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Research Note

Restriction of Host Range by the *sym2* Allele of Afghan Pea Is Nonspecific for the Type of Modification at the Reducing Terminus of Nodulation Signals

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***Rhizobium leguminosarum* bv. *viciae* strains producing lipo-chitin oligosaccharides (LCOs) that are O-acetylated at the reducing terminus are required for nodulation of wild pea cultivars originating from Afghanistan that possess the recessive *sym2*^{A} allele. The O-acetylation of the reducing sugar of LCOs is mediated by the bacterial *nodX* gene, which presumably encodes an acetyltransferase. We found that for nodulation on Afghan pea cultivars and *sym2*^{A} introgression lines the *nodX* gene can be functionally replaced by the *nodZ* gene of *Bradyrhizobium japonicum*, which encodes a fucosyltransferase that fucosylates the reducing terminus of LCOs. The structure of the nodules, which were induced with normal frequency, was typical for effective pea nodules, and they fixed nitrogen with the same efficiency as nodules induced by *nodX*-carrying strains.**

Within the cross-inoculation group of *Rhizobium leguminosarum* bv. *viciae* a cultivar specificity exists in that some primitive pea (*Pisum sativum*) cultivars originating from the Middle East (e.g., Afghanistan, Iran, Turkey, Israel; here, however, collectively called Afghan peas), are not nodulated by the ordinary European and North American strains but require *R. leguminosarum* bv. *viciae* strains from the Middle East for the symbiosis (Govorov 1928, 1937; Lie 1978). The resistance of Afghan peas to nodulation was found to be controlled by the *sym2*^{A} allele, which is involved in early stages of the infection process (Geurts et al. 1997; Kozik et al. 1995; Lie 1984). *R. leguminosarum* bv. *viciae* strains able to nodulate Afghan peas were isolated first from soils of Turkey (e.g., strain TOM; Winarno and Lie 1979), and later from different geographic regions of the world (Denmark, China, India, Morocco, Yugoslavia, Russia) (Ma and Iyer 1990). It was shown that the ability to nodulate Afghan peas in strain TOM is conferred by the *nodX* gene, which is located downstream of the *nodABCIJ* genes, indicating a gene-for-gene relationship (Davis et al. 1988; Geurts et al. 1997). The function of the host-specific gene *nodX*, which is present in all *Rhizobium* strains nodulating Afghan peas, is to O-acetylate lipo-chitin oligosaccharides (LCOs; also called Nod factors) at their reducing terminus (Firmin et al. 1993; Dénarié et al. 1996; Spaink 1996). As a consequence, it has become clear that the acetylation of the reducing terminus of Nod factor of *R. leguminosarum* bv. *viciae* is necessary to achieve infection on *sym2*^{A}-harboring peas, leading to successful nodulation (Firmin et al. 1993; Geurts et al. 1997; Kozik et al. 1995).

In order to test the structural requirements of Nod factors for nodulation of peas containing the *sym2*^{A} allele, we have constructed a set of strains carrying additional *nod* genes on separate plasmids. As a uniform background for the introduction of *nod* genes, *R. leguminosarum* bv. *viciae* strain 248 was used, which nodulates European peas (homozygote *sym2*^{C}) efficiently but fails to nodulate pea lines homozygote for *sym2*^{A}. The following genes were introduced into strain 248 on plasmids of different incompatibility groups: the *nodX* gene from *R. leguminosarum* bv. *viciae* strain TOM, the *nodZ* gene from *Bradyrhizobium japonicum*, which links a fucosyl group to the reducing terminus of LCOs (Stacy et al. 1994; Quinto et al. 1997), and the regulatory gene *nodD* FITA (flavonoid independent transcription activation), which activates *nod* genes in the absence of flavonoids (Spaink et al. 1989). To check the influence of copy number of the plasmid, we have used plasmids pMW1071 and pMW2102, which contain the *nodX* gene on replicons of the IncP and IncW groups, respectively (Table 1). The presence of introduced *nod* genes in transconjugant strains was in all cases confirmed by thin layer chromatography of ^{14}C-labeled LCOs as previously described (López-Lara et al. 1995; Spaink et al. 1995) and by

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polymerase chain reaction (data not shown). As expected, the introduction of *nodD* FITA gene into strain 248 resulted in the synthesis of LCOs even in the absence of inducer (data not shown).

To test whether the transconjugant strains are able to nodulate *sym2*^{A}-harboring peas, the two Afghan pea lines L2150 (also known as cv. Afghanistan) and L6559 and the *sym2*^{A} introgression line 37(1)2 were inoculated in a gravel-based nodulation assay (Raggio and Raggio 1956). Line 37(1)2 resulted from crossing of the European line NGB1238 with the Afghan line L2150, followed by six to seven selfcrosses with selection of plants with nodulation-minus phenotype upon inoculation by European strains. Nodules were scored 3 weeks after inoculation (Table 2). The *nodX*-containing strains induced nodules on all pea lines tested. The copy number of the vector containing the *nodX* gene did not have a significant effect on nodulation. Surprisingly, strains that contained the *nodZ* gene also were able to elicit nodules on *sym2*^{A}-harboring peas. The wild-type pea lines and the introgression line 37(1)2 formed a slightly reduced number of nodules upon inoculation by strain 248 harboring only the *nodZ* gene, but when the *nodD* FITA gene was added, the number of nodules reached a value similar to that obtained after inoculation by *nodX*-carrying strains (Table 2). Furthermore, in the presence of the FITA *nodD* gene these lines formed larger nodules on the main root whereas without the FITA *nodD* gene the number of nodules on lateral roots was larger. The lower number of big nodules on a main root in case of the introduction of the *nodZ* gene alone might indicate some delay in nodulation leading to preferential formation of nodules on the lateral roots. A positive effect of the *nodD* FITA gene was not observed in the case of the *nodX*-containing strains. The enhancement of nodulation, in case of co-introduction of *nodD* FITA with the *nodZ* gene, could be the result of overcoming a limited *nod* gene expression and subsequent Nod factor production. The strain harboring the combination of *nodZ* and *nodX* genes on separate plasmids displayed slightly decreased nodulation.

To determine the relative number of bacteria harboring plasmids inside the nodules, we have isolated bacteria from nodules and tested the frequency of antibiotic resistance. About 70 to 80% of isolated clones were resistant to the tested antibiotics. Since the IncP and IncW plasmids used in this study are lost relatively rapidly in the absence of antibiotics (data not shown) these results indicate that plasmids carrying *nodX* or *nodZ* genes conferred a selective advantage during the infection process.

The gene *nodO* encodes a secreted protein that is not involved in LCO synthesis or secretion but it may partially compensate the lack of genes participating in LCO modification (Downie and Surin 1990; Economou et al. 1994; van Rhijn et al. 1996; Sutton et al. 1994). Wild-type strain 248, harboring an active *nodO* gene, sporadically triggers infections on *sym2*^{A} introgression lines, leading to the formation of functional nodules (Table 2), whereas a *nodO* mutant is absolutely unsuccessful in triggering successful infections (Geurts et al. 1997; Sutton et al. 1994). We have tested whether *nodO* contributes to the nodulation ability of the strains producing fucosylated Nod factors. To this end we introduced the plasmid pMP2450 carrying the *nodZ* gene into strain 248 with a defective *nodO* gene. The analysis of the NodO effect was performed with a nodulation assay in which the pea plants were grown on perlite instead of gravel. In this assay the number of nodules obtained is higher than on gravel, facilitating the detection of a *nodO*-related phenotype. The cultivar Rondo, homozygote for *sym2*^{C}, and the introgression line Rondo-*sym2*^{A} were inoculated with the strains 248, 248.pMW1071 (*nodX*), and 248.pMP2450 (*nodZ*) as well as with their *nodO*::Tn*5* counterparts. Near-isogenic lines Rondo-*sym2*^{C} (cv. Rondo) and the backcross line Rondo-*sym2*^{A} were described by Kozik et al. (1995). Introgression line Rondo-*sym2*^{A} resulted from crossing of pea L2150 (cv. Afghanistan) to European cultivar Rondo-*sym2*^{C} with subsequent three backcrosses to Rondo-*sym2*^{C}. This line contains less introgressed DNA of Afghan line L2150 when compared with line 37(1)2.

Nodules were scored 3 weeks after inoculation (Table 3). From the results of nodulation experiments it is apparent that in the presence of *nodO* there is no difference in nodulation efficiency between the *nodX*- and the *nodZ*-harboring strains; 248*nodZ* nodulates the Rondo-*sym2*^{A} introgression line as efficiently as it does Rondo-*sym2*^{C}. In the absence of *nodO*, the *nodZ*-harboring strain was also able to elicit nodules on Rondo-*sym2*^{A}, although at a slightly lower frequency when compared with 248*nodO*::Tn*5*.pMW1071(*nodX*) (Table 3). Therefore, we can conclude that the presence of a fucosyl decoration at the C6 position of the reducing terminal glucosamine of the Nod factors is sufficient to overrule the block on nodulation independently from *nodO*.

Cross sections of mature nodules were examined by light microscopy. The structure of nodules induced by *nodZ*- and *nodX*-harboring strains was very similar and typical for normal nitrogen-fixing nodules (Fig. 1A and B). Central tissue of

Table 1. Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Relevant characteristics	Source or reference
Rhizobium leguminosarum		
248	*R. leguminosarum* bv. *viciae* wild type	Josey et al. 1979
248 *nodO*::Tn*5*	1391 carrying pRL1JI*nodO*$_{94}$::Tn*5*	Geurts et al. 1997
1391	248 Rif^r, cured from its plasmid pRL1JI	Schlaman et al. 1992
Plasmids		
pRL1JI	Sym plasmid of *R. leguminosarum* bv. *viciae* strain 248	Johnston et al. 1978
pRK2013	IncColE1, Tra$^+$, Km^r	Ditta et al. 1980
pMP604	IncP, contains *nodD* FITA, Tc^r	Spaink et al. 1989
pMP1604	IncW, contains *nodD* FITA, Spec^r	López-Lara et al. 1996
pMP2450	IncP, contains pA-*nodZ*, Tc^r	López-Lara et al. 1996
pMW1071	IncP, contains pA-*nodX*, Tc^r	Kozik et al. 1995
pMW2102	IncW, contains pA-*nodX*, Spec^r	Geurts et al. 1997

[a] Abbreviations: Tc^r, Specr, Rif^r, and Km^r: tetracycline, spectinomycin, rifampicin, and kanamycin resistance, respectively; pA, promoter of *nodA* gene of *R. leguminosarum* bv. *viciae;* Inc, plasmid incompatibility group; *nodO94*::Tn*5*, Tn*5* mutation in the *nodO* gene; Tra$^+$, region of conjugation transfer; Rlv, *R. leguminosarum* bv. *viciae; nodD* FITA (flavonoid independent transcription activation), hybrid *nodD* gene able to induce *nod* gene expression in the absence of flavonoids (Spaink et al. 1989). Rhizobial strains were grown on B$^-$ medium (van Brussel et al. 1977). Transconjugants were selected on B$^-$ media supplemented with 2 mg of tetracycline per l for IncP plasmids or with 100 mg of spectinomycin per l for IncW plasmids.

the nodules representing the nitrogen-fixing zone had a dark color due to the presence of fully occupied, bacteroid-containing cells (enlarged parenchyma cells whose cytoplasm was packed with bacteroids) (Fig. 1A and B). Bacteroid-containing cells had a prominent central vacuole, which is typical for pea nodules. Beneath the endodermis, normal vascular bundle structures surrounding the central tissue are present (Fig. 1). To confirm that nitrogen fixation in nodules did take place, the acetylene reduction activity was measured with plants of the introgression line 37(1)2 as representative. The results show that nodules elicited on pea plants by *nodX*- and *nodZ*-carrying transconjugant strains were able to fix nitrogen with comparable efficiency (Table 2). The total nitrogenase activity correlated in general with the total number of nodules and was maximal in nodules elicited by the strains 248.pMW1071(*nodX*) and 248.pMP2450(*nodZ*).pMP1604(*nodD* FITA) (Table 2). The acetylene reduction activity in the negative control strains 248 and 248.pMP1604(*nodD* FITA) was relatively high, presumably since the very few nodules formed in these cases were very large. Thus, nodules elicited on *sym2*^{A}-harboring peas by *nodZ*-carrying strains are efficient nitrogen-fixing organs structurally indistinguishable from wild-type, nitrogen-fixing pea nodules.

In this work we have shown that fucosylation of the reducing terminus of Nod factors confers on the bacteria an ability to nodulate peas carrying the *sym2*^{A} allele. The mechanism of Nod-factor perception by a leguminous host plant remains unclear. Basically, there could be two possible ways that a plant perceives LCOs, with different modifications. First, differently decorated LCOs may fit to different plant receptors. In this case, the stringent requirements for LCO structure should be dictated by more than one receptor. Second, different Nod factors might be recognized by the same receptor but their stability may vary depending on the host plant. There is evidence that decorations of Nod-factor backbone such as *nodH*-mediated sulfation, *nodEF*-mediated acylation, and others may improve their stability against plant chitinases that cause degradation of LCO molecules (Staehelin et al. 1994). Our results show that in the case of Afghan peas (*sym2*^{A} allele) the requirements for LCO structures are not very strict, since apparently a fucosyl group can functionally replace the structurally different *O*-acetyl group for infection and nodulation. This observation is not in favor for the hypothesis of involvement of the modifications of the reducing terminus for specific receptor-ligand interaction, but it rather seems to support the second possibility: increased stability of LCOs toward plant chitinases. On the other hand, studies on the degradation rate of mono-acetylated Nod factors by European and Afghan peas did not reveal any differences in degrading activity between root exudates of *sym2*^{A}- and *sym2*^{C}-containing lines, suggesting that Afghan peas do not possess specific chitinase activity that destroys monoacetylated LCOs faster than doubly acetylated LCOs. To get a better insight into the mechanisms of host range restriction by Afghan peas, it would be interesting to compare in more detail (preferably in situ) the relative stability of mono- and double-acetylated Nod factors toward degradation by plant enzymes in *sym2*^{A} and *sym2*^{C} homozygous backgrounds.

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Table 3. Nodule formation on near-isogenic pea lines upon inoculation with *Rhizobium* strains harboring additional *nod* genes[a]

***R. leguminosarum* bv. *viciae* strain/plasmid**	**Rondo-*sym2*^{A}**	**Rondo-*sym2*^{C}**
248	2 ± 1 (n = 8)	50 ± 4 (n = 8)
248.pMW1071(*nodX)*	51 ± 4 (n = 8)	50 ± 5 (n = 8)
248.pMP2450 (*nodZ)*	50 ± 4 (n = 8)	48 ± 2 (n = 7)
248*nodO*::Tn*5*	0 (n = 18)	46 ± 2 (n = 18)
248*nodO*::Tn5.pMW1071(*nodX*)	51 ± 4 (n = 18)	41 ± 3 (n = 18)
248*nodO*::Tn5.pMP2450(*nodZ*)	28 ± 2 (n = 18)	45 ± 3 (n = 17)

[a] Deviations are given for the number of plants indicated. Use was made of a perlite-based assay. For this assay, pea seeds were surface sterilized (15 min commercial bleach, 15 min 7% H_2O_2, thoroughly washed several times with sterile water) and sown in modified Leonard jars, which consist of a plastic (coffee) beaker of about 100 ml filled with perlite (Lie et al. 1988). This beaker is put into a 360-ml preservation jar, which serves as the reservoir for the nutrient solution (Fahraeus 1957). A foam plastic wick is inserted through a slit made in the bottom of the beaker. Before use, the Leonard jars were kept for 5 days at 70°C. After sowing, the pea seeds were inoculated with 2 ml of freshly grown rhizobia of OD_{620} = 0.1, and covered with a layer of sterilized, fine gravel to prevent contamination.

Table 2. Number of nodules and levels of nitrogen fixation in wild-type Afghan and *sym2*^{A} introgression pea lines inoculated with isogenic *Rhizobium leguminosarum* bv. *viciae* strains[a]

	Afghan pea line		**Introgression line**	**Acetylene reduction**
***R. leguminosarum* bv. *viciae* strain/plasmid**	**L2150**	**L6556**	**37(1)2**	**(µMol/plant)**
248	0	0	1 ± 1	2.3
248.pMP1604 (*nodD* FITA)	0	0	2 ± 2	3.4
248.pMW1071 *(nodX)*	9 ± 3	9 ± 2	16 ± 8	20.6
248.pMW1071 (*nodX)*.pMP1604 *(nodD* FITA)	11 ± 2	5 ± 2	19 ± 8	9.2
248.pMW2102 (*nodX)*	11 ± 2	2 ± 1	20 ± 7	15
248.pMW2102 (*nodX)* .pMP604 (*nodD* FITA)	6 ± 1	7 ± 2	13 ± 6	12
248.pMP2450 (*nodZ)*	8 ± 2	7 ± 2	5 ± 1	7.6
248.pMP2450 (*nodZ)*.pMP1604 *(nodD* FITA)	15 ± 2	15 ± 5	22 ± 6	15.9
248.pMP2450 (*nodZ)*.pMW2102 *(nodX)*	6 ± 1	2 ± 1	15 ± 6	6.8

[a] Nitrogen fixation data was obtained with introgression line 77(1)2. A minimum of six plants were grown in grown in a gravel-based assay. For this assay seeds of pea (*Pisum sativum* L.) were surface sterilized for 5 to 7 min in concentrated sulfuric acid, thoroughly washed several times with sterile water, and allowed to germinate on minimal medium solidified with agar. Three-day-old seedlings were transferred into sterile 5-l glass beakers filled with red gravel and watered with Raggio nutrient solution (Raggio and Raggio 1956). Each pea plant was inoculated with 500 ml of a suspension of the freshly grown rhizobia in Jensen medium (van Brussel et al. 1982) diluted up to an OD_{620} value of 0.1.

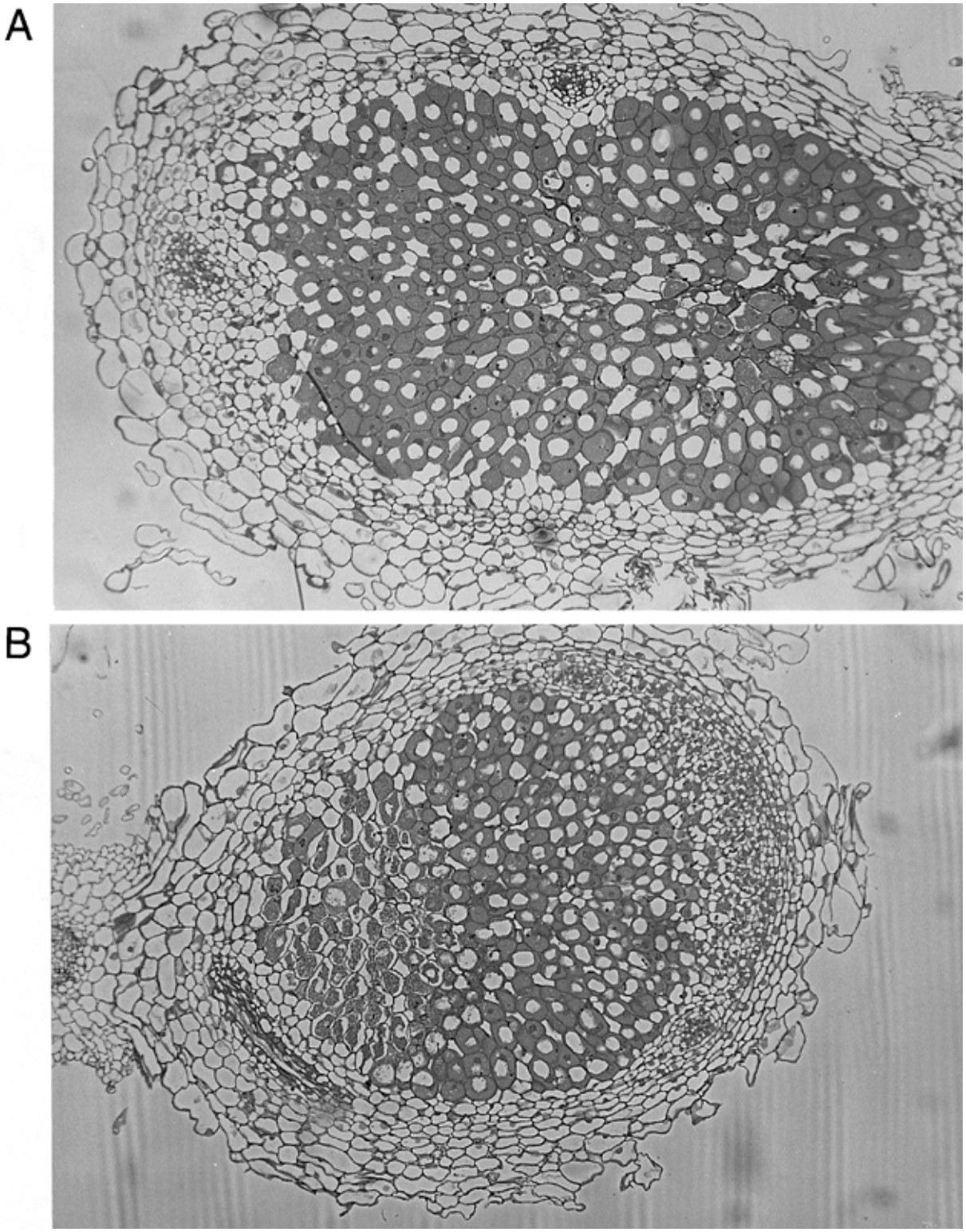

Fig. 1. Cross sections of mature pea nodules elicited on Afghan pea line L2150 by (**A**) *nodZ*- and (**B**) *nodX*-harboring derivatives of *Rhizobium leguminosarum* strain 248. The central tissue of the nodules representing the nitrogen-fixing zone is occupied by bacteroid-containing cells. Nodulation assays were performed as described previously (Hooykaas et al. 1977; van Brussel et al. 1982).

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