SUPPLEMENTAL MATERIAL AND METHODS

Casparian strip visualization.

After immunolocalization of MtNramp1-HA in roots and subsequent analysis by confocal microscopy, these sections were stained using the berberine-aniline blue method (Brundrett et al., 1988). Briefly, sections were incubated with 0.1% berberine hemi-sulphate (Sigma) in water for 1 h. After washing with water, samples were incubated in 0.5% aniline blue in water for 30 min and then rinsed with water. Finally, sections were transferred to 0.1% $FeCl_3$ in 50% glycerol for several minutes and mounted in the same solution. Images were obtained using a confocal laser-scanning microscope (Leica SP8, Wetzlar, Germany).

Iron distibution visualization

Roots and nodules from 28 dpi wild type and *nramp1-1* plants were collected and fixed in 0.25% glutaraldehyde, 4% formaldehyde, 2.5% sucrose in 50 mM potassium phosphate buffer (pH 7.4) at 4 ºC overnight. Sample were then dehydratated using ethanol series and embedded in LR-White resin (London Resin Company Ltd, UK) (Rodríguez-Haas et al., 2013). Finally, nodules were placed in gelatine capsules, filled with resin and polymerized at 60ºC for 24 h. Serial thin sections (1µm) were cut with a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria) fitted with a diamond knife. Sections for nodule structure analysis were stained with a mixture of 1% (w/v) toluidine blue in aqueous 1% sodium borate and 1% (w/v) methylene blue in water. Iron distribution was visualized using the Perl-DAB method (Roschzttardtz et al., 2009). Direct observation of sections was performed under a Zeiss Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany) with an attached digital camera (Leica DFC 420C, Heerburgg, Switzerland). A minimum of three nodules and roots per treatment and three sections per sample were examined.

Brundrett M, Enstone D, Peterson C (1988) A berberine-aniline blue fluorescent staining procedure for suberin, lignin, and callose in plant tissue. Protoplasma **146:** 133-142

Rodríguez-Haas B, Finney L, Vogt S, González-Melendi P, Imperial J, González-Guerrero M (2013) Iron distribution through the developmental stages of *Medicago truncatula* nodules. Metallomics **5:** 1247-1253

Roschzttardtz H, Conejero G, Curie C, Mari S (2009) Identification of the endodermal vacuole as the iron storage compartment in the Arabidopsis embryo. Plant Physiol **151:** 1329-1338

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure S1. *MtNramp* gene family expression. Gene expression of the corresponding *MtNramp* gene in nitrogen-fertilized roots (NFR), denodulated roots (DR) and nodules (Nod) relative to the internal standard gene *ubiquitin carboxyl-terminal hydrolase*. Data are the mean ± SE of three independent experiments.

Supplemental Figure S2. *MtNramp1-HA* yeast complementation assay. Yeast strain BY4741 was transformed with the pYPGE15 empty vector, while BY4741-derived BR4742 strain mutated in *smf1* was transformed with either pYPGE15 alone, or with pYPEG15 containing *MtNramp1* CDS, or with pYPGE15 harbouring *MtNramp1* CDS with a C-terminal 3xHA tag. Serial dilutions (10x) of each transformant were grown for 3 days at 30ºC on SD Glucose media with all the required amino acids. pH was buffered with 50 mM MES and Mn levels were kept low with 6.25 mM EGTA. Mn-replete positive controls were obtained by supplementing the plate with 500 µM $MnSO_4$.

Supplemental Figure S3. Antibody-specificity control for immunolocalization. Cross section of a 28 dpi *M. truncatula* nodule infected with *S. meliloti* constitutively expressing GFP (green) and transformed with a vector expressing *MtNramp1* under the regulation of its endogenous promoter. MtNramp1 localization was determined using an Alexa 594-conjugated antibody (red). DNA was stained with DAPI (blue). Infection threads are indicated with asterisks. Scale bar represents 100 µm.

Supplemental Figure S4. Expression in the different *M. truncatula* nodule zones. (A) *MtNramp1***.** (B) Nodule-specific MATE transporter *Medtr6g081400*. ZI indicates zone I; ZIId, distal zone II (region closest to zone I); ZIIp, proximal zone II; IZ is the interzone between zone II and zone III; and ZIII, zone III. Data were obtained from the Symbimics database (https://iant.toulouse.inra.fr/symbimics).

Supplemental Figure S5. MtNramp1-HA localization in the root. (A) Confocal microscopy localization of MtNramp1-HA in a cross section of a 28 dpi *M. truncatula* root transformed with a vector expressing *MtNramp1* under the regulation of its endogenous promoter. MtNramp1 position was determined using an Alexa 594-

conjugated antibody (red). DNA and xylem was stained with DAPI (blue). (B) Bright-field image of the cross section. (C) Overlay of panels A and B. (D) Close-up of overlaid MtNramp1-HA localization in *M. truncatula* transformed roots (detected with an Alexa 594-conjugated antibody in red) and bright-field image. (E) Casparian strip localization (blue) detected with the berberine-aniline blue method. (F) Overlay of panels D and E. Scale bars represent 50 µm (panels A-C) or 10 µm (panels D-F).

Supplemental Figure S6. Phenotype of *nramp1-1* under non-symbiotic conditions. (A) Growth of representative plants. Scale bar represents 1 cm. Fe concentrations in nutritive solutions used are indicated below. (B) Fresh weight of shoots and roots. Data are the mean ± SE (n = 5 plants). (C) Chlorophyll content. Data are the mean ± SE (n = 3 plants). (D) Iron content in roots and shoots of wild-type and *nramp1-1* plants under two different iron fertilization levels. Data are the mean ± SE of two sets of five pooled plants. * indicates significative differences ($p \leq 0.05$). (F) *MtFRO1* expression in wild type and nramp1-1 plants under two different iron fertilization levels. Data are the mean ± SE of three independet experiments. * indicates significative differences ($p \leq 0.05$).

Supplemental Figure S7. **Iron complementation of *nramp1-1* phenotype.** (A) Growth of representative plants. Scale bar represents 1 cm. (B) Fresh weight of shoots and roots. Data are the mean ± SE (n = 8 plants). (C) Nodule number per plant. 100 % = 4.25 nodules/plant. Data are the average ± SE (n=8-10 plants). (D) Nitrogenase activity in 28 dpi nodules. Acetylene reduction was measured in duplicate from two sets of five pooled plants. Data are the mean ± SE. 100% = 61.17 nmol ethylene $h^{-1}g^{-1}$.

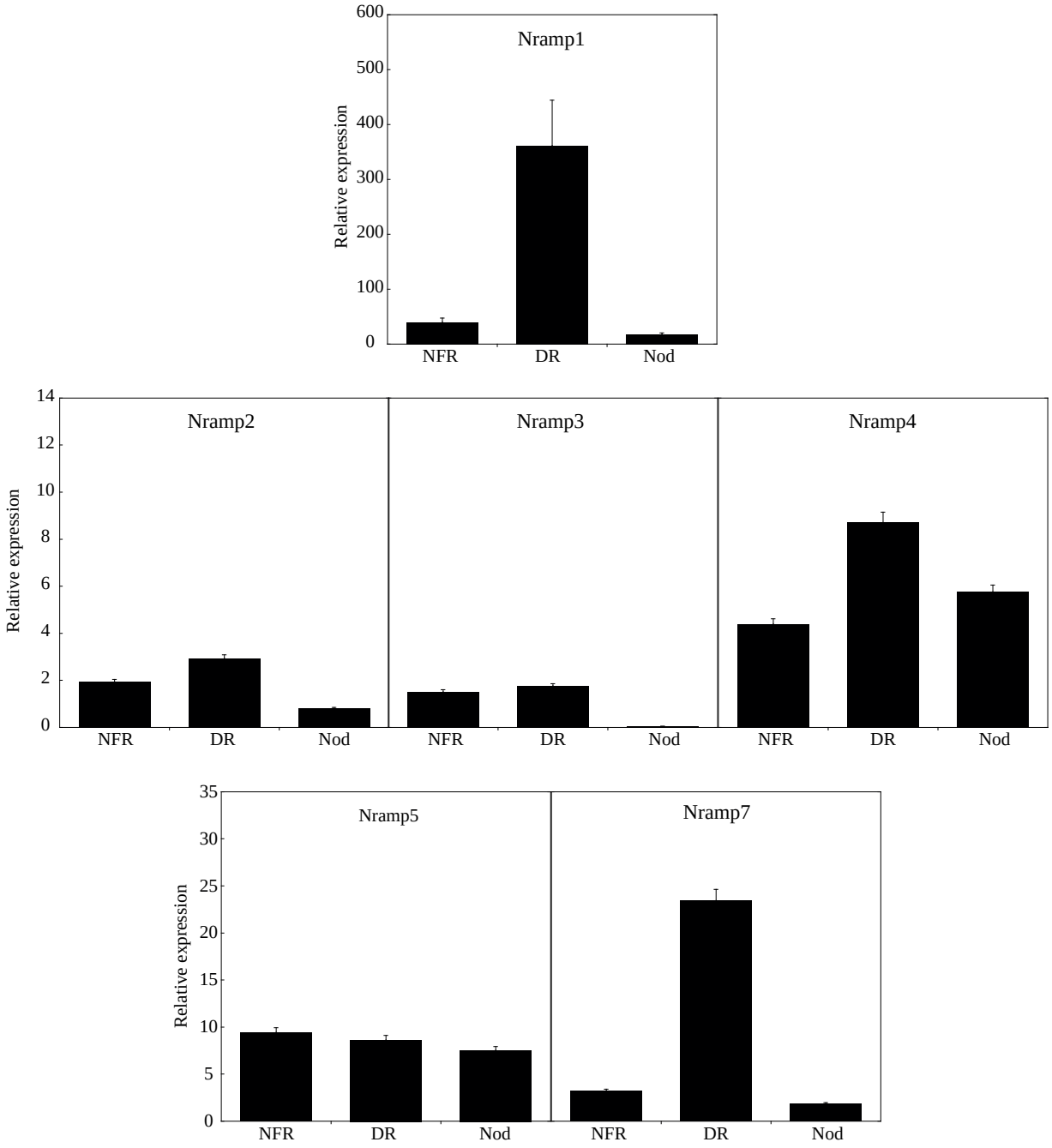
Supplemental Figure S8. Iron distribution in roots and nodules of wild type and *nramp1-1* plants. (A) wild type *M. truncatula* nodule and (B) *nramp1-1 M. truncatula* nodule. Left panels show reference sections stained with toluidine blue and methylene blue, the boxed regions represent the approximate area corresponding to the Perl-DAB-stained (right panel) which shows the iron distribution. Nodule zones are indicated. (C) Iron distribution in wild type *M. truncatula* root. Left panel shows a root section stained with toluidine blue and methylene blue, the boxed region represents the approximate area corresponding to the Perl-DAB-stained right panel. (D) Iron distribution in *nramp1-1 M. truncatula* root. Left panel shows a reference section stained with toluidine blue and methylene blue, the boxed region represents the approximate área

corresponding to the Perl-DAB-stained right panel. Arrowheads indicate regions of iron accumulation. (E) Close-up view of Perl-DAB stained *nramp1-1* root (corresponding to the boxed area of the right panel in D). Arrowheads indicate regions of iron accumulation. Scale bars represent 100 μm (A-C, and left panel D), 50 μm (right panel D), or 10 μm (E).

Supplemental Figure S9. Non-symbiotic phenotype *nramp1-1* under Mn deficiency conditions. (A) Growth of representative plants. Scale bar represents 1 cm. Manganese concentrations in nutritive solutions used are indicated below. (B) Fresh weight of shoots and roots. Data are the mean ± SE (n = 5 plants). (C) Chlorophyll content. Data are the mean ± SE (n = 3 plants). (D) Manganese content in roots and shoots of wild-type and nramp1-1 plants under two different manganese fertilization levels. Data are the mean ± SE of two sets of five pooled plants. * indicates significative differences ($p \leq 0.05$).

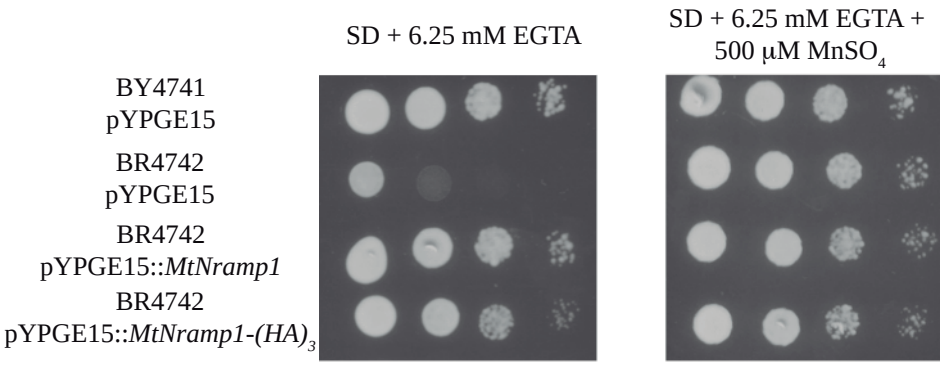
Supplemental Figure S10. Symbiotic phenotype of *nramp1-1* under two different Mn concentrations (A) Growth of representative plants. Scale bar represents 1 cm. (B) Close view of representative nodules of each M. truncatula line. Scale bar represents 1 mm. (C) Fresh weight of shoots and roots. Data are the mean ± SE (n = 6-8 plants). (D) Nodule number per plant. 100 % = 4.21 nodules/plant. Data are the average ± SE (n= 7 plants). (E) Nitrogenase activity in 28 dpi nodules. Acetylene reduction was measured in duplicate from two sets of five pooled plants. Data are the mean ± SE. 100% = 26.69 nmol ethylene h-1g-1. (F) Mn content (ppm) in 28 dpi plants. Bars indicate the average ± SE of two sets of 5 transformed plants. * indicates significant differences ($p \leq 0.05$).

SUPPLEMENTAL FIGURE S1



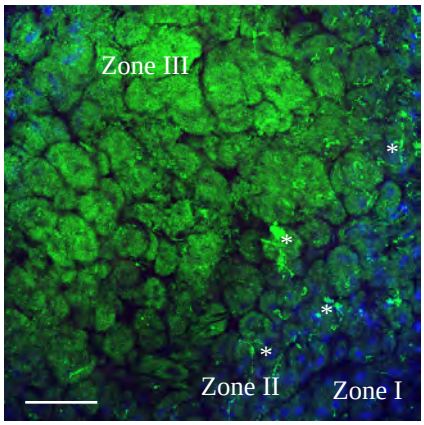
Supplemental Figure S1. *MtNramp* gene family expression. Gene expression of the corresponding *MtNramp* gene in nitrogen-fertilized roots (NFR), denodulated roots (DR) and nodules (Nod) relative to the internal standard gene *ubiquitin carboxyl-terminal hydrolase*. Data are the mean ± SE of three independent experiments.

SUPPLEMENTAL FIGURE S2



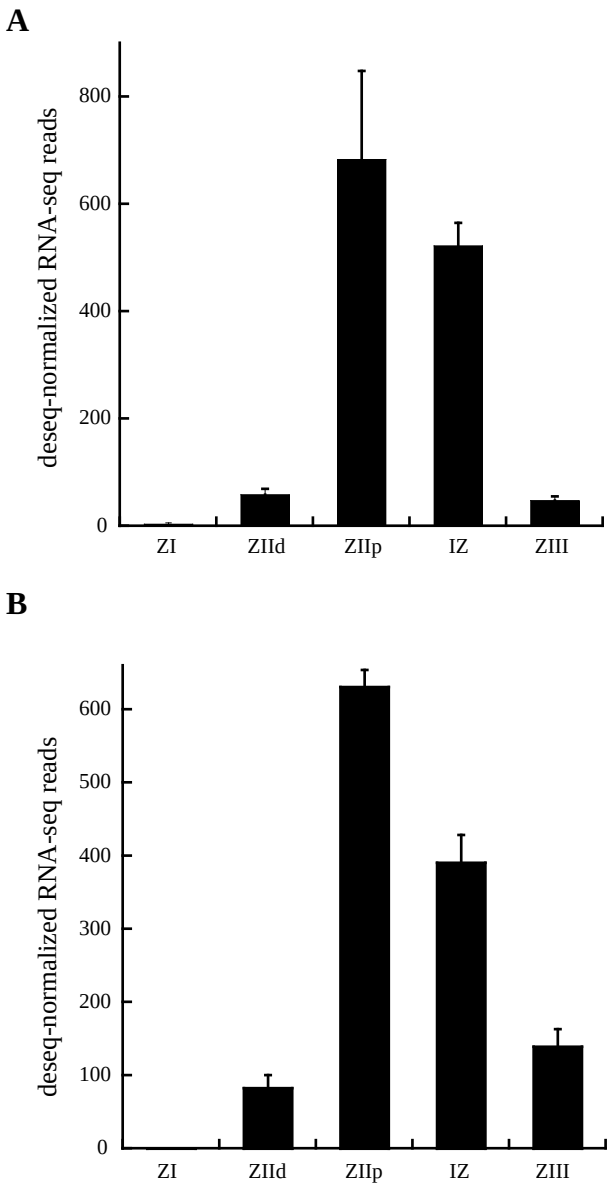
Supplemental Figure S2. *MtNramp1-HA* yeast complementation assay. Yeast strain BY4741 was transformed with the pYPGE15 empty vector, while BY4741-derived BR4742 strain mutated in *smf1* was transformed with either pYPGE15 alone, or with pYPEG15 containing *MtNramp1* CDS, or with pYPGE15 harbouring *MtNramp1* CDS with a C-terminal 3xHA tag. Serial dilutions (10x) of each transformant were grown for 3 days at 30ºC on SD Glucose media with all the required amino acids. pH was buffered with 50 mM MES and Mn levels were kept low with 6.25 mM EGTA. Mn-replete positive controls were obtained by supplementing the plate with 500 µM $MnSO_4$.

SUPPLEMENTAL FIGURE S3



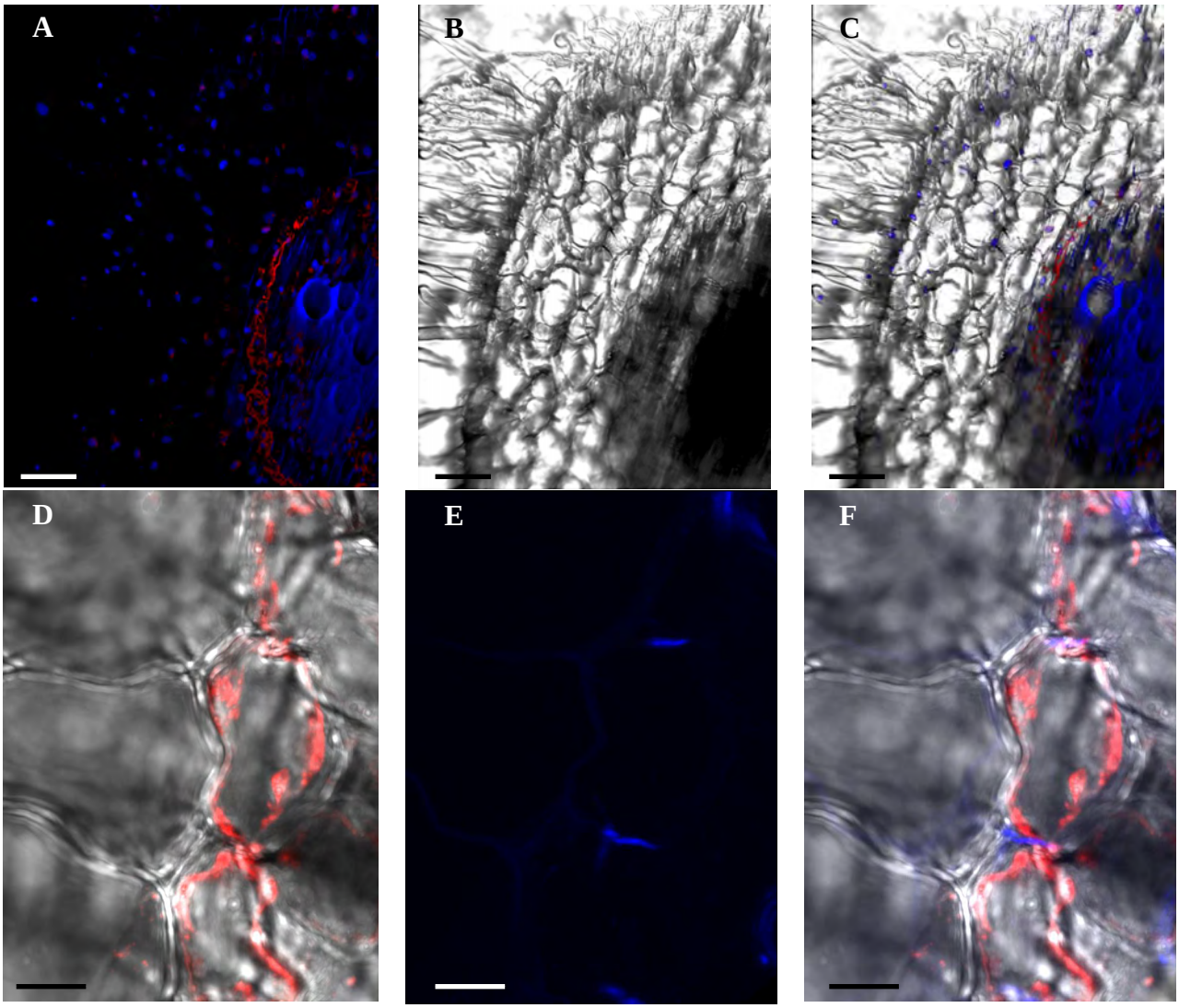
Supplemental Figure S3. Antibody-specificity control for immunolocalization. Cross section of a 28 dpi *M. truncatula* nodule infected with *S. meliloti* constitutively expressing GFP (green) and transformed with a vector expressing *MtNramp1* under the regulation of its endogenous promoter. MtNramp1 localization was determined using an Alexa 594-conjugated antibody (red). DNA was stained with DAPI (blue). Infection threads are indicated with asterisks. Scale bar represents 100 μm.

SUPPLEMENTAL FIGURE S4



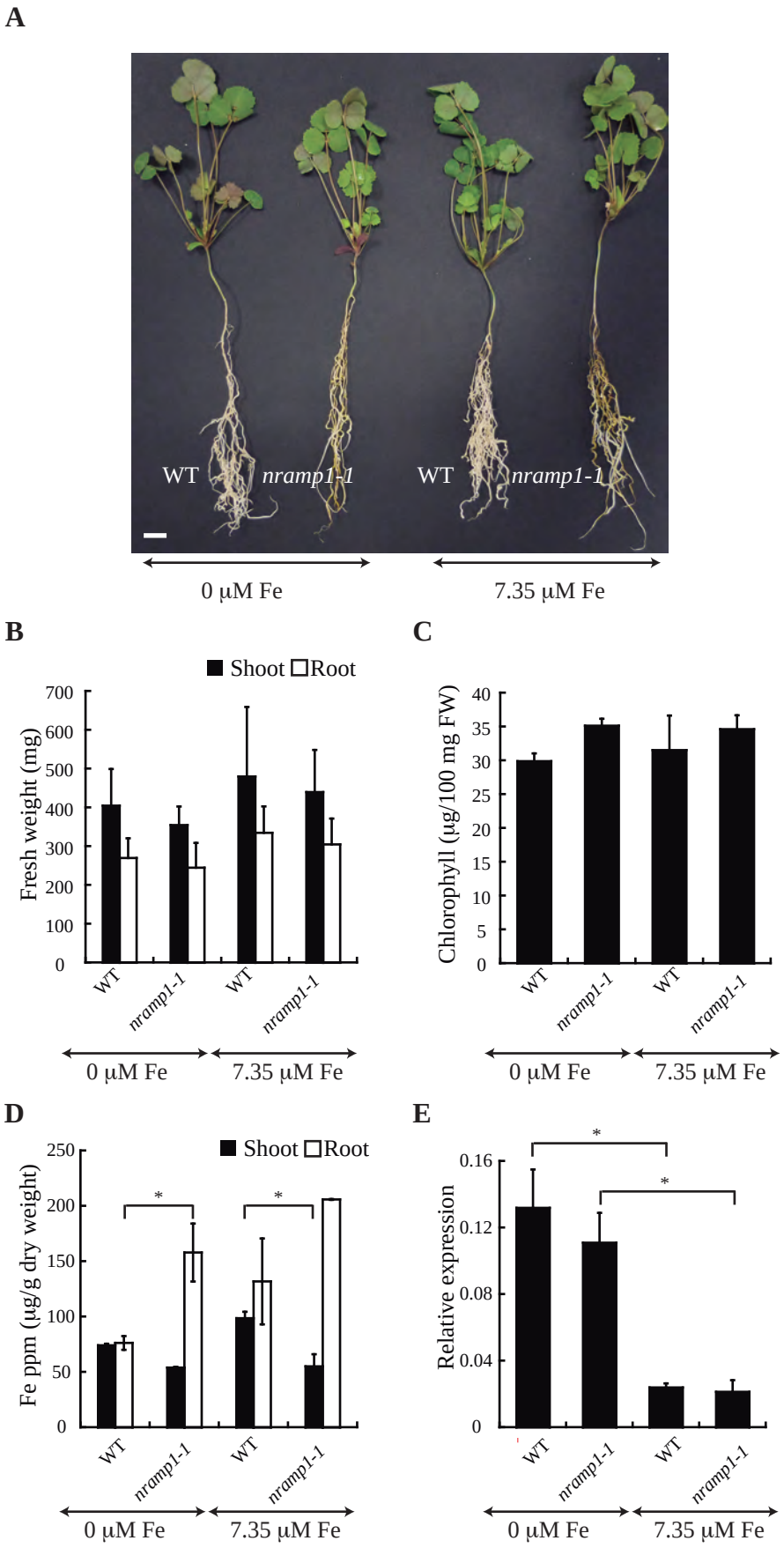
Supplemental Figure S4. Expression in the different *M. truncatula* nodule zones. (A) MtNramp1. (B) Nodule-specific MATE transporter *Medtr6g081400*. ZI indicates zone I; ZIId, distal zone II (region closest to zone I); ZIIp, proximal zone II; IZ is the interzone between zone II and zone III; and ZIII, zone III. Data were obtained from the Symbimics database (https://iant.toulouse.inra.fr/symbimics).

SUPPLEMENTAL FIGURE S5



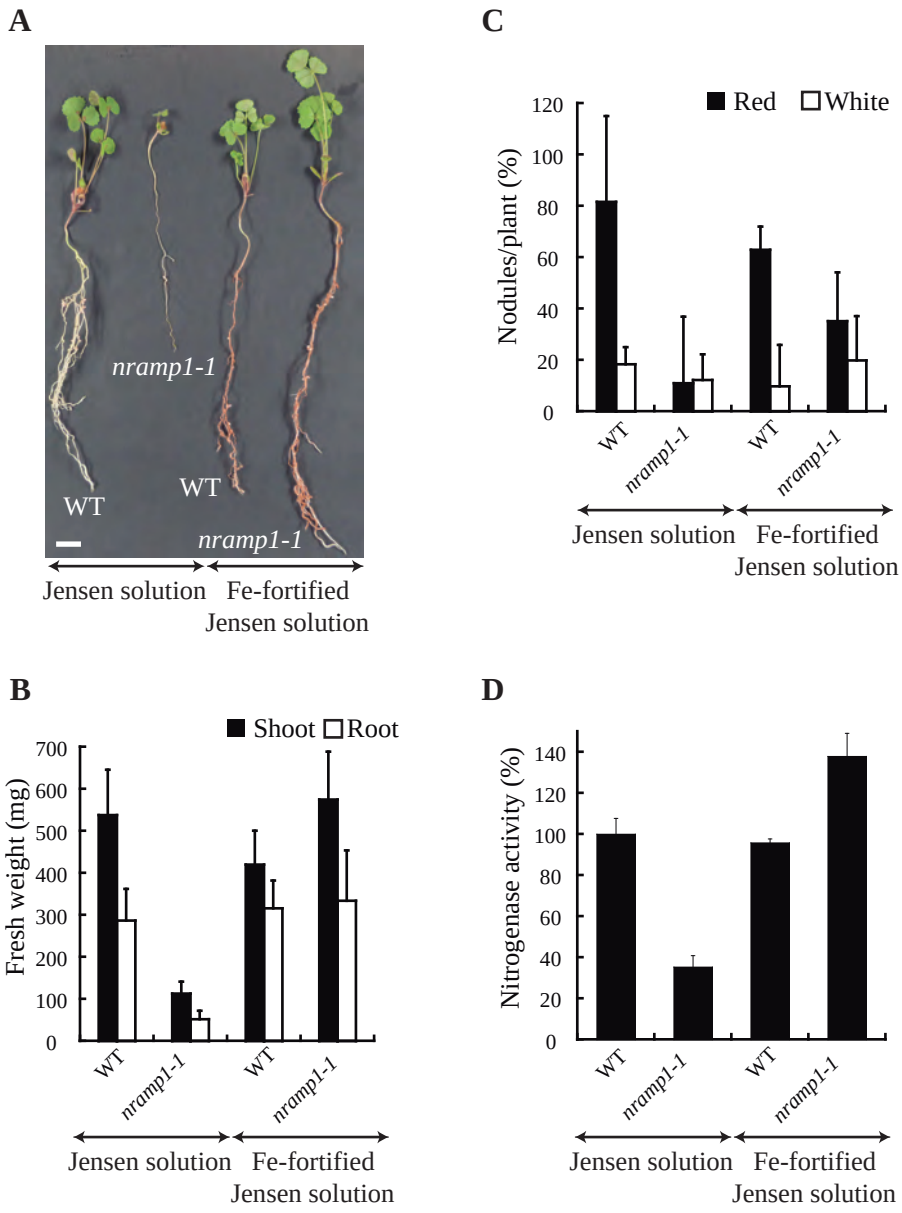
Supplemental Figure S5. MtNramp1-HA localization in the root. (A) Confocal microscopy localization of MtNramp1-HA in a cross section of a 28 dpi *M. truncatul*a root transformed with a vector expressing *MtNramp1* under the regulation of its endogenous promoter. MtNramp1 position was determined using an Alexa 594-conjugated antibody (red). DNA and xylem was stained with DAPI (blue). (B) Optical image of the cross section. (C) Overlay of panels A and B. (D) Close-up of overlaid MtNramp1-HA localization in *M. truncatula* transformed roots (detected with an Alexa 594-conjugated antibody in red) and optical image. (E) Casparian strip localization (blue) detected with the berberine-aniline blue method. (F) Overlay of panels D and E. Scale bars represent 50 μm (panels A-C) or 10 μm (panels D-F).

SUPPLEMENTAL FIGURE S6



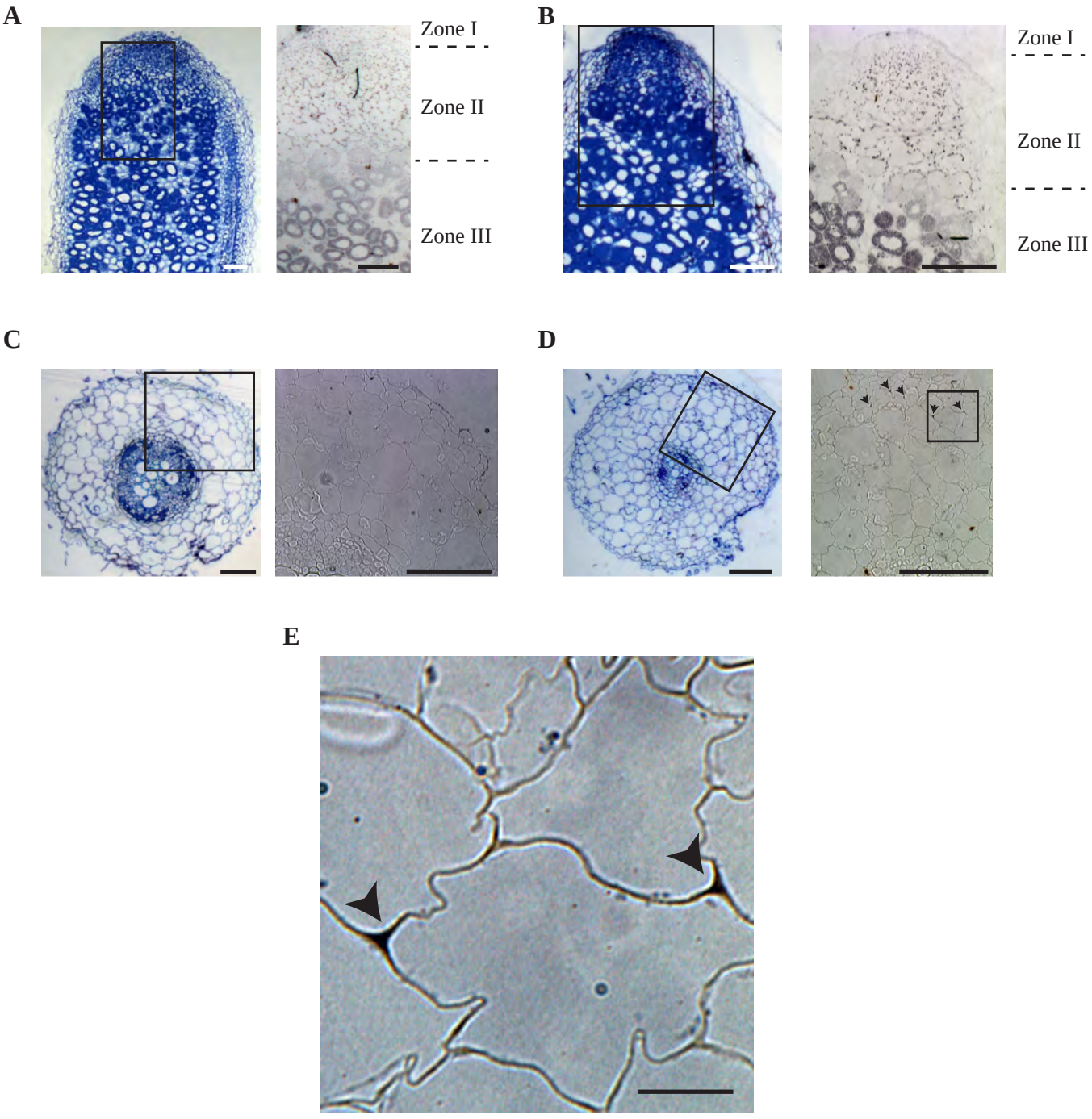
Supplemental Figure S6. Phenotype of *nramp1-1* under non-symbiotic conditions. (A) Growth of representative plants. Scale bar represents 1 cm. Fe concentrations in nutritive solutions used are indicated below. (B) Fresh weight of shoots and roots. Data are the mean ± SE (n = 5 plants). (C) Chlorophyll content. Data are the mean ± SE (n = 3 plants). (D) Iron content in roots and shoots of wild-type and *nramp1-1* plants under two different iron fertilization levels. Data are the mean ± SE of two sets of five pooled plants. * indicates significative differences ($p \leq 0.05$). (F) *MtFRO1* expression in wild type and nramp1-1 plants under two different iron fertilization levels. Data are the mean ± SE of three independet experiments. * indicates significative differences ($p \leq 0.05$).

SUPPLEMENTAL FIGURE S7



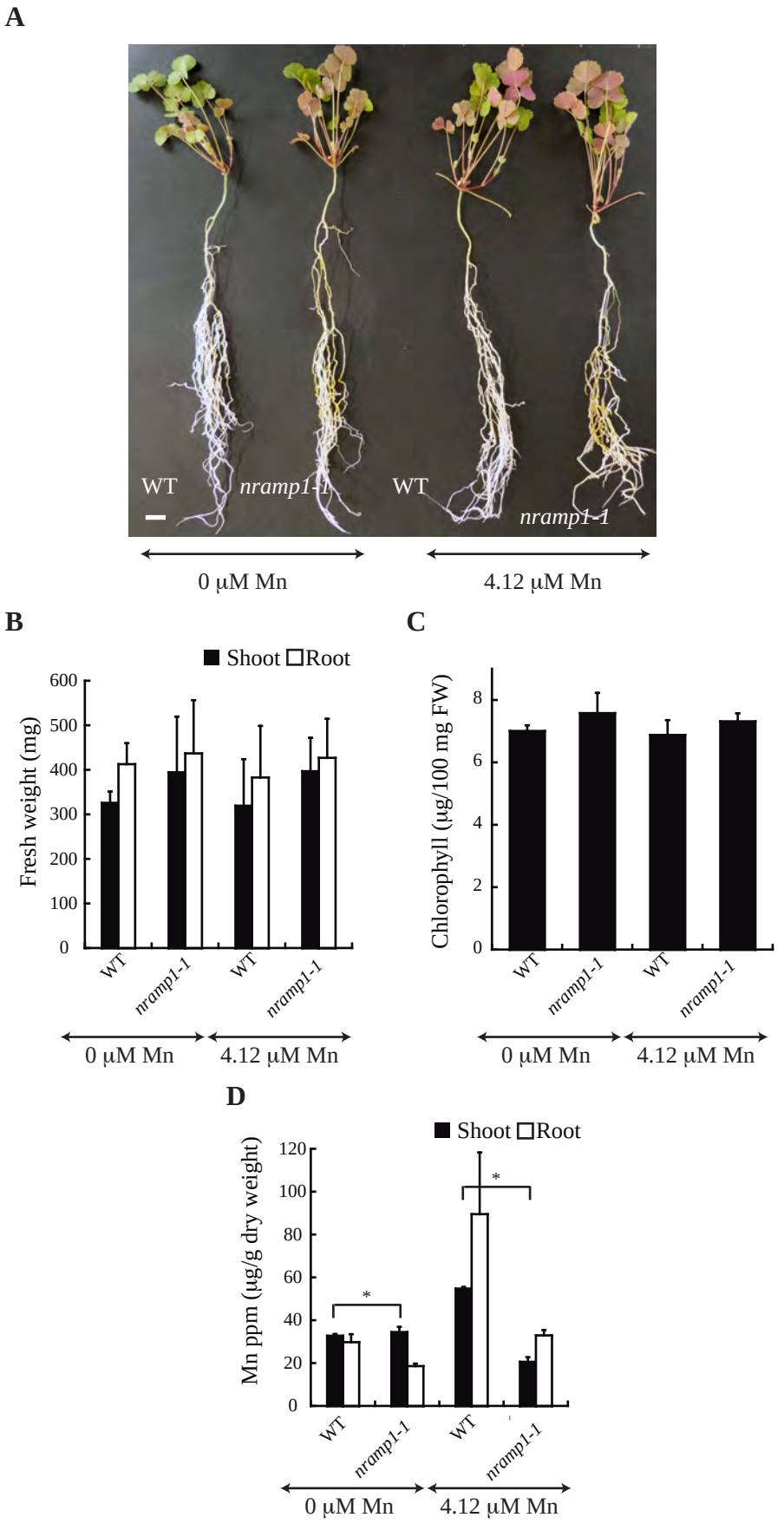
Supplemental Figure S7. Iron complementation of *nramp1-1* phenotype. (A) Growth of representative plants. Scale bar represents 1 cm. (B) Fresh weight of shoots and roots. Data are the mean ± SE (n = 8 plants). (C) Nodule number per plant. 100 % = 4.25 nodules/plant. Data are the average ± SE (n=8-10 plants). (D) Nitrogenase activity in 28 dpi nodules. Acetylene reduction was measured in duplicate from two sets of five pooled plants. Data are the mean ± SE. 100% = 61.17 nmol ethylene $h^{-1}g^{-1}$.

SUPPLEMENTAL FIGURE S8



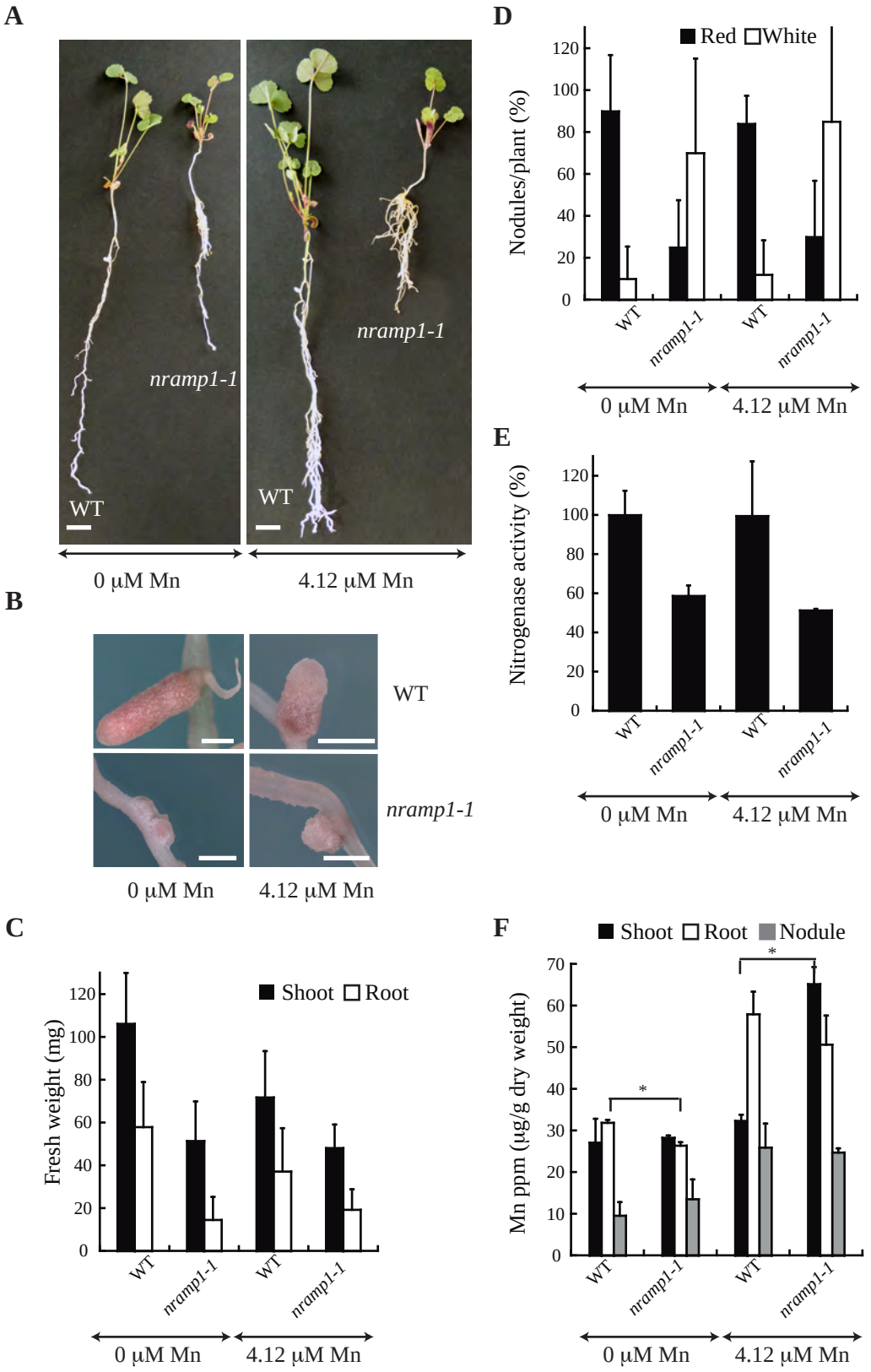
Supplemental Figure S8. Iron distribution in roots and nodules of wild type and *nramp1-1* plants. (A) wild type *M. truncatula* nodule and (B) *nramp1-1 M. truncatula* nodule. Left panels show reference sections stained with toluidine blue and methylene blue, the boxed regions represent the approximate area corresponding to the Perl-DAB-stained (right panel) which shows the iron distribution. Nodule zones are indicated. (C) Iron distribution in wild type *M. truncatula* root. Left panel shows a root section stained with toluidine blue and methylene blue, the boxed region represents the approximate area corresponding to the Perl-DAB-stained right panel. (D) Iron distribution in *nramp1-1 M. truncatula* root. Left panel shows a reference section stained with toluidine blue and methylene blue, the boxed region represents the approximate área corresponding to the Perl-DAB-stained right panel. Arrowheads indicate regions of iron accumulation. (E) Close-up view of Perl-DAB stained nramp1-1 root (corresponding to the boxed area of the right panel in D). Arrowheads indicate regions of iron accumulation. Scale bars represent 100 μm (A-C, and left panel D), 50 μm (right panel D), or 10 μm (E).

SUPPLEMENTAL FIGURE S9



Supplemental Figure S9. Non-symbiotic phenotype of *nramp1-1* under Mn deficiency conditions. (A) Growth of representative plants. Scale bar represents 1 cm. Manganese concentrations in nutritive solutions used are indicated below. (B) Fresh weight of shoots and roots. Data are the mean ± SE (n = 5 plants). (C) Chlorophyll content. Data are the mean ± SE (n = 3 plants). (D) Manganese content in roots and shoots of wild-type and *nramp1-1* plants under two different manganes fertilization levels. Data are the mean ± SE of two sets of five pooled plants. * indicates significative differences ($p \leq 0.05$).

SUPPLEMENTAL FIGURE S10



Supplemental Figure S10. Symbiotic phenotype of *nramp1-1* under two different Mn concentrations (A) Growth of representative plants. Scale bar represents1 cm. (B) Close view of representative nodules of each *M. truncatula* line. Scale bar represents 1 mm. (C) Fresh weight of shoots and roots. Data are the mean ± SE (n = 6-8 plants). (D) Nodule number per plant. 100 % = 4.21 nodules/plant. Data are the average ± SE (n= 7 plants). (E) Nitrogenase activity in 28 dpi nodules. Acetylene reduction was measured in duplicate from two sets of five pooled plants. Data are the mean ± SE. 100% = 26.69 nmol ethylene $h^{-1}g^{-1}$. (F) Mn content (ppm) in 28 dpi plants. Bars indicate the average ± SE of two sets of 5 transformed plants. * indicates significant differences ($p \leq 0.05$).