INVITED REVIEW

GRAPEVINE FANLEAF VIRUS: STILL A MAJOR THREAT TO THE GRAPEVINE INDUSTRY

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SUMMARY

Grapevine fanleaf virus (GFLV) is responsible for fanleaf degeneration, which is one of the most severe virus diseases of grapevines worldwide. GFLV causes substantial crop losses, reduces fruit quality and shortens the longevity of grapevines in the vineyard. GFLV is transmitted specifically from grapevine to grapevine by the ectoparasitic nematode *Xiphinema index*, and belongs to the genus *Nepovirus* in the family *Comoviridae*. Since the discovery of the nematode vector in the late 1950's and the identification of GFLV as the agent responsible for fanleaf degeneration in the early 1960's, a wealth of information has been accumulated on its transmission, biological properties and serological characteristics, as well as on the structure and expression of the GFLV genome. Although dissemination of the virus through propagation material has been drastically reduced over the past two decades by implementing rigorous certification schemes and establishing quarantine facilities, effective strategies are still needed to control GFLV in naturally infected vineyards. Recently, significant progress has been made on the elucidation of the function(s) of most GFLV proteins, in particular those involved in critical steps of the virus multiplication cycle, including RNA replication, cell-to-cell movement, and transmission by *X. index*. New insights have also been gained into the population structure and genomic variability among isolates from naturally infected vineyards, which have opened new avenues for designing alternative strategies to control this destructive virus. This review article offers a comprehensive overview of the most significant advances made over the past 15 years on GFLV and discusses novel control strategies for one of the major threats to the grapevine industry worldwide.

Key words: GFLV, fanleaf degeneration, grapevine, biological properties, serological characteristics, genome structure, genome expression, population structure, genetic variability, cell-to-cell movement, replication, nematode-mediated transmission, *Xiphinema index*, control, transgenic resistance.

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INTRODUCTION

Grapevine fanleaf virus (GFLV) is responsible for fanleaf degeneration, which is the most severe virus disease of grapevines (Raski *et al*., 1983; Bovey *et al*., 1990; Martelli and Savino, 1990). This disease occurs worldwide in almost all temperate regions where *Vitis vinifera* and hybrid rootstocks are cultivated. It has been reported in Asia, Africa, Europe, New Zealand, South Australia, North America, and South America (Bovey *et al*., 1990; Martelli and Savino, 1990). GFLV can be widespread in some viticultural regions. For example, this virus is estimated to affect around 2,000 hectares (6% of the total acreage cultivated with grapes) in the Champagne region of France. Crop losses caused by GFLV vary from moderate to high, affecting more than 80% of the crop depending on the virulence of the virus isolate, the susceptibility of the grapevine variety, and environmental factors (Bovey *et al*., 1990; Martelli and Savino, 1990). Fruit quality is also altered by GFLV with a substantial decrease in sugar content and titratable acidity. In the vineyard, GFLV is transmitted from grapevine to grapevine by the ectoparasitic nematode *Xiphinema index* (Hewitt *et al*., 1958).

GFLV belongs to the genus *Nepovirus* of the family *Comoviridae* (Mayo and Robinson, 1996). It has isometric particles of 28 nm in diameter, which result from the assembly of a single protein species of Mr 56,000 (Quacquarelli *et al*., 1976). The genome of GFLV consists of two single-stranded positive-sense RNAs (Quacquarelli *et al*., 1976). Since the discovery of GFLV and its nematode vector in the early 1960's and late 1950's, respectively, a wealth of information has been accumulated on the transmission, biological and serological properties, genome structure and expression, as well as on strategies to eliminate GFLV in grapevine propagation material (for a review see Martelli *et al*., 2003). Recently, significant progress has been made on the elucidation of the function(s) of most GFLV proteins, in particular those involved in critical steps of the

virus multiplication cycle, including RNA replication, cell-to-cell movement, and transmission by *X. index*.

This review article offers a comprehensive overview of our current knowledge on GFLV, with a special emphasis on the molecular aspects of the function(s) of viral proteins, in particular those involved in cell-to-cell movement, replication, and nematode-mediated transmission. Based on recent studies on the population structure and genetic variability of GFLV isolates from naturally infected vineyards, we will discuss alternative environmentally-sound strategies to control this destructive virus, including perspectives on the use of transgenic material.

DISCOVERY

More than 160 years ago the fanleaf degeneration disease of grapevines, with which GFLV is associated, was described and its soil-borne nature suspected (report from 1841 by the same author in Cazalis-Allut, 1865). As early as 1902, the implication of a pathogen similar to the *contagium vivum fluidum* (Beijerinck, 1898) was hypothesized (Baccarini, 1902). The first experimental evidence, which showed that fanleaf degeneration is contracted from diseased vineyard soil, was obtained by Petri (1918). Vuittenez (1975) showed that soil fumigation with different agrochemicals, prior to planting, could be effective in controlling fanleaf degeneration. Subsequently, *Xiphinema index*, the ectoparasitic nematode vector of GFLV, was identified (Hewitt *et al.*, 1958). This was the first report on the transmission of a plant virus by a nematode vector. In 1960, GFLV was successfully transmitted to experimental herbaceous hosts by mechanical inoculation of sap from diseased grapevines (Baldacci *et al.*, 1960; Cadman *et al.*, 1960; Vuittenez, 1960). Cadman and colleagues identified the long-suspected viral agent responsible for fanleaf degeneration by electron microscopy after partial purification from infected *Chenopodium amaranticolor* (Cadman *et al.*, 1960). These authors also partially characterized some of the biological and serological properties of GFLV (Cadman *et al.*, 1960). Back transfer of GFLV from infected herbaceous hosts to grapevine was attempted but no symptom development was reported in the natural host (Cadman *et al.*, 1960). Only in 1962 did Hewitt and colleagues fulfill Koch's postulates by using *X. index* to transmit GFLV to *C. amaranticolor* and back from *C. amaranticolor* to grapevines in which typical symptoms developed (Hewitt *et al.*, 1962). These experiments demonstrated conclusively that GFLV is the causal agent of fanleaf degeneration disease. In 1963, typical symptoms of fanleaf degeneration were reproduced in grapevines following graft transmission from GFLV-infected *C. amaranticolor* and the virus was successfully transferred by sap transmission from symptomatic grapevines to *C. amaranticolor* (Dias, 1963). It is worth noting that this controversial discovery in the early 1960's (controversial because phylloxera was believed to be involved in fanleaf degeneration) prompted some visionary scientists to create the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG). This nonprofit scientific organization founded in 1964 promotes exchange and collaboration worldwide on grapevine diseases caused by viruses and virus-like agents (http://www.racchangins.ch/doc/fr/chercheurs/collab_ext/ICVG/index/html).

SYMPTOMATOLOGY, HOST RANGE, AND SEROLOGY

GFLV-infected grapevines often show a patchy distribution in diseased vineyards, as a result of a plant-to-plant virus transmission by the nematode vector *X. index* and its limited movement in the soil (Fig. 1A). GFLV causes a variety of symptoms in grapevines that differ in type and severity (Martelli, 1993). Leaves become distorted and asymmetrical with sharply toothed margins, closer primary veins, and an open petiolar sinus. These typical foliar symptoms resemble a fan, hence, the name of the virus and the disease. Other foliar symptoms include chlorotic mottling, yellow mosaic with partially or completely chrome-yellow leaves, and vein-banding with light-green to chrome-yellow chlorotic bands along the veins. Obscure speckles and small yellow spots are also observed (Raski *et al.*, 1983). Foliar symptoms develop early in the spring and persist throughout most of the vegetative season (Martelli, 1993), despite some fading during hot summers. Canes can also be malformed, showing short internodes, double nodes, fasciations, and zigzag growth between nodes (Raski *et al.*, 1983). However, leaf and cane malformations may not always be prominent (Martelli, 1993). Remarkably, endocellular tubular structures, which resemble bars crossing the lumen of vascular elements, are highly characteristic internal GFLV symptoms. These structures consist of a pectic core surrounded by a cellulose sheath encrusted with lignin, suberin, or cutin depending on the tissue. Endocellular tubular structures are readily visible in lignified shoots and basal internodes. Thus, they are good indicators of the presence of GFLV (Martelli and Savino, 1990).

Crop losses can be from moderate (10%) to very high (>80%) (Fig. 1B). The reduction in crop yield can even result in a total loss of production (Raski *et al.*, 1983; M. Fuchs, unpublished observations). Fruit clusters are reduced in size and number, and their ripening is irregular (Martelli and Savino, 1990) (Fig. 1B). Infected berries are uneven in size with numerous small and seedless individuals, some of which may not mature.

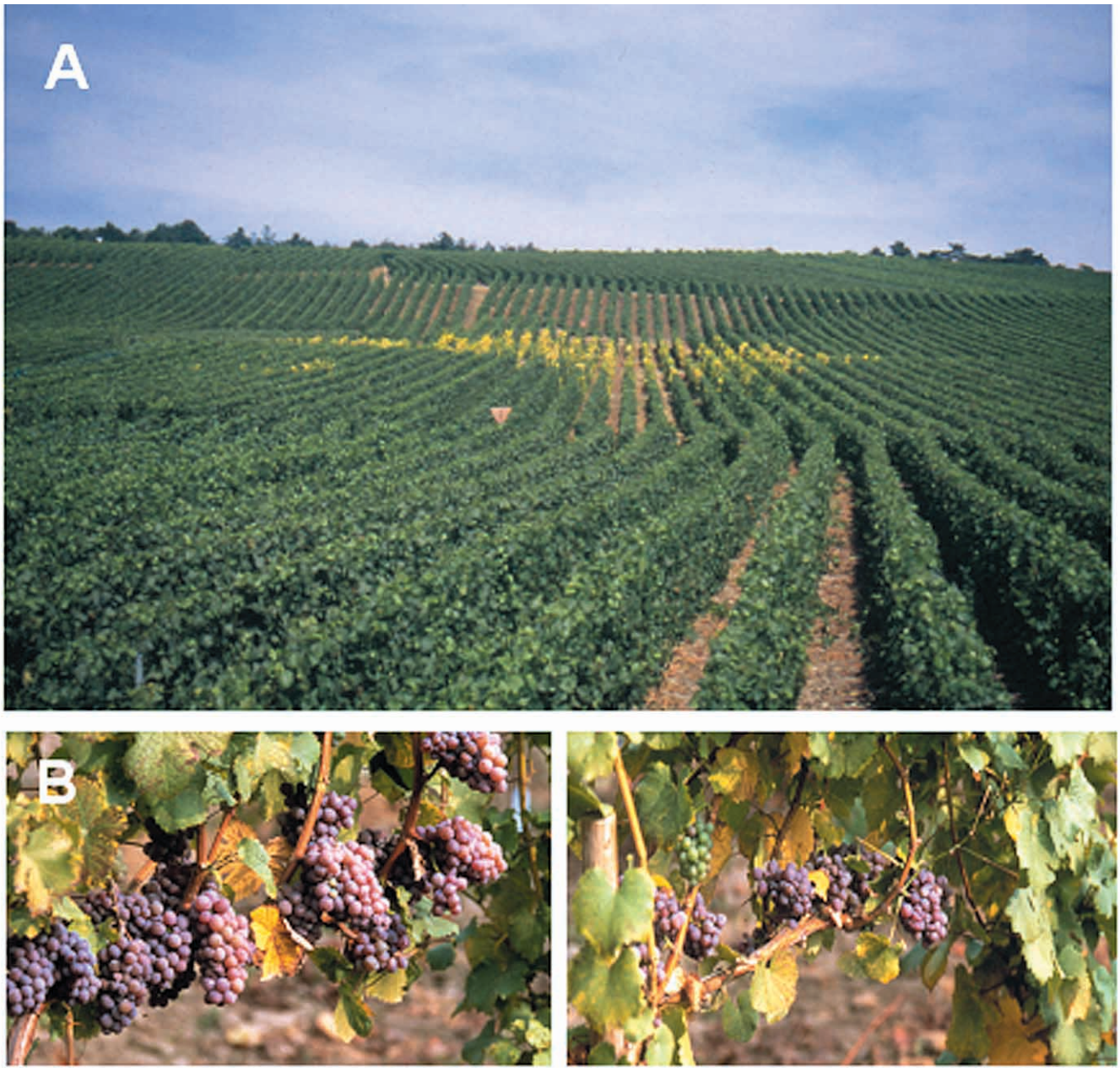

Fig. 1. (**A**) Patchy distribution of grapevines infected by *Grapevine fanleaf virus* in a Chardonnay vineyard in the Champagne region in France as a result of a plant-to-plant virus transmission by the ectoparasitic nematode *Xiphinema index*, and (**B**) Comparative production of a healthy (left) and a GFLV-infected (right) grapevine cv. *Vitis vinifera* Savagnin rose non aromatic, Klevener de Heiligenstein.

Fruit quality is affected due to a decrease in sugar content and titratable acidity. The rooting ability of rootstocks and the graft take of scions are both substantially reduced in GFLV-infected material. Also, the productive life of GFLV-infected vineyards is significantly reduced (15-20 years instead of 30-40 years or longer).

Vitis spp. are by far the major natural hosts of GFLV, although the virus can occasionally be detected in weeds in the vineyard (Horvath *et al*., 1994; Izadpanah *et al*., 2003). GFLV is readily transmitted to herbaceous plants by inoculation of grapevine sap but the experimental host range is limited to species in the families *Amaranthaceae*, *Chenopodiaeae*, *Cucurbitaceae*, *Leguminoseae*, *Solanaceae*, and *Fabaceae*.

GFLV particles are relatively good immunogens because polyclonal antisera with titers of 1/20,000 can be obtained (Etienne *et al*., 1990). Interestingly, GFLV is a serologically homogeneous virus species with limited serological variability reported in tests using polyclonal antisera (Savino *et al*., 1985) or monoclonal antibodies

(Huss *et al.*, 1987). GFLV is distantly related serologically to *Arabis mosaic virus* (ArMV) (Cadman *et al.*, 1960). GFLV can be detected routinely by enzyme-linked immunosorbent assay of various grapevines extracts (leaves collected during spring, rootlets, cortical scrapings from mature canes, petioles).

GENOME STRUCTURE AND EXPRESSION

GFLV formerly classified in the Nepovirus group (Harrison *et al.*, 1971), a collection of viruses with no taxonomic significance, now belongs to the genus *Nepovirus* in the family *Comoviridae* (Wellink *et al.*, 2000). Virus particles are polyhedral with a diameter of 28 nm and consist of three serologically indistinguishable density components called top (T), middle (M), and bottom (B) (Quacquarelli *et al.*, 1976; Brown *et al.*, 1995; Mayo and Robinson, 1996). T component particles are empty shells, M component particles contain RNA2, and B component particles contain both RNA species (Quacquarelli *et al.*, 1976). The genome of GFLV consists of two single-stranded positive-sense RNA species with a molecular weight of $2.4 \cdot 10^6$ for RNA1 and $1.4 \cdot 10^6$ for RNA2 (Quacquarelli *et al.*, 1976). Infectivity requires both RNA1 and RNA2 (Quacquarelli *et al.*, 1976).

The two genomic RNAs carry a small covalently linked viral protein (VPg) at their 5' extremity and a poly(A) stretch at their 3' end (Pinck *et al.*, 1988) (Fig. 2). RNA1 is 7,342 nt long and contains a single open reading frame of 6,855 nt, extending from nts 243 to 7097 (Ritzenthaler *et al.*, 1991). RNA2 is 3,774 nt long with a single open reading frame of 3,330 nt, extending from nts 233 to 3562 (Serghini *et al.*, 1990). Each genomic RNA codes for a polyprotein, denoted P1 for the Mr 253,000 polyprotein encoded by RNA1 (Ritzenthaler *et al.*, 1991) and P2 for the Mr 122,000 polyprotein encoded by RNA2 (Serghini *et al.*, 1990). Both polyproteins are proteolytically processed into functional proteins by the RNA1-encoded proteinase (Margis *et al.*, 1991; Margis and Pinck, 1992) (Fig. 2).

Five maturation products referred to as 1A (Mr 46,000), $1B^{Hel}$ (Mr 88,000), $1C^{VPg}$ (Mr 3,000), $1D^{Pro}$ (Mr 24,000), and $1E^{Pol}$ (Mr 92,000) from the N- to the C-terminus of polyprotein P1 are generated by *cis* processing at Cys/Ala, Cys/Ser, Gly/Glu, and Arg/Gly cleavage sites, respectively (Pinck *et al.*, 1991; Ritzenthaler *et al.*, 1991; Margis *et al.*, 1994) (Fig. 2). Sequence comparisons between RNA1 of GFLV and RNAs of closely related viruses, including nepo-, como-, and picornaviruses, reveal signature sequences that provide indications of the function of some of these proteins. Thus, protein 1A is a putative proteinase cofactor (Ritzenthaler *et al.*, 1991). Protein $1B^{Hel}$ involves an NTP-binding domain and is a putative helicase, and protein $1E^{Pol}$ is the putative RNA-dependant RNA polymerase (Ritzenthaler *et al.*, 1991). In addition, experimental evidence demonstrated that protein $1C^{VPg}$ is the virus genome-linked protein (Pinck *et al.*, 1991), and protein $1D^{Pro}$ a chymotrypsin-like cysteine proteinase (Margis *et al.*, 1991; Margis and Pinck, 1992).

Three final maturation products referred to as $2A^{HP}$ (Mr 28,000), $2B^{MP}$ (Mr 38,000) and $2C^{CP}$ (Mr 56,000) from the N- to the C-terminus of polyprotein P2 are generated by *trans* processing at Cys/Ala and Arg/Gly cleavage sites, respectively (Serghini *et al.*, 1990; Margis *et al.*, 1993b) (Fig. 2). Protein $2A^{HP}$ is required for RNA2 replication and could act as a homing protein by leading RNA2 or polyprotein P2 to a perinuclear area corresponding to the virus replication sites (Gaire *et al.*, 1999). Protein $2B^{MP}$ is the movement protein (MP) and a constituent of tubular structures that protrude from the cell wall and are observed in modified plasmodesmata of GFLV-infected cells (Ritzenthaler *et al.*, 1995b). Protein $2C^{CP}$ is the single coat protein species (Serghini

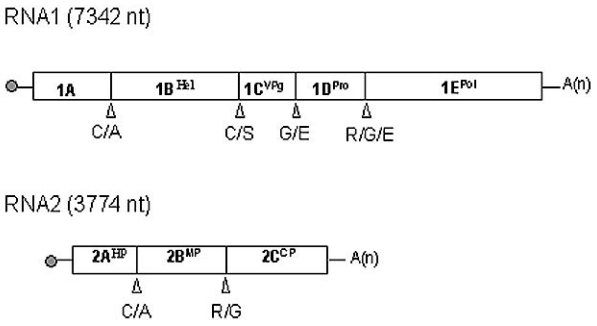

Fig. 2. Genetic organization of *Grapevine fanleaf virus* strain F13 RNA1 and RNA2. Open reading frames are represented by wide-open boxes and the VPg is represented by a grey circle. The 5' and 3' noncoding regions are represented by black lines. Each RNA codes for a polyprotein that is cleaved into functional proteins: Hel, helicase; VPg, viral genome-linked protein; Pro, proteinase; Pol, RNA-dependent RNA polymerase; HP, homing protein; MP, movement protein; CP, coat protein. Open triangles indicate the cleavage sites.

et al., 1990; Margis *et al.*, 1993b) that assembles into the virus capsid (Quacquarelli *et al.*, 1976). We recently constructed a three-dimensional model of the GFLV capsid (Andret-Link *et al.*, 2003) by using the crystal structure of *Tobacco ringspot virus*, the type member of the genus *Nepovirus* (Chandrasekar and Johnson, 1998). It shows the GFLV capsid composed of 60 copies of protein $2C^{CP}$ with domains arranged in a pseudo T = 3 icosahedral surface lattice folded into three trapezoid-shaped-β-barrel domains (designated C, B, and A from the N- to the C-terminus, respectively) covalently linked together (Andret-Link *et al.*, 2003). The B and C domains lie side-by-side around the capsid threefold axes

and the prominent protrusion along the fivefold axes is formed by the A domain (Andret-Link *et al.*, 2003).

In vitro transcripts produced from full-length cDNA of RNA1 and RNA2 are infectious *in planta* (Viry *et al.*, 1993). These biologically active transcripts are useful tools for investigating the function(s) of GFLV-encoded proteins and interaction of GFLV with its nematode vector (Margis *et al.*, 1991; 1993b; Gaire *et al.*, 1999; Belin *et al.*, 1999, 2001; Andret-Link *et al.*, 2004).

Satellite RNA molecules are associated with some GFLV isolates (Pinck *et al.*, 1988; Saldarelli *et al.*, 1993). The satellite RNA associated with GFLV strain F13, denoted RNA3, has the same 5' and 3' terminal structures as the two genomic RNAs (Pinck *et al.*, 1988). RNA3 is 1,114 nt long and contains a single open reading frame of 1026 nt, extending from nts 15 to 1040 (Fuchs *et al.*, 1989). A consensus sequence (UG/UGAAAAU/AU/AU/A) in the 5' untranslated region is found in RNA3 and the genomic RNAs of GFLV, and of several other nepoviruses (Fuchs *et al.*, 1989). RNA3 requires the two genomic RNAs for its encapsidation and replication (Pinck *et al.*, 1988; Hans *et al.*, 1992, 1993). The RNA3-encoded protein P3 is highly hydrophilic and extremely basic (calculated pHi of approximately 11) with a Mr of 37,275 (Fuchs *et al.*, 1989). Protein P3 is detected in subcellular membrane fractions and nucleus-enriched fractions but not in cytoplasmic or cell wall fractions (Moser *et al.*, 1992). Protein P3 seems to be required for the replication of RNA3 (Hans *et al.*, 1992, 1993).

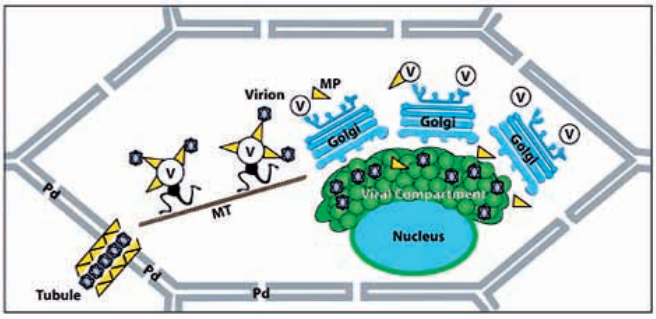

Fig. 3. Schematic representation of the replication, intracellular trafficking, and cell-to-cell movement steps of *Grapevine fanleaf virus*. After cell entry and decapsidation of GFLV particles, the two viral genomic RNAs are translated and replication occurs on endoplasmic reticulum (ER)-derived membranous vesicles that accumulate within the viral compartment at the nuclear periphery. RNA1- and RNA2-encoded polyproteins are proteolytically processed by the $1C^{Pro}$ protease within the viral compartment where mature proteins accumulate, except the movement protein $2B^{MP}$ that seems to be rapidly transported to the cell periphery. It is suggested that $2B^{MP}$ could be transported together with the CP or viral particles on Golgi-derived secretory vesicles (V) along microtubules (MT). Once at the cell periphery at sites probably related to plasmodesmata (Pd), the MP self assembles into tubules through which viral particles move from cell-to-cell. Elements of the figure are not drawn to scale.

REPLICATION

Plant RNA viruses replicate in association with membranes originating from different sources such as the endomembrane system, chloroplasts, mitochondria, etc.. GFLV-infected cells show a massive proliferation of membranes that generally accumulate in the nuclear periphery to form a so-called viral compartment (Fig. 3). Using cells expressing the green fluorescent protein (GFP) targeted to different cell compartments as a marker, it has been established that the membranes of the viral compartment originate essentially from the endoplasmic reticulum (ER) (Ritzenthaler *et al.*, 2002). Immunoconfocal microscopy experiments further demonstrated that this perinuclear compartment is the site of GFLV replication since it contained double-stranded replicative forms, newly synthesized viral RNA, and the RNA1-encoded VPg (Ritzenthaler *et al.*, 2002). GFLV replication depends also on *de novo* lipid synthesis and is sensitive to brefeldin A (a fungal metabolite known to disrupt the secretory pathway and to induce the formation of an ER-Golgi hybrid compartment in tobacco cells), suggesting that the COP (coat protein-coated vesicles that transport proteins from organelle to organelle) vesicular trafficking mechanism could recruit ER-derived membranes (Ritzenthaler *et al.*, 2002). The polyprotein P1 encoded by RNA1 is required for viral RNA replication and is responsible for the formation of the viral compartment, although the maturation products(s) responsible for the recruitment of vesicles from the ER is/are not characterized. The development of specific antibodies to each of the five RNA1-encoded proteins, including the VPg (Margis *et al.*, 1993a), will enable us to perform subcellular detection assays and *in situ* localization experiments, and thus reveal further details of the replication of GFLV.

RNA2 is replicated *in trans* by the RNA1-encoded replication machinery and protein 2A was shown to be necessary but not sufficient for RNA2 replication. Expression of a 2A:GFP fusion protein in healthy or GFLV-infected cells demonstrated that protein 2A accumulates in the viral compartment and its subcellular distribution is affected by the replication process. It was therefore suggested that protein 2A could mediate the transport of nascent P2-RNA2 complexes from their initial location in the cytosol to the perinuclear replication sites (Gaire *et al.*, 1999).

CELL-TO-CELL MOVEMENT

As described above, GFLV replication probably takes place in the perinuclear area where all replication pro-

teins accumulate. From there, GFLV particles have to find their way to the cell periphery and probably to plasmodesmata through which they move to invade neighboring uninfected cells. The early stage of GFLV movement can therefore be separated into two distinct steps: intracellular movement from the perinuclear site of RNA synthesis and virus assembly to the cell periphery, and then intercellular movement across the cell wall.

The identification of protein 2B as the movement protein (MP) was the first significant achievement in the study of the GFLV transport mechanism (Ritzenthaler *et al.*, 1995a, b). The highly stable MP does not accumulate in the viral compartment (Ritzenthaler *et al.* 1995b, 2002) but forms virion-filled tubules that either protrude from the surface of infected protoplasts or are embedded within highly modified plasmodesmata in infected cells (Ritzenthaler *et al.*, 1995a, b). Both MP and CP are needed for GFLV movement but not replication, as shown by the use of RNA2 deletion mutants and chimeric GFLV/ArMV RNA2 constructs (Belin *et al.*, 1999; Gaire *et al.*, 1999). More recently, the establishment of a transgenic tobacco BY-2 cell line expressing GFP:MP has enabled us to demonstrate that no other viral protein apart from the MP is required for tubule formation (Laporte *et al.*, 2003). In addition, this transgenic cell line allowed the spatio-temporal analysis of GFLV MP intracellular transport and assembly into tubules. By using various inhibitors, it was established that intracellular MP transport requires a functional secretory pathway and intact microtubules to cross cell walls through plasmodesmata, whereas tubule formation *per se* is cytoskeleton-independent (Laporte *et al.*, 2003). Interestingly, MP:GFP was also shown to accumulate within the cell plate in dividing cells. Together with results of biochemical analyses, these data revealed that the MP behaves as an intrinsic membrane protein and further suggest that the MP could be transported intracellularly at the surface of Golgi-derived secretory vesicles (Laporte *et al.*, 2003) (Fig. 3). Once in the immediate proximity of plasmodesmata at the cell periphery, the MP self assembles into tubules, possibly with the help of cellular factor(s) that remain(s) to be identified and could play a nucleator role. Tubules are unidirectional structures with their base embedded in the cross-walls and their tip hanging free within the cytoplasm. Tubule growth is polar and involves the addition of newly synthesized MP subunits only at the base of the tubule embedded in the cell wall, thus allowing the protrusion of the tubule into the cytoplasm of the neighboring cell (Laporte *et al.*, unpublished results) (Fig. 3).

Compared to the MP transport, less information is available on the transport of GFLV particles. However, based on strong similarities in virus spread between GFLV and *Cowpea mosaic virus* (CPMV), the cell-to-cell movement of GFLV probably involves MP-CP or MP-virion interactions during both the intracellular and intratubular transport processes (Ritzenthaler *et al.*, 1995a,b; Belin *et al.*, 1999; Carvalho *et al.*, 2003). This short distance movement represents the first step in systemic infection of a whole plant (Ritzenthaler *et al.*, 1995a,b).

POPULATION STRUCTURE AND GENETIC VARIABILITY OF FIELD ISOLATES

Theoretically, GFLV has a great potential for genetic variation because it infects its natural host for long periods of time and its replication process is error-prone since no proof-reading correction mechanism is associated with the RNA1-encoded RNA-dependent RNA polymerase. Therefore, each GFLV isolate is expected to consist of a population of genetically related variants termed a quasipecies. Naraghi-Arani *et al.* (2001) demonstrated the quasispecies nature of GFLV populations by analyzing 14 isolates from eight California vineyards. More recently, we investigated the population structure and genetic variability within 347 GFLV isolates from a naturally infected Chardonnay vineyard in the Champagne region in France (Vigne *et al.*, 2004a,b). Interestingly, 55% of the 347 GFLV isolates tested had a population structure consisting of one predominant restrictotype and 33% of the isolates sequenced (17 of 51) had a population structure with at least two distinct haplotypes (Vigne *et al.*, 2004a). Although the variability in the CP gene ranged from 0.5 to 13.8% at the nucleotide level, limited heterogeneity was found at the amino acid level (0.2-6.9%), indicating a strong genetic stability in the GFLV CP gene (Vigne *et al.*, 2004a).

Sequences of the CP gene of 51 isolates revealed that two major haplotypes were predominant in the population analyzed (Vigne *et al.*, 2004a). Interestingly, five recombinant isolates were identified among the 347 GFLV isolates analyzed (Vigne *et al.*, 2004a) and some of these are being analyzed to determine if they have biological properties similar to those of other isolates or new features such as changes in vector specificity, expanded host range or increased pathogenicity. No obvious differences in vigor, symptom expression, or disease incidence have been detected so far between grapevines infected with recombinant or non-recombinant isolates (Vigne *et al.*, 2004a,b).

NEMATODE TRANSMISSION

GFLV is transmitted from grapevine to grapevine by the ectoparasitic dagger nematode *Xiphinema index* from the family *Longidoridae* in the order *Dorylaimida* (Hewitt *et al.*, 1958; Raski *et al.*, 1983; Brown *et al.*, 1995). Unlike a number of other nematode vectors, *X. index* does not transmit any other virus besides GFLV

(Raski *et al*., 1983; Brown *et al*., 1995; Brown and Weischer, 1998; Wyss, 2000). Although *X. italiae* has also been reported as a vector of GFLV (Cohn *et al*., 1970), this finding has never been confirmed (Martelli, 1975; Catalano *et al*., 1992). Thus, it is unlikely that *X. italiae* acts as a specific vector of GFLV (Brown *et al*., 1995). Consequently, *X. index* is probably the sole vector of GFLV in the vineyard and the transmission of GFLV by *X. index* is characterized by a specific and complementary association (Brown and Weischer, 1998). *X. index* feeds on growing root tips and acquires GFLV particles upon feeding (Hewitt *et al*., 1958; Raski *et al*., 1983; Wyss, 2000). The transmission process is mediated by the ability of *X. index* to ingest GFLV particles from a virus source grapevine, retain virions at specific retention sites within its feeding apparatus, and subsequently infect a recipient vine by release of virus particles from the retention sites.

X. index males are rare, females reproduce parthenogenetically, and adults develop through four juvenile stages (Raski *et al*., 1983). Like other nepoviruses, GFLV can be acquired and transmitted by both juvenile and adult forms of the vector. However, GFLV is not passed transovarily through nematode eggs (Taylor and Raski, 1964; McFarlane *et al*., 2002). Electron microscope studies of viruliferous *X. index* revealed GFLV particles in monolayers adhering to the cuticular lining of the oesophageal tract from the most anterior part of the odontophore to the posterior part of the oesophageal bulb (Taylor and Robertson, 1970). Consequently, GFLV is lost from the nematode after moulting (which occurs between each of the four stages of the juvenile development) during the replacement of the external cuticle including the lining of the oesophagus (McFarlane, 2003). After moulting, if the nematode feeds again on roots of GFLV-infected grapevines, it regains its viruliferous potential (Raski *et al*., 1983). Remarkably, GFLV particles can be acquired by a nematode from infected rooted grapevine cuttings after as little as 5 min feeding and a similarly short period of time is needed to transmit the virus to healthy rooted grapevine cuttings during feeding by a viruliferous nematode (Das and Raski, 1968; Alfaro and Goheen, 1974; Mayo *et al*., 1994).

The molecular determinants involved in the transmission process of several plant viruses by their nematode vectors are under investigation in several laboratories (for a review see McFarlane, 2003). In our laboratories, we are studying the transmission process of GFLV by *X. index*. Previous studies have shown that the viral determinants of the specificity of nepovirus transmission map to RNA2 (Harrison *et al*., 1974; Harrison and Murant, 1977). To delineate such determinants on GFLV RNA2, we engineered chimeric RNA2 by exchanging cDNA sequences between GFLV and ArMV, another nepovirus transmitted by *Xiphinema diversicaudatum* but not by *X. index* (Jha and Posnette, 1959). By using transcripts of chimeric GFLV/ArMV RNA2, we demonstrated that the CP is the sole viral determinant responsible for the specific spread of GFLV by *X. index* (Andret-Link *et al*., 2004; Belin *et al*., 2001).

Limited information is available on the survival of nematodes and the persistence of GFLV particles in viruliferous *X. index* from naturally infected vineyards, in particular from fallowed fields. Also, limited information is available on the infectious potential of nematode populations. Earlier data indicated a correlation between surviving grapevine roots present in the soil, even after the removal of infected plants, and persistence of *X. index* (Vuittenez *et al*., 1969). Also, a five percent GFLV infection rate was reported after five years of fallow in a naturally infected vineyard (Vuittenez *et al*., 1969). However, *X. index* held in moist sterile soil in the absence of host plants remains viruliferous for up to eight months (Taylor and Raski, 1964). Recently, we developed a reliable, rapid, sensitive and user-friendly RT-PCR assay to detect GFLV in a single *X. index* individual (Demangeat *et al*., 2004). By using this assay GFLV was detected in *X. index* isolated from contaminated vineyard soils that had been stored for four years at 7 or 20°C in the absence of host plants (Demangeat *et al*., 2003). The high survival rate of *X. index* under adverse conditions (high temperature and lack of host plants) and the retention of GFLV in viruliferous *X. index* over extended periods of time (over four years) severely limit the efficacy of GFLV control by the eradication of indigenous nematode populations in diseased vineyards, e.g. uprooting, soil disinfestation with nematicides, and fallow over short periods of time.

LIMITED EFFICACY OF TRADITIONAL CONTROL MEASURES

The economic impact of GFLV can be dramatic for the grape industry, especially in old vineyards with a long history of GFLV incidence. GFLV causes severe losses by reducing yield, affecting fruit quality, and decreasing resistance to adverse environmental conditions. Diseased plots need to be replaced more often than healthy plots because GFLV-infected grapevines have a shorter productive life. Also, the rate of contamination of replants that are used to replace uprooted GFLV-infected grapevines in diseased vineyards is fairly high. Since contamination occurs at an early developmental stage, these replants will seldom be productive (M. Fuchs, unpublished information).

GFLV can be disseminated by several means, including indigenous *X. index* populations, seeds, and human activities such as grafting and soil transfer (Martelli *et al*., 2003). GFLV transmission through seeds is only occasional, although the virus is detected in pollen of in-

fected grapevines (Martelli *et al*., 2003). The transfer of soil containing viable roots of uprooted GFLV-infected grapevines and/or surviving viruliferous *X. index* allows GFLV dissemination over short and long distances. This is especially critical in regions where grapevines are traditionally grown on hillsides, steep slopes, and terraces when the impacts of runoffs and erosion need to be countered by soil transfers. However, the main worldwide dissemination of GFLV is via grafting and extensive exchange of propagation material. Interestingly, GFLV is apparently native to *V. vinifera* and probably originated in the same area as grapevines, i.e. the Caucasus area between the Black and Caspian seas (Raski *et al*., 1983). GFLV, and probably *X. index*, are likely to have been disseminated from this center of origin throughout Europe by successive generations of explorers and subsequently from Europe to the rest of the World. Remarkably, the presence of GFLV in grapevine propagation material has been greatly limited in most viticultural regions of the world because of the implementation of rigorous certification schemes and the establishment of quarantine facilities. Thus, significant progress has been made in restricting long distance spread of GFLV.

Nevertheless, despite the implementation of effective measures to control GFLV dissemination over long distances, the virus remains a recurrent problem in established diseased vineyards. Here the control strategy consists of breaking the natural cycle of the nematode-virus complex through cultural practices and soil disinfestation. If GFLV is present in a field but *X. index* is not, the only control measure to consider is replanting because GFLV-free replants will permanently eradicate the virus. Similarly, if *X. index* is present but GFLV is not, the use of GFLV-free material will keep a vineyard healthy. However, when both *X. index* and GFLV are present in vineyards, effective control strategies are very difficult to implement. Control strategies are mainly the eradication or reduction of *X. index* populations, currently by soil disinfection (Raski *et al*., 1983; Taylor and Brown, 1997). However, although effective in shallow soils, nematicides are usually of limited efficacy in deep clay soils. Also, GFLV-infected vineyards often do not respond to chemical treatments because the nematode population density is low (Esmenjaud *et al*., 1992), and nematodes can survive and retain GFLV in unfavorable conditions for many years (Demangeat *et al*., 2003). In addition, the agrochemicals used as nematicides have acute toxicities. Therefore, their use is prohibited in several countries because of potential adverse environmental effects (Burrows *et al*., 1998; Abawi and Widmer, 2000). Due to their limited efficacy and environmental impact, some grapevine growers no longer use nematicides routinely. Prolonged fallow for at least ten years after careful removal of root debris (Vuittenez *et al*., 1969), in combination with crop rotation, can effectively eradicate *X. index* since the host range, on which this nematode species can reproduce and survive, is essentially limited to grapevines and figs (Siddiqi, 1974; Wyss, 2000). However, this strategy is economically not acceptable in premium vineyards. Thus, fallow is usually limited to 1-2 years instead of the recommended ten. Also, crop rotation is virtually impossible in most viticultural regions, including in high premium vineyards. Sometimes, GFLV-infected grapevines are treated with systemic herbicides before uprooting in order to destroy surviving roots that could serve as virus reservoirs, thus delaying contamination of replants (Descottes and Moncomble, 1995). The identification and use of plants other than grapevines with potential detrimental effect on *X. index* populations is an alternative being investigated in some laboratories. Such plants could be used by intercropping or as mulch before replanting.

The development of a hybrid rootstock with resistance to GFLV and *X. index* would be an ideal strategy to control GFLV (Raski *et al*., 1983). However, so far, no useful sources of resistance against the virus have been identified in wild or cultivated grapevines (Raski *et al*., 1983; Lahogue and Boulard, 1996). Thus, conventional breeding for resistance to GFLV is currently not a feasible alternative. Breeding grapevine varieties for resistance to *X. index* is another approach to limit GFLV transmission. Tolerance to *X. index* has been identified in Muscadine grapes (Bouquet, 1981; Meredith *et al*., 1982; Raski *et al*., 1983; Harris, 1983; 1988; Walker *et al*., 1985, 1994; Malan and Meyer, 1993; McKenry *et al*., 2001). *V. munsoniana* has also been reported to be tolerant to *X. index* feeding (Staudt and Weischer, 1992). Nematode-tolerant grapevine species are poor hosts for *X. index* since roots are attacked very rarely, as a consequence of reduced feeding efficacy. Thus, the reproduction of nematodes is limited or even inhibited, and *X. index* populations decrease overtime. Although *X. index*-tolerant rootstocks could be of practical interest, they are unlikely to solve the problem of GFLV transmission. Indeed, root cells of *Muscadinia rotundifolia* respond to nematode feeding with a hypersensitive reaction. Therefore, a rapid decline of attacked cells may prevent GFLV particles from spreading into neighboring cells after injection by the nematodes during salivation but *X. index*-tolerant rootstocks, in particular *M. rotundifolia* and *V. munsoniana*, do not completely exclude GFLV (Staudt and Weischer, 1992). In spite of the identification of tolerant species, resistance to *X. index* has not yet been achieved in elite grapevine hybrid rootstocks or varieties.

There is a major need to develop GFLV-resistant grapevines given the limited efficacy of current control strategies, the difficulties of developing grapevine material with useful tolerance to *X. index*, the severity of the fanleaf degeneration disease, and the increasing demand for sustainable and environmentally safe viticultural

practices. Alternative control methods are being considered. Cross protection has been evaluated as a potential approach to control GFLV directly. This strategy is based on the use of mild strains of GFLV or ArMV, which is closely related to GFLV, in rootstocks to protect grapevines against indigenous GFLV strains. Cross protection gave promising results on the experimental host *Chenopodium quinoa* (Huss *et al.*, 1989). Mild strains of GFLV and ArMV have been identified among numerous field isolates (Legin *et al.*, 1993), transferred by heterologous grafting to various rootstocks grafted with *V. vinifera* scions, and evaluated in naturally infected vineyards. Although a 3-4 year protection is observed across most field experiments (M. Fuchs, personal observation), the limited efficacy of cross protection with the currently available mild strains makes this approach not useful for a perennial crop like grapevine.

GENETIC ENGINEERING OPENS NEW AVENUES FOR CONTROL

Recent progress on unraveling the genomic structure and expression of GFLV, and on determining the structure and genetic variability of GFLV populations from naturally infected vineyards has opened new avenues for alternative strategies to control GFLV. Genetic engineering is an attractive alternative way to develop GFLV-resistant rootstocks, especially since specific virus resistance traits can be inserted directly into desirable elite material (Fuchs, 2003). Several reports have documented the development of transgenic grapevines for resistance to GFLV (Krastanova *et al.*, 1995; Mauro *et al.*, 1995; Xue *et al.*, 1999) after high levels of resistance have been reported in transgenic *Nicotiana benthamiana* expressing the CP gene of GFLV (Bardonnet *et al.*, 1994). GFLV-derived genes, mainly the coat protein ($2C^{CP}$), movement protein ($2B^{MP}$), and RNA-dependent RNA polymerase ($1E^{Pol}$) genes, have been engineered and transferred into rootstocks, *V. vinifera*, and interspecific hybrids by using complete, truncated, sense or antisense, translatable or untranslatable gene constructs (Krastanova *et al.*, 1995; Mauro *et al.*, 1995; Xue *et al.*, 1999). Resistance to GFLV can be evaluated in transgenic grapevines by grafting, biolistic inoculation, protoplast electroporation or nematode transmission (Valat *et al.*, 2000, 2003a,b). Resistance to GFLV in transgenic rootstocks expressing the GFLV CP gene has been recently reported after a three-year trial in a naturally infected vineyard in France (Vigne *et al.*, 2004b). These data are very promising since they suggest that transgenic grapevines are likely to be of practical interest for the control of GFLV. Field trials over extended periods of time will need to be conducted to evaluate the durability of the resistance. Other strategies, including the development of plantibodies or the use of inverted-repeats of GFLV-derived genes with a central hairpin structure, are under investigation (Fuchs, 2003).

Our laboratories are involved in environmental safety studies to identify risks associated with the large-scale release of transgenic grapevines engineered for GFLV resistance. Identifying potential risks and assessing their impact on the environment is a first step towards the safe deployment of GFLV-resistant transgenic grapevines to ensure the development of sustainable and environmentally friendly viticultural practices. To determine if transgenic grapevine rootstocks expressing the GFLV CP gene favor the development of GFLV recombinant isolates through recombination events between transgene transcripts and RNAs from indigenous GFLV isolates, we characterized numerous isolates from transgenic and conventional grapevines (Vigne *et al.*, 2004a,b). Our study indicated that transgenic grapevines did not favor the development of GFLV recombinant isolates to a detectable level (Vigne *et al.*, 2004a,b). So far, our risk assessment studies and resistance evaluation clearly indicate that not only could GFLV-resistant transgenic material be effective for GFLV control but it could also be environmentally friendly. Thus, GFLV-resistant transgenic grapevines could allow sustainable production while preserving the environment.

To address social and consumer concerns on the use of transgenic crops in France, the Institut National de la Recherche Agronomique (INRA) initiated a wide consultation and promoted pro-active and transparent dialogues between the scientific community, growers, nursery managers, consumer groups and other stakeholders (http:www.inra.fr/Internet/directions/SED/science-gouvernance/ITA-Vignes/index.html). The GFLV-resistant transgenic grapevines were used as case study. This initiative has opened a window of opportunity to transfer scientifically-based information on benefits and potential environmental risks of the use of GFLV-resistant transgenic grapevines. A critical appraisal of the usefulness of the technology to eliminate a major threat to the grape industry will be needed for the successful use of GFLV-resistant transgenic grapevines by growers in the near future.

CONCLUSIONS AND PERSPECTIVES

Although considerable progress has been made in the last decades on understanding the transmission and life cycle of GFLV, and on elucidating the structure and expression of its genome, GFLV remains a serious problem in naturally infected vineyards. The establishment of quarantine facilities and the implementation of rigorous certification schemes based on improved serological and molecular diagnostic tools have resulted in significant progress in controlling the spread of GFLV through grapevine propagation material.

Recently, significant progress has been made in the elucidation of the function of most GFLV proteins and their interactions with cell components, in particular those involved in key steps of the virus multiplication cycle. Also, insights into the population structure and genetic variability within GFLV isolates have been gained. Such information has helped the design of alternative control strategies against this destructive virus. Promising results have been reported on the resistance to GFLV in transgenic rootstocks. Given that the engineered resistance is stable and durable, transgenic rootstocks could be used by growers with their favorite elite scion varieties - a situation reminiscent of the resolution of the phylloxera crisis in the early years of the 20th century when hybrid rootstocks saved the wine industry worldwide.

Transfer of transgenic grapevines to growers will now depend on open and pro-active dialogues between the scientific community and growers to facilitate a scientifically-based evaluation of the benefits and real impact on the environment. Along this line, it is critical to develop opportunities that enable both the independent assessment of this new technology by the scientific community and the communication of accurate and trustworthy information to growers and consumers.

Some major breakthroughs on GFLV research over the past 15 years have paved the way for the development of effective and environmentally friendly control strategies against GFLV, which, more than 160 years after its description and 40 years after its discovery, continues to be a major threat to the grapevine industry worldwide. Let's hope for a timely transfer of some of these strategies to growers in the near future.

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