A strain-specific segment of the RNA-dependent RNA polymerase of grapevine fanleaf virus determines symptoms in *Nicotiana* species

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Factors involved in symptom expression of viruses from the genus *Nepovirus* in the family *Secoviridae* such as grapevine fanleaf virus (GFLV) are poorly characterized. To identify symptom determinants encoded by GFLV, infectious cDNA clones of RNA1 and RNA2 of strain GHu were developed and used alongside existing infectious cDNA clones of strain F13 in a reverse genetics approach. *In vitro* transcripts of homologous combinations of RNA1 and RNA2 induced systemic infection in *Nicotiana benthamiana* and *Nicotiana clevelandii* with identical phenotypes to WT virus strains, i.e. vein clearing and chlorotic spots on *N. benthamiana* and *N. clevelandii* for GHu, respectively, and lack of symptoms on both hosts for F13. The use of assorted transcripts mapped symptom determinants on RNA1 of GFLV strain GHu, in particular within the distal 408 nt of the RNA-dependent RNA polymerase (1E Pol), as shown by RNA1 transcripts for which coding regions or fragments derived thereof were swapped. Semi-quantitative analyses indicated no significant differences in virus titre between symptomatic and asymptomatic plants infected with various recombinants. Also, unlike the nepovirus tomato ringspot virus, no apparent proteolytic cleavage of GFLV protein 1E Pol was detected upon virus infection or transient expression in *N. benthamiana*. In addition, GFLV protein 1E Pol failed to suppress silencing of EGFP in transgenic *N. benthamiana* expressing EGFP or to enhance GFP expression in patch assays in WT *N. benthamiana*. Together, our results suggest the existence of strain-specific functional domains, including a symptom determinant module, on the RNA-dependent RNA polymerase of GFLV.

**INTRODUCTION**

Mechanisms of symptom expression following plant–virus interaction are poorly understood although remarkable advances have been made for a few viruses (Culver & Padmanabhan, 2007; Dunoyer & Voinnet, 2005; Whitham & Wang, 2004). Several viral proteins such as the movement protein (Lewsey et al., 2009; Rao & Grantham, 1995; Scholthof et al., 1995), the helper component-proteinase (HC-Pro) (Shiboleth et al., 2007), the RNA-dependent RNA polymerase (RdRp) (Kagiwada et al., 2005; Padmanabhan et al., 2005), the capsid protein (CP) (Heaton et al., 1991; Zhu et al., 2005) and pathogenicity proteins (Jupin et al., 1992; Pfeffer et al., 2002) have been identified as symptom determinants. Many viral proteins involved in symptom expression such as the HC-Pro of potyviruses, 2b of cucumoviruses, P19 of tombusviruses, P0 of poleroviruses, 130K protein of tobamoviruses and CP of carmoviruses are virus-encoded suppressors of RNA silencing (VSRs) (Voinnet, 2005). The precise role of VSRs in eliciting symptoms remains unclear although the VSR activity itself does not always play a direct role (Díaz-Pendón & Ding, 2008; Haviv et al., 2012).

The GenBank/EMBL/DDBJ accession number for the GFLV-GHu RNA1 sequence is JN391442.

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Our knowledge of symptom determinants and VSRs of viruses from the genus *Nepovirus*, family *Secoviridae*, is very limited. Recovery, a phenomenon whereby the initial symptomatic infection is followed by attenuation or elimination of symptoms in newly emerging leaves, was first described 85 years ago for a nepovirus (Wingard, 1928). RNA silencing, an innate plant defence mechanism, is associated with this phenomenon (Jovel *et al.*, 2007, 2011; Ratcliff *et al.*, 1997). However, whereas VSRs are well described in most plant virus genera, their identification in nepoviruses is pending.

Nepoviruses have a bipartite single-stranded positive-sense RNA genome (Sanfaçon *et al.*, 2009), and earlier studies with pseudorecombinants from distinct strains of raspberry ringspot virus (RpRSV) showed that: (i) RNA2 causes systemic yellowing in *Petunia hybrida*, (ii) RNA1 is responsible for the severity of systemic symptoms in *Chenopodium quinoa* and (iii) both RNA species determine the type of local lesions in *C. quinoa* (Harrison *et al.*, 1974). Similar conclusions were obtained with tomato black ring virus (TBRV) (Harrison & Murant, 1977). More recently, the 5’ untranslated region (5’UTR) of grapevine chrome mosaic virus (GCMV) was shown to induce necrotic symptoms in *Nicotiana* spp. when expressed from a heterologous viral vector. However, because typical GCMV symptoms were not reproduced, this sequence may not act as a dominant determinant of symptomatology (Fernandez *et al.*, 1999).

*Grapevine fanleaf virus* (GFLV) is a member of the nepovirus subgroup A (Sanfaçon *et al.*, 2009). This virus causes fanleaf disease, one of the most detrimental viral diseases of grapevines worldwide (Andret-Link *et al.*, 2004a). The two GFLV genomic RNAs are covalently linked at their 5’ end to a viral genome-linked protein (VPg) and possess a poly(A) tail at the 3’ end. Each RNA contains a single ORF flanked by 5’- and 3’UTRs. Encoded polyproteins are proteolytically processed into functional proteins by the RNA1-encoded proteinase (1DPro) (Andret-Link *et al.*, 2004a). RNA1 codes for proteins involved in replication and polyprotein maturation, whereas RNA2 codes for proteins involved in virus movement and RNA encapsidation (Fig. 1). Both genomic RNA species are necessary for systemic plant infection (Viry *et al.*, 1993).

GFLV strains GHu and F13 show a differential reaction on *Nicotiana benthamiana* and *Nicotiana clevelandii*, two systemic hosts. Whilst strain GHu induces vein clearing on *N. benthamiana* and chlorotic spots on *N. clevelandii*, strain F13 causes an asymptomatic infection on both *Nicotiana* spp. These distinct phenotypes were exploited to investigate the GFLV determinants of symptomatology using cDNAs of strains GHu and F13 and transcripts derived thereof in a reverse genetics approach. Here, we show that the 3’ end of the GFLV-GHu RdRp coding region determines symptoms in *N. benthamiana* and *N. clevelandii* but does not seem to act as a VSR or to be proteolytically processed.

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**RESULTS**

**Phenotypic and genetic differences between GFLV strains F13 and GHu**

GFLV strains F13 and GHu induced a persistent systemic infection on *N. benthamiana* and *N. clevelandii*, as
indicated by double-antibody sandwich (DAS) ELISA in upper uninoculated leaves (data not shown). Whilst inoculated leaves remained asymptomatic with both strains, GFLV-GHu caused a symptomatic infection on *N. benthamiana* that usually started with a mild vein chlorosis at 6 days post-inoculation (p.i.) followed by a partial leaf chlorosis and mottling that vanish by 20 days p.i. In *N. clevelandii*, GFLV-GHu induced chlorotic spots at 6 days p.i.; these persisted for at least 20 more days. In contrast, no symptoms were visible for GFLV-F13 on *N. benthamiana* or *N. clevelandii* (Fig. 2).

The nucleotide sequence of GFLV-F13 RNA1 (Ritzenhailer *et al.*, 1991) and RNA2 (Serghini *et al.*, 1990) as well as of GFLV-GHu RNA2 (Vigne *et al.*, 2008) has been reported previously. Prior to investigating the viral symptom determinants, the full-length cDNA of GFLV-GHu RNA1 was synthesized by immunocapture (IC)-reverse transcription (RT)-PCR. Its sequence was 7342 nt like the RNA1 of strain F13 (GenBank accession no. NC_003615). The 242 nt RNA1 5'UTR of GFLV-GHu contains predicted stem–loop motifs (GYGT-TAAAGAAACRC) that are also present on the RNA2 5'UTR (Vigne *et al.*, 2008). RNA segments corresponding to polyprotein P1 of GFLV-GHu and -F13 share 88/93 % identity at the nucleotide/amino acid levels, respectively, particularly 90/91 % in the 1A-coding region, 89/97 % in the 1B*Hel* coding region, 90/100 % in the 1C*VPg* coding region, 89/97 % in the 1D*Pro* coding region and 85/88 % in the 1E*Poly* coding region. This identity range is similar for the other four GFLV strains for which complete RNA1 sequence information is available (GenBank accession nos JX513889, JF968120, GQ332373 and GQ332372) (Lampecht *et al.*, 2012; Mekuria *et al.*, 2009). Intraspecies recombination was detected in GFLV-GHu RNA1 in the 3' end of the 1E*Poly* coding region and 3'UTR (nt 6701–7313) with strain F13 as a major parent and an unknown minor parent using RDP v3.41 (Martin *et al.*, 2005). A similar recombination site was previously reported for GFLV strain SAPCS3 (Lampecht *et al.*, 2012).

**Symptom determinants in *Nicotiana* spp. map to GFLV-GHu RNA1**

Infectious cDNA clones of GFLV-GHu RNA1 (pG1) and RNA2 (pG2) were produced and used alongside infectious cDNA clones of GFLV-F13 RNA1 (pF1) and RNA2 (pF2) (Viry *et al.*, 1993) to identify symptom determinants (Fig. 1). The integrity of the four cDNA clones was verified by DNA sequencing. A few single-nucleotide polymorphisms (SNPs) were found, most of which were silent, and only 15 affected the amino acid composition (Tables S1 and S2, available in JGV Online). In agreement with previous reports (Andret-Link *et al.*, 2004b; Schellenberger *et al.*, 2010), *in vitro*-synthesized transcripts derived from plasmids pF1 and pF2 were infectious and caused an asymptomatic infection in *N. benthamiana* and *N. clevelandii*, as did WT GFLV-F13 (Table 1, Fig. 2). Importantly, G1/G2 transcripts reproduced the symptoms

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**Fig. 2.** Systemic symptoms induced by WT GFLV-GHu and -F13 and synthetic and chimaeric viruses on *N. benthamiana* (a) and *N. clevelandii* (b) at 6–12 days p.i. See legend of Fig. 3 for a description of the viruses.
observed with GFLV-GHu in *N. benthamiana* and *N. clevelandii* (Table 1, Fig. 2).

To map the GFLV-GHu symptom determinants, the phenotype induced by transcripts of assortants F1/G2 and G1/F2 was investigated. Both combinations caused a systemic infection, but only G1/F2 displayed typical GHu symptoms on the two hosts, whilst F1/G2 remained asymptomatic (Table 1, Fig. 2). These results indicated that GFLV-GHu RNA1 encodes the symptom determinants. Consequently, GFLV RNA2 was excluded from further investigation.

### The 3’ end of the GFLV-GHu 1E\textsuperscript{Pol} coding region elicits symptoms on *Nicotiana* spp.

To further delineate the symptom determinants on GFLV-GHu RNA1, a series of chimaeric RNA1 cDNAs between strains GHu and F13 was created. Plasmids pF1s and pG1s were first produced with an extraneous *SnaB* restriction site to facilitate the engineering of chimaeric RNA1 constructs. Transcripts derived from these two plasmids induced the same reactions as strains GHu and F13 on *N. benthamiana* and *N. clevelandii* (Fig. 3). Eight chimaeric RNA1 constructs were then produced by exchanging full-length or partial fragments of coding regions between the cDNAs of strains F13 and GHu using *SnaB* and other restriction sites (Fig. 3). Transcripts derived thereof produced systemic infections on herbaceous hosts when inoculated with either F2 or G2, as indicated by DAS-ELISA performed on apical leaves at 12–16 days p.i. (data not shown).

Transcripts G1(5’1AB)\textsubscript{F}, F1(1E3)\textsubscript{G} and F1(1E)\textsubscript{G} caused symptoms on *N. benthamiana* and *N. clevelandii*, whilst transcripts F1(5’1AB)\textsubscript{G}, F1(1BCD)\textsubscript{G} and G1(1E)\textsubscript{F} were asymptomatic (Fig. 3), indicating that the region corresponding to the 1E\textsuperscript{Pol} coding region of GFLV-GHu is the sole symptom determinant in these two hosts (Fig. 3). Symptomatic recombinant viruses caused typical GFLV-GHu symptoms, although the onset of symptom development varied from 7 to 10 days p.i. Furthermore, the exchange between the 5’ (nt 4623–6650) and 3’ (nt 6663–7094) parts of the 1E\textsuperscript{Pol} coding region indicated that only transcripts F1(1E\textsubscript{Cter})\textsubscript{G} induced typical GHu symptoms, whilst no symptoms were observed for transcripts F1(1E\textsubscript{Nter})\textsubscript{G} (Figs 2 and 3). These findings are consistent with the fact that the distal 432 nt of the GFLV-GHu 1E\textsuperscript{Pol} coding region carry the symptom determinants. As the first 24 of these 432 nt are strictly conserved between strains F13 and GHu, we concluded that the ultimate 408 nt or 136 aa of GFLV-GHu protein 1E Pol (residues 689–824).

### Characterization of GFLV-GHu protein 1E\textsuperscript{Pol}

The complete GFLV-GHu protein 1E\textsuperscript{Pol} has identities ranging from 39 to 84 % with the RdRp of several viruses in the family Secoviridae. The seven conserved signature RdRp motifs (195-TPKDERL\textsuperscript{201}, 216-GTRFLSVPLAYNL\textsubscript{V}KF\textsuperscript{L}-FSRLLMKK\textsuperscript{239}, 248-QVGPNSREW\textsuperscript{256}, 267-CDYKAFDGL-IT\textsuperscript{283}, 328-TEAGIPS\textsubscript{C}GALTVV\textsubscript{L}NS\textsuperscript{344}, 372-LITYGDDN\textsubscript{V}F\textsuperscript{382} and 427-LEFKRG\textsubscript{3}E) commonly described for plant viruses (Chisholm *et al.*, 2007) are located upstream of the distal 136 aa of GFLV-GHu protein 1E\textsuperscript{Pol} (residues 689–824). Interestingly, the latter 136 residues have no significant match with known proteins, except with protein 1E\textsuperscript{Pol} of arabis mosaic virus (GenBank accession no. NC_006057) and grapevine deformation virus (GenBank accession no. HE613269), two other subgroup A nepoviruses (48–60 % amino acid identities). Furthermore, putative helix–loop–helix proteins, RNA recognition motif and zinc fingers were found within the GFLV-GHu protein 1E\textsuperscript{Pol} C terminus with...
the protein domain annotation SMART (Letunic et al., 2012) but E-values were poor (4 × 10⁻³ to 3 × 10⁻⁴).

A putative proteolytic cleavage site was identified within GFLV-GHu and -F13 protein 1EPol at position G¹⁷⁰⁴/E¹⁷⁰⁵ using conserved motifs described previously (Andret-Link et al., 2004a). This site could theoretically release a 13.2 kDa C-terminal peptide and a 79 kDa N-terminal peptide. To test whether the GFLV-GHu protein 1EPol segment responsible for symptom development or is proteolytically cleaved at the C-terminal region like the RdRp of tomato ringspot virus (ToRSV) (Chisholm et al., 2007), Western blots were performed on total proteins from infected plants and agroinfiltrated patches with an antiserum specific to GFLV protein 1EPol. These antibodies detected the mature protein of about 92 kDa with no intermediate maturation product in plants infected with GFLV-GHu or -F13 (Fig. 4a). The transient assay based on agroinfiltration was then used to characterize protein 1EPol in the presence or absence of GFLV protease 1DPro. Again, only a GFLV-GHu protein 1EPol of the expected size was detected and no shorter product was visualized (Fig. 4a), whether the protease was co-expressed or not. The functionality of GFLV-1DPro was confirmed by cleavage of the tagged construct EGFP–2B MP containing a C/A cleavage site between the reporter EGFP and protein 2B MP (Fig. 4b).

To verify that the apparent lack of maturation was not due
to a loss of immunogenicity of the N terminus of protein 1EPol that is recognized by the 1EPol antiserum, a 1E–EGFP fusion was produced. Again, no additional cleavage of protein 1EPol from GFLV-GHu was detected using anti-EGFP mAbs, regardless of whether the proteinase was added or not (Fig. 4b). These results showed that symptom expression probably does not result from a GFLV-GHu protein 1EPol maturation and subsequent release of a hitherto unidentified product by GFLV protein 1DPro or an endogenous proteinase in planta. Despite a higher relative accumulation of GFLV-GHu protein 1EPol following ectopic expression compared with viral infection (Fig. 4a), no symptoms were observed in agroinfiltrated leaves of N. benthamiana.

**GFLV protein 1EPol does not seem to be a VSR**

To test whether GFLV-GHu protein 1EPol has VSR activity, several agroinoculation patch assays were employed in N. benthamiana. In the first procedure, silencing of EGFP in transgenic N. benthamiana was pre-established using tobacco rattle virus (TRV) RNA2–EGFP as an inducer of gene silencing (Fig. 5a, upper panel). In the second procedure, EGFP silencing was established from a cauliflower mosaic virus (CaMV) 35S expression cassette (Fig. 5a, lower panel). In both assays, agroinfiltration with GFLV-GHu 1EPol did not result in significant elevation of EGFP fluorescence, and protein 1EPol of GFLV-GHu and -F13 behaved similarly to the cucumber mosaic virus (CMV) CP, a non-VSR control, whilst known VSRs, e.g. 2b of CMV and p24 of grapevine leafroll-associated virus 2 (GLRaV-2), showed elevated fluorescence, as expected. In a third procedure using WT N. benthamiana, neither GHu nor F13 protein 1EPol boosted expression of GFP, as did CMV 2b, although both GFLV proteins were readily detected in agroinfiltrated patches by Western blotting (Fig. 5b). These three independent experiments indicated no detectable VSR activity associated with protein 1EPol of GFLV-GHu and -F13.

**Symptom expression in Nicotiana spp. is not associated with GFLV accumulation**

To determine whether symptom expression was related to virus titre, the level of accumulation of WT, mutated and chimaeric viruses was determined in infected N. benthamiana and N. clevelandii at 13 days p.i. by semi-quantitative DAS-ELISA. In a preliminary assay, crude sap of healthy N. benthamiana and N. clevelandii was spiked with known amounts of purified virions and assessed by testing serial dilutions of GFLV-GHu and -F13. No statistically significant difference in the detection of strains GHu and F13 was obtained (data not shown), validating our semi-quantitative DAS-ELISA procedure.

In N. benthamiana, GFLV accumulation ranged from 18±4 to 58±23 µg g⁻¹ in symptomatic plants infected with strains GHu, G1/G2, G1/F2, F1(1E)CF/F2, and F1(1ECter)CF/F2, and from 24±6 to 35±3 µg g⁻¹ in asymptomatic plants infected with strains F13, F1F2, F1G2, G1(1E)CF/F2, and F1(1EnTer)CF/F2. In N. clevelandii, the accumulation range was 37±13 to 69±23 µg g⁻¹ in symptomatic plants compared with 35±8 to 50±24 µg g⁻¹ in asymptomatic plants. Data analyses with Student’s t-test indicated no statistically significant correlation between virus titre and symptom expression (P=0.3913 for N. benthamiana and P=0.1204 for N. clevelandii). These results were corroborated by Northern blotting using riboprobes specific to RNA1 and RNA2 (data not shown).

**DISCUSSION**

In this study, the 3’ end of the GFLV-GHu 1EPol coding region was identified as a symptom determinant in N. benthamiana and N. clevelandii by using a reverse genetics approach with infectious clones of two GFLV strains that exhibit a differential phenotype. To our knowledge, this is the first report on a viral gene determinant of symptom expression for a member of the genus Nepovirus, family Secoviridae. To date, the symptom determinants identified for secovirids are proteins involved in replication and polyprotein maturation such as type III helicase, 3C-like cysteine proteinase (Fan et al., 2011; Gu & Ghabrial, 2005) and type I polymerase (this study).

The GFLV-GHu RdRp segment involved in symptomatology in N. benthamiana and N. clevelandii is located between residues 689 and 824, which are downstream of the canonical polymerase domains (residues 22–526) (Chisholm et al., 2007). This situation is similar to some potex- and cucumoviruses. For example, a single amino acid at the RdRp C terminus, located away from the polymerase domain of potato virus X (PVX), determines symptoms in Nicotiana spp. (Kagiwada et al., 2005). For Plantago asiatica mosaic virus, a single RdRp C-terminal residue is responsible for the phenotype on N. benthamiana with Cys¹⁵⁴ inducing necrosis and Tyr¹⁵⁴ an asymptomatic infection (Ozeki et al., 2006). In the case of GFLV, the RdRp segment (408 nt and 136 aa) involved in symptom determination is extremely variable among GFLV strains with only 62–77 % and 60–74 % nucleotide and amino acids identities, respectively. To further narrow the segment eliciting symptoms, short stretches of 45 residues were exchanged between GFLV-GHu and -F13 at the 1EPol C terminus. Unfortunately, transcripts thereof failed to induce systemic infection in the presence of functional RNA2 transcripts (data not shown).

No proteolytic cleavage of GFLV protein 1EPol was documented in transient expression assays and in infected plants by Western blot analyses, in spite of a putative cleavage site (G⁸⁰⁴/E⁸⁰⁶) that could theoretically release a C-terminal 1EPol peptide of 13.2 kDa in the region involved in symptom determination. Because a 92 kDa protein corresponding to a mature 1EPol was always detected, regardless of the assay and experimental conditions, a functional protein
1EPol in GFLV-infected N. benthamiana does not seem to be truncated, unlike that of ToRSV (Chisholm et al., 2007).

GFLV symptom expression in N. benthamiana and N. clevelandii is not correlated with a higher virus titre. This neutral association between virus accumulation and symptom expression is also known for brome mosaic virus (Rao & Grantham, 1995), PVX (Kagiwada et al., 2005) grapevine virus A (Haviv et al., 2012) and tobacco mosaic virus (Mansilla et al., 2009). In contrast, a positive correlation between virus titre and symptom expression was shown for bean pod mottle virus (Gu & Ghabrial, 2005) and CMV (Du et al., 2008; Kim & Palukaitis, 1997).

Several viral pathogenicity determinants are VSRs (Wang et al., 2012). For example, CMV 2b induces disease symptoms by interfering with microRNA-regulated turnover of host mRNAs (Lewsey et al., 2007) and HC-Pro targets the miR167 that regulates the AUXIN RESPONSE FACTOR 8 in Arabidopsis thaliana (Jay et al., 2011). Remarkably, many VSR genes are overprinted on either the N-terminal (P69 and P0) or C-terminal (2b of CMV and 2B of flock house virus) region of viral RdRp genes. The unique coupling of RNA replication and VSR functions can create gene modules for evolution and adaptation (Li & Ding, 2006). The GFLV-GHu 1EPol is a symptom determinant with no apparent potent VSR activity, as shown by three independent procedures, although we cannot exclude a weak VSR activity, as reported for the CP of another member of the family Secoviridae (Cañizares et al., 2004), or a tissue-specific VSR activity such as for p31 of beet necrotic yellow vein virus (Rahim et al., 2007). Viral symptom determinants are mostly proteins; however, nucleotide sequences can also affect symptom expression (Hirata et al., 2003; Rodriguez-Cerezo et al., 1991; Wang & Simon, 2000). For example, a single silent mutation located downstream of the polymerase domain at the 3′ end of the RdRp gene of apple stem grooving virus is responsible for symptom attenuation (Hirata et al., 2003). Also, three stem–loops in the 5′ UTR of RNA2 of GCMV, a subgroup B nepovirus, induce severe necrotic symptoms in several Nicotiana spp. when expressed from a PVX vector but do not act as dominant symptom determinants (Fernandez et al., 1999). The possible effect of the stem–loops in the 5′ UTR of GFLV-GHu RNA2 (Vigne et al., 2008) in symptom expression was ruled out in this study upon analysis of RNA1 and RNA2 assortants (Fig. 2). More work is needed to determine whether amino acids or nucleotide stretches are responsible for GFLV-GHu symptoms on N. benthamiana and N. clevelandii. We hypothesize that the mechanism(s) of symptom induction by the GFLV-GHu 1EPol coding region, the central role of protein 1EPol in virus replication and hitherto undiscovered functions associated with protein 1EPol might involve multiple interactions with host cellular factors. A better characterization of the functional domains on the GFLV 1EPol coding region and elucidation of symptom determinism in a wider array of hosts, including in grapevine, the natural host, are likely to be necessary to elucidate the mechanism of symptom expression.

Fig. 4. Western blot analysis of GFLV protein 1EPol accumulation in N. benthamiana infected with GFLV-F13 or -GHu at 7 days p.i. and in N. benthamiana agroinfiltrated with a native or an EGFP-fused GFLV-GHu protein 1EPol co-expressed with tomato bushy stunt virus P19 in the presence (+) or absence (−) of GFLV proteinase 1DPol at 3 days p.i. Proteins were separated by SDS-PAGE and analysed by Western blotting using an anti-1E Pol serum (a) or anti-GFP mAbs (b). The EGFP/2BMP construct was used to verify GFLV 1DPol activity. Arrowheads indicate expected protein sizes. H, healthy plant; CBS, Coomassie blue-stained proteins.
METHODS

Virus strains and viral cDNA clones. GFLV-F13 (Ritzenthaler et al., 1991; Serghini et al., 1990) and -GHu (Vigne et al., 2008) were isolated from naturally infected grapevines and propagated in C. quinoa, a systemic host. Full-length cDNA clones of GFLV-F13 RNA1 and RNA2 were used for in vitro synthesis of infectious transcripts (Viry et al., 1993). The full-length cDNA of GFLV-GHu RNA1 was amplified by IC-RT-PCR using primers GH1f and GH1r (Table S3) and crude extracts from infected C. quinoa leaves (Vigne et al., 2004). All RT-PCRs and PCRs were carried out using the Phusion RT-PCR (Finnzymes) or High Fidelity PCR (Roche Applied Science). To obtain pG1, the PCR amplicon was cloned into SalI/XmaI-digested pUC18 (Fig. 1). The full-length RNA2 of GFLV-GHu in plasmid pG2 was obtained by RT-PCR in a three cloning steps approach (Fig. 1) using total RNA extracted from infected C. quinoa leaves with an RNeasy Plant Mini kit (Qiagen), and appropriate primers (Table S3, Fig. 1).

Development of chimaeric GFLV-F13 and -GHu RNA1. Plasmids pF1 and pG1 were used as templates to develop chimaeric cDNAs of GFLV RNA1 by exchanging cDNA parts of strains F13 and GHu. For cloning purposes, a SnaBI restriction site was introduced first into both plasmids (Fig. 3) by site-directed mutagenesis with overlap PCR extension (Ho et al., 1989) using primer pairs G40/G43 and G42/G44 (Table S3). The corresponding products were mixed and amplified by PCR with primer pair G40/G42, yielding a 5799 nt fragment that was cloned in the corresponding plasmid after XhoI/NotI digestion (Fig. 3). The same strategy was used to engineer plasmids with a complete or fragmented chimaeric 1E Pol coding region [pF1(1E)G, pG1(1E)F, pF1(1E) enter G and pF1(1E)Cter G] (Table S4, Fig. 3). Plasmids pF1(5’1AB)G, pG1(5’1AB)F, pF1(1BCD)G and pF1(1E3)G were obtained by cloning DNA fragments digested with appropriate restriction enzymes (Fig. 3).

In vitro transcription of GFLV cDNA clones and mechanical inoculation of plants. Transcripts of WT, mutated and chimaeric
GFLV-F13 and -GHu RNA1 and WT GFLV RNA2 were obtained from corresponding cDNAs by in vitro transcription with an mMESSAGE mMACHINE T7 kit (Ambion) after linearization with appropriate restriction enzymes (Fig. 1). C. quinoa and N. benthamiana plants were inoculated mechanically with transcripts of GFLV RNA1 and RNA2 or combinations thereof (Viry et al., 1993). Symptoms were monitored at 6–14 days p.i. and systemic infection was assessed in uninoculated apical leaves at 2 weeks p.i. by DAS-ELISA (Vigne et al., 2004). Tissue from infected C. quinoa was used as inoculum for symptom monitoring experiments on N. benthamiana and N. clevelandii.

Characterization of GFLV-GHu RNA1, GFLV progeny and infectious clones by sequencing. The complete nucleotide sequence of GFLV-GHu RNA1 was determined from overlapping cDNA fragments obtained by IC-RT-PCR using specific primers (Table S4). The 5′- and 3′-terminal sequences were obtained by 5′/3′RACE (Roche Applied Science). The complete nucleotide sequence GHu RNA1 (GenBank accession no. JN391442) was used to design primers GH1f and GH1r (Table S3).

The progeny of chimaeric viruses F1(1E)G and F1(1ECter)G was amplified by RT-PCR using specific primers GH1f and GH1r (Table S3). The amplified products were sequenced (Eurogentec) and used to design primers G9/G10, G11/G12, G13/G14 and G15/16 for the PCR steps (Table S4).

Symptoms were monitored at 6–14 days p.i. and systemic infection of GFLV RNA1 and RNA2 or combinations thereof (Viry et al., 1993) were further diluted 100-fold and DAS-ELISA absorbance values were measured using a Synergy2 microplate reader (BioTek) set for ELISA.

Analysis of GFLV accumulation by semi-quantitative DAS-ELISA. GFLV detection by DAS-ELISA was performed using specific serological reagents (Bioreba). Samples were ground in 25 mM sodium phosphate buffer (pH 7.4) at a 1:10 ratio (w/v). Leaf extracts were further diluted 100-fold and DAS-ELISA absorbance values were compared with known amounts of purified GFLV serially diluted in sap of healthy plants. Semi-quantitative DAS-ELISA data were compiled, and statistical analyses were used to determine significant relationships between virus accumulation and symptom expression using Student’s t-test and ANOVA analyses with a program written in 

(Ahaka & Gentleman, 1996).

Transient expression of GFLV proteins in plants. Plasmids pMD32-1E F13 and pMD32-1E GHu were obtained by PCR amplification using pF1 and pG1 as templates and primers 146/148 and 107/108, respectively (Table S5), to add an attB1 sequence and an ATG at the 5′ end, and an attB2 sequence at the 3′ end of the 1EPro coding region. Amplicons were introduced by Gateway cloning into the pDONR/Zeo vector (Invitrogen) to produce pDZ-1E F13 and pDZ-1E GHu, which were further recombined into the destination vectors pMD32 (Curtis & Grossniklaus, 2003). To produce plasmid pK7-1E GHu-EGFP, PCR was carried out using pG1 and primers 108/109 to remove the stop codon before sequential recombination into plasmids pDonRZeo and pK7FWG2. All plasmids were introduced into Agrobacterium tumefaciens strain GV3101 pMP90 and used for agroinfiltration in the presence of tomato bushy stunt virus protein P19.

The GFLV-F13 1DPro coding region flanked by attB1 and ATG sequences at the 5′ end, and attB2 and TAA sequences at the 3′ end was PCR amplified from pF1 using primers 191/189 (Table S5), and subsequently recombined into pDONR/Zeo and pMD32 vectors. The resulting plasmid, pMD32-1DPro, was used for expression of GFLV-F13 protease 1DPro. The control pK7EGFP/2RMP clone allowed synthesis of a chiamaeric protein encompassing the complete EGFP, the last 9 aa of GFLV-F13 protein 2AHP with a functional C/A cleavage site, and the GFLV-F13 protein 2BMP.

Protein extraction and Western blot analysis. Total N. benthamiana proteins were extracted from agroinfiltrated leaf patches (3–5 days p.i.) or infected leaves (10–14 days p.i.) in PBS (pH 7.4) in the presence of protease inhibitors (Complete; Roche) in a Preccell tissue grinder (Bertin Technologies). Proteins (20 μl) were resolved by SDS-PAGE (10% acrylamide) and electrotransferred onto Immobilon PVDF membranes. Specific GFLV 1EPro rabbit antibodies (Eurogentec) raised against peptide HPVPSKTSFMKVPDELG corresponding to conserved aa 29–49 of GFLV-GHu and -F13 protein 1EPro were used in Western blot analysis at a 1:2000 dilution. Goat anti-rabbit–HRP (1:12 500) and a Lumi-Light chemiluminescence system (Roche) were used for antigen detection. The anti-GFP mAB (diluted 1:1000) was from Clontech.

VSR assays. The GFLV-GHu (1E GHu) and -F13 (1E F13) 1EPro coding region was amplified by PCR from pG1 and pF1, respectively. CMV 2b (Choi et al., 2008) and GLRaV-2 p24 (Chiba et al., 2006) were used as VSR controls, and the CP gene of CMV (Namba et al., 1991) was used as a negative control. GLRaV-2 p24 was obtained by one-step RT-PCR using appropriate primers and total RNA of infected Vitis vinifera cv. Chauvou.

Digested DNA products were subcloned into the plant expression vector pEPT8Bp or pEPT8MAC2 modified by Ling et al. (1997) to contain BgII and BclI restriction sites flanking a double CaMV 35S promoter, or to contain NotI within the CaMV 35S cassette, respectively. Individual virus coding regions within the CaMV 35S cassette (Table S5) were then restricted and ligationed into the binary vector pGA482G (Pang et al., 2000). The full-length EGFP (a derivative of S65T GFP; Clontech) coding sequence was cloned into TRV RNA2 (pTRV2) (Ratcliff et al., 2001) to produce pTRV2-EGFP. Binary vectors were mobilized into A. tumefaciens strains CS8Z707 (EGFP, 1E F13, 1E GHu, CMV CP and p24) or GV3101 (CMV 2b and pTRV2-EGFP) via electroporation. Plasmids pBinGFP, pBin2b and an empty pBin were also used in co-infiltration experiments on WT N. benthamiana plants.

N. benthamiana expressing EGFP were obtained by A. tumefaciens-mediated transformation using strain CS8Z707 containing pGA482G-EGFP. Regenerated plants were self-pollinated through T5. High and homogeneous levels of EGFP expression were confirmed by confocal microscopy and quantitative fluorescence measurements in transgenic line 12 (T12) through each generation (data not shown). Leaves of T12 plants were agroinfiltrated with constructs containing genes of known VSRs, various controls and 1EPro of GFLV-F13 or -GHu. For the experiments using a TRV-derived EGFP construct, 13 days elapsed between TRV infiltration and individual gene infiltration. Subsequently, 5 days elapsed between infiltration of individual virus coding regions and sample collection. Samples (2 cm²) were collected from each agroinfiltrated leaf, ground in phosphate buffer (pH 7.5) using a TissueLyser (Qiagen), and 100 μl homogenates were added to three wells per sample in a 96-well microplate. Fluorescence was measured using a Synergy2 microplate reader (BioTek) set for excitation at 484 nm and emission at 508 nm. Technical replicates were averaged together to produce a sample output. Sample output measurements were compared in SAS (SAS Institute) by ANOVA, and significant differences (P<0.05) were assigned using Fisher’s least significant difference post-hoc test.

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