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ORIGINAL PAPER

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Characterisation of new symbiotic *Medicago truncatula* (Gaertn.) mutants, and phenotypic or genotypic complementary information on previously described mutants

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Abstract From a pool of *Medicago truncatula* mutants—obtained by gamma-irradiation or ethyl methanesulfonate mutagenesis—impaired in symbiosis with the N-fixing bacterium *Sinorhizobium meliloti*, new mutants are described and genetically analysed, and for already reported mutants, complementary data are given on their phenotypic and genetic analysis. Phenotypic data relate to nodulation and mycorrhizal phenotypes. Among the five new mutants, three were classified as [Nod^{+} Fix^{-} Myc^{+}] and the mutations were ascribed to two loci, *Mtsym20* (TRV43, TRV54) and *Mtsym21* (TRV49). For the two other new mutants, one was classified as [$Nod^{-/+}$ Myc^{+}] with a mutation ascribed to gene *Mtsym15* (TRV48), and the other as [Nod^{-} $Myc^{-/+}$] with a mutation ascribed to gene *Mtsym16* (TRV58). Genetic analysis of three previously described mutants has shown that [$Nod^{-/+}$ Myc^{+}] TR74 mutant can be ascribed to gene *Mtsym14*, and that [$Nod^{-/+}$ $Myc^{-/+}$] TR89 and TRV9 mutants are ascribed to gene *Mtsym2* (*dmi2*). Using a detailed analysis of mycorrhizal phenotype, we have observed a delayed typical arbuscular mycorrhizal formation on some mutants that present thick lens-shaped appressoria. This phenotype was called [$Myc^{-/+}$] and mutants TR25, TR26, TR89, TRV9, P1 and Y6 were reclassified as [$Myc^{-/+}$]. Mutant P1 was reclassified as [$Nod^{-/+}$] because of a late nodulation observed on roots of this mutant.

Keywords *Medicago truncatula* · Mutagenesis · *Sinorhizobium meliloti* · Mycorrhizal fungus · Symbiosis

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Introduction

More than 10 years ago, *Medicago truncatula* (Gaertn.) was proposed as a model plant for the genetic analysis of nodulation and other traits. This species is a diploid, $2n=16$, with a small genome, a high level of synteny with temperate legumes, and offers possibilities of regeneration from cell culture, and genetic transformation (Barker et al. 1990; Cook 1999).

As in other legume species, atmospheric nitrogen fixation takes place in *M. truncatula* root nodules resulting from a symbiotic interaction between host plant and *Sinorhizobium* spp bacteria. Nodules originate in root cortical cells of legume plants. Their development involves a series of steps commencing with the dedifferentiation of cortical cells and culminating in the formation of a functional nitrogen-fixing nodule. Nodule ontogeny is predominantly determined by the plant genome (Gresshoff 1993), but chemical signals provided by both the host plant and the symbiotic bacteria induce expression of specific genes in both partners (Verma 1992; Dénarié and Cullimore 1993; Wais et al. 2000). The dissection of such a complex biological process can be facilitated by the isolation of bacterial or plant mutants affecting the numerous steps in the cascade of events leading to nodule development and functioning. This strategy has been recently illustrated by the isolation of three mutated genes in *M. truncatula*, involved both in rhizobium and mycorrhizal symbioses, *dmi1* (Ané et al. 2004), *dmi2* (Endre et al. 2002) and *dmi3* (Lévy et al. 2004). Plant mutants with altered rhizobium symbiosis characteristics have been identified in studies of spontaneous or induced mutations of several legume species such as pea (*Pisum sativum*), faba bean (*Vicia faba*), soybean (*Glycine max*), alfalfa (*Medicago sativa*), chickpea (*Cicer arietinum*), and *Lotus japonicus* (for examples, see: Carroll et al. 1985; Duc and Messager 1989; Egli et al. 1989; Duc 1995; Szcyglowski et al. 1998; Paruvangada and Davis 1999). Complementation tests in pea have identified more than 30 loci determining a modified nodulation phenotype (M. Sagan and G. Duc, unpublished data). Some of these mutations in pea also modify features of another

important root symbiosis based on mycorrhizal fungi (Duc et al. 1989).

A mutagenesis program using gamma rays led to the isolation of non-nodulating [Nod^-], nodulating but non-fixing [Nod^+ Fix^-] and supernodulating [Nod^{++}] *M. truncatula* mutants from cv. Jemalong line J5 (Sagan et al. 1998). Further screening of the same mutant population has now yielded additional mutants. Another ethyl methanesulfonate (EMS) mutagenesis program on *M. truncatula* (Penmesta and Cook 2000; Catoira et al. 2000) has also yielded several symbiotic mutants. In this study, we report on: (1) the phenotypic description and genetic analysis of five new symbiosis mutants: TRV43, TRV48, TRV49, TRV54, TRV58; (2) the genetic analysis of three mutants already phenotypically described: TR74, TR89, TRV9 (Sagan et al. 1998), and (3) complementary data on phenotypic description, mainly on mycorrhizal characterisation of seven previously described mutants: TR25, TR26, TR89, TRV9, TRV25 (Sagan et al. 1995, 1998), P1, and Y6 (Catoira et al. 2000).

Materials and methods

Plant material

Apart from P1 and Y6, all *M. truncatula* mutant lines studied originate from a set of nodulation mutants obtained after gamma ray irradiation in cv. Jemalong (line J5). The mutagenesis treatment procedure has been described previously (Sagan et al. 1995). The mutants detected in the M2 generation were further selfed for three generations and only stabilised mutants with uniform mutant phenotypes in the M5 generation were kept for this work. Mutants coded as TRx were isolated from distinct M2 progenies, whereas mutants coded as TRVx were isolated from a pool of M2 progenies. P1 and Y6, kindly provided by C. Gough and J. Dénarié (INRA-CNRS Toulouse, France), originate from EMS-mutagenised seeds of Jemalong (Penmetsa and Cook 2000) and have been previously characterised (Catoira et al. 2000).

Characterisation of nodulation phenotype

Seeds of the different lines and their crosses were scarified, surface-sterilised, and sown in plastic trays on sterilised sand. They were inoculated twice with *Sinorhizobium meliloti* strain 2011, once just after sowing and again 2 days later. Plants were grown in an insect-proof greenhouse with a 15 h day photoperiod, a minimum day temperature of 18°C, and a minimum night temperature of 15°C. Ventilation was assured for temperatures higher than 24°C. Plants were irrigated daily with a nitrogen-free nutrient solution containing (in mg l^{-1}): PO_4H_2 K, 584; SO_4K_2, 348; $MgSO_4 \cdot 7H_2O$, 246; H_3BO_3, 2; $MnSO_4 \cdot H_2O$, 1.8; $ZnSO_4 \cdot 7H_2O$, 0.22; $CuSO_4 \cdot 5H_2O$, 0.1; MoO_3, 0.83; and 60µl l^{-1} MASQUOL-ATE Fe 2500 (Synthron, Swillington, UK) (2.5% Fe). The growth substrate used was sand as described previously (Sagan et al. 1998). Following the procedure of visual observation of the root system described in this previous work, plants were screened at 5 and 10 weeks after sowing. In comparison with the wild-type [Nod^+ Fix^+] phenotype, characterisation led to the classification of mutant phenotypes as [Nod^-] (absence of visual nodule emergence on roots at 5 and 10 weeks), [$Nod^{-/+}$] (late nodulation observed at 10 weeks), and [Nod^+ Fix^-] (numerous small and pale nodules at 5 weeks, inefficient in fixing N_2).

Characterisation of mycorrhizal phenotype

Seeds of *M. truncatula* were scarified for 2 min in 98% sulphuric acid, rinsed five times with water and surface disinfected for 10 min in 3.5% calcium hypochlorite. After rinsing five times with water, seeds were germinated for 2 days at 20°C in the dark, on Petri dishes containing 0.3% agar. Seedlings were then transferred to 200 ml pots containing a growth substrate composed of a mixture of 2/3 (v/v) gamma-irradiated clay loam soil (from Domaine d'Epoisses, INRA Dijon) and 1/3 (v/v) sand containing high-density mycorrhizal propagules of *Glomus intraradices* (Nicol. & Gerd.) Gerd. & Trappe or *Glomus mosseae* Nicol. & Gerd.. Plants were watered every day. These conditions have been optimised to allow a rapid and intense mycorrhizal colonisation. Roots were collected for estimation of mycorrhizal colonisation after 2 and 4 weeks of growth and were stained with Trypan blue (Phillips and Hayman 1970). Mycorrhizal colonisation was estimated following two steps:

1. The stained entire root system for each plant was observed under a stereomicroscope to evaluate the global quality of the colonisation and the presence of atypical arbuscular mycorrhizal (AM) structures. When abnormal AM colonisation was reported, the corresponding root sample was processed further in step 2.
2. Thirty root segments of 1 cm length were randomly sampled for each root system. A stereomicroscope at ×40 magnification, giving a field corresponding to half a root segment was used in order to have a set of two measurements for each root fragment and a set of 60 measurements per root system. For each microscope field, mycorrhizal root length intensity were evaluated using 11 classes (from 0 to 10) and was noted l_1 for field 1, l_2 for field 2,…l_n for field n; the percentage of cortical cells containing arbuscules was evaluated using six classes (from 0 to 5) and was noted a_1 for field 1, a_2 for field 2,…a_n; For each root sample, we calculated: FI, the frequency of infection, $FI=f_1+f_2+\ldots+f_{60})10/60$ where $f_n=1$ if $l_n>0$ and $f_n=0$ if $l_n=0$.; LI, the percentage of mycorrhizal intensity in length, $LI=(l_1+l_2+\ldots+l_{60})$ 10/60; AI the percentage of cortical cells containing arbuscules, $AI=(a_1l_1 + a_2l_2 +\ldots+ a_{60}l_{60})20/60$.

Genetic analysis

After screening, the M5 plants selected for nodulation defects were potted individually and grown in a nutrient solution containing 13 meq l^{-1} mineral nitrogen. These plants were crossed with the wild type line J5, and the resulting F1 and F2 progenies were grown and characterised for their nodulation phenotype. The mutants were grouped into classes according to their nodulation phenotype and different mutants within a mutant class were crossed in all possible combinations in order to identify the complementation groups. A wild phenotype in the F1 progeny from two recessive mutants indicates complementation and, conversely, mutations were considered allelic when inter-crosses yielded a mutant phenotype. All possible combinations of crosses were not achieved: reference mutants [Nod^-] (TR25, TRV25) and [Nod^+ Fix^-] (TR3, TR36, TR183) (Sagan et al. 1998) were used to represent already identified complementation groups, and as soon as a mutant was ascribed to a given group of complementation, only one mutant was kept as representative of this group for further tests of the new mutants.

Results

The efficiency of the gamma ray mutagenesis procedure that produced this set of new mutants impaired in symbiosis with *S. meliloti*, has been reported previously (Sagan et al. 1995, 1998). In contrast with the reference line J5, leaves of all mutants show yellowish symptoms of nitrogen starvation 5 weeks after sowing and inoculation with *S. meliloti* strain 2011 when grown under conditions lacking mineral nitrogen. These symptoms disappear in the presence of an adequate mineral nitrogen source and such a treatment allows these mutants to grow to maturity and set viable seeds.

Phenotypic characterisation of mutants

Classification of mycorrhizal colonisation

Among the mutants tested, we observed three different phenotypes of mycorrhizal colonisation. Phenotype 1 was similar to the wild type J5. All mutants expressing this phenotype were called [Myc^+]. Phenotype 2 consisted of formation only of appressoria with no intercellular hyphae developing from them. These typical thick lens-shaped appressoria have been described previously (Sagan et al. 1998; Calantzis et al. 2001). All mutants expressing this phenotype were called [Myc^-]. Phenotype 3 showed these typical appressoria (thick lens-shaped with no intercellular hyphae developing from them) and normal appressoria leading to the formation of arbuscules on the same root system. The proportion of arbuscule formation varied depending on the mutants, the mycorrhizal fungus and on the time after inoculation. All mutants expressing this phenotype were called [$Myc^{-/+}$].

Mutant [Myc^-]

TRV25 was the only mutant of our study belonging to the mycorrhizal phenotype 2, presenting only lens-shaped appressoria with no further development of AM colonisation, regardless of the time after inoculation or the mycorrhizal fungus involved (Table 1). This mutant expresses complete absence of visual nodules on the roots after inoculation with *S. meliloti* strain 2011, when observed at 5 and 10 weeks after sowing and is therefore classified as [Nod^-].

Mutant [$Myc^{-/+}$]

TR25, TR26, TRV58 and Y6, which belong to mycorrhizal phenotype 3, were classified as [Nod^-]. TR89, TRV9, and

Table 1 Colonisation parameters of *Medicago truncatula* mutants and the wild-type J5, 2 and 4 weeks after inoculation with *Glomus mosseae* (*Gm*) or *Glomus intraradices* (*Gi*). *FI* Frequency of infection, *LI* percentage of root length colonised with typical AM structures, *AI* percentage of cortical cells containing arbuscules. Data represent means of three replicates. For each mycorrhizal parameter, means followed by different letters are significantly different according to Tukey-Kramer statistical analysis at $P \leq 0.05$

		J5	TR25	TR26	TR89	TRV9	TRV25	TRV58	P1	Y6
FI										
2 weeks	Gm	88 h	0 a	0 a	21 c	22 c	0 a	0 a	20 c	0 a
	Gi	90 h	28 cd	30 d	51 ef	48 ef	0 a	18 c	52	20 c
4 weeks	Gm	100 i	3 b	4 b	53 f	49 ef	0 a	2 b	41 e	2 b
	Gi	100 i	36 de	34 de	62 fg	64 g	0 a	32 de	58 fg	33 de
LI										
2 weeks	Gm	64 j	0 a	0 a	16 de	19 ef	0 a	0 a	18 def	0 a
	Gi	71 j	12 de	13 de	24 ef	19 def	0 a	10 d	23 ef	11 d
4 weeks	Gm	83 k	3 c	3 c	40 gh	39 g	0 a	2 bc	36 g	1 b
	Gi	99 l	28 fg	25 f	51 i	50 hi	0 a	19 ef	48 hi	27 fg
AI										
2 weeks	Gm	52 m	0 a	0 a	9 de	10 def	0 a	0 a	8 c	0 a
	Gi	60 m	8 cd	7 cd	16 f	14 ef	0 a	6 c	17 fg	9 cde
4 weeks	Gm	72 n	1 b	2 b	30 ik	28 hi	0 a	1 b	25 ghi	1 b
	Gi	90 o	20 gh	18 fg	43 lm	38 kl	0 a	10 def	38 kl	22 ghi

P1, which also belong to mycorrhizal phenotype 3, were scored as [Nod$^-$] at 5 weeks, but developed a single or several nodules 10 weeks after sowing in greenhouse culture, in the presence of *S. meliloti* and absence of mineral nitrogen. For this reason, they were then classified as [Nod$^{-/+}$]. The few nodules were pinky, normally shaped and have nitrogen-fixing ability, as confirmed by the fact that plants became green again at the end of flowering.

Detailed observation of mycorrhizal phenotype of the two sets of mutants of type 3, TR25, TR26, TRV58 and Y6 on the one hand, and TR89, TRV9 and P1 on the other, are described in Table 1. They appear to belong to two different mycorrhizal subgroups: for each mycorrhizal parameter FI, LI and AI, for each fungus and for each time after inoculation, one mutant from one subgroup is significantly different to any one mutant from the other subgroup, but not significantly different to any one mutant inside the subgroup (see Table 1). For the first subgroup, formation of typical AM structures is less intense than in the second subgroup. Interestingly, this is related to rhizobium symbiosis, since the first subgroup is [Nod$^-$], while the second is [Nod$^{-/+}$].

For all the mutants belonging to mycorrhizal phenotype 3, the mycorrhizal fungus *G. intraradices* forms significantly much more typical AM structures than *G. mosseae*.

Mutant [Myc$^+$]

TR74 and TRV48 were identical to J5 and classified as mycorrhizal phenotype 1. They form late nodules and are therefore classified as [Nod$^{-/+}$], but the few nodules produced are not able to fix nitrogen. On TR74, nodule development is blocked at an early stage—beginning of nodule emergence on roots—according to the nodule development stages described previously in *P. sativum* (Sagan and Gresshoff 1996). At this stage, nodules show differentiation of the peripheral tissue containing the initiation of vascular bundles. In TRV48, the stage of blockage of nodulation is intermediate, with late but regular emergence of nodules.

Mutant TRV43, TRV49 and TRV54 were also recorded with mycorrhizal phenotype type 1. They expressed numerous small, pale, non nitrogen fixing nodules, which could be observed as soon as 5 weeks after sowing. They were classified as [Nod$^+$ Fix$^-$] phenotype.

Genetic analysis of the mutants

F1 generation

The analysis of F1 progenies from mutants × J5 and J5 × mutant crosses was performed in the presence of a nitrogen-free nutrient solution in order to maximise expression of nodulation phenotype. For all mutants studied, the F1 plants were indistinguishable from the wild-type parental line J5, indicating for all loci a full dominance of the wild type allele (Table 2).

F2 generation

The distribution of F2 plants by nodule type yielded two classes resembling the parental phenotypes. The F2 progenies obtained from reciprocal crosses obtained for each mutant studied were comparable in their distribution. The chi-square tests on bulked data from reciprocal crosses did

Table 2 Inheritance of mutant phenotypes after crosses with parental line J5. Number of plants with wild and mutant phenotype in F1 and F2 generation. Results of $\chi 2$ test of 1:3 (mutant:wild distribution) in F2 are given

Mutant	Phenotype	Generation	Number of crosses	Wild	Mutant	% Mutants	$\chi 2$ (1/3)	*P*
[Nod$^-$] and [Nod$^{-/+}$] mutants								
TR74	Nod$^{-/+}$ Myc+	F1	2	5	0			
		F2	2	196	68	25.76	0.081	>0.5
TR89	Nod$^{-/+}$ Myc$^{-/+}$	F1	3	12	0			
		F2	3	397	141	26.21	0.419	>0.5
TRV9	Nod$^{-/+}$ Myc$^{-/+}$	F1	4	22	0			
		F2	4	427	145	25.35	0.037	>0.5
TRV48	Nod$^{-/+}$ Myc$^+$	F1	7	29	0			
		F2	4	254	61	19.36	5.33	>0.1
TRV58	Nod$^-$ Myc$^{-/+}$	F1	5	9	0			
		F2	4	279	78	21.87	1.89	>0.5
[Nod$^+$ Fix$^-$] mutants								
TRV43	Nod$^+$ Fix$^-$ Myc$^+$	F1	6	14	0			
		F2	4	107	29	21.32	0.980	>0.5
TRV49	Nod$^+$ Fix$^-$ Myc$^+$	F1	6	17	0			
		F2	4	20	11	35.48	1.816	>0.2
TRV54	Nod$^+$ Fix$^-$ Myc$^+$	F1	6	6	0			
		F2	4	204	72	26.08	0.17	>0.5

Table 3 Inheritance of non-nodulating and late nodulating mutant phenotypes after reciprocal crosses: allelism test on [Nod$^-$] and [Nod$^{-/+}$] mutant lines. + Wild phenotype, − mutant phenotype, (number of F1 plants)

Cross	Phenotype	TR25	TRV25	TR74	TRV48
TR89	Nod$^{-/+}$ Myc$^{-/+}$	−(44)	+(19)	+(19)	
TR74	Nod$^{-/+}$ Myc$^+$	+(21)	+(13)		
TRV9	Nod$^{-/+}$ Myc$^{-/+}$	−(2)	+(13)	+(10)	
TRV48	Nod$^{-/+}$ Myc$^+$	+(29)	+(21)	+(30)	
TRV58	Nod$^-$ Myc$^{-/+}$	+(13)	+(17)	+(6)	+(18)

not reject the 3:1 hypothesis, indicating a monogenic recessive determination of all eight mutant nodulation traits under investigation (Table 2).

Complementation tests

The mutants were split into two groups: (1) non-nodulating mutants [Nod$^-$] or mutants having late developing nodules [Nod$^{-/+}$], and (2) nodulating, non-fixing lines [Nod$^+$ Fix$^-$]. In each of these groups, reciprocal crosses were performed, and the results are presented in Tables 2 and 3.

For [Nod$^-$] or [Nod$^{-/+}$] mutants, four loci were deduced (Table 3). Each locus includes one or several mutant alleles as follows:

Mtsym2 [Nod$^{-/+}$] phenotype, includes alleles of TR89 and TRV9. This group also includes already describes alleles TR25 and TR26 as complementation group A (Sagan et al. 1998)
Mtsym14 [Nod$^{-/+}$] phenotype, includes allele of TR74
Mtsym15 [Nod$^{-/+}$] phenotype, includes allele of TRV48
Mtsym16 [Nod$^-$] phenotype, includes allele of TRV58

These four loci are distinct from the already described TRV25 locus [complementation group B (Sagan et al. 1998) and *dmi3* (Catoira et al 2000)] that we designate *Mtsym13*.

For [Nod$^+$ Fix$^-$] mutants, two new loci were identified (Table 4), each locus including one or several mutant alleles as follows:

Mtsym20 Includes alleles of TRV43 and TRV54
Mtsym21 Includes allele of TRV49

These two loci are distinct from already described groups (Sagan et al. 1998). In this previous work, TR3, TR36 and TR183 were ascribed to groups C, D and E, respectively, that we now name *Mtsym17*, *Mtsym18* and *Mtsym19*.

Table 4 Inheritance of ineffective nodulated mutant phenotype after reciprocal crosses: allelism test on Fix-mutants lines. + Wild phenotype, − mutant phenotype, (number of F1 plants)

Cross	TR3	TR36	TR183	TRV43
TRV43	+(8)	+(13)	+(22)	
TRV54	+(5)	+(15)	+(6)	−(7)
TRV49	+(20)	+(16)	+(17)	+(17)

Discussion

From a pool of previously induced nodulation mutants obtained after gamma-irradiation treatment in *M. truncatula* cv. Jemalong-line J5, we have analysed mutants that form ineffective nodules, that have late nodule development, or no nodules at all. We emphasise that mutants coded as TRx carry true independent mutations isolated from distinct M2 progenies, whereas mutants coded as TRVx were isolated from a pool of M2 progenies and consequently their independency has still to be checked.

Phenotypes of F1 progenies between mutant and wild type J5 plants, and the pattern of segregation of F2 families, show that each mutant is determined by a recessive allele at a single gene with observable Mendelian inheritance. The same situation is described in the literature for numerous induced symbiotic mutants of different species.

Genetic analysis of five new, and three already described mutants, has shown that several loci are involved in controlling nodulation initiation, development and fixative function. The five mutated alleles responsible for [Nod$^-$] or [Nod$^{-/+}$] phenotypes in these *M. truncatula* mutants were assigned to four loci. Locus *Mtsym2* was something of a "hot spot" mutation providing two of these alleles plus the previously analysed mutants TR25, TR26 (Sagan et al. 1998) and P1 (Catoira et al. 2000), which were shown to belong to the same complementation group (named A and *Dmi2*, respectively, in these latter papers).

The three mutated alleles responsible for the [Nod$^+$ Fix$^-$] phenotype were assigned to two loci we called *Mtsym20* and *Mtsym21*. For this phenotype, these genes can be added to the previously reported complementation groups C, D, and E (Sagan et al. 1998). We assign the names *Mtsym17*, *Mtsym18*, *Mtsym19*, respectively, to the corresponding genes. Table 5 gives a summary of the results obtained to date for the gamma mutagenesis program performed on *M. truncatula*. Ten loci have already been identified that result in impaired rhizobium symbiosis. As a comparison, a mutagenesis program performed on pea (INRA, Dijon, France), has identified 19 mutated loci for impaired rhizobium symbiosis. The fact that a 3-fold larger M2 population was screened in pea may easily explain the different number of mutants between the two mutagenesis programs for the two species.

As was discovered in pea (Duc et al. 1989), some *M. truncatula* mutants have been shown to have a joint depressive effect on rhizobium and mycorrhizal symbioses (Sagan et al. 1998) demonstrating that common genes are involved in both symbioses. The present work brings additional data, showing that at least three loci in *M. truncatula*, *Mtsym2*, *Mtsym13* and *Mtsym16*, exert joint control over rhizobium and mycorrhizal symbioses. In pea, at least four loci inducing such a symbiotic phenotype are presently available (Gianinazzi-Pearson et al. 1991; G. Duc and M. Sagan, unpublished data).

Table 5 Summary of complementation groups and phenotypes of symbiosis-defective mutants from the gamma-ray mutagenesis program (Sagan et al. 1995, 1998) of *M. truncatula* cv Jemalong line J5. In addition, phenotypic complementary information is given in this study on two previously described *M. truncatula* mutants (Catoira et al. 2000) from ethyl methanesulfonate (EMS) mutagenesis: P1 [$Nod^{-/+}$ $Myc^{-/+}$] (*dmi2*), Y6 [Nod^{-} $Myc^{-/+}$] (*dmi1*)

Name of gene (synonyme)	Phenotype		Name of mutants
	Nod	Myc	
Mtsym2 (*dmi2*)	[−]	[−/+]	TR25[a], TR26[a]
	[−/+]	[−/+]	TR89[a,b], TRV9[a,b]
Mtsym13 (*dmi3*)	[−]	[−]	TRV25
Mtsym14	[−/+]	[+]	TR74[b]
Mtsym15	[−/+]	[+]	TRV48[c]
Mtsym16	[−]	[−/+]	TRV58[c]
Mtsym17	[+] Fix−	[+]	TR3, TR9, TR13, TR62, TR69, TR79, TRV15
Mtsym18	[+] Fix−	[+]	TR36
Mtsym19	[+] Fix−	[+]	TR183
Mtsym20	[+] Fix−	[+]	TRV43[c], TRV54[c]
Mtsym21	[+] Fix−	[+]	TRV49[c]

[a]Phenotypic complementary information in this study
[b]Genetic complementary information in this study
[c]New description of mutant in this study

The present report provides more details on the mycorrhizal phenotypes of these mutants regarding the quantitative or qualitative aspects of the symbiotic defect. At the *Mtsym2* locus, TR25, TR26 and P1 mutants were previously described as [Myc^{-}] (Sagan et al. 1998; Catoira et al. 2000) and TR89 and TRV9 as [Myc^{+}] (Sagan et al. 1998). In this work, we observed a [$Myc^{-/+}$] phenotype for all of these latter mutants, with a higher proportion of cortical cells containing arbuscules for TR89, TRV9 and P1 than for TR25 and TR26. This different result may be attributed to (1) the use of a very "efficient" inoculum in this new experiment, especially *G. intraradices*, which gives a very rapid and intense colonisation thus facilitating visualisation of arbuscules, (2) the fact that, in previous observations, the "search image" was different, since the concept of [$Myc^{-/+}$] had not been defined, so the observation of arbuscules in TR89 and TRV9 led us to classify their phenotype as [Myc^{+}] without requiring the presence of lens-shaped appressoria.

It is interesting to point out that typical AM structures are significantly more intense on [$Myc^{-/+}$] mutants when colonised with *G. intraradices* than with *G. mosseae*, although this difference is much less pronounced on the wild-type J5. This indicate that the mutants do not have exactly the same behaviour depending on the mycorrhizal fungus, and reinforces the finding that colonisation patterns in mycorrhiza-defective mutants could vary when inoculated by different species of AM fungi (Gao et al. 2001).

This work has detected leaky phenotypes for both symbioses, which brings a warning about accurate phenotyping and adequate phenotypes and gene nomenclature. Such phenomena may explain some discrepancies in the literature on the characterisation of symbiotic mutants. Our results emphasise the value of precise quantitative parameters to measure the status of mycorrhiza colonisation, evaluated according to strain and environment effects at different stages of plant development. The same concern applies to rhizobium symbiosis. For example, the P1 mutant previously recorded as [Nod^{-}] (Catoira et al. 2000) was reclassified as [$Nod^{-/+}$] in this study.

Other mutagenesis programs are presently being conducted in several laboratories searching for symbiotic mutants of *M. truncatula*. They have yielded [Nod^{-}] mutant phenotypes similar to those described in this work. With its solid [Nod^{-} Myc^{-}] phenotype, TRV25 is presently a unique mutant at a locus we call *Mtsym13* [previously named B (Sagan et al. 1998) or *Dmi3* (Catoira et al. 2000)].

Clarification and standardisation of the nomenclature of phenotypes and mutated loci already published appears necessary. The complexity is illustrated by the fact that in a recent review (Marsh and Schultze 2001), different stages of mycorrhizal colonisation for the Myc^{-} phenotype are defined. In our study, we introduce the concept of phenotypic "instability", which leads us to name [$Myc^{-/+}$] or [$Nod^{-/+}$] the symbiotic phenotypes. There is also a need to confront mutants from different mutagenesis programs. Whether [Nod^{-} $Myc^{-/+}$] *Mtsym16* group of allelism (Table 5) corresponds to the *Dmi1* group to which Y6 belongs (Catoira et al. 2000), and whether [Nod^{-} Myc^{+}] groups *Mtsym14* and *Mtsym15* correspond to the NSP and *HCL* groups published previously (Catoira et al. 2000, 2001), remains to be explored.

In addition to the [Nod^{-}] or [$Nod^{-/+}$] mutants, the discovery of two new loci conferring a [Nod^{+} Fix^{-} Myc^{+}] phenotype enlarges an attractive resource for the analysis and isolation of genes implicated in the establishment and functioning of root symbioses. Their identification in the model species *M. truncatula* will help their discovery in other legumes. This strategy recently proved efficient in isolating gene homologous to pea *dmi3* (Lévy et al. 2004).

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