Presence of the effector gene *hopA1* in strains of *Pseudomonas syringae* pv. *actinidiae* isolated from France and Italy

J.L. Vanneste¹, D.A. Cornish¹, J. Yu¹, C. Audusseau², S. Paillard², C. Rivoal² and F. Poliakoff²

¹The New Zealand Institute for Plant & Food Research Limited, Ruakura Research Centre, Private Bag 3123, Hamilton 3240, New Zealand

²Agence Nationale de Sécurité Sanitaire - Laboratoire de la Santé des Végétaux, 7 rue Jean Dixmèras, 49044 Angers, France

Corresponding author: Joel.Vanneste@plantandfood.co.nz

Abstract *Pseudomonas syringae* pv. *actinidiae* (Psa) causes bacterial canker of kiwifruit, a disease that had limited economic impact in Italy before the outbreak of 2009. The strains isolated from Italy since 2008 have different characteristics to those isolated in Japan or Korea or those isolated earlier than 2008 in Italy. This study showed that strains of Psa isolated from France had the *cts* haplotype I and a BOX-PCR electrophoretic pattern similar to that of strains isolated from Italy after 2008. The effector gene *hopA1*, which was found previously by PCR to be present only in the strains of the *cts* haplotype I, was also present in other strains of Psa isolated from Italy. If this effector plays a role in the interaction between Psa and kiwifruit, then the difference in virulence observed between strains of different *cts* haplotypes cannot be attributed to the presence of this effector.

Keywords bacterial canker of kiwifruit, *cts* haplotypes, *gltA*, *hrmA*, *hopPsyA*, *Pseudomonas syringae* pv. *tomato*.

INTRODUCTION

Pseudomonas syringae pv. *actinidiae* (Psa), which causes bacterial canker of kiwifruit, was isolated for the first time in Japan in 1984 (Takikawa et al. 1989). Soon after, the disease was discovered in Korea (Koh et al. 1994) and in Italy (Scortichini 1994). In Italy the disease had limited economic impact until a recent outbreak in 2009 (Vanneste et al. 2011b). Since 2009, the disease has devastated kiwifruit in the Latina province, which is the major kiwifruit growing area in Italy. The strains of Psa isolated from that outbreak are different from those isolated from the previous outbreaks that occurred in Italy, and from those isolated from Asia (Japan and Korea) (Ferrante & Scortichini 2010; Vanneste et al. 2010, 2011b).

In particular, the DNA sequence of the *cts* gene (which codes for a citrate synthase and is also known as *gltA*) of the strains isolated from Italy since 2008 differs by two base pairs from that of the Asian strains or the strains isolated from Italy before 2008. The *cts* haplotype of the strains isolated recently from Italy has been called *cts* haplotype I and the other haplotype has been called *cts* haplotype A (Vanneste et al. 2010). Furthermore, a consistent correlation between *cts* haplotype and the electrophoretic pattern obtained after BOX-PCR has also been demonstrated (Vanneste et al. 2010).

A DNA band of the expected size for the *hopA1* gene (formerly called *hrmA* and *hopPsyA*) was

detected by polymerase chain reaction (PCR) only in the strains of Psa isolated from Italy after 2008, i.e. strains of the *cts* haplotype I (Ferrante & Scortichini 2010). These authors concluded that those strains carried the *hopA1* effector gene. Effector proteins are synthesised by plant pathogenic bacteria and injected in the plant cell via a type III secretion system (Jones & Dangl 2006). In the plant cell these effectors can be detected by host resistance proteins, leading to an effector-triggered immunity (no disease) or they can suppress the plant innate immunity, leading to disease (Jones & Dangl 2006). Therefore the presence of the effector HopA1 in only one of the *cts* haplotypes could have some implication on the virulence of that haplotype and, as a consequence, its economical impact.

In June 2010 Psa was found for the first time in France (Vanneste et al. 2011a). The first goal of this study was to determine whether the strains of Psa isolated from the recent outbreak in France were similar to those isolated from Italy since 2008. The second goal of this study was to determine whether the *hopA1* gene was present in all the strains that shared the *cts* haplotype I.

MATERIALS AND METHODS

Strains and media

All the strains were maintained on King's B medium (King et al. 1954) at 28°C. The strains used in this study, including those isolated during the course of the study, are presented in Table 1. Isolations were made by teasing out infected tissues in sterile water and streaking the resulting suspension on plates of nutrient agar supplemented with 5% sucrose (NSA medium). The strains were purified twice at 28°C on King's B medium before being characterised as described below. Production of a green or blue diffusible fluorescent pigment on King's B medium was observed under ultra-violet light (366 nm).

Plant material analysed for presence of Psa

Leaves with small angular necrotic leaf spots collected in commercial kiwifruit orchards in France or in Italy were washed or teased out in sterile distilled water and the resulting solution

was spread either on nutrient agar supplemented with 5% sucrose (NSA) or on King's B medium containing 5 ml/litre of a 1% solution of cycloheximide. Small fragments of canes with cankers were also teased out in sterile distilled water before being plated on NSA or King's B medium supplemented with cycloheximide. After 48 h of incubation at 28°C, Psa-like colonies were purified twice at 28°C on King's B medium before being characterised.

Characterisation of strains of *Pseudomonas* isolated from kiwifruit

Biochemical and molecular characteristics were used to distinguish Psa from other bacterial species and from other *P. syringae* pathovars. All strains of Psa induce a hypersensitive reaction (HR) when infiltrated in tobacco plants, do not have a cytochrome c oxidase or an arginine dehydrolase, do not hydrolyse esculin, do not rot potato and do not induce ice nucleation. The ability of a bacterial strain to induce HR when injected into tobacco plants was tested as described previously (Vanneste et al. 1990). Inoculum prepared in sterile water was infiltrated in the intercostal area of young and fully expanded leaves using an 18-gauge sterile needle. Absence of a cytochrome c oxidase, a criterion that differentiates *P. syringae* from other species of plant pathogenic fluorescent *Pseudomonas*, was determined using Test Oxidase™ (Pro-Lab Diagnostic, Richmond Hill, ON, Canada). For this assay, *P. fluorescens* strain A506 (Wilson & Lindow 1993) was used as a positive control and *P. syringae* pv. *syringae* strain ICMP3523 was used as a negative control. Production of an arginine dehydrolase under anaerobic conditions and the ability to hydrolyse esculin were determined as described by Lelliot et al. (1966). Inability to utilise arginine is a characteristic shared by all strains of *P. syringae*, while hydrolysis of esculin is a characteristic of some pathovars only. The ability to rot potato was tested as described earlier (Vanneste et al. 2010). The strains to be tested were inoculated by toothpick into the flesh of a potato slice that had been previously surface disinfected. Water was used as a negative control and *Pectobacterium*

sp. strain JLVK201 isolated from kiwifruit was used as a positive control. The test was read after 48 h of incubation at 28°C. Ability to induce ice nucleation was tested after 30 s and 1 min at -5°C as described by Lindow et al. (1978).

Identification and characterisation of *Psa* by polymerase chain reaction (PCR)

Total DNA was isolated with a cell disruptor FastPrep®-24 from MP™ using the ZR fungal/bacterial DNA kit™ from Zymo Research, USA. PCRs were performed on an Eppendorf Mastercycler® Gradient. The PCRs for either identification of *Psa* or amplification and sequencing of the *cts* gene were carried out in a final volume of 30 µl of 1 × PCR buffer supplied with the DNA polymerase containing 50 to 100 ng of DNA of the strain of interest, 10 pmol of each primer, 200 µM of each dNTP and 1 U of i-Taq™ from iNtRON Biotechnology, Inc. A negative control, in which the DNA solution was replaced by the same volume of water, and a positive control, in which the DNA was that of a strain already identified as *Psa*, were included for each experiment. The PCRs for detection and sequencing of the *hopA1* gene were carried out in a final volume of 20 µl using the Maxime PCR PreMix (i-Taq) from iNtRON Biotechnology, Inc. according to the manufacturer's instructions. Each 20 µl reaction contained 10 pmol of each primer and 50 to 100 ng of genomic DNA.

To identify strains of *Psa*, the primers *PsaF1* (5'-TTTGTCTTGCACACCCGATTTT-3') and *PsaR2* (5'-CACGCACCTTCAATCAGGATG-3') were used as they yield an amplicon of 280 bp with all the strains of *Psa* tested so far (Rees George et al. 2010; Vanneste et al. 2010). The thermal cycling programme of Rees-George et al. (2010) was employed as modified by Vanneste et al. (2010). For BOX-PCR fingerprinting, the BOX primer BOXA1R (5'-CTACGGCAA GGCGACCTGACG-3') was used with the BOX-PCR thermal cycling programme described by Louws et al. (1994). The *cts* gene was amplified using the primers *cts-Fp* (5'-AGTTGATCATC GAGGGCGCWGCC-3') and *cts-Rp* (5'-TGAT CGGTTTGATCTCGCACGG-3') following

the protocol published by Sarkar & Guttman (2004). The *hopA1* gene was amplified using the primers *hopA1-F* (5'-CGGCAAGAGGTAC GAGATTC-3') and *hopA1-R* (5'-TTCAATGCC TTTAGCGTGTG-3') following the thermal cycling programme described by Ferrante & Scortichini (2010).

The PCR products were separated by horizontal gel electrophoresis using a Biokey Super Screener 120 Electrophoresis system (Innovation Sciences Limited, Dunedin, New Zealand) on 1% or 2% agarose containing 10 ng/ml of ethidium bromide. From each PCR, 2 to 30 µl was loaded on the agarose gel and the DNA bands were visualised under UV light. On each gel, a DNA ladder (ZR 1 kb DNA ladder from Zymo Research, USA) was used for size comparison.

The *cts* gene was sequenced using the primers *cts-Fs* (5'-CCCGTCGAGCTGCCAATWCTGA-3') and *cts-Rs* (5'-ATCTCGCACGGSGTRTTGAAC ATC-3') published by Sarkar & Guttman (2004). The primer *hopA1F* was used for sequencing of the *hopA1* amplicon.

DNA sequencing was carried out at the Waikato DNA Sequence Facility. DNA sequences were resolved using a 3130XL Genetic Analyzer System fitted with 50 cm capillary arrays (Applied Biosystems) loaded with POP-7 polyacrylamide matrix (Applied Biosystems). DNA templates were prepared using Big Dye v3.1 terminator chemistry (Applied Biosystems). When required, DNA sequences were compared with DNA sequences deposited in GenBank® from the National Centre for Biotechnology Information (NCBI) using the programme BLAST 2.2.25 (Zhang et al. 2000). Multiple alignments were performed with AlignX from VECTOR NTI 11 (Invitrogen).

RESULTS AND DISCUSSION

Isolation and identification of strains of *Psa* from infected material

Strains that exhibited all the characteristics of *Psa* (i.e. induced HR when infiltrated in tobacco plants, did not have a cytochrome c oxidase or an arginine dehydrolase, did not hydrolyse esculin,

Table 1 List of strains of *Pseudomonas syringae* pv. *actinidiae* used in this study.

Strain number	Host plant	Country of origin	Year of isolation	Source
ICMP 9854 ¹	<i>Actinidia deliciosa</i>	Japan	1989	Y. Takikawa
ICMP 9855	<i>A. deliciosa</i>	Japan	1989	Y. Takikawa
K-Psa 2	<i>A. sp.</i>	Korea		GM Balestra
ISPAVE-B-019 ²	<i>A. deliciosa</i>	Italy	1994	M. Scortichini
ISPAVE-B-020 ³	<i>A. deliciosa</i>	Italy	1994	M. Scortichini
CRA-FRU 10.22	<i>A. chinensis</i>	Italy	2008	M. Scortichini
I.6.5.10.4-4	<i>A. chinensis</i>	Italy	2010	This study
I.23.6.10.UOM-1	<i>A. chinensis</i>	Italy	2010	This study
I-Psa 8	<i>A. chinensis</i>	Italy	2008	GM Balestra
I 25.10.10.6.6	<i>A. chinensis</i>	Italy	2010	This study
I-9.4.10-E4	<i>A. chinensis</i>	Italy	2010	JL Vanneste
LNPV 36.43 ⁴	<i>A. deliciosa</i>	France	2010	This study
LNPV 36.45	<i>A. deliciosa</i>	France	2010	This study
LNPV 36.48	<i>A. deliciosa</i>	France	2010	This study
LNPV 36.47	<i>A. deliciosa</i>	France	2010	This study
LNPV 36.74	<i>A. chinensis</i>	France	2010	This study
LNPV 36.79	<i>A. chinensis</i>	France	2010	This study

¹All the strains with an ICMP number were obtained from the International Collection of Microorganisms from Plants (ICMP) held by Landcare Research New Zealand.

²Received as NCPPB 3871.

³Received as NCPPB 3873.

⁴All the strains with a LNPV number are deposited at the Plant Health Laboratory (Anses-France).

did not rot potato, did not induce ice nucleation and produced a 280 bp amplicon after PCR with the primers Psaf1/R2) were isolated from infected tissues from French orchards located in the Aquitaine Region and Rhone-Alpes Region, and from Italian orchards located in the Latina region (Table 1). These strains were used in all the following experiments. The strains isolated from France or Italy were slightly fluorescent on King's B medium. Psa has been described as non-fluorescent on that medium (Takikawa et al. 1989), but some strains of Psa isolated earlier, including those isolated from Italy after 2008, are slightly fluorescent on this medium. Therefore, the lack of fluorescence on King's B medium cannot be used to identify potential strains of Psa.

Molecular characterisation of Psa strains isolated from France and Italy

The DNA sequence of the *cts* gene from the three strains isolated from Italy was 100% identical to that of strains of Psa isolated from Italy after 2008. These three strains had the *cts* haplotype I. They also had a BOX-PCR electrophoretic pattern similar to that of *cts* haplotype I strains (Figure 1). So far, all the strains isolated from Italy since 2008 have the *cts* haplotype I (Ferrante & Scortichini 2010; Vanneste et al. 2010, 2011b). The strains ISPAVE-B-019 and ISPAVE-B-020 isolated in 1992 in Italy have the *cts* haplotype A. Although no strains of haplotype A have been isolated in Italy since 2008, not enough strains have been analysed since 2008 to conclude that the *cts* haplotype A has completely disappeared from Italy.

Figure 1 Agarose gel electrophoresis of total DNA from strains of *Pseudomonas syringae* pv. *actinidiae* after polymerase chain reaction (PCR) using the primer BOX A1R. Lane 1 and 13: DNA ladder (ZR 1 kb DNA ladder from Zymo Research, USA), Lane 2: *P. s.* pv. *actinidiae* I-9.4.10-E4 (*cts* haplotype I), Lane 3: *P. s.* pv. *actinidiae* ICMP 9855 (*cts* haplotype A), Lane 4: *P. s.* pv. *actinidiae* I.6.5.10.4-4, Lane 5: *P. s.* pv. *actinidiae* I.25.10.10.6.6, Lane 6: *P. s.* pv. *actinidiae* I.23.6.10.UOM-1, Lane 7: *P. s.* pv. *actinidiae* LNPV 36.43, Lane 8: *P. s.* pv. *actinidiae* LNPV 36.45, Lane 9: *P. s.* pv. *actinidiae* LNPV 36.48, Lane 10: *P. s.* pv. *actinidiae* LNPV 36.47, Lane 11: *P. s.* pv. *actinidiae* LNPV 36.74 and Lane 12: *P. s.* pv. *actinidiae* LNPV 36.79. The arrows indicate the differences in the BOX-PCR electrophoretic pattern between the strains of *P. s.* pv. *actinidiae* that have the *cts* haplotype I from those which have the *cts* haplotype A.

The strains isolated from France also have the *cts* haplotype I and exhibit the same BOX-PCR electrophoretic pattern as all the strains isolated from Italy since 2008. This suggests that the French outbreak and the Italian outbreak of 2009 might be linked.

Using the primers *hopA1F/hopA1R*, no amplicon of the expected size (441 bp) was obtained with any of the Japanese or Korean strains examined. For some of those strains, a slightly longer amplicon was obtained. However,

sequencing of those amplicons revealed they were not related to *hopA1* (data not shown). Under the same conditions an amplicon of 441 bp was found for all the strains isolated from Italy and France (Figure 2). This includes the strains ISPAVE-B-019 and ISPAVE-B-020, which were isolated before 2008, and which are of a different *cts* haplotype than the other strains isolated from Italy. This is in contradiction with results published earlier by Ferrante & Scortichini (2010), who did not find any amplicon of 441 bp when using the DNA of those two strains, and using the same primers and the same thermal cycling programme. The only difference between the two experiments is the thermal cycliser. It might be that the annealing temperature between those two machines is different enough to lead to amplification of *hopA1* in only one case. To ensure that the fragment of DNA obtained after PCR was related to the *hopA1* gene, the amplicons of all the strains analysed were sequenced and analysed. The partial sequences (391 bp) of the *hopA1* amplicon from all the *Psa* strains analysed (including ISPAVE-B-019 and ISPAVE-B-020) were 100% identical to each other, and 97% similar to the *hopA1* gene from *P. syringae* pv. *tomato* DC3000 (GenBank accession number AE016853.1). This is consistent with all those strains carrying the gene *hopA1*. If the *hopA1* gene is expressed and functional in all those *Psa* strains, then there is no strict correlation between *cts* haplotype and presence of the *hopA1* gene. Furthermore, any differences in virulence between strains of these different *cts* haplotypes could not be explained solely by the presence or absence of this effector gene.

CONCLUSIONS

The strains of *Psa* isolated from France share the same *cts* haplotype and BOX-PCR pattern as the strains isolated in Italy after 2008. Although in itself this does not prove the origin of the pathogen in France, it does suggest a connection between the Italian and the subsequent French outbreaks.

Presence of DNA sequences similar to that of *hopA1* in all *Psa* strains isolated from Italy and

Figure 2 Agarose gel electrophoresis after polymerase chain reaction (PCR) of total DNA of *Pseudomonas syringae* pv. *actinidiae* strains using primers specific for *hopA1*. Lane 1: DNA ladder (ZR 1 kb DNA ladder from Zymo Research, USA), Lane 2: *P. s.* pv. *actinidiae* I-Psa 8, Lane 3: *P. s.* pv. *actinidiae* I.23.6.10.UOM-1, Lane 4: *P. s.* pv. *actinidiae* I-9.4.10-E4, Lane 5: *P. s.* pv. *actinidiae* ISPAVE-B-019, Lane 6: *P. s.* pv. *actinidiae* ISPAVE-B-020, Lane 7: *P. s.* pv. *actinidiae* LNPV 36.43, Lane 8: *P. s.* pv. *actinidiae* LNPV 36.74, Lane 9: *P. s.* pv. *actinidiae* ICMP 9854, Lane 10: *P. s.* pv. *actinidiae* ICMP 9855, Lane 11: *P. s.* pv. *actinidiae* K-Psa 2.

France is consistent with those strains carrying that effector gene. If the HopA1 effector plays a role in the interaction between Psa and kiwifruit, then the difference in virulence observed in the field and in the laboratory (data not shown) between strains of different *cts* haplotypes cannot be attributed to the presence of this effector.

ACKNOWLEDGEMENTS

This research was supported by ZESPRI International Ltd; Plant Health Laboratory – French Agency for Food, Environmental and Occupational Health & Safety; and The New Zealand Institute for Plant & Food Research Limited – KRIP project 09-01.

REFERENCES

- Ferrante P, Scortichini M 2010. Molecular and phenotypic features of *Pseudomonas syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow kiwifruit (*Actinidia chinensis*) in central Italy. *Plant Pathology* 59: 954–962.
- Jones JD, Dangel JL 2006. The plant immune system. *Nature*. 444: 323–329.
- King EO, Ward MK, Raney DE 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory Clinical Medicine* 44: 301–307.
- Koh YJ, Cha BJ, Chung HJ, Lee DH 1994. Outbreak and spread of bacterial canker in kiwifruit. *Korean Journal of Plant Pathology* 10: 68–72.
- Lelliot RA, Billing E, Hayward AC 1966. A determinative scheme for the fluorescent plant pathogenic *Pseudomonads*. *Journal of Applied Bacteriology* 29: 470–489.
- Lindow SE, Amy DC, Upper CD 1978. *Erwinia herbicola*: a bacterial ice nucleus active in increasing frost injury to corn. *Phytopathology* 68: 523–527.
- Louws FJ, Fulbright DW, Stephens CT, de Bruijn FJ 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* 60: 2286–2295.
- Rees-George J, Vanneste JL, Cornish DA, Pushparajah IPS, Yu J, Templeton MD, Everett KR 2010. Detection of *Pseudomonas syringae* pv. *actinidiae* using polymerase chain reaction (PCR) primers based on the 16S-23S rDNA intertranscribed spacer region and comparison with PCR primers based on other gene regions. *Plant Pathology* 59: 453–464.
- Sarkar SE, Guttman D. 2004. Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Applied and Environmental Microbiology* 70: 1999–2012.

- Scortichini M 1994. Occurrence of *Pseudomonas syringae* pv. *actinidiae* on kiwifruit in Italy. *Plant Pathology* 43: 1035–1038.
- Takikawa Y, Serizawa S, Ichikawa T, Tsuyumu S, Goto M 1989. *Pseudomonas syringae* pv. *actinidiae* sp. nov., the causal bacterium of canker in kiwifruit in Japan. *Annals of the Phytopathological Society of Japan* 55: 437–444.
- Vanneste JL, Kay C, Onorato R, Yu J, Cornish DA, Spinelli F, Max S 2011b. Recent advances in the characterisation and control of *Pseudomonas syringae* pv. *actinidiae*, the causal agent of bacterial canker on kiwifruit. *Acta Horticulturae* (in press).
- Vanneste JL, Paulin JP, Expert D 1990. Bacteriophage Mu as a genetic tool to study *Erwinia amylovora* pathogenicity and hypersensitive reaction on tobacco. *Journal of Bacteriology* 172: 932–941.
- Vanneste JL, Poliakoff F, Audusseau C, Cornish DA, Paillard S, Rivoal C, Yu J 2011a. First report of *Pseudomonas syringae* pv. *actinidiae* the causal agent of bacterial canker of kiwifruit on *Actinidia deliciosa* in France. *Plant Disease* (in press).
- Vanneste JL, Yu J, Cornish DA 2010. Molecular characterisations of *Pseudomonas syringae* pv. *actinidiae* strains isolated from the recent outbreak of bacterial canker on kiwifruit in Italy. *New Zealand Plant Protection* 63: 7–14.
- Wilson M, Lindow SE 1993. Interactions between the biological control agent *Pseudomonas fluorescens* A506 and *Erwinia amylovora* in pear blossoms. *Phytopathology* 83: 117–123.
- Zhang Z, Schwartz S, Wagner L, Miller W. 2000. A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* 7: 203–14.