Characterization of the Genome of Soybean Chlorotic Mottle Virus

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SUMMARY

The DNA of soybean chlorotic mottle virus (SoyCMV) has been isolated. When analysed by gel electrophoresis, the native DNA formed multiple bands resembling DNA from other caulimoviruses. The viral DNA was cloned in bacterial vectors. Cloned DNA was used to map restriction sites on the SoyCMV DNA. Comparison of cloned DNAs of SoyCMV and cauliflower mosaic virus (CaMV) by Southern transfer hybridization revealed the absence of significant sequence homology. Electrophoresis in denaturing gels showed that SoyCMV DNA had three single-strand discontinuities ('gaps'), one in one strand and two in the other, and the positions of these gaps were mapped on the viral DNA. Potential primer sites for reverse transcription were detected in the cloned DNA by sequence analysis of regions around the gap sites. The results obtained definitely establish SoyCMV as a member of the caulimovirus group and suggest a replication mechanism for this virus similar to that described for CaMV.

INTRODUCTION

Soybean chlorotic mottle virus (SoyCMV) is considered to be a member of the caulimovirus group on the basis of the morphology of its particles and of cytoplasmic inclusion bodies (Iwaki et al., 1984). The host range of SoyCMV differs from those of other caulimoviruses (Hull, 1984; Maule, 1985), being limited to only four plant species belonging to the family Leguminosae (Iwaki et al., 1984).

In this paper we report on the isolation, molecular cloning and physical mapping of the DNA of SoyCMV. The experiments described show that SoyCMV contains a circular, double-stranded DNA of approximately 8150 base pairs with single-strand interruptions ('gaps') typical of the caulimoviruses. In addition, the DNA of SoyCMV is compared with that of cauliflower mosaic virus (CaMV) by Southern hybridization, and nucleotide sequences of regions near the gap sites in cloned SoyCMV DNA are presented which reveal potential primer sites for reverse transcription of the viral DNA.

METHODS

Isolation of virion DNA. SoyCMV was originally isolated from naturally infected soybean (Glycine max) plants (Iwaki et al., 1984) and was maintained in bean (Phaseolus vulgaris cvs. Kintoki or Pinto) plants. The virus was purified from infected leaves as described (Iwaki et al., 1984), omitting the chloroform/butanol extraction and the sucrose gradient centrifugation steps. The resulting virus suspension was directly treated as described by Gardner & Shepherd (1980) to purify the viral DNA. The yield of viral DNA was about 1 μg per gram of leaf tissue.

Restriction enzymes. Restriction endonucleases and T4 DNA ligase were purchased from Anglian Biotechnology or Boehringer Mannheim. Incubations were according to the suppliers' recommendations.

Labelling. Labelling of 5' termini of virion DNA with T4 polynucleotide kinase (P-L Biochemicals) and [γ-32P]ATP (New England Nuclear) was done according to Hull et al. (1979). Bands of circular DNA were cut from low melting point agarose gels and purified by phenol extraction prior to kinase labelling. Recessed 3' termini of fragments of cloned SoyCMV DNA were labelled ('filled in') using the Klone fragment of DNA polymerase I (Anglian Biotechnology) and [α-32P]dATP (New England Nuclear) according to Drouin (1980). Nick translations were performed as described by Maniatis et al. (1982). DNA polymerase I was from Anglian Biotechnology.

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Gel electrophoresis. DNAs were separated by electrophoresis in agarose gels in TAE buffer (40 mM-Tris-acetate, 2 mM-EDTA, pH 8.0) or in polyacrylamide gels in TBE buffer (89 mM-Tris-borate, 2 mM-EDTA, pH 8.3). DNA fragments were isolated from agarose gels following the 'freeze-squeeze' procedure of Tantz & Renz (1983). The strands of viral DNA or DNA fragments were separated by electrophoresis in alkaline agarose gels according to McDonnell et al. (1977).

Molecular cloning. SoyCMV DNA digested with SacI was ligated into the SacI site of plasmid pUC18 (Yanisch-Perron et al., 1985). Viral DNA cut with SalI was ligated into the SalI site of pUC18. The ligation mixtures were used to transform Escherichia coli strain TG2 (Gibson, 1984). Viral DNA cut with ClaI was inserted into the Acel site of pUC8 (Vieira & Messing, 1982) and used to transform strain JM105 (Yanisch-Perron et al., 1985). Plasmids from active β-galactosidase-producing transformants were isolated by the method of Birnboim & Doly (1979) and further analysed by restriction enzyme digestions. Plasmids containing the full-size SoyCMV genome were isolated according to Birnboim & Doly (1979), followed by CsCl density gradient centrifugation. Subclones were made in M13 mp vectors according to Messing (1983). Sequencing of subcloned DNA was according to Sanger et al. (1980).

Southern transfer hybridization. Non-denaturing agarose gels containing DNA fragments of the full-size clones were treated with 0.25 M-HCl for depurination of the DNA, transferred to Gene Screen Plus Hybridization Membrane (New England Nuclear) by capillary blotting and probed with nick-translated DNA fragments. Subsequent hybridization was in 0.9 M-sodium chloride, 0.09 M-sodium citrate (6 × SSC) at 65 °C. Final washing was in 2 × SSC at 65 °C.

RESULTS

Native virion DNA

Native SoyCMV DNA, subjected to prolonged gel electrophoresis, formed multiple bands similar to DNA prepared from established members of the caulimovirus group (Fig. 1 b). In most cases SoyCMV DNA preparations also contained considerable amounts of contaminating RNA, which could be removed by ribonuclease A treatment prior to electrophoresis (not shown). Upon digestion of SoyCMV DNA with restriction enzymes ClaI, SacI, SalI or XhoI, which all cut the DNA at single sites (see below), single bands were formed comigrating with the fastest migrating band of uncut DNA (Fig. 1, lanes c to f). This result demonstrated that the fastest migrating band of SoyCMV DNA comprises linear molecules, while, similar to CaMV DNA, the slower migrating bands must be formed by knotted or twisted and open circular forms of the virion DNA (for recent reviews on CaMV DNA structure see Covey, 1985, and Howell, 1985). Comparison with the migration of marker DNA fragments (Fig. 1 a) gave a size estimate of 8.2 kilobase pairs (kbp) for SoyCMV DNA.

Denatured virion DNA

A common feature of caulimovirus DNAs is the presence of single-strand discontinuities ('gaps') (see Covey, 1985; Howell, 1985; Maule, 1985). The presence of such discontinuities can be determined by electrophoresis in a denaturing gel in which the DNA separates into a number of single-stranded fragments.

Electrophoresis in alkaline agarose gels separated SoyCMV DNA into three distinct bands, demonstrating single-strand gaps (Fig. 2). The single-stranded molecules, named α, β and γ, had estimated molecular sizes of about 8.2, 4.7 and 3.5 kilobases, respectively. This indicates the presence of one gap in one strand and two gaps in the other.

Molecular cloning and physical mapping

Full-length clones of SoyCMV DNA were obtained by insertion into pUC plasmids. Virion DNA was cloned at its unique ClaI, SacI or SalI sites, resulting in clones pSCla, pSSac and pSSal. Viral insert fragments of 8.2 kb were excised from pSSac and pSSal by cutting with SacI and SalI respectively. The 8.2 kb fragment of ClaI clone pSCla could only be excised by cutting with PstI and BamHI, since the ClaI site in the viral sequence was lost as a result of cloning into an AccI site. Sites for PstI and BamHI were not present in SoyCMV DNA (see Fig. 4) but were flanking the AccI site in the polylinker sequence of pUC8.

The viral moieties of the three full-length clones were isolated from agarose gels and cut with ClaI, SacI, and SalI. All three viral inserts contained the same ClaI–SalI, ClaI–SacI and SacI–
DNA of SoyCMV

Fig. 1. Electrophoresis in 1.1% agarose gel of native and restriction nuclease-cut DNAs. (a) λ DNA cut with HindIII; (b) uncut SoyCMV DNA; (c) SoyCMV DNA cut with ClaI; (d) with SacI, (e) with SalI and (f) with Xhol. All samples were treated with RNase A. Sizes of marker fragments are indicated.

Fig. 2. Electrophoresis of SoyCMV DNA in alkaline 1.1% agarose gel. (a) λ DNA cut with HindIII; (b) SoyCMV DNA. After the run the gel was neutralized and stained with ethidium bromide. Sizes of marker fragments in lane (a) and positions of the denatured fragments (α, β, γ) of SoyCMV DNA in lane (b) are indicated.

SalI fragments with sizes of 1.4 kb, 0.8 kb and 0.6 kb, respectively (Fig. 3). This unequivocally demonstrated the circularity of the SoyCMV DNA.

To construct a detailed restriction map of SoyCMV DNA the SacI insert of pSSac and the SalI insert of pSSal were cut with several restriction enzymes. Either the full-length inserts (prior to digestion) or smaller fragments generated by digestion were labelled by filling in recessed 3' termini using the Klenow polymerase and [α-32P]dATP. The labelled smaller fragments were also used for further digestions with other restriction enzymes. Digests were electrophoresed in agarose and polyacrylamide gels. Markers for size estimation of the fragments were λ DNA cut with HindIII and plasmid pBR322 cut with Sau3AI (autoradiograms not shown). In addition, precise (relative) positions of a number of sites were determined by partial sequencing of subclones of pSSac and pSSal (sequencing data not shown). The final restriction map of SoyCMV DNA is shown in Fig. 4. A comparison of this map with those of other cauliimoviruses (Covey, 1985) reveals that SoyCMV DNA has no, or only a few restriction sites in common with them.

Mapping of the gaps

Circular DNA of SoyCMV, purified from low melting point agarose gel, was kinase-labelled, cut with several restriction enzymes and electrophoresed in alkaline agarose gels. By comparison of the obtained fragment patterns with the restriction map we were able to determine the polarity of SoyCMV DNA and the position of the 5' termini of the viral DNA in the map. As an example Fig. 5 shows the patterns for the endonucleases PvuII and SalI. While this autoradiogram shows the separated α, β and γ strands with calculated lengths of 8.15, 4.62 and
3.53 kilobases respectively, digestion with SalI prior to electrophoresis produced labelled single-stranded fragments of 3.53, 2.59 and 0.74 kilobases, and digestion with PvuII single-stranded fragments of 3.03, 2.15 and 1.28 kilobases. Based on such data all three gaps (G1 to G3) were mapped as indicated in Fig. 4.

Southern transfer hybridization

To investigate more precisely the possible sequence homology between SoyCMV DNA and the DNA of CaMV, these DNAs were compared by Southern hybridization. Plasmid pCa305, which contains about 1.4 copies of the CaMV genome (Grimsley et al., 1986), was used as the source for a CaMV probe. Cloned SoyCMV DNA showed no significant hybridization when probed with labelled cloned CaMV DNA (Fig. 6a to h). Cloned CaMV DNA did not hybridize to labelled inserts of SoyCMV clones (Fig. 6i to p). In both cases the pUC18 vector fragments of the SoyCMV clones pSSac and pSSal gave a faint signal in the autoradiogram (Fig. 6, lanes g, h and m, n) which was probably due to a slight contamination of the viral insert fragments, used as probe, with fragments of the bacterial vector. The lack of significant cross-hybridization...
between the viral fragments strongly suggests that SoyCMV and CaMV are very distinct members of the caulimovirus group.

Potential primer sites near the discontinuities

In a replication model including reverse transcription of the viral DNA the occurrence of single-strand discontinuities in CaMV virion DNA has been explained. Potential primer sites for minus (−) and plus (+) strand synthesis have been detected near the 5′ termini of the α (minus) strand and β and γ (plus) strands. The (−) strand primer site adjacent to discontinuity G1 of CaMV DNA is a 14-nucleotide sequence perfectly corresponding to the 3′ terminal sequence of plant tRNA\textsuperscript{Met}. Adjacent to G2 and G3 in CaMV DNA, purine-rich regions have been found which show some similarities to primer sites for (+) strand synthesis in retroviruses (see reviews by Maule, 1985; Covey, 1985; Howell, 1985). We have searched for such potential primer sequences near the gap sites in cloned SoyCMV DNA.

Since we had determined the positions of the 5′ termini of the α, β and γ strands of SoyCMV DNA in the map (Fig. 4), we were able to construct subclones that were expected to contain the gap sites. By sequencing a clone containing gap site G1 a short sequence of 12 nucleotides was...
found to correspond to the 3' terminal sequence of tRNA^{Met} (Canaday et al., 1980) at a distance of about 50 bp from the XbaI site at map position 0.05 (Fig. 7). As done by Pfeiffer & Hohn (1983) for the CaMV sequence we propose to indicate the nucleotide corresponding to the 3' terminal residue of tRNA^{Met} as number one in the map of SoyCMV DNA. In clones containing gap sites G2 and G3 we detected close to the KpnI site at position 4.75 and the HindIII site at position 1.12 very purine-rich regions in the (+) strand, both starting with GAGGAGGG (Fig. 7). These purine-rich sequences were situated very close to the positions of the 5' ends of the β and γ strands in the map of SoyCMV DNA. By analogy to CaMV we suggest that these sequences represent primer sites for reverse transcription of the SoyCMV DNA.

DISCUSSION

We have isolated and characterized the DNA of SoyCMV, a recently described new member of the caulimovirus group (Iwaki et al., 1984). Among nine definitive and five tentative members of this group (Hull, 1984; Maule, 1985) only SoyCMV is reported to infect Leguminosae. The assignment of SoyCMV to the caulimovirus group, which was based on the morphology of the virus particles and of cytoplasmic inclusion bodies, is strongly supported by the results presented in this paper.
Fig. 6. Agarose gel electrophoresis and Southern transfer hybridization of cloned SoyCMV and CaMV DNAs. The SalI digest of CaMV clone pCa305 (b, k), the SacI digest of SoyCMV clone pSSac (c, j) and the SalI digest of SoyCMV clone pSSal (d, i) were electrophoresed in 1% agarose gels. (The two SalI fragments of pCa305 of 10.4 kb and 8.0 kb both contain CaMV-specific sequences. The 8-0 kb fragment contains the full-size genome of CaMV, the 10.4 kb fragment the vector and only a part of the virus genome.) Marker lanes (a, l) show λ DNA cut with HindIII. After capillary blotting on Gene Screen Plus lanes (a) to (d) were probed with the nick-translated 8-0 kb CaMV-specific SalI fragment of pCa305 (autoradiogram, e to h). Lanes (i) to (l) were probed with the labelled SoyCMV-specific inserts of clones pSSac and pSSal (autoradiogram, m to p). The pUC18 vector fragments of clones pSSac and pSSal and sizes in kilobase pairs of the fragments in lanes (a) and (l) are indicated.

Fig. 7. Sequence comparison of regions in the (+) strands around the gaps in CaMV DNA and around the putative gap sites in cloned SoyCMV DNA. Sequences thought to delimit primer sites for reverse transcription of CaMV DNA are underlined. The sequences corresponding to the 3'-terminal part of tRNA™ are indicated by dots. Open boxes show the identical left hand parts of polyuridine tracts in regions containing the gap sites G2 and G3 in SoyCMV DNA. Relevant restriction sites and their position in the map of cloned SoyCMV DNA are indicated. Black triangles give the positions of the fixed 5' termini at the gaps of CaMV DNA.

We have shown that SoyCMV virions contain a circular double-stranded DNA of 8-15 kbp, which is slightly larger than DNAs of other caulimoviruses (see Hull, 1984). In both denaturing and non-denaturing agarose gels SoyCMV DNA shows band patterns characteristic for caulimoviruses. The DNA has three single-strand discontinuities ('gaps'), which results, upon denaturation, in three single-stranded molecules (α, β and γ) of 8-15, 4-62 and 3-53 kbp. As for all caulimoviruses examined so far, one of the strands contains only one gap. This strand probably
corresponds to the minus (−) strand of CaMV, i.e. the strand that is transcribed (see Covey, 1985).

With cloned DNA of SoyCMV a detailed physical map, including restriction and gap sites, was constructed which shows no similarity with those of other caulimoviruses (see Covey, 1985). Moreover, a full-size cDNA insert appeared to be infectious to P. vulgaris plants in a preliminary infectivity test (data not shown). Infectious cDNA has also been reported for CaMV (Howell et al., 1980) and carnation etched ring virus (CERV), figwort mosaic virus (FMV) and mirabilis mosaic virus (MMV) (Donson & Hull, 1983).

In Southern transfer hybridization experiments no significant sequence homology was detected between cloned DNAs of SoyCMV and CaMV. Since little or no homology has also been reported between the DNAs of CaMV, CERV, FMV, MMV, thistle mottle virus and dahlia mosaic virus (Donson & Hull, 1983; Richins & Shepherd, 1983), this result strengthens the idea that the various members of the caulimovirus group have diverged widely during evolution.

Sequencing of subcloned SoyCMV DNA containing regions around gap site G1 revealed a sequence of 12 nucleotides exactly corresponding to the 3' terminal part of plant tRNA_{Met}, which in the case of CaMV is thought to act as a primer for the (reverse transcriptional) synthesis of the (−) strand (Guilley et al., 1983; Pfeiffer & Hohn, 1983; Hull & Covey, 1983; Turner & Covey, 1984). By sequencing regions around gap sites G2 and G3 we discovered very purine-rich tracts in the (+) strand which could possibly represent primer sites for (+) strand synthesis as proposed for CaMV (Pfeiffer & Hohn, 1983; Hull & Covey, 1983; Maule & Thomas, 1985). Based upon the identification of these potential (+) and (−) strand primer sites in SoyCMV DNA, a mechanism for replication of the viral DNA similar to that proposed for CaMV (for review, see Maule, 1985) is very likely.

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