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Behavior of Pea Nodulation Mutants as Affected by Increasing Nitrate Level

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Abstract

Nodule number and size, acetylene-reducing activity, leghemoglobin content in nodules, and nitrogen content in the shoot were measured in pea symbiotic mutants inoculated with *Rhizobium leguminosarum* bv. *viceae* strain 128C30 and cultivated on a range of nitrate levels in rooting medium. The abundantly nodulating mutants RisfixC and RisfixV, and the ineffective mutant RisfixO were compared to the parental cultivar 'Finale'. RisfixO displayed nodule initiation and nodule growth suppression similar to the wild-type plants, indicating that nodule effectiveness is not substantial for both endogenous and nitrate regulation of nodulation. RisfixC was seen to produce effective and nitrate-resistant nodulation in abundance, confirming its classification as a new supernodulating pea mutant. The nitrate resistance was apparent not only in nodule initiation, but also in nodule growth and specific nitrogenase activity. Shoot nitrogen content was markedly increased in RisfixC, indicating carbohydrate depletion or a regulatory fault. Nodulation in RisfixV was abundant like in RisfixC, but the nodules possesed only traces of nitrogenase activity, and their number sharply diminished with nitrate level increase. The small and nitrate-independent size of RisfixV nodules suggests that their development had been arrested before nitrate level affected nodule growth. The hypernodulation of RisfixV is supposed to result from the early deterioration of nodules of this mutant, which releases the suppression of further nodule initiation by the established nodules.

Keywords: C_2H_2-reduction, hypernodulation, nitrate response, *Pisum sativum* L., *Rhizobium* symbiosis, supernodulation

Abbreviations: DM = dry mass, FM = fresh mass, SARA = specific acetylene-reducing activity of nodule tissue [μmol h^{-1} (g nodule DM)$^{-1}$], SEM = standard error of mean

1. Introduction

Symbiosis between *Rhizobium* and plants is localized in the symbiotic root nodules of legume hosts. As shown repeatedly (e.g., Kneen and LaRue, 1984; Jacobsen, 1984; Carroll et al., 1985; Duc and Messager, 1989), symbiotic nodule development can be interrupted by plant mutations. The nodulation mutations lead either to the absence of nodule formation in the non-nodulating (Nod^-) mutants, or to plants forming ineffective nodules (Nod^+Fix^-). A third type of nodulation mutants is caused by mutations influencing the frequency of nodule initiation. They are denoted supernodulating (Carroll et al., 1985) or hypernodulating (Gremaud and Harper, 1989) mutants. A characteristic property of the supernodulating mutants is their insensitivity to nitrate and the released autoregulation of nodule number. In the wild-type plants, nitrate regulates nodule number negatively (e.g., Carroll and Gresshoff, 1983; Nelson, 1987), as well as nodule size (Carroll and Gresshoff, 1983), leghemoglobin content, and the activity of nitrogenase (Bisseling et al., 1978; Nelson, 1987). Long-term action of high levels of nitrate leads to root nodule senescence (Chen and Phillips, 1977).

We have reported two mutants forming elevated number of nodules (Novák et al., 1990), which had been revealed in a large set of pea nodulation mutants (Engvild, 1987). The nodulation of mutant RisfixV appeared to be sensitive to nitrate, while a putative supernodulator RisfixC was nitrate-resistant. We decided to study in detail the nodulation and nodule activity response in both these mutants to the exogenous nitrate in comparison with the wild-type plants and with the ineffective mutant RisfixO from the same mutant collection. RisfixO was included to distinguish the effect of nodule activity on nodulation.

2. Materials and Methods

Plants

The studied pea lines RisfixC, RisfixO, and RisfixV originate from the set of 50 nodulation and fixation mutants of pea (*Pisum sativum* L.), obtained by chemical mutagenesis from cv. 'Finale'. All three lines were originally described as Nod^+Fix^- (Engvild, 1987).

Plant cultivation and inoculation

Surface-sterilized seeds were germinated for 3 days at 28° C. Seedlings were inoculated with 3 mL of suspension of *Rhizobium leguminosarum* bv. *viceae* strain 128C30 (Hup^+ strain; Nelson and Salminen, 1982) containing 7×10^7 bacteria per plant, and cultivated in perlite supplied with nutrient solution under 16/8 hr light/dark period, 21/14° C temperature, and 77/87% of relative humidity (Škrdleta et al., 1980; Škrdleta et al., 1991). Nutrient solution (Škrdleta et al., 1980) was changed twice a week. The used levels of nitrate in nutrient solution were 0, 2.5, 5.0, 10.0 and 20.0 mmol NO_3^- L^{-1}. Constant ionic strength was kept with Cl^- ions.

Plant evaluation

Plants were harvested 31–35 d after germination, at the stage of blossom. Plants (n = 4) were evaluated for nitrogenase activity, nodule appearance, nodule number, nodule FM, shoot DM, and nitrogen concentration in the shoot. Nitrogenase activity was assayed as acetylene reduction by detached nodulated root system. Immediately after harvesting, roots were incubated in 100 mL flasks at 22° C. Ethylene was determined by gas chromatography in samples taken 33 min after acetylene (10%, v/v) injection (Škrdleta et al., 1987). The nitrogen content in dried and homogenized plant tissues was determined with a CHN analyzer (LECO Co.) by the automated Dumas method. Leghemoglobin content was determined in nodules detached from 25–27 d old plants grown on 0.625 mmol NO_3^- L^{-1} according to Wilson and Reisenauer (1963). Nodules were stored at −20° C before assay. The leghemoglobin content was determined in nodules elicited with *Rhizobium leguminosarum* bv. *viceae* strain 248, inducing nodules of the same phenotype as the strain 128C30.

3. Results

Leghemoglobin content in nodules

In contrast to the large pink nodules of 'Finale', containing 0.454±0.052% (mean ± SEM) of leghemoglobin on nodule FM basis when grown at 0.625 mmol NO_3^- L^{-1}, plants of RisfixO form white or greenish nodules of medium size, containing 0.062±0.006% of leghemoglobin only. Nodules of RisfixC were of normal appearance, but smaller than the wild-type, more abundant, uniformly distributed throughout the root system, and containing 0.257±0.021% of leghemoglobin. Nodules of RisfixV showed premature senescence, therefore, the evaluated plants of RisfixV possessed only about 10% of the active pink nodules. The spatial distribution of the nodules was uniform

like in RisfixC. Leghemoglobin content in RisfixV, representing the mean from both types of nodules, was 0.125±0.013%.

Nodule number

Without nitrate, RisfixC and RisfixV plants formed substantially (3.7 and 3.2×) more nodules than the wild-type (Table 1), whereas the nodulation of the ineffective mutant RisfixO was only slightly more abundant. In course of nitrate increase, nodule number both in the wild-type plants and RisfixO continuously decreased. RisfixC behaved as a typical supernodulating mutant, since its nodule number was not reduced at high nitrate concentrations. Nodulation of RisfixV corresponded either to RisfixC or to the wild-type depending on nitrate concentration.

Nitrate suppression was more pronounced when followed by the nodulation index (Table 1), that included plant growth response to improving nitrogen supply. Growth response was particularly expressed in the ineffective mutants RisfixO and RisfixV.

Nodule size

The wild-type nodules were generally of larger size than the ineffective nodules of RisfixO (Table 2). Both RisfixC and RisfixV formed smaller nodules than the wild-type.

Table 1. Nodulation in pea symbiotic mutants RisfixC, RisfixV, and RisfixO and in the wild-type plants of cv. 'Finale' as affected by nitrate level in nutrient solution. Data indexed with the same letter fall into the same confidence interval as determined by multiple range test at 95% confidence level.

Pea line	Nitrate level [mmol L^{-1}]				
	0	2.5	5	10	20
	Nodule number per root				
Finale	137^{a}	154^{a}	79^{a}	44^{a}	40^{a}
RisfixO	154^{a}	$211^{a,b}$	163^{a}	53^{a}	11^{a}
RisfixC	$513^{c,d}$	$838^{f,g}$	1070^{g}	790^{f}	$840^{f,g}$
RisfixV	$437^{b,c}$	$757^{e,f}$	$542^{c,e}$	145^{a}	69^{a}
	Nodulation index [nodules (g shoot DM)$^{-1}$]				
Finale	$94^{a,b}$	$86^{a,b}$	$34^{a,b}$	15^{a}	17^{a}
RisfixO	392^{c-e}	202^{a-c}	124^{a-c}	$27^{a,b}$	6^{a}
RisfixC	637^{e-f}	748^{f}	652^{e-f}	616^{e-f}	529^{d-f}
RisfixV	1470^{g}	673^{f}	300^{b-d}	$75^{a,b}$	$52^{a,b}$

Table 2. Nodule characteristics in pea symbiotic mutants and in the wild-type plants of cv. 'Finale' as affected by nitrate in nutrient solution. Indexing as in Table 1.

Pea line	Nitrate level [mmol L^{-1}]				
	0	2.5	5	10	20
	Single nodule DM [μg $nodule^{-1}$]				
Finale	820^{c}	$700^{b,c}$	$1410^{d,e}$	$900^{c,d}$	$200^{a,b}$
RisfixO	420^{a-c}	$170^{a,b}$	130^{a}	70^{a}	100^{a}
RisfixC	$270^{a,b}$	$170^{a,b}$	$180^{a,b}$	$180^{a,b}$	130^{a}
RisfixV	120^{a}	120^{a}	110^{a}	110^{a}	100^{a}
	SARA [μmol C_2H_4 h^{-1} (g nodule DM)$^{-1}$]				
Finale	372.0^{f}	238.5^{e}	$164.2^{d,e}$	$114.3^{b,d}$	93.0^{a-d}
RisfixO	$<0.1^{a}$	$<0.1^{a}$	$<0.1^{a}$	$<0.1^{a}$	$<0.1^{a}$
RisfixC	$142.2^{d,e}$	$115.0^{b,c}$	$117.5^{b,d}$	123.9^{d}	65.4^{a-d}
RisfixV	$2.0^{a,b}$	2.7^{a-c}	0.9^{a}	1.3^{a}	1.1^{a}

Table 3. Effect of nitrate level in nutrient solution on the nitrogen content in the shoot of pea symbiotic mutants and the wild-type plants of cv. 'Finale'. Indexing as in Table 1.

Pea line	Nitrate level [mmol L^{-1}]				
	0	2.5	5	10	20
	Nitrogen content [% of DM]				
Finale	4.15^{i-k}	4.32^{g-j}	3.50^{e-h}	3.04^{e-h}	3.23^{e-i}
RisfixO	1.30^{a}	2.60^{b-e}	2.88^{d-h}	2.84^{c-g}	3.20^{e-i}
RisfixC	6.14^{m}	$5.49^{l,m}$	4.41^{j-k}	4.21^{i-k}	3.92^{h-k}
RisfixV	1.91^{a-d}	2.40^{b-e}	2.70^{c-f}	3.02^{e-h}	4.14^{i-k}

Like nodule initiation, growth of the wild-type nodules was regulated by nitrate (Table 2). As follows from the comparison of nodule number and single nodule mass traits, nodule growth was inhibited by nitrate even in advance of nodule initiation. The inhibitory action of nitrate on nodule size in RisfixC was less pronounced, although still detectable. In contrast, nitrate effect was not observable in RisfixV nodules.

Activity of nodules

The functional state of nodules was characterized by specific nitrogenase activity assayed as SARA (Table 2). On low nitrate, SARA of RisfixC was markedly lower than the wild-type value. In view of the higher susceptibility of the wild-type to nitrate, SARA of the RisfixC nodules became comparable

to the wild-type value beginning from approximately 7.5 mmol nitrate L^{-1}. RisfixO lacked detectable activity, and the SARA of RisfixV was negligible.

Nitrogen content in the shoot

N content reflected nitrate supply in both ineffective mutants, being stable or even decreasing in both effective lines (Table 3). In RisfixC, N content increased in comparison with the wild-type plants at all nitrate levels.

4. Discussion

In the presented work, we have shown the influence of exogenous nitrate level on the nodulation and nodule activity of three nodulation mutants, each of them carrying a different type of nodule formation disturbance.

RisfixC is a supernodulator

The nodulation behavior of line RisfixC fits the definition of a supernodulating mutant (Carroll et al., 1985); Delves et al., 1987), confirming its preliminary characterization (Novák et al., 1990). Not only the number of nodules, but also nodule size and SARA showed only a weak response to the external nitrate concentration, forming the nitrate-tolerant symbiosis (nts) phenotype (Carroll et al., 1985). Other supernodulating mutants have been described in soybean (Carroll et al., 1985) and french bean (Park and Buttery, 1988). The first supernodulating mutant of pea, nod3 from cv. 'Rondo', was obtained by Jacobsen and Feenstra (1984), and four mutants of this type have been described by Duc and Messager (1989). Consequently, RisfixC appears to be the 6th supernodulating mutant of pea reported till now.

A hypothesis has been raised that the elevated nodule number in supernodulating mutants is conditioned by the absence of or a fault in the shoot-derived signal (Delves et al., 1986; Gresshoff et al., 1988), which is supposed to regulate nodule initiation cooperatively with nitrate (Day et al., 1987). The formation of this systemic regulatory signal can be revealed in split-root experiments (Olsson et al., 1989).

Another regulatory factor has been shown to be still active in the supernodulating mutants of soybean (Caetano-Anollés and Gresshoff, 1991a; Caetano-Anollés et al., 1991). This factor mediates the short-range suppression of nodule priomordia development by the established nodules. However, judging from the nitrate resistance of nodulation in RisfixC, the systemic response is disturbed in this mutant (Fig. 1).

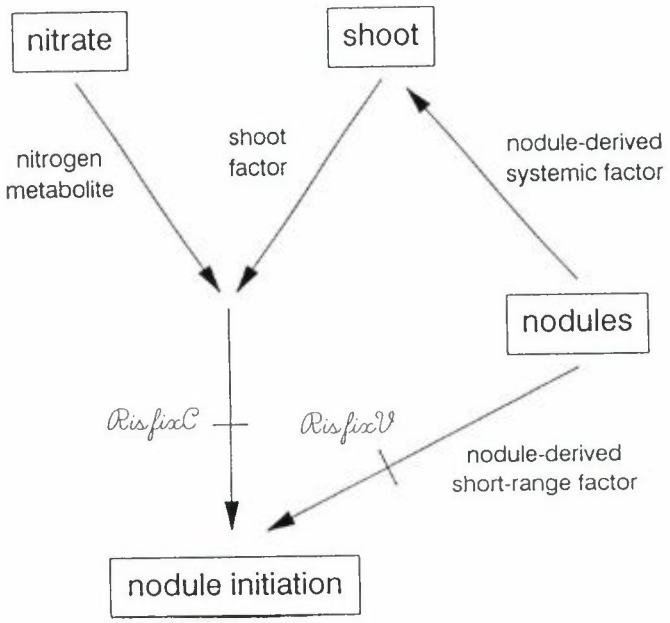

Figure 1. Putative localization of RisfixC and RisfixV mutants onto the links of the negative control of nodule initiation by the plant and nitrate

The small size of RisfixC nodules can be ascribed to the high nodule number per plant (Gresshoff et al., 1988). The generally lower SARA in the nodules of supernodulating mutants was pointed out by several authors and explained by the limited photosynthetic capacity of the plant (Jacobsen and Feenstra, 1984; Carroll et al., 1985) or by increased basal resistance for O_2 diffusion into nodules (Schuller et al., 1988). Photosynthate deprivation is supposed to condition nodule immature state, associated with lower bacteroid number (Day et al., 1987). The smaller nodule size of RisfixC supports the decreased bacteroid number notion, while lower leghemoglobin content is consistent with both nodule immaturity and higher O_2 diffusion resistance.

The increased stability of SARA in RisfixC is remarkable. Obviously, the mutation inactivates the action of nitrate not only on the nodule morphogenesis, but also on the bacteroid functioning. It is conceivable that the regulation of both traits includes a common step. The SARA stability has been already found in the supernodulating mutant of soybean nts382 (Day et al., 1987), and ascribed to the weak O_2 diffusion resistance response to NO_3^- (Schuller et al., 1988). On the other hand, increased resistance against nitrate penetration has been considered in pea mutant nod3 (Rosendahl et al., 1989).

By interpreting SARA data, it is necessary to take into account the sensitivity of the closed system assay to the acetylene-induced decline of the acetylene-reducing activity and the errors originating from the measurement of the detopped roots (Minchin et al., 1986). As shown by Schuller et al. (1988), the acetylene response is dependent both on the plant genotype and nitrate level, being less pronounced and less influenced by nitrate in the supernodulator nts382 than in the wild-type plants. Nevertheless, the estimate of the size and orientation of the potential error originating from the differences in SARA decline, as based on the published data (Schuller et al., 1988; Rosendahl et al., 1989), supports the differences in SARA found among the genotypes, as well as the notion about the stability of SARA in RisfixC.

The high N content in RisfixC shoots can be explained by carbohydrate depletion of the shoot in consequence of high energetic demands of excessive nodulation. Alternatively, it might be a consequence of a fault in the internal N level sensing, which could be the primary lesion leading to the nts phenotype. Increased N content has been also pointed out in a soybean supernodulating mutant nts382 (Delves et al., 1987).

RisfixV is a hypernodulating mutant

Mutant RisfixV, which forms abundant nodules, but is still subjected to nitrate inhibition, can be denoted as hypernodulating or copious-nodulating (Park and Buttery, 1988). Such a phenotype corresponds to pea plants carrying *nod1* and *nod2* natural alleles (Gelin and Blixt, 1964; Jacobsen and Feenstra, 1984). Apparently, in this mutant we face a block in the feedback regulation of nodule initiation by the established nodules, but not a block in the systemic/nitrate regulation (Fig. 1). It is not clear which role the observed early and fast degradation of the internal nodule tissue plays in the disruption of the feedback in RisfixV. The damage area might include the site that is responsible for the production of the nodulation inhibitor proposed by Caetano-Annollés et al. (1991). This mutation-associated nodule tissue necrosis could mimic nodule excision, which has been shown to release the feedback control of the arrested infections (Nutman, 1952; Caetano-Anollés et al., 1991). The constant and small size of RisfixV nodules suggests that the block of nodule development precedes the nitrate action on nodule growth in this mutant.

Ineffective nodulation is regulated like the wild-type

As shown by the comparison of the ineffective mutant RisfixO to the wild-type plants, the fault in N_2 fixation does not lead to the increase in nodule

number in the extent of RisfixC or RisfixV. Consequently, the nodule effectiveness is not essential for the production of both the endogenous regulatory factors, the absence of which is assumed to cause the abundant nodulation in the above mutants. Since the ineffective mutant RisfixO reacts to nitrate in a similar way as 'Finale', the nitrate action on nodule initiation is independent of nodule effectiveness as well.

Both conclusions are in accord with the findings of Caetano-Anollés et al. (1990). The "empty" nodules, induced by exopolysaccharide deficient mutants of *Rhizobium meliloti* in alfalfa, were able to suppress systemically the development of further rhizobial infections. Ineffective nodules are active also in the non-systemic nodulation control as evidenced by the excision of the "empty" nodules (Caetano-Anollés and Gresshoff, 1991b). Similarly, both autoregulation and the nitrate suppression of nodule initiation are preserved in spontaneous nodules formed by some alfalfa lines without rhizobia (Gresshoff et al., 1991). It appears that bacterial genes *nodABC* dependent changes in the root are sufficient to elicit the systemic autoregulatory response in soybean (Takats, 1990) and alfalfa (Caetano-Anollés and Bauer, 1988). The presented observation provides an independent line of evidence that the major factors regulating nodule initiation (Caetano-Anollés and Gresshoff, 1991a) are generated and act independently of the effective symbiosis.

Our data on RisfixO also show that the suppression of nodule growth by nitrate is independent of nodule activity as well, and that nitrate acts before the developmental block is expressed in this mutant. On the contrary, the impact of the effective symbiosis consists rather in the stimulation of nodule growth. The opposite effects suggest that the nitrate inhibitory action on the nodule organogenesis is not mediated by those nitrogen compounds that are common for both pathways of nitrogen assimilation. This notion is further supported by the similar nodulation response to nitrate in the wild-type plants and RisfixO, regardless of the varying origin and level of their internal N.

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