Detection of Two Strains of Grapevine Virus A

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ABSTRACT

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Two isolates of grapevine virus A (GVA), mechanically transmitted from leafroll-affected grapevine cultivars to *Nicotiana benthamiana* and designated SA646 and SA36, were purified from in vitro node cultures of *N. benthamiana* and reinoculated into *N. benthamiana* seedlings. Symptom development differed reproducibly between the two isolates. At 3 wk postinoculation, the *N. benthamiana* inoculated with GVA isolate SA646 showed a systemic dwarfing with vein clearing of the tertiary and smaller veins, whereas those inoculated with isolate SA36 showed a systemic dwarfing with a pronounced interveinal chlorosis. The strains were not serologically distinguishable using either of two polyclonal antisera.

Grapevine virus A (GVA), a member of the subgroup II closteroviruses (7), was isolated once from *Nicotiana clevelandii* A. Gray following mechanical inoculation from a grapevine with stem pitting symptoms (4). Later attempts at mechanical transmission to *N. clevelandii* from grapevine sap failed, but successfull transmission was obtained using mealybugs (11,12). The reproducible mechanical transmission of GVA from grapevine to a herbaceous host has recently been achieved (10). The herbaceous plant used was *N. benthamiana*, and success was attributed to the use of in vitro shoot tip cultures as inoculum.

GVA has been shown serologically to be widespread (7,8) and may be more closely associated with grapevine leafroll (GLR) disease than with stem pitting (5,6,7,12,14). GVA has been partially characterized (1,3,4), but no report has yet been published concerning the existence of strains of this virus. One comparison of two isolates has previously been conducted (3), but in that study both isolates produced similar symptoms in manually inoculated *N. benthamiana*. The objective of this communication is to report, for the first time, the existence of two biologically distinct strains of GVA.

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MATERIALS AND METHODS

Initial isolation of GVA strains. The GVA isolates in this study were from two grapevine cultivars: *Vitis vinifera* L. 'Limberger' and 'Muller-Thurgau.' Both were GLR-affected, corky bark-free, and rupestris stem pitting-free, based on indexing results obtained with the woody indicators *V. vinifera* 'Pinot Noir', LN-33 (Couderc 1613 × Thompson Seedless), and *V. rupestris* 'St. George.' The Muller-Thurgau was known to be infected with grapevine fanleaf virus. Shoot tip cultures of these cultivars were initiated and maintained in vitro using media and procedures described elsewhere (9). GVA was mechanically transmitted from these in vitro shoot tip cultures to *N. benthamiana* (10). Approximately 3 wk after inoculation, the Limberger-inoculated (or Muller-Thurgau-inoculated) *N. benthamiana* were established in vitro as node cultures, using

media and procedures previously published for the culture of *N. tabacum* 'Xanthi-nc' (13). The proliferating *N. benthamiana* node cultures were stored at −80 C when their fresh weight reached about 20 g.

GVA purification and inoculation of purified virus. In vitro node cultures of symptomatic *N. benthamiana*, stored at −80 C in 20-g lots, were used as starting material for virus purification, as they have consistently proved suitable for this purpose in our laboratory. The procedure used for the purification of GVA was a modification of that published by Conti et al (4). Cultures were finely ground in liquid nitrogen and transferred to a blender. Three volumes (w:v) of TM buffer (TM = 0.05 M Tris-HCl, pH 7.8, 0.01 M $MgSO_4$) were added, and the mixture was homogenized for 20 sec. The homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 3,300 g for 10 min in a Sorvall RC-5B centrifuge using a GSA rotor. The supernatant was collected and Triton X-100 was added to 2% (v:v). The mixture was stirred on ice for 45 min. Sodium chloride was then added to 0.2 M, polyethylene glycol (PEG 8000) to 6% (w:v), and 2-mercaptoethanol to 0.2%, and the mixture was stirred on ice for a further 90 min. This mixture was centrifuged at 12,000 g for 20 min in a GSA rotor. The supernatant was discarded and the pellet was resuspended in 10 ml TM buffer. The suspension was centrifuged at 5,000 g for 10 min in a SS 34 rotor, and the supernatant was collected. Three milliliters of supernatant was placed on top of a step gradient consisting of 4 ml each of 20, 30, 40, and 50% sucrose in TM buffer. The gradient had been prepared the day before and kept at 8 C overnight. The gradients were centrifuged at 65,000 g for 3.5 hr in a Beckman Model L ultracentrifuge using a SW25.1 rotor. A broad virus-containing band was then located using bottom illumination and withdrawn using a needle and syringe. The virus suspension was diluted fourfold with 0.05 M phosphate buffer, pH 7.0, and the virus was pelleted by centrifugation at 65,000 g for 4 hr in a Beckman type 30 rotor. The virus pellet was resuspended in 3.5 ml of 0.1 M Tris-HCl, pH 7.2, 0.01 M $MgCl_2$; 0.53 g of cesium sulfate was then added and dissolved by agitating on a vortex mixer. This preparation was then layered onto a 1-ml pad of 53% cesium sulfate in 0.1 M Tris-HCl, pH 8.2, 0.01 M $MgCl_2$, and centrifuged at 140,000 g for 18 hr in a Beckman SW 50.1 rotor. The virus band was located using bottom illumination and recovered with a needle and syringe. The purified virus was dialyzed overnight against 2 L of TM buffer and pelleted through a 1-ml cushion of 20% sucrose in TM buffer by centrifugation for 4 hr at 92,000 g in a SW 50.1 rotor. The pellet was resuspended in 0.02 M phosphate buffer, pH 7.2. The isolate from the Limberger-inoculated *N. benthamiana* was designated SA646, and that from the Muller-Thurgau-inoculated *N. benthamiana* was designated SA36.

Purified virus from each isolate was resuspended in 0.01 M potassium phosphate buffer containing 2.5% nicotine, final pH 9.5 (2), and was inoculated into three seedlings of *N. benthamiana* at the six-leaf stage. Control plants were inoculated with buffer only. Plants were maintained in the greenhouse and monitored daily for symptom development.

Purified preparations of SA646 and SA36 were also resuspended in 0.02 M phosphate buffer, pH 7.2, so that their A_{260} had a value of 0.295. Further dilutions (10^{-1} and 10^{-2}) of these suspensions were then prepared using the same buffer. Each virus at each of the three concentrations was then diluted 1:1 with nicotine-containing phosphate buffer and inoculated into four *N. benthamiana* seedlings. Control plants were inoculated with buffer only.

Antiserum preparation and immunosorbent electron microscopy (ISEM). A rabbit was inoculated subcutaneously with 0.5–0.8 ml of a 1:1 emulsion of purified SA646:Freund's complete adjuvant on July 12 and August 4, 11, and 18. It was bled 1 wk after the final injection. ISEM similar to that described elsewhere (15) was used to identify GVA, using this antiserum and anti-GVA rabbit serum generously provided by D. J. Engelbrecht. Extracts for this procedure were prepared by grinding selected leaves from systemically infected *N. benthamiana* in a mortar with 10 volumes (w:v) of 0.06 M potassium phosphate buffer, pH 7.0 (6-PB). Antiserum coating of grids (backed with Formvar-carbon films) and subsequent virus particle trapping and decoration on appropriate droplets of GVA antiserum (dilution 1:1,000 and 1:100 in 6-PB) or extract were for 3, 1, and 0.5 hr, respectively. Grids were washed with a stream of buffer (about 1 ml) between treatments and in a stream of distilled water after the last step, then stained with 2% uranyl acetate. Grids were examined with a JEOL JEM-100C electron microscope.

Particle length determinations. The instrument was calibrated using a JBS #401A grating replica (J. B. EM Services Inc., Quebec). Virus was trapped on anti-GVA serum-sensitized grids. Fifty-two and 67 particles from leaf homogenates of SA646- and SA36-infected *N. benthamiana*, respectively, were measured; 59 and 46 particles from purified preparations of SA646 and SA36, respectively, were also measured. For normal length calculations, all particles belonging to size classes (20 nm increments) between 600 and 860 nm were included. This corresponded to 96, 97, 95, and 74% of the particles from the SA646 leaf homogenate, SA36 leaf homogenate, SA646 purified preparation, and SA36 purified preparation, respectively. The normal length of the particles in each preparation was calculated using the formula: normal length = $\Sigma\ (f_i x_i)/\Sigma f_i$, where x_i is the midpoint of the size class and f_i is the number of particles within that class.

RESULTS AND DISCUSSION

The purified SA646 and SA36 fractions contained only GVA, based on electron microscopic examination of "dips" and ISEM. Symptom development in the SA646-inoculated *N. benthamiana* differed from that in the SA36-inoculated *N. benthamiana* as follows. At 9–12 days postinoculation, a systemic interveinal chlorotic flecking was observed on the leaves of the SA36-inoculated plants, whereas no symptom was apparent on the SA646-inoculated plants. At 3 wk postinoculation, the SA36-inoculated *N. benthamiana* showed a systemic dwarfing and a very pronounced interveinal chlorosis, whereas the SA646-inoculated *N. benthamiana* showed a systemic dwarfing with vein clearing of the tertiary and smaller veins (Fig. 1). The difference in symptom development between the SA646- and the SA36-inoculated *N. benthamiana* was most evident at this

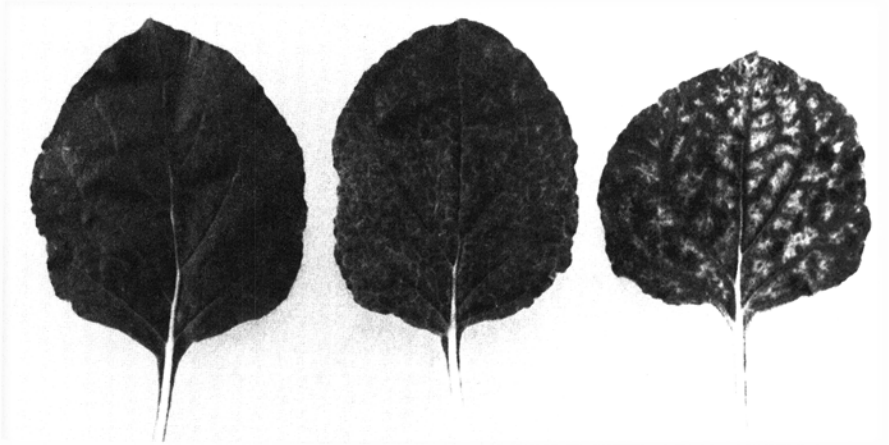

Fig. 1. Leaves from *Nicotiana benthamiana* plants inoculated with (L to R) buffer only, purified SA646 GVA, and purified SA36 GVA.

stage and persisted for another 2 wk. At 5–6 wk postinoculation, the difference in the symptoms induced by the two isolates was minimal. By this time, the SA646-inoculated *N. benthamiana* also showed an interveinal chlorosis, and seedlings were senescent. Inoculation of *N. benthamiana* seedlings with purified GVA from each isolate was repeated twice more, with essentially identical results. No symptoms developed in the *N. benthamiana* inoculated with buffer only.

No viruslike particles were detected by ISEM in the leaves of the buffer-inoculated *N. benthamiana*. Based on "decoration" tests, only GVA was observed in leaf dip preparations from symptomatic leaves of *N. benthamiana* that had been inoculated with either SA646 or SA36. No grapevine fanleaf virus was detected by ELISA in the purified preparations of SA36 or SA646 or in the *N. benthamiana* inoculated with these preparations. SA646 and SA36 thus appear to represent two strains of GVA, SA36 being more virulent than SA646. A purified preparation of SA36 virus with an A_{260} value of 0.295 produced symptoms on four out of four inoculated *N. benthamiana* plants within 15 days postinoculation. A 10^{-1} dilution of this preparation produced symptoms on three out of four inoculated plants, also within 15 days. With both of these SA36 virus concentrations, the symptoms consisted of interveinal chlorosis. With a 10^{-2} dilution, only one out of four inoculated plants developed symptoms. These appeared only after 22 days postinoculation and consisted of both vein clearing and interveinal chlorosis. No disease symptoms were observed on the *N. benthamiana* inoculated with SA646 at an A_{260} value of 0.295 or with 10^{-1} or 10^{-2} dilutions thereof. The observation that SA36 virus at a high dilution produced both vein clearing and interveinal chlorosis indicates that the type of symptom induced by GVA on *N. benthamiana* is partly dependent on the virus concentration. The observation that the two isolates (SA36 and SA646) at the same A_{260} value of 0.295 elicited different responses in *N. benthamiana*, indicates that the preparations possessed different infectivity. As both isolates were prepared by the same method, and as they were both diluted in the same buffer to the same absorbance value, the difference in infectivity of the two preparations is presumably a consequence of SA36 being a more virulent strain than SA646.

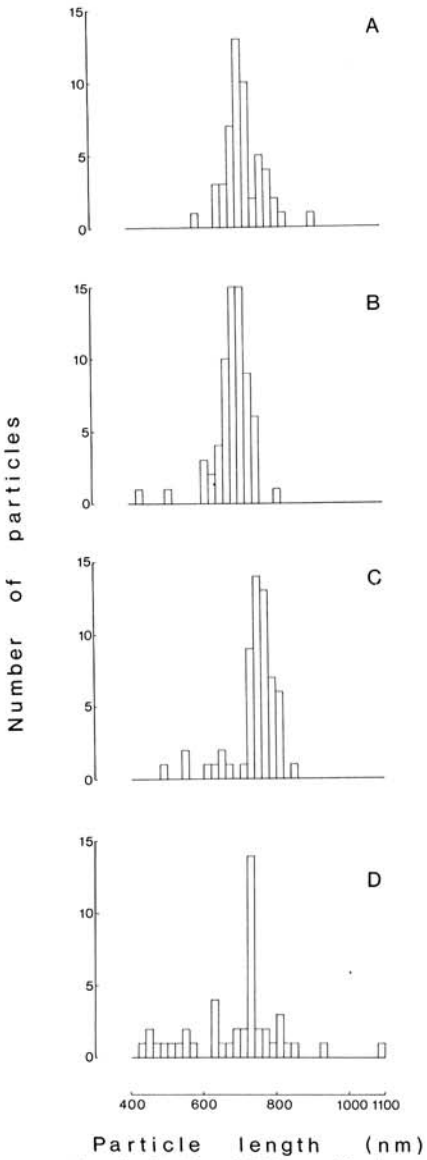

Fig. 2. Size distribution of GVA particles from leaf homogenates of *N. benthamiana* inoculated with purified SA646 (**A**) and SA36 (**B**) and from purified preparations of SA646 (**C**) and SA36 (**D**).

A difference was observed between SA646 and SA36 in the normal length of the particles, when measured in leaf dip preparations or when measured in the purified fractions (Fig. 2). In leaf dip preparations, the normal length of SA646 was 725 nm and that of SA36 was 696 nm. In the purified fractions, the normal length of SA646 was 754 nm and that of SA36 was 729 nm. Published values for the length of GVA are close to 800 nm (4,8,12). The values reported here for the normal length of GVA were clearly lower than those reported to date. This may simply reflect differences in measurement techniques. Although it was interesting to note that the SA36 particles were shorter than the SA646 particles in both sets of measurements taken, the differences were small and are probably not significant. The pitch of purified SA646 and SA36 GVA was 3.6–3.7, which is within the range (3.6–4.0) of published values (4). No noticeable difference was found between SA646 and SA36 in the degree of decoration obtained in ISEM, using either rabbit polyclonal antiserum prepared against purified SA646 or the rabbit anti-GVA serum obtained from D. J. Engelbrecht.

To the best of our knowledge, only one comparison of GVA isolates has been reported to date (3). One of the isolates in that study was that initially reported by Conti et al (4), and the other had been transmitted from a leafroll- and stem pitting-affected grapevine to *N. clevelandii* using mealybugs. Both of those isolates were cultured in *N. clevelandii* and were transmitted by manual inoculation to *N. benthamiana*, where they induced similar symptoms. In the study reported here, the purified GVA isolates induced reproducibly different symptom development in *N. benthamiana*.

Both GVA isolates used in this study were from leafroll-affected grapevines. This observation is consistent with the possibility that GVA may be involved, at least in some instances, in the etiology of GLR, but definitive evidence on this point is still lacking.

ACKNOWLEDGMENT

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