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# Characterisation of a novel ilarvirus causing grapevine angular mosaic disease

**Sobhi M. Girgis · Frederic P. Bem · Chrysostomos I. Dovas · Athanassios Sclavounos · Apostolos D. Avgelis · Mina Tsagris · Nikos Katis · Panayota E. Kyriakopoulou**

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**Abstract** Unusual symptoms were observed on 'Baresana' x 'Baresana' *Vitis vinifera* hybrid vines in the Grapevine Variety Collection of the Grapevine Institute, Athens. The affected vines showed sharp angular mosaic on leaves, along the veins and in vein angles, malformations, abortive flowers or very few berries with smaller, wrinkled and non-germinating seeds, as well as gradual decline, severe stunting and death of the vine. Serological tests on diseased vines for the presence of 13 known grapevine viruses gave negative results. An infectious agent was transmitted mechanically to several herbaceous indicator plants. Koch's Postulates were fulfilled, and the agent, proven to be a virus, was named *Grapevine angular mosaic virus* (GAMV). Serological tests have been developed for the virus. The most conserved polymerase region showed significant similarity of GAMV with members of subgroup 1 of the *Ilarvirus* genus; however ML phylogenetic analysis could not support its clustering within this subgroup. GAMV differs serologically and in particle morphology from *Grapevine line pattern virus* (GLPV) a putative

S. M. Girgis
Laboratory of Biotechnology of Agricultural Products-Institute of Technology of Agricultural Products,
National Agricultural Research Foundation,
14123 Lykovryssi,
Athens, Greece

F. P. Bem
Laboratory of Virology, Benaki Phytopathological Institute,
14561 Kiphissia,
Athens, Greece

C. I. Dovas
Laboratory of Microbiology and Infectious Diseases,
School of Veterinary Medicine,
Aristotle University of Thessaloniki,
54124 Thessaloniki, Greece

A. Sclavounos · P. E. Kyriakopoulou (✉)
Department of Plant Pathology,
Agricultural University of Athens,
11855 Athens, Greece
e-mail: pek@aua.gr

A. D. Avgelis
Plant Virus Laboratory,
National Agricultural Research Foundation,
71110 Herakleion,
Crete, Greece

M. Tsagris
Institute of Molecular Biology and Biotechnology,
71110 Herakleion, Greece

M. Tsagris
Department of Biology, University of Crete,
71000 Herakleion,
Crete, Greece

N. Katis
Laboratory of Plant Pathology,
Aristotle University of Thessaloniki,
54124 Thessaloniki, Greece

member of the *Ilarvirus* genus that infects grapevine. It is proposed that GAMV is a novel member of the *Ilarvirus* genus.

**Keywords** Grapevine virus · New ilarvirus · Virus identification

## Introduction

Grapevine is a major crop in Greece and the Mediterranean Basin. Viruses cause significant losses in grapevine, both in Greece (Avgelis 1987; 1998; Mavraganis et al. 1987; Rumbos 1992) and internationally (Hewitt 1968, 1970; Bovey et al. 1980; Martelli 1993) with more than 50 virus species, belonging to 20 genera, known to infect the crop (Martelli 1993, 2003).

In 1994, a new disease was observed on seven out of 55 'Baresana' x 'Baresana' hybrid vines, established since 1989 in the Grapevine Variety Collection of the Grapevine Institute of Athens, N.A.G.R.E.F., Greece. The original 'Baresana', a white table cultivar, was introduced from Italy. The symptoms of the new disease consisted of polygonal mosaic on the leaves connected with veins and vein angles, leaf asymmetry and little leaf, starting to occur after the first flush of growth (Fig. 1a, b, c). The affected vines showed gradual decline and severe stunting or death. They produced no or very few inflorescences, and exhibited flower abortion (Fig. 1d), partial necrosis of fruit clusters, reduced berry size and small wrinkled seeds. These symptoms were observed throughout the years in the vineyard, as well as in the vegetative progeny of the original seven affected vines established in other sites. The original seven (V1–V7) affected vines declined gradually and two died by the year 2000. The disease was initially named 'grapevine paraveinal mosaic' (Kyriakopoulou and Girgis 1996; Girgis et al. 2001) and later 'grapevine angular mosaic' (Girgis et al. 2003).

Based on its symptomatology and the negative results of serological tests on diseased vines against 13 grapevine viruses, it was suggested that the disease is caused by a novel virus which was designated as *Grapevine angular mosaic virus* (GAMV) (Girgis et al. 2000; Girgis 2002).

The present work verifies GAMV as the causal agent of grapevine angular mosaic disease. In addition, efficient purification and detection methods were developed. Phylogenetic analysis places GAMV in the genus *Ilarvirus* and more specifically in its subgroup 1, along with *Tobacco streak virus* (TSV), *Strawberry necrotic shock virus* (SNSV) (Tzanetakis et al. 2004), *Blackberry chlorotic ring spot virus* (BCRV) (Jones et al. 2006), and *Parietaria mottle virus* (PMoV) (Scott et al. 2006).

## Materials and methods

### Virus isolate and host range

The isolate of the virus used in the study was obtained from symptomatic 'Baresana' x 'Baresana' grapevine (V2) after mechanical inoculation onto *Gomphrena globosa* and passage through single local lesions to *Chenopodium quinoa* on which it was subsequently maintained.

Grapevine leaf tissue was homogenised in 0.1 M sodium phosphate buffer pH 7.3 with nicotine 2.5% (vol/vol) and the extract was applied on carborundum powder (600 mesh)-dusted leaves. After inoculation, plants were transferred in a greenhouse (20–24°C) and observed daily for symptom development. Plant species used in host range studies are listed in Table 1. When no symptoms were observed 2–3 weeks post-inoculation (p.i.), inoculated and non-inoculated leaves of the plants were back-tested by inoculating *C. quinoa* for possible latent infections.

### Graft and aphid transmission studies

Symptomatic 'Baresana' x 'Baresana' plants were top-grafted with scions from healthy grapevine *Vitis rupestris* San George indicator plants, and kept under greenhouse conditions. Two aphid species, *Myzus persicae* and *Aphis fabae*, maintained on pepper plants (*Capsicum annuum*), were tested for virus transmission in the non-persistent manner. Adults or last instar nymphs, starved for 1–2 h, were placed on virus-infected *C. quinoa* plants and allowed acquisition access times of 30 s, 1 min, 2 min, and 3 min, respectively. Groups of five individuals per acquisition access time were transferred to a healthy indicator *C. quinoa* plant and allowed inoculation access time of 2 h. After completion of the inoculation access period, the test

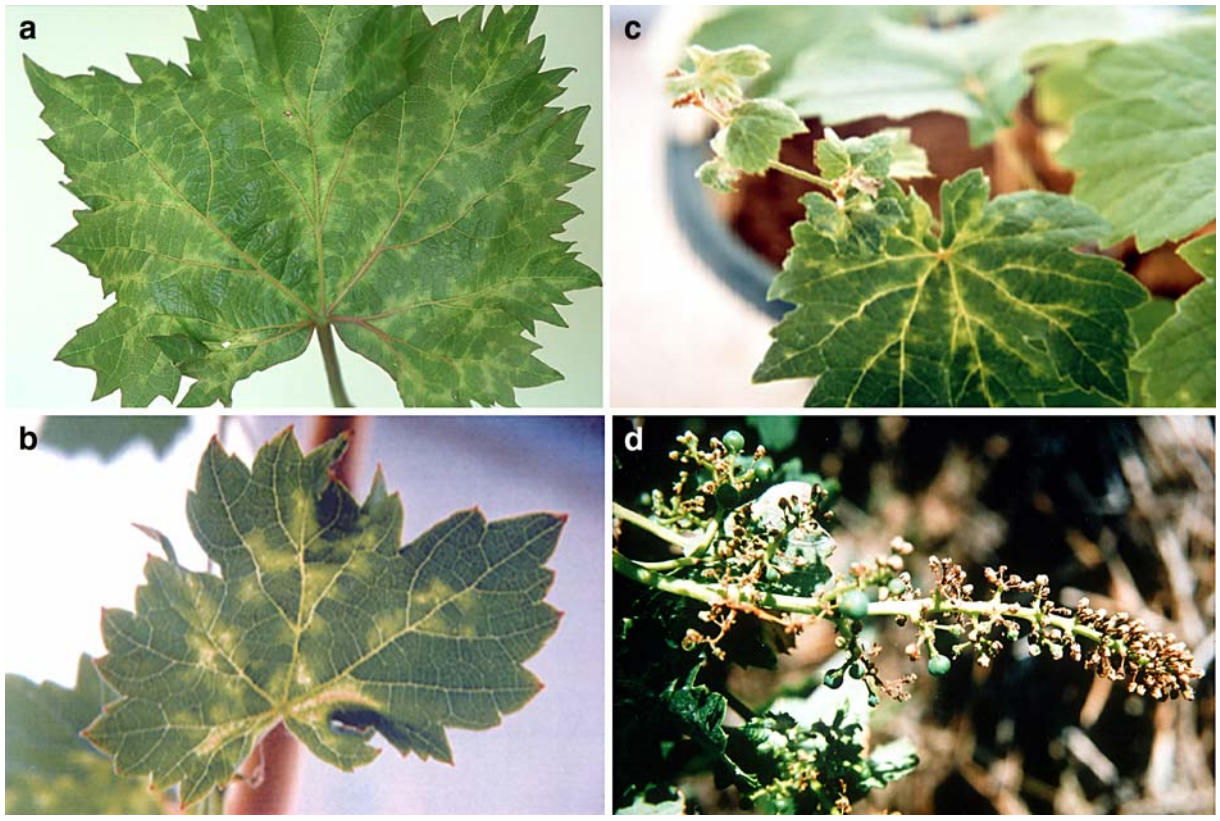


**Fig. 1** **a**. Polygonal mosaic connected with veins and vein angles; **b**. Leaf asymmetry and; **c**. Little leaf symptoms induced by GAMV in Baresana × Baresana grapevine hybrid; **d**. Flower drop induced by GAMV in Baresana × Baresana hybrid vine

plants were sprayed with insecticide (Confidor, Bayer Hellas, Athens) and transferred to the greenhouse for symptom expression.

## Purification

Three purification methods, the $Mg^{2+}$-bentonite method of Dunn and Hitchborn (1965), Steere's method (1959) for nepoviruses, and the Ong and Mink method (1989) for ilarviruses, were tested. The last method was adopted after a modification. Briefly, 100 g of infected *C. quinoa* plants harvested 3 weeks p.i. were blended (2.5 ml $g^{-1}$ tissue) in 0.02 M sodium phosphate buffer, pH 8.0, containing 0.01 M sodium diethyldithiocarbamate (DIECA) and 0.01 M sodium thioglycolate. The homogenate was centrifuged at 8,000*g* for 20 min, at 4°C. The supernatant was filtered through cheesecloth, brought to pH 4.5 with glacial acetic acid, and immediately increased to pH 5.3 with 3 M sodium hydroxide. The latter step was of utmost importance in the virus purification procedure in order to achieve maximum yield. The solution was kept for 30 min at room temperature, and clarified by centrifugation at 35,000*g* for 20 min, at 4°C. The virus was precipitated by ultracentrifugation at 75,000*g* for 4.5 h, at 4°C. The pellet was left overnight at 4°C to resuspend in 0.02 M sodium phosphate buffer, pH 8.0, and the suspension subsequently clarified by centrifugation at 10,000*g* for 15 min, at 4°C. Further purification was achieved by centrifugation for 4 h at 180,000*g* at 4°C in 10–40% sucrose density gradients prepared in 0.02 M sodium phosphate buffer, pH 8.0. The single UV absorbing zone was recovered and the virus concentrated by ultracentrifugation.

## Electron microscopy and coat protein size determination

Carbon-coated grids, floated for 1 h on a small drop of purified virus preparation and negatively

stained with 1% sodium phosphotungstate, pH 7.0 (Hill 1984), were examined in a Jeol 1200 EX electron microscope. For the molecular weight determination of the virus coat protein, 10 μg of purified virus preparation were loaded on 10% SDS-polyacrylamide gel (Sambrook et al. 1989) using tris-glycine buffer, pH 8.3 (25 mM tris base, 250 mM glycine, 0.1 SDS). After electrophoresis, the protein bands in the gels were fixed and stained with 0.025% Coomassie brilliant blue R-250.

## Antibody production

Antiserum against the virus was prepared by injecting rabbits intramuscularly with purified virus preparations (0.5 mg virus) emulsified in an equal volume of Freund's incomplete adjuvant. A total of eight injections were applied to the rabbit in 2-day intervals, with a resting period of one week between the 6$^{th}$ and 7$^{th}$ injections. The immunised rabbit was bled 12 days after the last injection. The antiserum titre was determined using the microprecipitin test. The serological methods double-diffusion Ouchterlony (Hill 1984), F(ab')$_2$-ELISA and DAS-ELISA (Clark and Adams 1977) were adopted. DAS-ELISA was performed on diseased vines (taking leaf tissue in May and bark scrapings from canes in December) and purified virus, using antibodies prepared against the virus, as well as antibodies against 13 tested grapevine viruses (*Grapevine fan leaf virus*/GFLV, *Grapevine leaf roll-associated virus 1*/GLRaV-1, *Grapevine leaf roll-associated virus 3*/GLRaV-3, *Grapevine leaf roll-associated virus 5*/GLRaV-5, *Grapevine leaf roll-associated virus 7*/GLRaV-7, *Grapevine virus* A/GVA, *Grapevine virus* B/GVB, *Grapevine fleck virus*/GFKV, *Alfalfa mosaic virus*/AMV, *Arabis mosaic virus*/ArMV, *Raspberry ring spot virus*/RpRSV, *Carnation latent virus*/CLV and *Tomato black ring virus*/TBRV.

## Molecular studies

Total RNA was extracted according to the method of Rott and Jelkmann (2001), using symptomatic grapevine leaves, *C. quinoa* plants infected with the virus, as well as purified virus preparations. Two degenerate primers, 'IlpoF': 5′-YTCIAMRTTYGAYAART CICA-3′ and 'IlpoR': 5′-GGYTGRTTRTGIG GRAAYTT-3′ were designed according to Dovas et al. (2004), after comparative analysis of published *Ilarvirus* sequences corresponding to the most conserved RNA-dependent RNA polymerase (RdRp) region. cDNA and PCR reactions were prepared as previously described (Maliogka et al. 2004). The PCR programme consisted of initial denaturation at 94°C for 2 min; 35 cycles of 30 s at 95°C, 30 s at 46°C and 25 s at 72°C, followed by an extension step at 72°C for 2 min. Amplifications were carried out in an Eppendorf Mastercycler gradient (Eppendorf, Germany). The reaction products were analysed by electrophoresis in 1.5% agarose gels in TAE buffer, stained with ethidium bromide and visualised under UV light.

Amplicons were cloned onto pCR 2.1 plasmid vector (Invitrogen, Netherlands). Plasmid DNA was extracted from bacterial cells using the 'Nucleobond' plasmid purification system (Macherey-Nagel, Germany) and both strands of the inserted DNA were sequenced. Sequencing was performed on an ABI Prism 3700 DNA Analyser.

Homologous partial amino acid sequences from *Bromoviridae* species corresponding to the amplicon sequence were aligned using the ClustalX programme Thompson et al. 1997). Maximum likelihood phylogenetic trees were reconstructed with PhyML (Guindon and Gascuel 2003). The appropriate amino acid substitution model used was Blosum62 + dΓ and was selected using PROTTEST version 1.3 with the Bayesian information criterion (BIC) (Abascal et al. 2004). The reliability of the phylogenetic hypothesis was assessed using non-parametric bootstrap (NPB) analysis.

## Koch's Postulates

For the fulfilment of Koch's Postulates, 2 month-old healthy grapevine self-rooted explants, from tissue culture, were transferred and rooted in soil (*in vivo*) in the glasshouse at 23–25°C. They were mechanically inoculated using pollen homogenate from *C. quinoa* plants infected with purified virus preparation. This inoculum was chosen to avoid possible mixed infection with other viruses. The grapevine test plants developed symptoms 10 weeks p.i. at 23–25°C. After symptom development, they were tested for the presence of the virus by mechanical inoculation on *C. quinoa* and by DAS-ELISA.

## Results

### Transmission studies

Mechanical transmission was done with difficulty. Repeated attempts to isolate GAMV on *C. quinoa* test plants with manual inoculation using infected grapevine sap (in 0.1 M sodium phosphate buffer, pH 7.3) were unsuccessful. However, the virus was isolated with difficulty in 1 out of 50 inoculated *G. globosa* seedlings, giving small (2 mm), round, purple local and systemic lesions with yellow halo and necrotic centres. This virus isolate (V2) was used for virus purification, and successfully transferred by manual inoculation from *G. globosa* lesions to *C. quinoa* seedlings giving small, chlorotic, local lesions 6 days p.i., followed by systemic mottle and little leaf symptoms 20 days p.i. Three out of five *C. quinoa* seedlings became infected when manually inoculated with pollen extracted (in 0.1 M sodium phosphate buffer, pH 7.3) from infected Baresana x Baresana grapevine on *C. quinoa* plants.

One year after graft inoculation of *V. rupestris* San George, vein flecking was observed on the leaves and symptomatic leaves gave positive reactions in DAS-ELISA tests using antibodies prepared against the virus. The aphid species *M. persicae* and *A. fabae* failed to transmit the virus from infected *C. quinoa* plants to *C. quinoa* seedlings.

Attempts to test transmission of the virus using seed from GAMV-affected vines of 'Baresana' x 'Baresana' were unsuccessful, due to the non-germinating seeds. However, 30% (48 out of 150) of the seedlings obtained from infected *C. quinoa* seeds developed symptoms typical of GAMV infection and they tested GAMV-positive by mechanical inoculation to *C. quinoa* test plants.

### Host range

The virus was successfully transmitted onto seven out of 15 different indicator plants inoculated with infected *C. quinoa* leaf extract (Table 1). All hosts were found both locally and systemically infected.

**Table 1** Host range and symptomatology of GAMV

| Species | Symptoms | |
|---|---|---|
| | Inoculated leaves | Uninoculated leaves |
| Amaranthaceae | | |
| *Gomphrena globosa* | NLL | PL |
| Chenopodiaceae | | |
| *Chenopodium amaranticolor* | - | - |
| *Chenopodium quinoa* | CLL | Mo, Li |
| *Chenopodium murale* | CLL | Mo |
| Graminae | | |
| *Zea mays* | - | - |
| Leguminosae | | |
| *Phaseolus vulgaris* cv. Pinto | - | - |
| *Vicia faba.* cv *minor* | - | - |
| Solanaceae | | |
| *Datura stramonium* | - | - |
| *Nicotiana benthamiana* | NLL | Mo |
| *Nicotiana clevelandii* cv. Gray | - | - |
| *Nicotiana glutinosa* | NLR | Mo |
| *Nicotiana rustica* | - | - |
| *Nicotiana tabacum* cv. Samsun. | NLL | Mo |
| *Nicotiana tabacum* cv. Xanthi nc | NLR | Mo |
| *Petunia hybrida* | - | - |

NLL= necrotic local lesions, CLL= chlorotic local lesions, PL= purple lesions, Mo= mottle, Li= little leaf, NLR= necrotic local rings, - = non-infected

**Table 2** Effect of pH of the acidified extract during incubation on GAMV yield

| pH of the extract | Virus yield /100 g *C. quinoa* leaves |
|---|---|
| 4.5 | 0.30 mg |
| 4.7 | 0.62 mg |
| 4.8 | 1.90 mg |
| 5.0 | 4.30 mg |
| 5.3 | 5.00 mg |
| 5.5 | Poor clarification |

## Purification, electron microscopy and coat protein size determination

By applying the original Ong and Mink (1989) purification method, a final virus yield of 0.3 mg per 100 g leaf tissue was achieved. However, when the original method was modified by inserting one more step (the immediate increase of the pH of the acidified extract from pH 4.5 to 5.3, before incubation for final clarification) virus yield of 5 mg per 100 g of infected *C. quinoa* leaves was achieved (Table 2). The modified Ong and Mink (1989) method yielded 5 mg virus per 100 g of tissue. Virus concentration in purified preparations was estimated using their absorbance at 260 nm, $A^{0.1\%}_{1\,cm}$=5.1 similar to that of TSV. The 260/280 ratio of the purified virus was 1.52.

Electron microscope examination of negatively stained purified preparations revealed a high concentration of uniform quasispherical virus particles of about 29 nm diam (Fig. 2). The coat protein obtained from purified virus particles migrated as a single species with an estimated molecular mass of 30 kDa (Fig. 3).

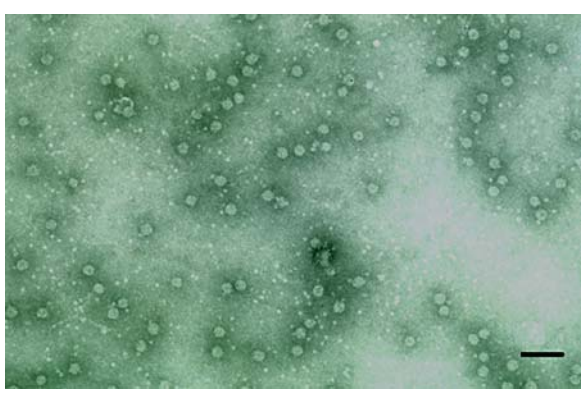

**Fig. 2** Electron micrograph of GAMV particles negatively stained with 1% sodium phosphotungstate, pH 7. Bar represents 100 nm

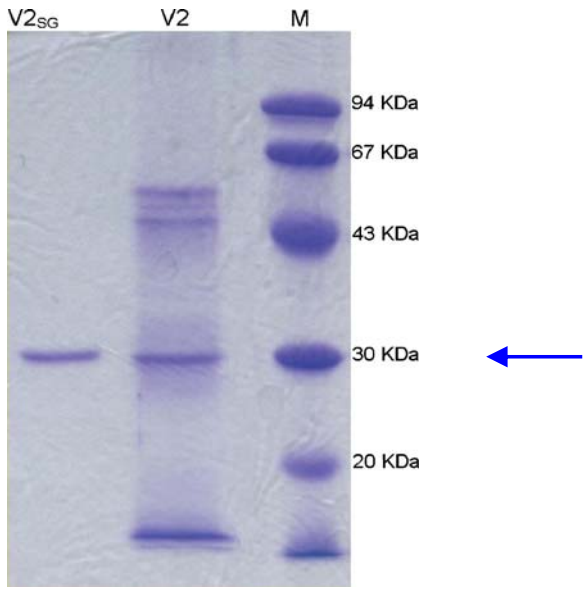


**Fig. 3** Electrophoresis of GAMV coat protein in 10% SDS-polyacrylamide gels. Line $V2_{SG}$: Purified virus preparation (after 10–40% sucrose gradients centrifugation); line V2: Partially purified virus preparations; line M: Marker proteins

## Serology

The antiserum obtained gave a titre of 1/1024, as determined in the microprecipitin test. IgG prepara-

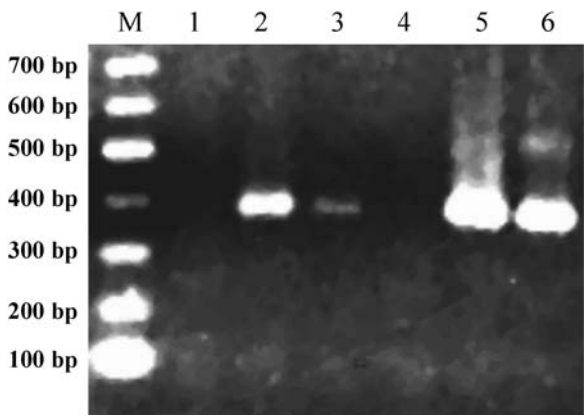


**Fig 4** Agarose gel electrophoretic analysis of PCR products obtained, using RdRp primers F and R. Lines 2 and 3: From GAMV-infected *C. quinoa* and grapevine sap respectively; line 5: From purified GAMV preparations; line 1: From healthy *C. quinoa* sap; line 4: from healthy grapevine sap; line 6: From *Spinach latent virus*-infected *C. quinoa* sap; M: 100 bp DNA ladder

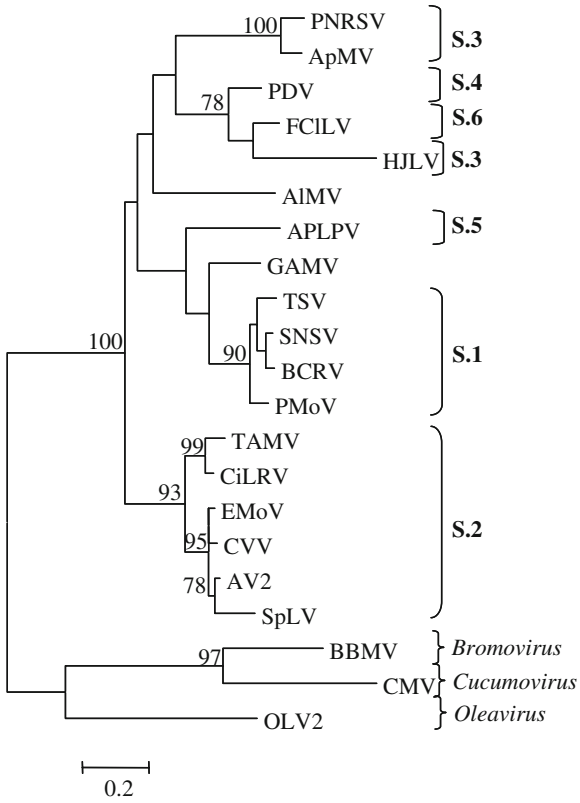


**Fig. 5** Phylogenetic tree inferred from selected members of the *Bromoviridae* family with maximum likelihood analysis, using published homologous partial amino acid sequences corresponding to the amplified partial RdRp region of GAMV. The viruses included, with acronyms and accession numbers in parenthesis, are: *Tobacco streak virus,* TSV (P89678), Strawberry necrotic shock virus, SNSV (NC008707), Blackberry chlorotic ringspot virus, BCRV (Q2QF34), *Parietaria mottle virus,* PMoV (Q9RK13), *Citrus leaf rugose virus,* CiLRV, (Q66107), *Tulare apple mosaic virus,* TAMV (Q99HR0), *Spinach latent virus,* SpLV, (O57162), *Elm mottle virus,* EMoV (Q88167), *Asparagus virus 2,* AV2 (O41889), *Citrus variegation virus,* CVV (O41891), *Prunus necrotic ringspot virus,* PNRSV (Q91NQ0), *Humulus japonicus latent virus,* HJLV (Q6JE41), *Apple mosaic virus,* ApMV (Q9QGG9), *Prune dwarf virus,* PDV (Q91NQ3), *American plum line pattern virus,* APLPV (Q997A3), *Fragaria chiloensis latent virus,* FC1LV (Q5VI74), GAMV (Q6PNM5), *Olive latent virus 2,* OLV2 (Q83944), *Broad bean mottle virus,* BBMV (Q65383) and *Cucumber mosaic virus,* CMV (P06012). The numbers above each branch are the non-parametric bootstrap (NPB) probabilities. Only values with $P>0.7$ are shown. The tree was midpoint rooted. Numbering of the different subgroups of ilarviruses has been done following the grouping that best fits the one described by the ICTV (Fauquet et al. 2005)

tions gave titres of at least 100 ng $ml^{-1}$ in DAS-ELISA and of at least 50 ng $ml^{-1}$ in $F(ab')_2$-ELISA. The seven original symptomatic 'Baresana' x 'Baresana' vines (V1–V7) tested by DAS-ELISA gave a positive reaction using antibodies prepared against the virus. In contrast, 48 asymptomatic 'Baresana' x 'Baresana' and 100 neighbouring vines of other varieties in the same grapevine collection gave a negative reaction. The serological tests on the original diseased vines and on purified virus preparations were negative against antisera of 13 tested grapevine viruses: GFLV, GLRaV-1, GLRaV-3, GLRaV-5, GLRaV-7, GVA, GVB, GFKV, AMV, ArMV, RpRSV, CLV, TBRV.

## Molecular studies

A 381 bases-long fragment, corresponding to the most conserved part of the RdRp gene, was successfully amplified using the degenerate *Ilarvirus*-specific primers (Fig. 4). Sequencing of the cloned amplicon revealed its respective viral origin. The nucleotide sequence was submitted to the Genbank database under the accession number AY590305. The consensus partial RdRp amino acid sequence of GAMV showed the highest identity with homologous RdRp regions of TSV (76.3%), SNSV (79.8%), BCRV (76.3%) and PMoV (77.2%). Maximum likelihood phylogenetic analysis confirmed that GAMV is a novel virus classified within the genus *Ilarvirus* (Fig. 5). However, although the partial RdRp amino acid sequence of GAMV had high sequence similarity to members of subgroup 1,

**Fig. 6** Symptoms on grapevine inoculated with *Grapevine angular mosaic virus* (GAMV)

there was no bootstrap support for its clustering with the subgroup 1 of ilarviruses.

Fulfilment of Koch's Postulates

Koch's Postulates were fulfilled as (a) all symptomatic 'Baresana' x 'Baresana' vines were found infected by the virus, (b) the original symptomatology was obtained in one out of five grapevine explants inoculated with the isolated virus (Fig. 6), and (c) the virus was detected in these last plants using bioassays and serology.

## Discussion

The aim of this study was to determine the etiology of a grapevine disease with a novel symptomatology named 'grapevine angular mosaic' (Girgis 2002). The virus which was persistently isolated from all the symptomatic grapevine plants was named GAMV (Girgis et al. 2000; Girgis et al. 2003; Girgis 2002). The fulfilment of Koch's Postulates applied for the isolated virus, especially the reproduction of the original symptomatology and the re-isolation of the virus in grapevine, proved that the new disease was caused by GAMV. In serological tests, none of the 13 tested grapevine viruses was detected in the symptomatic vines, an indication that suggested a previously undescribed pathogen. Initial studies of GAMV, regarding its particle shape and size, seed and pollen transmissibility and response to ilarvirus purification method, indicated that the virus was a possible member of the genus *Ilarvirus*.

Comparing the GAMV properties in sap (data not shown) with those of other members of the genus *Ilarvirus* (Hull 2002), the virus was found to be more stable. GAMV differs significantly from *Grapevine line pattern virus* (GLPV) which is associated with grapevine line pattern disease (Martelli and Lehoczky 1993) and is the only other putative ilarvirus reported to infect grapevine. GAMV has uniform quasispherical particles of 29 nm diam, in contrast to GLPV that has particles of three different sizes (25, 35 and 100 nm), a higher thermal inactivation point and longevity in vitro. An efficient purification method of GAMV was developed and a high titre antiserum was prepared for its detection.

Molecular work, by performing a molecular comparison of a 381 bp fragment of the ilarviruses RdRp domain of RNA 2 showed that GAMV was genetically closest to SNSV (79.8%), and to (PMoV), with an amino acid sequence similarity 77.2%.

The present work describes a new virus, GAMV, which is a new member of the genus *Ilarvirus*, and is the first proven ilarvirus naturally infecting grapevine. The described symptomatology has not been observed in grapevine in other parts of Greece. It is thus possible that the disease is limited to the above mentioned varietal collection, and was probably introduced into the collection with the importation of the 'Baresana' variety and its hybrids.

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