A *Medicago truncatula* Tobacco Retrotransposon Insertion Mutant Collection with Defects in Nodule Development and Symbiotic Nitrogen Fixation 1[W][OA]

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A *Tnt1*-insertion mutant population of *Medicago truncatula* ecotype R108 was screened for defects in nodulation and symbiotic nitrogen fixation. Primary screening of 9,300 mutant lines yielded 317 lines with putative defects in nodule development and/or nitrogen fixation. Of these, 230 lines were rescreened, and 156 lines were confirmed with defective symbiotic nitrogen fixation. Mutants were sorted into six distinct phenotypic categories: 72 nonnodulating mutants (Nod−), 51 mutants with totally ineffective nodules (Nod+ Fix−), 17 mutants with partially ineffective nodules (Nod+ Fix+/−), 27 mutants defective in nodule emergence, elongation, and nitrogen fixation (Nod+/− Fix−), one mutant with delayed and reduced nodulation but effective in nitrogen fixation (dNod+/− Fix+), and 11 supermodulating mutants (Nod+ Fix+/−). A total of 2,801 flanking sequence tags were generated from the 156 symbiotic mutant lines. Analysis of flanking sequence tags revealed 14 insertion alleles of the following known symbiotic genes: *NODULE INCEPTION* (NIN), *DOESN'T MAKE INFECTIONS*3 (DMI3/CCaMK), *ERF REQUIRED FOR NODULATION*, and *SUPERNUMERARY NODULES* (SUNN). In parallel, a polymerase chain reaction-based strategy was used to identify *Tnt1* insertions in known symbiotic genes, which revealed 25 additional insertion alleles in the following genes: *DMI1, DMI2, DMI3, NODULATION SIGNALING PATHWAY1* (NSP1), NSP2, SUNN, and SICKLE. Thirty-nine Nod− lines were also screened for arbuscular mycorrhizal symbiosis phenotypes, and 30 mutants exhibited defects in arbuscular mycorrhizal symbiosis. Morphological and developmental features of several new symbiotic mutants are reported. The collection of mutants described here is a source of novel alleles of known symbiotic genes and a resource for cloning novel symbiotic genes via *Tnt1* tagging.

Legumes have played a crucial role in sustainable agriculture for thousands of years because of their ability to reduce atmospheric N\textsubscript{2} to ammonia via symbiosis with nitrogen-fixing bacteria called rhizobia.

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This enables them to grow well in nitrogen-poor soils without the addition of organic or industrial nitrogen fertilizer. Symbiotic nitrogen fixation (SNF) in legumes injects around 50 million tons of nitrogen into agricultural systems each year, which sustains not only legume production but also subsequent nonlegume crops (Smil, 1999). Legume seeds from many different bean and pea species are important sources of food for humans, feed for animals, and raw materials for industry (Kinney, 1998; Graham and Vance, 2003). Legumes also serve as forage for animals and are touted as potential feedstock for the biofuel industry. SNF in legumes takes place in nodules, specialized organs that develop mainly on roots following signaling between rhizobia in the soil and the plant. In most members of the papilionoid subfamily (Papilionoideae), which includes beans, lupins, and medicas,
Rhizobia colonize cells of developing nodules via infection threads, which begin in hair cells of the root epidermis and, ultimately, deposit the bacteria in cortical cells via endocytosis. This process results in a unique “organelle” called the symbiosome that consists of one or more bacteria surrounded by a plant membrane (Udvardi and Day, 1997; Brewin, 2004). Bacteria (and symbiosomes) divide until they pack the infected cells and eventually differentiate into nitrogen-fixing bacteria. Plant cells also differentiate during nodule development and provide rhizobia with a carbon source(s) and other nutrients for metabolism, assimilate ammonium produced by the bacteria into organic compounds, and export these nitrogen compounds to the rest of the plant (Udvardi and Day, 1997; Prell and Poole, 2006; White et al., 2007). The nodule as a whole provides a low-oxygen environment that is crucial for bacteroid nitrogen fixation (Ott et al., 2005). Nodule development and plant and bacterial cell differentiation for SNF involves global reprogramming of gene expression in both organisms (Becker et al., 2004; Colebatch et al., 2004; Benedito et al., 2008; He et al., 2009; Høgslund et al., 2009; Karunakaran et al., 2009; Libault et al., 2010; Severin et al., 2010).

Bacterial and plant model species have been used over the past few decades to identify genes that are essential for SNF (Fuhrmann and Hennecke, 1984; Noti et al., 1986; Roche et al., 1996; Radutoiu et al., 2003; Arrighi et al., 2006; Mergaert et al., 2006). Rhizobial model species were chosen largely on the basis of the economic importance of their specific legume host species, with a long-term objective of improving the efficiency of SNF in crop and pasture legumes. Unfortunately, most of the agriculturally important legume species are poor model systems for legume genetics, because they have large genomes, are difficult to transform, and are out-crossing and/or polyploid. As a result, two species, *Medicago truncatula* and *Lotus japonicus* (Handberg and Stougaard, 1992; Cook, 1999; Udvardi et al., 2005), were chosen as models for legume genetics and genomics because of their inbreeding nature and small, diploid genomes, among other attributes. A variety of mutant populations have been developed for these two species (Tadeg et al., 2009) using chemical (ethyl methanesulfonate [EMS]; Benaben et al., 1995; Szczygłowski et al., 1998; Penmetsa and Cook, 2000), physical (fast neutron bombardment and γ-rays; Rogers et al., 2009), and DNA-insertion (transferred DNA [T-DNA] and transposons; Scholte et al., 2002; d’Erfurth et al., 2003a, 2003b, 2006) mutagenesis. Approximately 50 plant genes have been shown to be required for the establishment of SNF and for controlling the extent of nodule formation, mainly from EMS mutants via map-based cloning of defective genes, and have recently been reviewed in detail (Oldroyd and Downie, 2008; Ferguson et al., 2010; Kouchi et al., 2010; Murray, 2011; Oldroyd et al., 2011). Many of these are involved in bacterial signal perception and transduction, consequent regulation of gene transcription, and induction of nodule development (Catoira et al., 2000; Endre et al., 2002; Amor et al., 2003; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Ané et al., 2004; Mitra et al., 2004a; Smit et al., 2005; Arrighi et al., 2006; Kanamori et al., 2006; Andriankaja et al., 2007; Marsh et al., 2007; Riely et al., 2007; Saito et al., 2007; Charpentier et al., 2008). Others are required for infection and the accommodation of rhizobia in cells of developing nodules (Hayashi et al., 2010; Wang et al., 2010; Horváth et al., 2011; Murray et al., 2011), while a few are required for nodule metabolism (Krusell et al., 2005; Hakoyama et al., 2009, 2012; Yendrek et al., 2010). Another group of genes have been shown to control nodule number through either root- or shoot-derived signals by a long-distance feedback regulation mechanism called autoregulation of nodulation (Krusell et al., 2002; Nishimura et al., 2002; Schnabel et al., 2005; Murray et al., 2007; Penmetsa et al., 2008; Okamoto et al., 2009; Miyazawa et al., 2010; Mortier et al., 2010; Reid et al., 2011; Schnabel et al., 2011). Despite past successes, map-based cloning approaches are time consuming and resource intensive. Newly developed DNA-insertion mutant populations provide a rapid way of linking mutant phenotypes to defective genes and are poised to revolutionize the discovery of genes required for SNF, via both forward and reverse genetics (Tadeg et al., 2005; Young and Udvardi, 2009; Urbanski et al., 2012).

DNA-insertion mutagenesis using T-DNA, transposons, or retrotransposons for gene disruption or activation is a valuable tool for functional genomics and has the major advantage of identifying mutated genes quickly using the inserted DNA sequence as a “tag” (Schauer et al., 1998; Krysan et al., 1999; Alonso et al., 2003; d’Erfurth et al., 2003a). T-DNA tagging has been deployed most effectively in the nonlegume Arabidopsis (*Arabidopsis thaliana*) because this species is easy to transform and propagate in large numbers (hundreds of thousands of plants) in a limited area (Krysan et al., 1999; Alonso et al., 2003). Legumes are larger and more difficult to transform, so T-DNA mutagenesis has found limited application in this family of plants (Schauer et al., 1998; Martirani et al., 1999; Scholte et al., 2002; Brocard et al., 2006; Yano et al., 2009). DNA transposons have also been tested in legumes: the maize (*Zea mays*) Ac/Dc system in *Lotus* (Thykaer et al., 1995; Fajuelo et al., 1996) and soybean (*Glycine max*; Mathieu et al., 2009); the En/Spm and Tag1 systems in *Medicago* (d’Erfurth et al., 2003b, 2006); and the rice (*Oryza sativa*) MITE mPing element in soybean (Hancock et al., 2011). However, they tend to insert in A/T-rich regions, often outside of gene-coding sequences, are readily inactivated, and, more importantly, can be excised, resulting in the loss of tag from mutated genes. Retrotransposons are mobile DNA elements that transpose via an RNA intermediate in a “copy-and-paste” manner (Kumar et al., 1999). Unlike DNA transposable elements, retrotransposition causes stable mutations that are passed onto progeny during seed-
to-seed propagation (d’Erfurth et al., 2003a). Several retrotransposons have been characterized in legumes, including the *Lotus Retrotransposon1* (LORE1a), an endogenous member of the *Gypsy* superfamily that has been shown to display gametophytic transposition (Madsen et al., 2005); the *Medicago Retroelement1-1* (MEREL-1) in *Medicago*, which was identified as an insertion in the **NODULATION SIGNALING PATHWAY2** (NSP2) symbiotic gene (Rakocèvic et al., 2009); and the *Cicer arietinum Retroelement1* (CARE1), a TY3-Gypsy-like long terminal repeat retroelement in chickpea (Rajput and Upadhyaya, 2010). Long terminal repeat retrotransposons from tobacco (*Nicotiana tabacum*; Tnt1 and Tto1) and rice (*Tos17*) have been used to generate insertional mutants in Arabidopsis (Hirochika et al., 2000; Okamoto and Hirochika, 2000) and in *Medicago* (d’Erfurth et al., 2003a; Tadege et al., 2008).

The *Medicago Tnt1* insertion mutant population at the Samuel Roberts Noble Foundation is the largest collection of DNA-insertion mutants of all legumes. It was established from a starter parental line, Tnk88-7-7 (d’Erfurth et al., 2003a), containing approximately five copies of the Tnt1 retroelement transferred into wild-type R108 by *Agrobacterium tumefaciens*-mediated transformation. To date, almost 20,000 independent insertion lines have been generated by somatic embryogenesis of Tnk88-7-7 leaf explants (Fig. 1). Of the 9,300 lines that have been subjected to preliminary phenotypic screening, approximately 30% showed visible symbiotic phenotypes, consistent with a previous report on a sample of 3,237 lines (Tadege et al., 2008). A total of 9,320 photographs of phenotypes taken at the time of screening can be found at the mutant database (http://bioinfo4.noble.org/mutant). Here, we present the results of a detailed characterization of mutants affected in nodulation and SNF.

## RESULTS

### Isolation of Tnt1-Insertion Symbiotic Mutants

In preparation for the communal screening workshops, 12 plants from each Tnt1 insertion line were germinated on filter paper and transferred to soil. Approximately 1,500 lines were screened every year. The plants were grown under low nitrogen (0.5 mM KNO₃) and, after 6 weeks of exposure to the *Sinorhizobium meliloti* field isolate B1, were uprooted to observe nodules. Out of 9,300 lines, 317 lines exhibited stunted growth under these conditions and recovered when transferred to soil and provided with high-nitrogen fertilizer (2 mM KNO₃ and 2 mM NH₄NO₃). Segregating progeny of R0 and R1 seeds from 230 of these lines were subjected to rescreening under symbiotic conditions to confirm their phenotypes. In some cases, progeny from putative mutants were further rescreened to confirm their symbiotic phenotype. Detailed analysis of symbiotic phenotypes was facilitated by the use of *S. meliloti* strain Sm2011 transformed with a hemA::LacZ reporter gene construct. For the classification of symbiotic phenotypes, we used both nodule development and overall plant appearance features as described in Table I. Rescreening confirmed symbiotic phenotypes in 156 lines. In 21 instances, we distinguished multiple independently segregating symbiotic phenotypes in a line, presumably due to independent insertions affecting more than one symbiotic gene. Thus, the number of confirmed mutants was 179. In total, we isolated 72 nonnodulating and early infection mutants (Nod–), 51 mutants that developed ineffective, non-nitrogen-fixing white nodules (Nod+ Fix–), 17 mutants with pale-pink nodules that are less efficient in nitrogen fixation compared with the wild type (Nod+ Fix+/–), 27 mutants defective in nodule emergence, elongation, and nitrogen fixation (dNod+/>– Fix–), and one mutant with delayed and reduced nodulation but effective in nitrogen fixation (dNod+/– Fix+; Supplemental Table S1). We also isolated 11 mutants impaired in the negative feedback of nodule initiation and development, so-called supernodulators. In our growth conditions, these nodules are smaller and less effective in nitrogen fixation, as indicated by their pale pink appearance when compared with wild-type nodules (Nod+ Fix+/–). Representative mutants of each phenotypic category are shown in Figure 2, and a complete list of mutants and phenotypic features is included in Supplemental Table S1. All confirmed mutants were grown to maturity to produce sufficient seeds as a community resource.

### Identification of Tnt1 Insertions in Known Symbiotic Genes

We recovered 2,801 genomic sequences flanking Tnt1 insertion sites (flanking sequence tags [FSTs]) from the 179 symbiotic mutants using three different PCR-based protocols (Ratet et al., 2010). Notably, FSTs corresponding to the original insertions from the parental starter line were often not recovered, indicating that FST recovery was less than complete. The number of recovered FSTs ranged between four and 50, but the actual number could be higher. FSTs for each of these lines can be found in the *Medicago truncatula* Mutant Database using the specific line number as a search term (http://bioinfo4.noble.org/mutant). The set of FSTs generated from confirmed symbiotic mutants reveals insertions in three of the known genes involved in Nod factor (NF) signaling and nodule organogenesis: **DOESN’T MAKE INFECTIONS3** (DMi3/CaMk; NF1757), **NODULE INCEPTION** (NIN; tnk148, NF0117, NF0440, NF0825, NF1263, NF1277, NF1317, NF2640, NF2700, and NF2728), and **ERF REQUIRED FOR NODULATION1** (ERN1; NF1390; Fig. 3; Supplemental Tables S1 and S4).

In addition to FST sequencing, we used a PCR-based approach with nested Tnt1- and gene-specific primers to screen for novel insertion alleles of known genes (Fig. 3A; Cheng et al., 2011). This reverse screening was conducted on DNA extracted from all mutants
with confirmed Nod− or Nod++ symbiotic phenotypes. In this way, we identified additional alleles for most of the nod factor signaling pathway genes: DMI1/POLLUX (NF0359, NF0493, and NF3128), DMI2/SYMIRK (NF1449 and NF8854), DMI3/CaMK (NF0028, NF0600, and NF0692), NSP1 (NF0848, NF1594, and NF0692), NSP2 (NF0208, NF0351, NF0584, NF0814, and NF2811), and NIN (NF0532, NF3019, and NF3046). Overall, the largest number of insertion alleles (13) was found for NIN. Tnt1-insertion alleles were also identified for the ethylene-insensitive SICKLE (SKL) and the CLAVATA1-like Leu-rich receptor kinase SUPERNUMERARY NODULES (SUNN) genes that control nodule number. FST sequencing yielded two insertions in SUNN (NF1526 and NF2629), while PCR reverse screening on plants displaying a supernodulation phenotype recovered one skl allele (NF2085) and five additional alleles of SUNN (NF0984, NF1709, NF1858, NF3352, and Tnk100). Overall, eight of 11 supernodulation mutants can be accounted for by Tnt1 insertions in either SKL or SUNN. The remaining lines (NF0299, NF2262, and NF3447) may harbor insertions in novel genes controlling nodule number.

Most (71%) of the insertions in known genes identified by FST sequencing were also recovered by PCR reverse screening of DNA extracted from mutants with confirmed Nod− and Nod++ phenotypes (Fig. 3; Supplemental Table S1). They include seven insertions in NIN as well as one each for DMI3, ERN1, and SUNN.

**Isolation of Potentially New Nod− Mutants**

In our growth conditions, with a top layer of calcined clay (Turface) and a bottom layer of potting mix (Metro-Mix) as substrate, we isolated 41 mutants with a Nod− phenotype but no Tnt1 insertions in known nodulation genes. To ensure that the Nod− phenotypes were independent of growth substrate, we took 32 of these potentially novel Nod− mutants together with mutants affected in known nodulation genes and
repeated nodulation assays using a Turface:vermiculite mixture (3:1) as substrate. All the known Nod− mutants used as controls (nin, dmi1, and nsp1) failed to nodulate, as expected. Twenty-four of the other 32 mutants tested also failed to nodulate and displayed either absence of root hair curling in the infectible zone of roots or excessive root hair curling, with or without visible bacterial microcolonies (data not shown). Eight mutants isolated from lines NF0342, NF0438, NF0673, NF1457, NF1859, NF2811, NF5654, and NF5794 produced very small bumps resulting from cortical cell divisions in the root, indicating a later block in the nodulation pathway. No infection threads were observed to penetrate past the epidermis or the outer cortical cells into nodule primordia; therefore, we classified these as Nod− mutants (Fig. 4).

Among the potentially new Nod− mutants, several unusual infection phenotypes were observed (Fig. 4). In contrast to the wild-type infection of R108 depicted in Figure 4, A and B, in the mutant isolated from line NF0438, which eventually developed barely visible bumps (Fig. 4C), infection threads bifurcated, fragmented, and produced unusual sac-like structures (Fig. 4D). Rhizobia were released prematurely from these infection threads, apparently in the epidermis rather than the underlying cortex (Fig. 4E). Infection threads were never observed to reach the dividing inner cortical cells of the nodule primordium, as seen in the wild type (Fig. 4A). A peculiar phenotype was observed in NF2629, where rhizobial microcolonies attached to the root surface, while root hairs did not deform or curl as in the wild-type (Fig. 4F). No rhizobia

| Phenotype | Nodule Development, Appearance, and Infection Status | Overall Plant Development, Leaf Color | Presence of Fungal Structures (Hyphae, Appressoria, Arbuscles, Vesicles) |
|-----------|------------------------------------------------------|--------------------------------------|-------------------------------------------------|
| Wild type | Elongated, infected, and intense-pink nodules due to the accumulation of leghemoglobin, an indicator of efficient nitrogen fixation | Well-developed plants with healthy shoots and root systems, dark-green leaves, suggesting no nutrient deficiencies | |
| Nod−      | Absence of emerged nodules at 21 d post inoculation but may later develop a few small white bumps and a range of infection blocks; absence or reduction of root hair deformation; infection threads may form but terminate prematurely inside root hairs and do not penetrate past the epidermis or outer cortical cells into nodule primordia | Stunted plants with pale-green or yellowish leaves, due to severe nitrogen starvation | |
| Nod+ Fix− | White, infected nodules, inefficient nitrogen fixation | Stunted plants with pale-green or yellowish leaves, due to severe nitrogen starvation | |
| Nod+ Fix+/− | Infected nodules with pale-pink appearance due to less leghemoglobin, suggesting less efficient nitrogen fixation than in the wild type | Plant development is stunted but the yellowing of leaves is not as severe as in Nod− and Fix− mutants | |
| Nod+/− Fix− | Few small, infected bumps, or small inefficient nodules, visible at 21 d post inoculation | Stunted plants with pale-green or yellowish leaves, due to severe nitrogen starvation | |
| dNod+/− Fix+ | Delayed nodulation; very few pink, efficient nodules at 21 d post inoculation | Plant development and yellowing of leaves are less severe than in Nod− and Fix− mutants | |
| Nod++ Fix+/− | Supernodulators; numerous smaller, pale-pink nodules, suggestive of less efficient nitrogen fixation | Plant development is stunted, but the yellowing of leaves is not as severe as in Nod− and Fix− mutants | |
| Myc+      | All types of fungal structures observed more than 60% of the time | | |
| Myc−      | Fungal structures observed less than 30% of the time: mostly hyphae, few appressoria, no arbuscles | | |
| Myc+/-    | Fungal structures observed between 30% and 60% of the time | | |
were observed on the root surface of the Nod− mutant from NF0359. However, uncontrolled cell divisions were seen in segments of roots that are normally susceptible to rhizobia infection. In these segments, root hairs were deformed but not tightly curled (Fig. 4G). Intercellular infection appeared to occur in NF5794, as shown by blue staining from β-galactosidase activity in rhizobia cells (Fig. 4H). Patches of bacteria on the root surface and between cells generally accompanied regions of extensive cell division and root thickening. One or two small nodules eventually developed on this mutant. A Nod− mutant with extremely short and branched roots was isolated from line NF2853. No root hair deformations were observed on this mutant, and rhizobial β-galactosidase activity was detected only at sites of lateral root emergence, reminiscent of the crack entry mode of infection observed on Sesbania rostrata (Goormachtig et al., 2004; Fig. 4I). In mutants depicted in Figure 4, J to L, infections failed to proceed beyond the epidermis and brown material accumulated around the site of infection, consistent with a plant defense response (Veereshlingam et al., 2004). Excessive development of vascular tissue accompanied uncontrolled cortical cell division in the nodulation mutant of line NF5654 (Fig. 4M).

Arbuscular Mycorrhizal Phenotypes of Confirmed Nod− Mutants

The more ancient symbiotic association between plants and arbuscular mycorrhizal (AM) fungi shares several components (DMI1/POLLUX, DMI2/SYMRK, DMI3/CCaMK, and IPD3/CYCLOPS) of the NF signaling pathway of rhizobial symbiosis (Kistner et al., 2005). To facilitate the identification of potentially new components of the common symbiotic pathway and of novel nodulation-specific genes, the AM symbiosis was assessed on mutants that displayed a Nod− phenotype at 21 d post inoculation with rhizobia. After surveying the nodulation phenotype, plants were transferred to phosphate-deprived substrate and inoculated with a mixture of Glomus intraradices and Glomus mossae. Fungal structures were inspected at 2 and 4 weeks post inoculation. Thirty-nine Nod− mutants were phenotyped. Eight of these developed some nodules (Supplemental Table S2). It is not known whether this was due to delayed nodulation that is independent of AM infection or to a positive interaction between nodulation and mycorrhization. These mutants were classified as Nod+ Fix− (NF0134, NF0151, NF0428, and NF0440) and Nod+/− Fix− (NF0235, NF0302, NF1457, and NF2811). In total, 25 mutants failed to establish a successful mycorrhizal symbiosis (Myc−; although some hypheae and appressoria formed, no arbuscules were observed), five mutants had less severe defects and produced vesicles but no arbuscules (Myc+/−), and nine mutants were normal Myc+ (Supplemental Table S2). Of the 31 Nod− mutants, six contained Tnt1 insertions in the known common symbiotic genes, DMI1, DMI2, and DMI3, and displayed the expected Myc− phenotype. Many of the mutants with Tnt1 insertions in specialized nodulation genes (NIN, NSP1, NSP2, and ERN1) did not display the expected Myc+ phenotype (Supplemental Table S2). Similarly, an nsp2 mutant in the A17 genetic background was recently reported to have reduced mycorrhizal colonization (Maillet et al., 2011). However, given the additional Tnt1 insertions in each of our mutants, we cannot exclude the possibility that the aberrant mycorrhizal phenotypes were due to defects in unknown genes. Six mutants with defective AM phenotypes from lines NF0445, NF0549, NF0577, NF0662, NF2853, and NF3057 may contain insertions in novel common symbiotic genes or may have independent mutations in nodulation and mycorrhization genes. Also interesting are the Nod− Myc+ mutants of lines NF0342, NF0438, NF1241, and NF3037, which point toward potentially novel nodulation genes. A few examples of representative AM symbiotic phenotypes in Tnt1-insertion mutants are shown in Figure 5.
Figure 3. Tnt1 insertions in known symbiotic genes. A, Gene coverage of the mutant collection was assessed by identifying Tnt1 insertions in known symbiotic genes using nested Tnt1 forward (T-F1 and T-F2) and reverse (T-R1 and T-R2) and gene-specific forward (GS-F1 and GS-F2) and reverse (GS-R1 and GS-R2) primers. Gene-specific primers were designed to amplify the entire gene plus 300 to 400 bp of the 5’ and 3’ untranslated regions. B, Schematic representations of Tnt1 insertion sites in known genes as determined by PCR reverse screening (black arrowheads) and by thermal asymmetric interlaced-PCR FST sequencing (white arrowheads) in lines with confirmed symbiotic phenotypes. Exons are depicted as black boxes, and introns and untranslated regions are represented by lines. Exact insertion sites are given in Supplemental Table S1.

Isolation of New Mutants Impaired in Nodule Morphogenesis and Nitrogen Fixation

Among the 179 confirmed symbiotic mutants, 96 are impaired in some aspect of nodule development and/or nitrogen fixation, with 51 being Nod+ Fix−, 17 Nod+ Fix+/-, 27 Nod+/– Fix−, and one dNod+/- Fix+. Some mutants in these categories caught our attention because of their lack of resemblance to published symbiotic mutants. A few representative phenotypes are shown in Figure 6. Visible defects, as seen in nodule sections, included extremely low nodule occupancy in the Nod+ Fix− mutant from line NF0063 (Fig. 6B); early senescence in mutants of lines NF2496, NF4608, and NF1320 (Fig. 6, D, F, and J); persistence of infection thread networks throughout the nodule (NF5039, NF0440, NF4928; Figure 6, C, G, and I); and impairment in nodule emergence and elongation in mutants from NF0342 and NF0134 (Fig. 6, E and H). All the mutants depicted in Figure 6 had altered nodule zonation, and in mutants of lines NF2496, NF4608, NF1320, rhizobia appeared to be degraded prematurely, which was accompanied by the accumulation of brown material. These mutants were backcrossed to wild-type R108, and the analysis of segregating populations derived from the symbiotic mutants of lines NF0063, NF2496, NF4608, and NF4928 points toward single, monogenic recessive mutations in each case (Supplemental Table S6). FST sequencing and gene-specific PCR analysis will be helpful to identify disrupted genes that may confer each mutant phenotype.
Although not exhaustive at this time, our FST collection from the 156 confirmed symbiotic mutant lines includes sequences of many nodule-expressed genes, as determined from the Gene Expression Atlas (Benedito et al., 2008), which are reasonable candidates in our search for new symbiotic genes (Supplemental Table S4). These include 24 nodule-specific FSTs, some of which correspond to known symbiotic genes such as NIN (nine alleles) and DMI3 (one allele). Eleven of the remaining FSTs point to interesting candidates for defective genes that could account for the associated Nod+ Fix−, Nod+ Fix+/−, and Nod+− Fix− phenotypes. They include genes for a Glc transporter in NF1285 (Medtr6g006140.1), a Ser/Thr protein kinase in NF0218 (Medtr8g088740.4), an endo-1,3(4)-β-glucanase 2 in NF1807 (Medtr7g013170.1), a hexose transporter in NF3151 (Medtr1g104780.1), and a phosphoinositide-phospholipase C in NF0217 and NF2496 (Medtr4g085800.1; the latter being from studies in the P. Ratet and M. Udvardi laboratories). Finally, FSTs were found in three nodule-specific genes in Nod− mutants that are being investigated in our laboratories: a cystathionine β-synthase domain-containing gene (Medtr6g052300.1) in line NF1391, a nodule-specific lipoygenase gene (Medtr8g018570.1) in NF0495, and a WRKY transcription factor 73 (Medtr7g009730.1) in NF0493.

Figure 4. Symbiotic phenotypes of the wild type (R108) and potentially new Nod− Tnt1-insertion mutants. Plants were inoculated with the S. meliloti Sm2011 strain containing the chimeric hemA::LacZ gene and grown for 21 d post inoculation on Turface: vermiculite (2:1). Samples were harvested at different time points after inoculation, histochemically stained for β-galactosidase activity, and visualized as whole mounts. Rhizobia stain blue. A and B, Wild-type R108 infection in nodule primordium (A) and mature nodule (B). Successful infection was visualized at 5 d post inoculation, with branching infection threads (arrow) into the nodule primordium (NP), and the mature nodule was visualized at 15 d post inoculation. C to E, Symbiotic phenotype in the mutant isolated from NF0438. C, Root segment with numerous infections and nodule primordia that do not develop into nodules after 21 d post inoculation. D, Closeup imaging reveals highly branched and interrupted infection threads (arrows) alternating with sac-like structures (arrowheads) inside roots hairs. E, Infection thread maintenance is disrupted (arrow) and rhizobia are prematurely released in epidermal cells (arrowheads). F, Rhizobial microcolonies are seen on the root surface of the mutant isolated from NF2629 in the absence of root hair infection and cortical cell division. G, Extensive and uncontrolled cell division is seen in the nodule window on roots of the mutant isolated from NF0359, while root hairs undergo deformation but no curling (arrows). H, Blue stain for β-galactosidase activity in the mutants of NF5794 delineates cell shapes, suggestive of intercellular infection. I, The only sites of β-galactosidase activity in the mutant isolated from NF2853 are where some lateral roots emerge. J to L, Symbiotic phenotypes of Nod− mutants isolated from NF1859 (J), NF0673 (K), and NF2811 (L) display infections arrested at the root epi- demis, cortical cell division, and nodule primordia development but no nodule morphogenesis at 21 d post inoculation. M, Nod− phenotype in NF5654 with arrested infections, extensive and irregular cortical cell division (arrows), and proliferation of the vascular tissue (seen also in the inset), which gives the appearance of nodule bumps. Accumulation of brown material, suggestive of defense-like responses, is seen in most mutants (C, G, and I-M, arrowheads). Bars = 100 μm.
DISCUSSION

Here, we have described a large set of novel Medicago symbiotic mutants that were isolated from a Tnt1-insertion population. Progeny from 230 lines with putative symbiotic mutants were phenotyped carefully, yielding 179 symbiotic mutants with defects in nodule development or function, including 72 Nod−, 51 Nod+ Fix−, 27 Nod+ Fix−, 17 Nod+ Fix−/, one dNod+ Fix−, and 11 Nod++ mutants. Past work on the genetics of SNF in M. truncatula and L. japonicus focused mainly on the molecular identification of genes required for nodule development, which resulted in the identification of at least 10 essential genes, DMI1/POLLUX, DMI2/SYMRK, DMI3/CCaMK, NFP/NFR5, LYK3/NFR1, NSP1, NSP2, NUP85, NUP133, ERN1, NIN, and LIN/CERBERUS, and two, SKL and SUNN, that are required for negative feedback regulation of nodule numbers (Schauser et al., 1999; Éndre et al., 2002; Oldroyd and Long, 2003; Radutoiu et al., 2003; Ané et al., 2004; Mitra et al., 2004b; Schnabel et al., 2005; Arrighi et al., 2006; Kanamori et al., 2006; Andriankaja et al., 2007; Marsh et al., 2007; Middleton et al., 2007; Saito et al., 2007; Penmetsa et al., 2008; Kiss et al., 2009; Yano et al., 2009). To gauge the extent of mutagenesis in the Medicago Tnt1-insertion population, we focused on the Nod− and Nod++ mutants and determined how many of the known nodulation genes were represented by Tnt1-insertion alleles in these two sets of mutants. We tested the following genes: NFP, DMI1, DMI2, DMI3, NSP1, NSP2, NIN, ERN1, SUNN, and SKL. F斯塔nd PCR-based analyses of Nod− and Nod++ mutants revealed Tnt1 insertions in all but one of these, NFP (Fig. 3; Supplemental Table S1). The number of insertion alleles for each nodule gene ranged from one to 13, with an average of four alleles per gene. These results indicate near-saturation

Figure 5. Examples of AM phenotypes in Tnt1-insertion symbiotic mutants. Tnt1-insertion mutants defective in nodulation (Nod−) were inoculated with a mixture of soil and G. intraradices- and G. mossae-colonized root pieces. To visualize fungal structures (Arb, arbuscles; h, hyphae; v, vesicles), roots were cleared with 10% KOH at 95°C and subsequently stained with trypan blue. The wild-type Myc+ phenotype is shown in R108 (A). Myc+ phenotypes were also observed in NF0825 (C), which carries a Tnt1 insertion in NIN, and in NF0848 (D), which has a Tnt1 insertion in NSP1. Defective mycorrhization (Myc−) is seen in mutants NF0028 (B) and NF0814 (E), which have Tnt1 inserted in DMI3 and NSP2, respectively. An example of a Myc− mutant with no Tnt1 insertion linked to a known symbiotic gene is shown in F (NF0549). Bars = 100 μm.

Figure 6. Potentially new Fix− symbiotic mutants isolated from the Tnt1-insertion mutant population. Nodules of wild-type R108 (A) and Fix− mutants isolated from lines NF0063 (B), NF5039 (C), NF2496 (D), NF0342 (E), NF4608 (F), NF0440 (G), NF0134 (H), NF4928 (I), and NF1320 (J) were harvested from plants grown in Turface and Metro-Mix layers at 21 d post inoculation with Sm2011-hemA::LacZ. After 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining and clearing with 70% ethanol, nodules were sliced into 50-μm-thick sections and photographed. Rhizobia stain blue. Bars = 100 μm.
mutagenesis of Medicago genes in the Tnt1 population, in line with theoretical calculations and experimental data (Krysan et al., 1999; Tadege et al., 2009). Importantly, insertions in known nodulation genes account for less than one-half of the Nod− mutants that we isolated, which means that novel nodulation genes may be found in the remaining mutants. Alternatively, somatic embryogenesis, which was used to trigger Tnt1 retrotransposition during establishment of the mutant population, can also mobilize endogenous DNA transposons, as described in sugarcane (Saccharum officinarum; Suprasanna et al., 2010), and retrotransposons, such as MERE1-1 (Rakocevic et al., 2009). Thus, it is possible that some of the remaining Nod− mutants may be accounted for by non-Tnt1 mutant alleles of known nodulation genes. Genetic complementation testing will be required in the future to determine which of the remaining Nod− mutants mark novel nodulation genes. Additionally, not all the known genes that could cause Nod− phenotypes when mutated were tested for Tnt1 insertions by PCR reverse screening. While it is possible that some of the potentially new Nod− symbiotic mutants may be accounted for by insertions in untested genes such as LYK3, NUP85, and NUP133, it is unlikely that this is the case for all 41 mutants. In fact, the microscopic and histological phenotypes of some of the potentially novel Nod− mutants appear to be unlike any of the known Nod− mutants (Fig. 4), so it seems likely that novel nodulation genes will be discovered among the remaining 41 Nod− mutants.

Only a few genes have been cloned that are required for nodule function rather than nodule development per se (Kevei et al., 2007; Arrighi et al., 2008; Hakoyama et al., 2009, 2012; Kiss et al., 2009; Yokota et al., 2009; Haney and Long, 2010; Lefebvre et al., 2010; Miyahara et al., 2010; Wang et al., 2010; Yendrek et al., 2010; Horváth et al., 2011; Ovchinnikova et al., 2011). Although many Fix− and Fix+/− mutants have been described in the literature (Benaben et al., 1995; Bright et al., 2005; Starker et al., 2006; Teillet et al., 2008; Maunoury et al., 2010), many of these come from EMS populations. Map-based cloning of such mutant alleles remains arduous, despite complete or nearly complete genome sequences for the model species. From 44 of the mutant lines defective in nodule development and nitrogen fixation, we recovered 90 FSTs in nodule-expressed genes, 11 of which are nodule specific (Supplemental Table S4), and none of them correspond to known symbiotic genes. Indeed, many of the Fix− mutants exhibited microscopic phenotypes that have not been described before. FST sequences obtained from these mutants represent an obvious starting point for identifying and eventually cloning the underlying genes.

Nodulation mutants have been instrumental in the discovery of genes required for AM symbiosis, by virtue of the fact that Nod factor and AM symbioses share some common signaling, and other genes required for microbial colonization of plant cells (Catoira et al., 2000; Ané et al., 2004; Lévêque et al., 2004; Morandi et al., 2005). Interestingly, AM symbiosis defects (Myc− and Myc+/−) were found in 30 of the 39 Nod− Tnt1 mutants tested here (Supplemental Table S2). Twenty-one of these can be accounted for by known common symbiosis genes. Therefore, it is possible that novel common symbiosis genes will be discovered using the Tnt1 mutants described here.

Our current methods of recovering genomic sequences adjacent to sites of Tnt1 integration yield fewer than half of all Tnt1 insertion sites (Ratet et al., 2010; Cheng et al., 2011). This explains why more Tnt1 alleles of known nodulation genes were found in the Nod− and Nod++ mutants by PCR screening than by FST sequencing in this study. We are currently working on improving FST recovery from Tnt1 mutants, in part by utilizing next-generation sequencing. Recovery of FSTs associated with most, if not all, of the Tnt1 insertions in a given mutant will facilitate the identification of novel genes required for symbiosis.

Finally, the symbiotic mutants described here resulted from screening of less than one-half of the current Medicago Tnt1-insertion population, involving participation of researchers from twelve organizations (Supplemental Table S5). Annual community screening of the remaining mutant lines will uncover hundreds of additional symbiotic mutants, which will lead to the discovery of more genes required for Nod factor and AM symbioses.

MATERIALS AND METHODS
Plant Material
Tnt1 insertion mutants were regenerated by tissue culture from line Tnk88-7-7, as described previously (D’Errthly et al., 2003a; Tadege et al., 2008). Seeds (15–20 seeds per line) from putative symbiotic mutants isolated during research community screening workshops at the Samuel Roberts Noble Foundation and control R108 seeds were scarified with concentrated sulfuric acid, rinsed five times with sterile distilled water, and sterilized with a solution containing 30% commercial bleach and a few drops of Tween 20. Sterilized seeds were extensively rinsed with sterile water over a period of 5 to 6 h. Fully imbibed seeds were placed on sterile petri dishes and vernalized for 3 d at 4°C in the dark. Subsequent seed germination was carried out in the dark at room temperature. Seedlings with 1- to 2-cm-long radicles were planted into 7-cm Leach Cone-tainers (Stuewe & Sons) containing a bottom layer of sterilized vermiculite (Sun Gro Horticulture). To reevaluate nodule phenotypes of confirmed Nod− mutants, an alternative growth substrate consisting of a 2:1 mixture of Turface and vermiculite (Sun Gro Horticulture) was used. At the time of planting, seedlings were fertilized with one-half-strength B&O solution containing 2 mM KNO3 (Broughton and Dilworth, 1971).

Nodulation and Mycorrhization Assays
Five days after planting, each seedling was inoculated with a 50-mL suspension (optical density at 600 nm = 0.03) of Sinorhizobium meliloti strain Sm2011 carrying the hemA::lacZ reporter (Ardourel et al., 1994) prepared in one-half-strength B&O solution with 2 mM KNO3. No further fertilization was applied to inoculated plants prior to harvesting at 15 and 21 d post inoculation, although plants were watered regularly with deionized water. For mycorrhization assays, S. meliloti–inoculated mutants with confirmed Nod− phenotypes were transferred to a mixture of sand and TerraGreen (1:3) and were grown under a 16-h daylight regime. A mixture of soil and Glomus intraradices and Glomus mosseae–colonized root pieces was used as inoculum, as described previously (Reddy et al., 2007). Samples were analyzed at 2 and 4 weeks post inoculation.
Phenotypic Analysis, Histochemical Staining, and Imaging

At the time of harvesting, visible phenotypes were recorded as Nod−, Nod+ Fix−, Nod+ Fix+, Nod− Fix+, Nod+ Fix−/−, Nod+/−, Nod+/+ Fix−, and Nod++ Fix+/+ by comparing with the wild type and according to the criteria detailed in Table I. Whole-mount nodulated root images were captured using an Olympus SZX12 stereomicroscope equipped with a DXM1200 digital camera (Nikon Instruments). Histochemical staining with 5-bromo-4-chloro-3-indolylβ-D-galactopyranoside was performed as described previously (Boivin et al., 1990; Pislaru and Dickstein, 2007). The Rhizobium-infectible zone of root segments was visualized in whole mounts, while detached nodules were embedded in 5% low-staining with trypan blue. The presence of various fungal structures (hyphae, appressoria, arbuscules, and vesicles) was assessed using the gridline intersection method (Giovannetti and Mosse, 1980). A total of 100 intersections were observed per sample per time point. If a particular fungal structure was observed more than 60% of times, it was called present (Mycc+), and if it was observed less than 30% of times, it was called absent (Mycc−). The (Mycc+/−) designation is for the recognition of the respective fungal structure between 30% and 60% of times (Table I; Supplemental Table S2).

Photomicrographs were acquired using an Olympus BX41 compound microscope equipped with an Olympus DP72 camera. All digital micrographs were processed using Adobe Photoshop.

FST Recovery and PCR Reverse Screening

Recovery of FSTs adjacent to Tnt1 insertion sites was carried out using thermal asymmetric interlaced-PCR (Lu and Whittier, 1995; Cheng et al., 2011), touch-down-PCR, and inverse-PCR (Ratet et al., 2010), as described. All resulting PCR products were pooled for each Tnt1 line, purified with the PCR Purification Kit (Qiagen), and ligated into the pGEM-T Easy cloning vector for sequencing.

Supplemental Data

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

Supplemental Table S1. Nodulation symbiotic phenotypes in the Tnt1-insertion mutant collection.

Supplemental Table S2. AM phenotypes in confirmed Nod− mutants.

Supplemental Table S3. Primers used for PCR reverse screening.

Supplemental Table S4. Nodule-expressed candidates identified by FST sequencing in confirmed Tnt1-insertion mutants.

Supplemental Table S5. Participation of the research community in annual screenings for symbiotic mutants in the Medicago Tnt1-insertion population.

Supplemental Table S6. Segregation of symbiotic phenotypes in mutant × wild type (R108) crosses.

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