**SUPPLEMENTARY DATA TO**

# "A minimal genetic passkey to unlock many legume doors to root nodulation by rhizobia"

**by Jovelyn Unay and Xavier Perret**

University of Geneva, Department of Botany and Plant Biology, Sciences III, 30 quai Ernest-Ansermet, CH-1211 Geneva, Switzerland

**Correspondence to:** Xavier.Perret@unige.ch

**Table S1.** Strains and plasmids used in this study.

| Strain | Characteristics | Source / Reference |
|---|---|---|
| ANU265 | Derivative of NGR234 cured of its symbiotic plasmid pNGR234a, $Sp^R$ | Morrison et al. (1983) |
| ANU265::pMiniSym1 | ANU265 transconjugant carrying pMiniSym1, $Sp^R$, $Km^R$ | This work |
| ANU265::pMiniSym2 | ANU265 transconjugant carrying pMiniSym2, $Sp^R$, $Km^R$ | This work |
| ANU265::pMiniSym2-Gus | ANU265 transconjugant carrying pMiniSym2-Gus, $Sp^R$, $Km^R$ | This work |
| CBM832 | *Cupriavidus taiwanensis* strain that fixes nitrogen with *Mimosa pudica* | Marchetti et al. (2010) |
| CMG6 | Proficient *Mesorhizobium ciceri* strain isolated from *Cicer arietinum* | Ben Romdhane et al. (2007) |
| *E. coli* DH5α | F- Φ80*lac*ZΔM15, Δ(*lac*ZYA-*arg*F), *rec*A1, *end*A1, *hsd*R17, *pho*A, *sup*E44, *gyr*A96 *rel*A1 | Woodcock et al. (1989) |
| *E. coli* NEB 10-beta | DH10B derivative for cloning large plasmids, *endA1-* | NEB (Ipswich, MA, USA) |
| MAFF303099 | Strain of *Mesorhizobium japonicum* that is proficient on several *Lotus* spp. | Kaneko et al. (2000) |
| NGR234 | $Rif^R$-derivative of the *S. fredii* strain isolated from *Lablab purpureus* by M.J. Trinick | Stanley et al. (1988) |
| NGR234::pXPrpsL426 | NGR234 transconjugant carrying pXPrpsL426, $Rif^R$, $Sp^R$ | Fumeaux et al. (2011) |
| Sm1021 | *Sinorhizobium meliloti* strain 1021 proficient on *Medicago sativa* cv. Gemini | Capela et al. (2001) |
| WSM419 | *Sinorhizobium medicae* strain that fixes nitrogen with *M. truncatula* cv. Jemalong | Reeve et al. (2010) |
| **Plasmid** | **Characteristics** | **Source / Reference** |
| pBluescript II KS+ (pKS) | ColEI-based phagemid, *lac*α$Z^+$, $Ap^R$ | Stratagene |
| pHP45 Ω Km | pHP45 carrying the kanamycin resistant ($Km^R$) Omega interposon | Fellay et al. (1987) |
| pMiniSym1 | 7,282 bp RK2-based, mobilisable, low copy number vector for mini-symbiotic plasmids | This work |
| pMiniSym2 | pMiniSym1 with *nodD1* and *nodABCIJS* genes/promoters of NGR234, $Km^R$ | This work |
| pMiniSym2-Gus | pMiniSym2 with GusA constitutive expression, $Km^R$ | This work |
| pKS-PrpsL-GUS-1 | pKS with a 2,313 bp *Pst*I-*Sac*I fragment with the *uidA* gene and *rpsL* promoter of pXPrpsL426, $Ap^R$ | This work |

**Table S1.** Continuation

| Plasmid | Characteristics | Source / Reference |
|---|---|---|
| pNB3-nodD1-10 | pKS with NGR234 *nodD1* and native promoter as 1,347 bp *Spe*I fragment, $Ap^R$ | This work |
| pNB8-nodABCIJ-30 | pKS with NGR234 *nodABCIJ* and native promoter as 4,996 bp *Spe*I fragment, $Ap^R$ | This work |
| pNB12-nodS-4 | pKS with NGR234 *nodS* and native promoter as 1,224 bp *Spe*I fragment, $Ap^R$ | This work |
| pRK2oriT-1 | pKS with pRK7813 origin of transfer (*oriT*) as 410 bp *Spe*I fragment, $Ap^R$ | This work |
| pRK2oriV-1 | pKS with pRK7813 origin of replication (*oriV*) as 854 bp *Spe*I fragment, $Ap^R$ | This work |
| pRK2trfA-7 | pKS with pRK7813 *trfA* and native promoter as 1,782 bp *Kpn*I fragment, $Ap^R$ | This work |
| pRK2013 | $Tra^+$ helper plasmid for tri-parental mobilisation, $Km^R$ | Figurski and Helinski (1979) |
| pRK7813 | Broad host-range IncP1 and RK2 based costramid, $Tc^R$ | Jones and Gutterson (1987) |
| pSpsAB-4 | pKS with NGR234 *spsAB* and native promoter as 2,374 bp *Spe*I fragment, $Ap^R$ | This work |
| pXB182 | Lorist2 based cosmid covering a section of pNGR234a with the *nodABCIJ* genes, $Km^R$ | Perret et al. (1991) |
| pXB1027 | Lorist2 based cosmid covering a section of pNGR234a with the *nodSU* genes, $Km^R$ | Perret et al. (1991) |
| pXB1357 | Lorist2 based cosmid covering a section of pNGR234a with the *nodD1* gene, $Km^R$ | Perret et al. (1991) |
| pXPrpsL426 | pRG960 with *rpsL* promoter of NGR234 driving constitutive expression of *uidA*, $Sp^R$ | Fumeaux et al. (2011) |

## References

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**Table S2.** Oligonucleotides and templates for PCR amplification and cloning of gene blocks in separate constructs.

The *Spe*I (5'-A'CTAGT-3') and *Kpn*I (5'-GGTAC'C-3') restriction sites used for cloning amplicons are underlined.

| **Target** | **Primer** | **Sequence** (5′ to 3′) | **Template** | **Cloned as** (bp) | **In construct** |
|---|---|---|---|---|---|
| NB8 + *nodABCIJ* | NB8-nodABCIJ-For | gggactagtagcggtattagc | pXB182 | 4,996 | pNB8-nodABCIJ-30 |
| | NB8-nodABCIJ-Rev | gtaactagtaggccatgtgctc | | | |
| NB12 + *nodS* | NB12-nodS-For | gaactagtctccatcacatccacc | pXB1027 | 1,224 | pNB12-nodS-4 |
| | NB12-nodS-Rev | cgactagttattccgcttctcc | | | |
| *nodD1*+ NB3 | nodD1-NB3-For | gaactagtgctgcgcataggc | pXB1357 | 1,347 | pNB3-nodD1-10 |
| | nodD1-NB3-Rev | ttactagtggcaaggctgttgc | | | |
| *PrpsL* + *uidA* | PrpsL-GUS-For | gaaactagtatgaccatgattacg | pKS-PrpsL-GUS-1 | 2,594 | pMiniSym2-Gus |
| | PrpsL-GUS-Rev | caactagtgggaagggcgatc | | | |
| *oriV* | RK2oriV-For | caactagtcgctgaatgt | pRK7813 | 854 | pRK2oriV-1 |
| | RK2oriV-Rev | caactagtcagtgagcgagg | | | |
| *oriT* | RK2oriT-For | cccttactagttggcttgg | pRK7813 | 410 | pRK2oriT-1 |
| | RK2oriT-Rev | ggactagtaagataccaggcg | | | |
| *spsAB* | spsAB-For | ctactagtcctggacttcgtcg | NGR234 gDNA | 2,374 | pSpsAB-4 |
| | spsAB-Rev | gcactagtgggtggctatagg | | | |
| *trfA* | trfA-For | gtgggtaccgagcgatactga | pRK7813 | 1,782 | pRK2trfA-7 |
| | trfA-Rev | ggtggtacccagcggaagc | | | |

**Table S3**. Primers and templates for amplifying gene blocks for Gibson assemblies and resulting constructs, with nucleotides matching templates underlined.

| Gene block | Primer | Sequence (5′ to 3′) | Template | Construct |
|---|---|---|---|---|
| Omega-Km | GB-OmegaKm-For | tcaaggcgttagcccatccatggggttcgatacgttagcg | pHP45 Ω Km | pMiniSym1 |
| | GB-OmegaKm-Rev | gcttcgctcagtatcgctcggcgacgttggtgaccttgatcc | | |
| *oriT* | GB-OriT-For | gtttaaacactagtcctaggggcgcgccatccgcttgccctcatctg | pRK2oriT-1 | pMiniSym1 |
| | GB-OriT-Rev | gcagtaacgggatgggcgatggtgtatccaacggcgtcagc | | |
| *oriV* | GB-OriV-For | gctgacgccgttggatacaccatcgcccatcccgttactgc | pRK2oriV-1 | pMiniSym1 |
| | GB-OriV-Rev | cgatggaacgggtgcgtgaccagtgagcgaggaagcggaag | | |
| *spsAB* | GB-spsAB-For | cttccgcttcctcgctcactggtcacgcacccgttccatcg | pSpsAB-4 | pMiniSym1 |
| | GB-spsAB-Rev | cgctaacgtatcgaaccccatggatgggctaacgccttga | | |
| *trfA* | GB-trfA-For | gggcgcgcccctaggactagtgtttaaaccccctcgcacctttggtcgc | pRK2trfA-7 | pMiniSym1 |
| | GB-trfA-Rev | ggatcaaggtcaccaacgtcgccgagcgatactgagcgaagc | | |
| *nodD1* | GB2-nodD1-For | cgctaacgtatcgaaccccagacgaaatggcaggagcggt | pNB3-nodD1-10 | pMiniSym2 |
| | GB2-nodD1-Rev | agcaggagaagcggaataacaatggtcggagtgtggcatg | | |
| *nodS* | GB2-nodS-For | ttcagtcggagcacatggcctgtgatttcgacatggatgc | pNB12-nodS-4 | pMiniSym2 |
| | GB2-nodS-Rev | catgccacactccgaccattgttattccgcttctcctgct | | |
| *nodABCIJ* | GB2-nodABCIJ-For | tcaaggcgttagcccatccagcggtattagcttcattgcc | pNB8-nodABCIJ-30 | pMiniSym2 |
| | GB2-nodABCIJ-Rev | gcatccatgtcgaaatcacaggccatgtgctccgactgaa | | |
| pMiniSym1 | GB2-OmegaKm-For | accgctcctgccatttcgtctggggttcgatacgttagcg | pMiniSym1 | pMiniSym2 |
| | GB2-spsAB-Rev | ggcaatgaagctaataccgctggatgggctaacgccttga | | |

**Table S4.** Primers used in qPCR to determine pMiniSym2 and pNGR234a copy numbers in ANU265 and NGR234, respectively.

| **Primer** | **Sequence** (5′ to 3′) | **Gene** | **Replicon** | **Amplicon** (bp) |
|---|---|---|---|---|
| rpoC-F | GAGAAGTGCGGTGTCGAAGT | *rpoc* | chromosome | 98 |
| rpoC-R | TTCAGGAACCAGATGTGGGC | | | |
| rpsL-F | GAACTCGGCTCTGCGTAAGG | *rpsL* | chromosome | 98 |
| rpsL-R | AGTGCTCCTGAAGGTTGTGG | | | |
| nodB-For | TTTGACGACGGTCCTAACCC | *nodB* | pMiniSym2/pNGR234a | 100 |
| nodB-Rev | TAGCATAAGCCCCGATGACG | | | |
| nodD1-F | GAACAGCTTCGCTCTGGGA | *nodD1* | pMiniSym2/pNGR234a | 100 |
| nodD1-R | TTCCGATGAGCCAGATGAGC | | | |

**Table S5.** Comparing the copy numbers of pNGR234a and pMiniSym2 plasmids.

The plasmid copy number (PCN) was obtained using the following formula $2^{[Ct(Chr)-Ct(plas)]}$ where the averaged Ct's for the chromosome (Chr) and for plasmid (plas) were obtained by combining Ct values for the chromosomal *rpoC* and *rpsL* genes and plasmid *nodB* and *nodD1* genes, respectively. Genomic DNA (gDNA) was extracted from NGR234 and ANU265::pMiniSym2 cells harvested at $OD_{600}$ 0.8 and grown in either RMS or TY liquid media, using 50 μg/ml kanamycin (ANU265::pMiniSym2) or rifampicin (NGR234). For each gene, qPCR was performed in triplicate and using five gDNA concentrations (from 0.156 to 40 ng) as template. Primer sequences used for amplifying the *rpoC*, *rpsL*, *nodB* and *nodD1* target genes are listed in Table S4.

| | | | Chromosome | | Plasmid | | |
|---|---|---|---|---|---|---|---|
| Strain | Medium | gDNA (ng) | Ct(Chr) | StDev [Ct(Chr)] | Ct(plas) | StDev [Ct(plas)] | PCN |
| NGR234 | RMS | 40 | 15.72 | 0.25 | 15.30 | 0.40 | 1.34 |
| | | 10 | 17.39 | 0.05 | 17.11 | 0.38 | 1.22 |
| | | 2.5 | 19.35 | 0.26 | 18.79 | 0.37 | 1.48 |
| | | 0.625 | 21.65 | 0.37 | 21.21 | 0.42 | 1.36 |
| | | 0.156 | 23.63 | 0.38 | 23.14 | 0.42 | 1.41 |
| | TY | 40 | 17.88 | 0.09 | 17.59 | 0.35 | 1.22 |
| | | 10 | 19.97 | 0.19 | 19.63 | 0.32 | 1.27 |
| | | 2.5 | 21.76 | 0.19 | 21.45 | 0.39 | 1.23 |
| | | 0.625 | 23.88 | 0.34 | 23.72 | 0.42 | 1.12 |
| | | 0.156 | 25.88 | 0.39 | 25.35 | 0.37 | 1.44 |
| ANU265::pMiniSym2 | RMS | 40 | 16.47 | 0.07 | 16.49 | 0.31 | 0.99 |
| | | 10 | 18.58 | 0.24 | 18.48 | 0.37 | 1.07 |
| | | 2.5 | 20.73 | 0.34 | 20.41 | 0.32 | 1.25 |
| | | 0.625 | 22.61 | 0.31 | 22.42 | 0.35 | 1.14 |
| | | 0.156 | 24.74 | 0.31 | 24.34 | 0.30 | 1.32 |
| | TY | 40 | 17.36 | 0.08 | 16.51 | 0.15 | 1.80 |
| | | 10 | 19.23 | 0.25 | 18.28 | 0.13 | 1.94 |
| | | 2.5 | 21.18 | 0.34 | 20.20 | 0.09 | 1.97 |
| | | 0.625 | 23.36 | 0.43 | 22.30 | 0.08 | 2.08 |
| | | 0.156 | 25.27 | 0.37 | 24.32 | 0.09 | 1.94 |

**Figure S1.** pMiniSym2 confers to ANU265 a robust nodulation capacity on cowpea, siratro and *Leucaena leucocephala*. Photographs of shoots and corresponding roots of *Vigna unguiculata* cv. Red Caloona **(a)**, *Macroptilium atropurpureum* cv. Siratro **(b)** and *L. leucocephala* **(c)** plants that were harvested 28 days post-inoculation with NGR234, ANU265::pMiniSym2 or ANU265. Scale bars, 1 cm.

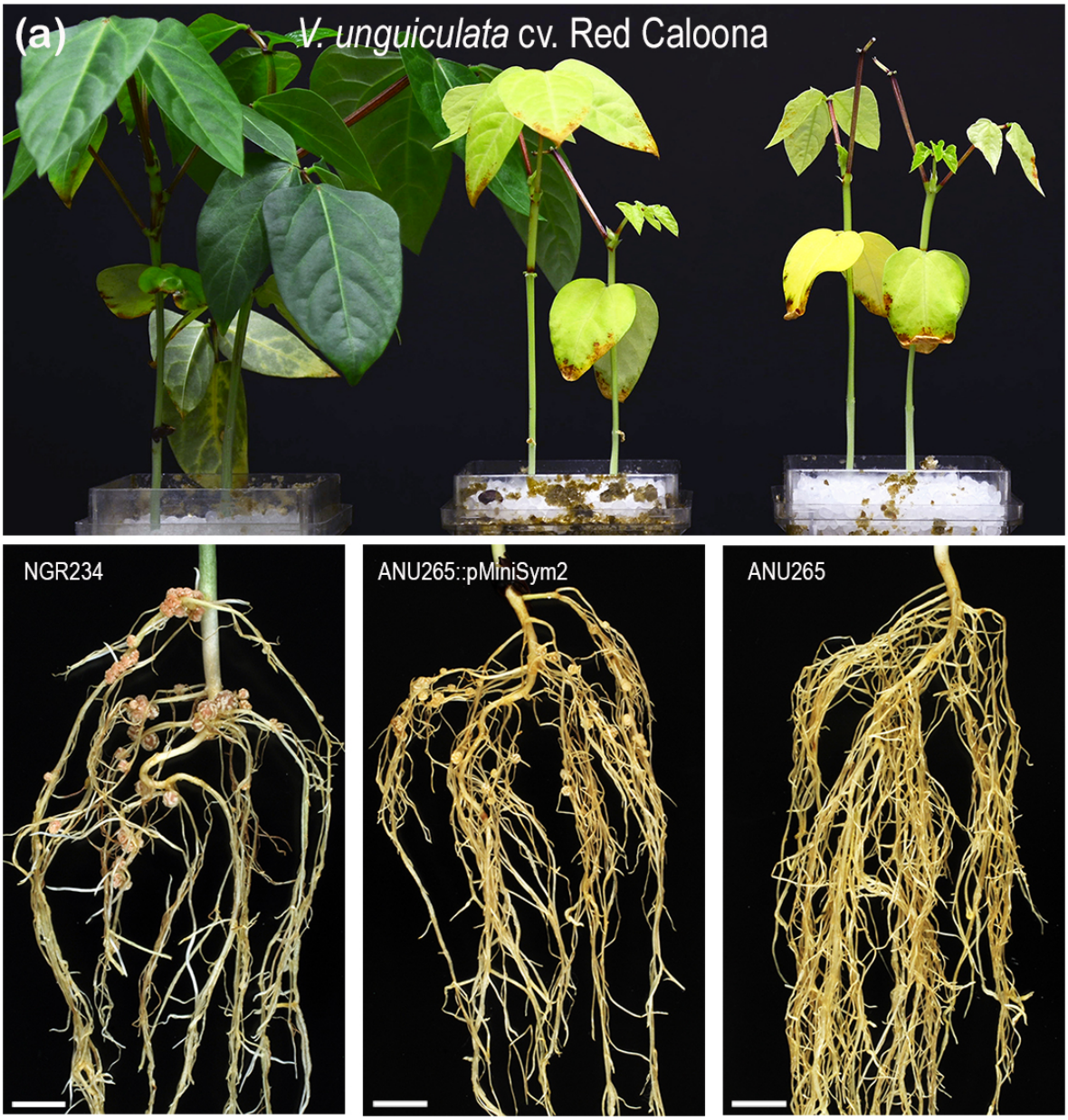


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**Figure S1.** Continuation

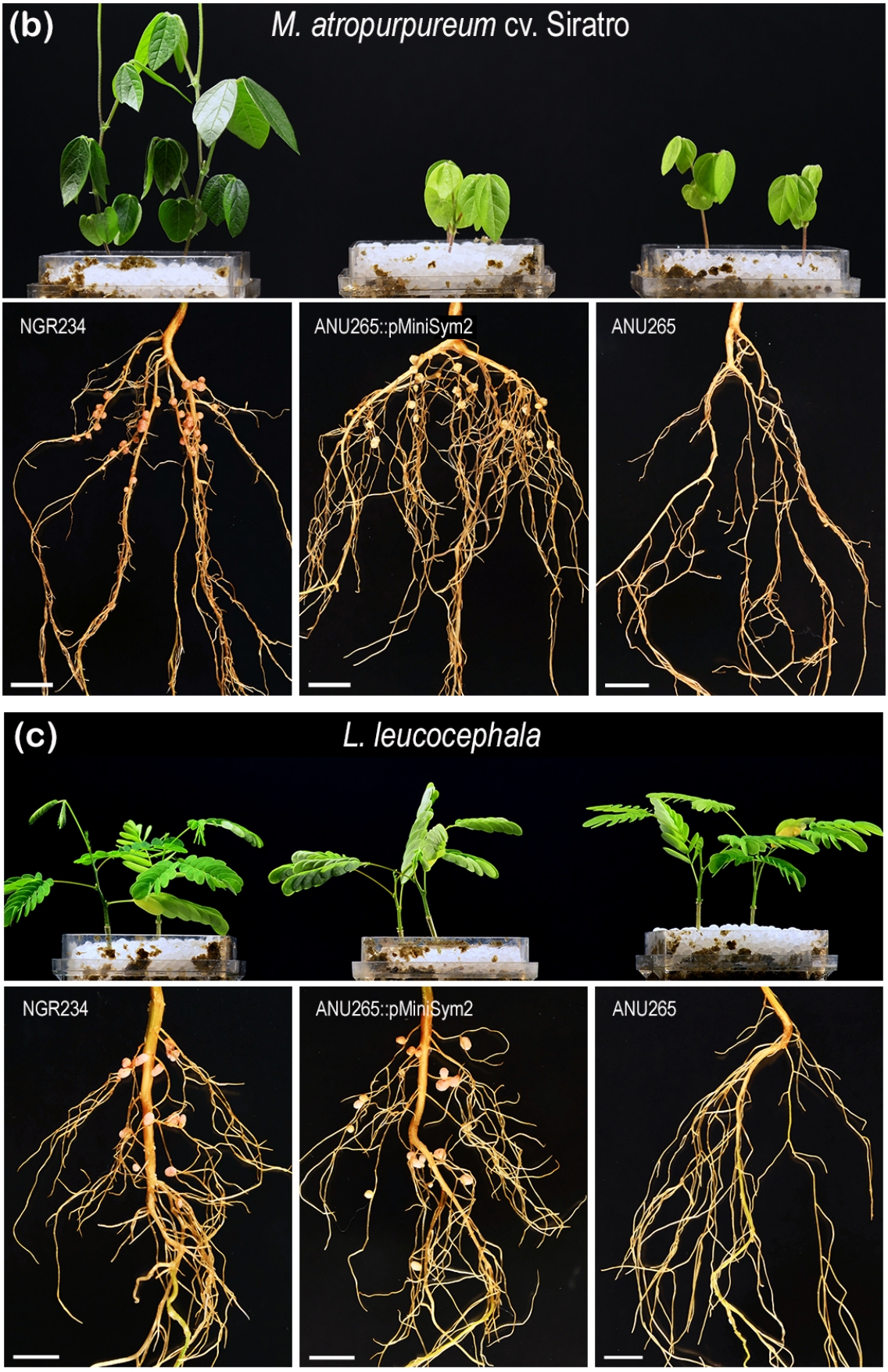


**Figure S2.** Capacity of ANU265::pMiniSym2-Gus to colonise root nodules is host-dependent. Sections of nodules formed on roots of *Vigna unguiculata* cv. Red Caloona, *Macroptilium atropurpureum* cv. Siratro and *Leucaena leucocephala* 28 days post-inoculation with ANU265::pMiniSym2-Gus or NGR234::pXPrpsL426 transconjugant strains. Nodule sections were photographed after staining for β-glucuronidase activity. More than a dozen nodules per inoculum and from several roots were examined per treatment. White circles delimit patches of Gus-stained cells occasionally observed in cowpea nodules formed by ANU265::pMiniSym2-Gus. By contrast, Gus-stained regions of siratro nodules infected by the pMiniSym2-Gus transconjugant were more frequently observed. All nodules formed by NGR234::pXPrpsL426 and those formed by ANU265::pMiniSym2-Gus on *L. leucoephala* were stained throughout infected zones. Scale bars, 1 mm.

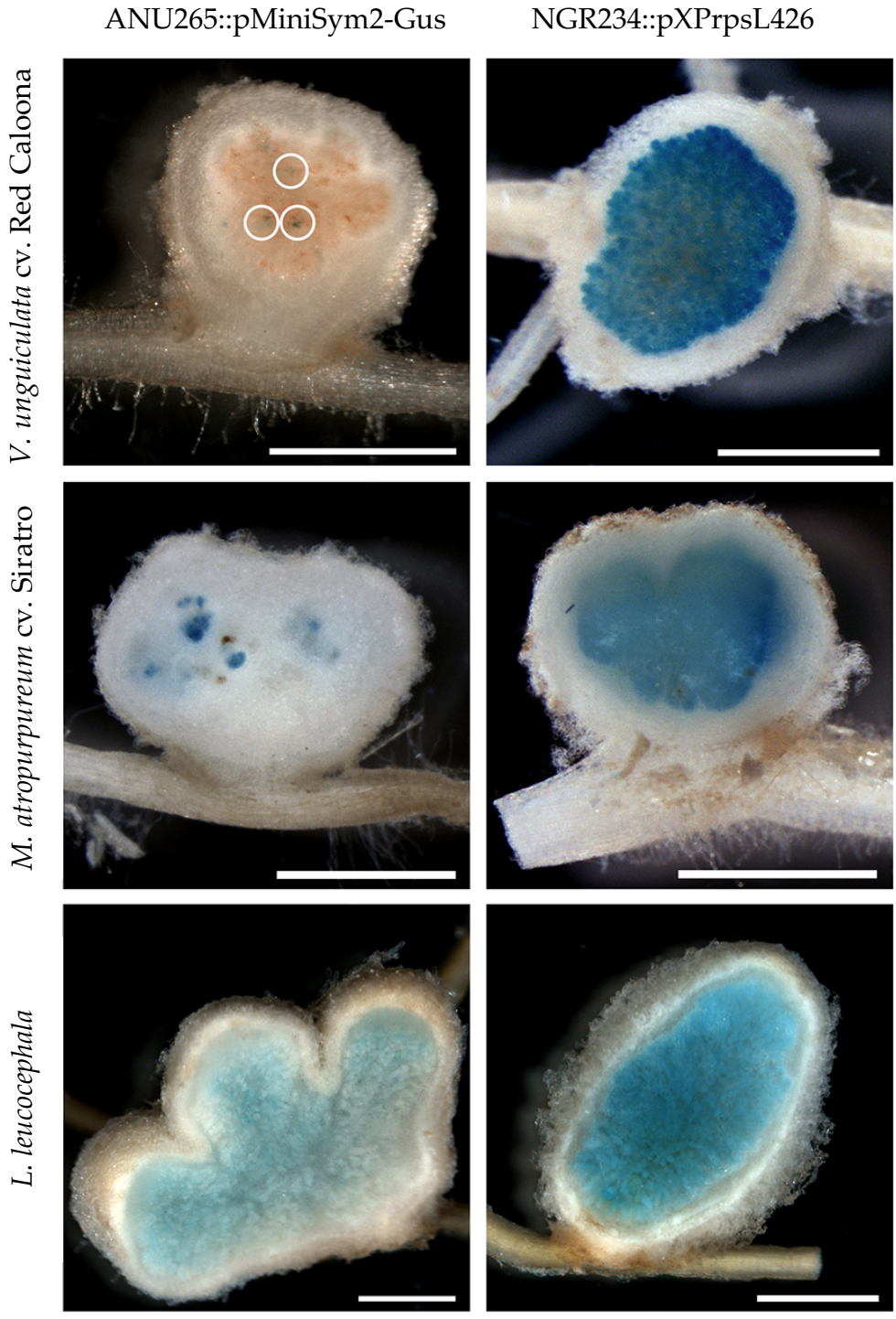


**Figure S3.** Aborted colonisation of root nodules by ANU265::pMiniSym2 correlates with presence of plant phenolic compounds. Nodules of *Vigna unguiculata* cv. Red Caloona and *Macroptilium atropurpureum* cv. Siratro formed 28 days after inoculation with ANU265::pMiniSym2 or NGR234. 300 μm thick nodule sections were photographed before (**left**) and after staining (**right**) for phenolic compounds. At least six nodules were stained per treatment. Nodule formed by ANU265::pMiniSym2 on cowpea was sectioned across the root vertical axis. Scale bars, 1 mm.

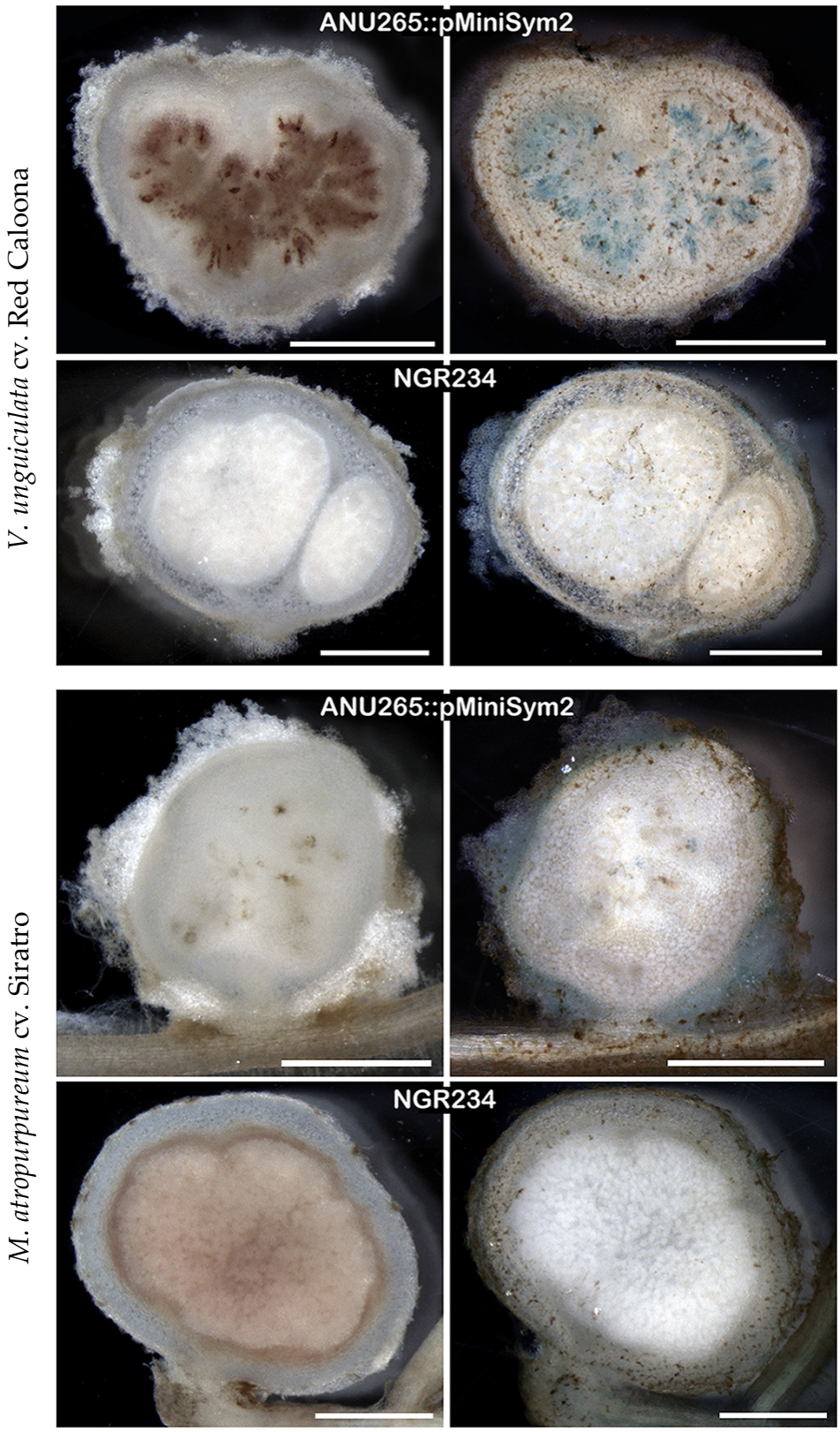


**Figure S4.** ANU265::pMiniSym2-Gus cannot establish persistent intracellular colonies in nodule cells of *Vigna unguiculata* cv. Red Caloona. Sections of cowpea nodules at 15 dpi with NGR234::pXPrpsL426 (**a to d**) or ANU265::pMiniSym2-Gus (**e** to **h**). Semi-thin sections (1 µm) (panels **a** and **e**) were stained with methylene blue and fuchsine prior to microscopy observation. Details of plant and bacteria cellular ultra-structures can be seen in electron micrographs shown in panels (**b**) to (**d**) and (**f**) to (**h**). White frames in panel (**f**) correspond to the enlarged sections shown in panel (**g**), which shows intracellular bacteria one of which appears to have lost cell integrity and to be degraded (black arrow), and in panel (**h**) where an abnormal infection thread or pocket can be seen. ***bc***, bacteroids; ***cw***, plant cell wall; ***er***, endoplasmic reticulum; ***ib***, intracellular bacteria; ***it***, infection thread; ***it****, abnormal infection thread or infection pocket; ***iz***, infected zone; ***n***, nucleus; ***pbm***, peribacteroid membrane; ***r***, root; ***s***, starch granules; ***v***, vascular bundles. More than six nodules collected on several roots were processed per treatment. Scale bars are 200 µm in (**a**) and (**e**), 5 µm in (**b**) and (**f**), and 2 µm in (**c**, **d**, **g** and **h**).

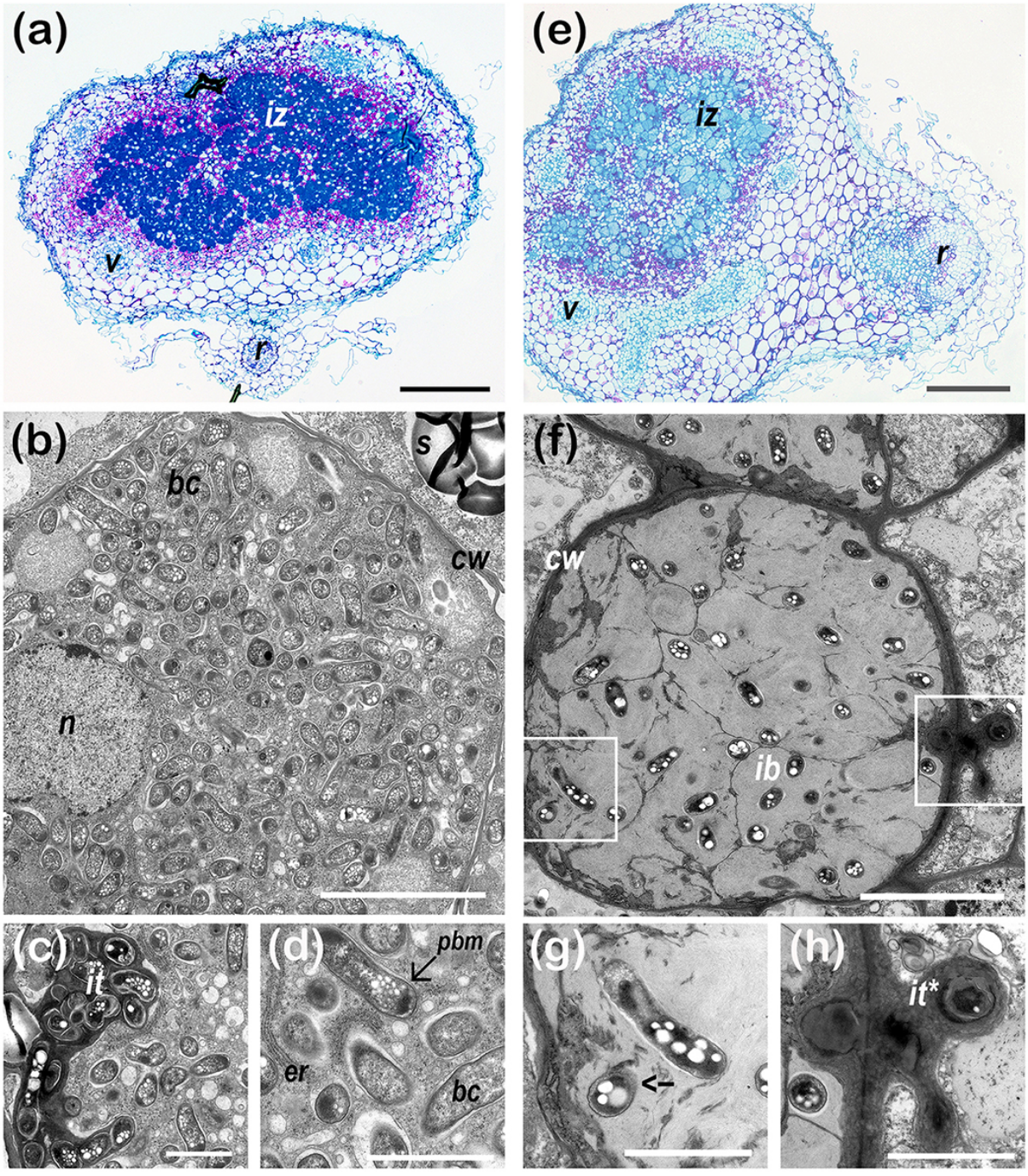


**Figure S5.** In nodules of *Macroptilium atropurpureum* cv. Siratro, the ANU265::pMiniSym2-Gus transconjugant fails to establish persistent intracellular colonies. At 15 dpi, nodules of siratro formed by NGR234::pXPrpsL426 (panels **a** and **b**) or ANU265::pMiniSym2-Gus (**c** and **d**) were examined by electron microscopy. Micrographs of nodule cells infected by NGR234::pXPrpsL426 (**a** and **b**) show numerous bacteroids that occupy the plant cytoplasm. Intracellular forms of NGR234::pXPrpsL426 are surrounded by peribacteroid membranes, thus forming proficient symbiosome units (panel **b**). By contrast, plant cells infected by ANU265::pMiniSym2-Gus have lost shape and internal cell structures, with several intracellular bacteria being enclosed into vesicles much larger than normal symbiosomes and occasionally showing signs of degradation (**c**). Higher magnification of one necrotic-like lesion with bacteria at various stages of degradation (**d**). ***bc***, bacteroids; ***cw***, plant cell wall; ***er***, endoplasmic reticulum; ***ib***, intracellular bacteria; ***pbm***, peribacteroid membrane; ***s***, starch granules. Scale bars are of 5 (**a** and **c**) and 2 μm (**b** and **d**).

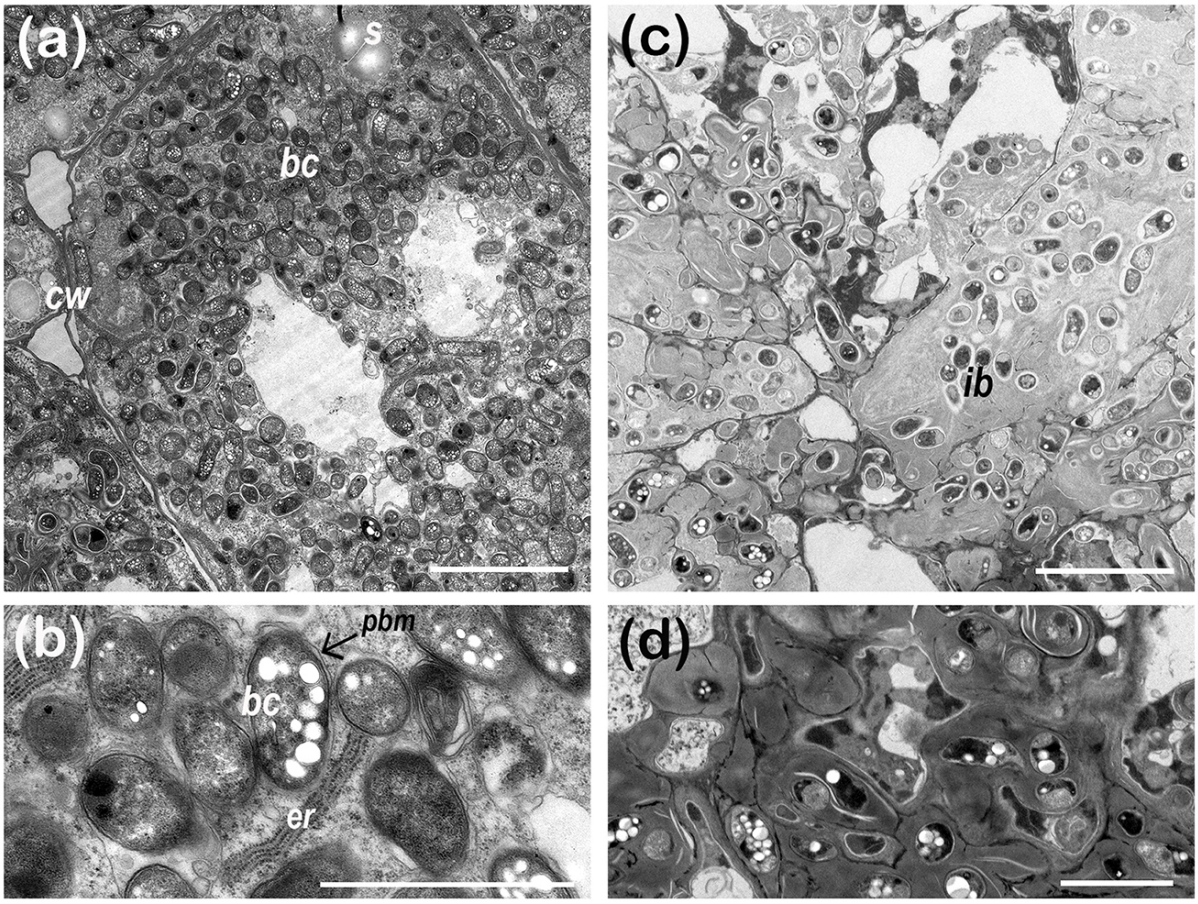
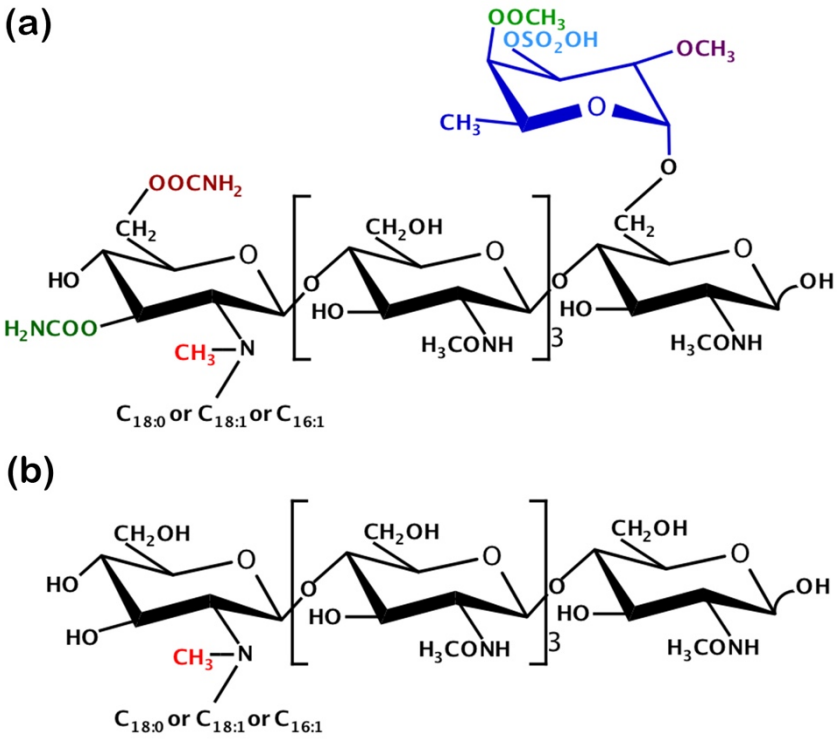


**Figure S6.** Structures of the Nod-factors produced by NGR234 (**a**) and of those predicted to be secreted by ANU265::pMiniSym2 (**b**). In NGR234, a number of enzymes encoded by the symbiotic plasmid pNGR234a modify the chore structure of Nod-factors (NF) shown in black below (for reviews see Perret et al. 2000 and Brougton et al. 2000). The changes resulting from the activity of these NF-modifying enzymes are highlighted as follows: in dark red for 6-*O*-carbamoylation by NodU; dark blue for 6-*O*-fucosylation by NodZ; light blue for *O*-sulfurylation by NoeE; purple for 2-*O*-methylation by NoeI; light green for *O*-acetylation by NolL; dark green for 3 or 4-*O*-carbamoylation by NolO; and red for *N*-methylation by NodS. The remaining NoeJ (mannose-1-phosphate guanyltransferase), NoeK (phosphomanomutase), NoeL (GDP-mannose 4,6-dehydratase) and NolK (fucose synthase) enzymes are involved in the synthesis of GDP-L-fucose, the substrate for NodZ activity. Except for NodS, all of the NF-modifying enzymes listed above are missing in ANU265::pMiniSym2. Hence, the NF secreted by the pMiniSym2 transconjugant were predicted to be methylated pentamers of *N*-acetylglucosamine that should carry the same fatty acids (C18:1, C18:0 or C16:1) as those found on NF of NGR234.

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