Hort Innovation

Final Report

Innovative Solutions to Management of Tospoviruses of Vegetable Crops

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Innovative Solutions to Management of Tospoviruses of Vegetable Crops – VG14063

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Content

Summary

Tospoviruses infect a large number of horticultural crops, causing losses across the supply chain at all levels. Species such as Tomato spotted wilt virus (TSWV) and Capsicum chlorosis virus (CaCV) are present in Australia, while Groundnut bud necrosis virus (GBNV) and others are impacting crops oversees and represent a significant biosecurity threat.

Existing methods for virus control in horticultural species are limited to natural resistance, prevention via hygiene, and control of insect vectors; once the virus gains a foothold, there is no currently available commercial equivalent of a pesticide to rid crops of its presence. Modern biotechnology offers new hope by supercharging the plant's innate antiviral processes in a directed manner. Artificial microRNAs (amiRNAs) are a tactic that uses a small stretch of the virus's own genome to direct the plant to attack the virus, making it highly specific and unlikely to affect beneficial off-target species.

In this project, genomic sequence information for many tospoviruses was compiled forming an important source of information. New software was developed to aid this process for viruses where sequence information didn't currently exist. AmiRNA constructs were designed to target all virus strains within a species, including those in Australia and abroad. Comprehensive testing demonstrated that amiRNA constructs induced host resistance to the targeted tospovirus. With multiple constructs developed for tospoviruses yet to reach Australia, this allows for a rapid response should this biosecurity threat eventuate.

A key aspect of this project has been leveraging next-generation sequence data to provide a much greater understanding of tospovirus/plant interactions. This includes in-depth knowledge of the plant's inherent ability to directly attack the virus via RNA silencing, how this response varies between different plants and tospovirus types, and how the host plant modulates expression of its own genes under virus-induced stress with the virus-activated small RNA pathway. This understanding can feed in to breeding programs, as important genes and regulatory pathways involved in resistance have been identified.

Keywords

Tospovirus; Tomato spotted wilt virus; TSWV; Capsicum chlorosis virus; CaCV; amiRNA; capsicum; small RNA; RNAi, virus resistance

Introduction

Tospoviruses infect a broad range of staple and horticultural crops. Transmitted by thrips, annual losses due to tospoviruses are estimated to be over $1 billion worldwide. These viruses cause significant losses to the supply chain at the nursery, grower and consumer level. The thrips-tospovirus pest complex is one of the most economically important agricultural production constraints among vegetable growers, and has been the focus of this research project.

Tospoviruses are listed under high risk category in terms of both entry and impact in The Vegetable Industry Biosecurity Plan (2011) by Plant Health Australia. Specifically, the tospoviruses listed are: Watermelon silver mottle virus (WSMoV), Groundnut bud necrosis (GBNV), and watermelon bud necrosis, infecting squash, pumpkin, pepper, eggplant, capsicum, cucumber & melon. These viruses are prevalent in other countries such as India and the information on the local isolates has not been readily available to Australian researchers. The Plant Health Australia report states that Capsicum chlorosis virus (CaCV) have already shown the ability of tospoviruses to establish in Australia, particularly as the major insect vectors *Thrips palmi* and *Frankliniella shultzi* are present. Tomato spotted wilt virus has long been present in Australia (first described in tomato in 1915), and has caused significant losses.

To date, tospovirus control methods have been somewhat limited, involving crop and farm hygiene, insecticidal control of the thrips vector, use of tospovirus-free planting material, and use of resistant crop varieties. Modern biotechnology techniques are offering new approaches to generating virus resistance outside of what can be obtained through traditional breeding techniques.

Transgenic approaches, where additional genetic material is introduced, allow for highly specific targeting of particular pathogenic viruses. These methods generally prime the host plant's inbuilt virus-defence RNAi machinery against key components of the virus's genome, limiting the virus's capacity to gain a foothold and overcome the plant's innate defences. There are, however, limitations to some transgenic approaches for providing broad-spectrum defence against the diversity of viral strains in a particular species (e.g. TSWV). Double-stranded RNA constructs are a frequently used approach, though these constructs have a may cover non-conserved regions and have a greater chance of unintended off-target effects on beneficial organisms, including pollinating bees, owing to their length. Artificial microRNAs (amiRNAs) circumvent these potential downsides due to their minimal mature length of 21-22 nt; short conserved regions among virus strains are easier to identify and target, and the possibility off off-target impacts are much reduced.

Methodology

The key to an effective amiRNA approach is comprehensive genomic and transcriptomic sequence information capturing the genetic diversity of the target virus. For this project, public repositories and private sources were systematically searched and accessed to collate sequence information for major tospovirus species, including TSWV, CaCV, GBNV, INSV, WBNV, WSMoV, MYSV and ZLCV. Novel bioinformatics approaches that leverage small RNA deep sequence data were developed to correct reference sequences though identification of variant nucleotides in the viral strains infecting the sequenced host plant. Nucleocapsid (N) gene sequencing was also carried out on CaCV isolates from prime horticulture regions across Queensland.

With this trove of curated sequence information, a bioinformatics software package developed for this project was able to identify conserved regions in the N genes across the diversity of viral strains within each tospovirus species. Unsurprisingly, there is a greater degree of diversity between tospovirus species than within, so a pan-tospovirus region of at least 21 nt in length, which would allow a single construct to target all tospoviruses, could not be detected.

Once conserved regions were identified, mature amiRNA target identification was carried out according to published principles (Weigelworld; http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). To overcome the lack of conserved regions between tospovirus species, a tandem construct engineering approach was undertaken in addition to the use of single-target constructs. The single construct method allowed for a single pre-miRNA to be expressed by a strong promotor (35S). For tandem constructs, two pre-miRNAs were expressed by the same promotor. This system was set up in such a way that the addition of pre-miRNAs to the construct is essentially plug-and-play. Simple directional cloning can add additional amiRNAs to the existing two, though the limit of multi-amiRNA targeting was not identified in the project. Importantly, such an approach to designing and engineering multi-amiRNA constructs proved simple and likely applicable to many other viruses affecting the horticultural industry.

Subsequent to construct engineering, transient expression of the introduced pre-miRNA/s of interest allowed for rapid analysis of the expression and processing of the mature amiRNA/s. For effective amiRNA degradation of target transcripts, a sufficient abundance of mature amiRNAs need to be loaded into the RISC complex. By using the strong constitutive promotor 35S to drive expression of the miR159 pre-miRNA backbone, easily detectable levels of mature amiRNAs were generated, suggesting expression was sufficiently high. Though the designed amiRNAs contained no homology to the endogenous *Arabidopsis* mir159 present in the pre-miRNA backbone, processing of the mature amiRNAs into the desired 21 nt length was successful. The use of multi-amiRNA constructs was also validated, with expression and correct processing of each discrete amiRNA evident. These data reinforced the construct engineering approach adopted for this project, demonstrating there were no technical limitations generating tospovirus-targeting amiRNAs.

In addition to demonstrating strong amiRNA expression and functional amiRNA processing, our transient system in *N. benthamiana* allowed for determination of whether the amiRNA provided resistance to target tospoviruses. Using this approach, resistance to TSWV and CaCV was demonstrated using single and tandem constructs. This resistance validated not only the construct design and engineering, but the selection of the appropriate targets in the tospovirus small (S) RNA segment.

Stable transformation of amiRNA constructs into tospovirus-affected species is the mechanism by which the resistance is introduced. *N. benthamiana* provides a useful model for Solanaceae horticultural crops, as transformation protocols for it and related species exist in the literature. Though significant optimisation of materials and protocols was required achieve transgenic adult plants, the approach was successful. As with the transient transformation approach, tandem constructs expressing discrete amiRNAs against TSWV and CaCV were stably integrated and healthy adult plant regenerated following tissue culture. Again, expression of the amiRNAs was strong, and processing of the mature amiRNA evident.

Two trials to test TSWV resistance in healthy transgenic *N. benthamiana* plants expressing the desired amiRNAs and grown under glasshouse conditions were undertaken. The first trial demonstrated TSWV resistance of transgenic plants via amiRNA against the TSWV N gene, with no TSWV amiRNA-expressing plants showing symptoms or positive ELISA results (n = 4) and four control plants displaying symptoms or positive ELISA results (n = 6) nine days post-inoculation. The second trial demonstrated that no plants expressing the TSWV N gene amiRNA displayed symptoms (n = 4), while four control plants showed typical symptoms (n = 4) nine days post-inoculation. Detection of the TSWV N gene by qPCR on challenged plants of the second trial confirmed that only control plants became infected. Importantly, this tospovirus species resistance was generated from the one tandem construct expressing two discrete amiRNAs.

In addition to generating tospovirus-resistant plants via a transgenic approach, an important aspect of this project was to gain a detailed and comprehensive understanding of how tospoviruses and horticultural crops interact. An increased understanding of the molecular nature of viral pathogenicity along with the mechanism behind the plant's response will likely provide essential information to plant breeders, even if a transgenic approach to resistance turns out to be undesirable.

Small RNA deep sequencing shone new light on the function of the host plant's RNAi machinery, both in how it responded directly to the virus, and how it targeted siRNAs to its own transcripts. Infection of capsicum with either TSWV or CaCV generated similar overall viral siRNA (vsiRNA) abundance. Hotspots were readily apparent in both vsiRNA profiles, and may point toward areas of interest for amiRNA targeting in future. For *N. benthamiana*, TSWV-targeting siRNAs were slightly more abundant than CaCV in the LAB strain, and readily apparent in the WA strain. The LAB strain of *N. benthamiana* is highly sensitive to many plant viruses, though its response to tospovirus infection via the generation of vsiRNAs was not greatly affected. This indicates that generation of vsiRNAs is only one aspect of virus resistance, and likely not solely sufficient if not induced in the correct manor (as is the case with introduced amiRNAs and dsRNA). This is supported by analysis of tospovirus-resistant and sensitive tomato, where the resistant variety had insufficient viral load to generate a significant number of vsiRNAs. The likely conclusion is that the plant's amiRNA machinery must be strongly activated prior to the virus gaining a foothold. Small RNA sequencing can detect large amounts of vsiRNAs once the virus does have a foothold, but these are not sufficient to actually rid the plant the virus, though may aid in survivability.

Reports have demonstrated that plants produce vsiRNAs not only against viral transcripts, but virus-activated siRNAs (vasiRNAs) against endogenous transcripts in response to infection (Cao *et al*., 2014). VasiRNAs have been thought to play a role in gene regulation, though their occurrence has not been widely reported outside of *Arabidopsis*, and many aspects of their biogenesis and function remain to be elucidated. Here we demonstrated that vasiRNAs are abundant in CaCV- and TSWV-infected Solanaceae, and that they primarily target a distinct subset of endogenous transcripts. In *Arabidopsis*, the RNA polymerase RDR1 has been implicated in vasiRNA biogenesis. We demonstrated that *N. benthamiana* without a functional RDR1 is unable to produce vasiRNAs, while the WA strain with a functional RDR1 generates them abundantly, indicating RDR1 is likely required for vasiRNA biogenesis in many higher plants.

The effect of tospovirus-generated vasiRNAs on the expression of their target transcripts is not as clear cut as has been previously reported. In *N. benthamiana* WA and LAB strains, there is not a strong consistent negative correlation between vasiRNA abundance and gene expression, as would be expected. These results may be confounded by other differences between the two *N. benthamiana* strains, though it is clear that any effect of vasiRNAs is likely to be, in general, subtle at the transcript expression level. It should however be noted that even minimal changes in transcript expression can have significant physiological effects under certain circumstances.

Interestingly, many of the transcripts affected are either ribosomal protein-encoding transcripts, or transcripts for proteins located in the endoplasmic reticulum, which are involved in protein processing and folding. It is feasible that co-localisation with many viral transcripts themselves makes these transcripts targets for vasiRNAs. Taken together, the evolutionary persistence of this pathway may provide benefit to the plant by modulating gene expression under large viral load, which may aid survival of the plant by not overexerting its protein expression and processing facilities. As such, these pathways could be of interest to plant breeders, as natural sources of variation could provide increased resistance to tospoviruses and other viruses.

Taken together, the outputs of this project demonstrate that resistance to tospoviruses can be readily engineered into horticultural Solanaceae crops, and that this resistance is likely to be highly effective against tospoviruses that already exist in Australia, and those that may yet come. Resistance against tospoviruses such as GBNV, that are not currently present in Australia, could be rapidly introduced into breeding lines if required via a transgenic approach. If the transgenic pathway remains unviable, potentially key genes and pathways identified in this project that respond to tospovirus infections may also be important areas of focus for plant breeders.

Outputs

1. Sequence information and bioinformatic analysis for tospoviruses of relevance to Australia generated and delivered.

2. Small RNA profiling and target genes identified in the infected host for improved understanding of host-pathogen interaction and downstream applications.

3. Artificial microRNA constructs ready and available for use against a range of Tospoviruses including those that are a biosecurity threat to Australia, including TSWV, CaCV and GBNV.

4. Model Solanaceae lines with enhanced virus resistance by expression of artificial microRNAs engineered.

5. Technology in place to manage Tospovirus associated problems in other crops

6. Valuable international links established for biotechnological application for crop improvement

7. Research outcomes to be published in peer reviewed international journals – two manuscripts have been prepared and are ready for submission.

8. Engagement with global audiences through presentation of work at international conference.

Outcomes

Scientific

- Generation of model plant lines with enhanced virus resistance by expression of artificial microRNAs
- Demonstration of the efficacy of amiRNA approach as a proof of principle and provision tools for manipulation of virus resistance in additional crop plants.
- Development of a technique that could be broadly applicable to other priority crops that are host to these viruses such as chillies, cucurbits, lettuce, eggplant, potato and onion (bulb and seed crops)
- Global advantage to Australian vegetable producers and processing industry to be the world leaders in using the cutting technology of RNA silencing to control viral resistance
- Scientific knowledge base improved and information generated on the efficacy of novel transgenic approaches for pathogen control
- Host-virus interaction investigated at molecular level and targets identified to augment reeding for virus resistance.

Economic

- Cutting edge technologies developed and validated to create outputs that maximize the economic potential of vegetable industries on a sustainable basis.
- Enhanced research program and activities to manage virus diseases of economic significance to Australia,
- Generation of potentially valuable intellectual property

Social

- Novel approach to deliver a sustainable product of value to horticulture industry in terms of resistance to viruses.
- New links formed, knowledge gained, resources generated and skills acquired.

Environmental

- Major impact on agriculture and environmental management, areas that are designated as national research priorities.
- This new technology will improve bio-safety, reduce any possible environmental impact (sustainable agriculture) and provides us with a powerful tool for management of plant viruses.
- Long-term cost effective, environmentally friendly management of virus control

Monitoring and evaluation

The Monitoring and Evaluation Framework underpinning the project relied on milestones arising from agreed project plan activities for the research services with achievement-based criteria. Timely milestone reporting and adherence to the defined budget was strictly followed. The project was completed on time with all milestones successfully achieved. The team held regular 3- monthly scientific meetings at which progress in all project areas was reviewed. Regular communication was also held with Prof Pappu at Washington State University and Denis Persley, Department of Agriculture and Forestry, Queensland.

The project started with Bioseeds India as potential partners with work on generating transgenic capsicum and Chilli plants to be done in India. However, due to policy and regulations about Genetic Modification in India. Bioseeds India pulled out of the project in second year. This was discussed with Hort Innovation and milestone and budget variation request was approved. The transgenic work to show proof of concept was done in Mitter Lab using model plant species as part of the revised milestone.

The key measure of success achieved for vegetable industry was delivery of sequence information on Tospsovirus isolates, molecular insights into host- virus interaction and proof of concept for artificial microRNA technology to impart resistance to tospoviruses.

Recommendations

- Artificial microRNAs can be exploited to generate resistance against tospoviruses through genetic modification. These amiRNAs can also be stacked, thus providing resistance to multiple species in a single construct. Though highly effective, such an approach is on the proviso that GMOs become publically acceptable. If not, non-GMO methods such as topical amiRNA application should be examined.
- This project has identified key genes in horticultural species including capsicum and tomato that are regulated via the vasiRNA pathway upon tospovirus infection. Genes such as *RDR1*, which act in the pathway itself, along with target genes regulated by vasiRNAs may be important to breeding strategies to increase innate resistance to tospoviruses and other viruses affecting horticultural species.
- Further genomic studies should be undertaken on the vasiRNA gene regulation pathway to examine how it can be used most effectively to increase virus resistance, and how widespread it is amongst horticultural species outside of the Solanaceae family.

Refereed scientific publications

Journal article

Fletcher, SJ., Peters, JR…Mitter, N. 2018. Tospovirus infection of Solanaceae species triggers activation of endogenous genes that serve as substrates for RDR1-dependent generation of small interfering RNAs. Manuscript prepared.

Fletcher, SJ., Peters, JR…N Mitter, N. 2018. Artificial microRNA for broad-range resistance to multiple tospovirus species. Manuscript prepared.

Paper in conference proceedings

Mitter, N. 2017. Invited talk on 'Innovative Crop Protection Products – Responsible Agriculture'; Australian Pesticides and Veterinary Medicines Authority (APVMA Science Day, Canberra).

Mitter, N. 2016. Keynote guest speaker at Tasmanian Division of the Australasian Plant Pathology Society, Seminar day, April 2016, Hobart, Tasmania (Fully funded by APPS).

Mitter, N. 2016. Plenary speaker at 6th International Conference on "Plant, Pathogens and People" with mission "Challenges in Plant Pathology to benefit humankind", New Delhi, India

Mitter, N. 2016. Invited expert on panel for 'Management of viruses using transgenic approaches' at one day Virology Symposium organised by Indian Virological Society, 5 December 2015, New Delhi, India.

Mitter, N. 2015. Keynote presentation 'Harnessing the power of RNA interference for crop protection' at 27th Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2015), Bangkok, Thailand.

Mitter, N. 2015. Virus-derived small RNAs associated with Tomato spotted wilt virus infection in peanut, Xth International Symposium on Thysanoptera & Tospoviruses, California, USA.

Mitter, N.2015. Harnessing artificial microRNAs for resistance to tospoviruses: The way forward, Xth International Symposium on Thysanoptera & Tospoviruses, California, USA.

Ramesh, SV., Mitter, N., Pappu, HR. 2015. Transcriptome-wide identification of host genes targeted by a Tomato spotted wilt virus-derived small interfering RNAs reveals host-dependent differential down-regulation, Xth International Symposium on Thysanoptera & Tospoviruses, California, USA.

Raikhy, G., Mitter, N., Pappu, HR. 2015 Characterization of transcriptional activity of Tomato spotted wilt virus RNAs Xth International Symposium on Thysanoptera & Tospoviruses, California, USA.

Fletcher, SJ., Peters, JR., Mitter, N. 2018 Tospoviruses specifically modulate host gene expression via virus activated small interfering RNAs, 11th International Congress of Plant Pathology in Boston, USA. Abstract accepted.

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Intellectual property, commercialisation and confidentiality

No project IP, project outputs, commercialisation or confidentiality issues to report

New Project IP of artificial microRNA constructs targeting Tospoviruses

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