Medicago truncatula *esn1* Defines a Genetic Locus Involved in Nodule Senescence and Symbiotic Nitrogen Fixation

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Symbiotic interaction between *Medicago truncatula* and *Sinorhizobium meliloti* results in the formation on the host roots of new organs, nodules, in which biological nitrogen fixation takes place. In infected cells, rhizobia enclosed in a plant-derived membrane, the symbiosome membrane, differentiate to nitrogen-fixing bacteroids. The symbiosome membrane serves as an interface for metabolite and signal exchanges between the host cells and endosymbionts. At some point during symbiosis, symbiosomes and symbiotic cells are disintegrated, resulting in nodule senescence. The regulatory mechanisms that underlie nodule senescence are not fully understood. Using a forward genetics approach, we have uncovered the *early senescent nodule 1* (*esn1*) mutant from an *M. truncatula* fast neutron-induced mutant collection. Nodules on *esn1* roots are spherically shaped, ineffective in nitrogen fixation, and senesce early. Atypical among fixation defective mutants isolated thus far, bacteroid differentiation and expression of *nifH*, *Leghemoglobin*, and *DNF1* genes are not affected in *esn1* nodules, supporting the idea that a process downstream of bacteroid differentiation and nitrogenase gene expression is affected in the *esn1* mutant. Expression analysis shows that marker genes involved in senescence, macronutrient degradation, and remobilization are greatly upregulated during nodule development in the *esn1* mutant, consistent with a role of *ESN1* in nodule senescence and symbiotic nitrogen fixation.

Legumes are the third-largest family of flowering plants, including many important forage and grain crops such as alfalfa (*Medicago sativa*) and soybean (*Glycine max*). Legumes are capable of establishing symbiotic interactions with soil bacteria, collectively called *Rhizobia*, for fixing atmospheric dinitrogen in root nodules. The development of nodules is triggered by the rhizobial Nod factors, lipochito-oligosaccharides that are thought to be perceived by receptor-like kinase complexes of compatible host plants (Oldroyd et al. 2011). During compatible symbiosis, rhizobia enter growing root hairs and travel through transcellular, cell-wall-bound infection threads

toward nodule primordial cells formed from root cortical cells. The processes of rhizobial invasion and nodule primordial development occur in parallel. When infection threads reach nodule primordial cells, rhizobia are released into the cytoplasm of plant cells in a process resembling endocytosis (Limpens et al. 2009). In the cytoplasm, rhizobia are enclosed by a plant-derived membrane, the peribacteroid or symbiosome membrane, which facilitates exchange of metabolites and signals between the symbiotic partners. At some points, nodule cells senesce, and nitrogen fixation and symbiotic relationships are lost (Puppo et al. 2005).

Two types of nodules, determinate and indeterminate, can be classified. Determinate nodules such as those of *Lotus japonicus* and some tropical legumes do not have an apical meristem and are round shaped. The central fixation zone contains infected and uninfected cells. In older nodules, senescence starts from the center and gradually moves toward the periphery of nodules. On the other hand, indeterminate nodules as observed in *M. truncatula* and other temperate legumes are cylindrical and consist of a gradient of developmental zones of different cell types (Vasse et al. 1990). Zone I contains a persistent apical meristem composed of dividing cells that are not infected by rhizobia. Zone II is the infection zone, in which rhizobia are released from infection threads and enter plant cells. In infected cells, rhizobia are enclosed by the symbiosome membrane, together forming symbiosomes. Within the symbiosome, rhizobia differentiate to form elongated bacteroids and this process is controlled by nodule-specific cysteine-rich (NCR) peptides (Van de Velde et al. 2010; Wang et al. 2010). In the Interzone II-III, rhizobia fully differentiate to nitrogen-fixing bacteroids. Zone III is the fixation zone, where bacteroids reduce atmospheric nitrogen to ammonium. In mature nodules, a senescence zone (zone IV) is established proximal to the fixation zone. Symbiosomes and host cells are being disintegrated in this zone. Nodule senescence starts gradually in the proximal-distal direction and reaches the distal part of the nodule, resulting in a conical-shaped senescence zone in indeterminate nodules. In the proximal end of senescing nodules is zone V, where saprophytic rhizobia proliferate in disintegrated plant cells (Timmers et al. 2000).

In nodules, oxygen-binding leghemoglobin functions to maintain a microaerobic environment necessary for bacteroid nitrogenase activity. During developmental senescence, nodules change from red to green due to the accumulation of bilirubin after leghemoglobin degradation (Roponen 1970). Transcriptional and phenotypic analyses reveal that developmental senescence of nodules is a gradual process that involves carbon sink

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*The e-Xtra logo stands for “electronic extra” and indicates that five supplementary figures and one supplementary table are published online and that Figures 3 and 5 appear in color online.

to-source transition and transcriptional activation of a large number of genes associated with stress and defense responses (De Michele et al. 2009; Van de Velde et al. 2006). Nodule life span is not only regulated by developmental programs but also influenced by various stress conditions and deficiencies in symbiotic interactions between host and endosymbiont (Puppo et al. 2005). The induced nodule senescence progresses much more rapidly than developmental senescence and lacks signs of nutrient remobilization (Perez Guerra et al. 2010).

What signals trigger developmental senescence of nodules is not yet clear. In *M. truncatula* and other monocarpic legumes, pod filling coincides with the senescence of nodules (Bethlenfalvay and Phillips 1977; Lawn and Brun 1974). It has been suggested that a decrease in the ascorbate-glutathione antioxidant pool and the carbon/nitrogen ratio inside the nodule would trigger an abscisic-acid-mediated signaling pathway, leading to massive proteolysis, nutrient remobilization, and nodule senescence (Puppo et al. 2005). Genetic studies have identified eight *Medicago* mutants clustered into seven complementation groups: *does not fix nitrogen (dnf)*1 to *dnf*7, with defective symbiotic nitrogen fixation (Mitra and Long 2004). Of these mutants, two have been cloned recently (Bourcy et al. 2013; Wang et al. 2010). *DNF1* encodes a subunit of a signal peptidase required to target NCR peptides to the symbiosomes (Van de Velde et al. 2010). *DNF2* (*MTR_4g085800*) has several alternatively spliced transcripts. A predominate one encodes a phosphatidylinositol-specific phospholipase C (PI-PLC) X domain (PI-PLCXD) with an N-terminal signal peptide. It belongs to a family of proteins widely spread in plants, with multiple members in each species (Bourcy et al. 2013). However, the PI-PLCXD proteins differ from the canonical PI-PLC proteins due to lack of other PI-PLC functional domains (Bourcy et al. 2013). Despite differences in protein functions, bacteroid differentiation and nitrogen fixation are compromised in both *dnf1* and *dnf2* mutants (Bourcy et al. 2013).

Understanding the mechanisms underlying nodule senescence might lead to agronomic improvements of quality and biomass of seed and leaves of legume crops. *M. truncatula* and *L. japonicus* have emerged as two genetic model species for legume studies (Cannon et al. 2006; Young et al. 2011). Here, we report isolation and characterization of the *M. truncatula* *early senescent nodule1 (esn1)* mutant from a fast neutron-deletion mutant population (Chen et al. 2010; Peng et al. 2011; Wang et al. 2006). The *esn1* mutant is defective in biological nitrogen fixation and nodule development associated with early nodule senescence. Detailed phenotypic studies provide new insights of symbiotic processes affected by the *esn1* mutation. Furthermore, analysis of marker gene expression substantiates the phenotypic observations, consistent with a role of *ESN1* in nodule senescence and symbiotic nitrogen fixation in *Medicago* spp.

RESULTS

Isolation and genetic characterization of *M. truncatula* *esn1* mutant defective in nodule development and symbiotic nitrogen fixation.

M. truncatula seed of the 'Jemalong A17' genetic background were radiated by fast neutron at the optimal dosage level of 32.5 Gy (Wang et al. 2006). M2 seed were germinated and grown under a limited nitrogen fertilizer condition for 3 to 5 days, then inoculated with *Sinorhizobium meliloti* Sm2011 (Boivin et al. 1990; Penmetsa and Cook 1997, 2000). At 35 days postinoculation (dpi), plants with yellow leaves and stunted shoots were selected and examined for defects in symbiotic nitrogen fixation and nodule development. Putative mutant plants were allowed to grow in nitrogen-rich soil and set seed.

M3 seedlings were then grown and inoculated with *S. meliloti* Sm2011 under the same limited-nitrogen condition to confirm symbiotic phenotypes of the M2 plants. Of the confirmed symbiotic mutants, we selected line FN9669 for detailed phenotypic characterization.

At 21 dpi, FN9669 plants were yellowish and severely stunted compared with wild-type (WT) plants (Jemalong A17) (Fig. 1A to C). However, 1 month after transplanting to nitrogen-rich soil, FN9669 plants were completely rescued and their growth was indistinguishable from WT plants (Supplementary Fig. S1). When grown under the nitrogen-replenish condition, FN9669 plants were comparable with WT plants in all developmental processes, including leaf, flower, pod, seed, and primary and lateral root development (Supplementary Figs. S2 and S3). These results indicate that yellow leaves and stunted growth of shoots of FN9669 plants grown under the rhizobial inoculation condition were caused by defective biological nitrogen fixation. In contrast to WT plants, which developed cylindrical nodules with purple color (due to the presence of functional leghemoglobin) on their roots, FN9669 mutant plants only developed small spherical nodules, most of which were white or green (due to degradation of leghemoglobin) (Fig. 1D to G). Noticeably, some nodules on FN9669 roots were pale pink, suggesting the presence of functional leghemoglobin in these nodules. Based on the observed symbiotic defects (also see below) and the nomenclature rule developed for *M. truncatula* (VandenBosch and Frugoli 2001), we named and registered FN9669 as *esn1*.

To characterize the nature of the *esn1* mutation, we generated F2 populations from cross-pollination between *esn1* and its WT parent (Jemalong A17). We observed a ratio of 3:1 segregation of WT and mutant nodulation phenotypes in F2 populations, indicating a single recessive locus for the *esn1* mutant phenotype (Table 1). Consistent with this, F3 plants derived from a heterozygous F2 plant also displayed a 3:1 ratio of segregation of WT and *esn1* mutant phenotypes (Table 1).

Early nodule development was normal in *esn1* mutant.

Detailed time-course experiments showed that nodules on both WT and *esn1* roots appeared white, pale pink, and pink at 4, 6, and 8 dpi, respectively (Fig. 2A to F). At 12 and 14 dpi, however, nodules developed on *esn1* roots appeared greenish, in contrast to WT nodules, which developed dark pink color over the same period of time (Fig. 2G to J). To further characterize the nodule development phenotype of *esn1*, we grew and inoculated WT and *esn1* seedlings with *S. meliloti* Sm2011 strains constitutively expressing *lacZ* (*hemA::lacZ*) or *mGFP* (*hemA::mGFP*) reporter genes. Results from microscopic analyses showed that infection threads were not distinguishably different between the WT and *esn1* mutant (Supplementary Fig. S4A and B). The appearance and number of nodules were similar between the *esn1* mutant and WT plants in the first few days after inoculation with rhizobia (Fig. 2A to F; Table 2). Laser confocal microscopic analysis of sections from more than 200 nodules collected at 9 dpi showed similar presence of green fluorescent protein (GFP)-labeled rhizobia in nodule-infected cells in the *esn1* mutant and WT plants (Fig. 3A and B). Consistent with these observations, semi-thin sections of nodules collected at 9 dpi showed that *esn1* nodules developed meristem, infection, and fixation zones, similarly to WT nodules (Fig. 4A and B). These results indicate that early nodule development appeared normal in the *esn1* mutant compared with the WT.

Early senescence of *esn1* nodules.

Laser confocal microscopic analysis showed that a dark area due to lack of GFP fluorescence was present in the center of

some *esn1* nodules 10 dpi (Fig. 3D). The dark area was larger and the number of nodules losing GFP fluorescence was increasingly higher in older *esn1* nodules (11 and 14 dpi) (Fig. 3F and H). At 14 dpi, *esn1* nodules lost most GFP fluorescence (Fig. 3H). By contrast, this phenotype was not observed in WT nodules at the same developmental stages (Fig. 3C, E, and G). In agreement with the GFP fluorescence results, the number of green nodules was higher when nodules age in the *esn1* mutant (Table 2). These results suggest that *esn1* nodules may undergo early senescence in comparison with WT nodules.

Table 1. Segregation analysis of the *Medicago truncatula* early senescent nodule 1 (*esn1*) mutant

Segregating populations	Wild type	<i>esn1</i>	χ^2 ^a
<i>esn1</i> × wild type; F2	95	44	3.28
<i>esn1</i> (+/-) selfing; F3	256	67	3.12

^a The χ^2 was calculated based on 3:1 ratio of segregation; *P* > 0.05 when χ^2 < 3.84.

At 16 dpi, the number of nodules slightly increased on *esn1* roots compared with the WT (Table 2). At 28 dpi, the number of nodules greatly increased on *esn1* roots (Table 2). In addition, nodules were distributed throughout the primary and lateral roots, including a region near the root tip in the *esn1* mutant (Supplementary Fig. S5B; arrows). We also noticed that white, pink, and green nodules occurred sequentially along the distal-proximal axis of roots in the *esn1* mutant. In WT plants, however, the majority of nodules developed in the mature region of roots.

Laser confocal images further showed that GFP-labeled rhizobia inside intact infected cells in zone II-III were elongated in *esn1* nodules similarly as in WT nodules, suggesting that bacteroid differentiation occurred in nodule infected cells in the *esn1* mutant (Fig. 5A and B). On the other hand, semi-thin longitudinal sections of nodules 11 dpi showed that symbiosomes were disintegrated in most infected cells at the proximal end of the fixation zone in the *esn1* mutant (Fig. 4C and D). In younger *esn1* and WT nodules, however, infected cells were

Fig. 1. *Medicago truncatula* early senescent nodule 1 (*esn1*) mutant phenotypes. **A**, Growth of *esn1* (left) and wild-type (WT; right) seedlings 21 days postinoculation (dpi) with *Sinorhizobium meliloti* Sm2011, showing stunted root and shoot growth of the *esn1* mutant. Top-down views of **B**, the *esn1* mutant and **C**, WT seedlings, showing nitrogen deficiency phenotype of the mutant. Nodules developed on **D**, *esn1* mutant and **E**, WT roots were white and pink, respectively. Close-up views of **F**, *esn1* and **G**, WT nodules.

Table 2. Number and coloration of nodules in wild-type (WT) and early senescent nodule 1 (*esn1*) mutant

DPI	WT				<i>esn1</i>			
	White	Pink	Green	Total	White	Pink	Green	Total
6	7.1 ± 1.1	5.1 ± 0.57	0	12.7 ± 0.78	7.7 ± 0.67	6.2 ± 0.57	0	13.9 ± 0.99
8	5.7 ± 0.83	17.1 ± 1.14	0	23.1 ± 1.50	5.2 ± 0.83	14.6 ± 0.89	0	19.8 ± 1.00
10	2.8 ± 0.63	21 ± 1.8	0	23.8 ± 2.08	3.7 ± 0.64	20.4 ± 1.45	0.08 ± 0.28	24.2 ± 1.64
12	0.08 ± 0.28	30.6 ± 8.68	0	31.2 ± 2.4	0	12.5 ± 1.33	13.6 ± 1.57	26.1 ± 2.56
14	0.08 ± 0.28	32.4 ± 2.65	0	32.5 ± 2.67	0.9 ± 0.29	11.2 ± 0.74	18.3 ± 1.14	30.4 ± 1.46
16	0.33 ± 0.14	30.1 ± 1.79	0	30.4 ± 1.80	3.3 ± 0.70	11.8 ± 0.76	21.8 ± 1.02	36.3 ± 1.43
28	3.1 ± 1.68	32.8 ± 1.40	0	39.6 ± 2.19	11.8 ± 2.20	21.4 ± 1.98	61.0 ± 3.74	94.2 ± 4.43

^a Mean ± standard deviation; *n* = 12; DPI = days postinoculation.

similarly filled with intact symbiosomes (Figs. 3A and B, 4A and B, and 5).

Transmission electron microscopy (TEM) was used to identify ultrastructural changes in nodule cells. TEM images clearly showed that rhizobia entered nodule cells through infection threads and were enclosed by symbiosome membranes (Fig. 6A and B). Within symbiosomes, elongated bacteroids were present in both WT and *esn1* nodules (Fig. 6A and B). However, ultrastructural features of infected cells were already different between WT and *esn1* nodules 9 dpi. Compared with symbiosomes in infected cells in WT nodules, an increased number of symbiosomes appeared to be packed in infected cells in *esn1* nodules 9 dpi (Fig. 6A and B). In addition, a large number of starch granules were present at the periphery of infected cells in *esn1* nodules but not in WT nodules (Fig. 6A and B, arrows). At 11 dpi, infected cells close to the center of the fixation zone in *esn1* nodules clearly showed signs of degradation and, in addition, a large number of saprophytic rhizobia within apparently enlarged infection threads were released into disintegrated infected cells (Fig. 6D). These were in striking contrast to WT nodules, in which infected cells were filled with intact symbiosomes and did not show signs of degradation at this stage (Fig. 6C). Thus, these results support the conclusion that *esn1* nodules undergo early senescence.

Defective symbiotic nitrogen fixation in the *esn1* mutant.

The *esn1* mutant was originally isolated as being defective in nodule development and symbiotic nitrogen fixation (Fig. 1).

Fig. 2. Time-course observation of nodule phenotypes. *Medicago truncatula* wild-type (WT) and *early senescent nodule 1 (esn1)* mutant seedlings were inoculated with *Sinorhizobium meliloti* Sm2011 strain and inoculated roots were observed in a time-course experiment. Representative images of **A**, WT and **B**, *esn1* mutant roots 4 days postinoculation (dpi); **C**, WT and **D**, *esn1* mutant 6 dpi; **E**, WT and **F**, *esn1* mutant 8 dpi; **G**, WT and **H**, *esn1* mutant 10 dpi; and **I**, WT and **J**, *esn1* mutant 12 dpi. **K**, WT nodules 105 dpi, showing green senescing zone. Scale bars: 500 μ m.

However, the presence of elongated bacteroids in symbiosomes of infected cells and some pale pink-colored nodules on *esn1* roots suggests that *esn1* nodules may have nitrogen fixation activity. To test this, we measured nodule nitrogenase activities using an acetylene reduction assay. The results showed that *esn1* nodules exhibited a negligible level of the nitrogenase activity compared with WT nodules 9, 12, and 14 dpi (Fig. 7). No acetylene reduction activities were detected in uninoculated roots (mock) and roots inoculated with the *S. meliloti* mutant of the *nifH* gene (Fig. 7). These results confirm that the *esn1* mutant is ineffective in symbiotic nitrogen fixation.

To test whether the defect in symbiotic nitrogen fixation was caused by lack of expression of nitrogenase in *esn1* nodules, we

Fig. 3. Laser confocal microscopic analysis of nodule development. *Medicago truncatula* wild-type (WT) and *early senescent nodule 1 (esn1)* mutant seedlings were inoculated with the *Sinorhizobium meliloti* Sm2011 strain harboring *hemA::mGFP* reporter gene. Longitudinal sections of nodules were observed under a laser confocal microscope and representative images were shown. **A**, WT and **B**, *esn1* mutant nodules 9 days postinoculation (dpi); **C**, WT and **D**, *esn1* mutant nodules 10 dpi; **E**, WT and **F**, *esn1* mutant 11 dpi; and **G**, WT and **H**, *esn1* mutant 14 dpi. Scale bar: 500 μ m.

examined the expression of the rhizobial *nifH* gene (TC192496; Supplementary Table S1) in nodules 9 dpi. Surprisingly, quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis showed that the expression level of *nifH* in nodules at the onset of senescence was not different between the WT and the *esn1* mutant (Fig. 7; Student's *t* test, $P > 0.3$). Next, we examined the expression level of the host *Leghemoglobin* gene (*LHG*), which is required to maintain a low-oxygen environment inside nodules necessary for nitrogenase activity (Becana and Klucas 1992). Quantitative RT-PCR results showed that *LHG* expression was not different between WT and *esn1* nodules (Fig. 8; Student's *t* test, $P > 0.3$). *DNF1* (TC180904) encodes a signal

Fig. 4. Semithin sections of wild-type (WT) and *early senescent nodule 1* (*esn1*) mutant nodules. *Medicago truncatula* **A**, WT and **B**, *esn1* mutant nodules 9 days postinoculation (dpi) were sectioned longitudinally and stained with toluidine blue, showing development of meristem, invasion, and infection zones, and infected and noninfected cells in both WT and *esn1* mutant. **C** and **D**, Longitudinal sections of **C**, WT and **D**, *esn1* mutant nodules 11 dpi, showing degradation of symbiosomes and infected cells at the proximal region of the *esn1* nodule. Scale bars: 100 µm.

Fig. 5. Laser confocal microscopic analysis of wild-type (WT) and *early senescent nodule 1* (*esn1*) mutant nodules. **A**, WT and **B**, *esn1* mutant seedlings were inoculated with *Sinorhizobium meliloti* Sm2011 strain harboring the *hemA::mGFP* reporter gene. Longitudinal sections of nodules 9 days postinoculation were used to observe rhizobial differentiation in symbiosomes in infected cells. This showed the presence of elongated bacteroids in *esn1* nodules similarly as in WT nodules. The number of symbiosomes in infected cells appeared to be much higher in *esn1* than WT nodules. Scale bar: 25 µm.

peptidase required for terminal differentiation of rhizobia in symbiosomes by targeting NCR peptides to rhizobial membrane and cytosol (Van de Velde et al. 2010). The expression data also showed that *DNF1* expressed normally in *esn1* nodules 9 dpi (Fig. 8; Student's *t* test, $P > 0.05$). These results suggest that the symbiotic defects of the *esn1* mutant were not caused by compromised expression of *nifH*, *LHG*, and *DNF1* genes. Next, we examined the expression of the rhizobial *bacA* gene, which encodes an integral membrane protein required for chronic infection of *Medicago* plants by rhizobia during symbiosis (Haag et al. 2011). Quantitative RT-PCR results showed that the *bacA* expression was increased by approximately 30% in *esn1*

Fig. 6. Transmission electron microscopy (TEM) analysis of wild-type (WT) and *early senescent nodule 1* (*esn1*) mutant nodules. TEM images of **A**, WT and **B**, *esn1* mutant nodules 9 days postinoculation (dpi). Elongated bacteroids within symbiosomes and traces of infection threads with undifferentiated rhizobia were clearly present in both WT and *esn1* mutant nodules. In *esn1* nodules, however, a large number of starch granules (arrows) were visible at the periphery of infected cells, which were packed with symbiosomes. TEM images of **C**, WT and **D**, *esn1* mutant nodules 11 dpi. In contrast to WT infected cells, which were filled with intact symbiosomes, infected cells of the *esn1* mutant nodule appeared to be disintegrated, with lots of vesicles and membrane debris. Infection threads appeared to be enlarged, releasing a large number of saprophytic rhizobia into the degraded infected cells.

Fig. 7. Acetylene reduction assay of nitrogenase activities of wild-type (WT) and *early senescent nodule 1* (*esn1*) mutant nodules. WT and *esn1* mutant roots were inoculated with the *Sinorhizobium meliloti nifH* mutant or *S. meliloti* strain and acetylene reduction assays were carried out 9, 12, and 14 days postinoculation (dpi). WT and *esn1* uninoculated roots were used as mock controls. Shown are means $\pm$ standard error; $n = 5$.

Fig. 8. Quantitative analysis of *nifH*, *Leghemoglobin*, *DNF1*, and *bacA* gene expression in *early senescent nodule 1 (esn1)* nodules. Wild-type (WT) and *esn1* nodules 9 days postinoculation with *Sinorhizobium meliloti* Sm2011 were subjected to quantitative reverse-transcription polymerase chain reaction analysis of gene expression. Three biological replicates each with three technical repeats were used. Shown are means $\pm$ standard deviation; $n = 3$.

nodules 9 dpi compared with WT counterparts (Fig. 8; Student's *t* test, $P < 0.0001$). This result is in agreement with the TEM analysis that showed proliferation of rhizobial invasion in *esn1* nodules compared with WT nodules (Fig. 6A and B).

Expression of early senescence and defense markers in *esn1* nodules.

Our physiological and morphological analyses indicated that *esn1* nodules undergo early senescence. To investigate the senescence process, we compared expression levels of early senescence and defense marker genes during nodule development (De Michele et al. 2009; Kardailsky and Brewin 1996; Kasprzewska 2003; Perez Guerra et al. 2010; Van de Velde et al. 2006). Quantitative RT-PCR results revealed that the expression of a cysteine protease gene (TC179181) was extremely low and not significantly changed in inoculated roots 0, 3, and 6 dpi between *esn1* and WT plants (Fig. 9A). However, its expression was increased by 133-, 313-, and 128-fold in *esn1* nodules 9, 12, and 16 dpi, respectively, in contrast to its expression level being not much changed in WT nodules within the same time period (Fig. 9A).

Similarly, a chitinase gene (TC176669) involved in plant defense responses was significantly upregulated in 9-, 12-, and 16-day-old nodules in the *esn1* mutant compared with that in WT plants (Fig. 9B). By contrast, the expression level of the

Fig. 9. Upregulation of early senescence and nutrient remobilization marker genes in *early senescent nodule 1 (esn1)* nodules. Wild-type (WT) and *esn1* mutant seedlings were inoculated with *Sinorhizobium meliloti* Sm2011. Root and nodule tissues at 0, 3, and 6 days and 9, 12 and 16 days, respectively, after rhizobial inoculation were collected and subjected to quantitative reverse-transcription polymerase chain reaction (RT-qPCR) analysis of genes encoding an A, cysteine protease; B, chitinase; C, nuclease; D, purple acid phosphatase; E, nucleoside transporter; and F, oligopeptide transporter. Three biological replicates each with three technical repeats were used. Shown are means $\pm$ standard error; $n = 3$.

chitinase gene was very low in both WT and *esn1* mutant roots 0, 3, and 6 dpi (Fig. 9B).

Early senescence of *esn1* nodules involves degradation of cellular components and nutrient remobilization.

Phenotypic analysis indicated that, in *esn1* nodules, early senescence started from the center and propagated toward the periphery and proximal end of nodules (Figs. 3 and 4). This process is reminiscent of developmental senescence observed in older nodules of *Medicago* plants (Perez Guerra et al. 2010; Van de Velde et al. 2006). During developmental senescence of WT nodules, genes involved in protein, nucleic acid, lipid, and sugar degradation and transport are transcriptionally induced to facilitate sink-to-source transition (Van de Velde et al. 2006). To further characterize the *esn1* early senescence process, we examined the expression of genes involved in nucleic acid degradation and nutrient remobilization (De Michele et al. 2009; Van de Velde et al. 2006).

Quantitative RT-PCR analysis indicated that a nuclease gene (TC182576) was increased by more than 4-, 15-, and 8-fold in *esn1* nodules compared with WT nodules 9, 12, and 16 dpi, respectively, although its expression was very low in both WT and *esn1* mutant roots in the first 6 dpi with rhizobia (Fig. 9C).

A purple acid phosphatase gene (XM_003625750) was increased by 14-, 168-, and 47-fold in *esn1* nodules compared with WT nodules 9, 12, and 16 dpi, respectively (Fig. 9D). Similarly, a nucleoside transporter gene (BE319374) and an oligopeptide transporter gene (TC186114) were all significantly upregulated in *esn1* nodules compared with WT counterparts 9, 12, and 16 dpi (Fig. 9E and F), although their expression was very low in WT and *esn1* mutant roots in the first 6 dpi with rhizobia (Fig. 9D to F).

DISCUSSION

Most leguminous plants are capable of establishing symbiotic relationships with the soil bacteria rhizobia and, as a result, develop nodules on their roots, in which biological nitrogen fixation takes place. Because of this, legumes not only can grow in nitrogen-poor soil but also improve soil nitrogen status, contributing significantly to agriculture. At some point, nodules undergo developmental senescence and lost nitrogen-fixation activities. In temperate legumes such as *M. truncatula* and other monocarpic legumes, nodule senescence coincides with pod filling (Bethlenfalvay and Phillips 1977). Exogenous application of nitrogen during pod filling increases seed yield and quality (Merbach and Schilling 1980). Thus, understanding the mechanisms of nodule senescence might lead to improved quality and yield of legume crops.

In this study, we isolated and characterized a new nodulation mutant from *M. truncatula*, *esn1*. The *esn1* mutant was isolated as being defective in symbiotic nitrogen fixation from a *Medicago* deletion mutant collection generated by fast neutron bombardment. Phenotypic characterization indicates that the *esn1* mutant is also defective in late-stage nodule development. In contrast to WT plants, which develop cylindrical nodules with a persistent apical meristem, *esn1* mutant plants develop small, spherical nodules with a nonpersistent meristem. Interestingly, unlike most fixation defective (*fix*⁻) mutants isolated thus far, phenotypic characterization reveals that early stages of nodule development, including infection thread and symbiosome development; bacteroid differentiation; and expression of *LHG*, *nifH*, and *DNF1* genes, appeared to be normal in the *esn1* mutant.

Microscopic studies of *esn1* nodules showed the presence of the meristem, infection, and fixation zones. Several observations support the conclusion that nodules developed on *esn1*

mutant roots undergo early senescence. First, symbiosomes and infected cells are being degraded starting approximately 9 dpi. The degradation appeared first from the nodule center and propagated to the periphery and proximal end of nodules. By contrast, nodules developed on WT roots did not show signs of senescence until approximately 45 dpi under our growth condition. Second, early senescence markers—a cysteine protease gene and a chitinase gene related to plant defense responses—were greatly upregulated in *esn1* nodules compared with WT nodules 9 dpi. Third, several genes related to macromolecule degradation and nutrient remobilization were also greatly upregulated in *esn1* nodules compared with WT nodules, suggesting that the early senescence processes in *esn1* nodules, at least in part, resemble those of WT nodules that undergo developmental senescence but not those induced by environmental stresses such as prolonged dark treatments (Matamoros et al. 1999; Perez Guerra et al. 2010). Fourth, although the expression level of *LHG*, *DNF1*, and *nifH* genes appears to be normal in *esn1* mutant nodules 9 dpi, *esn1* nodules exhibited a negligible level of the nitrogenase activity as measured by the acetylene reduction assay. Finally, the number of nodules was much higher and the distribution of nodules was broader in the *esn1* mutant than WT plants 1 month after inoculation with rhizobia.

Most *fix*⁻ mutants have an increased number of nodules. However, the *esn1* mutant appeared to be different from several other *fix*⁻ mutants reported thus far. In particular, in young *esn1* nodules, the meristem, infection, and fixation zones were developed; infected cells were filled with intact symbiosomes with differentiated bacteroids; and plant *LHG* and *DNF1* and rhizobial *nifH* genes were expressed similarly as in the WT counterparts; however, very little nitrogenase activities could be detected in *esn1* mutant nodules and *esn1* nodules showed signs of senescence as early as 9 or 10 dpi. In a recent study, phenotypic characterization of a number of *M. truncatula* nodulation mutants and *S. meliloti* mutants identified four classes of mutant nodules (Maunoury et al. 2010). Nodules of the first class, including those from TR36, TR183, TRV36, and TRV43 mutants in the Jemalong J5 background and those induced by *S. meliloti* mutants in the *nifH*, *nifA*, *fixG*, *fixJ*, *fixK*, and *lpsB* genes, were elongated similarly as WT nodules, with zonation and bacterial invasion and differentiation in large infected cells (Maunoury et al. 2010). However, 2 weeks after inoculation, nodules of these mutants exhibited a senescence zone. In comparison, WT nodules exhibited a senescence zone 2 weeks later. Nodules of the second class, induced by the *S. meliloti* mutant in the *bacA* gene, were white and either small or elongated. However, these nodules contained differentiated and infected symbiotic cells. Although nodules of the *esn1* mutant share some characteristics of the class 1 and 2 mutant nodules, they remained small under the growth condition tested. Another feature of the *esn1* mutant was the coloration of nodules. We observed sequential and recurrent white, pale pink, and green nodules along the distal-proximal root axis in *esn1* mutant plants, suggesting that new nodules were continuously being developed and then senesced in the *esn1* mutant. In comparison, nodules of *fix*⁻ mutants have been reported to be white, pale pink, or brown, the latter of which suggests strong disease responses (Bourcy et al. 2013; Maunoury et al. 2010; Pislariu et al. 2012; Veereshlingam et al. 2004). It has been reported that an amino acid cycling between legumes and rhizobia is critical for symbiotic nitrogen fixation (Lodwig et al. 2003). Mutations in *aap* and *bra*, which both encode ABC-type broad specificity amino acid transporters in *Rhizobium leguminosarum* bv. *vicia*, results in 30 to 50% reduction in the nitrogenase activity in pea nodules inoculated with the mutant rhizobial strain compared with those inoculated with the corre-

sponding WT strain. Interestingly, the pea nodules inoculated with the mutant rhizobial strain are pale pink, similar to some nodules developed in the *esn1* mutant inoculated with the WT rhizobial strain. In *L. japonicus*, which forms determinate nodules, a *fix⁻* mutant, *fen1*, forms small and pale pink nodules and exhibits nitrogen deficiency symptoms under symbiotic conditions (Hakoyama et al. 2009). *FEN1* encodes homocitrate synthase for biosynthesis of homocitrate, a component of the iron-molybdenum cofactor of the nitrogenase complex. These observations indicate that homocitrate derived from the host cells is essential for efficient and continuing synthesis of the nitrogenase system in the endosymbionts. On the other hand, mutations in the *L. japonicus SEN1* gene, which encodes an integral membrane protein homologous to soybean (*G. max*) nodulin-21; CCC1, a vacuolar iron/manganese transporter of *Saccharomyces cerevisiae*; and VIT1, a vacuolar iron transporter of *Arabidopsis thaliana*, result in lack of nitrogen fixation and compromised symbiosome or bacteroid differentiation (Hakoyama et al. 2012; Suganuma et al. 2003).

These observations suggest that a hitherto unknown process downstream from the *LHG*, *DNF1*, and *nifH* gene expression was most likely affected in the *esn1* mutant. Analyses of marker gene expression revealed that genes involved in nodule senescence were greatly upregulated in *esn1* nodules at the time when signs of senescence started to appear; suggesting that, at that point, the symbiotic interaction between the partners was changed to a senescence process that did not invoke strong disease responses. In addition, genes involved in macro-nutrient degradation and remobilization are upregulated in the *esn1* mutant. These results indicate that the senescence process in *esn1* nodules was similar to the developmental senescence of WT nodules but unlike the induced nodule senescence that leads to rapid cell death (Perez Guerra et al. 2010).

It is not clear what triggers nodule senescence. During biological nitrogen fixation, bacteroids resided in symbiosomes in infected cells “fix” the atmospheric dinitrogen to provide the host with reduced nitrogen and ammonium and, in turn, receive carbon and energy supplies from the host. This process is mutually beneficial but energy intensive for the host. It has been shown that, when metabolite and signal exchanges between the symbiotic partners at the symbiosome membrane interface break down, biological nitrogen fixation will be lost and symbiosomes and, eventually, host cells will be disintegrated by the endogenous defense-like response of the host cell (Banba et al. 2001; Kiers et al. 2003; Oono et al. 2011).

Genetic mapping of the *esn1* locus is in progress. Molecular cloning of the *ESN1* gene in the near future will shed light on the role of *ESN1* in legume-rhizobial symbiosis and nodule senescence.

MATERIALS AND METHODS

Plant materials and growth conditions.

The *M. truncatula* FN9669 mutant was isolated from a fast neutron-induced deletion mutant collection (Chen et al. 2010; Peng et al. 2011; Wang et al. 2006). WT (*M. truncatula* Jemalong A17) and mutant plants were germinated and grown in glasshouses with a photoperiod of 16 and 8 h of light and darkness, respectively, and temperatures of 25°C (day) and 20°C (night). Briefly, seed were scarified with concentrated H₂SO₄ for 8 min and rinsed five times with distilled water. Seed were imbibed in distilled water with gentle rotation for 5 to 7 h at ambient temperature, plated on petri dishes with filter papers and vernalized at 4°C for 24 h, then incubated in the dark at room temperature for 24 h for germination. Seedlings with 1- to 2-cm-long radicals were planted into 72-well plastic trays (11 by 21.44 in.) containing an autoclaved mixture of

perlite and sand (3:1). Seedlings were fertilized with nitrogen-limited solution I (ISV). One week after planting, seedlings were inoculated with *S. meliloti* suspension prepared in ISV solution (optical density at 600 nm = 0.05). Plants were watered with the ISV solution daily.

Segregation analysis.

F₂ plants derived from crosses between WT and FN9669 mutant plants were germinated and inoculated with *S. meliloti* strains as described above. Nodulation phenotype was examined up to 6 weeks after inoculation. Some WT-like plants from the F₂ population were allowed to set F₃ seed. Phenotypic analysis was also carried out in F₃ populations of heterozygous plants.

Acetylene reduction assay.

Acetylene reduction assays were performed essentially as previously described (Wych and Rains 1978). Briefly, plant roots 9, 12, and 14 dpi were detached, placed into airtight 12-ml tubes with rubber seals, and injected with 0.5 ml of acetylene. After 16 h of incubation at 28°C in the dark, 1 ml of air was withdrawn from each tube and analyzed using a gas chromatograph (Agilent 7890A GC System). *S. meliloti nifH* strain Rm1492 (Dickstein et al. 1988) was used as a negative control.

Microscopic observations.

For infection thread observation, plant roots 4 dpi with the *S. meliloti* Sm2011/pXLDG4 strain carrying the *hemA::lacZ* reporter (Ardourel et al. 1994; Boivin et al. 1990) were fixed as previously described (Veereshlingam et al. 2004). For GFP studies, nodules from plants inoculated with the *S. meliloti* Sm2011 *hemA::mGFP* strain (Fournier et al. 2008) were washed with distilled water, embedded in 6% agarose, and sectioned with Vibratome 1000 Plus (Leica Biosystems Nussloch GmbH, Nussloch, Germany). Roots or nodule sections (70 µm thick) were mounted on glass slides and observed with Nikon Optiphot-2 Microscope with Nikon Digital Sight DS-R1 Camera or Leica TCS SP2 AOBs laser confocal scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) using standard settings for GFP.

Toluidine blue staining and TEM.

WT (A17) and mutant (FN9669) nodules 9 and 11 dpi were fixed in 2.5% (vol/vol) glutaraldehyde and 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, U.S.A.) in phosphate-buffered saline (PBS, pH 7.2) for 12 h at 4°C. Samples were then washed with PBS, dehydrated in a graded ethanol series, and embedded in LR White resin (London Resin Co. Ltd., Reading, Berkshire, UK). The resin was polymerized at 55°C for 3 days. Then, 0.5- and 0.1-µm sections were cut with a diamond knife on Leica EM UC7 ultramicrotome (Leica Mikrosysteme GmbH, Vienna, Austria).

For toluidine blue staining, 0.5-µm semithin sections were placed onto glass slides, stained with 1% (wt/vol) Toluidine Blue O (with 1% [wt/vol] sodium borate) for 5 min, and observed under Nikon Optiphot-2 (Nikon Corporation, Tokyo).

Ultrathin sections (0.1 µm thick) were put onto formvar-carbon-coated copper grids and stained with 2% uranyl acetate for 30 min followed by staining with Sato's lead for 2 min. Specimens were observed under a Zeiss 10A (Carl Zeiss AG, Oberkochen, Germany) or JEOL 2000-FX (JEOL, Tokyo) transmission electron microscope operated at 80 kV.

Quantitative RT-PCR.

Plant tissues include root segments harvested at 0 and 3 dpi; nodules and nodule bumps with roots at 6 dpi; and nodules at

9, 12, and 16 dpi. Total RNA samples were extracted using the RNeasy plant mini kit (Qiagen USA, Valencia, CA, U.S.A.), following the supplier's instructions. An additional DNase treatment was performed on columns using RNase-Free DNase Set (Qiagen) to remove residual genomic DNA. RNA quality and quantity were measured using a NanoDrop 1000 (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.). Total RNA (1.5 µg) was used for cDNA synthesis using an Omniscript RT kit (Qiagen), and the oligo (dT)₁₈ primer for plant genes and a nine-nucleotide random primer for bacterial genes. Quantitative PCR was performed with the ABI PRISM 7900 HT sequence detection system (Life Technology, Grand Island, NY, U.S.A.) and SYBR Green (Sigma, St. Louis) for detection in a total reaction volume of 10 µl. Reactions were done in a 10-µl reaction volume on a 384-multiwell plate. Three independent experiments with three technical repeats were done for each gene. For plant genes, the amounts of transcripts were normalized to the transcript level of a housekeeping gene, encoding serine/threonine protein phosphatase 2A (corresponding to the *A. thaliana* homolog, At1g13320) (Czechowski et al. 2005; Lefebvre et al. 2010). For rhizobial genes, *smPNP* (GenBank accession number CP002740) encoding a polyribonucleotide nucleotidyltransferase was used as the internal reference (Martens et al. 2008). The relative expression level was calculated by the 2^{-ΔΔCT} method (Livak and Schmittgen 2001).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Medicago truncatula Gene Nomenclature website:
www.genome.clemson.edu/affiliated_cugi/medicago
 EMBO Practical Course on the New Plant Model System *Medicago truncatula*: www.isv.cnrs-gif.fr/embo01/manuels/index.html
 Leica Microsystems website: www.leica.com