Isolation and Characterization of *Pseudomonas syringae* subsp. *savastanoi* Mutants Defective in Hypersensitive Response Elicitation and Pathogenicity

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**Abstract**

Olive strain ITM317 of *Pseudomonas syringae* subsp. *savastanoi*, the causal agent of 'Olive and Oleander knot disease' was mutagenized by random transposon (*Tn5*) insertion. Of the 1.400 transconjugants, four were altered in their ability to induce a hypersensitive reaction (HR) on tobacco; Southern blot analysis showed that a single copy of the *Tn5* element was present in their chromosomes. In particular, mutants ITM317–69, ITM317–1010 and ITM317–1194 did not elicit HR whereas mutant ITM317–916 induced a variable response. When assayed for pathogenicity on olive, mutants ITM317–916 and ITM317–1010 induced knots comparable both in size and morphology to those caused by the parental strain. Prototropic mutant ITM317–1194, still able to produce indole-3-acetic acid and cytokinins, did not cause any knot formation on olive; furthermore, it was unable to multiply in host tissue. Auxotrophic mutant ITM317–69 caused the formation of smaller-sized knots and its prototrophic revertant fully regained the parental phenotypes, suggesting that a single *Tn5* insertion had a pleiotropic effect on the mutated phenotypes. *Tn5*-containing *EcoRI* fragments from mutants ITM317–69, ITM317–916, ITM317–1010 and ITM317–1194 were cloned into the plasmid vector pBR322. Hybridization of these clones with the *hrp* gene cluster of *P. syringae* pv. *syringae* strain 61 was not detected. These results suggest that genes different from those of the above gene cluster might be involved in the interaction of *P. syringae* subsp. *savastanoi* with olive and with the non-host plant tobacco.

**Zusammenfassung**

Isolierung und Charakterisierung von *Pseudomonas syringae* subsp. *savastanoi*-Mutanten, die keine Hypersensitivitätsreaktion hervorrufen und nicht pathogen sind


**Introduction**

*Pseudomonas syringae* subsp. *savastanoi* (ex Smith, 1908) Janse (1982) is a plant pathogenic bacterium which causes disease on oleander (*Nerium oleander* L.) and some Ole-
aceae [olive (Olea europea L.), ash (Fraxinus excelsior L.), privet (Ligustrum japonicum Thumb.) and jasmin (Jasminus officinalis L.)]. Even though the above pathogen has recently been reclassified as Pseudomonas savastanoi pv. savastanoi (Gardan et al., 1992), the present authors prefer to use the old denomination because it is still unclear whether P. s. pv. savastanoi includes the strains isolated from oleander and ash, classified as P. s. pv. nerii and P. s. pv. fraxini, respectively (Young et al., 1996). In any case, the strains isolated from oleander and olive can be differentiated on the basis of their host specificity. In particular, after infecting olive plants with oleander strains, typical hyperplastic symptoms (i.e. knots) form at the sites of inoculation, whereas after infecting oleander plants with olive strains necrosis is often induced (Iacobellis et al., 1994; Surico et al., 1985). All the strains are able to elicit a hypersensitive reaction on non-host plants, such as tobacco (Janse, 1982).

Knot formation depends on the ability of virulent strains of the pathogen to produce indole-3-acetic acid (IAA) and cytokinins (Smidt and Kosuge, 1978; Comai et al., 1982; Surico et al., 1985; Surico and Iacobellis, 1992; Iacobellis et al., 1994). The production of the two classes of phytohormones by Pseudomonas syringae subsp. savastanoi strains accounts for their virulence but not for their pathogenic capability (Iacobellis et al., 1994).

Although several studies have provided significant information on the genetic organization and metabolic regulation of phytohormone production (Comai et al., 1982; Yamada et al., 1985; Powell and Morris, 1986; Glass and Kosuge, 1988), no data on pathogenicity determinants, other than phytohormones, are available concerning this plant–pathogen interaction.

In the last decade, the development of transposon mutagenesis (Mills, 1985) and recombinant DNA procedures has led to the identification of hrp (hypersensitive reaction and pathogenicity) genes in several non-tumorogenic, gram-negative bacterial pathogens. These genes are required for a successful plant–pathogen interaction. Their inactivation by transposon insertion has a pleiotropic effect: Hrp− mutant bacteria are unable to elicit a hypersensitive reaction (HR) on both non-host plants and resistant cultivars of a susceptible host species; they are impaired in their ability to grow in host-plant tissues and to cause disease symptoms (Willis et al., 1991). So far, hrp genes, typically clustered in bacterial genomes, have been shown to be present in phytopathogens of the genera Pseudomonas,Ralstonia,Xanthomonas and Erwinia (Bonas, 1994; Willis et al., 1991), which mainly cause necrotic symptoms on their respective host plants. More recently, similar genes have been identified in Erwinia chrysanthemi (Bauer et al., 1994) and in Erwinia herbicola pv. gypsophilae (Nizan et al., 1997), which cause soft rot and hyperplasia of the infected tissues, respectively.

In order to evaluate whether hrp-like genes are also required in the interaction between P. s. subsp. savastanoi and olive, the olive strain ITM317 (Surico et al., 1985) was mutagenized with a transposable genetic element (transposon Tn5) (Berg, 1989). Here the isolation and characterization of mutants altered in their ability to elicit HR (HR− mutants) on tobacco and to induce symptoms on olive is reported.

Materials and Methods
Bacterial strains, plasmids and culture conditions
The bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli strains were grown overnight in LB agar or LB broth (Sambrook et al., 1989) at 37°C. Plasmids pBR322 (Bolivar, 1978) and pGS9 (Selvaraj and Iyer, 1983) were carried in E. coli HB101 (Boyer and Roulland-Dussoix, 1969), and pBluescript SKII (Stratagene, La Jolla, CA, USA) in TG1 (Gibson, 1984). Recombinant plasmid derivatives of pBR322 and pBluescript SKII were propagated in E. coli EasypoHs H (Eugorantec, Belgium), and in E. coli TG1, respectively. Plasmid pRK7813 (Jones and Gutterson, 1987) was carried in E. coli DH1 (Hanahan, 1983) and its recombinant derivatives were propagated in E. coli JM83 (Yanisch-Perron et al., 1985).

P. s. subsp. savastanoi strains were grown at 26°C on King’s medium B (KB) (King et al., 1954), 523 medium (Kado et al., 1972) or Woolley’s medium (Woolley et al., 1955), as indicated.

Miller’s minimal A medium (Miller, 1972) was used to screen for auxotrophic mutants among the kanamycin-resistant (Kmr′) P. s. subsp. savastanoi transconjugants. Antibiotics in selective media were used in the following amounts (µg/ml): ampicillin (Ap), 100; chloramphenicol (Cm), 50; kanamycin (Km), 20; rifampicin (Rif), 100; streptomycin (Sm), 50; tetracycline (Tc), 10, unless otherwise stated.

Transposon mutagenesis and identification of auxotrophic mutants
The suicide plasmid pGS9 (Selvaraj and Iyer, 1983) was used to deliver transposon Tn5 (which confers kanamycin resistance) by conjugation into a spontaneous rifampicin-resistant (Rif′) derivative (ITM317R) of P. s. subsp. savastanoi strain ITM317. Strain ITM317R was shown to be indistinguishable from its parental strain with regard to IAA and cytokinin production, ability to induce HR on tobacco and pathogenicity on olive.

The donor strain HB101 (pGS9) and the recipient strain ITM317R were grown to late log phase in LB broth containing Km and Cm and in KB broth amended with Rif, respectively. The donor and recipient cells were sedimented by centrifugation (5,000 g) and resuspended in an aliquot of 523 broth; their suspensions were adjusted to 106 and 107 colony forming units (cfu)/ml, respectively. Twenty microlitre aliquots of each suspension were mixed and spotted on 412. After 19 h incubation at 18°C, the bacterial mass was suspended in aliquots of KB broth amended with Rif and Km, respectively. Twenty microlitre aliquots of each suspension were mixed and spotted on 412. After 20 h incubation at 29°C, the bacterial mass was suspended in aliquots of KB broth, plated on KB agar containing Rif and Km and then incubated for 48–72 h at 26°C. The colonies resistant to Km (Km′) were isolated and cultured in duplicate on KB containing Km. Each set of plates was incubated at 25 and 37°C, respectively.

The Km′ colonies, which failed to grow at 37°C, were
screened for fluorescent pigment production on KB agar. Then, they were evaluated for auxotrophy on minimal A agar medium and for their ability to grow on KB amended with Sm or Cm.

The K^r derivatives of P. s. subsp. savastanoi that failed to grow on minimal A agar were further characterized for specific nutritonal requirements using the method of Carlton and Brown (1981).

The frequency of reversion to prototrophy of auxotrophic and HR^− mutant strain ITM317–69 was determined by growing it on KB medium to the stationary phase. The culture was centrifuged (5000 g), the pellet was rinsed twice with minimal A medium and the suspension containing about 2 × 10^9 cfu/ml was plated on minimal A agar. The prototrophic revertants were subsequently tested for sensitivity to Km.

**Hypersensitivity assays**

The Km^r Cm^s (chloramphenicol-sensitive) mutants of P. s. subsp. savastanoi strain ITM317R were screened for hypersensitive reaction on tobacco (Nicotiana tabacum, cv. Samsun) plants measuring about 15 cm in height, grown for 21 days in a growth chamber at 24 °C, 75% relative humidity, with a 15 h light cycle (Philips, Eindhoven, The Netherlands, TDL 32 W, General Electric, Hendersonville, NC, USA, KM 20 W). The parental strain ITM317R was used for comparison.

The bacteria were grown to log phase on KB agar and then suspensions containing about 3 × 10^8 cfu/ml were injected into three leaves of three tobacco plants following the method of Baker et al. (1987). The plants were then observed for the development of tissue collapse within 24 h. The mutant found to induce a variable HR response was further assayed as above by using suspensions containing 1.8 × 10^6 to 1.5 × 10^7 cfu/ml as determined by a viable cell count on KB agar.

**Pathogenicity tests**

The mutants of P. s. subsp. savastanoi strain ITM317R, which failed to induce a typical HR, were tested for pathogenicity on olive (cv. Nocellara del Belice) and oleander plants, as previously described (Surico et al., 1985). Wounds made in the bark of 1-year-old olive stems were inoculated with 3 µl of bacterial suspensions containing about 1 × 10^9 cfu/ml. Then, about 50 µl of the above suspensions were injected into the second internode below the apex of 1-year-old oleander shoots. The pathogenicity tests were repeated three times.

The population dynamics of P. s. subsp. savastanoi ITM317R parental strain and its mutant derivatives were determined in olive plants. For this purpose, 1-year-old stems were inoculated as above with about 10^5 cfu/inoculation site. At different times for up to 15 days, the inoculated tissues were collected, ground in sterile distilled water and 0.1 ml aliquots of serially diluted suspensions were plated on KB agar. The above experiments were repeated three times.
and its HR− mutants “Table 1 to produce IAA and with restriction endonuclease EcoCloning of Tn

Germany# The DNAs used as probes were labelled with purchased from Boehringer Mannheim “Mannheim
described by Sambrook et al “0878# Calf intestine alkaline phosphatase and molecular weight markers were

labelling
digoxigenin
digoxigenin, as probes. Transformants were cultured in KB broth supplemented with kanamycin for four con-
secutive cycles of 24 h each. The final culture was serially diluted and plated on KB agar containing kanamycin;
then individual colonies were grown on KB agar, with and without tetracycline, to identify the Tc− (Tc-sensitive) colonies that had lost the plasmid vector and were potential marker-exchanged mutants. The above colonies were tested for HR elicitation on tobacco, as described in the specific paragraph.

Production of phytohormones

The ability of P. s. subsp. savastanoi strain ITM317R and its HR− mutants (Table 2) to produce IAA and cytokinins in culture was assessed as previously described (Surico et al., 1985). The bacteria were grown with shaking in 500 ml Erlenmeyer flasks containing 150 ml of Woolley’s medium (Woolley et al., 1955) supplemented with 0.5 mM L-tryptophan. In the case of the auxotrophic mutant ITM317-69, the medium was also amended with 0.5 mM methionine. After 6 days’ incubation, the cells were removed by centrifugation and the resulting supernatants were extracted four times with an equal volume of ethyl acetate at pH 2.8 and subsequently at pH 8.5. The organic extracts were evaporated under reduced pressure at 30°C and the residues dissolved in methanol. The IAA content in the acidic extracts was evaluated using the colorimetric method of Gordon and Weber (1951). The cytokinin activity of the basic extracts was detected by using the cucumber cotyledon bioassay (Fletcher et al., 1982).

DNA manipulation

Restriction enzyme digestions, agarose gel electrophoresis, and DNA ligation were performed as described by Sambrook et al. (1989). Calf intestine alkaline phosphatase and molecular weight markers were purchased from Boehringer Mannheim (Mannheim, Germany). The DNAs used as probes were labelled with 32P, applying the random primer method of Feinberg and Vogelstein (1983). Southern blot analyses were performed as described by Sambrook et al. (1989). DNA labelling, hybridization and detection were also carried out using a non-radioactive DIG-labelling and detection kit (Boehringer Mannheim) according to the manufacturer’s instructions. The genomic DNA of P. s. subsp. savastanoi strains was extracted by applying the procedure of Glass and Kosuge (1988). Preparations of plasmid DNA from P. s. subsp. savastanoi and E. coli strains were made as described by Jacobellis et al. (1991) and Sambrook et al. (1989), respectively.

Cloning of Tn5-containing fragment from P. s. subsp. savastanoi HR− mutants

Genomic DNA from mutants was digested to completion with restriction endonuclease EcoRI, and DNA frag-
Results

Transposon (Tn5) mutagenesis

The Km' transconjugants of *P. s. subsp. savastanoi* ITM317R strain were obtained at frequencies ranging from $6.0 \times 10^{-4}$ to $1.0 \times 10^{-6}$ per recipient cell. The transconjugants produced fluorescent growths on KB, were resistant to Sm and Rif, and most of them (about 94%) were sensitive to chloramphenicol (Cm'). The sensitivity of transconjugants to Cm and their resistance to Km (conferring on Tn5) indicated the absence of the pGS9 plasmid in the cells and, consequently, the insertion of Tn5 in the genome. Only transconjugants resistant to Km and sensitive to Cm were further characterized. Southern blot analysis of randomly selected transconjugants, using pGS9 as a probe, showed that a single transposon (Tn5) insertion had occurred in different loci (data not shown).

Seventeen out of the 1400 ITM317R::Tn5 transconjugants tested were unable to grow on minimal A agar medium (Miller, 1972). The analysis of the nutritional requirements of these auxotrophs showed that they required adenine (one strain), asparagine (one strain) arginine (five strains), cysteine (two strains), isoleucine and valine (one strain) and methionine (four strains) for growth. The nutritional requirements of the three remaining auxotrophs could not be determined by using the procedure used in this study.

Identification of HR− mutants

A total of 1400 Km' Cm' transconjugants were tested for elicitation of HR on tobacco. Four failed to elicit a typical HR (Table 2). In particular three of these, namely ITM317−69, ITM317−1010 and ITM317−1194, did not elicit HR when suspensions containing about $3 \times 10^8$ cfu/ml were used. On the contrary, under the same assay conditions, transconjugant ITM317−916 was found to induce a variable response. In fact, in the different tobacco plants it elicited either no response or a weak HR, characterized by a partial collapse of the infiltrated area. Moreover, in some assays, it gave a response comparable with that caused by the parental strain. Further investigations in this regard, revealed that the HR behaviour of the parental strain ITM317R could be distinguished from that of its mutant derivative ITM317−916 when suspensions containing $7.5 \times 10^7$ cfu/ml or less were used: under these assay conditions, the above mutant failed to induce HR in all cases (Fig. 1).

Characterization of HR− mutant strains

The relevant characteristics of the HR− mutants are described in Table 2.

HR− mutants, evaluated for nutritional requirements, were observed to have different phenotypes: mutants ITM317−916, ITM317−1010 and ITM317−1194 were prototrophs, whereas mutant ITM317−69 was unable to multiply on minimal medium and required methionine to grow.

When assayed for pathogenicity on olive plants, HR− mutants behaved differently, e.g. mutant strain ITM317−1194 did not induce knot formation (Fig. 2, Table 2) whereas mutants ITM317−916 and ITM317−1010 induced knots comparable to those caused by the parental strain ITM317R (Table 2). The auxotrophic mutant strain ITM317−69 was found to cause reduced size knots with an atypical morphology (Fig. 3). In that case overgrowths appeared only on the surface of the inoculated wounds and they were greener and softer than those caused by the parental strain. The above differences were mainly evident in the first phase of symptom development (25 days after inoculation) and were attenuated about 2 months after inoculation (data not shown).

Mutant strain ITM317−69 showed a low reversion rate ($1 \times 10^{-8}$), indicating the stability of the Tn5 insertion. As expected, the naturally occurring revertant ITM317−69R was sensitive to Km, elicited a HR on tobacco and fully regained pathogenicity on olive (Fig. 3). Southern blot analysis of genomic DNA from ITM317−69R, digested with EcoRI restriction endonuclease and hybridized with pGS9, confirmed the absence of the Tn5 element in its genome.

Parental strain ITM317R and its mutants ITM317−916, ITM317−1010, ITM317−1194 and ITM317−69 behaved differently when evaluated for their ability to grow in olive tissues (Fig. 4). In particular, mutants ITM317−916, ITM317−1010 and the parental strain ITM317R multiplied in a similar pattern in the above tissues. On the contrary, the growth of mutant ITM317−1194 was markedly reduced (Fig. 4). The growth of mutant ITM317−69 was altered, e.g. the population size of the above mutant decreased soon after the inoculation but 9 days later the number of viable cells started to increase (Fig. 4). Similar results were obtained in three different experiments.

When inoculated on oleander shoots, *P. s. subsp. savastanoi* parental strain ITM317R as well as its HR− mutants were avirulent, since they did not cause any overgrowth of the inoculated tissues (Table 2).

Finally, mutant strain ITM317−916, ITM317−1010, ITM317−1194 produced in culture levels of IAA and cytokinins as high as those of the parental strain (Table 2). On the contrary, mutant strain ITM317−69 accumulated a reduced level of phytohormones in culture, but it also showed a reduced ability to grow in the Woolley's medium (data not shown).

Physical characterization of Tn5 insertions and cloning of Tn5-containing fragments

To determine if a single Tn5 insertion had occurred into the genome of mutants ITM317−69, ITM317−916, ITM317−1010 and ITM317−1194, their total DNA preparations were digested to completion with EcoRI or HindIII restriction endonucleases. The DNA fragments were then separated by agarose gel electrophoresis and hybridized with $^{32}$P labelled pGS9 (carrying Tn5). EcoRI and HindIII digested DNAs of mutant strains showed one and three DNA fragments, respectively, which hybridized with the probe. These findings showed that a single Tn5 insertion had occurred in the genome and, furthermore they are in agreement with the fact that Tn5 does not contain any EcoRI recognition sites but two HindIII sites (Jorgensen et al., 1979). In the case of HindIII-digested
DNA, one of the three signals of hybridization was common to all the mutants and corresponded to the 3.4 Kb HindIII fragment within the Tn5 element (Fig. 5). The other two DNA fragments varied in size depending on the mutants, thus indicating that the single Tn5 insertion had occurred in different loci of the genome (Fig. 5). As expected, no hybridization signal was observed in the DNA of the parental strain ITM317R (Fig. 5). When plasmid DNA preparations of P. s. subsp. savastanoi ITM317R and its HR− mutants were hybridized with the above probe, no hybridization was observed (data not shown), indicating that transposon insertion occurred in the chromosome.

EcoRI DNA fragments containing Tn5 from the four HR− mutants ITM317−69, ITM317−916, ITM317−1010 and ITM317−1194 were cloned into the plasmid vector pBR322 and the resulting recombinant plasmids were named pITM−69, pITM−916, pITM−1010, and pITM−1194, respectively (Table 1). EcoRI digestions of the above recombinant plasmids and Southern blot analysis, using the 3.4 Kb HindIII Tn5 fragment as a probe, confirmed that the clones contained the expected EcoRI fragments. The sizes of these EcoRI fragments were estimated to be as follows: pITM−69, 11.7 Kb; pITM−916, 21 Kb; pITM−1010, 7.9 Kb; and pITM−1194, 11.7 Kb (Fig. 6).
**Pseudomonas syringae** subsp. **savastanoi** HR− and Hrp− Mutants

**Fig. 3** Growth curves of *Pseudomonas syringae* subsp. *savastanoi* ITM206R and its mutant derivatives ITM317–916, ITM317–1010, ITM317–1194 and ITM317–69 in olive stem tissues. Similar results were obtained in three separate experiments.

**Fig. 4** Southern blot analysis of *Hin* III-digested genomic DNAs from Tn4-mutant strains of *Pseudomonas syringae* subsp. *savastanoi* ITM317R hybridized with 32P-labelled pGS8 (carrying Tn5). Lanes: 1, parental strain ITM317R; 2, mutant ITM317–916; 3, mutant ITM317–1010; 4, mutant ITM317–69; 5, mutant ITM317–1194

Similar results were obtained with the clone pRK7813E11.7, obtained by cloning the above EcoRI fragment from mutant ITM317–1194 into the plasmid vector pRK7813 (Table 1). When probed under high stringency conditions, none of the above EcoRI Tn5-containing fragments hybridized with plasmid pHIR11, a clone carrying the hrp gene cluster of *P. s. pv. syringae* 61 (Huang et al., 1988, 1991) (Fig. 7). On the contrary, similar experiments, using EcoRI-digested genomic DNA from *P. s. subsp. savastanoi* ITM317R showed the presence of sequences of DNA homologous to the above probe (Fig. 8).

**Marker-exchange mutagenesis**

The recombinant plasmid pRK7813E11.7 was introduced into the wild-type strain ITM317 of *P. s. subsp. savastanoi* by electroporation; then, after growing on KB with kanamycin, Km’ Tc’ colonies were obtained. These results suggest that Tn5 had been inserted into the genome of the above colonies and that the plasmid vector had been lost. However, when the above potential marker-exchanged colonies were assayed for the elicitation of HR on tobacco, none of them showed the expected HR− phenotype.

**Discussion**

By single transposon insertion in different loci of the chromosome of *P. s. subsp. savastanoi*, four mutants altered in their ability to elicit HR on tobacco were obtained; in two of them their pathogenicity on olive was also altered. In particular, mutant ITM317–1010 did not elicit HR, even when highly concentrated bacterial suspensions (i.e. 3 × 10^7 cfu/ml) were used, whereas mutant ITM317–916 showed an attenuated HR-inducing ability since it was able to elicit a variable HR under the same conditions. Further investigations in this regard showed that the latter mutant did not elicit HR when bacterial suspensions containing less than 7.5 × 10^7 cfu/ml were used. The variability of the HR assay results has been reported by other authors (Bauer and Beer, 1991) and may be due, at least in part, to the different sensitivity levels of individual tobacco plants or leaves. Nevertheless both of the above HR− mutants retained their full capability to cause disease symptoms on olive. These findings suggest that although the inactivated genes accounted for the hypersensitive reaction in different ways, they might not be involved in pathogenesis. This event seems particularly true for mutant ITM317–1010 which showed a distinct HR− phenotype.

Mutant ITM317–69, auxotrophic for methionine, did not elicit HR on tobacco; on olive it induced knots which were smaller than those caused by the parental strain and they were atypical in their morphology. The reduced ability of the above mutant to multiply in olive tissues, as ascertained in this study, and the possible relatively low level of phytohormones in planta might account for this behaviour. The fact that the prototrophic revertant of ITM317–69, obtained by the spontaneous excision of transposon Tn5, fully regained the wild-type phenotypes suggests that the single Tn5 insertion accounted for auxotrophy, for failure to induce HR and for attenuated virulence on olive. However, the possible relationship between auxotrophy and the above phenotypes remains to be determined. In this regard, Miller and Panopoulos (1993) isolated a Hrp− mutant from *P. syringae* pv. *phaseolicola* which required methionine to grow, but the authors concluded that auxotrophy per se did not account for the Hrp− phenotype.
Finally, mutant ITM317–1194 showed a typical Hrp− phenotype. In fact it did not induce HR and did not cause knots on olive, although it produced phytohormones in culture in same amounts as the parental strain ITM317R, and was not able to multiply in host plant tissues. Noteworthy in this regard is the observation that both the parental and mutant strain showed similar growth pattern in culture, on a complex medium as well as on a minimal medium. Attempts to marker-exchange the Hrp− mutation of ITM317–1194 into the parental strain were unsuccessful. The above failure might be due to the fact that, as ascertained during this study (data not shown), the DNA flanking the Tn5 insertion in ITM317–1194 showed sequences which hybridized to other regions of the genome. Consequently, it cannot be ruled out that in the examined marker-exchanged mutants the Tn5 transposon had been inserted into those other regions of the genome.

The genes inactivated in the four mutants of *P. s. subsp. savastanoi* strain ITM317 analysed in this study seemed to be different from those of the *hrp* gene cluster from *P. s. pv. syringae* 61. In fact, sequences homologous to the *hrp* gene cluster of *P. s. pv. syringae* were found in the genome of ITM317R, but they were physically distinct from the four mutated genes. Although no direct evidence is yet available, it can be surmised that also in *P. s. subsp. savastanoi* the latter genes might be important in the interaction of this pathogen with olive. Moreover, similarities between the amino acid sequences of the *hrpS* and *hrpR* gene products of *P. s. pv. phaseolicola* and the sequences in *P. s. subsp. savastanoi* have been reported (Mindrinos et al., 1990) but, to the best of the authors’ knowledge, the function of these genes in the latter pathogen has not yet been studied.

In conclusion, the results reported in this paper suggest that in *P. s. subsp. savastanoi* olive strain ITM317, besides genes homologous to the *hrp* genes of *P. s. pv. syringae*, at least four other genes are involved in the elicitation of the hypersensitive response on the non-host plant tobacco, and two of these genes seem also to be involved in pathogenicity. It also seems clear that the inactivated genes in mutants described in this study do not account for the host-range determination. In fact, none of the mutants was virulent on oleander. The availability of the
mutants of *P. s.* subsp. *savastanoi* here described and the clones bearing the Tn5-containing DNA fragments now provide a basis for the characterization of these genes. The possible relationship of the four inactivated genes and the region of DNA homologous to the *hrp* gene cluster of *P. s.* pv. *syringae* strain 61 remains to be elucidated.

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**Literature**


