

TRANSGENIC ROOTSTOCKS EXPRESSING GFLV COAT PROTEIN GENE IN A THREE YEARS FIELD TRIAL; RESISTANCE ASSESSMENT, IMPACT ON GFLV DIVERSITY AND EXCHANGES BETWEEN ROOTSTOCK AND SCION

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Summary

Transgenic rootstock lines expressing the full-length translatable coat protein (CP) gene of *Grapevine fanleaf virus* strain F13 (GFLV-F13) are under evaluation in an open-field trial. Control plants were contaminated after nematode-mediated transmission of GFLV after three years. In contrast, viral infection of non-transgenic scion (*Vitis vinifera* cv. Pinot Meunier) was delayed in vines grafted onto transgenic rootstocks. Sequencing of CP genes from GFLV isolates from this field, suggests that CP gene-expressing transgenic rootstocks do not influence significantly natural viral biodiversity. Transgenic-derived expression products were detected in GM rootstocks. However, their systemic movement was not detected suggesting that the scions remain truly wild-type. All experiments were co-constructed and followed within a Local Steering Committee. Thus, this field trial is a unique support for the assessment of environmental impact of genetically-modified plants (GMPs) and a survey of public perception of GMPs.

INTRODUCTION

The grapevine fanleaf degeneration, the major virus disease for the viticulture worldwide, is caused mainly by nepoviruses transmitted in a semi-persistent manner by longidorid ectoparasite nematodes. This disease is causing to French grapevine industry ca. 1 billion € losses per year with a prevalence of 60% of the total grapevine acreage (Fuchs *et al.*, 2006). *Grapevine fanleaf virus* (GFLV, genus *Nepovirus*, family *Comoviridae*) is the main aetiological agent and is transmitted by *Xiphinema index*. The control of fanleaf disease relies currently on prophylactic measures, cultural practices (fallows over ten years following devitalization of vines) and certification programs. Despite these measures and since the ban in Europe of some environmentally unfriendly agrochemicals, fanleaf disease remains an expanding pandemic leading to a technical deadlock in control strategies.

To date, no dominant nor recessive resistance genes toward GFLV have been found in grapevine. The implementation of genetic engineering opened up new strategies based on rootstock-mediated resistance, to develop virus-resistant grapevines (Laimer *et al.*, 2009). Transgenic rootstocks expressing the coat protein (CP) gene of GFLV-F13 strain have thus been obtained in the early 1990s (Mauro *et al.*, 1995). Their resistance in vineyard conditions has been assessed in an open-field trial in Champagne during 3 years (1996-1999). Facing strong public rejection, the trial had to be stopped. However,

preliminary results suggested a lower incidence of GFLV infection in 3 transgenic lines, out of 18 tested (Vigne *et al.*, 2004a). Relying on an Interactive Technological Assessment approach (Joly and Rip, 2007), we designed experimental conditions for an open-field trial (2005-2009) with a Local Steering Committee (LSC) and met public acceptance so far in Europe (Masson, 2007).

The aim of this field experiment was firstly to evaluate the level of resistance toward GFLV infection in vineyard conditions and secondly to assess the potential environmental impact of transgenic CP gene-expressing rootstocks on molecular variability of the GFLV population. A key question addressed by the LSC concerned putative exchanges between genetically-modified (GM) rootstock and non-GM scion. Preliminary data about the long-distance movement of mRNAs and proteins will be presented.

MATERIAL AND METHODS

Transgenic lines and field trial conditions: the gene encoding the full-length translatable CP of GFLV-F13 has been inserted in 41B rootstock hybrid (Mauro *et al.*, 1995), the *Neomycin phosphotransferase II* (NPTII) gene being used as a selection marker. The 5 transgenic lines G68, G77, G206, G219 and G240 were selected for the trial, subsequently green-grafted onto the wild-type Pinot Meunier cultivar. A total of 70 transgenic vines were used, 50 of them being planted in a *X. index*/GFLV infested soil that had been transferred from a fanleaf-infested vineyard into the experimental plot. During the whole experiment, inflorescences were systematically pruned and stored at -80°C for subsequent analyses. Resistance level was evaluated by DAS-ELISA on leaves of the scion in June each year.

DAS-ELISA: GFLV and NPTII proteins were detected respectively with specific anti-GFLV immunoglobulins from our laboratory and with commercial anti-NPTII antibodies (5Prime→3Prime, Inc.).

IC-RT-PCR-RFLP: GFLV particles of positive ELISA samples were immunotrapped and the full-length CP gene of viral RNA was subsequently amplified by RT-PCR as previously described (Vigne *et al.*, 2004b). The resulting fragments were characterized by RFLP using *EcoRI* and *SpyI* digestions.

Cloning and sequence analyses: PCR products with different RFLP patterns were selected and cloned using the pGEM-T vector system (Promega). Clones corresponding to putative variants were subsequently sequenced on both strands and further analyzed with Vector NTI software (Invitrogen). The phylogenetic tree was constructed with Mega4 software using the Neighbour-Joining algorithm.

Transgene-expressed mRNAs detection: Total RNAs were extracted from frozen leaves and inflorescences using RNeasy Plant Mini Kit (Qiagen). CP and NPTII mRNAs were detected by specific RT-PCR.

RESULTS AND DISCUSSION

The progression of infection of vines, investigated by ELISA, was remarkable, from 3% positive vines in 2007 to 70% in 2008, for the non-transgenic controls, indicating a high nematode transmission efficiency of GFLV, though nematode-infested soil had been transported. Although all grapevines were asymptomatic in June 2008, DAS-ELISA performed on the leaves showed infection rates, ranging from 30% in the transgenic lines, to 70% in the controls. Though showing no resistance, our preliminary data suggest a delay in infection, in 2008. The ELISA results of June 2009 will be presented and discussed.

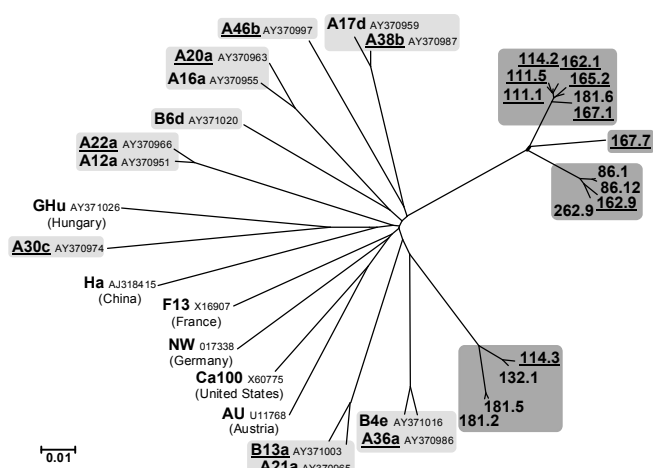


Figure 1: unrooted phylogenetic tree reconstructed from the 1515 bp CP sequences of 35 GFLV variants from various geographic origins (including Champagne -light gray- and Colmar -dark gray- field experiments) and isolated from transgenic (underlined) and non-transgenic vines. Accession numbers are given for sequences retrieved from Genbank.

It has been shown previously that transgenic grapevine rootstocks expressing the GFLV CP gene did not favor the development of detectable GFLV recombinants or specific molecular variants from the Champagne trial (Vigne *et al.*, 2004b). In the present study, we confirm that transgenic rootstocks expressing CP do not promote the emergence of GFLV variants, after 3 years. Interestingly, the GFLV population structure from the Alsatian soil (this experiment) reveals, as preliminary results, two new clusters, and a genetic diversity different from that displayed by the Champagne isolates (Figure 1). The frequency of mixed infections seems to be higher than that of single ones,

whatever the transgenic status of the vines. More data are nevertheless required to estimate the magnitude of the population structure and of the diversity of GFLV isolates in this field trial.

No translocation of the transgene-expressed products (CP and NPTII mRNAs and NPTII protein) has been detected between the GM rootstock and wild-type Pinot Meunier scion. This evidence is provided for the first time in a perennial crop in a long-term field trial. These original results are in accordance with those obtained for transgenic watermelon rootstock expressing the CP of *Cucumber green mottle mosaic virus* (*Tobamovirus* genus), where no translocation of CP mRNA or protein was detected in the scion (Youk *et al.*, 2009).

Through this field experiment, resistance toward GFLV, environmental impact of transgenic CP gene-expressing rootstocks, and systemic movement of transgenes products are being efficiently assessed. We proposed with the LSC to broaden our study on the impact of transgenic rootstocks on soil bacteria and GFLV genome using metagenomic approaches. An extension of duration of this trial aiming to address these scientific questions will be submitted. Our data, when confirmed at completion of the trial, may help refining guidelines for GMO release in Agriculture.

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