Note

Effects of the Hemolymph from *Bombyx mori*, Immunized with *Escherichia coli*, on the Proliferation of Plant Pathogenic Bacteria

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Insects do not have an antigen-antibody reaction, but have unique self-defence mechanisms such as an inducible antibacterial immune system.^{1,2)} For example, the silkworm, *Bombyx mori*, have been reported to contain lysozyme³⁾ and cecropin type antibacterial proteins.^{1,4)} In general, cecropin type proteins have wide antibacterial spectrum against Gram-positive and Gram-negative bacteria.¹⁾ It should be interesting if such insect antibacterial protein genes could be used to establish transgenic plants that are resistant to pathogenic bacteria.

In the course of efforts to generate such transgenic plants, we first examined the effects of *B. mori* hemolymph, immunized with *Escherichia coli*, on the proliferation of several plant pathogenic bacterial species. In this note, we present data which show strong inhibitory effects of the hemolymph on the growth of these bacteria.

Nine bacterial species were used as the plant pathogens. These bacteria were first grown on PPGA⁵⁾ culture plates at 25°C. These plates contained boiled extracts of 200 g potato (per liter); peptone, 5 g/l; glucose, 5 g/l;

Table I. PROLIFERATION RATIO BETWEEN BACTERIAL CULTURES CONTAINING CONTROL OR *E. coli* IMMUNIZED HEMOLYMPH

The proliferation ratio was the percentage of the optical density of the culture containing immunized hemolymph to the value of the culture containing normal hemolymph. Total volume of the culture was 100 μ l. Experiments were done in triplicate (Exp. I, Exp. II, and Exp. III). In these experiments non-immunized normal hemolymph did not show antibacterial activity. a) to i) indicate following symptoms; a) crown gall, b) bacterial canker of tomato, c) bacterial soft rot, d) bacterial rot (necrotic leaf spot), e) bacterial grain rot of rice, f) bacterial wilt, g) bacterial blight of mulberry, h) black rot of cabbage, and i) bacterial leaf blight of rice. Numbers in parentheses are the mean values of optical density at 600 nm of the control culture with normal hemolymph.

Bacteria	Hemolymph (μ l)	Proliferation ratio (%)				Optical density
		Exp. I	Exp. II	Exp. III	Mean	
<i>Agrobacterium tumefaciens</i> N1001 ^{a)}	10	0.0	9.0	10.3	6.4	(0.634)
<i>Clavibacter michiganense</i> p.v. <i>michiganse</i> ^{b)}	10	0.0	14.6	29.6	14.7	(0.355)
<i>Erwinia carotovora</i> subsp. <i>carotovora</i> 1393 ^{c)}	10	1.8	19.7	6.6	9.3	(0.581)
<i>Pseudomonas cichorii</i> NL7630 ^{d)}	10	0.0	8.2	8.5	5.5	(0.812)
<i>Pseudomonas glumae</i> N1169 ^{e)}	10	116.2	81.9	93.7	97.2	(0.348)
	50	74.9	37.6	21.5	44.2	(0.711)
<i>Pseudomonas solanacearum</i> N1023 ^{f)}	10	102.7	94.4	69.5	88.8	(0.397)
	50	73.3	70.7	48.1	63.5	(0.530)
<i>Pseudomonas syringe</i> p.v. <i>mori</i> S6804 ^{g)}	10	3.3	11.0	12.6	8.9	(0.478)
<i>Xanthomonas campestris</i> p.v. <i>campestris</i> N1076 ^{h)}	10	6.3	18.8	9.6	11.5	(0.587)
<i>Xanthomonas campestris</i> p.v. <i>oryzae</i> N1086 ⁱ⁾	10	1.3	2.0	19.4	7.5	(0.560)

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$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 3 g/l; KH_2PO_4 , 0.5 g/l; NaCl, 3 g/l; and agar, 15 g/l. The pH value of this medium was about neutral without adjusting. To prepare *B. mori* hemolymph immunized with *E. coli*, 20 5th instar larvae of silkworm (Tokai $\times$ Asahahi), reared on an artificial diet (Nihon-Nosanko Co., Ltd.), were kept on ice and then injected in the abdominal legs with $10\ \mu\text{l}$ (1×10^6 cells) of *E. coli* DH1⁶⁾, washed and suspended in saline (130 mM NaCl/5 mM KCl).⁷⁾ Twenty hrs later,⁷⁾ hemolymph was collected by cutting off an abdominal leg and stored in ice-cooled polypropylene test tubes containing a few crystals of phenylthiourea. The collected hemolymph was heated at 100°C for 5 min and centrifuged at $10,000 \times g$ for 5 min with a Sakuma centrifuge (Model M-150). The supernatant was pooled and stored at -30°C . As a control the hemolymph from uninjected silkworm larvae was prepared under the same conditions. To examine the inhibitory effects of immunized *B. mori* hemolymph on the bacterial proliferation, fresh bacterial cultures on PPGA plates were inoculated and diluted in the same culture medium (about 5×10^6 cells/ml). Ninety μl of each culture containing each bacterial species was transferred separately to a 96-hole microtiter plate and $10\ \mu\text{l}$ of control or *E. coli* immunized *B. mori* hemolymph was added to each culture (total $100\ \mu\text{l}$). In the case of *Pseudomonas glumae* N1169 and *Pseudomonas solanacearum* N1023, the dose effect of the hemolymph was also tested using a medium containing 50% hemolymph (total $100\ \mu\text{l}$). The culture was incubated at 25°C for 2 to 3 days with shaking. The optical density of the bacterial concentration was measured at 600 nm by a Bio-Rad EIA Reader Model 2550.

It is obvious that *B. mori* hemolymph immunized with *E. coli* contains factors that strongly inhibit the proliferation of plant pathogenic bacteria (Table I). However, the extent of inhibition differs considerably depending upon the bacterial species. Seven out of 9 species were strongly affected by immunization (5.5 to 14.7% proliferation ratio). The other two bacterial species are relatively resistant (44.6% and 64.0% ratio at a higher dose of hemolymph).

To date five types of proteins with antibacterial activity have been reported to be important in the self-defence of insects against bacteria. These are lysozyme,⁸ cecropin,¹⁾ attacin,¹⁰⁾ dipterocin,¹¹⁾ and insect defensin¹²⁾ type proteins. Recently Morishima *et al.* have reported that by immunization with bacteria *B. mori* induces in hemolymph at least 6 antibacterial proteins that show the typical

characteristics of cecropins: heat-stability, low molecular weight, and bactericidal action against bacteria.⁷⁾ The inhibitory effect on proliferation of plant pathogenic bacteria might result from the antibacterial activity such as cecropin type proteins but not lysozyme, since lysozyme has a very narrow antibacterial spectrum against bacteria.¹⁾ However, it should be clarified hereafter if isolated cecropins really contain an antibacterial activity against plant pathogenic bacteria to see if cecropin type genes are suitable candidates to generate transgenic plants that have a regulated antibacterial immune system against these bacteria.

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