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sym 13—A Gene Conditioning Ineffective Nodulation in *Pisum sativum*[1]

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ABSTRACT

Treatment of *Pisum sativum* (L.) cv. 'Sparkle' with ethyl methanesulfonic acid (EMS) produced a stable mutant, E135F, which forms small, white, ineffective nodules. These nodules exhibit histological zonation typical of an indeterminant nodule, *e.g.* meristematic, early symbiotic, late symbiotic, and senescent zones. Compared with the nitrogen fixing nodules of the parent, the zones are smaller and the nodules senesce prematurely. Bacteroids in E135F are less elongated and less differentiated than those in 'Sparkle.' The E135F mutant forms ineffective nodules when inoculated with nine different effective strains of *Rhizobium leguminosarum* and also when grown in a soil containing effective strains. The ineffective phenotype of E135F is under monogenic recessive control; the gene is designated *sym* 13. *sym* 13 was located on chromosome 2 by linkage with genes for shikimic dehydrogenase and esterase-2. The original selection E135F carried another mutation in heterozygous form at a separate locus, yielding some homozygous recessive nonnodulating progeny, E135N, in later generations. This indicates that EMS treatments may cause mutations at more than one *sym* gene. The gene conditioning non-nodulation in E135N was designated *sym* 14. It mapped to a locus on a different part of chromosome 2 by linkage to the gene for fumarase. The data demonstrate that *sym* genes are not necessarily closely linked.

Legume mutants defective in symbiotic nitrogen fixation provide a means of analyzing the contributions of the host to nodule development and function. Previously described mutants include those which form no or few nodules, ineffective nodules, or superabundant nodules (18). Variants of crimson clover (*Trifolium incarnatum*) (16), soybean (*Glycine max* (L.) Merr.) (2, 20), and alfalfa (*Medicago sativa* L.) (14) with ineffective nodules have been discovered in breeding programs. In addition, ineffective lines of chickpea (*Cicer arietinum* L.) (1), faba bean (*Vicia faba* L.) (4), and pea (*Pisum sativum* L.) (3, 15) have been obtained by induced mutagenesis.

This report describes an ineffective mutant of pea obtained by the use of mutagen EMS.[3] The mutant forms ineffective nodules when infected with strains of *Rhizobium leguminosarum* which effectively nodulate the parent cultivar. The stage at which nodule arrest is observed in this plant mutant is similar to that elicited on normal hosts by some ineffective (Fix^-) rhizobial mutants. This suggests that interruptions in nodule development, whether mediated by plant or bacterial mutants, occur only at a number of distinct stages.

MATERIALS AND METHODS

Plant Culture

Seeds of pea, *Pisum sativum* L. cv 'Sparkle,' were originally obtained from Rogers Bros. Seed Co. (Twin Falls, ID) and further inbred at the Boyce Thompson Institute. M_2 lines E135F[4] and E136, with white ineffective nodules were observed together during a search for nonnodulating mutants (10). To test for nodulation ability, peas were planted in coarse vermiculite in individual Cone-tainers (Ray Leach Cone-tainer Nursery, Canby, OR) and subirrigated with N-free nutrient (9), or with that nutrient containing 5 mM KNO_3. Seedlings were inoculated 4 DAP with *R. leguminosarum* 128C53 (Nitragen Co., Milwaukee, WI) and harvested 21 DAP and the roots and nodules examined. Plants were grown in light rooms at a 16 h/8 h, 20°C/15°C light/dark regime.

Effect of Rhizobial Strain

The nine strains of *Rhizobium leguminosarum* used were grown as described previously (9). Strains 128C53, RL300, ATCC 10004, PRE, PF2, TOM, 510P, 511P, and BB54b all form effective nodules on cv 'Sparkle.' The last four strains also nodulate *P. sativum* cv 'Afghanistan' (9).

Seeds were surface-sterilized, planted in sterile 180-mL Dispo bottles containing vermiculite and nutrient solution, and inoculated with the test strains (9). Roots were examined 21 DAP.

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[3] Abbreviations: EMS, ethylmethane sulfonic acid; *Est-2*, esterase 2; DAP, days after planting; *Skdh*, shikimic dehydrogenase.

[4] Seed of *P. sativum* E135F has been submitted to the Plant Introduction Service of the U.S. Department of Agriculture, Northeast USDA Germplasm Resources, New York State Agricultural Experiment Station, Geneva, NY 14456; to the Wiatrowo Pea Gene Bank, Wiatrowo, 62-100 Wagrowiec, Poland; and to the Nordiska Genbanken for jordbauksoch tradgardsvaxter, Box 41, 230-53 Alnarp, Sweden.

Table I. *Genetic Analysis of Mutant E135F Demonstrating Presence of Two Mutations Conditioning Ineffective Nodulation (sym 13) and Nonnodulation (sym 14)*

Cross No. 4543 was between an ineffective nodulating M_4 E135 and 'Sparkle.' Five F_1 plants were grown to maturity and their F_2 progeny were examined for nodulation and nodule color. χ^2 values indicated no significant deviation from the hypothesized ratios.

Population	No. of F_1 Lines	No. of Plants Observed			Closeness of Fit to Expected Ratios
		Pink nodules (%)	White nodules (%)	No nodules (%)	
'Sparkle'		10	0	0	
E135-1-10-X		0	6	1	
F_1	(one pod)	6			
F_2	3 (pooled)	38 (81%)	9 (19%)		3:1 $\chi^2 = 0.86$
F_2	2 (pooled)	25 (60%)	7 (17%)	10 (24%)	9:3:4 $\chi^2 = 0.20$

Genetic Analysis

Reciprocal crosses were made between ineffective lines E135F and E136 and between each of these lines and 'Sparkle.' The F_1 and F_2 generations and parental controls were scored for nodulation and nodule color after growth in Cone-tainers with strain 128C53. In some experiments, effectiveness was confirmed by measuring C_2H_4 production from plants placed in 500-mL jars containing 2.5% (v/v) C_2H_2. Nonnodulating line E135N was also crossed with 'Sparkle' and with other nonnodulating pea mutants in our collection (8).

Chromosomal Mapping

Lines E135F and E135N were crossed with two nodulating tester lines, A83-22-4c and 86-2-2, differing from each other in genetically mapped morphological and allozyme characteristics (21, 22). In addition, crosses were made between E135F and the chromosome 2 marker line B686-403. The F_2 generations were grown in Cone-tainers as above, inoculated with strain 128C53, and at 21 DAP were scored for effective nodulation and segregating morphological and allozyme polymorphism.

'Sparkle' and mutants derived from it have a fumarase allozyme (*Fum* locus) with a 'fast' mobility, whereas that enzyme in cv. 'Afghanistan' is 'slow' on electrophoretic gels. F_2 progeny of crosses between E135N and 'Afghanistan' were grown in sterile Dispo bottles and inoculated with strain TOM which nodulates 'Sparkle' and 'Afghanistan' but not E135N.

Linkages were established with the 'Linkage I' program on an Apple II Plus computer (17).

Field Test

'Sparkle' and E135F were planted at the Cornell Turf Grass Farm, Ithaca, NY, on 13 May 1986. The soil, Arkport silt loam, was known to contain effective *R. leguminosarum*. Maize had been grown on the plot in the previous year and available soil nitrate was low (7.2 mg/kg). Plants were harvested 32 DAP, nodules were counted, nitrogenase was detected by C_2H_2 reduction, and shoot N was measured by Kjeldahl analysis.

Microscopy

Nodules of 'Sparkle' and E135F were collected and prepared for light and electron microscopy as described previously (6, 7).

RESULTS

Two M_2 seedlings with white nodules, E135F and E136, were observed together during a search for nonnodulating mutants (10). The 'Sparkle' parent seed (M_1) had been mutagenized by treatment with 1% EMS for 1 h. The M_2 selections were grown to maturity and the ineffective character confirmed in the M_3 generation.

F_1 and F_2 progeny from E135F × E136 formed only ineffective nodules, indicating that these lines had mutant alleles at the same locus. Only E135F was used for further analysis.

Some nonnodulating E135F progeny were observed in the M_3 generation. This indicated that the gene conditioning nodulation had been carried as a heterozygote in the M_2. Analysis of an E135F × 'Sparkle' backcross (Table I) confirmed that E135F carried a hidden mutation affecting nodulation. F_2 families from some backcross F_1 segregated only for fixation, whereas sibling lines segregated for both nodulation and fixation in the ratio expected for two unlinked genes. Monogenic recessive control of nodulation was confirmed by

Table II. *Genetic Analysis of Ineffective Mutant E135F (sym 13) Established by Three Crosses to the Parent 'Sparkle'*

Acetylene reduction assays confirmed that all plants with pink nodules had nitrogenase activity while those with white nodules did not. The χ^2 values for the F_2 segregation ratios indicated no significant deviation from the expected 3:1 ratio.

Population	Generation	No. of Plants Observed		Closeness of Fit to Expected 3:1 Ratio
		Effective	Ineffective	
'Sparkle'	Parental	20	0	
E135F	Parental	0	33	
'Sparkle' × E135F	F_1	15	0	
Cross No. 4576	F_2	81	26 (24%)	$\chi^2 = 0.028$
Cross No. 5648	F_2	51	12 (19%)	$\chi^2 = 0.635$
Cross No. 5649	F_2	41	15 (27%)	$\chi^2 = 0.095$

Table III. *Joint Segregation Data for the Genes sym 13, Shikimic Dehydrogenase (Skdh), and Esterase 2 (Est-2) from F_2 Progeny of Two Reciprocal Crosses between Tester Line A83-22-4c and Ineffective Mutant E135F*

Parental phenotypes are A83-22-4c: Fix$^+$, slow *Skdh*, fast *Est-2*; and E135F: Fix$^-$, fast *Skdh* and slow *Est-2*. + = dominant phenotype or homozygous for fast allozyme. − = recessive phenotype or homozygous for the slow allozyme. [H/H = heterozygous allozyme pattern. + = parental phenotypes].

Loci	No. of Plants in Each Class						Total Progeny	χ^2			Recombination Frequency
	−/−	−/H	−/+	H/−	H/H	H/+	+/−	*sym* 13 +/H	Allozyme +/+	Joint	+ SE
sym 13/*Skdh*	0	2	15[x]	9[x]	30	2	58	0.57[a]	2.83[b]	40.0*	0.06 + 0.03
sym 13/*Est-2*	12[x]	4	1	2	28	10[x]	57	1.17[a]	1.17[b]	27.7*	0.13 + 0.05

[a] Expected 3:1 ratio. [b] Expected 1:2:1 ratio. [x] Parental type. * Significant deviation from random assortment, $P < 0.01$.

crosses of a stable E135N nonnodulating line with 'Sparkle' and with the nodulating tester lines.

Genetic analysis of a back cross of nonfixing E135F to the parent 'Sparkle' and crosses to other effectively nodulating lines demonstrated that the ineffective character is conditioned by homozygous recessive alleles. Effective is dominant to ineffective in the F_1 and segregates 3:1 in the F_2 (Table II), indicating control of the phenotype by a single gene. The gene is designated *sym* 13.

sym 13 assorted independently of segregating marker loci on chromosomes 1, 3, 5, and 7 (data not shown). Statistically significant deviation ($P < 0.001$) from random assortment was observed for *sym* 13 and the chromosome 2 marker loci *Skdh* and *Est-2* (Table III). Each of the markers individually fit the expected one-way segregation ratios. *sym* 13 is 6 and 13 map units distant from the genes for shikimic dehydrogenase and esterase-2, respectively, defining a map unit as 100 × recombination frequency (Fig. 1). Linkage of *sym* 13 to *Est-2* and *Skdh* was confirmed in crosses with tester line B686-403 (data not shown).

Crosses between E135N and other non-nodulating lines in our collection (8, 10) indicated that it was nonallelic with any previously named *sym* locus (data not shown). The new locus defined by E135N was designated *sym* 14.

The gene conditioning nonnodulation, *sym* 14, assorted independently of all the marker loci in the two tester lines, including nonlinkage to *Skhd* and *Est-2*. But, in crosses between E135N and 'Afghanistan,' joint segregation ratios for *sym* 14 and *Fum* deviated significantly from random assortment ($P < 0.001$). We estimate the *sym* 14 is 2 map units from *Fum*.

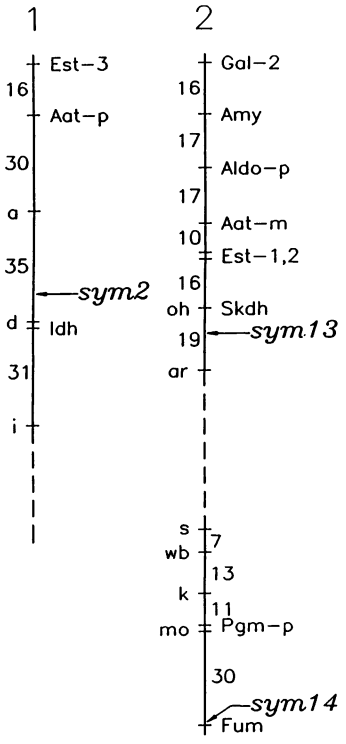

Figure 1. Location of *sym* 2, *sym* 13, and *sym* 14 on chromosomes of *P. sativum*. The approximate locations on chromosome 2 are estimated from the recombination frequencies in Tables III and IV.

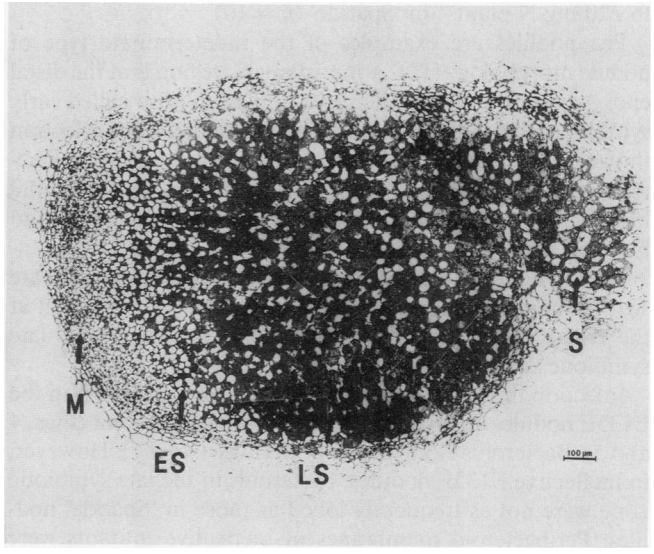

Figure 2. Light microscope montage of a longitudinal section of an effective nodule of 'Sparkle' harvested 4 weeks after inoculation. Four distinct developmental zones are present: M (meristematic zone); ES (early symbiotic or early invasion zone); LS (late symbiotic or elongate bacteroid zone); and S (senescent zone).

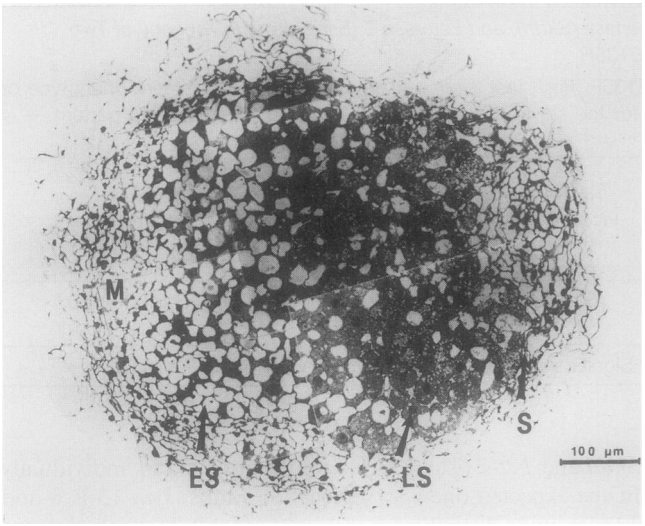

Figure 3. Light microscope montage of a longitudinal section of an ineffective nodule of mutant E135F. This nodule was harvested at the same age as the wild-type nodule. Although the same developmental zones are present, the size of each, especially the late symbiotic zone (LS), is reduced.

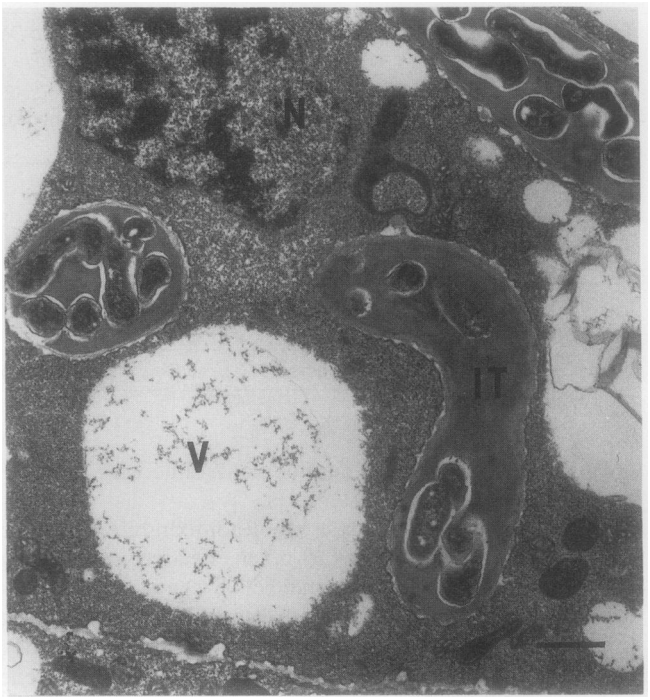

Figure 4. Transmission electron micrograph of a 'Sparkle' nodule cell from the ES zone. A branched infection thread (IT) is evident as are several vacuoles (V) and the host cell nucleus (N). Bar = 1 μm.

The nine strains of *R. leguminosarum* all induced 80 to 150 nodules on E135F, as many as they did on 'Sparkle.' But, in contrast to 'Sparkle,' the nodules were small and pale. Nitrogenase (C_2H_2) activity was absent whereas 'Sparkle' averaged 4.7 μmol C_2H_4 $plant^{-1}h^{-1}$.

By 32 DAP in an unfertilized plot, 'Sparkle' plants averaged 62 nodules, and had an average acetylene reduction activity of 2.3 μmol $plant^{-1}h^{-1}$. E135F plants averaged 22 nodules and had no detectable nitrogenase. The mutant plants were smaller, and their shoots contained 70 mg N $plant^{-1}$ compared to 220 mg N $plant^{-1}$ in 'Sparkle' ($n = 10$).

Pea nodules are examples of the indeterminate type of nodule morphology (12). A meristematic region is at the distal end. Adjacent to that is the thread invasion (also called early symbiotic) zone in which rhizobia are released from infection threads into the plant cells. In the late symbiotic zone, bacteroids differentiate and begin to fix nitrogen. The proximal histological zone is the senescent region in which plant and bacterial cells degenerate (Fig. 2).

E135F nodules resemble wild type in zonation but are significantly smaller in size (Fig. 3). A meristem is present at the distal end, but the dimensions of the early and late symbiotic zones are reduced.

Infection thread development and release of rhizobia in the E135F nodules appeared similar to that of the parent (Figs. 4 and 5). Bacteroids elongated in both (Figs. 6 and 7). However, in ineffective E135F nodules, bacteroids in the late symbiotic zone were not as frequently lobed as those in 'Sparkle' nodules. Peribacteroid membranes in ineffective mutants were frequently detached from the bacteroids. The bacteroids also had electron dense cytoplasms. These are both cytological markers indicating bacteroid degeneration (Fig. 7).

As in 'Sparkle,' the proximal end of the E135F nodule is senescent (Fig. 3).

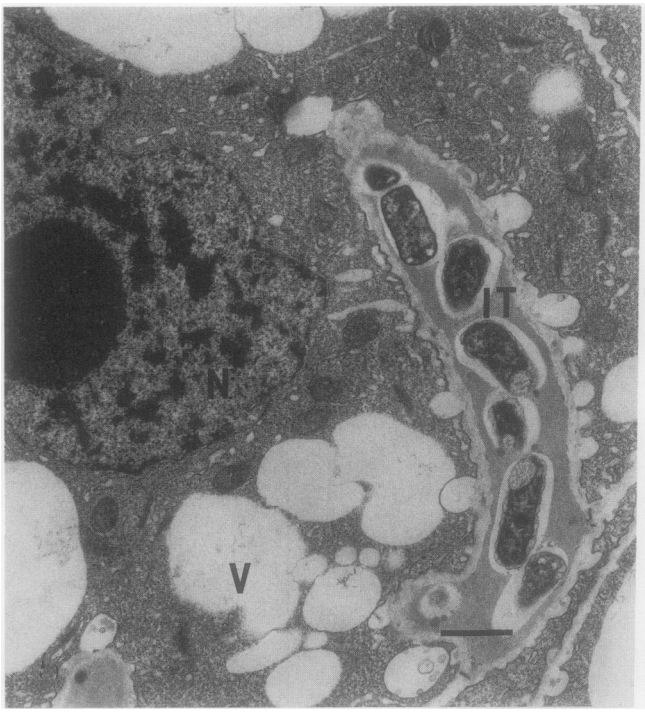

Figure 5. Transmission electron micrograph of an ineffective mutant E135F nodule cell from the ES zone. The host nucleus (N) and vacuoles (V) are present as is an infection thread (IT) with rhizobia. Bar = 1 μm.

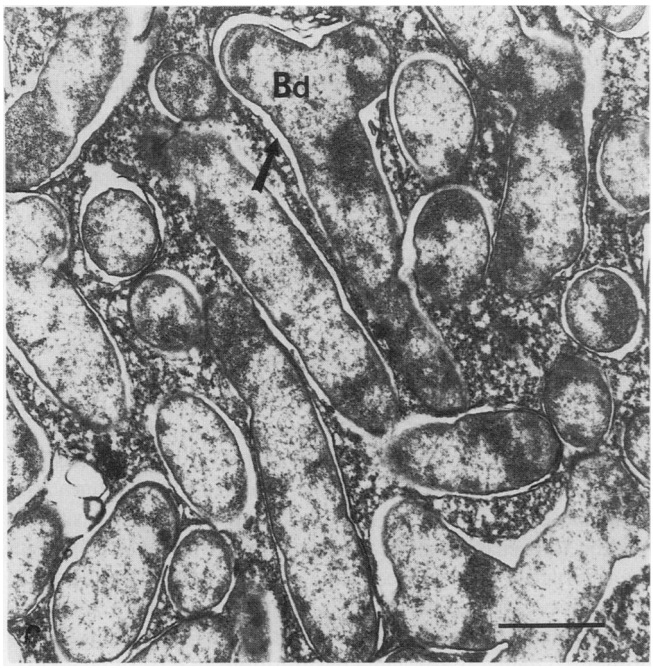

Figure 6. Transmission electron micrograph of bacteroids within a 'Sparkle' nodule cell from the LS zone. The bacteroids (Bd) are elongate and lobed. The peribacteroid membrane (*arrow*) is closely appressed to each individual bacteroid. Bar = 1 μm.

DISCUSSION

E135F is a stable nodulating, non-fixing mutant line of *Pisum sativum* cv. 'Sparkle.' The mutation is not strain dependent; nodules are ineffective with nine strains of tested rhizobia as well as in the field. The phenotype is stable in the field as well as in controlled lab situations, indicating that the ineffectiveness is not an artifact dependent on artificial growth conditions. Thus, the mutant E135F can be used in studies on crop physiology, such as in estimating by difference how much nitrogen is fixed by peas. Moreover, the E135F phenotype is not dependent on the genetic background of 'Sparkle,' because it is expressed in the F_2 progeny of crosses with tester lines. Monogenic recessive control of ineffective nodulation is documented both by back-crosses of E135F with 'Sparkle' (Table I) and in crosses with tester lines (Table II). *Sym* 13 is the first gene governing ineffective nodules found by mutagenesis of cv 'Sparkle' (10). An ineffective mutant was also obtained in *P. sativum* cv. 'Rondo' by EMS mutagenesis (15). However, that mutant gene is not allelic to *sym* 13 (JG Postma, personal communcation).

Our discovery of E135F and E136 with white nodules was a chance observation. By contrast, Duc and Messager (3) searched for nodulating, non-fixing pea mutants by using shoot chlorosis as an indicator of ineffective nodules. They obtained six independently derived stable ineffective mutants. All the F_1 plants obtained from reciprocal crosses among those mutants were nodulating and effective, indicating that there are at least six nonallelic plant genes involved in nodule effectiveness in pea. This suggests that the study of many mutants may be necessary to elucidate all the plant's genetic contribution to nodule function.

The ineffective mutant selection E135F harbored a cryptic mutation effecting nodulation. The segregation data (Table I) indicated that these two *sym* genes were unlinked; it is thus apparent that EMS can cause mutations at several locations in the genome of an individual pea. It is relatively easy to generate stable symbiosis mutants of pea (3, 10) and other species (18). It is tempting to use such mutant selections immediately for physiological studies. However, our results indicate that, before experimentation, mutant lines should be backcrossed to the parent to reveal hidden recessive mutations or eliminate undetected mutations at other loci.

The pair of tester lines used in this study were developed with different alleles at over twenty isozyme loci (21, 22). In addition, each tester line possesses distinctive morphological mutations. By crossing our *sym* mutants of 'Sparkle' to both the tester lines, a joint segregation pattern of the unmapped *sym* gene with loci covering approximately 75% of the known pea linkage map can be established. It should be noted that this efficient approach is valid regardless of the genotype of the parent line (here 'Sparkle') because, if the parent shares alleles with one tester line, it will necessarily differ from the other tester line. Use of these lines and line B686-403 showed that *sym* 13 was on chromosome 2.

The position of the *sym* 14 locus could not be determined with the two tester lines. However, crosses with an inbred line

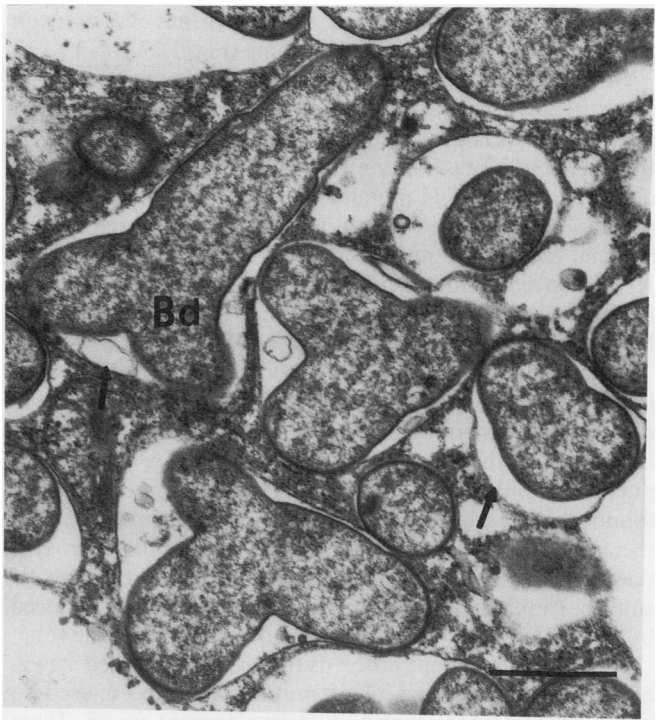

Figure 7. Transmission electron micrograph of bacteroids within a mutant E135F nodule cell from the LS zone. The bacteroids (Bd) are not as elongate as those in 'Sparkle' nodule cells; their cytoplasm is also more heterogeneous. The peribacteroid membrane (*arrow*) ia pulled away from the bacteroid and the host cell cytoplasm in electron dense. Bar = 1 μm.

Table IV. *Genetic Analysis of Nonnodulating E135N*

Joint segregation data for the genes *sym* 14 and *Fum* from the F_2 progeny of a cross between 'Afghanistan' and E135N. The parental phenotypes are 'Afghanistan': nod$^+$, 'fast' fumarase and E135N: nod$^-$, 'slow' fumarase. − = recessive phenotype or homozygous for the slow allozyme; H = heterozygous.

Loci	No. of Plants in Each Class						Total		Joint Segregation		Recombination Frequency + SE
	+/−	+/H	+/+	−/−	−/H	−/+	Progeny	*sym* 14	*Fum*	Joint	
sym 14/*Fum*	19	27	0	0	1	11	58	1.19[a]	2.27[b]	52.12*	0.02 + 0.02

[a] Expected 3:1 ratio. [b] Expected 1:2:1 ratio. * Significant deviation from random assortment, $P < 0.001$.

of 'Afghanistan,' which has an easily resolved allozyme variant of fumarase, revealed a linkage between *Fum* and *sym* 14 (Table IV). *Fum* is currently placed on *Pisum* linkage group 2, but it may be on another chromosome (23). If *Fum* and *Skdh* are on the same chromosome, they are so far apart that they display random assortment. The relatively close linkage between *Skdh* and *Sym* 13 (6 map units) and between *Fum* and *sym* 14 (2 map units), along with the fact that markers 20 map units towards *Skdh* from *Fum* still assort independently (23) imply that *sym* 13 and *sym* 14 must also assort independently.

Moreover, neither *sym* 13 nor *sym* 14 are linked to *sym* 2. This is a recessive gene, found in primitive peas from Afghanistan, which conditions strain-specific nodulation (9). *sym* 2 has been mapped to chromosome 1 by its linkage to *d* (axil pigment) (24) and to *idh* (isocitric dehydrogenase) (8). Inclusively, the data established that some *sym* genes of *P. sativum* are not even on the same chromosome (Fig. 1).

On the parental cultivar 'Sparkle', nodules have a normal structure like that previously reported (12). The phenotype of E135F nodules is similar to that of alfalfa nodules formed by the host-conditioned mutant in_1 (19) or that of nodules induced by *nif*A or *nif*H mutants of *R. meliloti* (6, 7). In those nodules, the bacteroid or late symbiotic zone is reduced in size and most of the nodule consists of senescent tissue. Like *R. meliloti nif*A mutant bacteroids, bacteroids of effective *R. leguminosarum* strains inhabiting E135F nodules elongate but not to the extent as they do in wild type.

The ineffectiveness of E135F nodules was confirmed by lack of acetylene reduction. In addition, no nitrogenase was detected on Western blots from *R. leguminosarum* bacteroids isolated from these nodules (N Suganuma, TA LaRue, unpublished results) Furthermore, very little leghemoglobin was found in E135 nodules, and the activities of glutamine synthetase, phospho*enol*pyruvate carboxylase, glutamate synthase, and other proteins were markedly reduced. These results are similar to those for ineffective alfalfa nodules induced by *R. meliloti nif* H mutants (13) and with the in_1 gene (5).

Nodule development proceeds through well defined stages, and both bacterial and plant mutants have been used to observe the stages at which nodule development arrests. There appears to be a good correlation between the stage at which nodulation is blocked and the structural appearance and number/amount of proteins expressed by both symbionts. For example, nodules arrested before rhizobial invasion into host tissues contain mRNAs for early nodulins only, whereas infected, but Fix$^-$ nodules, contain mRNAs for leghemoglobin, sucrose synthase, and other late nodulins (11). Interestingly, plant or bacterial mutants interfere with nodule development at similar, if not identical, stages. This would suggest that there are a limited number of steps at which bacteria and plant closely interact, most likely by an exchange of signals leading to a cascade of gene expression. If there is a flaw in the interaction, the subsequent stage in nodule development does not occur, and the next set of genes is not expressed.

We do not know how *sym* 13 functions in nodule development; a defect in this gene has multiple effects. In fact, very few plant genes have been examined with regard to nodulation and nitrogen fixation. An elucidation of *sym* 13 gene function may give us insight into the interaction between *Rhizobium* and legumes which results in an effective nitrogen-fixing symbiosis.

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