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The genetic interaction between non-nodulation and supernodulation in soybean: an example of developmental epistasis

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Summary. The interaction between three non-nodulation mutants (nod49, nod772 and nod139) and a supernodulation mutant (nts382) of soybean was studied by analysing the progeny from crosses between these mutants. Previously it had been shown that the non-nodulation mutants arose from single mutation events and that nod49 and nod772 are allelic, whereas nod139 represents another gene required for nodulation. Analysis of progeny from crosses between nts382 and the wild type showed that this mutant also arose from a single mutation. Complementation tests demonstrated that the mutation responsible for supernodulation in nts382 is not allelic to either of these non-nodulation characters, and that it segregates independently. Progeny were identified that were homozygous for both supernodulation and non-nodulation, and these plants were incapable of nodulation. Thus, non-nodulation is epistatic over supernodulation and this is discussed in terms of the developmental blockage in the two mutant types. The identification and confirmation of these double mutants of the supernodulation and non-nodulation mutations are described. Although the non-nodulation mutations behave as recessive characters in a wild-type background, these mutations are incompletely dominant in a genetic background homozygous for supernodulation. The significance of these results to the understanding of nodule ontogeny is discussed.

Key words: Non-nodulation mutants – Supernodulation mutant – Epistasis – Autoregulation – Soybean

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Introduction

The nitrogen-fixing root nodule symbiosis in legumes is influenced by genetic (Ohlendorf 1985) and environmental factors (Dart 1977). The clear demonstration of the importance of host genetic attributes controlling nodulation has come with the isolation of legume mutants that have altered numbers of nodules (Carroll and Mathews 1989). In soybean, for example, both supernodulation and non-nodulation mutants have been identified.

The early events in the encounter between the microsymbiont and the legume include colonisation of the rhizosphere, bacterial attachment to the root surface and infection thread formation (Vincent 1980; Pueppke 1986). The usual mechanism of entry by the rhizobia is through infection threads formed in the immature root hairs near the root tip (Turgeon and Bauer 1982). Nodules are formed by the differentiation and division of cells in the root cortex (Calvert et al. 1984; Mathews et al. 1989b). The major steps in soybean nodule ontogeny are illustrated in Fig. 1. The earliest discernible events in the differentiation of the soybean root cortex are cell divisions in those cells immediately subjacent to the root epidermis. These centres of differentiation, called subepidermal cell divisions, may or may not have an associated infection thread in a distal hair. Only divisions with actual infections are capable of developing into nodules, but even then the number of infections in wild-type legumes is much greater than the number of nodules that are formed (Bauer 1981).

In soybean, most infections do not develop beyond a relatively early stage of cell division, prior to the formation of nodule meristems (Calvert et al. 1984; Pueppke and Payne 1987; Mathews et al. 1989b). It has also been observed that the development of an infection into a nodule is less probable if earlier rhizobial infections have

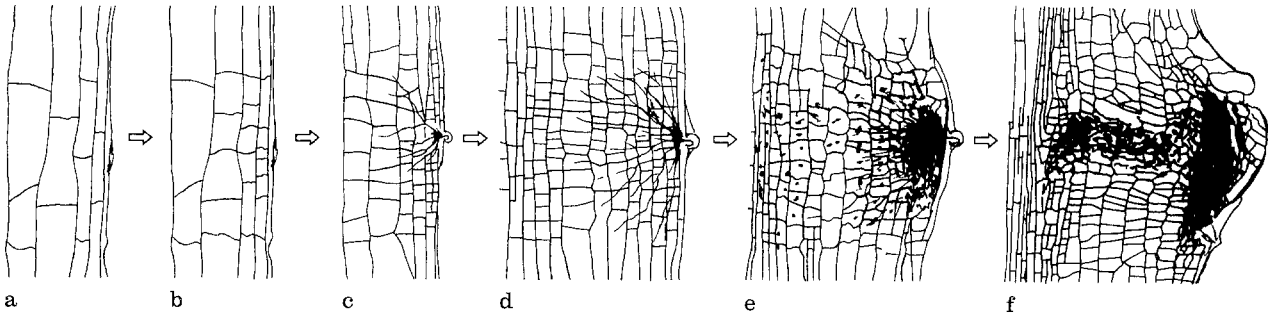

Fig. 1 a–f. Diagramatic representation of nodule ontogeny in soybean (drawn from micrographs published by Calvert et al. 1984 and Mathews et al. 1989 b). **a** Outer root cortex of an uninoculated root (the root epidermis is represented by the thin cell layer on the right-hand side of the figure). **b** Division of outer root cortex cells immediately subjacent to the root epidermis. **c** Progression of cell divisions in the outer root cortex and infection thread formation (in the case of actual infections, but not pseudoinfections, which are cell divisions without an infection thread). **d** Divisions extend to the inner root cortex. **e** Formation of a nodule meristem. **f** Differentiation of vascular tissue and nodule emergence. In wild-type legumes, the number of actual infection events greatly exceeds the number of nodules that are formed. The non-nodulating mutants of soybean are defective in the ability to initiate subepidermal cell divisions. In the supernodulating mutant nts382, a greater proportion of actual infections develop into nodules as compared to the wild-type parent (Mathews et al. 1989 b)

occurred on the root (Pierce and Bauer 1983). This phenomenon has been termed autoregulation of nodulation (Carroll et al. 1985 a, b) and it is responsible for curtailing the number of nodules that are formed on the root.

As mentioned earlier, soybean mutants are available that either lack nodulation (Williams and Lynch 1954; Carroll et al. 1986; Mathews et al. 1989 a) or have greatly increased numbers of nodules (Carroll et al. 1984, 1985 a, b). The latter category have been termed supernoduling mutants and are defective in autoregulation of nodulation. With the exception of the naturally occurring non-nodulation mutant rj_1 (Williams and Lynch 1954), all of these mutants arose after mutagenesis. The developmental blockage (Fig. 1; Mathews et al. 1989 b) and the nature of inheritance (Mathews et al. 1989 a) of the non-nodulation mutants has been described recently. The mutants of soybean that are allelic to rj_1 (nod49 and nod772), and rj_1 itself, have only a few centres of subepidermal cell divisions. These centres are devoid of infection threads and do not usually develop beyond the very early stages of nodule ontogeny. In nod139, subepidermal cell divisions or infection threads were never observed (Mathews et al. 1989 b). This mutant is not allelic to the rj_1 mutation and represents a separate gene required for nodulation (Mathews et al. 1989 a). In a genetic background where wild-type autoregulation of nodulation is operative, all of the non-nodulation characters behave as simple monogenic Mendelian recessives (Mathews et al. 1989 a). The supernodulating mutant nts382 behaves as a recessive (Carroll et al. 1985 b, 1988). While it has a similar number of subepidermal cell divisions to the wild type, a much greater proportion of these develop into nodules (Fig. 1; Mathews et al. 1989 b).

Grafting experiments have indicated that the supernodulation character in nts382 is shoot controlled while the non-nodulation characters are root controlled. Furthermore, the inability of the supernodulation shoot to suppress the non-nodulation phenotype in the non-nodulation mutants has been demonstrated (Delves et al. 1986). In this paper, the nature of inheritance of nts382 is described in more detail. The genetic interaction between the two extreme nodulation characters was studied by analysing the progeny from crosses between nts382 and the non-nodulation mutants. The identification and confirmation of the double mutants of supernodulation and non-nodulation are also described. The results presented here are of relevance to our understanding of autoregulation and the procedures used for isolating mutants defective in nodulation.

Materials and methods

The soybean cv Bragg and its non-nodulating mutants (Carroll et al. 1986; Mathews et al. 1989 a), and the supernodulation mutant nts382 (Carroll et al. 1985 a, b) were used. The F_1 plants of the crosses between nts382 and nod49, nod772 or nod139 (Table 1) were grown in the glasshouse, screened for the nodulation phenotype and then allowed to produce F_2 families. Eight to ten F_2 seeds were then planted in 25-cm diameter pots filled with a 2:1 mixture of sand and vermiculite. All seeds (including representatives of the parental lines) were inoculated at planting with *Bradyrhizobium japonicum* strain USDA110 (10^8 cells · seed^{-1}) and once again at the same rate a week after germination. The seedlings were watered with 5 m*M* KNO_3-supplemented nutrient solution (Herridge 1977) twice a week. For the first 2 weeks after planting, the plants received half strength of all the nutrients, except $CaCl_2$, which was dispensed at full strength. Full-strength nutrient solution was administered in the following 4 weeks. Plants were harvested 6 weeks after germination and scored for the F_2 segregation of the different nodulation phenotypes. The size of individual F_2 families ranged from 52 to 271 plants.

Selected F_2 plants were saved and selfed to obtain F_3 seeds. Segregation ratios, nodulation and plant growth parameters of the F_3 progeny were recorded. To identify the presence of a supernodulating shoot on a non-nodulating plant, scions of the selected F_3 segregants were grafted onto Bragg root stocks using the wedge-grafting technique. The terminal 6–8 cm of the shoots of the selected plants were grafted on 10-day-old Bragg rootstocks. Bragg and nts382 shoots grafted on Bragg rootstocks served as control grafts. The pattern of nodulation on the Bragg rootstock was observed. The wild-type pattern of nodulation was characterized by restricted nodulation mainly at the crown region of the root, whereas the nts382 phenotype displayed nodules over the entire root system from the top of the root to the root apex of both the primary and lateral roots.

Table 1. F_1 complementation analysis of the non-nodulation mutants nod49, nod772 and nod139 and the supernodulation mutant nts382. Plants were scored for nodulation 6 weeks after germination and inoculation with *Bradyrhizobium japonicum* strain USDA110. Reciprocal crosses gave identical results

♂ \ ♀	Bragg	nts382
Bragg	+	+
nod49	+	+
nod772	+	+
nod139	+	+
nts382	+	++

\+ Normal nodulation
++ Supernodulation

Results

Monogenic inheritance of the nts382 character

In confirmation of earlier research, the recessive nature of inheritance of nts382 was demonstrated by analysing progeny derived from reciprocal crosses between the mutant and its wild-type parent cultivar, Bragg. All eight F_1 plants tested from this cross were wild type for nodulation, and the 225 F_2 progeny segregated 165 wild type : 60 mutant, which approximates a 3 : 1 ratio. All the F_3 progeny derived from supernodulating F_2 plants displayed the supernodulation character, thereby demonstrating monogenic inheritance and indicating that the supernodulating mutant nts382 arose from a single mutation event.

F_2 segregation of crosses between the supernodulation and non-nodulation mutants

The F_1 progeny of the crosses between nts382 and either nod49, nod772 or nod139 had the wild-type pattern of nodulation (Table 1). At least eight F_1 progeny were screened for each cross and reciprocal crosses yielded identical results. Their root systems and the manner of nodulation on the roots were indistinguishable from the wild-type Bragg roots. Three phenotypic classes were always obtained in the F_2 families at a ratio of 9 wild type: 3 enhanced nodulation: 4 non-nodulation, instead of the classical 9 : 3 : 3 : 1 ratio, thus suggesting that the nodulation phenotype of the double mutant was non-nodulation (Table 2). Chi-square analysis indicated a high level

Table 2. F_2 segregation of progeny obtained from crosses between non-nodulating mutants nod49, nod772 or nod139 and the supernodulation mutant, nts382. The segregation ratio was 9 wild type: 3 supernodulation: 4 non-nodulation phenotypes, thus indicating a recessive epistatic gene interaction. The observed values were tested for goodness-of-fit to the expected by Chi-square analyses

Cross (♀ × ♂)	Cross no.	F_2 segregation			Calculated χ^2 (9:3:4)[a]	*P*
		Wild type	Enhanced nodulation	Non-nodulation		
nod49 × nts382	1	105 (106.875)[b]	40 (35.625)	45 (47.500)	0.702	0.704
	2	66 (73.688)	30 (24.563)	35 (32.750)	2.160	0.340
nts382 × nod49	1	156 (152.438)	51 (51.508)	64 (67.678)	0.193	0.908
	2	36 (37.125)	14 (12.375)	16 (16.500)	0.263	0.877
nod772 × nts382	1	83 (82.125)	25 (27.375)	38 (36.600)	0.277	0.871
	2	67 (68.063)	20 (22.688)	34 (30.250)	0.800	0.690
nts382 × nod772	1	99 (90.000)	24 (30.000)	37 (40.000)	2.325	0.313
nod139 × nts382	1	57 (58.000)	21 (19.500)	26 (26.000)	0.154	0.926
	2	86 (74.813)	20 (24.938)	27 (33.250)	3.825	0.148
nts382 × nod139	1	28 (29.250)	7 (9.750)	17 (13.000)	2.060	0.357
	2	74 (64.125)	18 (21.375)	22 (28.500)	3.536	0.171

[a] Calculated χ^2 values indicated the segregation ratios were not significantly different from the expected ratio (9 : 3 : 4) at 5% critical value; tabulated χ^2 for 2 *df* is 5.99
[b] Expected sizes of phenotypic classes for 9 : 3 : 4 segregation are listed in brackets

Table 3. F_3 segregation of the progeny derived from some F_2 plants with enhanced nodulation obtained from crosses between the supernodulation mutant nts382 and the non-nodulation mutants. The progeny segregated into one extreme supernodulation: two intermediate supernodulation: one non-nodulation progeny. Chi-square was used to test the goodness-of-fit of the observed and the expected values. Other F_2 progeny with enhanced nodulation bred true

Cross (♀ × ♂)	F_3 segregation				Calculated χ^2 (1:2:1)[a]	P
	F_3 family	Extreme supernodulation	Intermediate supernodulation	Non-nodulation		
nts382 × nod49	1	4 (6.250)[b]	12 (12.500)	9 (6.250)	2.040	0.361
	2	4 (6.000)	13 (12.000)	7 (6.000)	0.917	0.632
	3	3 (5.500)	12 (11.000)	7 (5.500)	1.636	0.441
nts382 × nod772	1	38 (32.250)	68 (64.500)	23 (32.250)	3.868	0.145
	2	26 (27.250)	59 (55.500)	26 (27.250)	0.441	0.802
nts382 × nod139	1	5 (4.750)	10 (9.500)	4 (4.750)	0.157	0.925
	2	10 (9.500)	22 (19.000)	6 (9.500)	0.789	0.674

[a] Calculated χ^2 values indicated that the segregation ratios were not significantly different from the expected ratio (1:2:1) at the 5% critical value; tabulated χ^2 for 2 *df* is 5.99

[b] Expected sizes of phenotypic classes for 1:2:1 segregation are listed in brackets

Table 4. Nodulation of F_3 segregants of F_2 supernodulating plants obtained from the cross between nts382 and nod49, nod772 or nod139. Plants were grown in sand-vermiculite (2:1) mixture and watered with 5 m*M* KNO_3-supplemented plant nutrient solution. Plants were harvested 6 weeks after planting. Data are means of eight plants ±SE

Cross (♀ × ♂)	F_3 nodulation phenotype	Nodule no. · plant^{-1}	Nodule dry weight · plant^{-1}
nts382 × nod49	Extreme supernodulation	158 ± 13	88 ± 6
	Intermediate supernodulation	103 ± 7	40 ± 5
	Non-nodulation	0	0
nts382 × nod772	Extreme supernodulation	370 ± 89	246 ± 26
	Intermediate supernodulation	217 ± 21	178 ± 12
	Non-nodulation	0	0
nts382 × nod139	Extreme supernodulation	420 ± 39	253 ± 15
	Intermediate supernodulation	300 ± 33	211 ± 29
	Non-nodulation	0	0

of significance of the observed values to the expected 9:3:4 ratio, and this conclusion was confirmed by subsequent experiments presented below.

Segregation of the supernodulation genotype in the F_3 and identification of double mutants

Since the mutation in nts382 is recessive, all the F_2 plants with enhanced nodulation were homozygous at this locus. At the other locus of interest (i.e. the non-nodulation locus), these plants were either homozygous wild type or heterozygous for the non-nodulation mutation: nod49, nod139 or nod772. Some enhanced nodulation plants obtained in the F_2 were true breeding, while others segregated in the F_3 into three phenotypic classes in the ratio of one extreme supernodulation: two intermediate supernodulation: one non-nodulation (Table 3). Although the supernodulation categories were similar, they could be distinguished on both the number and the pattern of nodulation on the roots and total nodule dry weight per plant (Table 4). For the progeny obtained from crosses between nts382 and nod49, e.g., the extreme category which produced only supernodulating progeny in subsequent generations had on average 158 nodules · plant^{-1} 6 weeks after planting, distributed in a beaded manner along the entire length of the tap and lateral roots. Furthermore, all laterals were covered with nodules and were similar to the supernodulating parent nts382. The intermediate category had a lower mean number of nodules with 103 nodules · plant^{-1} (Table 4), and the nodules were distributed at a lower density on both the laterals and tap root. The nodulation of the F_3 nts382 × nod49 segregants was different from the nts382 × 139 or nts382 × nod772 F_3 segregants, since the former experiment was done under different seasonal conditions. The intermediate supernodulators on selfing gave three phenotypes in the F_4 generation at the ratio of one non-nodulation: two intermediate supernodulation: one extreme supernodulation. From Table 4, it is clear that the nodule dry weight for the intermediate category was significantly lower than the true-breeding homozygous supernodulators. The non-nodulation segregants derived from plants with an intermediate phenotype were selected as the double mutants and were designated as DM49, DM772 and DM139 for the double mutants obtained from crosses between the supernodulation mutant nts382 and the non-nodulation mutants nod49, nod772 or nod139, respectively. The double mutants bred true for the non-nodulation character. The same results were obtained for the reciprocal crosses of the mutants.

Table 5. Nodule number of the grafts between the double mutant DM49 shoots and Bragg rootstocks. Mutant nts382 and Bragg shoots grafted on Bragg rootstocks served as control. The terminal portion of the shoots were grafted on 10-day-old Bragg roots. The grafts were watered with 5 m*M* KNO_3-supplemented plant nutrient solution twice a week and harvested 4 weeks after grafting. Data are means of eight plants ±SE

Graft (scion/rootstock)	Nodule no. · plant^{-1}
Bragg/Bragg	155± 18
nts382/Bragg	478±133
DM49/Bragg	660± 39

Verification of the double mutants

Double recessive mutants were selected in the F_3 by selfing the F_2 segregants with enhanced nodulation as described above. The shoots (scions) of these non-nodulating F_3 plants, when grafted onto the wild type Bragg (rootstock), produced supernodulation. In the case of DM49 (Table 5), the nodule number per plant of this graft was similar to the nts382 control grafted on Bragg (660±39 versus 478±133 nodules · plant^{-1}), and the Bragg controls had 155 nodules · plant^{-1}. The root, shoot and plant dry weights of the double mutant graft were similar to the nts382 controls and much lower than the Bragg control grafts. Likewise, the root length of the Bragg rootstock with the double mutant scion was similar to the nts382 control graft on Bragg and much lower than the Bragg control graft (data not shown).

Discussion

The non-nodulation mutants are not allelic with the supernodulation mutant nts382 and these different types of mutants complement one another in the F_1 to yield the wild type phenotype (Table 1). More importantly, the F_2 progeny of the crosses between the supernodulation mutant, nts382, and the non-nodulation mutants segregated into three phenotypic classes of 9 wild type: 3 supernodulation: 4 non-nodulation, denoting that the two genes (supernodulation and any non-nodulation character) are unlinked and show interaction in the form of epistasis. One-quarter of the non-nodulation class were the double recessive mutants. Thus, the expected 9:3:3:1 ratio was modified, since the double mutant was epistatically placed with the non-nodulating group, confirming that supernodulation did not suppress the non-nodulation root. Previous results showed that a nts382 shoot on a non-nodulation root resulted in non-nodulation (Delves et al. 1986). The genetic analysis described here provided an additional verification of this interactive phenomenon.

Closer examination revealed that there were two categories of supernodulation in progeny derived from crosses between nts382 and the non-nodulating mutants. When the F_2 plants with enhanced nodulation were saved to obtain the F_3 segregation ratios, three phenotypes were observed in some of these families at the ratio of one extreme supernodulation: two intermediate supernodulation: one non-nodulation. In other words, two categories of enhanced nodulation were observed. The extreme supernodulating plants had the characteristic nts382 pattern of nodulation, wherein large numbers of nodules completely covered the entire tap and lateral roots. These plants were true breeding for supernodulation and were, therefore, homozygous at both loci. In contrast, the intermediate supernodulating plants had a lower number of nodules compared to the supernodulators, and the nodules were randomly distributed on the root system at a lower density. The plants with intermediate supernodulation segregated into one extreme supernodulation: two intermediate supernodulation: one non-nodulation in the subsequent generation. The non-nodulating plants from these F_3 families carried the supernodulation allele in a homozygous condition and were classified as double mutants. Supernodulation of Bragg roots by the double mutant scions confirmed that non-nodulation is epistatic over supernodulation. The observed segregation of families derived from plants with intermediate supernodulation gave a good fit to the observed values when tested by Chi-square analysis (Table 3), and indicated that (i) the homozygous double mutant was non-nodulating, (ii) plants homozygous wild type for the non-nodulation locus and homozygous for nts382 were supernodulating, and (iii) plants heterozygous for the non-nodulation locus and homozygous for the *nts*382 allele were intermediate supernodulating.

The non-nodulation mutants nod49 and nod772 are blocked at the early infection stage of nodulation, namely at the initial stage of cortical cell division (Fig. 1). Subepidermal cell divisions in these mutants are rare and are restricted to pseudoinfections (divisions not associated with infection threads), while nod139 does not have any form of subepidermal cell divisions nor infection threads (Mathews et al. 1989b). The supernodulation mutant nts382 is altered in its autoregulation mechanism, resulting in an unrestricted number of nodules on the root system. The number of infection threads in this mutant is similar to the wild type but a greater proportion of these infections in nts382 are successful in the formation of nodules (Mathews et al. 1989b). The double mutants (DM49, DM772 and DM139) of the crosses between nts382 and either nod49, nod772 or nod139 have a non-nodulation phenotype. Here, the non-nodulation gene in these mutants epistatically suppresses the supernodulation gene. This relationship can be explained in terms of the defects in nodule ontogeny of the two

mutant types and is an example of developmental epistasis. Here, the alteration in the autoregulatory response in nts382 is masked by the blockage of the non-nodulation mutants at the earlier subepidermal cell division stage, thereby resulting in a non-nodulation phenotype in the double mutants.

It has been reported that the non-nodulation characters described here are recessive and that plants heterozygous for any of these defects display wild-type nodulation (Mathews et al. 1989a). However, in a genetic background that is homozygous for supernodulation and thereby lacks autoregulation, plants heterozygous for a non-nodulation locus produce intermediate, but not extreme supernodulation. These observations suggest that either there is some degree of physiological interaction between supernodulation and non-nodulation and/or that nod49, nod139 and nod772 mutations are indeed incompletely dominant, but this nature of inheritance is masked by autoregulation in the wild type. Based on the first possibility, it is probable that both the supernodulation and the non-nodulation mutations affect the rate of subepidermal cell differentiation (Mathews et al. 1989b). In addition, the results may also indicate incompletely dominant inheritance of the non-nodulation characters at the microscopic level of observation. In a background where autoregulation is operative, plants heterozygous for a non-nodulation allele could have less infections than wild-type plants, but there may still be sufficient infections to enable a wild-type nodulation phenotype. In plants that are heterozygous for non-nodulation and homozygous for supernodulation, a reduced number of infection events would result in a decreased number of nodules compared to nts382 since, without autoregulation, a considerable portion of infections result in nodules. Anatomical analysis of F_1 plants obtained from crosses between the non-nodulation mutants and the wild type would confirm or deny this hypothesis.

It is clear from this discussion that autoregulation may limit the detection of host mutants defective in nodulation (i.e. in the formation of subepidermal cell divisions and infections), in that leaky mutants may not be detected in a screen. With the existence of autoregulation, perhaps only null mutants will be reliably detected. Mutations may occur in nodulation genes and result in less infections, but these will be indistinguishable from the wild type in nodule number due to autoregulation. Leaky mutants defective in their ability to form infections could be more easily detected in a supernodulation background lacking autoregulation. This point is of relevance to both plant and bacterial geneticists who are interested in fragmenting nodule ontogeny by mutagenesis.

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