BARD

FINAL REPORT

PROJECT NO. IS-3109-99

**The Rugose Wood Disease of Grapevine: Etiology,
and Virus Resistance in Transgenic Vines**

M. Mawassi, A. Rowhani, D.A. Golino, A. Perl, E. Tanne

2003

6584 6

**BARD Final Scientific Report
Cover Page**

Date of Submission of the report: _____ **November 15, 2003**

BARD Project Number: _____ **IS-3109-99**

**Project Title: The Rugose Wood Disease of Grapevine: Epidemiology, Etiology , and Virus
Resistance in Transgenic Vines**

Investigators

Institutions

Principal Investigator (PI):

Munir Mawassi

A.R.O. The Volcani Center

Co-Principal Investigator (Co-PI):

Adib Rowhani

UC Davis, CA

Collaborating Investigators:

Golino D.A.

UC Davis, CA

Perl A.

A.R.O. The Volcani Center

Tanne E.

A.R.O. The Volcani Center

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Vitivirus, *Grapevine virus A*, resistance, post transcriptional gene silencing, cryopreservation

Budget: IS: \$150,000

US: \$150,000

Total: \$300,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)			4	4
Submitted, in review, in preparation			1	1
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses*			2*	2
Abstracts			2	2
Not refereed (proceedings, reports, etc.)				

* Two PhD students were devoted to this research: Nurbol Galiakparov and Wang Qiaochun.

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings			1	1
Longer Visits (Sabbaticals)				

Description of Cooperation:

This research is based on complementary cooperation between the Volcani Center and the UC Davis laboratories. The cooperation was involved sharing information and products such as experimental protocols, dsRNA extractions of GVA, GVB, GLRaV-1, GLRaV-2, and GLRaV-3. Information regarding sequences and primers of these viruses, and strategies for RT-PCR analysis were also exchanged.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Abstract

Rugose wood is a complex disease of grapevines, which occurs in all growing areas. The disease is spread in the field by vector transmission (mealybugs). At least five elongated-phloem-limited viruses are implicated in the various rugose wood disorders. The most fully characterized of these are *Grapevine virus A* (GVA) and GVB, members of a newly established genus, the *vitivirus*. GVC, a putative *vitivirus*, is much less well characterized than GVA or GVB. The information regarding the role of GVC in the etiology and epidemiology of rugose wood is fragmentary and no sequence data for GVC are available.

The proposed research is aimed to study the etiology and epidemiology of rugose wood disease, and to construct genetically engineered virus-resistant grapevines. The objectives of our proposed research were to construct transgenic plants with coat protein gene sequences designed to induce post-transcriptional gene silencing (PTGS); to study the epidemiology and etiology of rugose wood disease by cloning and sequencing of GVC; and surveying of rugose wood-associated viruses in Californian and Israeli vineyards.

In an attempt to experimentally define the role of the various genes of GVA, we utilized the infectious clone, inserted mutations in every ORF, and studied the effect on viral replication, gene expression, symptoms and viral movement. We explored the production of viral RNAs in a GVA-infected *Nicotiana benthamiana* herbaceous host, and characterized one nested set of three 5'-terminal sgRNAs of 5.1, 5.5 and 6.0 kb, and another, of three 3'-terminal sgRNAs of 2.2, 1.8 and 1.0 kb that could serve for expression of ORFs 2-3, respectively.

Several GVA constructs have been assembled into pCAMBIA 2301, a binary vector which is used for *Agrobacterium* mediated transformation: GVA CP gene; two copies of the GVA CP gene arranged in the same orientation; two copies of the GVA CP gene in which the downstream copy is in an antisense orientation; GVA replicase gene; GVA replicase gene plus the 3' UTR sequence; and the full genome of GVA. Experiments for transformation of *N. benthamiana* and grapevine cell suspension with these constructs have been initiated. Transgenic *N. benthamiana* plants that contained the CP gene, the replicase gene and the entire genome of GVA were obtained. For grapevine transformation, we have developed efficient protocols for transformation and successfully grapevine plantlets that contained the CP gene and the replicase genes of GVA were obtained. These plants are still under examination for expression of the transgenes. The construction of transgenic plants with GVA sequences will provide, in the long run, a means to control one of the most prevalent viruses associated with grapevines.

Our many attempts to produce a cDNA library from the genome of GVC failed.

For surveying of rugose wood associated viruses in California vineyards, samples were collected from different grape growing areas and tested by RT-PCR for GVA, GVB and GVD. The results indicated that some of the samples were infected with multiple viruses, but overall, we found higher incidence of GVB and GVA infection in California vineyards and new introduction varieties, respectively. In this research we also conducted studies to increase our understanding of virus - induced rootstock decline and its importance in vineyard productivity. Our results provided supporting evidence that the rootstock response to virus infection depends on the rootstock genotype and the virus type. In general, rootstocks differ widely in virus susceptibility. Our data indicated that a virus type or its combination with other viruses was responsible in virus-induced rootstock decline. As the results showed, the growth of the rootstocks were severely affected when the combination of more than one virus was present.

Achievements

Achievements obtained in the research by the Israeli team:

Cloning and of GVA genes: Several GVA constructs have been assembled into pCAMBIA 2301, a binary vector which is used for *Agrobacterium* mediated transformation (Fig. 1):

GVA CP gene; two copies of the GVA CP gene arranged in the same orientation; two copies of the GVA CP gene in which the downstream copy is in an antisense orientation; GVA replicase gene; GVA replicase gene plus the 3' UTR sequence; and the full genome of GVA.

Gene functions (Galiakparov *et al.*, 2003, *Virology* 306, 42-50). In an attempt to experimentally define the role of the various genes of GVA, we utilized the infectious clone, inserted mutations in every ORF, and studied the effect on viral replication, gene expression, symptoms and viral movement.

Subgenomic RNA production (Galiakparov *et al.*, 2003, *Virology* 312, 434-448). We explored the production of viral RNAs in a GVA-infected *Nicotiana benthamiana* herbaceous host, and characterized one nested set of three 5'-terminal sgRNAs of 5.1, 5.5 and 6.0 kb, and another, of three 3'-terminal sgRNAs of 2.2, 1.8 and 1.0 kb that could serve for expression of ORFs 2-3, respectively. Neither 3'- nor 5'-terminal sgRNAs which would correspond to ORF5 were detected, suggesting that expression of this ORF occurs via a bi- or polycistronic mRNA. Apparently, the production of the 5'- and 3'-terminal sgRNAs was controlled by sequences upstream of the 5'-terminus of each of ORFs 2-4. Detection of both plus- and minus-strands of the 5'- and 3'-terminal sgRNAs, though in different levels of accumulation, suggested that each of these *cis*-acting elements is involved in production of four RNAs: a 3'-terminal plus-strand sgRNA which could act as an mRNA, the corresponding 3'-terminal minus-strand RNA, a 5'-terminal plus-strand sgRNA, and the corresponding 5'-terminal minus-strand RNA.

Transformation of *N. benthamiana*: We performed transformation of *Nicotiana benthamiana* with the constructs described above. *N. benthamiana* leaf explants cut axenically were cultivated with *A. tumefaciens* strain EHA 105 containing the recombinant binary construct of pCAMBIA:GR19. Transformed tissue was selected for kanamycin-resistance and regenerated into plants. To reduce the possibility of selecting chimeric plants, shoots were taken from kanamycin-resistant callus, and rooted in the presence of kanamycin and whole plants were

regenerated. The genomic DNA was extracted and analyzed by PCR using specific primers. The lines which show specific PCR products were selected for continuing the experiments. In addition, a histochemical GUS assay was conducted. We obtained 16 plants which were transformed with GVA CP gene, one plant with the full GVA genome, and 20 plants with GVA replicase. Positive lines were selected and potted into pre-moistened turf and the pots were quickly placed in a plastic bag for 5-7 days until to bring in ambient humidity. Pots were transferred to growth chamber and gradually bags were removed. An additional 6-8 weeks after potting, seeds were harvested when completely dried. Fresh greenhouse-grown plant leaves were collected to extract genomic DNA. Southern blot analysis showed that these plants were transformed with GVA sequences.

Transformation of grapevine: (Wang et al., in preparation). Development of a basic transformation protocol: Factors affecting transformation efficiency, such as co-cultivation, antibiotics and culture conditions were studied. The preliminary results showed that a 3-days co-cultivation was the best. Transformed cell suspensions were more sensitive to Kanamycin than Paramomycin, and therefore, Paramomycin was used for selection of transformed cells. Selection in liquid medium was much more effective than on solid medium, while germination of embryo from transformed cells was much better on solid than in liquid.

Development of novel methods for transformation: Compared with the most frequently used transformation method, different methods, i.e. transformation of encapsulated cells and transformation of recovered cells from cryopreservation were tested. It appeared that transformation of recovered cells from cryopreservation would improve induction of secondary embryos and conversion of germinated embryos to whole plantlets and plant regeneration of transformed cells.

We are examining the transformation efficiency by GUS assay. Some of the transformation experiments are in advanced process and we have small plantlets that are GUS positive (Fig. 2). Additional analysis, such as Southern- and Northern blot analysis, will be carried out as soon as we will have sufficient plant material. We found that paromomycin was much more effective in selection of transformed cells than kanamycin. Embryo conversion rate of transformed cells was also higher in paromomycin selection than in kanamycin selection. Stable transformation with GVA CP was developed using cryopreserved cell suspensions. Cryopreserved cells retained their competence for genetic transformation. Although there were no differences between non-

cryopreserved and cryopreserved cells in transformation efficiency, conversion rates of transformed cells to embryos and plantlets were significantly higher with cryopreserved cell suspensions than with non-cryopreserved ones.

Achievements obtained in the research by the US team:

Cloning and sequencing of GVC and development of a detection methodology. Our many attempts to produce a cDNA library from the genome of GVC failed. The majority of sequences we obtained were related to GLRaV-2, GVA, or GVB. Limited supply of antiserum was available for GVC from the same source in Canada. We used this antiserum in ELISA and found a grapevine source which tested positive. An isolate, LR 109 isolate, which was detected by ELISA with GVB antiserum was also infected with a number of other viruses including GLRaV-2, -3, grapevine fleck virus (GFKV) and grapevine rupestris stem pitting associated virus (GRSPaV).

Surveying of rugose wood associated viruses in California vineyards. Samples were collected from different grape growing areas in California and tested by RT-PCR for GVA, GVB and GVD. The results from our survey are summarized in Table 1. Some of the samples were infected with multiple viruses, but overall as the data showed, we found higher incidence of GVB and GVA infection in California vineyards and new introduction varieties, respectively.

Mixed infection and the rugose wood complex –young vine decline in California. The main objective of these studies was to increase our understanding of virus - induced rootstock decline (VIRD) and its importance in vineyard productivity. In this experiment, 17 different rootstock and scion varieties were selected and inoculated with a single or combination of different viruses in 6 different treatments (Table 2). In the first year, data on shoot length and pruning weight showed that Freedom and Harmony growth was reduced by as much as 90% in treatments 4, 5, and 6 (Fig. 4) while some others were showing high degrees of resistant including AXR, Ramsey, Riparia Gloir, 3309 and 1616C (Fig.3). In addition, percent survival was reduced in all varieties except AXR. Virus effect on bud take was variable and not strongly correlated with growth reduction. This data provides supporting evidence that the rootstock response to virus infection depends on the rootstock genotype and the virus type. In general, rootstocks are differ widely in virus susceptibility.

In a different trial two rootstocks, Freedom and Kober 5BB, were selected and inoculated with 16 combinations of single and mixed virus infections. Virus treatments, sources, and virus

types are listed in Table 3. As the results indicated (Fig. 5), GLRaV-2 and especially its combination with a vitivirus (GVA, GVB, or GVC) caused more severe damages to these rootstock. These data indicated that a virus type or its combination with other viruses was also responsible in virus-induced rootstock decline. As the results showed, the growth of the rootstocks were severely affected when the combination of more than one virus was present (Fig. 5, treatments 1-10). As it is evident, in all these 10 treatments always GLRaV-2 and one of the vitiviruses (GVA, B, or C) were present. In treatments 11-15 and 17, single virus isolate or combination of other viruses were present, and the rootstocks did not show any adverse growth response.

Further experiments have been designed and executed in order to include more controls, e.g., single isolates of GLRaV-2, -3, and GVB and combination of double infections including these viruses (Table 4). Rootstocks included in this experiment are Freedom and Kober 5BB. This experiment is in progress and no data is available to present.

Appendix

Tables	9
Figures	12
Detailed description of the research activities and results	16
Cloning and of GVA genes	16
Functional analysis of GVA genome	16
Characterization of GVA subgenomic RNAs	17
Transformation of <i>Nicotiana benthamiana</i>	18
Transformation of grapevine	19
Cloning and sequencing of GVC and development of a detection methodology	22
Surveying of rugose wood associated viruses in California Vineyards	23
Mixed infection and the rugose wood complex-the case of young vine decline in California	24
Papers in press	27
Published papers	43

I. TABLES

Table 1. Results from the survey of some California vineyards and quarantine and newly introduced grapevine varieties and clones. The Table shows the incidence of GVA, GVB and GVD in different vineyards and also materials imported from several other countries in the past 3 years. All samples were tested by RT-PCR using specific pair of primers designed for the detection of each virus.

Virus	Varieties from California			New Introductions		
	No. Tested	No. Pos.	% Pos.	No. Tested	No. Pos.	% Pos.
GVA	1450	45	3.1	646	57	8.8
GVB	1499	103	6.8	646	31	4.8
GVD	1236	14	1.1	646	34	5.2

Table 2. Virus treatments inoculated to each of 17 grape varieties. 40 plants were budded/ treatment/variety and planted in 5 blocks with 8 plants/block.

Treatment #	Virus Type(s)	Virus Source
1	none - negative control	not grafted
2	none - negative control	healthy control
3	GLRV-3	LR101
4	GLRV-1, GLRV-2, GVB	LR102
5	GFkV, GLRaV-2, GLRV-3, GRSPaV, GVC	LR109
6	GLRV-2,GVB	CB100

Table 3. Virus treatments, types and virus sources inoculated to compare effects of single and multiple virus infections on growth of Freedom and Kober 5BB. 40 plants/treatment were budded and planted in 5 blocks with 8 plants/block.

Treatment #	Virus Type(s)	Virus Source
1	GLRaV-2, GLRaV-3, GFKV, GRSPaV, GVC	LR 109
2	GVB, GLRaV-2	CB100
3	GLRaV-1, GLRaV-2, GVB, GFKV	LV 92-2
4	GLRaV-1, GLRaV-2, GVA, GFKV	LV 91-2
5	GLRaV-2, GVB, GRSPaV	AsMo 100
6	GLRaV-2, GLRaV-3, GVA, GVB	LV 92-6
7	GVB, GLRaV-1, GLRaV-2	LR102
8	GLRaV-2, GVB	LV 91-1
9	GLRaV-2, GVB, GFKV, GRSPaV	Fleck 101
10	GLRaV-2, GVB, GFKV	LV 92-1
11	GLRaV-4, GRSPaV	LR 106
12	GRSPaV, GFKV	RSP 109
13	GFLV, GRSPaV	GFLV 102
14	GVB	CB120
15	GRSPaV	RSP 100
16	None-Negative Control	Healthy
17	None-Negative Control	Not Grafted
18	GLRaV-3	LR101

Table 4. Virus treatments, types and virus sources inoculated to compare effects of single and multiple virus infections on growth of Freedom and Kober 5BB. 40 plants/treatment were budded and planted in 5 blocks with 8 plants/block.

Treatment	Virus Type(s)	Virus Source
1	None - negative control	not grafted
2	None - negative control	Healthy
3	GVB, GLRV-1, GLRV-2 - positive control	LR102
4	GVB, GLRV-2 - positive control	CB100
5	GVB	CB120
6	GLRV-2	LV93-11
7	GLRV-3	LR101
8	GVB + GLRV-2	CB120 + LV93-11
9	GVB + GLRV-3	CB120 + LR101
10	GLRV-2 + GLRV-3	LV93-11 + LR101
11	GVB, GLRV-2 + GLRV-3	CB100 + LR101

II. FIGURES

Figure 1. Schematic maps of GVA constructs assembled for being used in transformation experiments.

Figure 2. Different Stages in transformation of grapevine cells

Figure 3

Figure 4

Figure 5

III. DETAILED DESCRIPTION OF THE RESEARCH ACTIVITIES AND RESULTS

(A) Cloning and of GVA genes:

Several GVA constructs have been assembled for their use for transformation of grapevines and induction of PTGS defense mechanism:

Constructs with GVA CP gene: As we proposed, we utilized the CP gene as the sequence to induce PTGS defense machinery in grapevines. For this purpose we assembled two constructs, each containing two copies of the GVA CP gene in tandem. In one construct the two copies were in the same orientation (head to tail) (Fig. 1). The second construct contained the downstream copy in an antisense orientation, thus enabling the formation of a double-stranded RNA (dsRNA) molecule upon transcription (tail to tail) (Fig. 1). An intron sequence was introduced in between the two GVA CP gene copies (Fig. 1). This later sequence is expected to form a loop structure that will enable dsRNA formation. GVA CP gene copies were obtained by RT-PCR and initially cloned separately in pBluescript vector using specific oligonucleotide primers and the DNA full-length cDNA clone as template. Next, the two copies were assembled into pCass2 vector under the 35-S promoter and transcriptional terminator. The cassette [35-S promoter:two copies of GVA CP gene :terminator] was then isolated and cloned into pCAMBIA 2301, a binary vector which is used for *Agrobacterium* mediated transformation.

Constructs with GVA Replicase gene: We assembled two constructs that contain the cDNA of the entire ORF1 (replicase) (Fig. 1). In one construct the 3' UTR sequence was also included. These two cDNAs were further cloned into pCass2 vector under the 35-S promoter and transcriptional terminator. The cassette [35-S promoter:GVA ORF1 or GVA ORF1 + 3' UTR:terminator] was then isolated and cloned in the binary vector pCAMBIA 2301 for *Agrobacterium* mediated transformation.

(B) Functional analysis of GVA genome

A manuscript describing the results of this section has been published (Galiakparov *et al.*, 2003, *Virology* 306, 42-50)

GVA carries five open reading frames (ORFs). Only the coat protein ORF has been experimentally identified as such, the roles of some of the other ORFs have been deduced by sequence homology to known genes. The construction of a full-length, infectious clone of GVA has been previously reported. In an attempt to experimentally define the role of the various genes of GVA, we utilized the infectious clone, inserted mutations in every ORF, and studied the effect on viral replication, gene expression, symptoms and viral movement. Mutations in ORF 1 abolished RNA replication. Mutations in ORF 2 did not affect any of the aforementioned parameters. Mutations in ORFs 3 and 4 restricted viral movement. Mutations in ORF 5 rendered the virus asymptomatic, and partially restricted its movement.

(C) Characterization of GVA subgenomic RNAs:

A manuscript describing the results of this section has been published (Galiakparov *et al.*, 2003, *Virology* 306, 42-50).

GVA, a species of the recently established genus *Vitivirus*, consists of an ~ 7.3-kb single-stranded RNA genome of positive polarity, organized into five open reading frames (ORFs). The virus, which is closely associated with the grapevine rugose wood disease complex, has been poorly investigated genetically. We explored the production of viral RNAs in a GVA-infected *Nicotiana benthamiana* herbaceous host, and characterized one nested set of three 5'-terminal sgRNAs of 5.1, 5.5 and 6.0 kb, and another, of three 3'-terminal sgRNAs of 2.2, 1.8 and 1.0 kb that could serve for expression of ORFs 2-3, respectively. Neither 3'- nor 5'-terminal sgRNAs which would correspond to ORF5 were detected, suggesting that expression of this ORF occurs via a bi- or polycistronic mRNA. The 5'-terminal sgRNAs were abundant in dsRNA enriched extracts. Cloning and sequence analysis of the 3' end of 5.5 kb 5'-terminal sgRNA and the 5' end of the 1.8 kb 3'-terminal sgRNA, suggested that a mechanism other than specific cleavage was involved in production of these sgRNAs. Apparently, the production of the 5'- and 3'-terminal sgRNAs was controlled by sequences upstream of the 5'-terminus of each of ORFs 2-4. Detection of both plus- and minus-strands of the 5'-and 3'-terminal sgRNAs, though in different levels of accumulation, suggested that each of these *cis*-acting elements is involved in production of four RNAs: a 3'-terminal plus-strand sgRNA which could act as an mRNA, the corresponding 3'-terminal minus-strand RNA, a 5'-terminal plus-strand sgRNA, and the corresponding 5'-terminal minus-strand RNA.

(D) Transformation of *Nicotiana benthamiana*:

Agrobacterium-mediated transformation of plants has been adapted to many plant species. The wide applicability of this transformation method is a result of several factors: the broad host range of *Agrobacterium*, the regeneration responsiveness of many different explant tissues and the utility of a wide range of selectable marker genes. *Nicotiana benthamiana*, is a herbaceous plant that can be easily transformed and mechanically inoculated by GVA. We proposed to use this plant to estimate the frequencies of PTGS induction by the gene constructs described above. Leaf explant transformation of tobacco is the paradigm for *Agrobacterium*-mediated transformation of plant tissues and subsequent selection and regeneration of transgenic plants. This system permits efficient gene transfer, selection and regeneration to be coupled together in a simple process.

We performed transformation of *N. benthamiana* with GVA CP gene (single copy). We constructed chimeric gene with coding sequence of the GVA CP gene (GR-19) fused to the enhanced CaMV-35S promoter and NOS terminator. *N. benthamiana* leaf explants cut axenically were cultivated with *A. tumefaciens* strain EHA 105 containing the recombinant binary construct of pCAMBIA:GR19. Transformed tissue was selected for kanamycin-resistance and regenerated into plants. To reduce the possibility of selecting chimeric plants, shoots were taken from kanamycin-resistant callus, and rooted in the presence of kanamycin and whole plants were regenerated. As a preliminary verification that the putative transgenic plants, we extracted genomic DNA and performed PCR using primers deduced from both the GVA CP gene and the neomycin phosphotransferase II (npt II) sequence. The lines which show specific PCR products were selected for continuing the experiments. In addition, a histochemical GUS assay was conducted.

After six subsequent transformation experiments, we have obtained 23 putative transgenic plants that have been selected for being resistant to kanamycin. Those lines were further tested by rooting capability on presence of kanamycin. sixteen out of the 23 lines were rooted and grown well. Plant pieces were taken from kanamycin-resistance lines both for histochemical GUS activity and for genomic DNA extraction to perform PCR analysis, using GVA CP- and npt II-specific primes. All 16 lines tested positive by PCR. Twelve of 16 lines were GUS positive. Positive lines were selected and potted into pre-moistened turf and the pots were quickly placed

in a plastic bag for 5-7 days until to bring in ambient humidity. Pots were transferred to growth chamber and gradually bags were removed. An additional 6-8 weeks after potting, seeds were harvested when completely dried. Fresh greenhouse-grown plant leaves were collected to extract genomic DNA. Southern blot analysis showed that these plants were transformed with GVA sequences.

(E) Transformation of grapevine: (Wang *et al.*, in preparation).

Grapevine cultivars may differ in their susceptibility to virus diseases, but no native resistance gene has been identified. Genetic transformation seems to be the only promising means for introducing virus resistance to the existing elite grapevine cultivars. Embryogenic cell suspensions are the most frequently used tissues for transformation system in grapevine. Although a significant progress has been made in developing transformation systems for both rootstocks and cultivars, *Agrobacterium*-mediated transformation efficiency of embryogenic cells and their conversion to transgenic plants are still very low. *Agrobacterium*-mediated transformation of plants has been adapted to many plant species. The wide applicability of this transformation method is a result of several factors: the broad host range of *Agrobacterium*, the regeneration responsiveness of many different explant tissues and the utility of a wide range of selectable marker genes. In this research we developed efficient systems using cryopreserved grapevine cell suspensions for transformation with GVA sequences.

Cryopreservation of grapevine shoot tips

Cryopreservation offers long-term storage capability, maximal stability of phenotypic and genotypic characteristics of stored germplasm, and minimal storage space and maintenance requirements. To date, cryopreservation has been widely applied to a great number of plant species from temperate to tropical regions.

Although there have been a few reports on cryopreservation of grapevine shoot tips, survival of cryopreserved shoot tips was low (24%). Cryopreserved shoot tips could not regenerate into plants. Manipulation was complicated. A simplified and efficient method needs to be developed, which can also be applicable for different cultivars or rootstocks belonging to different *Vitis* species.

In vitro-grown shoot tips of grapevines (*Vitis L.*) were successfully cryopreserved by encapsulation-dehydration and vitrification. An optimal survival of cryopreserved shoot tips was achieved when encapsulated shoot tips were dehydrated to 15.6 and 17.6% water content for the LN33 hybrid and cv. Superior, respectively. Comparison between the effects of dehydration with silica gel and by air drying on cryopreserved shoot tips, showed that survival was dependent on water content, not on dehydration method. The thawing method markedly affected survival of cryopreserved shoot tips, and thawing at 40°C for 3 min was found best. The presence of BA in the recovery medium was essential for survival. While a BA concentration of 4 µM was found optimal for recovery of control shoot tips, 1 and 2-4 µM produced the best recovery of shoot tips cryopreserved by vitrification and encapsulation-dehydration, respectively.

Cryopreservation of grapevine cell suspensions and subsequent plant regeneration: (Wang et al., Plant Cell, Tissue and Organ Culture 2003, in press) Since somatic embryogenesis of *Vitis vinifera* L. was first reported, using the unfertilized ovules as explants, many studies have been contributed to develop efficient methods for somatic embryogenesis from various *Vitis* species as well as for important cultivars of *Vitis vinifera* L. and their hybrids. Somatic embryogenesis has a potential application to rapid clonal propagation of the stock cultivars and has been shown to be the most frequently regenerative system in transformation studies.

The retention of the embryogenic potential of such cultures during the long-term maintenance has proved to be difficult. Embryogenic cultures usually lose their morphogenetic potential or differentiated completely into germinating embryos when cultured on a solid medium. Also the yield and quality of embryos were not stable at each subculture time. Liquid medium can also be used for the long-term maintenance of embryogenic cell suspensions, but embryos produced via liquid culture often yielded morphologically abnormal plantlets. Browning of media and finally a total loss of the embryogenic tissue have also been reported in a liquid culture system. In addition, frequent subculture of embryogenic cultures, which has been found beneficial in preventing browning and promoting embryo development is costly and laborious, and may have a risk of accumulation of somaclonal variation with increasing time. These are the main drawbacks that greatly limit the possible application of long-term solid or liquid embryogenic cell suspensions to rapid clonal propagation and genetic transformation of grapevines.

Cryopreservation has generally been considered as an ideal means of avoiding the loss of embryogenic potential during the repeatedly subculture and of evading the accumulation of somaclonal variation during long-term maintenance of embryogenic cultures.

With encapsulation-dehydration, an optimal viability (78%) of cryopreserved cells was achieved when encapsulated cells were precultured with 1 M sucrose for 2 to 4 days and dehydrated by air drying to 20.6% water content. MGN solid medium containing 2.5g/l AC was found to promote viability of cryopreserved cells. Although cryopreserved cells showed a 5-day lag phase in regrowth, and their increase in fresh weight was only half that of control cells, their growth pattern was the same as that of control cells after two subcultures, following re-establishment in cell suspensions. Cryopreservation significantly promoted embryogenesis and subsequent plant germination, compared with both the control and dehydration treatments.

With encapsulation-vitrification, an optimal viability (76%) of cryopreserved cells was obtained when cell suspensions were precultured with a final sucrose concentration of 0.75 M and dehydrated with PVS2 at 0°C for 270 min. Biochemical analysis showed that preculture with sucrose concentrations caused changes in levels of total soluble protein and sugars in cell suspensions. Morphologies of cells recovered from cryopreservation were identical to those from control cells. Although the increase of cryopreserved cells in fresh weight was significantly lower than that of control cells, their growth pattern was the same as that of control cells after two subcultures, following re-establishment in cell suspensions. Encapsulation-vitrification was also applied to cryopreservation of another rootstock B41 (*V. vinifera* x *V. Berlandieri*) and 4 cultivars (*Vitis vinifera* L.).

Transformation of grapevine embryogenic cell suspension with GVA sequences: Although significant progress has been made in developing transformation systems for both rootstocks and cultivars of grapevines, an efficient *Agrobacterium*-mediated transformation of embryogenic cells and their conversion to transgenic plants are still very low. Transformation procedure considerably decreased the induction of secondary embryos and conversion of germinated embryos to whole plantlets with the worst case found that transformed cells failed completely to regenerate.

Development of a basic transformation protocol: Factors affecting transformation efficiency, such as co-cultivation, antibiotics and culture conditions were studied. The preliminary results

showed that a 3-days co-cultivation was the best. Transformed cell suspensions were more sensitive to Kanamycin than Paramomycin, and therefore, Paramomycin was used for selection of transformed cells. Selection in liquid medium was much more effective than on solid medium, while germination of embryo from transformed cells was much better on solid than in liquid.

Development of novel methods for transformation: Compared with the most frequently used transformation method, different methods, i.e. transformation of encapsulated cells and transformation of recovered cells from cryopreservation were tested. It appeared that transformation of recovered cells from cryopreservation would improve induction of secondary embryos and conversion of germinated embryos to whole plantlets and plant regeneration of transformed cells

Currently, we have ongoing transformation experiments of grapevine cell suspension with the following sequences (Fig. 1):

- 1) GVA coat protein sequence
- 2) Duplicated copies of the GVA coat protein gene ligated tail to head
- 3) Duplicated copies of the GVA coat protein gene ligated head to head, separated by a sequence of an intron.
- 4) The complete cDNA of the GVA.
- 5) The complete cDNA of ORF1 (replicase).
- 6) The complete cDNA of ORF1 (replicase) with the 3' UTR sequence.

For transformation, we are using improved methods that we developed for this project as described above (cryopreserved cell suspensions). We are examining the transformation efficiency by GUS assay. Some of the transformation experiments are in advanced process and we have small plantlets that are GUS positive (Fig. 2).

We found that paromomycin was much more effective in selection of transformed cells than kanamycin. Embryo conversion rate of transformed cells was also higher in paromomycin selection than in kanamycin selection. Stable transformation with GVA CP was developed using cryopreserved cell suspensions. Cryopreserved cells retained their competence for genetic transformation. Although there were no differences between non-cryopreserved and

cryopreserved cells in transformation efficiency, conversion rates of transformed cells to embryos and plantlets were significantly higher with cryopreserved cell suspensions than with non-cryopreserved ones.

(F) Cloning and sequencing of GVC and development of a detection methodology

We obtained the grapevine virus C (GVC) source from Saad Masri, Center for Plant Health, Sidney, B.C., Canada. This is the same isolate that described by Paul Monett. Our PCR test indicated that this source also co-infected with grapevine leafroll associated virus type 2 (GLRaV-2), grapevine virus A (GVA), and grapevine virus B (GVB). Virus was propagated in *Nicotiana bentamiana*. Our many attempts to produce a cDNA library from the genome of GVC failed. Attempts included: random priming, immunocapture-RT-PCR (using the polyclonal antibody produced by Paul Monett), and use of oligo-dT. Each experiment was repeated few times using either dsRNA or total RNA extracted from the host plant. Large number of clones obtained from the above mentioned methodologies were screened and sequenced. The majority of sequences we obtained were related to GLRaV-2, GVA, or GVB (with over 91-97% homology with published sequences) and a small number were not virus related sequences.

Limited supply of antiserum was available for GVC from the same source in Canada. We used this antiserum in ELISA and found a grapevine source (LR 109 isolate) which tested positive. Unfortunately this particular isolate was also infected with number of other viruses including GLRaV-2, -3, grapevine fleck virus (GFKV) and grapevine rupestris stem pitting associated virus (GRSPaV).

(G) Surveying of rugose wood associated viruses in California vineyards

Samples were collected from different grape growing areas in California and tested by reverse transcription-polymerase chain reaction (RT-PCR) for GVA, GVB and grapevine virus D (GVD). Samples were from costal areas, central valley, Napa and Sanoma areas and foot hills. These samples were from production vineyards. In addition, most of the quarantine and new introductions delivered to Foundation Plant Services (FPS) at UC Davis for processing were also tested for the above mentioned viruses. The quarantine materials were mostly from France, Italy, Portugal, Russia and few Middle East countries. The results from our survey are summarized in Table 1.

Some of the samples were infected with multiple viruses, but overall as the data showed, we found higher incidence of GVB and GVA infection in California vineyards and new introduction varieties, respectively.

(H) Mixed infection and the rugose wood complex – the case of young vine decline in California

The main objective of these studies was to increase our understanding of virus - induced rootstock decline (VIRD) and its importance in vineyard productivity. Specifically, the objectives are: 1) to measure the effect of VIRD on economically important rootstocks; 2) to determine the degree of synergism in double and multiple infections as compared to single infections of the corresponding viruses demonstrated to be involved in VIRD.

Seventeen different rootstock and scion varieties were inoculated with 6 virus treatments. The varieties in this experiments are: Freedom, AXR, Harmony, SO4, Kober 5BB, Cabernet Franc, Chardonnay, Ramsey, Riperia Gloire, 3309C, 1103P, 140Ru, 420A, Teleki 5C, 1613C, 1616C, and Schwarzmann. Scion varieties were inoculated to demonstrate our unproven observation that the same virus combination which can cause VIRD may be minor problems on own-rooted *V. vinifera*. If this is true, it will be an important consideration in the choice of planting stocks in those parts of the world where there is an ongoing transition from own-rooted vineyards to vineyards propagated on rootstock.

Treatments, virus types and virus sources are listed in Table 2. Treatments 1 and 2 are negative controls. Treatment 3 is a single infection of GLRV-3. The other three treatments (4, 5, and 6) are various combinations of two or more viruses that occurred naturally.

Chipbudding was done by grafting dormant buds from the virus source vines into potted, green growing rootstock and scion varieties. Two inoculum buds were grafted into each plant to increase the chance of virus transmission to the rootstock. They healed in a greenhouse for 3 weeks and were hardened off in a shadehouse before planting. They were planted in 5 blocks and 8 plants/treatment/block for a total of 240 plants per rootstock. Budtake was read 1 to 2 months after grafting. Reading involved removing the budding rubber and tape, cutting into the bud to see whether it was dead (brown) or alive, and pulling on the bud to note the degree of attachment. After bud take was read, the inoculum buds were removed. Plants in which neither bud was grafted successfully were removed from the dataset.

Length of the longest shoot for each plant was measured in the Spring. Pruning weight for each plant was measured during the dormant season. The collected data allowed us to categorize the varieties into four categories (grades A, B, C and D) according to how much difference is observed between the healthy and virus-inoculated plants of each variety. More resistant varieties or grade A, tentatively included AXR, Ramsey, Riperia Gloire, 3309C and 1616C (Fig. 3). Tolerant varieties, or grade B, included 140Ru and 1103P and were defined as those in which plants in one virus treatment were 80 to 90% of the size of healthy control. Susceptible varieties, or grade C, included Teleki 5C, Kober 5BB, SO4, Chardonnay and Cabernet Franc. In these varieties, in at least one virus treatment in at least one parameter (pruning weight or longest shoot), plants were 50 to 75% of the size compared to healthy. Very susceptible varieties, or grade D, included Freedom, Harmony, 1613C, 420A, and Schwarzmann (Fig. 4).

In this experiment, 17 different rootstock and scion varieties were selected and inoculated with a single or combination of different viruses in 6 different treatments (Table 2). In the first year, data on shoot length and pruning weight showed that Freedom and Harmony growth was reduced by as much as 90% in treatments 4, 5, and 6 (Fig. 4) while some others were showing high degrees of resistant including AXR, Ramsey, Riperia Gloir, 3309 and 1616C (Fig.3). In addition, percent survival was reduced in all varieties except AXR. Virus effect on bud take was variable and not strongly correlated with growth reduction. This data provides supporting evidence that the rootstock response to virus infection depends on the rootstock genotype and the virus type. In general, rootstocks are differ widely in virus susceptibility.

In a different trial two rootstocks, Freedom and Kober 5BB, were selected and inoculated with 16 combinations of single and mixed virus infections. Virus treatments, sources, and virus types are listed in Table 3. As shown in the list, there are two negative controls (treatments 1 and 2), and rest of the treatments are from sources that were either singly infected or had multiple infection.

Inoculation was done by chip budding as described above. Forty plants were budded per treatment per rootstock. They were planted in the field in 5 blocks and 8 plants/treatment/block. Bud take was recorded as described above. Longest shoot length and pruning weight were measured for both rootstocks.

In this experiment, the two most susceptible rootstocks were selected and inoculated with 16

different viruses and virus combinations (Table 3). As the results indicated (Fig. 5), GLRaV-2 and especially its combination with a vitivirus (GVA, GVB, or GVC) caused more severe damages to these rootstock. These data indicated that a virus type or its combination with other viruses was also responsible in virus-induced rootstock decline. As the results showed, the growth of the rootstocks were severely affected when the combination of more than one virus was present (Fig. 5, treatments 1-10). As it is evident, in all these 10 treatments always GLRaV-2 and one of the vitiviruses (GVA, B, or C) were present. In treatments 11-15 and 17, single virus isolate or combination of other viruses were present, and the rootstocks did not show any adverse growth response.

Further experiments have been designed and executed in order to include more controls, e.g., single isolates of GLRaV-2, -3, and GVB and combination of double infections including these viruses (Table 4). Rootstocks included in this experiment are Freedom and Kober 5BB. This experiment is in progress and no data is available to present.

V. PAPERS IN PRESS

ORF 5 of Grapevine Virus A Encodes a Nucleic Acid-Binding Protein and Affects Pathogenesis.

^{1,2}Galiakparov, N., ¹Tanne, E., ¹Mawassi, M., ¹Gafny R., and ²Sela I.*

¹Agricultural Research Organization, The Volcani Center, Department of Virology, Tolkovski Laboratory, Bet Dagan 50250, Israel. ²The Hebrew University, Faculty of Agricultural, Food and Environmental Quality Sciences, Institute of Plant Sciences, Virus Laboratory, Rehovot 76100, Israel

*Corresponding author. Telephone: 972-8-9489377. Fax: 972-8-9473402. E-mail: sela@agri.huji.ac.il

Word count of Summary: 142

Word count of article: 2950

Number of Figures: 4

Number of Tables: None

SUMMARY

A previous functional analysis of the genome of grapevine virus A (GVA) was not conclusive as to the role of open reading frame 5 (ORF 5). This ORF encodes a 10-kDa protein (p10) carrying two distinct domains: a basic, arginine-rich domain and a zinc-finger domain. P10 was cloned and expressed in *Escherichia coli*, and was shown by northwestern assays to interact with nucleic acids. In-frame deletion of the basic region abolished p10's nucleic acid-binding capability, whereas substitution of cysteine residues by serine in the zinc-finger domain did not affect binding. These mutations were inserted into the full-length infectious clone. It has been shown that ORF 5 mutations do not affect replication of GVA-RNA. However, plants inoculated with the aforementioned mutations did not develop symptoms, and western blot analysis revealed markedly reduced expression of the movement protein (the product of ORF 3).

INTRODUCTION

Grapevine virus A (GVA) is a member of the genus *Vitivirus* (Martelli *et al.*, 1997). Its genome consists of a single-stranded, positive-sense RNA of 7349 nucleotides and carries five open reading frames (ORFs). Two different isolates have been hitherto sequenced: Is151 (Minafra *et al.*, 1994), and PA3 (Galiakparov *et al.*, Genbank accession # AY167577). Infectious clones were made from both GVA isolates. The Is151 clone was unstable (Saldarelli *et al.*, 2000), while that of PA3 was stable and suitable for further molecular studies (Galiakparov *et al.*, 1999). Following functional analysis (Galiakparov *et al.*, *Virology*, in press), ORFs 1, 3 and 4 were assigned the functions of a replicase, a movement protein (MP) and a coat protein (CP), respectively. The function of ORF 2 remained unsolved, and mutations in ORF 5 restricted viral movement to only a few layers of cells around the initial site of infection. ORF 5 encodes a putative 10-kDa protein (p10), with no homology to any other protein in the database. As demonstrated below, there are substantial differences in the p10 sequences of the two GVA isolates. P10 of PA3, but not of Is151, carries an arginine-rich domain, and both isolates carry a zinc-finger domain. ORF 5 of grapevine virus B (GVB), another member of the *Vitivirus* genus, encodes a protein, which shares homology with nucleic acid-binding proteins of various plant viruses (Minafra *et al.*, 1994). However, the ORF 5 product of GVB is only remotely

homologous to the ORF 5 products of other vitiviruses (see below). Here we present experimental evidence that p10 of PA3-GVA is a nucleic acid-binding protein, and we elucidate the domains responsible for nucleic acid binding. We also demonstrate that ORF 5 mutations limit viral accumulation and prevent symptom appearance.

MATERIALS AND METHODS

Cloning and mutagenesis of ORF 5. ORF 5 of PA3-GVA (bases 6174 to the end of the sequence) was subcloned in pBluescriptKS+ (Stratagene) between the PstI and Sall restriction sites, and the resulting construct was designated pBluePst/Sall. A single-stranded pBluePst/Sall DNA was isolated from *E. coli* strain JM109 after infecting with the R408 helper phage as described by Vieira (1987), and was used for primer-mediated point mutations. Mutations were performed using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega). Three clones were generated by mutagenesis using primers in which cysteine codons at p10 positions 25, 27, and 37 were replaced by serine codons (serine codons are underlined):

5'GAATGTGTCTAAGTGTGGTG3' (corresponding to nts 7076-7095),

5'GTAAGTCTGGTGCTATAATG3', (corresponding to nts 7084-7103) and

5'AATGAGGACTCCAAGTCC3' (corresponding to nts 7110-7127). The resultant clones were designated pBluePst/Sall-1C2S, pBluePst/Sall-2C2S and pBluePst/Sall-3C2S, respectively. The

basic domain of p10 was deleted by PCR. A part of full-length clone pGVAN3, from the beginning of the MP ORF (position 5642) to the end of the basic domain (position 7062), was amplified by PCR using the primers 5'CGACGCAGCATATGTCGCACGGAG3' and GCCTACGGCGCGCAAACGTGG3' (BssHII site underlined). This PCR product was cut with PstI and BssHI. Another fragment was generated by PCR using primer

5'CGCTGCGCGCATGAATGTGTGTAAG3' (corresponding to nts 7065-7088, BssHII site underlined), and a primer representing the 3' end of the GVA genome:

5'AAGTCGACGTG(T*30)GTCATAGTATGACAACCTAGC3' (corresponding to nts 7331-end, Sall site underlined, T*30 means 30 thymidine residues). The resultant PCR product corresponded to a segment of p10 starting at the end of the basic domain to the end of the GVA genome, and was cleaved by BssHI and Sall. Both digested fragments were cloned into pBluescriptKS+ between the PstI and Sall sites in a single ligation reaction. The obtained clone

was designated pBluePstI/SalI Δ ARM. To express GVA recombinant protein in *E. coli*, native and mutated forms of p10 in the plasmids: pGVAN3, pBluePst/SalI-1C2S, pBluePst/SalI-2C2S, pBluePst/SalI-3C2S and pBluePstI/SalI Δ ARM were amplified by PCR using the primer 5'GAAGATACATATGGATGACCCATC3' (corresponding to nts 7004-7024; NdeI site underlined) and the 3'-end primer. PCR products were digested by NdeI and SalI and inserted into the same restriction sites of the expression vector pET22b(+) (Novagen, Inc.), resulting in plasmids pETp10, pETp10-1C2S, pETp10-2C2S, pETp10-3C2S and pETp10- Δ ARM, respectively. In addition, The PstI-SalI fragments bearing the aforementioned p10 mutations were used for substituting the corresponding sequences in the full-length infectious clone pGVAN3, producing the full-length plasmids pGVA1C2S, pGVA2C2S, pGVA3C2S and pGVA Δ ARM.

Expression of native and mutated forms of p10. A fresh colony of *E. coli* BL21(DE3) (Novagen, Inc.) transformed with pET22b(+) and carrying ORF 5, or its mutated forms, was incubated in 2 ml LB medium at 37°C to an OD₆₀₀ of 0.5. A 1-ml aliquot of medium was transferred to another tube and protein expression was induced by addition of isopropyl- β -D-thiogalactopyranoside to a final concentration of 1 mM, and the cells were further grown for 3 h at 37°C.

Protein extraction and nucleic acid-binding assay. Following induction, cells were collected by centrifugation at 4000g and the pellets were resuspended in 100 μ l of water followed by the addition of 100 μ l of 2X Laemmli loading buffer (Laemmli, 1970). After boiling for 5 min, proteins were separated by electrophoresis in denaturing SDS polyacrylamide gels (SDS-PAGE) and electroblotted onto a nitrocellulose membrane using the BioTransMidi system (GelmanSciences). Northwestern blot analysis was performed as described previously (Gramstat *et al.*, 1990). Briefly, the membranes were incubated twice for 30 min in a denaturation solution (6 M urea, 0.1% Nonidet P-40) followed by incubation in renaturation buffer (10 mM Tris-HCl pH 7.8, 50 mM NaCl and 1X Denhardt solution) for 1 h with two changes at room temperature. After renaturation, the membranes were incubated with radiolabeled probes in the renaturation buffer for three h at room temperature. Membranes were then washed 3 times (15 min each) in washing buffer (10 mM Tris-HCl pH 7.8 and 200 mM NaCl) and autoradiographed. Single-stranded RNA (ssRNA) probes were synthesized by T7 RNA polymerase in the presence of [α -³²P]-UTP with linearized pGVAN3 or pBuescriptKS+ as templates. A double-stranded DNA

(dsDNA) probe was produced by PCR using the plasmid pBluescriptKS and M13 forward and reverse sequencing primers in the presence of [α - 32 P]-dATP. A ssDNA probe was obtained by alkaline denaturation of the labeled PCR product.

Full-length transcripts, plant inoculation, protein extraction and western blotting. Native and ORF 5-mutated forms of the full-length infectious clone were linearized by cleaving with Sall, and RNA transcripts were produced as described previously (Galiakparov *et al.*, 1999). Systemic leaves from *Nicotiana benthamiana* plants infected with the *in vitro* transcripts were collected 20 days post-inoculation and total proteins were extracted, followed by immunoblot analysis, as described by Robinson *et al.* (1997).

RESULTS

There are substantial differences in the sequences of ORF 5 of Is151-GVA and PA3-GVA. The start codon of the computer-deduced ORF 5 in PA3 is located immediately after the stop codon of the CP gene, whereas isolate Is151 carries an intergenic region of five nucleotides between ORF 4 and ORF 5, resulting in a different N-terminal region in the p10 of these two isolates. Multiple protein sequence alignment of the 3' proximal ORFs of viruses from the *Vitivirus* genus showed that the N-terminus of this protein is conserved among the PA3 isolate of GVA, grapevine virus D (GVD) and heracleum latent virus (HLV), less so for Is151-GVA and even less for GVB (Fig. 1). As shown in Figure 1, the N-terminal portion of PA3 (but not of Is151) carries a basic, arginine-rich motif (ARM; KRRRARR). This is followed by a typical zinc-finger motif (C-X-C-X₄-H-X₄-C, where X is any amino acid) occurring in the two GVA isolates. The GVA zinc-finger domain is similar to the metal-binding domain (C-X₂-C-X₄-H-X₄-C) of the retroviral *gag*-gene encoding a nucleic acid-binding protein (Green and Berg, 1989). The product of GVB-ORF 5 has also been suggested to include a putative nucleic acid domain (Minafra *et al.*, 1994). The nucleic acid-binding capacity of PA3-GVA-p10 was determined experimentally. ORF 5 was amplified by PCR and cloned into the bacterial expression vector pET22b, resulting in the recombinant plasmid pETp10. To test for nucleic acid binding, total protein was extracted from *E. coli* cells transformed with pET22b (an insertless plasmid) or with the recombinant plasmid pETp10. The protein extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane. After denaturation and subsequent renaturation of the membrane-bound proteins, a nucleic acid-binding assay was performed with a radiolabeled ORF 5-specific ssRNA probe, and binding of RNA was observed (Fig. 2A). In addition, a non-GVA,

ssRNA probe, as well as ss- and dsDNA probes, also bound to p10 (Fig. 2, B-D). Therefore, p10 binds nucleic acids in a non-specific manner. The addition of EDTA, a metal-chelating agent, to the northwestern assay had no effect on the nucleic acid-binding properties of p10 (data not shown).

To determine whether ARM or the putative metal-binding, zinc-finger domain are involved in the binding of nucleic acids, several mutations were introduced into ORF 5. Plasmid pETp10- Δ ARM carries p10 with an in-frame deletion of the basic region between residues 14 and 21. Plasmids pETp10-1C2S, pETp10-2C2S and pETp10-3C2S have point mutations in p10 where cysteine residues at positions 25, 27 and 37 have been changed to serine residues, respectively. Mutated forms of p10, along with the wild type and the insertless pET22b plasmid, were expressed in *E.coli*, and proteins were subjected to the northwestern assay. Figure 3A shows a Coomassie-stained gel, demonstrating that the protein load of p10 and its mutated forms was similar in all lanes. The binding experiments with 32 P-labeled ORF 5 RNA showed that the deletion of ARM (an in-frame mutation) abolishes the nucleic acid-binding capacity of p10, while substitution of the cysteine residues in the zinc-finger domain does not abolish its binding activity (Fig. 3B).

To investigate the effect of these mutations on the life cycle of the virus, the aforescribed mutated forms of ORF 5 were transferred to the full-length infectious clone pGVAN3 (Galiakparov *et al.*, 1999). RNA transcripts from the non-mutated, full-length pGVAN3, and its mutated forms (cysteine to serine substitutions in pGVA1C2S, pGVA2C2S, pGVA3C2S and an in-frame ARM deletion in pGVA Δ ARM), were used for mechanical inoculation of *N. benthamiana* leaves. The inoculated plants were monitored for at least 20 days for systemic symptom development. *N. benthamiana* plants inoculated with the wild-type transcript GVAN3 showed symptoms 6 days after inoculation. No symptoms could be detected on plants inoculated with the mutated transcripts. The most sensitive method for detecting GVA infection is a western blot analysis of MP (Rubinson *et al.*, 1997). This analysis showed the presence of the MP in systemic leaves of plants inoculated with transcripts of all of the aforescribed mutated clones (Fig. 4, lanes 3-6). However, the level of MP expression was considerably reduced relative to that of the wild type (Fig. 4, lane 1). Although antibodies were raised against the recombinant p10, detection of p10 by western blot failed, possibly due to its low concentration and/or low immunogenicity.

N. benthamiana protoplasts were inoculated with the aforescribed transcripts. Western blots with CP antiserum and quantitative RT-PCR with GVA-specific primers indicated that GVA accumulation levels in protoplasts inoculated with the wild type or the ORF 5-mutated transcripts are similar in all cases (data not shown).

DISCUSSION

The product of ORF 5 (p10) of the GVA isolate PA3 carries two distinct motifs near its N-terminus: ARM and zinc finger. The zinc-finger motif is conserved in all protein products of the 3' proximal ORFs of vitiviruses. Although zinc-fingers are considered to be involved in protein-nucleic acid binding, mutation analysis with PA3-GVA-p10 indicated that only the ARM is responsible for nucleic acid binding. The protein products of ORF 5 of Is151-GVA and GVB do not carry the ARM motif, while, based on sequence analysis, they are considered nucleic acid-binding proteins (Saldarelli *et al.*, 1996; Minafra *et al.*, 1997). This issue needs to be resolved experimentally.

In a previous paper (Galiakparov *et al.*, *Virology*, in press), we showed that PA3-GVA-p10 is required for symptom appearance. In that paper, ORF 5 was turned entirely into a non-sense mutation by substituting the initial methionine, or by causing frame-shift mutations. Symptoms did not develop, apparently due to inhibition of GVA accumulation beyond a few cell layers from its point of entry. In the present paper, we inserted only in-frame mutations, which resulted in the same phenotype, i.e. lack of symptom appearance. It is worth noting that the phenotype developed regardless of the nucleic acid-binding capacity of the mutant. As indicated in both papers, virus replication and accumulation in single cells (protoplasts), as well as CP and MP expression, were not hampered by any of the ORF 5 mutations. The only observation distinguishing wild-type GVA from all the tested mutations, was the restricted movement of the virus.

Although, p10 exhibits no sequence similarity to the nucleic acid-binding proteins of viruses from other groups, it carries structural motifs, which are exhibited by those proteins. These proteins play a role in replication, expression of other viral genes and pathogenesis. One of the most studied nucleic acid-binding proteins is the cysteine-rich protein encoded by the *yb* gene of the barley stripe mosaic virus (BSMV). This *yb* protein is not required for viral replication and movement, but is required for efficient accumulation of CP and replicase, and affects

pathogenesis (Petty *et al.*, 1990; Donald and Jackson, 1994). Another extensively studied cysteine-rich protein is p14 of the beet necrotic yellow vein virus (BNYVV). The p14 null mutations decreased accumulation of RNA2 up to 50 times *in cis* and the viral CP *in trans* (Hehn *et al.*, 1995). Also, null mutations of p14 reduced the size of local lesions by regulating expression of the triple gene block (Gilmer *et al.*, 1992). A common denominator of PA3-GVA-p10, BSMV-γb and BNYVV-p14 is the fact that a C-carrying motif is responsible for pathogenesis.

P10 does not affect viral replication or expression of any of its genes. However, it resembles many transcription factors by carrying the ARM and the zinc finger motif. At this stage, it may be postulated that p10 modulates some host response to GVA infection.

REFERENCES

- Donald, R. G. K., and Jackson, A. O. (1994). The barley mosaic virus γ b gene encodes a multifunctional cysteine-rich protein that affects pathogenesis. *Plant Cell* **6**, 1593-1606.
- Galiakparov, N., Tanne, E., Sela, I., and Gafny, R. (1999). Infectious RNA transcripts from grapevine virus A cDNA clone. *Virus Genes* **19**, 235-242.
- Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K., and Jonard, G. (1992). Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA2. *Virology* **189**, 40-47.
- Gramstat, A., Courtpozanis, A., and Rohde, W. (1990). The 12 kDa protein of potato virus M displays properties of a nucleic acid-binding regulatory protein. *FEBS Lett.* **276**, 34-38.
- Green, L. M. and Berg, J. M. (1989). A retroviral Cys-Xaa₂-Cys-Xaa₄-His-Xaa₄-Cys peptide binds metal ions: spectroscopic studies and a proposed three-dimensional structure. *Proc. Natl. Acad. Sci. USA* **86**, 4047-4051.
- Hehn, A., Bouzoubaa, S., Bate, N., Twell, D., Marbach, J., Richards, K., Guilley, H., and Jonard, G. (1995). The small cysteine-rich protein P14 of beet necrotic yellow vein virus regulates accumulation of RNA2 *in cis* and coat protein *in trans*. *Virology* **210**, 73-81.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature* **227**, 680-685.
- Martelli, G. P., Minafra, A., and Saldarelli, P. (1997). Vitivirus, a new genus of plant viruses [news]. *Arch. Virol.* **142**, 1929-1932.
- Minafra, A., Saldarelli, P., Grieco, F., and Martelli, G. P. (1994). Nucleotide sequence of the 3' terminal region of the RNA of two filamentous grapevine viruses. *Arch. Virol.* **137**, 249-261.

Minafra, A., Saldarelli, P., and Martelli, G. P. (1997). Grapevine virus A: nucleotide sequence, genome organization, and relationship in the *Trichovirus* genus. *Arch. Virol.* **142**, 417-423.

Petty, I. T. D., French, R., Jones, R. W., and Jackson, A. O. (1990). Identification of barley stripe mosaic virus genes involved in viral RNA replication and systemic movement. *EMBO J.* **9**, 3453-3457.

Rubinson, E., Galiakparov, N., Radian, S., Sela, I., Tanne, E., and Gafny, R. (1997). Serological detection of grapevine virus A using antiserum to a nonstructural protein, the putative movement protein. *Phytopathology* **87**, 1041-1045.

Saldarelli, P., Minafra, A., and Martelli, G. P. (1996). The nucleotide sequence and genomic organization of grapevine virus B. *J. Gen. Virol.* **77**, 2645-2652.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nuc. Acids Res.* **22**, 4673-4680

Vieira, J., and Messing, J. (1987). Production of single-stranded plasmid DNA. In "Methods in Enzimology" Vol. 153, pp. 3-11. Academic Press, San Diego, CA.

Legends to Figures

Figure 1. Alignment of proteins encoded by 3' proximal ORFs of GVA, GVB, GVD and HLV. The alignment was generated by the program Clustal W (Thompson *et al.*, 1994).

Figure 2. Northwestern blot analysis of p10. Total proteins from *E. coli* transformed with pETp10 (lane 1) or pET22b (lane 2) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and incubated with RNA and DNA probes: RNA transcribed from the pGVAN3 clone (A), RNA probe transcribed from empty pBluescriptKS+ plasmid (B), ssDNA obtained by denaturation of dsDNA probe (C), and dsDNA synthesized by PCR from empty pBluescriptKS+ plasmid (D).

Figure 3. Northwestern analysis of the mutated forms of p10. Extracts from *E. coli* transformed with pETp10- Δ ARM (lane 1), pETp10 (lane 2), pET22b (lane 3), pETp10-1C2S (lane 4), pETp10-2C2S (lane 5) and pETp10-3C2S (lane 6) were separated by SDS-PAGE (panel A, Coomassie-stained gel) and transferred to a nitrocellulose membrane. Blots were incubated with RNA probe transcribed from clone pGVAN3 and autoradiographed (panel B). Lane M, molecular mass markers (New England Biolabs, broad-range protein markers).

Figure 4. Immunoblot detection of MP (frame B) in extracts from *N. benthamiana* plants inoculated with transcript from pGVAN3 (lane 1), pGVA1C2S (lane 3), pGVA2C2S (lane 4), pGVA3C2S (lane 5) and pGVA Δ ARM (lane 6) clones. Lane 2: extracts from mock-inoculated plant. Frame A: Coomassie-stained gel. Lane M, molecular mass markers.

ACKNOWLEDGMENT

This study was supported by BARD (project no. IS-3109-99).

Fig.1

Fig. 2

Fig. 3

Fig. 4

IV. PUBLISHED PAPERS

- Galiakparov, N., Tanne, E., Sela, I., and Gafny, R. (2003). Functional analysis of the grapevine virus A genome. *Virology* 306, 42-50..
- Galiakparov, N., Goszczynski, D. E., Che, X., Batuman, O., Bar-Joseph, M., and Mawassi , M. (2003). Two classes of subgenomic RNA of *Grapevine virus A* produced by internal controller elements. *Virology* 312, 434-448.
- Wang, Q., Mawassi, M., Sahar, N., Li, P., Violeta, C., Gafny, R., Sela, I., Tanne, E., Perl, A. (2003). Cryopreservation of grapevine (*Vitis* spp.) embryonic cell suspensions by encapsulation-vitrification. *Plant Cell. Tissue and Organ Culture* 4550, in press.
- Wang, Q., Li, P., Batuman, O., Gafny, R., and Mawassi , M. (2003). Effect of benzyladenine on recovery of cryopreserved shoot tips of grapevine and citrus cultured *in vitro*. *Cryo-Letters* 24, 293-302.
- Galiakparov, N., Tanne, E., Mawassi , M., Gafny, R., and Sela, I. (2003). ORF 5 of *Grapevine virus A* encodes a nucleic acid-binding protein and affects pathogenesis. *Virus Genes* (in press).