VI21163: Keeping one step ahead of Psa adaptation

Cover note

Background:

The genome of Pseudomonas syringae pv. Actinidiae (Psa) can be altered by the acquisition of new genes, by deletion of genes or by a mutation(s) on certain genes. These genetic changes happen often in the natural world and often they are harmless. However, sometimes these changes to the genome can be beneficial to Psa and enable it to become more virulent or pathogenic.

We have seen that Psa can rapidly adapt to new conditions. A good example of this has been the widespread appearance of tolerance to copper. In addition, there have also been seasons where Psa infection is higher than usual. Therefore, it is important to monitor Psa strains from commercial kiwifruit orchards to look for genetic evidence of Psa adapting to cultivars or current on-orchard treatments for Psa control. Detecting change in the genome early allows time to consider whether actions are required to limit spread of a new Psa variant, or if different Psa control strategies or management practices are needed.

Aim:

This work aimed to investigate whether there was any evidence of Psa adaption to the tolerant cultivar “Zesy002” (Gold3) either via increased pathogenicity or via development of resistance to Psa control products. Sequenced Psa genomes were analysed for the acquisition or deletion of new genes, or mutations of existing genes that might increase the pathogenic or environmental fitness of Psa.

The genomes analysis included:

- 462 Psa genomes from a range of geographical locations.
- 151 isolates from the 2020 season, including copper resistance survey samples (99) and grower provided samples (52).
- 158 genome sequences from previous surveys carried out by Plant & Food Research.
- 153 publicly available Psa genomes sequenced by the University of Otago between 2012 and 2017.

Key findings:

- There was no evidence for the accumulation of mutations in specific genes within the wider Psa orchard population, nor was there evidence for the domination of a particular isolate.
- The vast majority of newly sequenced Psa isolates have at least one new element acquired by horizontal gene transfer. Most of these are associated with resistance to copper. New plasmids were also found - only one of these showed signs of accumulation in the on-orchard Psa population.
- No significant change in the genes known to be involved in pathogenicity was observed.
Conclusions:

This bi-annual genome survey of Psa strains ensures early detection of any genetic changes that are likely to impact kiwifruit in the future. In this sampling round, no significant changes in the genes known to be involved in pathogenicity was observed, which gives confidence that the Psa genome hasn’t adapted significantly to impact the Gold3 cultivar. However, there is an increase in new genetic elements that aid in conferring resistance to copper. Therefore, genetic mutations on the Psa genomes, resistance genes and new genetic elements will continue to be monitored in the future surveys.
VI21163 Keeping one step ahead of Psa adaptation

Templeton M, Hemara L, Andersen M, Arshed S

December 2021
Confidential report for:

Zespri Group Limited
VI21163

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PUBLICATION DATA


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Executive summary

VI21163 Keeping one step ahead of Psa adaptation

Templeton M, Hemara L, Andersen M, Arshed S
Plant & Food Research Auckland
December 2021

Rationale

*Pseudomonas syringae* pv. *actinidiae* (Psa) has largely been controlled by the introduction of *Actinidia chinensis* var. *chinensis* ‘Zesy002’, and on-orchard practices such as copper and Actigard™ applications. We have seen previously, however, that Psa can rapidly adapt to new conditions with a good example being the widespread appearance of resistance to copper. In addition, there have also been seasons where Psa infection is higher than usual.

It is therefore important to monitor commercial kiwifruit orchards to determine whether there is any genetic evidence of Psa adapting to ‘Zesy002’ or on-orchard treatments for Psa control. We analysed a total of 462 Psa genome sequences. To achieve this we sequenced the genomes of 151 isolates of Psa that were collected during orchard surveys in the 2020 growing season. This included 99 isolates collected by RJ Hill Laboratories Limited (Hill laboratories) for the Zespri copper resistance survey and 52 isolates submitted by growers through Hill Laboratories. These were combined with genome sequences from previous surveys carried out by PFR. In addition, 153 sequences from previous orchard surveys conducted at the University of Otago between 2012 and 2017 that have recently become publically available were included in these analyses.

Aims:

The aim of this work was to investigate whether there is any evidence for the on-orchard adaptation of Psa. We explored this by:

- Analysing the pattern of single nucleotide polymorphisms (SNPs, i.e. single nucleotide mutations) to determine whether SNPs are appearing uniformly across the Psa genomes or whether particular variants of Psa are increasing in frequency in the orchard population of Psa.
- Determining whether there have been instances of acquisition or deletion of new genes that might increase the pathogenic or environmental fitness of Psa.
Findings

- The SNPs appear to be more or less uniformly distributed across the Psa genome. There is no evidence for the accumulation of SNPs in specific genes within the wider Psa orchard population, nor is there evidence for the domination of a particular isolate.

- Psa has proved to be highly adept at acquiring new genes from other organisms in its environment. Indeed, the vast majority of newly sequenced isolates have at least one new element acquired by horizontal gene transfer. Most of these are associated with resistance to copper. New plasmids were also found, only one of these (p64-like) showed signs of accumulation in the on-orchard Psa population.

- We did not find any significant changes in the complement of genes known to be involved in pathogenicity.

- A new element previously discovered in a single Kiwifruit Property Identification Number (KPIN) site in the 2018 survey was not observed in the 2020 survey. It is unlikely that this element presents a beneficial adaptation.

Overall conclusions

We have found no evidence for the accumulation of specific SNPs or genetic elements in the Psa orchard population that standout above the background genetic changes. A new genetic element has been identified and others are likely to continually enter the orchard population. Likewise, SNPs will continue to accumulate, both these events should continue to be monitored.

We did not find any obvious genetic differences between the isolates sent to us by growers and those collected in the sample year by Hill Laboratories.

Recommendations

- Pathogenicity test selected isolates from the 2020 survey including those with the p64-like plasmid and some of the isolates provided by growers.

- Continue biennial surveys of kiwifruit orchards to assess potential acquisition of new elements, changes in the genetics of copper resistance and adaptation to new and existing cultivars.

- We have generated a large comprehensive collection of Psa genomes from a wide range of geographical locations. However, our sampling has been purposive and would benefit from the input of a biostatistician to ensure that the sampling is representative.

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1 Introduction

The original incursion of *Pseudomonas syringae* pv. *actinidiae* (Psa) into New Zealand was by a single isolate, several of which were sequenced in 2011 and were shown to be clonal (Templeton et al. 2015; Poulter et al. 2018; McCann et al. 2013, 2017). Since that original incursion, a number of other Psa isolates from New Zealand orchards were sequenced because they had developed resistance to copper. Genes encoding resistance to copper were discovered present either on Integrative Conjugative Elements (ICEs) or plasmids (Colombi et al. 2017). This indicated the ability of Psa to rapidly adapt to on-orchard treatments, and raised the question of whether it is able to adapt to the new plantings of *Actinidia chinensis* var. *chinensis* ‘Zesy002’ in particular.

The aim of this project was to determine whether there is any genetic evidence that Psa is adapting further to on-orchard treatments or to ‘Zesy002’. This project follows on from Zespri contract VI20084 (Templeton MD 2020) and two Kiwifruit Royalty Investment Programme-funded projects where two orchard surveys were carried out in parallel with the GoldFutures project.

To achieve this aim we analysed the genome sequences of 462 Psa isolates. This included 151 isolates of Psa collected in the 2020 season by RJ Hill Laboratories Limited (Hill Laboratories). This comprised 99 isolates collected as part of the annual copper survey, including those that had various levels of resistance to copper and those that were susceptible. In addition, a further 52 isolates were provided by growers who were concerned that the efficacy of Psa control on their orchards was not as high as expected. We included in this analysis the genomes of 148 isolates of Psa collected during surveys carried out in 2017 and 2018 from kiwifruit orchards participating in the GoldFutures project. In addition, a further 153 isolates sequenced by the University of Otago between 2012 and 2017 have become available and were included in the analysis.

New plasmids were also identified during these studies, but it has not been determined whether they have a direct benefit to the pathogenicity of Psa. However, if they were beneficial to Psa, it would be expected that their frequency in the Psa population would increase over time.

The aim of this work was to investigate whether there is any evidence for the on-orchard adaptation of Psa. We explored this by:

- Analysing the pattern of single nucleotide polymorphisms (SNPs, i.e. single nucleotide mutations) to determine whether SNPs are appearing uniformly across the Psa genomes or whether particular variants of Psa are increasing in frequency in the orchard population of Psa.
- Determining whether there have been instances of acquisition or deletion of genes that might increase the pathogenic or environmental fitness of Psa.
2 Methodology

Psa isolates were obtained from Hill Laboratories. These isolates were collected by Hill Laboratories as part of their contract for monitoring copper resistance in kiwifruit orchards. We requested a broad range of collection sites and an even split between isolates with some copper resistance and those that were still sensitive. In addition to these isolates, we requested Kiwifruit Vine Health (KVH) prompt growers who felt that Psa was not responding to management treatments to provide infected leaves to Hill Laboratories. The metadata for these isolates, including their copper resistance status, is found in Appendix 1. While we believe we have a large comprehensive collection of Psa genomes collected across a wide range of geographical locations, our sampling has been purposive and was not specifically designed by a biostatistician.

DNA extraction:

DNA was extracted using standard procedures (Andersen et al. 2018). The sequencing of all Psa genomes was managed by Auckland Genomics (The University of Auckland, New Zealand) who contracted the Microbial Genome Sequencing Center (https://www.migscenter.com/) which delivered sequencing results of very high quality.

Bioinformatic analysis:

Paired-end reads were assembled using SPAdes (settings: -k 21,33,55,77,99,127 –careful; version 3.14.0; Bankevich et al. 2012). These contigs were then annotated with Prokka, using the Psa3 V-13 protein model to preferentially annotate from (version 1.3; https://github.com/tseemann/prokka; Seeman, 2014). A bioinformatics pipeline was developed to analyse the sequence data (Figure 1).

Figure 1. A flow chart describing the bioinformatics pipeline used to analyse the sequence data. Sequencing reads were mapped to the reference strain Psa ICMP 18884, and single nucleotide polymorphisms identified (green pathway) using SNIPPY. Deletions (blue pathway) were identified using CNVnator. New genes, incorporated by horizontal gene transfer, (orange pathway) were identified by analysing unmapped reads.

Snippy (version 4.6.0; https://github.com/tseeman/snippy) was used to map reads to the reference genome of Psa3 V-13 (ICMP 18884). Snippy-core produced a core SNP alignment. Gubbins identified recombinant regions in this alignment, producing a filtered alignment of 2732 bp (version 2.4.1;
RAxML was used to generate a maximum-likelihood phylogenetic tree with 100 bootstrap replicates (version 8.2.12; -f a -# 100 –m GTRCAT; Stasmatakis 2014. The phylogeny and associated metadata were visualised with the R package ggtree (Yu et al. 2017). Only bootstrap support values of 50 or above were visualised.

Large gene deletions were identified by CNVnator (version 0.4.1). These deletions were confirmed by the SPAdes de novo assemblies (Abyzov 2011).

Reads that did not map to the reference Psa V-13 genome, as output by snippy, were assembled using SPAdes1 (version 3.14.0). This branch of the pipeline will identify any new genes and elements incorporated by horizontal gene transfer. These assembled contigs were then annotated with Prokka2 (version 1.3; https://github.com/tseemann/prokka; default settings). All reads from each sequence were therefore analysed by this pipeline.

To identify which element(s) have been incorporated into each isolate, BLAST+ (Camacho et al. 2009) was used to build a custom BLAST database of all ICEs, plasmids and other elements that have previously been found in Psa. These sequences were either found in and downloaded from GenBank or present in our in-house database.

An additional database of all effectors from P. syringae was also generated from Supplementary Figure 3 from Dillon et al. 2019. These databases were used to BLAST the assembled unmapped contigs with BLASTn (version 2.10.1+). New elements (not present in the custom database) were identified by visual comparison and inspection of the assembled contigs of unmapped reads from each isolate.
3 Results

3.1 Collection of isolates

Isolates from the 2020 growing season were collected by Hill Laboratories as part of their contract for surveying for copper resistance in Psa. In addition, we requested that growers who felt the control of Psa on their orchards was not as effective as expected send infected leaves to Hill Laboratories. Psa was isolated from these leaves and tested for sensitivity to copper.

We have now surveyed Psa over three growing seasons, 2017, 2018 and 2020 (Figure 2). The surveys represent a broad sampling in terms of geography and host of isolation.

![Figure 2. Locations of Psa sampling by provincial area by year. The number of isolates from each region is given in the coloured circles.](image)

In addition, the Psa sequences from surveys carried out by the University of Otago between 2012 and 2017 have been made publically available (PRJNA527140). Collectively, these data provide a comprehensive resource for monitoring the adaptation and evolution of Psa on New Zealand kiwifruit orchards since the beginning of the incursion (Table 1).

<table>
<thead>
<tr>
<th>Season of collection</th>
<th>Isolate numbers</th>
<th>‘Zesy002’ %</th>
<th>“Hayward” %</th>
<th>Other %</th>
<th>Resistant to copper (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017</td>
<td>78</td>
<td>81</td>
<td>19</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>2018</td>
<td>70</td>
<td>79</td>
<td>21</td>
<td>0</td>
<td>47</td>
</tr>
<tr>
<td>2019 (isolated from flower buds)</td>
<td>10</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>2020 (Hill Laboratories copper resistance survey)</td>
<td>99</td>
<td>30</td>
<td>68</td>
<td>2</td>
<td>73</td>
</tr>
<tr>
<td>2020 (Provided by growers)</td>
<td>52</td>
<td>60</td>
<td>21</td>
<td>19</td>
<td>67</td>
</tr>
<tr>
<td>2012–2017 University of Otago</td>
<td>153</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
3.2 The analysis of single nucleotide polymorphisms in Psa orchard isolates

The rate of SNP mutation in Psa determined from our previous report (VI20084; Templeton MD 2020) is relatively low at approximately 0.6 SNPs/genome/year. This low rate of mutation is confirmed in our current survey. If particular SNP mutations were to be advantageous to Psa we would expect these SNPs to start dominating the population of orchard isolates of Psa. We would further expect to see this domination reflected in the structure of the phylogenetic tree generated by these SNPs.

When we analyse the location of SNPs in the Psa orchard isolates we find they are distributed more or less evenly and randomly throughout the genome (Figure 3). No individual SNPs were present in the population at a high frequency. Most notably, it was rare that a particular SNP was present in more than one gene. This strongly indicates that we are not seeing beneficial SNP mutations that might be spreading throughout or dominating the Psa orchard population.

![Figure 3. The distribution of single nucleotide polymorphism (SNP) across the Pseudomonas syringae pv. actinidiae (Psa) genome. SNPs at the integrative conjugative element (ICE) locus (5390845-5512114bp) have been filtered/masked out from the visualisation, due to the high level of variation between ICES.](image)

This observation is supported by analysis of the phylogenetic tree generated from this SNP analysis (Appendix 2). The tree has very little structure, and there is no evidence that a single isolate is dominating the tree. Where structure or discrete clades are observed, these tend to be from a particular geographic location and are likely to be a function of sampling rather than the evolution of a dominant isolate.

3.3 Monitoring the incorporation of plasmids and other elements in Psa orchard isolates

It had previously been observed that in addition to ICE elements and some plasmids that confer resistance to copper in Psa, two new plasmids (p47 and p64) with unknown roles were observed at a relatively low frequency (Templeton and Arshed, 2020). We concluded that the low-level presence of p47 and p64 (20 and 7% respectively) tended to suggest that they were unlikely to be of significant overall benefit to Psa, but that their frequency in the population should continue to be monitored. The frequency of p47 and p64 in both the 2020 survey and historical isolates sequenced by the University of Otago was determined (Figure 4). The frequency of the p47-like family of plasmids has varied...
considerably over the past 8 years but now appears to be trending downward. It is not present in a significant (10% as opposed to 20–60% in other surveys) frequency in the samples sent to us by concerned growers. The frequency of the p64 family of plasmids has increased in 2020 to above 20% and it appears to be trending upwards.

Figure 4. The frequency of plasmids p47 and p64 in *Pseudomonas syringae* pv. *actinidiae* (Psa) orchard isolates. The frequency of each plasmid was determined for each growing season. The 2020 season was split into isolates collected by Hill Laboratories as part of their copper resistance survey (2020H) and those provided by growers (2020G).

In our previous report a new element (NE01) was identified on ‘Zesy002’ vines in a single orchard (Kiwifruit Property Identification Number 3610) in South Auckland. The element was inserted into the Psa chromosome and was 56.9 kb in length with 74 predicted genes. Virtually all the genes encoded putative novel proteins and no information about the function of the element could be predicted by bioinformatic analysis. We screened this element and four others that have been found at low frequency in the surveys conducted between 2016 and 2020. The NE01 element was not found in any surveys apart from the original 2018 detection (Table 2). The eight novel plasmids are only rarely and sporadically detected, with only pSP14 at a frequency of 10%. This indicated they are unlikely to be of any concern to the industry at present, but nevertheless should continue to be monitored in the future.

Table 2. The frequency of novel plasmids discovered in *Pseudomonas syringae* pv. *actinidiae* (Psa) orchard isolates.

<table>
<thead>
<tr>
<th>Season</th>
<th>NE01</th>
<th>pG_320 mega</th>
<th>p18_X01</th>
<th>p18_X02</th>
<th>p18_X02a</th>
<th>p65-like</th>
<th>pSP14-like</th>
<th>P1256-like</th>
<th>PS7-1M-like</th>
</tr>
</thead>
<tbody>
<tr>
<td>2016</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>2017</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>7</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>2018</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2019</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>2020H</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>2020G</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
4 Discussion/conclusions

4.1 Evidence for positive selection of genes in Psa

SNPs in the Psa orchard samples appeared to be randomly distributed throughout the genome. No single individual SNP was present in the population at a high frequency. This finding strongly indicates that, at present, SNPs that might potentially be beneficial to Psa are not accumulating or selectively spreading throughout the orchard population. This finding is now based on several years of orchard surveys. There did appear to be SNPs that are specific for particular kiwifruit growing regions, and individual SNPs were restricted to a single KPIN; these SNPs may be useful for tracking the progression and movement of Psa isolates.

Previously, we have proposed that by measuring the ratio of SNPs that give rise to changes in the amino acid sequence (Ka) divided by the number of SNPs that give rise to synonymous mutations (Ks; no change in amino acid sequence of the ORF), we can determine whether particular genes might be under positive selection. This analysis would be of interest with the current dataset because it would suggest whether these mutations were of a selective advantage to Psa. As commented in our previous report (VI20084; Templeton MD 2020), because the number of SNPs recorded is still relatively low, it was not possible to do this analysis with any statistical rigor.

This analysis provides details on the numbers of SNPs in the Psa orchard population compared with isolates sequenced earlier in the epidemic. It provides an important baseline for the rate of background SNP accumulation and therefore Psa variation within New Zealand orchards. We can use these data to detect any abnormally high variation rates should they appear in subsequent surveys and/or to refine future surveys in terms of sampling strategies.

4.2 Acquisition of new genes

For Psa the most likely means for adaption is to acquire new genes by horizontal gene transfer. It has been observed that Psa is particularly adept at acquiring genes by horizontal gene transfer (Colombi et al. 2017). Our results from the 2020 survey indicated that almost every isolate that was sequenced has encountered at least one horizontal gene transfer event, be that acquisition of new ICE elements or one of the several types of plasmids that appear to be circulating in kiwifruit orchards. The concern for us would be if we observed a particular plasmid or element dominating the Psa population in commercial orchards. This would indicate that presence of the plasmid might be conferring a competitive advantage to those isolates, and this might pose a risk.

Two plasmids p47 and p64 are found at a significant frequency in Psa. They have been present in some isolates of Psa for between six and seven years. The frequency of p47 has varied over the past seven years and appears to be trending down. This suggests it is not being positively selected for, although it appears to have been present at high frequencies between 2013 and 2017, and we do not think that it is dominating the Psa population. For this reason we do not think that its presence is poses a significant risk. There has been an increase in frequency of p64 over the past four years up to 20% in the 2020 survey. It would be prudent to continue to monitor the frequency of isolates with this plasmid and to pathogenicity test them to determine whether they are more virulent.
Several plasmids and other elements in addition to p47 and p64 have been periodically observed in the Psa orchard population (Table 2). We raised concern about one of these (NE01) in our previous report (Templeton and Arshed, 2020). This element has not been detected again, and thus is now unlikely to be of concern. Other rare elements do not appear to be increasing in frequency.

An explanation for the presence of these plasmids and elements and why they remain in the population is that many have systems that prevent them from being lost by the host bacterium once they have been acquired (Fraikin et al., 2020). This enables the plasmids and elements to persist in a population, if they have little detrimental effect on isolates that possess them.

4.3 Isolates supplied by growers in 2020

We analysed the 52 isolates sent in by growers and tested for copper resistance through Hill Laboratories. Sixty seven percent were copper resistant and of these, 12 (35%) showed resistance to the highest level of copper (1.92 mM). This compares to 24% of the copper resistant isolates from the Hill Laboratories survey which were resistance to the highest concentration of copper. There is thus a slightly larger proportion of the highest level of resistance to copper amongst the grower-supplied isolates. We did not observe anything unique or different from the other orchard isolates in the additional plasmid content or SNP analysis of these isolates.

5 Recommendations

- Pathogenicity test selected isolates from the 2020 survey including those with the p64-like plasmid and some of the isolates provided by growers.

- Continue biennial surveys of kiwifruit orchards to assess potential acquisition of new elements, changes in the genetics of copper resistance and adaptation to new and existing cultivars.

- We have generated a large comprehensive collection of Psa genomes from a wide range of geographical locations. However, our sampling has been purposive and would benefit from the input of a biostatistician to ensure that the sampling is representative.
6 References


Poulter RTM, Ho J, Handley T, Tayaroa G, Butler MI 2018. Comparison between complete genomes of an isolate of Pseudomonas syringae pv. actinidiae from Japan and a New Zealand isolate of the pandemic lineage. Scientific Reports 8: 10915. DOI:10.1038/s41598-018-29261-5.


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