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Formation of Nodule-like Component on Oat Root

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Nodule-like structures were formed on oat roots when the roots treated with the cell wall degrading enzymes, Cellulase Onozuka RS, Driselase and Pectolyase Y-23, infected with *Rhizobium leguminosarum* biovar *trifolii* H1t1 in the presence of polyethylene glycol. A variety of nodule-like structures developed. Electron microscopic observations revealed that *Rhizobium* was localized in the intercellular spaces among oat root cells, but no evidence for the presence of the rhizobia in the cells was obtained.

Introduction

Rhizobia infect and form nodules on the roots of their leguminous hosts. This host specificity has been extensively studied[1]. Interactions of genetically engineered *Rhizobium* transconjugants with seedlings of maize and rice resulted in root hair curling, but not in nodulation[2]. The observation that a mixture of Cellulase and Pectolyase was able to degrade the cell wall at the apices of root hairs of a wide range of crops, including legumes[3], suggested the possibility that such enzymatic treatment of roots of heterologous legumes or crops could overcome the restrictions of host specificity. The treatment of the root hairs of white clover with a cell wall degrading enzyme enabled *R. loti* to induce the formation of nitrogen fixing nodules[4]. Nodular structures have been induced on rice roots by the treatment of the roots with a mixture of hydrolytic enzyme in the presence of polyethylene glycol (PEG)[5]. The nodule-like structure was observed by light microscopy and transmission electron microscopy (TEM). The report showed that several rhizobia were associated with the outer surface and were seen inside the peripheral cells of spherical nodular structures.

In this study, we report our attempts to form nodular structures on the roots of oat by treatment with a mixture of cell wall degrading enzymes and by inoculation with *R. leguminosarum* biovar *trifolii* H1t1 in the presence of PEG.

Materials and Methods

1. *Rhizobium and culture*

Rhizobium leguminosarum biovar *trifolii* H1t1 was incubated in the dark, with shaking, for 48 h (25°C; 160 rev./min.) in tryptone-yeast extract (TY) medium[6]. Bacteria were centirifuged (8,000× g; 10 min.) and then the pellet was washed three times with Fåhraeus medium[7]. The concentration of bacteria was adjusted to 5×10^8 cells/m*l* in Fåhraeus medium.

2. *Plant materials*

Seeds of oat (*Avena sativa*) were sterilized with 5.0% (v/v) solution of sodium hypochlorite for 15 min. and then washed three times with sterile water. The seeds were germinated on the surface of paper soaked with sterile water, at 27°C, in the dark for 3 days.

3. *Treatment of seedlings*

The methods described previously were modified[4]. The roots of seedlings were treated with sterile solution that consisted of 1.0% (w/v) Cellulase Onozuka RS (Yakuruto Honsya, Tokyo, Japan), 1.0% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan), 1.0% (w/v) Driselase (Kyowa Hakko Kogyo, Tokyo, Japan) and 6.0% (w/v) mannitol (pH 6.2) for 20 min. at 25°C. The treated roots of oat were washed three times with sterile water and then inoculated with 1.0 m*l* of suspension of *R. leguminosarum* biovar *trifolii* H1t1 and 1.0 m*l* of sterilized 25% (w/v) polyethylene glycol (PEG 6000; Wako Pure Chemical Industries, Ltd., Osaka, Japan), in 50 mM calcium chloride, for 25 min. The plants were placed on the surface of Fåhraeus agar medium[7] in 9 cm (diameter) petri dishes. The samples were washed three times with potassium phosphate buffer, pH 7.0 (at intervals of 10 min.). The samples were fixed with 2.0% (v/v) glutaraldehyde (4°C; 2h) and postfixed with 1.0% (w/v) osminium tetroxide (4°C; 16h). Fixed nodules were washed twice with phosphate buffer and then dehydrated through an acetone series (10, 30, 50, 70, 90, 95 and 100% (v/v)). The samples were enclosed in Spurr's resin. Thin sections were stained with 2.0% (w/v) aqueous uranyl acetate for 10 min. at 25°C and then with lead citrate for 5 min. and then they were examined by TEM (Type HU-11, Hitachi Co. Ltd, Tokyo Japan). Sections for light microscopy were stained with 1.0% (w/v) metylene blue in 1.0% (w/v) sodium borate (25°C; 5 min.).

Results

Nodule-like structures were observed on the oat roots (**Fig. 1**). A variety of nodule-like structures developed on these roots. Some were short and elongated (**Fig. 1-A**), some were long and elongated (**Fig. 1-B**) and some were setting-pin shape (**Fig. 1-C**). The short and elongated nodules were observed most frequently (**Table 1**). The structures were not formed after treatment of the root with only 1.0% (w/v) Cellulase Onozuka, 1.0% (w/v) Pectolyase Y-23 or 1.0% (w/v) Driselase in the presence of PEG. Seedlings treated with PEG also did not form nodules (**Table 2**).

Sections of the nodular structures were observed by light microscopy (**Fig. 2**). The structures consisted of irregular shaped cells.

Ultrastructual examination of the nodular structures confirmed the presence of rhizobia in the interspaces between the root cells. A large number of the bacteria was observed in the spaces (**Fig. 3**). Among the three types of nodular structures, rhizobia were observed only in the short and elongated nodules (**Table 1**).

Discussion

Our observation that a mixture of Cellulase Onozuka RS, Pectolyase Y-23 and Driselase decomposed the cell walls of the root hairs of oat suggests that such enzymatic treatment of oat roots could overcome the host specificity in legume-*Rhizobium* symbiosis. In fact, *R. loti* nodulated a heterologous legume, white clover, and induced the formation of nitrogen-fixing nodules, when the roots of seedlings were pretreated with cell wall decomposing enzymes[4]. Bacteroids were observed in the nodules. This result implies that the cell wall structure of the root surface or root hairs is the barrier that normally prevents the establishment of a symbiotic relationship between *R. loti* and the heterologous legume, white clover. Once *R. loti* enters into the internal tissue, the bacteria can,

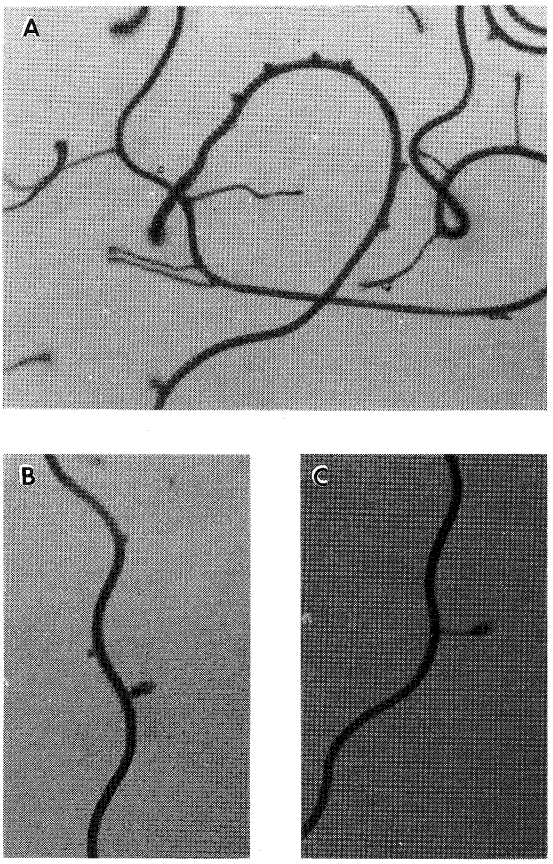

Fig. 1 Nodule-like structures induced after the treatment with cell wall degrading enzymes on root of oat.
(A) Short elongated shape, (B) Long elongated shape, (C) Shape of setting-pin (× 2.0)

Table 1. Existence of *Rhizobium* in the variety of nodules.

	short	long	setting-pin
number/13 roots*	13	2	6
bacteria (interspace)	+	−	−
bacteria (cytoplasm)	−	−	−

*The number of nodule-like structures obtained from 13 plants.
Note; +: exist, − : non-exist.

Table 2. Formation of nodule-like structures in oat after the treatment with cell wall degrading enzymes in the presence of PEG.

Cellulase +PEG	Pectolyase +PEG	Driselase +PEG	Cellulase+Pectolyase +Driselase+PEG	PEG
−	−	−	+	−

Note; + : formation of nodule-like structure, −: none.

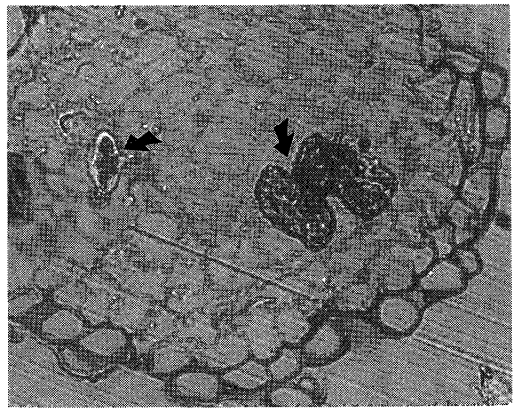

Fig. 2 The observation of nodule-like structure of the short elongated shape by light microscopy.
Arrows show a structure densed in methylene blue (×400).

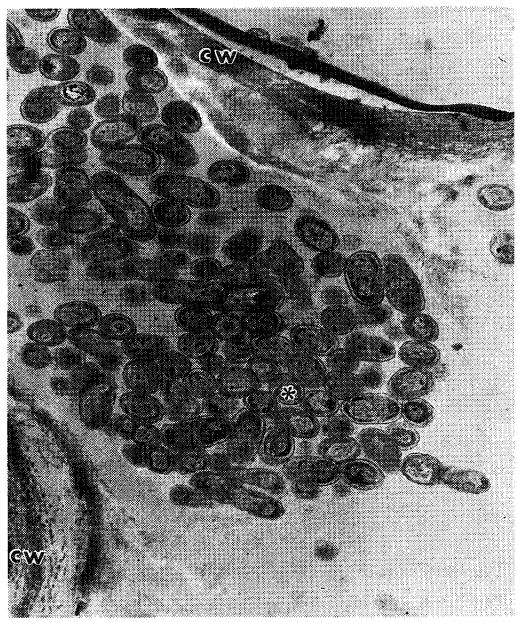

Fig. 3 The observation of nodule-like structure of the short elongated shape by TEM.
Rhizobia (*) are found among the intercellular space. CW shows the cell wall (× 4, 000).

apparently, infect the cells that make the host tissue, such as the cortical tissue, and develop normal nodules. In the case of oat tissues, treatment of roots with cell wall degrading enzymes enabled the rhizobia to penetrate into internal tissues. The bacteria were found, however, only in the intercellular spaces among the root cells (**Fig. 3**). That is, the rhizobia cannot invade the cells of the internal tissues, and the cell walls in the internal tissues are also a barrier for to the establishment of a symbiotic relationship in oat.

No nodular structures were formed after treatment with each of cell wall degrading enzyme, even in the presence of PEG (**Table 2**). The combination with enzymes is necessary for removing the barrier to nodulation even though the enzymes alone did not generate nodule-like structures (data not shown). PEG is also important for stimulating formation of nodules. Although PEG accentuated

entry through cracks in the root's surface has been claimed[5], its role remains to be resolved. Rhizobia were reported to infect rice roots treated with the same enzymes[5]. The structures were also of several types; the bacteria were associated with the outer surface and they were found in the peripheral cells of the nodular strucures. The differences among the results for white clover[4], oat (this paper), and rice[5] may be due to the differences in composition of the cell walls of these plants.

Acknowledgements

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References

1) Broughton, W. T., 1978. J. Appl. Bacteriol., **45**: 165-194.
2) Plazinski, J., R. W. Innes, B. G. Rolfe, 1985. J. Bacteriol., **163**: 812-815.
3) Cocking, E. C., 1985. Bio/Technology, **3**: 1104-1106.
4) Al-Mallah, M. K., M. R. Davey, E. C. Cocking, 1987. Bio/Technology, **5**: 1319-1322.
5) Al-Mallah, M. K., M. R. Davey, E. C. Cocking, 1989. J. Exp. Bot., **40**: 473-478.
6) Miller, J. H., 1972. In "Experiments in Molecular Genetics" p. 433, Cold Spring Harbor, New York.
7) Fåhraeus, G., 1957. J. Gen. Microbiol., **16**: 374-381.

《和文要約》

アベナにおける根粒様構造体の形成

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アベナの根毛を，細胞壁分解酵素である1% (w/v) セルラーゼ，1% (w/v) ドリセラーゼ，1% (w/v) ペクトリアーゼで処理し，25% (w/v) ポリエチレングリコール (PEG 6000) 存在下で根粒菌（クローバー菌；*Rhizobium leguminosarum* biovar *trifolii* H1t1）を感染させ，3種類の形態を持つ根粒様構造体を形成させることができた．透過型電子顕微鏡による観察によれば，根粒菌は構造体の細胞間隙に多く存在した．しかし，細胞内での存在は認められなかった．