Double-stranded DNA of Soybean Chlorotic Mottle Virus

Tadaaki HIBI*, Mitsuro IWAKI**, Yasuo SAITO**,
Jan VERVER*** and Rob GOLDBACH***

Abstract

The nucleic acid of soybean chlorotic mottle virus (SoyCMV) was isolated and analysed. CsCl equilibrium density gradient centrifugation and thermal denaturation kinetics revealed that the nucleic acid is a double-stranded DNA. Electrophoresis in agarose gels separated SoyCMV DNA into multiple bands, similar to those of cauliflower mosaic virus (CaMV) DNA, which indicated that this DNA consisted of both linear and circular molecules. The existence of these different topological forms was confirmed by electron microscopy. Using six different restriction endonucleases a physical map was constructed which showed the SoyCMV genome to be a circular DNA molecule of approximately 8,200 base pairs long. The results obtained support the previous assignment of SoyCMV to the caulimovirus group.

(Received June 3, 1986)

Key words: SoyCMV, caulimovirus, DNA, physical mapping.

Introduction

Recently, a new disease of soybean, named soybean chlorotic mottle, has been detected in Japan⁷⁾. The pathogen was found to be a virus, denoted soybean chlorotic mottle virus (SoyCMV), which consisted of spherical particles with a diameter of approximately 50 nm and which could be transmitted by mechanical inoculation, but not by aphids, to four different *Leguminosae* species. Characteristic symptoms included chlorotic mottling and stunting of the plants.

Although SoyCMV is serologically not related to cauliflower mosaic virus (CaMV) or carnation etched ring virus (CERV), the virus is considered to be a new member of the caulimovirus group on the basis of its particle structure and the formation of cytoplasmic inclusion bodies⁷⁾. By analytical ultracentrifugation, thermal denaturation kinetics, electron microscopy and electrophoretic analysis, we now demonstrate that SoyCMV contains a circular, double-stranded DNA. In addition, we have determined the cleavage sites for six different restriction endonucleases. The resulting physical map confirms that SoyCMV DNA is circular, with a size of approximately 8,200 base pairs.

*Laboratory of Applied Microbiology, National Institute of Agrobiological Resources, Tsukuba Science City, Ibaraki 305, Japan 農林水産省農業生物資源研究所

** Division of Microbiology, National Institute of Agro-Environmental Sciences, Tsukuba Science City, Ibaraki 305, Japan (Y. Saito: Present address; Food and Fertilizer Technology Center for the Asian and Pacific Region, Agriculture Building, 14 Wen Chow Street, Taipei, Taiwan) 農林水産省農業環境技術研究所

*** Department of Molecular Biology, Agricultural University, De Dreijen 11, 6703 BC Wageningen, The Netherlands.

Materials and Methods

Preparation of virus and extraction of virus nucleic acid. SoyCMV was propagated in bean leaves (*Phaseolus vulgaris* cv. Kintoki). 20 days after mechanical inoculation the virus was purified from leaf material according to the procedure for CaMV⁵⁾ and modified as described previously⁷⁾. Nucleic acid was extracted from purified virus by the method for CaMV DNA¹²⁾, except that 1 mg/ml of proteinase K instead of pronase was used in the presence of 1% SDS. CaMV DNA, used as reference, was similarly prepared from purified CaMV.

Equilibrium density gradient centrifugation. Equilibrium density gradient centrifugation was performed according to the method of Vinograd and Hearst¹⁴⁾. Nucleic acid (final concentration, about 0.7 μ g/ml) was mixed with CsCl (initial density, 1.704 g/cm³) in 0.01 M Tris-HCl buffer, pH 8.5, containing 1 mM EDTA and centrifuged for 48 hr at 44,000 rpm and 25 C using MSE Centriscan 75 analytical ultracentrifuge equipped with ultraviolet optics.

Thermal denaturation. Thermal denaturation kinetics of SoyCMV DNA in 1 $\times$ SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0) was followed by determining the rate of increase in absorbance at 260 nm in a thermal block of a Gilford 2400 S spectrophotometer equipped with a Gilford 2527 thermoprogrammer set at a heating rate of 0.50 C/min from 30 to 100 C.

Electron microscopy of nucleic acid. SoyCMV DNA was prepared for electron microscopy by the benzyldimethylalkylammonium chloride (BAC)-droplet method¹⁵⁾ from a solution containing 0.4 μ g/ml of DNA, 2 $\times$ 10⁻⁴% BAC, 40% formamide and 12 mM tri-ethanolamine, pH 7.4, stained with 1 mM uranyl acetate and shadowed with platinum-palladium. The specimens were examined in a Hitachi H 500 electron microscope and photographed at a magnification of 14,100 $\times$. Phage ϕ X 174 RF-DNA was used as an internal standard. Negatives were enlarged 9 times and molecular lengths were measured with a Numonics electronic graphics calculator.

Infectivity assay of nucleic acid. Aliquots of SoyCMV DNA solutions in 1 $\times$ SSC were mechanically inoculated on each of both primary leaves (100 μ l per leaf) of five bean seedlings at concentrations of 4, 20 or 100 μ g/ml, respectively, by rubbing the leaves, previously dusted with Carborundum, with a cotton piece soaked in inoculum. The inoculated plants were grown in a glasshouse and development of symptoms were examined 47 days after inoculation.

Restriction endonuclease treatment and gel electrophoresis. Restriction endonucleases, *Sac*I and *Sal*I (from Boehringer Mannheim GmbH) and *Acc*I, *Bgl*II, *Pvu*II and *Sca*I (from New England Biolabs) were used according to the manufacturer's instructions. Restriction endonuclease digests or untreated DNA were analysed in horizontal agarose slab gels in Tris-acetate buffer (40 mM Tris-HCl, 1 mM EDTA, 5 mM sodium acetate, pH 7.8) or, alternatively, in Tris-borate buffer (89 mM Tris, 89 mM borate, 2.5 mM EDTA, pH 8.3) under submarine conditions. *Hind*III and *Eco*RI endonuclease fragments of *E. coli* λ phage DNA were used as molecular weight markers. DNA bands were visualised by staining in ethidium bromide.

Results

Buoyant density and thermal denaturation kinetics of SoyCMV DNA

The buoyant density of SoyCMV nucleic acid in CsCl was estimated to be 1.688 g/cm³ (Fig. 1), indicating it to be double-stranded DNA rather than RNA. From this density a GC content of 28.0% can be deduced²⁾. Upon thermal denaturation in 1×SSC SoyCMV nucleic acid showed a sharp melting transition with a hyperchromicity of 38.4

Fig. 1. Optical density profile of SoyCMV DNA upon equilibrium density gradient centrifugation in CsCl. The DNA was mixed with CsCl in 0.01 M Tris-HCl buffer (pH 8.5) containing 1 mM EDTA to produce a solution of density 1.704 g/cm³. The sample was centrifuged at 44,000 rpm for 48 hrs at 25 C in an analytical ultracentrifuge and the optical density profile was measured at 260 nm.

Fig. 2. Thermal denaturation kinetics of SoyCMV DNA. Approximately 70 μ g/ml of DNA was suspended in 1 $\times$ SSC and the increase in absorbance at 260 nm was followed at a heating rate of 0.50 C per minute.

% (Fig. 2) indicating once more that the SoyCMV genome consisted of double-stranded DNA. The DNA melted at a T_m of 81.6 C, corresponding to a GC content of 30.0%⁹, a value which agrees rather well with the estimate from the CsCl centrifugation. Under the same conditions (1×SSC), the T_m and GC content of CaMV DNA have been estimated to be 87.2 C and 43.5%, respectively¹².

Gel electrophoresis of native DNA.

Electrophoresis of SoyCMV DNA preparations in 1% agarose gels resulted in the resolution of several distinct bands, similar and almost comigrating with those of CaMV DNA (Fig. 3 and 5). It has been reported that the multiple bands of CaMV DNA represent different topological forms and that under similar electrophoretic conditions as used here the fastest migrating band contained linear molecules while the others contained circular molecules⁶. This, together with the migration of bands formed by DNA cut at a single site by restriction endonucleases (see below), indicates that the fastest migrating band of SoyCMV DNA also contains linear molecules, with an approximate size of 8,200 base pairs, while the slower migrating bands represent circular forms.

Electron microscopy of SoyCMV DNA

Electron microscopical analysis of native, unfractionated SoyCMV DNA confirmed the

Fig. 3. Electrophoresis in a 1 % agarose gel of native DNA of SoyCMV (lane 1) and CaMV (lane 2), using Tris-borate buffer.

Fig. 4. Electron micrographs of linear (a), relaxed open circular (b and c) and twisted circular (d and e) molecules of SoyCMV DNA. The bar represents 200 nm.

presence of both linear and circular molecules (Fig. 4), similar as reported for CaMV DNA preparations^{5,11,13}). The circular molecules predominated over the linear ones and were composed of many highly twisted molecules and a few relaxed open circular forms. Although the length of the molecules prepared with BAC as spreading carrier were slightly shorter than those prepared with cytochrome C, the approximate size of relaxed open circular molecules and linear molecules was for both 8 kilobase pairs (kbp) when roughly calculated on the ratio of the mean contour length of each ten molecules to those of ϕ X174 RF-DNA marker molecules.

Infectivity of SoyCMV DNA

Purified SoyCMV DNA was tested for its infectivity on bean plants, *Phaseolus vulgaris* cv. Kintoki, a local lesion host of the virus (Table 1). The infectivity of SoyCMV DNA appeared significantly lower than that of isolated CaMV DNA¹²⁾ and to be almost the same as that of isolated CERV DNA⁸⁾.

Table 1. Infectivity of extracted SoyCMV DNA^{a)}

Conc. of SoyCMV DNA (μ g/ml)	No. of infected plants / No. of inoculated plants
4	0/5
20	1/5
100	2/5

a) Quantities of 100 μ l of SoyCMV DNA solutions were inoculated on each of both primary leaves of five different bean plants (*Phaseolus vulgaris*), and development of symptoms was examined 47 days after inoculation.

Table 2. Fragment sizes of the cleavage products of SoyCMV DNA by six different restriction endonucleases

Enzymes			Fragments (kbp)				
1	2	3					
<i>Sac</i> I	—	—	8.2				
<i>Sal</i> I	—	—	8.2				
<i>Sac</i> I	<i>Sal</i> I	—	7.6	0.6			
<i>Sal</i> I	<i>Sac</i> I	—	7.6	0.6			
<i>Sac</i> I	<i>Pvu</i> II	—	6.4	1.1	0.65		
<i>Sal</i> I	<i>Pvu</i> II	—	6.4	1.2	0.55		
<i>Sac</i> I	<i>Sca</i> I	—	4.9	2.9	0.4		
<i>Sal</i> I	<i>Sca</i> I	—	4.3	2.9	1.0		
<i>Sac</i> I	<i>Acc</i> I	—	3.1	3.3	1.2	0.6	
<i>Sal</i> I	<i>Acc</i> I	—	3.7	3.3	1.2		
<i>Sal</i> I	<i>Acc</i> I	<i>Sca</i> I	3.0	2.7	1.2	1.0	0.3
<i>Sac</i> I	<i>Bgl</i> II	—	4.4	3.3	0.45		
<i>Sal</i> I	<i>Bgl</i> II	—	5.0	2.7	0.45		
<i>Sac</i> I	<i>Sal</i> I	<i>Bgl</i> II	4.4	2.7	0.6	0.45	

Construction of a physical map of SoyCMV DNA

To confirm the circularity of the SoyCMV genome and to obtain a reliable estimate of its size a restriction endonuclease map of SoyCMV DNA was constructed. The products of restriction endonuclease digestion of SoyCMV DNA are listed in Table 2. Two single cutting enzymes were obtained, *i.e.* *Sac*I and *Sal*I. As shown in Fig. 5 each of

Fig. 5. Electrophoresis in a 1.1 % agarose gel of native and restriction endonuclease-cut DNA of SoyCMV, using Tris-acetate buffer. (1) Bacteriophage λ DNA cut with *Hind*III; (2) SoyCMV DNA uncut; (3) SoyCMV DNA cut with *Sac*I; (4) SoyCMV DNA cut with *Sal*I.

Fig. 6. The physical map of SoyCMV DNA. The cleavage sites for *Acc*I, *Bgl*II, *Pvu*II, *Sac*I, *Sal*I and *Sca*I are indicated. *Sac*I and *Sal*I each cut at a single site. The unique *Sac*I site is arbitrarily chosen as zero point of the map. The numbers indicate the length in kbp.

these enzymes produce a single DNA band (lane 3 and 4) exactly comigrating with the fastest migrating band of uncut DNA (lane 2). This, again, indicates, that the lowest band in native DNA represents linear molecules (see also Fig. 3). Using the *Hind*III fragments of phage λ DNA (Fig. 5, lane 1) as marker a size of 8.2 kbp was calculated for the linearised molecules (Table 2). Further digestion of *Sac*I- or *Sal*I-linearised molecules with enzymes cutting at two (*Bgl*II, *Pvu*II, *Sca*I) or three (*Acc*I) sites resulted in an unambiguous, circular physical map of SoyCMV DNA (Fig. 6). Based on all estimates (Table 2) a definite length of 8,200 bp could be established. Although preliminary experiments indicate that SoyCMV DNA contains single-stranded discontinuities in both strands (data not shown) these have not yet been mapped. Therefore, for the present the unique *Sac*I site has been arbitrarily chosen as zero point of the map.

Discussion

In this paper we showed that the genome of SoyCMV consists of a double-stranded DNA molecule, having a length of approximately 8,200 base pairs. From electron microscopical analysis, electrophoretic behaviour of native DNA and the constructed restriction map, it can be deduced that the genome of SoyCMV is, in principle, circular. Preliminary results indicate the presence of several single-stranded discontinuities in native SoyCMV DNA, as has been found for other caulimoviruses. Further analyses of these discontinuities are in progress. All results support the previous assignment of this virus to the caulimovirus group, which was mainly based on morphological and biological evidence⁷. The restriction map of SoyCMV DNA (Fig. 6) reveals no similarities to the maps of any of the other caulimoviruses^{3,4,10}. This, together with the characteristic host range of the virus⁷, indicates that SoyCMV is a distinct member of the group. The size of SoyCMV DNA as estimated from restriction enzyme digests (8.2 kbp, *i. e.* mol. wt. 5.45×10^6) is significantly larger than that of the other caulimoviruses so far analysed (7.8 - 8.0 kbp, for a recent review see Covey¹¹). It will be of great interest to study the structure and genetic properties of SoyCMV DNA in more detail and to gain more insight in the genetic basis of the important difference in host range compared to all other caulimoviruses.

We would like to thank Prof. Dr. A. van Kammen for stimulating discussion, Philip Schyns for help in the restriction enzyme experiments, Rijndert de Fluiter for art work and Gré Heitkönig and Marie-José van Neerven for typing the manuscript.

Literature cited

1. Covey, S. N. (1985). In *Molecular Plant Virology*, vol. II (Davies, J. W. ed.) CRC Press, Boca Raton, Florida. pp. 121-159.
2. De Ley, J. (1970). *J. Bacteriology* 101 : 738-754.
3. Donson, J. and Hull, R. (1983). *J. gen. Virol.* 64 : 2281-2288.
4. Hull, R. and Donson, J. (1982). *Ibid.* 60 : 125-134.
5. Hull, R. and Shepherd, R. J. (1977). *Virology* 79 : 216-230.
6. Hull, R. and Howell, S. H. (1978). *Ibid.* 86 : 482-493.

7. Iwaki, M., Isogawa, Y., Tsuzuki, H. and Honda, Y. (1984). *Plant Disease* 68 : 1009-1011.
8. Lawson, R. H. and Civerolo, E. L. (1978). *Phytopathology* 68 : 181-188.
9. Marmur, J. and Doty, P. (1962). *J. Mol. Biol.* 5 : 109-118.
10. Richins, R. D. and Shepherd, R. J. (1983). *J. gen. Virol.* 124 : 208-214.
11. Russel, G. L., Follett, E. A. C., Subak-Shape, J. H. and Harrison, B. D. (1971). *Ibid.* 11 : 129-138.
12. Shepherd, R. J., Bruening, G. E. and Wakeman, R. J. (1970). *Virology* 41 : 339-347.
13. Shepherd, R. J. and Wakeman, R. J. (1971). *Phytopathology* 61 : 188-193.
14. Vinograd, J. and Hearst, J. H. (1962). *Fortschr. Chem. org. Naturstoffe* 20 : 372-422.
15. Vollenweider, H. J., Sogo, J. M. and Koller, Th. (1975). *Proc. Nat. Acad. Sci. USA* 72 : 83-87.

和 文 摘 要

日比忠明・岩木満朗・斎藤康夫・Jan VERVER・Rob GOLDBACH: ダイズ退緑斑紋ウイルスの2本鎖 DNA の性状

ダイズ退緑斑紋ウイルス (SoyCMV) より核酸を抽出し、その理化学的性状を解析した。その結果、塩化セシウム平衡密度勾配遠心による分析および熱変性曲線の測定によって、SoyCMV の核酸は2本鎖 DNA であることが明らかにされた。また、アガロースゲル電気泳動による分析と電顕観察の結果から、このウイルス DNA には線状分子と環状分子があることが認められた。さらに、6種類の制限酵素を用いて制限酵素地図を作成したところ、SoyCMV のゲノムは約8,200塩基対から成る1分子の環状 DNA であることが示された。以上の結果から SoyCMV は caulimovirus グループに属する新たなウイルスであることが確認された。