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Loss of a Plasmid in *Pseudomonas syringae* pv. *eriobotryae* is Correlated with Change of Symptoms

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Abstract

Pseudomonas syringae pv. *eriobotryae* is a causal agent of stem canker of loquat. All of the strains which produced dark brown pigment harboured three plasmids of approximately 32, 39, and 85 megadaltons (Mdal). Twenty-eight derivative strains cured of the 85 Mdal plasmid were obtained from NAE57 and NAE34 by five or six times subcultures as the elevated temperature (32 C). All of the cured strains induced symptoms with no callus-like tissue on the inoculated stems of loquat, whereas 28 derivative strains that still harboured 85 Mdal plasmid induced symptoms with the development of the similar callus-like tissue as those of parent strains. Since the loss of the 85 Mdal plasmid was always accompanied by change in symptoms, it is strongly suggested that the 85 Mdal plasmid in *P. syringae* pv. *eriobotryae* plays an important role in the development of symptoms.

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Key words: *Pseudomonas syringae* pv. *eriobotryae*, plasmid, symptoms.

INTRODUCTION

In several pathovars of *Pseudomonas syringae*, plasmid-born virulence or other traits have been reported[1–3,10,12]. *Pseudomonas syringae* pv. *eriobotryae* is a pathogenic bacterium causing stem canker of loquat. The canker bacteria have been classified into three groups on the basis of the producibility of dark brown pigment and pathogenicity to leaves of loquat plant[9]. Group A strains produce no pigment, and are not pathogenic to leaves. Group B strains produce no pigment, and are pathogenic to leaves. Group C strains produce dark brown pigment, and are not pathogenic to leaves. Preliminary experiment showed that agarose gel electrophoretic profiles of plasmid DNA isolated from the strains belonging to the group C were almost similar. Therefore, indigeneous plasmids observed in these strains were expected to play an important genetic role. The present study was carried out in order to investigate the genetic functions of the plasmids in the group C strains.

MATERIALS AND METHODS

Bacterial strains and media. All the strains of *P. syringae* pv. *eriobotryae* except M8812 were kindly supplied by Dr. A. Morita, Nagasaki Fruit-Tree Experiment Station, Japan, and were originally isolated from canker of loquat stems collected from various localities in Japan. The strain M8812 was isolated in this laboratory from a diseased leaf of loquat.

Bacteria were routinely grown at 25 C in YP medium (yeast extract 5 g, polypepton 10 g, NaCl 5 g, glucose 1 g, distilled water 1 liter, pH 7.0) or PS medium (decoction of 250 g potato 1 liter, $Na_2HPO_4 \cdot 12H_2O$ 2 g, $Ca(NO_3)_2 \cdot 4H_2O$ 0.5 g, polypeptone 5 g, sucrose 20 g, pH 7.0). Both of the media solidified with 1.5% agar were used for plating bacterial suspensions or culture of inoculum.

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Isolation of plasmid DNA. Plasmid DNA was isolated by modified procedure of Kado and Liu[6]. The cells in 1.5 ml of YP cultures were centrifuged, and suspended in 20 μl of SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris-HCl). The cells were lysed by adding 70 or 100 μl of lysing solution containing 1% sodium dodecyl sulfate (SDS) and 0.2 N NaOH. The solution was mixed gently until it became clear, and 150 μl of phenol-chloroform solution (1:1) was added. After shaking, the mixture was centrifuged at 10,000 rpm for 10 min. Ten μl of aqueous phase was mixed with 2.5 μl of tracking dye (25% sucrose, 5 mM sodium acetate, 0.05% bromophenol blue, 0.1% SDS) and loaded onto a 0.7% horizontal agarose gel. After electrophoresis, gel was stained with ethidium bromide (0.5 μg/ml) and visualized on an ultraviolet light transilluminator.

Molecular weight of plasmids was estimated by measuring their electrophoretic mobilities in comparison with those of the standard plasmids, including RP4(36 Mdal)[11], pAS8Tc^{s}repl::Tn7(52 Mdal)[10], and the large (124 Mdal) and small (29.8 Mdal) plasmids from *Agrobacterium radiobactor* 84[8].

Curing of plasmid DNA. *P. syringae* pv. *eriobotryae* had optimum growth temperature of 25–26 C and a maximum growth temperature of 32 C. Bacterial strains were grown in 5 ml of YP medium at 32 C for 72 hr, and then 0.1 ml of the cell suspension was transferred to 5 ml of the fresh same medium and shake-cultured at 32 C for 72 hr. After five or six times of subculturing under the same conditions, the cultures were diluted with sterile distilled water and plated on YP agar or PS agar medium. After 2 or 3 days of incubation at 25 C, same colonies which changed in color very slightly were picked up and restreaked on the same medium. These clones resulted from streaking on the medium were subcultured for examination of their plasmid content.

Curing test with acridine orange, mitomycin C and SDS were performed according to the method of Comai and Kosuge[3].

Inoculation test. Cultures on YP agar medium were collected and suspended in sterile distilled water to give a concentration of about 10^{9} cells/ml. One year old stems of loquat tree were wiped with cotton wool soaked in 70% ethyl alcohol. After drying, a drop of the bacterial suspension was placed on the stems and pricked several times with a needle. The inoculated stems were covered with polyethylene bag for 24 hr to keep high humidity. The inoculated plants were incubated in growth chamber at 25 C (30,000 lux), and observed for the development of symptoms during one year after inoculation.

RESULTS

Plasmid content of P. syringae pv. eriobotryae

Plasmid DNA profiles of *P. syringae* pv. *eriobotryae* are shown in Fig. 1 and Table 1. NAE64 and several other strains (not presented) belonging to the group A harboured three plasmids of approximately 25, 52 and 60 Mdal. M8812 and a few other strains (not presented) belonging to the group B harboured three or four plasmids, with molecular weight approximately ranging from 8.5 to 45 Mdal. All of the strains belonging to the group C harboured three plasmids approximately 32, 39, and 85 Mdal. Additional 28 Mdal plasmid was present in NAE34 and NAE38.

Thus, plasmid DNA profiles were different among the three groups.

Isolation of derivative strains cured of 85 Mdal plasmid

Various treatments were tested for curing of plasmids in NAE57 and NAE34. Curing of plasmids was observed only after exposure of cells to elevated growth temperature *i.e.*, 32 C (Table 2, Fig. 2). Eighteen colonies from NAE57 and ten colonies from NAE34 cured of 85 Mdal plasmid were obtained. These cured colonies were slightly translucent. A slight change in colony color induced by curing of 85 Mdal plasmid could be recognized for the first time when there was some overlap between the cured and uncured colonies. Therefore, it was impossible

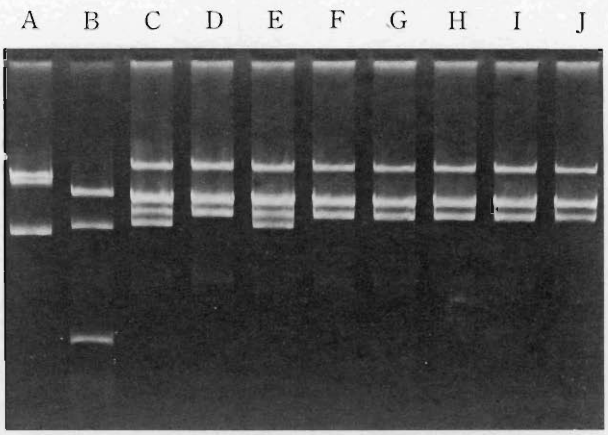

Fig. 1. Agarose gel electrophoresis of plasmid DNA isolated from *Pseudomopas syringae* pv. *eriobotryae*. (A) NAE64, (B) M8812, (C) NAE34, (D) NAE36, (E) NAE38, (F) NAE-57, (G) NAE87, (H) NAE101, (I) NAE118, and (J) NAE133.

Table 1. Plasmids of *Pseudomonas syringae* pv. *eriobotryae*

Bacterial strain	Type of group	Molecular weight of plasmids ($\times 10^6$)	No. of plasmids
NAE64	A	25, 52, 60	3
M8812	B	8.5, 27, 45	3
NAE34	C	28, 32, 39, 85	4
NAE36	C	32, 39, 85	3
NAE38	C	28, 32, 39, 85	4
NAE57	C	32, 39, 85	3
NAE87	C	32, 39, 85	3
NAE101	C	32, 39, 85	3
NAE118	C	32, 39, 85	3
NAE133	C	32, 39, 85	3

Table 2. Curing of 85 Mdal plasmid in *P. syringae* pv. *eriobotryae* by various treatments

Strain	Treatment	Concentration (μg/ml)	No. of colonies	
			Screened	Cured
NAE57	Acridine orange	50	100	0
NAE57	Mitomycin C	0.1	70	0
NAE57	SDS	5,000	80	0
NAE57	Elevated temp. (32 C)		18 a)	18
NAE34	Elevated temp. (32 C)		10 a)	10

a) Colonies which changed in color slightly were picked up.

to select a cured colony from the many well-separated colonies on the plate.

Two derivative strains were cured of 32 Mdal plasmid without any alteration of colony. Although a great number of colonies were investigated, no derivative strains which lost 39 Mdal plasmid could be detected. All the derivative strains cured of 85 or 32 Mdal plasmid still produced dark brown pigment.

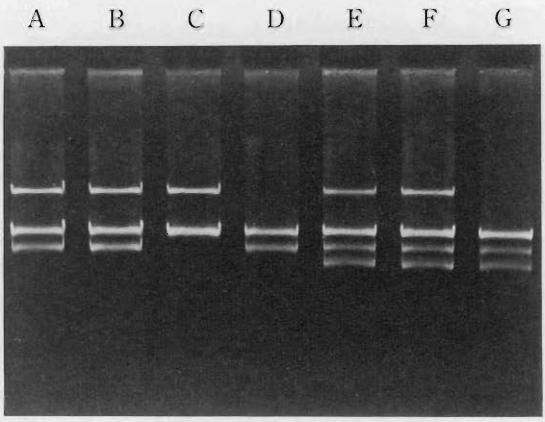

Fig. 2. Agarose gel electrophoretic profiles of plasmid DNA extracted from NAE57, NAE34 and their derivative strains obtained by subcultures at the elevated temperature. (A): NAE57 (parent strain), (B)–(D): Derivative strains obtained from NAE57. B, Strain that harboured the same plasmids as that of parent strain; C, Strain that cured of 32 Mdal plasmid; D, Strain that cured of 85 Mdal plasmid. (E): NAE34 (parent strain). (F)–(G): Derivative strains obtained from NAE34. F, Strain that harboured the same plasmids as that of parent strain; G, Strain that cured of 85 Mdal plasmid.

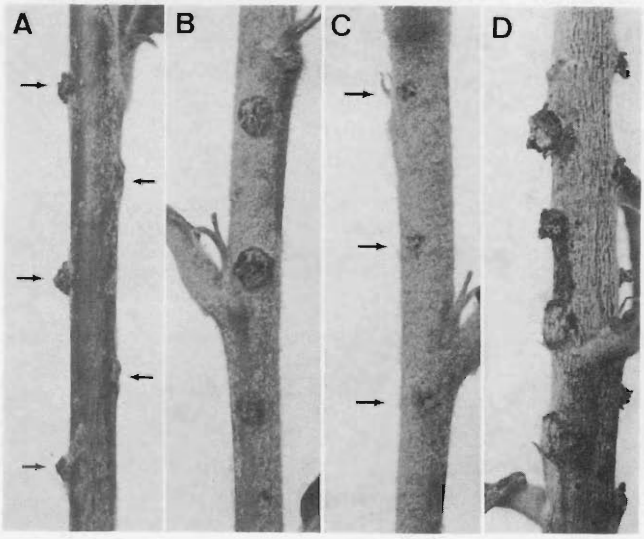

Fig. 3. Symptoms on loquat stem induced by the derivative strains that harboured the 85 Mdal plasmid and the cured derivative strains obtained from NAE57. A) Symptoms 50 days after inoculation with the derivative strains that harboured 85 Mdal plasmid (left side) and the cured derivative strains (right side). Arrows, inoculated sites. B) Symptoms 50 days after inoculation with the derivative strains that harboured 85 Mdal plasmid. Photographed from the front. C) Symptoms 50 days after inoculation with the cured derivative strains. Photographed from the front. Arrows, inoculated sites. D) Symptoms 250 days after inoculation with the derivative strains that harboured 85 Mdal plasmid (the upper two symptoms) and the cured derivative strains (the lower two symptoms).

Symptoms of the derivative strains that harboured 85 Mdal plasmid and the derivative strains cured of 85 Mdal plasmid

Eighteen and ten derivative strains still harbouring 85 Mdal plasmid after exposure of cells to elevated temperature were obtained from NAE57 and NAE34, respectively and used as a control strains of inoculation test. The two parent strains, NAE57 and NAE34, and those 28 derivative strains that haboured 85 Mdal plasmid including two strains cured of 32 Mdal plasmid were inoculated on the loquat stems. Development of symptoms induced by these strains was similar. First, it was evident as browning, and then callus-like tissue developed at inoculated parts from 2 or 3 weeks after inoculation (Fig. 3. A, B). The protruded callus-like tissue was dried and turned dark brown after several months of incubation, and could be sloughed off easily (Fig. 3, D).

On the other hand, the symptoms induced by the derivative strains cured of 85 Mdal plasmid were different from those of the strains that harboured this plasmid. Callus-like tissue was never formed on the loquat stem. All of the inoculated parts remained brown and slightly swelled even at 50 days after inoculation (Fig. 3. A, C). The margin of the lesion became definite after that (Fig. 3. D).

Symptoms of the derivative strains that harboured 85 Mdal plasmid and the cured derivative strains were sometimes accompanied by gummy exudation.

Search for phenotypic traits on 85 Mdal plasmid

Since the change in symptoms is not a useful selective marker, an attempt was made to detect another marker encoded on 85 Mdal plasmid. Derivative strains that harboured 85 Mdal plasmid were examined for phenotypic differences compared with cured isolates. The following properties were examined: resistances to ampicillin, streptomycin, kanamycin, tetracycline, copper and mercury; utilization of carbohydrates (*e.g.*, xylose, fructose, glucose, mannose, maltose, lactose, rhamnose, galactose, raffinose, saccharose, sorbose, arabinose, sorbitol, inositol and mannitol); degradation of organic acid (tartaric acid, lactic acid, nicotinic acid, galacturonic acid and citric acid); hypersensitivity in tobacco; antibiotic activity against *Geotricum candidum*[4]; congo red binding ability[10]; salt agglutination (*e.g.*, NaCl, $BaCl_2$, $FeCl_2$); morphological analysis of lipopolysaccharide by polyacrylamide gel electrophoresis[5]. No differences in these phenotypes were observed between the strains that harboured 85 Mdal plasmid and the cured strains.

Indoleacetic acid (IAA) was extracted according to the procedure of Liu and Kado[7], and analyzed by thin layer chromatography. The average amount of IAA in the culture filtrates of a derivative strain that haboured 85 Mdal plasmid and cured strain were 3.1 and 2.8 μg per ml, respectively. No significant difference was found between two derivative strains. Parents strains (NAE57 and NAE34) and all of their derivative strains were resistant to 5-methyltryptophan.

DISCUSSION

Each strain of *P. syringae* pv. *eriobotryae* belonging to the group A, B and C harboured three or four plasmids. The profiles of plasmid DNA isolated from strains belonging to the group C were similar, notwithstanding these strains were collected from various locality in Japan. To investigate the genetic functions of these plasmids, curing tests were performed with various treatments. Strains NAE57 and NAE34 were not cured of their plasmids with acridine orange, mitomycin C or SDS that used generally as plasmid curing agents. Only growth at elevated temperature resulted in the loss of 85 or 32 Mdal plasmid. All the cured derivative strains still produced dark brown pigment, suggesting that the pigmentation of these strains was not controlled by 85 or 32 Mdal plasmid.

There was a remarkable difference in the symptom developments between the derivative strains that harboured 85 Mdal plasmid and the cured strains. No other markers expressed

by 85 Mdal plasmid could not be detected, and tagging of 85 Mdal plasmid with transposon Tn7 was also unsuccessful (data not shown). Therefore, reintroduction of 85 Mdal plasmid into the cured derivative strains by conjugation or transformation could not be undertaken. However, the finding in this experiment showed that loss of 85 Mdal plasmid is always accompanied by change in symptoms. This strongly suggests that the 85 Mdal plasmid in *P. syringae* pv. *eriobotryae* plays an important role in the development of symptoms on loquat stems.

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和 文 摘 要

上運天 博：*Pseudomonas syringae* pv. *eriobotryae* のプラスミドの欠落に伴う病徴の変化

P. syringae pv. *eriobotryae* はビワがんしゅ病の病原細菌であり，供試した褐色色素産生菌株はすべて，分子量がそれぞれ約 32, 39, 85 メガダルトン (Mdal) の 3 個のプラスミドを共通に有していた。生育適温より高い温度 (32 C) で数回継代培養することにより，NAE57 および NAE34 から 85 Mdal プラスミドが欠落した 28 菌株を得た。これら 85 Mdal プラスミド欠落株 28 菌株と継代培養後もなお 85 Mdal プラスミドを保有していた 28 菌株をビワの茎に接種した結果，85 Mdal プラスミド保有株はすべて親株と同様にカルス様組織が発達した病徴を示したのに対し，85 Mdal 欠落株はすべてカルス様組織を伴わない病徴を示すようになった。85 Mdal プラスミドの欠落は常に病徴の変化を伴っており，85 Mdal プラスミドが *P. syringae* pv. *eriobotryae* の病徴進展に重要な役割を果たしていることが強く示唆された。