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ORIGINAL RESEARCH

In situ localization of *Grapevine fanleaf virus* and phloem-restricted viruses in embryogenic callus of *Vitis vinifera*

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Abstract In grapevine, somatic embryogenesis is particularly effective in eliminating several important virus diseases. However, the mechanism whereby regenerated somatic embryos are freed of the viruses is not clear. The distribution of *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus*-3 (GLRaV-3) and *Grapevine virus A* (GVA) in embryogenic callus of grapevine was investigated by *in situ* hybridization using digoxygenin-labelled oligonucleotide probes. Four months after culture initiation, in callus originated by GFLV-infected explants we observed a mosaic of infected and uninfected cells, with high concentrations of viruses in some cell groups in peripheral zones of the callus. In addition some abnormal somatic embryos showed a high hybridization signal. In callus originated by GVA- and GLRaV-3-infected explants the viruses were concentrated in few cells surrounded by areas of virus-free cells. The two viruses were generally localized in different clusters of cells inside the callus and the levels of infection were lower than those observed in GFLV-infected callus. No virus was detected in callus nor in somatic embryos after 6 months of culture. The results highlight the difficulties of some viruses at stably invading callus tissues and the differential ability of GFLV to spread in the callus cells compared to the phloem-limited viruses.

Keywords Grapevine viruses · *In situ* hybridization · Oligonucleotide probes · Somatic embryos · Virus movement

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Introduction

Grapevine (*Vitis vinifera* L.) is grown worldwide and is one of the most important horticultural crop for its global production value. Despite the efforts of scientists and nurserymen, viral diseases are still a major threat to this crop. Sixty different documented viruses infect grapevine (Martelli 2009), which is far greater than the number of viruses known in any other single perennial crop. It has been estimated that global losses due to viral infections of grapevines are over 1 billion US dollars (Mckenzie and Pathirana 2007). The most harmful and widespread ones are fanleaf degeneration, leafroll and rugose wood. *Grapevine fanleaf virus* (GFLV), a soil-borne *nepovirus*, is the main causal agent of grapevine fanleaf disease, which in sensitive cultivars can cause rapid death of young plants or a progressive decline over several years. GFLV is spread both via propagating material and the nematode vector *Xiphinema index* (Andret-Link et al.

2004). At least eight serologically distinct viruses, *Grapevine leafroll-associated virus* -1, -2, -3, -4, -5, -6, -7, -9 (GLRaVs), have been proposed as associated with leafroll disease (Martelli et al. 2002; Alkowni et al. 2004). Rugose wood can be divided into four distinct disorders based on symptoms expressed on specific *Vitis* indicators: Kober stem grooving, LN 33 stem grooving, Corky bark and Rupestris stem pitting. *Grapevine virus A* (GVA) is thought to be involved in Kober stem grooving disease (Chevalier et al. 1995).

The most important strategy to control viral diseases in grapes is preventive and consists of planting virus-free vines during vineyard establishment. Propagation material with optimal sanitary status is produced through certification schemes, which use reliable techniques for eliminating viruses associated with most common and harmful diseases. Meristem tip culture is particularly effective in eliminating phloem-limited viruses, while thermotherapy is normally required for the elimination of viruses that readily invade plant meristems, such as *nepovirus*. Somatic embryogenesis, usually adopted to regenerate plantlets in biotechnological breeding programs, successfully eradicated viruses from *Citrus* (D'Onghia et al. 2001), sugarcane (Parmessur et al. 2002) and cacao (Quainoo et al. 2008). In grapevine it efficiently eliminated several phloem-limited viruses (Goussard et al. 1991; Gambino et al. 2006) and recently two *nepovirus*, GFLV (Gambino et al. 2009) and *Arabis mosaic virus* (Borroto-Fernandez et al. 2009). The mechanism whereby regenerated somatic embryos are freed of some viruses is not clear. It was reported that the phloem-limited viruses are able to invade initially the callus derived from anther and ovary cultures (Gambino et al. 2006), but translocation of these viruses from infected tissue to somatic embryos was not observed (Goussard et al. 1991; Gambino et al. 2006). Although GFLV presence was detected in some grapevine somatic embryos, the eradication of this virus was obtained with a success percentage close to 100% (Gambino et al. 2009).

In this work, we used *in situ* hybridization to elucidate the distribution patterns of GFLV, GVA and GLRaV-3 in callus originated from floral explants of grapevine, and to better understand the mechanism(s) underlying the differentiation of uninfected plantlets from the embryogenic callus.

Materials and methods

Plant material

Embryogenic cultures were initiated from flower explants of two virus-infected clones of grapevine (*Vitis vinifera*) cultivars: Provinè infected by GFLV and Lumassina infected by GVA and GLRaV-3. Immature stamens (anthers plus filaments) and pistils (ovaries plus styles, stigmas and receptacle) were isolated from inflorescences collected in vineyards and indirect somatic embryogenesis was induced as previously described (Gribaudo et al. 2004). Below we refer to these explants simply as anthers and ovaries. Floral explants were cultivated on a callus induction medium containing 4.5 μM 2,4-D and 8.9 μM BAP. After 3 months, callus explants were transferred to an embryo differentiation medium containing 10 μM NOA, 1 μM BAP, 20 μM filter-sterilized IAA, and 0.25% activated charcoal. Cultures were kept at 26°C in the dark.

Virus detection

The viral status of the mother plants was analyzed by multiplex RT-PCR (Gambino and Gribaudo 2006) sampling phloem scraped from mature canes collected during winter pruning and tissues from inflorescences at the start of the culture. Single mature somatic embryos and small clusters of embryos were analyzed by multiplex RT-PCR approximately one year after culture initiation.

In situ hybridization

Different types of callus were collected 4 and 6 months after culture initiation. Before the paraffin-embedding of callus for histological sections, the friable samples were embedded in 3% agar to preserve the original morphology of the callus and the cell arrangement within the samples. Samples were then fixed in 4% paraformaldehyde in PBS (130 mM NaCl; 7 mM Na_2HPO_4; 3 mM NaH_2PO_4, pH 7.4) overnight at 4°C. For the first 30–40 min, samples were fixed under vacuum to facilitate infiltration with the fixative. Thereafter, the fixative was removed by washing in saline solution (150 mM NaCl) for 15 min and the tissues were dehydrated successively in solutions of 30, 50, 70,

80, 95 (in 150 mM NaCl), 100% ethanol and 100% xylene for 2 h each step. Finally, the samples were embedded in Paraplast (Paraplast plus, Sigma-Aldrich, St. Louis, MO) at 60°C. Eight μm-sections were obtained with a rotative microtome Leitz, transferred on slides treated with 100 μg/ml poly-L-lysine (Sigma), and dried overnight on a warming plate at 40°C.

Oligonucleotides were used as probes and 3′end-labelled by tailing with the DIG Oligonucleotide Tailing Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. For each virus we designed two short (30-mer) antisense oligonucleotides complementary to different conserved regions of the virus genome as reported in Table 1. To increase the sensitivity of the *in situ* hybridization procedure we used a mixture of the two oligonucleotide probes for each virus.

The sections were deparaffinized in xylene and rehydrated through an ethanol series from 100 to 30% followed by a rinse in water for 5 min each step, washed twice in PBS for 5 min and twice in PBS containing 100 mM glycine for 5 min. The sections were treated for 15 min in PBS containing 0.3% Triton X-100 and washed in PBS (2×5 min). The slides were then incubated with proteinase K (1 μg/ml in 100 mM Tris-HCl, and 5 mM EDTA pH 8.0) at 37°C for 30 min, post-fixed for 5 min at 4°C in 4% paraformaldehyde in PBS. After two rinses in PBS the sections were treated (2×5 min) with 0.1 M triethanolamine (Sigma) pH 8.0 containing 0.25% v/v acetic anhydride (Sigma). After three rinses in water, the sections were dehydrated in an ethanol series from 30 to 100% and overlaid with an hybridization buffer containing: 2X SSC (0.3 M NaCl, 30 mM sodium citrate), 1X Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2 mg/ml bovine serum albumin), 10% dextran sulfate, 50 mM phosphate buffer pH 7.0, 50 mM DTT, 250 μg/ml yeast tRNA, 100 μg/ml polyadenylic acid, 500 μg/ml denatured and sheared salmon sperm DNA and 47% formamide. After an incubation of 2 h at 37°C, hybridization was carried out overnight at 37°C with the above described hybridization buffer containing a total of 225 ng of mixed oligonucleotide probes per ml of solution. After hybridization, slides were washed at 37°C in 2X SSC (2×15 min), in 1X SSC 0.1% SDS (2×15 min) and finally in 0.25X SSC 0.1% SDS twice for 15 min.

After being rinsed in TBS (100 mM Tris-HCl, pH 7.5, 400 mM NaCl) for 10 min, slides were treated with 0.5% blocking reagent (Roche) in TBS for 1 h, incubated for 2 h with the anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche) diluted 1:1,000 in 0.5% BSA in TBS. The detection of the probes was performed using the chromogenic substrates nitroblue tetrazolium and 5-bromo-4-cloro-3-indolyl phosphate (NBT/BCIP) resulting in a blue colour precipitate that was viewed under a light microscope. The slides were washed in TBS (3×5 min) and incubated 10 min in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). The sections were covered with colour solution [45 μl of NBT (75 mg/ml in 70% dimethylformamide) and 35 μl of BCIP (50 mg/ml in dimethylformamide) were added to 10 ml of detection buffer] and incubated overnight in the dark. The colour reaction was stopped by washing the slides in water; the sections were then dehydrated through an ethanol series followed by Bioclear (Bio-Optica, Milano, Italy), and mounted with Bio-mount (Bio-Optica).

For each hybridization experiment we used sections from healthy plants as negative controls. Infected samples incubated without the probes or without the anti-DIG antibody were used as technical negative controls.

Results

Callogenesis was obtained from anther and ovary cultures of Provinè and Lumassina, with different efficiencies depending on genotype and explant type. The highest percentage of explants differentiating somatic embryos was recorded for the anthers of Lumassina (21.3%). Different types of callus were observed, as previously reported (Gambino et al. 2006): non-embryogenic watery and soft callus (Fig. 1a, d, g, j), and granular pre-embryogenic or embryogenic callus (Fig. 1b, e, h, k). Five callus samples of each type were collected 4 months after culture initiation; additionally, 5 embryogenic callus samples (Fig. 1c, f, i, l) were collected 2 months later (6 months from culture initiation).

Multiplex RT-PCRs on mature canes and floral explants confirmed the virus infection of the field plants used as source material. In the somatic embryos

Table 1 Oligonucleotide probes and sequence location within the viral genome

Target	Sequences 5'-3'		Location[a]	Gene
GFLV	Oligo 1	AGCCTTATAATCACAATTGTATCCGACATC	5424-5453	RNA-dependent RNA polymerase
	Oligo 2	GATACCCTACAAGTATTCGAAGTGAAATCG	3157-3186	Coat protein
GLRaV-3	Oligo 1	ATCTTTGACGTAAAAATACCCTCTCCTACG	909-938	ORF 1 papain-like protease
	Oligo 2	GATACTTTCCCTGGCTCGTTAATAACTTTC	13658-13687	ORF 6 coat protein
GVA	Oligo 1	GATCATTCCTCATCGTCTGAGGTTTCTAGT	7257-7286	ORF 5 putative RNA binding protein
	Oligo 2	ATCTTAACTTCCTTTGGTTCTGAGTGCGAT	5687-5716	ORF 3 movement protein

[a] The reference accession numbers (NCBI) for determining the olinucleotides positions are: NC_003623 and NC_003615 for GFLV, NC_004667 for GLRaV-3 and DQ855087 for GVA

tested 1 year after culture initiation, viruses were never detected in any of the samples.

Histological observations

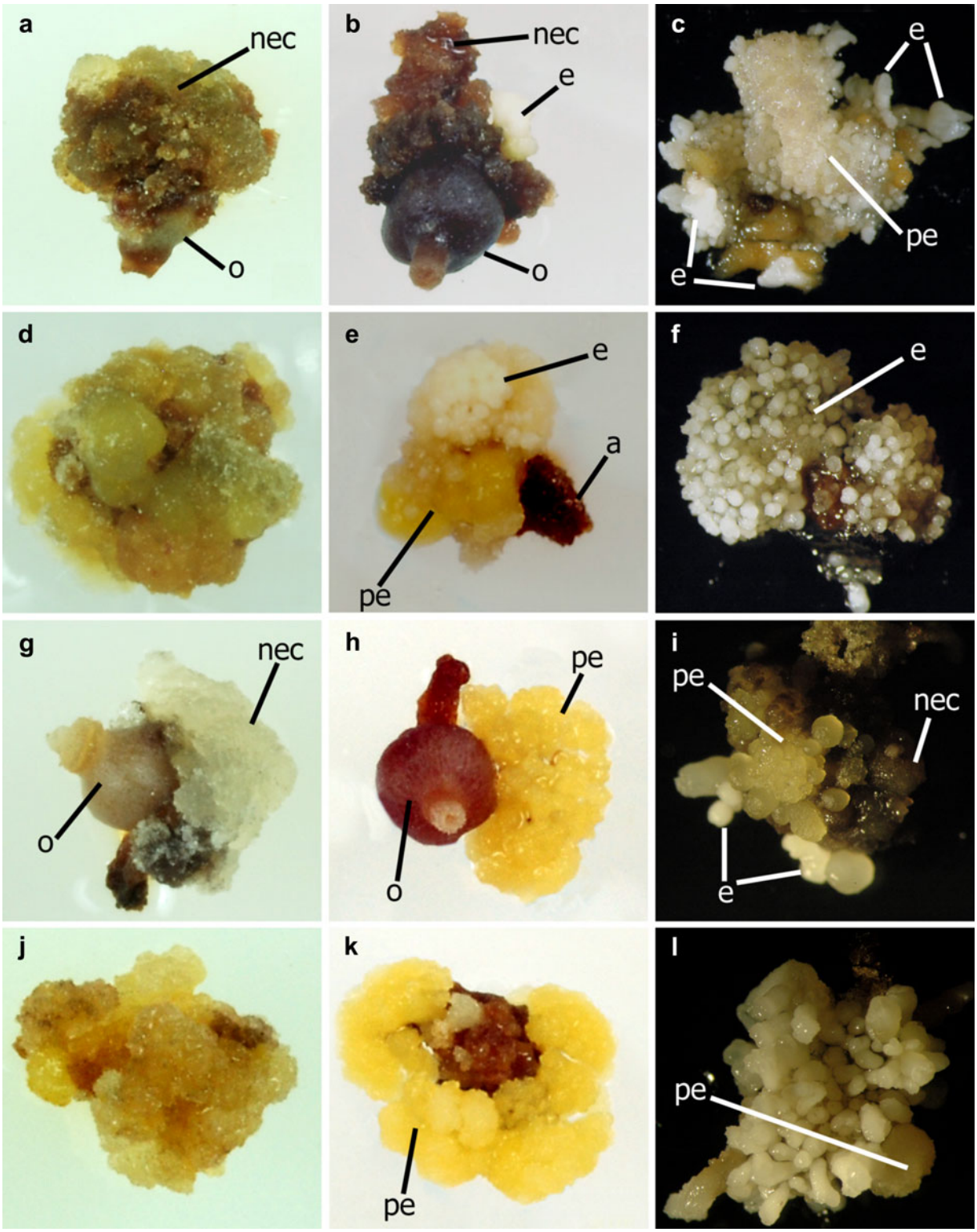
After 4 months of culture, we observed a proliferation of embryogenic or non-embryogenic callus at the receptacle region of several immature ovary explants. The original ovary explants, when still visible, were generally brown and compact (Fig. 1a, b, g, h). Microscopic observations of serial sections of the explants showed that a large part of the ovary wall was degenerated, with tannin deposition (Fig. 2a, b, e). Most embryo sacs were also degenerated; cell divisions were not observed in the ovules, whereas callus proliferated from the parenchyma of the receptacle (Fig. 2e, i). In anther explants, callus was produced from the cells of the connective tissues located between the two lobes of the anther close to the filament attachment zone, from the filaments and from epidermal cells of the anther. The pollen sacs remained almost intact and were still visible after several months of culture (Fig. 2k and Fig. 4b, c, e), while the microspores degenerated.

No evident morphological difference was noted between embryogenic cultures originated from ovaries or from anthers, with the exception of many small tracheid-like elements observed in the ovary-derived callus (Fig. 4h). Cells of the newly formed callus were characterized by small size, dense cytoplasm, absence of vacuoles or tannin deposition, prominent nucleus and thick cell walls, and could be clearly distinguished from those of original explants and from cells of old callus. Proembryos developed in the superficial zone of the newly formed callus (Fig. 3a, b) and numerous embryos were produced. The embryos developed asynchronously and passed through the globular, heart-shaped, torpedo and cotyledonary stages, with various embryo developmental stages present at the same time in the embryogenic callus (Fig. 3d, e and Fig. 4f). No vascular connection between neighbouring embryoids or between grapevine somatic embryos and the parent tissues was observed. After 6 months of culture, in the embryogenic explants the proportion of undifferentiated callus was much reduced compared to the volume of embryogenic tissues (Fig. 1c, f, i, l).

Localization of GFLV in grapevine callus

In situ hybridizations were carried out using as probes two oligonulceotides complementary to different regions of GFLV sequences. We used short oligonucleotides instead long RNA probes to increase the ability of the probes to enter the tissues and to hybridize the target RNA. The specificity and efficiency of the probes were checked for each hybridization experiments with several kind of controls (Figs. 2j, 3f, g). In the negative controls no deep-blue colour precipitate was present, although in the young tissues of pre-embryogenic masses and embryos a low background, i.e. a pale-blue colour, could be observed in correspondence to the cell walls (Fig. 3d, e,). Despite this

Fig. 1 Phenotypes of callus of *V. vinifera* Provinè and Lumassina 4 and 6 months after culture initiation. Four months after culture initiation: non-embryogenic **a** and embryogenic callus **b** from ovaries, and non-embryogenic **d** and embryogenic callus **e** from anthers of Provinè; non-embryogenic **g** and embryogenic callus **h** from ovaries, and non-embryogenic **j** and embryogenic callus **k** from anthers of Lumassina. Six months after culture initiation: embryogenic callus from ovaries **c** and anthers **f** of Provinè, and embryogenic callus from ovaries **i** and anthers **l** of Lumassina. a, anther; e, embryos; nec, non-embryogenic callus; o, ovary; pe, proembryogenic tissues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background, the comparison of the control slides from healthy plants (Fig. 3g) with the areas where the viruses were clearly present (with high intensity of blue colour precipitate) helped to decide if the hybridization technique worked correctly.

After 4 months of culture the callus originated from the Provinè ovary tissues showed high GFLV-infection levels and several sectors of the callus as well as some somatic embryos were infected. In the original explants, the ovary cells were rich in tannin depositions and the hybridization signals were restricted to single isolated cells (Fig. 2b, h). The receptacle surrounding the immature ovary showed high-intensity hybridization signals with the virus mainly concentrated in peripheral areas (Fig. 2i, l). In anther-derived callus, GFLV was less abundant (Fig. 2k).

In the callus originating from both virus-infected ovaries and anthers, we observed substantially a mosaic of infected and uninfected cell groups. The highest GFLV hybridization signal was concentrated in some groups of cells in peripheral zones of the callus, surrounded by virus-free cells (Fig. 2c, f, h). Hybridization signals were evident in peripheral areas of pre-embryogenic masses (Fig. 3a, b) and in some globular embryos. In addition GFLV seems to be concentrated in abnormal large embryos (Fig. 2d). In the analyses carried out 2 months later, i.e. after 6 months of culture, we did not observe any hybridization signal in callus (Fig. 3c, e) or somatic embryos (Fig. 3d, e).

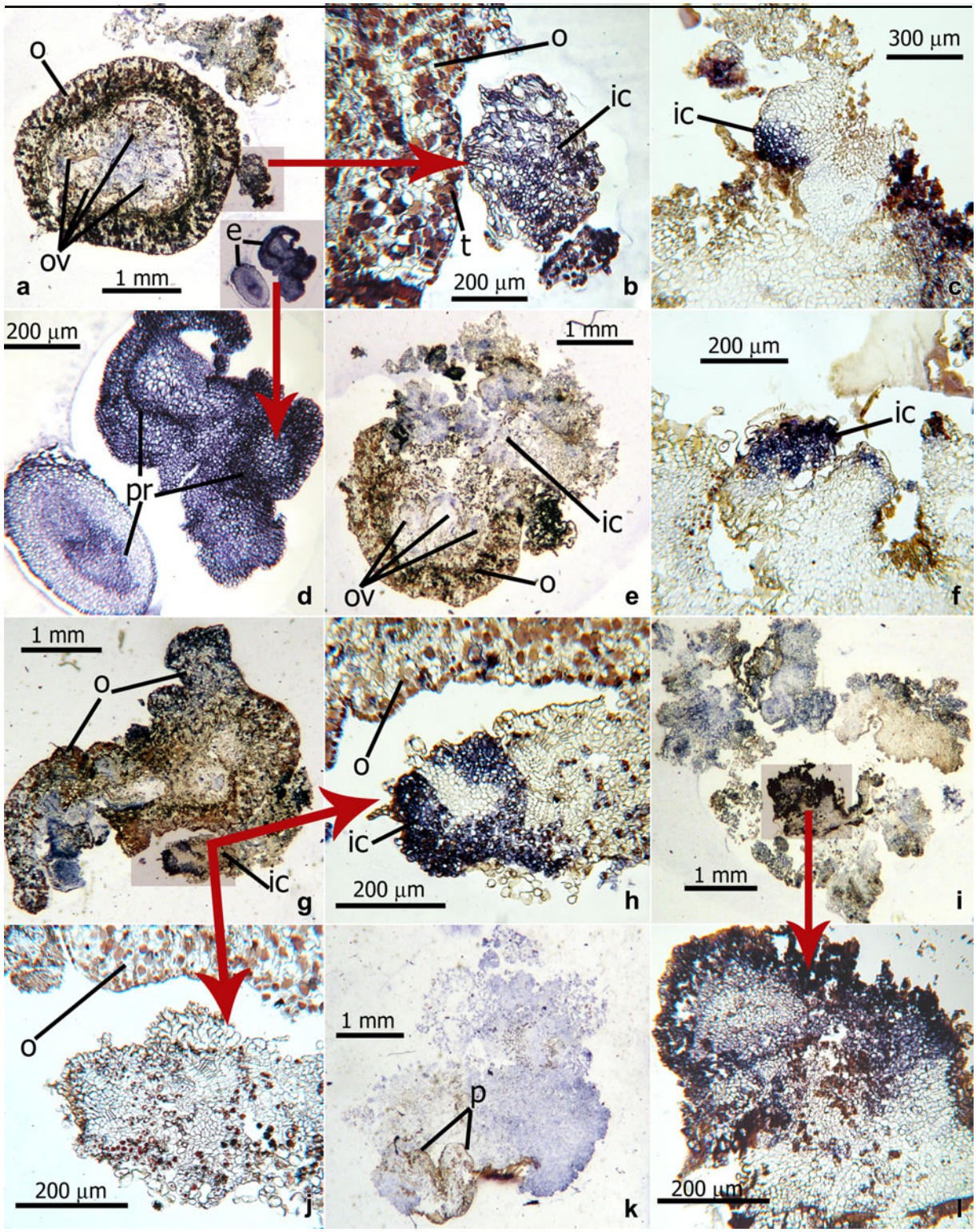
Localization of GVA and GLRaV-3 in grapevine callus

Two oligonucleotides complementary to different regions of the genomes of GVA and GLRaV-3 were used as probes for *in situ* hybridizations. The specificity and efficiency of the probes were checked by using several controls as described above. As reported for GFLV, a pale blue colour background was observed in correspondence to cell walls in pre-embryogenic masses and embryo tissues of healthy controls. However, also for this probes, the comparison of the control slides from healthy plants and the areas where the viruses were clearly present allowed to confirm that the hybridization technique worked correctly. Since the flower explants of Lumassina were infected by both GVA and GLRaV-3, the callus originated from these explants was separately analyzed for the detection of each virus using contiguous sections of the same tissues.

Fig. 2 Microscopic imaging of GFLV-infected callus of Provinè 4 months after culture initiation. The blue colour indicates the presence of target viral RNA. Embryogenic callus from ovaries is in panels **a** and **e**. The area inside the box in panel **a** is enlarged in panel **b** to show a closer view of the infected cells in the callus, the cells of the ovary rich in tannin depositions and the single isolated GFLV-infected cells. A second area inside the box in panel **a** is enlarged in panel **d** to show abnormal virus-infected somatic embryos. Non-embryogenic callus from anthers is in panels **c** and **f**, with high hybridization signal concentrated in clusters of cells in the peripheral zone of the callus. Non-embryogenic callus from ovary is in panel **g**; the box is enlarged in panel **h** to show infected cells in the callus, and in panel **j** to show a negative technical control with the section incubated without the anti-DIG antibody. A section of the receptacle surrounding the ovary together with callus is in panel **i**: the box is enlarged in panel **l** to show a closer view of GFLV-infected receptacle. Anther-derived callus with the pollen sacs is in panel **k**. e, somatic embryos; ic, infected callus tissues; o, ovary; ov, ovules; p, pollen sacs; pr, procambium; t, tannin depositions

After 4 months of culture, despite the partial degeneration of the tissues, the original explants showed hybridization signals. As observed for Provinè, the ovaries were rich in tannin depositions and the hybridization signals were restricted to few single isolated cells (Fig. 5e, f). In the receptacle blue precipitates were present in correspondence to vascular components and parenchymatic cells (Fig. 4a, d), and hybridization signals were visible inside the anther locules (Fig. 4c). In contiguous sections of ovaries (Fig. 4a, d) and of anthers (Fig. 4b, c, e) we generally observed higher concentrations of GLRaV-3 than of GVA, probably reflecting different concentrations of the viruses in the original explants. The ovary infection levels were lower compared to those observed in GFLV-infected ovaries of Provinè. In callus originated from explants infected by GLRaV-3 and GVA, infected and uninfected cells were visible (Fig. 5). However, unlike the GFLV-infected callus, in the callus of Lumassina we did not observe high concentrations of viruses in the peripheral zones. GVA and GLRaV-3 were concentrated in few cells surrounded by wide areas of virus-free cells (Fig. 5a, b, c, d), and when we examined contiguous sections of the same tissue, the two viruses were generally localized in different groups of cells (Fig. 5c, d).

After 4 months of culture, no somatic embryo or pre-embryogenic mass showed hybridization signals,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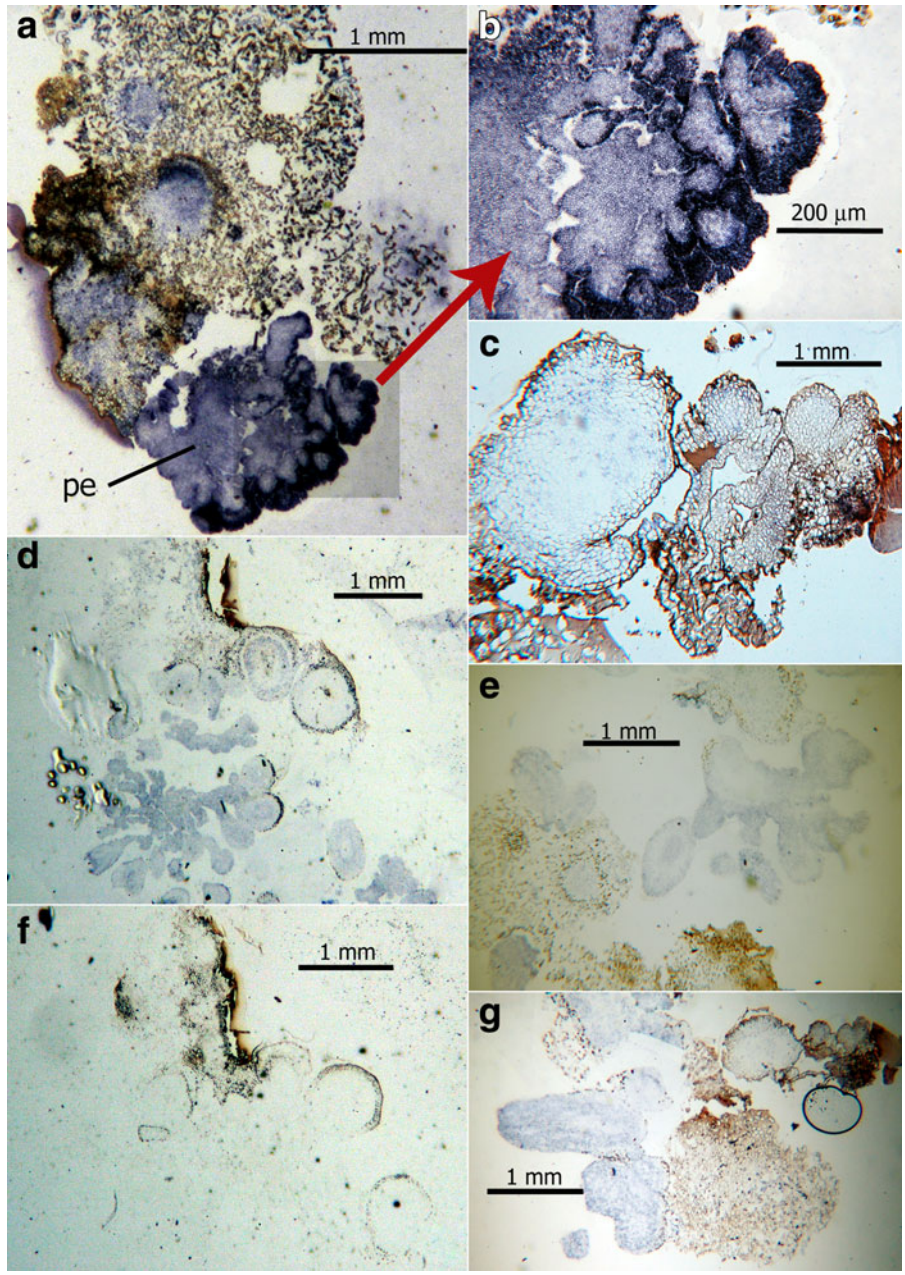

Fig. 3 Microscopic imaging of GFLV-infected callus of Provinè, 4 and 6 months after culture initiation. The blue colour indicates the presence of target viral RNA. Four months after culture initiation: embryogenic callus from anther is in panel **a**: the area inside the box is enlarged in panel **b** to show a closer view of infected-cells of proembryogenic tissues. Six months after culture initiation: section of ovary-derived callus **c** showing no hybridization signal; somatic embryos from ovary **d** and anther **e**. It is possible to observe a low background in correspondence to the cell walls of the embryos: the same is observable in the negative control i.e. section from healthy plants **g**. Negative technical control with the section incubated without the probes is in panel **f**. pe, proembryogenic tissues

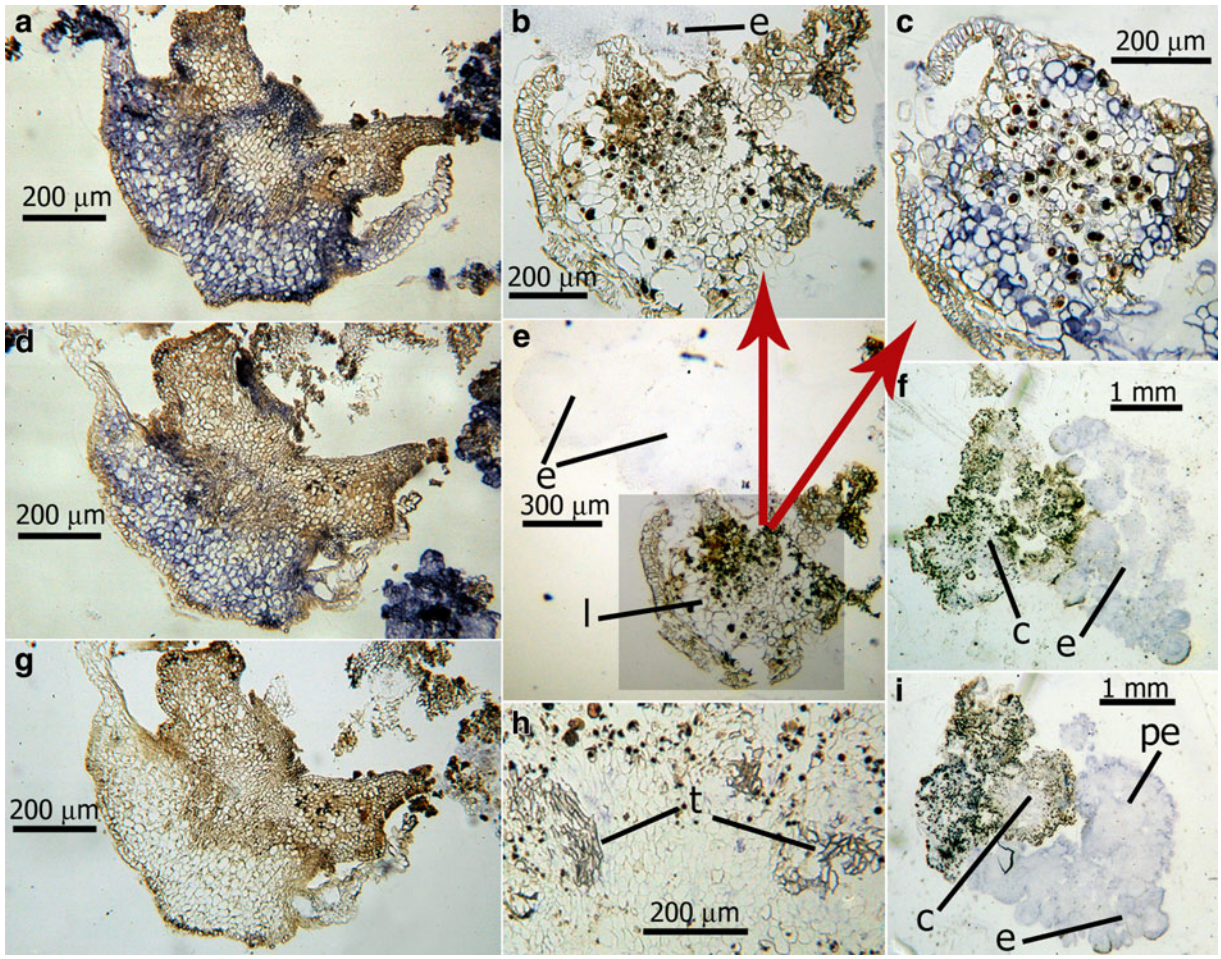

Fig. 4 Microscopic imaging of callus of Lumassina infected by GVA and GLRaV-3, 4 and 6 months after culture initiation. The blue colour indicates the presence of target viral RNA. Four months after culture initiation: section of receptacle hybridized with probes for GLRaV-3 **a**, for GVA **d** and negative control incubated without the anti-DIG antibody **g**. Section of the anther locule and somatic embryos hybridized with GVA-probes is in panel **e**; the area inside the box is enlarged in panel **b** to show a closer view of anther locule, and in panel **c** where the same tissue was hybridized with GLRaV-3-probes. Six months after culture initiation: embryogenic callus from ovary hybridized with probes for GLRaV-3 **f** or for GVA **i**. Small tracheid-like elements in the ovary-derived callus are visible in panel **h**. c, callus tissues; e, somatic embryos; l, anther-locules; pe, proembryogenic tissues; t, tracheid-like elements

nor the samples collected after 6 months of culture (Fig. 4f, i).

Discussion

Indirect somatic embryogenesis is technically more difficult compared with other sanitation techniques and is largely genotype-dependant, but has proven very effective in elimination of the most important grapevine viruses (Goussard et al. 1991; Gambino et al. 2006, 2009; Borroto-Fernandez et al. 2009). In our previous studies, the assays for several phloem-limited viruses (GLRaV-1, GLRaV-3, GVA and GRSPaV) and for GFLV indicated that the original explants (anthers and ovaries) were infected. The percentages of infected callus explants varied according to their origin, with more frequent virus detection in ovary-derived callus than in anther-derived callus (Gambino et al. 2006, 2009), probably because of the larger size of ovaries that may entail a higher initial viral inoculum with consequently more

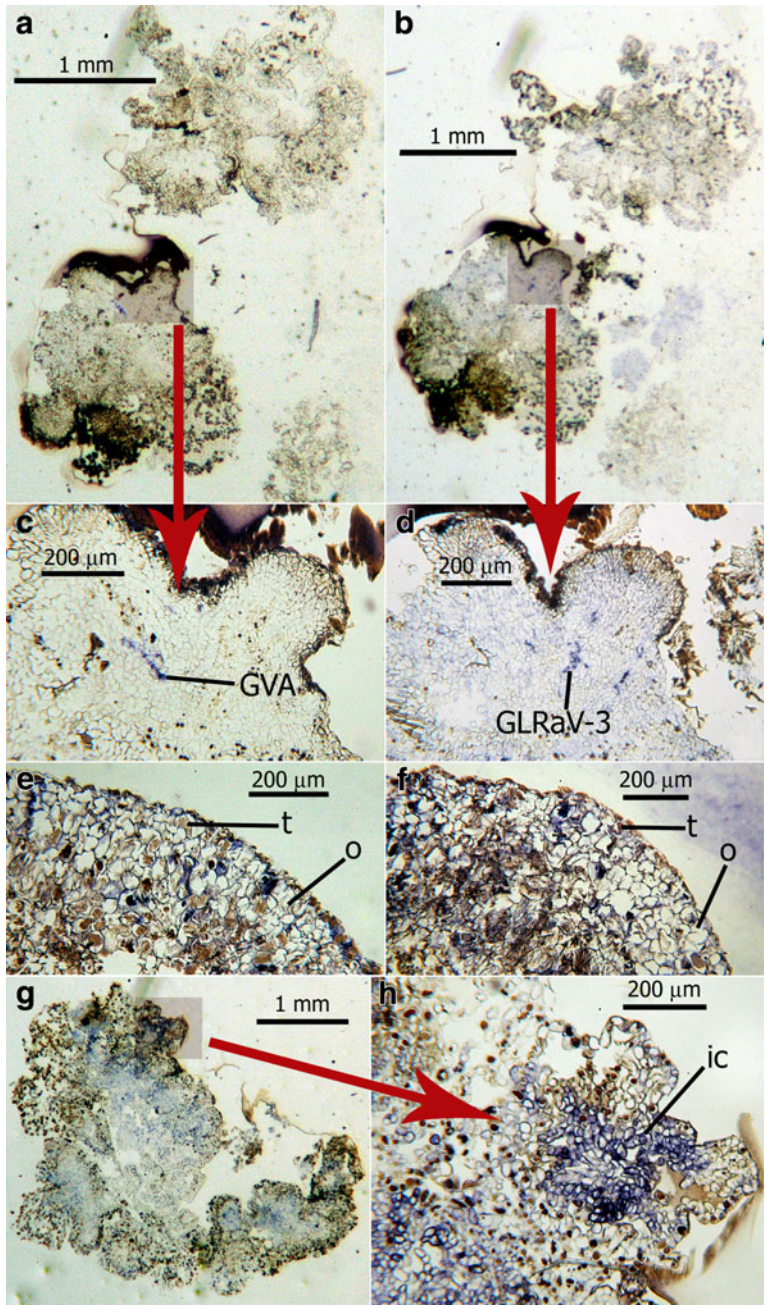

Fig. 5 Microscopic imaging of callus of Lumassina infected by GVA and GLRaV-3, 4 months after culture initiation. The blue colour indicates the presence of target viral RNA. Callus from ovary hybridized with probes for GVA is in panel **a** and for GLRaV-3 in panel **b**. The area inside the box in panel **a** is enlarged in panel **c** to show a closer view of GVA infected-cells, and the area inside the box in panel **b** is enlarged in panel **d** to show a closer view of GLRaV-3 infected-cells. Cells of the ovary rich in tannin depositions and single isolated GVA-infected **e** and GLRaV3-infected cells **f** are visible. Callus from anther hybridized with probes for GVA is in panel **g**: the area inside the box is enlarged in panel **h** to show a closer view of GVA infected-cells. ic, infected callus tissues; o, ovary; t, tannin depositions.

frequent and easier virus diffusion in ovary-derived callus.

Results reported in the scientific literature are quite controversial about survival and spread of viruses in callus. It is known that virus level in cultured cells can be very low and sometimes the virus may be lost (Walkey 1991). Salati et al. (1993) detected closteroviruses in callus cultures from two of five infected grapevines. Scagliusi et al. (2002) and Infante et al. (2008) found that GLRaV-3 and GFLV respectively were present in callus from infected grapevine plants, and that the viruses persisted through many successive generations. In our experimental conditions, the three analyzed viruses initially spread in the callus tissues, and we observed higher accumulation levels of GFLV in groups of cells compared with GVA and GLRaV-3.

The distribution of viruses in callus and the regeneration of healthy embryos/plantlets are related to mechanisms of virus movement in the tissues, and most probably to the characteristics of the callus and its evolution after several months of culture. The three viruses analyzed in this work have different abilities to invade the plant tissues. GFLV, a *nepovirus*, has a rapid diffusion in the tissues. The replication of the two viral genomic RNAs of GFLV occurs on endoplasmic reticulum (ER)-derived membranes vesicles that accumulate within the viral compartment of the host cell at the nuclear periphery (Ritzenthaler et al. 2002). The intracellular transport of movement protein (MP), from the perinuclear site of RNA synthesis to cell periphery, requires a functional secretory pathway and intact microtubules (Andret-Link et al. 2004). The results reported by Laporte et al. (2003) reveal the requirement of a functional cytoskeleton for the proper delivery of MP: the disruption of cytoskeleton did not prevent tubule formation, but had profound effects on the sites of tubule assembly. In the dividing cells during cytokinesis, the MP accumulates almost exclusively within the cell plate (Laporte et al. 2003). The preferential targeting of the tubules to the youngest cross walls is relevant for the movement of the virus in the plant and could explain the ability of GFLV to invade young cells and meristematic tissues.

Unlike GFLV, GVA and GLRaV-3 are phloem-restricted viruses. The replication of GLRaV-3, as other member of family *Closteroviridae*, is associated to a membrane derived from the ER, and the infection results in a drastic remodelling of the ER network that is transformed into a large vesicular factory of viral RNA (Dolja et al. 2006). The intracellular transport of functional virions involve the endomembrane trafficking pathway supported by a integral cytoskeleton (Prokhnevsky et al. 2005). The cell-to-cell movement machinery of C*losteroviridae* member involves five proteins, among which only one, the ER transmembrane protein p6, can be considered a conventional MP, while HSP70h is the only movement-associated protein that accumulates in plasmodesmata and plays an important role in the virion translocation to and through plasmodesmata (Dolja et al. 2006). GLRaV-3 replicates preferentially in the phloem and the cycle of colonization is relatively slow compared to that of other virus families (Dolja et al. 2006). The cytopathological features of GVA, a *vitivirus*, consist of cell wall thickenings, proliferation and accumulation of endomembranes and vesicular evaginations of the tonoplast protruding into the vacuole (Martelli et al. 2007). GVA has a single MP that is a member of the 'p30-like' superfamily typified by the 30-kDa movement protein of TMV; this protein was localized by gold immunolabelling in the cell walls and plasmodesmata (Saldarelli et al. 2000).

As already reported (Gambino et al. 2006) for phloem-limited viruses, regeneration of healthy embryos and plantlets began even when at least a sector of the callus was still infected. Although GVA and GLRaV-3 are phloematic viruses, they could enter the newly formed callus possibly through the plasmodesmata and/or the sieve elements associated to small tracheid-like elements often observed in callus. However their level of infection was low, probably because of their slow movement outside the phloem. After a few months of culture they seemed unable to further spread to new callus cells or survive in the old ones, as no hybridization signal was detected in 6 months-old callus. Pelah et al. (1994) suggested that the disappearance of a tobacco phloematic virus during callus culture was due to a disruption of some cell-to-cell connections, resulting in islands of infected cells in the midst of uninfected tissue, and/or to the competition between the rate of cell division and that of virus replication. Similarly, Scagliusi et al. (2002) observed that the distribution of GLRaV-3 particles appeared to be uneven in grapevine callus, with high concentrations of viruses in some groups of cells. The characteristics of cells of the grapevine

callus and the culture environment could be obstacles for virus movement. In our protocol for somatic embryogenesis induction, high levels of growth regulators (BAP, NOA and IAA) are used for prolonged periods of time. There are indications that treatments with cytokinins have detrimental effects on some viruses, as well as high concentrations of IAA (Clarke et al. 1998). Plant growth regulators interact with the key components of cytoskeleton: they can change the spatial organizations of microtubules and microfilaments, affect gene expression for principal cytoskeletal proteins and change some cytoskeleton-dependent processes, i.e. vesicular transport (Klyachko 2003). In addition, Zhang and John (2005) observed in prolonged suspension culture of *Nicotiana plumbaginifolia* an increased level of cyclin dependent kinase A (CDKA;1) that obstacles plant regeneration by preventing assembly of the preprophase band cytoskeleton. As described above, the closteroviruses require a functional cytoskeleton for proper delivery of MP. The phytohormones that interact with the cytoskeleton may distort the cell-to-cell movement of the viruses and thereby hinder them to move into cells of callus. In addition to the simultaneous presence of non-infected and infected cells in the callus, we observed that GLRaV-3 and GVA were not present in somatic embryos regenerated from callus at least partially infected by these viruses, thus confirming previous results (Goussard et al. 1991; Gambino et al. 2006). As a rule, phloem-limited viruses are not seed-transmissible as they cannot enter zygotic embryos; seed transmission via the embryo and via nucellar tissues seems to require infection of meristematic tissue prior to cytological exclusion of the embryo from the mother plant (Bos 1999). The cell-wall thickening and the disappearance of plasmodesmata, which lead to the isolation of the zygotic embryo, have also been observed in grapevine differentiated cells giving rise to somatic embryos (Faure et al. 1996). This lack of vascular contact between embryos may hinder the spread of viruses restricted to vascular tissues, from infected tissues to somatic embryos. Likewise we did not observe any vascular connection between the parent tissues and the secondary embryos (that usually differentiate in the hypocotyl zone of the primary somatic embryo), as already reported by Newton and Goussard (1990).

Conversely, callus deriving from GFLV-infected explants after 4 months of culture showed relatively high infection levels, with hybridization signals concentrated in peripheral areas of the callus. This presence in peripheral zones may be related to the young age of those cells, given the ability of GFLV to rapidly invade young cells and meristems. Although infected embryos were also present in our cultures, we regenerated only one GFLV-infected plant out of more than 70 embryo-derived plantlets (Gambino et al. 2009 and unpublished results). Apparently GFLV could invade undifferentiated callus and embryos in the early stages of development, but these infected embryos were not—or only in few cases—converted into plants. Abnormal somatic embryos can be frequently observed in grapevine embryogenic cultures, and abnormal embryo morphogenesis has been found to be linked to low germination efficiency, as reviewed by Martinelli and Gribaudo (2009). We observed a possible association between GFLV infection and abnormal embryos that were not converted in plants, as already observed by Popescu et al. (2003) in abnormal somatic embryos infected by *Grapevine fleck virus*. High levels of virus infections may help the malformations of somatic embryos and therefore hamper their germination; however currently we do not have sufficient evidences to definitely corroborate this hypothesis. In addition, in our protocol generally the primary embryos developed from callus provided a long-term source of somatic embryos by secondary embryogenesis. The primary embryos infected by GFLV may have a limited attitude to secondary embryogenesis and/or young secondary embryos regenerated from the primary GFLV-infected embryos may escape virus invasion. A similar situation was indeed observed by Quainoo et al. (2008) in somatic embryos of cacao: the *Cocoa swollen shoot virus* was gradually excluded from primary to secondary embryos.

The present work reports the difficulties of some grapevine viruses in stably invading callus tissues, highlighting the differential ability of GFLV to spread in callus cells compared to the phloem-limited viruses. Moreover, it confirms the effectiveness of somatic embryogenesis in eliminating some of the most important grapevine viruses.

References

Alkowni, R., Rowhani, A., Daubert, S., & Golino, D. (2004). Partial characterization of a new ampelovirus associated

with grapevine leafroll disease. *Journal of Plant Pathology, 86*, 123–133.

Andret-Link, P., Laporte, C., Valat, L., Ritzenthaler, C., Demangeat, G., Vigne, E., et al. (2004). *Grapevine fanleaf virus*: still a major threat to the grapevine industry. *Journal of Plant Pathology, 86*, 183–195.

Borroto-Fernandez, E. G., Sommerbauer, T., Popowich, E., Schartl, A., & Laimer, M. (2009). Somatic embryogenesis from anthers of the autochthonous *Vitis vinifera* cv. Domina leads to *Arabis mosaic virus*-free plants. *European Journal of Plant Pathology, 124*, 171–174.

Bos, L. (1999). *Plant viruses, unique and intriguing pathogens*. Leiden: Backhuys.

Chevalier, S., Greif, C., Clauzel, J.-M., Walter, B., & Fritsch, C. (1995). Use of an immunocapture-polymerase chain reaction procedure for the detection of *Grapevine virus A* in Kober stem grooving-infected grapevines. *Journal of Phytopathology, 143*, 369–373.

Clarke, S. F., Burritt, D. J., Jameson, P. E., & Guy, P. L. (1998). Influence of plant hormones on virus replication and pathogenesis-related proteins in *Phaseolus vulgaris* L. infected with white clover mosaic potexvirus. *Physiological and Molecular Plant Pathology, 53*, 195–207.

D'Onghia, A. M., Carimi, F., De Pasquale, F., Djelouah, K., & Martelli, G. P. (2001). Elimination of *Citrus psorosis virus* by somatic embryogenesis from stigma and style cultures. *Plant Pathology, 50*, 266–269.

Dolja, V. V., Kreuze, J. F., & Valkonen, J. P. T. (2006). Comparative and functional genomics of closterovirus. *Virus Research, 117*, 38–51.

Faure, O., Aarrouf, J., & Nougarède, A. (1996). Ontogenesis, differentiation and precocious germination in anther-derived somatic embryos of grapevine (*Vitis vinifera* L): proembryogenesis. *Annals of Botany, 78*, 23–8.

Gambino, G., & Gribaudo, I. (2006). Simultaneous detection of nine grapevine viruses by multiplex RT-PCR with coamplification of a plant RNA internal control. *Phytopathology, 96*, 1223–1229.

Gambino, G., Bondaz, J., & Gribaudo, I. (2006). Detection and elimination of viruses in callus, somatic embryos and regenerated plantlets of grapevine. *European Journal of Plant Pathology, 114*, 397–404.

Gambino, G., Di Matteo, D., & Gribaudo, I. (2009). Elimination of *Grapevine fanleaf virus* from three *Vitis vinifera* cultivars by somatic embryogenesis. *European Journal of Plant Pathology, 123*, 57–60.

Goussard, P. G., Wiid, J., & Kasdorf, G. G. F. (1991). The effectiveness of *in vitro* somatic embryogenesis in eliminating fanleaf virus and leafroll associated viruses from grapevines. *South African Journal of Enology and Viticulture, 12*, 77–81.

Gribaudo, I., Gambino, G., & Vallania, R. (2004). Somatic embryogenesis from grapevine anthers: identification of the optimal developmental stage for collecting explants. *American Journal of Enology and Viticulture, 55*, 427–430.

Infante, R., Fiore, N., & Seibert, E. (2008). Preservation of *Grape fanleaf virus* on callus culture of *Vitis vinifera* cv. Cabernet Sauvignon. *Acta Phytopathologica et Entomologica Hungarica, 43*, 101–108.

Klyachko, N. L. (2003). Phytohormones and cytoskeleton. *Russian Journal of Plant Physiology, 50*, 426–430.

Laporte, C., Vetter, G., Loudes, A.-M., Robinson, D. G., Hillmer, S., Stussi-Geraud, C., et al. (2003). Involvement of the secretory pathway and the cytoskeleton in intracellular targeting and tubule assembly of *Grapevine fanleaf virus* movement protein in tobacco BY-2 cells. *Plant Cell, 15*, 2058–2075.

Mckenzie, M., & Pathirana, R. (2007). Micrografting grapevine for virus indexing. In S. M. Jain & H. Häggman (Eds.), *Protocols for micropropagation of woody trees and fruits* (pp. 259–266). Netherlands: Springer.

Martelli, G. P. (2009). Grapevine virology highlights 2006–09. Extended abstract of 16th ICVG Meeting, Dijon, France, 15–23

Martelli, G. P., Agranovsky, A. A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R. H. A., et al. (2002). The family *Closteroviridae* revised. *Archives of Virology, 147*, 2039–2044.

Martelli, G. P., Adams, M. J., Kreuze, J. F., & Dolja, V. V. (2007). Family *Flexiviridae*: a case study in virion and genome plasticity. *Annual Review of Phytopathology, 45*, 73–100.

Martinelli, L., & Gribaudo, I. (2009). Strategies for effective somatic embryogenesis in grapevine (*Vitis* spp.): an appraisal. In K. Roubelakis-Angelakis (Ed.), *Grapevine molecular physiology & biotechnology* (2nd ed., pp. 461–494). Dordrecht: Springer.

Newton, D. J., & Goussard, P. G. (1990). The ontogeny of somatic embryos from *in vitro* cultured grapevine anthers. *South African Journal of Enology and Viticulture, 11*, 70–75.

Parmessur, Y., Aljanabi, S., Saumtally, S., & Dookun-Saumtally, A. (2002). Sugarcane yellow leaf virus and sugarcane yellows phytoplasma: elimination by tissue culture. *Plant Pathology, 51*, 561–566.

Pelah, D., Altman, A., & Czosnek, H. (1994). *Tomato yellow leaf curl virus* DNA in callus cultures derived from infected tomato leaves. *Plant, Cell, tissue and Organ Cultures, 39*, 37–42.

Popescu, C. F., Buciumeanu, E. C. & Visoiu, E. (2003). Somatic embryogenesis, a reliable method for *Grapevine fleck virus* free grapevine regeneration. Extended abstract of 14th ICVG Meeting, Locorotondo, Italy, 243

Prokhnevsky, A. I., Peremyslov, V. V., & Dolja, V. V. (2005). Actin cytoskeleton is involved in targeting of a viral Hsp70 homolog to the cell periphery. *Journal of Virology, 79*, 14421–14428.

Quainoo, A. K., Wetten, A. C., & Allainguillaume, J. (2008). The effectiveness of somatic embryogenesis in eliminating the *Cocoa swollen shoot virus* from infected cocoa trees. *Journal of Virological Methods, 149*, 91–96.

Ritzenthaler, C., Laporte, C., Gaire, F., Dunoyer, P., Schmitt, C., Duval, S., et al. (2002). *Grapevine fanleaf virus* replication occurs on endoplasmic reticulum-derived membranes. *Journal of Virology, 76*, 8808–8819.

Salati, R., Golino, D., Rowhani, A., Willitis, N. & Gonsalves, D. (1993). Detection of grapevine closterovirus associated with leafroll and corky bark in vitro using F(ab')2 ELISA. Extended Abstracts of the 11th ICVG Meeting, Montreux , Switzerland, 142–143

Saldarelli, P., Minafra, A., Castellano, M. A., & Martelli, G. P. (2000). Immunodetection and subcellular localization of the proteins encoded by ORF 3 of grapevine viruses A and B. *Archives of Virology, 145*, 1535–1542.

Scagliusi, S. M. M., Vega, J., & Kuniyuki, H. (2002). Cytopathology of callus cells infected with *Grapevine leafroll-associated virus* 3. *Fitopatologia Brasileira, 27*, 384–388.

Zhang, K., & John, P. C. L. (2005). Raised level of cyclin dependent kinase A after prolonged suspension culture of *Nicotiana plumbaginifolia* is associated with more rapid growth and division, diminished cytoskeleton and lost capacity for regeneration: implications for instability of cultured plant cells. *Plant Cell, Tissue and Organ Culture, 82*, 295–308.

Walkey, D. G. A. (1991). *Applied plant virology*. London: Chapman and Hall.