

植物病原性 *Pseudomonas syringae* pathovars から葉面細菌へのプラスミドの in planta 接合伝達

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In Planta Conjugal Transfer of Plasmids from Phytopathogenic *Pseudomonas syringae* Pathovars into Epiphytic Bacteria

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Abstract

In planta conjugal transfer of a native plasmid pBPW1 of *Pseudomonas syringae* and a broad host range plasmid RSF1010 among plant-associated bacteria was investigated. The plasmid pBPW1::Tn7 harbored in *P. syringae* pv. *atropurpurea* NIAES 1309 transferred into epiphytic *Pseudomonas viridiflava* Ko8 at a high frequency by *in planta* mating on Italian ryegrass. The transfer frequency was much higher than that of *in vitro* mating. The plasmid pBPW1::Tn7 also transferred into epiphytic *Erwinia herbicola* DW1 and Mei3 in Italian ryegrass, although it did not replicate in Mei3 strain. In mulberry leaves, RSF1010 plasmid was mobilized by pBPW1 at a high frequency from *P. syringae* pv. *mori* Ni27 into *E. herbicola* DW1 and Mei3, showing that pBPW1 also transferred and replicated in DW1 strain. Thus, *in planta* conjugal transfer of these plasmids between plant-pathogenic *P. syringae* pathovars and popular epiphytic bacteria was confirmed by mating on Italian ryegrass and mulberry.

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Key words: *In planta* gene transfer, *Pseudomonas syringae*, *Pseudomonas viridiflava*, *Erwinia herbicola*, conjugative plasmid.

INTRODUCTION

In planta gene transfer among bacteria is of major importance in the understanding of how plant-associated bacteria genetically interact each other and evolve. However, so little work has been reported concerning this subject. Direct evidence for *in planta* transfer of a native plasmid between plant-associated bacteria was demonstrated in the following studies; transfer of tumor inducing plasmid (Ti) from *Agrobacterium tumefaciens* into non-pathogenic *Agrobacterium* on plants^{2,3}, transfer of coronatine-inducing plasmid (pCOR1) from *Pseudomonas syringae* pv. *atropurpurea* to the avirulent strain of this bacterium on Italian ryegrass⁹, and transfer of copper resistance plasmid between *P. syringae* pv. *syringae* strains on bean plants¹². Moreover, Yano and Fujii (1983) reported on transmissible agent conferring resistance to streptomycin in *P. syringae* pv. *lachrymans* although its plasmid nature was not established¹³.

As for *in planta* conjugal transfer among plant-associated bacteria using drug resistance plasmid (R plasmids) originated from clinical bacteria, some works were reported. Lacy (1978) described the transfer of RP1 plasmid between *Erwinia chrysanthemi* strains on maize plant⁴. Similar results were demonstrated in mating between *P. syringae* pv. *syringae* and *P. syringae* pv. *phaseolicola* on excised bean plants⁵ or among *P. syrin-*

gae, *Erwinia herbicola* and *Erwinia amylovora* in detached pear blossoms⁶. *In planta* transfer of RP4 in field level was demonstrated in mating between *Xanthomonas campestris* pv. *corylina* and *Erwinia herbicola* in hazelnut tissues⁷.

In the present study, *in planta* conjugal transfer of the native plasmid pBPW1 of *P. syringae*¹¹ and mobilization of RSF1010 by pBPW1 between *P. syringae* pathovars and popular epiphytic bacteria, *P. viridiflava* and *E. herbicola* are demonstrated.

MATERIALS AND METHODS

Bacterial strains and plasmids. Used bacteria and plasmids, and their relevant characteristics are shown in Table 1. Epiphytic bacteria were isolated by the following procedures; One gram of fresh leaves were submerged in 20 ml of sterile distilled water and shaken violently for 30 min to remove bacteria, and leaf washings were plated on modified LB medium (polypeptone 10 g, yeast extract 5 g, NaCl 10 g, agar 15 g, distilled water 1 liter) and cultured at 25°C. Many species of epiphytic bacteria were isolated from 15 species of plants. Three strains were selected in the present study since they are able to multiply in wounded leaves of Italian ryegrass and/or mulberry when co-inoculated with plant-pathogenic bacteria. One strain, an epiphytic bacterium named Ko8 isolated from *Brassica campestris*

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L (Komatsuna), was identified as *Pseudomonas viridiflava* from the following properties; Gram reaction negative, Fluorescent pigment production on King's B medium, Arginine dihydrolase absent, Oxidase-negative, Utilization of carbohydrates; Sucrose(-), Trehalose(-), Lactose(-), Sorbitol(+), Inositol(+), Dulcitol(-), Green pigment production on PPGA medium, no growth at 40°C and other properties obtained from API 20NE identification kit (profile index; 0457451). The strain Ko8 did not induce HR (hypersensitive reaction) on tobacco leaves.

Two yellow pigmented strains, an epiphytic bacterium named DW1 isolated from mulberry leaves and a bacterium named Mei3 isolated from mulberry pyralid (*Glyphodes pyloalis*) were identified *Erwinia herbicola* from the following properties; results from API 20E identification kit (profile index 1005133), Acid production from carbohydrates; Inositol(-), Raffinose(-), Cellobiose(-), Glycerol(-). Both strains did not induce HR on tobacco leaves.

Rifampicin resistant mutants of *P. viridiflava* Ko8 (Ko8 Rif) was obtained by direct selection on LB medium supplemented with 20 µg/ml of rifampicin.

In planta and in vitro mating. Potted Italian ryegrass or mulberry trees were inoculated with donor and recipient bacterial strains (approximately 10⁸ cfu/ml) using the needle prick method. Inoculated plants were kept in a green house at around 25°C. At several days interval, inoculated puncture zones were cut off from each plants, homogenized in ca. 2 ml of sterile distilled water, and plated on selective media. In mating between *P. viridiflava* Ko8 Rif and *P. syringae* pv. *atropurpurea* NIAES 1309 (pBPW1::Tn7), LB medium supplemented with streptomycin (20 µg/ml) and rifampicin (20 µg/ml) was used as a selective medium. Recipient cells were counted on LB medium supplemented with Rif (20 µg/ml). In the case of the mating between *E. herbicola* recipient and streptomycin resistant donor, *P. syringae* pv. *mori* Ni27 (pBPW1, RSF1010), selection of transconjugants was performed in LB medium supplemented with streptomycin by incubating at 37°C. Recipient cells were counted on LB medium at 37°C.

In vitro mating was performed on membrane filters (Millipore Co.), overnight at 25°C. Selection was performed by above-mentioned methods, excepting the

mating with recipient *P. syringae* pv. *mori* Ni27 using a minimal tartrate medium¹⁰⁾ supplemented with streptomycin (20 µg/ml).

Plasmid detection. Plasmid DNA isolation was performed as described by Birnboim and Doly¹¹⁾ and analyzed by agarose gel (0.9%) electrophoresis with Tris-borate buffer (89 mM Tris-base, 2.5 mM disodium EDTA, 8.9 mM boric acid, pH 8.0).

RESULTS

In vitro and in planta mating between Pseudomonas syringae pathovars and Pseudomonas viridiflava

First, *P. syringae* pv. *atropurpurea* NIAES 1309 (pBPW1::Tn7) was mated with recipient *P. viridiflava* Ko8 Rif on membrane filter. The plasmid pBPW1::Tn7 transferred into the recipient strain at a frequency of 5.7×10^{-6} per a recipient cell. Next, the transconjugant Ko8 Rif (pBPW1::Tn7) was mated with recipient *P. syringae* pv. *mori* Ni27. The plasmid transferred into the

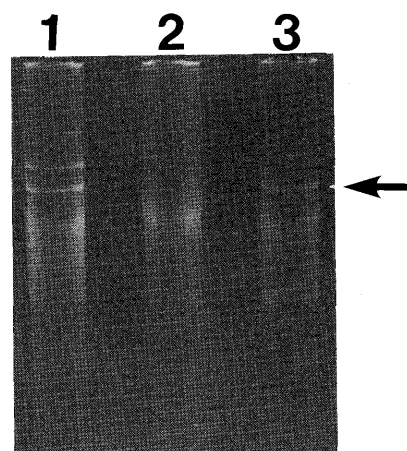


Fig. 1. Agarose gel electrophoresis of pBPW1::Tn7 transferred into *P. viridiflava* from *P. syringae* pv. *atropurpurea* by mating in Italian ryegrass. Lane 1, a donor strain *P. syringae* pv. *atropurpurea* NIAES 1309 (pCOR1, pBPW1::Tn7); Lane 2, a recipient strain *P. viridiflava* Ko8 (no plasmid); Lane 3, transconjugant *P. viridiflava* Ko8 (pBPW1::Tn7). An arrow indicates pBPW1::Tn7 plasmid.

Table 1. Bacterial strains and plasmids

Strain or (plasmid)	Relevant characteristics and origin
<i>P. syringae</i> pv. <i>atropurpurea</i> NIAES 1309 (pBPW1::Tn7)	SM ^r , TP ^r ; See reference (10)
<i>P. syringae</i> pv. <i>mori</i> Ni27	Wild type; See reference (10)
<i>P. syringae</i> pv. <i>mori</i> Ni27 (pBPW1, RSF1010)	SM ^r SU ^r ; See reference (10)
<i>P. viridiflava</i> Ko8 Rif	Rif ^r , Wild strain was isolated from <i>Brassica campestris</i> leaves
<i>Erwinia herbicola</i> Mei3	Isolated from a mulberry pyralid
<i>Erwinia herbicola</i> DW1	Isolated from mulberry leaves

SM^r, TP^r, SU^r, Rif^r represent resistance to streptomycin, trimethoprim, sulfonamid and rifampicin, respectively.

Table 2. Transfer frequency of pBPW1::Tn7 into *Pseudomonas viridiflava* Ko8 from *Pseudomonas syringae* pv. *atropurpurea* by *in planta* (Italian ryegrass) mating

Period (days) of <i>in planta</i> mating				
0	1	3	6	8
ND	ND	2.8×10^{-3}	7.7×10^{-2}	5.9×10^{-2}

Figures show transfer frequency of plasmid per recipient cell.

ND: Not detected.

Table 3. Transfer frequency of pBPW1::Tn7 plasmid or Tn7 into *Erwinia herbicola* strains from *Pseudomonas syringae* pv. *atropurpurea* by *in planta* (Italian ryegrass) mating

Recipient	Period (days) of <i>in planta</i> mating					Transferred DNA
	0	1	3	5	8	
DW1	ND	1.2×10^{-3}	5.4×10^{-1}	3.2×10^{-2}	1.4×10^{-1}	pBPW1::Tn7
Mei3	ND	ND	1.2×10^{-3}	3.2×10^{-4}	2.5×10^{-3}	Tn7

Figures show transfer frequency of the plasmid or transposon per a recipient cell.

ND: Not detected.

strain Ni27 at a frequency of 1.4×10^{-4} per a recipient cell. Moreover, Ni27(pBPW1::Tn7) was mated with Ko8 Rif again and the plasmid transferred into Ko8 at a frequency of 1.2×10^{-3} per a recipient cell. The presence of pBPW1::Tn7 plasmid in recipient cells was confirmed by electrophoresis (data not shown) and the appearance of antibiotics resistance (streptomycin and trimethoprim) originated from Tn7.

Thus, the consecutive transfer of pBPW1::Tn7 among *P. syringae* pathovars and *P. viridiflava* was confirmed although transfer frequencies varied in each mating combination.

Then, *P. syringae* pv. *atropurpurea* NIAES 1309 (pBPW1::Tn7) was mixed with *P. viridiflava* Ko8 Rif and the mixture was inoculated into Italian ryegrass. The transfer of pBPW1 into *P. viridiflava* Ko8 Rif was found on 3 days later (Fig. 1), but was not detected on 0 and 1 day after inoculation. As shown in Table 2, transfer frequency of the plasmid tended to increase during 8 days after inoculation, showing much higher frequency than that (5.7×10^{-6} per a recipient cell) of *in vitro* mating in the same mating combination. Transfer frequency at 8 days of *in vitro* mating was maintained in the similar levels.

In planta mating between *Pseudomonas syringae* pv. *atropurpurea* and *Erwinia herbicola* on Italian ryegrass

In planta transfer of pBPW1::Tn7 into *E. herbicola* on Italian ryegrass was investigated using two recipient strains. In the first mating set, *P. syringae* pv. *atropurpurea* NIAES 1309 (pBPW1::Tn7) was mated with *E. herbicola* DW1. The plasmid transferred into the recipient strain at a very high frequency (10^{-1} to 10^{-2}) on 3, 5 and 8 days after inoculation, although it was not detected or detected only at a lower frequency on 0 or 1 day after inoculation (Table 3, Fig. 2).

In the another mating set, *E. herbicola* Mei3 was used as a recipient. In this case, the transconjugants were obtained on 3 days later after inoculation at much lower

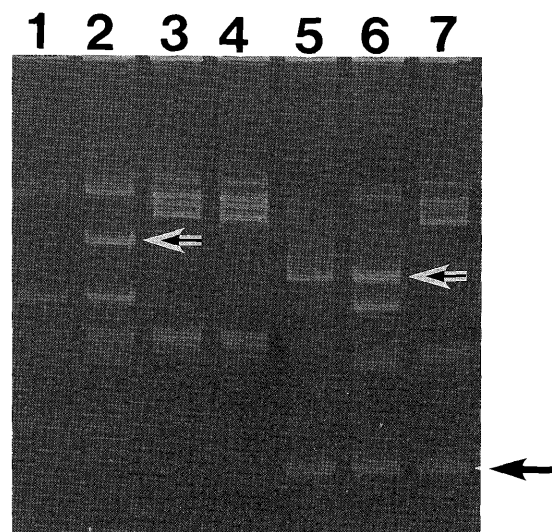


Fig. 2. Agarose gel electrophoresis of plasmid DNA transferred into *Erwinia herbicola* strains from *Pseudomonas syringae* pathovars by *in planta* mating. Lane 1-4, *In planta* (Italian ryegrass) mating between *Pseudomonas syringae* pv. *atropurpurea* NIAES 1309 (pBPW1::Tn7) and *Erwinia herbicola* DW1 or Mei3 strain; Lane 1, DW1 wild type; Lane 2, DW1 acquired pBPW1::Tn7 (39 Mdal, arrow); Lane 3, Mei3 wild type; Lane 4, Mei3 acquired only Tn7 (pBPW1 do not replicate). Lane 5-7, *In planta* (mulberry) mating between *Pseudomonas syringae* pv. *mori* Ni27 (pBPW1, RSF1010) and *Erwinia herbicola* DW1 or Mei3 strain; Lane 5, a donor strain Ni27 (pBPW1, RSF1010); Lane 6, DW1 acquired both plasmids of pBPW1 (30 Mdal, arrow) and RSF1010 (5.5 Mdal, arrow); Lane 7, Mei3 acquired PSF1010 (5.5 Mdal, arrow), wild types of DW1 and Mei3 are shown at lane 1 and lane 3, respectively.

frequency (1.2×10^{-3}) than that (5.4×10^{-1}) of the first mating set of 1309 (pBPW1::Tn7)/DW1. In any trans-

Table 4. Mobilizing transfer frequency of RSF1010 plasmid from *Pseudomonas syringae* pv. *mori* into *Erwinia herbicola* strains by *in planta* (mulberry) mating

Recipient	Period (days) of <i>in planta</i> mating			
	0	1	3	8
DW1	ND	2.2×10^{-2}	3.9×10^{-2}	3.1×10^{-2}
Mei3	ND	2.9×10^{-4}	1.0×10^{-2}	1.2×10^{-1}

Figures show transfer frequency of the plasmid per a recipient cell.

ND: Not detected.

conjugants, however, pBPW1::Tn7 was not detected. Since Tn7 markers of streptomycin and trimethoprim resistance were maintained in the transconjugants, it was thought that pBPW1 transferred but did not replicate in the strain Mei3. That shows that pBPW1 behaved as a suicidal plasmid in this strain. Transfer frequencies of pBPW1::Tn7 by *in vitro* mating into the recipient strains DW1 was about 10^{-4} and that of Tn7 into the recipient strain Mei3 was about 10^{-6} , showing much lower frequencies than those of *in planta* mating.

In planta mating between *Pseudomonas syringae* pv. *mori* and *Erwinia herbicola* on mulberry

In planta transfer of a broad host range plasmid RSF1010 by mobilization using pBPW1 was investigated by two sets of inoculation experiments. In the first set, *P. syringae* pv. *mori* Ni27 (pBPW1, RSF1010) was mated with *E. herbicola* DW1. The plasmid RSF1010 transferred into recipient cells on 1 days later after inoculation and the high transfer frequency (10^{-2}) was maintained during 8 days (Table 4). The plasmid pBPW1 co-transferred was detected in some transconjugants after electrophoresis (Fig. 2). In the another mating set using the recipient strain Mei3, high frequencies of transfer of RSF1010 were also confirmed on 3 and 8 days after inoculation. However, pBPW1 plasmid was not detected after electrophoresis in any transconjugants as shown in the previous experiment of Table 3.

DISCUSSION

In the present study, *in planta* transfer of a native plasmid pBPW1 of *Pseudomonas syringae* among plant-associated bacteria such as *P. syringae* pv. *atropurpurea*, pv. *mori*, *P. viridiflava* and *Erwinia herbicola* was confirmed. Although *P. syringae* pathovars and *P. viridiflava* are pathogenic to some species of plants, they are able to grow epiphytically on a wide variety of plant host. *E. herbicola* is one of the most popular epiphytic bacteria and these *E. herbicola*-group bacteria including *Erwinia ananas* were the most dominant flora on mulberry leaves in our recent work (our unpublished data). The confirmation of gene transfer at a high frequency among these popular epiphytic and pathogenic bacteria may be significant from the view of evolution of plant pathogenic bacteria. Although this is the first report of *in planta* transfer of a native plasmid into *E. herbicola*, a work using a non-native plasmid, drug-resistant RP4 originated from clinical bacteria, has been reported.

Manceau *et al.* (1986) showed that the transfer of RP4 took place from *Xanthomonas campestris* pv. *corylina* into *E. herbicola* when inoculated in hazelnut tissues⁷. These results suggest that gene exchange mediated by plasmid among plant-associated bacteria may be a common event under natural field conditions. To know whether it is "a common event" or not, however, more information are needed. For example, how population of highly and widely conjugative plasmid like pBPW1 used in this experiment exists in nature and how often bacterial conjugation occurs in natural habitats, and other informations will be required.

The plasmid pBPW1 is a naturally found self-transmissible plasmid of *P. syringae* pv. *tabaci* BR2¹¹ and transferred into many species of bacteria which includes enteric bacteria and several pseudomonads^{8,11}. In this study, some strains of *E. herbicola* and *P. viridiflava* were newly confirmed to be a host bacterium of pBPW1 plasmid. In recipient *E. herbicola* Mei3, however, the plasmid did not replicate after transferring. Similar results have been obtained in the recipient strains of *P. syringae* pv. *mori* and *P. syringae* pv. *eriobotryae*¹⁰.

In planta transfer frequencies on Italian ryegrass were much higher than those of *in vitro* mating. Similar results were described in some reports. Lacy (1978) showed that the transfer frequency of RP1 between marked *Erwinia chrysanthemi* strains when inoculated to maize plants was 10^{-2} to 10^{-3} per donor comparing to 10^{-5} per donor in parallel *in vitro* matings⁴. Transfer frequencies of RP1 between *E. herbicola* or *P. syringae* as the donor and an *E. amylovora* recipient on detached pear blossoms were much higher than that of *in vitro* mating⁶. The reasons of "accelerating of transfer frequency in plants" are still uncertain. Ti plasmid of *Agrobacterium tumefaciens* are highly repressed for conjugal transfer on media, but genes involved in conjugational transfer are activated by octopine produced in plants after inoculation^{2,3}. The pCOR1 plasmid of *P. syringae* pv. *atropurpurea* is also mobilized by pBPW1 only at *in planta* mating, suggesting that some plant factors may induce mobilizing transfer of pCOR1⁹. Some unknown plant substances may play a role in accelerating transfer frequency of the plasmid. In other possibilities, physiological or physical state of bacterial bodies growing *in planta* may be more suitable for plasmid transfer than that of *in vitro* culture. More information are required to prove these possibilities.

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和 文 摘 要

佐藤 守・森長晴子・西山幸司：植物病原性 *Pseudomonas syringae* pathovars から葉面細菌へのプラスミドの *in planta* 接合伝達

Pseudomonas syringae のプラスミドである pBPW1 と広宿主域プラスミド RSF1010 の植物病原細菌と葉面細菌間の *in planta* 接合伝達について調べた。*P. syringae* pv. *atropurpurea* NIAES 1309 内のプラスミド pBPW1::Tn7 はイタリアンライグラスでの *in planta* 接合によって受容菌の *Pseudomonas viridiflava* Ko8 に高頻度で移行した。その伝達頻度は *in vitro* 接合よりはるかに高かった。また、pBPW1::Tn7 はイタリアンライグラス上で *Erwinia herbicola* DW1 と Mei3 にも移行したが、DW1 中では複製されず Tn7 のみ転移した。一方、クワにおいては、pBPW1 の可動化により RSF1010 が *P. syringae* pv. *mori* から *E. herbicola* DW1 と Mei3 に高頻度で伝達した。この場合も pBPW1 は DW1 では複製しなかった。以上のように、これらプラスミドが *P. syringae* の2つの pathovar と自然界に普通に存在する2種の葉面細菌との間で *in planta* 接合伝達を起こすことが明らかになった。