Isolation of *Frankia* from the root nodules of *Elaeagnus macrophylla* and *Alnus sieboldiana* and their infectivities to the host plants

HIDEO SASAKAWA*, HIROSHI KAWAI,
NORIKATSU TAKAHASHI and SIZUKA TANIGAWA

Faculty of Agriculture, Okayama University, Okayama 700-8530, Japan

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We isolated endophytic *Frankia* from the root nodules of *Elaeagnus macrophylla* and *Alnus sieboldiana* and examined the infectivity of the isolates to the host plants.

One strain from *E. macrophylla* and 13 strains from *A. sieboldiana* were isolated by the simple method of suspending the nodule homogenate directly in liquid Qmod medium. Hyphae, sporangia and vesicles, which are morphological characteristics of *Frankia*, were observed in the *E. macrophylla* isolate. However, only hyphae and sporangia were observed in the *A. sieboldiana* isolate.

The *E. macrophylla* isolate formed nodules on the roots of the host plant and the average acetylene reduction activity (ARA) was $17.7 \pm 6.9 \mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} (\text{g fresh wt nod.})^{-1}$. Eleven strains isolated from *A. sieboldiana* formed nodules on the root of the host plants, but 2 strains did not nodulate. The average ARA was $4.22 \pm 3.73 \mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} (\text{g fresh wt nod.})^{-1}$.

Key words: actinorhizal plants, *Frankia*, infectivity, isolation, root nodules

Introduction

Most actinorhizal plants, which form nodules on their roots after infection with actinomycete *Frankia*, are capable of achieving higher rates of nitrogen fixation^{4,5,9,14,15} and the estimated amount of nitrogen fixed by those plants annually per unit area is comparable to that fixed by legumes^{8,10}.

Actinorhizal plants can grow on barren soils deficient in nitrogen nutrients and some of them are used for the recovery of vegetation and improvement in the fertility of wastelands. For example, *Alnus* and *Elaeagnus* have been widely used for land reclamation of industrial wastelands, for stabilization, and for the

improvement of the fertility of degraded forest soils in Europe²). In Japan, *Myrica rubra* has been planted with pine trees in liparite soil in the Seto Inland Sea area of western Japan. The planting of *Myrica rubra* improved the soil pH and fertility, and promoted the growth of the pine trees¹⁷). *Alnus* species such as *A. firma* and *A. sieboldiana* are commonly used as “fertilizer trees” to accelerate the growth of the target trees during forestation. Thus, introduction of actinorhizal plants is expected to be useful for the recovery of vegetation and fertility improvement in problem soils. For this purpose, it is necessary to select *Frankia* strains which have higher infectivity, forming many nodules with high nitrogen-fixing activity.

Here, we report the isolation of endophytic *Frankia* from the root nodules of *E. macrophylla* and *A. sieboldiana*, morphological characteristics of the isolated strains, their infectivity to the host plants, and the ni-

* Corresponding author; e-mail: sasakawa@ccews2.cc.okayama-u.ac.jp

trogen-fixing activity of the nodulated plants.

Materials and Methods

Frankia isolation

We previously isolated a *Frankia* strain from *M. rubra* nodules with different size mesh screens⁹⁾. Here, we isolated *Frankia* from the root nodules of *E. macrophylla* and *A. sieboldiana* by a simple method. Nodules were thoroughly washed with running tap water to remove soil, rinsed three times with distilled water and then nodule lobes were cut with a razor blade. The lobes were surface-sterilized with Na-hypochlorite (available chlorine approximately 5%) solution for 7 min and rinsed with distilled water 5 times. The lobes were further washed with 0.1% (v/v) Tween 80 solution and sterilized water, and again surface-sterilized with Na-hypochlorite solution. After being rinsed with sterilized water 5 times, each lobe was immersed in 0.15 mL of 50 mM sodium phosphate buffer (pH = 7.5) containing 1% (w/v) polyvinyl pyrrolidone (K30)(Isizu Chemical Co.) for 15 min and ground with a glass rod in a test tube. The homogenate was then suspended directly in 3 mL of liquid Qmod medium¹¹⁾ in another test tube and incubated at 28°C in the dark.

Photomicrography

An aliquot of isolated *Frankia* was transferred onto a slide glass and stained with one drop of 0.5% fuchsin (basic) solution for 1 min at room temperature. After putting a cover glass on the sample, distilled water was gently injected between the slide and the cover glass and excess dye was removed carefully with filter paper. The stained samples were observed with an Olympus photomicroscope.

Inoculation test for host plants

The seeds of *E. macrophylla* were germinated and grown in a mixture of autoclaved vermiculite and perlite (7:3) at room temperature. One 2-month-old seedling was transplanted into each plastic pot (12 cm diameter, 10 cm depth) containing autoclaved vermiculite. A *Frankia* strain was propagated and collected as previously described¹³⁾ and diluted to 1,000-fold of packed cell volume with N-free Arnon-

Hoagland solution¹⁾. Twenty milliliters of the *Frankia* cell suspension was supplied, then the seedlings were cultured for 4 months at 25°C under artificial light conditions ($100 \mu\text{E m}^{-2} \text{s}^{-1}$) and a day/night regime of 14 h/10 h. N-free Arnon-Hoagland solution was supplied at one-week intervals.

About 100 *A. sieboldiana* seeds per pot were sown. The pots contained autoclaved vermiculite. The seeds were then cultured under artificial light conditions ($250 \mu\text{E m}^{-2} \text{s}^{-1}$) and a day/night regime of 14 h/10 h at 25 °C. Two weeks after germination, the seedlings were thinned to leave 10 vigorously growing seedlings per pot. They were then supplied 20 mL of a *Frankia* cell suspension diluted to 500-fold of packed cell volume with N-free Arnon-Hoagland solution. The seedlings were cultured for 2 months under the same conditions as described above. N-free Arnon-Hoagland solution was supplied at one-week intervals.

Measurement of nitrogen-fixing activity

Nitrogen-fixing activities were measured by the acetylene reduction method for intact plants in *E. macrophylla* and for nodulated roots in *A. sieboldiana*. The intact *E. macrophylla* seedling was fixed at the stem portion with greasy clay in a hole bored in the center of a rubber stopper and the root portion was introduced into a 200 mL glass bottle. The gap between the glass bottle and rubber stopper was sealed with greasy clay to prevent gas leakage. The nodulated roots of an *A. sieboldiana* were in a 110 ml Erlenmeyer flask and sealed tightly with a rubber stopper. Gas phases in the containers were adjusted to contain 10% acetylene and incubated at 25°C under room light conditions. After a 1 h incubation, the ethylene produced was measured as described previously¹⁴⁾.

Results and Discussion

Isolation of *Frankia*

Several hundred nodule lobes were used in the isolation of endophytic *Frankia*. Test tubes contaminated with bacteria or fungi were eliminated over the 10 days from the commencement of incubation. The percentage of such contaminated test tubes was approximately 40% to 50%. The uncontaminated ones were fur-

ther incubated. After a 1 month incubation of the nodule lobe homogenate, colonies of filamentous bacteria, which were clearly different from fungi, ap-

Fig. 1. Photomicrograph of the *Frankia* strain, Ema 1, isolated from root nodules of *Elaeagnus macrophylla*. Arrow (↓) and arrowhead (▼) indicate the sporangium and the vesicle, respectively. Bar = 10 μ m.

peared in some test tubes. Under microscopical observation, 1 strain from *E. macrophylla* and 13 strains from *A. sieboldiana* were isolated. Hyphae, sporangia and vesicles which are morphological characteristics of *Frankia* were observed in the *E. macrophylla* isolates (Fig. 1). However, only hyphae and sporangia were observed in the *A. sieboldiana* isolates (Fig. 2). We designated the strain isolated from *E. macrophylla* as Ema1 and numbered the strains isolated from *A. sieboldiana* from As1 to As13. Although the formation of vesicles is an important parameter in identifying morphologically if the isolate is *Frankia*, some strains form vesicles only when they are cultured in a defined nutrient medium with a limited supply of nitrogenous compounds and express nitrogen-fixing activity^{3,9,16}. Fukumoto et al.⁵ isolated an infective *Frankia* strain from *A. sieboldiana* and reported that only sporangia were observed microscopically. These findings suggest that *Frankia* from *A. sieboldiana* does not form or forms very few vesicles in pure culture.

Fig. 2. Photomicrograph of *Frankia* strains isolated from root nodules of *Alnus sieboldiana*. Numbers 1 to 6 indicate strains As1 to As6 and arrows indicate sporangia. Bar = 10 μ m.

Fig. 3. Growth and nodules of *Elaeagnus macrophylla* formed by inoculation of *Frankia* strain Ema1. Fig. 3A shows the growth of plants inoculated with the *Frankia* strain Ema1. The left pot is an uninoculated control. Fig. 3B shows nodules formed on a plant. Plants were cultured for 4 months after inoculation. Arrows indicate nodules. Bar = 10 mm.

Inoculation test

Each isolate was inoculated onto host plants growing in vermiculite. The growth of inoculated *E. macrophylla* plants was more vigorous than that of uninoculated plants (Fig. 3A) and many nodules were formed on the roots of the seedlings inoculated with the Ema1 strain (Fig. 3B). The average ARA of 6 plants was $17.7 \pm 6.9 \mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} (\text{g fresh wt nod.})^{-1}$ (Table 1). This value was 40 times higher than that of the nodules of *Eleagnus pungens* formed by inoculation of *Frankia* isolated from the nodules of the host plant⁷⁾. This large difference in ARA between the nodules of *E. macrophylla* and *E. pungens* may be related to the age of the nodules used in the determination of ARA. However, there is a possibility that the *Frankia* strain we isolated is more infective and forms nodules with higher ARA activity than the strains isolated from *E. pungens* by Fukumoto *et al.*⁷⁾. The Ema1 strain formed vesicles in pure culture (Fig. 1). In contrast, no vesicles were observed in pure culture of the *Frankia* strains from *E. pungens*⁷⁾. The formation of vesicles in pure culture may also be related to the formation of nodules with higher ARA activity.

Figure 4 shows the growth of *A. sieboldiana* seedlings inoculated with 13 isolates and the nodulated plants. The growth of the seedlings was more vigorous after inoculation of the 11 strains, except for As3 and As7, than the uninoculated seedlings (Fig. 4A). Many root nodules were formed on the roots of seedlings showing active growth (Fig. 4B). Nodule formation, ARA, and shoot fresh weight after inocula-

Table 1. Acetylene-reducing activity of *Elaeagnus macrophylla* seedlings 6 months after inoculation with a *Frankia* strain Ema1

plant	ARA		nodule fr wt
	$\mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} \text{ plant}^{-1}$	$\mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} (\text{g fr wt nod.})^{-1}$	mg
1	1.12	11.3	99
2	1.55	14.1	110
3	0.96	24.6	39
4	0.78	14.7	53
5	0.73	28.1	26
6	1.07	13.5	79
av. $\pm$ SD	1.04 ± 0.30	17.7 ± 6.9	68 ± 34

Fig. 4. Growth and nodules of *Alnus sieboldiana* formed by inoculation with *Frankia* strains. Fig. 4A shows the growth of plants inoculated with *Frankia* strains As1 to As13. The left most pot is an uninoculated control. Fig. 4B shows nodules formed on a plant. Plants were cultured for 2 months after inoculation. Arrows indicate nodules. Bar = 10 mm.

Table 2. Acetylene-reducing activity of *Alnus sieboldiana* seedlings 2 months after inoculation with *Frankia* strains

strain	nodule No.	nodule fr wt	ARA		shoot fr wt
		mg	nmol C ₂ H ₄ h ⁻¹ plant ⁻¹	μmol C ₂ H ₄ h ⁻¹ (g fr wt nod) ⁻¹	
As 1	19 ± 6*	70 ± 37	186 ± 94	2.66 ± 0.53	1.461 ± 0.545
As 2	23 ± 4	37 ± 12	71 ± 64	1.87 ± 1.47	0.908 ± 0.218
As 3	0	0	0	0	0.028 ± 0.007
As 4	37 ± 16	33 ± 19	183 ± 164	4.96 ± 3.27	0.748 ± 0.361
As 5	43 ± 12	43 ± 16	329 ± 296	7.19 ± 6.10	1.005 ± 0.338
As 6	33 ± 4	39 ± 15	302 ± 498	5.54 ± 6.91	1.041 ± 0.173
As 7	0	0	0	0	0.035 ± 0.016
As 8	57 ± 25	43 ± 22	110 ± 53	3.00 ± 1.79	0.962 ± 0.380
As 9	65 ± 21	46 ± 15	179 ± 135	3.52 ± 1.58	0.731 ± 0.179
As10	60 ± 24	42 ± 21	289 ± 211	6.72 ± 2.77	0.875 ± 0.349
As11	70 ± 27	45 ± 19	179 ± 94	4.47 ± 2.42	1.141 ± 0.407
As12	33 ± 11	29 ± 15	91 ± 69	2.88 ± 1.40	0.910 ± 0.283
As13	30 ± 10	41 ± 24	125 ± 74	3.05 ± 0.81	0.821 ± 0.420
average	43 ± 24	42 ± 23	187 ± 223	4.22 ± 3.73	0.890 ± 0.451

* Numbers indicate average ± SD of 7 plants.

tion of the 13 strains are summarized in Table 2. Eleven strains produced nodules on *A. sieboldiana* seedlings but 2 strains, As3 and As7, did not. The average ARA of the *A. sieboldiana* nodules was $4.22 \pm 3.73 \mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} (\text{g fresh wt nod.})^{-1}$, which is equivalent to the value reported previously for *A.*

sieboldiana^{5,6)} and *M. rubra*¹³⁾. Judging from both nitrogen-fixing activity (ARA) shown in Table 2 and the growth rate in pure culture (data not shown), As5 and As10 are the most effective strains of *A. sieboldiana*.

Root nodules were not formed on the roots of the host plants after inoculation of the As 3 and As7

strains. Lechevalier and Lechevalier¹²⁾ classified frankiae into two broad groups, A and B. Group A is very heterogeneous and contains strains that may not be infective to the host from which they were isolated and most of these strains grow rapidly. In contrast, Group B is far more homogeneous and strains in this group grow relatively slowly while being infective to their original host plants. Both As3 and As7 grew relatively rapidly compared to other strains isolated from *A. sieboldiana* (data not shown). Furthermore, the hyphae of As3 and As7 had greater diameters than those of other infective strains and the ability of sporangia formation was lost due to subculture (data not shown). Accordingly, they may be frankiae which lack infectivity to the host plant and can be classified as Group A or as other actinomycetes which differ from frankiae. Comparison of their characteristics, including DNA homology, against the infective *Frankia* strains is required to determine whether they are frankiae or not.

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