Cross-Protection as Control Strategy Against *Grapevine fanleaf virus* in Naturally Infected Vineyards

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ABSTRACT

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The efficacy of cross-protection at mitigating the impact of *Grapevine fanleaf virus* (GFLV) on grapevines (*Vitis vinifera*) was assessed in two naturally infected vineyard sites. Test vines consisted of scions grafted onto rootstocks that were healthy or infected by mild protective strains GFLV-GHu or *Arabis mosaic virus* (ArMV)-Ta. Challenge GFLV infection via the nematode *Xiphinema index* was monitored over nine consecutive years in control and ArMV-Ta cross-protected vines by double-antibody sandwich-enzyme-linked immunosorbent assay using GFLV-specific antibodies, and in GFLV-GHu cross-protected vines by characterizing the coat protein gene of superinfecting isolates by immunocapture-reverse transcription-polymerase chain reaction-restriction fragment length polymorphism. Results were consistent with a significantly reduced challenge infection rate in cross-protected vines compared with control vines, more so in those protected with GFLV-GHu (19 versus 90%) than with ArMV-Ta (40 versus 65% in field A and 63 versus 90% in field B). However, the two mild strains significantly reduced fruit yield by 9% (ArMV-Ta) and 17% (GFLV-GHu) over 8 years and had a limited effect on fruit quality. Therefore, in spite of a great potential at reducing the incidence of challenge field isolates, cross-protection with natural mild protective strains GFLV-GHu and ArMV-Ta is not attractive to control GFLV because the negative impact on yield is a limiting factor for its deployment.

Grapevine fanleaf virus (GFLV) is responsible for fanleaf degeneration, the most severe virus disease of grapevines (1). This virus is present in the majority of vineyards where *Vitis vinifera* grapes and French-American rootstocks are grown. GFLV reduces plant vigor and the productive lifespan of vineyards. It also incites severe yield losses (up to 80%) and alters fruit quality (1). GFLV is transmitted from vine to vine by the ectoparasitic nematode *Xiphinema index*.

Control strategies against GFLV in infected vineyards are essentially directed against its nematode vector (1). Roguing of GFLV-infected vines can limit the spread of the disease, and soil disinfection with nematicides prior to planting can reduce *X. index* populations. These strategies are of limited efficacy because viruliferous *X. index* can survive in infested soils over extended periods of time even in the absence of any host plant

(1,10). Rootstocks with resistance to *X. index* feeding have been developed, but they do not prevent GFLV spread into scions (1,38). Therefore, there is a need to develop alternative approaches to mitigate the impact of GFLV (1).

Cross-protection has been explored as a biological strategy to manage plant viruses (8,14,19,20,25,27). This phenomenon relies on the use of mild virus strains to protect plants from economic damage caused by severe virus strains (14,20,25). The mechanism of cross-protection is not well characterized, although competition for host factors, differential spatial distribution of distinct viruses and virus strains with regard to exclusion mechanisms (12,16,25,28), and post-transcriptional gene silencing (PTGS) in terms of RNA-mediated resistance (26,43) have been suggested.

Mild isolates of GFLV and *Arabis mosaic virus* (ArMV), a closely related nepovirus (11), were identified by comparative performance analysis of grapevines infected with different strains (22) and by comparative symptom severity screening on *Chenopodium quinoa*, a systemic herbaceous host for both viruses. The successful use of the mild strains GHu of GFLV and Ta of ArMV to protect plants from challenge infection with severe

GFLV strains was shown in *C. quinoa* (17). Protection with mild GFLV strains against GFLV infection was also achieved in *Gomphrena globosa* (6). These earlier results suggested that cross-protection with mild strains of GFLV and ArMV could be effective to protect grapevines from GFLV infection. To validate data obtained in herbaceous plants, we assessed the protective ability of mild strains GFLV-GHu and ArMV-Ta in two naturally GFLV-infected vineyard sites. We report here on the potential of these two mild protective viral strains at reducing the rate of GFLV challenge infection and at mitigating the impact of GFLV on fruit yield and quality.

MATERIALS AND METHODS

Mild protective virus strains. Strains GHu of GFLV (17) and Ta of ArMV (21) were used as protective strains based on mild symptoms in *C. quinoa* (17) and limited impact on grape vigor and yield (22), respectively, in comparison with more severe viral strains. These two viral strains were propagated in *C. quinoa* and transferred onto the certified rootstock Kober 5BB (*Vitis berlandieri* × *V. riparia*) clone 259 by in vitro heterologous grafting (4).

Plant material. Test plants consisted of certified *V. vinifera* cv. Gewürztraminer clone 643 grafted onto rootstock Kober 5BB clone 259. Graft-indexing on selective *Vitis* sp. indicators and double-antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) were used to ascertain the sanitary status of the test material (18). At the beginning of the cross-protection field experiments, rootstocks were either healthy or infected by GFLV-GHu or ArMV-Ta.

Field sites and experimental layout. Areas of homogeneous topographic and soil conditions were selected for field trials in two naturally GFLV-infected commercial vineyards, one in Ammerschwihr (field A) and the other in Bennwihr (field B), two localities 15 km apart in the Alsace region of France. The presence of GFLV and its nematode vector *X. index* were both confirmed in the two experimental vineyard sites prior to planting. However, ArMV and its nematode vector *X. diversicaudatum* (17) were not found in the two vineyard sites. No soil disinfection was done with nematicides after uprooting

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established vines in fall 1992 in field A and in fall 1994 in field B, because one of the objectives of the field experiments was to determine the reaction of test vines to natural transmission of GFLV by its nematode vector.

In field A, 112 healthy vines and 48 cross-protected vines infected with ArMV-Ta were planted in June 1993, making a total of 168 plants, including eight border plants on one side of the experimental site. Both treatments (healthy vines and cross-protected vines infected with ArMV-Ta) were assigned to two blocks in a split plot design. Each block had 80 plants, 56 healthy and 24 cross-protected, in 14 and 6 groups of four plants each, respectively, that were randomized within each block. Vines were planted in four rows of 40 plants each, spaced 1 m apart within rows and 2 m between rows.

In field B, 100 vines were planted in August 1995 for each of the three treatments (healthy vines, GFLV-GHu cross-protected vines, and ArMV-Ta cross-protected vines), making a total of 300 (3 × 100) plants. Treatments were assigned to two blocks in a split plot design. Each block had 150 plants, 50 per treatment, in 10 groups of five plants each randomized within each block. Vines were planted in five rows of 60 plants each, with the same spacing as in field A.

The location of vines in fields A and B was identified by coordinates *x,y*, with *x* being the row number and *y* the position within row *x*. For example, vine 1.13 was in position 13 within row 1.

Monitoring virus infection in test plants over time. The presence of GFLV was assessed by visually monitoring symptom development, i.e., foliar mosaic, deformation, vein chlorosis, plant stunting,

and short internodes (1). The virus was also monitored in samples of apical leaves of each test plant (five to six leaves per plant) for which the rootstock was initially healthy or infected by ArMV-Ta by DAS-ELISA with anti-GFLV immunoglobulins produced in our laboratory (18). Serological tests were performed from 1998 to 2006 in the two vineyard sites in the spring (June). Samples were considered positive if their optical density (OD_{405nm}) readings were at least twice those of healthy controls ± 20%.

An immunocapture–reverse transcription–polymerase chain reaction–restriction fragment length polymorphism (IC-RT-PCR-RFLP) assay with *StyI* was developed to differentiate the coat protein (CP) gene of protective GFLV strain GHu and challenge field GFLV isolates, as described previously (31,33). It was used to monitor GFLV infection in plants initially infected with GFLV-GHu in field B in 2004 and 2006.

In parallel to GFLV tests, the presence of ArMV-Ta was assayed in fields A and B on a yearly basis by DAS-ELISA using anti-ArMV immunoglobulins produced in our laboratory. Infected samples were identified as described above for GFLV.

Genetic variability of the CP gene of mild protective virus isolates. The CP gene of mild strains GFLV-GHu and ArMV-Ta was characterized by IC-RT-PCR. DNA amplicons were subsequently cloned into plasmid pGEM-3Zf(+) and sequenced, as described previously (31,33). Nucleotide sequences were analyzed with the VectorNTI software package. The program CLUSTAL W was used for alignment of nucleotide sequences (30).

Analysis of fruit yield and quality. The number of clusters was counted at harvest in field B for each group of five plants per treatment, and clusters were weighted. In addition, berry samples were randomly collected for each group of five plants per treatment and crushed with a hand press to measure sugar content with a hand temperature-compensating refractometer and to evaluate titratable acidity by titration with 0.1 N NaOH to pH 7.0. Data on fresh fruit yield, cluster number, and fruit maturity indices were analyzed from 1999 to 2006.

Statistical analyses. Data collected on virus infection of test plants, fruit yield, and fruit quality were summarized to obtain descriptive statistical parameters. Analysis of variance was used to detect treatment (cross-protected versus controls) differences and relationship between variables with SAS (Statistical Analysis System, SAS Institute Inc., Raleigh, NC). Disease progress was analyzed using a generalized linear model with a logit link function $\eta_i = \log(\hat{y}_i / 1 - \hat{y}_i)$ where η_i is modeled as $\eta_i = \tau_i^T \beta$ with $\hat{y}_i$ being the expected proportions showing infection and having uncorrelated binomial errors. The β are

treatment effects (parameters) and the τ_i are rows of the design matrix (explanatory variates). For variables related to fruit yield and quality, a mixed effect repeated measurements model was fitted with treatment (cross-protected versus control) and time as fixed effects and random experimental units. Contrasts among treatments using the means least squares (LS) method were used to compare differential effects on field performance due to mild protective virus isolates.

RESULTS

Characterization of the *StyI* CP gene polymorphism of GFLV-GHu and field GFLV isolates. An IC-RT-PCR-RFLP assay was developed to characterize the CP gene of mild protective strain GHu of GFLV and challenge field GFLV isolates. This molecular assay was necessary to determine the rate of superinfection of vines initially protected with GFLV-GHu because anti-GFLV immunoglobulins used in DAS-ELISA did not discriminate GFLV-GHu and field GFLV isolates (data not shown).

The 1.5-kb CP gene of GFLV-GHu (GenBank accession no. AY371026) digested with *StyI* generated five fragments of 510, 394, 350, 279, and 43 bp (Fig. 1, lane 1). The smallest fragment is not visible in Figure 1 but is deduced from *in silico* sequence analysis. Some field GFLV isolates from GFLV-GHu cross-protected vines had a more complex polymorphism (Fig. 1, lanes 2 to 5). For example, the banding pattern of isolate 2.35 (Fig. 1, lane 2) consisted of a 790-bp *StyI* fragment in addition to those typical of GFLV-GHu. Also, the CP gene of isolate 5.47 (Fig. 1, lane 5) had *StyI* fragments of 680, 450, and 380 bp in addition to those of GFLV-GHu. The *StyI* CP gene polymorphism was further used to assess the rate of GFLV challenge infection in cross-protected vines initially infected with GFLV-GHu.

Effect of cross-protection on the rate of challenge GFLV infection. Cross-protection trials were established in 1992 and 1994 in two naturally GFLV-infected vineyard sites. Infection of test vines following *X. index*-mediated GFLV inoculation was monitored over time. Symptom development was used early on as an indicator of disease progress and severity. However, this approach was not sufficiently reliable to discriminate mild symptoms in cross-protected vines and discrete symptoms in control vines at an early infection stage. Therefore, starting in 1998, the rate of GFLV infection was monitored on a yearly basis by DAS-ELISA in vines initially infected with ArMV-Ta in fields A and B, and by IC-RT-PCR-RFLP in vines initially infected with GFLV-GHu in field B.

The percentage of infected plants increased over a 9-year period (1998 to 2006) (Fig. 2). GFLV incidence was generally lower in field A (65% infected con-

Fig. 1. *StyI* polymorphism characterization of the coat protein gene of *Grapevine fanleaf virus* (GFLV) isolates from the Bennwihr site by immunocapture–reverse transcription–polymerase chain reaction–restriction fragment length polymorphism. Lane 1: unchallenged GFLV-GHu in vine 4.8; lanes 2 to 5: GFLV-GHu and field GFLV isolates in challenged vines 2.35, 4.7, 4.11, and 5.47, respectively; lane H: healthy vines; lane M: 100-bp DNA size standard (Promega).

trol vines) than in field B (90% infected controls) (Fig. 2), with odds of infection being half (0.5 ± 0.09) in field A relative to field B. However, regardless of vineyard site, the cumulative infection rate was reduced in cross-protected vines compared to control vines. In field A, a 40% (19 of 48 plants) infection rate was obtained in vines preinoculated with ArMV-Ta while 65% (70 of 108 plants) of the controls were infected in 2006 (Fig. 2A). Similarly, in field B, vines cross-protected with GFLV-GHu (19%, 19 of 100 plants) and ArMV-Ta (63%, 63 of 100 plants) had a reduced infection rate relative to control vines (90%, 90 of 100 plants) (Fig. 2B). ANOVA indicated no significant field $\times$ treatment (cross protection) interaction ($F = 0.002$; $df = 352$; $P = 0.96$). Most of the variation detected was due to the main effect of cross-protection ($F = 31.26$; $df = 353$; $P < 0.0001$), and to a lesser extent, to field site ($F = 7.003$; $df = 354$; $P = 0.0085$), confirming the beneficial impact of cross-protected vines at reducing the rate of challenge infection.

Odds of GFLV infection were quantitatively 2.5 ± 0.42 times greater for control than for ArMV-Ta-treated vines with 2.51 and 0.54 as coefficients fitted by the model for treatment [$\log$ -odds ratios = $\exp(0.922)$] and field site [$\log$ -odds ratios = $\exp(-0.6169)$], respectively. In addition, in field A, 29% of the controls were infected with GFLV in 1999, and this percentage doubled by 2003 (60%), while only 31% of ArMV-Ta cross-protected vines were infected by GFLV in 2003 (Fig. 2A). In field B, 53% of the control vines were infected with GFLV in 2000 and 84% in 2004, while only 52% of the ArMV-Ta cross-protected vines were infected by GFLV in 2004 (Fig. 2B). Since a similar infection rate was achieved with a 4-year lapse in control versus ArMV-Ta cross-protected vines, cross-protection delayed GFLV infection by 4 years.

Effect of cross-protected vines on fruit yield. A comparative fruit yield analysis in field B indicated that cross-protected vines had a marked negative impact relative to control vines from 1999 to 2001, and to a lesser extent, in 2004, causing a 5 to 60% reduction (Fig. 3A). In 2002 to 2003 and 2005 to 2006, a positive or neutral impact on yield was observed in cross-protected vines (Fig. 3A). These data are likely explained by the fact that cross-protected vines were infected prior to the start of the field trial, hence yield was reduced early in the experiment. In contrast, control vines were infected gradually after planting, depending on the effectiveness of nematode-mediated GFLV transmission. ANOVA revealed a significant treatment $\times$ year interaction ($F = 14$; $df = 48$; $P < 0.0001$). Over 8 years (1999 to 2006), ArMV-Ta and GFLV-GHu induced an overall 9 and 17% yield reduction relative to control vines (Table 1). Differences in yield between

GFLV-GHu cross-protected and control vines (t value = -5.03 ; $df = 48$; $P < 0.0001$) and between ArMV-Ta cross-protected and control vines (t value = -2.81 ; $df = 48$; $P = 0.0071$) were significant, confirming that cross-protected vines depressed fruit production.

The number of clusters per vine was higher overall for cross-protected compared to control vines from 1999 to 2006 (Table 1). ANOVA revealed a significant treatment $\times$ year interaction ($F = 5.27$; $df = 48$; $P < 0.0001$) with most of the variation due to year ($F = 25.36$; $df = 48$; $P <$

0.0001) rather than cross-protection ($F = 1.78$; $df = 48$; $P = 0.180$). Differences of LS means between ArMV cross-protected and control vines (t value = 1.73 ; $df = 48$; $P = 0.0894$) and between GFLV-GHu cross-protected and control vines (t value = 1.51 ; $df = 48$; $P = 0.1382$) were not significant. These data indicated that cross-protected vines had no impact on production in terms of cluster number.

Overall, the mean cluster weight was lower for cross-protected than for control vines throughout the cross-protection trial, except for ArMV-Ta cross-protected vines

Fig. 2. Disease progress from 1998 to 2006 in cross-protected and control vines in naturally *Grapevine fanleaf virus* (GFLV)-infected vineyard sites in **A**, Ammerschwahr and **B**, Bennwihr in the Alsace region in France. The presence of GFLV in control and cross-protected vines infected with *Arabis mosaic virus* (ArMV)-Ta was determined by double-antibody sandwich-enzyme-linked immunosorbent assay with anti-GFLV immunoglobulins, and in cross-protected vines infected with GFLV-GHu by immunocapture-reverse transcription-polymerase chain reaction-restriction fragment length polymorphism.

in 2005 and 2006 (Fig. 3B). Vines initially infected with GFLV-GHu had a cumulative 20% reduction in cluster weight from 1999 to 2006, and those infected with ArMV-Ta showed a 14% reduction (Table 1). ANOVA indicated a significant treatment $\times$ year interaction ($F = 21.49$; $df = 48$; $P < 0.0001$). The variation observed was due to the combined effect of year ($F = 73.30$; $df = 48$; $P < 0.0001$) and cross-protection ($F = 28.31$; $df = 48$; $P < 0.001$). Differences in LS means between ArMV-Ta cross-protected and control vines were significant (t value = -5.16 ; $df = 48$; $P < 0.0001$) as well as between GFLV-GHu cross-protected and control vines (t value = -7.32 ; $df = 48$; $P < 0.0001$). Together, cross-protected vines had a negative impact on cluster weight. Since the number of clusters per vine was not affected, our data are consistent with smaller clusters in cross-protected relative to control vines.

Effect of cross-protected vines on fruit quality. Sugar content and titratable acidity in fruit juice were measured from 1999 to 2006 in field B as indicative of fruit quality. An overall increase in sugar level was observed in berries of cross-protected vines compared to control vines, although

there were yearly fluctuations (Fig. 3C). Vines cross-protected with ArMV-Ta had a cumulative 2% increase in sugar content, and those initially infected with GFLV-GHu had a 3% increase (Table 1). ANOVA revealed a treatment $\times$ year interaction ($F = 5.80$; $df = 48$; $P < 0.0001$). Most of the variation observed in sugar content was due to year ($F = 70.32$; $df = 48$; $P < 0.0001$) and, to a lesser extent, to cross-protection ($F = 6.56$; $df = 48$; $P < 0.003$). Differences in LS means between GFLV-GHu cross-protected and control vines (t value = 3.59 ; $df = 48$; $P = 0.0008$) were significant, but those between ArMV-Ta cross-protected and control vines ($F = 2.24$; $df = 48$; $P = 0.0297$) were not. These

data indicated that cross-protected vines had limited, if any, impact on sugar content in fruit juice.

Titrate acidity fluctuated greatly from year to year (Fig. 3D). Vines cross-protected with ArMV-Ta had increased acidity values compared to controls for half of the data points (1999, 2002, 2005, and 2006), while GFLV-GHu cross-protected vines had decreased acidity values for most years, except 2002. ANOVA revealed no significant treatment $\times$ year interaction ($F = 1.54$; $df = 48$; $P = 0.1334$), with most of the variation due to year ($F = 279.28$; $df = 48$; $P < 0.0001$) but not to cross-protection ($F = 1.01$; $df = 48$; $P = 0.3720$). Over time, the impact of cross-

Table 1. Cumulative effect of mild protective virus strains on fruit yield and quality of grapevines in a naturally *Grapevine fanleaf virus* (GFLV)-infected vineyard site in Bennwihr from 1999 to 2006

Cross-protective viral strain ^b	N	Impact on fruit yield and quality ^a				
		Yield (kg/vine)	No. cluster/vine	Weight/cluster	Sugar	Acidity
ArMV-Ta	100	-9**	+5	-14**	+2	0
GFLV-GHu	100	-17**	+4	-20**	+3*	-3

^a Percent increase (+) or decrease (–) relative to mean values of control vines. Significant differences of least squares means are indicated (* = $P < 0.05$; ** = $P < 0.0001$). N = number of vines tested.

^b ArMV = *Arabis mosaic virus*.

Fig. 3. Effect of cross-protection from 1999 to 2006 in the Bennwihr site on **A**, fruit yield per vine, **B**, cluster weight, **C**, sugar content in fruit juice, and **D**, fruit juice titratable acidity. Data are expressed as percent increase or decrease relative to control vines for cross-protected vines initially infected with *Arabis mosaic virus* (ArMV)-Ta or *Grapevine fanleaf virus* (GFLV)-GHu.

protected vines was neutral from 1999 to 2006, with no significant differences in LS means (t value = 0.47; df = 48; P = 0.6389 for ArMV-Ta and t value = -0.93; df = 48; P = 0.3595 for GFLV-GHu). Together, cross-protected vines had no impact on titratable acidity in fruit juice, and most of the variation observed was due to seasonal fluctuations.

Genetic stability of the CP gene of mild protective strains GFLV-GHu and ArMV-Ta. The genetic stability of the 1.5-kb CP gene of mild strains GFLV-GHu and ArMV-Ta was examined over time by IC-RT-PCR combined with cloning and sequencing to better characterize the reaction of cross-protected plants to GFLV infection. A 99.5% nucleotide identity was obtained by comparing the CP gene nucleotide sequence of ArMV-Ta and GFLV-GHu from vines grown in the greenhouse and in field B over 10 years. These results were consistent with the genetic stability of the CP gene over a decade. Similar data were reported previously for other GFLV strains (32).

DISCUSSION

GFLV is widespread in major viticultural regions of the world and causes severe losses (1). It is estimated to affect 65% of the grape acreage in France (540,000 ha) with a severe incidence in 30% (270,000 ha) (9). Presently, control strategies against GFLV are not satisfactory. Soil disinfection with nematicides is of limited efficacy to eradicate *X. index* populations (1). In addition, this approach is costly and can also contaminate groundwater supplies and affect nontarget soil microorganisms (1). Rootstocks resistant to *X. index*, in particular VR 039-16 (37) and RS-3 (3), are used in California to mitigate the impact of GFLV. However, this material does not prevent the translocation of GFLV from rootstocks to scions following feeding of viruliferous nematodes, although a severe decline in fruit set does not generally occur in grafted vines (38).

Cross-protection, a phenomenon whereby a mild virus strain can protect plants from challenge infection by a more virulent strain, is an attractive strategy to mitigate the impact of viruses, in particular in perennial crops. It was first described by McKinney in 1929 (24). Success of cross-protection has been documented in citrus against *Citrus tristeza virus* (8), in cucurbits against *Zucchini yellow mosaic virus* (19,21,39), and in papaya and cucurbits against *Papaya ringspot virus* (27,40,42), among others. In the case of nepoviruses, previous studies have shown that herbaceous plants can be protected from infection with severe virus strains (6,7,11,13, 15,17,36). Protection against nepoviruses is expressed as delayed challenge infection (6,11,15,17,36), absence of challenge virus symptoms (7,11,13,36), reduced accumula-

tion of challenge virus (7,11,17), or inhibition of challenge virus multiplication (17).

In the present work, we examined the ability of two mild virus strains, one of GFLV and one of ArMV, to control GFLV in two naturally infected vineyard sites. Our data demonstrated that those strains significantly reduced infection by challenge GFLV isolates in the two vineyard sites, in particular when GFLV-GHu was used as the protective strain. However, in spite of their protective ability, the two mild strains had a substantial negative impact on fruit yield. Thus, cross-protection with ArMV-Ta and GFLV-GHu appears to be of limited practical interest, if any, to control GFLV because a detrimental impact on production is not attractive to the grape and wine industry.

The fact that a higher protection rate was achieved against GFLV with GFLV-GHu rather than with ArMV-Ta is consistent with cross-protection being more efficient if the protective and challenge virus strains are similar or related (20,25). In contrast, cross-protection is less or not effective if the two virus strains are less related or unrelated (11,13,15). Also, cross-protection often does not work well if the two viruses are inoculated simultaneously (20). For GFLV, 3 to 9 days or 20 days were required for the establishment of cross-protection against challenge strains after the protective strain has infected *C. quinoa* (17) and *Gomphrena globosa* (6), respectively. This sequential infection is conceivably of practical interest for a perennial crop like grapevine because rootstocks can be infected with a mild strain several months or even years prior to exposure of grafted vines to challenge virus strains in the vineyard.

The mechanism of cross-protection has not been deciphered, in particular for GFLV. Cross-protection was initially ascribed to competition for host factors. Indeed, protective and challenge virus isolates could compete for the same host factors that are indispensable in the virus multiplication cycle (15,20,25). Other studies suggest the implication of a differential spatial distribution and an exclusion mechanism, whereby a plant cell already infected with a virus strain cannot be superinfected by other strains (20,28,29) or by another virus (12). Also, PTGS is active in RNA-mediated cross-protection (26,43). Regardless of the mechanism of cross-protection, the fact that it only occurs between closely related viruses implies that viral nucleotide sequence similarity is required for competition. Our results support this hypothesis because the rate of GFLV infection was markedly lower in vines initially infected with GFLV-GHu (19%) than in those initially infected with ArMV-Ta (40 to 65%). Furthermore, if PTGS were active in our cross-protected vines, a high nucleotide sequence identity between the mild protective and challenge

virus strains would be expected for efficient RNA degradation. Since GFLV-GHu is an interspecies recombinant between GFLV and ArMV with part of the RNA2-encoded 2A^{HP} and 2B^{MP} genes of ArMV origin and the 2C^{CP} gene of GFLV origin (34), it is conceivable that the CP gene might be involved in the protective mechanism. However, more work is needed to validate this hypothesis.

The CP gene of mild strain ArMV-Ta, as well as of GFLV-GHu, was genetically stable over the course of the cross-protection trial in the Bennwihr site, as shown by comparative sequence data. Therefore, natural mutations in this viral gene that could have precluded protection of test plants are unlikely to account for a high infection rate of vines cross-protected with ArMV-Ta. Instead, monitoring the accumulation of the protective strain ArMV-Ta over time indicated a drastic reduction in ArMV 13 years postplanting in the Ammerschwihr site (from 100% to 65% of the cross-protected vines) and 11 years postplanting in the Bennwihr site (from 100 to 36% of the cross-protected vines). This reduction could result from a displacement of ArMV-Ta by indigenous GFLV strains, as shown previously in *C. quinoa* (17), maybe as a result of poor competitiveness of the mild protective strain. Although at a lower extent, a similar lower level of GFLV-GHu was observed 11 years after planting by IC-RT-PCR-RFLP in a few cross-protected vines (4%, 4 of 100) in the Bennwihr site.

Mild protective strains GFLV-GHu and ArMV-Ta were isolated from field infected vines, and subsequently were propagated and selected on *C. quinoa* (17,22). These strains could be engineered for improved cross-protection potential by reducing their pathogenicity and increasing their competitiveness. For viruses different from GFLV, treating virions with mutagenic agents (41) or low temperature (19), or mutagenizing full-length cDNA clones of genomic viral RNA (23,42) are other approaches used to develop mild protective strains. For GFLV, variants with reduced virulence and increased competitiveness could be engineered from full-length infectious cDNA clones (35) by reverse genetics and mutagenesis. GFLV cDNA clones have been successfully used to unravel GFLV-*X. index* interactions (2), cell-to-cell and long distance movement, and processing of the RNA2-encoded polypeptide (4,5) as well as determining the biological properties of recombinant variants (32). They could be further used to engineer a mild protective chimeric GFLV strain for improved cross-protection potential, as described for a cucumovirus (43) and potyvirus (23,42).

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