Evolution of a wild-plant tobamovirus passaged through an exotic host: Fixation of mutations and increased replication

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Abstract

Tobamovirus is a group of viruses that have become serious pathogens of crop plants. As part of a study informing risk of wild plant virus spill over to crops, we investigated the capacity of a solanaceous-infecting tobamovirus from an isolated indigenous flora to adapt to new exotic hosts. Yellow tailflower mild mottle virus (YTMMV) (genus Tobamovirus, family Virgaviridae) was isolated from a wild plant of yellow tailflower (Anthocercis littoria, family Solanaceae) and initially passaged through a plant of Nicotiana benthamiana, then one of Nicotiana glutinosa where a single local lesion was used to inoculate a N. benthamiana plant. Sap from this plant was used as starting material for nine serial passages through three plant species. The virus titre was recorded periodically, and 85% of the virus genome was sequenced at each passage for each host. Six polymorphic sites were found in the YTMMV genome across all hosts and passages. At five of these, the alternate alleles became fixed in the viral genome until the end of the experiment. Of these five alleles, one was a non-synonymous mutation (U1499C) that occurred only when the virus replicated in tomato. The mutant isolate harbouring U1499C, designated YTMMV-a, increased its titre over passages in tomato and outcompeted the wild-type isolate when both were co-inoculated to tomato. That YTMMV-a had greater reproductive fitness in an exotic host than did the wild type isolate suggests YTMMV evolution is influenced by host changes.

Key words: virus evolution; host adaptation; nucleotide substitution; experimental evolution rate; virus ecology.

1. Introduction

Species of genus Tobamovirus probably co-evolved with the angiosperms 140–120 million years ago (Gibbs 1999). Sometime after agriculture began, a number of tobamoviruses emerged from wild plants to become pathogens of crops, especially in solanaceous and cucurbitaceous species (Lartey et al. 1996). Tobacco mosaic virus (TMV) was the first plant virus described (Mayer 1886; Ivanovsky 1892; Beijerinck 1898) and purified (Stanley 1935), eventually lending its name to the genus Tobamovirus. TMV originated in the Americas (Holmes 1951), where the majority of Nicotiana species evolved. It has spread to all key tobacco growing regions of the world where it still causes significant yield losses (Shen et al. 2013).

Yellowtail mild mottle virus (YTMMV) is a tobamovirus discovered recently in southern Western Australia (W.A.) in two endemic solanaceous species, Anthocercis littorea Labill. (yellow tailflower) (Wylie et al. 2014) and A. ilicifolia Hook. (red-striped tailflower) (Li et al. 2015). The incidence of YTMMV in W.A. in...
wild plant hosts has not been investigated but studies on the experimental host range of YTMMV showed that it systemically infected all the Australian Nicotiana species tested, and the North American N. tabacum, but not the Peruvian N. glutinosa in which it induced local lesions. Other species systemically infected were Capsicum annum (capsicum and chilli), Solanum betaceum (tamarillo), S. lycopersicum (tomato), S. melongena (aubergine), S. nigrum (black nightshade), Physalis angulata (balloon cherry), P. peruviana (cape gooseberry), and P. philadelphica (tomatillo) (Li et al. 2015; Wylie et al. 2015). Symptoms ranged from mild mosaic (tomato) to death (one cultivar of capsicum) (Fig. 1). Seedlings were 4–6 weeks old at inoculation. At each passage, a cohort of six plants of the same species was inoculated with YTMMV, and leaf tissue was macerated and used as inoculum. dsRNA was extracted from each plant, and the YTMMV IR was amplified and sequenced using IR primers only.

2 Materials and methods

2.1 Virus passaging

YTMMV-infected A. littorea leaf tissue (Cervantes isolate, GenBank accession NC_022801) was inoculated to Nicotiana benthamiana accession RA-4 seedlings and then to a N. glutinosa seedling in a glasshouse. A single local lesion was isolated from a N. glutinosa leaf, crushed in cold 0.1 M phosphate buffer (pH 7.0), and applied to a healthy N. benthamiana seedling. N. benthamiana was used as the “primary passage host” because it quickly displayed clear symptoms of YTMMV infection compared with other species such as tomato plants that display less obvious symptoms.

The YTMMV isolate was passaged nine times through each of the host lineages: S. lycopersicum cv Money Maker (tomato), N. benthamiana laboratory accession RA-4, and alternating plants of S. nigrum (black nightshade) and N. benthamiana RA-4 (Fig. 1). Seedlings were 4–6 weeks old at inoculation. At each passage, a cohort of six plants of the same species was inoculated with the same inoculum. Inoculum was made by taking one young leaf from each of the six plants in a cohort, pooling and macerating them in 0.1 M phosphate buffer (pH 7.0) with a pinch of celite, and inoculating six more seedlings. This procedure was repeated every 16 days for nine passages. Plants were spaced so that there was no physical contact within and between cohorts. Cohorts were named by passage number (e.g. P1 for the first passage) and host name, i.e. R for N. benthamiana RA-4, T for tomato and M for the mixed lineage of black nightshade and N. benthamiana RA-4 (Fig. 1). At 14 dpi, leaf tissue was collected from all six plants of cohorts P1, P3, P4M, P4R, P4T, P5M, P5T, P6M, P6T, P7R, P7T, P8M, P8R, P8T, P9M, P9T, P10R, G10T, P11M, P11T, P12M, P12R, and P12T (Fig. 1), and most (84.2%) of the viral genome sequence determined by direct Sanger sequencing of RT-PCR products. RT-PCR was done using eight sets of virus-specific primers (Supplementary Table S1).

Two experiments were done to determine if the YTMMV U1499C mutant isolate, from now on referred to as YTMMV-δ, would re-occur at the third passage through tomato (P6T). In the first experiment, inoculum made by taking virus-infected leaves from P4T was passaged twice through cohorts of six tomato plants and the intervening region (IR; the region between the methyltransferase and helicase domains in ORF1 of the viral genome) was sequenced at each passage. In the second experiment, P5T tomato plants’ inoculum (the passage before the dominance of YTMMV-δ) was inoculated to eleven tomato plants, respectively. At 14 dpi, the IR of each of the eleven plants was sequenced to determine the identity of the nucleotide at position 1499 (Fig. 2).

A competition was set up between the wild-type isolate (YTMMV-Cervantes) and YTMMV-δ to determine their relative fitness in different hosts. An inoculum mixture was made by combining the two isolates in a 1:1 ratio and this was used to inoculate six plants each of N. benthamiana RA-4 and S. lycopersicum cv Money Maker. Fourteen days after inoculation, dsRNA was extracted from each plant, and the YTMMV IR was amplified and sequenced directly to assess the presence of the U1499C mutation.

Figure 1. Outline of passage experiments. Twelve passages were done. Nine passages (P4 – P12) were done with cohorts of six plants, either alternating black nightshade and N. benthamiana accession RA-4 (P4M – P12M), N. benthamiana RA-4 (P4R – P12R), or tomato (P4T – P12T). Leaf tissues were collected from cohorts that were underlined for further analysis. Cohorts with solid lines were sequenced with the eight primers. Those underlined with dotted lines were sequenced using IR primers only.
2.2 dsRNA extraction, cDNA synthesis, and PCR amplification

Nucleic acids were extracted from leaves pooled from all six plants per cohort and enriched for dsRNA using a cellulose-based method (Morris and Dodds 1979). cDNA was synthesised using GoScript™ reverse transcriptase (Promega) using a random primer (Supplementary Table S1) according to the manufacturer’s recommendations. The replicate genome (spanning the methyltransferase (Met), IR, helicase (Hel), and RNA-dependent RNA Polymerase (RdRp) domains), the movement protein and the coat protein (CP) genes of the YTMMV genome were amplified from each sample using eight primer pairs (Supplementary Table S1). Cycling conditions were 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 55 °C for 45 s, 72 °C for 10 min using GoTaq® Green mastermix (Promega) according to the manufacturer’s instructions. Amplicons were sequenced directly using the same primers as used in the PCRs. To identify reverse transcriptase and Taq DNA polymerase-induced sequence mis-incorporations, independent cDNA-synthesis, amplification and sequencing reactions and PCR and sequencing were repeated two to three times for 182 of the 228 sequencing reactions done. All amplicons were sequenced directly using the same primers as used in the PCRs. To identify reverse transcriptase and Taq DNA polymerase-induced sequence mis-incorporations, independent cDNA-synthesis, amplification and sequencing reactions and PCR and sequencing were repeated two to three times for 182 of the 228 sequencing reactions done. All amplicons were sequenced in both directions, and primer sequences were removed prior to analysis.

2.3 Data analysis

Data analysis was done using Geneious v8.1.7 (Biomatters Ltd) software. Where present, primer sequences were trimmed off and remaining sequences edited manually if required. Non-contiguous sequences were concatenated before alignment using ClustalW (Thompson et al. 1994) with gap open cost of 200 and gap extension cost of 6.66.

2.3.1 Nucleotide sequence analysis

As a result of the small number of observed changes in the genome after passaging, no correction (i.e. determining the evolution model) was done. Calculation of the substitution rate of YTMMV (substitutions/site/year) was done using TipDate v1.2 (Rambaut 2000) with the Mobyle platform set to default parameters (Néron et al. 2009). YTMMV Cervantes (NC_022801) from the original host plant was used as the root of the tree.

2.4 Relative quantitative measure of YTMMV isolates

Changes in virus titre were measured using quantitative RT-PCR (RT-qPCR). Double-stranded RNA extracted from 100 mg of leaf material (as above) for each sample along with a CP forward and reverse primer (sequence CTCAGAATGCC AGAAAACACTG and CGAATTTAACCAGCGACTGA) to amplify an amplicon of 149 nt of the YTMMV CP region using SensiFAST™ SYBR® No-Rox One-Step Kit from Bioline (Australia). A cytochrome oxidase gene sequence was amplified using primers 5’- CGTCCGATTTCCAGATTCA-3′ and 5’-CAACTCCCGATATATA AGAGCCAAAACCTG-3′ and used as an internal reference. Each reaction was done in triplicate and carried out in a Qiagen (Corbett) RotorGene™ 3000. A melt curve was generated from 72° to 95 °C to detect primer dimers or plant genomic DNA. Each experiment was repeated two times.

The ΔACq method was carried out to compare relative gene expression of the CP of the virus populations before and after the U1499C mutation had occurred for these plant varieties. Cq values were generated in Rotor-Gene Q Series software (v6.1.93).

Using the delta method (Casella and Berger 2002), it can be shown that fold changes of ΔACq can be estimated by \( 2^{\Delta \text{Cq}} = 2^{(\text{Cq}_{\text{target}} - \text{Cq}_{\text{reference}})} \), with corresponding 95% confidence intervals.

2.5 Symptom development

After the 12 passages had been done, we compared responses to infection of the wild-type YTMMV-Cervantes and YTMMV-6, on different solanaceous plants. Both isolates of YTMMV were inoculated separately onto leaves of Capsicum annuum cv Green Giant, C. annum cv Black Pearl, S. betaceum, N. benthamiana RA-4, and S. lycopersicum cv Money Maker. Symptoms on the inoculated plants were scored at 25 dpi. A symptom severity index (ssi) was used to assess the symptoms with indices defined as: 0, healthy plant with no visible disease symptoms; 1, mild symptoms including mild chlorosis, stunting, leaf curling; 2, moderate symptoms of chlorosis, stunting and leaf curling; 3, severe stunting, chlorosis, leaf curling visible, dark veins on leaves sometimes visible; and 4, whole plant death.

Virus-infected leaf material (100 mg) obtained from the N. benthamiana cohort P12R and the tomato cohort P12T was inoculated separately to seven solanaceous plants. Both isolates of YTMMV were inoculated separately onto leaves of Capsicum annuum cv Green Giant, C. annum cv Black Pearl, S. betaceum, N. benthamiana RA-4, and S. lycopersicum cv Money Maker. Symptoms on the inoculated plants were scored at 25 dpi. A symptom severity index (ssi) was used to assess the symptoms with indices defined as: 0, healthy plant with no visible disease symptoms; 1, mild symptoms including mild chlorosis, stunting, leaf curling; 2, moderate symptoms of chlorosis, stunting and leaf curling; 3, severe stunting, chlorosis, leaf curling visible, dark veins on leaves sometimes visible; and 4, whole plant death.

3. Results

3.1 Mutations

Most of the virus genome, consisting of 5,370 nucleotides (84.2% of the YTMMV genome) was sequenced for most cohorts. Sequences were aligned with the genome sequence of YTMMV-Cervantes (NC_022801). Across all lineages and passages, six polymorphic sites were found (Table 1). At nt 5790, SNPs were dominant for a cohort (12T). Four synonymous SNPs, three in the replicase (nt 2737, 3853, 4414) and one in the CP gene (nt 5876) became fixed when the virus was first passaged from its natural host A. littorea to a N. benthamiana plant, suggestive that these may be host specific.
Symptom development was observed until 20 dpi. At 20 dpi cum, chilli, inoculated separately onto leaves of tamarillo, tomato, capsicum, chilli, N. benthamiana RA-4, and N. glutinosa plants (n = 6). Symptom development was observed until 20 dpi. At 20 dpi plants of most species from both groups had developed similar symptoms typical of wild-type YTMMV infection (Table 2). The exception was N. glutinosa, where the number of local lesions induced by YTMMV-δ per leaf at three dpi was only 15.18% (±8.89%) of those induced by YTMMV-Cervantes passed once through N. benthamiana (Fig. 3).

### 3.2 Sequence analysis

The overall YTMMV substitution rate was estimated to be $1.752 \times 10^{-5}$ substitutions/site/year. An experiment was carried out where equal amounts of tomato leaf materials infected with YTMMV-Cervantes and YTMMV-δ were macerated, mixed and inoculated to N. benthamiana and tomato seedling plants (n = 6) to determine if one virus strain would dominate the population in the two host backgrounds. Fourteen days after inoculation in tomato plants, only YTMMV-δ was detected in all six plants tested, whereas, in the six N. benthamiana plants, only YTMMV-Cervantes was detected, as determined by sequence analysis.

#### 3.2.1 Quantitative analysis of YTMMV-Cervantes and YTMMV-δ

Quantitative RT-PCR using the ΔCq method was carried out to compare relative amounts of YTMMV CP in plants before and after inoculation.
after the U1499C mutation occurred in P6T. In this experiment, it was assumed that the amount of CP was proportional to the titre of the virus in the sample. RT-qPCR revealed that YTMMV in the final cohort of tomato (P12T) was 90.14-fold (95% confidence interval of 87.94- to 92.35-fold) and 6.82-fold (95% confidence interval of 4.68- to 8.97-fold) greater than in P3 and P6T plants, respectively (Table 3), showing a trend of increasing virus titre in later passages. The role of U1499C in this response is yet to be determined.

4. Discussion
4.1 Mutation and virus titre
In this experiment, an isolate of YTMMV from a wild host plant was passaged through a N. benthamiana plant, and from it to a N. glutinosa plant, a local lesion host. A single local lesion was isolated from it, and inoculum derived from this lesion was used in a series of passages to cohorts of Australian (N. benthamiana) and non-Australian (black nightshade and tomato) solanaceous plants. The majority of the YTMMV genome was sequenced at each passage and for each host lineage, and nucleotide substitutions recorded. In general, negative selection acted against change in the YTMMV genome.

Four SNPs (T2737C, C3853A, C4414T, and T5876C) became fixed in the replicate and CP early after the first passage from the natural host of YTMMV Anthocercis littorea to the first N. benthamiana plant (P1), revealing that those wild-type alleles may be wild host specific.

The U-to-C substitution that became dominant in the viral IR (U1499C) only in tomato at P6T was present until the last passage in the experiment (P12T). The tobamovirus IR contains the binding site where the 130k and 180k replicase proteins interact to form replication complexes which allow the synthesis of negative-stranded RNAs (Kawamura-Nagaya et al. 2014). These negative-strand RNAs then act as templates for which genomic RNA is replicated. The IR is also believed to play a role in suppressing post-transcriptional silencing (Goregaoker et al. 2001; Ichiki et al. 2005). However, the titre of YTMMV in tomato increased 13.22 (±8.54)-fold from P3 to P6T before the U1499C allele in the IR became dominant, which suggests that the allele did not influence the increasing virus titre. After U1499C had become dominant, virus titre continued to increase in tomato at a lesser rate (6.82 (±6.00)-fold) from P6T to P12T.

The virus present in leaves in P4T and P5T had the dominant wild-type U-allele at nt 1499, but by the 6th passage, U1499C had become dominant. P4T and P5T leaf tissue were used to repeat the passages to a fresh cohort of tomato plants to observe if the U1499C allele once again became dominant. Surprisingly, the result of the P4T inoculation was the appearance of a new dominant non-synonymous mutation, G1497A, adjacent to nt 1499T. The result of repeating the P5T inoculation was the appearance of U1499C in the majority of the plants tested. Both results suggested that positive selection is acting on the IR when YTMMV is infecting tomato.

An isolate of tobacco etch potyvirus was passaged 15 times through a new host, Capsicum annum, and its original host, N. tabacum (Agudelo-Romero et al. 2008). A mutation occurred in the HC-Pro region (V414A) at passage 15, but only in the pepper plants (Agudelo-Romero et al. 2008). The mutation resulted in more aggressive symptoms in pepper, but the mutant was less virulent and had lower titre in tobacco (Agudelo-Romero et al. 2008). Similarly, YTMMV-δ (U1499C) replicated at a higher level in tomato at passage 12 than it did when it first encountered tomato at passage 4. It was unexpected to find that the wild-type YTMMV-Cervantes consistently induced far greater local lesions on N. glutinosa leaves than did YTMMV-δ, which replicated in greater numbers in tomato. YTMMV-δ may induce a milder programmed cell death response in N. glutinosa.

The greater selective advantage of YTMMV-δ over YTMMV-Cervantes in tomato was confirmed in a competition experiment between the two isolates co-infected to tomato and N. benthamiana plants. In tomato plants, YTMMV-δ became the dominant strain, whereas YTMMV-Cervantes was the dominant strain in N. benthamiana.

4.2 Substitution rate
The substitution rate for YTMMV upon encountering new host species is probably an over-estimation of the natural substitution rate of the virus (Raney et al. 2004; van der Walt et al. 2008). The natural rate of nucleotide substitution was not tested in A. litoris, the natural host, because seed of this species could not be induced to germinate. Experimental substitution rates are usually greater than rates that occur in nature where they are subjected to more complex selection processes (van der Walt et al. 2008). In this experiment, the rate of nucleotide substitution YTMMV was estimated to be 1.752 × 10⁻⁵ substitutions/site/year, similar to rates obtained for dsRNA plant viruses (Kearney et al. 1999; Fargette et al. 2008; Simmons et al. 2008; Pagán et al. 2010; Fraile et al. 2011) and tobamoviruses, which are recorded to be 1.3 × 10⁻³ and 8 × 10⁻⁵ (Kearney et al. 1999; Pagán et al. 2010), which are within the range observed for YTMMV.

Host change is one of the primary drivers for evolution of many pathogens, including viruses (Woolhouse et al. 2001; Lajeunesse and Forbes 2002; Lalić et al. 2011). In this experiment, it was shown that multiple passages through an exotic host species led to the fixation of a dominant allele. This suggests that the allele was strongly selected for in tomato but not in N. benthamiana. It is unknown what function this allele has because it does not appear to be associated with increased virus titre in tomato. In the context of the natural environment of YTMMV, where there exist many species of solanaceous plants, both indigenous and introduced, the possibility remains that YTMMV could extend its host range.

Viruses adapted to wild plants may face greater selection pressures than those infecting agricultural plants because of the inherent genetic heterogeneity in wild hosts compared to cultivars: compatible hosts and vectors may be widely spaced, making transmission problematic, and competition from other viruses may be frequent (Fraile et al. 1997). In this study, only the impact of host change on viruses is studied. However, it is important to acknowledge that adaptation is not only limited to changes in within-host multiplication, but also the optimisation of between-host transmission, which was not done in this study. The findings suggest that YTMMV is capable of adapting to an exotic host in a very short time. Further studies on the optimisation of between-host transmission should also be done to confirm YTMMV adaptation in the exotic host. This has implications for the understanding tobamovirus evolution as well as spillover of wild-plant tobamoviruses to new hosts.

Acknowledgements
This study was funded in part by Australia Research Council Linkage grant LP110200180 and by a studentship granted to SHK by Murdoch University.
Data availability
All sequence data from viruses isolated from hosts during passages are available in the supplementary document.

Supplementary data
Supplementary data are available at Virus Evolution online.

Conflict of interest: None declared.

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