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Short communication

Citrus viroid II variants associated with 'Gummy Bark' disease

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Abstract

A viroid etiology for citrus gummy bark (CGB) disease of sweet orange is supported by the similarity of symptom expression to cachexia disease of mandarins and tangelos caused by the hop stunt viroid (HSVd) related citrus viroid II (CVd-II), as well as the detection of CVd-II variants in CGB infected Washington navel and Dörtyol sweet orange, a Turkish cultivar. A survey was made of 67 clones of CVd-II related variants recovered from severe CGB symptomatic and non-symptomatic trees of the same cultivars growing in close proximity. Only CVd-IIa, a non-cachexia inducing variant, was found in non-symptomatic Washington navel trees and no CVd-II variants were recovered from the Dörtyol control. CGB infected sources contained a number of CVd-II related variants with the predominant species detected closely related to CVd-IIc, a known cachexia inducing viroid. Biological activity of representactive variants from CGB sources was determined by transmission to citron (*Citrus medica*) as well as by bioassay on the indexing host for cachexia, Parson's Special mandarin (*Citrus reticulata*).

Citrus gummy bark (CGB) was first described in sweet orange (*Citrus sinensis*) as a disease causing phloem discoloration (Nour-Eldin, 1956, 1959). When it was later determined that gummy deposits were present on the outer layers of bark tissue, the term 'gummy bark of sweet orange' was applied (Nour-Eldin, 1968, 1980). Scrapping the bark exposes localized spots or a line of reddish-brown, gum-impregnated tissue around the scion circumference especially visible near the bud union. The discoloration and gumming may extend above the bud union to the main branches of the sweet orange while in severe infection dark streaks of gum-impregnated tissue may also be observed in longitudinal sections.

The most frequent occurrence of citrus gummy bark disease has been in North Africa and the Middle East on the cultivars Baladi, Aboussoura, Valencia, Hamlin, Washington navel, Sukkary, Khalili white, Egyptian blood and Sanguinoval (Bové, 1995). CGB has also been reported to affect Akay Sekeri, Trablus and Kozan in Pakistan. In Turkey, the cultivars Dörtyol, Kozan and Trablus and Washington navel are affected. A survey made of CGB and cachexia in the citrus growing area of the Eastern Mediterranean region of Turkey documented the occurrence of gummy bark symptoms in 28% of the sweet orange plantings which was also 'widely infected with cachexia' (Cinar et al., 1993). It is unusual to detect cachexia in sweet orange; however, a possible source for this infection might be traced to the high frequency (50%) of cachexia in Satsuma and Clementine mandarins in the same area that are grown in close proximity or interplanted with sweet orange.

The causal agent of citrus gummy bark disease has been transmitted experimentally by bud inoculation from infected trees to sweet orange; however, symptoms are visible only after an incubation period of 5–10 years (Nour-Eldin, 1968; Roistacher, 1991; Önelge et al., 1996). The etiology of citrus gummy bark disease is unknown, but viroids

have been suggested as a possible causal agent (Rostacher, 1991; Önelge et al., 1996). Symptoms of CGB bear similarities to cachexia, a disease specific to mandarins and tangelos (Childs, 1952) caused by variants of the hop stunt viroid (HSVd) related CVd-II (Semancik et al., 1988). Thus, it has been suggested that a variant of this viroid may also be either the causal agent or a factor directly involved in CGB disease expression.

Three viroids (citrus exocortis viroid [CEVd], CVd-I, CVd-III) of the five reported in citrus (Duran-Vila et al., 1988) have been found in non-symptomatic field sources of Washington navel and Dörtyol analyzed as controls for CGB sources (Önelge et al., 1996). In this study, CGB infected trees contained the complete range of citrus viroids which also included CVd-II and CVd-IV. Since CEVd, CVd-I and CVd-III were detected in non-symptomatic sources and with the infrequent occurrence of CVd-IV which has not been correlated with any citrus disease, a case can be made for a CVd-II related viroid as the most probable agent in support of a viroid etiology for citrus gummy bark disease. A survey is reported here of variants of CVd-II found in Washington navel and Dortyol sources of citrus gummy bark disease to test the validity of the postulated viroid etiology of the disease.

Field sources of 15–20 year old Washington navel and Dörtyol sweet orange on sour orange rootstocks expressing severe symptoms of citrus gummy bark were collected in the Çukurova region in Turkey. Comparable non-symptomatic trees growing in close proximity to the infected trees were employed as control materials. Budwood from these sources were graft inoculated to citron for amplification of the viroid profiles present in these materials. Viroid enriched nucleic acid preparations were obtained from citron extracts 6–12 weeks post-inoculation as previously described (Semancik et al., 1988).

Partially purified viroid preparations were amplified by RT-PCR using CVd-II specific primers complementary to residues 271–288 (IICI) and homologous to 283–302 (IIHI) (Reanwarakorn and Semancik, 1998). Purified PCR products were ligated into SP72 vector and transformed to *E. coli* DH5 α strain. Clones were initially analyzed for nucleotide differences by single-stranded conformational polymorphism (SSCP) prior to sequencing (Palacio and Duran-Vila, 1999). All clones recovered from CGB positive tissues were sequenced while only six of the 27 from control sources were analyzed since all had the same SSCP profile. A total of 46 clones were sequenced in the survey.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA software version 2.1 (Kumar et al., 2001). The minimum evolution based distance method, Neighbor Joining (NJ), was utilized due to the limited number of parsimonious informative sites of the relatively small and conserved genomes of CVd-II variants. The Jukes–Cantor estimate of the number of nucleotide substitutions per site (d) range was $0 \leq d \leq 0.049$ supporting the use of the p-distance model for the NJ method. Since the viroid sequence alignment produced a series of distinct insertion/deletion events the gap sites were included in the phylogenetic analysis using the pairwise-deletion option. Bootstrap analysis (10,000 replicates) assessed the reliability of the constructed phylogenetic tree which was rooted on the outgroup CEVd (Nei and Kumar, 2000).

Viroid cDNA clones sequenced were transcribed (MEGAscript, Ambion) as monomeric cDNA clones, inoculated to citron and subsequently analyzed for positive transmission by extraction and/or imprint hybridization 6–12 months post-inoculation (Semancik et al., 1988; Palacio-Bielsa et al., 2000). Citrons positive for CVd-II transmission were used as inoculum sources for bioassays on Parson's Special mandarin (PSM) and Washington navel. Two independent tests were preformed, in California and Turkey, on PSM with nine replicates. About 18–24 months post-inoculation bark was stripped from an area around the bud union and branching of the PSM scion for evaluation of the cachexia reaction of gumming and stem pitting. A field trial to test for the CGB reaction on Washington navel is in progress at the University of Çukurova, Adana, Turkey.

The relationships of CVd-II related clones recovered from the CGB symptomatic and control samples from both cultivars were determined by nucleotide sequence homology and biological activity in comparison with previously characterized variants. The six clones from the non-symptomatic sources were all recovered from Washington navel and found to be identical to CVd-IIa. However, no CVd-II variants were recovered from the Dörtyol control source. This

inconsistency might not be totally unexpected since the Parent Washington navel and the oldest propagations made from this source have been shown to contain only a single viroid, CVd-IIa (J.S. Semancik, unpublished). From this, all propagations from old-line sources of Washington navel made prior to the indexing for viroids initiated in the 1980s would be expected to harbour CVd-IIa. This variant does not induce any severe disease symptoms, but bark cracking on *Poncirus trifoliata* rootstock has been attributed to CVd-IIa (Roistacher et al., 1993). This condition does not result in economic damage to trees or crop but to the contrary sweet orange with CVd-IIa exhibit an enhancement in commercial performance (Semancik et al., 2002).

About 20 clones from each of the gummy bark infected cultivars were characterized by molecular properties and transmissibility (Table 1). The molecular size range (296–303 nt.) is within that known for citrus viroid II variants. Only three of the 13 clone types were common to both cultivars; however, the 296 nt. species (type A) was predominant in both cultivars representing 16/40 clones recovered.

Clones not transmissible to citron were typically single species types and comprised only about 20% of the total number recovered. Because of this low frequency and the lack of transmissibility to citron, a host known to amplify all viroids from citrus, these clones were disregarded from further consideration as relevant to CGB disease. In addition, sequence analyses of the clones non-transmissible to citron did not reveal any common nucleotide change or unusual mutations that might suggest a relationship to the lack of transmissibility. Budwood from infected citrons was employed as inoculum for cachexia bioassay on the indexing host Parson's Special mandarin (Roistacher et al., 1973). The major (16/32) clone type, A, as well as the minor (2/32) type, E, were the only variants that induced cachexia positive reactions (Table 1).

Transmissible clone types were viewed against the phylogenetic background of known CVd-II variants including the non-cachexia inducing species (CVd-IIa) and the causal agents of severe (CVd-IIb, CVd-IIc) and mild (Ca909) cachexia. The distinction in relationship between the severe cachexia and the non-cachexia variants is apparent in Figure 1.

The close relationship of clone type D with CVd-IIa might be expected assuming propagation from the old-line Parent Washington navel lineage containing CVd-IIa. The other clone types (B, C, and F) may represent selections from CVd-IIa by the cultivar Dörtyol. The majority (18/32) of the transmissible CGB clones are strongly cachexia

Table 1. Citrus viroid-II related clone types by sequence variations derived from gummy bark sources

Clone type	CGB sources		Size (nts.)	Citron transmission	PSM[c] bioassay
	WN[a]	DS[a]			
A	11[b]	5	296	Positive	++++
B	4	2	303	Positive	–
C	0	4	300	Positive	–
D	2	0	302	Positive	–
E	0	2	300	Positive	++++
F	1	1	303	Positive	–
G	0	2	298	Negative	NT[d]
H	0	1	298	Negative	NT
I	1	0	295	Negative	NT
J	1	0	299	Negative	NT
K	0	1	299	Negative	NT
L	0	1	299	Negative	NT
M	1	0	301	Negative	NT

[a] WN – Washington navel, DS-Dortyol sweet.
[b] Number of clones.
[c] Parson Special Mandarin severe (++++) or negative (–) cachexia reaction.
[d] Not tested.

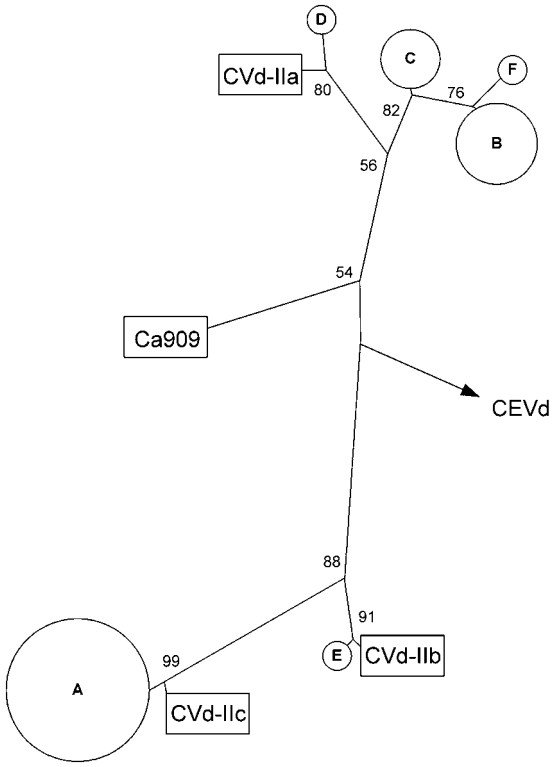

Figure 1. Phylogenetic tree representing the relationships among CVd-II variants (boxed) and related clones (circled) from citrus gummy bark (CGB) infected Washington navel and Dörtyol sweet orange. The rooted tree was constructed by genetic distances using the Neighborhood-joining method (MEGA 2.1 package). Citrus exocortis viroid (CEVd) (arrow indicates length not proportional) was used both as the root and outgroup. Numbers represent bootstrap percentage values based on 10,000 replicates. Circle diameters are directly proportional to the population of each clone type (A–F).

positive but represented by only two clone types, A with a very close relationship to CVd-IIc and E with an affinity to CVd-IIb. A clear predominance of the A type (16/18) is evident. Although a stronger indication can be drawn from the Washington navel than the Dörtyol gummy bark sources, the finding of an identical principal variant detected in both diseased cultivars is essential in support of the proposition of a viroid etiology.

A 'cachexia cassette' of 5–6 nucleotides found in the variable domain is directly related to induction of severe symptoms (Reanwarakorn and Semancik, 1998; Palacio and Duran-Vila, 2000). While CVd-IIb and CVd-IIc contain the cachexia cassette, a number of sequence distinctions are evident in the terminal (T1) and pathogenic (P) domains. However, Ca909, although accepted as a cachexia variant on the basis of a very mild reaction on the cachexia indexing host does not contain this defining cassette. Therefore, in the absence of any report of association of Ca909 with cachexia disease symptoms in the field, the question remains if the reaction on Parson's Special mandarin is sufficient to designate this variant as a 'cachexia-inducing' agent.

The very mild cachexia isolate, Ca909, is clearly defined from the non-cachexia variant but more importantly also as a distinct genetic lineage from the known severe cachexia inducing variants. This is supported by the Jukes–Cantor estimate of gene tic distance calculated between Ca909 and CVd-IIb ($d=0.021$), CVd-IIc ($d=0.031$) as well as with the non-cachexia CVd-IIa ($d=0.017$) variant. The relationship between Ca909 and CVd-IIa as determined by the estimated genetic difference is identical to the value between the two cachexia variants CV-IIb and CVd-IIc.

Specific nucleotide changes detected in the PSM positive type A and E clones from the non-cachexia variant, CVd-IIa, are compared with those of two severe and the mild cachexia variants in Table 2. The principal CGB clone type A is characterized by a striking identity to CVd-IIc and clone type E with CVd-IIb with the exception of the retention of an adenine [A] in position 153 of the T2 domain. This single change constitutes the only distinction of the PSM positive CGB clones from known severe cachexia variants. The relationship of both clone types (A and E) to the mild cachexia Ca909 is remote having in common only two nucleotides in the central conserved region (CCR) and a three nucleotide cluster in the P domain of clone type A.

The data collected in this survey indicates that no highly distinct cachexia-like variant could be identified as a specific gummy bark disease-inducing agent. However, the high frequency of detection of clone type A as the principal CVd-II related variant found in both symptomatic Washington navel and Dörtyol suggests a possible relationship with CGB. With the virtual identical genome to CVd-IIc, the gummy bark symptom may constitute an expression of cachexia disease in sweet orange. Critical to this conclusion is the verification that the clone type A does induce the characteristic symptoms under controlled field-

Table 2. Location of nucleotide changes of citrus viroid-II (CVd-II) Parson Special Mandarin (PSM) gummy bark positive variants when compared to citrus cachexia variants

CVd-II variants	CVd-IIa[a]	—	—	CVd-IIb	CVd-IIc	Ca-909
CGB clone type	—	A	E	—	—	—
PSMreaction	–	++++	++++	++++	++++	+/–
Domain	Nucleotide					
T1	26	G → A	G → AA	G → AA	G → A	
	48–49	+G			+G	
	53	–G	G → A	G → A	–G	
	56	–G			–G	
	57					–A
P	58					G → U
	245	–C			–C	–C
	246	–U	U → G	U → G	–U	–U
	247	–U			–U	–U
	250	A → G			A → G	
	256–257	+C			+C	
CCR	206–207	+C	+C	+C	+C	+C
	209	–G	–G	– G	–G	–G
	107	A → G	A → G	A → G	A → G	
	109	–A	– A	–A	–A	
V[b]	115	–A	– A	–A	–A	
	188	–U	– U	–U	–U	
	193	U → C	U → C	U → C	U → C	
	196	C → U	C → U	C → U	C → U	
T2	153			–A	–A	A → U

[a] Reanwarakorn and Semancik (1998).
[b] Nucleotide changes in variable domain associated with the 'cachexia cassette'.

testing. The required lengthy incubation period for symptom production to the resolution of this causal relationship, although problematic, is currently in progress.

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