

植物内における *Pseudomonas syringae* pv. *atropurpurea* の病原性遺伝子の菌株間移行

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In planta Transfer of the Gene(s) for Virulence between Isolates of *Pseudomonas syringae* pv. *atropurpurea*

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Abstract

A phytotoxin (coronatine)-producing ability of *Pseudomonas syringae* pv. *atropurpurea* NIAES 1309 is associated with 58 Mdal plasmid designated as pCOR1. A conjugative plasmid pBPW1::Tn7 from *P. syringae* pv. *tabaci* BR2 was introduced into the strain NIAES 1309. When the strain was mated with avirulent strains derived from NIAES 1309 in Italian ryegrass, pBPW1::Tn7 was transferred to the avirulent strain at a high frequency. Twenty strains out of 894 trans-conjugants contained the pCOR1 plasmid co-transferred with pBPW1::Tn7. All of these strains produced coronatine and induced typical symptoms on Italian ryegrass. That is, *in planta* transfer of virulence genes between isolates of phytopathogenic pseudomonad was confirmed.

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Key words: *Pseudomonas syringae* pv. *atropurpurea*, plasmid, phytotoxin, transfer of virulence genes.

INTRODUCTION

It has been reported that the transfer of virulence genes from a pathogenic strain to a non-pathogenic strain of *Agrobacterium* occurs *in planta*^{4,5)} due to the transfer of plasmid (Ti plasmid) which codes for virulence^{10,11)}. Such a phenomenon, however, has never been reported in plant pathogenic bacteria other than *Agrobacterium*. Here, we report that virulence-related genes located on a plasmid were transferred *in planta* to an avirulent strain from a virulent strain among plant pathogenic pseudomonads.

Pseudomonas syringae pv. *atropurpurea* NIAES 1309 is the causal agent of halo blight of Italian ryegrass (*Lolium multiflorum* Lam). The bacterium produces a phytotoxin designated as coronatine responsible for developing symptoms^{3,6)}. Coronatine producing ability of the bacterium is associated with 58 Mdal plasmid designated as pCOR1⁹⁾. The strain NIAES 1309 could accept a conjugative plasmid pBPW1::Tn7 from *P. syringae* pv. *tabaci* BR2 by mating and acquired a donor ability for pBPW1 plasmid⁸⁾. On the other hand, coronatine-minus avirulent mutants of the strain NIAES 1309 in which the pCOR1 plasmid was excluded were isolated from Italian ryegrass⁷⁾.

This paper reports the transfer of the genes for virulence between the isolates of *Pseudomonas syringae* pv. *atropurpurea* by mating the virulent strains harbouring pBPW1 plasmid with the avirulent strains above mentioned in the leaves of Italian ryegrass.

MATERIALS AND METHODS

Bacterial strains. The donor strain NIAES 1309 (pBPW1::Tn7) is a rifampicin-sensitive spontaneous mutant from the rifampicin-resistant strain as described previously⁹⁾. The

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strain is pathogenic to Italian ryegrass producing coronatine, and it has the following properties; resistant to streptomycin (Sm, 20 $\mu\text{g/ml}$) and trimethoprim (Tp, 500 $\mu\text{g/ml}$) coded by Tn7, "aggregation positive" in King's B broth⁸), rough colony type and sensitive to rifampicin (Rif, 100 $\mu\text{g/ml}$). Two recipient strains, designated as Cor-#60 rif and (Cor-#60 rif)::Tn5 (Sato and Tsuyumu, unpublished), were used. Neither strains are able to produce coronatine nor to induce symptoms on Italian ryegrass as a result of curing pCOR1, although they are able to multiply in the plant⁷). These strains are characterized by a smooth colony type and "aggregation negative" compared with the donor strain. Moreover, they were distinguished clearly from the donor strain for the resistance to antibiotics (Rif for Cor-#60 rif, Rif and Kanamycine, 20 $\mu\text{g/ml}$ for (Cor-#60 rif)::Tn5).

Mating in plants. Donor and recipient cells were suspended in distilled water at the concentration of approximately $1.0 \times 10^{10}/\text{ml}$, mixed in the ratio of 1:1, and inoculated to young leaves of potted Italian ryegrass by using the needle prick method. Inoculated plants were kept in a greenhouse at around 25 C. At 4 to 16 days after inoculation, ten inoculated puncture zones were cut from each plant, homogenized in ca. 2 ml of sterile water, and plated on King's B medium supplemented with Sm and Rif, or Rif alone. Colonies with resistance to these antibiotics were isolated from the plates.

Detection of plasmids. Plasmid DNA was isolated according to the procedure described by Birnboim and Doly¹⁾ and analyzed by electrophoresis at 18 mA and 100 volts in 0.8% agarose gel with Tris-borate buffer (89 mM Tris-base, 2.5 mM disodium EDTA and 8.9 mM boric acid, pH 8.0) as described previously⁹⁾.

Bioassay of coronatine production. Coronatine production in the bacterium was assayed by the method described previously⁶⁾. Young culture of bacteria was inoculated to the small pieces of potato tuber placed on the moistened filter paper in a petri dish. Coronatine production was checked by the hypertrophic growth of inoculated tuber pieces after 3 to 5 days of incubation at 25 C.

RESULTS AND DISCUSSION

Transfer of pBPW1::Tn7 and pCOR1 plasmids into avirulent strains from virulent strains in Italian ryegrass

Two set of mating experiments using either Cor-#60 rif or (Cor-#60 rif)::Tn5 as recipient cells were conducted. The donor strain NIAES 1309 (pBPW1::Tn7) alone was also inoculated to the plant as a control. Symptoms began to appear 3 days after inoculation. Samples were collected 4 to 16 days after inoculation, and the homogenized mixtures of them were plated on King's B/Sm/Rif medium to select recipient cells (Rif resistant) which had acquired pBPW1::Tn7 (Sm resistant). As shown in Table 1, colonies with resistance to Sm and Rif were obtained at high frequencies ranging from 8.9×10^{-1} to 6.6×10^{-2} per recipient in the experiment using

Table 1. Frequency of transfer of pBPW1::Tn7 and pCOR1 plasmids into avirulent strains from virulent strains of *P. syringae* pv. *atropurpurea* NIAES 1309 by *in planta* (Italian ryegrass) conjugation

Donor	Recipient	Duration of conjugation	Transfer frequency of pBPW1::Tn7 per recipient	Proportion of transconjugants harbouring both plasmids	Co-transfer frequency per recipient
NIAES 1309 (pBPW1::Tn7)	Cor-#60 rif	4 days	1.8×10^{-1}	4/211 (1.9%)	3.4×10^{-3}
		6	6.6×10^{-2}	1/144 (0.7%)	4.6×10^{-4}
		16	8.9×10^{-1}	2/144 (1.4%)	4.0×10^{-3}
NIAES 1309 (pBPW1::Tn7)	(Cor-#60 rif)::Tn5	4	1.0×10^{-1}	3/213 (1.4%)	1.4×10^{-3}
		6	5.8×10^{-2}	10/182 (5.5%)	3.2×10^{-3}

Cor⁻#60 rif as a recipient. Recipient cells were isolated on King's B/Rif medium at the concentration of ca. 7.0×10^6 /ml in the mixture. Similar results were obtained in another set using (Cor⁻#60 rif)::Tn5 as a recipient. In contrast, no bacteria could be isolated from plants inoculated with donor cells alone on the selective medium (King's B/Rif/Sm). Colonies thus obtained were purified twice on the same medium and were characterized.

All the colonies with resistance to the selective antibiotics obtained in two sets of mating experiments harboured the plasmid pBPW1::Tn7. These colonies were, then, tested for the detection of the pCOR1 plasmid which may be mobilized by the conjugative plasmid pBPW1. As a result, 0.7% to 5.5% (average 2.2%) colonies examined contained both plasmids, and frequencies of co-transfer were estimated from 4.0×10^{-3} to 4.6×10^{-4} per recipient (Table 1, Fig. 1). Twenty strains thus obtained could not be distinguished from the parent recipient strain in their properties such as colony type, aggregation test and Rif or Rif/Km resistance. They acquired, however, new properties such as Sm/Tp resistance coded by pBPW1::Tn7 and coronatine-producing ability coded by pCOR1 plasmid. Typical symptoms were induced when these strains were inoculated into Italian ryegrass (Table 2). It is unlikely that these strains were derived from spontaneous mutation of the donor cell NIAES 1309 (pBPW1::Tn7, pCOR1)

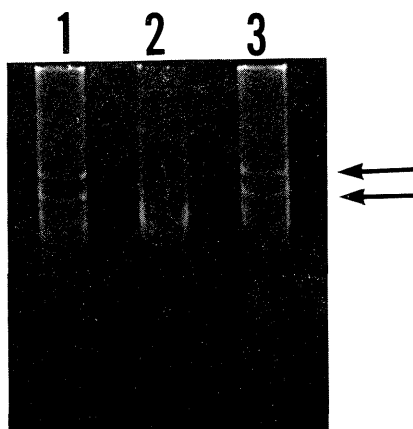


Fig. 1. Agarose gel electrophoresis of plasmid DNA from *P. syringae* pv. *atropurpurea* strains used *in planta* mating experiments.

Slot 1: donor strain NIAES 1309 (pCOR1, pBPW1::Tn7), Slot 2: recipient strain Cor⁻#60 rif, Slot 3: transconjugant Cor⁻#60 rif (pCOR1, pBPW1::Tn7), pCOR1 is the upper and pBPW1::Tn7 is the lower band.

Table 2. Characterization of transconjugants harbouring the pCOR1 and pBPW1::Tn7 plasmids

Strains (plasmids)	Ability to produce coronatine	Ability to induce symptoms	Resistance to antibiotics ^{a)}				Aggregation in King's B broth
			Rif	Sm	TP	Km	
Cor ⁻ #60 rif (pCOR1, pBPW1::Tn7) (7 strains)	+	+	+	+	+	-	-
Cor ⁻ #60 rif::Tn5 (pCOR1, pBPW1::Tn7) (13 strains)	+	+	+	+	+	+	-

a) Rif, Sm, TP, Km stand for rifampicin (100 µg/ml), streptomycin (20 µg/ml), trimethoprim (500 µg/ml) and kanamycin (20 µg/ml), respectively.

from the following reasons. The frequency of occurrence of spontaneous Rif resistant mutants in NIAES 1309 (pBPW1::Tn7, pCOR1) was estimated to range from 1.5×10^{-6} to 8.0×10^{-7} on King's B medium, and the frequency of *in planta* was estimated to less than 1.5×10^{-6} since none of Rif resistant mutant was isolated from plants at 4 days after inoculation. Moreover, the frequencies of occurrence of spontaneous Km resistant mutants in the strain was estimated to less than ca. $1.0\text{--}2.0 \times 10^{-9}$. The frequency of double mutation (Rif and Km) was, therefore, estimated to less than ca. 10^{-15} ($10^{-6} \times 10^{-9}$). Nevertheless, these transconjugants harbouring the both plasmids were obtained at frequencies of 10^{-3} to 10^{-4} per recipient cell (Table 1) and per donor cell (it was confirmed that the donor cell could multiply at much the same level as the recipient cell in another *in planta* mating experiments). The value is 10^{2-3} times ($10^{-4}/10^{-6}$ to $10^{-3}/10^{-6}$) or 10^{11-12} times ($10^{-4}/10^{-15}$ to $10^{-3}/10^{-15}$) higher than those of spontaneous Rif or Rif/Km respectively. It has been reported that the plasmid pBPW1::Tn7 induced cell aggregation in many transconjugants (17 isolates out of 19 isolates) when it was introduced into NIAES 1309 from the donor strain *P. syringae* pv. *tabaci* BR2⁸⁾. The phenomenon, however, was not observed in the present study using NIAES 1309 (pBPW1::Tn7) as a donor cell.

From the foregoing results, it is evident that the pBPW1 plasmid is effective on inducing transfer of pCOR1 plasmid from one *Pseudomonas* strain to another in the plant. According to our unpublished data, it was uncommon that pBPW1 mobilized pCOR1 to recipient cells on medium (not *in planta*). These facts suggest that some inducers for the activation of the expression of mobilization-related genes of the pCOR1 plasmid may be present in the leaves of Italian ryegrass. The pBPW1 plasmid used in this experiment is the indigenous plasmid of plant pathogenic *Pseudomonas* (*P. syringae* pv. *tabaci*). Since conjugative plasmid could be detected in many phytopathogenic bacteria including pseudomonads,⁹⁾ the above mentioned phenomenon may occur among plant pathogenic pseudomonads in plants in the field. Our results also provide an additional evidence of plasmid control of coronatine production in *P. syringae* pv. *atropurpurea*, which is the first report in phytopathogenic bacteria that phytotoxin production is controlled by plasmid⁹⁾.

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和 文 摘 要

佐藤 守: 植物内における *Pseudomonas syringae* pv. *atropurpurea* の病原性遺伝子の菌株間移行

イタリアンライグラスかさ枯病菌 *Pseudomonas syringae* pv. *atropurpurea* NIAES 1309 株の植物毒素 (コ

ロナチン) 産生能は, pCOR1 と命名された 58 メガダルトンのプラスミドの支配を受ける。この菌株に *P. syringae* pv. *tabaci* BR2 の伝達性プラスミド pBPW1::Tn7 を導入したのち, NIAES 1309 の非病原性変異株と混合し, イタリアンライグラスに接種した。その結果, 両菌株は植物内で接合をおこし, pBPW1::Tn7 は高頻度で非病原性株に移行した。これらトランスコンジュガント 894 株のうち 20 株は, pCOR1 をも共に保有していた。これら 20 株は, すべてコロナチン産生能力を有し, かつイタリアンライグラスに病原性を示すようになった。すなわち, 植物病原 *Pseudomonas* としては初めて植物内での病原性遺伝子の菌株間移行が確認された。