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Recent Advances in Phytopathogenic Mycoplasmology

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ABSTRACT

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The plant pathogenic mycoplasmas (MLOs) are known to cause diseases in a wide variety of economically important plants around the world. Diseases caused by MLOs are often referred to as "yellows" diseases because symptoms include yellowing, stunting, shoot proliferation, and phyllody. MLOs in plants resemble animal mycoplasmas, which are wall-less prokaryotes. Since these microorganisms have yet to be grown in culture, progress in MLO research has been slow. Classification of MLOs has not been possible because of a lack of information on their nutritional requirements, membrane composition, genome organization, and detection and identification techniques. During the past several years, advancements in immunology and biotechnology have greatly improved our methods for studying the MLOs and have also increased our knowledge of the classification and of evolutionary origin of these organisms. Recently, researcher on MLO has progressed in three areas: 1. techniques for detecting and identifying these organisms in infected plants have improved; 2. the phylogeny of MLOs is better understood; and 3. molecular data has been used in an attempt to provide relevant means to group MLOs associated with various plant yellows diseases. Rapid, specific, and sensitive techniques have been developed for MLO detection. These include monoclonal antibodies, DNA probes, and polymerase chain resctions (PCR). By using sequence data from analyses of 16s rRNA and 16s rRNA oligonucleotide catalogs, phylogenetic trees have been constructed to show relationships among bacteria, mollicutes, and MLOs. It has become possible to group MLO clusters by employing dot-blot hybridization analyses and restriction fragment length polymorphism (RFLP) with specific MLO-DNA fragments. Coincidentally, comparison studies of restriction patterns of DNA fragments amplified from variable regions of 16s RNA have established similar MLO clusters. To make definitive biological characterizations of these microorganisms, however, more research is needed.

Key words: mycoplasma, detection, identification.

INTRODUCTION

Plant pathogenic mycoplasmalike organisms (MLOs) cause diseases of a wide array of economically important plants around the world. In the past twenty five years since their discovery (12), MLOs have been shown to be associated with over 300 plant yellows diseases (35). Symptoms of infected plants include yellowing of the leaves, shortening of internodes, physiological change of the floral parts, such as phyllody and general stunting of the plants. For many years, plant diseases caused by MLOs were thought of as viral origin, but electron microscopy revealed the presence of cell-wall free microorganisms in the phloem. These microorganisms resemble mycoplasmas that cause human and animal diseases and that are classified in the class Mollicutes.

Although morphologically indistinguishable between mycoplasmas, and the plant pathogenic MLOs have not been given a precise taxonomic position yet. The major reason is, up to date, all attempts to culture these organisms *in vitro* have not been successful. Thus, not only information on their nutritional requirements, membrane composition, and genome organization is lacking but methods in detecting and disease diagnosis are often met with difficulty. During the past several years, however, advancements in immunology and biotechnology have greatly improved our methods to study the MLOs with which new investigations can be carried out without relying on a pure MLO culture.

Three areas of research on MLOs have been progressed in recent years: 1. detection of MLOs in infected plants, 2. better understanding of the phylogeny of MLOs, and 3. meaningful grouping of MLOs associated with various plant yellows diseases.

DETECTION AND IDENTIFICATION

MLOs are transmitted from plant to plant in nature by insect vectors. In the host plants, they are restricted to grow in the sieve tubes of the phloem. Generally, MLO titer in herbaceous hosts is higher than that in woody plants in which they are often unevenly distributed. In the past, diagnosis of MLO-associated disease, relying upon symptom identification and vector insect transmission, has been mostly presumptive. Electron microscopic observation of the presence of MLOs in the sieve tubes of infected plant is the only means of positive diagnosis. Disease diagnosis and MLO detection are important for the investigation of epidemiology and the establishing of control measures. Means of detecting and identifying MLOs in their insect vectors and plant hosts, that are rapid, sensitive and specific are therefore urgently needed. Three techniques have been developed for MLO detection. These include monoclonal antibodies, DNA probes and polymerase chain reactions.

Monoclonal antibodies (MAbs) to MLOs that are associated with aster yellows, peach eastern-X disease, maize bushy stunt, grapevine yellows disease, ash yellows and elm yellows were produced in our laboratory (3,5,14,15,19,31). MLO-purification methods were developed (18) and modified (15,19) which have greatly facilitated the immunization process and increased the number of hybridomas that secrete specific MAbs. Indirect ELISA and immunofluorescence (IF) techniques have been developed (31,32) for *in vitro* and *in situ* detection of MLOs. The IF test was improved recently which has reduced the total test time to less than 30 min (15). Other serological methods, such as dot blot immuno-assay (4) and tissue blot immunoassay (33) can also be adapted for MLO detections.

Of great interest in recent years, a new approach has been developed toward detection and diagnosis of MLO-associated disease by the application of recombinant DNA techniques. Randomly cloned fragments of MLO DNA have been used as probes in nucleic acid hybridization assays. Which can it provide a reliable and sensitive means of detecting MLOs in infected plants and vector insects (2,4,7,8,9,16, 17,20,21,22,23,25,28). Although several methods have been used to extract MLO-DNA from the infected plant hosts, bisbenzimide-CsCl buoyant density gradient centrifugation developed by Kollar *et al.* (21) is by far the simplest. It separates MLO-DNA from host plant DNA as a well resolved band due to the low G + C content of the MLO-DNA. The availability of such enriched MLO-DNA facilitates the preparation of genomic DNA probes for disease diagnosis.

In 1988, Saiki and co-workers (39) used the *in vitro* enzymatic gene amplification to detect sickle cell hemoglobin genes in human genome. This technique is also known as the polymerase chain reaction (PCR), and has provided a most sensitive detection of DNA molecules. Deng and Hiruki (10,11) reported that primers synthesized according to the sequence of cloned genomic DNA of clover proliferation (CP) MLO could specifically amplify CP MLO DNA fragments from crude nucleic acids extracted from CP-infected plants. Using the conserved regions as primers in PCR experiments, Ahrens and Seemuller (1) amplified a 558-bp fragment of the 16s RNA gene which was shown to be shared by 17 isolates of MLOs. After 24 cycles of PCR, this amplification product was obtained from all MLO-infected periwinkles but no product was amplified from healthy plant DNA. Although a similar sized DNA product was detected from the healthy DNA after 40 cycles of amplification, it could be distinguished from the MLO fragments by restriction fragment length polymorphism (RFLP) analysis with Alu I digestion. Recently, Chen and co-workers (4) were successful to use PCR in detecting MLOs from grapevines infected with grapevine yellows (GY) MLO. The titer of GY-MLO was extremely low in the grapevines so that results of other detecting methods could be misleading. They used primers designed from a 550-bp genomic DNA of GY-MLO, and successfully a MLO-specific DNA product from as little as 10^{-2} pg of total DNA extracted from diseased plants.

In a comparison study using monoclonal antibodies, DNA probes and PCR for the detection of GY-MLO, Chen *et al.* (4) concluded that immuno-assay with monoclonal antibodies was specific and very rapid for diagnosing infected periwinkles. However, it was useless for grapevines perhaps because the titer of GY-MLO was so low and/or its distribution was so uneven in the plant tissues. The DNA probe has increased the sensitivity of detection. Nevertheless, in the dot blot hybridization analysis, 10 ng of total DNA from infected periwinkles was required for positive detection of GY-MLO. At least 2,000–3,000 fold of total DNA extracted from diseased grapevines was needed for positive signals. In addition, dot blot hybridization was time consuming. PCR, on the other hand, needs only a few molecules of DNA templates. Thus, it provides a significant advantage for detecting target organism in low concentration. In terms of sensitivity, PCR has increased the detectibility of GY-MLO one million times as compared with that of the cloned DNA fragments.

PHYLOGENY STUDIES

The class Mollicutes is a group of prokaryotes that lack a cell wall. The families of mollicutes are distinguished by their morphological characteristics, cholesterol requirements and abillities to metabolize certain compounds. Plant pathogenic mycomplasmalike organisms (MLOs) have been tentatively placed into the class Mollicutes because they, like animal mycoplasmas, are also wall-less prokaryotes. Since MLOs have not been cultured axenically their nutritional requirements are unknown and thus, it is also not possible to classify MLOs with the cholesterol requiring mycoplasmataceae or with the non-cholesterol requiring acholeplasmataceae.

To study the phylogenetic relationships of wall-less prokaryotes many workers have taken the approach of analyzing their ribosomal RNA (rRNA) genes (13,34,36, 37,38,43,44,45). The prokaryotic 16s rRNA gene is highly conserved and the conserved regions are often shared by many divergent microoganisms. Comparative analysis of the 16s RNA partial sequences or the full-length 16s rRNA genes have increased our understanding of the evolution of prokaryotes as well as the phylogenetic relationships among them. Kuske and Kirkpatrick (24) cloned and sequenced the 16s rRNA gene from the severe strain of the western aster yellows MLO (SAY-MLO), which is approximately 1,535 bp long, with a G + C content of 47%. Sears *et al.* (42), working with an MLO pathogen of *Oenothera hookeri* (evening primrose)(O-MLO), isolated and characterized its DNA. They showed that the G + C content of O-MLO DNA is less than 30% and that of 16s rRNA is 48%. Lim and Sears (29) cloned and sequenced the 16s rRNA gene and adjacent regions of the O-MLO for comparison with the analogous sequences of *Escherichia coli, Bacillus subtilis, Mycoplasma capricolum* (an animal mycoplasma) and *Anacystis nidulans* (a bluegreen alga). The plant MLO sequence was most similar (80% homology) to the 16s rRNA of *M. capricolum.* However, when subsets of their 16s rRNA sequences are compiled and compared, *M. capricolum* appeared to be more closely related to *B. subtilis* indicating MLOs and animal mycoplasmas may be only remotely related. When the tRNA gene of O-MLO was used for comparison study, Lim and Sears (29) suggested that there was an early evolutionary divergence of plant pathogenic MLOs and animal mycoplasmas. Accordingly, a phylogenetic tree was constructed (Fig. 1). In addition, they consulted the oligonucleotide catalog to make sequence comparisons among MLO, Mycoplasmataceae, Spiroplasmataceae, Acholeplasmataceae and Clostridial bacteria. The 16s rRNA signature of the MLO was most similar to that of the Acholeplasma suggesting that the MLOs were more closely related to the acholeplasmas than to mycoplasmas. Their result was further substantiated by the study of Kuske and Kirkpatrick (24). Phylogenetic relationships between SAY-MLO and other prokaryotes were determined by 16s rRNA gene sequence analyses. The SAY-MLO 16s rRNA gene sequence was most similar to that of O-MLO (99.6%), followed by the western X-MLO. The three MLO sequences were more homologous to each other than to the 16s rRNA sequence of any other prokaryote. A phylogenetic dendrogram was constructed (Fig. 2) which also showed

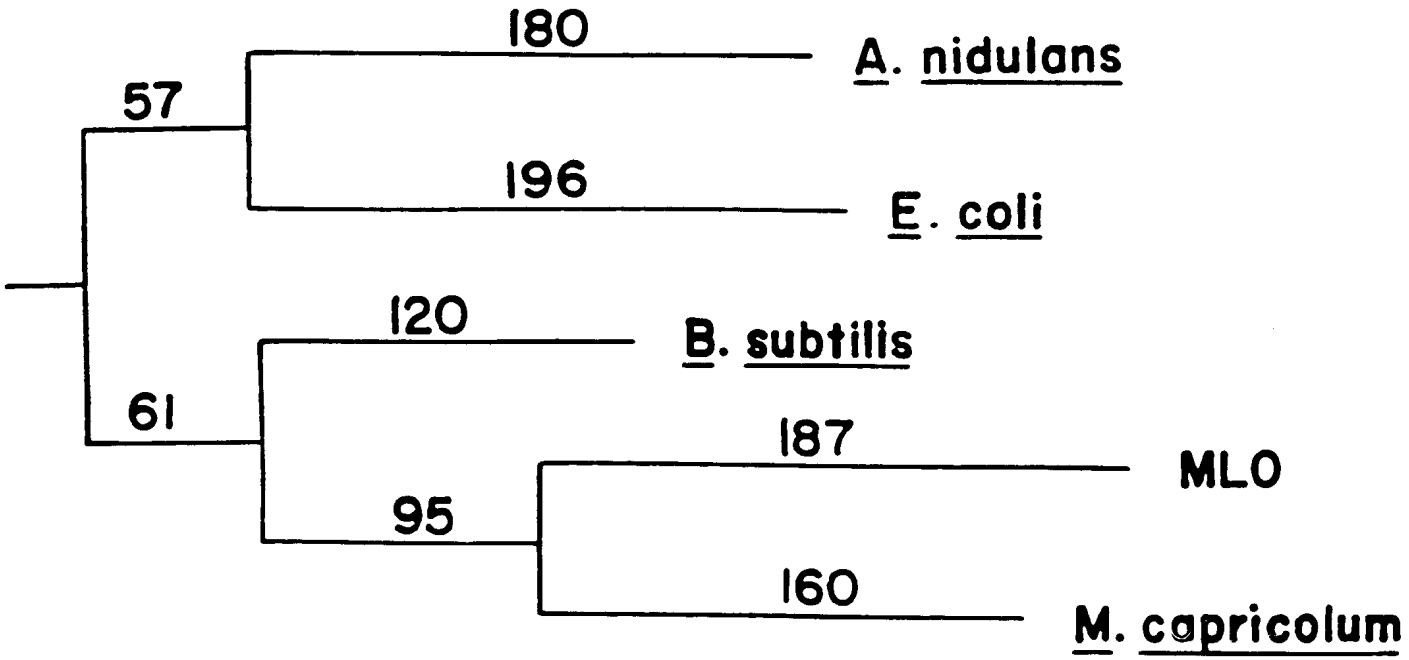

Fig. 1. Phylogenetic tree according to analyses of the 16s rRNA gene sequences among *E. coli, Bacillus subtilis, M. cupmicolum* and *Anacystis nidulans.* The number of each branch indicates the number of base substitutions in the 16s rRNA on the branch. (Copy from Lim and Sears, J. Bacteriol 171:5901-5906, 1989)

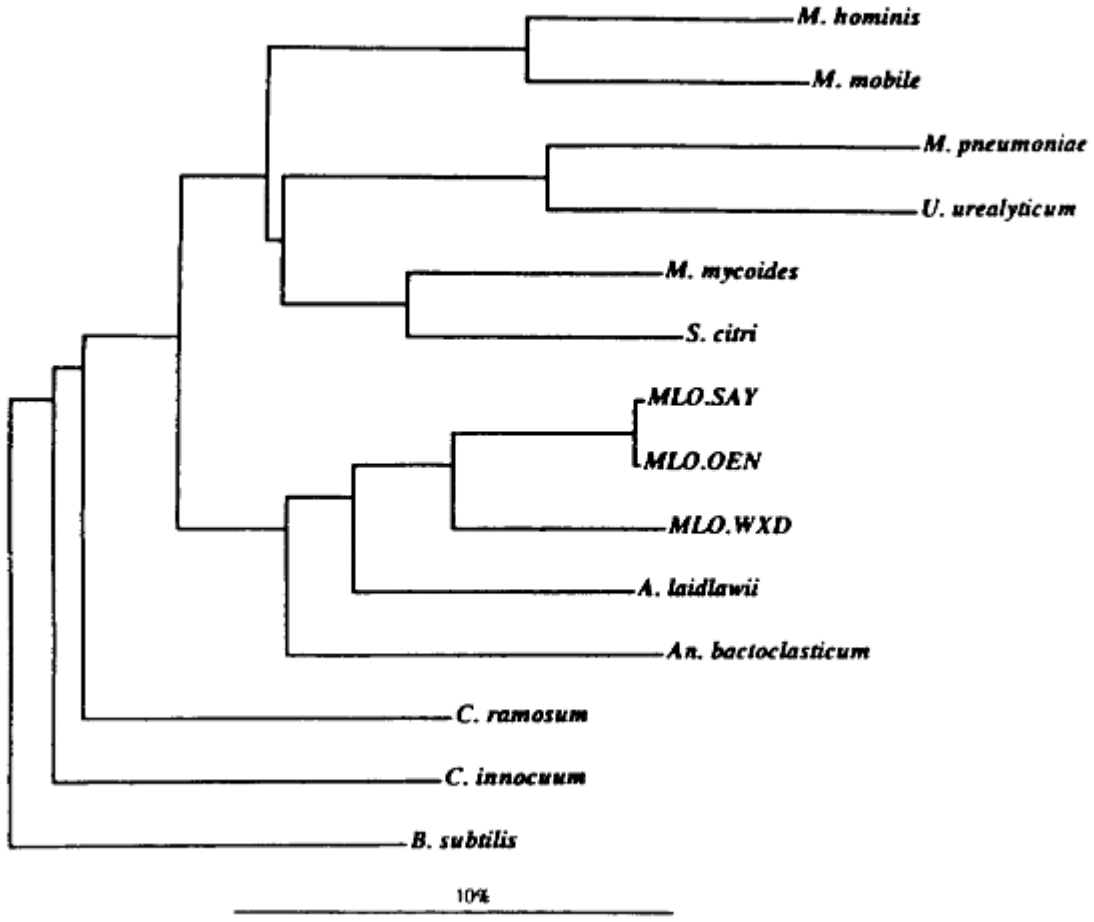

Fig. 2. Phylogenetic dendrogram constructed from evolutionary distance matrices by analyzing 16s rRNA sequences of MLOs and comparing them with other Mollicutes and bacteria (Copy from Kuske and Kirkpatrick, Int. J. Stst. Bacteriol. 42:226-233, 1992)

a close evolutionary relatedness between MLOs and acholeplasmas.

Recently, Lim and Sears (30) have cloned and sequenced a segment of a ribosomal protein operon from O-MLO and *Acholeplasma laidalawii*. The amino acid sequences from the two protein genes also supported their earlier study with 16s rRNA gene analyses that the MLOs were more closely related to Acholeplasmas than to Mycoplasmas. In addition, they have examined the codon usage of protein genes. Unlike the animal mycoplasmas and spiroplasmas, the MLO and *A. laidlawii* probably do not use UGA as tryptophan codon. Whether or not UGA is used as a termination codon is difficult to conclude.

GROUPING OF MLOs

The use of cloned MLO DNA fragments as molecular probes has not only permitted detection and identification of MLOs in their plant and insect hosts but also investigation of interrelations among MLOs associated with various yellows diseases. Lee and Davis employed reciprocal dot hybridizations using cloned DNA probes from a number of MLOs to study the genetic relatedness among these organisms. Based on the shared sequence homology they proposed the aster yellows (AY) MLO Strain Cluster (25) to include the known eastern and western AY strains and those yellows disease agents under designated names other than AY. Recently, in addition to the dot blot hybridization Lee *et al.* (27) analyzed 15 MLOs in the AY strain cluster with Southern hybridization and restriction fragment length polymorphism (RFLP) using AY-MLO DNA probes. A total of 22 cloned chromosomal DNA probes from 3 different MLOs were used for RFLP analyses to differentiate subclusters. From these results a dendrogram was constructed according to the similarity coefficients between various AY strains (see Fig. 3). They could be broadly grouped into 3 types, each of which exhibited a unique RFLP pattern. Thus, these strains were closely related to one another but distantly related to other MLOs which were not in the AY-MLO cluster.

Using the same broad based reciprocal dot hybridization analyses, Lee and Davis (26) compared various different yellows disease agents and obtained several genomic strain clusters: aster yellow MLOs, peach-X disease MLOs, ash yellows MLOs, clover proliferation MLOs and elm yellows MLOs. Ahren and Seemuller (1) investigated 17 isolates of plant yellows

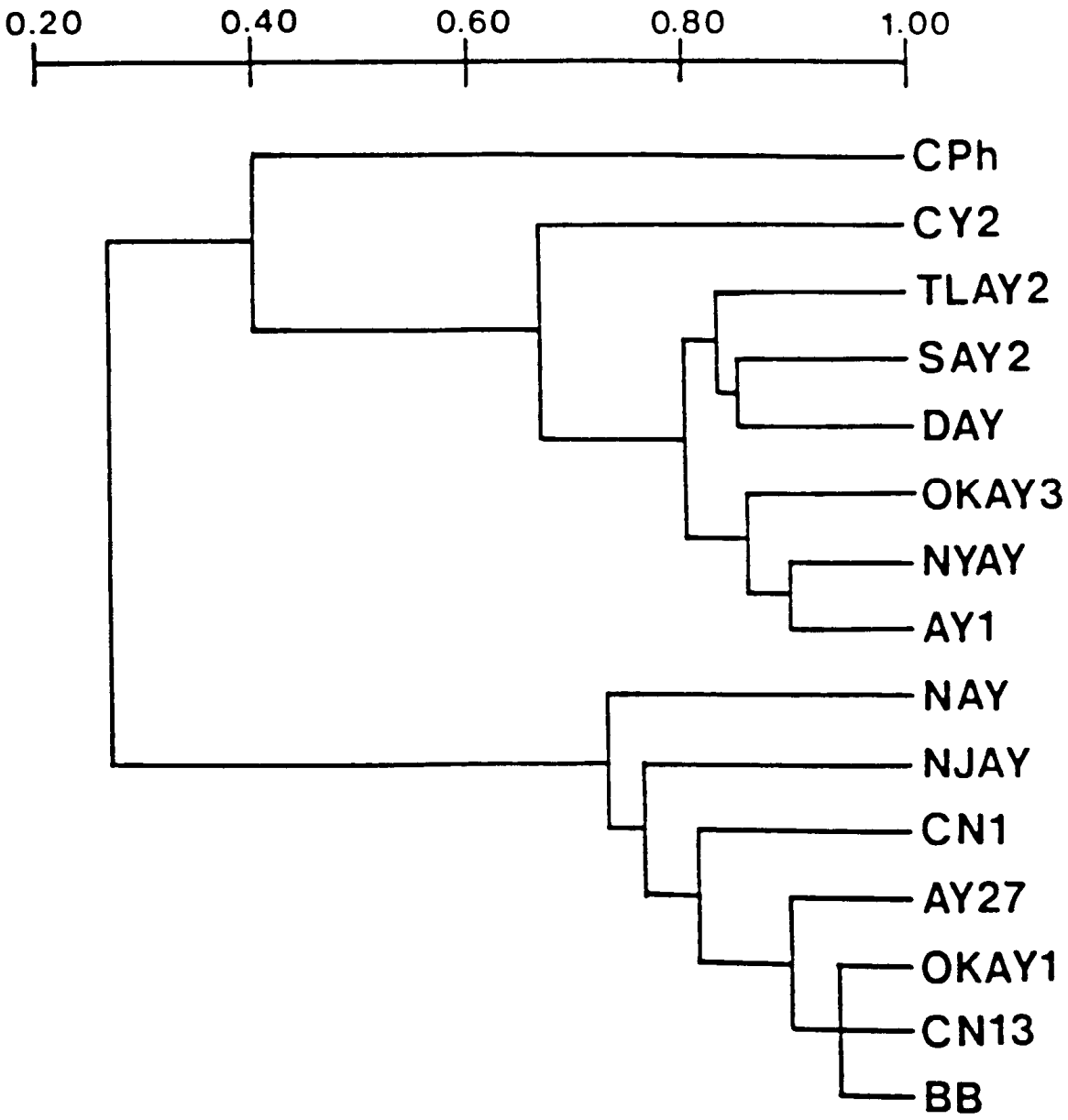

Fig. 3. Dendrogram obtained by cluster analysis of similarity coefficients, F. Derived from restriction fragment length polymorphism analysis of strains in the aster yellows MLO strain cluster. Scale refers to the similarity index. Cph, clover phyllody; Cy2 chrysanthemum yellows; TLAY2, Tulelake aster yellows; SAY2, severe aster yellows; DAY, dwarf aster yellows; OKAY and OKAY3, strains of Oklahoma aster yellows; NYAY, New Yord aster yellows; AY 1, Maryland aster yellows; MAY, eastern strain of aster yellow; NJAY, New Jersey aster yellows; CN 1 and CN 13, strains of periwinkle little leaf; AY 27, Alberta aster yellows; BB, tomato big bud. (Copy from Lee *et al.* Phytopathology 82:977-986, 1992).

MLOs maintained in periwinkle and 9 from field grown woody plants. Using oligonucleotide primers designed from conserved regions of the 16s rRNA gene of the O-MLO in PCR, they were able to amplify a fragment of MLO DNAs prepared from all the diseased plants. *Alu* I digestion of the amplified DNA followed by PAGE showed four distinct RFLP patterns. When analyzing the *Alu* I restriction sites, the four patterns corresponded to four groups of restriction maps. Group I was represented by O-MLO, apple proliferation MLO (isolate AT), aster yellows MLO, clover phyllody MLO (Europe) and sandal spike MLO. Group II was represented by cherry molieres disease MLO and stolbur disease MLO. Group III was represented by apricot chlorotic leaf roll MLO, grapevine flavescence doree' MLO, plum leptonecrosis MLO, peach yellow leaf roll MLO (western X-disease MLO), and blueberry witches' broom MLO. Group IV was represented by ash yellows MLO and elm yellow MLO. Schneider and coworkers (40,41) have extended this investigation to 49 MLOs. When the PCR products were digested with *Alu* I and *Rsa* I restriction endonucleases, 7 different restriction patterns were obtained. Group I consisted of 75% of the MLOs studied, of which most were isolated

from herbaceous hosts. They included aster yellows MLOs and clover phyllody MLO (European). Group II included apricot chlorotic leaf roll and plum leptonecrosis MLOs. Group III was represented by ash yellows MLO and group IV, elm yellows MLO. Group V included Apple proliferation MLO and MLOs occurring in several stone fruit trees in Europe. Group VI was represented by the Western X-disease MLO, blueberry witches' broom MLO, grapevine yellows MLO (Italian isolate) and safflower phyllody MLO. Group VII was represented by the sugarcane white leaf MLO and Bermuda grass white leaf MLO. According to sequence analyses and comparisons, the 16s rRNA gene fragment of the same group exhibited 97.8% to 99.5% homology while those of different groups were 89.6% to 92% homologous.

CONCLUSION

There is a clear requirement to detect and identify plant pathogenic MLOs in many situations as new yellows diseases being discovered and their epidemiological information urgently needed. In the past, vector relationships and disease symptom expressions were the basis for disease diagnosis without pathogen identification. Recently, increased knowledge in the biology of mollicutes has helped plant pathologists to improve ways to purify the MLOs for serological tests. The advent of modern biotechnology has made it possible to isolate MLO-specific DNA from infected plants and insect vectors. Thus, not only MLO detection methods have been greatly improved but tentative classification or grouping of various yellows agents became possible. Comparison of MLOs with other prokaryotes in the class Mollicutes has revealed new phylogenetic information. It appears that MLOs are closely related to acholeplasmas and both of them probably belong to the Anaeroplasma subgroup or clade (Fig. 2). Since MLOs are remotely related to the animal mycoplasmas the name "mycoplasma-like" may be a misnomer. In 1992, a proposal that the plant pathogenic MLOs be named "Phytoplasmas" was presented to the International Committee on Systematic Bacteriology (ICSB) Subcommittee on the Taxonomy of Mollicutes at the 9th Congress of International Organization for Mycoplasmology (IOM) in Ames, Iowa. Nevertheless, the ultimate characterization of this group of microorganisms will probably have to wait until they can be cultured *in vitro*.

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摘　　要

陳澤安．1993．植物病原菌質學之研究近況．植病會刊2:195-202．(美國紐澤西州羅格斯大學植物病理系)

植物病原似菌質體(MLOs)在世界各地之許多重要經濟植物上均可造成病害，此類病害因常造成植物之黃化、矮化、枝條增生，及花器葉化等病徵，故此類病害多歸爲“黃萎型病害”。植物病原似菌質體與動物性菌質體一樣均不具細胞壁，但因植物病原似菌質體迄今仍無法被培養，故其研究進展便相對的較爲遲緩，復因此群微生物在養份需求，胞膜成份，基因組成，及偵測鑑定技術等方面之資訊均不完備，而致植物病原似菌質體之分類工作仍無法進行。直至近幾年來，拜免疫學及生物科技長足進展之賜，許多更佳之研究方法也陸續的被延用，且大幅的提昇我們對似菌質體在分類及演化上之瞭解。最近，對似菌質體之重要研究進展包括：(1)有關偵測及鑑定此群微生物之技術已有進步；(2)似菌質體間之演化相關性已較爲明瞭；(3)分子生物學之資訊已被用於各植物病原似菌質間之分群與歸類工作上。在對植物病原似菌質體之快速及精確偵測方法之建立中已先後研發出單源(元)抗體，DNA探針及聚合酶連鎖反應技術(PCR)。而藉由對16s rRNA之核苷酸序列之分析，已能在細菌，菌質體，似菌質體之間建立其在演化樹上之相關性及相關位置。而利用特定似菌質體之DNA片段從事點漬染及內鑑識酶酵解長度多樣性(RFLP)之分析，對各似菌質體進行分群之工作已然可行，而且此些分群之結果與利用16s rRNA之PCR增殖片段的酵解圖譜所得之分群結果互爲一致。然而欲對此群似菌質體之生物特性作更徹底及明確之瞭解，則需更多之努力及探討。

關鍵詞：似菌質體。