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Full Length Research Paper

Insights on genetic diversity and phylogenetic analysis of Hop stunt viroid (HSVd) population from symptomatic citrus tree in Tunisia

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Hop stunt viroid (HSVd) variants from cachexia symptomatic citrus tree were subjected to retro-transcription and DNA amplification (RT-PCR), cloning and sequencing. Here we report genetic diversity and phylogenetic analysis of HSVd Tunisian isolate. Our study revealed obvious polymorphism within Tunisian isolates and high similarity with Japanese variants. Neighbor-joining analysis was carried out on the new HSVd-citrus sequences together with 44 previously described HSVd isolates from citrus and one from grapevine. The phylogenetic analysis showed that Tunisian isolates were clustered into 4 different groups (CVd-IIa, b, and c and grapevine group). Furthermore, the predicted secondary structure was scrutinized to be more understanding on how the nucleotide change affects variable (V) and pathogenicity domain (P).

Key words: Hop stunt viroid (HSVd), citrus, cachexia, phylogeny, secondary structure, Tunisia.

INTRODUCTION

Viroids are single-stranded, circular RNAs of 246 to 401 nucleotides (nt) that infect plants are the smallest nucleic acid-based pathogens and the smallest self replicating genetic units known. They are covalently closed molecule with a high degree of self-complementation resulting in a compact folding (Flores et al., 2005). In fact, this RNA folding represents, together with the induced symptoms in susceptible plants, one of the few identifiable phenotypes of most viroids (Sanjuàn et al., 2006). Hop stunt viroid (HSVd) (295 to 303 nucleotides) is the only member of *Hostuvioid* genus within the family *Pospiviroidae* (Flores et al., 2005) and is found in a wide range of hosts, including hop, cucumber, grapevine, citrus, plum, peach, pear (Shikata, 1990), apricot and almond (Astruc et al., 1996; Cañizares et al., 1999). Citrus host contains three type of HSVd variant (CVd-IIa, CVd-IIb, CVd-IIc) (Sano et al., 1988; Semancik et al., 1988; Levy and Hadidi, 1993).

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CVd-IIa (302 nt), is the non-pathogenic strain and only induces an extremely mild bark cracking in trifoliate orange (*Poncirus trifoliate*) rootstock under field condition (Roistacher et al., 1993). CVd-IIb (298–299 nt) and CVd-IIc (295–296 nt) are the causal agents of citrus cachexia disease and induce severe gumming, discolouration and stem-pitting symptoms on the indexing host Parson's Special mandarin (PSM) (*Citrus reticulata*) (Semancik et al., 1988; Reanwarakorn and Semancik, 1999). *Hostuviroid* adopt *in vitro* a rodlike or quasi-rodlike secondary structure of minimal energy with five structural domains: left-terminal domain (TL), pathogenicity domain (P), central conserved region (CCR), a variable domain (V) and a right terminal domain (TR). Only six nucleotides appear to determine symptom expression (Reanwarakorn and Semancik., 1998) between cachexia (CVd-IIb) and non-cachexia (CVd-IIa) strain located in variable domain (V) at the right of the C domain (A107→G, A109→Δ, A115→Δ, C197→U, U194→C, U189→Δ). The folding of RNA sequences into secondary structures is a simple but biophysically well- grounded and powerful model for studying the mapping relationships between genotype

and phenotype (Fontana, 2002). As well known, viroids should be able to manipulate many of the cellular functions in their own benefit, and they do so by acquiring a well-defined secondary structure (Sanjuàn et al., 2006). Therefore, viroids secondary structure can be taken as a proxy to study their fitness and virulence. So it is very useful to study mutation-secondary structure relationship.

Considering present research, we know that grapevine HSVd isolate can be founded in citrus hosts. The present work describes the molecular characterization of HSVd isolates from two symptomatic citrus varieties in Tunisia and provides information regarding the variability found within each isolate and population structure. The variability among isolates from USA, Spain, China, Australia and Japan was also studied.

MATERIALS AND METHODS

Plant and viroid sources

Plant material was collected in the field from two principal citrus-growing areas in Tunisia (Cap-Bon and north Tunisia). 38 citrus plants were used in this study, showing few or all symptoms of cachexia disease. Variety used in this study were: Maltaise demi-sanguine, Maltaise sanguine, Maltaise blonde, clementine, Valencia late, common mandarin, Cassar clementine, Lunari lemon, Eureka lemon, Washington navel and Citonier arbi.

Reverse transcription-polymerase chain reaction (RT-PCR) for full lentgth amplification

Total RNA was extracted following the method reported by Turturo et al. 2005 except for the silica particle absorption (providing high quality of RNA). cDNAs were synthesized by using the high-capacity cDNA Reverse Transcription kit as suggested by the supplyer (Applied Biosystems). PCR amplification reactions were carried out with the following primer pairs: HSVd-83M-Rev: 5'-AACCCGGGGCTCCTTTCTCA-3' and HSVd-78P For: 5'AACCCGGGGCAACTCTTCTC-3' specific for full-length HSVd cDNA amplification as previously described by Sano et al. (2001) using Expend high fidelity PCR System (Roche). The amplified PCR products were analysed on 1.5% agarose gels stained with ethidium bromide.

Cloning and sequencing of RT–PCR products

RT–PCR products, obtained from the full length amplification protocol using 83M or 78P primers, were purified and ligated into pGEM-T-easy vector (Promega) and recombinant plasmids from transformed cells were subjected to PCR and restriction analysis to verify the presence of an insert of the expected size. Twelve randomly selected clones from each HSVd isolate were sequenced with an ABI PRISM DNA sequencer (Perkin-Elmer, Boston, MA, USA).

Northern-blot and dot-blot hybridization of HSVd with oligodeoxyribonucleotides

Total RNA of citrus tree extracted by TNA extraction as reported by Dalmay et al., 2000 were electro transferred from the gel to positively charged Nylon membranes (Hybond-N, Amersham) at 200 mA for 1 h using modified TBE buffer (40 mM Tris, 40 mM boric acid, and 1 mM EDTA, pH 8.3). The membranes were hybridized at 68°C in DIG-hybridization buffer (Roche) with riboprobes corresponding to the full length HSVd genome RNAs of both polarities. After overnight hybridization, the membranes were washed twice with 2X SSC plus 0.1% SDS for 10 min at room temperature, once with 0.1X SSC plus 0.1% SDS at 65°C for 15 min and examined with a bio-image analyzer (Fujifilm FLA-5100).

Phylogenetic analysis and determination of secondary structures

Multiple alignment of sequences was performed using clustalW (Ver.1.74) program (Thompson et al., 1994) and nucleotide distances were estimated considering alignment gaps and using the Jukes and Cantor's method (Jukes and Cantor, 1969) for correction of superimposed substitutions with the Molecular Evolutionary Genetics Analysis (MEGA) software (Ver. 4.0) (Tamura et al., 2007). Phylogenetic relationships among HSVd sequence variants were evaluated using neighbour joining (NJ) implemented through MEGA 4.0, and bootstrap analysis (1000 replicates) was performed to assess the reliability of the constructed phylogenetic tree. Possible secondary structures were calculated with the MFOLD (Zuker, 1999) and CLC RNA Workbench package downloaded from the World Wide Web (version 4.4, http://www.clcrnaworkbench.com/).

RESULTS

HSVd detection

Of the 38 samples tested by dot-blot hybridization, 34 were positive for HSVd proving high incidence of HSVd infection in our citrus varieties. Results obtained by dot-blot hybridization were confirmed via northern-Blot and RT-PCR amplification.

Genetic diversity of HSVd within each isolates from Tunisia

In the present study, three citrus trees presented a severe and mild cachexia symptoms (citronier arbi1, citronier arbi2 and Washington navel) were chosen to investigate genetic diversity. After RT-PCR amplification and cloning, twelve clones from each isolate were randomly chosen for sequencing. A total of 36 were sequenced, the length of new HSVd variants are ranged between 295 and 301 nt, which was in agreement with previous reports on this viroid. The 12 sequence variants of Washington navel consisted of eight type of sequence variants. In the case of citronier arbi1, from 12 sequenced variants, three type of sequence was founded (HSVd1.7, HSVd1.6 and HSVd1.5); the master sequence was HSVd1.7 (54%). It should be noted that master sequence in this study signify the preponderant haplotype. Similarly, from citronier arbi2 two sequences variants are repeated (HSVd2.6 and HSVd2.7), only one was predominant (HSVd2.6). In view of this result we can conclude that genetic diversity of

Table 1. Sequence variants of Hop stunt viroid (HSVd) from *Tunisian citrus* analyzed in this work. Nucleotide differences with the closest sequences are indicated. + = insertion, = deletion.

Clone	Size(nt)	Closest HSVd variant	Nucleotide difference from closest sequence
HSVd1.5	299	AY536521	+C79 +A114 +T187
HSVd1.7	300	FJ716175	A27→G ΔA27
HSVd2.6	296	AF213349	ΔA27
HSVd2.7	297	HQ447057	C190→T
17F-HSV	298	FJ465506	A57→T ΔG58 ΔA72 T193→C G207→C A208→G G209→A C300→T
3R-HSV	297	AB211242	A57→T ΔG58 ΔA72 ΔG111
11R-HSV	295	GQ246200	A57→T ΔG58 ΔA72 ΔC105 C298→T ΔC299 ΔT300
15R-HSV	299	AF131252	ΔA71
16F-HSV	297	FJ716215	A57→T G107→A +A109 +A115 C299→T
1R-HSV	300	FJ716207	A58→T ΔG59 ΔA73 ΔT189 T194→C ΔC207 ΔG208 A209→G G210→C C244→G G245→C
4F-HSV	296	GQ246200	A57→T +C214 C299→T
5F-HSV	295	AF131252	ΔA24 G26→A ΔA71 A246→G G251→C C297→T ΔC298 ΔC298

citronier arbi2 isolate was the lowest than both previous isolates. The overall sequence homology among the 36 sequences in this study was ranged between 94% and 100%. Comparison of all sequences obtained in this work with CVd-IIa, CVd-IIb and CVd-IIc revealed twelve characteristic variations in the Tunisian isolates localized mainly in the P and V domain: ±A 27; G55→A; C107→G; G157→C; C209→G; ΔG 219; C246→G; G247→C; C249→T; ΔT 250; G259→C; ±T263. In addition, each variant were compared with the closest variant founded in Genbank (Table 1). In the light of this evaluation, we can assume that Tunisian variants have similarity with a great number of variants around the world.

Sequence polymorphism

Genetic diversity of twelve HSVd variants from this study was carried out together with the 44 reported HSVd sequence from USA, Spain, Japan, China and those previously reported in Tunisia (Table 2). All of these sequences were multiple-aligned with the CLUSTAL W program with minor manual adjustments resulting in 307 positions including the gap. A total of 49 variable sites were found in HSVd, 36 of which parsimoniously informative nucleotide sites in which more than two clones have the same mutation and 13 of which were singleton sites. Generally, singleton variants may represent true polymorphisms or may result from PCR artefact. For this reason, we used a high fidelity DNA polymerase to minimize the artefacts during PCR amplification and most of polymorphic positions found were identical to other polymorphic positions previously reported. Thus, we hypothesize that they are naturally occurring mutations. The nucleotide diversity estimated from the nucleotide distances of all HSVd population was 0.02196. Moreover, Pi (nucleotide diversity) estimated from HSVd population of this study (0.0275) was intermediate between HSVd population from Japan (0.0355) and those previously reported in Tunisia (0.0274) (Table 3). This results show that the original HSVd source recovered from field-grown contained a heterogeneous population. In the case of new Tunisian variants, the number of variable sites reached 27, with 17 parsimony informative sites. There was the highest value comparing to those from other country. The sequence variations of our HSVd variants were high with 11 haplotypes detected out of 12 analyzed sequences, yielding a haplotype diversity of 0.985. The average number of nucleotide differences (K) was estimated to be 7.80 but for the new Tunisian variants were 7.879 (Table 4). Our HSVd variant shows also the greatest value of minimum recombination, event (Rm = 4) (DnaSP 5.10 (Rozas et al., 2003). (Table 4) which explain the observed heterogeneity described previously. The selective neutrality tests show that the test of Tajima as the test of Fu and Li has negative and significant value for new Tunisian variant and when all group was taken together (Table 5), suggesting an excess of external mutations. As well, the rapid expansion of the viroid sequences was strongly supported by the negative D-value. As well known, during evolution and diversification of viroids, selection may have been driving viroid populations toward regions of sequence space, wherein neutrality is the highest possible.

Phylogenetic analysis

The sequences obtained were submitted to GenBank, and a phylogenetic analysis was carried out on the new 12 sequences variants and 44 others previously reported in Tunisia, USA, Spain, China and Japan. As the viroid sequence alignment produced a series of distinct addition or deletion events, the gap sites were included in the

Table 2. Listing and geographical location of HSVd isolates characterized in this study or available at the GenBank.

Isolate	Original host	Geographic origin	Accession numbers	References
Ca1, Ca2	Citronier Arbi	Manouba (Tunisia)	GU825979- GU825969	This work
WN	Washington navel	Cap bon (Tunisia)	HQ386721-HQ386728	This work
X-712	Salzara satsuma	Spain	AF213499- AF213496	Palacio-Bielsa et al. (2004)
X-715	Cajel sour orange	Spain	AF213500- AF213502	Palacio-Bielsa et al. (2004)
X-707	Prior Lisbon lemon	California	AF213491- AF213494	Palacio-Bielsa et al. (2004)
X-704	Old-line Navel orange	California	AF213487- AF213490	Palacio-Bielsa et al. (2004)
X-701	Cachexia 114	Corsica, France	AF213486	Palacio-Bielsa et al. (2004)
Cl	clementinier	Tunisia	AY143169- AY143170	Elleuch et al. (2006)
CA909	Citrus medica	USA	AF131252	Reanwarakorn and Semancik (1999)
Ca903	Citrus medica	USA	AF131251	Reanwarakorn and Semancik (1999)
CVd-IIa	Citrus medica	USA	AF131248	Reanwarakorn and Semancik (1999)
KS14	Ueno Wase	Japan	AB211242- AB211243	Ito et al. (2006)
CC-G	Newhall navel orange	Jiangxi or China	FJ716210- FJ716211	Wang et al. (2010)
CC-H	Newhall navel orange	Guizhou／China	FJ7162112-FJ716216	Wang et al. (2010)
CC-E	Yiyuan No. 73-6 jincheng	Sichuan／China	FJ716195	Wang et al. (2010)
CC-A	Eureka lemon	Chongqing／China	FJ716172	Wang et al. (2010)
CC-D	Ehime Kashi No. 22 mandarin	Hunan／China	FJ716188- FJ716189	Wang et al. (2010)
Tu HSVd 2-7	Thompson Seedless	China	DQ371459	Guo et al. (2006)

Table 3. Sequence polymorphism among Hop stunt viroid (HSVd) isolates from different country.

Origin	No. clones sequence	Variable sites	Parsimoniously informative nucleotide sites	Singleton sites	Nucleotide diversity
This work	12	27	17	10	0.027548
China	11	11	6	5	0.011302
Spain	17	23	14	9	0.022833
Japan	2	10	0	10	0,03550
USA	7	19	10	9	0.024788
Tunisia	4	19	8	8	0.027178
All	53	49	36	13	0.021966

phylogenetic analysis using the pairwise-deletion option. Phylogenetic analysis of all the above 44, plus CVd-IIa variant (X69518), cachexia variant (AF131249), CVd-IIc variant (AF131250) and CEVd B variant (K00965) used as outgroup, showed that the sequences were apparently

Table 4. Nucleotide diversity of *Hop stunt virioid* (HSVd) isolates from Tunisia, China, Spain, USA and Japan.

Parameter	HSVd This work	HSVd Tunisia	HSVd China	HSVd Spain	HSVd USA	HSVd Japan	All
No. of analyzed sequence	12	4	11	17	7	2	56
H	11	4	6	13	7	2	30
Pi	0,02755	0.02717	0,01146	0,02334	0,02527	0,03469	0,01956
Hd	0,985	1,000	0,800	0,963	1,000	1,000	0,964
K	7,879	7,800	3,345	6,507	7,238	10,000	5,169
Rm	4 (58/111, 196/199, 199/254, 254/305)	1 (209/303)	0	1 (54/195)	2 (26/108, 197/208)	0	3 (59/109 196/199, ,199/220)

H = number of haplotypes; Pi (JC) = nucleotide diversity; Hd = haplotype diversity; K = average number of nucleotide differences; Rm = minimum recombination events.

Table 5. Results of neutrality test for each HSVd population. Asterisks indicate that the test of Tajima and those of Fu and Li were negative and significant when all groups were considered.

Test name	HSVd This work	HSVd Tunisia	HSVd China	HSVd Spain	HSVd USA	HSVd Japan	All
Tajma's D	-0,37903 P > 0.10	-0,32423 P > 0.10	-0,08568 P > 0.10	-0,00007 P > 0.10	0,24019	-	-1,02184 P > 0.10
Fu and Li's D	-0,27088 P > 0.10	0,03603 P > 0.10	-0,09386 P > 0.10	-0,47788 P > 0.10	0,21786	-	-1,09185* P > 0.10
Fu and Li's F	-0,34074 P > 0.10	-0,03848 P > 0.10	-0,10378 P > 0.10	-0,39544 P > 0.10	0,24543	-	-1,27080 * P > 0.10
Fu's F	-3,665	-0,678	-0,267	-3,806	-2,182	-	-16,839*

grouped into several clusters (Figure 1). Four of the 12 cDNA clones from Tunisia were included in the CVd-IIb group, each variant were clustered into an independent clade even though the bootstrap percentage value was below 63%, confirming their sequence differentiation with reported sequences, whereas the 4 variants had greater homology with X-715-2 isolate (AF213502) which presents the typical V domain of cachexia variants. Interestingly, one clone (HSVd1.7) had 100% identity compared with cachexia variant from Japan (AB211242). In addition, the same variant was recently reported in China (FJ716175) (Wang et al., 2010), Iran (GQ246200) and Spain.

A second cluster, formed by CVd-IIa group, contained 2 cDNA clones from Washington navel isolate, two clementine variant previously reported by Elleuch et al. (2006) and two other variants recently reported by Wang et al. (2010). Four cDNA clones (17F-HSV, 16F-HSV, HSVd1.5 and HSVd2.6) clustered in CVd-IIc group. Our results show an equal distribution of variants to the three group. Surprisingly, three HSVd types three

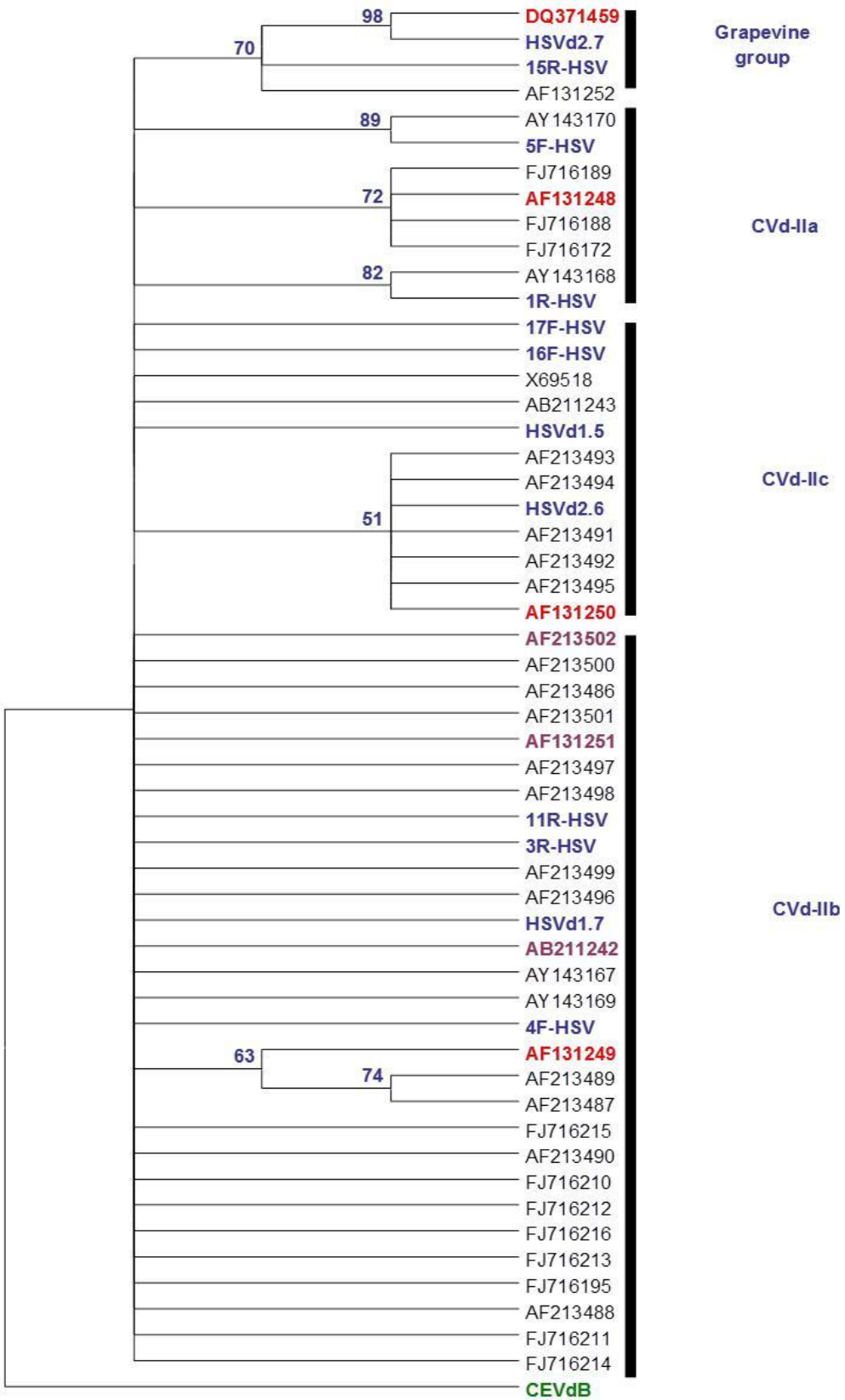

Figure 1. Neighbour-joining tree of all Hop stunt viroid (HSVd) cDNA clones. All HSVd sequences were divided into four main clusters CVd-IIa, CVd-IIb and CVd-IIc and a grapevine group. CEVd was used as outgroup. Numbers represent bootstrap percentage values based on 1000 replicates and values below 70 were collapsed.

groups. Surprisingly, three HSVd types of sequence variants such as: CVd-IIa, CVd-IIb and CVd-IIc have been detected in Washington navel isolate, demonstrating a complex infection involving three sequence variants in natural infections. Finally, two variants belonging to "Citronier arbi 2" and "Washington navel" were clustered in Grapevine group. It is worth noting that the sequence of HSVd2.7 and DQ371459 forming a distinct sub-cluster with 98% of identity, the second sequence was founded in grapevine of 100 year old in China. Our results support previous study suggesting the possible grapevine origin of citrus viroid (Sano et al., 2001). To summarize, phylogenetic analysis showed that new Tunisian HSVd variants was heteregeneous which mean a high level of polymorphism and a clear similarity with Japanese and Chinese variants was observed.

Genetic diversity on proposed secondary structure

Like all members of *Pospiviroidae,* HSVd viroid adopts in vitro a rod-like or quasi-rodlike secondary structure of minimal energy with 5 structural domains (CCR, TL, TR, V, and P). The predicted secondary structures of minimum free energy were determined by CLC RNA Workbench package downloaded from the World Wide Web (version 4.4, http:/www.clcrnaworkbench.com/). All "master sequences" appeared to fold into a rod-like structure with base pairing ranging from 66 to 71.8%. However, the minimum free energies of the secondary structure for the new Tunisian cachexia isolates are always higher (ranging from -121.2 to -125.9 Kcal/mol) than those of non-cachexia isolates (ranging from -105.5 to -118.0 Kcal/mol), indicating a lower stability of the rod-like conformation of cachexia sequences. Our results are in agreement with the previous work on HSVd citrus isolates by Palacio-Bielsa et al., (2004). The base pairing of V domain which contains five discriminating nucleotides from cachexia and non-cachexia, presents structural differences that may affect their geometry and stability. In majority of cases, the secondary structure analysis of the V domain shows two loops separated by a short helical region (Figure 2). In the cachexia structure the two loops contain a single mismatched nucleotide, while one of the loops of the non-cachexia structures containing two mismatched nucleotides. In Figure 2, some variant clustered by phylogenetic study in CVd-IIa and grapevine group shows a typical structure of cachexia V domain. We observe that some base changes does not affect secondary structure, the two single mismatched nucleotide was preserved. *In silico* analysis on the predicted secondary structures of the non-cachexia variants suggested that some of the sequence variations have influence on the structure but we note also the persistence of the two characteristic loops of V domain. HSVd1.5 variant show a different secondary structure from the other with a big terminal loop, furthermore HSVd2.7 identical to grapevine variant founded in china have a bifurcation at the end of the secondary structure. Cruciform structures were detected, for the variants clustered into the two pathogenic groups (CVd-IIb and CVd-IIc).

DISCUSSION

Genetic structure of HSVd populations must always be characterized to understand the way of their evolution. Therefore, it is essential to control cachexia and cachexia-associated HSVd variants and eventually their transmission and expression on sensitive and resistant hosts. Here, twelve new variants from three citrus tree, two of them present severe symptoms of cachexia (Ca1 and Ca2), and the third (Washington navel) have mild symptoms. Our study suggests that Tunisian HSVd variants was very heterogeneous, with 12 new mutations and a high value of minimum recombination event (Table 4), this is the highest value than those obtained from the other countries around the world. As assumed previously by Amari et al., (2001), recombination events are more frequent on HSVd and intra-specific recombination could be a general mechanism in the evolution of viroids (Candresse et al., 1997).

As stated above, it has been proposed that the five nucleotide motif in V domain was responsible for discriminating pathogenic and non-pathogenic HSVd isolates (Palacio-Bielsa and Duran-Vila, 2000; Palacio-Bielsa et al., 2004). Recently, mutagenesis experiments provided the evidence of this observation and confirm that the "cachexia expression motif" plays a major role in inciting cachexia symptoms, and one base change within this motif affect symptom severity and may even suppress symptom expression (Serra et al., 2008). The U197→C transition responsible for the shift from severe to mild symptoms (Ragozzino et al., 2004; Ito et al., 2006) is also present in HSVd2.6 variant. The mild symptoms observed on Washington navel tree, can be explained by the presence of heterogeneous haplotype. Phylogenetic analysis shows that 5R-HSV and 1R-HSV were clustered into CVd-IIa group, 17F-HSV and 16F-HSV were clustered into CVd-IIc group, 4F-HSV, 3R-HSV and 11R-HSV were clustered into CVd-IIb. Moreover, previous study shows that effect of cachexia variants on indexing hosts such as PSM can be seriously moderated by the presence of a mixed infection. Thus, the severe reaction induced by CVd-IIb was markedly reduced by "viroid interference" in the presence of CVd-IIa (Pina et al., 1991; Semancik et al., 1992). So, our study strongly supports this assumption. In the other hand, some of the reactions previously attributed to cachexia isolates may have been the result of using poorly characterized graft-transmissible agents containing a mixture of variants (Reanwarakorn and Semancik, 1999). In the case of citronier arbi 2, presence of grapevine strain in citrus tree support a previous hypothesis suggesting that US citrus cachexia type isolates could be recombinants between HSVd

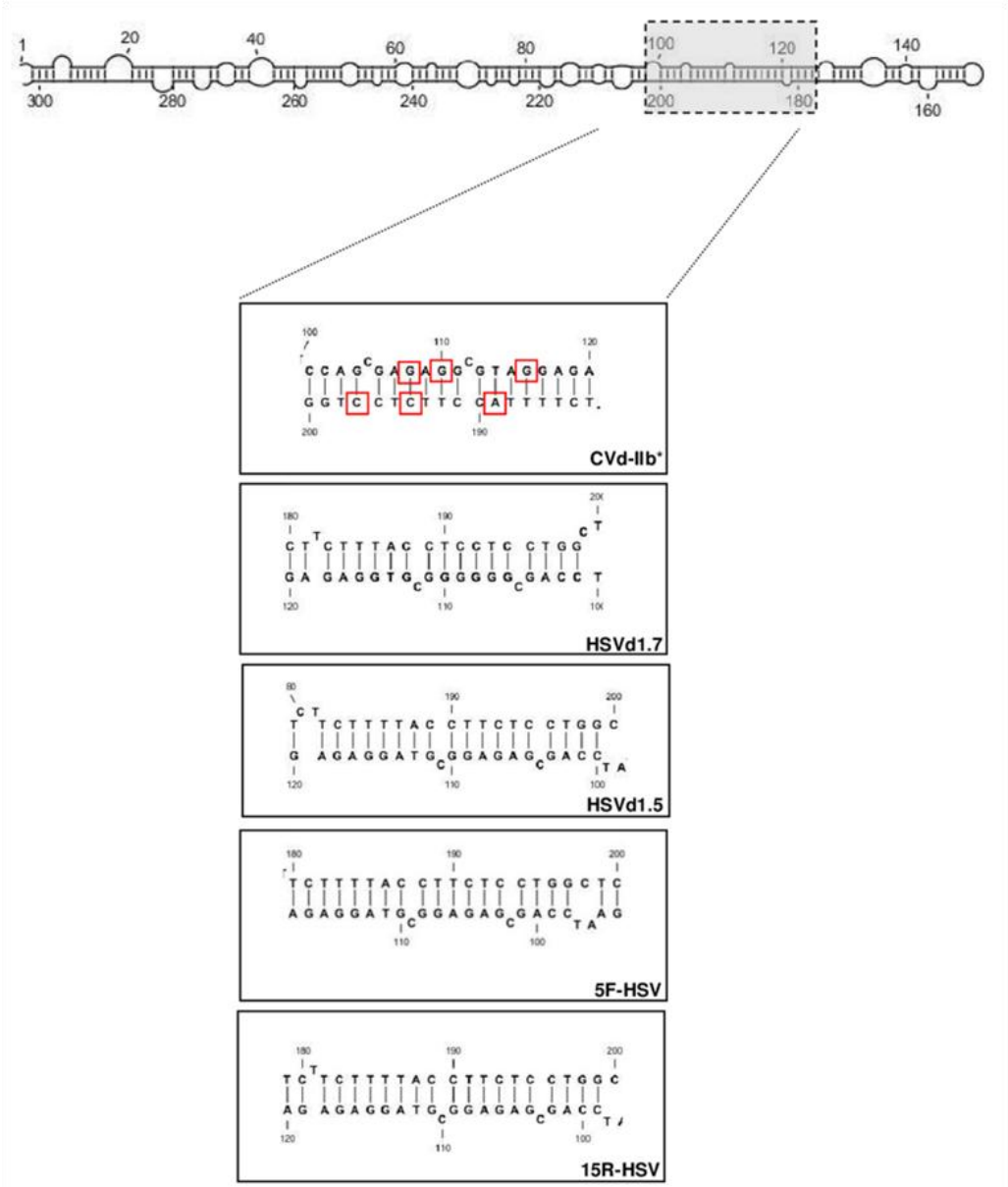

Figure 2. Predicted secondary structure of HSVd1.7 variant. Variable domain (V) of cachexia, compared to those obtained in this work. Characteristic nucleotide changes of the "cachexia expression motif" are boxed in red. Samples with asterisk (*) indicate those obtained from Genbank.

grapevine and citrus-cucumber group (Sano et al., 2001). Presence of grapevine strain in citrus is perhaps caused by the close proximity of citrus and grapevine crop growing, emphasizing the need to control the sanitary status of grapevine even though HSVd is considered to be latent in this crop. Recently, Kawaguchi-Ito et al. (2009) identified that cultivated grapevines as a symptomless reservoir in which HSVd can evolve and be transmitted to hop crops to cause epidemics. Surprisingly, HSVd1.7 variant have a great homology with the cachexia variant from Japan (AB211242) (Ito at al., 2006) and reported recently in Tunisian pomegranate (Gorsane et al., 2010). This is the first report of cachexia strain in fruit tree except citrus. More, phylogenetic analysis shows a great homology with Japanese and Chinese HSVd variant, this result could be explained by the frequent

plant exchange between those countries or alternatively, by a parallel evolution of the viroid molecule.

As can be seen, when all the 56 variants were compared, most of the variability is located on the pathogenic (P) and variable (V) domains. On the other hand, both TR and TL domains are regions of very low or no variability, respectively, which suggests that they have a key role in the viroid life-cycle; thus, our results are in agreement with those previously reported. In the case of type member of *Pospiviroidae* (PSTVd), three nucleotide substitutions in the left terminal loop of the TL domain of resulted in absence of systemic infection (Hu et al., 1997). Nevertheless, Reanwarakorn and Semancik (1999) suggest that nucleotide sequence is not the sole factor regulating the expression of biological activity of viroids by conformational homology derived from dissimilar nucleotide sequences. Therefore, RNA folding represents an invaluable tool for studying the evolution of robustness (Fontana, 2002): folding can be seen as a phenotype encoded by an RNA sequence and the thermodynamics rules governing folding as the genotype-to-phenotype map. However, viroid fitness critically depends on the right folding of their genomes. In fact, natural variation usually involves compensatory changes that preserve the secondary structure; examples are abundant (reviewed in Diener, 2001). In addition, site-directed mutagenesis experiments with PSTVd have shown that only double mutants in the hairpin II (important for replication) that did not alter the native rod-like structure were viable and stably recovered after several passages in tomato plants (Qu et al., 1993). By contrast, single mutants were either nonviable or reverted to the wild-type sequence (Qu et al., 1993). In our study, HSVd1.5 is the only variant showing a co-variation in the V domain. Our new sequences show a very stable rod-like secondary structure, the cachexia sequence shows as reported above by Amari et al., 2001 a cruciform structure with high level of energy that probably still unstable.

In conclusion, incidence of HSVd infection in citrus tree in Tunisia appears very important. By this study, we wished-for investigate HSVd population from field grown citrus presenting cachexia symptoms. In the present work, an HSVd isolate recovered from Washington navel (mild cachexia symptoms) was characterized as a heterogeneous population which did not contain a predominant haplotype and may act as reservoirs to infect other host. In the case of citronier arbi1 and citronier arbi2 (presenting severe cachexia symptoms), HSVd populations appear as very homogeneous with one predominant haplotype. So, CEVd and CVd-V was founded in citronier arbi2 explaining the severity of symptoms. However, predominant variant in this isolate was clustered in grapevine group. New Tunisian variant was very heterogeneous, an excess of singleton was supported by negative D value of tajima's test. Consequently, our study allowed evidence of rapid evolution of HSVd. More cDNA clones should be selected for further analyses of viroid population. In the meanwhile, infectious dimeric from HSVd1.5 and HSVd1.7 variants should be synthesized and co-inoculated to investigate their molecular interactions for explaining the potential mechanism.

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