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Supporting Online Material for

A Nodule-Specific Protein Secretory Pathway Required for Nitrogen-Fixing Symbiosis

Dong Wang, Joel Griffitts, Colby Starker, Elena Fedorova, Erik Limpens, Sergey Ivanov, Ton Bisseling, Sharon Long*

*To whom correspondence should be addressed. E-mail: SRL@stanford.edu

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Supporting Online Material

Materials and methods

Plasmids and vectors. Constructs for complementation and promoter activity were first cloned into the pCR8/GW/TOPO vector, and later recombined with destination vectors using the Gateway technology (Invitrogene). For complementation assays, a 7.8 KB fragment of the *DNF1* gene, including a 3 KB promoter, was cloned into pMDC100 (*1*). The same fragment was modified slightly to allow the in-frame fusion between the last exon of *DNF1* and the GFP coding sequence of PMDC107 (*1*). 57 bp of the first exon was included to generate the DNF1 promoter fragment. For the other promoter fragments, sequences up to 3 KB upstream of the transcriptional start sites were cloned. Promoter::GUS reporters were derived from pMDC163. For studying the membrane identity markers the constructs *pUBQ3:GFP-MtRab7A1* and *pUBQ3:GFP-MtVTI11* were used as described in Limpens et al. (*2*).

Plant transformation and nodulation. Plasmids were transformed into *Agrobacterium rhizogenes* strain MSU440, ARqua1, or K599, and hairy root transformations were carried out according to Boisson-Dernier et al. (*3*). Transgenic roots and nodules were selected based on antibiotic resistance or GFP or DsRED1 expression. For nodulation *Sinorhizobium meliloti* strain Sm2011, Sm2011-GFP (*4*), Sm2011-mRFP (*5*), Rm1021-lacZ, or Rm1021-mCherry were used.

Light and Confocal microscopy. For fluorescence study, transgenic nodules were hand-sectioned using double-edged razorblades and mounted on microscope slides in 0.1 M phosphate buffer (pH7.4) containing 25 mg/ml sucrose. Transgenic roots and sectioned nodules were further analyzed on a ZEISS LSM 510 confocal laser scanning microscope. For promoter activity assays, nodules were first incubated in GUS staining buffer, briefly fixed with glutaraldehyde for 2 hours, hand-sectioned using double-edged razorblades, and then incubated in the presence of magenta-Gal for lacZ.

WT and *dnf1* nodules were fixed in 4% paraformaldehyde with 3% glutaraldehyde in 50 mM phosphate buffer (pH7.4) and embedded using Technovit 7100 (Heraeus Kulzer). 2 μm sections were cut using a Reichert-Jung 2035 microtome. Sections were counterstained using 0.05% toluidine blue and viewed with a Nikon Optiphot-2 microscope.

Electron Microscopy. For the analysis of the structure of the *dnf1* nodules the tissue was fixed in 4% paraformaldehyde with 3% glutaraldehyde in 50 mM phosphate buffer (pH7.4), postfixed with 1% of OsO_4, embedded in LR white resin according to the supplier recommendations and polymerised at 60 ^{o}C. Thin sections (60 nm) were cut using a Leica Ultracut microtome. The sections were contrasted with 2% aqueous uranyl acetate and lead citrate and examined using a JEOL JEM 2100 TEM electron microscope equipped with a Gatan US4000 4K x 4K camera.

Supplemental Figure legend

fig. S1 Low magnification light microscopy analysis of WT and *dnf1* nodules.

Longitudinal sections were produced through a WT nodule (**A**) and a *dnf1* nodule (**B**) 12 dpi. The meristem (m) is present at the distal part. z2 is the infection zone where rhizobia are released followed by division and differentiation of symbiosomes. z3 is the fixation zone. Stage 1 bacteroids are only in 1-2 host cell layers in (**A**) but are in many more cells in (**B**). Scale bars: 50 μm.

fig. S2 Characterization of *dnf1* nodules using membrane identity markers. (**A**) Confocal image of a *dnf1* nodule transformed with *pUBQ3:GFP-MtRab7A1* 10 dpi. GFP-MtRab7A1 marks the symbiosome membrane (arrowhead) and the tonoplast, which is here best seen surrounding the invading infection thread (it). (**B**) Confocal image of a

dnf1 nodule transformed with *pUBQ3:GFP-MtVTI11* 10 dpi. GFP-MtVTI11 marks the tonoplast but not the symbiosomes (arrowhead). The rhizobia are expressing mRFP (red). v, vacuole; n, nucleus. Scale bars in (a) and (b): 10 μm.

fig. S3 *DNF1L* expression pattern in various tissues. Values are based on microarray data from the *Medicago truncatula* Gene Expression Atlas. Note the different scale compared with Fig. 3: the expression levels are much lower than that of *DNF1*.

Fig. S4 *DNF1* promoter activity in young nodules. *DNF1* promoter::GUS transgenic roots were inoculated with Rm1021, and stained for GUS activity in the nodule 4 dpi. Scale bar: 100 μm.

fig. S5 *DNF1* promoter activity in older nodules. *DNF1* promoter::GUS transgenic roots were inoculated with Rm1021, and stained for GUS activity in the nodule 28 dpi. Scale bar: 1 mm.

Supplemental References

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4. E. Limpens *et al.*, *Science* **302**, 630 (2003).
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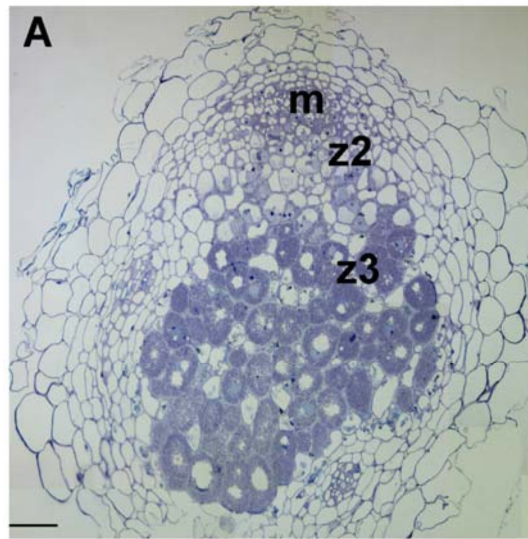
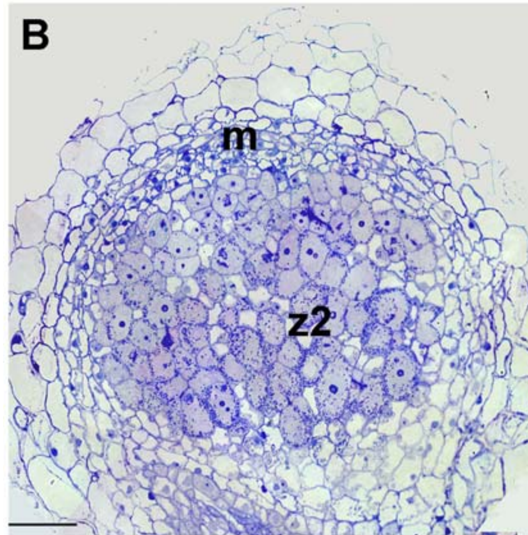
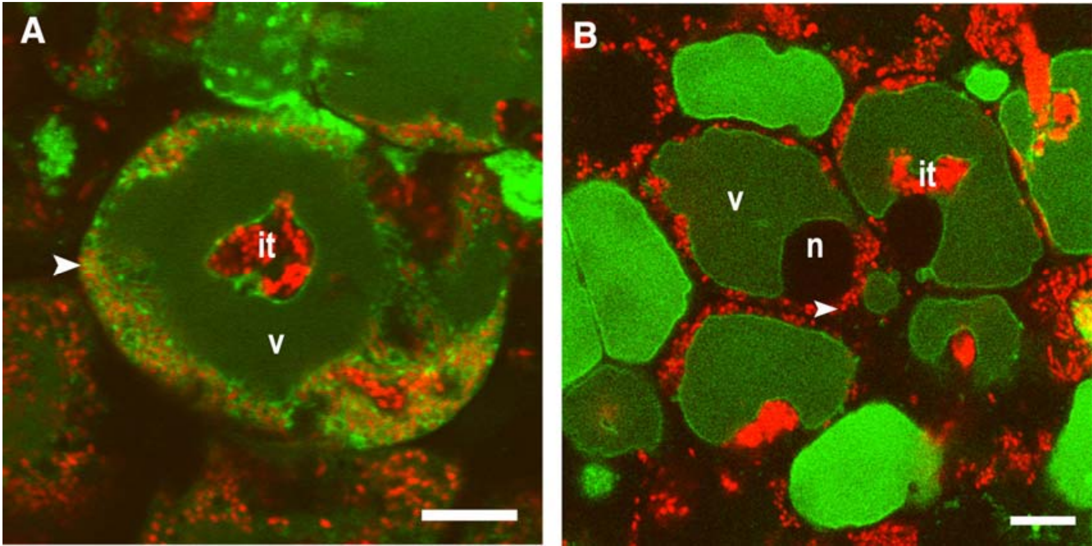
Wang et al. fig. S1

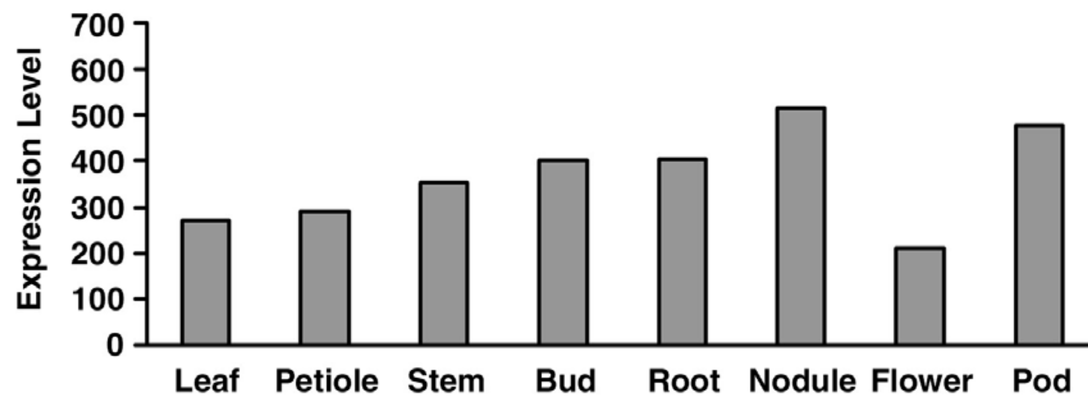




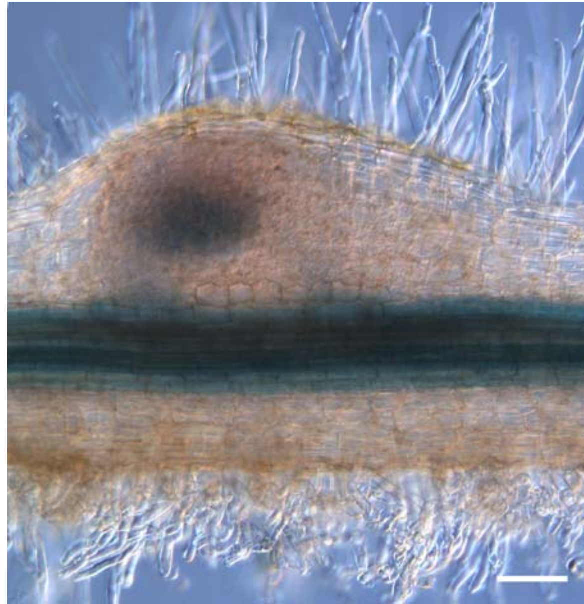
Wang et al. fig. S2



Wang et al. fig. S3



Wang et al. fig. S4



Wang et al. fig. S5