Biology and chemistry of an Umbravirus like 2989 bp single stranded RNA as a possible causal agent for *Opuntia* stunting disease (engrosamiento de cladodios) - A Review.

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ABSTRACT

Perhaps the most economically important disease of *Opuntia ficus indica* fruit cacti in Mexico is the "engrosamiento de cladodios" or macho disease. The symptoms of this disease, which has been suggested to be caused by a phytoplasma, are severe stunting of cladodes, flowers and fruits. In the mid-1980s this disease appeared in commercial cactus fruit orchards of D'Arrigo Bros near Gonzalez, California. It was performed more than 30 PCR-based tests for viruses as well as various extraction methods and polymerase chain reaction (PCR) tests for phytoplasmas but were unable to find any of the known viruses or mycoplasmas in the strongly symptomatic cactus with this disease. As almost all plant viruses go through a replication phase involving double stranded RNA (dsRNA), a dsRNA extraction was performed and a dsRNA species of about 600 bp identified. Then, reverse-transcribed the dsRNA, amplified the resultant cDNA by PCR, and cloned and sequenced the 600 bp fragment that were identified in symptomatic tissue. When this sequence was compared to translated DNA in the National Center for Biotechnology Information (NCBI) nucleotide data base (BLAST analysis) it was most similar to the Tobacco bushy top virus (E score of $2e^{-39}$), which is a single stranded RNA virus with no DNA intermediate. Primers made from this 630 bp fragment were used to extend this sequence to 2989 sequence. This sequence appears to be a full-length sequence with three open reading frames (ORF) and is shorter than the closest class of viruses, the Umbraviruses that can be spread by mechanical transmission and by aphids. It was not possible to transmit the virus or symptoms mechanically. Over a six-year period using traditional PCR, this virus was found in hundreds of symptomatic cacti but not in non-symptomatic pads. RT-PCR has found low levels of this virus on non-symptomatic cladodes (3.7 fg) on a symptomatic plant and much higher concentrations (1×10^2 to 1×10^5 fg) on symptomatic cladodes from the same plant. Black bean aphids (*Aphis*

fabae), that are the vector for a closely related Umbravirus known as groundnut rosetta virus, have been routinely found on the unopened flowers of cactus. This Umbravirus was found in aphids feeding on symptomatic cladodes. As Umbraviruses cannot infect plants without a companion Luteovirus, that provides the protein coat for the Umbravirus, degenerate Luteovirus primers were used and a probable incomplete Luteovirus-like 4797 bp sequence was found on aphids feeding on symptomatic cactus. This Luteovirus was not found in *Opuntia* cladodes using PCR. A micro RNA assembly of six pooled symptomatic *Opuntias* did not find a contig that spanned the 4797 putative Luteovirus sequence, but some fragments as large as 44 bp were exact matches to the Luteovirus. As Umbraviruses occur throughout the plant but Luteoviruses only occur in the phloem, lower Luteovirus concentrations would be expected. Two successive one hour 60°C heat treatments eliminated these symptoms on new growth that was also PCR negative. A 5839 bp Potexvirus was found in some of these cladodes but its presence was not correlated with any symptoms. Similar symptomatic cacti in Italy, South Africa and Mexico should be examined with these primers and dsRNA to see if similar correlations between presence/absence of this fragment and symptomatic plants can be obtained. It is suggested that this disease be known as OSD (*Opuntia* Stunting Disease).

Keywords: Virus, aphids, Luteovirus, Potex virus, "engrosamiento", cactus.

INTRODUCTION

A disease of commercial fruit cacti (*Opuntia ficus indica*) that produces stunted cladodes and its fruit is commonly known as "engrosamiento de cladodios" (cladode swelling) or "macho" and while absent in Texas and Argentina, is present in South Africa and California and is perhaps the most serious disease of commercial fruit cactus production in Mexico (Pimienta, 1990; Perales-Segovia, 2018). Fucikovsky *et al.* (2011), have estimated that about 60% of the plants of six years of age and on, do not produce any fruits, or the fruits are very small.

We were able to transmit the symptoms of this disease by grafting, thus confirming the disease was not caused by edaphic conditions but rather a biological agent. The causal agent has been suggested to be a phytoplasma (Pimienta, 1990; Cueto, 2002; and Bertaccini *et al.*, 2007). Phytoplasmas contain small genomes (about 500 kb) (Lefting and Kirkpatrick, 2003), do not have a cell wall, only occur in the phloem, and are transmitted by leafhoppers (Lee *et al.*, 1998). Lee *et al.* (2004) have provided a comprehensive classification system for phytoplasmas based on the sequences of the 16S rRNA gene sequence.

As this disease was important in California cactus pear production, in 2004 research began at D'Arrigo Bros, in Salinas, CA to identify the causal agent for this disease. A commercial pathology laboratory (www.calspl.com) was contracted and used PCR and/or ELISA assays to examine the following 23 viruses or groups and found all of them to be negative: Arabis Mosaic, Beet curly top virus, Begomo virus group, Bromovirus group, Carlavirus virus group, Caumovirus group, Closterovirus group, Cucumber mosaic virus, Oianthovirus group, IIar virus group, Luteovirus group, Nepovirus group, Potexvirus group, Potyvirus group, Tobacco

mosaic virus, Tobacco ring spot, Tobacco streak, Tobamovirus group, Tomato ring spot, Tomato spotted wilt virus, and Tospovirus group. This laboratory also ground infected cactus tissue in buffer and mechanically inoculated three seedlings of each of the following species *Phaseolus vulgaris, Nicotiana glutinosa, Nicotiana tobaccum xanthii, Nicotiana benthamiana*, and *Cucumis Sativus* but was unable to transmit the symptoms. This lab also tested for Xyella by PCR and found that to be negative.

Some early work in Mexico suggested that this disease was caused by a phytoplasma (Pimienta, 1990; Cueto, 2002). Recently two works appeared in Mexico, Hernandez *et al.* (2009) and Fucikovsky *et al.* (2011) that used DNA extraction, nested primers and DNA sequencing that suggested that the "engrasamiento" was caused by a phytoplasma. This latter work also observed the phytoplasma in various weeds and chinch bugs (*Chelinidea* sp.) and the brown garden snail (*Helix aspersa)*. Neither chinch bugs nor snails have been found to be associated with phytoplasmas (Kirkpatrick, University California Davis, personal communication, 2012) suggesting the possibility that perhaps there were errors in the phytoplasma methodology.

Following the negative results with virus testing, various extraction techniques and sets of nested primers (two sequential PCR reactions with the second reaction using primers inside the first set to increase sensitivity and specificity) were used to test for the presence of phytoplasmas (Bertaccini *et al.*, 2007). While Bertaccini *et al.* (2007) was able to find phytoplasmas in *Opuntias* from D'Arrigo Bros plantations, despite collaboration with two phytoplasma laboratories (Kirkpatrick, UC Davis and Ing-Ming, Lee, USDA Beltsville), after several years effort it was not possible to detect phytoplasmas in symptomatic *Opuntias* in California by use of PCR with traditional and nested primers for phytoplasmas. In 2008, a search was begun to look for other causal agents.

For this reason, it was decided to reexamine the possibility that a virus was the causal agent using Valverde's (1990) innovative technique to screen for double stranded RNA in *Opuntias*. Valverde's (1990) rationale was "Single-stranded RNA viruses compose approximately 90% of all known plant viruses. During their replication in plant cells, dsRNA is produced as an intermediate product and is consistently present when a plant is infected with an ssRNA virus, regardless of the host". Thus, screening for dsRNA is a general approach to find any unknown plant virus and that by dsRNA extraction, conversion of dsRNA to complementary DNA, PCR, and sequencing, one could detect unknown viruses.

MATERIALS AND METHODS

This review along with new information on micro sequencing, is a synthetic summary of three papers published in regional symposia in Monterrey, Mexico (Felker *et al.*, 2009; Felker *et al.*, 2010; Felker *et al.*, 2012). Sections of text, tables and figures are reproduced here with the grateful permission of Professor Rigoberto Vazquez, the symposium editor.

1. Initial small-scale method dsRNA extraction of cactus

The dsRNA purification procedure was as described by Valverde *et al.* (1990). Briefly, 3.5 g of tissue was homogenized with a mortar and pestle in grinding buffer. The homogenate was extracted with a mixture of phenol, bentonite and SDS, mixed with ethanol, and bound to cellulose columns Whatman CF-11 (Whatman, Clifton, NJ), that were formed in the barrel of a 20 ml syringe. After washing to remove ssRNA and DNA from the cellulose, purified dsRNA was eluted and precipitated. The concentrated dsRNA was then analyzed by agarose gel electrophoresis. The dsRNA was prepared for reverse transcription as described (Potgieter, 2009) by ligating a specially designed, self-priming "anchor primer" (5-/5Phos/GGA TCC CGG GAA TTC GGT AAT ACG ACT CAC TAT ATT TTT ATA GTG AGT CGT ATT A-3) to the dsRNA.

The ligated dsRNA was then denatured, and cDNA was generated by reverse transcription with the Retroscript kit (Ambion, Austin, TX.) according to the manufacturer's instructions. The cDNA was subsequently amplified with the Amplitaq Gold Kit (Applied Biosystems, Foster City, CA) and the primer PC2 (5' –p-CCGAATTCCCGGGATCC-3') (Potgieter *et al.*, 2009). The reaction consisted of 5 µl cDNA template, a 200 nM concentration of primer and a reaction buffer containing 1.8 mM $MgCl_2$, 200 µM each deoxynucleoside-5'-triphosphate, and 2.5 U Amplitaq Gold DNA polymerase.

The cycling parameters for the reaction consisted of one cycle at 94°C for 5 min, one cycle at 72°C for 1 min (to fill-in incomplete cDNA ends) followed by 35 cycles of 94°C for 30 s, 67°C for 30 s, and 72°C for 4 min and a final extension step of one cycle of 72°C for 5 min.

Amplified fragments were resolved on a preparative 1% agarose gel, and the 600 bp amplicon was excised and purified by use of the QIAquick Gel Extraction Kit protocol (QIAGEN Inc., Valencia, CA), according to the manufacturer's instructions. The purified amplicon was subsequently cloned into the pCR2.1TOPO vector (Invitrogen, Carlsbad, CA) and transformed into chemically competent TOP10 cells (Invitrogen, Carlsbad, CA). Clones were screened by DNA sequence analysis performed by cycle sequencing with BigDye v3.0 chemistry (Applied Biosystems, Foster City, CA) and M13 forward and reverse oligonucleotide primers. Reactions were analyzed on an Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA), and data was assembled with the Sequencher analysis software (Gene Codes Corporation, Ann Arbor, MI).

2. Method of obtaining 5' end and 3' end virus sequence to complete an existing fragmentary sequence.

2a. Obtaining viral RNA from cactus: Approximately 100 g of cactus pad (designated AR3 WS1) chlorenchyma layer was chopped, frozen in liquid N_2, and ground into powder in a mortar. To obtain dsRNA (virus replicative form) a scaled-up modification of the method of Mo Xiaohan (personal communication) was used. Briefly, the tissue was stirred vigorously in a

mixture of extraction buffer, water-saturated phenol, chloroform, β-mercaptoethanol, and bentonite, before centrifugation. The aqueous layer was extracted again with water-saturated phenol. The aqueous layer was adjusted to 17 % Ethanol and mixed with Whatman CF-11 cellulose powder to bind dsRNA to the cellulose. The cellulose was vortexed in wash buffer (with 17% ethanol) and pelleted three times to remove undesired forms of nucleic acid. The cellulose was then poured into a column (a syringe plugged with porous Miracloth) and washed with additional wash buffer before eluting the dsRNA in elution buffer (lacking ethanol). The dsRNA was precipitated with sodium acetate and isopropanol, redissolved in TE, and treated with RNase-free DNase (Roche) to remove any remaining DNA before a second precipitation with sodium acetate and ethanol. The final product was redissolved in TE. On an agarose gel, it was found to be heavily contaminated by what appeared to be ribosomal and other ssRNA species (not shown). So RNA was size-fractioned on an agarose gel and areas representing some faint banding at about 3-3.5 kb and 6-8 kb were collected for further experimentation. The former was used for the experiment below.

2b. Amplifying virus from the prepared RNA: This is a modification of the method of Potgieter *et. al.* (2009) and utilizes the self-complimentary oligo PC3-T7loop (PC3) (5'-p-GGATCCCGGGAATTCGGTAATACGACTCACTATATTTTTATAGTGAGTCGTATTA-3') to prime the reverse-transcription of the dsRNA from its outer termini, to make cDNA copies of the full-length RNA strands. This is followed by PCR with a primer (PC2, 5'-p-CCGAATTCCCGGGATCC-3') that recognizes part of the PC3 oligo sequence, to amplify the cDNAs. Although this method alone should generate full length amplified cDNAs, it did not have satisfactory results with the procedure as originally designed, possibly because of the impurity of the dsRNA preparation.

Therefore, the following modification was examined: The 630 bp fragment of amplified viral cDNA previously obtained was used as a model for designing PCR primers specific to the sequence. These "gene specific" PCR primers were designed to extend the existing sequence in either the 5' or 3' direction, using PC2 as the 2nd primer in each case. So the Potgieter method was modified as shown in Figure 1. The modification, using a gene specific primer, occurs in step E. Note that the elements of the drawings are not drawn to scale.

   A. PC3 DNA oligos are ligated to the termini of dsRNA strands by T4 RNA Ligase.
   B. Reverse transcriptase synthesizes cDNA complimentary to the RNA strands from the folded-over 3'end of the PC3 oligo.
   C. RNAse H removes the RNA from the paired DNA-RNA strands, leaving 2 DNA strands that are complimentary to each other.
   D. The complimentary DNA strands are annealed, and in a pre-PCR step, the loop is unfolded and the single stranded ends are filled in with DNA by the thermostable DNA polymerase iProof™ (BioRad).
   E. During PCR, the gene-specific primers and PC2 prime the synthesis of new complimentary strands of DNA. Although the PC2 primers in theory could copy both stands, it was found that the reaction favored the shorter product representing the 3'

end of the original RNA as shown. A separate reaction was done to amplify the 5' end with a different gene-specific primer.

As the PCR continues, thousands of copies of the shorter product of PC2 and the gene-specific primer are made. These can be cloned and the nucleotide sequence determined.
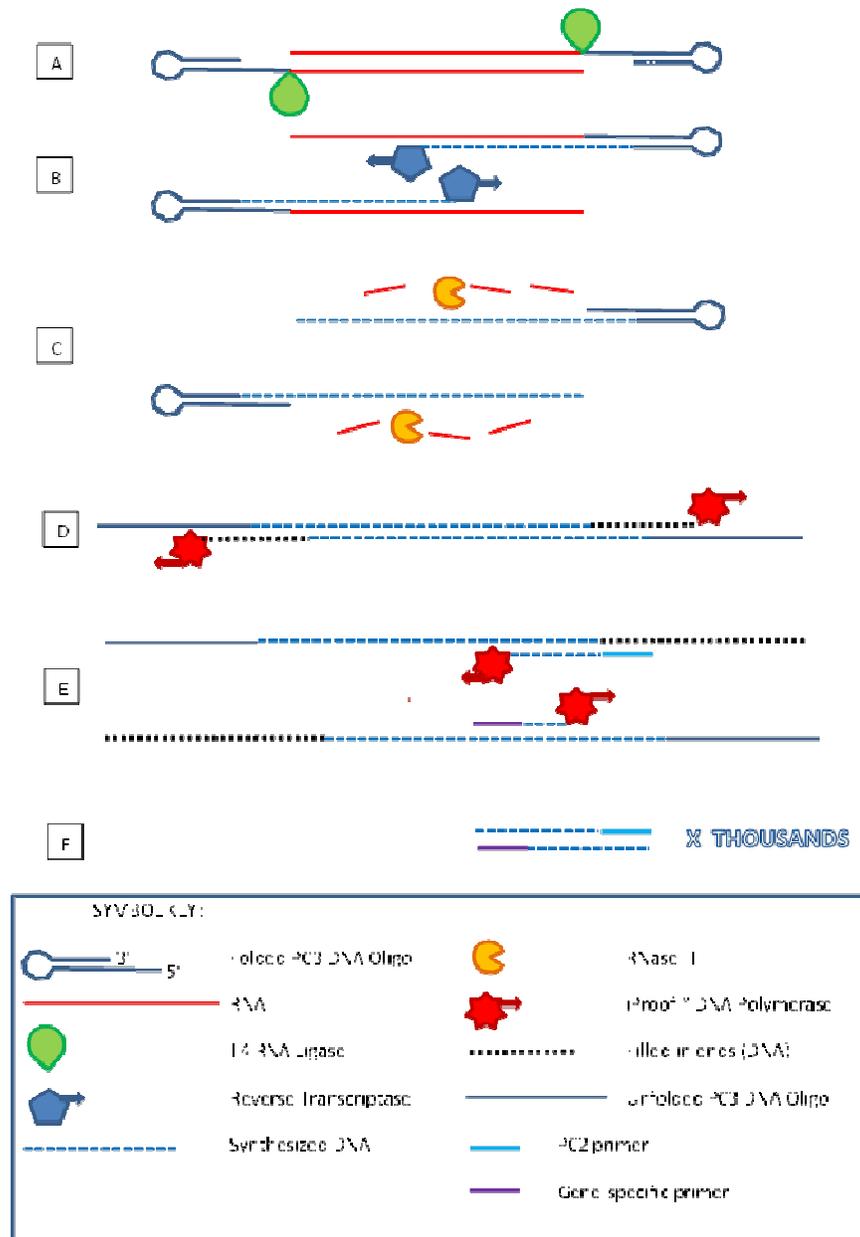
Figure 1. Diagrammatic representation of the procedure by which cDNA corresponding to viral dsRNA was obtained. Drawings are not to scale.

Once the 5' and the 3' end sequences were determined, new primers were made representing the most distal termini [not including the PC2 sequence that was still attached to the 5' and 3' amplicons (amplified DNA products)] so that the entire sequence could be amplified. Successful amplification of the entire sequence between the two termini demonstrated that the 5' and 3' end sequences actually did come from the same molecule.

The putative full-length amplicons were cloned and sequenced. A consensus sequence was produced using the sequences of three clones. It should be noted that the putative full length could be slightly shorter than true full length for at least 2 reasons: 1) some degradation of the termini of the original dsRNA could have occurred before ligating the PC3 loop, and 2) the dsRNA that was amplified might represent a sub-genomic viral fragment that includes most, but not all, of the genome.

3. Identification of Umbravirus Open Reading Frames (ORFs)
Vector NTI software (Invitrogen) was used to search the 2,989-base sequence for open reading frames (ORFs) larger than 100 codons (300 base pairs) in length. The triplets ATG and GTG were designated as start codons and the standard triplets TAA, TGA, and TAG as stop codons. Predicted ORFs were evaluated by BLAST analysis to determine their relationship to known viral sequences.

4. Field method for Umbravirus identification with RNA extraction, cDNA synthesis, primers, and PCR.

1. Take 3 cores of cactus with a 7 mm id stainless steel sharpened coring tube.
2. Cut off the green chlorenchyma with a new razor blade on a disposable paper plate, discard the white parenchyma, and insert 6 chlorenchyma sections from the 3 cores into a 2 ml centrifuge tube.
3. Use a clean 6 mm diameter plunger to compress the 6 chlorenchyma cores thus releasing internal cell contents.
4. Use a 1 ml tuberculin or insulin syringe without the needle to remove the liquid from the compressed chlorenchyma sections.
5. Transfer up to 200 µl of the viscous liquid to a new tared 2 ml centrifuge tube.
6. Put on dry ice and store or ship to laboratory.
7. Upon arrival add 1200 µl of RNaequous Lysis/Binding buffer (Ambion) containing RNAase inhibitors to 120 µl of the recently thawed extract, allow to thaw and vigorously vortex.
- Centrifuge at top speed (10,000 – 14,000 x g) for 5 min at room temperature and in general follow RNAaequous procedure at the following website. http://tools.invitrogen.com/content/sfs/manuals/cms_055306.pdf
- Use supernatant to continue with standard RNA extraction.
- Add equal volume (about 800 µl) of 64 % ethanol to each sample. Add lysate/ethanol mixture to assembled filter cartridge. A maximum of 700 µl can be loaded onto the cartridge. Up to 2 ml can be passed through without clogging (3 repeats).
- Centrifuge 15 s to 1 min at full speed, repeat, Discard flow-through.
- Apply 700 µl Wash solution1 to the cartridge.

- Centrifuge 15 s to 1 min at full speed.
- Add 500 µl Wash solution-2. Spin as above.
- Repeat with a second aliquot of 500 µl Wash solution 2.
- After discarding the wash solution, repeat spin to remove traces.
- To elute: Put the filter cartridge into a fresh collection tube.
- Pipet 50-100 µl preheated elution solution to cartridge.
- Centrifuge for 30 s at RT, 10,000-15,000 x g.
- Repeat elution procesure with the second aliquot.

8. Two step reverse transcriptase PCR protocol using Superscript TM III (this is more expensive than other reverse trancriptases but is generally acknowledged to be the most reliable RT). Preferably use 1-2 µg RNA but as little as 50-100 ng can be used. The volume can be increased but then also other reagents proportionally. Add 1 µl OligoT (50 µM) or Random decamers (50 ng/µl), or gene-specific primers (2 µM). Add 1 µl dNTP mix (Superscript kit) to total of 10 µl. Spin briefly, heat up for 5 min at 95°C, then cool on ice (4°C).

For 5 reactions the cDNA Synthesis Mix contained; 10x RT buffer-10 µl, 25 mM $MgCl_2$ -20 µl, 0.1 M DTT-10 µl, RNaseOUT (40 U/ml) 5 µl and SuperScript III RT (200 U/µl) 5 µl. The total volume was 50 µl. Add cDNA Synthesis Mix to each RNA/primer mixture. Mix gently, spin, and incubate as follows: Random hexamer primers;10 min at 25°C, followed by 50°C for 50 min. Incubate for 85°C for 5 min to inactivate RT, cool on ice 4°C. Store at -20°C or continue with PCRs, for standards introduce 3-5 µl of RT reaction.

9. Normal PCR or quantitative PCR is done on the reverse transcriptase mixture using one of the primer sets found in Table 1. The conditions for the normal PCR were: Initial heat up 2 min 95°C, 35 cycles with 30 s 95°C, 30 s 55°C, and 1 min 72°C, followed by 7 min at 72°C and extension at 4°C. Primer stock is 5 pmol/µl. 1 µl of each primer is used in a total volume of 15 µl PCR Rxn. 1-3 µl cDNA is introduced into each PCR Rxn. The reaction mix contained; 3 µl 5x Green GoTaq Reaction Buffer, 1µl 3 µM primer 1, 1µl 3 µM primer 2, 1µl 10mM dNTP, 0.2 µl 25mM $MgCl_2$, 0.15 µl Tag Polymerase (5 U/µl), 1-3 µl DNA template and H_2O to final volume of 15 µl.

The qPCR program here consists of an initial denaturation 1 min at 95°C , then 40 cycles with 15 s initial denaturation, followed by 1 min annealing/extension at 55°C or 60°C. Separate annealing and extensions are not used. PCR reactions are in 20 µl, with 1 µl cDNA, 1 µl primer/probe mix (see below) and 10 µl TaqMan GTXepress reaction mix from ABI. The rest is water. The TaqMan probe+primers are premixed to probe - 4 nMoles, primers - 8 nMoles each.

10. Take 5 µl of the PCR product and place on a 1% agarose gel with Tris Acetate EDTA buffer (TAE) for 10-15 min.

11. Take 10 µl of the PCR product and treat with ExoSAP to eliminate unincorporated primers and dNTPs.
(http://www.affymetrix.com/estore/browse/brand/usb/product.jsp?productId=131310#1_1

12. DNA sequencing was performed on a 3730 ABI machine.

Table 1. Summary of primers used for normal and qPCR for Umbravirus in *Opuntias*.

Forward 5' to 3'	Reverse 5' to 3'	Fluorescent (qPCR)	Comments	Size bp
GAT CCA ACC CAC CGA TGG AAG CCT	*CGT ACG CGT AGG AAG ATT CTT TGC AGG AG*		Original primer developed after first dsRNA was found.	623
GTG AAG GGT GTT GAG GCT GA	TTG CCC AGA GCA GTG TCC ATG		Primers changed due to improved sequence information. KEP	443
CCA ATC CCA TCT GCG TTT CT	CCG TAG CAT GCC AGT GGA A	TGC AAA TCA AAT CTG GAC GCA TCG A	qPCR. The probe is labeled on the 5' end with 6-FAM, and on the 3' end with Iowa Black Fluorescent Quencher.	
CCA ATC CCA TCT GCG TTT CT	CCG TAA CAT GCC AGT GGA A	6'FAM - TGC AAA TCG AAT CTG GAC GCA TCA A - BHQ	Reverse primer changed from Tine due to new sequence data.	66
TTAGCAAAGG ACCTCACGAA TG	CGAATGCTCTCTG GAAAGACAA		New primer developed in Open Reading Frame 2 (ORF2).	276
GCCGAACCA CTCTCTTCCT AAG			Primer used for reverse transcriptase prior to PCR using ORF2 primers	
GTGAAGGGT GTTGAGGCTG A	TTGCCCAGAGCAG TGTCCATG		More reliable primer KEP	
GGGTAATTAT GGATTTAAAT GAAACCCATG	GGGTCGCCACCG AGTCATTGGAGCA		Full length Umbravirus KEP	2989

In order to measure the quantity of virus in the plant tissue, a Taqman probe-based quantitative PCR assay was developed. The forward primer was CCAATCCCATCTGCGTTTCTC, the taqman probe was 6-Fam-TGCAAATCAAATCTGGACGCATCGA-BHQ (reverse and complement of the virus sequence) and the reverse primer was CCGTAGCATGCCAGTGGAAACA (reverse and complement of the virus sequence). In some experiments, an absolute quantification approach was employed by including a standard curve consisting of serial dilutions of a plasmid containing the cloned 630 bp virus fragment. The qPCR analyses were conducted on an Applied Biosystems 7900HT Sequence Detection System. The manufacturer's universal cycling conditions were employed.

5. Initial Isolation of virus from Aphids feeding on cactus flowers.

For the isolation of the virus from cactus flowers, on 29 August 2009, aphids were brushed off the tops of flowers from three plants (Figure 2) from Salinas, California into a test tube containing pellets of dry ice and then shipped to the University of Albany. These frozen aphids (38.8 mg total) were extracted by the RNA extraction procedure for plants, run on a gel, the 630 bp size fragments excised from the bands and sequenced.

Figure 2. Black bean aphids (*Aphis fabae)* on an unopened floral bud of *Opuntia ficus indica*. The dark shiny mature aphids that lay the young live can be seen as well as the light tan recently aphids that recently emerged from the adults. The white empty casts can be seen as well as some of the winged variants of these black bean aphids.

6. Methods for Luteovirus PCR amplification and sequence extension in aphids.

While the Umbravirus exists in the entire plant, Luteoviruses only exist in the phloem (Chomič *et al*., 2010), resulting in a much lower concentration in the entire plant and thus requiring more sensitive methods for Luteovirus detection than for Umbraviruses *i.e*. nested PCR. As aphids transmitting a Luteovirus would not have complicated mucilage that occurs in cactus and would not be dilute in the plant in only the phloem, the search for Luteovirus was

concentrated in the aphids feeding on infected cactus. To ensure there were no problems in RNA extraction, reverse transcriptase and PCR, PCR positive controls were employed. These included primers for an *Opuntia* Cinnamate 4 Hydroxylase (C4H) a widespread enzyme involved in lignin synthesis, and the constitutive Elongation Factor (ELF) in aphids. The Chomic *et al.*, 2010 degenerate primers for Luteoviruses were used. The thermocycling conditions of Chomič *et al.* (2010) of 95°C for 2 min, then 40 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 30 s, followed by 7 min at 72°C were used for all PCR reactions.

In September 2012, 7 months after aphids from symptomatic plants were brought into the greenhouse for aphid transfer experiments (see aphid transfer trials in Results section), RNA was extracted from aphids growing on young tender cactus growth ("nopalitos") that were being grown in the greenhouse. Using the Chomič *et al.*, 2010 degenerate primers C2F1 fwd x C2R1 Rev and C2F2 fwd x C2R3 rev, amplicons were observed from this extraction. Using the Umbravirus primers UORF bands, an amplicon was also observed.

After finding Luteovirus sequences from blasting these initial amplicons, 46 new primers were designed from conserved regions of the Turnip yellow virus X83110 and other closely related Luteoviruses, to obtain the full-length Luteovirus sequence. After continuing this effort, 23 primer pairs were used to cover the entire sequence.

Table 2. Primers for positive controls and degenerate primers to find Luteoviruses in symptomatic cactus and aphids growing on symptomatic cactus.

Forward 5' to 3'	Reverse 5' to 3'	Primer for Reverse transcriptase	Comments	Size bp
AAGATCTGCCAAG AGGTCAAGG	CACCATGCGT TCACCAATAT CT	5'-AGCTCAAAGTTC TGGACCAACC-3'	Cinnamate 4 Hydroxylase (C4H) gene used as positive control for PCR function in Cactus	488
TCCACCAACCTTG ACTGGTACAA or AACATGATTGAGA GGTCCACC	ACCAGGGTG GTTCATGATG AT		Elongation factor used as positive control for PCR function in insects. EF1	316
TTAATGAATACGG TCGTGGGTA	CTCTTCCCAA AATTCCGGTT		Chomič *et al.*, 2010 degenerate primer for Luteovirus LF1a	377
ACGATCAATGGAA GAAGACGACC	AAGACAATCT CGCGGGAAG TTC		Chomič *et al.*, 2010 degenerate primer for Luteovirus. Nested LF1	229

7. Methods for RNA extraction and purification for micro cDNA sequencing.

Six *Opuntias* were selected from a greenhouse plant that was the source of the Luteovirus and strongly symptomatic mature plants of four varieties on two separate D'Arrigo Bros

ranches. Stainless steel cork borers as described above were used to obtain cores from which the mucilage rich parenchyma region was eliminated with a razor blade. The chlorenchyma core sections were extracted using a Trizol-based method to avoid losses of micro RNAs that would have occurred in column purification methods. Approximately 5 µg of each RNA from each of the six sources were mixed, precipitated with ethanol, washed and stored on 75% ethanol and shipped on dry ice to the sequencing facility in Shanghai.

The micro RNA assembly used a non-filtered approach and was done in the laboratory of Cushman using Spades software that provided the most useful information. The SPAdes assembler (Bankevich *et al.*, 2012) used a set of k-mers to iterate its assembly, which was set to 15, 17, 19, 21, 25, 27, 29, 31, 33, and 35. The assembly produced a single contig fasta file containing the resulting assembly for each dataset. The assemblies were compared by performing BLAST with ViralZone in Expasy (Hulo *et al.*, 2011) to check their identity. The original reads were mapped using Bowtie2 (Langmead and Salzberg, 2012) against the assemblies and ViralZone virus reference genome to identify the coverage in order to assess the assembly qualities.

RESULTS AND DISCUSSION

1. Symptoms of pad swelling disease

As the symptoms of this "pad swelling disease" have not been described in detail, here we describe typical symptoms. The symptoms of the disease generally appear slowly over a period of months or years. A single 4 m tall and wide plant may have branches that grow normally and produce normal sized fruits as well as branches whose new vegetative and floral growth is severely stunted. Plants that have had the disease for several years have thickened, rounded cladodes and hence the common name translated into English as pad thickening disease. Normal "spineless" commercial *Opuntia* fruit types do not have 20-25 mm long spines arising from each areole as do spiny species, but they have numerous small solitary spines about 5 mm long arising from the margins and/or flat surfaces of the cladodes. In contrast infected plants often have no spines.

For many mature symptomatic plants, one side of the plant, or at least some of the cladodes, are non-symptomatic. It has been found that by propagating new plants by selective harvest of non-symptomatic cladodes, it is usually possible to obtain some plants that no longer possess symptoms of the disease. For plants with high value, such as progeny of crosses, non-symptomatic cladodes were taken from sections of the plant that were non-symptomatic and planted in a randomized complete block trial with 4 single plant replicates. After 4 years, 2 of the four plants were symptomatic and two were strongly symptomatic (Figure 3).

Figure 3. Two plants propagated from non-symptomatic cladodes from the same mature partially symptomatic plant 1-29-21 that was derived from seed. On the left is a symptomatic cladode about 1 year after planting and on the right is a non-symptomatic plant 4 years after planting.

The economic loss of this disease results from low production and stunting of the fruits. Fruits from infected plants have no edible pulp and may be 20 g or less as opposed to 120 g for the smallest commercial size class. In severely affected plants there are no fruits. If floral buds occur, they may only be 20 mm long versus 50 mm or longer for uninfected cactus. Aphid transfer experiments indicate that it is easier to get aphids established on small young buds that have a floral cap about 10 mm in diameter than larger diameter flowers. Spiders aggressively seek out aphids on infested flowers in the greenhouse and possibly could be used as a biological control agent in the field.

The disease is commonly known in Mexico as "chatilla" or "engrosamiento de cladodios" (pad swelling disease) and "macho" in California. In 18 years of research in South Texas this disease was never observed. Similarly, the disease does not occur in the Phoenix, Arizona area nor in the low elevation sites in northern Mexico such as Monterrey. The widespread problems with this disease in high elevations of Zacatecas, Mexico and cool coastal regions California, suggests that there is some high temperature limit to establishment of this disease. It is proposed that this disease be known as *Opuntia* Stunting Disease (OSD) and this designation will be used throughout the paper.

2. Greenhouse trial to transfer virus and symptoms from symptomatic to non-symptomatic potted *Opuntias* and non-cactus host plants.

In 2012 many trials were undertaken to transfer the *Opuntia* stunting disease between symptomatic and non-symptomatic plants in the greenhouse and in the field. Unopened floral caps from symptomatic cactus with aphids in the field were appressed to similar unopened

buds of non-symptomatic cactus in the field and greenhouse. In March of 2012, black bean aphids were brought from the field to inoculate the cactus and various non-cactus hosts. Plants that supported very good growth of black bean aphids included crimson clover, turnips, red beets, shepherd's purse and plants that supported little growth of aphids included cowpeas, celery, yellow beets, and Chenopodium. However, no symptoms were transferred to non-symptomatic cactus and as judged by PCR, no Umbravirus was transferred to any of the non-cactus host plants.

These trials also involved establishing alternate rows of 20 potted symptomatic and 20 non-symptomatic cladodes of two cactus varieties in the greenhouse with crimson clover (*Trifolium incarnatum* L.) also planted in the pots. In the spring and summer of 2012 many aphids, cactus and non-host plants were assayed for Luteoviruses by PCR without success. However, aphids harvested September 18, 2012 from young tender vegetative growth, known as "nopalitos" were positive for both the Umbravirus and the Luteovirus. As the lifespan of a reproductive female is about 10-20 days (Akca *et al.*, 2015), it is unlikely that the aphids harvested in September were the same aphids introduced from the field 5 months earlier, and that the Luteovirus originated from outside the greenhouse. The aphids harvested in September were the ones from which the Luteovirus sequence described below was obtained.

3. Effect of 60°C one-hour heat treatment of cladodes on OSD symptoms and presences of Umbravirus

As heat treatment is sometimes used to eliminate viruses in meristem culture, due to the heat tolerance of *Opuntias*, it was attempted to eliminate the OSD in important *Opuntia* progeny using heat treatment of cladodes. Preliminary experiments examined heat tolerance of mature cladodes at 45, 50, 55, 60 and 65°C temperatures for one hour in a forced air-drying oven and found that the 60°C - 1 h treatment caused little tissue necrosis and stimulated new shoot production.

In Figure 4, it is illustrated an example of successive heat treatment of an original cladode and a subsequent cladode. Thus, there is a correlation between disappearance of the symptoms and disappearance of the Umbravirus. In spite of the inability to infect the *Opuntias* and cause the symptoms, the elimination of both symptoms and positive PCR results, would lend support to the Umbravirus as a causal agent for the symptoms.

4. Initial Double stranded RNA extraction of symptomatic cactus.

After dsRNA extraction of symptomatic tissue, a faint band of approximately 600 bp was obtained. After reverse transcription, PCR amplification, cloning, and sequencing, it was found that this RNA fragment was 633 bp in length. When this sequence was compared to those in the National Center for Biotechnology Information (NCBI) nucleotide data base using a nucleotide query (BLASTn), a short 97 bp subfragment that was similar to a Fig Luteovirus

JJW-2008 RNA-dependent RNA polymerase (RdRp) (gb|FJ211075.1|) was found that had a score of E score of 2^{-08}). When the NCBI data base for translated nucleotides was searched after this sequence was translated to amino acids (tBLASTx) the best fit was for Tobacco bushy top virus (E score of $2e^{-39}$), a single stranded RNA virus of the Umbravirus genus with no DNA stage. The complete sequence for this Tobacco bushy top virus has been reported (Mo *et al.*, 2003) from China, where the symptoms were initially mistaken for a phytoplasma.

First this cladode was incubated for 1 hr a 60 C.

The cladodes that emerged from a second round of heat treatment of the daughter cladode were PCR negative

This lightly symptomatic cladode was taken from the cladode that was submitted to 1 hour at 60 C.

Figure 4. After one hour at 60°C the previously severely stunted pad produced a slightly symptomatic pad. When this pad matured and was subjected to a second round of one hour 60°C treatment it produced non-symptomatic "nopalitos".

5. Presence of Black bean aphids (*Aphis fabae)* on unopened cactus floral buds

Umbraviruses can be transmitted mechanically or by aphids (Taliansky and Robinson, 2003). If they are transmitted by aphids, they first must be encapsidated with a coat protein produced by a companion virus known as a Luteovirus. Umbraviruses can multiply in the plant without the Luteovirus after mechanical transmission, but they cannot be transmitted by aphids without this coat protein. The aphids for this transmission are very specific. The Tobacco bushy top virus is reported to be transmitted by the green aphid (*Myzus persicae*).

An Umbravirus that is slightly less related to the 600 bp fragment (E score of 4e^{-36}), the groundnut Rosetta virus (Taliansky *et al.*, 1996), is only transmitted by the *Aphis craccivora* known commonly as the cowpea aphid. This cowpea aphid is prevalent on winter legumes in California (http://www.ipm.ucdavis.edu/PMG/r1301511.html). These aphids have also been frequently observed on the caps of *Opuntia* flowers several days before they open (Figure 2) and on the flowers of *Opuntias* that were symptomatic for this "macho disease". Entomologists from the California Department of Food and Agriculture determined that the species of aphids on the *Opuntias* was the black bean aphid (*Aphis fabae).*

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Comparison of presence/absence of OSD symptoms and presence absence of Umbravirus before and after 1 hr -60C heat treatment.

Umbra ORF2 1 2 3 4 4

KEP463x493 1 2 3 4 4
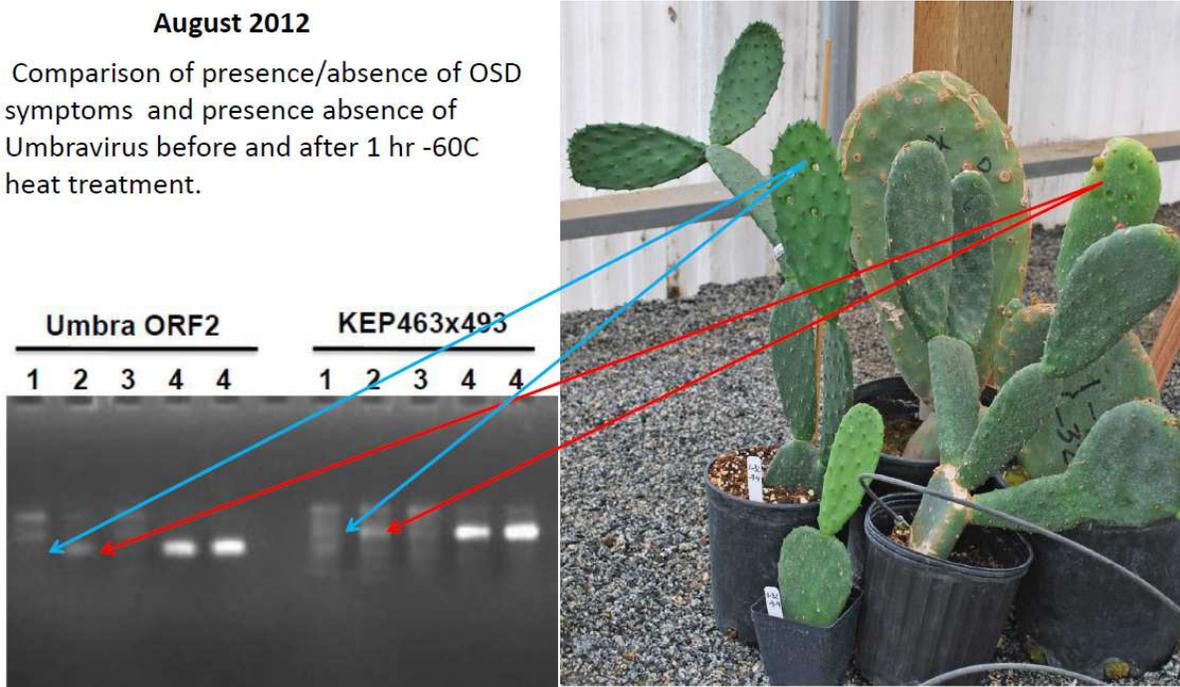
Figure 5. The stunted pad from which the red arrows originated was positive for the Umbravirus using primer ORF2 (276 bp) and KEP 463-493 primer (443 bp). A pad originating from a second round of the 60°C - 1 h treatment had healthy rapidly expanding new growth, was negative for OSD symptoms and had no positive amplicon using either of two primer pairs.

6. Measurements of the Umbravirus concentrations in field cactus with various degrees of symptoms.

Great differences in the severity of this disease were apparent from one side of the plant to the other. Rather than just rely on the presence/absence of bands on an agarose gel (which has a visual detection limit in the ng range), it was important to develop a quantitative assay for the amount of RNA present in ranges of symptomatic and non-symptomatic cladodes from the same plant. Using reverse transcriptase to convert the RNA to cDNA and then using many cycles to amplify the DNA (reverse transcriptase-quantitative PCR or RT-qPCR) and by

using serial dilutions of the cloned 630 bp virus fragment, a standard curvewas obtained that was linear from 1 ng (10^{-9}) to 10 ag (10^{-18}) with a slope: $y = -2.4193x + 37.483$ with an R^2 of 0.972, where: y = threshold cycle PCR, and x = mass in attagrams (10^{-18}).

A major problem in avoiding this disease in commercial plantings is the ability to select cladodes for propagation in which the virus is absent. This is potentially complicated by the fact that non-symptomatic cladodes could have the virus present that could later be transmitted to other non-infected plants in the field. To test this hypothesis, RT-qPCR was used to measure the amount of the virus in various cladodes of a plant in the field in which one side was non-symptomatic with normal sized cladodes and flowers, and the other side strongly symptomatic.

The virus concentrations were determined in two strongly symptomatic cladodes, two weakly symptomatic cladodes and 4 non-symptomatic cladodes as can be seen in Figure 6. Here it can be seen that the very stunted, strongly symptomatic cladodes had virus concentrations of about 10^2 fg, that the adjacent weakly symptomatic cladodes had much higher concentrations of 0.95 $\times 10^4$, 3.94 $\times 10^5$ and an adjacent non-symptomatic cladode also had high concentrations of 4.8 $\times 10^4$ fg. Two cladodes with normal sized blossoms more distant from the strongly symptomatic cladodes had very low values of 3.7 and 6.0 fg while one non-symptomatic cladode had much higher concentrations of 1.3 $\times 10^3$ fg. It is surprising that the highest virus concentration was not observed in the most strongly symptomatic tissues but rather in weakly symptomatic and non-symptomatic cladodes that were closest to the strongly symptomatic cladodes. Perhaps the strongly symptomatic cladodes have their overall physiological health sufficiently compromised that the virus cannot replicate optimally. The cladode with the largest number of normal size cactus pears and flowers had very low values of the virus. It has been frequently noted that when observing a row of cactus plants from a distance, that plants that have abundant fruits and flowers usually do not have any symptomatic cladodes, while plants that possess very few fruits on the top surfaces, often have a few symptomatic cladodes. Thus, non-symptomatic plants may be carriers for the virus and in selecting cladodes for propagation, it will be especially important to avoid any cladodes from a plant with symptomatic cladodes.

7. Full length sequence and open reading frames of the putative Umbravirus.

A contiguous 2,989 base sequence that may represent the full-length viral RNA has been found (Genbank accession MH579715). From 2011 to 2014 more than 100 experiments examined Umbravirus transmission and conditions for optimal Umbravirus detection in which primers from this 2,989 sequence were used to evaluate presence/absence of the symptomology, and in call cases, the presence of this sequence was correlated with presence of the symptoms.

In spite of the small size of the genome of plant viruses, they are able to code for more proteins than initially expected by translating multiple proteins from the same nucleotide sequence by reading various frameshifts of the same sequence. The tentative assignment of

open reading frames (ORF's) for the 2,951-base viral RNA is shown in Figure 7 along with amino acid translations of each ORF.

ORF 1, appears to be in a similar context to the ORF 1's of other Umbraviruses, based on where it starts in relation to the 5' end of the sequence, the context of the ATG (good Kozak sequence), and that this coding sequence overlaps the next large ORF, ORF 2. A second ORF, ORF 1.5, also overlaps with ORF 1 (Figure 7).
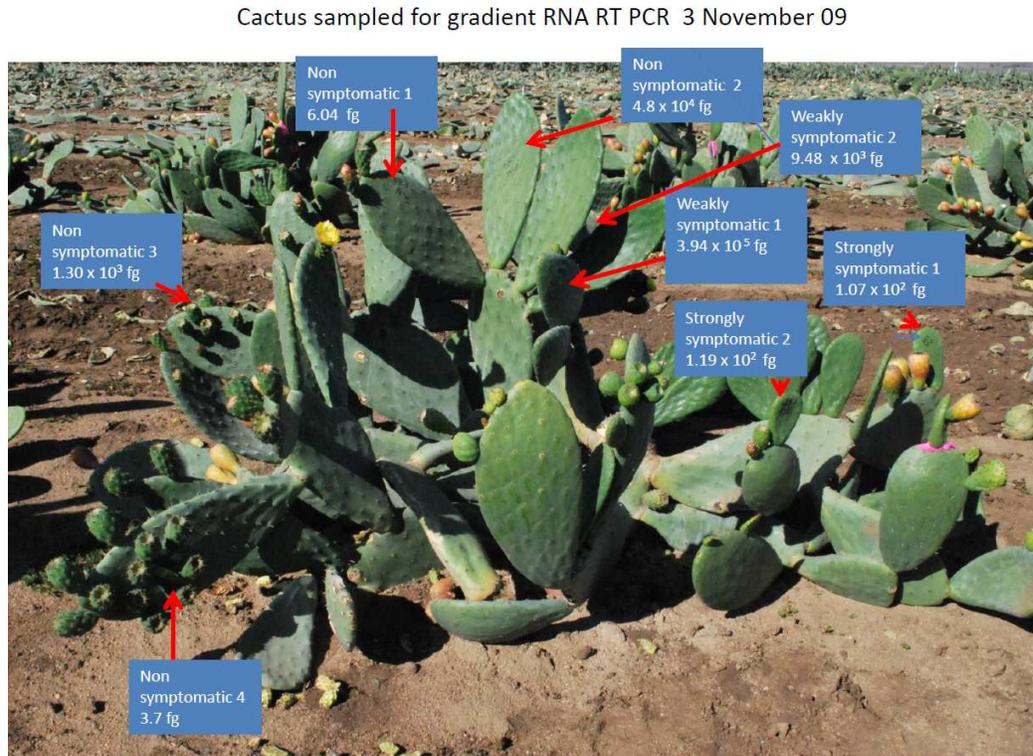
Figure 6. Concentrations of Umbravirus as determined by qPCR for cladodes of various levels of symptoms.
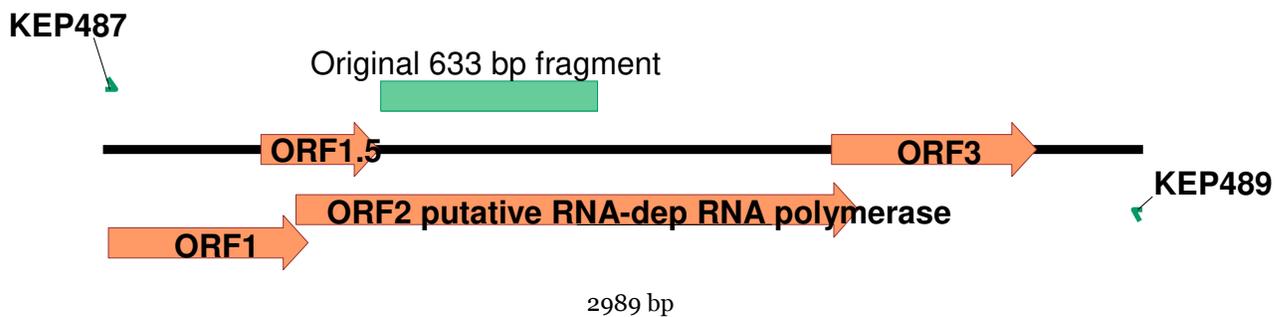
Figure 7. Tentative arrangement of Open Reading Frames (ORFs) on the Umbra-like virus sequence. ORFs larger than 100 codons are shown. KEP487 and KEP489 are the PCR primers used to amplify the 2,989 base sequence.

Translations of ORFs:

1. MRSRSRVSTPLGRMPKSRQLRRPCGLLPPSEMKRGASTRVFTVCSDWGRRVRGSGSASSC RTPTAPGSPARAKPTVFTVFVAAYSVLSIRQSRQLGQQSEACYDDTGYAIQRP

1.5. MDLNETHGYSSLWAQARKVLRAPASFTHSVVRAAHAWVRQQRSKNAIQLSEALGIILGNKVE GLLKRLPNPRRVAEKLVVSKGIDTLVDDWCSGHSNPATPEVGWALRLRDRFGLPPASEPTRL SGERWVLKQLNGVDPESWNAELRDEIAIQGIYAPGENAQIAAAAATLWLTPTFRDEAWSKHQ GFHSLQ

2. MEQAPGFSQFAVIGVDGLEARAVPLAAGLRLLPVPRPGQSRRSSQSLLLPTPCCQFVNHANS DNNLKRVMTTRVMQYKGRDPIQPTDESLRVLNQRFKRLYSSRPSTVYPLSYQGFLNCYQGR QLSRYSHAVEQLRRSALVRKDARVETFIKNEKFDWSVKGVEADPRAIQPRKPKFLAEVGRWF KPLERIIYKDLSKRLYGPEALPCIAKGFNALESGRILRAKWERFTNPICVSLDASRFDLHVSTGM LRFTHMIYNFYCKSPTLQRYLSWTLRNAGTASCKESSYAYEVKGRRMSGDMDTALGNCIIMT SLTWLMLDTLGIKHELFDNGDDCLFICEQHDVPSDQVIMKFFSDFGFVVKLEGVTSVFERIEFC QTSPVYTERGWLMCRNIKSLAKDLTNVNSCTGSAKEYTHWLRAVGKCGSVLNCGVPVFQSF HRMLERLGTNSRVERSVFWNCGLVNLIRGMKRDNDVTISTEARLSFQRAFGIDPAMQMAIEG YYDSVMGPLGKIRTSKWPIDLRKEFVLGREWFGDDVGALPL

3. MANRSAKGIRLRKRVVRRRRRRAATISTGLPMPIVPGRAAKASLVRETFIPASTTTGKEAVWAI WPINVGVFPQLVTAVSDPLYWRVTSVTIAMEPAMSTSTQHCGVGLATSGAYSSGEGFGNLFK YKRACNYTRRSPVGGNVMVKWPISMPYSLNDDAHKSTGLTAGIVIGVTNPGAITGLAWVEISL NVEYVMGT

BLAST analyses of ORFs 1 and 1.5 did not identify any highly similar known viral proteins. The best match of ORF 1 had an e value of 0.63 while the best value for ORF 1.5 had an e value of 1.1. A similar analysis of ORF 2 identified strong similarity with the RNA dependent RNA polymerases (RdRp's) for the Umbraviruses carrot mottle mimic virus (e value 1e -118) and tobacco bushy top virus (8e-115). While the similarity with Umbravirus RdRp was most strong, significant similarity to the RdRp's of other plant RNA viruses was observed (not shown). A sequence was also identified in the overlap between ORFs 1 and 2 that conforms to the consensus for a "slippery sequence" (often UUUAAAC). These sequences, which are found in Umbraviruses and other RNA viruses, mediate a frame shifting event that is necessary for the translation of RdRp's in these viruses.

Although the 5' end of this sequence generally conforms to the genomic arrangement of Umbraviruses, the 3' end of the sequence does not. In other Umbraviruses, there is a gap between the end of ORF 2 and the start of the next ORFs, and then those next ORFs (ORF 3 and ORF 4) are almost completely overlapping, but in different reading frames. In this sequence, the beginning of ORF 3 overlaps with the end of ORF 2, and there is no evidence of an overlapping ORF that would be comparable to ORF 4. A BLAST analysis of the putative ORF 3 did not identify any strongly similar proteins, but weak similarity to the coat protein of carnation mottle virus was seen (e value 0.2). This ssRNA Tombusvirus has a genome size of about 4,800 bases.

The fact that ORF 3 is most similar to a coat protein from another virus family suggests that perhaps this virus might not be an Umbravirus. The only molecular evidence to link this 2,989 sequence to the Umbraviruses is the similarity of the RdRp (ORF 2), but all ssRNA viruses would possess this gene. From the nucleotide clustal analysis, the only place where there is

really significant similarity among this 2,989 sequence and other genomes is in the RdRp (ORF 2), although it must be kept in mind that there is considerable variation even among closely related RNA viruses.

As Umbraviruses have genomes that are slightly larger than 4 kb in size, *i.e.*, carrot mottle mimic virus = 4,201 bp, (Gibbs *et al.*, 1996), pea enation mosaic virus-2 = 4,253 (Demler *et al.*, 1993), tobacco Bushy top = 4,152 (Mo *et al.*, 2003) and groundnut rosette virus = 4,019 (Taliansky *et al.*, 1996), it is less certain that it was indeed identified an Umbravirus in the cactus.

This size of the 2,951 bp fragment is similar to another type of viral RNA known as 'virus - associated RNA". Mo *et al.* (2011) purified virus particles from tobacco, then extracted the RNA and ran it on an agarose gel where 5 dsRNA bands were found. Two of the bands hybridized with probes for tobacco bushy top virus (4.2 kb) which is an Umbravirus, and tobacco vein distorting virus (6.0 kb), which is a member of the genus Polerovirus. Poleroviruses are known to act as helper viruses for Umbravirus infection by encapsidating the Umbravirus genomic RNA (in this case tobacco bushy top virus). However, these particles also contained a 2,971 bp dsRNA band that was isolated, sequenced, and found to contain two ORFs. This was designated as Tobacco bushy top disease-associated RNA.

Similar viral RNAs, *i.e.*, the beet western yellows virus ST9-associated RNA (Chin *et al.*, 1993), and carrot red leaf Luteovirus associated RNA (Watson *et al.*, 1998), and a Tobacco Bushy top associated RNA (Mo *et al.*, 2011) have been described. The clustal score for those virus combinations can be found in Table 3.

Table 3. Umbra-like viral RNA associated with OSD is the sequence described in this paper. Tobacco bushy top disease-associated virus (Mo *et al.*, 2011), Carrot red leaf virus-associated RNA (Watson *et al.*, 1998).

Name	Length	Name	Length	Clustal Score
Umbra-like viral RNA associated with OSD	2989	Tobacco bushy top disease- associated RNA	2971	40.0
Umbra-like viral RNA associated with OSD	2989	Carrot red leaf virus-associated RNA	2835	42.0
Umbra-like viral RNA associated with OSD	2989	Beet western yellows virus ST9-associated RNA	2843	43.0
Tobacco bushy top disease-associated RNA	2971	Carrot red leaf virus-associated RNA	2835	50.0
Tobacco bushy top disease-associated RNA	2971	Beet western yellows virus ST9-associated RNA	2843	58.0
Carrot red leaf virus-associated RNA	2835	Beet western yellows virus ST9-associated RNA	2843	50.0

The genomic organization of the tobacco bushy top disease associated virus was hypothesized to consist of only two ORFs, an ORF at the 5' end with unknown function and a 1,583 bp fragment for the RdRp (Mo *et al.*, 2011), which is essential for all RNA viruses.

8. Partial sequence for a Luteovirus from aphids on a symptomatic cactus.

Since Umbraviruses do not have a protein coat, and require a companion Luteovirus to encapsidate the Umbravirus genome (Falk *et al.*, 1999), various degenerate Luteovirus primers (Robertson *et al.*, 1991; Chomic *et al.*, 2010) were used to find a companion Luteovirus in cactus. Using the Chomič *et al.* (2010) degenerate primers for Luteoviruses as described in the methods on cDNA isolated from greenhouse grown aphids feeding on symptomatic cactus, a small amplicon was found and extended to 4,797 bp as seen below in Table 4 (Genbank accession MH579714).

As Luteoviruses do not infect and replicate in the vector aphids (Gray and Gildow, 2003) and as Akca *et al.* (2015) found the generation time in *Aphis fabae* to range from 19.5 days at 15°C to 10.4 days at 30°C, given the 7 months period between introduction of *A. fabae* from the field to the greenhouse and the extraction of RNA from aphids on symptomatic plants, the most likely scenario is the Luteovirus came from the symptomatic cactus.

Table 4. Sequence for Luteovirus associated with *Opuntia* Stunting Disease.

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GTTGATGCAATTTGTTGCTCACGACAACTTTCACACTCTAGAAGTAAGAAAAGTCAGGTACCTCCATTCACGAC
AAGTAACGTTTCTTTTAGCAGGTCTTTTGCTTAACATCGAACAATTCGTAAAAGCAATCAAAGAGCGCAACAAT
GAATTCAAAATTGATGTTTTTCTTCGCTCTCTGCTCTATCAGCTTCCTCTCCACCTCGGAGACCACGTCCACGA
TGACGTTAGGAAGTCTTTACTTGTCCCCGAACCAGAGCTTTGTGCCTGGTTCTCTTTACAAACGGGATATGCT
CCCGCCTCCACCTCAGGCCGTGTTAACTTATACGTGCCAGGAACCAAGACCTCTCGTAGAAGAATCATACAA
CGATCTCTTGCGAGCAATTTCTCAGAAAAGTTCAAGCGATTTCCAGAATGTTTATTCGTTGGCCTTGAACATTT
CCAGCGATTTCTGTCTATCTGGACTAGAGATGCTGAAAGACGCCTCTTTTCTGGCTGTCGAGAAATTCCTGTG
GGGTCTCACACGCTTGTGGAGCTCGCTAATCTTGGCGAGCTTCTCCGCGTTATGGTGGCTGATGAGCAATTT
CACAACTCCCGTCTTCTGTCTCGCCTTGCTGTACACTGTTACAAGATTTATGGTGAAGACGGTTTCATTTCTTT
TTGGAGGATTGCCAATCTGGATTATCTCGATTGTTTTCTCACTCCTGAAGAAATCCTTTTCAGCTCTTCGGTCT
ACACCGAAATGTTTGTATGAGAAGGCTATAGACGGTTTCAAGAGTTTCACTATCCCGCAGAGCCCCCCAAAAT
CTTGCGTGATTCCCATCACCCACGCAAGCGGAAACCACGCTGGCTACGCCAGCTGCGTTAAGCTGTACAATG
GAGAAAATGCGTTGCTGACAGCGACACACGTCTTACGTGATTGTCCCAACGCTATAGCTGTTTCCGCCAGAG
GGCTTCAAGCTCGGATTCCACTCGCTGAATTCAAAACAATCGCGAAATCCGACAAAGGTGATGTCACCCTTCT
TCGCGGCCCCCCCAACTGGGAAGGATTGTTAGGCTGCAAAGCGGCCAACGTCATTACCGCTGCCAACCTTG
CAAAATGCAAAGCAACTATTTACTCTCTTGAGAAGGACGGTTGGGCATGCGGCTACGCCGAGATAGTTGGTT
CAGATGGCACTGATGCCACGGTTCTGAGCCAGACAGAAGAAGGACACTCCGGAAGCCCCTACTTTAATGGCA
AAACCATTTTAGGGGTTCACTCAGGTGCAAGTAAGAAAAGAAATTACAATCTAATGGCACCAATCCCGTCCAT
CCCTGGGCTCACGAGCCCCACGTACGTGTTTGAGACAACCGCACCGCAAGGTCGGATTTTCGCACAAGAGG
ACATCGCCGAGATCGAGAATCTCTACTCAGAGTATAAACGATTGCAAAATTTCAAGTCCAAGACTGGAATGAC
CTGGGGCGATGTAAGCGAGGATGACGACATCTTCTTCGAAAGCAAAGAAGATTTTTCGGGAAACGGAGTGCG
CGGCGCCGACCGCGGAACAAACGGCGAAGGCAGCTCCACCCCAAAGACAAACAACGTCGATGGGAAAGAG
ATGATGGAGAAAATAATCTCGTCTCTAGTGGGAAAGATAAATCTCGAGAATATCGAGAAGAAAGTGGTCGAAC
AGATCTCCGCGAAAGCGATGAAGACACCAAGACCCAACCGCAGAAGAGCCCCAAAGAAACAGCCGGAGAGT
TCGAAAGATACTTCTCCTCACTCTACGACTGGGAGGTATCAACCTCCCCATGTGAGGTCCCCGGCTTCCGTC
ACTGCGGCAAGCTGCCCCAATACTACCACCCCAAGCAGAAGGAAGAATCACGCTGGGGGAAGACGCTCATC
GGGATCCATCCCGCATTGGGTGAGAAAACAAGCGGCTTCGGCTGGCCCAAGTTCGGCCCCGAAGCAGAACT
AAAGAGCCTGCGACTGCAGGCTTCACGGTGGCTGGAACGCGCCCAGTCCGCAGAATACCCTCTGACGCTGA
GAGGGAGCGTGTGATTCAAAAGACCGCAGATGTTTACCATCCTTGCCAAACCAATGGACCTGCGGCAACCCG
AGGAGGAACACTGACCTGGAACAACTTCATGATAGATTTCAAACAGGCCGTGTTCTCGCTAGAGTTCGATGCC
GGAATAGGCGTCCCCTATATTGCCTATGGCAAGCCCACGCACCGTGGGTGGGTAGAAGATCAGAGACTCCTC
CCAGTCCTAGCTCAGTTGACCTTCATCCGACTACAGAAGATGTTGGAGGTTAACTTCGAAGCCATGGGACCT
GAGGAGCTGATCCGGAACGGTTTGTGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
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NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNTGATCGTCCGCAACAGACTTACCATCGACCTCAACCCTGTTACAGAAAGATT
AAGATCCTGTTGGTTGAGATGTGTTTCAAACTCAGTTTTGTGTCTGAGTGATGGTACCCTTTTAGCCCAAACCT
ATCCGGGCGTTCAGAAAAGTGGGAGCTACAATACATCAAGCTCCAACTCCCGGATTAGAGTCATGGCCGCCT
TTCACACAGGTGCTGCCTGGGCTATGGCGATGGGTGATGATGCCCTCGAGTCCAATCCCGCTGACCTAGCA
GCGTATAAAAGACTAGGATTCAAGGTAGAGGTTTCCGGACAACTGGAATTCTGCTCTCACATTTTTAGAGCGC
CGGACCTCGCCCTCCCTGTGAACGAAAACAAAATGATCTACAAATTGATCTTTGGCTATGAACCAGGGAGCG
GAAACGCTGAGGTAGTTGCGAACTACTTGGCCGCTTGTTTCTCAGTTCTGAACGTGTTGCGGCACGATCCAG
CGTCCGTTGAACTTCTTTACTCGTGGTTAGTCGACCCAGTGCTACCACAAAAGATACCAGGAGAGTAAAGAAG
AGAAGAAGTTAGCCCTACATACGTAAGTTGCAAGTGAGGGAAACAAAGTCTTACACCCGAGTATAAACATAGA
TTACAAATTCCTAGCAGGCTTTTCCGCAGGATTCTTGTTGAGCATACCAGTTTCCACACTCGGATTATACATAA
TCTACCTGAGAATTTCAACCCACCTGAGATCAATTGTTAATGAATACGGTCGTGGGTAGAAGAACGATCAATG
GAAGAAGACGACCACGGAGGCAAACACGACGCGCTCAGCGCTCTCAGCCAGTGGTTGTGGTCCAAACCTCT
CGGGCAACACAACGCCGATCTAGACGACGACGAAGAGGTAATAACCGGACAAGAGGAGCTGTTCCTACCAG
AGGAGCAGGCTCAAGCGAGACATTTGTTTTCTCAAAAGACAATCTCGCGGGAAGTTCCAGCGGAGCAATCAC
GTTCGGGCCGAGTCTATCAGACTGCCCGGCATTCTCTAATGGAATACTCAAGGCCTACCATGAGTATAAAATC
TCAATGGTCATTTTGGAGTTCGTCTCCGAAGCCTCTTCCCAAAATTCCGGTTCCATCGCTTACGAGCTGGACC
CACACTGTAAACTCAACTCCCTTTCCTCAACTATCAACAAATTCGGGATCACAAAGCCCGGGAGGAGGGCGTT
TACAGCGTCTTACATCAACGGGACAGAATGGCACGATGTTGCCGAGGACCAATTCAGGATCCTCTACAAAGG
CAATGGTTCTTCATCGATAGCTGGTTCTTTCAGAATCACCATAAAGTGTCAATTCCACAACCCGAAATAGGTAG
ACGAGGAACCCGGCCCTAGCCCAGGGCCTTCTCCCTCTCCACAACCCACACCCCAAAAGAAATATCGTTTCA
TCGTCTATACTGGTGTCCCCGTGACCCGTATAATGGCTCAATCTACAGATGATGCCATCTCTTTGTACGACAT
GCCGTCTCAACGGTTTCGTTACATAGAGGACGAGAACATGAACTGGACGAACCTCGACTCTCGATGGTATTC
CCAGAATTCTTTGAAAGCCATCCCGATGATAATAGTACCAGTCCCTCAAGGTGAGTGGACTGTGGAAATTTCG
ATGGAGGGGTATCAGCCAACTTCAAGCACCACAGATCCTAATAAGGACAAACAAGATGGTCTCATTGCATACA
ATGATGACCTCAAGGAGGGTTGGAACGTGGGGGGTTTATAATAATGTGGAGATAACCAACAATAGGGCCGACA
ATACTCTCAAGTACGGCCACCCAGACATGGAACTCAATGGCTGTCATTTCAATCAAGGACAGTGTCTGGAGAG
AGATGGGGATTTGACTTGTCACATCAAAACAACTGGTGATGACGCCTCCTTCTTTATTGTTGGACCCGCCGTC
CAAAAGCAATCAAAGTATAATTACGCCGTTTCATACGGGGCTTGGACAGATCGGATGATGGAGATAGGATTGA
TAGCCATAGCACTTGGTGAACAAGGCTCATCCGGTTCCGCAAAGACAGAAAGACCAAAGAGAGCAGGGCACT
CCATGGCAGTCTCAACCTGGGAGACTA
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A BlastX search for similarities to the *Opuntia* consensus sequence found a considerable number of similar Luteoviruses, mainly related to beet, turnip and *Brassica* yellowing Poleroviruses. A phylogenic tree was calculated to show how this virus compared to other similar viruses, found that the *Brassica* yellows virus described in China was most closely related to the *Opuntia* virus (Figure 8), but many beet sequences were also closely related.

It is of interest to note that the Salinas Valley where the *Opuntias* are cultivated once had extensive areas of cultivated sugar beets in the early 20th Century and today Brassicas such as Broccoli and Broccoli rabe (*Brassica rapa*) are extensively cultivated. The disease was first noted in D'Arrigo commercial *Opuntia* fields in the 1980s and could have arisen from nearby extensive backyard *Opuntia* plantings (originally from Mexico) or perhaps from cultivated Brassicas or beets. While the sequence observed is significantly different from beets, possibly mutations in the beet RNA could have resulted in this *Opuntia* Luteovirus. As noted earlier, trials using the black bean aphid to transfer the symptoms to beets and turnips were unsuccessful. However, before this work was initiated, an extensive blow up of these symptoms only occurred in two locations of the 80-ha plantation, about 1 km apart, that were uniquely associated with a December planting of two adjacent winter legume cover crops, *i.e.* crimson clover (*Trifolium incarnatum)* and Santiago bur medic (*Medicago polymorpha* L).

Figure 8. After a Blastx was used to find similar sequences to the *Opuntia* Luteovirus consensus sequence MH579714, a phylogenic tree was built with UGENE using the PHYLIP neighbor joining method. The NCBI accession number for each virus were reported by NC004756 - Beuve and Lemaire (Unpub), AF352024- Hauser *et al.* (2002), HQ388351- Xiang *et al.* (2011), X13063- Veidt *et al.* (1988), X83110- Guilley *et al.* (1995), DQ132996- Stephan and Maiss (2006) and EF107543- Stevens and Vigano (2007).

9. Sequencing of micro RNA from symptomatic *Opuntias.*

For the 2,989 bp Umbra reported in this paper, 117 contigs were obtained covering the entire sequence, but no contigs were found for Luteovirus. The micro RNA had 123,418 reads that mapped to the Umbravirus sequence. While only 108 reads mapped to the 4,798 Luteovirus sequence above, albeit without contigs, the sequences occurred throughout the length of the Umbravirus. Some of the reads were 44 bp long and had an exact match to the Luteovirus sequence. As the Luteovirus only occurs in the phloem while the Umbravirus occur throughout the plant, a lower concentration of Luteovirus than Umbravirus would be expected. While Luteoviruses were obtained from aphids feeding on symptomatic plants, in over 2 years with many attempts, no Luteovirus sequences were found in the cactus. As such this preliminary finding of Luteovirus partial sequences in cactus vegetative tissue suggests the need for more micro RNA sequencing. However, Gray and Gildow (2003) have suggested that the inability of a virus to be detected in a recipient plant following a transmission assay may be due to a change in the ability of the virus to initiate or maintain a systemic infection.

10. Isolation of Potex-like viral RNA from plants with *Opuntia* Stunting Disease (OSD).

While searching for viruses related to OSD with dsRNA techniques, an RNA was detected that is similar to the genomes of potexviruses that have been identified in *Opuntias* (Koenig *et al.*, 2004; Sanches *et al.* 2015) and *Hylocereus* (Liou *et al.*, 2004) and may be related to the development of this disease. However, unlike the very strong correlation between OSD symptoms and the Umbravirus sequence, there is no direct correlation between this Potex virus and OSD symptoms.

As shown in Figure 9, the 5,839 bp sequence (Genbank MH579716) has high similarity to potexvirus sequences. Based on its size in relation to other potexviruses, it seems that this may represent a sequence slightly smaller than full length. Approximately five years before

the work on OSD began, it was obtained a short sequence (272 bp) from a slightly mottled *Opuntia* with completely different symptoms than OSD. Based on BLAST analysis, this sequence appears as though it may also be derived from a potexvirus. The potex-like viral RNA sequences isolated are shown in Figure 9 in green while the cactus virus X potex sequence (Koenig *et al.*, 2004) is shown in orange for comparison.

Cactus virus X AF30815   6614 bp
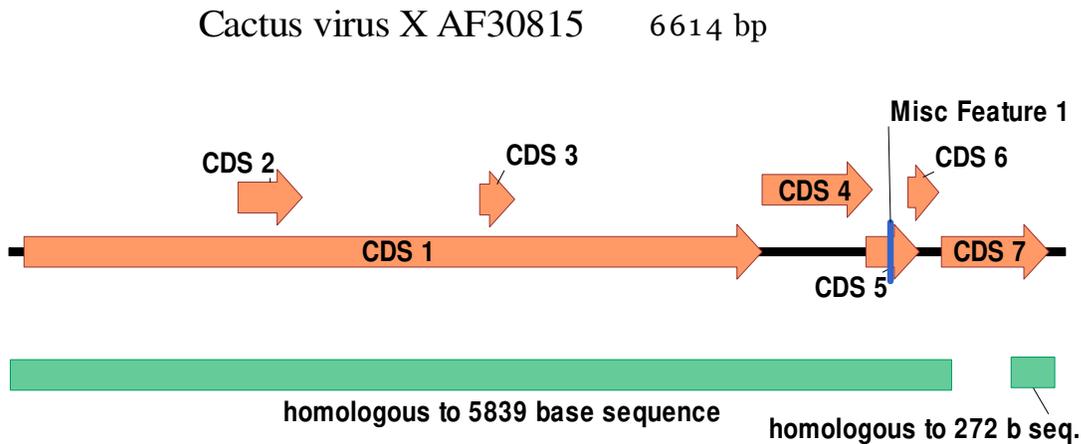
Figure 9. Nucleotide alignment of the potex cactus virus X (Koenig *et al.*, 2004) with a 5,839-base viral RNA sequence obtained from an *Opuntia* with OSD and a 272-base fragment from an *Opuntia* without OSD but with mottling.

The 5,839 base potex-like RNA that was obtained is most similar (lowest e value of 6e-179) to the sequence of the *Opuntia* X virus of Koenig *et al.* (2004) but with 69% nucleotide identity it would not appear to be an isolate of *Opuntia* X virus. The smaller 272 base fragment that was obtained previously also is most similar to the *Opuntia* X virus (lowest e value of 3e-39), and aligns with the coat protein of this virus. As this 272 bp piece has 74 % nucleotide identity with the *Opuntia* X virus, it would also not appear to be derived from an isolate of *Opuntia* X. The relatedness of the 272-base to the newly identified 5,839 base sequence is currently being evaluated. Possibly there are several Potex viruses in these plants with similar sequences or possibly there are two segments of the same sequence.

 An ORF analysis of the potex-like RNA was performed, the results of which were shown in Figure 4 along with the putative translations. The arrangement of the ORFs in this RNA is similar to the comparable region of the *Opuntia* Potex virus (shown in Figure 10). The ORF 1 sequence from the potex-like RNA is highly similar to the viral replicase of Potexviruses. This large protein has several discrete functional domains, including methyl-transferase, helicase, and polymerase domains. The sequences of ORFs 2 and 3 from the Potex-like RNA are very similar to the first two of three "triple gene block proteins" (TGBP) of Potexviruses. The first of these triple block proteins is a helicase (these viruses thus contain two proteins with putative helicase function). The Potex-like sequence that has been identified, ends after the 2nd ORF in the TGB, and thus may be incomplete.

The amino acid translation of the 272-base segment is shown in Figure 10. This translation aligned well with ORF 5 for the coat protein of *Opuntia* X virus and the Potex virus from *Hylocereus*.

ORF 1.5

ORF 3

ORF 1 Putative viral replicase

ORF 2

5839 bp
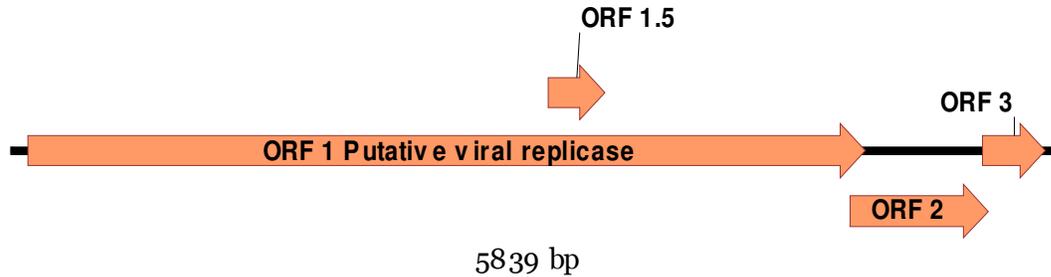
1. MARVREVMSALTDPSLKAVIQEEAYKSIKLSLEATKHTNPFAQTNESADVLENLGIMTNPLAVLPHTHAAD
   KALENDMYDVVSNYFDRSEPITFYYMKPGKLGRFRRGPQQRNYFINTIFEPKDVVRYPEATIYKSFHDQP
   CTTEKAFMGDTLHYWDPDHLAQLFMNSPKLNQLYATMVLPAEAYYKIDSLYPQIYTLKYVKEHFMYLPGG
   HAGGAYCHHRRQLGWLRIGKISRFGGRLTLTLQILESKAAHHLFLITRGDLQTPLYRTFTQSTPYVLLPRIF
   LPARYNCRTPIPLELASKMFMYCKSLGEVKMRDLYAKLRQMLPSKELVKYRGDDLVFITNYFYLISRLDSM
   TCFDQILSGNLLQKALRPVRAWFQEVAMKIFGRHEFTQLVEALQWTPIDLTFEVTIDQPSDWFTNMLNST
   FGPPLPNELNPSPKTPPGFETEDPTTEAANLFEAWDRINKIHGPPAPTTFPEEGTTTVMQEDTPSETHAE
   PAAPKELDAEATAAVQDDDEERPKPSWASEVSEEIPVQVQPEGTPASLPIKVSKPYPTEVYNAVHQLVM
   DTLPMPDKLKGRRAGLFSRNSKVKEYSYGKIKHVSSPWRDSLDQIGKMLDLDLSSFDHCLVQFFEDQALI
   PPHADNESLIQKHSRILTVSLGSALLTIQPRLSSSNAPPVSIPLEGCTVYVMEETCQDDFLHGIKALESGRL
   SLTFRRSVHFEKESFDHSLPWAAWIPILRSLGFRGNERQINPGDQSLIMPISEINNGLPKASCPTKEWTEL
   LSRLHRLPVPFHPSLERAKAFGSDIKNCRIGKLLTHQPEVWRNAFGRRTEEQPRELALSLIHGAGGSGKS
   YAIQDFLRAHPDINVTIILPTNELRLDWVSKLPATPLANIKTYEKALLSNVHEVVILDDYGKLPAGFVEALVC
   VSPALKLIIATGDSQQSVHHESNENSSIYKLPPLIQIVQPLCRYYINATHRNRKQLANMLGVYSENVGDFRI
   THGTSPIQGVHLLVPSIFKKTAFAEMGHKVSTYSGCQGITAERVQILLDQDTHLCTKQVLYTALSRAVHSI
   HFIDTGTKLNSFWEKLDATPYLKTFLRTIREEKLKEMEVVEPEIAEPAPPNTHFPVENSKNFFDNVTENMG
   EKFEREIFKADTGFSNCVQTEDQLVQMFAHQQAKDETLFWATIEARLTITNPKVNFTEFYNKKKIGEILFE
   NYKRAMQLPNDPVAFSRELWDVCADEVQKTYLSKPLHMIKNGQNRQSPDYPKNFITLFLKSQWVQKLEK
   LGLPKIKPGQTIASFHQITVMLYGTMARYLRRTRETFQPANIFINCERTPEDMSKWVREHWNFTRTSYAN
   DFTAFDQSQDGAMLQFEIMKARFFNIPEEVIEGYIDIKCNAEVFTGVLSIMRLTGEGPTFDANTECNIAFTH
   TKYNIPKECAQLFAGDDSALDFSPEVKPSFRLIETELSLTAKPVLKMQIQGGQWAEFCGMLITPLGVVKDPI
   KLWASWVRARQTGKLKEVADSYELDLCLAYQHRDLLQEVFDERQSEAHFNTVREIILYTKGRRLATYNG
1.5 MQRTATGSSWPTCWAFTLKMWEISGSPTELAQSKGCTFWSRPSSRKLRSLRWDTKCQLTQAVKASRL
   SVCKFSLTKTPTCAPNRFSTLHSPERSTQSTLLTQGPN
2. MKDKVRLTSTLCVKSSYTLKVDASPLTMDSNLALLTNLLTSKGFVRTNRPLSKPLVVHAVAGAGKSTVIRE
   FLQEVFNSSGQTLGIPDPPTLEGAYIKAATVPLSNHFNILDEYSVQPLRHSWDAVFSDPLQNPAHPVRPH
   FICTVSHRLGPEVCGLLRDLGISISSSGSRSQTVFRGGVFESELKGTLIALDEEIRDLLTAHALNPVCPHEV
   LGREFPVVTVLSSQPIPNIKDRSGLYIALTRATDELRILAPGF
3. MSSGSWPQGFRLTPPKDYTNSALALALGIGTALVIHFTRRSSLPFAGDLTHSLPHGGCYRDGTKSVTYN
   RPSTDHQPTFIISTLAILLPALIYLSSRFSSMFNSPKCAHCRAHMPN

The amino acid translation of the 272 bp segment found below aligned well with ORF 5 for the coat protein of Opuntia X virus and the Potex virus from Hylocereus.

ALQKFAAFDFFDGVLNEASLGPVDGLTRIPNEAERLANQTNRNVHLFESNAQKSRALTTSALVTKGLQGS
EPPRMQLLPSPEIISKGSRSSLPPGSNCEG

Figure 10. Tentative assignments of ORFs to the 5,839 base Potex-like RNA and translation of 272 base Potex-like fragment.

Potexviruses have interesting economic potential since they have been used for Virus Induced Gene silencing (VIGS) (Baulcombe, 1995), and as an expression vector to produce proteins in plants (Pogue *et al.*, 2002). For use as an expression vector, the gene to be produced is inserted between the last set of three proteins, known as the triple block proteins, and the virus coat protein. Whatever gene is present in this location will be produced wherever the virus is replicated in the plant. If the gene that is being produced by the virus is the same as that being transcribed by the plant, double stranded RNA will result and the plants inherent defenses will recognize this double stranded RNA and cut it into 22 bp long pieces, thus silencing expression of that gene.

Falk *et al.* (1999) found that whenever the beet western yellows virus ST9-associated RNA (Table 3) was present at the same time as the Beet western yellows virus (BWYV), the resulting Shepherd's purse plants showed severe stunting symptoms typical of the wildtype BWYV ST9 infection, and the BWYV titer was ca. 10-fold higher than in plants infected only by BWYV. Perhaps the combination of the *Opuntia* Potex-like RNA and the 2.9 kb Umbra-like RNA found here may act synergistically in the development of strong OSD symptoms.

CONCLUSIONS

The finding of the sequence of this 2,989 bp RNA fragment in Black bean aphids feeding on symptomatic cactus plants strengthens the case for these aphids being the vector of this virus. The development of reverse transcriptase quantitative PCR has observed approximately 10,000 times more virus in most strongly symptomatic *Opuntia* cladodes than the non-symptomatic ones, suggesting that this virus is responsible for, or closely associated with this disease. Detectable quantities of the Umbravirus occur in non-symptomatic cladodes on plants that also have symptomatic cladodes. This suggests the possibility that non-symptomatic cladodes might harbor latent Umbraviruses that might spread to other plants in the future. It will be important to develop tests for cladode planting stock to ensure the virus is not spread to additional blocks in the same fields or to new fields.

It was not possible to demonstrate transfer of Umbravirus symptoms or the 2.9 kb Umbravirus plasmid from 10 symptomatic cladodes growing adjacent to 10 non-symptomatic cladodes in the greenhouse with use of black bean aphids and the aphid host Crimson clover as an intercrop. It is not known if the aphids did not carry the virus from symptomatic to non-symptomatic plants or if the virus has a short lifetime in the body of the aphid. Thus, it was not possible to fulfill Koch's postulates.

A symptomatic cladode that was subjected to 60°C for one hour, potted and the new cladode was also subjected to 60°C for one hour, produced symptomless sprouts, that when repotted grew in into a 60 cm tall plant with no symptoms. Cladodes from the original symptomatic pad were positive by PCR for the Umbravirus but the non-symptomatic cladode was negative for the Umbravirus. Thus, while Koch's postulates cannot yet be verified, the correlation between elimination of the symptoms and virus with heat treatment supports a cause/effect relationship.

A Luteovirus has not been found in symptomatic cactus with use of conventional or nested primers. Perhaps this is due to the low titer since while the Umbravirus is present in the entire plant, the Luteovirus is only present in the phloem. Recently the powerful utility of deep sequencing of micro RNAs (Kreuze *et al.*, 2009; Wu *et al.*, 2010; Li *et al.*, 2012,) has shown potential to find previously unknown viruses. When this technique was used on a pool of 6 symptomatic *Opuntia*'s, contigs were not found to cover the putative Luteovirus sequence found in aphids feeding on *Opuntia*'s, but a 44 bp cDNA fragment was found which exactly matched the putative Luteovirus sequence. These results suggest the need for additional work with micro RNAs on this disease.

Molecular analyses of single stranded RNA viruses are more complicated than molecular analyses for pathogens that can be analyzed by DNA (bacteria, fungi, phytoplasmas) due to; the presence of RNAase that rapidly degrades RNA, the need to convert RNA to DNA, mucilage in the plant sample and the need to rigorously clean sample extraction devices to prevent contamination. A simple technique was developed using 7 mm stainless steel coring devices, 6 mm reusable plastic plungers, 1 ml disposable tuberculin syringes and 2 ml centrifuge tubes that permits maceration, extraction of extracts containing RNA and storage after addition of dry ice. Subsequent addition of RNA extraction buffer and PCR analysis can be conducted at any point later in time.

Using the RNA extraction described here, a comparison was made of normal PCR to qPCR with diluted Umbravirus plasmid. This experiment found that detection limit for the purified Umbravirus is about 1 fg (10^{-15} g).

A set of Umbravirus primers for routine PCR has been developed in open reading frame 2 of the Umbravirus that provides more specificity than the first primers developed from the original 630 bp fragment.

Recent reports have suggested that the Chatilla disease in Mexico is caused by a phytoplasma and loose correlations between amplification of DNA regions of phytoplasmas by PCR and symptoms have been obtained. DNA amplification using phytoplasma primers has also been obtained from snails and chinch bugs that have never been reported to harbor phytoplasmas. Amplification of DNA regions characteristic of phytoplasmas normally uses two sequential PCR amplifications (*i.e.* nested PCR) with a second set of primers inside the first set. This procedure can help with specificity. However, such incredibly sensitive amplification can result in very low level of phytoplasma contaminants being amplified yielding false positives. A highly regarded phytoplasma laboratory at the University of California Davis has not used nested PCR in 28 years due to possible contamination problems. As demonstrated in this paper, normal PCR can detect 1 fg of Umbravirus and it seems unlikely that significantly lower detection levels are required.

A high priority should be given to compare Umbravirus screening and phytoplasma screening in locations where phytoplasmas and macho symptoms are widely present. Possibly these

regions have both a phytoplasma and an Umbravirus. Identification of the causal agent is critical to identify the insect vector responsible (and thus control methods).

 A high priority should also be given to testing *Opuntia* germplasm collections and commercial nursery stock used to establish new plantations. It would be important to establish a certified source of non-infected Umbravirus and Phytoplasma *Opuntia* planting stock of current commercial varieties.

In work to find the complete sequence of the Tobacco bushy top virus, three RNA's of 4.5 kbp, 1.7 kbp and 0.9 kb were identified and sequenced. The 0.9 kb satellite-like dsRNA was the most abundant form of this virus in tobacco (Mo *et al.*, 2003). In addition to the presence of an Umbravirus, if the virus is transmitted by aphids (and not mechanically) there must be a helper Luteovirus present to encapsidate the Umbravirus with a protein coat. The 4,797 Luteovirus like sequence we obtained associated with Umbravirus is a candidate for the helping virus to provide the protein coat needed for Umbravirus infection.

The 5,839 bp Potex virus obtained has little or no association with the *Opuntia* stunting disease. However, this virus could be useful in inserting genes for transient expression (possibly to incorporate WUSCHEL and/or BABYBOOM genes to stimulate de novo shoot production from transformed callus) as described by Baulcomb *et al.* (1995).

While Suaste-Dzul *et al.* (2012) have reported new viruses and virus transmission, the symptoms after inoculation are not those of pad swelling disease and their photographs do not include cactus with "engrosamiento de cladodios".

Other works (Cueto, 2002; Bertaccini *et al.*, 2007) have reported that the causal agent for "engrosamiento de cladodios" is a phytoplasma. Perhaps in other very different environments, the causal agent is indeed a phytoplasma. It is suggested that the method described here be used to test symptomatic and non-symptomatic *Opuntia ficus indica* cladodes in other locations around the world to test for correlations with this Umbravirus RNA fragment.

In summary: (a) the presence of various portions of a 2,989 Umbra virus like sequence in virtually all of symptomatic cactus and no non symptomatic plants over a 2 year; (b) the presence of the 600 bp fragment in symptomatic pads but not in new plants propagated from a 60°C – 1 h heat treated cladode; and (c) the presence of the Umbravirus in black bean aphids feeding on symptomatic plants in the field, suggests that an Umbravirus is responsible for the "engrosamiento de cladodios" disease in California.

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