

Time-Course Studies on Virus Titer and DNA Component Ratio in Beans Infected with Bean Golden Mosaic Virus

Teresa L. Shock and Robert M. Goodman

Assistant plant pathologist (virology) and associate professor, respectively, Department of Plant Pathology and International Soybean Program (INTSOY), University of Illinois, Urbana 61801.

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ABSTRACT

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Systemically infected trifoliolate leaves of beans (*Phaseolus vulgaris* L. 'Top Crop') harvested at varying times after sap inoculation of primary leaves with bean golden mosaic virus (BGMV) yielded a maximum titer of 8 μg virus per gram (fresh weight) of tissue. The concentration of virus increased from the time when leaves were just beginning to develop symptoms (5-6 days after inoculation) to a peak (8-12 days after

inoculation) and then decreased rapidly. Virus titer in older leaves (> 3 wk after inoculation) with well developed symptoms was very low. The relative amounts of the two topological forms (linear and circular) of DNA present in BGMV were measured during the course of infection. The ratios obtained were between 1:3 and 1:5 (linear:circular). No variation was observed in this ratio with time of harvest after inoculation or with age of infected tissue.

Bean golden mosaic virus (BGMV) is a geminivirus (8,16) transmitted in nature by the tropical whitefly, *Bemisia tabaci* (9). The viral etiology of bean golden mosaic (2) or mosaico dorado (7), was established only in the 1970s (6,7) after the sap transmissibility of the agent was demonstrated (6,10,17). Sap transmissibility was for BGMV, and remains for other geminiviruses, an elusive property (8). Characterization of geminiviruses, their use in screening for disease resistance, and studies on the etiology of other important diseases possibly caused by geminiviruses would be aided by a better understanding of the details of virus infection.

Unlike the luteoviruses, which have never been successfully transmitted by sap inoculation, geminiviruses are not exclusively limited to conducting elements, or cells destined to become conducting elements, of the phloem (8). BGMV, beet curly top virus, and Euphorbia mosaic virus are found also in phloem-associated parenchymatous cells (3-5, 14,15) and two leafhopper-transmitted geminiviruses (maize streak virus and chloris striate mosaic virus) are found in all tissues of the leaf except the xylem vessels and the epidermis (1,12). This consideration suggests that, while tissue specificity might account for difficulties in sap transmission of some geminiviruses, for others (such as BGMV) other factors may be involved. We thus wanted to analyze the time-course of BGMV titer in systemically infected leaves of inoculated plants for clues as to why sap transmission is so difficult from leaves in the chronic stage of the disease, as well as to identify the time when virus concentration is highest. Because no sensitive infectivity assay is available for BGMV, we were limited to physical measurements of virus concentration.

Another interesting and as yet unresolved aspect of BGMV is the relationship between the geminate morphology of the virus particle and the structure of the viral genome. The single-stranded DNA of BGMV contains two components that can be resolved by rate-zonal sedimentation or polyacrylamide gel electrophoresis under denaturing conditions; the components were found to be a circular molecule and a linear molecule slightly shorter than the length of the DNA in the circle (18). A similar situation is found for maize streak and cassava latent viruses (11). It is not yet known definitely whether these viruses have a multipartite genome as was first

suggested for maize streak virus (1); the linear DNA molecules clearly could arise from limited endonuclease digestion of the circular molecules (11), but the nearly unit length of the BGMV linear DNA molecule could as well be accounted for by the packaging of an incomplete viral DNA replication product or by the multipartite genome hypothesis. We have therefore investigated the DNA component ratio during the course of BGMV infection as a means of searching for clues as to the possible biological significance of the two BGMV DNA species.

MATERIALS AND METHODS

Expanding primary leaves of 9- to 10-day-old bean plants (*Phaseolus vulgaris* L. 'Top Crop') were inoculated with BGMV and placed in a 2-m² EGC growth chamber (Environmental Growth Chambers, Chagrin Falls, OH 44022) held at the dew point. Light was provided for 16 hr daily from four 40-W fluorescent (Cool-white, General Electric Co.) and eight 60-W incandescent bulbs. Temperature was maintained at 32 C.

Samples were taken from the first systemically infected trifoliolate leaves at various times after inoculation of primary leaves and stored at -80 C. Our observations over the past 2 yr indicate the length of storage at -80 C does not affect either virus yield or the ratio of the two DNA species. In one experiment, additional samples were taken 17 days after inoculation from the uppermost expanded trifoliolate leaves to determine whether such leaves, which show the severe chronic symptoms of the disease, contained virus. Several representative leaflets were also taken with each sampling for analysis of dry weight.

Virus was purified from all samples by the method described previously (10) except that the final clarification step before density gradient centrifugation was omitted. Sucrose density gradients were run in a Beckman SW 27 rotor at 25,000 rpm for 4 hr and scanned with an ISCO density gradient fractionator. The amount of virus present in sucrose density gradient fractions was estimated by integrating to obtain the area (A) beneath the scan curve according to Simpson's Rule:

$$A = (h/3) (y_0 + 4y_1 + 2y_2 + 4y_3 + \dots + 2y_{n-2} + 4y_{n-1} + y_n)$$

in which h = volume, and y = absorption at 254 nm.

Virus fractions from sucrose density gradients were concentrated

by ultracentrifugation (Beckman 40 rotor, 35,000 rpm, 2.5 hr). The virus was dissolved in thrice distilled water and its DNA was extracted by adjusting to 0.3 N NaOH, and incubating the mixture for 30 min at room temperature. The mixture was then layered on linear alkaline sucrose density gradients (5–20% sucrose) containing 0.3 N NaOH, 0.7 M NaCl, and 1 mM EDTA. Gradients were run at 20 C, either for 20 hr at 25,000 rpm in a Beckman SW 27.1 rotor, or for 15 hr at 35,000 rpm in a Beckman SW 41 Ti rotor. Gradients were scanned and the amount of DNA was estimated as described above for virus.

DNA samples from sucrose density gradients were dialyzed against thrice-distilled water, concentrated by freeze-drying, and the DNA fractions were analyzed by electrophoresis as previously described (18). Relative quantities of the two DNA species, hereafter referred to as linear to circular DNA species ratio, were calculated.

RESULTS AND DISCUSSION

Bean seedlings inoculated with BGMV on their expanding primary leaves (9–12 days after seeding, depending on season) and subsequently held at 32 C, exhibited symptoms on the first set of emerging trifoliolate leaves (first leaves) 5 days after inoculation. The amount of virus present in these systemically infected leaves rose to a maximum of between 5 and 8 $\mu\text{g/g}$ tissue (fresh weight) 8–12 days after inoculation (Fig. 1). The leaf water content was determined in all experiments by measuring the ratio of dry weight to fresh weight; this value was consistently between 0.10 and 0.12. The rapid and early rise in virus titer was in all cases followed by a similarly rapid decline in titer so that by 16 days after inoculation virus titer was often less than that observed on day 5 or 6 when symptoms were first apparent. The symptoms induced in trifoliolate leaves that developed after the first one, which was the one used for the experiments reported in Fig. 1, were invariably more severe than those induced on the first leaf. The uppermost of these leaves, harvested 17 days after inoculation at a stage of leaf development corresponding to that of first leaves at 8–10 days after inoculation, contained approximately 1 μg virus per gram of tissue. Thus, the virus concentration in young leaves at the chronic stage of infection appeared not to rise to the levels found in first leaves. The virus concentration in chronic leaves 17 days after inoculation was similar to that found in first leaves on that day and considerably lower than the concentration in first leaves at the corresponding stage of leaf development.

The ratio of micrograms of DNA recovered to micrograms of virus originally collected was typically 18–20%. This represents DNA recovery of nearly 100% based on chemical and biophysical estimates of BGMV DNA content (S. Haber and R. M. Goodman, unpublished). The proportion of linear DNA as a percentage of total DNA varied from 17 to 26%. These values are in the same range as similar data accumulated over the past 2 yr in other experiments in which BGMV DNA was purified for other purposes. No time-course-related trends were apparent in comparisons of ratios from younger vs older infected tissue (Table 1). The proportion of linear DNA from virus obtained from later developing leaves harvested at day 17 was 22%.

The viral titer curve indicated by results of our experiments confirms and, in part, may explain results of earlier attempts to transmit BGMV by sap inoculation. Inoculation results in fewer or no infected plants if first trifoliolate leaves are allowed to develop symptoms for ≥ 2 wk before they are used to prepare inoculum. The most striking symptoms appear on trifoliolate leaves that develop 3–6 wk after inoculation and from such leaves sap transmission efficiency is low or nil. Our results suggest that virus titer is low in leaves with well developed symptoms. In an earlier study, we noted that BGMV particles are most obvious in tissues examined by electron microscopy 8–12 days after inoculation; virus particles are either reduced in number or dispersed and thus hard to see in older tissues (15). In the absence of an infectivity assay, we are unable to determine whether the decrease in sap transmissibility from tissues harvested at later stages of infection is due to the apparent reduction in the absolute number of virus particles

present in the tissues or to changes in the specific infectivity of the virus or decreased efficiency of virus extraction from such tissues.

The absence of time-course-related changes in the relative proportions of circular and linear DNA components implies that the linear DNA is either present in the virus particles or generated in a consistent fashion by the method used to extract DNA from the particles. The consistency of these ratios does not aid in choosing between these possibilities, but does appear to rule out hypothetical mechanisms involving such factors as leaf age or time of inoculation.

The consistent proportion of linear to circular DNA components (20% average) suggests several possibilities. The linear form may be an artifact derived from breakage of the circular DNA in a reproducible way during purification. Alternatively, the linear molecule may be a necessary portion of the genome which, together with the circular form, constitutes the minimal infective genome. The unequal proportions of circular and linear components might in this case be analogous to the situation with multicomponent RNA plant viruses with unequal molar ratios of nucleic acid components (13). A third hypothesis is that the linear molecule may be the

TABLE 1. Effect of time after inoculation on the relative amount of linear bean golden mosaic virus DNA

Days after inoculation	Linear DNA (% of total DNA) ^a		
	Expt. 1	Expt. 2	Expt. 3
5	...	17	...
6	24
7	...	18	...
8	24	...	18
12	22	18	...
16	19	26	...

^aData are for DNA extracted from virus purified from systemically infected trifoliolate bean leaves. Plants were inoculated on their primary leaves. Circular and linear DNA were resolved and relative amounts measured in 2.5% polyacrylamide gels in 8 M urea as described in the text. Data given are from three separate experiments.

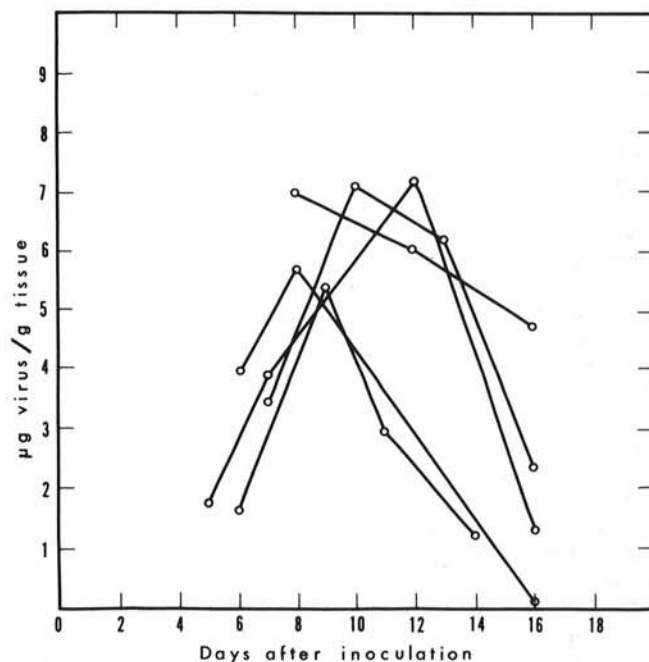


Fig. 1. Titer of bean golden mosaic virus (BGMV) in systemically-invaded trifoliolate bean leaves as a function of time after sap inoculation of primary leaves. Results shown are from five separate experiments and are based on tissue fresh weights. The first trifoliolate leaf to develop after primary-leaf-stage inoculation was harvested. Analysis was by UV absorption (254 nm) following sucrose density gradient centrifugation of partially purified preparations. All samples from each experiment were purified at the same time. Samples were stored at least 1 day at -80 C before purification.

immediate precursor of the circular molecule in the DNA replication cycle and may be packaged by mistake during virion assembly. If the last hypothesis is the correct explanation, it implies that DNA replication and virion assembly are closely linked and that circularity of the DNA is not a prerequisite of virion assembly. Our continuing investigation of the mechanism of BGMV replication is designed to test these hypotheses.

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