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Development of a rapid tool for the molecular characterisation of Psa haplotypes

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Executive summary

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The outcome of developing an effective rapid Psa-V detection method has been achieved.

Twenty-one potential loci that might be able to distinguish the virulent isolate of Psa present in New Zealand (Psa-V) from other isolates of Psa present in New Zealand (Psa-LV), from other closely related pathovars of *P. syringae* and from other related pseudomonads were identified and tested by genome comparisons, in various PCR assays, and sequenced in order to identify a set of loci that best suited the characteristics required for developing a highly specific and rapid PCR-based assay for Psa-V.

Over 170 primers were designed to amplify portions of these loci and included some specifically designed to test their performance in Psa-V specific assays. More than one hundred PCR products were also direct sequenced and the sequencing results compared. During the course of this project the draft genome sequences from a number of isolates of Psa and other pathovars of *P. syringae* became available through our own sequencing efforts (Psa isolates - phase 3) and those of other groups overseas (additional draft *P. syringae* genomes analysed in Baltrus et al. 2011). These additional sequences were used as and when they became available to accelerate development of the Psa-V specific assays.

Several promising loci were identified which contain polymorphisms specific to Psa-V. However, two loci in particular had characteristics that lend themselves particularly well to designing a robust specific assay (loci with major rearrangements or novel loci). Thus these two assays were tested against a significant number of related bacteria. This specificity testing corroborated the likely specificity of these tests for the Psa-V isolate.

We note that these test included several bacteria likely to be found on kiwifruit (such as *P. viridiflava* and *P. fluorescens*) and a number of bacteria known (or deduced by this analysis) to show “off-target” amplification for other PCR-based Psa detection assays, including the most specific published system described by Rees-George et al. (2010) (e.g. *P. syringae* pv. theae, *P. syringae* pv. morsprunorum and *P. syringae* pv. passiflorae). While it is always possible to subject an assay to a greater number of samples in order to test its specificity, all reasonable steps were taken to test these two assays.

Bacteria in the *Pseudomonas* complex do appear to evolve by sharing genes amongst one another, albeit at a low frequency. It should be noted, therefore, that the possibility of another bacterial strain existing somewhere that shares one of these characteristics, however remote, can never be completely ruled out. We recommend that an initial assay followed by a confirmation assay (the hopA1 and hopZ2b assays respectively) form the basis of the test for Psa-V. As the likelihood of another bacterial strain sharing both of these loci is even lower and we know that they are located in different parts of the genome (data analysis undertaken in the genome project), the combination of the two tests together provides a greater certainty of identification.

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1 Introduction

1.1 Background

The ability to characterise Psa haplotypes rapidly is essential information for management of the disease at orchard, regional and national levels. It is essential that these tools are both rapid and robust. This project is aimed at developing the base information and technologies necessary for commercial providers to be able to develop commercial haplotype testing services.

The strains of *Pseudomonas syringae* pv. *actinidiae* (Psa) isolated from New Zealand can be grouped in different categories based on a number of DNA sequences and DNA-based analysis. At the outset of this project, a correlation was known to exist between *cts* haplotype (a particular DNA sequence at the citrate synthase or *cts* gene in Psa), BOX PCR patterns, the presence or absence of some effectors (bacterial genes that are believed to suppress the plant's ability to resist bacterial infection) and the DNA sequence of some of those effectors.

More importantly, there seems to be a link between these various DNA-based markers and the amount of the virulent Psa (called the V isolate and abbreviated as Psa-V). The evidence for this link is quite strong for the *cts* haplotype but weaker for the other genes (as fewer isolates have been tested). We proposed the development of a rapid and simple DNA-based test for the V isolate using two phases of testing: a primary test based on one or more of the genome regions that differentiate the Psa-V isolate from other isolates (for example the *cts* haplotype), followed by a secondary test of the positive samples. This will enable the rapid delivery of a result to Kiwifruit Vine health (KVH), to enable the industry to use this information to make critical decisions on control measures.

At the outset of this research, the haplotype of a Psa strain was determined in several ways. A rapid commercial test was available; however, this testing had been associated with some apparent Psa-V false positives, suggesting that there may be issues with specificity. For this reason, verification by culturing bacteria was still recommended. Haplotypes can also be determined using BOX-PCR or by sequencing a number of specific DNA regions with variation. This latter method requires laboratories to obtain a pure culture of the strain first, a process that takes, under the best circumstances, over a week to complete. Once such a culture has been obtained, isolation of the DNA and the BOX-PCR or DNA sequencing takes two-three additional days. As an alternative and based on the correlations between *cts* haplotypes and other molecular markers, several PCR protocols other than the BOX-PCR could be used to determine the haplotype of the strain. However, replacing one PCR protocol by another one does not allow determination of the haplotype of a strain in a shorter period of time. Therefore, the challenge is not to replace one PCR/sequencing based protocol with another PCR method but to develop a protocol that allows determination of the haplotype of a strain without having to culture or purify Psa. This would require a test based on unique regions of the Psa-V genome.

This project was specifically aimed at the development of an improved method which allows the typing of Psa strains (determining to which haplotype a strain belongs and by extension its degree of virulence) from leaf and cane tissues, without the need to isolate and purify the bacteria.

The research was divided into five phases, listed below, with an emphasis on identifying more than one locus (region of a gene that has a particular DNA sequence) that could be used in a haplotyping test, in order to decrease the likelihood of a single test failing because of lack of specificity. Research on known loci started straight away, but the research also utilised whole

genome sequence data from a project funded by PFR CORE research programmes initiated at about the same time to sequence several isolates from various geographical regions. This information was utilised as soon as it and any other pertinent additional information in the public domain became available.

1.2 Key outcome

Outcome as listed in contract: An effective rapid Psa-V detection method

1.3 Key milestones

Key milestones as listed in the contract:

1. Initial base information available for commercial providers
2. Genome sequence data released (Psa whole genome sequence data will be provided on the basis that the information is used purely for the development of Psa diagnostic tools, which will then be provided free into the public domain by the developing party. The information provided must be not published or used for any other purpose)
3. Final report.

1.4 Research Plan

Phase 1: PCR specificity tests of primer loci versus Psa isolates

Phase 2: Amplification and sequencing of candidate effector haplotyping loci from Psa-V and other Psa isolates

Phase 3: Obtaining whole genome sequence data and analysis of Psa genome sequences to identify additional candidate effector and housekeeping haplotyping loci. Note this phase was not funded by this project. DNA sequencing costs were funded by the MSI-funded Better Border Biosecurity research programme, while the analysis of the data was funded by the Horticultural Genomics CORE-funded MSI research programme. The ZESPRI-funded project reported here would not have been able to deliver on its milestones in a timely fashion without this co-funding

Phase 4: Designing quantitative PCR (qPCR) assays, specificity tests of these assays versus Psa isolates and *P. syringae* pathovars and identification of key haplotype assay loci for further commercial laboratory testing

Phase 5: Validation testing of key haplotype assay loci in a commercial laboratory.

2 Methodology

2.1 Methodology background

Phases 1 and 2 were carried out before the acquisition of whole genome sequence from Psa-V and Psa-LV isolates. During this phase, a series of specific effector loci were targeted for sequence amplification and analysis in order to try to identify loci with polymorphisms (DNA sequence differences) that were specific to the V isolate and around which rapid and specific assays might be designed. The focus was necessarily on effector loci which are under selective pressure to change, as most housekeeping genes are too highly conserved to be able to identify polymorphisms that are specific to the V isolate of Psa and not present in any other closely related *P. syringae* pathovar. Phases 3 to 5 were carried out subsequent to the acquisition of whole genome sequence from Psa-V and Psa-LV isolates. This allowed us to look globally at the sequence data in order to identify the best loci on which to base rapid and

specific assays, and to include both effector loci and identify and test the most polymorphic housekeeping loci.

2.2 Methods and protocols

DNA was isolated from a series of bacterial isolates of Psa and/or obtained from New Zealand colleagues from Landcare and MAF and included a series of DNA samples from other pathovars of Psa and used in all phases of the research.

Phase 1: The initial stage involved Blast searches of a draft genome lodged on the NCBI database in the public domain (http://www.ncbi.nlm.nih.gov/nuccore/aeal00000000) derived from a Japanese isolate Psa. These searches used DNA and protein sequence data from effector loci derived from a publically available web page (http://www.pseudomonas-syringae.org/pst_func_gen2.htm) – this included effectors from other *P. syringae* pathovars. The next stage involved amplifying portions of these candidate effector loci from 4 different isolate groupings of Psa, namely virulent New Zealand isolates (identified as Psa-V), low virulence isolates from New Zealand (identified as Psa-LV), Japanese isolates (identified as Psa-J) and Korean isolates (identified as Psa-K). This information was then used to identify loci, or portions of loci, to sequence in phase 2 (see below). Phase 1 results identifying the loci and primers tested are summarised in Table 1.

Phase 2: The most promising subset of the loci tested in phase 1 was subjected to phase 2 analysis. This was achieved by designing primers based on the initial blast results and then sending these amplification products away to be DNA sequenced. When sequencing results were obtained, these sequences were compared with one another in order to look for a number of single nucleotide polymorphisms (SNPs) around which a Psa and/or Psa-V specific amplification protocol could be designed and tested. The focus was on regions that could meet two specifications (1) they amplified at least one Psa-V specific SNP and (2) they were bracketed by potential primer sites containing multiple SNPs compared with *other P. syringae* pathovars that have sequence information in the public DNA databases.

Phase 3: This phase involved obtaining whole genome sequence data and analysis of these Psa genome sequences to identify additional candidate effector and housekeeping haplotyping loci. As this was not funded from this project, only the data pertinent to designing the haplotyping loci are included in these results below. The sequence data on pertinent loci were shared among laboratories to speed up the development of the haplotyping test.

Phase 4: This phase involved designing quantitative PCR (qPCR) assays based on the analyses in phases 1, 2 and 3. The specificity of these assays was then tested against Psa isolates and a series of *P. syringae* pathovars and other bacterial isolates and this information was used to identify the key haplotype assay loci for further commercial laboratory testing.

Phase 5: This phase involved validation testing of the key haplotype assay loci identified from the research in phase 4. The validation was performed by collaboration with a commercial laboratory (Hill Laboratories Limited). A large number of commercial kiwifruit leaf and cane samples from which DNA was extracted previously by the commercial laboratory were the basis of the validation test. These tests (?) included samples identified by the initial Psa-V testing procedures as Psa-V, samples that tested negative or non-detected for Psa-V and Psa, and a few samples that had previously tested positive as Psa-LV. A copy of this report has already been received directly by KVH from Hill Laboratories Ltd, but is appended here again for completeness (Appendix 3).

3 Results

3.1 Results for phase 1

Ten loci and 28 primer combinations were tested in phase 1 of the project. The research involved amplifying portions of effector loci using various combinations of PCR primers tested by normal (end-point) PCR across representative sets of Psa isolates (Psa-V, Psa-J, Psa-K and Psa-LV). This phase aimed to identify PCR primers that could be used to compare the DNA sequences of all four groups of isolates (when the effector was present in all four) but also noted any indications of polymorphisms between the isolates (e.g. presence/absence or different sizes of amplification products) that it might be possible to exploit for development of an assay to distinguish the key isolates. This research identified six promising loci (highlighted below), which were taken into the next phase of testing (see below).

Table 1. The effector loci and primers tested in phase 1

Locus	Primer number	Primer name	Forward or Reverse Primer Sequence	Band amplified			
				Psa-V	Psa-LV	Psa-J	Psa-K
hrpK1	25	hrpK1-BF	AGCGGACAAGACCAAAGTTG	+	+	+	+
	26	hrpK1-BR	GCGGACCAGATTGCGCTCCT				
	23	hrpK1-AF	TGCGTATATCCAGTTCTCCC	+	+	+	+
	24	hrpK1-AR	CTTCTGGCGAGGAGTATTCG				
	5	hrpK1-1F	GACAGTGCCGACAAGGACT	+	+	+	+
	6	hrpK1-1R	ATCGGCGGTTTGCAGAGACT				
hopAF1	16	hopAF1-BF	TCTTCTGGCACAAGGTCTTC	+	+	+	+
	18	hopAF1-BR	TATGGGACTATGTATTTCAA				
	15	hopAF1-AF	TGCTAACTGAAAAATCACCC	+	+	+	-
	17	hopAF1-AR	AGACGCTTATCAAGCAGAAA				
	8	hopAF1-1F	CAAGCAGAAAGACGGCATC	+	+	+	+
	10	hopAF1-1R	GCACACGCGACAGCAATG				
	72	hopAF1C-1F	GCTCTGTAGCTCGTAGACGCGGG	+	+	+	+
	73	hopAF1C-1R	GACCGTCTAGTAGTAGCTATCGTG				
	74	hopAF1C-1AF	TCATCTCGTCTGATGCTCTGTAG	+	+	+	+
	73	hopAF1C-1R	GACCGTCTAGTAGTAGCTATCGTG				
AvrRPM1	11	RPM1A-1F	TTATCCAGTAAAGCGGCTCA	+	-	+	-
	12	RPM1A-1R	TGGATATTATTCCGGTTACG				
	13	RPM1B-1F	TTTGTCGACACATTGTGTCC	+	-	+	-
	14	RPM1B-1R	TTGATACCCAATGCGAGGAG				
AvrRPM2	15a	avrRPM2-F1	CATTCAATTTCAAGTGAGAGG	+	-	+	+
	16a	avrRPM2-R1	GGCGGGCTATGTTTAGATCC				
	15a	avrRPM2-F1	CATTCAATTTCAAGTGAGAGG	+	-	+	+
	17a	avrRPM2-R2	GTAATCGTCCCGCTTGCTCG				
hopB1	3	hopB1-1F	AGGCTATTATCCGCCAACCT	+	-	+	+
	4	hopB1-1R	TCTTGCAACAGGATGCTCAC				

Table 1 key: +=band ,-=No band. Note that absence of a product does not necessarily mean absence of any sequence homologous to the effector, as there could also be sequence polymorphisms in the priming sites.

Table 1. (continued) The effector loci and primers tested in phase 1.

Locus	Primer number	Primer name	Forward or Reverse Primer Sequence	Band amplified			
				Psa-V	Psa-LV	Psa-J	Psa-K
hopZ3	21	hopZ3-BF	GGATTAACCTGCCCCAGTTA	+	-	+	+
	22	hopZ3-6R	GTGTCCCTCGAGCATACATC				
	19	hopZ3-AF	TAACGAGGAAATCATGGCAG	+	-	+	+
	20	hopZ3-AR	GTGTAATAGCGTCTCTGCAT				
AvrE1	31	avrE1-BF	GTTTCGGGTCCTGGCGCAT	+	+	+	+
	32	avrE1-BR	AACGTCGCCAAGCCGATAAC				
	35	avrE1-C2F	TCGAAGTTGAATCGCCGAGC	+	+	+	+
	36	avrE1-C2R	TGAACCTCACCACCCCGTTC				
	37	avrE1-DF	AACCGCTTTCCAGCGCTTT	+	+	+	+
	38	avrE1-DR	TCAAGGGCTGAAACCAGC				
	39	avrE1-EF	GGCGACCTTGGTAGCGATGC	+	+	+	+
	40	avrE1-ER	ACCAGTACCGGCGGGCTTTA				
	29	avrE1-AF	TTGTCCTGGTCTGCGCCAT	+	+	+	+
	30	avrE1-AR	TAGCGCCAGGACCCGAAAC				
hopS2	43	hopS2-BF	CAGTGCCTGATTGAACAGC	+	+	+	+
	44	hopS2-BR	GTGAAAAAGTCTGGCGCTG				
	41	hopS2-AF	CCACAGAACGAAGTGATGC	+	+	+	+
	42	hopS2-AR	GCTGACACGGCTCAATAAC				
hopAN1	52	hopAN1-AF	TTTGCCCGGCGTCTCTGGC	+	+	+	+
	53	hopAN1-AR	AGAACCACCCGCTGACGCC				
	54	hopAN1-BF	GGCGTCAGCGGGTGGTTCT	+	+	+	+
	55	hopAN1-BR	ACCTGGCCAACATGCTGGTG				
hopY1	56	hopY1-AF	CCGCACAAGGCACAGAC	+	+	+	+
	57	hopY1-AR	TCGTCCCTTGGCAGACCTG				
	58	hopY1-BF	TGCTGAGTTTGCTGGGC	+	+	+	+
	59	hopY1-BR	TCACTGGTAGTTGATGCCCG				
hopAG1	60	hopAG1-AF	TGTTTCGCGAGGGCGAGG	+	+	+	+
	61	hopAG1 -AR	ACGCACCTTCCCGGCGAG				
	62	hopAG1 -BF	CCGGTTCTGTTTGTGCTGC	+	+	+	+
	63	hopAG1 -BR	GAAAGGCACGACTGGACG				
	64	hopAG1- CF	AACAAACCTGTGCGCATCG	+	+	+	+
	65	hopAG1 -CR	TCCTTGAGTGCATTGGTCC				

3.2 Results for phase 2

Six loci and 15 primer combinations were tested in phase 2 of the project. More than 100 PCR products from the four main groups of Psa isolates (Psa-V, Psa-J Psa-K and Psa-LV) were direct sequenced and the resulting sequences compared with one another to identify polymorphisms. This research identified two promising loci (highlighted below), which were taken into the next phase of testing (see phase 4 below).

Table 2. The most promising effector loci tested in phase 2

Locus	Primer No.	Primer name	Forward or Reverse Primer Sequence	Sequence Polymorphisms (compared with the sequence from Psa-V)			
				Psa -V	Psa -LV	Psa -J	Psa-K
hrpK1	25	hrpK1-BF	AGCGGACAAGACCAAAGTTG	P	Y	Y	N
	26	hrpK1-BR	GCGGACCAGATTGCGCTCCT				
	23	hrpK1-AF	TGCGTATATCCAGTTCTCCC	P	Y	Y	N
	24	hrpK1-AR	CTTCTGGCGAGGAGTATTCG				
	5	hrpK1-1F	GACAGTGCCGACAAGGACT	P	N	Y	N
	6	hrpK1-1R	ATCGGCGGTTTGCAGAGACT				
hopAF1	16	hopAF1-BF	TCTTCTGGCACAAGGTCTTC	P	Y	Y	Y
	18	hopAF1-BR	TATGGGACTATGTATTTCAA				
	72	hopAF1C-1F	GCTCTGTAGCTCGTAGACGCGGG	P	Y	Y	Y
	73	hopAF1C-1R	GACCGTCTAGTAGTAGCTATCGTG				
	74	hopAF1C-1F	TCATCTCGTCTGATGCTCTGTAG	P	Y	Y	Y
	73	hopAF1C-1R	GACCGTCTAGTAGTAGCTATCGTG				
AvrRPM1	13	RPM1B-1F	TTTGTCGACACATTGTGTCC	P	A	Y	A
	14	RPM1B-1R	TTGATACCCAATGCGAGGAG				
AvrRPM2	15a	avrRPM2-F1	CATTCAATTTCAAGTGAGAGG	P	A	Y	Y
	16a	avrRPM2-R1	GGCGGGCTATGTTTAGATCC				
	15a	avrRPM2-F1	CATTCAATTTCAAGTGAGAGG	P	A	Y	Y
	17a	avrRPM2-R2	GTAATCGTCCCGCTTGCTCG				
AvrE1	31	avrE1-BF	GTTTCGGGTCCTGGCGCAT	P	Y	Y	N
	32	avrE1-BR	AACGTCGCCAAGCCGATAAC				
	37	avrE1-DF	AACCGCTTTCCAGCGCTTT	P	Y	Y	Y
	38	avrE1-DR	TCAAGGGCTGAAACCAGC				
	39	avrE1-EF	GGCGACCTTGGTAGCGATGC	P	Y	N	N
	40	avrE1-ER	ACCAGTACCGGCGGGCTTTA				
	29	avrE1-AF	TTGTCCTGGTCTGCGCCAT	P	Y	N	N
	30	avrE1-AR	TAGCGCCAGGACCCGAAAC				
hopS2	43	hopS2-BF	CAGTGCCTGATTGAACAGC	P	Y	N	N
	44	hopS2-BR	GTGAAAAAGTCTGGCGCTG				
	41	hopS2-AF	CCACAGAACGAAGTGATGC	P	Y	N	Y
	42	hopS2-AR	GCTGACACGGCTCAATAAC				

Table 2 key: Y=polymorphisms compared with Psa-V present , N= polymorphisms compared with Psa-V not present. P =sequence present, A=sequence absent.

3.3 Results for phase 3

In this phase, a genome sequencing project was initiated by PFR and collaborators (Profs Paul Rainey from Massey University and David Guttman from University of Toronto). This phase was not funded by this project and therefore only the data pertinent to designing the haplotyping loci are included in this report. Genome data were searched by Blast using known effector sequences from other *P. syringae* pathovars. This phase identified five key effector loci (hopA1, hopZ2b, hopM1, hopAM1 and hopH1) for further analysis. A genome-wide search for housekeeping genes with the highest degree of polymorphisms was also undertaken by Rainey et al. (pers. comm.) and the ten most polymorphic loci were identified. The sequences from these loci were aligned with one another and homologous sequences from other closely related pathogens were identified in public domain databases by BlastN and BlastX searches. This analysis allowed us to develop a shortlist of five housekeeping loci (Gene4817, Gene900, recN, Gene1642 and Tuf1) that had sufficient variation and differences between the V isolate and other isolates and potential sites for developing Psa-specific or Psa-V specific PCR priming sites. These loci were subjected to further analysis as detailed in Table 3A and Table 3B below and the most promising loci (highlighted below) were added to the most promising loci identified from phase 2 above, and tested in phase 4 below. Note that sequence information for key sequences were shared with other New Zealand commercial laboratories actively working on developing detection assays, in order to accelerate the development of these assays for the industry.

Table 3A. List and characteristics of key housekeeping loci identified from Psa genome analysis.

Locus	Characteristic of gene	Primer Name (number)	Isolate Polymorphisms (when compared with the sequence from Psa-V)				
			Psa-V	Psa-LV	Psa-J	Psa-K	Comment other pathovars
Gene 4817	Gene variable between Psa isolates	4817_1F (158), 4817_2F (159), 4817_3F (160), 4817_4F (161), 4817_1R (162), 4817_2R (163), 4817_3R (164), 4817_4R (165)	P	Y	Y	Y	Among 10 most variable *P. syringae* (*P.s*) HK genes
Gene 900	Gene variable between Psa isolates	900_A1F (166), 900_A2F (167), 900_A1R (168), 900_A2R (169), 900_B1F (170), 900_B2F (171), 900_B1R (172), 900_B2R (173)	P	Y	Y	Y	Among 10 most variable *P.s* HK genes
RecN 4407	Gene variable between Psa isolates	recN_A1F (90), recN_A1R (91), recN_A2F (92), recN_A2R (93), recN_A3F (94), recN_A3R (95), recN_B1F (96), recN_B1R (97), recN_B2F (98), recN_B2R (99), recN_B3F (100), recN_B3R (101), recN_B4F (102), recN_B4R (103), recNC1F (110), recNC2F (111), recNC3F (112), recNC1R (113), recNC2R (114), recNC3R (115)	P	Y	Y	Y	Among 10 most variable *P.s* HK genes
Gene 1642	Gene variable between Psa isolates	G1642A1F (104), G1642A2F (105), G1642A3F (106), G1642A4F (107), G1642A1R (108), G1642A2R (109)	P	Y	Y	Y	Among 10 most variable *P.s* HK genes
Tuf1	Gene variable between Psa isolates	Tuf1F (68), Tuf1R (69), Tuf2F (66), Tuf2R (67), Tuf3F(66), Tuf3R (67)	P	Y	Y	Y	Common HK gene used for identifying bacteria

Table 3A key: Y=polymorphisms compared with Psa-V present , N= polymorphisms compared with Psa-V not present. P =Sequence present in Psa-V.

Table 3B. List and characteristics of key effector loci identified from Psa genome analysis.

Locus	Characteristic of gene	Primer Name (number)	Isolate Polymorphisms (compared with the sequence from Psa-V)				
			Psa-V	Psa-LV	Psa-J	Psa-K	Comment other pathovars
hopA1	Interrupted gene	HopA1F2 (83) HopA1R1 (84)	P	Y	A	Y	Unique interruption event
hopZ2b	Novel Psa-V effector	HopZ2bF2-L (116) , HopZ2bR2 (119)	P	A	A	A	Absent in most other pathovars
hopH1	Novel Psa-V effector	HopH1F1 (77), HopH1R1 (78), HopH1F2 (79), HopH1R2 (80)	P	A	A	A	Highly similar effectors common
hopAM1	Highly variable effector	hopAM1-F1 (139), hopAM1-R1 (140), hopAM1-R2 (141), hopAM1-F2 (142), hopAM1-R3 (143), hopAM1-R4(144), hopAM1-R5 (145)	P	A	Y	A	Identified as variable in Baltrus et al. (2011)
hopM1	Novel 5' region	hopM-F1(128),hopM1-R1(127), hopM1-R2(126),hopM1-R3(125), hopM1R4(136)	P	Y	Y	N	

Table 3B key: Y=polymorphisms compared with Psa-V present , N= polymorphisms compared with Psa-V not present. P =sequence present in Psa-V, A=sequence absent.

3.4 Results for phase 4

The analyses from phases 3 and 4 were joined together and the best loci for assay development were chosen based on the criterion of ease of interpretation and likelihood of specificity in qPCR. The simplest scenario looked for was the design of an assay that resulted in a presence/absence polymorphism. Ideally the locus or amplification strategy devised would also be unlikely to yield any amplification in "off target" pathovars of *P. syringae* or related bacterial species. As it was not certain that appropriate loci that had these characteristics could be identified, preliminary research was also performed with loci showing only single nucleotide polymorphisms (SNPs).

A qPCR assay that is able to distinguish SNP differences by high resolution melting (HRM) point differences between the amplicons in the various isolates of Psa and other pathovars of *P. syringae* showing "off target" amplification will need to be developed for loci that display only minor sequence variation. For these reasons, the first preference was loci that would be likely to amplify loci only in Psa-V and not in other isolates of Psa or other pathovars of *P. syringae.* In particular, loci that are unique to Psa-V and/or loci with gross rearrangements – where these rearrangements are only present in Psa-V - were preferred for further testing and development.

Amplicon priming sites were designed to minimise the likelihood of amplification from other pathovars. There are, however, constraints on the size of the amplified region that compromise the ability to use the most variable parts of a particular region as priming sites. Thus instances of amplification from "off-target" related bacteria are sometimes a possibility. Where other amplifications might be expected from these related organisms, amplified fragments were designed to usually contain additional variations, in order to be able to distinguish them from homologous amplicons should these occur because of inefficient priming with mismatches from other related pathovars. In order for these loci to be converted to commercial assays, they would require the ability to distinguish isolates by the different melting points (Tm) of the amplified qPCR products. While differentiation can often be achieved for pure bacterial cultures when these melting point temperatures are quite close, the added complication of having to perform these tests on plant DNA extractions in order for the assay to be suitably rapid suggests that these assays could be quite hard to interpret reliably in a commercial laboratory.

For these reasons, a simpler presence/absence (P/A) assay was preferred. Some preliminary research to test the feasibility of HRM assays in plant material was performed and this indicated that products with very close melting temperatures would indeed be hard to differentiate in a reliable manner. As the key was to make rapid progress in a test that could be offered to industry, some potential assays that would require HRM assays to be developed were therefore not always pursued.

Primers for the five effector and five housekeeping loci were redesigned to encompass polymorphisms between Psa and other pathovars of *P. syringae*. These redesigned primers were then retested across Psa-V, Psa-LV, Psa-J and Psa-K isolates using qPCR. Then the best loci (hopA1, hopZ2b, hopAF1, avrE1, hopM1, Tuf1) were tested by qPCR across the bacterial isolates listed in Appendix 1 for specificity. This analysis rapidly identified two key effector loci (hopA1, hopZ2b) as offering the best loci for designing a specific test. These loci showed the least amount of "off target" amplification when tested against the pathovar set in Appendix 1. At 65°C, none of the other pathovars produced qPCR products at Cq values below 35 cycles for the best primer combinations for these assays (83, 84 and 116,119 respectively). The data also suggested that only Psa-V was able to amplify products with these primer sets at Cq values below 35 cycles. In order to submit these loci to an even more rigorous test, these two key loci

(hopA1, hopZ2b) were also tested across an additional set of bacterial strains listed in Appendix 2. This included several more representative pathovars that are likely to occur on kiwifruit leaves (such as *P. viridiflava,* see notes in appendix keys).

Note that the 95-member *P. syringae* pathovar set used to test the specificity of the haplotype assays developed is listed in Appendix 1, while Appendix 2 lists the set of 52 additional bacteria used to test the specificity of the key haplotype assays developed.

Table 4. Quantitative PCR analysis of specificity of primer combinations.

Locus	Primer numbers	qPCR Ta	P/A[*1] or HRM candidate	Primer Name	Forward and Reverse Primer Sequence	Psa comparison with list of pathovars that amplify with Cq below 40 cycles		
						Amplifying pathovar	Cq	Tm[*2]
Psa ITS[*3]	F1 R2	65°c	P/A	PsaF3 PsaR4	ACC TGG TGA AGT TGG TCA GAG C CGC ACC CTT CAA TCA GGA TG	*P. syringae* pv. actinidiae	22.7	88.17
						P. amygdali	18.64	88.28
						P. syringae pv. ciccaronei	19.84	88.35
						P. syringae pv. helianthi	19.77	88.35
						P. syringae pv. raphiolepidis	18.43	88.3
						P. syringae pv. spinaceae	35.81	88.36
avrE1	48 51	60°c	HRM	avrE1-D5F avrE1-D5R	GAC GCG CTT TGC TCA ACC CAT AAG GGC TGA AAC TCA GCC ACC AG	*P. syringae* pv. actinidiae	21.15	85.02
						11 pathovars	15-30	85-2-86.5
						42 pathovars	>35	85-3-86.5
	49 51	60°c	HRM	avrE1-D6F avrE1-D5R	AAG ACG CGC TTT GCT CAA CCC AAG GGC TGA AAC TCA GCC ACC AG	*P. syringae* pv. actinidiae	21.37	85.08
						18 pathovars	15-30	85.6-86.5
						3 pathovars	>35	86.5
hopA1(V)	83 84	65°C	P/A	hopA1F2 hopA1R1	GCCTCGATGTCGGCGC ATTCGATAGAAGAACTTCTTTGCGTTT	*P. syringae* pv. actinidiae Psa-V only	22-24	84.9
						Amplification Psa-LV, Psa-J, Psa-K and other pathovars	none[*4]	-
hopA1(LV, K)	84 85	65°C	P/A	hopA1R1 hopA1F1	ATTCGATAGAAGAACTTCTTTGCGTTT GTTCCTGCACACGCTAAAGGAG	*P. syringae* pv. actinidiae Psa-LV and Psa-K	22-30	82.8-82.9
						7 other pathovars[*5]	20-26	82.5 - 82.9

Table 4 (continued). Quantitative PCR analysis of specificity of primer combinations.

Locus	Primer numbers	qPCR Ta	P/A[*1] or HRM candidate	Primer Name	Forward and Reverse Primer Sequence	Psa comparison with list of pathovars that amplify with Cq below 40 cycles		
						Amplifying pathovar	Cq	Tm[*2]
hopZ2b	81 82	55°C	HRM	hopZ2bF3 hopZ2bR3	CATCAAAACCCCGCACAAC GCCAGTCTCTTCAGGCTCTGG	*P. syringae* pv. actinidiae Psa-V only	27-30	80.3
						Psa-LV, Psa-J, Psa-K and other pathovars	34-38	79.0-79.7
	116 119	60°C	P/A	hopZ2bF2-L hopZ2bR2	ACAACTTCAGGCTACAATACTTACGC CTCAGGATGCGTTTCGGTTAC	*P. syringae* pv. actinidiae Psa-V		80.7
						P. syringae pv. coronafaciens	28.66	81
	117 120	60°C	P/A	hopZ2bF3' hopZ2bR3'	TACTCCAGAGCCTGAAGACAC GCCTCTTTAAGGGCAATAGCT	*P. syringae* pv. actinidiae	22.91	79.89
						P. cannabina	19.11	79.77
						P. syringae pv. coronafaciens	16.62	81.04
						P. tremae	16.29	80.75
	118 121	60°C	P/A	hopZ2bF4, hopZ2bR4	TTTCTAATAACACCCACTTAGC GTATGATGCGTAAGTATTGTA	*P. syringae* pv. actinidiae Psa-V	23	79.5
						P. syringae pv. coronafaciens	26	79.5
	116 119	65°C	P/A	hopZ2bF2-L hopZ2bR2	ACAACTTCAGGCTACAATACTTACGC CTCAGGATGCGTTTCGGTTAC	*P. syringae* pv. actinidiae Psa-V	23	80.5
						Amplification Psa-LV, Psa-J, Psa-K and other pathovars	none[*4]	-
hopM1	128 125	65°C	HRM	hopMF1 hopM1R3	ATGAGCGACATGAGAATCAATGT GCTGCTGGGAGCGACCGCATTG	*P. syringae* pv. actinidiae Psa-V, Psa-J Psa-K	24-25	86.6
						Amplifies in 5 pathovars	18-23	86.6-86.8
hopAF1	73 74	60°C	HRM	hopAF1C1R hopAF1C1AF	GACCGTCTAGTAGTAGCTATCGTG TCATCTCGTCTGATGCTCTGTAG	27 pathovars	15-35	81.4-82.2
Tuf1	68 70	60°C	HRM	Tuf1F Tuf3R	GTACGGAAGTAGAACTGTGG CGGCGTTCTGTTGCGCGGC	Amplified in several other pathovars and Psa-LV with an HRM peak close to 87.4 (Psa-V)		

Table 4 Key: [*1] P/A=presence absence polymorphism HRM=high resolution melting point polymorphism [*2] Note the exact Tm (melting point) is dependent on a number of factors including the qPCR equipment utilised and the exact composition of the qPCR amplification mixtures -therefore the actual Tm needs to be established empirically in each laboratory.[*3] The Psa ITS locus is the original locus used to identify Psa and is included here for comparison to the new loci – this assay is derived from Rees-George et al. (2010); the other Psa ITS primer set F3/R4 gave very similar results. [*4] No pathovars amplified with Cq values below 35 and a similar melting point. These tests included screening an additional set of bacterial isolates identified in Appendix 2. [*5]As this LV-specific amplification cross-amplifies in other "off-target" pathovars, additional test are required to provide additional proof that Psa-LV is present - the pathovar samples where these primers amplified a product were *P. syringae* pv. tomato ICMP7230, *P. syringae* pv. viburni ICMP3963, *P. syringae* pv. mori ICMP4331, *P. syringae* pv. ciccaronei ICMP5710, *P. syringae* pv. broussonetiae 13650, *P. cannabina* ICMP2823 and *P. amygdali* ICMP3918. The following genes that were HRM candidates were not pursued, as greater priority was placed on P/A candidates: Gene 4817, Gene 900, RecN, Gene 1642 and hopAM1

3.5 Results for phase 5

Phase 5 results are presented in Appendix 3 - a report submitted to KVH by Hill Laboratories Limited. This was an independent test in a commercial laboratory of the key assays developed in this project. These tests indicated that both loci performed well and could detect the presence of Psa-V using the DNA extraction procedures developed to detect Psa with ITS primers F1/R2 or F3/R4 (Rees-George et al. 2010). The testing of a significant number of positive and negative samples identified by the previous ITS-based qPCR test indicated there was a good correlation between the old and new test. As more than one hundred samples tested negative with both the old and the two new test loci, this suggest there is only a low probability that other organisms normally present on kiwifruit material are capable of interfering with the new tests. Note that the appendices referred to in this section have been amended by addition of the name 'Hill', to distinguish them from appendices with the same number in the rest of this report. Note furthermore that the comment in the Hill Laboratories Ltd report that the hopZ2b primers also detect *P. syringae* pv. *coronafaciens* were based on the experiments completed at that time. Further experiments at a higher temperature indicated that this pathovar does not amplify with these primers at the higher qPCR annealing temperature of 65°C.

4 Discussion

4.1 Interpretation of qPCR results

It should be noted that some pathovars did show late amplification of products with the two key primer combinations (at cycle numbers greater than 35 cycles), but for these samples the melting points were easily distinguishable from the melting point of Psa-V and there was a cycle number difference of at least 10 cycles between these amplifications and the amplification of a general bacterial locus (23S) included as a control to verify the amplification competence of the bacterial DNA samples. This indicates that this "off-target" amplification is very inefficient for these pathovars and should be able to be identified by unusual melting points. It should further be noted that some of the Psa-LV DNA samples also gave amplification at late cycles (greater than 35). In these cases, the product melting temperature was not distinguishable from the Psa-V melting point. After further extensive testing of these samples in collaboration with a commercial test provider (DNature), it became clear that these samples probably suffered from a very low amount of cross-contamination. They were produced in DNA extraction batches where Psa-V samples were also previously extracted in close physical proximity (as dictated by the internal rules instituted to restrict the laboratories where initial research with the Psa bacterium could be performed). New DNA extractions tested did not usually suffer from these late amplifying peaks. This is a problem with highly sensitive qPCR-based tests.

As for other qPCR-based Psa tests – a result in the region of 30-35 cycles should be repeated, whereas results with a cycle number of greater than 35 need to be interpreted with great care. These results do not necessarily mean a false positive, but could instead mean the amount of the organism present is below the ability of the test to be able to identify the presence of the organism conclusively, and therefore are best described as "not-detected". It should be noted that every test has its detection limit. More research would be needed to identify exactly how much further this detection limit could be driven down (i.e. the cycle number where a result is still called positive increased) in any particular laboratory setting. The observation that very low amounts of cross-contamination can also give a positive in these cycle ranges suggests it may be counter-productive to drive the detection down much further.

We noted a general tendency for amplification with the key hopA1 and hopZ2b primer sets to be 3-5 cycles behind the amplification with the original ITS primer sets. This is to be expected, as the ITS locus is believed to be a multi-copy locus – whereas the two effector loci hopA1 and hopZ2b are probably single copy loci. This does mean that there is a greater tendency for these new tests to give results in the 30-35 cycle zone where interpretation is more difficult. In cases where the cycle number at which a product is detected is high, backing up the result with the original ITS primer set could give greater confidence in returning a positive result, particularly if the difference in cycle number between the two new tests and the ITS-based test is within the 3-6 cycle range. If, however, the difference in cycle number between the tests is much greater than this, the possibility of interference from "off-target" organisms able to be detected by the ITS primer set (see results above) needs to be taken into account.

5 Appendices

5.1 Appendix 1.The *Pseudomonas syringae* pathovar set used to test the specificity of the haplotype assays developed.

code	Name	ICMP
amyg3918	*P. amygdali*	3918
avel9746	*P. avellanae*	9746
cann2823	*P. cannabina*	2823
cari2855	*P. caricapapayae*	2855
cich5707	*P. cichorii*	5707
ficu7848	*P. ficuserectae*	7848
meli6289	*P. meliae*	6289
frax7711	*P. savastanoi* pv. fraxini	7711
glyc2189	*P. savastanoi* pv. glycinea	2189
neri16943	*P. savastanoi* pv. nerii	16943
phas2740	*P. savastanoi* pv. phaseolicola	2740
phas637	*P. savastanoi* pv. phaseolicola	637
phas4324	*P. savastanoi* pv. phaseolicola	4324
phas5059	*P. savastanoi* pv. phaseolicola	5059
reta16945	*P. savastanoi* pv. retacarpa	16945
sava4352	*P. savastanoi* pv. savastanoi	4352
acer2802	*P. syringae* pv. aceris	2802
acti9617	*P. syringae* pv. actinidiae	9617
aesc8947	*P. syringae* pv. aesculi	8947
alis15200	*P. syringae* pv. alisalensis	15200
anti4303	*P. syringae* pv. antirrhini	4303
apii2814	*P. syringae* pv. apii	2814
apta459	*P. syringae* pv. aptata	459
atro4394	*P. syringae* pv. atrofaciens	4394
atrp4457	*P. syringae* pv. atropurpurea	4457
avii14479	*P. syringae* pv. avii	14479

Appendix 1 (continued).The *Pseudomonas syringae* pathovar set used to test the specificity of the haplotype assays developed.

code	Name	ICMP
brou13650	*P. syringae* pv. broussonetiae	13650
cast9419	*P. syringae* pv. castaneae	9419
cera17524	*P. syringae* pv. cerasicola	17524
cicc5710	*P. syringae* pv. ciccaronei	5710
cori12471	*P. syringae* pv. coriandricola	12471
cori9625	*P. syringae* pv. coriandricola	9625
cori9829	*P. syringae* pv. coriandricola	9829
cori12341	*P. syringae* pv. coriandricola	12341
coro3113	*P. syringae* pv. coronafaciens	3113
cory17001	*P. syringae* pv. coryli	17001
cunn11894	*P. syringae* pv. cunninghamiae	11894
daph9757	*P. syringae* pv. daphniphylli	9757
delp529	*P. syringae* pv. delphinii	529
dend9150	*P. syringae* pv. dendropanacis	9150
erio4455	*P. syringae* pv. eriobotryae	4455
garc4323	*P. syringae* pv. garcae	4323
garc5019	*P. syringae* pv. garcae	5019
garc4466	*P. syringae* pv. garcae	4466
garc3649	*P. syringae* pv. garcae	3649
heli4531	*P. syringae* pv. helianthi	4531
heli3263	*P. syringae* pv. helianthi	3263
heli827	*P. syringae* pv. helianthi	827
heli11933	*P. syringae* pv. helianthi	11933
hibi9623	*P. syringae* pv. hibisci	9623
lach3507	*P. syringae* pv. lachrymans	3507
laps3947	*P. syringae* pv. lapsa	3947
macu3935	*P. syringae* pv. maculicola	3935
macu2744	*P. syringae* pv. maculicola	2744
macu4981	*P. syringae* pv. maculicola	4981
macu11281	*P. syringae* pv. maculicola	11281
mell5711	*P. syringae* pv. mellea	5711
mori4331	*P. syringae* pv. mori	4331
mors18416	*P. syringae* pv. morsprunorum	18416
mors568	*P. syringae* pv. morsprunorum	568
mors4983	*P. syringae* pv. morsprunorum	4983
mors3897	*P. syringae* pv. morsprunorum	3897
myri7118	*P. syringae* pv. myricae	7118
oryz9088	*P. syringae* pv. oryzae	9088
papu4048	*P. syringae* pv. papulans	4048
pass129	*P. syringae* pv. passiflorae	129
pers5846	*P. syringae* pv. persicae	5846

Appendix 1 (continued).The *Pseudomonas syringae* pathovar set used to test the specificity of the haplotype assays developed.

code	Name	ICMP
phil8903	*P. syringae* pv. philadelphi	8903
phot7840	*P. syringae* pv. photiniae	7840
pisi2452	*P. syringae* pv. pisi	2452
prim18417	*P. syringae* pv. primulae	18417
porr8961	*P. syringae* pv. porri	8961
raph9756	*P. syringae* pv. raphiolepidis	9756
ribi3883	*P. syringae* pv. ribicola	3883
sesa763	*P. syringae* pv. sesami	763
spin16929	*P. syringae* pv. spinaceae	16929
stria3961	*P. syringae* pv. striafaciens	3961
stria4418	*P. syringae* pv. striafaciens	4418
stria4483	*P. syringae* pv. striafaciens	4483
syri3023	*P. syringae* pv. syringae	3023
taba2835	*P. syringae* pv. tabaci	2835
tage4091	*P. syringae* pv. tagetis	4091
thea3923	*P. syringae* pv. theae	3923
toma2844	*P. syringae* pv. tomato	2844
toma2841	*P. syringae* pv. tomato	2841
toma4263	*P. syringae* pv. tomato	4263
toma7230	*P. syringae* pv. tomato	7230
ulmi3962	*P. syringae* pv. ulmi	3962
vibu3963	*P. syringae* pv. viburni	3963
ziza8921	*P. syringae* pv. zizaniae	8921
trem9151	*P. tremae*	9151
viri2848	*P. sp*	2848
kiwi3272	*P. sp*	3272
kiwi11296	*P. sp*	11296
fluo3512	*P. fluorescens*	3512

Appendix key: Note samples viri2848, kiwi3272 and kiwi11296 are listed as *P. sp.* in this pathovar set but are classified as *P. viridflava* by some people, depending on their origin (see discussion in Rees-George et al. 2010).

5.2 Appendix 2. The additional fifty-two bacteria used to test the specificity of the key haplotype assays developed.

Template no.	Species name	Strain
1	*Agrobacterium rhizogenes*	ICMP3379
2	*A. rhizogenes*	ICMP8304
3	*A. rhizogenes*	ICMP8308
4	*Clavibacter michiganensis* subsp*. michiganensis*	ICMP2551
5	*Clavibacter michiganensis* subsp*. michiganensis*	ICMP2354
6	*Erwinia amylovora*	ICMP8865
7	*Erwinia amylovora*	ICMP12365
8	*Erwinia carnegeiana*	ICMP5701
9	*Erwinia carotovora* subsp*. carotovora*	ICMP11523
10	*Erwinia carotovora* subsp*. carotovora*	ICMP11524
11	*Erwinia chrysanthemi*	ICMP6926
12	*Erwinia chrysanthemi*	ICMP6928
13	*Erwinia herbicola*	EH252
14	*Erwinia herbicola*	D4
15	*Escherichia coli*	JM109
16	*Pseudomonas cichorii*	ICMP3521
17	*Pseudomonas cichorii*	ICMP5707
18	*Pseudomonas corrugata*	ICMP5819
19	*Pseudomonas corrugata*	ICMP8898
20	*Pseudomonas fluorescens* 5A	526
21	*Pseudomonas fluorescens* 5B	599
22	*Pseudomonas marginalis*	ICMP8127
23	*Pseudomonas marginalis*	ICMP9503
24	*Pseudomonas marginalis* 4A	754
25	*Pseudomonas syringae* pv. papulans	ICMP4043
26	*Pseudomonas syringae* pv. actinidiae	ICMP9617
27	*Pseudomonas syringae* pv. actinidiae	ICMP9855
28	*Pseudomonas syringae* pv. papulans	ICMP4055
29	*Pseudomonas syringae* pv. syringae	415
30	*Pseudomonas syringae* pv. syringae	ICMP2443
31	*Pseudomonas syringae* pv. syringae	ICMP3676
32	*Pseudomonas syringae* pv. syringae	ICMP3938
33	*Pseudomonas syringae* pv. syringae	ICMP4268
34	*Pseudomonas syringae* pv. syringae	ICMP4610
35	*Pseudomonas syringae* pv. syringae	ICMP5823

Appendix 2 (continued). The additional fifty-two bacteria used to test the specificity of the key haplotype assays developed.

Template no.	Species name	Strain
36	*Pseudomonas syringae* pv. tomato	ICMP3449
37	*Pseudomonas syringae* pv. tomato	ICMP4259
38	*Pseudomonas syringae* pv. tomato	ICMP4608
39	*Pseudomonas syringae* pv. tomato	ICMP9501
40	*Pseudomonas viridiflava*	ICMP8943
41	*Pseudomonas viridiflava*	ICMP8952
42	*Pseudomonas viridiflava*	ICMP11126
43	*Xanthomonas campestris* pv. pruni	2.4
44	*Xanthomonas campestris* pv. pruni	96.08
45	*Xanthomonas campestris* pv. phaseoli	ICMP2722
46	*Xanthomonas campestris* pv. phaseoli	ICMP3403
47	*Xanthomonas campestris* pv. populi	ICMP9367
48	*Xanthomonas campestris* pv. populi	ICMP9369
49	*Xanthomonas campestris* pv. vesicatoria	ICMP7383
50	*Xanthomonas campestris* pv. vesicatoria	18g
51	*Xanthomonas* sp.	36A
52	*Xanthomonas* sp.	36B

Appendix key: Template numbers 40, 41 and 42 are listed as *P. viridiflava* in this bacterial isolate set but may be classified as *P. sp.* by some people depending on their origin (see discussion in Rees-George et al. 2010).

5.3 Appendix 3. A report on the performance of two Psa-V assays submitted to KVH by Hill Laboratories Limited.

Validation of a PCR assay to detect the *Psa*-V haplotype in Kiwifruit plant tissue

Plant & Food Research Ltd and Hill Laboratories Ltd

September 21, 2011

Introduction

The plant pathogen *Pseudomonas syringae* pv. *actinidiae* (*Psa*) is causing significant losses for the New Zealand kiwifruit industry. In the last quarter of 2010, two strains of *Psa* – virulent (V) and low virulent (LV) – were identified in kiwifruit orchards. The virulent haplotype's prevalence is currently restricted to the Tauranga region while the low virulent haplotype has been discovered in most of the kiwifruit growing areas of New Zealand. The V-strain of Psa causes significant damage to kiwifruit plant while the LV-strain is believed to only cause leaf spotting.

A DNA-based test for *Psa* developed by MAF using the polymerase chain reaction (PCR) method and the primer sequences named F1/R2 cannot differentiate between the low virulent (LV) and virulent (V). This test was used extensively at the beginning of 2011 to identify *Psa*-infected orchards. Further resolution of LV or V infection required isolation and culturing of *Psa* bacteria before limited DNA sequencing at Landcare Research Ltd. This process took up to two weeks.

A more rapid two-test PCR method was developed and offered as a 48-hour turnaround service to the kiwifruit industry by Verification Laboratory Services Ltd in Te Puke. However, although this test can distinguish between the V and LV strains of *Psa*, it can produce false positive results by reacting with seven other types of pseudomonas bacteria.

Since significant financial decisions are being made on the basis of a Psa-V test result the kiwifruit industry identified a need to develop a *Psa*-V specific test. ZESPRI commissioned Plant and Food Research Ltd (PFR) to identify unique DNA sequences that could be used to develop DNA primers for a PCR assay that would be highly specific for Psa-V without cross reacting with other microorganisms found on kiwifruit.

Hill Laboratories Ltd has collaborated with PFR to assist in the laboratory validation of the new highly specific PCR test for the Psa-V strain.

This report summaries the validation methodology and validation result of two sets of DNA primers developed by PFR.

Validation Methodology

After development and selection of primers set based on *in silico* comparison of sequence uniqueness, Psa-V specific primers sets were incorporated into conventional-PCR or quantitative-PCR assay systems and optimized for PCR cycle temperatures, primer concentrations and nucleotide concentrations.

Plant and Food Research Ltd's laboratory assessed two primer sets (labeled 83/84 from the hopA1 gene, and hopZ2bF2/R2) by testing for cross reactivity against 95-isolated pseudomonas or related microorganisms from the International Collection of Microorganisms from Plants (ICMP) held at Landcare Ltd.

Concurrently, validation at Hill Laboratories entailed testing both primer sets against 90 plant extracts (leaves and canes) previous found to be either *Psa*-V or *Psa*-LV positive, and 108 plant extracts found to have no detectable *Psa*, using the original F1/R2 primers set developed by MAF.

For part of the validation, an additional primer '85' was added to the 83/84-primer set. This allowed simultaneous detection of the LV-strain. However, the '85' primer also cross reacts with seven other strains of pseudomonas. This additional primer was used to identify samples that were *Psa*-LV-like. We do not intend to use this additional primer in the final *Psa* test.

Validation Results

The 83/84-primer set was highly specific for Psa-V isolated organisms. None of the 95 ICMP organisms (see Appendix 1.Hill) tested by PFR against the 83/84 Psa–V specific primer set gave a positive response and gave a T_m (melt temperature) in the region expected for *Psa*-V in the optimized PCR assay.

Only one organism, *Pseudomonas syringae* pv*. coronafaciens* (causes blight on oats) cross reacted with the hopZ2bF2/R2 primer set.

In comparison, the original F1/R2 primer set, used at the onset of the orchard infections (and currently being used), cross reacted with *Pseudomonas syringae* pvs: *ciccaronei, helianthi, raphiolepidis,* and *spinaceae.* Although not tested in the laboratory, there is also *in silico* sequence evidence that the F1/R2 primer set cross reacts with *Pseudomonas syringae* pvs: *theae, morspronorum* and *passiflorae*. The F1/R1 primer set will not be used in the new assay.

DNA extracts from leaf and cane samples taken from orchards between February and September 2011 and previously tested for *Psa* using the original F1/R2 primer set were tested using both validation primer sets. Of the 90 samples that were *Psa* positive to the F1/R2 primer set, 73 were determined to be Psa-V positive to the 83/84 and 71 (2 samples had insufficient volumes) hopZ2bF2/R2 primer sets. Only one sample did not react to either primer set. Using the additional '85' primer, with the 83/84-primer set, the remaining 16 extracts were identified as

Psa-LV (or LV-like)[1]. (See Appendix2.Hill) The '85' primer cannot be added to the hopZ2bF2/R2 primer set to also detect *Psa*-LV.

Of the 90 plant extracts that tested positive to *Psa,* using the F1/R2 primer set (original set), 31 samples were also tested by Landcare for haplotype identification using DNA sequencing. Where LandCare was able to isolate and culture *Psa* bacteria and subsequently sequence DNA from these organisms, it confirmed the *Psa* haplotype results found when using the 83/84/85 and hopZ2bF2/R2 primer sets. Both laboratories identified 15 samples as *Psa*-LV (Appendix 3.Hill) and Landcare confirmed that another 14 samples identified as *Psa*-V by the 83/84/85 and hopZ2bF2/R2, were the virulent strain (Appendix 4.Hill). Two samples determined to be Psa-V using the 83/85 and hopZ2b primer sets did not produce Psa-positive bacteria colonies at LandCare. One of these samples was noted as showing signs of deterioration before attempts were made to isolate bacterial colonies. DNA sequencing requires isolation and culturing of viable bacteria. It is likely that *Psa*-V positive results obtained by PCR came from non-viable (dead) bacteria.

Plant extracts, found to have no detectable *Psa* using the original F1/R2 primers, were also testing using the 83/84/85 and hopZ2bF2/R2 primer sets. No *Psa*-positive PCR results were found. (See Appendix 5.Hill).

Conclusions and Recommendation

- Plant and Food Research has successfully developed one primer set (83/84) that is highly specific for Psa-V and another primer set (hopZ2bF2/R2) that cross reacts to only one other *P. syringae* pathovar found on oats.
- Extensive validation against 94 pseudomonas showed a very high degree of specificity of the 83/84 primer set for *Pseudomonas syringae* pv. *actinidiae*
- The 83/84-primer set test only requires one assay, and no confirmatory second assay, to detect *Psa*-V with a high degree of certainty.
- Addition of a third primer (85) to the 83/84 pair can give an indication of the *Psa*-LV haplotype although the 85 primer does cross-react with some other *Pseudomonas* organisms
- The hopZ2b primer set can also be used as a one-test *Psa*-V test, with a very slight possibility of cross reacting to one known other organism only found on oats.
- The recommendation is that the new one-test PCR method using the 83/84 primer set developed by Pant and Food Research is adopted to identify the *Psa*-V haplotype in kiwifruit plant tissue.

[1] Note the 85-primer, pairing with the 84-primer, cross reacts with *P. amygdale, P. cannabina, P. syringae* pvs *broussonetiae, ciccaronei, mori, tomato, viburni.*

Appendix 1.Hill

List of 95-isolated Pseudomonas or related microorganisms from the International Collection of Microorganisms from Plants (ICMP) held at Landcare Ltd used to validate assay specificity

#	Code	Name
I01	amyg3918	P. amygdali
I02	avel9746	P. avellanae
I03	cann2823	P. cannabina
I04	cari2855	P. caricapapayae
I05	cich5707	P. cichorii
I06	ficu7848	P. ficuserectae
I07	meli6289	P. meliae
I08	frax7711	P. savastanoi pv. fraxini
I09	glyc2189	P. savastanoi pv. glycinea
I10	neri16943	P. savastanoi pv. nerii
I11	phas2740	P. savastanoi pv. phaseolicola
I12	phas637	P. savastanoi pv. phaseolicola
I13	phas4324	P. savastanoi pv. phaseolicola
I14	phas5059	P. savastanoi pv. phaseolicola
I15	reta16945	P. savastanoi pv. retacarpa
I16	sava4352	P. savastanoi pv. savastanoi
I17	acer2802	P. syringae pv. aceris
I18	acti9617	P. syringae pv. actinidiae
I19	aesc8947	P. syringae pv. aesculi
I20	alis15200	P. syringae pv. alisalensis
I21	anti4303	P. syringae pv. antirrhini
I22	apii2814	P. syringae pv. apii
I23	apta459	P. syringae pv. aptata
I24	atro4394	P. syringae pv. atrofaciens
I25	atrp4457	P. syringae pv. atropurpurea
I26	avii14479	P. syringae pv. avii
I27	brou13650	P. syringae pv. broussonetiae
I28	cast9419	P. syringae pv. castaneae
I29	cera17524	P. syringae pv. cerasicola
I30	cicc5710	P. syringae pv. ciccaronei
I31	cori12471	P. syringae pv. coriandricola
I32	cori9625	P. syringae pv. coriandricola
I33	cori9829	P. syringae pv. coriandricola
I34	cori12341	P. syringae pv. coriandricola
I35	coro3113	P. syringae pv. coronafaciens
I36	cory17001	P. syringae pv. coryli
I37	cunn11894	P. syringae pv. cunninghamiae
I38	daph9757	P. syringae pv. daphniphylli
I39	delp529	P. syringae pv. delphinii
I40	dend9150	P. syringae pv. dendropanacis
I41	erio4455	P. syringae pv. eriobotryae
I42	garc4323	P. syringae pv. garcae
I43	garc5019	P. syringae pv. garcae
I44	garc4466	P. syringae pv. garcae
I45	garc3649	P. syringae pv. garcae
I46	heli4531	P. syringae pv. helianthi
I47	heli3263	P. syringae pv. helianthi

#	Code	Name
I48	heli827	P. syringae pv. helianthi
I49	heli11933	P. syringae pv. helianthi
I50	hibi9623	P. syringae pv. hibisci
I51	lach3507	P. syringae pv. lachrymans
I52	laps3947	P. syringae pv. lapsa
I53	macu3935	P. syringae pv. maculicola
I54	macu2744	P. syringae pv. maculicola
I55	macu4981	P. syringae pv. maculicola
I56	macu11281	P. syringae pv. maculicola
I57	mell5711	P. syringae pv. mellea
I58	mori4331	P. syringae pv. mori
I59	mors18416	P. syringae pv. morsprunorum
I60	mors568	P. syringae pv. morsprunorum
I61	mors4983	P. syringae pv. morsprunorum
I62	mors3897	P. syringae pv. morsprunorum
I63	myri7118	P. syringae pv. myricae
I64	oryz9088	P. syringae pv. oryzae
I65	papu4048	P. syringae pv. papulans
I66	pass129	P. syringae pv. passiflorae
I67	pers5846	P. syringae pv. persicae
I68	phil8903	P. syringae pv. philadelphi
I69	phot7840	P. syringae pv. photiniae
I70	pisi2452	P. syringae pv. pisi
I71	prim18417	P. syringae pv. primulae
I72	porr8961	P. syringae pv. porri
I73	raph9756	P. syringae pv. raphiolepidis
I74	ribi3883	P. syringae pv. ribicola
I75	sesa763	P. syringae pv. sesami
I76	spin16929	P. syringae pv. spinaceae
I77	stria3961	P. syringae pv. striafaciens
I78	stria4418	P. syringae pv. striafaciens
I79	stria4483	P. syringae pv. striafaciens
I80	syri3023	P. syringae pv. syringae
I81	taba2835	P. syringae pv. tabaci
I82	tage4091	P. syringae pv. tagetis
I83	thea3923	P. syringae pv. theae
I84	toma2844	P. syringae pv. tomato
I85	toma2841	P. syringae pv. tomato
I86	toma4263	P. syringae pv. tomato
I87	toma7230	P. syringae pv. tomato
I88	ulmi3962	P. syringae pv. ulmi
I89	vibu3963	P. syringae pv. viburni
I90	ziza8921	P. syringae pv. zizaniae
I91	trem9151	P. tremae
I92	viri2848	P. sp
I93	kiwi3272	P. sp
I94	kiwi11296	P. sp
I95	fluo3512	P. fluorescens

Appendix 2.Hill

Samples tested with primer sets: F1/R2 (original set), 83/84/85, and hopZ2b F2/R2.

Sample No.	Job Number	Sample Type	F1/R2 Primer Set Cq	83/84/85 Primer Set		HopZ2 Primers	
				Cq	Melt Profile	Cq	Melt Profile
1	893088/1	Leaf	14.98	20.67	V	19.94	V
2	893088/2	Leaf	18.40	23.34	V	22.75	V
3	893088/3	Leaf	16.26	22.87	V	21.61	V
4	893472/1	Leaf	18.66	18.63	V	17.42	V
5	893474/1	Leaf	17.13	17.96	V	18.37	V
6	893864/11	Leaf	24.32	27.19	LV	—	
7	893866/5	Leaf	21.21	25.89	V	25.22	V
8	893866/7	Leaf	19.47	24.02	V	24.17	V
9	893869/16	Leaf	27.95	30.85	V	30.27	V
10	893869/19	Leaf	25.21	30.95	V	29.59	V
11	893869/20	Leaf	28.70	35.41	V	32.88	V
12	896344/1	Leaf	19.01	22.7	LV	—	
13	896344/5	Leaf	17.81	26.38	LV	—	
14	896344/19	Leaf	25.40	27.51	LV	—	
15	896354/3	Leaf	19.42	22.09	LV	—	
16	896354/5	Leaf	21.61	23.8	LV	—	
17	896354/7	Leaf	20.08	20.56	LV	—	
18	896354/13	Leaf	21.09	23.05	LV	—	
19	896354/15	Leaf	20.90	23.4	LV	—	
20	896375/1	Leaf	28.50	33.22	V	31.30	V
21	896375/2	Leaf	28.56	33.22	V	33.39	V
22	896344/1	Leaf	18.41	22	LV	—	
23	896344/2	Leaf	27.51	30.99	LV	—	
24	896344/8	Leaf	20.16	21.43	LV	—	
25	896354/2	Leaf	29.17	30.99	LV	—	
26	896354/5	Leaf	30.31	31.47	LV	—	
27	896354/7	Leaf	20.26	21.44	LV	—	
28	897552/1a	Cane	19.07	18.67	V	16.48	V
29	897552/1b	Cane	12.26	19.24	V	19.11	V
30*	897552/1c	Cane	14.02	N/A	No	N/A	
31	899604/1a	Cane	19.33	23.77	V	20.11	V
32	899604/1b	Cane	19.28	21.31	V	19.67	V
33	899604/1c	Cane	16.99	17.36	V	17.20	V
34	905105/1	Leaf	29.70	31.77	LV	—	
35	911283/1b	Cane	17.78	20.55	V	20.19	V
36	911283/2a	Cane	16.88	20.59	V	20.85	V
37	911283/2b	Cane	18.06	22.54	V	21.71	V
38	911283/3b	Leaf	23.79	27.74	V	26.80	V
39	911283/4a	Cane	23.12	26.91	V	25.11	V
40	911283/4b	Cane	21.22	24.71	V	23.30	V
41	917213.1	Cane	16.08	20.69	V	20.02	V
42	917213.1	Cane	15.17	18.96	V	18.23	V
43	917213.1	Cane	16.15	16.22	V	16.13	V
44	917213.1	Cane	18.06	22.32	V	20.93	V
45	892189/1	Leaf	26.61	33.19	V	29.69	V
46	889372/1a	Cane	22.17	25.09	V	24.17	V
47	889372/1b	Cane	19.64	27.79	V	28.43	V
48	889372/1c	Cane	16.85	24.57	V	24.51	V
49	889372/2a	Cane	19.94	29.12	V	28.89	V
50	889372/2b	Cane	13.25	22.55	V	21.60	V
51	889372/2c	Cane	16.17	26.73	V	26.00	V

52	Cane (7)	Cane	17.25	28.44	V	27.29	V
53	889497/20	Leaf	28.18	33.68	V	34.56	V
54	889497/18	Leaf	26.56	33.32	V	31.91	V
55	889500/1	Leaf	23.06	31.17	V	30.78	V
56	887140/3	Leaf	25.04	33.68	V	32.60	V
57	888577/3	Leaf	24.83	34.04	V	‡	-
58	888577/2	Leaf	21.76	32.05	V	‡	-
59	888577/1	Leaf	24.83	36.46	V	32.84	V
60	887549/1	Leaf	19.45	22.41	V	25.19	V
61	886118/1	Leaf	25.42	32.56	V	31.51	V
62	886118/2	Leaf	28.79	33.29	V	32.95	V
63	886118/3	Leaf	27.32	35.09	V	32.80	V
64	886122/2	Leaf	12.67	34.52	V	20.88	V
65	886122/3	Leaf	30.69	30.28	V	39.04	V
66	886124/1	Leaf	23.56	35.22	V	29.87	V
67	886124/2	Leaf	27.95	19.18	V	34.92	V
68	886124/3	Leaf	22.04	28.22	V	27.67	V
69	885045/1	Leaf	23.16	29.33	V	28.77	V
70	885045/2	Leaf	22.25	28.30	V	27.77	V
71	885045/3	Leaf	22.27	27.71	V	28.14	V
72	885045/4	Leaf	23.64	30.65	V	29.75	V
73	902202/1a	Cane	14.24	20.4	V	17.85	V
74	902202/1b	Cane	14.30	18.19	V	16.56	V
75	902202/1c	Cane	13.24	18.33	V	16.95	V
76	902202/1d	Cane	14.66	17.88	V	18.17	V
77	902202/2a	Cane	15.41	19.84	V	21.13	V
78	902202.2	Cane	14.33	18.65	V	19.81	V
79	902202.2	Cane	13.32	17.02	V	16.75	V
80	902202.2	Cane	14.11	20.29	V	16.67	V
81	898876.2	Cane	14.57	21.56	V	24.29	V
82	898876.1	Cane	16.14	20.76	V	19.17	V
83	898876.1	Cane	14.37	19.01	V	16.40	V
84	898876.1	Cane	17.53	22.16	V	20.24	V
85	898876.2	Leaf	25.28	27.31	V	18.42	V
86	898876.1	Cane	17.14	20.94	V	20.10	V
87	898876.2	Cane	13.74	17.33	V	18.08	V
88	898876.3	Cane	15.11	20.09	V	16.70	V
89	898876.3	Cane	13.42	20.44	V	19.40	V
90	898876.3	Cane	15.32	19.37	V	19.32	V

‡ Insufficient sample

* Sample 30 was a “Not Detected” for 83/84/85 and hopZ2 primer sets

Appendix 3.Hill

Samples identified as Psa-LV by PCR using the 83/84/85 primer set and confirmed as Psa-LV by sequencing at LandCare.

Sample No.	Job No.	83/84/85 Primer Set		LandCare Tested*	LandCare Result
		Cq	Melt Profile		
6	893864/11	27.19	LV	Yes	Negative
12	896344/1	22.7	LV	Yes (.2, .5, .8 tested)	(.2 was LV)
13	896344/5	26.38	LV	Yes (.2, .5, .8 tested)	Negative (.2 was LV)
14	896344/19	27.51	LV	(.2, .5, .8 tested)	(.2 was LV)
15	896354/3	22.09	LV	Yes (.2, 3, 5, 7, 13, 14 tested)	LV
16	896354/5	23.8	LV	Yes (.2, 3, 5, 7, 13, 14 tested)	LV
17	896354/7	20.56	LV	Yes (.2, 3, 5, 7, 13, 14 tested)	LV
18	896354/13	23.05	LV	Yes (.2, 3, 5, 7, 13, 14 tested)	LV
19	896354/15	23.4	LV	Yes (.2, 3, 5, 7, 13, 14 tested)	(.2,3,5,7,13,14 were LV)
22	896344/1	22	LV	(.2, .5, .8 tested)	(.2 was LV)
23	896344/2	30.99	LV	(.2, .5, .8 tested)	LV
24	896344/8	21.43	LV	(.2, .5, .8 tested)	(.2 was LV)
25	896354/2	30.99	LV	Yes (.2, 3, 5, 7, 13, 14 tested)	LV
26	896354/5	31.47	LV	Yes (.2, 3, 5, 7, 13, 14 tested)	LV
27	896354/7	21.44	LV	Yes (.2, 3, 5, 7, 13, 14 tested)	LV

* Note: Not all samples from one orchard that were *Psa* positive by PCR were tested by Landcare. For example, Sample 13 (896344/5) was tested as sample '.5' (as were .2 and .8) by Landcare.

Appendix 4.Hill

Samples identified as Psa-V by PCR using the 83/84 and hopZ2b primer sets and confirmed as Psa-V by DNA sequencing at LandCare.

Sample No.	Job No.	83/84/85 Primer Set		hopZ2b Primer Set		LandCare Result	
		Cq	Melt Profile	Cq	Melt Profile	Psa PCR	Haplotype
71	885045.3	27.71	V	28.14	V	Positive	V
61	886118.1	32.56	V	31.51	V	Positive	V
64	886122.2	34.52	V	20.88	V	Positive	V
66	886124.1	35.22	V	29.87	V	Positive	V
56	887140.2	33.68	V	32.6	V	Positive	V
60	887549.1	22.41	V	25.19	V	Positive	V
59	888577.1	36.46	V	32.84	V	Positive	V
46	889372.1	25.09	V	24.17	V	Positive	V
49	889372.2	29.12	V	28.89	V	No Positives*	-
54	889497.18	33.32	V	31.91	V	Positive	V
55	889500.1	31.17	V	30.78	V	Positive	V
1	893088.1	20.67	V	19.94	V	Positive	V
7	893866.5	25.89	V	25.22	V	No Positives **	-
86	898876.1	19.01	V	16.4	V	Positive	V
31	899604.1	23.77	V	20.11	V	Positive	V
35	911283.1	20.55	V	20.19	V	Positive	V

*Could not isolate *Psa* colonies, as sample had deteriorated before isolation attempt.
** No colonies of *Psa* bacteria could be detected

Appendix 5. Hill

Samples tested using the 83/84-primer set, the hopZ2 primer set, and the original primer set (F1/R2) that gave “Not detected” with each primer set.

No.	Wks*	Sample	No.	Wks	Sample	No.	Wks	Sample	No.	Wks	Sample
1	75	32	**35**	77	13	**69**	82	3	**103**	84	2
2	75	33	**36**	77	14	**70**	82	4	**104**	84	3
3	75	34	**37**	77	15	**71**	82	5	**105**	84	5
4	75	35	**38**	77	16	**72**	82	6	**106**	84	8
5	75	36	**39**	77	17	**73**	82	7	**107**	84	9
6	75	37	**40**	77	18	**74**	82	8	**108**	85	3
7	75	38	**41**	77	19	**75**	82	9			
8	75	39	**42**	77	20	**76**	82	10			
9	75	40	**43**	77	21	**77**	82	11			
10	75	41	**44**	77	22	**78**	82	12			
11	75	42	**45**	111	1	**79**	82	13			
12	75	43	**46**	111	8	**80**	82	14			
13	75	44	**47**	111	13	**81**	82	15			
14	75	45	**48**	111	15	**82**	82	16			
15	75	46	**49**	111	18	**83**	83	21			
16	75	47	**50**	104	2	**84**	83	22			
17	76	4	**51**	103	1	**85**	83	23			
18	76	7	**52**	103	11	**86**	83	24			
19	76	10	**53**	102	4	**87**	83	25			
20	76	11	**54**	102	12	**88**	83	26			
21	76	12	**55**	86	10	**89**	83	27			
22	76	13	**56**	86	18	**90**	83	28			
23	76	14	**57**	86	47	**91**	83	29			
24	77	1	**58**	86	60	**92**	83	30			
25	77	2	**59**	85	81	**93**	83	32			
26	77	3	**60**	85	82	**94**	83	33			
27	77	4	**61**	85	86	**95**	83	34			
28	77	5	**62**	85	90	**96**	83	35			
29	77	6	**63**	79	21	**97**	83	36			
30	77	7	**64**	79	61	**98**	83	37			
31	77	8	**65**	79	28	**99**	83	38			
32	77	9	**66**	79	72	**100**	83	39			
33	77	10	**67**	93	4	**101**	83	40			
34	77	12	**68**	82	2	**102**	84	1			

* Test worksheet identification

6 References

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