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Occurrence and Relatedness of Extrachromosomal DNAs in Plant Pathogenic Mycoplasmalike Organisms

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DNA from mycoplasmalike organisms (MLOs) of herbaceous and woody plants as well as of leafhopper vectors was examined for the occurrence and interrelatedness of extrachromosomal DNAs. When MLO DNA, purified by repeated CsCl-bisbenzimide density gradient centrifugation, was analyzed in agarose gels stained with ethidium bromide, variable numbers of extrachromosomal DNA bands were observed in most organisms. Southern blots of undigested total DNA from infected plants probed with cloned extrachromosomal DNA fragments from four different MLOs revealed extrachromosomal DNA in almost all of the MLO isolates examined, including those from woody plants. Detection of extrachromosomal DNAs was sometimes difficult because of its low relative amount and/or insufficient homology to the probes. The ranges of hybridization of the probes from the four organisms with the extrachromosomal DNAs of the MLOs examined were very different. Also, the banding patterns obtained with a given probe varied considerably. No close correlation between the hybridization results and the symptoms induced by the isolates in periwinkle was found.

Additional keyword: MLO plasmids.

Mycoplasmalike organisms (MLOs) are associated with a great number of plant diseases. Recently, extrachromosomal DNA considered to be of plasmid nature was found in several MLOs. Davis *et al.* (1988) were the first to detect extrachromosomal DNA in a Florida strain of the maize bushy stunt (MBS) MLO. Cloned extrachromosomal DNA probes of this strain detected extrachromosomal DNAs of the MBS MLO in an isolate from Mexico but not in an isolate from Texas. Cloned extrachromosomal DNA from an MLO that caused phyllody of *Oenothera hookeri* Torr. & A. Gray hybridized to extrachromosomal DNA from plants infected with aster yellows (AY) (Sears *et al.* 1989). Kuske and Kirkpatrick (1990) characterized extrachromosomal DNAs that were present in three strains of western AY. Southern blot analysis, with cloned and native extrachromosomal DNAs from the severe strain (SAY) as probes, identified at least four different extrachromosomal DNAs in these three strains. Extrachromosomal DNAs were also detected in other but not all virescence-inducing MLOs from America and Europe, including MBS and the *Oenothera* MLO. No extrachromosomal DNA was detected in decline-inducing MLOs that infect sugarcane and bermudagrass or several herbaceous and woody dicots (Kuske *et al.* 1991). Bertaccini *et al.* (1990) detected extrachromosomal DNAs in the MLOs associated with chrysanthemum yellows, tomato big bud, and periwinkle little leaf, and in a strain of American eastern AY. Extrachromosomal DNAs were also detected in the MLOs associated with witches'-broom disease of pigeon pea (Harrison *et al.* 1991) and walnut witches'-broom (Chen *et al.* 1992).

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The objectives of this study were to exam MLOs for the presence of extrachromosomal DNAs and to determine whether such DNAs are related to each other; cloned extrachromosomal DNAs from four MLOs were used. Several decline-inducing MLOs from herbaceous plants as well as isolates from woody dicots were included in this work because extrachromosomal DNA, with the exception of the recently discovered presence in the walnut witches'-broom MLO (Chen *et al.* 1992), has not been reported in such MLOs. The relationship between presence and patterns of extrachromosomal DNAs and the symptoms induced by the respective MLO was also investigated.

MATERIALS AND METHODS

Sources of MLOs. Sources of the MLOs examined in this study are listed in Table 1. Isolate EAY was maintained in the greenhouse in *Coleus blumei* Benth. by cuttings, and KVV (clover phyllody) was collected in white clover (*Trifolium repens* L.) in the experimental field of the Dossenheim institute. All other isolates were maintained in the greenhouse in periwinkle (*Catharanthus roseus* (L.) G. Don) by graft transmission.

DNA isolation. We isolated DNA from healthy and diseased plants by using the CTAB method as described previously (Kollar *et al.* 1990). After precipitation, the DNA was washed several times with 70% EtOH, dried under vacuum, and dissolved in a small volume of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Such preparations are referred to as total DNA and were used in most Southern hybridization experiments. Agarose gel electrophoresis followed by staining with ethidium bromide (EtBr) was used for cloning and demonstrating extrachromosomal DNA bands. The MLO DNA was purified from infected plants by repeated bisbenzimide-CsCl density gradient

Table 1. Isolates of mycoplasmalike organisms (MLOs) examined for the presence of extrachromosomal DNA

MLO code	Disease/source	Country of origin	Collected and/or transmitted[a]
AAY	American aster yellows	Florida, USA	1
ACLR	Apricot chlorotic leaf roll	Spain	2
AKV	Virescence of *Aquilegia alpina*	Germany	3
AT	Apple proliferation	Germany	3
AV976	Aster yellows	Germany	3
AV2192	Aster yellows	Germany	3
AV2226	Aster yellows	Germany	3
AVUT	Aster virescence	Germany	3
AYW	Eastern American aster yellows	New Jersey	4
BVK	Leafhopper-born (*Psammotettix cephalotes*)	Germany	5
CACT	Proliferation of *Opuntia* sp.	?	6
CHRYM	Virescence of *Chrysanthemum frutescens*	Germany	3
COL	Latent in *Cuscuta odorata*	?	7
CVA	Leafhopper-born (species not determined)	Germany	5
CVB	Leafhopper-born (species not determined)	Germany	5
CVL	Catharanthus virescence	Peru	8
CVT	Catharanthus virescence	Thailand	9
DAY	Dwarf American aster yellows	California, USA	10
DEV	Virescence of a *Delphinium* hybrid	Germany	3
DIV	Virescence of *Diplotaxis erucoides*	Spain	11
EAY	Aster yellows	Germany	3
HYDF	Hydrangea phyllody	France	12
HYDP	Hydrangea phyllody	Belgium	13
KV	Clover phyllody	Germany	3
KVE	Clover phyllody	England	14
KVF	Clover phyllody	France	12
KVV	Clover phyllody	Germany	15
PLN-V6	Plum leptonecrosis	Italy	16
PRIVA	Virescence of primrose (*Primula* sp.)	Germany	3
PRIVB	Virescence of primrose (*Primula* sp.)	Germany	3
PRIVC	Virescence of primrose (*Primula* sp.)	Germany	3
PVM	Virescence of *Plantago coronopus*	Germany	3
PVW	Virescence of *Plantago major*	Germany	5
RV	Rape virescence	France	17
SAY	Severe American aster yellows	California, USA	10
SAFP	Safflower phyllody	Israel	18
SAS	Sandal spike	India	19
SBB	Big bud of *Solanum marginatum*	Ecuador	3
STOL	Stolbur of *Capsicum annuum*	Croatia	20
SUNHP	Sunhemp phyllody	Thailand	15
VAC	Witches'-broom of *Vaccinium myrtillus*	Germany	3

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centrifugation as described previously (Kollar *et al.* 1990). Such DNA is referred to as purified MLO DNA. DNA from *Spiroplasma citri* Saglio *et al.* was obtained by use of standard methods (Carle *et al.* 1983).

Gel electrophoresis and Southern blots. To demonstrate the presence of extrachromosomal DNAs in agarose gels, we electrophoresed 1–2 μg of purified MLO DNA in horizontal 0.6 or 1.0% agarose gels in Tris-acetate (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 3.0 V/cm for 16 hr at room temperature. The bands were visualized by irradiation with UV-light after they were stained with EtBr. For hybridization studies, the DNA was transferred to nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) following the procedure described by Maniatis *et al.* (1982). When total DNA from infected plants was used in Southern hybridization experiments, approximately 10 μg of DNA was electrophoresed and blotted as described above.

Cloning. Purified DNA from CVA (leafhopper-born), RV (rape virescence), and VAC (witches'-broom of *Vaccinium myrtillus* L.) MLOs was electrophoresed as described above. Extrachromosomal DNA bands were excised from the gel, and the DNA was recovered from the gel with the Geneclean kit (Bio-101, Vista, CA). Extrachromosomal DNA of CVA and RV MLOs was digested with *Hin*dIII and extrachromosomal DNA of VAC MLO with *Eco*RI restriction endonucleases according to the instructions of the manufacturer (BRL-Life Technologies, Gaithersburg, MD). Digested DNAs were electrophoresed in agarose gels, and bands were recovered from the gel as described above. Fragments of CVA extrachromosomal DNA were ligated into the *Hin*dIII site of plasmid Bluescript M13+ (Stratagene, La Jolla, CA), and fragments of extrachromosomal DNA of RV and VAC MLOs were ligated into the *Hin*dIII site or the *Eco*RI site of pUC18 (Boehringer-Mannheim, Indianapolis, IN), respectively; standard procedures were used. The ligation mixture was used to transform competent cells of *Escherichia coli* strains DH5α or XL1-blue (Stratagene), respectively, which were plated on LB medium containing ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and IPTG (isopropyl-β-D-thiogalactopyranoside). Recombinant plasmids were isolated by the method of Birnboim and Doly (1979). The inserts were excised from the cloning vector by digestion with the appropriate restriction enzyme, purified twice by gel electrophoresis, and recovered from the gel by the Geneclean method. The specificity of the inserts was tested by hybridization against DNA from healthy periwinkle, total MLO DNA of the homologous organism, and gel-purified high molecular weight chromosomal DNA from the homologous organism.

Cloning of extrachromosomal DNA of the SAY MLO has been described by Kuske *et al.* (1991). The cloned 4.9-kb *Eco*RI restriction fragment used in this study is designated PSA45.

Probes and hybridization. Extrachromosomal DNA inserts of clones from CVA, RV, SAY, and VAC MLOs were excised with the appropriate restriction enzyme, gel-purified as described above, and labeled with ^{32}P-dATP by random priming (Boehringer-Mannheim). Membranes were prehybridized in solutions containing 6× SSPE (0.9 M

NaCl, 60 mM Na_2HPO_4, 6 mM EDTA, pH 7.6), 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrollidone, 0.1% bovine serum albumin), 100 μg/ml of herring sperm DNA, and 0.5% sodium dodecyl sulfate (SDS) at 68° C for 2 hr and were hybridized in the same buffer and at the same temperature for at least 16 hr. After hybridization, the membranes were washed twice in 2× SSC (0.3 M NaCl, 0.1 M sodium citrate) and 0.1% SDS at room temperature for 10 min, followed by two washes with 0.2× SSC and 0.1% SDS at either 40° or 55° C for 30 min (low and moderate stringency, respectively). High stringency washes were performed in 0.1× SSC and 0.1% SDS at 68° C. After being washed, the blots were exposed to X-ray film (Kodak X-Omat) at −80° C with intensifier screens. In some instances, we stripped the blots of the probes by washing the membranes with boiling 0.1% SDS. After cooling to room temperature, the membranes were exposed to an X-ray film for verifying complete removal of the probe.

RESULTS

Grouping of MLO isolates. The isolates examined have been divided into four groups according to the most pronounced symptoms they induce in periwinkle (Table 2). The predominant symptoms induced by group I, which

Table 2. Presence of extrachromosomal DNAs and their hybridization with cloned extrachromosomal DNA probes

MLO[a]	Number of bands observed in EtBr-stained gels	Maximal numbers of extrachromosomal DNA bands[b]					
		CVA143	RV132	PSA45	VAC79	VAC131	VAC211
Group I[c]							
AAY	2–4	5/2	8/4	0/0	0/0	0/0	2/2
ACLR	...[d]	6/6	2/0	4/4	5/5	0/0	0/0
AKV	1–4	0/0	2/0	0/0	0/0	0/0	3/3
AVUT	...	...	...	5/4	...	...	...
CVA	6–12	12/5	3/2	4/0	2/2	0/0	0/0
CVB	7	10/3	3/3	0/0	1/1	1/1	3/3
CVL	3	0/0	3/3	4/3	0/0	0/0	0/0
CVT	5	...	6/-	...	...	...	...
DAY	...	2/2	...	...	...	...	...
HYDP	6	5/0	5/3	2/2	2/2	0/0	0/0
KV	7–11	8/5	5/5	7/6	1/1	0/0	0/0
KVE	2–5	...	...	5/3	...	...	...
KVF	4	...	...	4/3	...	...	...
KVV	...	...	...	5/4	...	...	...
PLN-V6	...	0/0	0/0	0/0	2/2	1/1	2/2
PRIVA	2	...	...	5/4	...	...	...
PRIVC	7–11	4/0	7/2	4/3	0/0	0/0	0/0
SAFP	2	0/0	1/0	0/0	0/0	0/0	0/0
SAS	3–5	0/0	9/1	0/0	0/0	1/0	1/0
SBB	2–7	1/0	7/0	0/0	4/4	4/3	4/4
STOL	0	0/0	0/0	4/0	0/0	0/0	0/0
SUNHP	4	...	...	...	...	...	...
Group II							
AV2192	0	0/0	0/0	4/0	0/0	0/0	0/0
AV2226	0	0/0	0/0	0/0	0/0	0/0	0/0
AYW	2	...	...	5/3	...	...	...
CACT	2	...	...	10/8	...	...	...
CHRYM	...	...	...	4/2	...	...	...
DIV	2–3	3/1	4/4	2/0	0/0	1/0	3/3
EAY	...	0/0	2/0	0/0	0/0	0/0	0/0
HYDF	2	...	...	5/4	...	...	...
PVW	6–8	1/0	7/0	5/4	0/0	0/0	0/0
RV	6–13	5/2	13/6	8/8	0/0	0/0	0/0
SAY	2	2/0	0/0	2/2	...	...	...
Group III							
AV976	...	...	...	1/0	...	...	...
DEV	2	0/0	2/1	3/2	0/0	0/0	0/0
PVM	4–10	5/2	4/3	5/4	0/0	0/0	0/0
Group IV							
AT	3	5/2	2/0	0/0	0/0	0/0	0/0
BVK	1–7	2/0	2/0	7/5	0/0	0/0	0/0
COL	3	2/1	2/1	11/3	0/0	0/0	0/0
PRIVB	...	6/2	5/5	5/1	0/0	0/0	0/0
VAC	3	8/1	3/1	0/0	2/2	2/2	2/2

[a] Mycoplasmalike organism. See Table 1 for MLO codes.
[b] Observed by Southern blot hybridization with probes. Numbers represent moderate/high stringency of the posthybridization washes.
[c] Group I, MLOs that cause virescence and shortened internodes in periwinkle. Group II, MLOs that cause virescence and etiolated and elongated internodes in periwinkle. Group III, MLOs that cause reduced flower size and etiolated and elongated internodes in periwinkle. Group IV, MLOs that cause reduced flower size and leaf and flower malformations in periwinkle.
[d] Not determined.

includes the agent of clover phyllody and stolbur, are virescence and phyllody as well as shortened internodes. Group II includes the organisms that cause the symptoms of typical AY isolates, such as virescence and phyllody with elongated and etiolated internodes. Group III is similar to group II but induces small and faintly colored flowers instead of virescence and phyllody. Group IV induces small and faintly colored flowers, but virescence, phyllody, internode elongation, and etiolation are absent. It corresponds to the decline MLOs proposed by Kuske *et al.* (1991). In contrast to the relatively homogenous symptoms induced by groups I–III, those of the isolates in group IV are more heterogenous because they induce different kinds of leaf and flower malformations.

Extrachromosomal DNA visualized in agarose gels. Purified MLO DNA of most of the isolates was examined for the appearance of extrachromosomal DNA bands in EtBr-stained agarose gels. With the exception of AV2192 (aster yellows), AV2226, and STOL (stolbur of *Capsicum annuum* L.), extrachromosomal DNA bands were detected in all of the samples tested (Table 2). The individual strains showed up to 13 bands with sizes that varied between 1.4 and 16 kb relative to supercoiled standards. In some cases, such as CVA, higher molecular weight bands were observed. Considerable differences in the patterns of the extrachromosomal DNA profiles were observed between the different isolates that were tested. None of the organisms had identical patterns, and bands common to several isolates did not often occur. There was also some variation in the number of bands when different DNA preparations from the same organism were analyzed, although the overall patterns were similar. Representative extrachromosomal DNA profiles are shown in Figure 1A.

Cloning of MLO extrachromosomal DNA. Several recombinant plasmids containing an insert of MLO extrachromosomal DNA were obtained from CVA, RV, and VAC MLOs. The following fragments of cloned extrachromosomal DNA were used in hybridization: one 4.3-kb fragment from CVA designated CVA143; one 1.3-kb fragment from RV designated RV132; and three fragments from VAC, 1.4, 1.7, and 3.9 kb, designated VAC79, VAC211, and VAC131, respectively. As shown by digestion of the corresponding open circular and ccc forms with *Hin*dIII restriction endonuclease, CVA143 is the entire sequence of an extrachromosomal DNA; the other inserts used are fragments of native extrachromosomal DNAs (data not shown).

Southern blot hybridization. Southern blot hybridization of the purified MLO DNAs shown in Figure 1A with probe RV132 resulted in banding patterns different from those obtained by gel electrophoresis. More bands were, for instance, detected in the SAS (sandal spike) sample, an equal number in RV and HYDP (hydrangea phyllody), and fewer in KV, PRIVC (virescence of primrose), and VAC (Fig. 1B). These differences are due to the specificity of the probe for the samples examined.

The results of Southern hybridization of undigested total DNA from infected plants with the six probes used are summarized in Table 2. Examples of blots hybridized with probes CVA143, RV132, and PSA45 are depicted in Figures 2 and 3 which, however, do not show all results obtained with these probes. As shown in Table 2, extrachromosomal DNAs were detected by Southern hybridization in all MLOs examined with the exception of isolate AV2226. With this isolate, all attempts with the different probes and increased amounts of DNA remained negative. In contrast, extrachromosomal DNA was detected with probe PSA45 in isolate AV2192, which is genetically and symptomatically closely related to AV2226 (B. Schneider,

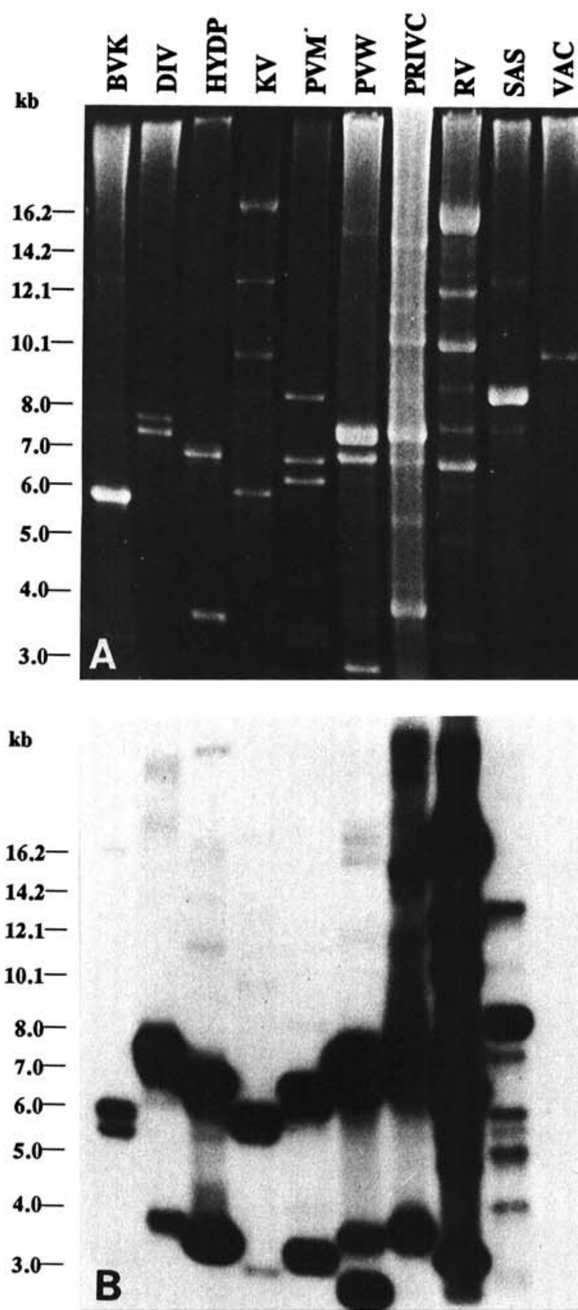

Fig. 1. Demonstration of extrachromosomal DNAs in purified, undigested DNA from 10 different mycoplasmalike organisms (MLOs) **A**, by agarose gel electrophoresis followed by staining with ethidium bromide and **B**, by hybridization of the blotted DNA with probe RV132. Posthybridization washes were at moderate stringency. With the two methods, different profiles were obtained with the DNA from most MLOs. See Table 1 for MLO code. SM, supercoiled DNA marker.

unpublished results), after the amount of DNA was increased. Also, the extrachromosomal DNAs present in the STOL MLO were detected with probe PSA45 only when high amounts of DNA were applied to the gel (data not shown).

The importance of the amount of applied DNA for the detection of extrachromosomal elements also became evident when blots of total DNA from infected plants were compared with blots of purified MLO DNA. For instance, after hybridization with probe RV132 and moderately stringent posthybridization washes, six bands were observed in the total DNA of RV-infected plants, whereas 13 bands were detected in purified MLO DNA of the same organism. The corresponding figures of total DNA versus purified MLO DNA were for PRIVC 2 and 7, for SAS 2 and 8, and for AAY 4 and 8 (Table 2; Figs. 1B,2B). However, it is possible that the higher number of bands present in MLO DNA might be partially due to the purification procedure during which some extrachromosomal elements were broken, thus giving linear forms that were not present in total DNA.

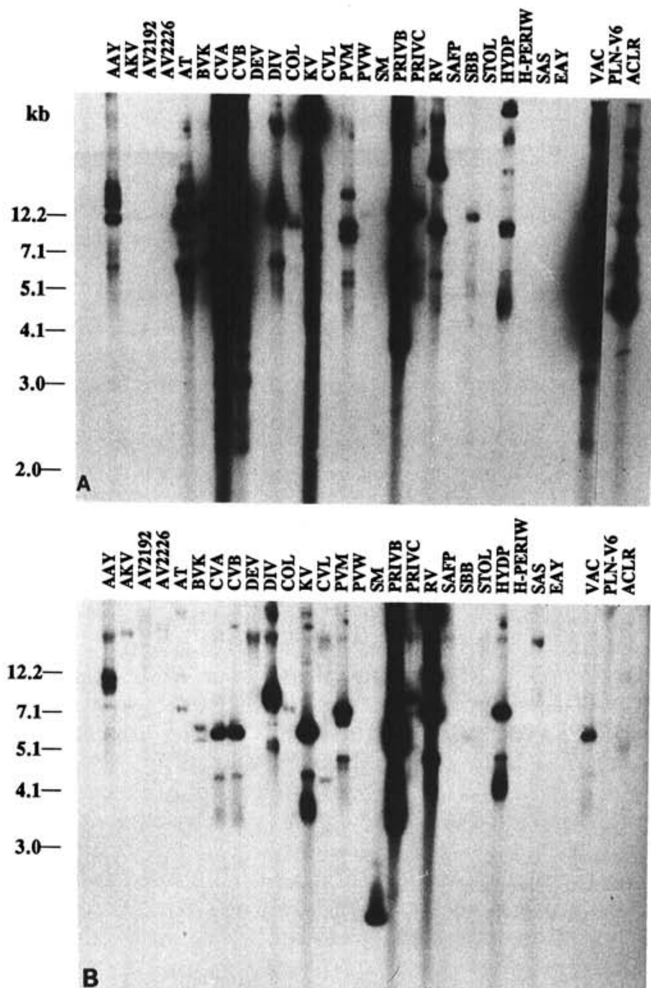

Fig. 2. Southern blot hybridization of undigested total DNA from infected plants **A,** with probe CVA143 and **B,** after removal of CVA143 with probe RV132. Posthybridization washes were at moderate stringency. See Table 1 for codes of the mycoplasmalike organisms (MLOs). SM, *Bam*HI- and *Pst*I-digested lambda DNA and undigested pUC18. H PERIW, healthy periwinkle.

Hybridization of the extrachromosomal DNA inserts with the extrachromosomal DNA of the isolates examined differed considerably (Table 2; Figs. 2,3). At moderate stringency, probe RV132 hybridized with extrachromosomal DNAs present in 82% of the nonhomologous isolates, whereas probes PSA45 and CVA143 hybridized with 68 and 64%, respectively. Extrachromosomal DNA was detected in many of the isolates by all three probes. The hybridization patterns were closest between CVA143 and RV132, which recognized in AAY, DIV (virescence of *Diplotaxis erucoides* (L.) DC), PRIVB, RV, and HYDP extrachromosomal DNAs with similar mobilities. Some of the extrachromosomal DNAs of PVM (virescence of *Plantago coronopus* L.), RV, and HYDP, which hybridized with CVA143 and RV132, were also detected by probe PSA45. Probes CVA143 and RV132 hybridized to most or all extrachromosomal DNA bands present in purified DNA of the homologous organisms in EtBr-stained agarose gel.

The three VAC probes cross-hybridized to a significantly lower extent with the extrachromosomal DNAs of the isolates examined than CVA143, RV132, and PSA45 (Table 2). They showed homology to only 19–27% of the other isolates. The hybridization patterns of the three VAC probes were similar but not identical. Bands detected by probes CVA143, RV132, and PSA45 were usually not detected by the VAC probes. The few exceptions include bands of the ACLR (apricot chlorotic leaf roll) MLO, which showed homology to both VAC79 and CVA143. In DNA from VAC-infected plants, all three VAC probes hybridized to two bands showing the same mobility, whereas CVA143 hybridized with eight. The VAC probes did not hybridize to extrachromosomal DNAs of the other isolates of group IV or, with the exception of DIV, to the isolates inducing internode elongation and etiolation.

In all hybridization experiments, the number of bands observed after high stringency washes was lower than the numbers seen after moderately stringent washing conditions

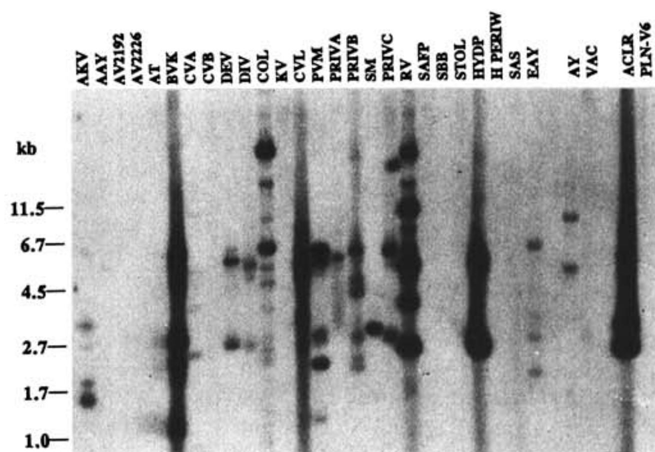

Fig. 3. Southern blot hybridization of undigested total DNA from infected plants with probe PSA45. The blot was washed at moderate stringency. The bands in the lanes of AKV and EAY remained despite strip-washing from a previous hybridization of the blotted DNA with pUC18. See Table 1 for codes of mycoplasmalike organisms (MLOs). SM, *Bam*HI- and *Pst*I-digested lambda DNA and undigested pUC18. H PERIW, healthy periwinkle. AY is isolate SAY.

(Table 2). However, for most isolates some bands remained after high stringency washes. This indicates the presence of homologous sequences in extrachromosomal DNAs of these organisms. In a few cases only, such as SAFP (safflower phyllody), STOL, AV2192, and AV976 when only faint bands were present at moderate stringency conditions, the hybrids were disassociated by high stringency washes.

None of the extrachromosomal DNA probes hybridized to DNA from healthy periwinkle or *C. blumei* or to DNA of *S. citri*.

DISCUSSION

Extrachromosomal DNAs were detected in virtually all of the 41 MLO isolates examined. These isolates were collected in different geographic areas, and they originated from leafhopper vectors as well as from several herbaceous and woody dicot plants. These results indicate that extrachromosomal DNAs do not only occur in MLOs inducing virescence symptoms as reported by Kuske *et al.* (1991), but are present also in decline-inducing agents. Detection of extrachromosomal DNA is difficult when its relative concentration is low or when the probe used does not have sufficient homology. For instance, extrachromosomal DNA has never been detected in purified MLO DNA of STOL, AV2192, and AV2226 by EtBr staining, although several attempts were made and large amounts of sample DNA were used. The failure of Kuske *et al.* (1991) to demonstrate extrachromosomal DNA with probe PSA45 in CVL (Catharanthus virescence; in that paper, CV) and AV2192 (AV) appears also to be a matter of the relative quantity of extrachromosomal DNA because such DNA was detected in these MLOs with probe PSA45 in this study.

The cloned extrachromosomal DNA fragments varied considerably in cross-hybridizing to extrachromosomal DNAs of the isolates examined. However, in most isolates extrachromosomal DNAs were detected by more than one of the probes. Poor hybridization due to insufficient homology with the probes was observed in some samples. For example, only one of the probes (PSA45) hybridized weakly at moderate stringency to extrachromosomal DNA of AV2192, STOL, and SAFP. Failure to detect extrachromosomal DNA in isolate AV2226 might not only be due to a low relative amount of extrachromosomal DNA but also to a low sequence homology of the probes. The lack of extrachromosomal elements in this MLO cannot be excluded either.

In EtBr-stained gels and Southern blot analysis, the extrachromosomal DNAs of the MLOs examined varied significantly in number and size. However, probes RV132, CVA143, and PSA45 hybridized with extrachromosomal DNAs present in the majority of the isolates. Also, RV132 and CVA143 hybridized with most or all extrachromosomal DNAs of very different sizes occurring in the RV and the CVA MLOs, respectively. These results indicate that the extrachromosomal DNAs of the same organism as well as of different organisms that might be only distantly related share homologous sequences. Homology between extrachromosomal DNAs of distinctly different MLOs (western AY and MBS) was also noted by Kuske *et al.* (1991).

There seems to be no close relationship between the type of symptoms induced in the host plants and the extrachromosomal DNA profiles. Exceptions are the symptomatically similar isolates CVA and CVB when probed with either CVA143 or RV132. In other cases, such as ACLR and PLN-V6 (plum leptonecrosis), which are, according to transmission results and symptom induction, considered closely related (Giunchedi *et al.* 1982), very different extrachromosomal DNA patterns were observed. Also, extrachromosomal DNAs of isolates from China aster (*Callistephus chinensis* (L.) Nees) appear to be quite different. The European isolates AV2192 and AV976 seem to possess little extrachromosomal DNA homology with aster isolates from North American or European non-aster isolates that induce AY-like symptoms in periwinkle. Differences were also observed among the clover phyllody isolates examined.

Despite these observations, there are differences in the specificity of the probes used; the probes were developed from MLOs allocated to three of the four symptom-inducing groups described. Probe CVA143 (group I) cross-hybridized with all MLOs of group IV and with about half of the organisms of groups I–III. It did not hybridize, or only weakly so, to extrachromosomal DNAs of the stolbur type MLOs SBB (big bud of *Solanum marginatum* L.) and STOL and to the AY isolates from China aster (AV2192, AV2226, EAY, and SAY). Probe RV132 (group I) cross-hybridized with extrachromosomal DNAs of almost all organisms of group I, with all of group IV, but not or only weakly with the "authentic" AY isolates. Probe PSA45, which derives from the SAY MLO (group II), cross-hybridized to the extrachromosomal DNAs of almost all MLOs inducing internode elongation and etiolation, the typical AY symptoms. It showed no or only low homology with the stolbur type isolates. The VAC probes (group IV) showed no homology to the other isolates of group IV and very little to groups II and III. However, they hybridized to several MLOs of group I. Despite these differences in the hybridization results, the extrachromosomal DNA probes appear to be less suitable for characterizing and classifying the MLOs than probes from chromosomal DNA selected for appropriate specificity.

Extrachromosomal DNA in MLOs is generally considered to be of plasmid nature (Davis *et al.* 1988; Sears *et al.* 1989; Kuske and Kirkpatrick 1990; Kuske *et al.* 1991), although viruslike particles have been observed in association with several MLOs (Ploaie 1971; Allen 1972; Gourret *et al.* 1973; B. Schneider, unpublished results). Double-stranded DNA present in *S. citri* was first described as a plasmid by Nur *et al.* (1986) but was later shown to be the replicative form of a virus (Bodin-Ramiro *et al.* 1990). Further work is necessary to elucidate the identity of the extrachromosomal elements present in MLOs.

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