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Influence of genes determining supernodulation on root colonization by the mycorrhizal fungus *Glomus mosseae* in *Pisum sativum* and *Medicago truncatula* mutants

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Abstract We evaluated two supernodulating *Pisum sativum* (pea) mutants of the wild type cv. Frisson and six supernodulating *Medicago truncatula* mutants of the wild-type cv. Jemalong line J5 for their ability to form endomycorrhizas. The six mutants of *M. truncatula* were shown to be allelic mutants of the same gene Mtsym12, whereas distinct genes (sym28 and sym29) are known to determine the supernodulation character of the P64 and P88 pea mutants, respectively. Mutant P88 of pea and the majority of the *M. truncatula* mutants were significantly more colonized by the mycorrhizal fungus *Glomus mosseae* than their corresponding wild types, 4 weeks and 30 days after inoculation, respectively. These differences were expressed essentially in transversal intensity rather than in length intensity of root colonization and appeared to correspond to an increase in arbuscule formation. Results are discussed in relation to the mutated genes and, in particular, whether the observed effects are due indirectly to plant physiological modifications or are a direct result of possible common factors of regulation of nodulation and mycorrhizal development.

Key words *Pisum sativum* · *Medicago truncatula* · Mycorrhizal colonization · Supernodulating mutants

Introduction

There is now evidence that legumes forming symbioses with mycorrhizal fungi or *Rhizobium* share expression of common genes during the establishment of the associations. Several genes have been identified which are induced during both symbiotic interactions: the early nodulin genes ENOD5 and ENOD12 in *Pisum sativum* L. (pea) (Albrecht et al. 1998), ENOD2 and ENOD40 in *Medicago sativa* L. (Van Rhijn et al. 1997) and the leghemoglobin gene VFL b29 in *Vicia faba* L. (Frühling et al. 1997).

The discovery of non-nodulating legume mutants (Nod^-; Myc^-) in which both symbioses are inhibited concomitantly has mainly contributed to reinforce the idea that rhizobial and mycorrhizal associations involve common mechanisms.

The majority of these non-nodulating mutants are blocked at the stage of appressorium formation when challenged by a mycorrhizal fungus, and have been described in *P. sativum, V. faba* (Duc et al. 1989; Gianinazzi-Pearson et al. 1991), *M. truncatula* (Gaertn.) (Sagan et al. 1995) and, recently, in *Lotus japonicus* (Wegel et al. 1998). In addition, supernodulating mutants characterised by increased nodule numbers and nitrate tolerance have been described in some legumes: *Glycine max* L. (Merr.) (Carroll et al. 1985; Akao and Kouchi 1992), *Phaseolus vulgaris* L. (Park and Buttery 1988), *V. faba* (Duc 1995) and *M. truncatula* (Sagan and Duc 1996).

It was of interest to evaluate whether these supernodulating mutants are affected in their ability to form endomycorrhizas. For this purpose, we compared several supernodulating mutants of *P. sativum* and *M. truncatula* with their respective wild-type genotypes for response to mycorrhizal colonization.

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Materials and methods

Biological material

Two supernodulating mutants of *P. sativum* L. and their corresponding wild-type genotype (cv. Frisson) were used in the experiments. The mutants P64 and P88 were obtained after ethyl methanesulfonate treatment (Duc and Messager 1989; Sagan

and Duc 1996) and appeared to be controlled by the two different genes sym28 and sym29, respectively (Sagan and Duc 1996).

The control wild-tye genotype of *M. truncatula* (Gaertn.) cv Jemalong line J5 and its supernodulating mutants TRV3, TRV16, TRV17, TRV22, TRV29 and TR122 were used in the experiments. These mutants, which can form 4–5 times more nodules than the control line, were obtained after gamma-ray irradiation. The mutagenesis technique and screening procedure were described by Sagan et al. (1995).

Two successive mutant screening experiments were applied to 214 individual M_2 families harvested on single M_1 plants and to a pool of M_2 families harvested on another group of 248 M1 plants which may have been contaminated by fallen pods (less than 1 %) from the first group of plants. TR122 was the first supernodulating mutant to be selected in a single M2 progeny of the first experiment. TRV3, TRV16, TRV17, TRV22 and TRV29 were selected in the second experiment. The supernodulation phenotype has been confirmed in selfed progenies of each mutant up to the M_5 generation for TR122 and the M_4 generation for the other mutants.

The arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol and Gerd.) Gerdemann and Trappe (BEG 12) was used in experiments involving mycorrhizal inoculation. The mycorrhizal inoculum was a soil (neutral, clay loam from Epoisses, INRA Experimental Station, Dijon) containing mycelium, root fragments and spores collected from a 3-month-old culture of *G. mosseae* on onion plants. This inoculum was previously shown to be free of *Rhizobium leguminosarum* and *R. meliloti* using an N-free Long Ashton nutrient solution to grow the supernodulating mutants TR122 and P88 for 6 weeks in calcined clay containing 30 % of the inoculum. No nodulation was observed.

Rhizobium meliloti strain 2011 was used in the genetic analysis of *M. truncatula* and inoculated, at planting, as a liquid bacterial suspension.

Genetic analysis of *Medicago truncatula* mutants

Crosses

J5 and the mutant TR122 were reciprocally pollinated after emasculation. F_1 plants from this cross were transplanted into pots after nodulation phenotype evaluation and were then selfed to produce F_2 progenies. The nodulation phenotype of the resulting F_2 plants was then scored with reference to the control lines J5 and TR122. The second set of mutants TRV3, TRV16, TRV17, TRV22 and TRV29 were reciprocally crossed with J5 for a dominance test and with TR122 for a complementation test. The nodulation phenotype of the resulting F_1 plants was then scored with reference to control lines J5 and TR122.

Plant growth conditions

Seeds of the different lines and their crosses were scarified, surface sterilized and sown in plastic recipients in sterilized sand. They were inoculated just after sowing and 2 days later with *R. meliloti*. Plants were grown in an insect-proof greenhouse with a 15-h day photoperiod, a minimal day temperature of 18 °C and a minimal night temperature of 15 °C. Ventilation was assured for temperatures higher than 24 °C. Plants were irrigated daily with an N-free nutrient solution containing 584 mg l^{-1} KH_2PO_4; 348 mg l^{-1} K_2SO_4; 246 mg l^{-1} $MgSO_4.7H_2O$; 2 mg l^{-1} H_3BO_3; 1.8 mg l^{-1} $MnSO_4.H_2O$; 0.22 mg l^{-1} $ZnSO_4.7H_2O$; 0.1 mg l^{-1} $CuSO_4.5H_2O$; 0.83 mg l^{-1} MoO_3 and 60 µl l^{-1} Masquol-ate Fe 2500 (Synthron) (2.5 % Fe).

Nodulation scoring

The parental lines and the F_1 and F_2 plants were visually screened for nodulation 35 days after sowing. Plants with 4–5 times more nodules than the control lines were scored as supernodulated as described in Sagan et al. (1995).

Experimental conditions for evaluating mycorrhizal infection

Seed disinfection and germination

Pea seeds were surface disinfected for 15 min in 3.5 % calcium hypochlorite and sown in the dark at 20 °C in germination boxes containing sterile vermiculite. Three days later, homogenous seedlings were transplanted into pots containing the mycorrhizal inoculum. *M. truncatula* seeds were scarified and sterilized for 15 min in 3.5 % calcium hypochlorite and germinated in soft water agar (0.3 %) for 3 days in the dark at 20 °C. Homogenous seedlings were then transplanted into pots containing the mycorrhizal inoculum.

Plant growth conditions

Five plants per genotype were individually grown in 200-ml black plastic pots in 150 ml substrate consisting of a mixture of 1/3 (v/v) mycorrhizal inoculum and 2/3 (v/v) calcined clay (Terra-green, Oil Dry Corporation). They were raised in a growth chamber with the following conditions: 16-h photoperiod, 70 % day and 80 % night relative humidity, 320 µmol m^2 s^{-1} light, 22 °C day and 19 °C night, and watered every 2 days with 20 ml of a modified Long Ashton nutrient solution (Hewitt 1966) containing 1616 mg l^{-1} KNO_3 and 20.8 mg l^{-1} $NaH_2PO4.$ $2H_2O$.

No *R. leguminosarum* bv *viciae* was inoculated to the *G. mosseae*-inoculated plants, in order to avoid an eventual secondary effect of nodulation on mycorrhizal infection, and also because a high concentration of nodules on roots hampers vizualization of mycorrhizal infection.

Estimation of endomycorrhizal colonization

Endomycorrhizal colonization was estimated after staining roots with trypan blue using a method derived from Phillips and Hayman (1970) in which the percentage of colonized root length was complemented by an estimation of transversal intensity of root colonization.

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 measures for each root fragment and a set of 60 measures per root system. For each microscope field, mycorrhizal root length intensity was evaluated using 11 classes (from 0 to 10) and was noted as l_1 for field 1, l_2 for field 2 etc. Transversal mycorrhizal intensity was evaluated using six classes (from 0 to 5), depending on the infection intensity observed transversally, and was noted as w_1 for field 1, w_2 for field 2 etc.

For each root sample, we calculated percentage of mycorrhizal intensity in length, *LI:*

$$LI = (l_1+l_2+...+l_{60})10/60$$

and percentage of transversal colonization intensity in the mycorrhizal part of the root, *TI:*

$$TI = (w_1l_1+w_2l_2+...+w_{60}l_{60})20/\ (l_1+l_2+...+l_{60})$$

and percentage of global (overall) intensity, *GI*:

$$GI = LI \times TI/100$$

Experimental protocol

Two independent experiments were performed: 1) with the pea cultivar Frisson and the supernodulating mutants P64 and P88, which were grown 46 days after inoculation with *G. mosseae* and harvested after 11, 22, 30 and 46 days to estimate mycorrhi-

Table 1 Nodulation phenotype in the F_1 and segregation of wild-type: supernodulation phenotypes in the F_2 derived from crosses between the parental line J5 and its supernodulated mutant TR122

Generation	Number of crosses	Number of wild-type plants	Number of supernodulated plants	Chi-square value for 3:1 ratio	Probability
F_1	3	20	0		
F_2	3	486	141	2.17	0.15

zal colonization and measure growth parameters; 2) with the wild-type cv. Jemalong line J5 of *M. truncatula* and 6 supernodulating mutants, which were harvested 4 weeks after inoculation with *G. mosseae* to estimate mycorrhizal colonization and measure growth parameters. Five single plant replicates were analysed per treatment. The data were statistically analysed by variance analysis after arcsin $\sqrt{}$ transformation for percentages. The mean values were then compared using the Tukey-Kramer test ($P \leq 0.05$).

Results

Genetic analysis of *M. truncatula* supernodulating mutants

F1 plants from the cross of TR122 with line J5 showed a wild-type phenotype, indicating the recessive character of the mutation in relation to the wild allele (Table 1). The calculated Chi square value on the F2 segregation from this cross is consistent with Mendelian inheritance, the supernodulation phenotype of TR122 being determined by a mutation in a single gene. We have named this gene controlling the supernodulating phenotype Mtsym12. The other five mutants crossed with J5 gave a wild-type phenotype, indicating that these mutations are also recessive. When crossed with TR122, they gave a supernodulated F1 progeny, indicating that they belong to the same complementation group (Table 2). It can be concluded from these results that TRV3, TRV16, TRV17, TRV22, TRV29 and TR122 are allelic mutants of the same Mtsym12 gene. Since 5 of these mutants were selected in the pool of M2 progenies of the second experiment with a possible contamination by pods of the first experiment, the mutation events may not be independent.

Table 2 Genetic analysis of new *Medicago truncatula* supernodulating mutants. F_1 phenotypes after dominance tests against the parental line J5 and complementation tests against the supernodulating mutant TR122

F_1 CROSS	Number of F_1 plants	F_1 nodulation phenotype
TRV3 × J5	8	Wild-type
TRV3 × TR122	22	Supernodulation
TRV16 × J5	15	Wild-type
TRV16 × TR122	3	Supernodulation
TRV17 × J5	17	Wild-type
TRV17 × TR122	8	Supernodulation
TRV22 × J5	8	Wild-type
TRV22 × TR122	9	Supernodulation
TRV29 × J5	9	Wild-type
TRV29 × TR122	34	Supernodulation

Evaluation of growth and mycorrhizal colonization parameters of *P. sativum* and *M. truncatula* supernodulating mutants

Plant growth parameters

The three genotypes of pea had the same shoot mass during the period of the experiment, the only significant difference ($P \leq 0.05$) being observed at 46 days for P88, which had a shoot mass 9.5 % lower than that of cv. Frisson (Fig. 1). The root masses were significantly smaller for the supernodulating mutants of pea than the wild type cv Frisson after 30 days growth.

Although the shoot masses of the different mutants of *M. truncatula* appeared generally lower than that of the wild-type line J5, the differences where not significant ($P \leq 0.05$), with the exception of TR122 (Fig. 2). The root masses of the *M. truncatula* mutants were generally significantly lower (about 35 % decrease) than the control line J5, except for TRV17 (Fig. 2).

Visual assessment of root development for the two plant species showed that overall root length was generally reduced in supernodulating mutants, and this appears to be the major feature related to the root mass decrease.

Mycorrhizal colonization parameters

For pea (Fig. 3), *G. mosseae* colonization in terms of root length (LI) appeared slightly, but not significantly, more intense in the two mutants from 22 days after inoculation onwards. The transversal intensity (TI) and the global intensity (GI) of mycorrhizal colonization were significantly higher in the P88 mutant from 30 days after inoculation onwards. Forty-six days after inoculation, this difference reached 36 % for the transversal intensity and 26 % for the global intensity. P64 did not differ from the wild type cv. Frissson in transversal intensity.

For *M. truncatula* (Fig. 4), there was a significant increase in all mycorrhizal colonization parameters 4 weeks after inoculation with *G. mosseae* in the supernodulating mutants TRV3, TRV16, TRV17, TRV22 compared with the wild-type line J5. For the other two supernodulating mutants, mycorrhizal colonization also appeared to increase, though not significantly, for all three parameters; in TRV29 the difference was only significant for TI and for TR122 the difference was only significant for GI.

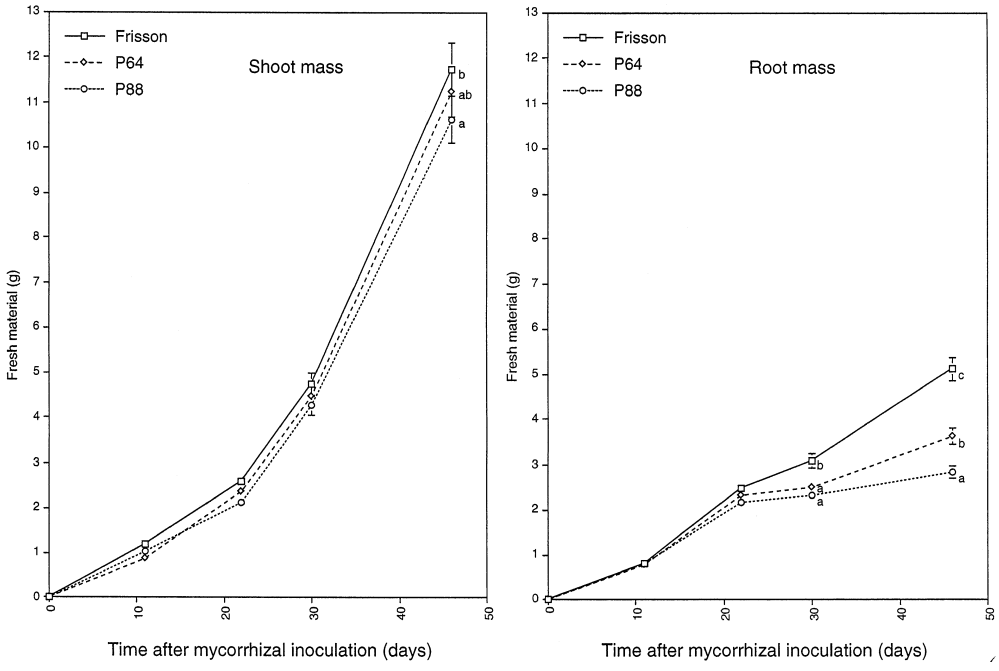

Fig. 1 Growth parameters of pea cv. Frisson and the two supernodulating mutants P64 and P88 raised in a growth chamber and harvested 11, 22, 30 and 46 days after inoculation with *Glomus mosseae*. Each time point represents the mean of five replicates. For each harvest, means associated with different letters are statistically different (Tukey-Kramer test) at $P \leq 0.05$. The absence of letters for a given harvest indicates no statistical difference between the corresponding means; *bars* standard errors

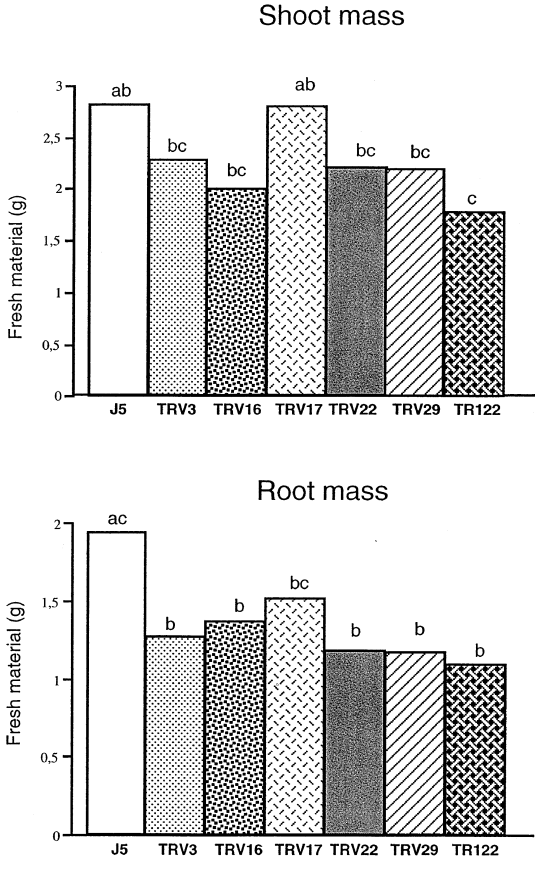

Fig. 2 Growth parameters of *Medicago truncatula* (cv. Jemalong) line J5 and six supernodulating mutants raised in a growth chamber and harvested 4 weeks after inoculation with *G. mosseae*. Each column represents the mean of five replicates. Means associated with different letters are statistically different (Tukey-Kramer test) at $P \leq 0.05$

Although the number of arbuscules was not quantified directly, a high density of arbuscules was consistently observed in the cortical tissue of the two plant species. Consequently, the increase in TI observed in hypernodulating mutants corresponded to an overall increase in arbuscule formation within the roots of these plants.

Discussion

The method used in this paper for estimating mycorrhizal colonization corresponds to that of Phillips and Hayman (1970) in terms of root length (LI) intensity. The global intensity (GI) corresponds to "M %" of Trouvelot et al. (1986), but is more precise. In the latter, the M % value is obtained from 30 notations (one per 1 cm root fragment) of a global visual estimation using 6 classes (0–5), whereas in the present work, the GI value was obtained by combining two sets of 60 notations based on a greater number of classes (two per 1-cm root fragment): the first set (LI) used 11 classes (0–10) and the second set (TI) used 6 classes (0–5).

For the majority of the supernodulating mutants of *P. sativum* and *M. truncatula* tested, we found that mycorrhizal colonization by *G. mosseae* was higher than in the corresponding wild-type genotypes cv. Frisson and cv. Jemalong line J5. Interestingly, these supernodulating mutants generally have a lower root mass which appears to be related to a shorter root length. This could have explained the increased percentage mycorrhizal colonization of roots. Indeed, the apparent percentage mycorrhizal colonization in terms of root length might have been higher in mutants

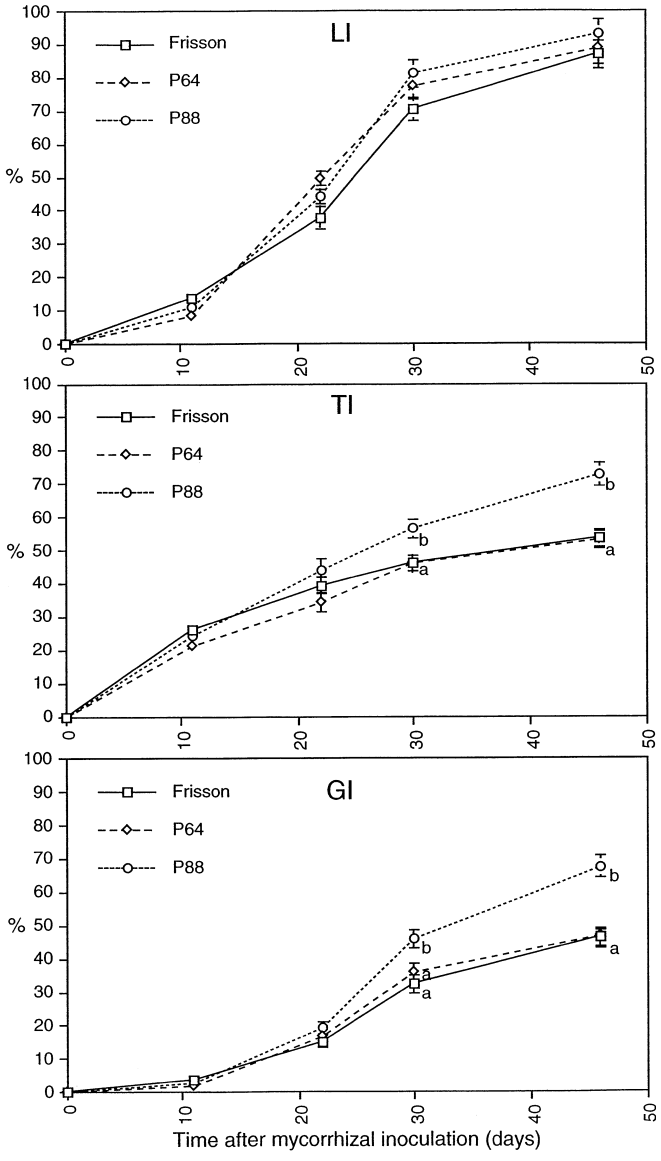

Fig. 3 Mycorrhizal colonization parameters of pea cv. Frisson and the two supernodulating mutants P64 and P88 raised in a growth chamber and harvested 11, 22, 30 and 46 days after inoculation with *G. mosseae*. Each time point represents the mean of five replicates. For each harvest, means associated with different letters are statistically different (Tukey-Kramer test) at $P \leq 0.05$. The absence of letters for a given harvest indicates no statistical difference between the corresponding means. (*LI* the percentage of root length colonized by the mycorrhizal fungus, *TI* the percentage of transversal root colonization intensity, *GI* the global coonization intensity, *bars* standard errors)

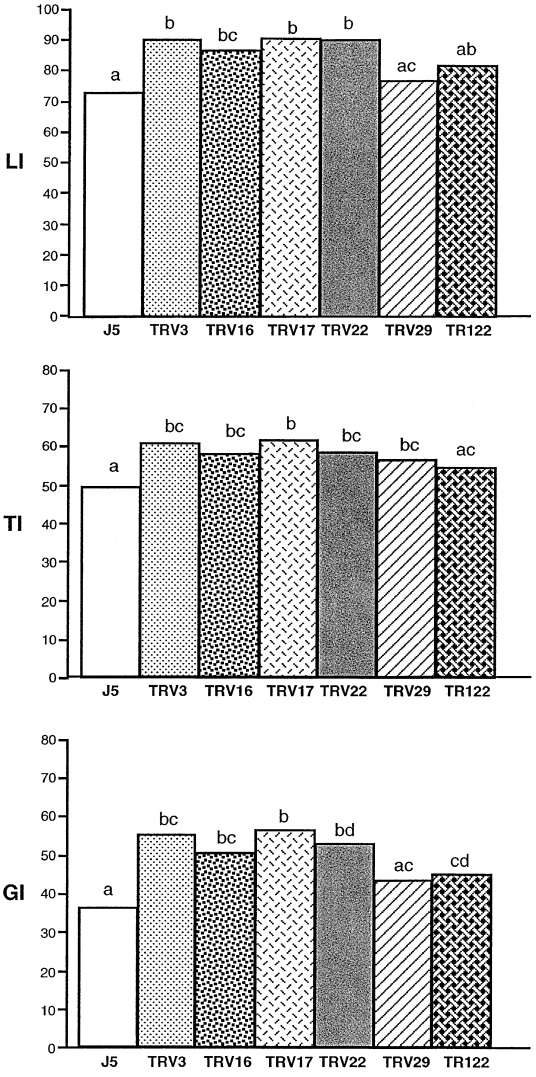

Fig. 4 Mycorrhizal colonization parameters of the *M. truncatula* line J5 (cv Jemalong) and six supernodulating mutants cultivated in a growth chamber and harvested 4 weeks after inoculation with *G. mosseae*. Each bar represents the mean of five replicates. Means associated with different letters are statistically different (Tukey-Kramer test) at $P \leq 0.05$. Abbreviations as in Fig. 3

simply because the roots were shorter. However, our results show no significant difference in mycorrhizal colonization in terms of root length (LI) for the pea genotypes; the most significant difference was observed for transversal colonization (TI). Thus there was a real overall increase in the development of the mycorrhizal fungus in the roots of supernodulating mutants compared with the wild-type genotypes. From our observations, the increase in transversal colonization intensity was due to an increase in the number of cortical cells containing arbuscules, so that higher mycorrhizal colonization in supernodulating mutants appears essentially to be due to an increase in arbuscule formation. For this reason, we have called the corresponding phenotype "Myc^{A++}".

The question now arises as to the factor responsible for this increase in mycorrhizal colonization which is associated with the supernodulating mutation. The two supernodulating pea mutants tested, P64 and P88, were affected at the level of two different nodulation genes, sym28 and sym29, respectively (Sagan and Duc 1996). Nothing is known about the function of these genes and only sym29 appears to enhance mycorrhizal colonization. It is interesting that the P88 mutant has

short internodes and it was suggested following F2 progeny characterization and grafting experiments that the same recessive gene controls supernodulation and the short internode character (Sagan and Duc 1996). This short internode character suggests a defect in the hormonal balance of the plant and good candidate for such a hormonal alteration would be gibberellic acid, which is known to play a role in internode elongation. Interestingly, gibberellic acid is a strong inhibitor of mycorrhizal colonization, especially arbuscule formation (El-Ghachtouli et al. 1996). Therefore, a decrease in endogenous levels of gibberellic acid in P88 could be involved in the observed increase in mycorrhizal colonization, particularly in the density of arbuscules. This hypothesis merits further evaluation.

In the case of the gene sym28, which also modifies stem architecture by inducing a fasciation in the upper part, its regulation of nodulation has been shown by grafting experiments to be shoot-induced as for sym29 but, in this case, regulation by sym28 appears specific to the *Rhizobium* symbiosis with no detectable effect on the mycorrhizal symbiosis.

The genetic analysis of the supernodulating *M. truncatula* mutants showed that all mutants are allelic of the Mtsym12 gene. All mutants exhibited 4–5 times higher numbers of nodules than the wild-type genotype. The fact that significant differences were measured between mutants like TR122 and TRV17 for plant growth characteristics and mycorrhizal response, is a strong argument for independent mutational events in these two mutants. Further genetic studies of allelic diversity are required. The fact that independent mutants simultaneously determine Nod^{++} and Myc^{A++} phenotypes supports the hypothesis that both symbiotic phenotypes are pleiotropic effects of the same mutated gene, but further genetic analyses are necessary.

In contrast to the pea P88 mutant and with the exception of the dry mass of TR122, we observed no difference in shoot morphology between *M. truncatula* mutants and the line J5. Thus, no evident hormonal effect appears to be associated with the supernodulated phenotype; however, the fact that the root length is reduced compared with the wild-type genotype does not exclude further kinds of hormonal effect.

This first indication of a relationship between the supernodulating character and improved ability to develop mycorrhiza in two leguminous species, *M. truncatula* and *P. sativum*, reinforces the idea of common factors in mycorrhizal and *Rhizobium* symbioses. Further investigations of the mechanisms involved in the two symbiotic interactions could include grafting experiments and molecular characterization of the mutated genes.

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