Horticulture Innovation Australia

# Final Report

## Breeding Capsicum for Tospovirus Resistance

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The Department of Agriculture and Fisheries (DAF)

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# Summary

A fresh-market capsicum improvement program has developed genetic resistances to two tospoviruses, Capsicum chlorosis virus (CaCV) and Tomato spotted wilt virus (TSWV) in new parent lines with commercial potential. Earlier research had produced resistant material that lacked advanced commercial attributes but collaboration with Syngenta Seeds has now enabled the development of elite resistant parent lines with greatly improved production and quality suitable for commercial F1 hybrid cultivar development.

Both CaCV and TSWV cause significant crop damage in the largest production areas of Queensland with typical annual crop losses of $15M. In South Australia TSWV is responsible for major losses in the protected cropping industry.

The inheritance and action of the CaCV resistance gene were characterised in previous research (VG02035), allowing for a straight-forward transfer in the current project to a range of improved Syngenta parent lines. The new lines were developed by a series of three or four backcrosses with selection for resistance at appropriate points in the breeding cycle. An additional strategy of generating dihaploid progenies was also introduced as a means of hastening the production of uniform, fixed parent lines.

Collaboration between the project's partners has led to the development of at least one useful DNA marker linked to the CaCV resistance gene. Association mapping in appropriate populations segregating for resistance identified several good markers, one of which appears to be highly predictive and robust. The use of DNA markers is critical in the efficient selection of resistant breeding lines and F1 hybrids, especially where physical assays for resistance are not possible.

A parallel program of F1 hybrid development by Syngenta, directed in particular towards broad adaptation in Queensland, has identified two excellent F1 cultivars. Although these are not CaCV-resistant, one parent in each has been included in the project's CaCV backcross conversion program so equivalent resistant F1 cultivars will be derived from this collaboration.

The specific outputs from the project include a range of advanced CaCV/TSWV resistant parent lines, identification of two new advanced F1 cultivars and their potential CaCV-resistant equivalents and at least one functional DNA marker for CaCV resistance. The introduction of this new material should provide an immediate improvement in agronomic performance and fruit quality as a result of the underlying attributes of the adapted parent lines; furthermore, host plant resistance to CaCV in particular should increase marketable yields by up to 15% in those production areas in Queensland where losses are most severe. The multiplier effects of this benefit are considerable in an industry valued conservatively at $160M.

The development of CaCV-resistant F1 hybrids should be completed by testing and selection of the most suitable parent genotypes in the relevant backcross populations. A relatively small amount of additional research will provide resistant parents for two F1 cultivars with potential.

Further research to more accurately map DNA markers for CaCV resistance is continuing. Although the identification and validation work for several candidate markers has been done, additional fine mapping will more accurately characterise the most suitable marker, allowing for the most efficient use in breeding programs.

# Keywords

Capsicum chlorosis virus; Tomato spotted wilt virus; capsicum breeding; DNA molecular markers

# Introduction

Commercial capsicum production occurs in all mainland States of Australia with Queensland the major field producer where the crop is valued conservatively at $160M. The majority of Queensland production is grown in the areas centered around Bundaberg, Bowen, Gumlu, Ayr and Stanthorpe, with some seasonal variation between production in northern and southern areas. The largest area of protected cropping for capsicum is on the North Adelaide Plain in South Australia.

Tomato spotted wilt virus (TSWV; Family *Bunyaviridae* Genus *Tospovirus* ) is the most prevalent virus in capsicums in Australia. It was identified in the early years of the 20th century and is now widely distributed in all states (Persley *et al.* 2006; Sharman and Persley 2006; Latham and Jones 1997). TSWV has a very wide host range among crop, weed and ornamental species. In recent decades it has become more damaging with the proliferation of a major vector species, *Frankliniella occidentalis* (western flower thrips) that has been implicated in severe epidemics in South and Western Australia (Coutts and Jones 2002). Resistance to TSWV in capsicum, operating as a hypersensitive response and controlled by the single dominant gene *Tsw*, has been found in several *C. chinense* lines (Black *et al.* 1991; Mouray *et al.* 1997). This resistance has been used to develop virus-resistant hybrids grown in several countries, including Australia. It has generally proven to be durable, although isolates of TSWV virulent towards the *Tsw* gene are being detected with increasing frequency worldwide (Roggero *et al.* 2002; Sharman and Persley 2006; Kenyon et al. 2014). In Australia the most notable example of *Tsw* failure is in the South Australian protected cropping industry (Sharman and Persley 2006).

By contrast, the tospovirus Capsicum chlorosis virus (CaCV) was first detected in capsicum and tomato in 1999 in Queensland and has been present in the state since 1992 (McMichael *et al.* 2002). Since then it has been identified in capsicum in Kununurra, WA (Jones and Sharman 2005) and reported in Thailand, Taiwan and China (Knierim *et al.* 2006). Although less widely distributed than TSWV, CaCV is significant in Bundaberg and the northern Dry Tropics of Queensland to the extent that it can become the predominant virus at certain times through the season. The effect of tospoviruses generally is a reduction in fruit size, distortion of fruit shape and scarring of the skin, resulting in large losses of marketable yield. Crop damage from tospoviruses in Queensland is estimated at $15M.

Previous research identified genetic resistance to CaCV in accession PI 290972 of the uncultivated species *Capsicum chinense (*Persley *et al.* 2006). The genetic basis for resistance was determined and resistance transferred to a series of bell capsicum breeding lines by conventional breeding. The resulting lines were greatly improved but commercially uncompetitive because they lacked sufficient fruit quality and good agronomic performance. Our collaboration with Syngenta Australia has enabled the continuing improvement of this material by the introduction of elite parent lines that provide a foundation for breeding advanced tospovirus-resistant F1 hybrid cultivars suitable for the Australian market.

Modern applied breeding programs rely on the use of marker-assisted selection for optimal efficiency. The development of a useful DNA marker for CaCV resistance was a priority in this collaboration where a successful mapping exercise was undertaken by the project partners to produce several markers that can be applied effectively to breeding populations.

# Methodology

## Virology

### Glasshouse bioassays

An efficient screening system was developed, allowing consistent detection of resistant genotypes or individuals in segregating populations following inoculation with either Tomato spotted wilt virus (TSWV) or Capsicum chlorosis virus (CaCV).

TSWV was propagated in tomato or capsicum and CaCV in capsicum cv. Yolo Wonder or *Nicotiana benthamiana*. Symptomatic leaves were ground in a cold mortar and pestle with cold 0.1 M phosphate buffer to which 0.1% sodium sulphite has been added immediately prior to use. Diatomaceous earth (Celite) was added to the inoculum which was then applied with a finger or a pad dipped in the inoculum. When phenotyping capsicum plants for CaCV reaction, plants were inoculated approximately five weeks after sowing when at the four to five leaf stage of growth. Plants were rinsed with water after inoculation to remove excess inoculum and abrasive. Appropriate susceptible and resistant lines were included in each experiment. Plants were then maintained in a glasshouse and monitored for local and systemic symptom development. The necrotic hypersensitive resistant response developed approximately five days after inoculation. Discrete lesions formed which then usually coalesced resulting in abscission of inoculated leaves.

### Development of CaCV resistance

1. **Confirmation of resistance in donor lines and evaluation of agronomic performance.**

   Two uniformly CaCV-resistant donor parent lines, A32-24-1 and A32-24-5, were contributed by DAF as starting points for the development of resistance in Syngenta's germplasm. The resistance of the parent lines was confirmed by glasshouse assay and the lines were included for evaluation in the initial field trial of available material.

2. **First testcrosses to yield F1 hybrids**.

   Thirty-six Syngenta parent lines with different combinations of disease resistances and other attributes were crossed to CaCV parents A32-24-1 and A32-24-2 by DAF in 2010 to provide 72 F1 testcrosses for field evaluation. Approximately 25000 seeds were produced in total by this exercise. The seeds were allocated to four field trials, confirmation of CaCV resistance by glasshouse assay, a first backcross cycle and shipment to Syngenta's laboratory for dihaploidisation.

## 3. Backcross breeding cycles to transfer CaCV resistance to elite Syngenta parent lines

Up to four backcross cycles were undertaken using female lines segregating for the CaCV resistance gene and elite, susceptible recurrent inbred male parents supplied by Syngenta Australia. The strategy was to transfer CaCV resistance to each of the elite parent lines. Two plants of the 36 initial F1 genotypes above were each crossed to their respective Syngenta parent line to generate approximately 60 seeds of the backcross 1 (BC1) generation. A population of 30 - 50 seeds of each BC1 family was assayed for resistance, enabling the selection of at least two plants per family that expressed a strong hypersensitive resistant reaction. Selection was undertaken either before or shortly after first flowers opened so that crosses to the recurrent parent could generate a BC2 generation directly. Unexpected losses and difficulties with selection or crossing resulted in 29 BC2 families. The cycle was repeated to produce 29 BC3 lines.

### Production of dihaploid genotypes

Dihapoid (DH) plants were developed from F1 crosses that carried the CaCV resistance gene in hybrid (heterozygous) form. The pollen derived from such plants is haploid, expressing only one of the alleles of any specific gene, and by an appropriate laboratory process, can be induced to form normal diploid plants that are true-breeding. In this way uniform parent lines can be obtained quickly, bypassing the need for six generations of inbreeding used in conventional breeding.

Dihaploids were first used to initiate a DNA marker program. After shipping all 36 F1 crosses to Syngenta's laboratory for DH production, one set of 219 DH lines comprising 4923 plants was received and assayed for CaCV resistance. It was anticipated that the resistant and susceptible lines identified would be submitted for genotyping and mapping analysis.

In a second application of DH material for breeding, two sets of DH lines from specific BC3 families have been generated for CaCV assays. These BC3 families are related to two standard F1 hybrids identified for commercial release shortly. Although they are not CaCV-resistant, some of the derived DH lines will be uniformly resistant and may be suitable for direct use as parents in F1 hybrids.

### DNA marker development

Two BC3 families were selected as mapping populations for the development of DNA markers linked to the CaCV resistance gene. The first comprised 639 and the second, 224 individuals. Leaves from each seedling were sampled at the first true leaf stage, freeze-dried and sent to Syngenta's laboratory for genotyping based on prior knowledge of likely genome location. The same plants were subsequently phenotyped as CaCV-resistant or susceptible based on typical hypersensitive resistant or systemic susceptible reactions three weeks after inoculation. An association mapping exercise then determined a series of putatively linked DNA markers with estimates of distance from the CaCV gene based on recombination frequencies. Despite some limitations inherent in the material studied, one marker in particular was identified as particularly valuable because it is relatively rare in germplasm. The phenotypes of a subset of

the lines were validated independently using the most favoured marker.

**Field evaluation of BC2 and BC3 lines**

In 2016, total yield, fruit number and fruit size were assessed and compared in each of five plants from unreplicated field plots of the BC2 and BC3 generations of two backcross families and a commercial F1 hybrid cultivar.

# Outputs

### New CaCV resistant breeding lines and F1 hybrids

The primary material outcomes of the project include a series of 29 backcross (BC) 3 lines with resistance to Capsicum chlorosis virus (CaCV) and a range of resistances to other pathogens such as Tomato spotted wilt virus and multiple races of bacterial spot (*Xanthomonas campestris pv. vesicatoria).* These lines were developed from crosses of elite Syngenta parents and a DAF CaCV donor parent. By creating essentially the same Syngenta lines with additional CaCV resistance, the project has provided potentially new resistant F1 hybrids with excellent adaptation and attributes for Australian conditions. There are two strategies for advancing the BC3 lines to provide such an outcome – two generations of inbreeding to produce uniformly resistant parents or the use of double haploids to generate fixed parent lines quickly. Both methods require screening and careful field evaluation of finished lines. In both cases there is a high probability of delivering excellent parents similar to the original elite material.

A parallel program of F1 development by Syngenta has identified two hybrids with excellent performance in Queensland conditions that will be brought forward for commercialisation in the next 12 months. Although these F1s are CaCV-susceptible, their parents were included in the CaCV program so they can be immediately fixed and introduced to equivalent new F1s carrying resistance. This work is in progress now and can be completed quickly.

### Knowledge of Tomato spotted wilt virus isolates

Isolates of TSWV were collected from capsicum crops in the Virginia area of SA from both *Tsw* and non-*Tsw* genotypes. All were virulent when inoculated to varieties with the *Tsw* gene. The distribution and incidence of TSWV in capsicum crops in Virginia indicated that the resistant breaking strain was widespread and dominant. Two isolates were used to inoculate *C. chinense* accessions in a search for resistance to the resistance breaking strain. All 46 lines inoculated developed systemic symptoms of TSWV.

### Development of new DNA markers for resistance to Capsicum chlorosis virus

The development of three DNA markers for CaCV resistance was a significant benefit from the project. All markers appear to be within a recombination distance of 1 centimorgan of the CaCV gene, indicating useful linkage for identification of resistance and selection of breeding material. The most reliable marker has been validated in breeding populations and predicts genotypes with high levels of accuracy.

### Scientific publications

Two scientific publications have arisen from this work and are referenced in the appropriate section below.

# Outcomes

### Impact of resistance to Capsicum chlorosis virus for industry

The development of new CaCV-resistant parents and F1 hybrids will make a tangible contribution to the productivity of field capsicum crops in Queensland in the medium term. We estimate that approximately 10% of marketable yield is lost at peak production periods when tospovirus is most damaging. Although the balance of CaCV and TSWV varies through the season and from one year to the next, the damage from CaCV alone is consistently high. The benefit to industry from CaCV resistance in cultivars will therefore be large, about $10M - $15M per year for Queensland industry and potentially more where future applications in other markets are included. So far CaCV has been found only in Queensland. If the disease becomes more widely distributed in other states of Australia in the future there will be a need for more resistant cultivars and the resistance will become more valuable.

### Tomato spotted wilt virus

The incidence of resistance-breaking isolates of TSWV in South Australia is causing concern. Although none of the germplasm screened in this project expressed resistance to these isolates, a better understanding of the virus was achieved. The study of TSWV indicated the need for more extensive screening of uncultivated germplasm and emphasised the current difficulty in importing capsicum accessions for research purposes.

### Development of DNA markers for CaCV resistance

A most important outcome of the project is the benefit derived from the identification, validation and application of three new DNA markers for CaCV resistance. One marker in particular appears to be highly predictive and reliable, enabling routine application in breeding programs. This development has immediate benefits for the increased flexibility and efficiency of selection across a wide range of potential programs. Because identification of resistance in germplasm and breeding lines is now independent of virus assays and can be undertaken at any location, selection is possible anywhere at any time. The application of a specific CaCV marker is obviously critical to the wider use of the resistance gene. One such potential outcome is breeding work directed towards chilli cultivars in the large markets of South East Asia where CaCV or similar viruses are damaging. Resistant chilli cultivars will be bred more easily in Asian locations with the availability of a DNA marker.

### Collaboration between project partners

The successful collaboration between DAF and Syngenta in the development of new breeding lines, F1 cultivars and DNA markers was built on good teamwork between the researchers in Queensland and overseas. DAF partners in several disciplines and locations in Queensland were effective in undertaking virus assays, hybridization, field trials and marker validation; the Syngenta partners included the supervising breeder and support staff in the US, their molecular biology team in the US and France and a regional team of trial and supporting staff in Australia. The most difficult logistical exercises were shipments of leaf samples overseas under tight time restrictions and seed imports or exports. As a result of this excellent collaboration, additional work to refine the application of DNA markers and planning for more fundamental research with collaborators from the University of Queensland are underway.

**Additional outcomes**

Tospoviruses have a propensity to develop new variants that are virulent on previously resistant cultivars. Instances of new damaging variants have been recorded in Europe and the United States where significant economic losses have arisen following breakdown of resistance. The researchers in this project have considered alternative solutions to the long-established cycle of resistance failure and introduction of new resistance to cultivars. The conventional solution is time-consuming, costly and seldom durable. As a result of the current project, a broader collaboration involving University of Queensland researchers with Tospovirus experience is considering more fundamental research that would seek a permanent solution to these regular failures of resistance.

# Evaluation and Discussion

**Development of parent lines and F1 hybrids with resistance to Capsicum chlorosis virus**

The development of improved CaCV F1 cultivars was a primary objective of the project that was addressed by two different breeding strategies. Initially a wide range of crosses between 36 elite Syngenta parents and 2 DAF resistant parents were constructed and field tested, demonstrating that none of the initial crosses was commercially competitive. All of the F1 crosses were confirmed as CaCV resistant in glasshouse assays. One resistant parent produced superior field performance and was selected as the donor parent for further breeding.

The primary breeding strategy adopted was a backcross procedure to transfer CaCV resistance to specific well-adapted Syngenta parent lines with additional resistances to diseases such as Tomato spotted wilt and bacterial spot. This method is ideally suited to programs such as this where a strong gene with dominant action conferring easily classified phenotypes is introduced. The previous research that established the inheritance of CaCV resistance was confirmed in each of the backcross populations evaluated here. Clear segregation for a single dominant gene was evident in each backcross cycle. Initially 36 F1 crosses were established for the backcross program, ultimately providing 29 backcross 3 populations segregating for resistance. In each cycle, a population was generated by crossing resistant individuals with the recurrent Syngenta parent, effectively reconstituting its background genotype as well as the introduced disease resistance gene.

A parallel program of F1 hybrid development by Syngenta was also implemented in association with specific CaCV breeding objectives. Although these F1s did not carry CaCV resistance, they expressed superior field performance, additional disease resistances, improved fruit quality attributes and adaptation that served as a benchmark for the objectives of the CaCV program. Two such potential F1 hybrids were identified in replicated trials and then assessed at on-farm sites with grower evaluations. As a result, they are now being advanced for commercial release. Furthermore, the female parents in each of the new F1s were included in the CaCV backcross program so that equivalent F1s with resistance can be developed. It is expected that suitable new parent lines can be delivered with a relatively modest amount of additional work. The feedback from this part of the program was critically important in successfully guiding the CaCV work.

Field evaluation of the two parent lines above demonstrated clearly the improvement in agronomic performance in the backcross 3 compared with the backcross 2 generations. For one parent, the marketable yield increased to the level of the standard commercial cultivar, due to a large increase in fruit size. The second parent also expressed a similar increase, in this case due to an increase in fruit number.

The second breeding strategy was to use double haploid (DH) lines that were generated by a laboratory procedure as fixed uniform parents obtained directly from F1 genotypes. The procedure is relatively quick, providing inbred parent lines without the lengthy delay involved with up to 7 generations of conventional inbreeding. This methodology was not initially successful for applied CaCV breeding but has been applied subsequently to the development of the two new F1 hybrids indicated previously. It is anticipated that screening of a large number of DH lines from these F1 hybrids will identify potential parents for equivalent CaCV-resistant F1 hybrids.

**Resistance to Tomato spotted wilt virus**

Capsicum cultivars with resistance to Tomato spotted wilt virus (TSWV) carry the *Tsw* gene which is included in many of the breeding lines of this project. In recent years there have been instances of emerging strains of TSWV that are virulent on *Tsw* genotypes, causing a break down of resistance. In Australia the commercially significant examples have been in South Australia.

Thirty accessions of the uncultivated species *C. chinense* were introduced from the United States and assayed for resistance to the most significant resistance-breaking isolate of TSWV in South Australia. None of these accessions expressed resistance. Because tospoviruses have a propensity to break host plant resistance it is important to monitor such developments.

**Development of DNA marker for resistance to Capsicum chlorosis virus**

A major objective of the project was the development of a DNA marker for CaCV resistance. A robust, functional marker contributes to large improvements in selection efficiency in applied breeding programs and allows selection in the absence of physical assays that require virus inoculation.

Collaboration between DAF and Syngenta researchers enabled the identification and development of several markers, one of which appears to be highly predictive and useful for breeding applications. An association mapping project based on phenotyping of resistance by DAF researchers and genotyping by Syngenta researchers identified three potential markers. An initial approach used Double haploid (DH) lines involving approximately 5000 seedlings from 219 lines that were classified as either uniformly resistant or susceptible and then genotyped. This had difficulties because of the inadequate structure of the original population. However a second approach using several different backcross lines was successful in identifying three markers closely linked to the CaCV gene (about 1% recombination between the gene and marker). One of the markers is rare in germplasm and is therefore valuable as an indicator of resistance. A number of refinements to the analysis have been undertaken and are continuing to overcome some initial limitations with the populations studied.

The most reliable marker was used to independently validate the phenotypes of a subset of a segregating backcross 3 population. The marker predicted the phenotype in about 97% of the individuals, establishing its value for selection in breeding populations. The errors could have been the result of misclassifications in phenotyping or genetic recombination. In another instance of its predictive value, the marker demonstrated that a line, putatively segregating for resistance, was in fact uniformly susceptible. The CaCV assay of this material failed to detect resistant plants, confirming the marker results. The development and application of a new marker such as this is a significant advance in the understanding of CaCV resistance and its application in new F1 hybrid cultivars for the Australian capsicum industry.

**Concluding remarks**

The collaboration with Syngenta Seeds and DAF has been successful in advancing non-commercial CaCV- resistant breeding lines to a range of elite parent lines. Two of these parents have been selected for development as CaCV-resistant F1 cultivars following Syngenta's decision to bring susceptible, related F1 cultivars to market. As a result of supporting work in this project, a useful DNA marker has been developed and applied in breeding programs.

# Recommendations

1. The female parent lines in the CaCV conversion program in Syngenta's two new F1 cultivars should be stabilized and hybridised to generate the equivalent resistant F1 hybrids. These hybrids should be evaluated and advanced for commercial release. A broader continuing program of F1 development should be supported.

2. The DNA markers identified in this project should be refined and validated for routine application to marker-assisted selection.

3. Additional work is planned with DAF in a further collaboration with Syngenta to refine the genomic mapping of the CaCV resistance gene. This work should be supported and funded.

4. Further fundamental research involving collaborators at Syngenta, DAF and University of Queensland is being planned. The intention is to explore long-term durability of tospovirus resistances at a molecular level. This work should be supported and funded.

5. Additional screening of capsicum germplasm for resistance to South Australian isolates of TSWV that are virulent on *Tsw* cultivars should be undertaken.

# Scientific Refereed Publications

Journal articles

The following paper has been submitted to the research journal PLOS One as a research article:

Widana Gamage, S., McGrath, D.J., Persley, D. M., Dietzgen, R. G., 2016. Transcriptome analysis of capsicum chlorosis virus-induced hypersensitive resistance response in Bell capsicum. *PLOS One*

The following paper has been published:

Gamage, S.W., Persley D.M., Higgins, C.M., Dietzgen R.G. (2015) First complete genome sequence of a capsicum chlorosis tospovirus isolate from Australia with an unusually large S RNA intergenic region. Archives of Virology **160**, 869-872.

# Intellectual Property/Commercialisation

An IP/Commercialisation agreement has been developed between DAF and Syngenta Australia to facilitate sharing of IP between the partners in the project. The agreement reflects IP relating to breeding lines, cultivars and molecular markers arising from the project. Royalty payments from the IP will be distributed in accordance with equity shares of the partners, Department of Agriculture and Fisheries, Syngenta Australia Pty. Ltd. and Horticulture Innovation Australia Ltd.

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