Pseudomonas gladioliを検出するためのDNAプローブ

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A Cloned DNA Probe for Detection of *Pseudomonas gladioli*

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Abstract

Six *Eco* RI digested-DNA fragments, 17.6-kb, 8.3-kb, 7.3-kb, 10.5-kb, 5.0-kb, and 8.7-kb, were selected from genomic DNA of *Pseudomonas gladioli* pv. *gladioli* MAFF 302545 to obtain specific probes of this bacterium. The 8.3-kb fragment hybridized to 56 of the 61 strains of *P. gladioli* but did not hybridize to 46 strains of *P. caryophylli, P. cepacia, P. glumae, P. plantarii* and *P. solanacearum* with dot blot hybridization. This fragment hybridized to genomic DNA from the 56 strains of *P. gladioli* producing a single band of 8.3-kb with Southern blot hybridization. This implies that these *P. gladioli* strains contain an 8.3-kb DNA fragment of *Eco*RI termini, which is common to these strains. These results indicate that the 8.3-kb DNA fragment is useful as a probe for detection and rapid identification of *P. gladioli.*

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Key words: specific probe, *Pseudomonas gladioli*

INTRODUCTION

Pseudomonas gladioli Severini 1913 causes stem base rot and corm rot of gladiolus, freesia, cymbidium, iris, onion and tulip, etc.[6], and some strains of this species are well known as an antagonist of pathogenic bacteria and fungi[1-3,8]. Although this species consists of two pathovars, pv. *gladioli* and pv. *alliicola*, it was difficult to distinguish them by bacteriological characteristics[6].

P. gladioli has been circumscribed in rRNA group II of *Pseudomonas* species which contains *P. caryophylli*, *P. cepacia* and *P. solanacearum*[11,12]. Of these, *P. gladioli* and *P. cepacia* are difficult to separate from each other on biochemical and physiological characteristics. *P. glumae* has been reported to be closely related to rRNA group II of *Pseudomonas* species[7]. *P. plantarii* is also reported to resemble *P. gladioli* pv. *gladioli* on the basis of DNA homology[5]. For the reasons stated above, identification of *P. gladioli* is time-consuming and laborious, consequently, the methods for rapid identification and detection of *P. gladioli* are urgently needed. DNA fragments specific to some plant pathogenic bacteria have been isolated to use as probes for DNA-DNA hybridization, which is more rapid, sensitive and specific than conventional methods for identification of these bacteria[13-15,17,18]. In the present study, the development of DNA probes for detecting *P. gladioli* are described.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. Bacterial strains used in this study are listed in Table 1. *P. gladioli* pv. *gladioli* MAFF 302545 was used to construct DNA library. This strain was isolated from a rice plant and capable of causing bacterial scab of gladiolus. These bacteria were cultured on PPGA slants[10] except that *P. solanacearum* was grown on PSA slants[16].

Total genomic DNA was extracted from bacteria by the method of MINIPREP using CTAB/NaCl (10% hexadecyltrimethyl ammonium bromide in 0.7 M NaCl) solution[4].

Construction of DNA library. Genomic DNA of *P. gladioli* pv. *gladioli* MAFF 302545 was purified as above and then digested with *Eco*RI. Digested DNA fragments were ligated into *Eco*RI digested pUC 18 plasmid vector by using Ligation kit (Takara Ltd.) and transformed the competent cells of *Escherichia coli* JM 109 (Takara Ltd.). Transformed *E. coli* JM 109 cells were selected on LB plates[9] containing 50 μg/ml ampicillin and each colony produced on plates was cultured overnight separately in LB containing 50 μg/ml ampicillin at 37°C. Transformed cells were stored at −40°C after addition of 20-40% glycerol (final conc.).

Extraction of plasmid containing genomic DNA fragments. Plasmid extraction was carried out according to the modified method of Asubel *et al.*[4] as follows: Transformed cells were suspended in 350 μl of STET (8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0), treated with lysozyme (10 mg/ml) and then incubated in boiling water for 40 sec. After centrifugation, plasmid DNA in supernatant was precipitated in isopropanol and stored at −20°C until use.

Selection of probes. Six hundred recombinant plasmids were tested to select specific probes. One μl of

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Table 1. Strains used in this study[a)]

Species	Strain	Other designation
Pseudomonas gladioli pv. *gladioli*	ICMP 3850^T, MAFF 301064, MAFF 301065, MAFF 301066, MAFF 301580, MAFF 301581, MAFF 301582, MAFF 301583, MAFF 301584, MAFF 301585, MAFF 301586, MAFF 301587, MAFF 301588, MAFF 301589, MAFF 301590, MAFF 301728, MAFF 301729, MAFF 301730, MAFF 302385, MAFF 302386, MAFF 302537, MAFF 302538, MAFF 302543, MAFF 302544, MAFF 302545,	
P. gladioli pv. *alliicola*	ICMP 2804^T	
P. gladioli pv. undetermined	MAFF 302408, MAFF 302409, MAFF 302410, MAFF 302411, MAFF 302418, MAFF 302419, MAFF 302420, MAFF 302424, MAFF 302425, MAFF 302426, MAFF 302427, MAFF 302428, MAFF 302429, MAFF 302430, MAFF 302431, MAFF 302432, MAFF 302433, MAFF 302434, MAFF 302435, MAFF 302436, MAFF 302515, MAFF 302516, MAFF 302517, MAFF 302518, MAFF 302519, MAFF 302520, MAFF 302521, MAFF 302522, MAFF 302523, MAFF 302524, MAFF 302525, MAFF 302526, MAFF 302527, MAFF 302533, MAFF 302534,	
P. caryophylli	ICMP 512^T, MAFF 301060, MAFF 301100, MAFF 301192, MAFF 301194, MAFF 301196, MAFF 301406, MAFF 301407, MAFF 301411, MAFF 301414,	
P. cepacia	MAFF 302528, MAFF 302529, MAFF 302530, MAFF 302531, MAFF 302532, A 4[b)], A 10, Pc 10, Pc 20, ALQ 8281, 86130	
P. glumae	MAFF 301169^T, MAFF 301094, MAFF 301099, MAFF 301171, MAFF 301386, MAFF 301388, MAFF 301441, MAFF 302465, MAFF 302552,	PDDCC 6355^T
P. plantarii	MAFF 301723^T, MAFF 302381, MAFF 302387, MAFF 302392, MAFF 302412, MAFF 302466, MAFF 302476, MAFF 302479, MAFF 302483,	ICMP 9425^T, JCM 5492^T
P. solanacearum biovar I	MAFF 302154^T,	ATCC 11696^T
biovar II	MAFF 301559,	
biovar III	MAFF 301492, MAFF 301860	
biovar IV	MAFF 301067, MAFF 301418, MAFF 301524, MAFF 301556	

a) T: Type strain or pathotype strain, MAFF: Ministry of Agriculture, Forestry and Fisheries of Japan, ATCC: American Type Culture Collection, ICMP: International Collection of Micro-organisms from Plant, JCM: Japan Collection of Microorganisms.

b) The 6 strains (No. A 4, A 10, Pc 10, Pc 20, ALQ 8281 and 86130) of *P. cepacia* were supplied by Dr. K. Tsuchiya in National Institute of Agrobiological Resources.

plasmid DNA solution (approximately 20 ng) was blotted on Optiblot nylon membrane (International Biotechnologies, Inc.). One μg each of total genomic DNA from *P. gladioli* pv. *gladioli* MAFF 302545, *P. caryophylli* ICMP 512, *P. cepacia* MAFF 302528, *P. glumae* MAFF 301169 and *P. plantarii* MAFF 301723 was labeled with [α-^{32}P]dCTP (specific activity, >3000 Ci/mmol) using Multiprime DNA Labelling System (Amersham International plc.) to use as probes for dot blot hybridization analysis.

Hybridization was carried out overnight at 68°C in hybridization buffer (7% SDS, 1% bovine serum albumin and 1 mM EDTA in 1 M sodium phosphate buffer pH 7.5). The membrane was washed three times for 30 min in washing buffer (1% SDS, 0.1% bovine serum albumin and 1 mM EDTA in 50 mM sodium phosphate buffer pH

Table 2. Dot blot hybridization between ^{32}P-labeled DNA fragments and 61 strains of *Pseudomonas gladioli*

Species	Strain	Source	Probe with ^{32}P-labeled DNA fragment[a)]					
			17.5-kb	8.3-kb	7.3-kb	10.5-kb	5.0-kb	8.7-kb
Pseudomonas gladioli pv. *gladioli*	ICMP 3850^T	Gladiolus	+	+	−	−	+	+
	MAFF 302385	*do.*	−	+	−	−	−	+
	MAFF 301064	Freesia	−	+	−	−	−	+
	MAFF 301065	*do.*	−	+	−	−	−	+
	MAFF 301066	*do.*	+	+	−	−	−	+
	MAFF 301580	Dendrobium	−	+	−	−	−	−
	MAFF 301581	*do.*	−	+	−	−	−	−
	MAFF 301582	*do.*	−	+	−	−	−	−
	MAFF 301583	*do.*	−	+	−	−	−	−
	MAFF 301584	Vuylstekeara	−	−	−	−	−	−
	MAFF 301585	*do.*	−	+	−	−	−	−
	MAFF 301586	*do.*	−	−	−	−	−	−
	MAFF 301587	*do.*	−	+	−	−	−	−
	MAFF 301588	Cymbidium	−	+	−	−	−	−
	MAFF 301589	*do.*	−	+	−	−	−	−
	MAFF 301590	*do.*	−	+	−	−	−	−
	MAFF 301728	Vanda	−	−	−	−	−	−
	MAFF 301729	*do.*	−	−	−	−	−	−
	MAFF 301730	*do.*	−	−	−	−	−	−
	MAFF 302537	Onion	+	+	−	−	−	+
	MAFF 302538	*do.*	+	+	−	−	−	+
	MAFF 302386	Rice	+	+	−	−	−	+
	MAFF 302543	*do.*	−	+	−	−	−	−
	MAFF 302544	*do.*	+	+	−	−	−	+
	MAFF 302545	*do.*	+	+	+	+	+	+
P. gladioli pv. *alliicola*	ICMP 2804^T	Onion	−	+	−	+	+	−
P. gladioli pv. undetermined	MAFF 302408	Adzuki bean	+	+	−	−	−	+
	MAFF 302409	*do.*	+	+	−	−	−	+
	MAFF 302410	*do.*	+	+	−	−	−	+
	MAFF 302411	*do.*	+	+	−	−	−	+
	MAFF 302418	Mung bean	−	+	−	−	−	−
	MAFF 302419	*do.*	−	+	−	−	−	−
	MAFF 302420	*do.*	−	+	−	−	−	−
	MAFF 302424	Cymbidium	−	+	−	+	+	−
	MAFF 302425	*do.*	−	+	−	+	+	−
	MAFF 302426	*do.*	−	+	−	+	+	−
	MAFF 302427	*do.*	−	+	−	+	+	−
	MAFF 302428	*do.*	−	+	−	+	+	−
	MAFF 302429	*do.*	−	+	−	+	+	−
	MAFF 302430	*do.*	−	+	−	+	+	−
	MAFF 302431	*do.*	−	+	−	+	+	−
	MAFF 302432	*do.*	−	+	−	+	+	−
	MAFF 302433	*do.*	−	+	−	+	+	−
	MAFF 302434	*do.*	−	+	−	+	+	−
	MAFF 302435	*do.*	−	+	−	+	+	−
	MAFF 302436	*do.*	−	+	−	+	+	−
	MAFF 302515	Tulip	−	+	−	−	−	−
	MAFF 302516	*do.*	−	+	−	−	−	−
	MAFF 302517	*do.*	−	+	−	−	−	−
	MAFF 302518	*do.*	−	+	−	−	−	−
	MAFF 302519	*do.*	−	+	−	−	−	−
	MAFF 302520	*do.*	+	+	−	−	−	+
	MAFF 302521	*do.*	−	+	−	−	−	−
	MAFF 302522	*do.*	−	+	−	−	−	−
	MAFF 302523	*do.*	−	+	−	−	−	−
	MAFF 302524	*do.*	−	+	−	−	−	−
	MAFF 302525	*do.*	−	+	−	−	−	−
	MAFF 302526	*do.*	−	+	−	−	−	−
	MAFF 302527	*do.*	−	+	−	−	−	−
	MAFF 302533	Soil	+	+	−	−	−	+
	MAFF 302534	*do.*	−	+	−	−	−	−

a) Symbols: +; positve, −; negative.

7.5) at 65°C. Autoradiography was performed on Fuji X-ray films.

Specificity of probes. Six recombinant plasmid DNAs, which strongly hybridized to *P. gladioli* pv. *gladioli* MAFF 302545, were digested with *Eco*RI and separated into insert DNA and vector DNA on 0.7% agarose gel electrophoresis. Insert DNAs were excised separately and extracted from the agarose by Bandprep (Amersham International plc.). Approximately 20 ng each of insert DNAs were used as probes for dot blot hybridization. One μl of total genomic DNA (1 μg/μl) from each strain of *P. gladioli, P. caryophylli, P. cepacia, P. glumae, P. plantarii* and *P. solanacearum* was blotted on the nylon membrane. Dot blot hybridization was performed to all strains with the 6 probes.

For Southern blot hybridization, 2 μg of each genomic DNA was digested with *Eco*RI, electrophoresed at 34 V for 4 hr in 0.7% agarose gel in TAE buffer (40 mM Tris-acetate 1 mM EDTA pH 8.0), and then transferred to nylon membrane. Hybridization was performed by the method as described above.

RESULTS

Cloning of DNA fragments

Of 600 recombinant plasmids, the six plasmids, pG 1, pG 312, pG 423, pG 479, pG 521, and pG 523, were selected which hybridized with ^{32}P-labeled genomic DNA from *P. gladioli* pv. *gladioli* MAFF 302545, but did not hybridize to those from four strain of other *Pseudomonas* spp. These plasmids contained single fragments, 17.5-kb, 8.3-kb, 7.3-kb, 10.5-kb, 5.0-kb, and 8.7-kb. Southern blot hybridization analysis showed that two insert DNAs, 7.3-kb and 10.5-kb, cross-hybridized with each other, but others did not (data not shown).

Dot blot and Southern blot hybridization

Each DNA probe from 6 recombinant plasmids hybridized to genomic DNA of 1 to 56 strains among 61 strains of *P. gladioli* as shown in Table 2. The 8.3-kb fragment from pG 312 was useful for detecting *P. gladioli* because it hybridized to almost all the strains of this species. Whereas the 6 probes failed to hybridize to the 3 strains from vanda and the 2 strains from vuylstekeara, and all the strains of other *Pseudomonas* spp. shown in Table 2 (data not shown).

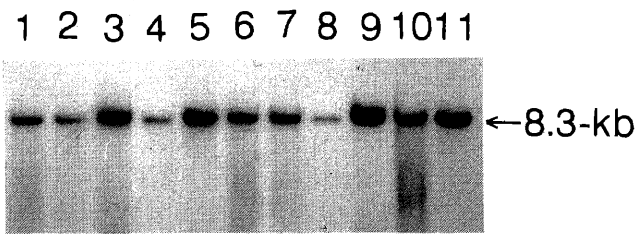

Fig. 1. Genomic Southern blot hybridization. Genomic DNA of *P. gladioli* strains were digested with *Eco*RI, and were hybridized with ^{32}P-dCTP labeled 8.3-kb fragment. lane 1: MAFF 302419, 2: MAFF 302420, 3: MAFF 302424, 4: MAFF 302425, 5: MAFF 302426, 6: MAFF 302427, 7: MAFF 302428, 8: MAFF 302429, 9: MAFF 302430, 10: MAFF 302431, 11: MAFF 302545.

The 8.3-kb, insert DNA in pG 312 hybridized to only 8.3-kb band in *Eco*RI-digested genomic DNA from 56 of *P. gladioli* strains which hybridized with dot blot hybridization. A part of data is shown in Fig. 1.

DISCUSSION

The 8.3-kb, insert DNA in pG 312 is useful for specific identification and detection of *P. gladioli*. The 8.3-kb insert DNA hybridized to a single band (8.3-kb) in *Eco*RI digested-genomic DNA from 56 of the 61 *P. gladioli* strains by Southern blot hybridization. This result indicates that 56 strains of *P. gladioli* contain a DNA sequence of 8.3-kb in length, and the sequence is considered to be a conservative region common to *P. gladioli* strains.

This 8.3-kb DNA probe hybridized to 56 of the 61 strains of *P. gladioli*, but did not hybridize to two strains of *P. gladioli* pv. *gladioli* (MAFF 301584, MAFF 301586) isolated from vuylstekeara and 3 strains (MAFF 301728, MAFF 301729, MAFF 301730) isolated from vanda. Investigation on bacteriological characteristics revealed that the strains from vuylstekeara showed identical patterns to those of *P. cepacia* MAFF 302528, *e.g.*, negative reaction in diffusible pigment production, gelatin liquefaction, and positive reaction in acid production from maltose, *etc.* Though the 3 strains from vanda were also similar to *P. gladioli* in bacteriological characteristics, the extent of each reaction was invariably lower than ordinary strains including type strain of *P. gladioli* used in this study. These facts imply that the three strains from vanda are genetically different from other strains of *P. gladioli*.

Five insert DNAs other than 8.3-kb fragment are inadequate to use as probes for identification and detection of *P. gladioli* even though they hybridized specifically to some of *P. gladioli* strains tested and did not hybridize to other species. Two of the five insert DNAs, 7.3-kb and 10.5-kb cross-hybridized with one another by random primer method, but showed different hybridization patterns to strains of *P. gladioli* except for MAFF 302545. They are considered to share common DNA sequences, although these sequences are not specific to all the strains of *P. gladioli*.

In conclusion, the 8.3-kb DNA fragment, isolated from genomic DNA of *P. gladioli* pv. *gladioli* MAFF 302545 is useful as a specific probe for rapid identification and detection of *P. gladioli*.

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和 文 摘 要

水野明文・対馬誠也・門田育生・西山幸司：*Pseudomonas gladioli*を検出するためのDNAプローブ

P. gladioli pv. *gladioli* MAFF 302545 の *Eco*RI 消化 DNA からショットガンクローニングにより，17.6 kb，8.3 kb，7.3 kb，10.5 kb，5.0 kb，8.7 kb の DNA 断片を得た。ドットハイブリダイゼーションにより，8.3 kb の DNA 断片は *P. gladioli* 61 菌株中 56 菌株と反応し，近縁細菌 5 種 47 菌株とは反応しなかった。しかし，その他 5 つの DNA 断片はこれら近縁の供試細菌とは反応しなかったが，*P. gladioli* に対しても一部の菌株としか反応しなかった。8.3 kb の DNA 断片と反応した 56 菌株のゲノミック DNA を *Eco*RI で消化し，サザンハイブリダイゼーションを行った結果，これらの菌株には 8.3 kb の位置に共通して 1 本のバンドが検出された。このことから，これらの菌株が 8.3 kb DNA 断片と塩基配列および *Eco*RI サイトが共通である極めて保存性の高い領域を保持していることが示唆された。これらの結果から，8.3 kb の DNA 断片は *P. gladioli* の検出および同定に有効であると考えた。