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ORIGINAL PAPER

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Effect of mutations in the pea genes *Sym33* and *Sym40*

I. Arbuscular mycorrhiza formation and function

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Abstract Two pea (*Pisum sativum* L.) symbiotic mutants SGEFix$^-$-1 (*sym40*) and SGEFix$^-$-2 (*sym33*) with abnormalities in infection thread development and function in symbiotic root nodules have been characterised in terms of mycorrhizal colonisation of roots, shoot and root biomass accumulation and shoot and root phosphorus (P) content. The mutation in gene *sym33* decreased mycorrhizal colonisation of roots (except arbuscule abundance in mycorrhizal root fragments, which increased) but did not change the effectiveness of mycorrhiza function. The mutation in *sym40* did not affect either of these processes. Both mutants showed differences in plant development compared with the wild-type line SGE. The mutants had delayed flowering and pod ripening, and shoot/root biomass ratios and P accumulation also differed from those of SGE. These observations suggest that the gene mutations cause systemic changes in plant development.

Keywords Plant–microbe interaction · Arbuscular mycorrhiza · *Pisum sativum* L. · Symbiotic genes · Colonisation

Introduction

Garden pea (*Pisum sativum* L.) is a leguminous plant establishing root symbioses with *Rhizobium leguminosarum* bv. *viciae* and arbuscular mycorrhizal (AM) fungi of the order Glomales. Both legume symbioses represent model systems for studying mechanisms of mutualistic plant-microbe interactions and for agricultural production. Many legume symbiotic mutants have been found to be impaired in the development of both root symbioses (Duc et al. 1989; Bradbury et al. 1991; Kolycheva et al. 1993; Balaji et al. 1994; Sagan et al. 1995; Shirtliffe and Vessey 1996; Wegel et al. 1998; Bonfante et al. 2000; Morandi et al. 2000; Senoo et al. 2000; Shrihari et al. 2000; Solaiman et al. 2000; Resendes et al. 2001). For example, two developmental stages of arbuscular mycorrhiza formation in pea are blocked by plant gene mutations (Gianinazzi-Pearson 1996). The Myc^{-1} mutant phenotype refers to a block occurring after appressorium formation in AM development (Duc et al. 1989; Kolycheva et al. 1993; Balaji et al. 1994; Gianinazzi-Pearson 1996), whilst the Myc^{-2} mutant phenotype refers to lack of arbuscule formation, where intracellular fungal growth is reduced to a few stumpy branches (Gianinazzi-Pearson 1996). Myc^{-1} mutations have no effect on development of external fungal mycelium (L.M. Jacobi, V.E. Tsyganov, A.Y. Borisov, unpublished results), and Myc^{-2} has no positive effect on host plant biomass accumulation or phosphorus uptake (I. Jakobsen, personal communication).

The present study aimed to characterise the AM phenotypes of the two pea mutants SGEFix$^-$-1 (*sym40*) and SGEFix$^-$-2 (*sym33*) defective in nodulation. SGEFix$^-$-1 (*sym40*) is blocked at the stage of infection droplet formation in nodule organogenesis, whilst SGEFix$^-$-2 (*sym33*) is impaired in the process of endocytosis and rhizobia are not released properly into the nodule cells (Tsyganov et al. 1998).

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Materials and methods

Plant material

SGEFix$^-$-1 (*sym40*) and SGEFix$^-$-2 (*sym33*), with defects in nitrogen-fixing symbiosis (NFS) formation (Tsyganov et al. 1994, 1998), and the wild-type line SGE (Kosterin and Rozov 1993) were used in the study. After isolation, mutant lines were checked for stability of their phenotypes in a series of generations and, after back-crossing with the wild-type line SGE, were re-isolated from the F_2 population to eliminate possible additional mutations and to equalise the genetic background of wild-type line and mutants. Only then were the mutants used in the experiments on phenotypic characterisation. Seeds within a narrow weight range

(99–100 mg of each line) were selected for the experiments, sterilised with concentrated sulphuric acid (20 min shaking at room temperature) and washed with distilled water.

Mycorrhizal fungus inoculation

Glomus intraradices Schenck and Smith isolate CIAM8 from the Collection of the All-Russia Research Institute for Agricultural Microbiology (registered in the European Bank of Glomales as isolate BEG144) was used to inoculate the plants in different growth conditions. The fungus was isolated from soil in a suburban field of St.-Petersburg (Gatchinsky district) and characterised as forming a highly effective AM symbiosis with many agricultural crops (Muromtsev et al. 1989). A root–soil mixture (15 g per plant) from a pot culture of *Sorghum vulgare* Pers. colonised by this fungus was used for inoculation. In variants without inoculant, a root/soil mixture similar to that described above was used from non-mycorrhizal *S. vulgare*.

Growth conditions and measurements

The humus horizon of a soddy podzolic, sandy loam, lea land soil with the following characteristics was used for plant growth: pH (KCl) 4.9; 3% organic matter; 1.26 mg/100 g (extraction with 0.2 N HCl); 7 mg/100 g K_2O available phosphorus (P) (extraction with 0.2 N HCl); 3.3 mg-equivalent/100 g hydrolytic acidity; 9.8 mg-equivalent/100 g base exchange materials. Air-dried soil sterilised by autoclaving (1 h, 140°C) was kept for a month to eliminate volatile toxic compounds and then mixed with sterile quartz sand (soil/sand 2:1). $CaCO_3$ (1.6 mg/kg) was added to the mixture to correct pH. One seed was planted per pot of 500 g sterilised soil/sand mixture.

For analysis of AM effectiveness in the mutants, the treatments were: (1) full mineral nutrition (NPK) with macro-elements according to Prianishnikov (Klechkovsky 1967) and micro-elements according to Hoagland (Valter and Pinevich 1938), or (2) bioavailable P in the form of $CaHPO_4$ at half dose according to Prianishnikov (Klechkovsky 1967). These nutrients were added into the soil/sand mixture.

Plants were grown in an HPS2000 growth chamber (Heraeus Vötch, Germany) (day/night 16/8 h, temperature 21°C, relative humidity of 75%, photon irradiance of 490 E/m^2 per s).

To analyse the effectiveness of AM in the mutants, it was necessary to standardise the physiological state of plants grown under different conditions. For this, material for analysis was collected at a specific late stage of plant development described as almost mature but not dry first pod. The mutant lines differed from the wild-type line SGE in timing of flowering and pod ripening, both developing more slowly. Therefore, each parameter used to estimate AM effectiveness was evaluated per day of growth to obtain an average rate of increase of all parameters during the chosen period of plant development. Measured parameters were weight and P (extraction with 0.2 N HCl) content of shoots and roots (separately).

AM development in roots was estimated according to Trouvelot et al. (1986). The parameters used were F% (frequency of mycorrhiza in the root system), M% (intensity of mycorrhizal colonisation in the root system), m% (intensity of mycorrhizal colonisation in mycorrhizal root fragments), A% (arbuscule abundance in the root system), a% (arbuscule abundance in mycorrhizal root fragments).

Statistical analysis

Six to ten plant replicates were used for analysis. Data were calculated by standard methods of variance analyses and comparison of two groups of data. SigmaStat for Windows version 2.3, SPSS Inc. software, was used for statistical analysis of the data.

Table 1 Days of growth of wild-type pea and two mutants before collecting material for analysis at the almost mature but not dry first pod stage of plant development (*Control* without inoculation and no additional mineral nutrition, *AM* inoculation with *Glomus intraradices*, *P* addition of $CaHPO_4$ to the soil/sand mixture, *NPK* full mineral nutrition)

Pea lines	Growth conditions			
	Control	AM	P	NPK
SGE (wild-type)	42	48	45	44
SGEFix⁻-1 (*sym40*)	57	61	60	53
SGEFix⁻-2 (*sym33*)	60	55	53	52

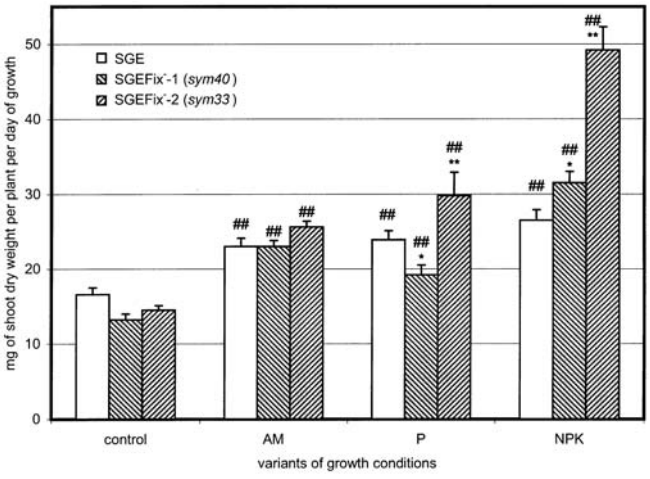

Fig. 1 Rate of shoot biomass increase of the wild-type pea line SGE and mutants SGEFix⁻-1 (*sym40*) and SGEFix⁻-2 (*sym33*) under different growth conditions (*Control* without inoculation and no additional mineral nutrition, *AM* inoculation with *Glomus intraradices*, *P* addition of $CaHPO_4$ to the soil/sand mixture, *NPK* full mineral nutrition, *##* mean values differ from those of the same pea line controls at $P \geq 0.99$; **, * mean values differ from corresponding values of other lines under the same growth conditions at $P \geq 0.99$, $P \geq 0.95$, respectively, *vertical bars* standard errors)

Results

Mutant lines used in the experiments differed from wild-type line SGE by their timing of flowering and pod ripening; they grew slower by several days under all growth conditions (Table 1). Analysis of the rate of biomass accumulation (Figs. 1, 2 for shoots and roots, respectively) and of the rate of P accumulation (Figs. 3, 4 for shoots and roots, respectively) at the almost mature but not dry first pod stage of development, under control conditions without inoculation and without additional mineral nutrition, showed no statistically significant differences between the lines. Therefore, this physiological stage was used for comparison of plant development under different experimental conditions.

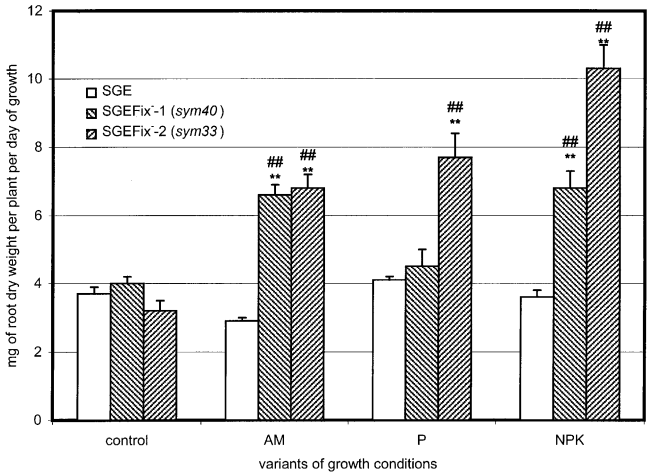

Fig. 2 Rate of root biomass increase of the wild-type pea line SGE and mutants SGEFix⁻-1 (*sym40*) and SGEFix⁻-2 (*sym33*) under different growth conditions. Symbols and abbreviations as in Fig. 1

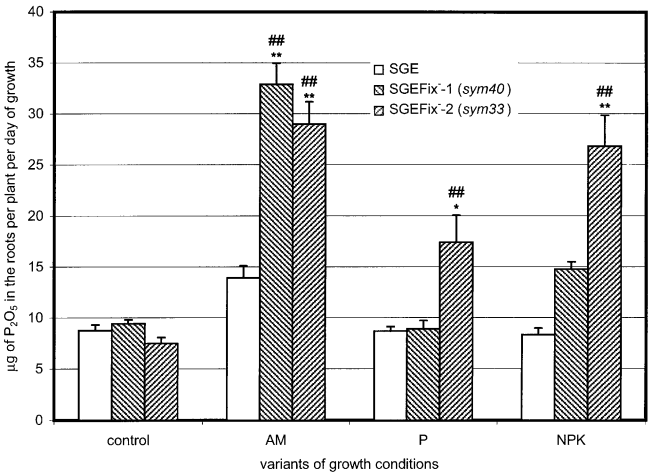

Fig. 4 Rate of P accumulation in roots of the wild-type pea line SGE and mutants SGEFix⁻-1 (*sym40*) and SGEFix⁻-2 (*sym33*) under different growth conditions. Symbols and abbreviations as in Fig. 1

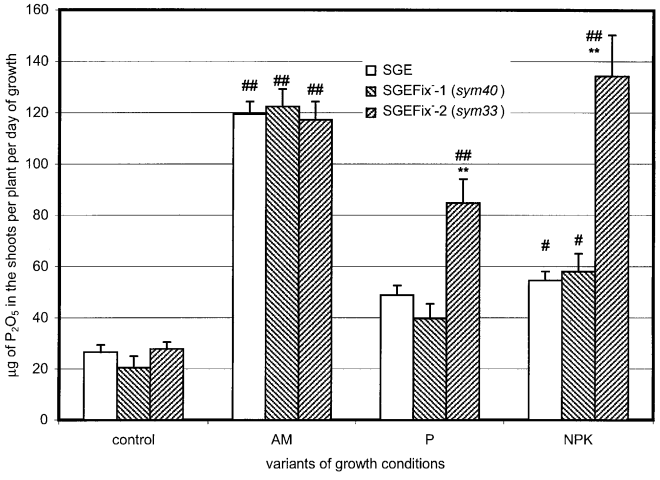

Fig. 3 Rate of phosphorus (P) accumulation in shoots of the wild-type pea line SGE and mutants SGEFix⁻-1 (*sym40*) and SGEFix⁻-2 (*sym33*) under different growth conditions. Symbols and abbreviations as in Fig. 1

Wild-type line SGE

All treatments delayed development of line SGE (Table 1), which had a high level of mycorrhizal colonisation (Table 2). AM formation in line SGE had a positive and significant effect on shoot biomass accumulation similar to that resulting from addition of bioavailable P or full mineral nutrition (NPK) to the substrate (Fig. 1). No statistically significant changes occurred in root biomass accumulation of line SGE as a result of mycorrhization or mineral nutrition (Fig. 2). Mycorrhization of line SGE plants increased P accumulation in the shoots more than fivefold, whereas NPK increased it twice. The addition of bioavailable P did not significantly affect this parameter (Fig. 3). P accumulation in the roots of this pea line did not significantly increase in response to AM formation or to additional mineral nutrition (Fig. 4).

Mutant line SGEFix⁻–1 (*sym40*)

All the experimental treatments, except NPK, slightly delayed development of SGEFix⁻-1 (*sym40*) (Table 1). This pea line developed a high level of mycorrhizal colonisation which did not differ significantly from that of the wild-type line SGE (Table 2). SGEFix⁻-1 (*sym40*) had improved biomass accumulation under all treatments, but the effect of P was less pronounced than that of AM. The addition of NPK gave a more pronounced increase in biomass accumulation than that caused by AM (Figs. 1, 2). This mutant, unlike the wild-type line SGE, accumulated approximately 1.5-fold more root biomass in presence of AM or NPK but no significant changes were observed with additional P (Fig. 2). Accumulation of P in shoots by this mutant line was similar to that of the wild-type line SGE under all experimental conditions (Fig. 3). Unlike the wild-type line SGE, P accumulation in roots was enhanced threefold as a result of AM formation. There were no significant changes in root P with additional mineral nutrition (Fig. 4).

Mutant line SGEFix⁻–2 (*sym33*)

All experimental treatments accelerated plant development in SGEFix⁻-2 (*sym33*), despite increased biomass accumulation of both shoots and roots compared with the control conditions. This behaviour distinguishes this mutant line from the other two lines. SGEFix⁻-2 (*sym33*) had a lower level of mycorrhizal colonisation than the wild-type SGE. The decreases ranged from 70% to 35% depending on the parameters, except for arbuscule abun-

Table 2 Comparison of mycorrhizal colonisation in pea wild-type line SGE and the symbiotic mutants SGEFix⁻-1 (*sym40*), SGEFix⁻-2 (*sym33*), respectively (*F%* frequency of mycorrhiza in the root system, *M%* intensity of mycorrhizal colonisation in the root system, *m%* intensity of mycorrhizal colonisation in mycorrhizal root fragments, *A%* arbuscule abundance in the root system, *a%* arbuscule abundance in mycorrhizal root fragments)

Pea lines	F%	M%	m%	A%	a%
SGE (wild-type)	94.16±2.98	66.74±3.82	70.35±2.48	37.27±3.23	55.34±3.11
SGEFix⁻-1 (*sym40*)	97.82±0.94	60.97±1.22	62.27±1.00	35.50±1.40	58.20±1.75
SGEFix⁻-2 (*sym33*)	59.00±4.70*	19.87±1.72*	33.62±1.19*	13.58±0.55*	69.52±5.55*

*Values differ significantly ($P \geq 0.95$) from corresponding values of other lines

dance in mycorrhizal root fragments (a%), which increased by 25% (Table 2). Plants of SGEFix⁻-2 (*sym33*) responded to AM formation and additional mineral nutrition with improved shoot biomass accumulation, the highest effect being with the addition of NPK (Fig. 1). Development of AM increased root biomass accumulation in this mutant, unlike the wild-type line SGE but similar to mutant SGEFix⁻-1 (*sym40*). An increase was also observed with the addition of bioavailable P and NPK (Fig. 2). Additional mineral nutrition had a much more pronounced effect on P accumulation in shoots of SGEFix⁻-2 than either SGE or SGEFix⁻-1, and the effect was nearly the same as that of AM development (Fig. 3). P accumulation in roots was also significantly increased in this mutant line SGEFix⁻-2 (*sym33*) by all experimental treatments. The largest increase was observed with AM but pronounced increases were also seen in response to additions of P and NPK (Fig. 4).

Discussion

The mutation in pea gene *sym33* involved in both NFS and AM symbioses was characterised phenotypically with respect to mycorrhizal colonisation and AM function. Mutation of this gene results not only in a decreased ability to perform endocytosis of rhizobia into nodule cell cytoplasm and in the formation of Fix⁻ nodules in NFS (Tsyganov et al. 1998), but also in decreased AM fungal colonisation of the root. However, this reduction in AM colonisation does not change the effectiveness of the AM symbiosis, perhaps due to increased arbuscule abundance in the mycorrhizal part of the roots. This is the first time that a plant gene has been described that controls NFS and AM development without affecting AM function. Unlike SGEFix⁻-2 (*sym33*), the Fix⁻ mutants of *P. sativum* (Duc et al. 1989), *Medicago sativa* L. (Bradbury et al. 1991) and *Phaseolus vulgaris* L. (Shirtliffe and Vessey 1996) described earlier are impaired in arbuscule formation (Marsh and Schultze 2001). Increased arbuscule development has been shown for the supernodulating pea mutant P88 (*sym29*) and for some allelic *Medicago truncatula* Gaertn. supernodulating *Mtsym12* mutants (Morandi et al. 2000). The latter authors attributed the increase in arbuscular mycorrhizal colonisation to possible defects in hormone balance and, in particular, to a decrease in the endogenous level of gibberellic acid in the pea mutant P88 (Morandi et al. 2000). Gibberellic acid is a strong inhibitor of mycorrhizal colonisation and especially of arbuscule formation (El-Ghachtouli et al. 1996). Because of the relative increase in arbuscule formation in mutant SGEFix⁻-2 (*sym33*), decreases in gibberellic acid may also be involved in the determination of its phenotype.

The shoot/root ratio estimates for biomass and phosphorus accumulation in the studied pea lines (Table 3) demonstrated that AM formation as well as NPK application can alter the pattern of wild-type plant development, in that shoots accumulated biomass and phosphorus faster than roots. These data are consistent with the results obtained earlier with pea (Estaun et al. 1987; Reinhard et al. 1992; Martensson and Rydberg 1994). Decreased root growth of mycorrhizal plants is generally explained by the AM fungi acting as an intense carbon sink, in spite of the fungal hyphae supplementing the root function (Smith and Read 1997). It is also important to note that AM development specifically increases cytokinin levels in plants and that cytokinin substantially influences shoot but not root growth (reviewed in Barker and Tagu 2000). Changes in shoot/root ratios in either pea mutant line were much less pronounced than those of the wild-type pea line or even negative. This, together with the delayed timing of flowering and pod ripening, suggests that mutations in *sym33* and *sym40*, in spite of their expression in roots (Tsyganov et al. 1998), have a pleiotropic effect and induce slight systemic changes in whole plant development due to defects in some hormonal regulatory mechanisms. These changes are particularly pronounced in the mutant SGEFix⁻-2 (*sym33*), which has the highest rate of biomass accumulation and a shorter period of plant development upon addition of NPK. The mutation in *sym33*, responsible for defective nodule development, reduced AM formation but not AM function and, thus, may affect some hormonal mechanisms controlling not only the development of both symbiotic systems but also plant growth and development.

Future research should throw light on the real nature of the mutation in *sym33*, especially its involvement in the plant hormone equilibria that play an important role in the regulation of development of symbiotic systems (Hirsch et al. 1997; Spaink 1997; Barker et al. 1998; Schultze and Kondorosi 1998; Barker and Tagu 2000). More extensive knowledge of the genetic control of such a hormonal regulatory mechanism would contribute to a better understanding of cellular interactions in root symbioses.

Table 3 Shoot/root ratios of rates of biomass increase and P accumulation in the wild-type pea line SGE and mutants SGEFix⁻-1 (*sym40*) and SGEFix⁻-2 (*sym33*) under various growth conditions (abbreviations as for Table 1)

Pea lines	Control		AM		P		NPK	
	Biomass	P	Biomass	P	Biomass	P	Biomass	P
SGE (wild-type)	4.5	3.0	7.9	8.6	5.8	5.6	7.4	6.6
SGEFix⁻-1 (*sym40*)	3.3	2.2	3.5	3.7	4.3	4.5	4.6	3.9
SGEFix⁻-2 (*sym33*)	4.5	3.7	3.8	4.0	3.9	4.9	4.8	5.0

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