FINAL REPORT 2015

For Public Release

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

CRDC Project Number: **DAQ1201**

Project Title: Surveillance and monitoring for endemic and exotic virus diseases of cotton

Project Commencement Date: 01/07/2011 **Project Completion Date:** 30/06/2015

CRDC Program: 2 Industry

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Date Submitted: ______________

Part 3 – Final Report

(The points below are to be used as a guideline when completing your final report.)

Background

1. Outline the background to the project.

Viral diseases of cotton are of economic significance in many parts of the world. Only two virus diseases have been reported from Australian cotton crops, Cotton bunchy top (CBT) and Tobacco streak virus (TSV). However, many of the most economically damaging virus diseases of cotton remain serious biosecurity threats. These include Cotton leaf curl disease (CLCuD), Cotton leaf roll dwarf virus (CLRDV; causing Cotton blue disease) and Cotton leaf crumple virus (CLCrV).

Insect vectors for Cotton leaf curl disease, Cotton leaf crumple and Cotton blue disease are common and widespread in many cotton growing regions of Australia. Surveillance in Australia for viral diseases of cotton will be important to help protect the cotton industry from these serious biosecurity threats. Unusual virus-like symptoms in NSW cotton crops such as tall sterile plants have also been observed by CSIRO staff in several locations. If found, transmissibility studies may be able to determine if the cause is pathogenic.

While significant progress has been made by previous researchers into several aspects of the biology of Cotton bunchy top disease, it is still unclear what natural alternative hosts this pathogen has in and around cotton crops. This information will be important to effectively minimise the risk of this disease entering crops. Results from current CRDC project (DAQ0002) indicate that while TSV appears to be currently restricted to Central Queensland, some of the alternative hosts such as Fleabane and Crownbeard are commonly found in many other cotton growing regions.

Cotton blue disease has caused economic losses in cotton from Brazil and Argentina but little is known about the diversity of the Asian or African strains. There is also currently no expertise in Australia for diagnostics of Cotton blue disease. It is important to identify the diversity of the viruses that cause Cotton blue disease to determine the likely effectiveness of resistance currently available.

Transgenic resistance to viruses has been successfully used in a range of plant species. It is possible that this may be a useful strategy to utilise in cotton as preparedness for an incursion of Cotton leaf curl disease.

Objectives

2. List the project objectives and the extent to which these have been achieved.

Objective 1. Survey Qld and NSW cotton crops for endemic and exotic virus diseases.

This objective has been achieved. Disease surveys were conducted at least once during each growing season from 2012 to 2015 in central Queensland. Surveys were also conducted in southern Qld and northern NSW cotton crops in each season.

Objective 2. Determine key alternative hosts of Cotton bunchy top virus and Tobacco streak virus in cotton

Based on the field observations made during this project and related CRDC-funded projects, this objective has been achieved. A range of alternative hosts were identified as detailed below. While it is possible that further alternative hosts may exist for CBTV, we believe the key alternative hosts of CBTV and TSV have been determined.

Objective 3. Dissemination of disease management recommendations and provision of virology diagnostic services

This objective has been achieved through the regular dissemination of disease management extension material via a range of media. Many samples have also been received from, and tested for agronomists, pathologists and collaborating researchers and surveillance staff.

Objective 4. Develop an assay and diagnostic standard for Cotton leaf roll dwarf virus (Cotton blue disease)

This objective has been achieved. A reliable assay for testing cotton for CLRDV was developed and incorporated into a draft National Diagnostic Protocol that has been submitted to the Subcommittee on Plant Health Diagnostic Standards for review and assessment in 2014 for assessment. However, it is likely that further improvements can be made to the diagnostic assay. In the process of validating the current assay, it was found that testing of hosts other than cotton (*G. hirsutum*) compromised the reliability of the results.

Objective 5. Provide support and preparedness for viral biosecurity threats

This objective has been achieved with the preparation of a full draft version of a Threat Specific Contingency Plan for Cotton blue disease caused by Cotton leafroll dwarf virus. This has been attached as a separate document included with this report.

Objective 6. Review of potential for transgenic resistance for CLCuD

This objective has been achieved. Dr Paul Campbell investigated the published literature and assessed the potential for development of transgenic resistance for CLCuD.

Objective 7. CLCuD Transgenic Resistance constructs developed

This objective has been achieved. Constructs have been produced. Further details are provided in a separate confidential report.

Methods

3. **Detail the methodology and justify the methodology used. Include any discoveries in methods that may benefit other related research.**

Objective 1. Survey Qld and NSW cotton crops for endemic and exotic virus diseases.

The main aim of this objective was to ensure that the surveillance method used provided a high reliability of detecting endemic and exotic virus diseases in commercial cropping regions. While it was of secondary importance to determine the incidence of endemic virus diseases, this was also done in situations where it appeared to be having an impact on the crop being inspected. To determine incidence, a randomised disease count method is preferred in which plants are inspected from a representative transect through the crop. In contrast, to maximise the probability of detecting any virus-affected plants, a more targeted surveillance method was adopted.

Where possible, a standardised disease survey method was used as developed by Dr Cherie Gambley in project DAQ0001 for the purposes of maximising the chances of detecting virus diseases in crops. Approximately 600 plants were inspected on the edge or corner of a block

most likely to be exposed to possible virus sources such as weeds, ratoon or volunteer cotton, or bushland upwind. Additional plants were also inspected outside the standardised count area while moving through the crop so that typically about 1000 plants were sighted at each count location.

This surveillance method was adopted to provide approximately 95 % confidence that a virus-symptomatic plant would be detected in the search area if 1 % of plants were virus-symptomatic. To provide this level of confidence, at least 300 plants need to be inspected (Sergeant 2015; Cameron and Baldock 1998). Total number of plants inspected was sometimes lower for the late season inspections due to the difficulty (and slower process) in checking larger plants.

Crops were inspected for any virus-like symptoms to check for endemic (Cotton bunchy top virus (CBTV) and Tobacco streak virus (TSV)) and exotic viruses (e.g. Cotton leaf curl disease (CLCuD), Cotton leaf roll dwarf virus (CLRDV; causing Cotton blue disease) and Cotton leaf crumple virus (CLCrV)). Representative samples of symptomatic plants were collected and tested either for TSV or CBTV depending on the observed symptoms. Occasional plants were also tested for exotic begomoviruses. TSV was tested for by Enzyme Linked Immuno-Sorbent Assay (ELISA) essentially as per the manufactures instructions (Reagent set SRA25500, Agdia, USA) to confirm that visual assessments were accurate. Representative TSV-infected samples from various locations were also tested for the two known TSV strains that occur in central Queensland, TSV-parthenium and TSV-crownbeard by strain-specific PCRs developed during the course of this project and the related GRDC project (DAQ00154) (Sharman et al. 2015). CBTV was tested for by reverse transcription PCR to distinguish between the two known strains, CBTV-A and CBTV-B using the multiplex PCR developed as part of this project (see Appendix 1).

Objective 2. Determine key alternative hosts of Cotton bunchy top virus and Tobacco streak virus in cotton

As part of the annual survey activities in Queensland cotton crops, likely alternative hosts of CBTV and TSV were collected from in and around crops in locations where these diseases were detected. Diagnostic testing was done as described for Objective 1 above.

An emphasis was placed on CBTV and this objective is being jointly reported between projects DAQ1201 and CRC1.1.30. Samples of a range of potential hosts were collected from a wide range of field locations in NSW and QLD. To test for other potential hosts of CBTV, field trials were conducted by Dr Lewis Wilson and Ms Tanya Smith in 2011, 2012 and 2013 at the ACRI to facilitate the natural aphid transmission of CBTV from infected spreader rows into a range of test plant species. The test species selected were mostly from the family Malvaceae and many occur naturally in the local area on or near to cotton farms. The samples from the ACRI field trial (Wee Waa) were jointly processed with the molecular diagnostics being conducted at the QDAF Ecosciences Precinct, Brisbane.

A ratoon nursery was established at ACRI in which spreader rows of CBTV-infected cotton plants were established and used as source plants to infect other test plants. We used gaps in the CBT ratoon cotton rows in which to plant these potential malvaceous hosts. In December or when the ratoon CBT cotton began to show symptoms of CBT, we infested the ratoon plants with aphids from a glasshouse culture. Once aphids were well established we moved populations from bunchy top cotton to the potential hosts, favouring the most bunchy plants for collecting from, until these hosts had well established aphid colonies. We also placed cages on the plants that excluded beneficial species to ensure aphids had a chance to colonise. Several weeks after infesting the potential hosts with aphids, we collected several leaves from

each plant and freeze dried them for later analysis in the QDAF laboratory. This approach was repeated over several cotton seasons.

Objective 3. Dissemination of disease management recommendations and provision of virology diagnostic services

Extension material, based on research data obtained, was prepared in collaboration with management and extension staff such as Susan Maas (CRDC) and Ngaire Roughley (QDAF) to help build grower and consultant awareness of viral disease threats to cotton. Virology support was also provided to extension material produced by CRDC and collaborators.

Objective 4. Develop an assay and diagnostic standard for Cotton leaf roll dwarf virus (Cotton blue disease)

An assay and diagnostic standard for Cotton leaf roll dwarf virus (cotton blue disease) was developed and optimised based on published protocols and virus sequence data. Samples of cotton displaying typical Cotton blue disease were dried, gamma irradiated and imported into Australia under DAF-Biosecurity Import Permit IP11002580. Samples were sourced from various locations. Dr Nelson Suassuna, EMBRAPA, provided 6 samples from two *Gossypium* species, *G. hirsutum* and *G. mustelinum* from Santa Helena de Goias, Brazil. Murray Sharman also travelled to Brazil in February 2012 as part of a DAFF funded training scholarship to learn more about field diagnosis of Cotton blue disease. Further representative samples were collected from the production areas around Primavera do Leste, Brazil. Murray also travelled to Thailand as part of the CRDC funded science exchange (project DAQ1201T) to inspect and collect samples of Cotton leafroll disease (a suspected polerovirus). The Thailand samples were subsequently shown to be CLRDV-infected. All these samples were used to develop and validate a diagnostic protocol for the specific detection of Cotton leafroll dwarf virus (see Appendix 2). The National diagnostic protocol for CLRDV is also included as a separate document with this report.

Objective 5. Provide support and preparedness for viral biosecurity threats

This objective was centred on the preparation of a threat specific contingency plan for Cotton blue disease caused by *Cotton leafroll dwarf virus*. The method used was similar to that used for a recently developed contingency plan for Cotton leaf curl disease (CRDC project DAQ0001, Dr Cherie Gambley) and also using guidelines provided in version 2.0 of PlantPlan by Plant Health Australia (PHA 2014). Published literature was reviewed to form the basis of the scientific content for the contingency plan and the National Diagnostic Protocol for CLRDV developed as part of Objective 4 was also incorporated into the contingency plan.

Objective 6. Review of potential for transgenic resistance for CLCuD

Dr Paul Campbell investigated the published literature to determine if a transgenic approach may be useful for protection against an incursion of Cotton leaf curl disease and in doing so determined a potentially novel approach for the development of transgenic constructs. The methods for this objective and results derived have intellectual property issues and are being reported separately.

Objective 7. CLCuD Transgenic Resistance constructs developed

The methods for this objective and results derived have intellectual property issues and are being reported separately.

Results

4. Detail and discuss the results for each objective including the statistical analysis of results.

Objective 1. Survey Qld and NSW cotton crops for endemic and exotic virus diseases.

A total of over 81,000 plants were inspected from within 142 standardised disease counts across seasons in 2011/12 to 2014/15 from production areas in central Queensland (Clermont, Emerald, Comet, Arcturus), Southern Queensland (Jimbour, Dalby, Chinchilla, Cecil Plains, Goondiwindi) and Northern NSW (Goondiwindi, Moree, Wee Waa, Gunnedah, Breeza) (Table 1). Additional plants were also inspected outside standardised counts while moving through the crop and this was occasionally effective for detecting trace levels of virus-infected plants or isolated patches within the crop. Hence, a combined surveillance approach is recommended which adopts both standardised counts on the crop edge where influx of virus is most likely and a more general observation further into the crop. The airborne habit of most virus insect vectors such as aphids and white flies means that infection loci are usually along a crop edge but can also be within the crop boundary.

As was also found in preceding project DAQ0002, TSV was only detected in cotton crops across central Queensland and was closely associated with the presence of TSV-infected parthenium or crownbeard, the two major alternative hosts for distinct TSV strains.

CBTV-infected plants were seen in all growing regions surveyed (Table 1). The surveillance strategy was effective at detecting diseased plants but it was important to also check surrounding volunteers and ratoons as they were often infected with CBTV but no infected plants were observed within the nearby crop. This was particularly apparent in surveys conducted in January and March 2013 when between 7 to 64 % of nearby volunteers were CBTV-infected at 7 different locations (see May 2013 Progress Report for further details).

Table 1. Details of virus survey sites and results of surveillance for endemic viruses TSV and CBTV.

Farm #	Nearest locality	GPS waypoint	Total plants inspected in disease count	TSV plants	CBTV plants	Month-Year
1	Emerald-east	WPms308	600	0 [A]	0	Dec-11
1	Emerald-east	WPms308	600	0	0	Dec-11
1	Emerald-east	WPms309	600	**3**	0	Dec-11
1	Emerald-east	WPcg194	600	0	**2**	Dec-11
2	Emerald-east	WPms241	300	**1**	**1**	Dec-11
2	Emerald-east	WPms241	300	0	**1**	Dec-11
2	Emerald-east	WPms311	600	**2**	0	Dec-11
2	Emerald-east	WPms312	600	0	0	Dec-11
3	Emerald-west	WPms313	600	0	**1**	Dec-11
3	Emerald-west	WPms314	600	**12**	**11**	Dec-11
3	Emerald-west	WPms214	600	0	**1**	Dec-11
4	Emerald-west	WPms32	600	**5**	**5**	Dec-11
4	Emerald-west	WPms318	600	**2**	0 [A]	Dec-11
5	Emerald-west	WPms53	600	**3**	0 [A]	Dec-11
6	Arcturus	WPms329	1000	**7**	0	Dec-11
6	Arcturus	WPms139	1000	**6**	0	Dec-11
7	Wee Waa-north	WPms359	600	0	**32**	Apr-12

7	Wee Waa-north	WPms359	600	0	0	Apr-12
8	Wee Waa-east	WPms362	600	0	**2**	Apr-12
9	Comet	WPms435	600	**1**	0	Dec-12
9	Comet	WPms436	600	**1**	0	Dec-12
9	Comet	WPms437	300	0	0	Dec-12
10	Arcturus	WPms329	300	**20**	**2**	Dec-12
10	Arcturus	WPms330	300	**10**	0	Dec-12
11	Emerald-west	WPms438	600	0	0 [A]	Dec-12
3	Emerald-west	WPms439	1000	0	0	Dec-12
3	Emerald-west	WPms444	1000	**3**	**5** [A]	Dec-12
3	Emerald-west	WPms314	1000	0	**1**	Dec-12
12	Emerald-east	WPms440	1000	**1**	**4**	Dec-12
12	Emerald-east	WPms441	600	**1**	0	Dec-12
12	Emerald-east	WPms442	600	0	**1**	Dec-12
1	Emerald-east	WPms443	600	**1**	0	Dec-12
13	Jimbour	WPms233-1	1000	0	0	Jan-13
13	Jimbour	WPms233-2	500	0	0	Jan-13
14	Jimbour	WPms446	1000	0	0	Jan-13
14	Jimbour	WPms447	1000	0	0	Jan-13
15	Warra	WPms448	1000	0	0	Jan-13
16	Chinchilla	WPms449	1000	0	**1**	Jan-13
16	Chinchilla	WPms450-1	500	0	0	Jan-13
16	Chinchilla	WPms450-2	500	0	0	Jan-13
17	Nandi	WPms451-1	1000	0	0	Jan-13
17	Nandi	WPms451-2	1000	0	**1**	Jan-13
18	Goondiwindi-north	WPms454	1000	0	0	Jan-13
18	Goondiwindi-north	WPms455	500	0	0	Jan-13
19	Goondiwindi-south	WPms456	1000	0	0	Jan-13
19	Goondiwindi-south	WPms457-1	750	0	0	Jan-13
19	Goondiwindi-south	WPms457-2	1000	0	0	Jan-13
20	Goondiwindi-south	WPms458	1000	0	0	Jan-13
20	Goondiwindi-south	WPms459	500	0	0	Jan-13
21	Goondiwindi-east	WPms460	500	0	0	Jan-13
22	Goondiwindi-east	WPms461-1	1000	0	0	Jan-13
22	Goondiwindi-east	WPms461-2	500	0	0	Jan-13
22	Goondiwindi-east	WPms462	500	0	0	Jan-13
23	Wee Waa-south	WPms473	317	0	0	Mar-13
23	Wee Waa-south	WPms474	200	0	0	Mar-13
24	Wee Waa-south	WPms475	-	-	-	Mar-13
24	Wee Waa-south	-	300	0	0	Mar-13
25	Moree-north	WPms477	303	0	0	Mar-13
25	Moree-north	WPms478	411	0	0	Mar-13
25	Moree-north	WPms479-1	-	-	- [A]	Mar-13
25	Moree-north	WPms479-2	200	0	0	Mar-13
25	Moree-north	WPms480	230	0	**54** [A]	Mar-13
26	Moree-north	WPms481	321	0	**18** [A]	Mar-13
26	Moree-north	WPms482	302	0	**2**	Mar-13
27	Baan Baa	WPms483	301	0	0	Mar-13
27	Baan Baa	WPms484	306	0	0 [A]	Mar-13
28	Boggabri	WPms485	306	0	0 [A]	Mar-13
28	Boggabri	WPms486	300	0	0	Mar-13
28	Boggabri	WPms487	300	0	0	Mar-13
29	Gunnedah-south	WPms489	315	0	0	Mar-13
29	Gunnedah-south	WPms490	364	0	0	Mar-13
30	Breeza	WPms491	300	0	0	Mar-13
30	Breeza	WPms492	316	0	**2** [A]	Mar-13
30	Breeza	WPms493	356	0	**1**	Mar-13
30	Breeza	WPms494	300	0	0	Mar-13
9	Comet	WPms558	610	0	0	Dec-13
9	Comet	WPms435	600	**3**	0	Dec-13
9	Comet	WPms436	490	0	0	Dec-13
31	Comet	WPms561	600	0	0	Dec-13
10	Arcturus	WPms562	600	**1**	0	Dec-13

12	Emerald-east	WPms440	300	**2**	**5**	Dec-13
12	Emerald-east	WPms573	300	0	0	Dec-13
12	Emerald-east	WPms573	300	0	**3**	Dec-13
12	Emerald-east	WPms574	300	0	**4**	Dec-13
32	Cecil Plains	WPms578	300	0	0	Jan-14
32	Cecil Plains	WPms579	400	0	0	Jan-14
32	Cecil Plains	WPms580	400	0	0 [A]	Jan-14
32	Cecil Plains	WPms582	400	0	0	Jan-14
33	Brookstead	WPms583	400	0	0	Jan-14
33	Brookstead	WPms584	400	0	0	Jan-14
33	Brookstead	WPms585	427	0	0	Jan-14
33	Brookstead	WPms587	400	0	0	Jan-14
34	Brookstead	WPms586	436	0	0	Jan-14
34	Brookstead	WPms588	600	0	0 [A]	Jan-14
34	Brookstead	WPms589	300	0	**7**	Jan-14
19	Goondiwindi-south	WPms590	315	0	0	Jan-14
19	Goondiwindi-south	WPms591	307	0	**2**	Jan-14
19	Goondiwindi-south	WPms593	300	0	0	Jan-14
20	Goondiwindi-south	WPms594	300	0	0	Jan-14
20	Goondiwindi-south	WPms595	300	0	**1** [A]	Jan-14
20	Goondiwindi-south	WPms596	550	0	0	Jan-14
20	Goondiwindi-south	WPms597	300	0	0	Jan-14
20	Goondiwindi-south	WPms598	300	0	0	Jan-14
20	Goondiwindi-south	WPms599	300	0	0	Jan-14
18	Goondiwindi-north	WPms600	300	0	0	Jan-14
18	Goondiwindi-north	WPms601	300	0	0	Jan-14
18	Goondiwindi-north	WPms602	300	0	0	Jan-14
18	Goondiwindi-north	WPms603	300	0	0	Jan-14
18	Goondiwindi-north	WPms604	300	0	0 [A]	Jan-14
1	Emerald-east	WPms308	313	**1**	**17**	Dec-14
1	Emerald-east	WPms668	346	**7**	**1**	Dec-14
1	Emerald-east	WPms669	320	**5**	**7**	Dec-14
1	Emerald-east	WPms670	518	**2**	**2**	Dec-14
5	Emerald-west	WPms672	454	**1**	**1**	Dec-14
5	Emerald-west	WPms672	502	0	0	Dec-14
5	Emerald-west	WPms673	328	0	**23** [A]	Dec-14
9	Comet	WPms678	304	**88**	0	Dec-14
9	Comet	WPms679	339	**15**	0	Dec-14
9	Comet	WPms680	304	**11**	0	Dec-14
9	Comet	WPms681	333	**1**	0	Dec-14
35	Dalby	WPms682	436	0	0	Feb-15
35	Dalby	WPms682	383	0	0	Feb-15
35	Dalby	WPms683	328	0	0	Feb-15
36	Nandi	WPms685	301	0	**7**	Feb-15
36	Nandi	WPms685	216	0	**10**	Feb-15
36	Nandi	WPms686	313	0	**1**	Feb-15
36	Nandi	WPms687	400	0	**1**	Feb-15
36	Nandi	WPms687	316	0	**3**	Feb-15
36	Nandi	WPms688	306	0	0	Feb-15
37	Cecil Plains	WPms690	406	0	0	Feb-15
37	Cecil Plains	WPms691	385	0	0	Feb-15
37	Cecil Plains	WPms692	336	0	0	Feb-15
34	Brookstead	WPms589	349	0	0	Feb-15
20	Goondiwindi-south	WPms693	415	0	**2**	Feb-15
20	Goondiwindi-south	WPms694	337	0	0	Feb-15
20	Goondiwindi-south	WPms458	362	0	0	Feb-15
20	Goondiwindi-south	WPms695	395	0	0 [A]	Feb-15
20	Goondiwindi-south	WPms696	351	0	0	Feb-15
20	Goondiwindi-south	WPms697	306	0	0	Feb-15
20	Goondiwindi-south	WPms697	300	0	0	Feb-15
38	Boomi-east	WPms698	353	0	0	Feb-15
38	Boomi-east	WPms699	303	0	0	Feb-15
38	Boomi-east	WPms700	368	0	0	Feb-15

38	Boomi-east	WPms701	354	0	0	Feb-15
39	Boomi-east	WPms702	440	0	0 [A]	Feb-15

[A] Virus infected plants observed outside standard disease count area, either within crop or nearby in volunteer or ratoon cotton.

Objective 2. Determine key alternative hosts of Cotton bunchy top virus and Tobacco streak virus in cotton

Tobacco streak virus.

There are two genetically distinct strains of TSV in central Qld, called TSV-parthenium and TSV-crownbeard in reference to the major alternative hosts they are most commonly found in. Studies into the natural host range of these two TSV strains have been part of this project and previous CRDC and GRDC funded projects. The data shown in Table 2 is a summary of all the known natural hosts confirmed by TSV strain-specific PCRs. Of the 42 hosts identified, most were infected with TSV-parthenium which supports other field observations that TSV-parthenium is the most important strain causing disease outbreaks in crops such as cotton, sunflower and mung beans.

Table 2. Summary of natural host range of TSV-parthenium (P) and TSV-crownbeard (C) strains identified during the current and previous projects. For most hosts there were multiple samples and a selection of localities is shown to represent the geographic distribution of samples.

Species	Common name	Strains detected		Nearest localities
Abelmoschus ficulneus	Native rosella	P		Mt McLaren, Emerald
Alysicarpus rugosus	Rough chainpea	P		Clermont
Amaranthus mitchellii	Boggabri weed	P		Emerald
Arachis hypogaea	Peanut	P	C	Emerald, Gogango
Bidens pilosa	Cobblers pegs	P		Clermont
Cajanus cajan	Pigeon pea	P		Clermont, Emerald, Comet
Carthamus tinctorius	Safflower	P		Mt McLaren
Cicer arietinum	Chickpea	P		Clermont, Gindi
Commelina benghalensis	Wandering-jew	P		Arcturus
Conyza bonariensis	Flax-leaf fleabane	P		Arcturus, Emerald
Corchorus trilocularis	Native jute	P		Mt McLaren
Crotalaria mitchellii subsp. *Mitchellii*	Yellow rattlepod		C	Emerald
Datura ferox	Fierce thornapple	P		Clermont
Datura leichhardtii	Leichhardt's Thornapple	P		Mt McLaren
Eclipta prostrata	White Eclipta		C	Arcturus
Glycine max	Soy bean	P		Emerald
Gossypium hirsutum	Upland cotton	P	C	Comet, Emerald, Theodore, Clermont
Gossypium sturtianum	Sturt's desert rose	P		Clermont
Helianthus annuus	Sunflower	P	C	Clermont, Emerald, Arcturus
Lactuca serriola	Prickly lettuce		C	Arcturus
Leptopus decaisnei	none	P		Clermont
Lupinus sp.	Lupin	P		Mt McLaren
Macroptilium lathyroides	Phasey bean		C	Emerald
Nicotiana megalosiphon subsp. Megalosiphon	none	P		Capella
Parsonsia sp.	Unknown	P		Mt McLaren
Parthenium hysterophorus	Parthenium weed	P		Aligator Ck, Clermont,

				Emerald, Injune
Phaseolus vulgaris	French bean	P		Mt McLaren
Phyllanthus sp.	Unknown	P		Clermont
Physalis lanceifolia	none	P		Mt McLaren
Senecio madagascariensis	Fireweed	P		Arcturus
Solanaceae sp	unknown	P		Clermont
Sonchus oleraceus	Annual sowthistle	P	C	Mt McLaren
Tephrosia sp.	Vetch sp.	P		Mt McLaren
Trichodesma zeylanicum	Camel bush	P		Clermont
Tridax procumbens	Tridax daisy		C	Emerald
Verbena bonariensis	Blue-top	P		Arcturus
Verbesina encelioides	Crownbeard	P	C	Emerald, Comet, Orion
Vicia faba	Faba bean	P		Mt McLaren
Vigna radiata	Mung bean	P		Orion, Emerald, Mt McLaren
Vigna radiata var. sublobata	Native mung bean	P		Clermont
Vigna unguiculata	Cowpea	P		Mt McLaren
Xanthium occidentale	Noogoora burr	P	C	Airdmillan, Arcturus, Orion

Cotton bunchy top virus

A generic PCR assay (Pol3630F / Pol3982R, Table 3) originally developed at the end of the preceding project (DAQ0002) has been shown to work reliably to detect CBTV and a range of other related poleroviruses from many different hosts and locations. This assay worked well for detection of the CBTV strain reported by Ellis et al. (2013), hereafter referred to as CBTV-A. It was through the use of this generic PCR that the second distinct CBTV strain, CBTV-B, was first detected in central Queensland. Based on the sequences derived from a number of samples of CBTV-A and CBTV-B, strain-specific PCRs were developed and have been used to accurately characterise the strains infecting samples as part of the host range studies and also the surveillance activities.

The results of studies into the alternative hosts of CBTV are being jointly reported between this project (DAQ1201) and project CRC1.1.30, lead by Dr Lewis Wilson. A combination of field collected samples, glass house inoculated samples and field trial samples were tested by CBTV strain-specific PCRs. More than 670 samples from 36 plant species were tested by PCR. From these, samples positive for at least one CBTV strain were found from 15 plant species from 5 families (Table 4).

As shown in Table 4, some cotton plants were found to have single infections of CBTV-A. However, these plants were either collected as non-symptomatic from nearby to symptomatic plants or were randomly collected with unknown symptoms status. From the samples that were collected as symptomatic, essentially all were positive for CBTV-B and about half of these samples also contained CBTV-A. This provided strong evidence for the close association of CBTV-B with disease symptoms and it appears that CBTV-A is sometimes also present as mixed infections probably because the aphids were carrying both viruses.

Table 3. Diagnostic PCR primers developed for the detection of virus groups, species and strains of CBTV and CLRDV.

PCR primer name	Sequence (5' – 3')	Target pathogen
Pol3630F	ATGAATACGGYCGYGGSTAGAA	CBTV, CLRDV and other poleroviruses
Pol3628F	TAATGAATACGGYCGYGGSTAG	CBTV, CLRDV and other poleroviruses
CBTVqld3870F	GCTGYTGGAAGAGGCTCAAATA	CBTV-B strain only
CBTV.B3148F	CCTGGAGGCTTATAAGTCTC	CBTV-B strain only
CBTVnsw3484F	GCCATAAGTCAAAACTAGGAA	CBTV-A strain only
CBTV.A3332F	CGCGTGCTTCAGCGTACTC	CBTV-A strain only
CLRDV3675F	CCACGTAGRCGCAACAGGCGT	CLRDV, Brazil, Thailand and Timor Leste
CBD3553F	GGTAGCAGTACCAATATCAACGT	CLRDV, standard strain
AtyCBD3476F	GTAGTGGACCTTTAGCCTTGTAA	CLRDV, atypical strain
CBTVcp_3'end_R	CTGGGGGGGRACTACCWTCTAC	3' end of CBTV coat protein gene
Pol3982R	CGAGGCCTCGGAGATGAACT	CBTV, CLRDV and other poleroviruses
Pol4021R	GGRTCMAVYTCRTAAGMGATSGA	CBTV, CLRDV and other poleroviruses

Table 4. Plant species found to be positive for one or both CBTV strains by strain-specific PCRs. Total number of plants tested shown and number of single or mixed infections detected. Samples collected from field, glasshouse or ratoon nursery.

Plant Family	Host species	Common name	Field (F), Glasshouse (GH), Ratoon nursery (RN)	CBTV-A only	CBTV-B only	CBTV-A/B mix	Total tested
Malvaceae	*Abutilon theophrasti*	Velvet leaf	F, GH	0	3	2	13
	Anoda cristata	Spurred anoda	F, GH, RN	0	3	4	7
	Gossypium australe	Desert rose	F, GH	0	1	0	10
	Gossypium hirsutum	Cotton	F, RN	12 [A]	117	89	310
	Hibiscus sabdariffa	Rosella	F, GH	1	2	0	13
	Hibiscus trionum	Bladder ketmia	RN	2	0	0	15
	Malva parviflora	Marshmallow	F, GH, RN	2	11	20	72
	Malva sp.	unknown		1	0	0	1
	Malvastrum coromandelianum	Malvastrum	GH	1	0	0	20
	Sida rhombifolia	Paddy's lucerne	F	0	1	0	15
	Gossypium sturtianum	Sturt's desert rose	F	4	0	5	18
Euphorbiaceae	*Chamaesyce hirta*	Asthma plant	F	0	3	0	18
Lamiaceae	*Lamium amplexicaule*	Deadnettle	RN	0	0	1	3
Fabaceae	*Medicago polymorpha*	Burr Medic	GH, F	1	0	0	8
Aizoaceae	*Trianthema portulacastrum*	Black or Giant pigweed	GH, RN	4	0	0	12

[A] Of the 12 cotton that were CBTV-A-only infected, 7 were collected from plants known to be non-symptomatic from Brookstead from within a small outbreak of CBTV, and at least further 4 were from random collections of roadside volunteers of unknown symptom status (collected as part of Dr Paul Grundy's project DAQ1301).

The intergenic region between the RNA dependant RNA polymerase (RdRp) gene and the Coat Protein (CP) gene is a well known site for both intra- and inter-species recombination for other poleroviruses (Silva et al 2008; Knierim et al 2010) and should be a good location to look for sequence diversity in CBTV samples. For this purpose, additional CBTV strain-specific primers (CBTV.A3332F and CBTV.B3148F, Table 3) were designed to amplify part of the RdRp gene, the entire intergenic region and entire CP gene when paired with downstream primer (CBTVcp_3'end_R, Table 3). The primers also amplify strain size-specific PCR products to enable identification of mixed infections and direct sequencing of strain-specific products. A total of 17 CBTV samples from various growing regions and representing a combination of both mixed –A/B infections and single –A or –B infections from various naturally infected hosts were tested using the new primers and resulting PCR products sequenced and compared by phylogenetic analysis (Fig 1).

Results in Figure 1 clearly show that all CBTV-B and –A samples group together as separate strains and there is very little diversity within each strain and there is no evidence of recombinations or further diversity. Within each CBTV strain, samples are approximately 99 % identical for the 865 bp overlap examined. However, CBTV-A and CBTV-B share less than 74 % nt identity over the same region. By comparison, both CBTV strains share between 70 % to 73 % identity to CLRDV (cotton blue disease). CBTV-A shares a higher nt identity with Chickpea chlorotic stunt virus (CpCSV) than it does with either CBTV-B or CLRDV. CBTV-A and CBTV-B differ by more than 30 % amino acid identity for the coat protein and Figure 2 clearly illustrates that CBTV-A is most closely related to CpCSV. A difference of greater than 10 % in this region is one of the demarcation criteria for different virus species. Along with the observed differences in symptom expression in cotton between CBTV-A and CBTV-B, these genomic differences suggest that these strains represent distinct species.

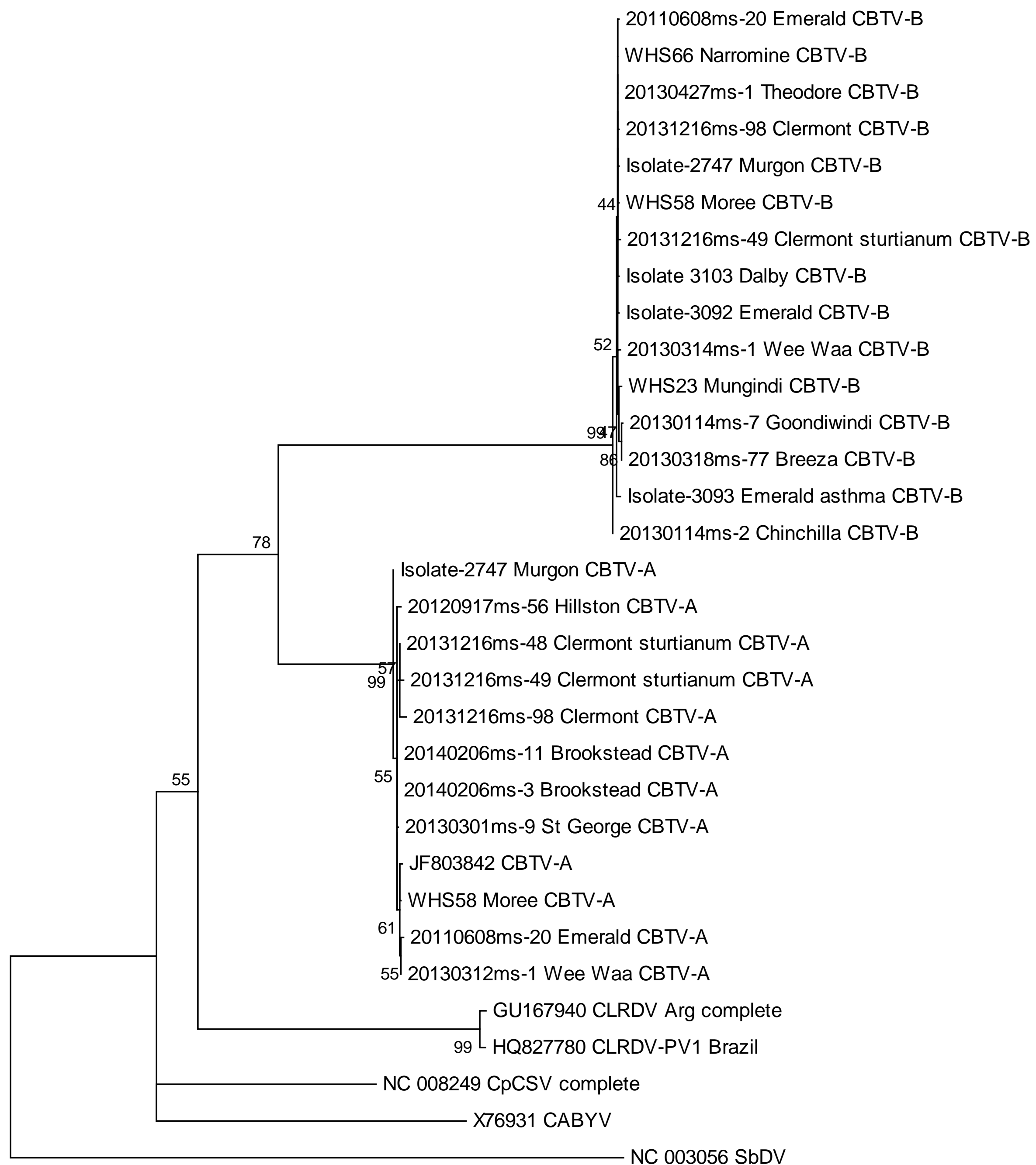

Fig. 1. Comparison of partial genome covering partial RdRp gene, complete intergenic region and complete coat protein gene from a range of CBTV-A and –B samples from different locations and hosts. Other related poleroviruses (with GenBank accession numbers) included in comparison are *Cotton leafroll dwarf virus* (CLRDV – cotton blue disease), *Chickpea chlorotic stunt virus* (CpCSV), *Cucurbit aphid-borne yellows virus* (CABYV) and *Soybean dwarf virus* (SbDV). CBTV-A reference isolate reported by Ellis et al. (2013) is shown as GenBank accession JF803842. Phylogram produced from Maximum likelihood nt analysis of an 865 bp overlap.

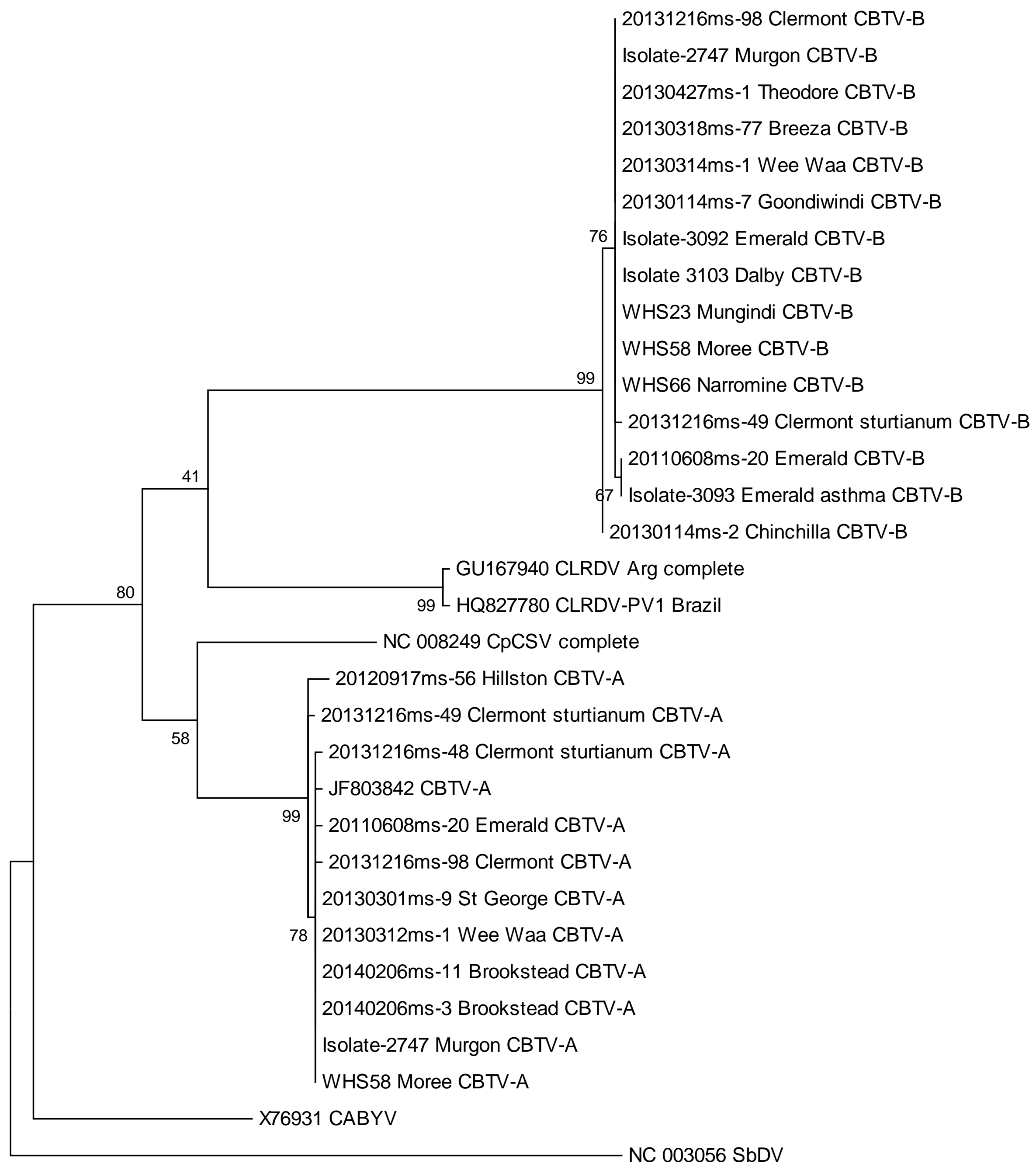

Fig. 2. Comparison of complete coat protein amino acid sequence from a range of CBTV-A and –B samples from different locations and hosts. Other related poleroviruses (with GenBank accession numbers) included in comparison are *Cotton leafroll dwarf virus* (CLRDV – cotton blue disease), *Chickpea chlorotic stunt virus* (CpCSV), *Cucurbit aphid-borne yellows virus* (CABYV) and *Soybean dwarf virus* (SbDV). CBTV-A reference isolate reported by Ellis et al. (2013) is shown as GenBank accession JF803842. Phylogram produced from Maximum likelihood amino acid analysis of a 201 aa overlap.

Objective 3. Dissemination of disease management recommendations and provision of virology diagnostic services

During the course of this project, related data has been published in the following peer reviewed publications:

- Ellis MH, Silva TF, Stiller WN, Wilson LJ, Vaslin MFS, Sharman M, Llewellyn DJ (2013) Identification of a new Polerovirus (family Luteoviridae) associated with cotton bunchy top disease in Australia. Australasian Plant Pathology 42 (3):261-269.
- Sharman M, Thomas JE (2013) Genetic diversity of subgroup 1 ilarviruses from eastern Australia. Archives of Virology 158 (8):1637-1647.
- Sharman M, Thomas JE, Persley DM (2015) Natural host range, thrips and seed transmission of distinct *Tobacco streak virus* strains in Queensland, Australia. Annals of Applied Biology 167 (2):197-207.

Disease management extension material was disseminated via a range of media including:

- Cotton bunchy top fact sheet co-authored with the CottonInfo team, available at: http://www.cottoninfo.com.au/publications/disease-idm-cotton-bunchy-top
- An article about the risk and management of CBTV was presented in the Winter and Spring 2011 editions of Spotlight magazine.
- Text and photos for the Cotton Symptom Guide for TSV and CBTV (Feb-2012).
- Co-authored CottonInfo Communique article about detection of Cotton leafroll dwarf virus from East Timor; available at: http://www.cottoninfo.com.au/publications/significant-virus-detection-east-timor
- Published article: Sharman M, Wilson L, Smith T, Grundy P, Webb M (2014). Cotton bunchy top disease and related biosecurity threats. The Australian Cottongrower, 35: 30-31.
- Contributed text and photos to the current Cotton Pest Management Guide, 2014-15.

Talks about the biology and management of CBTV were presented to growers and agronomists at Jimbour, Cecil Plains, Dalby, Brookstead and Goondiwindi. Talks or posters about research findings were also presented at yearly FUSCOM meetings, the Australian Cotton Conference (Gold Coast, 2012 and 2014) and the Australian Cotton Researchers Conference (Sept 2013).

Virology diagnostic support was provided to a range of industry members during the course of the project. Field samples of cotton were received from agronomist from QLD and NSW and tested for known endemic viruses and when negative, also for exotic begomoviruses. More than 35 samples collected by Dr Cherie Gambley as part of project DAQ1405 from the Kimberley region of WA were tested for poleroviruses (i.e. CLRDV and CBTV); all were negative. A further 16 samples collected from East Timor were tested and one *G. barbadense* was positive for CLRDV (see further details in Objective 4 below). Chickpea samples (a known host of CLRDV) were received from Northern Australian Quarantine Strategy inspector Dr Jane Ray from Kununurra; all were negative.

From 84 roadside cotton samples were received from Dr Paul Grundy, collected as part of project DAQ1301; 5 were CBTV-A-only, 19 were CBTV-B-only and 14 were CBTV-A/B mixed infection. Most of these samples were randomly collected and the fact that approximately 45 % of roadside plants were CBTV-infected indicates that these off-farm volunteers pose a significant threat as a source of virus and aphid vectors to move into production regions. This collaborative link with project DAQ1301 has also provided valuable information about the geographic distribution of CBTV strains.

Objective 4. Develop an assay and diagnostic standard for Cotton leaf roll dwarf virus (Cotton blue disease)

A National Diagnostic Protocol for the detection and diagnosis of Cotton leafroll dwarf virus was prepared and submitted to the Subcommittee on Plant Health Diagnostic Standards for review and assessment in 2014. In order to develop this diagnostic protocol, significant developmental work was required. This included sourcing typical and atypical disease samples from multiple locations in Brazil. An initial PCR assay worked well for these Brazilian samples but had to be modified to enable detection of CLRDV from Thailand samples (also collected in person). This represented the first confirmation of CLRDV from Thailand and south-east Asia. This diagnostic assay was then used to confirm CLRDV for the first time from Timor Leste from a single sample of *G. barbadense*.

As indicated in Figure 3, chickpea (*Cicer arietinum*) is a host of CLRDV in India (referred to by the synonymous name Chickpea stunt disease associated virus) and severe disease symptoms are reported by Indian researchers. In collaborative work with Dr Safaa Kumari for a GRDC funded project, CLRDV has now been detected in 3 diseased chickpea samples from Uzbekistan using the diagnostic assay developed in this project. This is the first record of CLRDV from Uzbekistan. The reported experimental host range of CLRDV from India includes several other Fabaceae species so it may be prudent that members of this plant family should be considered in surveys for CLRDV (e.g. in East Timor).

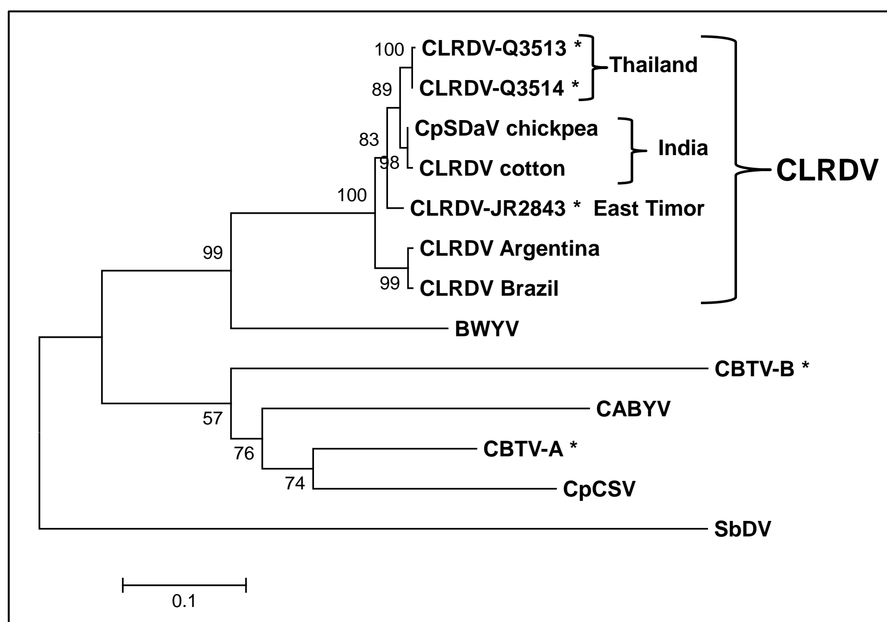

Fig. 3. Comparison of 560 bp overlap of coat protein gene for CLRDV, CBTV and other related poleroviruses. Samples marked with an asterisk (*) were characterised in this project. Abbreviations are: CLRDV – *Cotton leafroll dwarf virus*; BWYV – *Beet western yellows virus*; CBTV – *Cotton bunchy top virus* (strains –A and –B); CABYV – *Cucurbit aphid borne yellows virus*; CpCSV – *Chickpea chlorotic stunt virus*; and SbDV – *Soybean dwarf virus*. Phylogram produced from Maximum likelihood nt analysis.

As mentioned in the May 2014 PR, there were significant problems with non-specific reactions from the CLRDV-specific PCR (primers CLRDV3675F / Pol3982R) when Malvaceous hosts other than *G. hirsutum* were tested. On the other hand, this assay was the only combination of primers that readily detected CLRDV from the East Timor sample which appeared to have poor quality RNA template, so it is preferable to utilise this assay for CLRDV detection. To improve the sensitivity of the assay and reduce non-specific reactions, and hence increase confidence in the results, a two-step nested PCR strategy was tested with several primer options. This strategy uses an initial, larger PCR with degenerate primers (Pol3628F / Pol4021R, Table 3) that should work on most poleroviruses but is not CLRDV-specific and the resulting PCR product is then diluted and used as template in the CLRDV-

specific assay (CLRDV3675F / Pol3982R). This assay has been used with good results on 36 Malvaceae samples from the Kimberley region (collected by Dr Cherie Gambley, DAQ1405) with no positive detections. The same nested assay worked very well for CLRDV samples from Brazil, Thailand and East Timor. This nested PCR strategy greatly increased sensitivity and reduced problematic non-specific reactions (see November 2014 PR for further details). Further validation of this strategy should be done before it is incorporated into the National Diagnostic Protocol for CLRDV, then reviewed and tested by an independent national laboratory thereafter.

Objective 5. Provide support and preparedness for viral biosecurity threats

A complete draft of the Threat Specific Contingency Plan for Cotton blue disease caused by *Cotton leafroll dwarf virus* has been prepared and is included as a separate document to this report.

Objective 6. Review of potential for transgenic resistance for CLCuD

Dr Paul Campbell investigated the published literature and assessed the potential for development of transgenic resistance for CLCuD. This has been reported as an in-confidence appendix to the November 2013 Progress Report.

Objective 7. CLCuD Transgenic Resistance constructs developed

Progress against this objective has been included as a separate confidential document to this report.

Outcomes

5. Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

Results from this project have significantly increased our understanding of the natural and potential host range of CBTV and TSV in and around crops. While CBTV was found to infect 15 plant species from 5 families, the most common and important alternative host were cotton and *Malva parviflora*. The knowledge that volunteer and ratoon cotton are regularly infected with CBTV in and around cotton production areas provides an opportunity to target and eliminate the source of the disease to reduce the risk of CBTV and aphid vectors persisting between seasons. The development of CBTV diagnostic assays in this project has been used to greatly enhanced understanding of the diversity, distribution and importance of CBTV strains infecting cotton in Australia. This knowledge will be critical for implementing effective management strategies.

The major alternative hosts for two distinct TSV strains are Parthenium hysterophorus and Verbesina encelioides. Extensive surveillance in many production regions has shown that both TSV strains are currently restricted to the geographical range of these two weed hosts in central Queensland.

The surveillance and extension activities of this project have greatly increased awareness of exotic cotton viruses, particularly for *Cotton leafroll dwarf virus* (Cotton blue disease). The development of effective diagnostic assays and a threat specific contingency plan for CLRDV and increased understanding of the global distribution of CLRDV provided by this project enhances the industry's preparedness for this significant biosecurity threat.

The annual surveys for endemic (CBTV and TSV) and exotic viruses in different regions has provided definitive records for the presence of CBTV and TSV in various regions and also the absence data for exotic viruses.

This project has provided an assessment of the potential for transgenic resistance to protect the cotton industry from an incursion of CLCuD.

6. Please describe any:-
   a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);
   b) other information developed from research (eg discoveries in methodology, equipment design, etc.); and
   c) required changes to the Intellectual Property register.

The novel approach investigating transgenic resistance to CLCuD is described in detail in the separate confidential document included with this this report.

Conclusion

7. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?

The most important host of CBTV that commonly acts as a source of virus and aphids in and near to crops is volunteer and ratoon cotton. Effective control of these host plants between crops will greatly reduce the risk of subsequent disease spread in following seasons and in doing so also reduce the dependence on insecticide use to control CBTV outbreaks.

Two distinct strains or species of CBTV occur in all growing regions of Australia, CBTV-A (the original strain described by Ellis et al. (2013) and CBTV-B (investigated in detail for the for the first time in this project). Testing of many symptomatic cotton plants from many growing regions has clearly shown that CBTV-B is closely associated with Cotton bunchy top disease and CBTV-A by itself does not appears to induce obvious symptoms in cotton. Hence, CBTV-B is the most important strain that is associated with significant disease outbreaks.

Cotton leafroll dwarf virus (CLRDV – cotton blue disease) has now been detected for the first time in Thailand and Timor Leste. This greatly extends the known global distribution of this important biosecurity threat which is now known to be relatively close to the Australian mainland. Further consideration should be given to the potential hosts and incursion pathways for this virus (this is being considered in project DAQ1601). An accurate diagnostic assay for CLRDV has been developed and will be important for future surveillance and epidemiology studies.

Extension Opportunities

8. Detail a plan for the activities or other steps that may be taken:

(a) to further develop or to exploit the project technology.

- Further improvement and validation is planned for the diagnostic assay for CLRDV as part of the continuing project DAQ1601.

(b) for the future presentation and dissemination of the project outcomes.

- A Disease Note reporting the first detection of CLRDV from Thailand was submitted for publication in Australian Plant Disease Notes in May 2015 and has now been published in July 2015.

- A Disease Note reporting the first detection of CLRDV from Timor Leste has been prepared by Dr Jane Ray (NAQS) and has been submitted for publication in Plant Disease.
- A peer reviewed publication co-authored with several collaborators is planned to report the host range, diagnostic assays, geographic distribution and genetic diversity studies for CBTV in Australia. This could be published in an international journal such as Annals of Applied Biology.

(c) for future research.

- Further related research is being undertaken as part of project DAQ1601 to continue surveillance for CLRDV in northern Australia and to investigate the prevalence and potential host range of CLRDV in Timor Leste.
- Transformation of cotton historically relies on the use of a line that is not suitable for commercial production, so traditional breeding has to be used to bring the transgene into modern lines. This takes many generations, and thus is not suitable for the provision of 'rapid' resistance provision following incursions, or responding to resistance breaking strains. There are reports of genetic transformation of cotton utilising floral dips or similar technology, whilst these appear to work at low frequency, it may be possible to generate transgenic plants in the current lines, thus overcoming years of backcrossing to introduce the genes into commercially favourable lines. Investigation into these techniques and their applicability to the Australian cotton lines would be advantageous to the industry.

9. A. List the publications arising from the research project and/or a publication plan. (NB: Where possible, please provide a copy of any publication/s)

- Ellis MH, Silva TF, Stiller WN, Wilson LJ, Vaslin MFS, Sharman M, Llewellyn DJ (2013) Identification of a new Polerovirus (family Luteoviridae) associated with cotton bunchy top disease in Australia. Australasian Plant Pathology 42 (3):261-269.
- Sharman M, Thomas JE (2013) Genetic diversity of subgroup 1 ilarviruses from eastern Australia. Archives of Virology 158 (8):1637-1647.
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- Cotton bunchy top fact sheet co-authored with the CottonInfo team, available at: http://www.cottoninfo.com.au/publications/disease-idm-cotton-bunchy-top
- An article about the risk and management of CBTV was presented in the Winter and Spring 2011 editions of Spotlight magazine.
- Text and photos for the Cotton Symptom Guide for TSV and CBTV (Feb-2012).
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- Published article: Sharman M, Wilson L, Smith T, Grundy P, Webb M (2014). Cotton bunchy top disease and related biosecurity threats. The Australian Cottongrower, 35: 30-31.
- Contributed text and photos to the current Cotton Pest Management Guide, 2014-15.

B. Have you developed any online resources and what is the website address?

Part 4 – Final Report Executive Summary

Provide a one page Summary of your research that is not commercial in confidence, and that can be published on the World Wide Web. Explain the main outcomes of the research and provide contact details for more information. It is important that the Executive Summary highlights concisely the key outputs from the project and, when they are adopted, what this will mean to the cotton industry.

This project aimed to enhance and support the sustainability of the Australian cotton industry by: providing continued capacity in plant virology expertise and diagnostics, building industry awareness of viral disease threats, and developing contingency plans and preparedness for viral diseases that pose serious biosecurity threats to the Australian cotton industry.

Two genetically distinct strains of CBTV have been identified (CBTV-A and –B) from almost all growing regions. From a number of samples across most growing regions, there is low genetic diversity within CBTV strains –A and –B. However, these CBTV strains are about as different to each other as they are to *Cotton leafroll dwarf virus* (CLRDV – cotton blue disease). The CBTV-B strain is closely associated with typical CBT symptoms in cotton and while CBTV-A strain can infect cotton in the absence of CBTV-B, it does not appear to induce obvious symptoms. These symptom and genetic differences indicate these strains represent distinct virus species that commonly infect cotton in Australia. The development of CBTV diagnostic assays in this project has been used to greatly enhanced understanding of the diversity, distribution and importance of CBTV strains infecting cotton in Australia. This knowledge will be critical for implementing effective management strategies.

The major alternative hosts for two distinct TSV strains are Parthenium hysterophorus and Verbesina encelioides. Extensive surveillance in many production regions has shown that both TSV strains are currently restricted to the geographical range of these two weed hosts in central Queensland.

Cotton leafroll dwarf virus (CLRDV – cotton blue disease) has now been detected for the first time in Thailand and Timor Leste. This greatly extends the known global distribution of this important biosecurity threat which is now known to be relatively close to the Australian mainland. Further consideration should be given to the potential hosts and incursion pathways for this virus (this is being considered in a continuing CRDC funded project).

In collaboration with Dr Lewis Wilson and co-workers in project CRC1.1.30, a total of 15 hosts have been identified for one or both of the CBTV strains. Thirteen of these hosts were not known prior to this project. The most commonly identified hosts of CBTV in and around cotton growing areas is volunteer / ratoon cotton and *Malva parviflora* in some areas. Off-farm roadside volunteers, greater than one season old were commonly found with CBTV which indicates that they pose a significant threat as a long term source of virus (and aphids) that can move into cropping areas. This knowledge provides an opportunity to target and eliminate the source of the disease to reduce the risk of CBTV and aphid vectors persisting between seasons.

Appendix 1

CBTV-A/B multiplex-PCR (CBTVqld3780F / CBTVnsw3484F / Pol.cp3982R)

Should produce CBTV-A strain-specific band at ~ 500 bp and/or CBTV-B band at ~ 200bp.

Reverse Transcription (cDNA synthesis)

- To the tubes add:

	Vol per tube (1x)	Master mix **X**	**Check added**
Pol.cp3982R 10uM	1.0 µl		
ddH2O	3.5 µl		
Total nucleic acid extract (CTAB method)	1.5 µl		

- Heat to 80°C for 10min, chill on ice and spin down.
- From a master mix, add to tubes:

	Vol per tube (1x)	Master mix **X**	**Check added**
5×1st strand buffer	2.0 µl		
0.1M DTT	1.0 µl		
10mM dNTPs	0.5 µl		
SuperScript III 200U/µl (Invitrogen)	0.25 µl		
0.6ug/µl BSA	0.25 µl		

- Incubate at 55°C for 45min then 70°C for 10min, chill and spin down. Store on ice or at -20°C.

CBTV-A specific PCR

PCR Reaction Mix	**Vol per tube (1x)**	Master mix **X**	**Check added**
10 x PCR buffer (Invitrogen, Cat # 18038-067)	2.5 µl		
$MgCl_2$ (50 mM) (Invitrogen, Cat # 18038-067)	0.875 µl		
dNTPs (10 mM) (Invitrogen, Cat # 10297-018)	0.5 µl		
Primer: **CBTVqld3780F** (10µM)	0.3 µl		
Primer: **CBTVnsw3484F** (10µM)	0.7 µl		
Primer: **Pol.cp3982R** (10µM)	0.5 µl		
Taq (5U/µl) (Invitrogen, Cat # 18038-067)	0.2 µl		
H_2O	18.425 µl		
Template (cDNA)	1.0 µl		
Total:	**25.0 µl**		

PCR reactions were set up in 200 µl thin walled tubes
Cycling parameters: 95ºC for 1:00 min,
then 35 cycles of (95ºC for 15 sec, 62ºC for 20 sec, 57°C for 10 sec, 72ºC for 20 sec), then 1 cycle of 72ºC for 3:00 min.

Appendix 2

CLRDV specific RT-PCR (CLRDV3675F / Pol3982R)

This RT-PCR should detect both the typical and atypical strains of Cotton blue disease from Brazil, Thailand and East Timor. This PCR does not detect the two known strains of Cotton bunchy top virus (A and B) from Australia. Amplifies about half the 5' end of coat protein gene; expected size product **~307 bp**.

Reverse Transcription (cDNA synthesis)

- To the tubes add:

	Vol per tube (1x)	Master mix **X**	**Check added**
Primer: Pol3982R 10uM	1.0 µl		
ddH2O	3.5 µl		
Total nucleic acid extract (CTAB method)	1.5 µl		

- Heat to 80°C for 10min, chill on ice and spin down.
- From a master mix, add following to tubes:

	Vol per tube (1x)	Master mix **X**	**Check added**
5×1st strand buffer	2.0 µl		
0.1M DTT	1.0 µl		
10mM dNTPs	0.5 µl		
SuperScript III 200U/µl (Invitrogen)	0.25 µl		
0.6ug/µl BSA	0.25 µl		

- Incubate at 55°C for 45min then 70°C for 10min, chill and spin down. Store on ice or at -20°C.

CLRDV3675F / Pol3982R PCR

PCR Reaction Mix	**Vol per tube (1x)**	Master mix **X**	**Check added**
10 x PCR buffer (Invitrogen, Cat # 18038-067)	2.5 µl		
$MgCl_2$ (50 mM) (Invitrogen, Cat # 18038-067)	0.875 µl		
dNTPs (10 mM) (Invitrogen, Cat # 10297-018)	0.5 µl		
Primer: **CLRDV3675F** (10µM)	0.5 µl		
Primer: **Pol.cp.3982R** (10µM)	0.5 µl		
Taq (5U/µl) (Invitrogen, Cat # 18038-067)	0.2 µl		
H_2O	18.925 µl		
Template (cDNA)	1.0 µl		
Total:	**25.0 µl**		

PCR reactions in 200 µl thin walled tubes

Primers: **CLRDV3675F** (CCACGTAGRCGCAACAGGCGT)
Pol3982R (CGAGGCCTCGGAGATGAACT) – cDNA primer

Cycling parameters: 95ºC for 1:00 min, then 35 cycles of (95ºC for 15 sec, 62ºC for 20 sec, 56°C for 10 sec, 72ºC for 20 sec), then 1 cycle of 72ºC for 3:00 min.

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