The C$_2$H$_2$ Transcription Factor REGULATOR OF SYMBIOSOME DIFFERENTIATION Represses Transcription of the Secretory Pathway Gene VAMP721a and Promotes Symbiosome Development in *Medicago truncatula*

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INTRODUCTION

Symbiotic nitrogen fixation (SNF) by bacteria called rhizobia within legume nodules injects ~40 million tons of nitrogen into agricultural systems each year (Peoples et al., 2009) and is central to sustainable agriculture. Legume nodule development is initiated by an exchange of chemical signals between plant root cells and soil rhizobia (Murray, 2011; Oldroyd, 2013). In response to plant-secreted flavonoids, rhizobia produce and release nodulation (Nod) factors that are perceived by plant root hair cells, triggering asymmetric growth (curling) of the root hair cells and division of cortical cells (Limpens et al., 2003; Radutoiu et al., 2003). Rhizobia enter and traverse epidermal and underlying cortical cells via infection threads (ITs) and are eventually released, via endocytosis, into the cytoplasm of postmeristematic cells of the nodule primordium. This results in bacteria surrounded by a plant membrane, called the symbiosome membrane (SM), which creates a novel organelle termed the symbiosome (Goodchild and Bergersen, 1966; Robertson and Lyttleton, 1984; Roth and Stacey, 1989). Finally, rhizobia undergo a process of differentiation that involves induction of nitrogen fixation genes and repression of ammonium assimilation genes, making symbiosomes into ammonium-exporting organelles of the plant (Udvardi and Day, 1997; Udvardi and Poole, 2013).

Early molecular events involved in nodule initiation are quite well understood as a result of forward genetic studies of two model legumes, *Medicago truncatula* and *Lotus japonicus* (Popp and Ott, 2011; Oldroyd, 2013). SNF mutants can be divided into two major categories: mutants unable to form nodule primordia (Nod$^-$) and mutants that develop nodule-like structures with impaired nitrogen fixation (Nod$^+$/Fix$^-$). Mutations in the early recognition and signaling genes produce the Nod$^-$ phenotype (Oldroyd, 2013). Several genes have been implicated in IT growth and bacterial colonization of nodule cells, including Mt-Interacting Protein of DMI3 (IPD3)/Lj-CYCLOPS, Mt-VAPYRIN, the flotillin genes Mt-FLOT2 and Mt-FLOT4, Mt-SYMBIOTIC REMORIN1, a putative long coiled-coil protein (Mt-Rhizobium-directed polar growth), and pectate lyase (Lj-Nodulation pectate lyase) (Arrighi et al., 2008; Yano et al., 2008; Haney and Long, 2010; Lefebvre et al., 2010; Murray et al., 2011; Horváth et al., 2011; Xie et al., 2012). Relatively little is known about plant genes...
and proteins that act after bacterial endocytosis and are required for symbiosome development. A series of seven Nod+/Fix− mutants of *M. truncatula* designated as defective in nitrogen fixation (dnf1-7) provide some insight into these later processes (Starker et al., 2006). However, the defective genes in only two of these mutants have been characterized. *DNF1* encodes a subunit of the signal peptidase complex, which is required to process and target plant proteins to symbiosomes, and *DNF2* is a phosphatidylinositol-specific phospholipase C, the precise role of which remains unknown (Van de Velde et al., 2010; Wang et al., 2010; Bourcy et al., 2013). Reverse-genetic studies of *M. truncatula* have linked a few transcription factor (TF) genes to nitrogen fixation but not nodulation per se (i.e., mutants in these genes exhibit Nod+/Fix− phenotypes); these genes include alfalfa (*Medicago sativa*) Kruppel-like Zinc finger Protein2-1, Mt-Heme Activator Protein2.1 (HAP2.1), Mt-EFD, and a basic helix loop helix-TF involved in nodule vasculature development (Frugier et al., 2000; Combier et al., 2006; Vernié et al., 2008; Godiard et al., 2011). Forward genetic studies with *L. japonicus* have identified roles for the following proteins in the later stages of nodule and/or bacteroid development and differentiation: a sulfate transporter, Symbiotic Sulphate Transporter1; an ankyrin-repeat membrane protein, Ineffective Greenish Nodules1; homocitrate synthase, Failure in EnLargement of infected cells1; a putative iron transporter, SEN1; a syntaxin Syntaxin of plants71; and a citrate transporter, Multidrug And Toxic compound Extrusion1 (Krusell et al., 2005; Kumagai et al., 2007; Hakoyama et al., 2009, 2012a, 2012b; Takanashi et al., 2013).

*M. truncatula* forms indeterminate nodules that contain a persistent apical meristem (zone I), a bacterial invasion zone (zone II), interzone II-III, a nitrogen-fixing zone (zone III), and a senescence zone that is proximal to the root (zone IV, only present in older nodules; Vasse et al., 1990). In zone II, plant cells undergo successive rounds of endoreduplication, resulting in nuclear DNA content ranging from 2C to 64C (Mergaert et al., 2006). In the distal part of the invasion zone, bacteria are released from ITs and symbiosomes are formed. In this zone, bacteroids resemble their free-living counterparts and are called type I bacteroids (Vasse et al., 1990). Type I bacteroids undergo several rounds of cell division, followed by endoreduplication, which results in enlarged, type II bacteroids in the proximal region of zone II (Kereszt et al., 2011). In contrast with the 1C/2C DNA content of free-living *Sinorhizobium meliloti*, the DNA content of bacteroids peaks at 24C (Mergaert et al., 2006). In zone III, endoreduplication of both plant and bacterial cells ceases and type II bacteroids differentiate into type III bacteroids, which exhibit cytoplasmic heterogeneity that increases as type IV nitrogen-fixing bacteroids are formed (Vasse et al., 1990; Cebolla et al., 1999).

Endoplasmic reticulum and Golgi-derived vesicles fuse with the SM continuously during symbiosome maturation (Kereszt et al., 2011). Recently, two vesicle-associated membrane proteins, Mt-VAMP721d (for VESICLE-ASSOCIATED MEMBRANE PROTEIN 721d) and Mt-VAMP721e, were shown to be required for symbiosome formation (Ivanov et al., 2012). Numerous plant proteins are delivered to the symbiosome throughout its development. These include a small family of calmodulin-like proteins, Early Nodulin8, a nodule-specific esterase (Liu et al., 2006; Coque et al., 2008), and nodule-specific Cys-rich (NCR) peptides (Van de Velde et al., 2010). Some of the NCR peptides promote endoreduplication of type I bacteroids (Van de Velde et al., 2010). In the *M. truncatula dnf1* mutant impaired in the nodule-specific signal peptidase complex, bacteroid differentiation is halted at the type I stage because the signal sequences of NCR peptides cannot be cleaved and these peptides are unable to reach bacteroids (Van de Velde et al., 2010; Wang et al., 2010). In *dnf2* mutants, bacteroids are unable to complete differentiation and are subject to early senescence probably due to NCR peptide toxicity (Bourcy et al., 2013).

Symbiosome development culminates in nitrogen fixation at which stage many bacterial housekeeping genes inside the symbiosomes are repressed, while nitrogen fixation (*nif and fix*) genes are induced (Terpolilli et al., 2012). Micro-aerobic conditions inside nodules are not only prerequisite for activity of oxygen-labile nitrogenase in bacteroids, but also appear to be necessary for induction of bacterial *nif* and *fix* genes: Suppression of plant leghemoglobin gene expression in *L. japonicus* nodules via RNA interference resulted in higher oxygen concentrations inside nodules, lower bacterial *nif* and *fix* gene expression, and absence of nitrogenase activity (Ott et al., 2005, 2009). In *M. truncatula*, *S. meliloti* 2011 mutants in *nifH*, *nifA*, *fixG*, *fixJ*, and *fixK* give rise to nodules in which bacteroids are fully elongated (type IV), but exhibit early senescence (Maunoury et al., 2010). Similarly, *L. japonicus* Symbiotic Sulphate Transporter1 nodules, which are defective in transport of sulfate from the plant cell cytoplasm to the bacteroids, show low levels of nitrogen fixation (fix+/− phenotype) and early senescence (Krusell et al., 2005).

Interplay between different TFs regulates nodule development. Four TFs from *M. truncatula*, NODULATION SIGNALING PATHWAY1 (NSP1), NSP2, ERF REQUIRED FOR NODULATION1, and NODULE INCEPTION, have been found to act at early stages of nodule development in response to Nod factor signaling (Schauer et al., 1999; Oldroyd and Long, 2003; Kaló et al., 2005; Smit et al., 2005; Heckmann et al., 2006; Marsh et al., 2007; Middleton et al., 2007). Additionally, Mt-Nuclear transcription factor Y subunit alpha1 (Mt-HAP2-1) has been implicated in nodule meristem formation and nodule development (Combier et al., 2006). Only one TF, EFD, has been connected with symbiosome development, albeit indirectly and via an unknown mechanism (Vernié et al., 2008).

Nodule development and cellular differentiation involve changes in the expression of thousands of plant genes (Colebatch et al., 2004; Benedetto et al., 2008). Hundreds of TFs are expressed during nodule development, but the roles of only a few of these have been determined (see above). We have taken a systematic, reverse-genetic approach to elucidate the role of uncharacterized *M. truncatula* TF genes in SNF. We began by annotating 3692 TF genes in the genome (Young et al., 2011) and targeting several nodule-specific TFs for further study. Here, we present functional characterization of a nodule-specific Cysteine-2/Histidine-2 (C2H2) TF from *M. truncatula* that we have REGULATOR OF SYMBIOSOME DIFFERENTIATION (RSD).
RESULTS

**RSD Encodes a C₂H₂ TF with Nodule-Specific Expression**

Using the *M. truncatula* Gene Expression Atlas (MtGEA; Benedito et al., 2008), we identified a putative C₂H₂ TF gene (MtGEA: Mtr.38404.1.S1_at) with nodule-specific expression (see Supplemental Figure 1 online), which we named RSD for reasons explained below. Comparison of cloned cDNA and genomic DNA sequences indicated that the gene contains a single exon, which encodes a putative C₂H₂ TF of 151 amino acids (see Supplemental Figure 2 online).

Phylogenetic analysis clustered Mt-RSD with a protein from *L. japonicus* and two from soybean (*Glycine max*), which shared between 50 and 62% sequence identity with Mt-RSD (Figures 1A and 1B). Mt-RSD is also related to *Arabidopsis* SUPERMAN and *M. truncatula* PALMATE-LIKE PENTAFOLIATA1 (PALM1) proteins (Figure 1B), which regulate different aspects of development (Bowman et al., 1992; Chen et al., 2010). All of the proteins closely related to Mt-RSD share a single C₂H₂ DNA binding domain near the N terminus and a hexapeptide sequence (DLELRL), known as an EAR domain, at the C terminus (Figure 1A). The EAR domain confers transcriptional repression upon DNA binding proteins (Hiratsu et al., 2003; Ikeda and Ohme-Takagi, 2009).

To confirm and extend the RSD gene expression data from MtGEA (see Supplemental Figure 1 online), we measured transcript levels in roots and in nodules of genotypes A17 and R108 at different stages of development or in different nodule zones, using quantitative RT-PCR (qRT-PCR). Preliminary work had established that the onset of nitrogen fixation exhibited similar kinetics in these two genotypes under our experimental conditions (see Supplemental Figure 3 online). RSD transcripts were barely detectable in roots before and 2 and 4 d after inoculation (DAI) with rhizobia in the wild-type R108 genotype (Figure 2A). Transcript levels in developing nodules increased by 6 DAI and peaked at 8 DAI, with a level 4000-fold higher than in roots, before declining steadily at 10, 15, and 21 DAI (Figure 2A). Thus, RSD expression during nodule development was qualitatively similar in the R108 and A17 genotypes. Acetylene reduction assays and qRT-PCR showed that RSD transcript accumulated before the onset of nitrogen fixation (see Supplemental Figure 3 online; Figure 2A). RSD was expressed in all nodule zones at 28 DAI, with maximal levels in the invasion zone in A17 nodules (zone II) (Figure 2B). **HAP2.1**, Nodule-specific Cystine-Rich peptide001, ROP-GTPase, and DNF1 transcript levels were also measured in the five nodule zones to assess our nodule zonation data for RSD. Promoter-β-glucuronidase or in situ hybridization results for expression of these genes in nodules are available in the literature (Mergaert et al., 2003; Combier et al., 2008; Beals et al., 2009).

**Figure 1.** Protein Sequence, Conserved Domains, and Phylogenetic Analysis of RSD.

(A) Alignment of Mt-RSD amino acid sequence with that of putative orthologs in *L. japonicus* and soybean. Conserved amino acids are highlighted. The C₂H₂ DNA binding domain and EAR domain are marked.

(B) Unrooted phylogenetic unweighted pair group method with arithmetic mean tree of Mt-RSD homologs/orthologs in *M. sativa* (Ms), *Arabidopsis* (At), *Populus trichocarpa* (Pt), *G. max* (Gm), *L. japonicus* (Lj), and *Oryza sativa* (Os). Sequences were identified via BLASTP searches of the National Center for Biotechnology Information database of nonredundant sequences and aligned at the protein level using multiple sequence comparison by log expectation (see Supplemental Data Set 1 online). At-ZAP6, which has a C₂H₂ DNA binding domain but lacks the EAR domain, was used as an outgroup. Numbers represent the percentage of 1000 bootstrap replications to assess robustness of nodes. The asterisk indicates the nodulation-specific subgroup of proteins. The bar represents estimated amino acid change per sequence position.
genotype R108. qRT-PCR was used to measure expression data for each of the four marker genes (Figure 2B) genotype A17 at 28 DAI.

(B) nodules (6 to 21 DAI) the infection-susceptible zone of roots (0 to 4 DAI) and in developing (A) Temporal and Spatial Expression Pro

HAP2.1 and higher levels of transcripts for terzone II-III in our hand-sectioned material. Nonetheless, the some cross-contamination between the invasion zone and interzone II-III and the nitrogen- of (Mergaert et al., 2003). Our qRT-PCR data indicated a high level II-III and the nitrogen- transcripts of which were detected previously only in interzone three biological replicates are presented in each case.

RSD Is Required for Normal Nodule Development and SNF

To illuminate the function of RSD in nodules, we isolated two independent Tnt1 (for transposable element of Nicotiana tabacum cell type1) insertion rsd mutants of M. truncatula R108 and determined their symbiotic phenotypes. In the mutant line Noble Foundation (NF) 11265, Tnt1 was found in the open reading frame of RSD (rsd-1 allele), whereas in line NF3492, it was in the 3' untranslated region (UTR), 382 bp after the stop codon (rsd-2 allele; Figure 3A; see Supplemental Figure 2 online). Homozygous rsd mutants of both lines usually developed small, spherical, white nodules that turned brown within 3 weeks of inoculation with S. melloti strain 2011 (Figure 3B). rsd-1 was unable to fix nitrogen, which resulted in retarded growth relative to the wild type (Figures 3B and 3C). The rsd-2 mutant allele appeared to be weaker than that of rsd-1, as rsd-2 mutants produced occasional light pink nodules and fixed nitrogen, according to the acetylene reduction assay, albeit significantly less than the wild type (Figures 3B and 3C). The leakiness of the rsd-2 allele was likely due to the location of Tnt1 in the 3'-UTR of the gene. Subsequent work on rsd mutants focused on the presumptive complete loss-of-function allele rsd-1, which bears Tnt1 in the RSD coding sequence.

Despite the reduced nodule size of rsd-1 mutants (1.2 ± 0.2 mm compared with 2.3 ± 0.3 mm of the wild type at 21 DAI; n = 16 plants; ±sd), nodule number was unaffected at 21 DAI (11.4 ± 4.0 versus 11.2 ± 4.9 of the wild type; n = 22 plants).

Progeny of a heterozygous rsd-1 mutant segregated with an 81:24 ratio of wild-type (Fix+) to mutant (Fix-) phenotypes, fitting the 3:1 ratio expected of a monogenic, recessive mutation (χ² = 0.257; P > 0.05). Genotyping of Fix- individuals confirmed that all were homozygous for the rsd-1 Tnt1 insertion allele. A similar result was obtained after backcrossing the rsd-1 mutant to wild-type R108, self-pollinating a resultant rsd-1 heterozygote, and analyzing its progeny; 60 Fix+ and 21 Fix- individuals, again fitting the expected 3:1 ratio (χ² = 0.037; P > 0.05). To confirm that the Tnt1 insertion in the RSD gene caused the defective symbiotic phenotypes, we placed a wild-type copy of the gene under the control of the constitutive cauliflower mosaic virus 35S promoter (3SS-MtRSD) and expressed it in transgenic hairy roots of the rsd-1 mutant. Wild-type-like nodules containing fully elongated bacteroids developed on transformed roots of the mutant following inoculation with rhizobia (cf. Figures 3F and 3G with 3D and 3E and Supplemental Figures 4A to 4I online).

In contrast with the growth defect of rsd-1 mutants under symbiotic conditions with low soil mineral nitrogen (Figure 3B; see Supplemental Figures 5A and 5B online), rsd-1 mutants exhibited no significant differences in growth or development of vegetative or reproductive organs when grown in the presence of high soil mineral nitrogen (see Supplemental Figure 5 online).

RSD Affects Symbiosome and Bacteroid Development

No delay in nodule initiation and no defects in IT formation or propagation were observed in rsd-1 mutants (see Supplemental Figures 6A to 6C online). In addition, no significant differences in the numbers of bacterial microcolonies or ITS in the susceptible zone of roots were observed between rsd-1 and wild-type R108 plants (see Supplemental Figure 6C online). Staining of beta-galactosidase (lacZ)-expressing rhizobia revealed a slightly smaller population of bacteria in rsd-1 mutant nodules.

Figure 2. Temporal and Spatial Expression Profiles of Mt-RSD. (A) Expression profile of RSD during nodule development in M. truncatula genotype R108. qRT-PCR was used to measure RSD transcript levels in the infection-susceptible zone of roots (0 to 4 DAI) and in developing nodules (6 to 21 DAI).

(B) Relative transcript levels of RSD in different zones of nodules of genotype A17 at 28 DAI. HAP2.1, NCR001, ROP-GTase, and DNF1 relative transcript levels are included for comparison. Mean and so of three biological replicates are presented in each case.
compared with those of the wild type at 6 DAI and a substantially smaller population by 8 DAI (cf. Figures 4A and 4B with 4D and 4E). By 21 DAI, brown material, possibly polyphenolic compounds involved in plant defense, accumulated proximal to the invasion zone in the rsd-1 mutant, but not in the wild type (cf. Figures 4C and 4F).

Despite the retarded growth of developing rsd-1 nodules, no significant defect was apparent at the early stage in zone I (meristem) of the rsd-1 mutant in comparison to the wild type (cf. Figures 4C and 4F with Supplemental Figures 6D and 6E online).

However, the meristematic activity ceased rapidly in rsd-1, and nodules were usually spherical instead of cylindrical. Marked differences between the mutant and wild type were observed in the older cell layers of zone II (invasion) and in interzone II-III. Although release of bacteria from ITs and their subsequent division and differentiation into type I bacteroids in the distal part of zone II appeared normal in rsd-1 (Figures 5B and 5F; see Supplemental Figures 6F and 6G online), further differentiation reflected by bacteroid elongation was retarded in the mutant compared with the wild type (Figures 5B to 5D and 5F to 5H; see Figure 3. Symbiotic Phenotypes of rsd Mutants and Complementation by p3SS-GFP-MtRSD.

(A) Schematic representation of the Mt-RSD gene with Tnt1 insertion sites (arrowheads) in two independent lines. The black region represents the protein coding region, and UTRs are shown in gray. Bar = 100 bp.

(B) Wild-type R108 (left), rsd-1 (NF11265; middle), and rsd-2 (NF3492; right) at 21 DAI with S. meliloti strain 2011. Close-up views of root nodules of the wild type (left), rsd-1 (middle), and rsd-2 (right) are shown below.

(C) Acetylene reduction activity (ARA) of whole nodulated roots of R108, rsd-1, and rsd-2 at 15 DAI. Data are the mean of 41 plants for R108, 32 plants for rsd-1, and 12 plants for rsd-2. Vertical bars represent SE. n.d., not detected.

(D) to (G) Complementation of the rsd-1 (NF11265) mutant with a p3SS-GFP-MtRSD construct via Agrobacterium rhizogenes–mediated hairy root transformation. Stereomicroscope images under light ([D] and [F]) and fluorescent illumination ([E] and [G]). Bars = 1 mm.

(D) and (E) Spheroidal nodules on control roots transformed with empty vector at 28 DAI.

(F) and (G) Cigar-shaped nodules on roots transformed with p3SS-GFP-MtRSD at 28 DAI. The inset in (F) shows a typical, pink complemented nodule.
Supplemental Figures 6F to 6K online). The most striking difference between \( \text{rsd-1} \) and wild-type nodules was the presence of plant cells heavily packed with bacteria at the distal end of the invasion zone in the case of the mutant but not the wild type (cf. Figures 5A and 5E with 5B and 5F; cf. Supplemental Figures 6D and 6F with 6E and 6G online).

On the proximal (i.e., root) side of these cells, one or two layers of cells were packed with incompletely elongated bacteroids in the mutant (Figure 5F; see Supplemental Figure 6G online). Furthermore, the characteristic orientation of symbiosomes around the central vacuole of wild-type cells was not observed in \( \text{rsd-1} \) mutant cells (cf. Supplemental Figures 6J and 6K online). Overall, invasion zone II was larger in the mutant than in the wild type. Type II bacteroids reach lengths of up to 10 \( \mu \text{m} \) (Figures 5C and 5D) in wild-type nodules. However, no bacteroids longer than 5 \( \mu \text{m} \) were observed in \( \text{rsd-1} \) nodules (Figures 5G and 5H). Moreover, cytoplasmic heterogeneity (i.e., distinct electron-dense and translucent regions imaged by electron microscopy; Vasse et al., 1990) was not observed in bacteroids of mutant nodules. Instead, premature senescence of bacteroids occurred by 12 DAI proximal to zone II of mutant nodules (Figure 5E; see Supplemental Figure 7 online). To distinguish the bacteroid from the SM, we employed rhizobia expressing the mCherry (red) fluorescent protein and the membrane stain, FM1-43 (green). In zone II of \( \text{rsd-1} \) nodules, some cells showed mCherry-labeled bacteria within FM1-43-stained SMs, while other cells exhibited FM1-43-stained symbiosomes without mCherry-labeled bacteria, possibly indicating that bacteroid degradation began before disintegration of the SM (cf. Supplemental Figures 6H and 6I online).

To test the viability of bacteria inside \( \text{rsd-1} \) nodules, we used a live/dead staining procedure with a mixture of the two fluorescent nucleic acid dyes, SYTO9 and propidium iodide (PI; Haag et al., 2011). Live bacteria with intact cytoplasmic membranes are stained by membrane-permeable SYTO9 (green), while dead bacteria with degraded cytoplasmic membranes are stained by membrane-impermeable PI (red). As shown in Supplemental Figures 7A to 7C online, bacteria in wild-type cells stained green throughout the invasion and fixation zones. By contrast, green-stained bacteria were observed only inside ITs and in a few cells of the invasion zone in \( \text{rsd-1} \) nodules at 12 DAI (see Supplemental Figures 7F to 7H online). In the distal part of the invasion zone of \( \text{rsd-1} \) nodules, some cells contained both red- (dead) and green-stained (live) bacteria (see Supplemental Figure 7G online). All bacteria except those inside ITs appeared to be dead (stained red) in the proximal part of the invasion zone (see Supplemental Figure 7G online). Transmission electron microscopy showed that degrading symbiosomes, indicated by an enlarged symbiosome space containing multiple bacteria, occurred in plant cells containing intact nuclei (see Supplemental Figures 6K, 7I, and 7J online). Taken together, the microscopy results indicate that bacteria lose viability within the invasion zone of \( \text{rsd-1} \) mutant nodules but not as a consequence of plant cell senescence.

Given the microscopy data and the known correlation between cell size and ploidy, we measured the ploidy of both plant...
and bacteroid cells from rsd mutant and wild-type nodules. There was a slight shift to lower ploidy levels among plant cells harvested from rsd-1 mutants compared with the wild type (Figures 5I and 5J), indicating a reduction in endoreduplication in some of the mutant cells. Similarly, a shift in ploidy distribution to lower levels in bacteroids of the rsd-1 mutant indicated fewer rounds of endoreduplication in the mutant compared with the wild type (Figures 5K and 5L), consistent with the observed reduction in bacteroid size (cf. Figures 5C and 5D with 5G and 5H) and the rapid loss of bacterial viability noted above.

**Placing RSD within the Nodule Developmental Program**

To place RSD function into context relative to other genes that are required for nodule development and/or differentiation, we quantified Mt-RSD transcript levels in various plant mutants. Because RSD gene expression in the wild type commenced around 6 DAI (Figure 2A), after formation of nodule primordia, we excluded Nod- mutants that do not form primordia from this analysis. The mutant with the earliest nodulation defect that we analyzed was ipd3 (Benaben et al., 1995; Horváth et al., 2011). IpD3 mutants make small nodule-like structures in which bacteria are very rarely released from ITs.

RSD expression was significantly lower in the ipd3 mutant than in the wild type at 10 DAI (P ≤ 0.001) (Figure 6A). Likewise, RSD expression was significantly lower in dnf1, dnf5 (P ≤ 0.01), and dnf6 (P ≤ 0.05) mutants at 10 DAI (Figure 6A).

We examined the expression of RSD in Fix- nodules elicited by the bacterial mutants exoA, bacA, and fixJ (Leigh et al., 1985; David et al., 1988; Glazebrook et al., 1993; Oke and Long, 1999;
NCR peptides and rapid death of undifferentiated bacteria (Haag absence of BacA results in hypersensitivity toward antimicrobial differentate (Glazebrook et al., 1993; Oke and Long, 1999). The mutant bacteria show normal endocytosis but are unable to dif-

mitogen activation 

eExpression was barely detectable in nodules elicited by the RSD fails to invade nodules (Leigh et al., 1985; Mitra et al., 2004).

bacterial mutant that elicits nodule primordium formation but 

eexpression was not (see Supplemental Figure 8 online). Mitra et al., 2004).

Various Fix

Expression of

Figure 6. Expression of RSD in Various Fix− Nodules.

Relative transcript levels of Mt-RSD in different types of Fix− nodules measured at 10 DAI. Asterisks indicate a significant difference in RSD expression with respect to the Fix+ control: ***P ≤ 0.005, **P ≤ 0.01, and *P ≤ 0.001. Mean and so of three biological replicates are presented in each case.
(A) Various Fix− plant mutants.
(B) Various Fix− bacterial mutants in wild-type plants.

Mitra et al., 2004). exoA is an exopolysaccharide EPS I-deficient bacterial mutant that elicits nodule primordium formation but fails to invade nodules (Leigh et al., 1985; Mitra et al., 2004). RSD expression was barely detectable in nodules elicited by the exoA mutant (Figure 6B). Bacterial differentiation A (bacA) mu-
tant bacteria show normal endocytosis but are unable to differen-
tiate (Glazebrook et al., 1993; Oke and Long, 1999). The absence of BacA results in hypersensitivity toward antimicrobials NCR peptides and rapid death of undifferentiated bacteria (Haag et al., 2011). In the Fix− nodules elicited by the bacA mutant, RSD expression was significantly lower than in nodules con-
taining wild-type bacteria after 10 DAI (P ≤ 0.001), although transcript levels were substantially higher in nodules containing the bacA mutant than in those elicited by the exoA mutant (Figure 6B). FixJ is a response regulator, and fixJ mutants elicit wild-type-like nodules that fix nitrogen but undergo early se-

nencescence (David et al., 1988; Oke and Long, 1999). RSD ex-

pression was normal in these nodules at 10 DAI (Figure 6B).

We also measured the expression of leghemoglobin (Medtr1g011540.1) in all of the above-mentioned Fix− nodules. In dnf1, dnf5 (both plant mutants), and bacA (a bacterial mutant) nodules, expression of RSD was detectable, but expression of leghemoglobin was not (see Supplemental Figure 8 online). Likewise, leghemoglobin gene expression was not induced in the rsd-1 mutant, indicating that expression of RSD precedes that of leghemoglobin (see Supplemental Figure 8 online).

RSD Is a Nuclear Protein

To gain insight into the possible subcellular location of Mt-RSD protein, we transformed tobacco (Nicotiana tabacum) leaf epi-
dermal cells with DNA encoding a green fluorescent protein (GFP)-MtRSD fusion, which revealed a nuclear location for the protein (see Supplemental Figures 9A and 9B online). Expression of GFP alone in tobacco cells led to GFP fluorescence in both nucleus and cytoplasm (see Supplemental Figures 9C and 9D online).

RSD Acts as a Transcriptional Repressor when Expressed in Yeast

To test whether Mt-RSD, which contains an EAR (for Ethylene-

responsive element binding factor-associated Amphiphilic Re-

pression) repressor domain, can repress gene transcription, we used a yeast one-hybrid assay. Mt-RSD or Mt-RSD without the EAR domain—encoding sequence (MtRSDΔEAR) were cloned into vector pGBT9 between the Gal4 DNA binding domain (Gal4-DBD) and the activation domain of herpes simplex virus protein VP16 (Sadowski et al., 1988), generating effector constructs (Figure 7A). Another construct containing six Gal4-DNA binding motifs and the pCYC1 minimal promoter controlling the lacZ gene was used as reporter in transactivation assays (Figure 7A). After coexpressing reporter and effector constructs in yeast, β-galactosidase assays were performed. Mt-RSD repressed the transactivation activity of VP16 by over 90% (comparing pGBT9: MtRSD-VP16 to the control, pGBT9:GFP-VP16; Figure 7A), Removal of the EAR domain of Mt-RSD (pGBT9:MtRSDΔEAR-VP16) led to partial recovery of VP16 activity (Figure 7A). These results indicated that the EAR domain of Mt-RSD can act as a repressor of transcription and that additional amino acids in Mt-RSD may contribute to transcriptional repression.

Transcriptome Perturbations Resulting from Loss of Mt-RSD Function

To identify potential targets of RSD transcriptional repression, we performed transcriptome analysis of mutant and wild-type plants using Medicago Affymetrix GeneChips. Bearing in mind that RSD expression began around 6 DAI and reached a maxi-

nal level by 8 DAI (Figure 2A), we sought genes that were ex-

pressed at significantly higher levels in nodules of the rsd-1 mutant than in the wild type at both of these time points, using 0 DAI as a point of reference for each genotype (see Supplemental Data Set 1 online). Eleven genes satisfied this criterion (Table 1; see Supplemental Data Set 1 online). To test more broadly for a negative correlation between transcript levels of each of these 11 genes and RSD, we used the MtGEA nodule developmental data and nodule zonation data (see Methods; Table 1). Transcript levels of only one of the genes, VAMP721a, showed a moderate negative correlation (R^2 = 0.64) with RSD transcript levels (see Supplemental Figure 10 online). To validate the microarray data, we performed qRT-PCR to quantify RSD and VAMP721a gene expression in wild-type (R108) and rsd-1 roots and nodules at 6 and 8 DAI (Figure 7B). A strong negative correlation between RSD and VAMP721a expression was found
Furthermore, the upregulation ratio of \textit{VAMP721a} in \textit{rsd-1} nodules to that of wild-type R108 nodules at 6 and 8 DAI were 1.5- and 2.13-fold, respectively (Figure 7B), substantiating the microarray data. To identify potentially pleiotropic effects of loss of RSD activity, we also sought genes that were expressed at significantly lower levels in nodules of the \textit{rsd-1} mutant than in the wild type at 6 and/or 8 DAI (see Supplemental Data Set 2 online). In nodulated roots of wild-type plants, transcript levels of 1093 plant genes increased significantly (\(P \leq 0.05\)) and substantially (more than twofold increase/decrease in transcript level) by 6 DAI compared with the 0-DAI root control (see Supplemental Figures 11A to 11C and Supplemental Data Set 3 online). Far fewer (479) genes were induced at 6 DAI in nodulated roots of the \textit{rsd-1} mutant compared with mutant roots at 0 DAI (see Supplemental Figures 11A to 11C and Supplemental Data Set 3 online). By 8 DAI, 2003 genes were induced in the wild type and 1742 genes were induced in the mutant, relative to their respective 0-DAI controls (see Supplemental Figures 11A to 11C and Supplemental Data Set 3 online). Interestingly, 506 of the genes induced by 6 DAI in wild-type nodules were induced later, only at 8 DAI in \textit{rsd-1} mutant nodules (see Supplemental Figures 11A to 11C and Supplemental Data Set 3 online), including many nodule-specific \textit{NCR} genes (see Supplemental Figure 11D online) and other nodule-specific genes (see Supplemental Data Set 3 online). These microarray results were validated by qRT-PCR analyses of 22 genes, all of which gave qualitatively similar results (see Supplemental Figures 12B to 12E online).

Bacteroid differentiation leading to SNF involves many genes that are induced during nodule development (Oke and Long, 2003).
12A online). By contrast, no expression of the nifA and wild-type nodules at 15 DAI (see Supplemental Figure rsd
plant Supplemental Figure 12A online). Parallel measurements of expression of these genes in wild-type nodules at 15 DAI (see
revealed that a subunit of nitrogenase (Starker et al., 2006). qRT-PCR analysis expression (Starker et al., 2006); and nifH, which encodes a subunit of nitrogenase (Starker et al., 2006). qRT-PCR analysis revealed that bacA transcript levels were similar in bacteroids of rsd and wild-type nodules at 15 DAI (see Supplemental Figure 12A online). By contrast, no expression of the nifA and nifH genes was detected in the mutant, despite high levels of expression of these genes in wild-type nodules at 15 DAI (see Supplemental Figure 12A online). Parallel measurements of plant leghemoglobin gene expression in these nodules revealed high expression levels in the wild type but no expression in rsd-1 mutant nodules at 15 DAI (see Supplemental Figure 12A online).

Thus, loss of Mt-RSD function resulted in delayed or no induction of many nodule-specific plant genes as well as bacterial nif genes, some of which are crucial for bacteroid differentiation and SNF. However, given the likely role of RSD as a transcriptional repressor, we considered these effects pleiotropic and focused our attention on the Mt-VAMP721a gene as a putative target of Mt-RSD transcriptional repression.

RSD Binds to the Promoter of VAMP721a
To test the hypothesis that VAMP721a is a direct target of RSD repression, we performed a chromatin immunoprecipitation (ChIP)-PCR assay. First, we introduced a p35S-RSD-GFP construct into M. truncatula hairy roots. We then performed ChIP using an anti-GFP antibody, with three independently transformed hairy root systems, followed by quantitative PCR using several primer pairs designed to bind within and outside the VAMP721a promoter region. Quantitative PCR analysis with primer set 2, which amplified the region between −1645 and −1579 upstream (5’) of the ATG start codon revealed massive and significant enrichment of DNA compared with two other segments in the promoter region and one in exon 5 of the VAMP721a gene (Figure 7C). The sequence ACAGTGTC, and especially the AGT in its core, is known to bind C2H2 zinc-finger proteins from petunia (Petunia hybrida) and Arabidopsis thaliana (Takatsuji and Matsumoto, 1996; Dathan et al., 2002; Ren et al., 2007). We found three instances of the sequence AAAGTTC between positions −1641 and −2139 relative to the translation start codon of VAMP721a (Figure 7C). To confirm direct binding of RSD to the VAMP721a promoter, we performed an electrophoretic mobility shift assay (EMSA) using a 288-bp fragment (−1870 to −1581: two AAAGTTC-boxes are present in this region) as the DNA probe and purified His6-MtRSD protein (Figure 7D). RSD protein formed a single distinct complex with this 288-bp fragment, as evidenced by a single retarded band in the EMSA. Competition assays with 25- and 50-fold molar excess of nonlabeled DNA probe indicated specific binding of RSD to the VAMP721a promoter fragment (Figure 7D).

DISCUSSION
The data presented here reveal an important role of RSD in symbiosome development and nitrogen fixation in M. truncatula. To recapitulate, RSD was expressed in a nodule-specific manner, with massive transcript accumulation in nodules by 6 DAI, maximal transcript levels in the invasion zone (II) and high levels in the interzone (II-III) of mature nodules (28 DAI), the zones in which symbiosomes arise and differentiate prior to SNF in zone III (Figure 2). RSD was barely expressed in nodules of plants in which primordia formed, but no bacteria were taken up into symbiosomes, such as those formed by the bacterial exoA

### Table 1. Regression Analysis for Genes Expressed at Significantly Higher Levels in Nodules of the rsd-1 Mutant Than in the Wild

<table>
<thead>
<tr>
<th>MtGEA Probe Sets</th>
<th>Putative Annotation</th>
<th>MTG9</th>
<th>IMAGA3.5v4 Gene ID</th>
<th>Fold Change rsd-1 0 DAI/R108</th>
<th>Fold Change rsd-1 6 DAI/R108</th>
<th>Fold Change rsd-1 8 DAI/R108</th>
<th>Regression (R)</th>
<th>Regression Coefficient (R²)</th>
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<tr>
<td>Mtr.1810.1.S1_at</td>
<td>Hypothetical protein</td>
<td>BE206254</td>
<td>Not present</td>
<td>0.80</td>
<td>2.39</td>
<td>2.02</td>
<td>−0.33</td>
<td>0.11</td>
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<tr>
<td>Mtr.22685.1.S1_at</td>
<td>Ser hydroxymethyltransferase</td>
<td>IMAGA</td>
<td>Medt7g070020.1</td>
<td>1.02</td>
<td>3.06</td>
<td>3.36</td>
<td>0.43</td>
<td>0.19</td>
</tr>
<tr>
<td>Mtr.23318.1.S1.s_at</td>
<td>MDr family transposase protein, partial (61%)</td>
<td>Not present</td>
<td>1.02</td>
<td>48.53</td>
<td>49.62</td>
<td>0.60</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Mtr.29527.1.S1_at</td>
<td>Hypothetical protein</td>
<td>TC140926</td>
<td>Not present</td>
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<td>6.30</td>
<td>4.48</td>
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<tr>
<td>Mtr.40617.1.S1_at</td>
<td>maiA maleylacetoacetate isomerase</td>
<td>TC121481</td>
<td>ILLUM</td>
<td>contig_55748_1</td>
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<td>3.19</td>
<td>2.20</td>
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<td>IMAGA</td>
<td>Medtr3g109700.1</td>
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<td>Mtr.46130.1.S1_at</td>
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<td>TC119955</td>
<td>IMAGA</td>
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<tr>
<td>Mtr.48599.1.S1_at</td>
<td>Phosphatidylethanolamine binding protein</td>
<td>TC129531</td>
<td>IMAGA</td>
<td>Medtr7g104460.1</td>
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<td>2.00</td>
<td>−0.43</td>
</tr>
<tr>
<td>Mtr.48791.1.S1_at</td>
<td>PLAC8 family protein</td>
<td>TC123158</td>
<td>IMAGA</td>
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<td>2.44</td>
<td>4.39</td>
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<td>Mtr.583.1.S1_at</td>
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<tr>
<td>Mtr.7517.1.S1_at</td>
<td>Protein transport protein Sec24-like At3g07100</td>
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<td>4.02</td>
<td>3.65</td>
<td>0.23</td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

Transcript levels of each gene were plotted against the corresponding level for the RSD gene at each time point. Time points used for this analysis are indicated in Supplemental Figure 10 online. Blank cells in column three indicate that gene IDs were not available. MUDR, Mutator (MU) Class II family of transposons.
mutant and the plant idp3 mutant. On the other hand, Mt-RSD was expressed in nodules in which bacterial endocytosis was normal, but bacteria did not fully differentiate into nitrogen-fixing bacteroids, such as in nodules of the plant dnf1-7 mutant (Figure 6A) and nodules of wild-type plants elicited by bacterial bacA and fixJ mutants (Figure 6B). Furthermore, bacteroids failed to differentiate normally in rsd-1 mutant nodules as indicated by microscopy (Figures 4 and 5; see Supplemental Figure 6 online) and reduced endoreduplication (Figures 5I to 5L). Reduced bacterial enlargement in rsd-1 nodules was observed first in invasion zone II, coincident with the peak of RSD expression in wild-type nodules. Live-dead staining showed that rhizobia lost viability within the invasion zone of rsd-1 nodules (see Supplemental Figure 7 online). Nodules of rsd-1 mutants exhibited early nodule senescence, which is typical of symbioses formed by rhizobia mutants that fail to differentiate and/or cannot fix nitrogen (Starker et al., 2006; Van de Velde et al., 2006).

Mt-RSD is a member of the C2H2 family of plant TFs and contains not only the signature C2H2 domain, but also a conserved EAR domain (Figure 1A), which is known to confer transcriptional repressor activity on some TFs (Bowman et al., 1992; Takatsuji, 1999; Chen et al., 2010). Consistent with this, we found that the EAR domain contributed to transcriptional repression by Mt-RSD of a reporter gene in a yeast transactivation assay (Figures 7A and 7B). Removal of the EAR domain partially alleviated the repressive effect of Mt-RSD.

Transcriptome analyses (Supplemental Figure 11 and Supplemental Data Sets 1 to 3 online; Table 1) pointed to at least one potential target of RSD repression in M. truncatula, namely, the VAMP721a gene. Subsequently, we found that RSD binds to the promoter of VAMP721a, confirming the latter as a direct target of RSD repression (Figures 7C and 7D).

VAMP721a belongs to the R-SNARE family, members of which are generally located on the membranes of trafficking vesicles (V-SNAREs) and are required for vesicle fusion with specific target membranes via interaction with target t/Q-SNAREs (Lipka et al., 2007). Recently, it was found that two related v/R-SNAREs in M. truncatula, VAMP721d and VAMP721e, are required to form the SM during nodule development, via an exocytotic pathway (Ivanov et al., 2012). Reduction of VAMP721d and VAMP721e levels via RNAi in M. truncatula led to failure in both release of rhizobia from ITs and subsequent symbiosome proliferation, due to reduced cell wall dissolution around the IT at the site of bacterial entry into plant cells. The authors proposed that the cargo delivered by vesicles bearing VAMP721d and VAMP721e are important for the formation of a cell wall-free interface at the IT prior to symbiosome formation. VAMP721a, VAMP721d, and VAMP721e are strongly expressed in roots, but VAMP721a is repressed dramatically during nodule development, whereas VAMP721d and VAMP721e are not (Ivanov et al., 2012; Table 1, Figure 7B; see Supplemental Figure 9 online). Recently, it was found that the expression

**Figure 8. Hypothetical Mode of Action of Mt-RSD during Nodule Development.**

IT delivery and endocytosis of rhizobia (green) and biogenesis of symbiosomes in a single plant cell are depicted. Mt-VAMP721d and Mt-VAMP721e are required to target and fuse specialized vesicles, presumably containing cell wall–degrading enzymes, to the IT membrane, which leads to dissolution of the IT cell wall and bacterial endocytosis (Ivanov et al., 2012). These VAMPs may continue to deliver membrane and cargo to the symbiosomes as they proliferate. The endoplasmic reticulum and Golgi are involved in the biogenesis of these and other types of secretory vesicles. Mt-RSD blocks the production of Mt-VAMP721a (via transcriptional repression), eliminating an alternative secretory pathway that may compete with the pathway(s) required for symbiosome biogenesis or alternatively may act in plant defense. Mt-RSD is crucial for symbiosome development, possibly because of its direct effect on Mt-VAMP721a expression and vesicle trafficking or its indirect effects on other genes, such as the NCR genes, which encode proteins that are delivered to symbiosomes and affect bacteroid differentiation.
patterns of VAMP721a and RSD are mutually exclusive (Limpens et al., 2013). VAMP721a transcript was found in uninfected cells of the fixation zone, but not in the infected cells in which RSD was expressed specifically (Limpens et al., 2013). This indicates that repression of VAMP721a by RSD in infected cells may be crucial for normal symbiosome development.

One hypothesis to explain the symbiotic defect of the rsd-1 mutant is that VAMP721a directs vesicle traffic to an alternative target membrane(s), possibly the plasma membrane, at the expense of vesicle flow to the IT and SMs (Figure 8). Substantially reduced vesicle flow to these symbiotic membranes could compromise bacterial entry and/or symbiosome development, as observed in VAMP721d and VAMP721e RNA interference plants (Ivanov et al., 2012). Alternatively, VAMP721a-bearing vesicles, containing cargo different from that of VAMP721d- and VAMP721e vesicles, might fuse with symbiosomes in the rsd-1 mutant, delivering compounds that interfere with bacteroid differentiation and symbiosome function (Figure 8). Germane to this idea are the roles in plant defense of the closest homologs of Mt-VAMP721a in Arabidopsis, namely, At-VAMP721/722. These proteins are part of the secretory apparatus that targets vesicles containing cell wall-reinforcing material and other defense compounds to the plasma membrane at sites of fungal attack, which is an important component of non-host resistance in plants (Kwon et al., 2008). If Mt-VAMP721a plays an analogous role in plant defense in M. truncatula, it may be important that it is repressed during symbiosis with rhizobia to avoid delivery of defense compounds to the IT and/or developing symbiosomes.

At least two phases of plant transcriptional activity accompany differentiation and symbiosome formation during nodule development (Maunoury et al., 2010). Massive induction of secretory and endocytic pathway genes takes place during the first phase of gene activation (Maunoury et al., 2010). Mt-RSD is activated during this phase (Maunoury et al., 2010; Moreau et al., 2011). Loss of RSD activity in the rsd-1 mutant interferes with the induction of many genes that are normally induced during phases one and two, including many of the NCR genes. Although NCR genes are not targets of RSD repression, delayed/no expression of these genes in the rsd-1 mutant (see Supplemental Figure 11D and Supplemental Data Set 3 online) may contribute to the aberrant differentiation of bacteroids observed in rsd-1 nodules, given their known involvement in this process (Van de Velde et al., 2010).

Phylogenetic analysis of Mt-RSD and homologous sequences from other plant species identified one putative ortholog in L. japonicus and two in soybean (Figure 1), all of which are expressed in a nodule-specific manner (L. japonicus, Probeset chr1. CM0147.88 at is nodule specific according to the L. japonicus Gene Expression Atlas [Hegslund et al., 2009; Verder et al., 2013]; soybean, Glyma07g16290 and Glyma18g40360, are nodule specific according to SoyBase [Severin et al., 2010]). It also has been shown the putative ortholog of RSD in L. japonicus is expressed specifically in infected cells of nodules (Kouchi and Hata, 1995; Kumagai and Kouchi, 2003). Therefore, it is likely that the role of these proteins has been conserved among legumes during evolution and is not tied to terminal differentiation of bacteroids in M. truncatula.

In summary, we identified a member of the C2H2 family of TFs, Mt-RSD, as being crucial for symbiosome development and SNF in M. truncatula. This opens a new chapter in symbiosis research by identifying a plant TF involved in this process. We have shown that one of the direct targets of Mt-RSD is Mt-VAMP721a, and ectopic expression of this secretory pathway gene in the rsd-1 mutant could conceivably explain the symbiosome development defect of the mutant. On the other hand, we cannot exclude the possibility that other direct targets of RSD or indirect effects of the rsd-1 mutation are responsible for the complex mutant phenotype. Future work on VAMP721a and any other direct targets of RSD should help to clarify this.

Finally, our demonstration that Mt-RSD is a transcriptional regulator of plant secretory pathway genes provides an entry point into elucidating the genetic regulation of processes that are crucial for many aspects of plant development and response to the environment, including cytokinesis, embryogenesis, gravitropism, plant-microbe interactions, abscisic acid signaling, and abiotic stress responses (Lipka et al., 2007). It will be interesting to determine if other secretory pathway genes contain C2H2 TF binding domains in their promoters and whether transcript levels of such genes correlate negatively or positively with those of C2H2 TFs with or without EAR domains, respectively. Identification, characterization, and utilization of transcriptional regulators of plant secretory pathways will help us to understand better these important processes and to manipulate them to improve plant performance.

**METHODS**

**Plant Growth and Bacterial Strains**

Medicago truncatula ecotype R108 and homozygous rsd mutants (rsd-1 and rsd-2) were used. Seeds were scarified for 10 min in H2SO4 followed by sterilization for 3 min with 30% (v/v) commercial bleach containing a few drops of Tween 20. Surface-sterilized seeds were placed on inverted plates containing damp, sterile filter paper in the dark for 3 d at 4°C and 1 d at 20°C. Germinated seedlings were transferred to plastic cones containing a 2:1 ratio of vermiculite. Plants were cultivated under a 16-h light/dark regimen with 200 μE m−2 s−1 light irradiance at 21°C and 40% relative humidity. After 7 d of growth, plants were inoculated with 50 mL of Sinorhizobium meliloti (strains 1021 and 2011 with pXLD44 plasmid [Boivin et al., 1990] or 1022 with mCherry-expressing plasmid). S. meliloti was grown overnight in yeast mannitol broth liquid medium, with shaking at 250 rpm, to OD600 ≈ 1.0, pelleted by centrifugation, and resuspended in half-strength Broughton and Dilworth (Broughton and Dilworth, 1971) with 0.5 mM KNO3 at OD600 ≈ 0.02. For root transformation, ARqua1 Agrobacterium rhizogenes was used, while for Nicotiana benthamiana transient expression, Agrobacterium tumefaciens strain LBA4404 was used.

**Gene Identification and Phylogenetic Analysis**

The Mt-RSD gene was identified by its nodule-specific expression profile (probe set ID Mtr.38404.1.S1_at), using the MtGEA Web server (Benedito et al., 2008) available at http://mtgea.noble.org/v2/ (He et al., 2009). Alignment of the deduced amino acid sequences of Mt-RSD and other proteins of this family (see Supplemental Data Set 4 online) was performed using multiple sequence comparison by log expectation in the Geneious software suite (Biomatters). The phylogenetic tree was built using an unweighted pair group method with arithmetic mean with 1000 bootstrap replicates.
Insertional Mutant Screening, Genotyping, and Backcrossing

Generation of the *M. truncatula* Tnt1 insertional mutant population and growth of R1 seeds were described previously (Tadge et al., 2008). Reverse genetic screening for Tnt1 retrotransposon insertions in Mt-RSD was performed using a nested PCR approach (Cheng et al., 2011). Homozygous *rSD-1* and *rSD-2* plants were further isolated from R1 seeds by using the primers mentioned in Supplemental Data Set 5 online. Backcrossing of mutants with the R108 accession was performed as described (Taylor et al., 2011).

Complementation of the *rSD* Mutant Phenotype

The open reading frame of Mt-*RSD* was amplified from cDNA synthesized with total RNA extracted from mature nodules of the ecotype R108 using the Trizol RNA extraction method (Life Technologies) and Superscript III reverse transcriptase (Life Technologies). PCR products were cloned into Gateway destination vector pk7WGF2 (Karimi et al., 2002). This generated GFP-MtRSD fusion protein. The empty vector expressing only the GFP gene was used as a control for hairy root transformation. pk7WGF2 vectors harboring GFP-MtRSD or GFP sequences were transformed into *A. rhizogenes* strain ARAWa 1. Agrobacteria were infected on *M. truncatula* (R108) seedlings to generate composite plants (Boisson-Dernier et al., 2001). Transformed hairy roots were screened under UV light.

Nodulation Assays and Sample Collection

For nodulation time-course assays, susceptible zones were harvestable for 0- to 4-DAI samples. From 6 DAI onwards, only portions of the roots carrying visible nodules were excised and immediately frozen under liquid nitrogen. From 6 DAI onwards, only portions of the roots carrying visible nodules were excised and immediately frozen under liquid nitrogen. Nodulation assays were performed by inoculating 1-week-old plants with *S. meliloti* as described by Kakar et al. (2008). For qRT-PCR, reverse transcription was performed as described previously (Kakar et al., 2008). Transcript levels were normalized using the geometric average of three housekeeping genes, *Ubiquitin-Conjugating enzyme E2* (TC106312), *Polyprotein Converting Activity Tract-Binding protein* (TC111751) *Ubiquitin* (TC102473), whose transcript levels were very stable across all the samples analyzed. All primer sequences used in this analysis are given in Supplemental Data Set 5 online. For qRT-PCR analysis three technical replicates were used.

Phenotypic Analysis, Histochemical Staining, and Confocal and Transmission Electron Microscopy

Images of whole-mount nodulated roots were captured using an Olympus SZX12 stereomicroscope (Olympus) equipped with a Nikon DXM1200C digital camera (Nikon Instruments). Histochemical staining with X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) was performed as described previously (Boivin et al., 1990). Detached nodules were embedded in 5% low-melting-point agarose and sliced into 50-µm-thick sections with a 1000 Plus Vibratome (The Vibratome Co, Technical Product International). For confocal microscopy, sample preparation was done according to Haynes et al. (2004). Nodules were sliced using a vibratome. Forty-micrometer sections were prepared and stained with SYTO13, Calcofluor (Life Technologies), and/or FM1-43 (Life Technologies). Images were acquired with a Leica TCS SP2 AOBS confocal laser scanning microscope. For transmission electron microscopy, nodules were fixed with 3% glutaraldehyde (v/v) and 4% paraformaldehyde (v/v) in cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in LR White resin (London Resin Company). Serial 0.1-µm sections were cut with a diamond knife on a Leica EM UC7 ultramicrotome (Leica Microsystems). Ultrathin sections were then put onto carbon-coated copper grids and stained with 2% uranyl acetate followed by staining with Sato’s lead. Specimens were observed under a Zeiss 10A transmission electron microscope operated at 80 kV. Images captured onto negative films were scanned and digitalized. All digital micrographs were processed using Adobe Photoshop CS4.

In Situ Live/Dead Staining of Rhizobia

Live/dead staining was performed according to Haag et al. (2011). Briefly, wild-type R108 and *rSD-1* were grown and inoculated with *S. meliloti* strain Sm1021. Nodules were harvested 12 DAI and embedded in 6% (w/v) agarose. Nodule sections of 100-µm thickness were prepared with a 1000 Plus Vibratome (The Vibratome Co, Technical Product International) and incubated for 20 min in live/dead staining solution (1:1000 dilution of 1.67 mM SYTO9 and 18.3 mM PI [Molecular Probes; L7007] in 50 mM Tris, pH 7.0, buffer). Sections were removed from staining solution and mounted in deionized water for microscopy. Images were acquired with a Leica TCS SP2 AOBS confocal laser scanning microscope.

Acetylene Reduction Assay

Acetylene reduction assays were performed as described previously (Oke and Long, 1999). Plants were grown on a mixture of vermiculite and surface, watered with B&D solution containing 0.5 mM nitrate, and inoculated with rhizobia strain *Sm1021*. At 15 DAI, entire root systems were place into sealed test tubes for the assays.

Flow Cytometry

*M. truncatula* R108 and *rSD-1* mutant plants were cultivated and inoculated with *S. meliloti* in aeroponic factories. Measurements of the DNA content of plant nuclei and bacteroids were done according to Cebolla et al. (1999) and Mergaert et al. (2006), respectively. Briefly, freshly collected nodules at 15 DAI were sliced in 0.5 mL of Galbraith buffer containing 1% Triton X-100, nuclei were stained with 4. הפרמידון-2-
phenylindole (DAPI) (5 μg/mL), and 5000 nuclei were analyzed per measurement. In the case of bacteria, the total bacteroid population was analyzed. DNA content of nuclei and bacteria was measured with a MoFlo Astrios flow cytometer from Beckman-Coulter driven by Summit 6.1 software.

**Transient Expression in *N. benthamiana***

Suspensions of *Agrobacterium* strain LBA4404 carrying the p3SS-GFP-MtRSD construct or the empty vector (pK7WG2F) were infiltrated into leaves of *N. benthamiana*, as previously described (Goodin et al., 2002).

**Transcriptional Repression in Yeast**

The vector pGBT9 (Clontech Laboratories) was used to construct effector plasmids for transcriptional repression activity assays. The reporter yeast strain carrying six Gal4-UASs upstream of the lacZ reporter and the pGBT-9 vector in which Gal4-UAS is fused to the herpes simplex virus VP16-activation domain were described previously (Shen et al., 2012). Mt-RSD was cloned into pGBT9 as a fusion protein with Gal4-UAS or Gal4-UAS plus VP16 transactivation domain. The effector plasmid was transformed into the yeast reporter strain according to the yeast protocols handbook (Clontech). β-Galactosidase assays were performed as described in the Matchmaker one-hybrid system user manual (Clontech).

**ChIP**

ChIP was performed using GFP-MtRSD-overexpressing hairy roots. The tissues were pulverized in liquid nitrogen. Powdered tissue was taken in extraction buffer 2 (0.4 M Suc, 50 mM HEPES-KOH, pH 7.5, 5 mM β-ME, and PI cocktail) containing 1% formaldehyde. Cross-linking was done for 10 min at room temperature and was quenched by the addition of Gly to a final concentration of 125 mM. Following centrifugation of the cross-linked material at 4000g for 20 min, the pellet was resuspended in extraction buffer 3 (0.25 M Suc, 10 mM Tris-Cl, pH 8, 10 mM MgCl₂, 1% Triton X-100, 5 mM β-mercaptoethanol, and Complete protease inhibitor tablets [Roche; PI tablet]). This was followed by another centrifugation at 14,000g for 20 min. The pellet was resuspended in extraction buffer 3 (1.7 M Suc, 10 mM Tris-Cl, pH 8, 2 mM MgCl₂, 0.15% Triton X-100, 5 mM β-ME, and protease inhibitor cocktail) and centrifuged at 20,000g for 40 min. The resulting pellet was resuspended in sonication buffer (10 mM Tris-aminomethane-hydrochloride, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, 0.5% N-lauroylsarcosine, and PI tablet). This was followed by sonication (10 × 10-s pulses with 1-min interval between each pulse) to shear the chromatin. Cell debris was removed by another centrifugation at 20,000g for 40 min. To be used as the input sample, 100 μL of this clear supernatant containing chromatin was preserved for subsequent analysis. ChIP was performed with rabbit anti-GFP polyclonal antibody (Abcam) bound to Dynabead Protein-G magnetic beads (Life Technologies) for 8 h at 4°C. The beads were washed twice with high-salt wash buffer (500 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 20 mM Tris-HCl, pH 8, 2 mM EDTA, and PI tablet), followed by RIPA buffer (50 mM HEPES-KOH, pH 7.5, 500 mM LiCl, 1 mM EDTA, 1% Nonidet P-40 or Igepal CA-630, 0.7% Na-deoxycholate, and PI tablet) and Tris-buffered saline. Chromatin was eluted in elution buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, and 1% SDS) and de-cross-linked (including the inputs) at 65°C for 16 h. Cellular RNA and protein were then removed by RNase (Sigma-Aldrich) and protease-K (Applied Biosystems) treatment, respectively. DNA was then precipitated using 2.5 volumes of 100% ethanol and finally dissolved in 50 μL water. Target DNA enrichment for each sample was determined by real-time PCR (PCR primers are given in Supplemental Data Set 5 online) using the equation 2^((Ct of Input-Ct of ChIP)/(Ct stands for cycle threshold)). RSD-dependent DNA enrichment was calculated as the ratio of DNA enrichment in GFP-MtRSD-expressing roots to that in empty vector transformed roots.

**EMSA**

His₃,tagged Mt-RSD was expressed in *Escherichia coli* BL21 codon plus cells using pDEST17 vector and purified with the QiAexpressionist kit, following the manufacturer’s instructions (Qiagen). EMSA was performed with a Light Shift Chemiluminescent EMSA kit, following the manufacturer’s instructions (Pierce). Briefly, we used ~100 ng of purified recombinant protein and 20 fmol of biotin-labeled DNA fragment in a 30-μL reaction including 25 mM HEPES, pH 7.5, 50 mM KCl, 6.25 mM MgCl₂, 0.13% Nonidet P-40, 5% glycerol, and 500 ng double-stranded poly(dI/dC). After incubation, the mixture was loaded on a 6% polyacrylamide gel (Life Technologies) and run in 0.5% Tris Borate Ethylene diamine tetra-acetate at 4°C (120 V for 4 h). Biotin-labeled DNA fragment was generated using biotin-labeled forward primer. Primer sequences are listed in Supplemental Data Set 5 online.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At-ZIP6 (NP_176873), At-SUPERMAN (NP_188954), Os-C-H₂ (BAD31832), Gm-PALM1 (ADI60289), Gm-PALM2 (ADI60290), Ms-PALM1 (ADI60287), Mt-PALM1 (XP_003611481), At-TAC1 (NP_187540), At-C2H2-Znf (NP_199167), Pt-C2H2-Znf (XP_00233519), Mt-RSD1 (NP_001235396), Gm-RSD2 (NP_001235081), and Mt-RSD (R108). RSD-dependent DNA enrichment was calculated as the ratio of DNA enrichment in GFP-MtRSD-expressing roots to that in empty vector transformed roots.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Reduction in Wild-Type *M. truncatula* Ecotypes.

**Supplemental Figure 2.** RSD cDNA and Deduced Amino Acid Sequence from *M. truncatula* R108.

**Supplemental Figure 3.** Nitrogenase Acetylene Reduction Activity during Nodule Development of the Two *M. truncatula* Wild-Type Genotypes A17 and R108.

**Supplemental Figure 4.** Confocal Microscopy of RSD-Complemented rsd-1 Nodules.

**Supplemental Figure 5.** Effect of Full Nitrogen and *Sinorhizobium meliloti* on Wild-Type *M. truncatula* R108 and the rsd-1 Mutant.

**Supplemental Figure 6.** Rhizobial Infection Phenotype of rsd-1.

**Supplemental Figure 7.** Rhizobia in rsd-1 Nodules Undergo Rapid Death.

**Supplemental Figure 8.** Expression of leghemoglobin in Various Fix- Mutants.

**Supplemental Figure 9.** Localization of Mt-RSD in Tobacco Epidermal Cells.

**Supplemental Figure 10.** RSD Regulates VAMP721a (Medtr4g022570: IMGAG3.5) Expression.

**Supplemental Figure 11.** Nodulation-Induced and -Repressed Genes in the rsd-1 Mutant and Wild-Type Plants.
Supplemental Figure 12. Expression of Plant and Bacterial Genes in \textit{rsd-1} Mutant and Wild-Type Nodules.

Supplemental Table 1. List of Primers.

Supplemental Data Set 1. List of Genes Expressed at Significantly Higher Levels in Nodules of the \textit{rsd-1} Mutant Than in the Wild Type at Both 6 and 8 DAI.

Supplemental Data Set 2. List of Genes Expressed at Significantly Lower Levels in Nodules of the \textit{rsd-1} Mutant Than in the Wild Type at 6 and/or 8 DAI.

Supplemental Data Set 3. List of Genes Expressed during Wild-Type Nodule Development, but Delayed or Not Expressed in \textit{rsd-1} Nodules.

Supplemental Data Set 4. Text File of the Alignment Used for the Phylogenetic Analysis Shown in Figure 1.

Supplemental Data Set 5. List of Primers Used in This Study.

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The C$_2$H$_2$ Transcription Factor REGULATOR OF SYMBIOSOME DIFFERENTIATION Represses Transcription of the Secretory Pathway Gene VAMP721a and Promotes Symbiosome Development in Medicago truncatula

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