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Communication

Construction of an Infectious DNA Clone of Grapevine Geminivirus A and Its Biological Activity in *Nicotiana benthamiana* and Grapevine Plants

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Abstract: The pathogenicity of grapevine geminivirus A (GGVA), a recently identified DNA virus, to grapevine plants remains largely unclear. Here, we report a new GGVA isolate (named GGVA^{QN}) obtained from grapevine 'Queen Nina' plants with severe disease symptoms; the infectious clone of GGVA^{QN} (pXT-GGVA^{QN}) was also constructed. Infection assays indicated that *Nicotiana benthamiana* plants inoculated with GGVA^{QN} display upward leaf curling and chlorotic mottling symptoms. Direct injection of *Agrobacterium tumefaciens* cells carrying pXT-GGVA^{QN} into roots of in vitro-grown 'Red Globe' grape plantlets by syringe led to systemic infection with GGVA^{QN}, and the plants exhibited chlorotic mottling symptoms on their upper leaves, and downward curling, interveinal yellowing, and leaf-margin necrosis symptoms on their lower leaves. Our results provide insights into the pathogenicity of GGVA, and a simple and efficient inoculation method to deliver infectious viral clones to woody perennial plants.

Keywords: grapevine geminivirus A; infectious viral clone; pathogenicity; inoculation method

1. Introduction

Viral diseases in viticulture lead to serious reductions in yield quantity and quality. With the development of high-throughput sequencing technology, new viruses infecting grapevines have been identified. To date, more than 80 different viruses from 17 families and 34 genera have been identified in grapevines [1,2]. Grapevine geminivirus A (GGVA) was first reported in 2017 using high-throughput sequencing [3]. Since its initial characterization, GGVA has also been reported in China [4], South Korea [5], New Zealand [6], and India [7]. GGVA is a monoparticle, single-stranded circular DNA virus belonging to the genus *Maldovirus* in the family *Geminiviridae*. The complete genome of GGVA ranges from 2903 to 2907 nucleotides (nt) in length [8], including two open reading frames (ORFs) on the viral sense strand encoding V1 (coat protein) and V2 (putative movement protein), and four ORFs on the complementary strand encoding C1 (replication-associated protein), C2 (transcriptional activator protein), C3 (replication enhancer), and C4 (host-activated protein) [3].

Infectious clones of viruses are important research tools for the analysis of viral biology and pathogenic mechanisms. Infectious cDNA (DNA) clones of some grapevine viruses [8–18] have already been constructed. By using GGVA infectious clones, the pathogenicity of different GGVA isolates has been analyzed in *Nicotiana benthamiana* [8,16] and grapevine plants [16]. Those authors found that GGVA infection induces dwarfing and upward leaf curling symptoms in *N. benthamiana* plants, whereas grapevine plants agroinfiltrated with GGVA were asymptomatic [16].

Grapevine and other woody perennials are recalcitrant to mechanical inoculation. The main approaches for delivering infectious clones into grapevine plants include vacuum-assisted agroinfiltration [2,9,16] and agro-drenching [10,15,18]. However, these methods are complex and time-consuming, especially for the latter method which requires more than 20 days to establish a viral

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infection. In addition, the two methods result in relatively low survival and infection rates of the treated plants. Therefore, development of an efficient and simple experimental system to launch grapevine infections is needed.

In the present study, we constructed an infectious DNA clone of GGVA isolate QN (GGVA^{QN}), which was isolated from a grapevine 'Queen Nina' plant with severe disease symptoms, including malformation, upward curling of the leaf margins, vein clearing, and reduced leaf size. The biological activity of infectious clone of GGVA^{QN} (pXT-GGVA^{QN}) was analyzed in *N. benthamiana* and grapevine plants. In addition, we provide a simple, time-saving and highly efficient inoculation method to deliver an infectious clone of a virus into woody perennial plants, including grapevine.

2. Results

2.1. Construction of an Infectious Clone of GGVA Isolate QN

The recombinant plasmid pCE3-GGVA^{QN}, containing the full genome of GGVA^{QN}, was used as template. The full genome of GGVA^{QN} (2905 nt) was 100% identical to the isolate we obtained from grapevine 'Marselan' (GenBank accession no. PP397181), and shared 96.58% to 99.70% sequence identity with previous reported isolates.

To construct an infectious clone of GGVA^{QN}, two fragments covering 1.2× viral genome sequence amplified from pCE3-GGVA^{QN} and the linearized vector pXT1 (JN029690) [19] were assembled into circular plasmids (Figure 1), which were then transformed into competent *Escherichia coli* DH5a cells. Positive clones were identified by PCR, enzyme digestion and sequencing. The recombinant plasmid (pXT-GGVA^{QN}) was transformed into *Agrobacterium tumefaciens* GV3101.

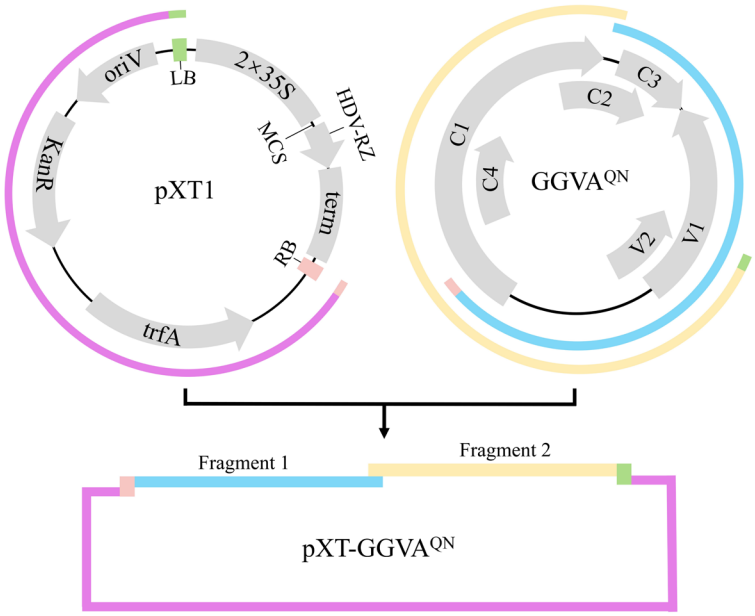

Figure 1. Diagram of the strategy used to build the infectious clone of grapevine geminivirus A isolate QN (pXT-GGVA^{QN}). Fragment 1, fragment 2 and the linearized pXT1 are marked in blue, yellow, and purple, respectively. The 5′ terminus of fragment 1 and the 3′ terminus of fragment 2 separately contain the T-DNA right border (RB) and left border (LB), marked in pink and green, respectively. MCS, multiple cloning site region; oriV, origin of vegetative replication; KanR, kanamycin resistance gene; trfA, trans-acting replication protein that binds to and activates oriV; term: NOS terminator; HDV-RZ, hepatitis delta virus ribozyme; 2×35S, two copies of the 35S promoter region.

2.2. Infectivity of GGVA^{QN} in N. benthamiana Plants

Six-week old *N. benthamiana* plants were agroinfiltrated with the infectious clone of pXT-GGVA^{QN}, and agroinfiltration of the empty vector pXT1 served as a control. At 7 days postinfiltration (dpi), the pXT-GGVA^{QN}-infiltrated leaves exhibited upward curling of the leaf edge, and symptoms

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of upward leaf curling together with chlorotic spots were observed on upper systemically infected leaves 21 dpi, whereas the control plants were asymptomatic (Figure 2a). All 37 *N. benthamiana* plants (10–13 plants, three repeats) were systemically infected with pXT-GGVA^{QN}, as evidenced by PCR and western blot assays showing the presence of GGVA^{QN} in the first systemically infected and upper new developing leaves of all GGVA^{QN}-inoculated plants at 3 and 6 dpi, respectively (Figure 2b). These results indicated that pXT-GGVA^{QN} can successfully infect *N. benthamiana* plants and elicits upward leaf curling and chlorotic spot symptoms.

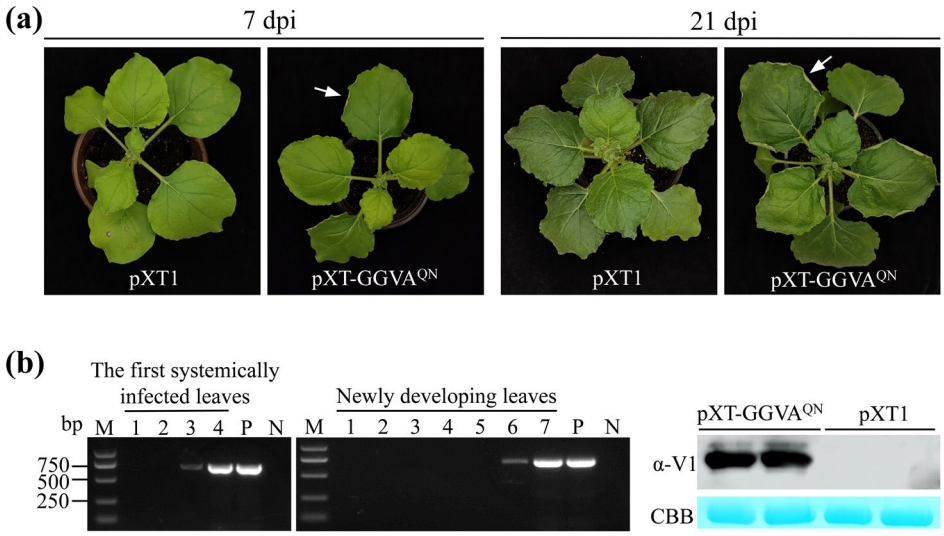

Figure 2. Systemic infection of GGVA^{QN} in *N. benthamiana* plants. (**a**) Symptoms induced by GGVA^{QN}. (**b**) PCR (left and middle panels) and western blot (right panel) assays confirm the presence of GGVA^{QN} in *N. benthamiana* plants. Lanes 1–7: samples collected 1 to 7 dpi, respectively. Upper newly developing leaves collected 6 dpi were used for western blot assay. P and N: positive and negative controls with pXT-GGVA^{QN} and ddH_2O instead of DNA, respectively. CBB, Coomassie brilliant blue staining confirmed equal loading.

2.3. Infectivity of GGVA^{QN} in Grapevine Plants

Before infection assays, the virological condition of the in vitro-grown 'Red Globe' grapevine plantlets, which had tested negative for 19 grapevine viruses, as well as grapevine leafroll-associated virus 4 (GLRaV-4) strains GLRaV-5, GLRaV-6, GLRaV-9, GLRaV-De, and GLRaV-Pr, were resubjected to RT-PCR and PCR assays. They were all free of the targeted viruses (Supplementary Figure S1).

We then used a very simple and efficient method to deliver pXT-GGVA^{QN} into grapevine plants. Roots of each in vitro-grown grapevine plantlet were directly injected with 0.5 mL of *Agrobacterium tumefaciens* cells carrying pXT-GGVA^{QN} by syringe and then transplanted into a pot (Figure 3a). Empty vector pXT1 served as a control. A total of 18 plants were inoculated with GGVA^{QN} in two repeat experiments (9 plants each), and 15 plants survived after transplanting. As shown in Figures 3b and 4a, about 2 month later, 'Red Globe' plants inoculated with GGVA^{QN} showed chlorotic mottling on the upper leaves; symptoms of downward curling and necrosis of the leaf margins, and interveinal yellowing were also observed on the lower leaves. Five months later, the color of interveinal tissues of the lower leaves of a few plants changed from yellow to red. All of these symptoms were absent in the corresponding control 'Red Globe' plants. All 15 surviving plants were systemically infected with GGVA^{QN}, as confirmed by the presence of GGVA^{QN} in the upper leaves detected by PCR and western blot assays 1 month after inoculation (Figure 4b).

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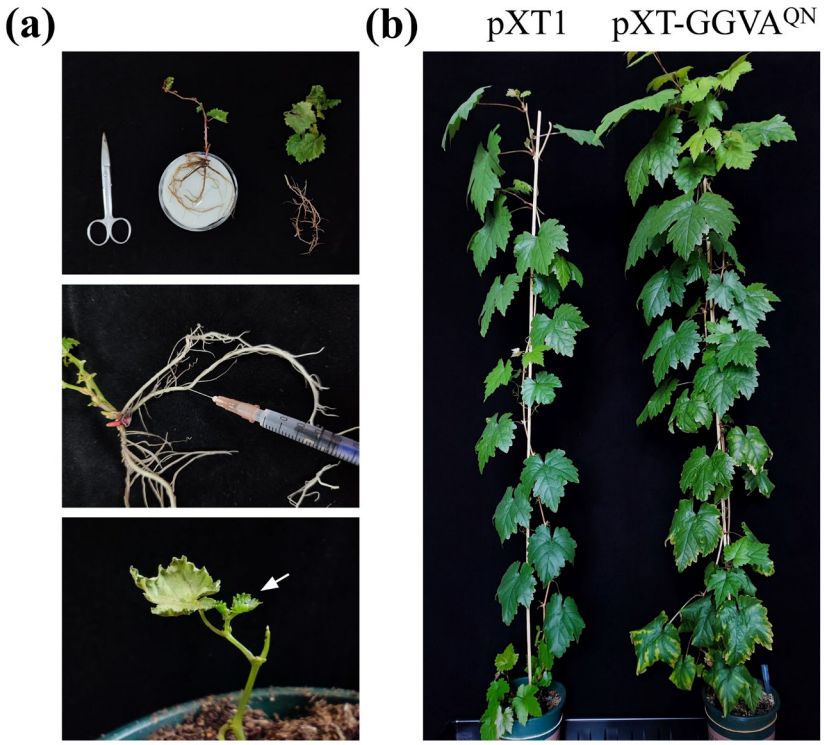

Figure 3. A simple, quick, and efficient method for delivering the infectious clone pXT-GGVA^{QN} into grapevine plants. (**a**) Diagram of the procedure for agroinfiltration. Arrow indicates the sprouting buds after inoculation and transplanting. (**b**) Symptoms induced by GGVA^{QN} in grapevine 'Red Globe' plants 4 months after inoculation and transplanting.

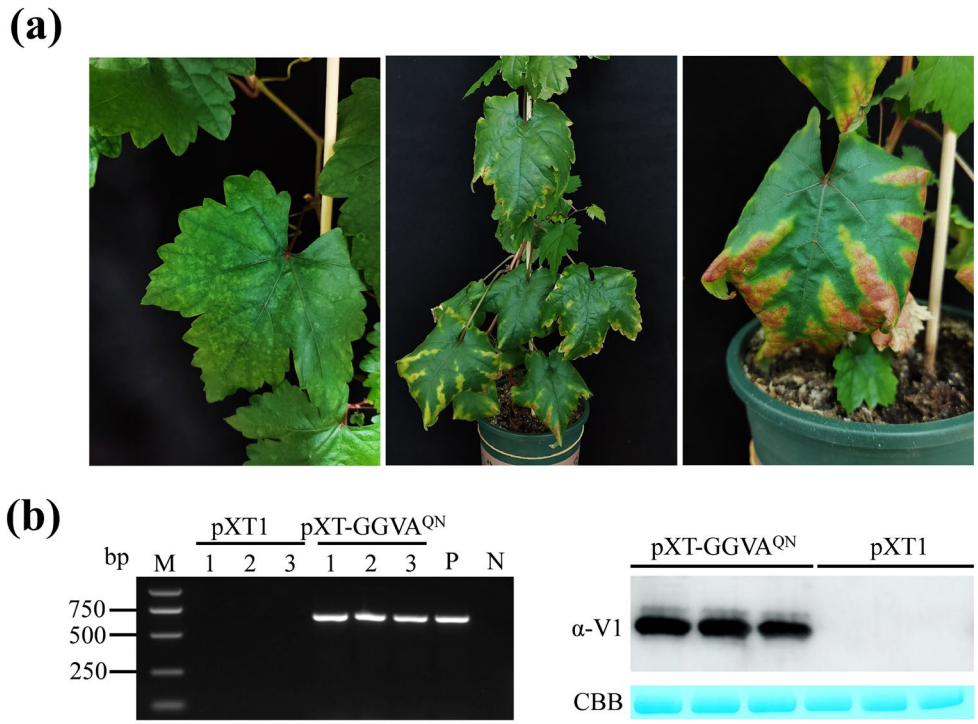

Figure 4. Systemic infection of GGVA^{QN} in grapevine 'Red Globe' plants. (**a**) Symptoms in upper (left panel) and lower (right panels) leaves of grapevine plants inoculated with pXT-GGVA^{QN}. (**b**) Systemic infection by GGVA^{QN} in grapevine 'Red Globe' plants was confirmed by PCR (left panel) and western

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blot (right panel) assays. Samples were collected from three pXT-GGVA^{QN}-inoculated and control plants 1 month after inoculation and transplanting, respectively. P and N: positive and negative controls with pXT-GGVA^{QN} and ddH_2O instead of DNA, respectively. GGVA accumulation was detected with α-V1 antiserum. CBB, Coomassie brilliant blue staining confirmed equal loading.

3. Discussion

GGVA was first found in 2017 in samples from both grapevine 'Nagano purple' plants with leaf chlorotic ringspot symptoms and asymptomatic 'Black Beet' plants [3]. Fan et al. [4] reported that most grapevine samples that were positive for GGVA were asymptomatic, except for a few samples with foliar ringspot symptoms. Similarly, Jo et al. [5] reported the detection of GGVA in all 16 individual grapevine plants representing 12 different cultivars, but only the cultivar Shine Muscat displayed obvious symptoms (leaf malformation, vein clearing, and yellowing). Infectious clones of three GGVA isolates—GGVA-17YM1 [8] and GGVA-76 and GGVA-93 [16]—all induced upward leaf curling and stunting symptoms in *N. benthamiana* plants, but grapevine (cultivars Colombard, Salt Creek, Cabernet Sauvignon, and Vaccarèse) plants infected with GGVA-76 or GGVA-93 did not display any obvious symptoms [16]. Therefore, our knowledge of the pathogenicity of GGVA to grapevine plants remains largely unclear.

In this study, we constructed an infectious clone containing the full genome of GGVA^{QN} to investigate its pathogenicity. *N. benthamiana* plants that were systemically infected with GGVA^{QN} showed symptoms of upward leaf curling and chlorotic mottling (Figure 2a) which were somewhat different from the upward leaf curling and stunting symptoms observed by Sun et al. [8] and Kuo et al. [16]. GGVA^{QN} shares 99.52%, 97.26%, and 98.45% sequence identity with GGVA-17YM1, GGVA-76, and GGVA-93, respectively. The inconsistency may not be due to the sequence differences between GGVA^{QN} and the three GGVA isolates; rather, other factors, such as the different ages of the *N. benthamiana* plants used for inoculation and fertilization, may be responsible.

The pathogenicity of GGVA^{QN} on grapevine was also investigated using in vitro-grown 'Red Globe' rooted plantlets. Upon GGVA^{QN} infection, all 'Red Globe' plants exhibited chlorotic mottling symptoms on their upper leaves, and symptoms of downward curling and necrosis of the leaf margins, and interveinal yellowing on the lower leaves 2 months after inoculation and transplanting (Figures 3b and 4a). In contrast, all control 'Red Globe' plants which were inoculated with empty vector pXT1 were asymptomatic. The in vitro-grown 'Red Globe' rooted plantlets used for infection assays were negative for GGVA, GLRaV-4 and its strains GLRaV-5, GLRaV-6, GLRaV-9, GLRaV-De, and GLRaV-Pr, and another 17 viruses reported in China. Therefore, the symptoms on 'Red Globe' plants were closely associated to GGVA^{QN} infection. Our results differ from those reported by Kuo et al. [16], where neither GGVA-76 nor GGVA-93 induced obvious symptoms on grapevine 'Colombard', 'Salt Creek', 'Cabernet Sauvignon', and 'Vaccarèse' plants, although the virological condition of these plants was unclear. Lovato et al. [12] reported that grapevine plants infiltrated with the infectious clone of grapevine Algerian latent virus exhibited different symptoms in different cultivars. Therefore, the inconsistency may be due to the different grapevine cultivars used in the two studies. Future research investigating the pathogenicity of GGVA^{QN} in different grapevine cultivars is warranted.

One important factor that hinders the research on viruses of woody perennial plants, including grapevine, is the lack of a reliable experimental system for delivering infectious clones. Two main approaches, vacuum-agroinfiltration and agro-drenching, are currently used to deliver infectious clones into grapevine plants. However, they are complex and time-consuming, and the achieved rate of infection is relatively low. In this study, we directly injected *A. tumefaciens* cells carrying the infectious clone pXT-GGVA^{QN} into roots of grapevine plantlets by syringe followed by transplanting the plantlets into pots, and all surviving plants (15 out of 18) were systemically infected with GGVA^{QN}. We also successfully launched the infection of rooted grapevine plantlets with infectious clones of grapevine Pinot gris virus (GPGV) and GLRaV-2 with this method (data not shown). Therefore, we provide a very simple, quick, and efficient method for delivering infectious clones into grapevine plants, with no vacuuming operation, taking only a few minutes to complete the

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infiltration, and resulting in 100% infection rate. To our knowledge, the experimental protocol reported herein is the most effective system for achieving grapevine infection with infectious clones of a virus, potentially facilitating grapevine virology research.

In conclusion, an infectious clone of GGVA^{QN} was constructed and its pathogenicity in *N. benthamiana* and grapevine plants was investigated. The results presented here provide important insights into GGVA pathogenesis, as well as a very simple and highly effective method for launching grapevine infection with the infectious clone of a virus.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Grapevine cv. Red Globe in vitro-grown rooted plantlets used in the infection assays were previously obtained from treatments of thermotherapy combined with meristem tip culture, and kept in our laboratory. The virological condition of these plantlets were investigated three times and were negative for GGVA and other viruses reported in China, including GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4 (including GLRaV-4 strains GLRaV-5, GLRaV-6, and GLRaV-9, GLRaV-De, and GLRaV-Pr), GLRaV-7, GLRaV-13, grapevine rupestris stem pitting-associated virus (GRSPaV), grapevine fleck virus (GFkV), grapevine fanleaf virus (GFLV), grapevine virus A (GVA), GVB, GVE, GPGV, grapevine fabavirus (GFabV), grapevine red blotch virus (GRBV), grapevine Syrah virus-1 (GSyV-1), grapevine berry inner necrosis virus (GINV), and grapevine red globe virus (GRGV).

In vitro-grown rooted grapevine plantlets were grown on woody plant medium supplemented with 30 mg mL^{-1} sucrose and 1 mg mL^{-1} indole-3-butyric acid. *N. benthamiana* plants were grown in individual pots containing commercial soil, and 6-week-old plants were used. All plant materials were grown at 26–31 °C with a 16-h light regime.

4.2. Construction of Infectious Clone of GGVA

The full genome of GGVA^{QN} isolate was PCR amplified from a sample collected from a grapevine 'Queen Nina' plant in Guangxi Province in Southwest China using a TOPO-Blunt Cloning Kit (Vazyme, China) with the primer pair GGVA-1678-F/GGVA-1678-R previously described [8]. The PCR products were cloned into the pCE3 Blunt Vector provided by the kit, and the recombinant plasmid pCE3-GGVA^{QN} was used as the template to construct the infectious clone of GGVA.

Construction of the infectious clone of GGVA^{QN} was performed essentially as described previously [16] with modifications. A fragment (the linearized pXT1 without the sequence of the 35S promoter, multiple cloning site region, ribozyme, and NOS terminator) (Figure 1a) was amplified using the binary vector pXT1 as template with primer pair F27/R27. Two fragments, each covering 0.6× viral genome sequence, were amplified from pCE3-GGVA^{QN} using primer pairs F25/R25 and F26/R26, with F25 and R26 containing a 30-bp sequence homologous to the T-DNA right border (RB) and left border (LB) on pXT1, respectively. The three fragments were assembled into circular plasmids using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA). Positive clones were identified by *Hind* III digestion (resulting in a 7197-bp fragment), and PCR with the primer pairs F25/R25 and F26/R26 for amplifying fragment 1 (1886 bp) and fragment 2 (1934 bp), respectively. Three positive clones were then sequenced to verify the correctness of the GGVA genome sequence. The recombinant plasmid pXT-GGVA^{QN} was delivered into competent *A. tumefaciens* strain GV3101 using the freeze–thawing method.

4.3. Inoculation

A. tumefaciens GV3101 carrying the infectious clone pXT-GGVA^{QN} was cultured to an optical density of 1.0 at 600 nm. *Agrobacterium* cells were harvested by centrifugation and resuspended in the infiltration buffer (10 mM MES/NaOH pH 5.8, 10 mM $MgCl_2$, 150 μM acetosyringone). Agroinfiltration of *N. benthamiana* plants was performed essentially as described previously [20].

For inoculation of in vitro-grown grapevine plantlets, rooted 'Red Globe' plantlets were removed from the culture jar and washed to remove the agar adhering to their base. Plantlets with

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pruned roots and leaves were placed on moist filter paper. A 0.5-mL aliquot of *A. tumefaciens* GV3101 cells carrying pXT-GGVA^{QN} (for each grapevine plantlet) was injected into the main root bark with a syringe, and the plantlet was transplanted in a pot (10 cm diameter) containing commercial soil (Figure 3a). These pots were placed in plates filled with a shallow layer of water. About 3 days after transplanting, new buds were observed, indicating that they had survived. We used 8–13 plants or plantlets for each treatment in each experiment.

All inoculated plants were fertilized once a week with water-soluble fertilizer (Peters Professional).

4.4. RT-PCR and PCR

The virological condition of the in vitro-grown grapevine plantlets was detected by RT-PCR and PCR, and the presence of GGVA in inoculated plants was assessed by PCR.

Primers used in the RT-PCR and PCR assays are listed in Supplementary Table S1. Primer pairs F6/R6, F19/R19, F20/R20, F25/R25, F26/R26 and F27/R27 were designed in this study, and others were described previously [21–33]. RNA and DNA were extracted from *N. benthamiana* leaves, or phloem scrapings of grapevine petioles or leaves of in vitro-grown grapevine plantlets using the CTAB method. Synthesis of cDNA (at 37 °C) was primed with a mixture of random primers using 500 ng total RNA with HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). A 1-μL aliquot of cDNA or DNA was used as a template in a 10 μL PCR, and PCR amplification was conducted as follows: DNA denaturation at 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 52–60 °C (depending on the specific primers used) for 30 s, and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. A negative control (ddH_2O instead of cDNA or DNA) was included in the PCR assays. Positive PCR products were randomly selected for sequencing to confirm their identity.

4.5. Western Blot Analysis

Plant protein extraction and western blot analysis were performed as detailed previously [20]. The dilution of anti-V1 antiserum (noncommercial) used in the western blot analysis was 1:2000. The hybridization signals were detected with High-sig ECL Western Blotting Substrate (Beyotime, China) according to the manufacturer's instructions. Coomassie brilliant blue staining confirmed equal protein contents in each lane.

Author Contributions: Conceptualization, Y.-Q.C., C.L. and S.-Z.Y.; Construction of GGVA clone and inoculation, C.L. and S.-Z.Y.; Virus detection, J.-Y. W. and Y.-S.X.; supplying the plant materials, X.-Q.Z., C.-L.F. and W.-H.Z.; Western blot analysis, H.-W.L.; Symptom observation and photography, C.L., X.-Q.Z., C.-L.F. and W.-H.Z.; writing—original draft preparation, Y.-Q.C.; writing—review and editing, Y.-Q.C., C.L., J.-Y. W. and Y.-S.X. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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