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Short Communication

Acetylation of a Fucosyl Residue at the Reducing End of *Mesorhizobium loti* Nod Factors is Not Essential for Nodulation of *Lotus japonicus*

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NodMl-V($C_{18:1}$, Me, Cb, AcFuc) is a major component of lipo-chitin oligosaccharides (LCOs), or Nod factors, produced by *Mesorhizobium loti*. The presence of a 4-*O*-acetylated fucosyl residue (AcFuc) at the reducing end has been thought to be essential for symbiotic interactions with the compatible host plant, *Lotus japonicus*. We generated an *M. loti* mutant in which the *nolL* gene is disrupted; *nolL* has been shown to encode acetyltransferase that is responsible for acetylation of the fucosyl residue. The *nolL* disruptant Ml107 produced LCOs that lacked acetylation of fucosyl residues as expected, but exhibited nodulation performance on *L. japonicus* as efficiently as the wild-type *M. loti* strain MAFF303099. We show that LCOs without acetylation of a fucosyl residue purified from Ml107 are also able to induce abundant root hair deformation and nodule primordium formation. These results indicate that NolL-dependent acetylation of a fucosyl residue at the reducing end of *M. loti* LCOs is not essential for nodulation of *L. japonicus*.

Keywords: *Lotus japonicus* — *Mesorhizobium loti* — Nod factors — Nodulation.

Initiation of the symbiotic interactions between *Rhizobium* bacteria and legume plants is mediated by rhizobial lipo-chitin oligosaccharides (LCOs), or Nod factors (Lerouge et al. 1990, Spaink et al. 1991). Various substitutions on the lipo-chitin backbone play essential roles in determining the strict host specificity. *Mesorhizobium loti*, the symbiont for the model legume, *Lotus japonicus*, produces LCOs composed mainly of NodMl-V($C_{18:1}$, Me, Cb, ACFuc) that is characterized by a 4-*O*-acetyl-fucosyl residue (AcFuc) at the reducing end (Lopez-Lara et al. 1995, Olsthoorn et al. 1998, Niwa et al. 2001). *Rhizobium etli*, a natural symbiont of *Phaseolus vulgaris*, and broad host range *Rhizobium* sp. NGR234, also produce acetyl-fucosylated LCOs (Cardenas et al. 1995), and are able to nodulate *L. japonicus* even though their nodulation efficiencies are not comparable with that of *M. loti* (Banba et al. 2001, Müller et al. 2001). Thus the presence of an acetyl-fucose in LCOs has been thought to be essential for determining the host specificity of *M. loti*. Purified NodMl-V($C_{18:1}$, Me, Cb, AcFuc) has been shown to be able to induce infection and nodule formation processes on *Lotus* plants (Lopez-Lara et al. 1995, Niwa et al. 2001). However, *M. loti* also produces substantial amounts of LCOs bearing fucose instead of acetyl-fucose at the reducing end, and deacetyl-fucosylated LCOs has been suggested to possess biological activities, because *Rhizobium leguminosarum* harboring *nodZ* from *Bradyrhizobium japonicum*, which encodes a fucosyl transferase, is able to nodulate *L. japonicus* (Bras et al. 2000). Therefore, the biological activities of individual LCO molecules, together with their possible cooperative action in the *Lotus* nodulation process (Ardourel et al. 1994, Minami et al. 1996), are still to be elucidated.

The *M. loti nolL* gene is shown to encode an acetyl transferase that acts specifically for fucosylated *N*-acetylglucosamine pentamers (Berck et al. 1999, Bras et al. 2000). The *M. loti* genome contains a single copy of *nolL* (Kaneko et al. 2000). To explore the biological significance of acetyl-fucose at the reducing end of *M. loti* LCOs, we generated a *nolL* disruptant of *M. loti* MAFF303099 in which the *nolL* coding region was replaced with a gentamicin resistance cassette (*aacC1*) from pMS246 (Becker et al. 1995). The resultant mutant Ml107 was transformed further with a plasmid pMP2112 that contains *nodD* from *R. leguminosarum* bv. *trifolii* (Lopez-Lara et al. 1995) to induce LCO production by naringenin.

The LCOs were isolated from culture medium containing 1 μM naringenin followed by purification by reversed-phase high-performance liquid chromatography (HPLC) as described previously (Niwa et al. 2001) with some modifications. In brief, crude LCOs were prepared by *n*-butanol extraction of the medium after about 16 h bacterial culture, dissolved in 85% acetonitrile after evaporation of *n*-butanol, and applied to a silica gel 60 column (MegaBondElut SI, 10 g/60 ml, Varian) pre-equilibrated with 85% acetonitrile. The column was washed extensively with 85% acetonitrile and then LCOs were eluted with 50% acetonitrile. The effluent was evaporated to dryness, dissolved in aliquots of 50% acetonitrile and subjected to HPLC with an octadodecyl silica column (Cosmosil ARII-5C18, 4.6 mm i.d.×150 mm, or 250 mm for preparation, Nakalai Tesque, Kyoto, Japan) with 40% acetonitrile as a solvent.

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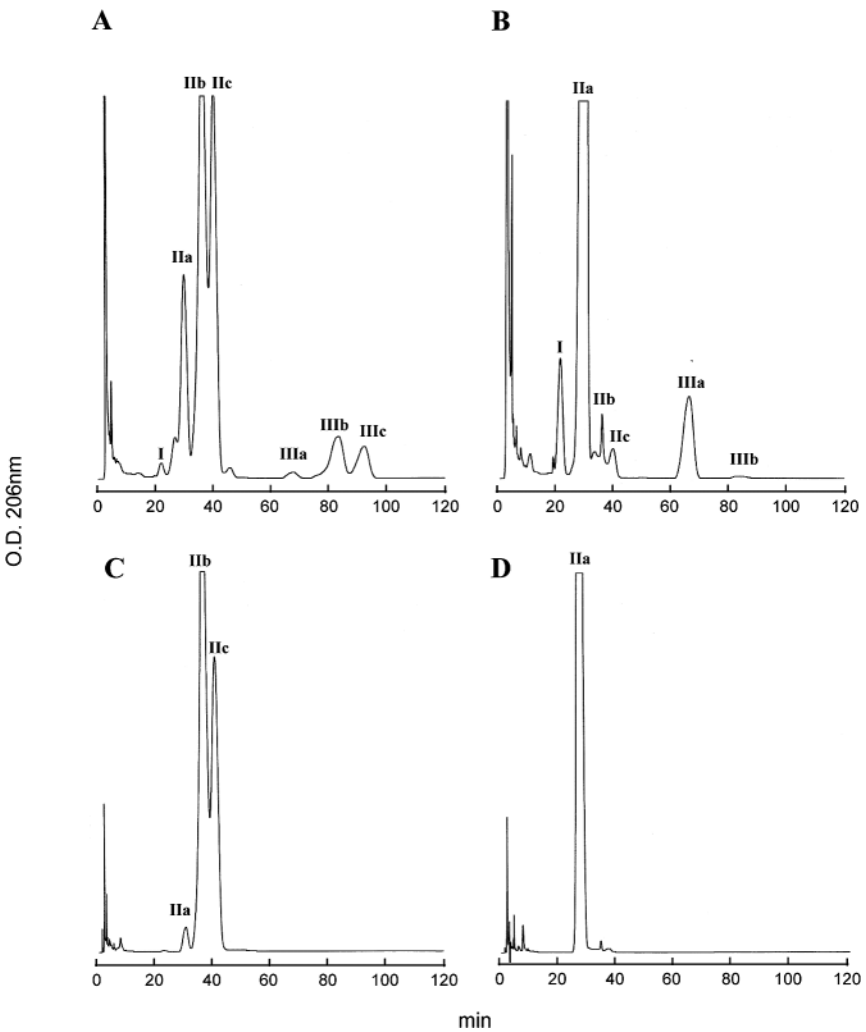

Fig. 1 High-performance liquid chromatography of lipo-chitin oligosaccharides (LCOs) from *Mesorhizobium loti* MAFF303099 (A) and the *nolL* disruption mutant Ml107 (B). Predicted structures for each identified peak are summarized in Table 1. Purified fractions of component IIb [NodMl-V($C_{18:1}$, Me, Cb, AcFuc)] from MAFF303099 and IIa [NodMl-V($C_{18:1}$, Me, Cb, Fuc)] from Ml107 are shown in (C) and (D), respectively.

The HPLC profiles of LCOs produced by MAFF303099 and Ml107 are shown in Fig. 1A and B, respectively. Structures of LCOs in each peak were determined by tandem mass spectrometry in combination with HPLC (LC-MS/MS) as described in Niwa et al. (2001). We identified three groups of LCO molecules, I, II and III, representing LCOs with C16:0, C18:1 and C18:0 acyl moieties at their non-reducing ends, respectively. Groups II and III each comprised three components with distinct HPLC retention times according to the combinations of substitutions at their reducing and non-reducing ends; they were fucosylated (designated 'a' in Fig. 1) or acetyl-fucosylated (b and c) at their reducing ends, and carbamoylated (a and b) at the non-reducing ends. Group I contained a single fucosylated and carbamoylated LCO and was a minor component. Group II represents the major fraction of *M. loti* LCOs and has an activity to induce root hair deformation and nodule primordium formation (Niwa et al. 2001). Group III that has also been shown to have strong biological activity, was identified as LCOs with an C18:0 acyl moiety. Although it was evident that acetyl-fucosylated LCOs are the major components of *M. loti* LCOs, substantial amounts of fucosylated LCOs were also detected. The amounts of fucosylated relative to acetyl-fucosylated LCOs tended to increase by prolonged culture of bacteria in the induction medium or during storage after preparation, suggesting that fucosylated LCOs come, in part, from autohydrolytic deacetylation of acetyl-fucosylated LCOs. The carbamoyl group at the non-reducing ends is also shown to be unstable during the preparation of LCOs (Lopez-Lara et al. 1995). The predicted structures of LCOs identified by LC-MS/MS are summarized in Table 1.

As expected, the *nolL* disruption mutant Ml107 produced deacetyl-fucosylated LCOs as super-abundant LCO molecules (Fig. 1B), clearly indicating that NolL is responsible for acetylation of fucose at the reducing ends of the lipo-chitin backbone, irrespective of the structure of the lipid at the non-reducing ends. However, small amounts of acetyl-fucosylated LCOs were still detected from the Ml107 culture. This may be due to non-specific acetyltransferase activities other than NolL. Disruption of *nolL* did not appear to have an effect on the quantity of LCOs produced, as judged from HPLC profiles.

Table 1 Predicted structures of LCOs produced by *M. loti* MAFF303099

Peak	$[M+H]^+$ mass		Predicted structure
	Observed	Nominal	
I	1,433.9	1,433	NodMl-V($C_{16:0}$, Me, Cb, Fuc)
IIa	1,459.8	1,459	NodMl-V($C_{18:1}$, Me, Cb, Fuc)
IIb	1,501.8	1,501	NodMl-V($C_{18:1}$, Me, Cb, AcFuc)
IIc	1,458.7	1,458	NodMl-V($C_{18:1}$, Me, AcFuc)
IIIa	1,461.7	1,461	NodMl-V($C_{18:0}$, Me, Cb, Fuc)
IIIb	1,503.8	1,503	NodMl-V($C_{18:0}$, Me, Cb, AcFuc)
IIIc	1,460.6	1,460	NodMl-V($C_{18:0}$, Me, AcFuc)

The positions of individual peaks are shown in Fig. 1.

We examined the nodulation performance of Ml107 in comparison with the wild-type *M. loti* strain MAFF303099. *Lotus japonicus* B-129 'Gifu' seeds were surface-sterilized, germinated and grown in sterilized vermiculite pots supplied with nitrogen-free B & D medium in an artificially lit growth cabinet controlled at 26°C (day, 16 h) and 24°C (night, 8 h) as described previously (Niwa et al. 2001). The plants were inoculated with *M. loti* MAFF303099 or Ml107 10 d after germination and harvested at time intervals from 6 to 22 d post-inoculation. Nitrogenase activity was measured by acetylene reduction assay of the nodulated roots by the method described in Kouchi et al. (1991).

Under the growth conditions used in this work, nodules became visible as small bumps 4–5 d after inoculation, and nitrogen fixation (acetylene reduction) activity was first detected by 8–10 d. Comparisons of symbiotic performance between *M. loti* wild-type MAFF303099 and the *nolL* disruption mutant Ml107 are summarized in Fig. 2. The results clearly indicate that there is no significant difference in nodule number per plant (Fig. 2A), nodule growth (Fig. 2B) or acetylene reduction activity (Fig. 2C) through 6 to 22 d post-inoculation. Plants infected with Ml107 grew as well as plants infected with the wild-type MAFF303099 (data not shown). Thus *nolL*, and consequently acetylation of fucose in *M. loti* LCOs, does not appear to be essential for symbiotic interaction with the host legume, *L. japonicus*.

Although the *nolL* disruptant *M. loti* Ml107 produced mostly fucosylated LCOs, it also produced small but significant amounts of acetyl-fucosylated LCOs (Fig. 1B). Therefore, we examined directly the biological activity of a fucosylated LCO purified from Ml107 culture. A peak of NodMl-V($C_{18:0}$, Me, Cb, Fuc) (peak IIa in Fig. 1B) was collected and further purified by HPLC with the same column conditions. This procedure was repeated three more times, and the final preparation contained a negligible amount of acetyl-fucosylated LCOs (Fig. 1D). This fraction was subjected to root hair deformation (HAD) assay and to a spot inoculation test for the activity of nodule primordium induction according to methods described previously (Niwa et al. 2001).

Representative results of the HAD assay are shown in Fig. 3. The concentration of purified LCOs in the rooting medium was adjusted approximately to 10^{-8} M as the absorption at 300 nm of 1.2×10^{-4} M cm^{-1} (Niwa et al. 2001). Purified NodMl-V($C_{18:1}$, Me, Cb, Fuc) induced abundant root hair deformation on *L. japonicus* roots, as did NodMl-V($C_{18:1}$, Me, Cb, AcFuc) purified from *M. loti* MAFF303099. Furthermore, NodMl-V($C_{18:1}$, Me, Cb, Fuc) induced nodule primordia when spot-inoculated on *L. japonicus* MG-20 root (Fig. 4). These results demonstrated that fucosylated LCOs have biological activities that are comparable with those of acetyl-fucosylated LCOs.

We have presented evidence that 4-*O*-acetylation of the fucosyl residue at the reducing ends of *M. loti* LCOs is not essential for symbiotic interactions with *Lotus* plants. Bras et al. (2000) showed that heterologous expression of NolL together with the fucosyl transferase NodZ in *R. leguminosarum* bv. *viciae* greatly improved the nodulation efficiency of *L. japonicus* as compared with the same bacterial species carrying only NodZ. They also described that *R. leguminosarum* carrying both NodZ and NolL was able to fix nitrogen sufficiently to support the growth of *L. japonicus*, but bacteria carrying only NodZ did not. In the present study, however, growth and nitrogen fixing activity of plants infected with the *nolL* disruptant Ml107 were comparable with those of plants infected

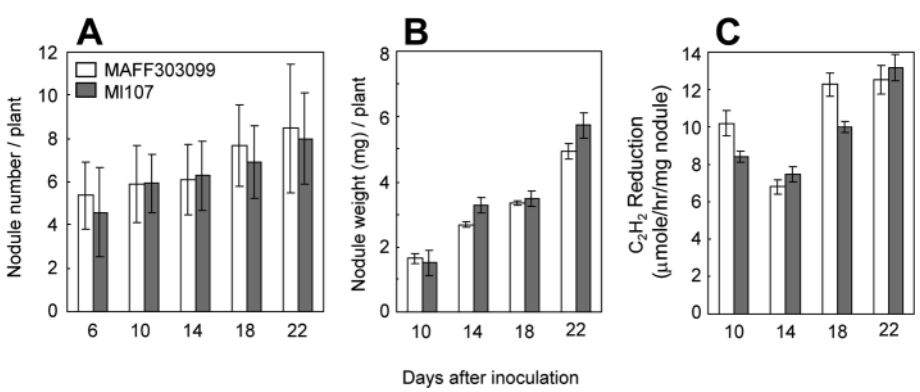

Fig. 2 Nodulation profiles of *Lotus japonicus* with *Mesorhizobium loti* MAFF303099 and the *nolL* disruption mutant Ml107. (A) Nodule number per plant; (B) nodule fresh weight per plant; (C) nitrogenase activity as measured by acetylene reduction activity. The data are given as means of 30 plants in (A) and (B), and are means of triplicate samples of 10 plants in (C). Error bars represent SEs.

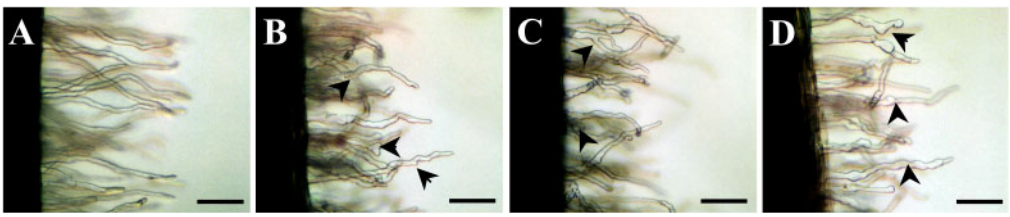

Fig. 3 Root hair deformation of *Lotus japonicus* after 24 h of incubation with purified LCOs at 10^{-8} M concentration. (A) Control without LCOs; (B) acetyl-fucosylated LCO, NodMl-V($C_{18:1}$, Me, Cb, AcFuc) purified from *Mesorhizobium loti* MAFF303099; (C) fucosylated LCO, NodMl-V($C_{18:1}$, Me, Cb, Fuc) from Ml107; (D) crude LCO mixture isolated from MAFF303099. Typically deformed root hairs are indicated by arrowheads.

with the wild-type *M. loti* MAFF303099 throughout the duration of the experiment (Fig. 2). Currently, we have no obvious explanation for this inconsistency. NolL-dependent acetylation of LCOs in the heterologous system is shown significantly to increase the quantity of LCOs produced (Bras et al. 2000). *Rhizobium leguminosarum* bv. *viciae* carrying NodZ and NolL can nodulate on *L. japonicus* roots, but its nodulation ability is still inferior to that of the compatible symbiont *M. loti*. Therefore, it is possible that the improvement of nodulation efficiency by additional introduction of NolL in *R. leguminsarum* carrying NodZ is simply due to the larger quantities of LCOs produced. Alternatively, *Lotus* plants may favor 4-*O*-acetyl fucosylated LCOs rather than de-*O*-acetyl fucosylated LCOs under suboptimal conditions.

Although 4-*O*-acetyl-fucosylated LCOs are the primary and major components of *M. loti* LCOs, they are not highly stable and have been shown to be easily deacetylated even under neutral pH (data not shown). The redundancy of *Lotus* plants with regard to the perception of 4-*O*-acetyl and de-*O*-acetyl fucosylated LCOs, therefore, meets the strategy of the host plants to accomplish an efficient symbiotic relationship with *M. loti* under various natural rhizosphere conditions.

Fig. 4 Formation of nodule primordia on *Lotus japonicus* MG-20 'Miyakojima' roots by purified LCO fractions (10–20 ng) at 7 d of the spot inoculation. (A) Acetyl-fucosylated LCO, NodMl-V($C_{18:1}$, Me, Cb, AcFuc) from *Mesorhizobium loti* MAFF303099; (B) fucosylated LCO, NodMl-V($C_{18:1}$, Me, Cb, Fuc) from Ml107.

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