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# Transgenic grapevine rootstock clones expressing the coat protein or movement protein genes of *Grapevine fanleaf virus*: Characterization and reaction to virus infection upon protoplast electroporation

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**Abstract**

The reaction to *Grapevine fanleaf virus* (GFLV) infection in 42 independent transgenic grapevine rootstock 41B clones expressing the coat protein (CP) or movement protein (MP) gene of GFLV was assayed by protoplast electroporation. Two of the 26 transgenic clones expressing the CP gene did not support the accumulation of GFLV MP to detectable levels, 12 accumulated substantially lower levels of MP, and 12 accumulated equivalent levels of MP relative to protoplasts of nontransformed controls at 72 h post-electroporation, as shown by Western blots with anti-MP γ-globulins. Interestingly, inhibition of MP accumulation was achieved against virions but not viral RNAs, and was dependent on the inoculum dose. No interference was observed with the multiplication of *Arabis mosaic virus*, which is closely related to GFLV, likely due to low nucleotide identity between the CP genes. Also, one of the 16 transgenic clones expressing the MP gene significantly reduced the accumulation level of GFLV CP at 72 h post-electroporation, as shown by DAS-ELISA with anti-GFLV γ-globulins. The potential of protoplast electroporation as rapid identification of GFLV-resistant grapevine clones at the cell level will be discussed relative to field screening for resistance at the plant level by nematode-mediated GFLV transmission.
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*Keywords:* Transgenic grapevine; Protoplast electroporation; *Grapevine fanleaf virus*; Coat protein; Movement protein; Resistance

## 1. Introduction

Fanleaf degeneration is one of the most important viral diseases of grapevines worldwide [1]. It causes a significant reduction in crop yield (up to 80%) and a progressive decline that reduces plant longevity or can even lead to plant mortality. Fanleaf degeneration is caused by several virus species from the genus *Nepovirus* in the family *Comoviridae*. The most important of them is *Grapevine fanleaf virus* (GFLV), which is vectored by the ectoparasitic nematode *Xiphinema index* [1].

The viral genome of GFLV is composed of two single stranded positive-sense RNAs, denoted RNA1 and RNA2, which carry a small covalently linked viral protein (VPg) at their 5′ extremities and a poly(A) stretch at their 3′ ends [1]. Each genomic RNA codes for a polyprotein, which is proteolytically processed into functional proteins. RNA1 codes for the proteins implicated in RNA replication and for the viral proteinase [1]. RNA2 codes for protein 2A, which is required for RNA2 replication, the movement protein (MP), and the coat protein (CP) [1].

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Current strategies to control fanleaf degeneration in vineyards are based on soil disinfection with nematicides and cultural practices, including plant devitalization, uprooting, removal of root debris, and prolonged fallow [1,2]. Active molecules of nematicides have acute toxicity and fumigation is ineffective in heavy soils since nematode populations are not completely eradicated. Based on the limitations of the current strategies, there is a need to develop novel, efficient, and environmentally friendly alternatives to control GFLV in grapevines. One approach is to develop GFLV-resistant rootstocks. This can be achieved by engineering virus resistance through the application of the concept of pathogen-derived resistance [3] or by developing tolerance to virus spread through conventional breeding approaches using *X. index*-tolerant germplasm [4]. Since Powell-Abel et al. [5] demonstrated that tobacco plants expressing the CP gene of *Tobacco mosaic virus* (TMV) are protected against this virus, numerous transgenic plants expressing various CP gene constructs and exhibiting virus resistance have been developed [6,7]. Namely, transgenic *Nicotiana benthamiana* expressing the GFLV CP gene were obtained and shown to delay the onset of infection following mechanical inoculation with GFLV [8]. Based on these promising results, the GFLV CP gene was introduced into several grapevine rootstocks and varieties to engineer resistance to GFLV [9–12]. For instance, the grapevine rootstock 41B (*Vitis vinifera* cv. Chasselas × *V. berlandieri*), which is extensively used in the Champagne region in France for its tolerance to limestone, was transformed with the CP gene of GFLV strain F13 [10]. Other GFLV-derived constructs such as the MP gene were also introduced into rootstock 41B (Valat and Burrus, unpublished). Recently, resistance to GFLV was reported in *V. vinifera* var. Chardonnay grafted onto transgenic rootstock 41B clones expressing the GFLV CP gene that were tested over a 3-year period in a naturally GFLV-infected vineyard [13].

Evaluation of resistance to GFLV in grapevines usually relies on nematode-mediated GFLV transmission under field or greenhouse conditions. This approach requires prolonged period of time, i.e. several months to a few years, to identify GFLV-resistant clones. Other screening techniques would be desirable for a faster delivery of GFLV to test plants and for a more timely selection of GFLV-resistant material. Protoplast electroporation with virions or viral RNAs is another way to inoculate grapevines with GFLV [14,15]. No information is available on the potential of protoplast electroporation, as alternative to nematode-mediated GFLV inoculation, to identify transgenic grapevine clones that can interfere with GFLV multiplication. The aim of our study was to investigate protoplast electroporation as a rapid screening technique of transgenic grapevine clones expressing the CP or MP gene of GFLV to identify material that reduces or inhibits the accumulation of viral proteins at the cell level. Our results will be discussed in regard to the usefulness of grapevine protoplast electroporation as rapid evaluation of GFLV resistance at the cell level relative to lengthy field screening at the plant level.

## 2. Materials and methods

### 2.1. Viruses and viral RNAs

GFLV strain F13 [16] and *Arabis mosaic virus* (ArMV) strain S [17] were propagated on the systemic herbaceous host *Chenopodium quinoa* and purified as described previously [18]. Purified GFLV and ArMV virions, and GFLV strain F13 RNAs extracted from purified virions [18] were used in protoplast experiments.

### 2.2. Plant material

Plants of the grapevine rootstock 41B (*Vitis vinifera* cv. Chasselas × *Vitis berlandieri*) clone 233 were used for *Agrobacterium tumefaciens*-mediated transformation. Establishment and maintenance of embryogenic cultures, and transformation procedures were as previously reported [10]. Briefly, embryogenic cells were cultured with *Agrobacterium tumefaciens* strain LBA4404 containing either plasmid pRCPI with the CP gene [10] or plasmid PCT172 with the MP gene of GFLV strain F13 (Fig. 1). Plasmids pRCPI and PCT172 were kindly provided to us by Dr. L. Pinck, IBMP, Strasbourg, France, and Prof. P. Coutos-Thevenot, Université de Poitiers, Poitiers, France, respectively. After 24 h of co-culture, embryogenic cells were transferred to liquid MS medium containing maltose (18 g/l) and glucose (4.6 g/l) (GM medium), supplemented with 5 μM β-naphthoxy acetic acid and cefotaxime (400 μg/ml), and cultured on paromomycin-containing medium. Transgenic 41B clones expressing the CP or MP gene of GFLV were obtained in independent experiments.

Nontransgenic and transgenic clones of were maintained in tissue culture by propagation of one-node cuttings every two-months on Murashige and Skoog medium (MS) [19]. Plants were grown in growth chambers under controlled light (16 h) and temperature (23 °C) conditions.

### 2.3. Characterization of the GFLV CP and MP transgenes by Southern blot and PCR

The integration of the GFLV CP and MP transgenes was detected in putative transgenic grapevines by PCR and Southern blot hybridization with total DNA extracted from young leaves of in vitro-grown plants using 1 g of fresh tissue [10]. PCR and Southern blot experiments were conducted separately with distinct DNA samples.

For PCR analysis of transgenic plants expressing the GFLV CP gene, primers CPfor (5′-$^{3000}$CGGGTGAGACTGCGCAAC$^{3017}$-3′) and CPrev (5′-$^{3572}$ GTCAGATACCCTAGACTG$^{3554}$-3′) were used to amplify a 572 bp fragment corresponding to the 3′ end of the CP gene. For transgenic plants expressing the GFLV MP gene, primers MPfor (5′-$^{1300}$TGCACCATAGGATCAGTACGT$^{1321}$-3′) and MPrev (5′-$^{1938}$ACTGAATCAGTATCCACAGTG$^{1917}$-3′) were used to amplify a 638 bp fragment corresponding to the central part of the MP gene. PCR reactions were performed with 0.3 μg

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