RESEARCH ARTICLE

Differential Responses to Virus Challenge of Laboratory and Wild Accessions of Australian Species of *Nicotiana*, and Comparative Analysis of *RDR1* Gene Sequences

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Abstract

*Nicotiana benthamiana* is a model plant utilised internationally in plant virology because of its apparent hyper-susceptibility to virus infection. Previously, others showed that all laboratory accessions of *N. benthamiana* have a very narrow genetic basis, probably originating from a single source. It is unknown if responses to virus infection exhibited by the laboratory accession are typical of the species as a whole. To test this, 23 accessions of *N. benthamiana* were collected from wild populations and challenged with one to four viruses. Additionally, accessions of 21 other *Nicotiana* species and subspecies from Australia, one from Peru and one from Namibia were tested for susceptibility to the viruses, and for the presence of a mutated RNA-dependent RNA polymerase I allele (*Nb-RDR1m*) described previously from a laboratory accession of *N. benthamiana*. All Australian *Nicotiana* accessions tested were susceptible to virus infections, although there was symptom variability within and between species. The most striking difference was that plants of a laboratory accession of *N. benthamiana* (RA-4) exhibited hypersensitivity to Yellow tailflower mild mottle tobamovirus infection and died, whereas plants of wild *N. benthamiana* accessions responded with non-necrotic symptoms. Plants of certain *N. occidentalis* species and subspecies from Australia, one from Peru and one from Namibia were tested for susceptibility to the viruses, and for the presence of a mutated RNA-dependent RNA polymerase I allele (*Nb-RDR1m*) described previously from a laboratory accession of *N. benthamiana*. All Australian *Nicotiana* accessions tested were susceptible to virus infections, although there was symptom variability within and between species. The most striking difference was that plants of a laboratory accession of *N. benthamiana* (RA-4) exhibited hypersensitivity to Yellow tailflower mild mottle tobamovirus infection and died, whereas plants of wild *N. benthamiana* accessions responded with non-necrotic symptoms. Plants of certain *N. occidentalis* accessions also exhibited initial hypersensitivity to Yellow tailflower mild mottle virus resembling that of *N. benthamiana* RA-4 but not from any of 51 other *Nicotiana* accessions, including wild accessions of *N. benthamiana*, demonstrating that the accession of *N. benthamiana* used widely in laboratories is unusual.
Introduction

The genus *Nicotiana*, family Solanaceae, comprises 64 described species, the best known and perhaps most infamous of which are *N. tabacum* and *N. rustica* from the Americas, which form the basis of the tobacco industry. The genus is divided into 13 sections, the largest of which is *Suaveolentes*. *Nicotiana* section *Suaveolentes* holds 30 species, 27 of which are endemic to Australia, two to the Pacific Islands and one to Africa [1, 2]. The species within *Suaveolentes* best known to the plant science community is *N. benthamiana*, an allotetraploid thought to originate from diploid parents [3, 4]. *N. benthamiana* occurs sporadically across approximately 3000 kilometres of northern Australia from longitudes 114°E to 140°E and between latitudes 14°S and 26°S. Like *Nicotiana* species from the Americas, the Australian members of the genus contain nicotine and other alkaloids that stimulate the human central nervous system, and these compounds made members of the genus important to the Aboriginal peoples of Australia [5]. From the leaves of various *Nicotiana* species and the related genus *Duboisia* they made *Pituri* (also known as *Tjuntiwari*, *Muntju*, *Pinkaraangu*, *Mingulba* and other names) [5, 6], a product made of dried or baked leaves and wood ash to form a wad that was held in the mouth between the teeth and gums [5, 7, 8]. *N. benthamiana* was not the most favoured species for making *Pituri*, but it was used when more desirable species were unavailable [8].

*N. benthamiana* is valued today not only because of its susceptibility to over 500 plant viruses [9], but also because of its susceptibility to infection by bacteria, fungi, oomycetes and nematodes [10, 11, 12, 13]. It is used for transient expression of transgenes through agroinfiltration, where *Agrobacterium tumefaciens* harbouring a T-DNA plasmid is introduced locally into a leaf. Transient local expression of genes from the T-DNA region by the plant enables studies in protein expression and regulation in the infiltrated leaf without the need to express transgenes stably in a whole plant [9, 12, 14, 15, 16]. As a reflection of its importance in the plant sciences, two draft genome sequences and transcriptome sequences of *N. benthamiana* have been released [16, 17, 18].

The evolutionary basis of the apparent hyper-susceptibility to viruses by *N. benthamiana* is unclear. Being highly susceptible to many viruses would seem at first glance to place the species on a fast track to extinction, but recent research has shown that viruses with long associations with wild plants are seldom severe pathogens [19]. Under experimental conditions, only a minority of plant viruses actually kill *N. benthamiana* plants; most infect with mild to moderate symptoms and often the plant is able to reproduce. The climate in which *N. benthamiana* grows may offer protection from severe virus-induced symptoms. *N. benthamiana* grows in Australia’s north where daytime temperatures can reach above 40°C (>104°F). The earliest report we could find in the scientific literature describing *N. benthamiana* and responses to virus infection showed how high growing temperatures ameliorated symptoms of Tobacco mosaic virus (TMV) infection [20]. Another suggestion is that wild populations of *N. benthamiana* live in zones relatively free of plant virus incidence, making resistance to viruses an unnecessary trait. Although virus surveys of wild plants have not been undertaken in most of the natural range of *N. benthamiana*, there is no reason to suppose that viruses are not present in the flora there. Further south in Western Australia, new viruses are regularly encountered in the indigenous flora [21, 22, 23, 24, 25, 26, 27, 28, 29, 30]. If natural populations of *N. benthamiana* are indeed highly susceptible to virus infection, might infection confer an evolutionary advantage under certain environmental conditions and/or at some stages of the life cycle? In the controlled conditions of the laboratory, *N. benthamiana* plants infected with Cucumber mosaic virus (CMV) lived about 20% longer under drought conditions than did uninfected plants [31], probably because virus-infected plants accumulate glycol, myo-inositol and other water stress-related protectants [32]. If the same phenomenon occurs amongst wild plants living in regions...
of variable water availability and seasonally arid conditions, such as occur in the areas that *N. benthamiana* naturally inhabits, it is conceivable that sub-lethal virus infections in later stages of the life cycle may enable plants to tolerate drought longer than uninfected plants, perhaps long enough to complete the life cycle.

A possible genetic basis to virus susceptibility in *N. benthamiana* was provided by Yang *et al.* [33] who identified that the RNA-dependent RNA polymerase I (*Nb-RDR1*) involved in small interfering RNA synthesis and virus resistance, contained a 72 nucleotide insertion mutation that introduced tandem stop codons. The mutant allele was referred to as *Nb-RDR1m* (*Nicotiana benthamiana* RNA-dependent RNA polymerase I mutant).

Using AFLP analysis Goodin *et al.* [12] showed that *N. benthamiana* accessions used in laboratories have a very narrow genetic basis. They named the five accessions gathered from laboratories around the world Research Accession (RA) 1–5 and concluded they were probably all derived from a single source. The source of the original laboratory accessions is not published. In collections of wild *N. benthamiana* lines held by Australian herbaria there exist specimens collected from different habitats, and these show variation in plant size and structure, leaf and flower shape, and other traits [12].

Other Australian *Nicotiana* species have been utilized by science to a much lesser extent than has *N. benthamiana*. The best known is *N. occidentalis*, where several accessions, for example B37 (also known as 37B), P-1, P12, and N1, are identified and have been used in virus-related studies [34, 35, 36, 37, 38]. These accession codes probably refer to members of *N. occidentalis ssp obliqua*, the most widespread subspecies in Australia. Although *N. hesperis* was described in 1960 [39] and accession 67A of this species has been cited in scientific reports up until the present day [38, 40, 41, 42], *N. hesperis* has not existed as a species since 1981 when it was reclassified as *N. occidentalis* subspecies *hesperis* [43]. This subspecies has a limited natural distribution, being restricted to northern coastal regions of Western Australia. We could find no records of scientific use of the third subspecies, *N. occidentalis ssp occidentalis*, also restricted to northern coastal and island sites in Western Australia. Despite recommendations that *N. cavigola*, *N. roslata*, *N. ingulba* (syn. *N. roslata ssp ingulba*), and *N. rotundifolia* are useful experimental hosts in the diagnosis of plant viruses [10], apparently none of these Australian species have been widely adopted for this purpose. The reason is unclear, but perhaps it is because of limited availability of their seed or because of the broader availability of *N. benthamiana*, *N. occidentalis* and non-Australian species such as *N. tabacum*, *N. clevelandii*, and *N. glutinosa* [44].

Here, we tested responses of laboratory and wild accessions of *N. benthamiana*, accessions of the three subspecies of *N. occidentalis*, accessions of 19 other Australian *Nicotiana* species, a South American *Nicotiana* species, and the sole African *Nicotiana* species to plant viruses. Partial *RDR1* gene sequences were obtained from some accessions, and we speculate further on the role of this gene in virus susceptibility and symptom development.

**Materials and Methods**

**Plants**

Plants were collected under licence and a Regulation 4 Authority issued by the Western Australian Department of Parks and Wildlife. Eighteen accessions of *N. benthamiana*, 26 accessions of *N. occidentalis*, including three of subspecies *hesperis*, 14 of subspecies *obliqua*, four of subspecies *occidentalis*, two *N. occidentalis* accessions for which the subspecies was not determined, three accessions *N. rotundifolia*, two accessions of *N. heterantha*, one accession of *N. umbratica*, and three *Nicotiana* accessions that were not identified to the species level were collected from multiple wild populations located in northern Western Australia (Table 1, Fig 1).
Table 1. Accessions used, showing species and their origins.

| Species                | Accession/Seed Line | Origin (if known)a      | Habitat (if known)           | Year collected (if known) | Reference |
|------------------------|---------------------|-------------------------|------------------------------|---------------------------|-----------|
| Chenopodium amaranticolor |                     |                         |                              |                           |           |
| C. quinoa              |                      | Peruvian Andes          | Montane                      |                           |           |
| Nicotiana africana     | SL6                 | Namibia                 |                              | 1983                      | [46]      |
| N. amplexicaulis       | SL7                 | Carnarvon Range, QLD    |                              | 1957                      | [46]      |
| N. benthamiana         | 17.19C              | Cleaverville Cove, WA   |                               |                           |           |
| N. benthamiana         | 17.23               | Hammsley Range, WA      | Cave entrance, inland riverbed| 2013                      | This study|
| N. benthamiana         | 17.24               | Millsteam-Chichester National Park, WA | Cliffs, inland riverbed | 2013                      | This study|
| N. benthamiana         | 17.26               | Millsteam-Chichester National Park, WA | Cliffs, inland riverbed | 2013                      | This study|
| N. benthamiana         | 17.27               | Millsteam-Chichester National Park, WA | Inland riverbed | 2013                      | This study|
| N. benthamiana         | 17.28               | Millsteam-Chichester National Park, WA | Inland riverbed | 2013                      | This study|
| N. benthamiana         | CI-1                | Cleaverville Cove, WA   | Coastal cliffs               | 2013                      |           |
| N. benthamiana         | HCK-1               | Honeymoon Cove, WA      | Coastal cliffs               | 2013                      | This study|
| N. benthamiana         | HCK-3               | Honeymoon Cove, WA      | Coastal cliffs               | 2013                      | This study|
| N. benthamiana         | KL-1                | Kalamina Gorge, Karijini National Park, WA | Cliffs, inland riverbed | 2013                      | This study|
| N. benthamiana         | KL-2                | Kalamina Gorge, Karijini National Park, WA | Cliffs, inland riverbed | 2013                      | This study|
| N. benthamiana         | KL-3                | Kalamina Gorge, Karijini National Park, WA | Cliffs, inland riverbed | 2013                      | This study|
| N. benthamiana         | KL-4                | Kalamina Gorge, Karijini National Park, WA | Cliffs, inland riverbed | 2013                      | This study|
| N. benthamiana         | KL-5                | Kalamina Gorge, Karijini National Park, WA | Cliffs, inland riverbed | 2013                      | This study|
| N. benthamiana         | Kx-1                | Knox Gorge, Karijini National Park, WA | Cliffs, inland riverbed | 2013                      | This study|
| N. benthamiana         | MTa-3               | Mt Augustus region, WA  | Rock overhang, inland riverbed| 2013                      | This study|
| N. benthamiana         | MTa-5               | Mt Augustus region, WA  | Rock overhang, inland riverbed| 2013                      | This study|
| N. benthamiana         | MTa-6               | Mt Augustus region, WA  | Rock overhang, inland riverbed| 2013                      | This study|
| N. benthamiana         | MTa-7               | Mt Augustus region, WA  | Rock overhang, inland riverbed| 2013                      | This study|
| N. benthamiana         | PPM-1               | Millsteam-Chichester National Park, WA | Cliffs, inland riverbed | 2013                      | This study|
| N. benthamiana         | PPM-2               | Millsteam-Chichester National Park, WA | Cliffs, inland riverbed | 2013                      | This study|
| N. benthamiana         | RA-4                |                         |                              |                           |           |
| N. benthamiana         | VL552B2.1           | Cleaverville Cove, WA   | Coastal cliffs               | 2012                      | This study|
| N. benthamiana         | VL552B2.2           | Cleaverville Cove, WA   | Coastal cliffs               | 2012                      | This study|
| N. cavicola            | SL9                 | Meekathara, WA          |                              | 1956                      | [46]      |
| N. excelsior           | SL11                |                         |                              | 1998/99                   | [46]      |
| N. forsteri (syn N. debneyi) | SL5              |                          |                              | 2003                      | [46]      |
| N. glutinosa           |                     |                         |                              |                           |           |
| N. goodspeedii         | SL13                | Port Augusta, SA        |                              | 1955                      | [46]      |

(Continued)
Table 1. (Continued)

| Species                  | Accession/Seed Line | Origin (if known)* | Habitat (if known) | Year collected (if known) | Reference |
|--------------------------|---------------------|--------------------|--------------------|---------------------------|-----------|
| N. gossei                | SL14                | Henbury, NT        |                    |                           | [46]      |
| N. heterantha            | Ft-2                | Fortesque River, WA| Inland riverbed    | 2013                      | This study|
| N. heterantha            | Ft-3                | Fortesque River, WA| Inland riverbed    | 2013                      | This study|
| N. heterantha            | SL33                |                    |                    | 2005                      | [46]      |
| N. maritima              | SL35                |                    |                    |                           | [46]      |
| N. megalosiphon          | SL1                 |                    |                    |                           | [46]      |
| N. occidentalis (or N. suaveolens) | SL15 | |                    |                           | [46]      |
| N. occidentalis          | Ci-1                | Cleaverville Plain, WA| Inland plain 2 km from coast | 2013 | This study |
| N. occidentalis ssp hesperis | NT-1     | Ashburton River, Nanutarra, WA | Inland, dry riverbed | 2013 | This study |
| N. occidentalis ssp hesperis | NT-4     | Ashburton River, Nanutarra, WA | Inland, dry riverbed | 2013 | This study |
| N. occidentalis ssp hesperis | NT-5     | Ashburton River, Nanutarra, WA | Inland, dry riverbed | 2013 | This study |
| N. occidentalis ssp obliqua | Ft-1         | Fortesque River, WA | Verge, public carpark | 2013 | This study |
| N. occidentalis ssp obliqua | MA-10     | Mt Augustus region, WA | Inland plain | 2013 | This study |
| N. occidentalis ssp obliqua | MA-11     | Mt Augustus region, WA | Inland plain | 2013 | This study |
| N. occidentalis ssp obliqua | MA-12     | Mt Augustus region, WA | Inland plain | 2013 | This study |
| N. occidentalis ssp obliqua | MA-4      | Mt Augustus region, WA | Inland plain | 2013 | This study |
| N. occidentalis ssp obliqua | MA-9      | Mt Augustus region, WA | Inland plain | 2013 | This study |
| N. occidentalis ssp obliqua | SL17     | Earsbiddy Hills, WA |                    | 1956                      | [46]      |
| N. occidentalis ssp obliqua | UK-1      | Tom Price region, WA | Inland plain | 2013 | This study |
| N. occidentalis ssp obliqua | UK-2      | Tom Price region, WA | Inland plain | 2013 | This study |
| N. occidentalis ssp obliqua | UK-3      | Tom Price region, WA | Inland plain | 2013 | This study |
| N. occidentalis ssp obliqua | UK-4      | Tom Price region, WA | Inland plain | 2013 | This study |
| N. occidentalis ssp obliqua | VL552B1.1 | Varanus Island, Lowendal Island group, WA | Coastal | 2013 | This study |
| N. occidentalis ssp occidentalia | CB-1    | Coral Bay, WA | Road verge, coastal township | 2013 | This study |
| N. occidentalis ssp occidentalis | BC-1     | Boundary Creek, Exmouth region, WA | Coastal dry riverbed | 2013 | This study |
| N. occidentalis ssp occidentalis | Br-1     | Bridled Island, Lowendal Island group, WA | Coastal | 2013 | This study |
| N. occidentalis ssp occidentalis | SC-2A    | Shothole Canyon, Cape Range National Park, WA | Inland, cliffs | 2013 | This study |
| N. occidentalis ssp occidentalis | TB-1     | Turquoise Bay, Cape Range National Park, WA | Coastal | 2013 | This study |
| N. rosulata ssp ingulba  | SL18                | Curtain Springs, NT |                    | 1953                      | [46]      |
| N. rosulata ssp rosulata | SL51                |                    |                    |                           | [46]      |
| N. rotundifolia          | SL20                |                    |                    |                           | [46]      |
| N. rotundifolia          | RG-1                | Murchison River, WA | Inland riverbed    | 2013                      | This study|
| N. rotundifolia          | HH-1                | Murchison River, WA | Inland riverbed    | 2013                      | This study|
| N. rotundifolia          | KB-1                | Kalbarri, WA       | Road verge in township | 2013 | This study |
| N. simulans              | SL19                | Wiluna, WA         |                    |                           | [46]      |
| N. simulans              | BrH-1               | Sampson Point, WA  | Road verge         | 2013                      | This study|
| N. simulans              | SL29                | Ayr, QLD           |                    | 1955                      | [46]      |
| N. suaveolens            | SL24                | Flinders Peak, VIC |                    | 2003                      | [46]      |
| N. truncata              | SL44                | Fish Hole Creek, SA|                    | 2005                      | [46]      |

(Continued)
Accessions referred to as ‘Seed Lines’ (SL) of 20 other Nicotiana species indigenous to Australia and one from Namibia (N. Africana, section Suaveolentes) were kindly provided as seed by Dr Edward Newbigin, University of Melbourne (Table 1). Plants of a laboratory accession of N. benthamiana, which we designated RA-4 after Goodin et al. [12], N. glutinosa (section Undulatae, naturally occurring from Bolivia to Peru), and Chenopodium amaranticolor (local lesion host native to South America) were already available. All plants were grown in a rotted bark and sand mix to which 5 g each of lime and dolomite and 40 g of slow release NPK fertiliser was added per 40 litres of potting mix. When the germination rate was low or uneven, seed was soaked overnight at room temperature in a solution of 100 mM gibberellic acid (GA4) to stimulate germination [45].

Viruses

Virus isolates used to challenge plants.

1. Yellow tailflower mild mottle virus isolate Cervantes (YTMMV, genus Tobamovirus, GenBank accession KF495565) was originally isolated from a wild plant of Yellow Tailflower (Anthocercis littoria, family Solanaceae) at Cervantes, Western Australia [30]. The plant was collected under a flora permit issued by the Western Australian Department of Parks and Wildlife.

2. Bean yellow mosaic virus isolate SW3.2 (BYMV, genus Potyvirus, GenBank accession JX156423) was originally isolated from a wild donkey orchid plant (Diuris longifolia, family Orchidaceae) at Brookton, Western Australia [29]. The plant was collected under a flora permit issued by the Western Australian Department of Parks and Wildlife.

3. Cucumber mosaic virus isolate SW-11 (CMV, subgroup II, genus Cucumovirus, GenBank accessions KM434204, KM434205, and KM434206) was isolated from a plant of Cymbidium species (family Orchidaceae) growing on private property belonging to co-author MGK Jones in Perth, Western Australia, with his permission.

4. Tomato spotted wilt virus isolate WA-7 (TSWV, genus Tospovirus, GenBank accessions KM365064, KM365065, and KM365066) was originally isolated from a seedling of tomato cv Money Maker (Solanum lycopersicum, family Solanaceae) purchased from a garden supply store in Perth, Western Australia. No specific permissions were required to collect this plant.

Virus isolates of BYMV, CMV and TSWV were maintained in plants of N. benthamiana RA-4 where they were subcultured every 110–140 days. The genome sequences of each virus isolate were fully or largely determined from double-stranded RNA enriched fractions from the systemic host, N. benthamiana. For YTMMV, leaf sap from the original wild host

| Species           | Accession/Seed Line | Origin (if known)a | Habitat (if known) | Year collected (if known) | Reference |
|-------------------|---------------------|--------------------|--------------------|---------------------------|-----------|
| N. umbratica      | Wea-1               | Weano Gorge, Karijini National Park, WA | Inland riverbed    | 2013                      | This study|
| Nicotiana sp      | MtG-2               | Mount Gould, WA    | Inland, dry riverbed | 2013                      | This study|
| Nicotiana sp      | MtG-4               | Mount Gould, WA    | Inland, dry riverbed | 2013                      | This study|
| Nicotiana sp ‘Corunna’ | SL23               | Corunna, WA        |                    | 2005                      | [46]      |

a NT, Northern Territory; QLD, Queensland; SA, South Australia; VIC, Victoria; WA, Western Australia

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Anthocercis littoria was used to inoculate a plant of N. benthamiana RA-4, and the isolate was subcultured every 20–30 days to a new plant before the previous host died.

Inoculation of Nicotiana plants

After germination, seedlings were grown to the 4-leaf stage before they were subjected to either mock inoculation with 0.1M phosphate buffer (pH7) and diatomaceous earth (Sigma Corp.) or inoculation as above with the addition of macerated leaf material from a virus-infected plant. Five to ten plants of each accession were used for each treatment, and an equal number were used for mock inoculations. Treated plants were grown in climate-controlled, insect-free greenhouses where they were provided with optimal growing conditions (22°C day and 17°C night temperatures, daily watering, weekly nutrient feeds).

Symptom category index and statistical analysis

Symptom development was recorded on infected plants every two to five days until 35 days post-inoculation (dpi). All plants were tested for the presence of systemic spread of virus in uninoculated young leaves at 35 dpi using virus-specific primers (S1 Table) in RT-PCR assays (below).

Plant symptoms and infections were also scored 35 dpi using a simple assessment of systemic infection and symptom severity indices as follows (Fig 2):

Fig 1. Nicotiana plants in their natural habitats in Australia. Top left, a N. benthamiana plant growing amongst rocks beside the Indian Ocean in the Pilbara region. Top right, a N. occidentalis ssp occidentalis plant growing on coastal spinifex grasslands near Roeburn. Lower left, a N. rotundifolia plant on a dry riverbed in the Murchison Region. Lower right, a group of N. occidentalis ssp obliqua plants growing at the base of a rock face in the Pilbara region.

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Fig 2. Symptom indices. A: Index 2, mild symptoms including faint mosaic, little stunting or leaf distortion. Example given is from N. benthamiana accession KL-1 infected with BYMV. B: Index 3, moderate symptoms of infection including strong mosaic and some leaf distortion and plant stunting. Example given is from N. benthamiana accession KL-1 infected with YTMMV. C: Index 4, severe necrosis affecting most of the plant. No flowers. Example given is from N. umbratica accession Wea-1 infected with YTMMV (left). Plant on the right is uninfected. D: Index 5, whole plant is dead.

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0. No systemic infection detected. Local necrotic lesions (NL) may present on inoculated leaves.

1. Systemic spread confirmed by RT-PCR. No visible symptoms of infection observed.

2. Systemic spread confirmed by RT-PCR. Mild symptoms of chlorosis, mosaic and/or leaf deformation evident. Slight stunting may occur. Ring patterns (rings) or small necrotic lesions (NL) sometimes visible.

3. Systemic spread confirmed by RT-PCR. Moderate symptoms of chlorosis, mosaic and/or leaf deformation. Moderate to significant stunting of growth and small necrotic lesions may be present. Flowers usually present.

4. Systemic spread confirmed by RT-PCR. Large necrotic lesions on leaf/stem tissue that affect more than half of the plant. Severe stunting. Plant remained alive but apparently not actively growing. No flowers present.

5. Systemic spread confirmed by RT-PCR. The plant was dead by 35 dpi

Confirmation of infection status

Inoculated plants were screened at 35 dpi for presence of the virus using virus-specific primers (S1 Table) in RT-PCR assays. The MyTaq One-Step RT-PCR kit (Bioline) was used to synthesise cDNA and amplify fragments of virus genomes in the presence of virus-specific forward and reverse primers from total RNA extracted from plants using the RNeasy Plant Mini kit (Qiagen) or a dsRNA enrichment method [47] modified by replacing Whatman CF11 cellulose powder with Machery Nagel MN100 cellulose powder. Virus-specific primers were used for YTMMV [30], BYMV [48], CMV [49], and TSWV [50] (S1 Table). Subsequent Sanger sequencing of amplicons was done using the amplification primers and with BigDye terminator V3.1 chemistry, and analysed with an Applied Biosystems/Hitachi 3730 DNA Analyzer. Sequences were edited manually in FinchTV (Geospiza) and aligned using ClustalW [51].

RDR1 gene sequencing

DNA was extracted from 100 mg young leaf tissue using DNeasy Plant Mini kit columns (Qiagen). Primers were designed to flank the region of the Nb-RDR1m allele 72 nt insertion mutation [33]. Three sets of primers were used. Primers RP1 and RP2 [33], which generated an amplicon of approximately 327 nt or 255 nt, depending on the presence of the insertion mutation. Primers RP120614 and RP220614 generated amplicons of 351 nt or 279 nt, and primers RP1new and RP2new generated amplicons of 389 nt or 317 nt (S2 Table). Amplification was done using GoTaq DNA Polymerase (Promega Corp). The parameters used for subsequent Sanger sequencing of amplicons were as described above.

Results

Significant differences (p<0.05) between plant responses to virus infection were recorded (Table 2, S2 Table, S3 Table, Fig 2, S1 Fig.).

YTMMV

There were significant differences (p<0.05) among the infected plants by YTMMV. This virus killed members of some of the Nicotiana species tested, and it had the highest overall mean
Table 2. Mean symptom severity index (Standard Deviation above 0 in parenthesis) of virus infection on plants expressed 35 dpi.

| Species* and subspecies (where known) | Accession/Seed line | YTMMV Symptom index | BYMV | CMV | TSWV | GenBank Accession RDR1 (partial) | indel present | Y/N |
|--------------------------------------|---------------------|---------------------|------|-----|------|----------------------------------|---------------|-----|
| *Chenopodium amaranticolor*          |                     | 0.0 (NL)            | 0.0  | 0.0 | 0.0  |                                 |               |     |
| *C. quinoa*                          |                     | 0.0 (NL)            | 0.0  | 0.0 | 0.0  |                                 |               |     |
| *Nicotiana africana*                 | SL6                 | 2.0                 | 1.4 (0.55) | 1.0 | 1.0  |                                 |               |     |
| *N. amplexicaulis*                   | SL7                 | 1.0                 | 2.0  | 2.0 | 2.0  | KM411324                        | N             |     |
| *N. benthamiana*                     | 17.19C              | 2.6 (0.54)          | 1.0  | 2.0 | 2.0  | KM411316                        | N             |     |
| *N. benthamiana*                     | 17.23               | 3.0                 |      |     |      | KM411311                        | N             |     |
| *N. benthamiana*                     | 17.24               | 3.0                 |      |     |      | KM411313                        | N             |     |
| *N. benthamiana*                     | 17.26               | 3.0                 |      |     |      | KM411325                        | N             |     |
| *N. benthamiana*                     | 17.27               | 3.0                 |      |     |      | KM411314                        | N             |     |
| *N. benthamiana*                     | 17.28               | 3.0                 |      |     |      | KM411315                        | N             |     |
| *N. benthamiana*                     | CI-1                | 2.0                 |      |     |      |                                 |               |     |
| *N. benthamiana*                     | HCK-1               | 3.8 (0.45)          |      |     |      |                                 |               |     |
| *N. benthamiana*                     | HCK-3               | 3.8 (0.45)          |      |     |      |                                 |               |     |
| *N. benthamiana*                     | KL-1                | 3.0                 | 2.2 (0.45) | 2.8 | 3.2 (0.45) | KM411351                      | N             |     |
| *N. benthamiana*                     | KL-2                | 3.0                 | 2.2 (0.45) | 2.8 | 3.2 (0.45) | KM411352                      | N             |     |
| *N. benthamiana*                     | KL-3                | 2.0                 | 3.0  | 3.0 | 3.0  | KM411349                        | N             |     |
| *N. benthamiana*                     | KL-4                | 2.0                 | 3.0  | 3.0 | 3.0  | KM411353                        | N             |     |
| *N. benthamiana*                     | KL-5                | 2.0                 | 3.0  | 3.0 | 3.0  | KM411319                        | N             |     |
| *N. benthamiana*                     | KL-1                | 3.0                 | 3.0  | 3.0 | 3.0  | KM411307                        | N             |     |
| *N. benthamiana*                     | MA-3                | 3.0                 |      |     |      | KM411321                        | N             |     |
| *N. benthamiana*                     | MA-5                | 3.0                 |      |     |      | KM411312                        | N             |     |
| *N. benthamiana*                     | MA-6                | 3.0                 |      |     |      | KM411304                        | N             |     |
| *N. benthamiana*                     | MA-7                | 3.0                 |      |     |      | KM411341                        | N             |     |
| *N. benthamiana*                     | PPM-1               | 3.0                 |      |     |      | KM411341                        | N             |     |
| *N. benthamiana*                     | PPM-2               | 3.0                 |      |     |      | KM411341                        | N             |     |
| *N. benthamiana*                     | RA-4                | 5.0                 | 3.0  | 3.0 | 3.0  | KM411308                        | Y             |     |
| *N. benthamiana*                     | VL552B2.1           | 3.0                 | 2.0  | 2.0 | 3.0  | KM411346                        | N             |     |
| *N. benthamiana*                     | VL552B2.2           | 3.0                 | 2.0  | 2.0 | 3.0  | KM411346                        | N             |     |
| *N. cavicola*                        | SL9                 | 5.0                 | 3.8 (0.45) | 2.0 | 4.2 (0.45) | KM411326                      | N             |     |
| *N. excelsior*                       | SL11                | 4.6 (0.55)          | 1.1  | 1.0 | 2.4 (0.55) |                                 |               |     |
| *N. forsteri*                        | SL5                 | 1.0                 | 1.0  | 3.0 | 3.0  | KM411327                        | N             |     |
| *N. glutinosa*                       | SL13                | 0.0 (NL)            | 0.0  | 0.0 | 0.0 (NL) | KM411328                       | N             |     |
| *N. goodspeedii*                     | SL14                | 1.0                 | 3.0  | 3.0 | 3.0  | KM411329                        | N             |     |
| *N. heterantha*                      | FI-2                | 2.4 (0.55)          |      |     |      |                                 |               |     |
| *N. heterantha*                      | FI-3                | 2.2 (0.45)          |      |     |      | KM411310                        | N             |     |
| *N. heterantha*                      | SL33                | 1.8 (0.45)          | 4.2 (0.55) | 4.2 | 3.4 (0.55) |                                 |               |     |

(Continued)
| Species and subspecies (where known) | Accession/Seed line | YTMMV Symptom index | BYMV Symptom index | CMV Symptom index | TSWV Symptom index | GenBank Accession | RDR1 (partial) | indel present |
|--------------------------------------|---------------------|---------------------|-------------------|-----------------|------------------|-----------------|---------------|--------------|
| *N. maritima*                        | SL35                | 3.0                 | 3.0               | 3.0             |                  |                 |               |              |
| *N. megalosiphon*                    | SL1                 | 2.0 (NL)            | 1.0               | 5.0             | KM411330         | N               |               |              |
| *N. occidentalis hesperis* (or *N. suaveolens)* | SL15                | 2.0                 | 1.0               | 4.0             | KM411318         | N               |               |              |
| *N. occidentalis*                    | CI-1                | 4.4 (0.55)          |                   |                 | KM411331         | N               |               |              |
| *N. occidentalis ssp hesperis*       | NI-1                | 4.0 (NL)            | 5.0 (NL)          | 5.0 (NL)        | KM411322         | N               |               |              |
| *N. occidentalis ssp hesperis*       | NI-4                | 4.0 (NL)            | 3.0 (NL)          | 4.0 (NL)        | KM411323         | N               |               |              |
| *N. occidentalis ssp hesperis*       | NI-5                | 4.0 (NL)            | 3.0 (NL)          | 5.0 (NL)        | KM411332         | N               |               |              |
| *N. occidentalis ssp obliqua*         | FI-1                | 4.4 (0.55)          |                   |                 | KM411333         | N               |               |              |
| *N. occidentalis ssp obliqua*         | MA-10               | 5.0                 |                   |                 | KM411322         | N               |               |              |
| *N. occidentalis ssp obliqua*         | MA-11               | 4.8 (0.45)          |                   |                 | KM411309         | N               |               |              |
| *N. occidentalis ssp obliqua*         | MA-12               | 4.8 (0.45)          |                   |                 | KM411323         | N               |               |              |
| *N. occidentalis ssp obliqua*         | MA-12               | 4.8 (0.45)          |                   |                 | KM411323         | N               |               |              |
| *N. occidentalis ssp obliqua*         | MA-4                | 5.0                 |                   |                 | KM411332         | N               |               |              |
| *N. occidentalis ssp obliqua*         | MA-8                | 5.0                 |                   |                 | KM411332         | N               |               |              |
| *N. occidentalis ssp obliqua*         | MA-9                | 4.4 (0.55)          |                   |                 | KM411331         | N               |               |              |
| *N. occidentalis ssp obliqua*         | SL17                | 5.0                 | 4.0               | 1.0             | 4.0              | KM411344        | N               |              |
| *N. occidentalis ssp obliqua*         | UK-1                | 4.8 (0.45)          |                   |                 | KM411344         | N               |               |              |
| *N. occidentalis ssp obliqua*         | UK-2                | 5.0                 |                   |                 | KM411343         | N               |               |              |
| *N. occidentalis ssp obliqua*         | UK-3                | 4.6 (0.55)          |                   |                 | KM411343         | N               |               |              |
| *N. occidentalis ssp obliqua*         | UK-4                | 5.0                 |                   |                 | KM411345         | N               |               |              |
| *N. occidentalis ssp obliqua*         | VLS52B.1.1          | 5.0                 | 2.0               | 2.0             | 5.0              | KM411337        | N               |              |
| *N. occidentalis ssp obliqua*         | MB-1                | 3.8 (0.45)          |                   |                 | KM411317         | N               |               |              |
| *N. occidentalis ssp obliqua*         | BC-1                | 5.0                 |                   |                 | KM411334         | N               |               |              |
| *N. occidentalis ssp obliqua*         | Br-1                | 5.0                 |                   |                 | KM411320         | N               |               |              |
| *N. occidentalis ssp obliqua*         | SC-2A               | 4.8 (0.45)          |                   |                 | KM411335         | N               |               |              |
| *N. occidentalis ssp obliqua*         | TB-1                | 5.0                 |                   |                 | KM411336         | N               |               |              |
| *N. rosulata ssp ingulba*             | SL18                | 5.0                 | 1.4 (0.45)        | 1.0             | 4.4 (0.55)       | KM411338        | N               |              |
| *N. rosulata ssp rosulata*            | SL51                | 4.8 (0.45)          |                   |                 | KM411339         | N               |               |              |
| *N. rotundifolia*                     | SL20                | 1                   | 1.0               | 4.0             | KM411305         | N               |               |              |
| *N. rotundifolia*                     | HH-1                | 5.0                 |                   |                 | KM411347         | N               |               |              |
| *N. rotundifolia*                     | KB-1                | 4.4 (0.55)          |                   |                 | KM411348         | N               |               |              |
| *N. rotundifolia*                     | RG-1                | 5.0                 |                   |                 | KM411348         | N               |               |              |
symptom severity index, 3.94, of the four viruses tested across all plant accessions (S3 Table, S4 Table). *N. benthamiana* laboratory accession RA-4 became wilted and chlorotic within 20 days of infection by YTMMV (Fig 3C), then died between 21 and 35 dpi In contrast, none of the wild accessions of *N. benthamiana* died upon infection with YTMMV. Instead, they exhibited moderate symptoms of mosaic and leaf distortion (Table 2, Fig 2B, Fig 3D). The following species and accessions exhibited severe symptoms of disease or died: *N. cavicola* SL9, *N. excelsior* SL11, all the *N. occidentalis* accessions tested, consisting of subspecies *obliqua* and *occidentalis* (accession codes Cl-1, CB-1, SC-2A, 17.3B, SL17, Mta-8, Mta-4, Mta-9, Mta-10, Mta-11, Mta-12, Ft-1, VL552B1.1, UK-4, UK-3, UK-2, UK-1, BC-1, Br-1, TB-1, BC-1) (Fig 2D), the two *N. rosulata* accessions tested, consisting of subspecies *ingulba* and *rosulata* (SL18 and SL51, respectively), two accessions of *N. simulans* (SL19, SL29), three of *N. rotundifolia* (KB-1, HH-1, RG-1), *N. umbratica* (Wea-1, Fig 2C), and a *Nicotiana* accession not identified to the species level (BrH-1). Of those that did not die, plants of *N. occidentalis* accessions CB-1, Ft-1, and CI-1, Mta-9, Mta-12, Mta-12, UK-1, UK-3, SC2-A grew new, often chlorotic and deformed shoots from axillary buds, some of which produced flowers and seed. Most of the other species tested exhibited moderate symptoms, the exceptions being accessions of *N. heterantha* (Ft-2, Ft-3, SL33) and *N. africana* (SL6), which exhibited mild symptoms. *N. glutinosa* plants

| Species* and subspecies (where known) | Accession/Seed line | YTMMV | BYMV | CMV | TSWV | GenBank Accession | RDR1 (partial) | indel present | Symptom index* |
|--------------------------------------|---------------------|--------|------|-----|------|------------------|---------------|--------------|----------------|
| *N. simulans*                        | SL19                | 5.0    | 2.0  | 2.0 | 4.2  | KM411306         | N             |              |                |
| *N. simulans*                        | SL29                | 5.0    | 2.2  | 2.2 | 4.8  | KM411340         | N             |              |                |
| *N. suaveolens*                      | SL24                | 2.0    | 2.0  | 3.0 |      | KM411306         | N             |              |                |
| *N. truncata*                        | SL44                | 2.0    | 2.0  | 3.0 |      | KM411342         | N             |              |                |
| *N. umbratica*                       | Wea-1               | 3.6    | 1.0  | 1.0 | 4.0  | KM411303         | N             |              |                |
| Nicotiana sp                         | BrH-1               | 5.0    |      |     |      | KM411333         | N             |              |                |
| Nicotiana sp                         | MtG-2               | 3.0    |      |     |      | KM411350         | N             |              |                |
| Nicotiana sp                         | MtG-4               | 3.0    |      |     |      | KM411303         | N             |              |                |
| Nicotiana sp ‘Corunna’               | SL23                | 1.0    | 1.0  | 4.0 |      |                 |               |              |                |

The presence (Y) or absence (N) of an insertion/deletion (indel) mutation in the *RDR1* is indicated. GenBank accession codes of partial *RDR1* coding sequences are given.

*YTMMV,* yellow tailflower mild mottle virus; *BYMV,* bean yellow mosaic virus; *CMV,* cucumber mosaic virus; *TSWV,* tomato spotted wilt virus.

*Mean symptom indices (Standard deviation)*

0. Systemic infection not detected
1. No visible symptoms of infection.
2. Mild symptoms of chlorosis, mosaic and/or leaf deformation evident. Slight stunting may be evident. Ring patterns or small necrotic spots sometimes visible. Little to no obvious impact on the numbers of flowers or viable seed produced.
3. Moderate symptoms of chlorosis, mosaic and/or leaf deformation. Moderate to significant stunting of growth and small necrotic patches may be present. Some flowers and seed may be produced.
4. Large patches of leaf/stem necrosis, severe stunting. Plant remained alive.
5. The plant was dead.

NL = necrotic lesion; rings = ring patterns

Table 2. (Continued)

| Species* and subspecies (where known) | Accession/Seed line | YTMMV | BYMV | CMV | TSWV | GenBank Accession |
|--------------------------------------|---------------------|--------|------|-----|------|-------------------|
|                                       |                     | Symptom index* |
| *N. simulans*                        | SL19                | 5.0    |
| *N. simulans*                        | SL29                | 5.0    |
| *N. suaveolens*                      | SL24                | 2.0    |
| *N. truncata*                        | SL44                | 2.0    |
| *N. umbratica*                       | Wea-1               | 3.6    |
| Nicotiana sp                         | BrH-1               | 5.0    |
| Nicotiana sp                         | MtG-2               | 3.0    |
| Nicotiana sp                         | MtG-4               | 3.0    |
| Nicotiana sp ‘Corunna’               | SL23                | 1.0    |

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symptom severity index, 3.94, of the four viruses tested across all plant accessions (S3 Table, S4 Table). *N. benthamiana* laboratory accession RA-4 became wilted and chlorotic within 20 days of infection by YTMMV (Fig 3C), then died between 21 and 35 dpi In contrast, none of the wild accessions of *N. benthamiana* died upon infection with YTMMV. Instead, they exhibited moderate symptoms of mosaic and leaf distortion (Table 2, Fig 2B, Fig 3D). The following species and accessions exhibited severe symptoms of disease or died: *N. cavicola* SL9, *N. excelsior* SL11, all the *N. occidentalis* accessions tested, consisting of subspecies *obliqua* and *occidentalis* (accession codes Cl-1, CB-1, SC-2A, 17.3B, SL17, Mta-8, Mta-4, Mta-9, Mta-10, Mta-11, Mta-12, Ft-1, VL552B1.1, UK-4, UK-3, UK-2, UK-1, BC-1, Br-1, TB-1, BC-1) (Fig 2D), the two *N. rosulata* accessions tested, consisting of subspecies *ingulba* and *rosulata* (SL18 and SL51, respectively), two accessions of *N. simulans* (SL19, SL29), three of *N. rotundifolia* (KB-1, HH-1, RG-1), *N. umbratica* (Wea-1, Fig 2C), and a *Nicotiana* accession not identified to the species level (BrH-1). Of those that did not die, plants of *N. occidentalis* accessions CB-1, Ft-1, and CI-1, Mta-9, Mta-12, Mta-12, UK-1, UK-3, SC2-A grew new, often chlorotic and deformed shoots from axillary buds, some of which produced flowers and seed. Most of the other species tested exhibited moderate symptoms, the exceptions being accessions of *N. heterantha* (Ft-2, Ft-3, SL33) and *N. africana* (SL6), which exhibited mild symptoms. *N. glutinosa* plants
Fig 3. Symptoms of infection. A. Leaf of *N. occidentalis ssp hesperis* Nt-5 infected with BYMV exhibiting small necrotic lesions within 7 days of infection. B. *N. simulans* SL19 exhibited small chlorotic rings (arrow) 15 dpi with BYMV. C. Symptoms of infection of YTMMV on *N. benthamiana* RA-4 20 dpi. D. *N. benthamiana* VLS2B2.1 exhibiting symptoms of leaf mottling and deformation 20 dpi with YTMMV. E. *N. benthamiana* Kx-1 (left) exhibiting chlorotic spots 20 dpi with BYMV. Plant on the right is uninfected.
responded with small necrotic local lesions on infected leaves but there was no systemic spread of the virus.

Seeds of *N. benthamiana* accessions VL552B2.1, 17.24 and 17.26 were collected from YTMMV-infected parent plants and from three mock-infected plants of the same accessions and germinated. Visual assessment was done at the two-leaf stage of approximately 300 seedlings from each batch. Seedlings derived from infected and uninfected parents appeared indistinguishable and none exhibited symptoms typical of YTMMV infection that were evident on parents. RT-PCR assays using YTMMV-specific primers were carried out RNA extracted from bulked leaf samples from each group of seedlings, and these failed to detect YTMMV.

**BYMV**

There were significant differences (p<0.05) among the infected plants by BYMV. All inoculated *Nicotiana* plants became infected, but none of the infected plants died. The range of responses expressed by systemically infected plants was from asymptomatic to severe (symptom indices 1–4) (Table 2, S2 Table, S3 Table, Fig 2A). Most *N. benthamiana* accessions tested, including RA-4, responded with similar mild to moderate symptoms. Plants of *N. benthamiana* accession Kx-1 and of *N. occidentalis* ssp *hesperis* (Nt-1, Nt-4, Nt-5) were unusual in that they exhibited small necrotic lesions on inoculated leaves (Fig 3A and 3E). Plants of *N. occidentalis* ssp *hesperis* accessions NT-1, Nt-4 and Nt-5 became severely symptomatic, but *N. occidentalis* ssp *obliqua*—VL552B1.1 plants remained mildly symptomatic, and no necrotic lesions were present. Another unusual symptom of BYMV infection was chlorotic ring patterns that occurred on leaves of all infected *N. simulans* SL19 plants (Fig 3B), but not on *N. simulans* SL29 plants. In plants of severely affected accessions of *N. goodspeedii* SL13 and *N. heterantha* SL33, the majority of leaves and apical meristems died. These showed signs of recovery when axillary buds emerged by about 35 dpi, but these remained deformed and chlorotic and few flowers, if any, were produced. None of the following exhibited visible symptoms, although they were all systemically infected: *N. amplexicaulis* SL7, *N. forsteri* SL5, *N. excelsior* SL11, *N. gossei* SL14, *N. rotundifolia* SL20, *N. umbratica* Wea-1, *N. africana* SL6, and *Nicotiana* species ‘Corunna’ SL23 (Table 2).

**CMV**

There were significant differences (p<0.05) among the infected plants by CMV. All *Nicotiana* plants tested became infected with CMV. In most cases, infected plants showed similar patterns of symptom development to those infected with BYMV (the mean difference in overall symptom index between BYMV and CMV was 0.56) (S2 Table). The notable exception was *N. occidentalis* ssp *hesperis* Nt-1, where all plants died, whereas the other two *N. occidentalis* ssp *hesperis* accessions exhibited moderate symptoms. The three accessions of *N. occidentalis* ssp *hesperis* tested (Nt-1, Nt-4, Nt-5) developed necrotic lesions. *N. occidentalis* ssp *obliqua* SL17 plants were asymptomatic. *N. cunicola* SL9 plants that were strongly symptomatic under BYMV infection responded with mild symptoms to CMV infection (Table 2). There were no rings induced by CMV infection on any plant tested.

**TSWV**

There were significant differences (p<0.05) among the infected plants by TSWV. All *Nicotiana* plants inoculated became infected, and plants of some accessions were killed by infection with TSWV (symptom index 5), and on all plants tested, overall symptoms of TSWV were more severe (mean symptom index of 3.49) than with BYMV or CMV infection (S2 Table, S3 Table). Generally, plants reacted with symptoms about one category higher on infection with TSWV.
than they did with BYMV or CMV. Exceptions were plants of *N. rotundifolia* SL20 and *Nicotiana* 'Corunna' SL23 that had symptoms three categories higher. Infected plants *N. occidentalis* ssp *hesperis* Nt-1, Nt-4, Nt-5 and SL15 initially exhibited local necrotic lesions on inoculated leaves and later symptoms were severe or plants died. *N. occidentalis* ssp *obliqua* accessions VL552B1.1 and SL17 did not show local lesions on inoculated leaves but later symptoms resembled those of *N. occidentalis* ssp *hesperis* accessions. Plants of *N. megalosiphon* SL1, *N. simulans* SL19 and SL29, *N. occidentalis* ssp *hesperis* SL15, *N. cavincola* SL9, *N. rotundifolia* SL20, and *Nicotiana* 'Corunna' SL23 all exhibited severe symptoms. TSWV did not spread systemically in *N. glutinosa*, instead plants responded with small necrotic local lesions on inoculated leaves.

**RDR1 gene sequence**

Fifty-one partial RDR1 gene fragments were sequenced and GenBank accessions assigned (Table 2), 19 of which were from *N. benthamiana* accessions and the rest from accessions of other *Nicotiana* species. Fifty sequences shared >94% nt identity with one another. The notable exception was laboratory accession *N. benthamiana* RA-4, which contained the identical 72 nt insertion mutation reported by Yang and colleagues [33]. The wild accessions of *N. benthamiana* tested did not contain this insertion or other insertions or deletions or translation stop codons in this gene region, nor did accessions of the other species tested (Table 2).

**Discussion**

Responses to infection by YTMMV were significantly different between new wild accessions of *N. benthamiana* and laboratory accession RA-4. Although both groups were equally susceptible to infection by YTMMV and to the other viruses tested, as defined by the ability of the virus to replicate and move systemically within the plant, the differences between them were in symptom responses. Every *N. benthamiana* RA-4 plant infected with YTMMV was dead by 21–35 dpi, whereas all plants of the new wild accessions responded with moderate symptoms, and none died. Additionally, YTMMV-infected plants of the wild *N. benthamiana* accessions produced viable seed. YTMMV was not detected from seedlings grown from seed from three infected *N. benthamiana* parent plants, indicating that seed transmission of YTMMV does not occur or it is rare. The insertion mutant *Nb-RDR1m* allele [33] was present in plants of our laboratory accession RA-4, which contained the identical 72 nt insertion mutation reported by Yang and colleagues [33]. The wild accessions of *N. benthamiana* tested did not contain this insertion or other insertions or deletions or translation stop codons in this gene region, nor did accessions of the other species tested (Table 2).

Previously, two groups [33, 52] researched the role of *Nb-RDR1m* in response to virus infection. Yang et al. [33] used virus-induced gene silencing to show that despite being truncated, *Nb-RDR1m* ameliorates virus-induced (Potato virus X, PVX, Potexvirus) symptom development. Both groups attempted to create the equivalent of *N. benthamiana* *Nb-RDR1* plants by complementing the mutant *Nb-RDR1m* with the functional ortholog from another species. Yang et al. [33] used the RDR1 from *Medicago truncatula* (*Mt-RDR1*), creating *Nb-RDR1m* +
Mt-RDR1 (hereafter called Mt-RDR1 plants) plants, while Ying et al. [52] used the N. tabacum Nt-RDR1 to create Nb-RDR1m + Nt-RDR1 (hereafter called Nt-RDR1 plants) plants. Both groups assumed that a functional transgenic RDR1 ortholog would be expressed in a dominant manner over the endogenous mutant Nb-RDR1m. Ying and colleagues [33] showed that expression of Nt-RDR1 did not suppress expression of endogenous Nb-RDR1m, Nb-RDR2 or Nb-RDR6 in the transgenic N. benthamiana lines developed. In the two studies [33, 52], responses to virus infection differed widely. After Cucumber mosaic virus (CMV, Cucumovirus), PVX or Potato virus Y (PVY, Potyvirus) infection, Yang et al. [33] demonstrated that Mt-RDR1 plants and Nb-RDR1m control plants responded similarly in terms of virus accumulation, viral RNA expression, and symptom severity. In contrast, Ying and colleagues [52] found that Nt-RDR1 plants infected with CMV, PVY and Plum pox virus (Potyvirus) displayed more severe symptoms, had higher virus accumulation, and greater viral RNA expression than did Nb-RDR1m control plants. Like the earlier study [33], our study showed that infection by the non-tobamoviruses we tested (BYMV, CMV, TSWV) did not induce greater symptom expression in Nb-RDR1 than in Nb-RDR1m plants. When challenged with tobamoviruses, Mt-RDR1 plants accumulated less TMV, Turnip vein-clearing virus and Sunn hemp mosaic virus, whereas Nb-RDR1m control plants were severely symptomatic [33]. Again, the responses reported by Ying and colleagues [52] differed markedly; transgenic Nt-RDR1 plants responded in a similar manner to Nb-RDR1m control plants after infection with TMV and Tomato mosaic virus. Our experiment with Nb-RDR1m and Nb-RDR1 plants and the tobamovirus YTMMV gave results more in line with those of Yang et al. [33] than of Ying et al. [52]. We found that plants with an apparently functional Nb-RDR1 were protected against tobamovirus-induced severe symptomology. We are aware that the Nb-RDR1 and Nb-RDR1m plants we used have slightly different genetic backgrounds, so traits other than RDR1 may be present, and these may also influence symptom response. Other genes known to be involved in antiviral RNA silencing include DCL2, DCL3, DCL4, DRB4, RDR6, SGS3, HEN1, AGO1 and AGO2 [18, 53], and these were not examined in the current study.

It is unclear why plant responses reported by us and by Yang et al. [33] differed so much from those of Ying et al. [52]. Possible reasons are the differences observed may relate to the sources of the RDR1 (M. truncatula vs N. tabacum vs N. benthamiana), or differences in virulence in the virus strains used. The latter explanation does not easily account for the differences seen in CMV symptomology; both Yang et al. [33] and Ying et al. [52] used genetically similar CMV subgroup I isolates (CMV-Fny and CMV-SD, respectively), whereas in this study we used a relatively dissimilar subgroup II isolate (CMV-SW-11).

Here we determined that there was no difference between Nb-RDR1 and Nb-RDR1m plants in terms of susceptibility to all the viruses tested, as defined by the ability of the virus to infect the plant systemically. Both Yang et al. [33] and Ying et al. [52] determined that Mt-RDR1 or Nt-RDR1 were still susceptible to the tobamoviruses they analysed. Therefore, from all these results it appears that symptom responses and possibly virus titre and viral RNA accumulation, but not susceptibility as such, is associated with Nb-RDR1 function.

Yang et al. [33] proposed that Nb-RDR1m is a recent mutation because its nucleotide sequence (other than the 72 nt insertion) closely resembles the N. tabacum Nt-RDR1, and because Nb-RDR1m is still inducible by phytohormone application and virus infection. Our finding that Nb-RDR1m was absent in the wild populations we tested tends to support the hypothesis that Nb-RDR1 is a recent mutation that has not had time to expand its range. On the other hand, it is possible that it was once more widely distributed but now remains in localised populations, perhaps because of tobamovirus epideemics. The sporadic distribution of N. benthamiana populations in northern Australia may help protect wild Nb-RDR1m populations from tobamovirus infection. Tobamoviruses have no known arthropod vectors, but because
they have extremely stable particles [54], potentially any creature that eats or contacts them might vector them. The existence of YTMMV, a Solanaceae-infecting tomatovirus, and Clitoria yellow mottle virus, a legume-infecting one [55], makes it possible that other tobamoviruses exist in the Australian flora, and that over time contact with *N. benthamiana* populations is likely to occur. Systemic necrosis responses in *Nb-RDR1m* plants and in other *Nicotiana* species to YTMMV infection may be examples of ‘field resistance’, where susceptible plants die quickly, thereby removing themselves as sources of infection and slowing virus spread within the population, but we find this scenario improbable because it should lead to the extinction of *Nb-RDR1m*. If the location of the *Nb-RDR1m* population that yielded the original laboratory accession were determined from historical documents, it would be of interest to elucidate natural distribution of *Nb-RDR1m* within it and surrounding populations to establish if its range is expanding or contracting. Similarly, analysis of the dynamics of the acute and persistent viral flora infecting natural *Nb-RDR1m* and *Nb-RDR1* populations would be of great scientific interest in revealing co-evolutionary strategies and ecological roles in natural plant/virus systems.

Ying *et al.* [52] proposed that *Nb-RDR1* has a dual role: that of silencing viral transcripts and of suppressing *Nb-RDR6*-mediated inhibition of systemic virus spread. Their hypothesis that the dysfunctional *Nb-RDR1m* evolved during a prolonged host-virus arms race to favour up-regulation of an *Nb-RDR6*-induced antiviral system seems unlikely given the apparent rarity of *Nb-RDR1m* in the wild, but this hypothesis can be tested by carrying out the distribution and ecology studies of natural *Nb-RDR1m* populations proposed above.

We propose two hypotheses to account for the prevalence in laboratories of the apparently rare-in-the-wild *Nb-RDR1m*. The first hypothesis is that the insertion mutation happened recently in a laboratory during the 70+ years that *N. benthamiana* has been utilised by plant biologists. Its discovery in a natural population would immediately discount this hypothesis. The second hypothesis is that seed from a wild *Nb-RDR1m* plant was collected from a natural population, perhaps amongst *Nb-RDR1* plants, and biologists selected the former because of its greater symptom responses to some viruses and perhaps other traits. Indeed, when we compare *Nb-RDR1m* plants with *Nb-RDR1* plants, the former appear to express several unusual traits, some of which may be considered as ‘domestication’ traits. For example, seed from *Nb-RDR1m* plants germinated quickly and evenly 1–3 weeks after harvest, whereas that of wild accessions usually required 6–20 weeks of storage (or GA4 treatment) after harvest before it germinated evenly. Uneven physical or physiological seed dormancy is a common survival strategy against desiccation in wild annual plants [56]. The leaves of *Nb-RDR1m* plants are relatively glabrous (hairless), have thin laminas, are paler green, smaller, and with petioles that lacked wings. Thin glabrous laminas enable more efficient virus inoculation. Leaves of wild accessions from inland populations were often covered with hard prickly hairs, had thicker laminas, larger leaf sizes, and sometimes they had winged petioles (e.g accessions MtA 3–7). Leaves from plants collected at coastal sites were glabrous with reflective, rugose, waxy laminas, and without winged petioles (e.g. accession VL552B2.1). Experiencing the same growing conditions under glass, *Nb-RDR1m* plants grew to about a metre in height, whereas plants of some wild accessions (e.g. accession PPM1) grew to two metres. *Nb-RDR1m* plants quickly produced many small white flowers, while wild accessions were generally slower to flower, had fewer but relatively larger flowers (e.g. accession PPM-1), and some were cream to yellow in colour, or tinged with purple pigment. Smaller, faster developing plants are more suited to scientific experiments done in confined spaces. We are cautious to attribute all the unusual traits seen in *N. benthamiana* RA-4 plants to human selection. *RDR1* influences small RNA expression, which in turn may influence expression of other genes [57, 58, 59, 60, 61].
The genetic homogeneity of the single available accession of *N. benthamiana* has enabled meaningful extrapolation of experiments done in different laboratories over 70 years. The presence of *Nb-RDR1m* has undoubtedly stimulated its acceptance as a model plant for virology and transient gene expression studies. The discovery reported here that the widely used laboratory accession is probably not representative of the species as a whole is surprising and exciting. This finding opens up possibilities of comparisons between accessions, the most obvious being comparative expression analysis of *Nb-RDR1m* and *Nb-RDR1* plants. There appears to be no sexual incompatibility between the few accessions we have already crossed using *N. benthamiana*-RA-4 as the female partner, and this is being tested more broadly within the species. Sexual incompatibilities do occur between some accessions of some species, for example *Arabidopsis thaliana* [62, 63].

Any effective biological model system should have available a collection of accessions with a range of genotypical and phenotypical characteristics. For example, *A. thaliana*, the most widely used model plant system, has over 300 natural accessions available from across Eurasia [64]. Until recently, *N. benthamiana* probably had only one. Although *N. occidentalis* has played second fiddle to *N. benthamiana* in virus research and expression studies, we envisage that availability of a broader range of subspecies and accessions will enable it too to become a useful model species.

**Supporting Information**

S1 Fig. A: Marginal means of symptom severity induced in 15 *Nicotiana* accessions systemically infected with four viruses (Tables 1, 2). Symptom indices of 0–5 represent a range of responses to inoculation from (1) systemic infected detected but no symptoms observed to (5) systemic infection detected leading to whole plant death. B: Overall comparison of the viruses assessed using marginal means of symptom severity induced in 15 *Nicotiana* accessions.

S1 Table. Virus specific primer sequences (5’ > 3’) used to confirm infection by Yellow tailflower mild mottle virus (YTMMV), Bean yellow mosaic virus (BYMV), Cucumber mosaic virus (CMV), and Tomato spotted wilt virus (TSWV).

S2 Table. Sequences (5’ > 3’) of primers used to amplify part of the *Nicotiana RDR1* gene. Numbers in parentheses represent the annealing coordinates of the primers on the *N. benthamiana Nb-RDR1m* sequence (GenBank accession AY574374).

S3 Table. Comparison symptom severity induced by four viruses on 75 *Nicotiana* plants using severity of symptoms as the dependent variable. This is a measure of relative symptom severity induced by each virus. Thus, mean differences >1 indicates overall greater symptom severity is induced by virus I than by virus J, while mean differences <1 indicates overall milder symptoms induced by virus I compared to virus J.

S4 Table. Overall symptom severity indices of four viruses infecting 75 *Nicotiana* plants (five plants each of 15 different *Nicotiana* accessions) as calculated by Tukey B analysis. Symptom indices of 0–5 represent a range of responses to inoculation from (1) systemic infected detected but no symptoms observed to (5) systemic infection detected leading to whole plant death. Thus, as indices of severity increase, symptom severity increases.
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Author Contributions

Conceived and designed the experiments: MJR HL SW. Performed the experiments: HL CZ SW. Analyzed the data: HL CZ SW SHK. Contributed reagents/materials/analysis tools: MJ VL. Wrote the paper: SW HL SI MJR VL MJ.

References

1. Knapp S, Chase MW, Clarkson JJ. Nomenclatural changes and a new sectional classification in *Nicotiana* (Solanaceae). Taxon. 2004; 53: 73–82.
2. Marks CE, Newbigin E, Ladiges PY. Comparative morphology and phylogeny of *Nicotiana* section *Sua-veolentes* (Solanaceae) in Australia and the South Pacific. Austral Syst Bot. 2011; 24: 61–86.
3. Clarkson JJ, Knapp S, Garcia VF, Olmstead RG, Leitch AR, Chase MW. Phylogenetic relationships in *Nicotiana* (Solanaceae) inferred from multiple plastid DNA regions. Mol Phylogen Evol. 2004; 33: 75–90.
4. Knapp S, Bohs L, Nee M, Spooner DM. Solanaceae—a model for linking genomics with biodiversity. Comp Func Gen. 2004; 5: 285–291.
5. Ratsch A, Steadman KJ, Bogossian F. The pituri story: a review of the historical literature surrounding traditional Australian Aboriginal use of nicotine in Central Australia. J. Ethnobiol Ethnomed. 2010; 6: 1–13. doi: 10.1186/1746-4269-6-1 PMID: 20089149
6. Cleland JB, Johnston TH. Aboriginal names and uses of plants at the Granites, Central Australia. Trans Royal Soc South Austr. 1939; 63: 22–26.
7. Johnston TH, Cleland JB. The history of the Aboriginal narcotic, pituri. Oceania. 1933; 4: 201–223.
8. Latz PK. Bushfires & bushtucker: Aboriginal plant use in Central Australia. Alice Springs: Iad Press. 1995.
9. Clemente T. *Nicotiana* (*Nicotiana tobacum, Nicotiana benthamiana*). In: Wang K, ed. Agrobacterium Protocols. Humana Press, 2006. pp. 143–154
10. van Dijk P, Van der Meer FA, Piron PGM. Accessions of Australian *Nicotiana* species suitable as indicator hosts in the diagnosis of plant virus diseases. Neth J Plant Pathol. 1987; 93: 73–85.
11. Horvath J. The role of *Nicotiana* species in plant virology with special regard to *Nicotiana benthamiana* Domini: A review. Acta Phytopathol et Entomolog Hungarica. 1993; 28: 355–377.
12. Goodin MM, Zaitlin D, Naidu RA, Lommel SA. *Nicotiana benthamiana*: its history and future as a model for plant-pathogen interactions. MPMI. 2008; 21: 1015–1026. doi: 10.1094/MPMI-21-8-1015 PMID: 18616398
13. Dubreuil G, Magliano M, Dubrana MP, Lozano J, Lecomte P, Favery B, et al. Tobacco rattle virus mediates gene silencing in a plant parasitic root-knot nematode. J. Exp. Bot. 2009; 60:4041–4050 doi: 10.1093/jxb/erp237 PMID: 19629337
14. Waterhouse PM, Helliwell CA. Exploring plant genomes by RNA-induced gene silencing. Nature Rev Genet. 2003; 4: 29–38. PMID: 12509751
15. Sparkes IA, Runions J, Kearns A, Hawes C. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. Nature Protocols. 2006; 1: 2019–2025. PMID: 17487191
16. Naim F, Nakasugi K, Crowhurst RN, Hilario E, Zwart AB, Hellens RP, et al. Advanced engineering of lipid metabolism in *Nicotiana benthamiana* using a draft genome and the V2 viral silencing-suppressor protein. PloS One. 2012; 7: e52717. doi: 10.1371/journal.pone.0052717 PMID: 23300790
17. Bombarely A, Rosli HG, Vrebalov J, Moffett P, Mueller LA, Martin GB. A draft genome sequence of *Nicotiana benthamiana* to enhance molecular plant-microbe biology research. MPMI. 2012; 25: 1523–1530. doi: 10.1094/MPMI-06-12-0148-TA PMID: 22876960

18. Nakasugi K, Crowhurst RN, Bally J, Wood CC, Hellens RP, Waterhouse PM. De novo transcriptome sequence assembly and analysis of RNA silencing genes of *Nicotiana benthamiana*. PloS One. 2013; 8: e59354. doi: 10.1371/journal.pone.0059534 PMID: 23555698

19. Roossinck MJ. Plant virus metagenomics: biodiversity and ecology. Ann Rev Genet. 2012; 46: 359–389. doi: 10.1146/annurev-genet-110711-155600 PMID: 22934641

20. McKinney HH, Clayton EE. Genotype and temperature in relation to symptoms caused in *Nicotiana benthamiana* by a draft genome sequence assembly and analysis of RNA silencing genes of *Nicotiana benthamiana*. PloS One. 2013; 8: e59354. doi: 10.1371/journal.pone.0059534 PMID: 23555698

21. Wylie SJ, Luo H, Wylie SJ, Coutts BA, Jones RAC, Jones MGK. A virus of an isolated indigenous flora spreads naturally to an introduced crop species. Ann Appl Biol. 2011; 159: 339–347. doi: 10.1111/j.1744-7348.2011.00453.x PMID: 21005991

22. Wylie SJ, Jones MGK. Characterisation and quantitation of mutant and wild-type genomes of *Hardenbergia mosaic virus* isolates co-infecting a wild plant of *Hardenbergia comptoniana*. Arch Virol. 2011; 156: 1287–1290. doi: 10.1007/s00705-011-1002-3 PMID: 21519930

23. Wylie SJ, Jones MGK. Hardenbergia virus A, a novel member of the *Betaflexiviridae* from a wild legume in South-west Australia. Arch. Virol. 2011; 156: 1245–1250. doi: 10.1007/s00705-011-0963-6 PMID: 21394605

24. Wylie SJ, Jones MGK. Multiple polyadenylated RNA viruses detected in pooled cultivated and wild plant samples. Arch Virol. 2012; 157: 271–284. doi: 10.1007/s00705-011-1166-x PMID: 22075920

25. Wylie SJ, Luo H, Li H, Jones MGK. Donkey Orchid Symptomless Virus: A Viral Disease of *Donkey Orchid* (Diuris species) in Australia. *Virus Res.* 2013; 171: 22–32. doi: 10.1016/j.virusres.2012.10.003 PMID: 23089850

26. Wylie SJ, Tan A, Li H, Dixon KW, Jones MGK. Caladenia virus A, an unusual new member of the *Potyviridae* from terrestrial orchids in Western Australia. Arch. Virol. 2012; 157: 2447–2452. doi: 10.1007/s00705-012-1452-2 PMID: 22139460

27. Wylie SJ, Luo H, Li H, Jones MGK. Yellow tailflower mild mottle virus: a new tobamovirus described from *Anthocercis littorea* (Solanaeaceae) in Western Australia. Arch Virol. 2012; 157: 271–284. doi: 10.1007/s00705-011-1166-x PMID: 22075920

28. Wylie SJ, Li H, Jones MGK. Donkey Orchid Symptomless Virus: A Viral ‘Platytypus’ from Australian terrestrial orchids. PLoS ONE. 2013; 8: e79587. doi: 10.1371/journal.pone.0079587 PMID: 24223974

29. Wylie SJ, Li H, Dixon KW, Richards H, Jones MGK. Exotic and indigenous viruses infect wild populations and captive collections of temperate terrestrial orchids (*Diuris* species) in Australia. Virus Res. 2013; 171: 22–32. doi: 10.1016/j.virusres.2012.10.003 PMID: 23089850

30. Wylie SJ, Li H, Jones MGK. Tomato marchitez scorch virus. Plant Dis. 2005; 89: 205–209. doi: 10.1094/PDIS-89-1-0205. PMID: 15079073

31. Verbeek M, Dullermans AM, Van den Heuvel JFJM, Maris PC, Van der Vlugt RAA. Tomato marchitez virus, a new plant picorna-like virus from tomato related to tomato scorch virus. Arch Virol. 2008; 153: 127–134. PMID: 17965923
39. Burbidge NT. The Australian species of *Nicotiana* L. (Solanaceae). Aust. J Bot. 1960; 8: 342–380.
40. Walton NJ, Belshaw NJ. The effect of cadaverine on the formation of anabasine from lysine in hairy root cultures of *Nicotiana hesperis*. Plant Cell Rep. 1988; 7: 115–118. doi: 10.1007/BF00270118 PMID: 24241546
41. Verhoeven JTJ, Roenhorst JW. Herbaceous test plants for the detection of quarantine viruses of potato. EPPO Bull. 2000; 30: 463–467.
42. Verbeek M, Dullemans AM, van Raaij HM, Verhoeven JTJ, van der Vlugt RA. Lettuce necrotic leaf curl virus, a new plant virus infecting lettuce and a proposed member of the genus *Torradovirus*. Arch Virol. 2014; 159: 801–805. doi: 10.1007/s00705-013-1835-z PMID: 24142269
43. Horton P. A taxonomic revision of *Nicotiana* (Solanaceae) in Australia. J. Adelaide Bot. Garden. 1981; 3: 1–56.
44. Angel CA, Hsieh YC, Schoelz JE. Comparative analysis of the capacity of tombusvirus P22 and P19 proteins to function as avirulence determinants in *Nicotiana* species. MPMI. 2011; 24: 91–99. doi: 10.1094/MPMI-04-10-0089 PMID: 20977306
45. Wünschová A, Be ová V, Vlašinová H, Havel L. Dormancy of *Nicotiana benthamiana* seeds can be broken by different compounds. Biologia. 2009; 64: 705–710.
46. Marks CE, Ladiges PY, Newbiggin E. Karyotypic variation in *Nicotiana* species. MPMI. 2011; 24: 1358–1372. doi: 10.1105/tpc.109.072058 PMID: 20400679
47. Wylie SJ, Coutts BA, Jones MGK, Jones RAC. Phylogenetic analysis of Bean yellow mosaic virus isolates from four continents: relationship between the seven groups found and their hosts and origins. Plant Dis. 2008; 92: 1596–1603.
48. Wylie S, Wilson CR, Jones RAC, Jones MGK. A polymerase chain reaction assay for cucumber mosaic virus in lupin seeds. Crop Pasture Sci. 1993; 44: 41–51.
49. Fukuta S, Ohishi K, Yoshida K, Mizukami Y, Ishida A, Kanbe M. Development of immunocapture reverse transcription loop-mediated isothermal amplification for the detection of tomato spotted wilt virus from chrysanthemum. J. Virol. Meth. 2004; 121: 49–55. PMID: 15350732
50. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acid Res. 1994; 22: 4673–4680. PMID: 7984417
51. Ying X-B, Dong L, Zhu H, Duan C-G, Du Q-S, Li D-Q, et al. RNA-dependent RNA polymerase 1 from *Nicotiana tabacum* suppresses RNA silencing and enhances viral infection in *Nicotiana benthamiana*. Plant Cell. 2010; 22: 1358–1372. doi: 10.1105/tpc.109.072058 PMID: 20400679
52. Garcia-Ruiz H, Takeda A, Chapman EJ, Sullivan CM, Fahlgren N, Brempelis KJ, et al. Arabidopsis RNA-dependent RNA polymerases and Dicer-like proteins in antiviral defense and small interfering RNA biogenesis during tumip mosaic virus infection. Plant Cell. 2010; 22: 481–496. doi: 10.1105/tpc.109.073056 PMID: 20190077
53. Fraile A, Escriu F, Aranda MA, Malpica JM, Gibbs AJ, Garcia-Arenal F. A century of tobamovirus evolution in an Australian population of *Nicotiana glauca*. J. Virol. 1997; 71: 8316–8320 PMID: 9343184
54. Wei K, Gibbs A, MacKenzie A. Citloria yellow mottle virus: a tobravirus from Northern Australia. Aust. Plant Dis Note. 2012; 7: 59–61.
55. Bewley JD, Bradford K, Hilhorst H. Seeds: physiology of development, germination and dormancy. Springer. 2012.
56. Yu D, Fan B, MacFarlane SA, Chen Z. Analysis of the involvement of an inducible *Arabidopsis* RNA-dependent RNA polymerase in antiviral defense. MPMI. 2003; 16: 206–216. PMID: 12650452
57. Hunter LJ, Westwood JH, Heath G, Macaulay K, Smith AG, MacFarlane SA, et al. Regulation of RNA-Dependent RNA Polymerase 1 and Isocorismate Synthase gene expression in *Arabidopsis*. PloS ONE. 2013; 8: e66530. doi: 10.1371/journal.pone.0066530 PMID: 23799112
58. Pandey SP, Baldwin IT. 2007. RNA-directed RNA polymerase 1 (RdR1) mediates the resistance of *Nicotiana attenuata* to herbivore attack in nature. Plant J. 2007; 50: 40–53. PMID: 17346266
59. Qi X, Bao FS, Xie Z. Small RNA deep sequencing reveals role for *Arabidopsis thaliana* RNA-dependent RNA polymerases in viral siRNA biogenesis. PloS ONE. 2009; 4: e4971. doi: 10.1371/journal.pone.0004971 PMID: 19308254
62. Alcázar R, García AV, Parker JE, Reymond M. Incremental steps toward incompatibility revealed by Arabidopsis epistatic interactions modulating salicylic acid pathway activation. PNAS. 2009; 106: 334–339. doi:10.1073/pnas.0811734106 PMID: 19106299

63. Alcázar R, García AV, Kronholm I, de Meaux J, Koornneef M, Parker JE, et al. Natural variation at Strubbelig Receptor Kinase 3 drives immune-triggered incompatibilities between Arabidopsis thaliana accessions. Nature Genet. 2010; 42: 1135–1139. doi:10.1038/ng.704 PMID: 21037570

64. Koornneef M, Alonso-Blanco C, Vreugdenhil D. Naturally occurring genetic variation in Arabidopsis thaliana. Ann Rev Plant Biol. 2004; 55: 141–172. PMID: 15377217