCCTACAAGAAGTGGGCCCTAAGTATGATGGTATCCATAACATCCGCCCGGGGTGACGACGCTGCTGTCCATCG 320
Chit5b GAATCCGACAAGAAGTGGGCCCTAAGTATGATGGAATCCGTAACATCCGTCCGCGGGTGACACGCTGCTATCCATCG 314
Chit5a GAATCCTACAAGAAGTGGGCCCTAAGTATGATGGTATCCATAACATCCGCCCGGGGTGACGACGCTGCTGTCCATCG 320

Chit5 AGGGGGTGGCAACAACGCCAACCCCTCTTCTCAGAGATGGCTAGCAGCAAAACAAAACCGTGCAGCTTTCATAAACTCCACCA 400
Chit5b AGGGGGTGGCAACAACGCCAACCCCTCTTCTCAGAGATGGCTAGCAGCAAAACAAAACCGCGCGAGCTTTCATAAACTCCACCA 394
Chit5a AGGGGGTGGCAACAACGCCAACCCCTCTTCTCAGAGATGGCTAGCAGCAAAACAAAACCGCGCGAGCTTTCATAAACTCCACCA 400

Chit5 TCCACGTGGCGCAAAAGTACGAGTTCAAATGGTCTTGAAGTGGGAGTGGCCAGGAGATGAAAAAGACATGTCTAAC 480
Chit5b TCCACGTGGCGCAAAAGTACGAGTTCAAATGGTCTTGAAGTGGGAGTGGCCAGGAGATGAAAAAGACATGTCTAAC 474
Chit5a TCCATGTGGCGCAAAAGTACGAGTTCAAACGGTCTTGAAGTGGGAGTGGCCGGGAGATGAAAAAGACATGTCTAAC 480

Chit5 CTTCGCTTGTGTATTTAAAGGAATGGTACAAGGCTTTGGTGGTGGAAAGCTAACACTAGTAGAAAAACACGTTTGTCTTAAAC 560
Chit5b CTTCGCTTGTGTATTTAAAGGAATGGTACAAGGCTTTGGTGGTGGAAAGCTAACACTAGTAGAAAAACACGTTTGTCTTAAAC 554
Chit5a CTTCGCTTGTGTATTTAAAGGAATGGTACAAGGCTTTCAGTGGTGGAAAGCTAACACTAGTAGAAAAACACGTTTGTCTTAAAC 560

Chit5 TCTGCTGTGTATTTAACTCCACGATTTCACTTATAGGTAATGGGCCCTAGGTCGTACCCGGTTCGAGCCATTCCGAAGT 640
Chit5b TCTGCTGTGTATTTAACTCCACGATTTCACTTATAGGTAATGGGCCCTAGGTCGTACCCGGTTCGAGCCATTCCGAAGT 634
Chit5a TCTGCTGTGTATTTAACTCCACGATTTCACTTATAGGTAATGGGCCCTAGGTCGTACCCGGTTCGAGCCATTCCGAAGT 640

Chit5 ACTTGGATTGGGCTAAGCCCAATGTGTTTGGATTTCATGGCCGCTGGGCCAACGAAACGGGCTTTAACGCCGCGTGTAT 720
Chit5b ACTTGGATTGGGCTAAGCCCAATGTGTTTGGATTTCATGGCCGCTGGGCCAACGAAACGGGCTTTAACGCCGCGTGTAT 714
Chit5a ACTTGGATTGGGCTAAGCCCAATGTGTTTGGATTTCATGGCCGCTGGGCCAACGAAACGGGCTTTAACGCCGCGTGTAT 720

Chit5 GACCCCAATTCTAACATAAGCACCAGATGTTATCGGGTCTGGATCGGGTGGGTCGCCGCCGAGAAACTGGTTA 800
Chit5b GATCCAAATCTAACATAAGCACCAGATGTTATCGGGTCTGGATCGGGTGGGTCGCCGCCGAGAAACTGGTTA 794
Chit5a GATCCAAATCTAACATAAGCACCAGATGTTATCGGGTCTGGATCGGGTGGGTCGCCGCCGAGAAACTGGTTA 800

Chit5 GGGTTTCCCGCTATACGGGCGGGCGTGGGAGCTGAAGGATCCGAATGATCATGGGTTGGGGCAAAAGCGGTGGTCCAG 880
Chit5b GGGTTTCCCGCTATACGGGCGGGCGTGGGAGCTGAAGGATCCGAATGATCATGGGTTGGGGCAAAAGCGGTGGTCCAG 874
Chit5a GGGTTTCCCGCTATACGGGCGGGCGTGGGAGCTGAAGGATCCGAATGATCATGGAGTGGGGCAAAAGCGGTGGTCCAG 880

Chit5 CAGTTGATACAGATGGTGTATGGACTATGATGAAATCTTGGTGTATAATAAGACACTGGTGCCAAAGGTTGTGTATGAT 960
Chit5b CAGTTGATACAGATGGTGTATGGACTATGATGAAATCTTGGTGTATAATAAGACACTGGTGCCAAAGGTTGTGTATGAT 954
Chit5a CAGTTGATACAGATGGTGTATGGACTATGGTGAATCTTGGTGTATAATAAGACACTGGTGCCAAAGGTTGTGTATGAT 960

Chit5 GAAGTGGCTGTGTCTTCTACTCTTATTCGGGCACACTTGGATCGGGTATGATGATGGACCCTCCATAACGAAGAAGGT 1040
Chit5b GAAGTGGCTGTGTCTTCTACTCTTATTCGGGCACACTTGGATCGGGTATGATGATGGACCCTCCATAACGAAGAAGGT 1034
Chit5a GAAGTGGCTGTGTCTTCTACTCTTATTCGGGCACACTTGGATCGGGTATGATGATGGACCCTCCATAACGAAGAAGGT 1040

Chit5 TCAATTTGCTCGGTCAATGGGCTGAAAGGGTATTTCTTTGGGCTATTGGCAAGGACAAAGGATGGACCATTCTCAAAAAC 1120
Chit5b TCAATTTGCTCGGTCAATGGGCTGAAAGGGTATTTCTTTGGGCTATTGGCAAGGACAAAGGATGGACCATTCTCAAAAAC 1114
Chit5a TCAATTTGCTCGGTCAATGGGCTGAAAGGGTATTTCTTTGGGCTATTGGCAAGGACAAAGGATGGACCATTCTCAAAAAC 1120

Chit5 AAGGTAAGACACCAATACGACAATACCCTTGTTTTACCTCTTTAATGATTGACAAAAA - -CACCTCTTTATAA 1198
Chit5b AAGGTAAGAAACCAATACGACAATACCCTTGTTTTACCTCTTTAATGATTGACAAAAA - -CACCTCTTTATAA 1194
Chit5a AAGGTAAGAAACCAATACGACAATACCCTTGTTTTACCTCTTTAATGATTGACAAAAA - -CACCTCTTTATAA 1198

Chit5 TATTAAATCAAATAAAACCTGTTTATAATTTTTTAAATTAATACATTTTGCATTTTCTTT - -ATAATATACATTTT 1276
Chit5b TATTAGCAAAATAAAACCTCTTTATAATTTTTTAAAT - AATACATTTTGCATTTTATTGATAATATACATTTT 1273
Chit5a TATTAGCAAAATAAAACCTCTTTATAAAATTTTTTAAAT - AATACATTTTGCATTTTATTGATAATATACATTTT 1277

Chit5 AACAAATTTCAATATTATGCTGTGACGAATCTGAATCCTCACTTTATAGAGTAAAGTAAAGCCATTACAATCACTGATTG 1356
Chit5b AACAAATTTCAATATTATCTGTGACGAGTCTGAATCCTCACTTTATAGAGTAAAGTAAAGCCATTACAATCACTGATTG 1353
Chit5a AACAAATTTCAATATTATCTGTGACGAGTCTGAATCTCACTTTATAGAGTAAAGTAAAGCCATTACAATCACTGATTG 1357

Chit5 AGTTGTTTTATTAGCTTTTCAAATTAAGTAAATCAACGTTAATGATTATGATGATTATTTTATTCTTTAAAAATGACTCTCT 1436
Chit5b AGTTGTTTTATTAGCTTTTCAAATTAAGTAAATCAACGTTAATGATTATGATGATTATTTTATTCTTTAAAAATGACTCTCT 1433
Chit5a AGTTGTTTTATTAGCTTTTCAAATTAAGTAAATCAACGTTAATGATTATGATGATTATTTTATTCTTTAAAAATGACTCTCT 1437

Chit5 TATTTTGAAGAGCTAGGTCCAGATGGTGAATAGTTGCAAAAATAAATCCCTTTGGTAAAGGCATTTCATGATCGTTATTT 1516
Chit5b TATTTTGAAGAGCTAGGTTCATATGGTGAATAGTTGCAAAAATAAATCCCTTTGGTAAAGGCATTTCATGATCGTTATTT 1513
Chit5a TATTTTGAAGAGCTAGGTCCAGATGGTGAATAGTTGCAAAAATAAATCCCTTTGGTAAAGGCATTTCATGATCGTTATTT 1517

Chit5 TGTTTTTT - ATTTAATTTTTTTCTAGATAAATGATTTCTAAAATAAATCTATAACATTGACCCAAAATATCCATTGTCA 1595
Chit5b TGTTTTTT - ATTTAATTTTTTTCTAGAAATGATTTCTAAAATAAATCTATAACATTGACCCAAAATATCCATTGTCA 1592
Chit5a TGTTTTTTATTATTAATTTTTTTCTAGATAAATGATTTCTAAAATAAATCTATAACATTGACCCAAAATATCCATTGTCA 1597

Chit5 ACAATTCATGACTCTCT - -TTTTCTACTTATTTTTAATTTCAATTTCTCATTTTACATATTTGTA - - - -GTATTA 1673
Chit5b ACAATTCATGACTCTCTCTTTTTCTAATTTTTAATTTCAATTTCTCATTTTACATATTTGTA - - - -GTATTA 1666
Chit5a ACAATTCATGACTCTCTCTTTTTCTACTTATTTTTAATTTCAATTTCTCATTTTACATATTTGTA - - - -GTATTA 1671

Chit5 AGATTTTTCTCTTTAGGAGAGGAGATGGTTTTTTATTATAATTTGATGGGTTTCTGCATTTTAAATGCAGGCAGAAAGT 1753
Chit5b AGATTTTTCTCTTTAGGAGAGGAGATGGTTTTTTATTATAATTTGATGGGTTTCTGCATTTTAAATGCAGGCAGAAAGT 1746
Chit5a AAATGTTCTCTTTAGGAGAGGAGATGGTTTTTTATTATAAATTTGATGGGTTTCTGCATTTTAAATGCAGGCAGAAAGT 1751

Chit5 CTTTAGATTTTCCATATAATAATGTTGATATTTGTTGTTGCTGTGTTTTTCTCATTGGCAGCTTCAAATGC 1833
Chit5b CTTTAGATTTTCCATATAATAATGTTGATATTTGTTGTTGCTGTGTTTTTCTCATTGGCAGCTTCAAATGC 1826
Chit5a CTTTAGATTTTCCATATAATAATGTTGATATTTGTTGTTGCTGTGTTTTTCTCATTGGCAGCTTCAAATGC 1831

Chit5 ATGGGGTACTGA 1846
Chit5b ATGGGGTACTGA 1839
Chit5a ATGGGGTACTGA 1844

Figure 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.

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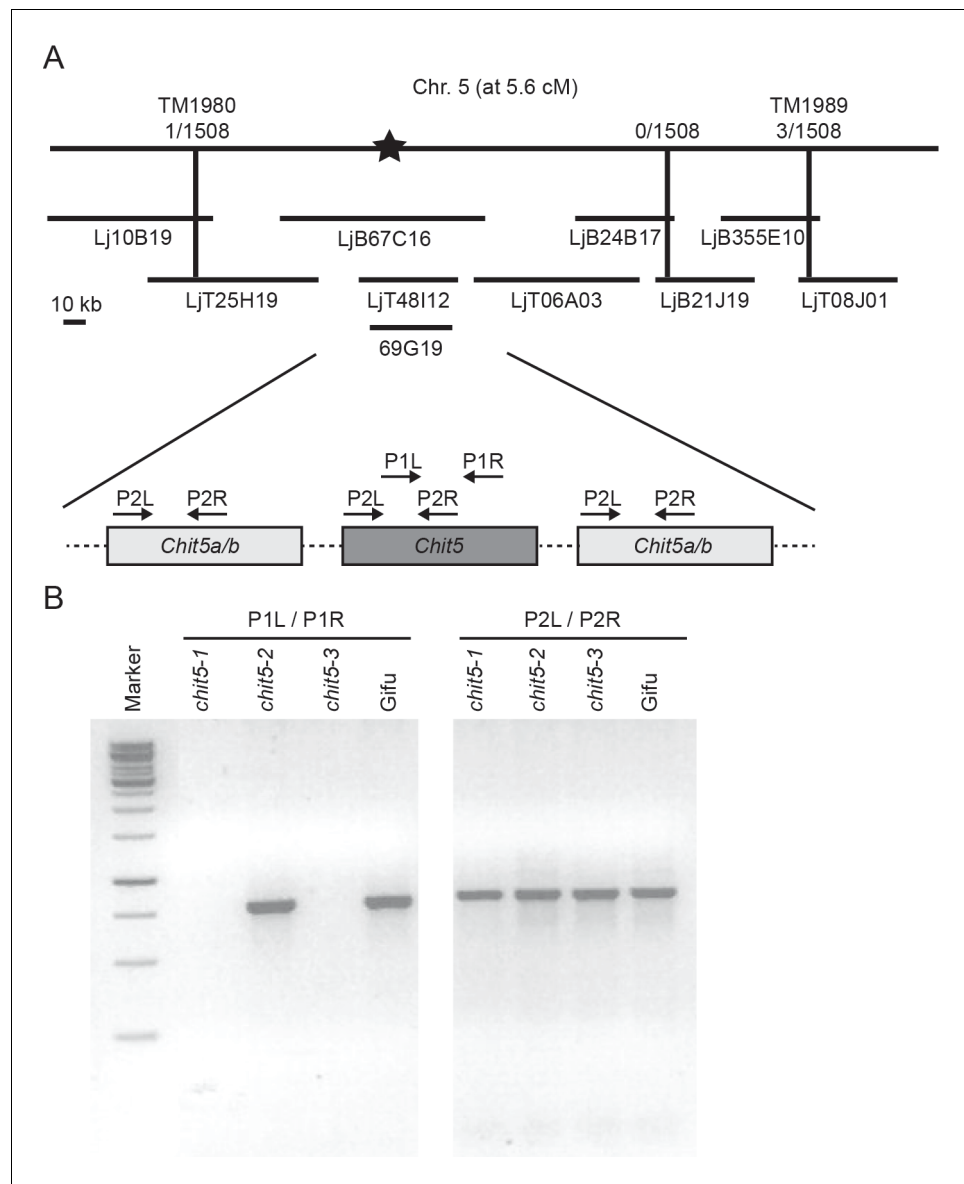


Figure 3—figure supplement 1. Map-based cloning of *Chit5* gene. (A) Positions of bacterial artificial chromosome (BAC) and transformation-competent artificial chromosome (TAC) clones from *L. japonicus* MG20 and BAC clone 69G19 from *L. japonicus* Gifu are shown. The closest markers with the number 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).

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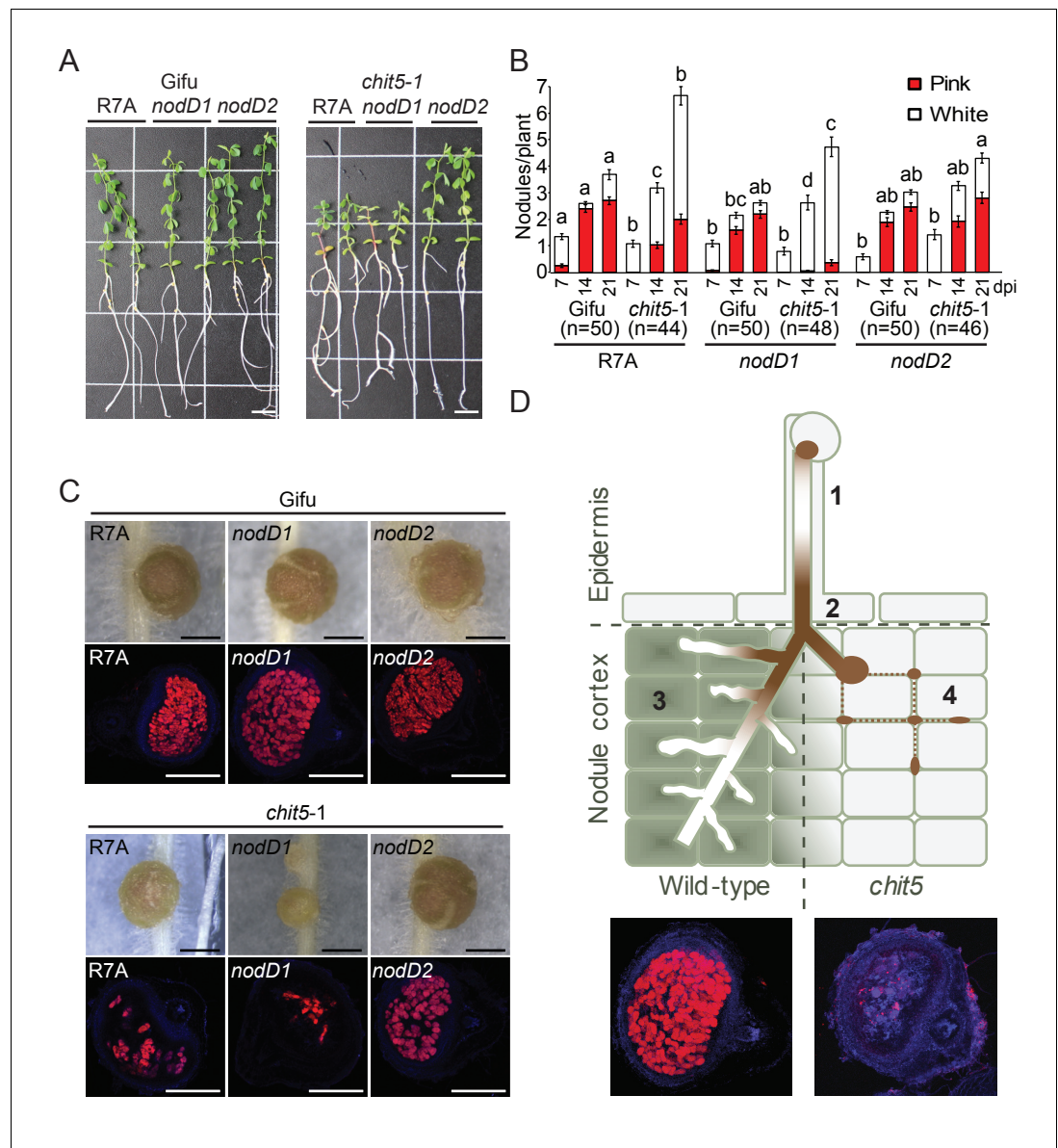


Figure 4. The symbiotic impairment of *chit5* mutants can be overcome by an *M. loti* R7A mutant affected in the regulation of Nod factor biosynthesis. (A) Representative images of Gifu and *chit5-1* plants inoculated with the indicated *M. loti* R7A strains 7 wpi. Scale bars are 1 cm. (B) Average number 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.

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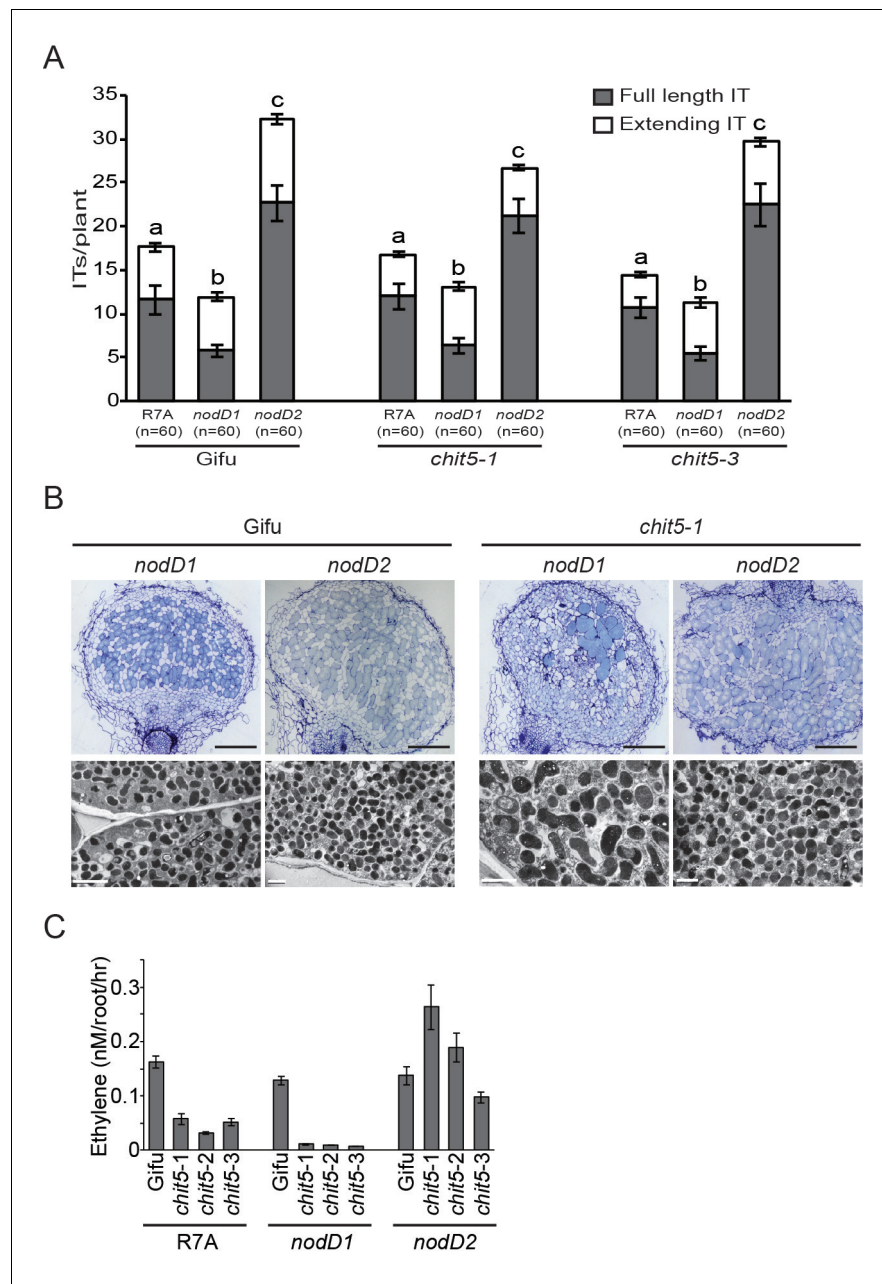


Figure 4—figure supplement 1. *chit5* mutant phenotypes with *M. loti* R7A mutants affected in the regulation of Nod factor biosynthesis. (A) Root hair infection thread formation on the indicated genotypes with *M. loti* R7A strains. 20 plant roots were cut into 1 cm pieces and 60 of these were analysed 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.

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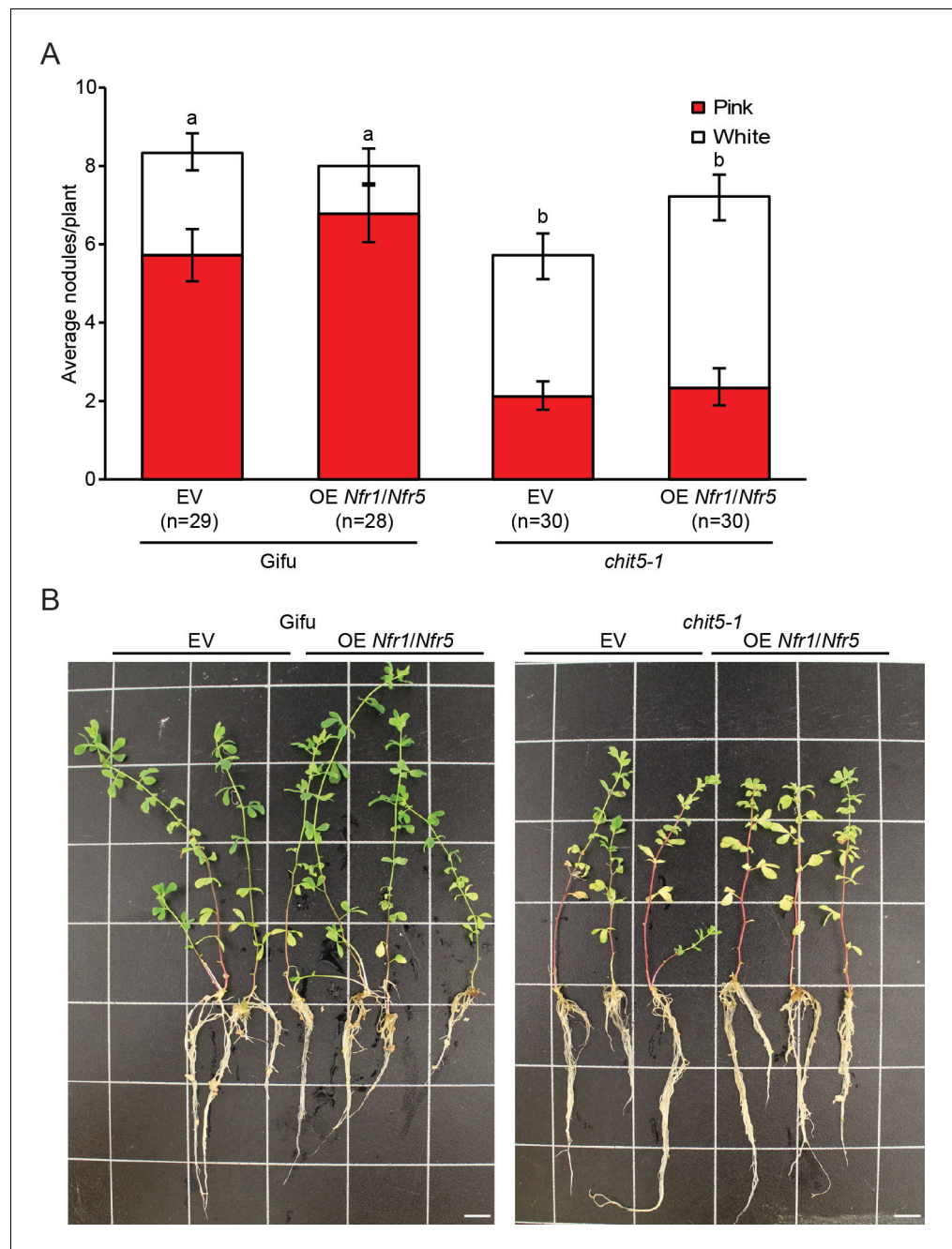


Figure 4—figure supplement 2. Overexpression of *Nfr1* and *Nfr5* does not rescue the symbiotic impairment of *chit5-1*. (A) Nodule counts on Gifu and *chit5-1* plants transformed with an empty vector control or the *Nfr1/Nfr5* over-expression construct 6 weeks post-inoculation with *M. loti* R7A. Error 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.

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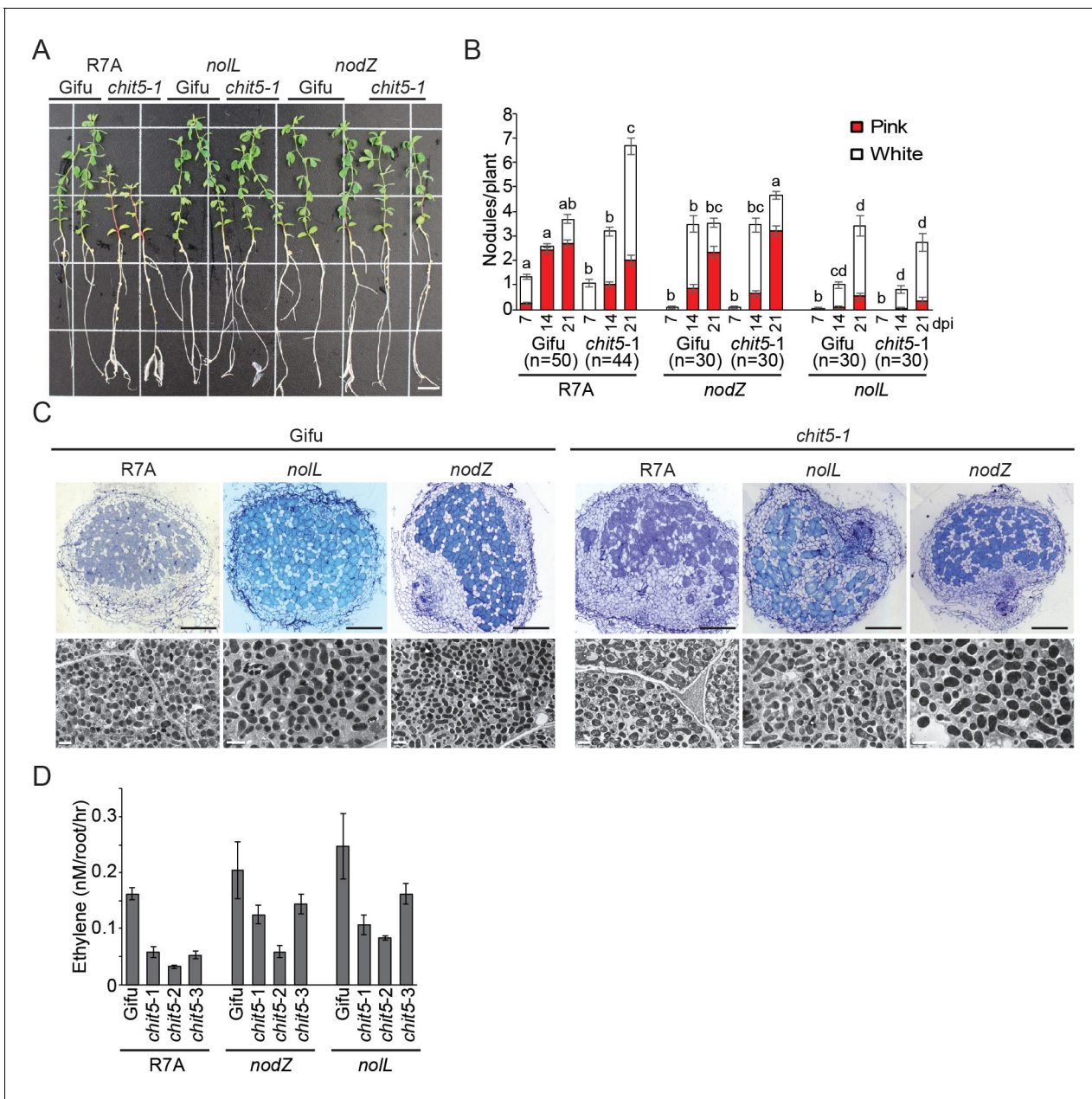


Figure 4—figure supplement 3. *chit5* mutant phenotypes with *M. loti* R7A mutants affected in the biosynthesis of fully decorated Nod factor. (A) Representative images of Gifu and *chit5-1* plants inoculated with the indicated strains 7 wpi. Scale bars are 1 cm. (B) Average number of pink and white 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 μ m for light microscopy images and 2 μ m 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. DOI: <https://doi.org/10.7554/eLife.38874.021>

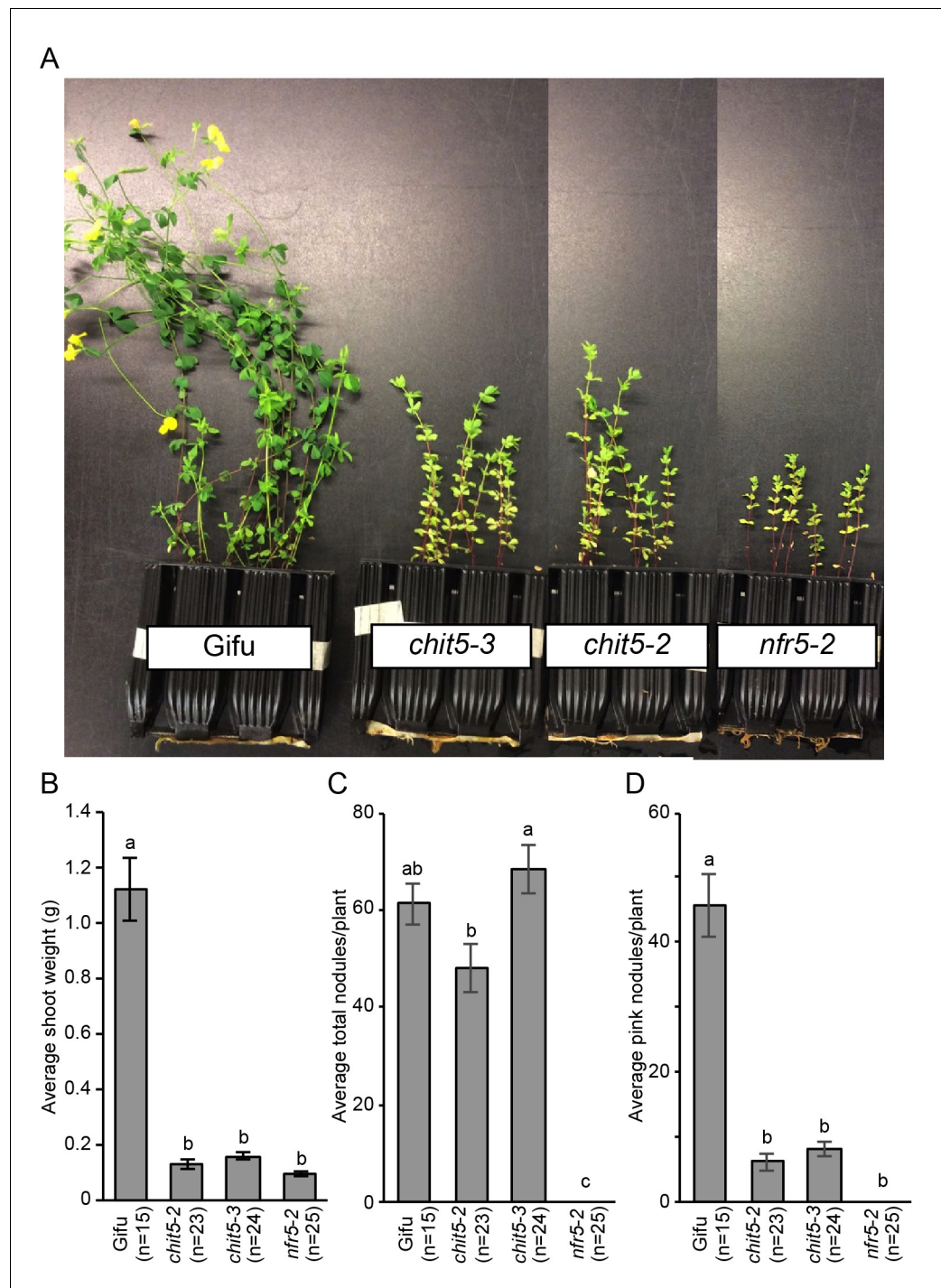


Figure 4—figure supplement 4. *chit5* mutants show a nitrogen-deficient phenotype when grown in soil. (A) Plant growth of the indicated genotypes when grown in soil containing its native microbiota for 9 weeks. (B) Shoot weight of plants after 7 weeks growth in soil. (C) Counts of total nodules formed 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.

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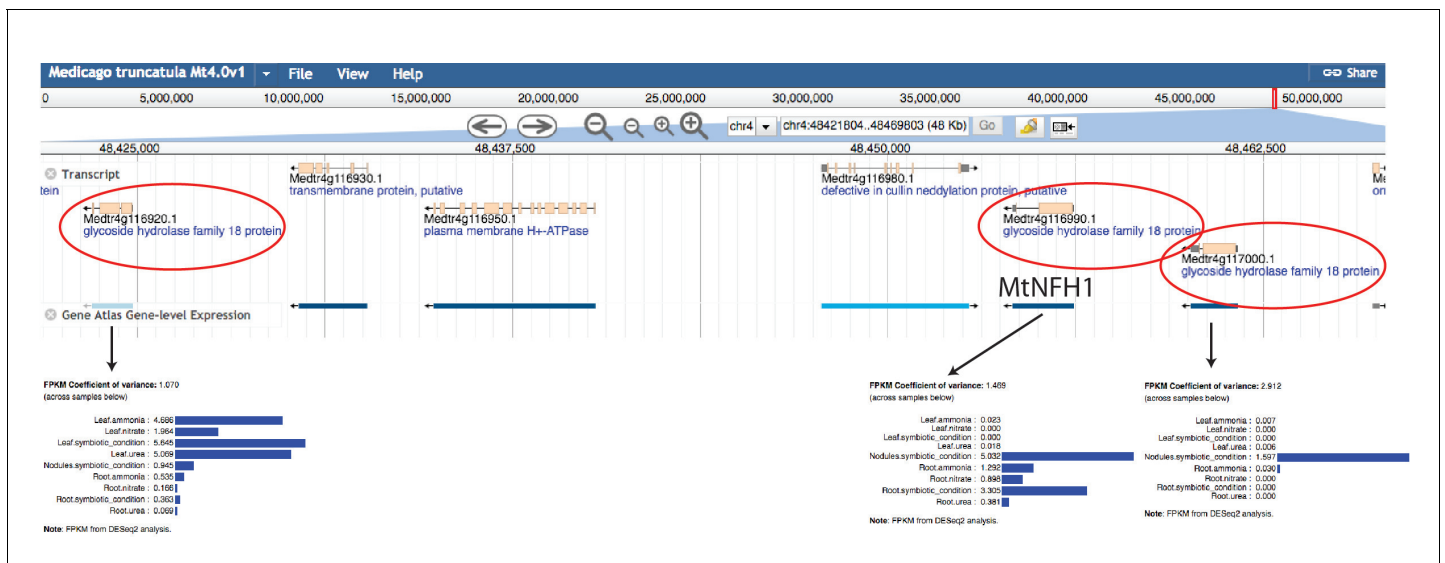
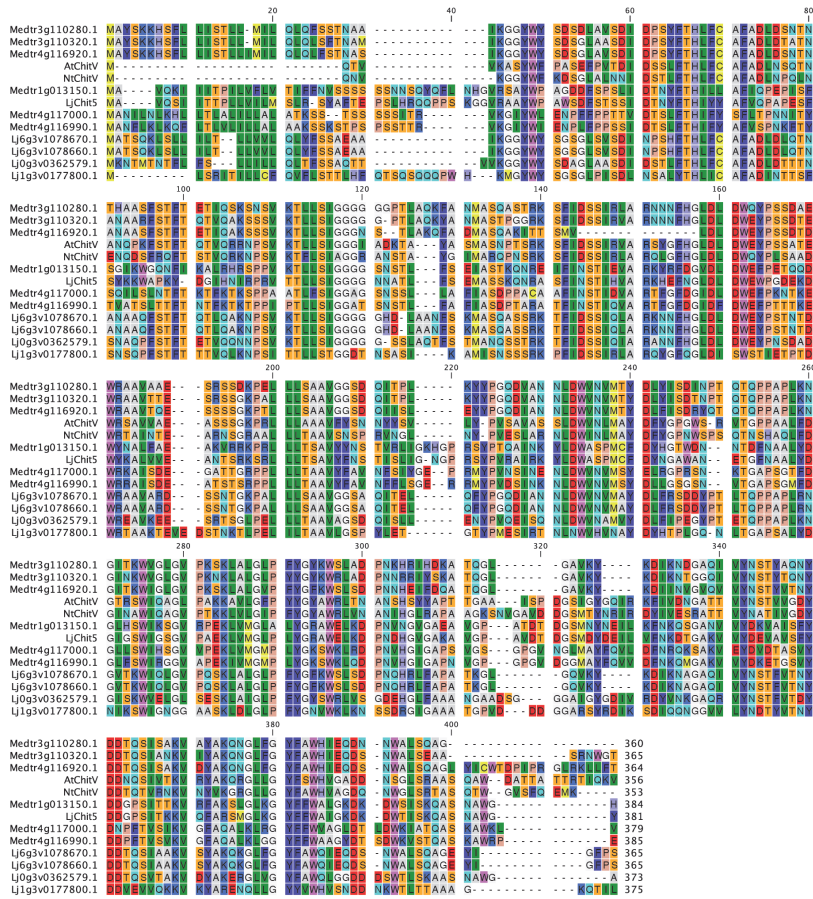


Figure 4—figure supplement 5. Genomic location and expression pattern of *MtNfH1* and the two close-located paralogs. The genomic information available on Phytozome 12 (<https://phytozome.jgi.doe.gov>) is given according to *Medicago truncatula* Mt4.0v1 version of the genome, and their expression level according to Gene Atlas expression tool. The three GH18 coding genes are marked (red circle) and their expression pattern is shown. DOI: <https://doi.org/10.7554/eLife.38874.023>

A



B

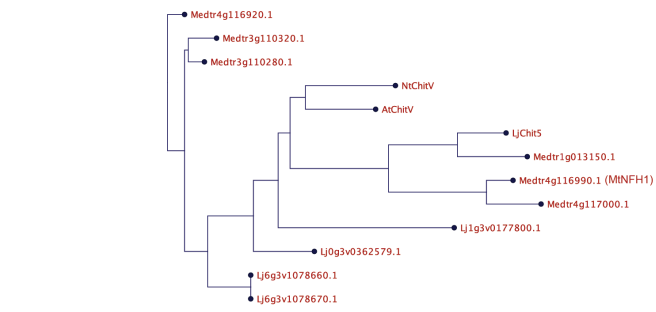


Figure 4—figure supplement 6. GH18 proteins from *M.truncatula* and *L.japonicus*. Alignment (A) and phylogenetic tree (B) of *Lotus* and *Medicago* proteins along with CHIT5 from *Arabidopsis* (AT4G19810.1) and *Nicotiana tabaccum* (XP_016468204.1). Protein sequences were obtained from <https://phytozome.jgi.doe.gov>, and <https://lotus.au.dk/>.

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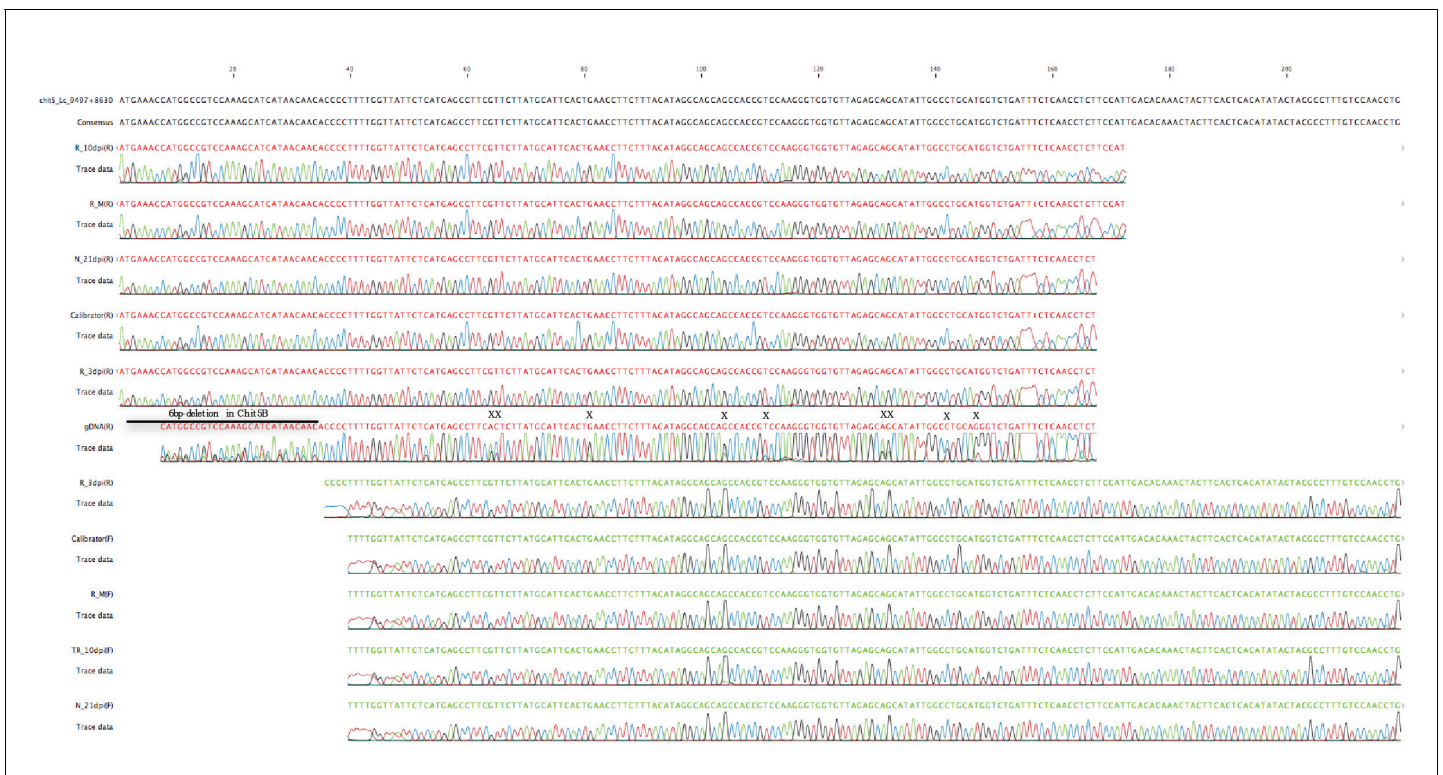


Figure 4—figure supplement 7. Sequencing traces from PCR products using genomic DNA (gDNA), cDNA from water treated roots (R_M), *M.loti* inoculated roots-3dpi (R_3dpi), -10dpi (R_10dpi) and nodules-21 dpi (N_21dpi) as template. Calibrator template contains a mixture of *L. japonicus* cDNAs from various 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*.

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