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Genetic analysis and complementation studies on a number of mutant supernodulating soybean lines

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Abstract. Genetic analysis was done on a number of nitrate tolerant supernodulating (*nts*) mutant soybean lines. These lines are altered in the autoregulation response, and each was isolated as a separate mutational event following chemical mutagenesis. Crosses were made between *nts* lines on a diallel pattern, and each was also crossed using *nts* lines as female parent, to wild-type nodulation cultivars. F_1 and F_2 data were analysed from each cross for nodulation type and number. No complementation was noted where *nts* lines were intercrossed, suggesting that in each line the same gene was affected. Where *nts* lines were crossed with wild-type cultivars all the F_1 progeny were wild-type, confirming that the *nts* gene is recessive and, with one exception, *nts*1116, all of the F_2 progeny segregated into a 3:1 wild-type to supernodulating phenotype, indicating that a single gene is involved. The hypernodulating line *nts*1116 gave a 1:1 ratio in its F_2 progeny when crossed with other *nts* lines. This line behaved as a dominant in the latter crosses. No wild-type segregants were recovered, therefore again no complementation took place. This line may be a leaky mutant with partial autoregulation as its segregation ratios do not fall into any of the obvious patterns.

Keywords. Supernodulating; soybean; complementation genetic analysis.

1. Introduction

Formation of the nitrogen fixing symbiosis between legume and bacteria is a closely regulated phenomenon, with nodule number, development and nitrogen fixing activity governed by both external and internal factors (Gresshoff and Delves 1986). In wild type soybeans *Glycine max* (L.) Merr. such as the cultivar Bragg, external nitrogen, in the form of soil nitrate, inhibits nodule formation. In addition the plant possesses its own internal "autoregulation" system which controls the amount of nodules allowed to develop on the root (see Pierce and Bauer 1983, Kosslak and Bohlool 1984).

A number of soybean mutants have been isolated (Carroll *et al* 1985a, b) that produce nodule numbers far in excess of wild-type cultivars and continue to nodulate even in the presence of an external nitrogen source. These mutants,

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termed supernodulating *n*itrate *t*olerant *s*ymbiosis or *nts* mutants, appear to have an altered autoregulatory system governed by shoot factors (Delves *et al* 1986).

A total of 15 *nts* mutants were isolated from M2 families; thus each one represents a separate mutational event. Preliminary analysis of M3 data, examined to indicate the probable mode of inheritance and genetically effective cell number, pointed to monogenic recessive inheritance (Carroll *et al* 1985a, 1987). Other legume mutants have been isolated which also show increased nodulation. Jacobsen and Feenstra (1984) reported a pea (*Pisum sativum* L.) mutant which also was nitrate tolerant and monogenic recessive, but supposedly root controlled, whereas the mutants described by Delves *et al* (1986) were shoot controlled.

Most reports of mutants altered in their symbiotic properties have been reported as recessive and monogenic, but have differed greatly in the number of complementation groups involved (Davis *et al* 1985; La Rue *et al* 1985). This paper describes experimentation to determine the dominant or recessive nature of genes involved in some of the soybean mutants reported by Carroll *et al* (1985) and the number of genes involved in the *nts* phenotype by means of complementation analysis and outcrosses to a number of wild-type cultivars.

2. Materials and methods

Plant material and culture

The following soybean (*Glycine max*) lines were used in the experiments described in this paper. The non-mutant wild-type cultivars: Bragg, Clark and 1029–a revertant from 382. The mutant *nts* lines derived from Bragg were: 182, 246, 382, 1116, 501, 733, 1007, 2062 and 2264.

Plants for the initial hybridisation were grown one per 25 cm diameter pot in a 3:1 sand/vermiculite mixture in a heated glasshouse in which temperatures were maintained between 16–28° during spring and summer. Lighting was by natural daylight and day length varied during the growing period. Nutrients were supplied three times per week until flowering and then daily during the flowering and pod-fill period to minimise flower abortion due to nutrient stress. A complex nutrient solution (Herridge's) (referred to in Delves *et al* 1986) supplemented with 7·5 mM KNO_3 was used and added to the pots until run–off. Plants were watered once daily in the moring until higher day temperatures necessitated 2 waterings per day to avoid wilting, when an additional watering was done with tap water in the late afternoon. Plants were inoculated with *Bradyrhizobium japonicum* strain CB1809 in peat culture (10^8 viable bacteria per pot) on planting and 4 days after planting to ensure nodulation.

Crosses were done in a diallel pattern between all of the mutant lines and between each mutant with one or more of the wild-type lines. In the latter cases, mutants were always used as the female line to enable easy identification of the crosses in the F_1 generation which were wild-type in their nodulation pattern assuming recessive inheritance.

Emasculation using very fine surgical forceps was done early in the morning before anther dehiscence took place. Crossing was usually completed by 10 a m. Flowers were selected in which the petals had emerged less than 5 mm from the

calyx, but were still tightly folded together. Petals were removed leaving the calyx intact and all the anthers were carefully extracted, checking that anther dehiscence had not taken place. Jewellers' magnifying glasses (× 5) were used by operators to ensure that the stigma was not damaged, as it is small, fragile and closely associated with the anthers at this stage.

Pollen from the selected male parent line was immediately dusted onto the stigma from an anther which had just dehisced. When lightly tapped on the back of a thumbnail, the anthers selected for crossing gave a dusting of fluffy yellow pollen. Plants were not watered until after crossing was completed for the day, otherwise dehisced anthers were difficult to find. After pollination, other flowers in the same cluster were removed and a tag was tied around the calyx to identify it, noting cross and data of pollination.

Plants were allowed to mature and the pods dried naturally. Watering with both nutrients and tap water continued until all pods involved in hybridisation on the plant were brown. When the seed was ripe each pod was harvested and bagged separately.

F_1 seed was regrown under the same conditions as described previously using 25 cm or 20 cm pots, depending on seed number and inoculated twice with *Bradyrhizobium*. Plants were allowed to grow through to seed and F_2 seed was collected from individual plants. F_2 seed from each original cross was then regrown to check segregation ratios. F_2 plants were grown as described previously, but in 25 cm diameter pots with up to twelve seeds per pot. Plants were again inoculated twice. Levels of KNO_3 in the nutrient were maintained at 7·5 mM to accentuate different levels of nodulation between wild-type and supernodulating segregants in each cross.

Whole plants were examined 6–8 weeks after germination and scored for nodule number and distribution pattern. Control plants of all lines used in the original crossing programme were grown under the same experimental regime. These included both supernodulating and wild-type lines.

3. Results

Mutant lines (as females) were crossed with wild-type controls (as males) to determine whether the genes involved in supernodulation phenotype were dominant or recessive in individual lines. The results (table 1) demonstrate that the seven different *nts* isolates behaved as a recessive in the F_1 and segregated as a single Mendelian gene in the F_2.

Data are given in table 2 for the number of nodules on F_2 wild-type and supernodulating segregants from the crosses described in table 1. The average number of nodules for each of the parent lines grown at the same time of year under a nutrient regime including 7·5 mM nitrate is also included in table 2.

In each case the data suggest that the genes involved in supernodulation are simple, single gene nuclear recessives. Supernodulating plants were not noted in any of the F_1 progeny. Where Clark was used as the pollen parent, all F_1s could be detected by the dominant purple colour of the hypocotyl at an early stage of growth, and later by the purple flower colour. All purple flowered plants were wild-type for nodulation.

Table 1. *Nts* (supernodulating) soybean lines crossed with wild type cultivars. F_1 and F_2 segregation data for nodulation phenotype.

Female parent supernodulating mutant (Sno)	Male parent wild-type (Wno)	F_1 progeny		F_2 progeny		Chi-square[a] value for 3 (Wno):1 (Sno) segregation Yates correction factor included
		Sno	Wno	Sno	Wno	
*nts*382	Clark	0	3	11	48	
*nts*382	1029	0	3	3	15	1.63
*nts*382	Bragg	0	3	1	4	
*nts*183	Clark	0	7	14	47	0.19
*nts*183	1029	0	2	1	7	
*nts*2062	Clark	0	3	9	30	0.02*
*nts*2062	Bragg	0	2	2	4	
*nts*733	Clark	0	1	1	5	0
*nts*733	1029	0	1	3	5	
*nts*246	Clark	0	6	5	30	1.60
*nts*501	1029	0	2	7	14	0.16
*nts*501	Clark	0	3	8	35	
*nts*2264	Bragg	0	1	0	1	0.93
*nts*2264	Clark	0	2	1	8	

Phenotype key: Sno = supernodulation; Wno = wild-type nodulation; Hno = hypernodulation and Nno = non-nodulation.
* Yates correction factor not used.
[a] Chi-square 0·05 level of significance 1 degree of freedom = 3·84. Therefore chi-square values listed in the table are not significant.

Table 2. Nodule number in segregating F_2 progeny from crosses of *nts* lines as female parent with wild-type as pollen parent.

Cross	Average nodule numbers per plant in			
	F_2 segregants		Mutant line	Wno parent
	Wno	Sno	Control	Control
382 × Clark	93 ± 19	860 ± 171	750 ± 133	58 ± 11
382 × 1029	70 ± 4	1095 ± 148		55 ± 16
183 × Clark	64 ± 14	1087 ± 195	930 ± 263	58 ± 11
183 × 1029	66 ± 7	920[a]		55 ± 16
2062 × Bragg	45 ± 8	910 ± 225	900 ± 227	65 ± 13
2062 × Clark	57 ± 11	920 ± 246		58 ± 11
733 × Clark	93 ± 39	1020[a]	820 ± 236	58 ± 11
733 × 1029	80 ± 19	827 ± 226		55 ± 16
246 × Clark	66 ± 24	718 ± 215	800 ± 214	58 ± 11
501 × 1029	59 ± 12	390 ± 188	450 ± 111	55 ± 16
2264 × Clark	87 ± 24	1300[a]	610 ± 122	58 ± 11

± Standard deviation, abbreviations as in table 1; [a] only one plant.

Results from the line *nts*1116 are treated separately in table 3. This line has been termed a hypernodulator (Hno) as its level of nodulation is not as extreme as the other *nts* mutants. In crossing programmes it behaved as a dominant when crossed with other *nts* lines, but as a recessive in crosses with wild-type cultivars. Segregation ratios in the F_2 approached a ratio of 1:1 when crossed with wild-type cultivars but gave a 45 (Hno):16 (Sno), when crossed with other *nts* lines.

Crosses made between the supernodulating lines are reported in table 4. In no case did a cross between two *nts* lines result in an F_1 plant which had expressed other than supernodulation, except where the hypernodulation line *nts*1116 was used as one of the parents. In the latter instance the F_1 plants were invariably hypernodulating. No reciprocal differences were detected, so data in this table do not differentiate between male and female parent lines used in each cross. Nodule numbers were not counted as the difference between the three levels of nodulation was quite clear, in addition supernodulating plants are smaller with a more fibrous root system as compared to both hypernodulation and wild type.

All F_1 plants in this programme were repotted and grown for F_2 seed. All F_2 seed were grown and the resulting plants checked for nodulation patterns. In all cases the F_2 plants from *nts* × *nts* F_1 seed showed the supernodulation pattern (data not shown). Results of F_2 plants from crosses involving *nts*1116 × other supernodulating lines are shown separately in table 3.

Not all crosses were successful, usually due to poor pollen production by the mutants, and in some cases only a small number of F_2 seeds were available for checking segregation ratios, due to less than ideal growing conditions during the winter months.

Table 3. Crosses involving *nts*1116 with wild-type and *nts* lines. Segregation of F_1 and F_2 progeny into nodulation type with nodule numbers.

Parents in cross and nodulation type		F_1 No. tested plus phenotype	F_2 Nodulation type segregants and (nodule number per plant)		
Female	Male		Wild-type	Hyper-nodulation	Super-nodulation
*nts*1116	Clark	2Wno	5 (64 ± 13)	6 (208 ± 21)	0
*nts*1116	Bragg	2Wno	2	1	0
*nts*1116	1029	4Wno	6 (54 ± 8)	14 (190 ± 40)	0
*nts*1116	*nts*2264	1Hno	0	23 (226 ± 49)	1 (472)
*nts*1116	*nts*1007	3Hno	0	7	1
*nts*382	*nts*1116	1Hno	0	1	1
*nts*733	*nts*1116	1Hno	0	5 (172 ± 29)	3 (738 ± 190)
*nts*501	*nts*1116	5Hno	0	9 (235 ± 61)	10 (489 ± 96)

± Standard deviation, abbreviations as in table 1.

Table 4. Complementation analysis F_1 data

	*nts*382	*nts*501	*nts*246	*nts*2062	*nts*2264	*nts*183	*nts*733	*nts*1007	Bragg
*nts*382	+++	+++	+++	+++	+++	+++	+++	+++	+
*nts*501	+++	+++					+++	+++	+
*nts*246			+++	+++	+++	+++			+
*nts*2062		+++		+++					+
*nts*2264	+++				+++				+
*nts*183			+++			+++	+++		+
*nts*733							+++	+++	+
*nts*1007	+++	+++						+++	+
*nts*1116	++	++	++	++	++	++	++	++	+
Bragg	+	+	+	+	+	+	+	+	+

Phenotypic classes: +++ = supernodulation, approximately 650 nodules/plant; ++ = hypernodulation, from 200–350 nodules/plant; + = wild-type nodulation, approximately 70 nodules/plant.

4. Discussion

From the data presented in this paper it is evident that expression of the supernodulation phenotype is controlled by a mutation in one gene, or one or more very closely linked genes, which behave as one complementation unit. This gene is recessive and simply inherited. All of the *nts* mutants described in this paper were isolated as separate mutational events (Carroll *et al* 1985b), suggesting that this gene is either a mutational "hot-spot" or only one gene can mutate to give supernodulation is soybean. The mutation frequency of this gene is high, with 15 *nts* mutants arising from a total of 2,300 M2 families (15 M2 seeds were scored per family) screened from the original EMS treated population.

There are differences between the *nts* lines in level of supernodulation expressed, despite all lines being in the same Bragg background. This is most probably due to the location of the mutation in the complementation group or to other genomic damage inflicted by the mutagen, as the various lines can be seen to vary in other respects, for example *nts*183 has male-sterility present in some segregants, 1007 and 733 give rise to dwarf plants. The *nts* line 1116 however shows a different pattern of inheritance. In each case, when crossed with the wild-type line, the resulting F_1 progeny were wild type, and the F_2 plants resulting from self-fertilisation of crossed F_1's segregated into both wild-type and hypernodulation patterns of nodule development.

As we consider that the supernodulating lines are altered in their autoregulation response (Carroll *et al* 1985a, b: Gresshoff and Delves 1986), in that they cannot regulate the amount of nodulation permitted, the line 1116 is probably a leaky mutant which is capable of a certain amount of autoregulation, which accounts for its increased, but not totally uncontrolled level of nodulation. When 1116 is crossed with other *nts* lines, again a partial autoregulation system is evident in the increased rather than supernodulation patterns seen in the F_1 plants. In the F_2, the plants segregate, as would be expected, into plants showing supernodulation and hypernodulation. Segregation ratios for 1116 do not fall into the same 3:1 pattern as is demonstrated by the F_2 progeny from *nts* × wild-type cross. The absence of wild-type segregants argues against *nts*1116 and *nts*382 being unlinked loci. The 3:1 Hno:Sno ratio of the 1116 × *nts* F_2 segregants argues for there being one or two closely linked loci. Hence it is suggested that *nts*1116 and *nts*382 are part of the same locus, *nts*1116 being leaky, presumably for the synthesis of inhibitor substances. All that can be concluded about *nts*1116 is that its inheritance is complex, as the segregation data do not indicate any of the simple inheritance patterns.

One of the problems associated with the interpretation of results from these experiments is to distinguish between self- and cross-fertilized seed where there are no readily available markers to allow identification of crossed F_1 seeds. Where crosses with wild-type parents were done, identification was simple as when the cultivar Clark was used, its purple hypocotyls and flowers (a dominant trait) conferred these characters on the F_1 as well as wild-type nodulation. In the F_2, purple flower colour segregated independently from the *nts* phenotype (data not shown). Other crosses of *nts* lines with wild type as pollen parents resulted in an F_1 with wild-type level of nodulation. All of the mutant lines were derived from the white (recessive) flowered cultivar Bragg which makes identification of crosses between *nts* mutants difficult as all are white flowered.

Where crosses were made with wild-type cultivars, 60% of all attempted crosses resulted in crossed F_1 seed. Crosses of *nts* mutants with wild type which showed no wild-type F_1 or F_2 segregants were counted as selfs and are not included in the data. It is therefore reasonable to suppose a similar level of success in *nts* × *nts* crosses and even if the level of successful crossing was reduced to 40%, it would still show non-complementation between lines, should non-allelic genes have been involved in producing the same supernodulation phenotype. In no case did this occur. Where crosses were done between *nts*1116 and other *nts* lines, again an average of 50% of the attempted crosses resulted in crossed F_1 seed. As in the wild type × *nts* crosses, the F_1 seed could be sorted into selfed and crossed seed by the nodulation pattern in the F_1 and segregation ratios in the F_2 plants.

In the mutants described in this paper, one mutation event in a single locus has resulted in a number of separately identified mutants, all of very similar phenotype and as yet there is no information as to why this gene may be particularly susceptible to alteration.

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