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**Etiology of the Rugose Wood Disease of Grape-
vine and Molecular Study of the Associated
*Trichoviruses***

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Project Title: Etiology of the rugose wood disease of grapevine and molecular study of the associated *trichoviruses*

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Abbreviations used in the report, in alphabetical order:

CP: coat protein, **GFP:** green fluorescent protein, **GVA:** grapevine virus A, **MP:** movement protein, **OR** open reading frame

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Refereed (published, in press, accepted)			2	2
Submitted, in review, in preparation			1	1
Invited review papers				
Book chapters				
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Master theses			2	2
Ph.D. theses			1	1
Abstracts			3	3
Not refereed (proceedings, reports, etc.)			1	1

Patent Summary (numbers)

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Submitted				
Issued (allowed)				
Licensed				

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings			1	1
Sabbaticals				
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- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

Unfortunately there was little cooperation between the two laboratories

Abstract

Rugose wood is a complex disease of grapevines, characterized by modification of the woody cylinder of affected vines. The control of rugose wood is based on the production of healthy propagation material. Detection of rugose wood in grapevines is difficult and expensive: budwood from tested plants is grafted onto sensitive *Vitis* indicators and the appearance of symptoms is monitored for 3 years. The etiology of rugose wood is complex and has not yet been elucidated. Several elongated clostero-like viruses are consistently found in affected vines; one of them, grapevine virus A (GVA), is closely associated with Kober stem grooving, a component of the rugose wood complex. GVA has a single-stranded RNA genome of 7349 nucleotides, excluding a polyA tail at the 3' terminus. The GVA genome includes five open reading frames (ORFs 1-5). ORF 4, which encodes for the coat protein of GVA, is the only ORF for which the function was determined experimentally.

The original objectives of this research were: 1- To produce antisera to the structural and non-structural proteins of GVA and GVB and to use these antibodies to establish an effective detection method. 2- Develop full length infectious cDNA clones of GVA and GVB. 3- Study the roll of GVA and GVB in the etiology of the grapevine rugose wood disease. 4- Determine the function of *Trichovirus* (now called *Vitivirus*) encoded genes in the virus life cycle.

Each of the ORFs 2, 3, 4 and 5 genes of GVA were cloned and expressed in *E. coli* and used to produce antisera. Both the CP (ORF 4) and the putative MP (ORF 3) were detected with their corresponding antisera in-GVA infected *N. benthamiana* and grapevine. The MP was first detected at an early stage of the infection, 6-12 h after inoculation, and the CP 2-3 days after inoculation. The MP could be detected in GVA-infected grapevines that tested negative for CP, both with CP antiserum and with a commercially available ELISA kit. Antisera to ORF 2 and 5 encoded proteins could react with the recombinant proteins but failed to detect both proteins in GVA infected plants.

A full-length cDNA clone of grapevine virus A (GVA) was constructed downstream from the bacteriophage T7 RNA polymerase promoter. Capped *in vitro* transcribed RNA was infectious in *N. benthamiana* and *N. clevelandii* plants. Symptoms induced by the RNA transcripts or by the parental virus were indistinguishable. The infectivity of the *in vitro*-transcribed RNA was confirmed by serological detection of the virus coat and movement proteins and by observation of virions by electron microscopy. The full-length clone was modified to include a gus reporter gene and gus activity was detected in inoculated and systemic leaves of infected plants. Studies of GVA mutants suggests that the coat protein (ORF 4) is essential for cell to cell movement, the putative movement protein (ORF 3) indeed functions as a movement protein and that ORF 2 is not required for virus replication, cell to cell or systemic movement. Attempts to infect grapevines by *in-vitro* transcripts, by inoculation of cDNA construct in which the virus is derived by the CaMV 35S promoter or by approach grafting with infected *N. benthamiana*, have so far failed.

Studies of the subcellular distribution of GFP fusion with each of ORF 2, 3 and 4 encoded protein showed that the CP fusion protein accumulated as a soluble cytoplasmatic protein. The ORF 2 fusion protein accumulated in cytoplasmatic aggregates. The MP-GFP fusion protein accumulated in a large number of small aggregates in the cytoplasm and could not move from cell to cell. However, in conditions that allowed movement of the fusion protein from cell to cell (expression by a PVX vector or in young immature leaves) the protein did not form cytoplasmatic aggregates but accumulated in the plasmodesmata.

Achievements

The main achievement in this project is the construction of a full-length cDNA of GVA. This was the first report of such a cDNA clone of any member of the *vitivirus* genus. The full-length cDNA clone from which infectious transcripts of GVA can be produced is a valuable tool that makes the genetic analysis of GVA feasible. One specific question that can now be addressed concerns the role of the 20 kDa. protein encoded by ORF 2. Since no homologous gene was found in viruses outside the *vitivirus* group, no information is available as to the function of this protein. Initial experiments utilizing ORF 2 mutants demonstrate that this ORF is dispensable for replication (the mutants replicate in protoplasts) and for cell to cell or long distance movement (Gus activity detected in inoculated *N. benthamiana* plants). Another intriguing question concerns the transmission of GVA by mealybugs: GVA is transmitted by several species of mealybugs, and *Pseudococcus longispinus* has been reported to transmit GVA in a semi-persistent manner. However, no information is available regarding the GVA proteins participating in or required for the transmission process.

GVA is closely related with Kober stem pitting of the rugose wood complex, but the Koch postulates could not be fulfilled. GVA is usually found in grapevines in a mixed infection with other filamentous viruses. Furthermore, purified GVA cannot be mechanically inoculated to grapevine. For these reasons, the exact role of GVA (and other *vitiviruses*) in the rugose wood syndrome is not clear. With an infectious cDNA clone at hand, alternative methods for inoculation, such as agroinfection or biolistic inoculation can be tested to determine whether the cloned virus can infect grapevine. Successful inoculation of grapevine would permit direct study of the role of GVA in the rugose wood disease. Our attempts to inoculate grapevine plantlets with virus derived from the infectious clone failed. However further experiments should be carried out, including the testing of other inoculation methods.

In this project we have devised research tools that put us in a position to gain basic knowledge on *vitivirus* biology and solving applied problems such as diagnosis and control. These tools include: Four recombinant proteins expressed and purified from *E. coli*. Improved antisera for detection of the coat protein and the movement protein. GFP fusion of 3 GVA ORFs and a full length cDNA from which infectious RNA can be produced. Using transient expression assay and expression by means of PVX vector, we

determined the subcellular localization of GFP fused to the GVA proteins encoded by ORFs 2, 3 and 4.

The antiserum for the GVA-MP proved to be a valuable diagnostic tool. Immunoblot analysis showed that the MP provides a sensitive mean of detection of GVA in infected grapevines. The MP antiserum proved to be superior both to the CP antiserum and to a commercially available ELISA kit based on anti-GVA antiserum. This is the first demonstration that a non-structural protein may be the preferred target for serological detection of a plant virus. While the CP of elongated plant viruses is present in several thousand molecules per genome, the non-structural proteins are thought to be produced in much smaller quantities. There may be several reasons for the more efficient detection of the MP than of the CP. The GVA-MP may be a more stable protein, which accumulates in infected cells while the CP concentration decreases as the virus titer decreases; alternatively, the MP may be more immunogenic, resulting in better antiserum production in the vaccinated animal.

Background

Rugose wood is a complex disease of grapevines, characterized by modification of the woody cylinder of affected vines. There are four different disorders, which show similar symptoms in the field, but which can be distinguished by indexing on specific *Vitis* indicators. The woody cylinder is typically marked by pits and/or grooves, which may occur on the scion, rootstock or both. Affected vines have impaired growth vigor and delayed bud opening, and they may decline and die within a few years (1). The control of rugose wood is based on the production of healthy propagation material (1). Detection of rugose wood in grapevines is difficult and expensive: budwood from tested plants is grafted onto sensitive *Vitis* indicators and the appearance of symptoms is monitored for 3 years (1). Much effort has been invested in developing serological and nucleic acid-based methods for detection of viruses implicated in the rugose wood complex (2,3).

The etiology of rugose wood is complex and has not yet been elucidated. Several elongated clostero-like viruses are consistently found in affected vines; one of them, grapevine virus A (GVA), is closely associated with Kober stem grooving, a component of the rugose wood complex (4,5). Initially, serological detection of GVA was found difficult because of the low immunogenicity of the virus and low concentration of particles in affected plants, but recently the method has been improved by the

introduction of monoclonal antibodies (6). PCR-based detection protocols have been developed which resulted in the more sensitive detection of GVA (4,7,8).

The genus *Vitivirus* include four definite species: grapevine virus A (GVA), grapevine virus B (GVB), grapevine virus D (GVD), and heracleum latent virus (HLV). Grapevine virus C (GVC) is a tentative species in the genus (9).

GVA has a single-stranded RNA genome of 7349 nucleotides, excluding a polyA tail at the 3' terminus. The GVA genome includes five open reading frames (ORFs) (Fig. 1). ORF1 encodes a 194 kDa polypeptide with conserved motifs of replication-related proteins of the "Sindbis-like" supergroup of positive-strand ssRNA viruses. ORF2 encodes a protein of 19 kDa, with no significant homology with any other known proteins. ORF 3 encodes a polypeptide of 31 kDa, with amino acid similarity to putative movement proteins (MPs) from the 30 K superfamily. The protein encoded by ORF 4 is the coat protein (CP). ORF 5 encodes a small protein of 10 kDa with no homology to other proteins. However, the corresponding ORF 5 of the closely related *Vitivirus* grapevine virus B (GVB) shares homology with small, 3' terminal polypeptides of various plant viruses that contain the zinc finger domain of nucleic acid binding proteins. To date, the coat protein gene is the only GVA gene to which a function has been experimentally assigned (10,11).

Methodologies and Materials

Standard molecular techniques were used for cDNA synthesis, amplification and cloning, for *in vitro* transcription, mechanical inoculation and for RNA and protein detection. The genes encoding ORFs 2-5 of the PA3 isolate of GVA were each cloned by means of RT-PCR and the corresponding proteins expressed in *E. coli*. Antisera to the recombinant proteins were raised in rabbits.

Construction of a full-length GVA-cDNA was done by simultaneous assembly of a number of independent full-length cDNA clones as described Yu and Wong (12) (Fig. 8).

The ORFs 2, 3 (putative MP), 4 (CP) and 5 were cloned in an expression vector between the CaMV 35S promoter and a transcription terminator as a fusion protein with GFP. The expression vector was constructed by modification of the multiple cloning sites of the pCASS 2 vector (13). Each of the constructs was devised to express the corresponding GVA proteins fused at its C-terminus to the N-terminus of GFP. Plasmid DNA was delivered to *N. benthamiana* leaves by particle bombardment and

examined 24 h later under an UV-fluorescence microscope and a confocal microscope. The genes encoding each of the ORFs fused to the sGFP gene were also cloned in a full-length cDNA clone of PVX and expressed in *N. benthamiana* plants by means of PVX infection.

Results and Discussion

Serological detection of grapevine virus a using antiserum to a non-structural protein, the putative movement protein (Israel).

The CP and MP genes of GVA isolate PA3 were cloned. The nucleotide sequences are accessible as Accession no. AF007415 at the GenBank database. The deduced amino acid sequences are presented in Figure 2. Comparison with the Is 151 isolate of GVA (10) revealed 88 and 78% identity of the CP and MP nucleotide sequences, respectively. The amino acid sequences of the CPs of the two GVA isolates have 97% identity and 99% similarity of amino acid sequences while the MPs share only 80% identity and 92% similarity.

Antisera induced in response to the two *E. coli*-produced proteins reacted specifically with GVA CP and the MP in infected *N. benthamiana* plants, as shown by Western blot analysis (Fig. 3).

To monitor the accumulation of GVA in infected tissue, inoculated leaves of *N. benthamiana* were collected at different times after inoculation, and total proteins were extracted and analyzed by immunoblotting (Fig. 3). The MP was first detected 0.5 day after inoculation, whereas the CP was first detected 3 days after inoculation. The specific time after inoculation at which the GVA proteins were detected varied between experiments. In several experiments the MP was detected 6 to 12 h after inoculation while the CP was detected 1 to 3 days after inoculation.

In order to examine the subcellular distribution of the GVA-MP, inoculated leaves were fractionated, 11 days after inoculation, into cell wall (CW), soluble proteins (S30), membrane- or organelle-bound proteins (P30), and Triton wash (Tw) fractions. The GVA-MP protein was detected in all fractions but was consistently most abundant (per gram fresh weight) in the S30 fraction (data not shown). Using an alternative method previously employed by Sato et al. (14) to study the subcellular distribution of *apple chlorotic leaf spot trichovirus*, protein extracts were fractionated to cell membrane, soluble and cell wall fractions. Again, most of the GVA MP was detected in the soluble protein fraction (data not shown).

Phloem shavings of rugose wood-affected grapevines (cv. Superior) from the Jordan Valley were extracted with ESB buffer and subjected to immunoblot analysis. Both the MP and the CP were detected in extracts of plants that showed symptoms (Fig. 4). In each case where GVA could be detected, the reaction with the MP antiserum appeared as a stronger band relative to the detection signal obtained with the CP antiserum (Fig. 4). GVA was not detected in three out of the eight symptomatic vines tested (Fig. 4). This may have been because of uneven distribution of GVA in the vine or low concentration of the virus, or because the plants were not infected by GVA. In a similar test of corky rugose wood-affected vines (cv. Thompson seedless) collected in the Lachis region, the MP could be detected in all five vines tested, while the CP could not be detected in the same extracts (Fig. 5). The same plants were also tested with a commercial ELISA kit for the detection of GVA (based on anti-GVA CP antiserum) and all five plants tested negative.

Figure 6 shows that phloem rich tissues - phloem shavings, petioles and older leaf veins - were the best source of material for testing for the presence of GVA-MP in grapevines. The MP was not detected in young leaves or in developing fruits.

To test whether detection of GVA by means of the MP antiserum is virus-specific, proteins were extracted from a grapevine (cv. Maressane), which had been tested by PCR and found positive for GVB and negative for GVA (data not shown). These proteins were tested with GVA-MP antiserum, which did not react with GVB (Fig. 7).

The protein encoded by ORF3 was hypothesized to be the MP because of its amino acid similarity to other viral MPs of the 30-kDa. family (10). The MP was detected at an early stage of infection in the inoculated leaves of *N. benthamiana* plants. Early expression of MPs was reported for other viruses such as cucumber mosaic virus (CMV) (15), alfalfa mosaic virus (16) and tobacco necrosis virus (17). In the present study, the GVA MP was detected, using two alternative cell-fractionation protocols, in all fractions but mainly in the soluble protein fraction (S30), whereas the MPs of several other plant viruses have been detected primarily in the cell-wall fraction (18-21). Recently it was demonstrated, by means of the same extraction methods used in the present work, that the distribution of CMV and TMV MP in infected plants is complex: the distribution was found to differ among viruses, among leaf positions and also between transgenic plants and virus infected-plants (22). More detailed study of

GVA-MP localization during different stages of infection is needed to determine whether GVA-MP has a distinct subcellular distribution compared with other viral MPs.

Immunoblot analysis showed that the detection of the putative MP may provide a sensitive means of detection of GVA in infected grapevines. The MP antiserum proved to be superior both to the CP antiserum and to a commercially available ELISA kit based on anti-GVA antiserum. To our knowledge this is the first demonstration that a non-structural protein may be the preferred target for serological detection of a plant virus. While the CP of elongated plant viruses is present in several thousand molecules per genome, the non-structural proteins are thought to be produced in much smaller quantities. There may be several reasons for the more efficient detection of the putative MP than of the CP: the GVA-MP may be a more stable protein, which accumulates in infected cells while the CP concentration decreases as the virus titer decreases. Alternatively, the MP may be more immunogenic, resulting in better antiserum production in the vaccinated animal.

Serological relations between GVA and GVB were recently demonstrated using antisera to GVA and GVB in Western-blot (23). Cross-reactivity was not detected by means of immunosorbent electron microscopy or ELISA (23,24). The finding that GVA-MP antiserum did not detect GVB in infected vine indicate additional advantage of using the MP as an antigen for detection of GVA infection.

Serological detection of GVA is difficult because of low virus accumulation, uneven distribution of the virus in the host tissue, and poor immunogenicity of the virus (25). The production of monoclonal antibodies to GVA and the construction of an ELISA method based on protein A sensitized plates provided more efficient serological detection of GVA (6,25). Recently developed PCR and IC-PCR detection methods for GVA ensure much more sensitive detection of GVA in infected vines (3). Although less sensitive than PCR, serological methods such as ELISA or dot-blot assays are more suitable for routine, large-scale indexing of plant viruses (25).

Infectious RNA transcripts from grapevine virus A cDNA clone (Israel).

To facilitate the construction of a full-length cDNA clone of the PA3 isolate, the 5' and 3' ends of the genome were cloned and sequenced. Sequence comparison showed no differences between the two GVA isolates in nucleotides 1 to 22, the sequence used to design the 5' oligonucleotide primer. Differing nucleotides were found at three positions in the 3' end of the virus used for the construction of primer the 3' primer. The

substitutions were C to A at position 7341, G to T at position 7344 and A to T at position 7346.

Initially, a full-length cDNA copy of the GVA genome downstream of the T7 RNA polymerase promoter was engineered in plasmid pPCR2.1, by sequentially linking restriction fragments from overlapping cDNA clones. However, *in vitro*-transcribed RNA from this clone was not infectious. We therefore employed the strategy termed "population cloning", devised by Yu and Wong (12), which allows the simultaneous assembly of a number of independent full-length cDNA clones.

Fig. 8 illustrates the strategy for construction of the full-length cDNA clones. The amplified cDNAs representing the 5' and the middle sections of the genome were ligated with the plasmid vector in a single reaction. Three resultant clones were used independently for further addition of the 3' section, as described in Fig. 8. Nine full-length clones were pooled in three groups of three clones each, which were used as substrates for *in vitro* transcription. Inoculation of the *in vitro*-transcribed RNA indicated that each of the three groups contained at least one infectious clone. Examinations of individual clones indicated that four out of six clones tested produced infectious RNA transcripts. One clone, designated pGVAN3 was selected for further characterization.

When *N. benthamiana* was mechanically inoculated with *in vitro*-transcribed RNA from clone pGVAN3, symptoms appeared 7-8 days post-inoculation. The symptoms included vein clearing, leaf curling, and mottling. The symptoms were indistinguishable from those caused by the native virus. The time of symptom appearance was the same for plants inoculated with RNA transcripts, viral progenies of pGVAN3 or by the native virus.

GVA was detected serologically in systematically infected leaves of *N. benthamiana* inoculated by N3 transcripts. Protein extracts were subjected to Western blot analysis with antisera to the capsid protein or to the putative movement protein (12). Both proteins were detected in plants inoculated by pGVAN3 transcripts (Fig. 9). The serological analysis confirmed the infectivity of the N3 transcripts and indicated that both the structural coat protein and the non-structural movement proteins are produced by the cloned virus. To confirm that the cDNA-derived virus can be assembled into mature virions, partially purified preparations were examined by electron microscopy. The virions produced from inoculation with infectious RNA transcripts were indistinguishable from those of the parental virus.

The natural host range of GVA is restricted to grapevine. The experimental host range was also narrow and comprised two herbaceous species, *N. benthamiana* and *N. clevelandii* (29). Inoculation of *N. clevelandii* with N3 transcripts produced similar symptoms to those in plants inoculated with the parental virus, thus confirming the infectivity on the second experimental host of GVA.

The full-length cDNA clone from which infectious transcripts of GVA can be produced is a valuable tool that makes the genetic analysis of GVA feasible. One specific question that can now be addressed concerns the role of the 20-kDa. protein encoded by ORF 2. Since no homologous gene was found in viruses outside the *vitivirus* group, no information is available as to the function of this protein (9). Another intriguing question concerns the transmission of GVA by mealybugs: GVA is transmitted by several species of mealybugs, and *Pseudococcus longispinus* has been reported to transmit GVA in a semi-persistent manner (30). However, no information is available regarding the GVA proteins participating in or required for the transmission process.

GVA was found to be closely related with Kober stem pitting of the rugose wood complex, but the Koch postulates could not be fulfilled (4,5,31). GVA is usually found in grapevines in a mixed infection with other filamentous viruses. Furthermore, purified GVA cannot be mechanically inoculated to grapevine. For these reasons, the exact role of GVA (and other *vitiviruses*) in the rugose wood syndrome is not clear. With an infectious cDNA clone at hand, alternative methods for inoculation, such as agroinfection or biolistic inoculation can be tested to determine whether the cloned virus can infect grapevine. Successful inoculation of grapevine would permit direct study of the role of GVA in the rugose wood disease.

Expression of GVA encoded genes: identification of subgenomic RNA subcellular localization of the proteins (Israel).

Examination of the genome structure of GVA suggests that the internal genes (ORFs 2-5) are expressed from subgenomic RNAs. The different RNA species present in GVA infected *N. benthamiana* were detected by Northern blot analysis. Double and single stranded RNA preparations were hybridized with molecular probes prepared from various regions of the GVA genome. A cDNA probe spanning the 300 3' nucleotides detected several RNA species. Ranging in size from 0.75 to 7.5 kb. (Fig. 10). The various RNA molecules of 2.1, 1.4, 0.9 and 0.75 kb. may represent subgenomic RNAs for expression of ORFs 2, 3, 4 and 5 (Fig. 10). The northern blot analysis is not

sufficiently accurate to determine the exact size of the various RNA species. Further analysis using cDNA probes derived from the various GVA ORFs and primer extension analysis is being used to achieve a more precise mapping of the subgenomic RNAs.

Northern blots of dsRNA preparations from GVA infected *N. benthamiana* revealed 3 molecules of sizes 7.6, 6.4 and 5.6 kb. (Table 1). A cDNA probe derived from the 3' terminus of the genome (Fig. 10) also detected the larger molecule representing the genomic RF. However, the 6.4 and 5.6 kb. dsRNAs were detected by the 5' probe but not by the 3' probe, suggesting these RNA molecules have a 5'-coterminous. Further analysis mapped the 3' terminus of the 6.4 RNA to the movement protein gene and the 5.6 molecule to the ORF2 gene. German et al., (32) have detected similar 5'-coterminous RNA molecules produced during apple chlorotic leaf spot virus infection. The role of these RNAs during virus infection is not clear.

Expression of GVA encoded genes: subcellular localization of the proteins (Israel).

As described above we have prepared antisera to the coat protein and the movement protein of GVA and have used these antisera to determine the subcellular localization of the two proteins. Antisera against the products of ORF 2 and ORF 5 were also prepared and used to detect the proteins in *N. benthamiana* plants infected with GVA. Both antisera failed to identify the appropriate proteins, probably due to a low accumulation of the proteins in the plants or low immunogenicity of the proteins.

To gain information on possible functions of the GVA encoded proteins, such as cell to cell movement, or on their subcellular localization, we have applied a transient expression assay of GFP-fusion proteins. ORFs 2, 3 (putative MP), 4 (CP) and 5 were cloned in an expression vector between the CaMV 35S promoter and a transcription terminator as a fusion protein with GFP. Each of the constructs was devised to express the corresponding GVA proteins fused at its C-terminus to the N-terminus of GFP. Plasmid DNA was delivered to *N. benthamiana* leaves by particle bombardment and examined 24 h later under UV-fluorescence microscope and a confocal microscope. Each of the genes was also cloned in an "infectious cDNA clone" of PVX. The PVX constructs were used as templates for transcription and the infectious RNAs were introduced to *N. benthamiana* by mechanical inoculation.

Free GFP was distributed in the cytoplasm and the nucleus. CP-GFP had a similar distribution as the free GFP with no specific subcellular localization. The ORF2-GFP fusion protein was cytoplasmatic but each cell contained 2-5 aggregates, possibly

located in the cytoplasm. The ORF2-GFP protein was absent or showed weak fluorescence in the nucleus, suggesting a certain type of localization. The MP-GFP fusion accumulated in numerous aggregates.

When the putative movement protein (ORF 3) fused to GFP was expressed in source type cells no movement from cell to cell was detected. The fused protein accumulated in the cytoplasm in a structure of small aggregates and occasionally near the cell wall. When the same fused protein was expressed in sink type cells or by the PVX vector there was movement of the protein from one expressing cell to others. Whenever movement occurred, there were no aggregates in the cell cytoplasm and the fused proteins tended to localize in a rod shape structure, transversing the cell walls. The bridge-like structures were most probably plasmodesmata. (Fig. 11)

These results support the assumption that the protein encoded by ORF 3 plays a role in the virus movement from cell to cell. Moreover, similar to other members of the 30 K superfamily ORF 3 product is localized to the plasmodesmata. However, the results indicate that in order to accumulate in the plasmodesmata and move from one cell to another, the movement protein of GVA might need the help of other viral components. Experiments with GVA-CP mutants described below, support this suggestion by showing that both the coat protein and the movement protein are required for cell to cell movement.

The fused proteins (ORFs 2, 3 and 4) were expressed in three *Nicotiana* species: *N. benthamiana*, *N. clevelandii* and *N. tabacum*. The first two are hosts of GVA while the last is a non-host. No differences in subcellular localization or accumulation were detected among the 3 species.

Assigning functions to GVA genes by site specific mutagenesis of the full-length cDNA clone (Israel).

The coat protein gene is the only GVA gene for which a function was experimentally assigned. Based on amino acid sequence homologies ORF 1 was suggested to encode the viral replicase and ORF 3 to encode the movement protein. No functions were suggested to the proteins encoded by ORF 2 and 5. The full length "infectious" cDNA was used to assign functions to the GVA encoded proteins. As a first step, mutations were introduced directly to the full-length cDNA and transcripts were inoculated to *N. benthamiana* protoplasts and plants. At a later phase, the GUS gene was introduced to the full-length clone as a marker gene. The expression of the

GUS gene enabled us to follow the virus spread in inoculated and systemic leaves. The various mutants tested so far and the effect of each mutation on GVA replication and movement are described in Fig. 12.

The results indicate that ORFs 2, 3 and 4 are dispensable for replication in protoplasts. In leaves inoculated by the mutant carrying gus insertion in ORF 3, GUS activity was detected only in single cells. These results demonstrate that the ORF 3 encoded protein indeed functions as a movement protein.

GUS activity was detected in both inoculated and systemic leaves of *N. benthamiana* plants inoculated with fs-ORF 2-gus or ns-ORF 2-gus transcripts, suggesting that the ORF 2 encoded protein is not required for both cell to cell and long distance movement (Figure 12). Further constructs with various mutations in ORF 2 are under construction and these will be used to explore the function of this gene.

GVA mutants lacking the CP gene (ORF 4) could replicate in protoplasts but could infect only single cells in inoculated plants, indicating that the CP functions in cell to cell movement.

Pathogenesis and movement of GVA in *N. benthamiana* (USA)

Inoculation of *in vitro* synthesized transcripts of GVA to *N. benthamiana* resulted in the development of systemic infection displaying severe mosaic symptoms. Virus was purified from these symptomatic leaves and found to contain flexuous rods characteristic of GVA. Availability of full-length cDNA clone for GVA is ideal for testing the genes that promote cell-to-cell and long distance movement characteristics. Green fluorescent protein (GFP) has been successfully used to monitor viral movement in many viral systems. We exploited this available technology to investigate and identify viral genes that promote short and long distance spread of GVA in *N. benthamiana*. We fused GFP sequences to putative movement protein gene using available restriction sites. Inoculation of *in vitro* transcripts containing the GFP fusion did not result in visible infection sites. Therefore we are now using several other alternative approaches to construct a biologically viable clones harboring GFP.

Replication of GVA in protoplasts: *N. benthamiana* protoplasts were isolated from approximately 3 weeks old plants using a procedure adapted to isolate protoplasts from barley plants. Protoplasts digested with enzyme were purified on sucrose and suspended in 0.55M mannitol, pH 5.9. Approximately 3×10^5 protoplasts were transfected with GVA RNA using PEG. Transfected protoplasts were kept under lights

for at least 24 hr prior to isolating RNA or virions. It was observed that more than 90% protoplasts were infected using the above procedure. Electron microscopic examination revealed the presence of numerous virions characteristic of GVA.

In vivo labeling of proteins synthesized during replication of GVA: One of versatile way of examining various proteins synthesized during replication of a given virus is to incorporate ³⁵S-Methionine into trasfected protoplasts. We have using this procedure to identify various proteins synthesized during GVA replication. Following PEG mediated protoplast transfection with transcripts of GVA, ³⁵S-Methionine was added to culture medium and incubated for 24 hr. Transfected protoplasts solution was mixed with a solution containing SDS, boiled for 5 min and subjected to fractionation of 16% PAGE. The gel was then dried and exposed to X-ray film to detect ³⁵S-Methionine proteins. The results indicate coat protein was the major component synthesized while other proteins could not be identified with confidence. We are currently refining this technique further to characterize other viral proteins synthesized during replication of GVA.

Coat protein defective clones replicate efficiently in protoplats: We synthesized RNA transcripts from a variant clone of GVA in which the coat protein gene was deleted. These RNA transcripts were inoculated to *N. benthamiana* protoplasts. Northern blot analysis of progeny RNA recovered from these transfected protoplasts revealed that replication of GVA proceeded efficiently and indistinguishable from that of wild type transcripts.

Coat protein of GVA is indispensable for whole plant infections: In vitro synthesized transcripts of GVA lacking the coat protein gene sequences were inoculated to *N. benthamiana* plants to verify whether coat protein is required for efficient movement. None of the inoculated plants displayed any visible symptoms. Northern blot analysis of progeny RNA recovered from these plants did not reveal accumulation of RNA characteristic of GVA.

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Publications resulting from the project

Rubinson, E., Galiakparov, N., Radian, S., Sela, I., Tanne, E. and Gafny, R..(1997) Serological detection of grapevine virus A using antiserum to a non-structural protein, the putative movement protein. *Phytopathology* 87(10): 1041-1045.

Galiakparov, N., Sela, I., Tanne, E. and Gafny, R. (1999) Infectious RNA transcripts from grapevine virus A cDNA clone. *Virus Genes* 19(3): 235-242.

Data Appendix

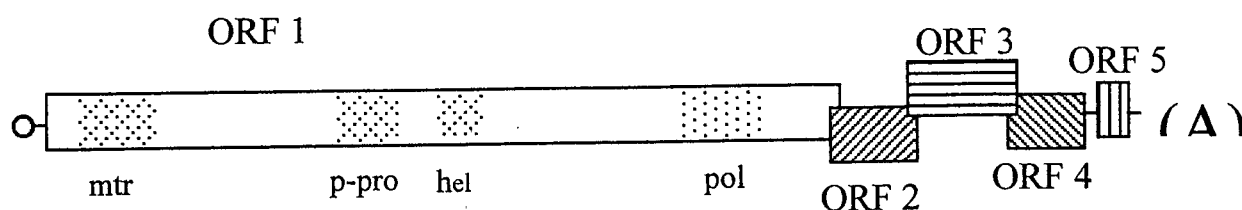


Figure 1. Genomic organization of grapevine virus A (GVA). ORF – open reading frame, mtr – methyl transferase, p-pro – papain proteinase, hel – helicase, pol – polymerase

A Coat protein

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MAHYAKRVEIRAIIEELVLAKATPTEDASESGYDRNMYLNTLFGYIALVG
-----Q--D-----

TSKKAVHYGEVDIVGPKASKKTGIDPRGKMVSELVGRMRTLSVAVSEGP
-----I-----

VKGATLRQMCEPFAQNAIDFLVLAEMGTYSQLATKMTRSGFKEPQVMFD
-----V-----

FASGLDLKALTQLQEAIVIAMHSRLFRTEGAKGVFNAQPSVGEQAVEI
-----S-I-----

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B Movement protein

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MSQEDSQATKGSPSPDPEIKIFNVTRNTKDLETNLSLHRGDVYDTIELIE
---G-LG---A-SFE-QD--V-H-K-S-R-----N-----

RVFPRRTKKCVVHKELVVQDGRVDCELDMDGGLDDIDETEPYLYHVGCI
K-----I---I-K-----D-I--E-----N-E-F-----

VVALMPHGKNLQGVVVEIDTRLQGWVRAGSLG.AVMDMSKPLSACADF
-----S---L---VD..G-SRISRTL-----

PGYFISTDLLNGYTLHLSITTTDLQFVDGVHPFSVQLMNIGRLCGDDMK
-----S---F--E---

TRYAVGEASKILHQNILNSQGDGEMIPRGVQVQKVPDTLVMPEVYETIKR
---IT-T--M-----TE----L-----F---R

LGLKTNGTLRQEGGDKGDRGTGAGESHAN
F-----R---N-RV-V---PT-

```

Figure 2. Comparison of the amino acid sequences of A, the coat protein and B, the movement protein of GVA-PA3 (upper sequence) and GVA-Is 151 (26) (lower sequence). Alignment was generated using the UWGCG Gap program (10). Dashes indicate identical amino acids; dots indicate gaps introduced for optimal alignment.

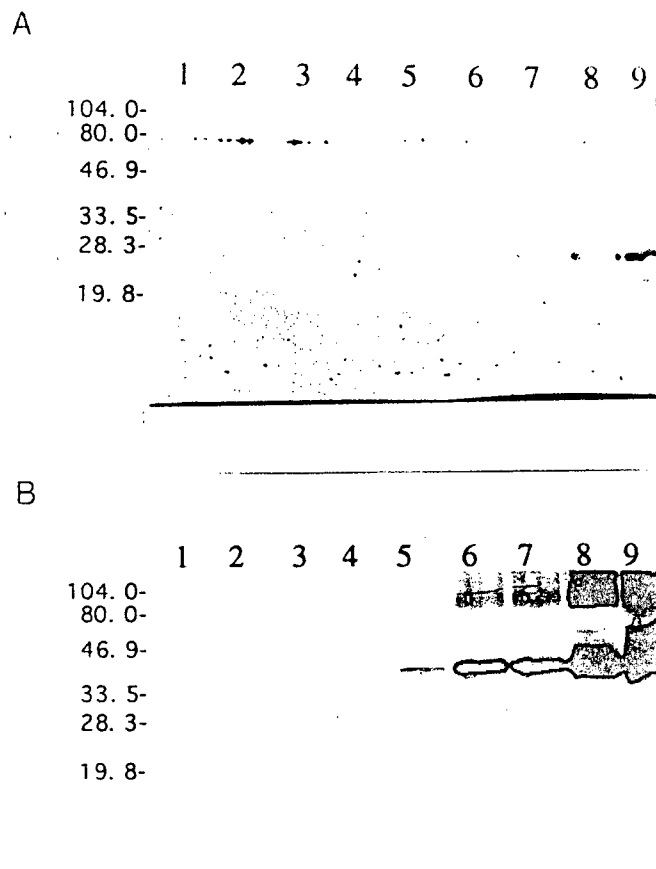
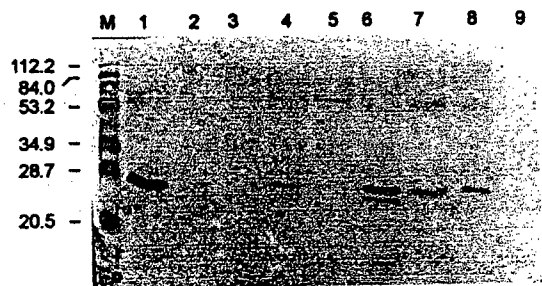


Figure 3. Time course of accumulation of GVA proteins in the inoculated leaf of *N. benthamiana* plants. **A**, immunoblot analysis using the coat protein antiserum. **B**, immunoblot analysis using the movement protein antiserum. Leaf tissue was extracted before inoculation (lane 1) and 12 h (lane 2), 1 day (lane 3), 2 days (lane 4), 3 days (lane 5), 4 days (lane 6), 5 days (lane 7), 7 days (lane 8) and 11 days (lane 9) after inoculation. Numbers on the left indicate the positions of molecular weight markers. The MP was detected, on the western blot membrane, as a faint band at 12 h (lane 2) which can not be discern in the photograph (**B**).

A



B

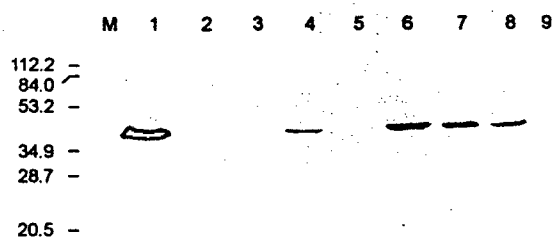
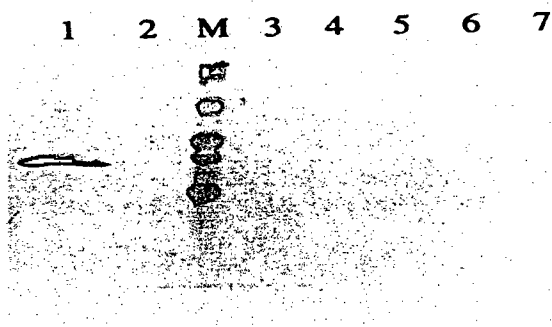


Figure 4. Detection of GVA in infected grapevines. Proteins were extracted from phloem scrapings of: lanes 1 to 8, grapevines (cv. Superior) showing stem pitting symptoms, collected in the Jordan Valley; lane 9, healthy vine used as control; lane M, molecular weight markers. **A**, immunoblot analysis using the coat protein antiserum. **B**, immunoblot analysis using the movement protein antiserum.

A



B

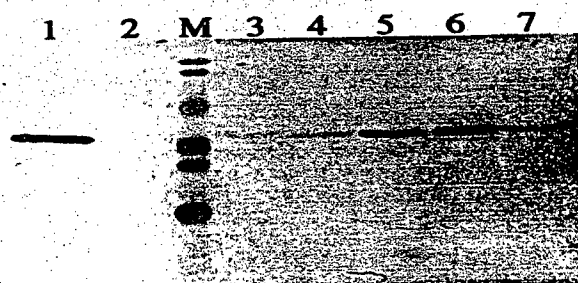


Figure 5. Detection of GVA in infected grapevines. Lane 1, proteins extracted from GVA-infected *N. benthamiana* plants; lane 2, proteins extracted from healthy *N.*

benthamiana plants (used as positive and negative controls, respectively); lanes 4 to 8, proteins were extracted from phloem scrapings of grapevines (cv. Thompson seedless) showing corky rugose wood symptoms, collected in the Lachish region; lane M, molecular weight markers. A, immunoblot analysis using the coat protein antiserum. B, immunoblot analysis using the movement protein antiserum.

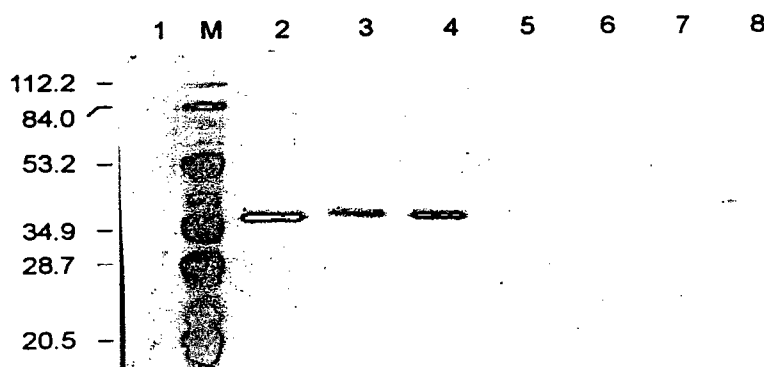


Figure 6. Immunoblot of total proteins and detection using antiserum to GVA movement protein. Lane 1, petioles of greenhouse grown, healthy vine used as a control; lanes 2 to 7, extracts from field-grown rugose wood-affected grapevine (cv. Superior): lane 2, phloem scrapings; lane 3, petioles; lane 4, veins of mature leaves; lane 5, young leaves; lane 6, cluster pedicles; lane 7, developing fruits. Lane 8, petioles from greenhouse grown vine used as a positive control. Lane M, molecular weight markers.

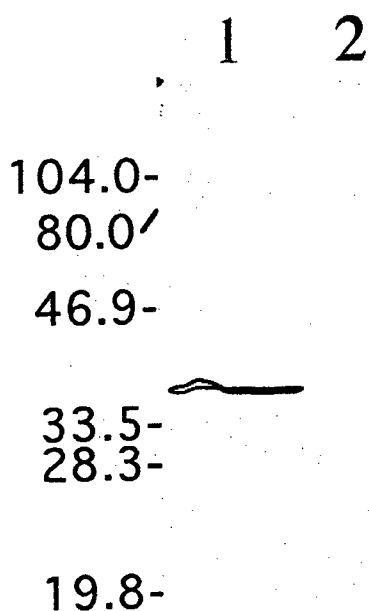


Figure 7. Immunoblotting of proteins extracted from phloem scrapings of: lane 1, GVA-infected grapevine (cv. Superior); lane 2, GVB-infected grapevine (cv. Maressane). Detection was performed with antiserum to GVA movement protein. Numbers on the left indicate the positions of molecular weight markers.

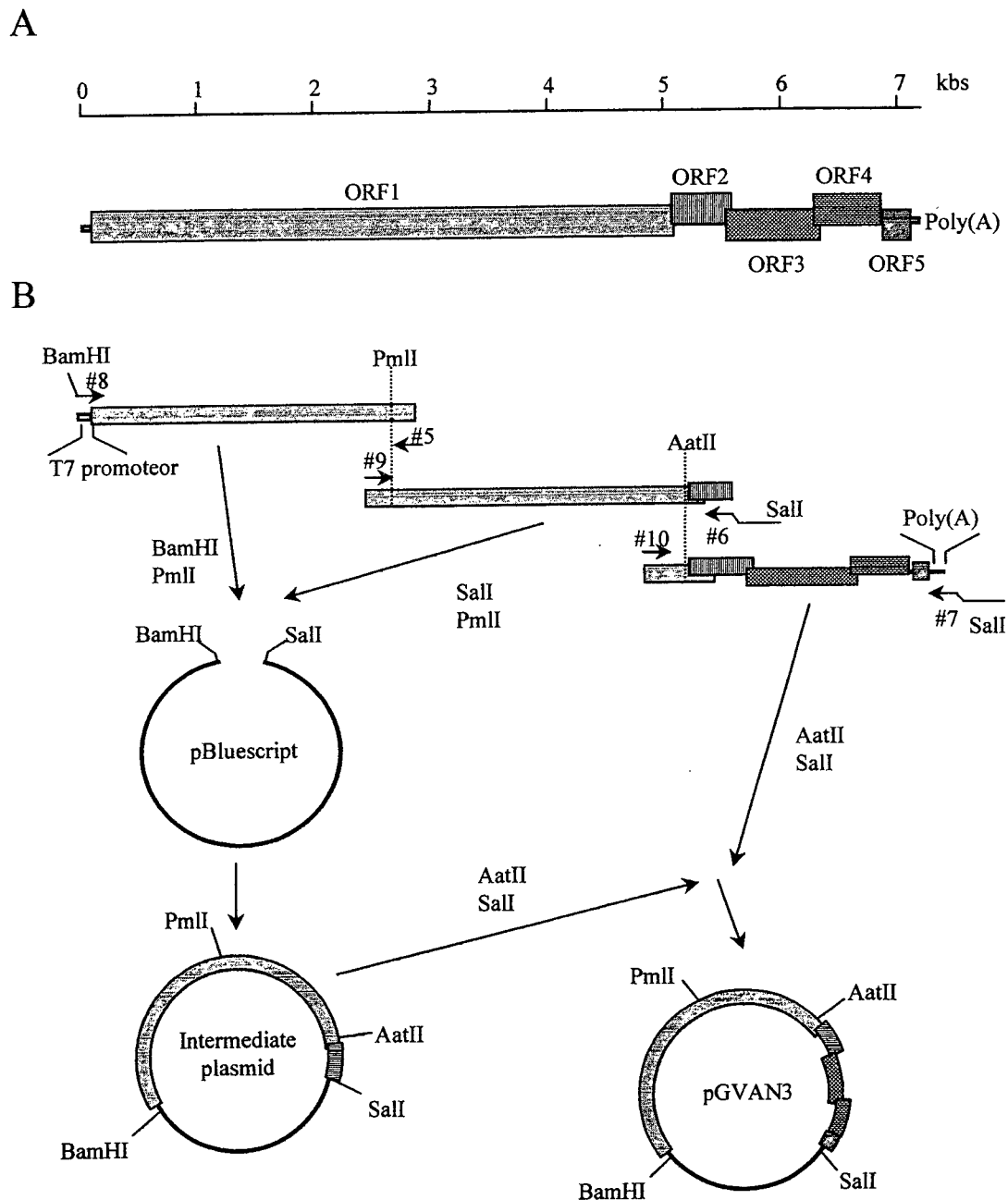


Figure 8. A- Schematic representation of the GVA genome (5,6). B- Construction of full-length cDNA clones of GVA. RT-PCR product representing the 5' and 3' sections were cloned into pBluescript by three-piece-ligation. Three resultant intermediate clones were used for construction of full-length clones by addition of the 3' section. For details see Materials and Methods.

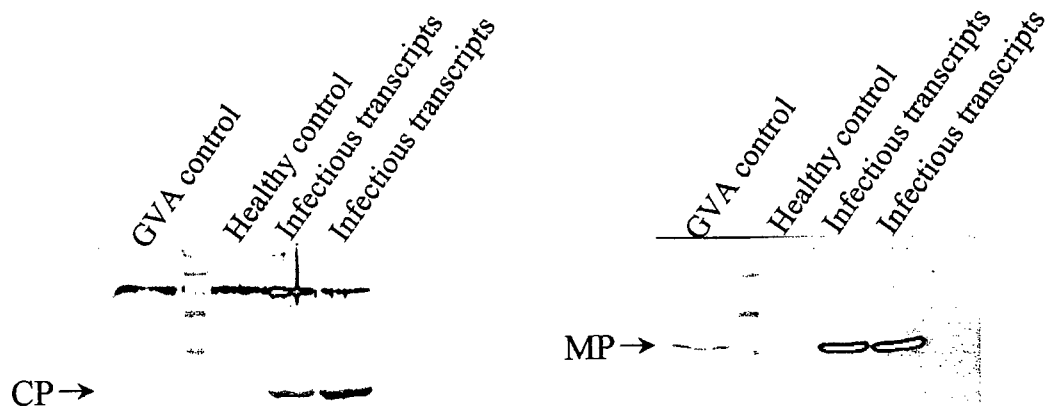


Figure 9. Detection of grapevine virus A movement protein (right panel) and coat protein (left panel) in *N. benthamiana* plants. Proteins extracted from uninfected plant or from plants inoculated with *in vitro*-transcribed RNA of cDNA clones pGVAN2 and pGVAN3. Proteins were detected by immunoblot analysis using antiserum to the movement protein or the coat protein.

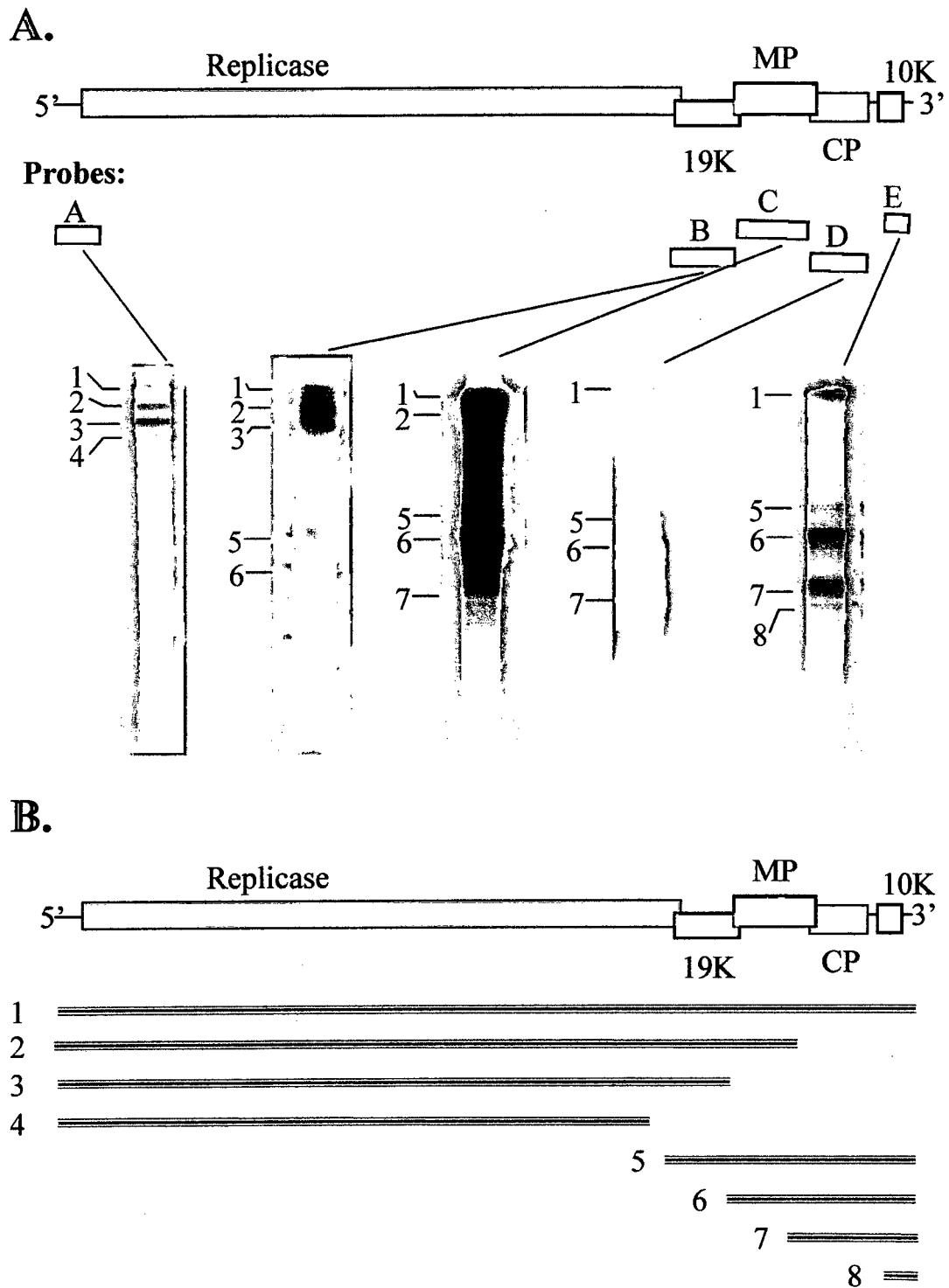


Figure 10. Subgenomic RNAs of GVA. A- Northern blot analysis of dsRNA extracted from GVA infected *N. benthamiana* plants. B- Schematic representation of the GVA subgenomic RNA molecules as interpreted from the Northern blots.

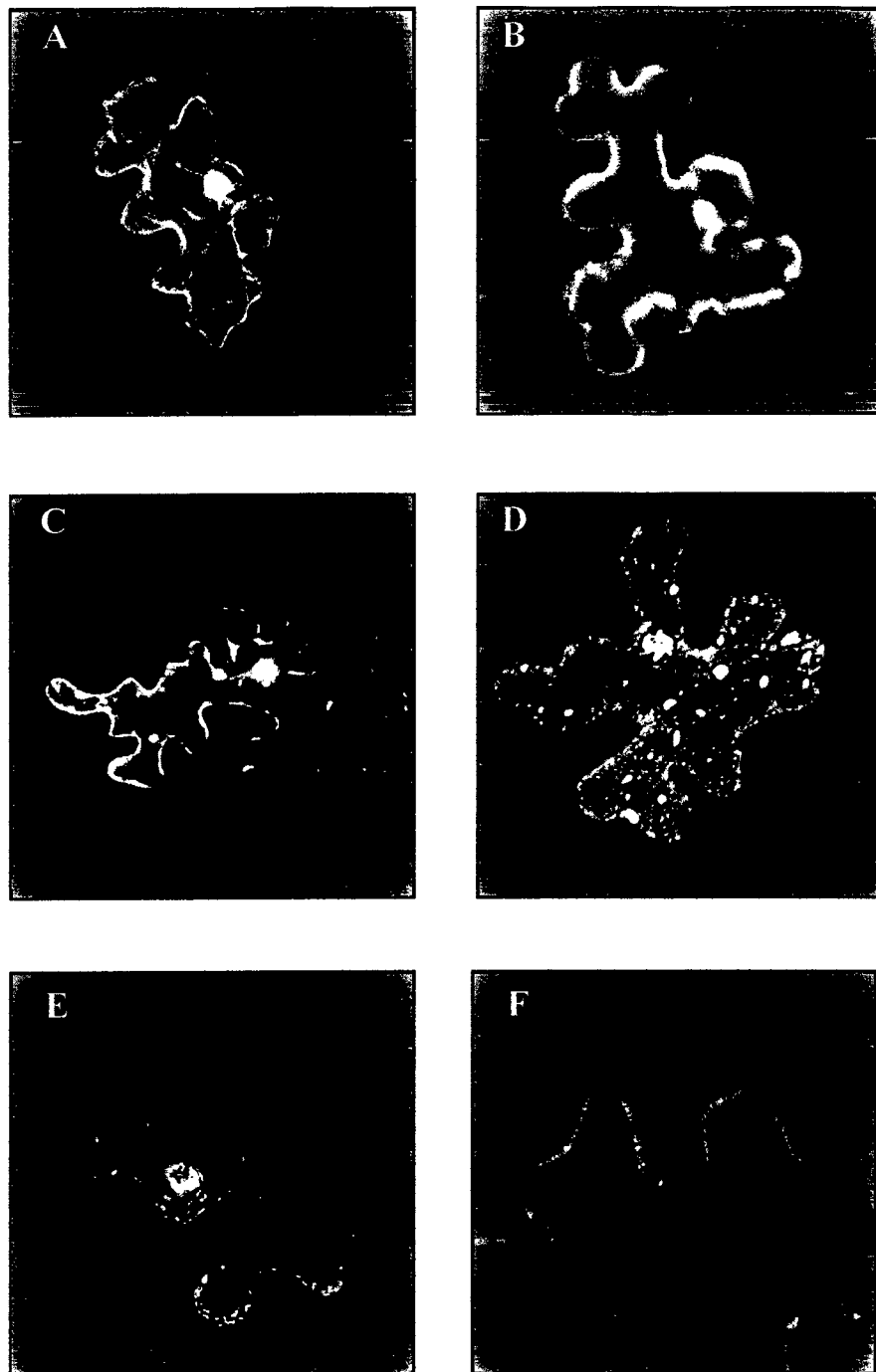


Figure 11. Accumulation of GFP fusion proteins in *N. benthamiana* mesophyll cells. (A)- free GFP., (B)- coat protein-GFP fusion., (C)- ORF 2-GFP fusion., (D), (E and F)- putative movement protein-GFP fusion. (A-D)- composite pictures of all the optical sections of each cell. (E, F) a single confocal section. (A-E) are cells from fully developed leaves, (F) a cell from young leaf. Plasmids carrying the respective gene constructs were introduced by particle bombardment. Cells were examined by a confocal microscope 24h after bombardment.

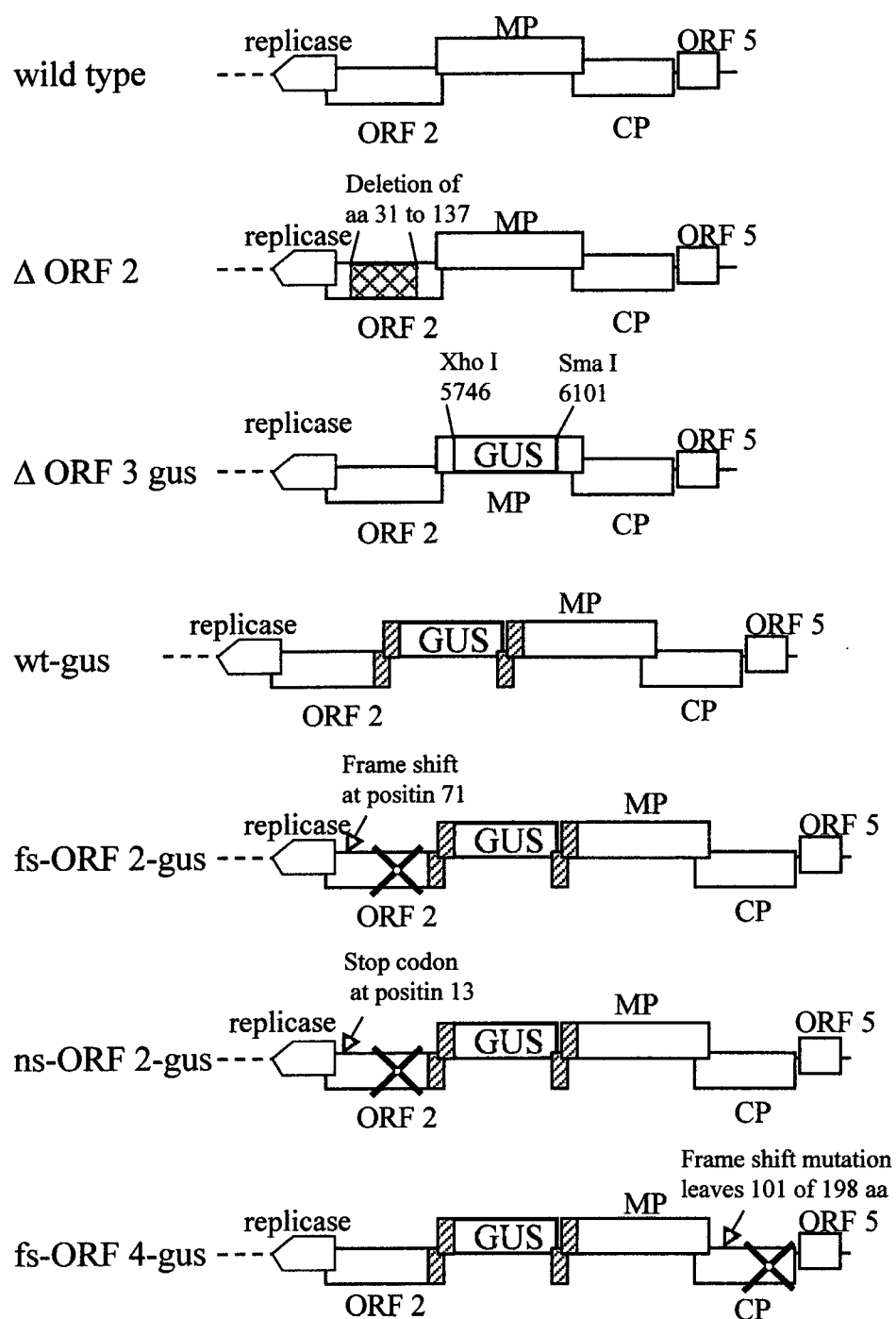


Figure 12. Schematic diagram of mutations introduced into thr GVA full-length cDNA clone.

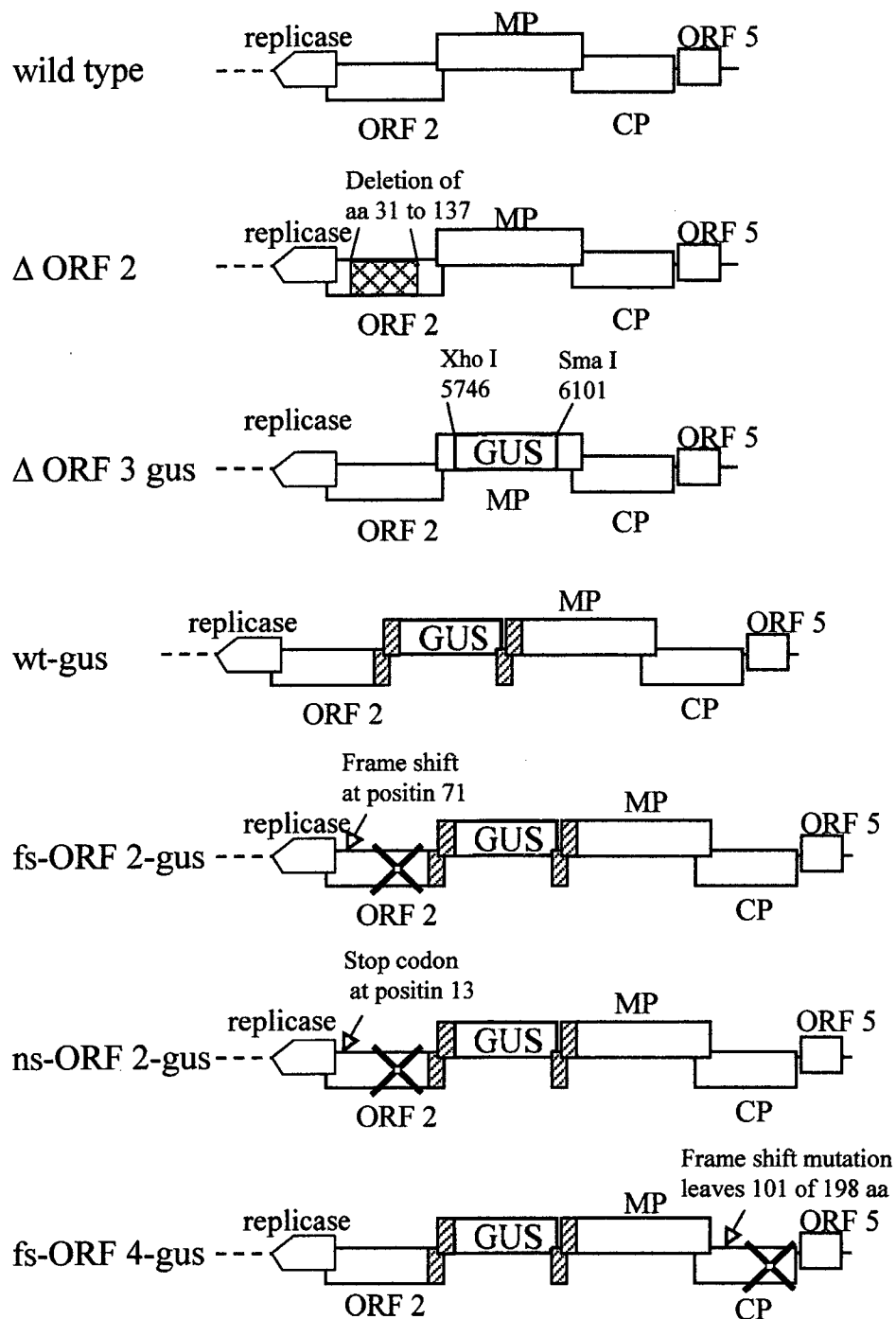


Figure 12. Schematic diagram of mutations introduced into thr GVA full-length cDNA clone.