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 (Diaz-Pendón & Ding, 2008; Haviv et al., 2012).
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.*, 2008). 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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**Fig. 1.** Schematic representation of GFLV (a) RNA1 and (b) RNA2 and infectious clones derived from GFLV-F13 (open boxes) and GFLV-GHu (shaded boxes). Large open boxes indicate open reading frames with the processed proteins above (Hel, putative helicase; VPg, viral protein genome-linked; Pro, proteinase; Pol, RdRp; HP, putative homing protein; MP, movement protein; CP, capsid protein). Small open boxes denote the 5‘ and 3‘ UTRs and black circle represents the VPg. Plasmids are named with letters and numbers corresponding to the GFLV strains (F for F13 and G for GHu) and to the RNA species (1 for RNA1 and 2 for RNA2). The T7 promoter is represented by a black arrow, and the position of the restriction sites used for cloning and/or linearization is indicated by dashed lines.

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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 (Ritzenthaler 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 (GYGTAAAAGAAACRC) 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\textsubscript{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\textsubscript{Pol} 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) (Lamprécht et al., 2012; Mekuria et al., 2009). Intraspecies recombination was detected in GFLV-GHu RNA1 in the 3’ end of the 1E\textsubscript{Pol} 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 (Lamprécht 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

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<sup>Po</sup> coding region elicits symptoms on *N. clevelandii* 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 SnaB1 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 SnaB1 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′AB)<sub>F</sub>, F1(1E3)<sub>G</sub> and F1(1E)<sub>G</sub> caused symptoms on *N. benthamiana* and *N. clevelandii*, whilst transcripts F1(5′AB)<sub>G</sub>, F1(1BCD)<sub>G</sub> and G1(1E)<sub>F</sub> were asymptomatic (Fig. 3), indicating that the region corresponding to the 1E<sup>Po</sup> 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<sup>Po</sup> coding region indicated that only transcripts F1(1ECter)<sub>G</sub> induced typical GHu symptoms, whilst no symptoms were observed for transcripts F1(1ENter)<sub>G</sub> (Figs 2 and 3). These findings are consistent with the fact that the distal 432 nt of the GFLV-GHu 1E<sup>Po</sup> 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 the GFLV-GHu 1E<sup>Po</sup> coding region elicit a symptomic phenotype (Table S1).

Transcripts F1(1E)<sub>G</sub> and F1(1ECter)<sub>G</sub> in the presence of F2 was characterized by IC-RT-PCR and sequencing to determine their stability. The nucleotide and amino acid sequence conservation is substantially lower (70.3 and 64.7 %, respectively) than within the complete 1E<sup>Po</sup> coding region (85.0 and 88.2 %, respectively) of GFLV strains GHu and F13. Reduced conservation is also observed with equivalent regions from other GFLV strains for which sequence information is available in GenBank with 62–77 % and 60–74 % nucleotide and amino acid identities, respectively (Lamprecht et al. 2012; Mekuria et al., 2009; Oliver et al., 2010).

The progeny of chimaeric viruses F1(1E)<sub>G</sub> and F1(1ECter)<sub>G</sub> in the presence of F2 was characterized by IC-RT-PCR and sequencing to determine their stability. The nucleotide sequence from the progeny of F1(1E)<sub>G</sub> in *N. benthamiana* and *N. clevelandii* was as expected. In contrast, one SNP (C<sup>6210</sup> instead of T<sup>6210</sup>) was observed on both hosts for the progeny of F1(1ECter)<sub>G</sub>. This SNP led to a S<sup>1999</sup>P change, restoring the original F13 sequence, which was mutated at this position in pF1, but without recovering the asymptomatic phenotype (Table S1).

**Characterization of GFLV-GHu protein 1E<sup>Po</sup>**

The complete GFLV-GHu protein 1E<sup>Po</sup> has identities ranging from 39 to 84 % with the RdRp of several viruses in the family *Secoviridae*. The seven conserved signature RdRp motifs (195TPKDERL<sup>201</sup>, 210GTRLFSVLAY215NLLRVKFL, 239SFSRLMKG<sup>259</sup>, 248QVVPNPSREV<sup>256</sup>, 270CDYKAFDGL-<sup>283</sup>IT, 328TEAGIPSGCALT<sup>344</sup>VLSN, 372LITYGDDNVF<sup>382</sup> and 427LEFLKRG<sup>433</sup>) commonly described for plant viruses (Chisholm et al., 2007) are located upstream of the distal 136 aa of GFLV-GHu protein 1E<sup>Po</sup> (residues 689–824). Interestingly, the latter 136 residues have no significant match with known proteins, except with protein 1E<sup>Po</sup> 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<sup>Po</sup> C terminus with

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<th>Inoculum&lt;sup&gt;+&lt;/sup&gt;</th>
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<th><em>N. clevelandii</em></th>
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<tr>
<td>WT F13</td>
<td>0 % (37)</td>
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<td>WT GHu</td>
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<sup>+</sup>Crude sap of *C. quinoa* infected with WT (F13 and GHu), synthetic (F1/E2 and G1/G2) and assportant (G1/E2 and F1/G2) GFLV was used as inoculum. Each RNA is designated a letter referring to strain F13 (F) or GHu (G), and a number to refer to RNA1 (1) or RNA2 (2).

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Table 1. Symptoms of WT, synthetic and assortant GFLV in systemically infected *N. benthamiana* and *N. clevelandii*

Results are shown as the percentage of infected plants showing vein clearing, mottling or discoloration (*N. benthamiana*) or chlorotic spots (*N. clevelandii*), and reacting positively to GFLV antibodies in DAS-ELISA. Numbers in parentheses correspond to the number of plants tested in at least three independent experiments. Note that asymptomatic plants reacted positively to GFLV antibodies in DAS-ELISA.
Fig. 3. Systemic symptoms induced by WT, mutated and chimaeric GFLV RNA1 on *N. benthamiana* and *N. clevelandii* in the presence of GFLV-F13 or -GHu RNA2 at 6–12 days p.i. The name of transcripts is the same as for plasmids (for example, F1 is the transcript from pF1). GFLV-F13 and -GHu sequences are in open and shaded boxes on the genetic map of RNA1, respectively. Restriction sites used for the creation of chimaeric clones are denoted. The presence (+) or absence (−) of viral symptoms in 5–20 plants in at least two independent experiments is indicated. The insertion of *Sna*BlI introduced a silent nucleotide modification at nt 4638 for GFLV-F13 RNA1 in pF1 and at nt 4640 for GFLV-GHu RNA1 in pG1 (nucleotide positions are given according to the GFLV-F13 RNA1 sequence, GenBank accession no. NC_003615). For each plasmid, the information in parentheses indicates the region that was swapped in a pF1 or pG1 backbone plasmid, and letters G and F as subscripts refer to the origin of the region exchanged with G standing for GHu and F for F13. For example, G1(5′1AB)F contains the 5′UTR, the 1A-coding region, and the 5′ part of the 1BHel-coding region of GFLV-F13 in a GFLV-GHu RNA1 cDNA in plasmid pG1.
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, 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)C/F2, and F1(1ECter)C/F2, and from 24 ± 6 to 35 ± 3 μg g⁻¹ in asymptomatic plants infected with strains F13, F1F2, F1G2, G1(1E)C/F2, and F1(1ENter)C/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 secpovirids 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 Cys1154 inducing necrosis and Tyr1154 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 (G1704/E1705) 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-Cerzo 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 1DPro 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/2B MP construct was used to verify GFLV 1DPro 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(1ENter)G and pF1(1ECter) G] (Table S4, Fig. 3). Plasmids pF1(5′AB)G, pG1(5′AB)F, pF1(1BCD)G and pG1(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 symptoms in *Nicotiana* are determined by the RdRp

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 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(1E)G was assessed in uninoculated apical leaves at 2 weeks p.i. by DAS-ELISA. All the cDNAs and plasmids were fully sequenced to verify integrity and helpful discussions, Drs S. Saito and E. Duche ˆne for statistical analyses were used to determine significant differences (P<0.05) were assigned using Fisher’s least significant difference post-hoc test.

**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 PreScylla tissue grinder (Bertin Technologies). Proteins (20 μl) were resolved by SDS-PAGE (10% acrylamide) and electroblotted onto Immobilon PVDF membranes. Specific GFLV 1E*Hu* rabbit antibodies (Eurogentec) raised against peptide HPVSTFSKMVKPDLEC corresponded to conserved aa 29–49 of GFLV-GHu and -F13 protein 1EPol were used in Western blot analysis at a 1:2000 dilution. Goat anti-rabbit–HRP (1:12500) 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) 1EPol coding region was amplified from 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., 1999) 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. Chauvex rose.

Digested DNA products were subcloned into the plant expression vector pEPT8_BB or pEPT8_MCS2 modified from Ling et al. (1997) to contain BsII and Bell 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 restriction digested and ligated 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 CSZ7270 (EGFP, 1E_F13, 1E_GHu, CMV CP and p24) or GV3101 (CMV 2b and pTRV2-EGFP) via electroporation. Plasmids pBinGFP, pBin2b and 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 C58Z707 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 1EPol 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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