A New *Medicago truncatula* Line with Superior in Vitro Regeneration, Transformation, and Symbiotic Properties Isolated Through Cell Culture Selection

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The understanding of how leguminous plants establish symbiosis with rhizobia is limited by the lack of genetic system in most legume plants. Here we propose a *Medicago truncatula* line suitable for genetic analysis of *Medicago-Rhizobium meliloti* symbiosis because of its high regeneration capacity and broad *R. meliloti* strain specificity. This line has been isolated by extensive in vitro screening of explants from several ecotypes of *M. truncatula* that are known to be autogamous and have small diploid genomes. One such derivative identified from ecotype 108-1 has gained the capacity to be readily regenerated in vitro. The derivative, called R108-1, like its parent, can establish effective symbiosis with several widely studied *R. meliloti* strains (Rm41, Rm2011, Rm1021, F51, and GR4). Importantly, the nodulation characteristics of the R108-1 line were not compromised by the in vitro selection procedure. We have further established the conditions for efficient transformation of R108-1 by co-cultivation with *Agrobacterium tumefaciens*. Thus, this in vitro-derived plant line has most of the favorable attributes, otherwise rare among legumes, of a complementary model plant system for investigating symbiosis at the genetic and the molecular levels.

Legumes are protein-rich plants widely cultivated for food and forage. The growth of legumes is independent of an external supplement of reduced nitrogen, because of the fixation of dinitrogen in symbiosis with rhizobia. This phenomenon is widespread, as 90% of the 3,000 legume species surveyed were reported to form nitrogen-fixing nodules (Allen and Allen 1981), and thus the ecological and agricultural importance of this process has fueled much of the incentive to study plant-microbe relations.

The development of nitrogen-fixing symbiosis is a complex process determined by both partners. One of the most extensively characterized systems, from the viewpoint of the bacterial partner, is the symbiosis between *Rhizobium meliloti* and

Medicago sativa (Hirsch 1992; Schultze et al. 1994). Many symbiotic genes of the bacterium, including all of the nodulation genes, have been characterized. Much is also known about the early bacterial signals, especially the Nod factors, in triggering nodule organogenesis in the roots of the host plant (Lerouge et al. 1990; Schultze and Kondorosi 1995; Truchet et al. 1991). Complementary research on the plant has been hindered by the lack of a genetic model. The perennial cultivated alfalfa is poorly suited for this purpose: it is a tetraploid; it is allogamous, with varying degrees of self-incompatibility; and it has a large genome, rendering the identification of recessive mutations and the cloning of their corresponding genes an arduous task. These drawbacks have led to the proposal of a diploid autogamous annual *Medicago* species, *M. truncatula* Gaertn. ecotype Jemalong, as a nominal model plant for genetic and molecular analyses of symbiosis (Barker et al. 1990). From about 100 *M. truncatula* ecotypes, Jemalong was selected primarily because of its transformability and in vitro regeneration capacity (Barker et al. 1990; Nolan et al. 1989; Thomas et al. 1992), and methods for these steps have recently been optimized (Chabaud et al. 1996). Various *R. meliloti* strains, however, may exhibit distinct genotype or cultivar specificity. For example, Jemalong shows limitations in nitrogen-fixing symbiosis with some European and American strains of *R. meliloti* (Synman and Strijdom 1980), including *R. meliloti* strain 41, which is used in our laboratory.

Therefore, we have continued to search for other *M. truncatula* ecotypes that could establish symbiotic relations with the commonly used laboratory strains of *R. meliloti*. The availability of different ecotypes in addition to Jemalong would also add to the flexibility of mapping mutations by restriction fragment length polymorphism (RFLP), a prerequisite for isolating specific genes by chromosome walking.

The diploid *Lotus japonicus* 'Gifu,' amenable to *Agrobacterium tumefaciens*-mediated transformation, has also been advanced as a model leguminous plant (Handberg and Stougaard 1992). In contrast to *M. truncatula*, *L. japonicus* forms determinate nodules, and thus the two plants might differ in the molecular events governing nodule organogenesis. Also, *R. loti*, the bacterial symbiont of *L. japonicus*, has not been the subject of detailed molecular and genetic analysis, as *R. meliloti* has been. As knowledge of the genetics of *R. loti* gradually improves (López-Lara et al. 1995; Scott et al. 1996), two parallel systems could be envisaged for the analysis of sym-

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biosis, one for investigating the development of indeterminate nodules (*M. truncatula*) and the other for determinate nodules (*L. japonicus*).

We report here on a large and systematic in vitro screening of explants derived from *M. truncatula* ecotypes Ghor, 108-1, 131-1, 139-2, and E4258 and the isolation of a derivative line of 108-1, which has a greatly enhanced or de novo capacity to form somatic embryos and can be efficiently regenerated into diploid fertile plants. This derivative, R108-1, can enter effective symbiotic relations with a wide range of *R. meliloti* strains. We further developed an efficient transformation procedure for R108-1 based on *Agrobacterium*-mediated gene transfer. Thus, the R108-1 line could be a valuable and complementary model plant accessible to both genetic and molecular techniques for the study of symbiosis.

RESULTS

General characteristics of *M. truncatula* ecotypes selected as potential candidates.

M. truncatula ecotypes 108-1, Ghor, 131-1, 139-2, and E4258 were chosen because of their low nuclear DNA content, about 0.5 pg per 1C nucleus (Blondon et al. 1994), or three times that of the haploid complement of the *Arabidopsis* genome (Bennett and Smith 1976); they are diploid ($2n = 16$) and self-fertilizing, thus producing remarkably uniform genetic lines. Mature plants are between 50 and 100 cm tall and 20 to 25 cm wide and can be grown at higher density. They grow well under standard greenhouse conditions (24 to 28°C during the day and 17°C at night, with a 16-h photoperiod and 30 to 60% humidity), in either soil or vermiculite, and 2,000 to 3,000 seeds per plant can be readily obtained. The seed-to-seed generation times of these ecotypes range from about 9 weeks (for Ghor) to about 12 to 14 weeks (for 108-1, 131-1, 139-2, and E4258), which under our experimental conditions are shorter than that of Jemalong. Seeds kept dry can be stored at 4°C for at least several years and still germinate at high frequency after disruption of the seed coat with sulfuric acid. Seeds of these ecotypes, like those of Jemalong (Barker et al. 1990), are sufficiently small (2 to 3 mm) that plantlets can be cultured aseptically in test tubes, allowing convenient nodulation assays and screening for early symbiotic mutant phenotypes in large numbers. All of these ecotypes—except E4258—can establish symbiotic relations with at least five different wild-type laboratory strains of *R. meliloti* (Rm41,

Rm2011, Rm1021, F51, and GR4) (see Materials and Methods; data not shown). Rm2011 was previously used as a symbiotic partner of Jemalong (Barker et al. 1990). The exception mentioned above, *M. truncatula* E4258, can form effective symbiosis only with Rm1021, F51, and GR4 (data not shown), and not with Rm41 or Rm2011 (see below).

In vitro selection of a plant line with high regeneration capability.

Since nothing was known concerning the potential in vitro regenerative capacity of these ecotypes, we initially tested explants from all five ecotypes under the in vitro conditions described for Jemalong (Nolan et al. 1989; Thomas et al. 1992) but were not successful. We thus systematically screened for somaclonal variants with acquired regenerative capacity by testing explants from each ecotype on at least 60 different combinations of synthetic media and culture conditions (data not shown). Briefly, brown calli were eventually recovered from one petiole and one root explant derived from ecotype 108-1, which had been cultured in the dark on SH basal medium (Schenk and Hildebrandt 1972) containing high concentrations of 2,4-D, similar to the proembryogenic callus induction (PCI) media described in Table 1 (data not shown). The calli were multiplied and were used as the source to establish subsequent steps in regenerating fertile plants by similar empirical tests under a range of culture conditions (data not shown; see Fig. 1A–D). Of more than 200 primary regener-

Table 1. Media used for different steps in plant regeneration of *Medicago truncatula* line R108-1^a

	PCI-4	PCI-1	EI	ED	SD	RF
Macronutrients	N6	N6	N6	N6	N6	0.5× SH
Micronutrients	SH	SH	SH	SH	SH	0.5× SH
Vitamins	SH	SH	SH	SH	SH	0.5× SH
2,4-D (mg/L)	4	1	...	...	...	...
NAA (mg/L)	...	...	...	0.01	...	...
BAP (mg/L)	0.2	0.2	...	1	...	...
Sucrose (g/L)	30	30	30	20	20	10
Phytigel (g/L)	3	3	3	...	...	...
Agar (g/L)	...	...	...	8	8	8
Augmentin (mg/L)	...	400	200	200	200	100

^a PCI-1 and PCI-4, proembryogenic callus induction media; EI, embryo induction medium; ED, embryo development medium; SD, shoot development medium; RF, root formation medium; SH, Schenk and Hildebrandt basal medium (Schenk and Hildebrandt 1972); NAA, α -naphthaleneacetic acid; BAP, 6-benzylaminopurine. Agar in all cases was kalys agar 575 (Magyoly Spindler, France); pH for all media was 5.8.

Fig. 1. Summary of steps in the regeneration of *Medicago truncatula* line R108-1 and its subsequent transformation by *Agrobacterium tumefaciens*-mediated gene transfer. **A**, Somatic proembryos induced from calli cultured on proembryogenic callus induction medium 1 (Table 1). **B**, Mature embryos subsequently induced by culturing explants on embryo induction medium without hormones for 3 weeks. **C**, Shoots from mature embryos cultured on shoot development medium. **D**, Portion of a root derived from an R108-1 regenerant showing development of effective nodules 2 weeks after infection with *Rhizobium meliloti* strain Rm41. Analysis of the transgenic plants was done by detection of β -glucuronidase (GUS) activity or by Southern genomic blotting. **E**, T1 kanamycin-resistant plant (progeny of a primary transformant [T0]) transformed with p35S GUS INT and stained with 5-bromo-4-chloro-3-indolyl glucuronide, showing intense GUS reporter gene activity in both leaves and roots, as expected for the constitutively expressed cauliflower mosaic virus 35S promoter. An untransformed regenerant used as a negative control in this same photograph showed no staining. Similar results were obtained with T1 plants transformed with pBDH001-16 (data not shown). **F**, Confirmation of the co-segregation of the GUS and plant selection markers in T1 plants. Southern blot analysis of two randomly selected progeny plants derived from T0 transgenic lines Mt34 (lanes 1 and 2) and Mt39 (lanes 3 and 4), which are resistant to kanamycin, and from line Mt16, which is resistant to hygromycin (lanes 5 and 6). Lane 7 is a positive control containing DNA isolated from tetraploid *Medicago sativa* subsp. *varia* transformed with p35S GUS INT in parallel with R108-1. As hybridization probes, a 700-bp fragment obtained by polymerase chain reaction (see Materials and Methods) amplified from the coding region of the neomycin phosphotransferase (NPTII) gene in p35S GUS INT (for the kanamycin-resistant plants) and the entire pBDH001-16 plasmid (for the hygromycin-resistant plants) were used. The estimated sizes (in kb) of the hybridizing bands are noted. Genomic DNA resolved by pulsed-field gel electrophoresis after digestion with rare-cutting restriction enzymes indicated that in every case the hybridization signals are associated with high-molecular-weight genomic fragments and thus are not due to contaminating plasmids; additional controls with untransformed in vitro regenerants showed no hybridization (data not shown).

ants obtained in three separate experiments, 75 to 89% of the plants remained diploid, as determined by flow cytometry (data not shown). However, only 1 to 2% of the somatic embryos recovered from explants were capable of regenerating into complete plants. To further select lines with increased embryogenic and regenerative capacity, one primary diploid regenerant was then recycled through a second round of a

similar in vitro regeneration regime. Among the secondary regenerants, one plant was identified as having very little restriction on the different types of explants capable of producing proembryonic calli in PCI-based medium (Table 2), and up to 50% of the embryos were capable of regenerating into complete plants. This secondary regenerant was morphologically indistinguishable from the parental 108-1, suggesting no

large chromosomal abnormalities had been induced by the selection process. This *in vitro*-selected derivative of the 108-1 ecotype, which was retained for all subsequent experiments, was named R108-1.

Nodulation characteristics of R108-1.

Because our major aim in developing a model plant is to use it in genetic and molecular analysis of nodule organogenesis and nitrogen fixation, we assessed the symbiotic performance of R108-1 with two widely studied wild-type strains of *R. meliloti*, Rm41 (Kondorosi et al. 1973) and Rm2011 (Rosenberg et al. 1981), using three different criteria—nodulation capability (the total nodule number and the ratio of pink, nitrogen-fixing nodules to white, non-nitrogen-fixing nodules), the dry weight of the green matter, and acetylene reduction. To interpret the data in a wider perspective, the parental 108-1 and other candidate ecotypes used in our preliminary studies were included in the comparisons (see Materials and Methods).

The location and shape of the nodules induced by Rm41 were similar in R108-1 and the parent 108-1 line (Fig. 1D)

Table 2. Regeneration capacity of different types of explants derived from *Medicago truncatula* line R108-1

Explant	Explants forming proembryonic calli (%) ^a
Hypocotyl	65.3 ± 3.0
Root	58 ± 4.6
Petiole	70 ± 8.3
Leaf	72 ± 3.2

^a Between 46 and 60 pieces of explants derived from the various tissues listed were incubated in proembryogenic callus induction medium containing 2,4-D (4 mg/L) and 6-benzylaminopurine (0.4 mg/L). The percentage (± SE) of explants that gave rise to at least one proembryonic callus was derived from three independent experiments. Similar frequencies of proembryonic calli were observed in the different types of explants when 6-benzylaminopurine was replaced with kinetin (0.4 mg/L).

and thus provide both morphological and primary inferences that the nodules on R108-1 are functional (see below). Rm2011 also established nitrogen-fixing symbiosis with both the parental and the selected lines; however, it induced fewer pink nodules on the both lines than Rm41 (Fig. 2A and B). Thus, the global nodulation behavior of R108-1 with these two *Rhizobium* strains has not been altered. However, both Rm41 and Rm2011 induced a higher percentage of pink nodules earlier (6 days after infection) on R108-1 than on 108-1. With respect to the total number of nodules, the regenerant showed about 15% lower efficiency in symbiotic relations with Rm41 but was enhanced by about 20% with Rm2011, relative to 108-1 (Fig. 2A and 2B). Under the same experimental conditions, Jemalong (only the transformable and regenerable genotype 2HA-1-9 was tested) (Nolan et al. 1989; Thomas et al. 1992) showed negligible symbiotic performance with either Rm41 (Fig. 2A) or Rm2011 (Barker et al. 1990; Fig. 2B), as only white Fix⁻ nodules were observed.

The efficiency of nitrogen-fixing symbiosis and its influence on plant growth was quantified on the basis of the dry matter yield (Vance et al. 1988). The dry weight of the green matter of R108-1, 108-1, Ghor, 131-1, and 139-2 was generally greater when these ecotypes were infected with Rm41 than when they were infected with Rm2011 (Fig. 2C). Although we cannot discount the possibility that these different plant ecotypes may be genetically different in their tolerance of nitrate limitation, their green matter yield is nonetheless positively correlated with the relative number of pink nodules induced by these two *R. meliloti* strains (Fig. 2A and B). Jemalong 2HA-1-9 produced only ineffective nodules, regardless of the bacterial partner (Fig. 2A and B) and was also weaker in growth under our conditions, as reflected in the lower dry green matter yield of the plant (Fig. 2C).

Finally, the effectiveness of nodules of R108-1 in nitrogen fixation was further confirmed by semiquantitative acetylene reduction assays (see Materials and Methods) diagnostic for nitrogenase activity in nodulated roots. Nodules from R108-1

Fig. 2. Development of nitrogen-fixing root nodules on *Medicago truncatula* line R108-1 and other ecotypes with *Rhizobium meliloti* strain Rm41 (A) and strain Rm2011 (B) as symbiotic partners. The total number of nodules (white columns in the background) and the number of pink (nitrogen-fixing) nodules were determined at the indicated times. Note the different scales for numbers of nodules in A and B. E4258 and 2HA-1-9 developed only large numbers of white nodules with either Rm41 or Rm2011. After 3 weeks, the green parts of these same plants were excised, and their dry weight was measured (C). The data are averages from five plants per ecotype tested in parallel in two independent repeats.

and 108-1 infected with Rm41 produced similar amounts of ethylene, in the range of 60 to 145 nmol per 10 mg of nodules (data not shown); nodules from both of these plants infected with Rm2011 were diminished in their capacity to reduce acetylene but also produced similar amounts, of 22 to 42 nmol per 10 mg of nodules. Also, by way of comparison, these same relative ranges of values from nodules infected with either Rm41 or Rm2011 were also observed for Ghor, 131-1, and 139-2 capable of producing pink nodules, while no ethylene production was detectable for E4258 and 2HA-1-9, with both producing only small, round, white nodules (data not shown).

Although each of the individual tests described above has inherent technical limitations, when taken together they are entirely consistent with the interpretation that R108-1 is not compromised in nodule development when tested with *R. meliloti* strains Rm41 and Rm2011. Also, we have only presented these particular data for simplicity; similar positive symbiotic relations were observed when R108-1 was infected with at least three other *R. meliloti* strains (F51, GR4, and Rm1021) (data not shown).

Plant transformation by co-cultivation with *A. tumefaciens*.

Since R108-1 has retained its capacity to form effective symbiosis with various bacterial partners, this plant line was used as starting material to develop a convenient transformation protocol and at the same time to simplify the in vitro regeneration regime described above. The essential steps of the transformation method based on co-cultivation with *Agrobacterium* are outlined in Figure 3. A medium with a high auxin content (PCI-4, Table 1) and darkness seem crucial as pre-culture conditions for stimulating callus formation on explants derived from hypocotyls, roots, petioles, and leaflets of R108-1 grown in vitro. We have also noted that N6 macronutrients are the most effective in stimulating later somatic embryogenesis and possibly shoot initiation, which might be attributed to their high ammonium and nitrate content (Handberg and Stougaard 1992). Explants were then infected by co-cultivation with *Agrobacterium* harboring either the p35S GUS INT plasmid (Vancanneyt et al. 1990) or the pBDH001-16 plasmid (Leung et al. 1991), which carry, respectively, the neomycin phosphotransferase (NPTII) and hygromycin phosphotransferase (HPTII) genes as plant-selectable markers. Because the p35S GUS INT plasmid is equipped with an intron-containing β -glucuronidase (GUS) reporter that is expressed only in plant cells (Vancanneyt et al. 1990), we used this plasmid to semi-quantitate the effects of bacterial culture density and duration of infection on the efficiency of transformation at the cell level. When explants were stained with the chromogenic substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) (Jefferson 1987) 1 week after co-cultivation, no significant differences in the number of transformed cells (scored as blue foci) were observed when the bacterial culture density was varied from $A_{600} = 0.2$ to $A_{600} = 2.0$. However, a period of 4 to 36 h of co-cultivation was required for optimal transformation (an average of five blue foci per explant, with 12 to 20 explant pieces scored per experiment), whereas no blue foci were observed when the co-cultivation time was less than 2.5 h. Vacuum infiltration (Bechtold et al. 1993) of explants for 3 to 20 minutes during co-cultivation also did not visibly enhance

transformation at the cell level, compared to treatment without vacuum (data not shown). Thus, for most of our transformation experiments, we generally used bacterial cultures either grown to or diluted down to $A_{600} = 0.5$ and a co-cultivation of 1.5 days in the dark with gentle shaking. The transformation of R108-1 appears to be indifferent to the genetic background of strains of *A. tumefaciens*, since a disarmed T-DNA-containing strain (GV2260) (Vancanneyt et al. 1990) and an intact T-DNA-containing strain (A281) (Hood et al. 1987) had similar transformation efficiency. Moreover, because transgenic R108-1 can be selected by resistance to either kanamycin or hygromycin, most of the existing *A. tumefaciens*-based gene transfer vectors can therefore be employed.

Infected explants were rinsed in water to remove excess bacteria and were then cultured on PCI-1 medium, with reduced auxin (Table 1), for 3 days, after which the explants were washed again in water supplemented with Augmentin (400 μ g/ml) to prevent bacterial overgrowth and then cultured on PCI-1 medium with either kanamycin (40 μ g/ml) or hygromycin (10 μ g/ml), depending on the particular vector used, to select transformed plant cells. Changes to fresh medium were made every 2 weeks. Calli appearing on explants were excised and transferred to embryo induction (EI) medium

Fig. 3. Summary highlighting essential points of the protocol for transformation of *Medicago truncatula* line R108-1 by co-cultivation with *Agrobacterium tumefaciens*. All media are described in Table 1.

(Table 1) placed under light; embryo induction usually required 3 to 4 weeks of subculturing. Embryos (appearing as dark green protrusions) were excised from calli for further development on embryo development (ED) medium, shoot development (SD) medium, and finally root formation (RF) medium to complete the regeneration of transformed plants (Table 1 and Fig. 1A–D). Selection on hygromycin appeared to be more stringent; on average, 90% of the explants (presumably untransformed) were killed during the first 3 weeks of culturing, but more than 80% of the embryos on the surviving explants eventually generated shoots. By comparison, selection on kanamycin was slower, with untransformed explants surviving up to 6 weeks of culturing and about 10% of the embryos eventually surviving to the shooting stage. In both cases, about 45 to 50% of the transformed shoots eventually formed roots.

Molecular and genetic analyses of transgenic plants.

Thirty-one kanamycin-resistant and fertile primary transformants (T0) carrying the p35S GUS INT plasmid were obtained (9 from vacuum infiltration and 22 from normal co-cultivation experiments). In every case, GUS expression could be detected in these transformants and also in kanamycin-resistant T1 progeny by staining with the chromogenic substrate X-Gluc (Fig. 1E). Further tests on genomic DNA iso-

lated from leaves of 14 randomly selected T0 plants by polymerase chain reaction (PCR) using oligonucleotide primers corresponding to the NPTII gene (see Materials and Methods) amplified the expected 700-bp fragment (data not shown).

Similarly, 104 hygromycin-resistant fertile T0 plants carrying the plasmid pBDH001-16 were obtained. Only 79 of them showed detectable GUS expression upon staining with X-Gluc (plants with no detectable GUS expression were not examined further). DNA isolated from three randomly selected GUS-positive plants were also verified by PCR using oligonucleotide primers corresponding to the GUS reporter gene, and in every case a band of approximately 700 bp was amplified (data not shown).

To analyze the genetic segregation of the plant selection markers, seeds from a portion of the above-mentioned selfed transgenic plants (11 from the p35S GUS INT and 8 from the pBDH001-16 experiments) were germinated on 0.5× SH medium supplemented with either hygromycin or kanamycin. Resistance to either kanamycin or hygromycin was a heritable dominant trait except in three lines carrying p35S GUS INT (Mt34, Mt39, and Mt41) (Table 3) which might have resulted from low expression of the integrated NPTII marker. The pattern of inheritance close to the theoretical Mendelian ratio of 3:1 (resistance to sensitivity to the antibiotics) also suggests most T0 transgenic plants harbored a single locus of T-DNA integration. Genomic blotting analysis of two T1 progeny each from lines deviating from the expected Mendelian segregation (Mt34 and Mt39, which were resistant to kanamycin, and Mt16, which was resistant to hygromycin) further confirmed the heritability of the T-DNA (Fig. 1F).

Table 3. Segregation analysis of the kanamycin resistance marker (kan^r) from plasmid p35S GUS INT and the hygromycin resistance marker (hyg^r) from plasmid pBDH001-16 in 100 T1 progeny plants derived from T0 transgenic lines after selfing

Transgenic line	kan ^r	hyg ^r	χ^2
p35S GUS INT			
Mt27	68	...	2.613 a
Mt29	72	...	0.48 a
Mt30	72	...	0.48 a
Mt31A	75	...	0.000 b
Mt33	70	...	1.333 a
Mt34	62	...	9.013 c
Mt39	56	...	19.253 c
Mt41	65	...	5.333 c
Mt53	75	...	0.000 b
Mt54	72	...	0.48 a
Mt55	75	...	0.000 b
pBDH001-16			
Mt9	...	75	0.000 b
Mt10	...	73	0.213 d
Mt12	...	75	0.000 b
Mt16	...	70	1.333 a
Mt23	...	75	0.000 b
Mt123	...	67	3.413 a
Mt148	...	75	0.000 b
Mt176	...	68	2.613 a

^a Seeds from each transgenic line were germinated on 0.5× SH basal medium (Schenk and Hildebrandt 1972) supplemented with either kanamycin (40 µg/ml) or hygromycin (10 µg/ml), and incubated at 24°C with a 16-h photoperiod. The number of resistant plants was scored after 3 weeks. Untransformed plants as controls in both cases failed to germinate under these conditions. The expected fraction (3/4) of kanamycin- or hygromycin-resistant plants was analyzed by χ^2 tests of goodness of fit assuming these selection markers behave as a single dominant locus: a, $0.1 \leq p \leq 0.5$; b, $p \geq 0.975$; c, $p < 0.05$; d, $0.5 \leq p \leq 0.9$. Segregation of the kanamycin resistance marker in lines Mt34, Mt39, and Mt41 (class c) transformed with p35S GUS INT deviates significantly from the expected ratio, which might reflect low expression of the neomycin phosphotransferase (NPTII) gene.

DISCUSSION

We have presented R108-1, an in vitro-selected derivative of ecotype 108-1 of *M. truncatula*, as a potential model plant partner for investigating the genetic and molecular processes underlying symbiosis with *R. meliloti*. This line has significantly enhanced or de novo regeneration capability and can be readily transformed by co-cultivation with standard *A. tumefaciens* strains. Moreover, effective interaction with a wider range of *R. meliloti* strains, large seed output, and the smallest genome size of the tested *M. truncatula* ecotypes (Blondon et al. 1994) are all favorable attributes not widely found among legumes.

The R108-1 derivative produces pink nodules slightly earlier than the parent line, regardless of whether Rm41 or Rm2011 was used as the symbiotic partner (6 days postinfection), and the relative total numbers of nodules formed are reversed with respect to the two bacterial strains (Fig. 2A and B). In qualitative terms, however, the general nodulation properties of R108-1 with these two *Rhizobium* strains are not compromised. In particular, R108-1 has retained its broad specificity, since it can still enter symbiotic relations with other commonly used strains of *R. meliloti* (data not shown). Its genome size is approximately 17% smaller than that of Jemalong (Blondon et al. 1994). Under our greenhouse conditions, R108-1 has a seed-to-seed generation time of 12 to 14 weeks, which is about 3 weeks shorter than that of Jemalong. Parenthetically, Ghor has the shortest seed-to-seed generation time (9 weeks) and the least expensive growth conditions of all the *M. truncatula* ecotypes that we have tested, and in this

respect it may yet provide a more convenient model than R108-1. However, Ghor has not yet been completely regenerated at present (our unpublished observations).

M. truncatula ecotype Jemalong was previously proposed as a nominal model plant for investigating symbiosis (Barker et al. 1990). During the last 6 years Jemalong lines with high symbiotic performance and regeneration capacity have also been developed (Chabaud et al. 1996). The joint use of the R108-1 and Jemalong lines may avail considerable flexibility to a genetic approach to understanding and dissecting the symbiotic processes. This would be analogous to the situation in *Arabidopsis*, in which different ecotypes are used for mapping mutations by RFLP, an important prerequisite for cloning their corresponding genes by chromosome walking. Second, the distinct strain-plant genotype specificity of the two lines appears to be a highly valuable tool for the identification of genetic determinants involved in bacterial invasion and the development of nitrogen-fixing nodules. Moreover, the expression of certain genes (or penetrance of mutant phenotypes) from one ecotype may vary when they are placed in a different ecotype, reflecting the existence of modifiers or regulatory loci in the different genetic background that can influence the expression of these genes. These modifiers can be mapped, like mutations, by intercrosses between different ecotypes and can be exploited as a convenient method to identify additional loci involved in regulating complex phenomena. In addition, Jemalong and R108-1 exhibit distinct morphological markers which can be used for following genetic crosses and for distinguishing progeny from parents. Figure 4 shows some of the obvious differences between Jemalong and R108-1, in leaf shape and pigmentation, seed pod morphology (marked by the presence or absence of spikes), and seed shape. Crosses between the recently available Jemalong line J5 and R108-1 have been performed in our laboratory (P. Ratet, unpublished), and the resulting progeny are being used for mapping morphological and embryogenic traits. Moreover, the parallel use of Jemalong and R108-1

lines could help in determining the locus (or loci) responsible for the Fix^- phenotype of nodules induced by Rm41 on Jemalong.

The transformation protocol described here for R108-1 requires about 5 months for the development of explants into fully rooted plants, which is the shortest period required for the regeneration of transformed legumes reported so far (Chabaud et al. 1996; Handberg and Stougaard 1992). However, a considerably faster regenerable variant is being identified by additional in vitro cycling method (T. H. Trinh, unpublished), which may be useful for developing insertional mutagenesis.

MATERIALS AND METHODS

Plasmids and bacterial strains.

The plasmid p35S GUS INT was maintained in *A. tumefaciens* strain GV2260 as previously described (Vancanneyt et al. 1990). The plasmid pBDH001-16 (Leung et al. 1991) was maintained in *A. tumefaciens* strain A281 (Hood et al. 1987).

R. meliloti strains Rm41 (Kondorosi et al. 1973), GR4 (Casadesús and Olivares 1979), F51 (Paaú et al. 1981), Rm2011 (Rosenberg et al. 1981), and Rm1021 (Meade et al. 1982) were grown in GTS medium (Kiss et al. 1979) at 30°C.

In vitro growth of plants.

M. truncatula seeds were pretreated with concentrated sulfuric acid for 20 min, rinsed several times with water, and then surface-sterilized for 20 min in a 50% solution of commercial bleach containing 12% sodium hypochlorite. They were then rinsed three times in sterile water and then germinated in 0.5× SH basal medium (Schenk and Hildebrandt 1972) supplemented with sucrose (10 g/L) and kalys agar (8 g/L). The plates were placed in the dark at 18 to 20°C for 3 days. Germinated seeds were then transferred to the same medium in Magenta vessels and cultured for 2 weeks (for plant transformation) or for 10 days in 150-mm-square petri plates

Fig. 4. Morphological markers distinguishing *Medicago truncatula* lines R108-1 and Jemalong, useful for following genetic crosses.

placed vertically, with aluminum foil covering the bottom half of the plate to protect plant roots from light (for nodulation assays). In both cases, cultures were maintained at 24°C with a 16-h photoperiod (60 to 100 $\mu\text{E m}^{-2} \text{s}^{-1}$).

Nodulation assays.

M. truncatula seedlings were grown under greenhouse conditions (22°C) in aeroponic tanks (Lullien et al. 1987) filled with 0.5× nutritive solution (NS) consisting of 274 mg of KNO_3 , 137 mg of KH_2PO_4 , 274 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 137 mg of $(\text{NH}_4)_2\text{SO}_4$, 1,095 mg of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 41 mg of Fe Na_2EDTA , 2.7 mg of KCl, 3 mg of H_3BO_3 , 1.7 mg of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.274 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.274 mg of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.137 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 0.137 ml of H_2SO_4 per liter, pH 5.8. Prior to assays, the plants (10 days old, with roots 2 to 6 cm long) were transferred to fresh 0.5× NS for 1 week and then to 0.25× NS for a further 3 days before inoculation with *R. meliloti* at $A_{600} = 0.015$. Three weeks after the infection, the green part of each plant was dried at 80°C for 2 days, and its weight was determined. Nitrogenase activity was assayed by acetylene reduction (Hardy et al. 1968) using gas chromatography with Porapak Q columns. Ten nodules per plant were used. The reaction was started by injection of 10% acetylene; after 30 min, 375- μl samples were withdrawn with calibrated syringes, and ethylene production was measured by gas chromatography (Hewlett-Packard 5890 series II). Pure acetylene and ethylene were used as internal standards. All experiments were done on five plants per eco-type per nodule factory and from a total of two repeats.

In vitro plant regeneration.

Explants from 108-1 grown in vitro were regenerated by culturing tissues on a medium combining N6 macronutrients (Chu 1978), SH micronutrients, and SH vitamins (Schenk and Hildebrandt 1972), with different concentrations of sucrose and phytohormones as required for particular regeneration steps (Table 1). Plants fully regenerated in vitro were transferred to the greenhouse (22°C during the day and 18°C at night, with a 16-h photoperiod and 40 to 60% humidity). Seeds from the regenerants were germinated, and plantlets were used for a second cycle of in vitro regeneration. The R108-1 line issued from this second round of selection, which showed increased regenerative capacity, robust growth, and high seed yield, was retained for all subsequent studies.

Determination of ploidy level.

Flow cytometry was used to determine the ploidy level of regenerated plants. Leaves and petioles were used as material and *Petunia* nuclei as the standard (Blondon et al. 1994).

Co-cultivation of explants with *A. tumefaciens* and selection of transgenic plants.

Agrobacterium strains carrying either pBDH001-16 or p35S GUS INT were grown overnight in liquid Luria-Bertani medium (10 g of Bacto-tryptone, 5 g of yeast extract, and 5 g of NaCl per liter, pH 7.2) containing appropriate antibiotics as described elsewhere (Leung et al. 1991; Vancanneyt et al. 1990). Cells were harvested by centrifugation and resuspended in liquid PCI medium (Table 1) to a density of $A_{660} = 0.5$ to $A_{660} = 0.6$, and 20-ml aliquots were used to infect explants of

R108-1 inside a sterile 90-mm petri plate. Augmentin (Beecham, Paris, France) was used to suppress the growth of *Agrobacterium*. Kanamycin monosulfate (40 mg/L) (Sigma) and hygromycin B (10 mg/L) (Boehringer, Mannheim, Germany) were added to the media to select plants transformed with p35S GUS INT or pBDH001-16, respectively, as required. Seeds derived from T0 plants were germinated in 0.5× SH medium (without hormones) containing either kanamycin or hygromycin as described above.

Assays for GUS activity in transgenic plants.

Different tissues from control and transgenic plants were stained overnight at 37°C in 2 mM X-Gluc and then were cleared by several washes in 70% ethanol (Jefferson 1987).

PCR analysis.

Single leaves were ground to a fine powder in liquid nitrogen. Then 300 μl of lysis buffer, containing 350 mM NaCl, 10 mM Tris-HCl, pH 7.6, 50 mM EDTA, 7 M urea, 1% *N*-laurylsarcosine (sodium salt), 1% polyvinylpyrrolidone (MW 40), and 14 mM β -mercaptoethanol, was added, and the mixture was incubated for 5 min at 50°C, followed by 10 min at room temperature with gentle shaking. The suspension was extracted twice with a mixture of equal volumes of phenol and chloroform (1:1 v/v) and once with chloroform. Nucleic acids were recovered by precipitation in ethanol and then resuspended in 200 μl of TE (10 mM Tris-HCl, pH 7.6, and 1 mM EDTA) containing RNase A (40 $\mu\text{g/ml}$).

PCR was performed in a volume of 25 μl consisting of 1× PCR buffer (Appligene), the four deoxyribonucleotides (200 μM each), the two relevant primers (0.5 μM each), 0.4 units of Taq DNA polymerase (Appligene), and 4 μl of the leaf extract described above. The mixture was heated for 30 sec at 94°C and then subjected to 25 cycles of amplification consisting of 30 s at 94°C, 60 s at 55°C, and 60 s at 72°C. The reactions were then concluded by a 2-min incubation at 72°C. An aliquot (routinely 2.5 μl) of the reactions was used for analysis of the amplified products by agarose gel electrophoresis. The primer pairs 5'-GAGGCGAGGCGGCTATGACTG-3' (nucleotides 201 to 222) and 5'-ATCGGGAGCGGCGATACCGTA-3' (nucleotides 900 to 879) were used to amplify the NPTII gene (Beck et al. 1982); for the PCR amplification of the GUS gene, the primer pairs 5'-GGCCAGCGTATCGTGCTGCG-3' (nucleotides 540 to 559) and 5'-GGTCGTGCA-CCATCAGCACG-3' (nucleotides 1242 to 1223) were used (Jefferson et al. 1986).

Isolation of genomic DNA and Southern blot analysis.

Genomic DNA from either leaves or entire plantlets was isolated essentially as described above for PCR analysis. DNA was digested overnight with *EcoRI*, and the fragments were resolved by electrophoresis in a 0.8% agarose gel and transferred by capillary action to Hybond-N membrane (Amersham) in a mixture of 1.5 M ammonium acetate and 0.2 N NaOH (pH ~9.0). After transfer, membranes were hybridized at 65°C overnight as previously described (Church and Gilbert 1984) with appropriate DNA probes radiolabeled by random hexamer primers (Feinberg and Vogelstein 1983) or by PCR amplification for three to five cycles in the presence of radioactive nucleotides using about 10 ng of DNA templates as starting material. After hybridization, the membranes were

then washed twice for 10 minutes each in 0.1% (w/v) sodium dodecyl sulfate, 15 mM NaCl, and 1.5 mM sodium citrate, pH 7, at room temperature and then again twice, for 20 minutes each, in the same washing solution at 55°C.

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