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REVIEW

Phytoplasma and phytoplasma diseases: a review of recent research

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Summary. Numerous yellows-type diseases of plants have been associated with wall-less prokaryote pathogens – phytoplasmas over the last 40 years. These pathogens are not grown in axenic culture till now so that advances in their study are mainly achieved by molecular techniques. Severe disease epidemics associated with phytoplasma presence have been described worldwide. These include coconut lethal yellowing in Africa and the Caribbean, grapevine yellows in major viticultural areas and various diseases affecting stone and pome fruit plants. Phytoplasma-infected plants exhibit symptoms suggesting a profound disturbance in the normal balance of growth regulators and also yellows symptoms, but very often the symptomatology is not diagnostic. Detection and characterization of phytoplasmas infecting different plant species are now possible with molecular methods, based on the study of 16S rDNA polymorphisms. Molecular diversity of phytoplasmas is also demonstrated by studying genes coding the ribosomal proteins S3, tuf, SecY, amp, imp and other genes. Four phytoplasma genomes have been fully sequenced, including those of two '*Candidatus* Phytoplasma asteris' strains, and those of strains of '*Ca*. P. mali' and '*Ca*. P. australiense'. Three of these genomes contain large amounts of repeated DNA sequence, and the fourth carries multiple copies of almost 100 genes. Considering that phytoplasmas have unusually small genomes, these repeats might be related to their transkingdom habitat and to their pathogenic activity. An outlook of recent findings in the field is also reported.

Key words: phytoplasma, plant-host interaction, detection, classification, plant diseases.

Introduction

Numerous yellows-type diseases of plants were once thought to be caused by viruses in view of their infective spread, their symptomatology, and the fact that they were transmitted by insects. The first demonstration that the etiological agents of yellows diseases could be wall-less prokaryotes rather than viruses caught the field of plant pathology by surprise more than forty years ago (Doi *et al.*, 1967). This discovery of a new group of plant pathogens related to bacteria prompted new studies which revealed that pleomorphic, wall-less prokaryotes occurred in the phloem of many plant species affected with yellows-type diseases. The term mycoplasma-like organisms (MLOs) was first used for these micro-organisms due to their morphological and ultrastructural similarity to mycoplasmas. MLOs and mycoplasmas both belong to the *Mollicutes* class, since they are prokaryotes without cell walls. However, in contrast to mycoplasmas, which cause an array of disorders in animals and humans, phytopathogenic MLOs resisted all attempts to culture them *in vitro* in cell-free media (Lee and Davis, 1986). Following the application of molecular technologies, however, the enigmatic status of MLOs amongst the prokaryotes was finally resolved. This led to the application of the trivial name "phytoplasma", and eventually to the designation of a new taxon for these organisms, named '*Candidatus* phytoplasma' (IRPCM, 2004).

Plants infected with phytoplasmas exhibit symptoms suggesting a profound disturbance in the normal balance of growth regulators, leading to: virescence/phyllody (development of green leaf-like structures instead of flowers), sterility of flowers, proliferation of axillary buds result-

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ing in "witches' broom" symptoms, abnormal internode elongation, and generalized stunting (Bertaccini, 2007). Phytoplasmas are transmitted by insects belonging to the families Cicadellidae, Cixidae, Psyllidae, Delphacidae and Derbidae (Weintraub and Beanland, 2006).

Recent molecular data on phytoplasmas have provided considerable insight into their molecular diversity and genetic interrelationships, which has in turn served as a basis for several comprehensive studies on phytoplasma phylogeny and taxonomy (Hogenhout *et al*., 2008). Some investigations, particularly those employing the sequence analysis of 16S rDNA, have shown that phytoplasmas constitute a coherent, genus-level taxon. In the monophyletic phytoplasma clade, groups and subgroups have been delineated, many of which are now considered species under the provisional status '*Candidatus*' for incompletely described prokaryotes (Murray and Stackebrandt, 1995). Several provisional species have been described, and rules for future putative species delineation have been defined (IRPCM, 2004).

The first comprehensive phytoplasma classification scheme was based on restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified 16S rDNA (Lee *et al.*, 1998a; 2000). This approach provided a reliable tool for broad differentiation among phytoplasmas. The system has classified phytoplasmas into 19 groups and more than 40 subgroups, and has become the most comprehensive and widely accepted phytoplasma classification system (Lee *et al.*, 2004a, b, 2006b; Arocha *et al.*, 2005; Al-Saady *et al.*, 2008).

Sensitive and accurate detection of these micro-organisms is a prerequisite for the study and management of phytoplasma-associated diseases. After their discovery, phytoplasmas were initially difficult to detect due to their low concentration, especially in woody hosts, and their erratic distribution in the sieve tubes of infected plants (Berges *et al.*, 2000). Electron microscopy represented the indexing procedure for phytoplasmas, together with graft transmission to healthy indicator plants. DNA-specific dyes such as DAPI were also applied. However, all these techniques were not able to differentiate phytoplasmas. Later, protocols for the production of enriched phytoplasma-specific antigens were developed, thus introducing serological detection techniques for the study of these pathogens in plants or insect vectors. Serological techniques have gained little application, however, due to difficulties in the production of antisera. Phytoplasma detection is now routinely carried out by different nucleic acid techniques based on the polymerase chain reaction (PCR). These techniques, developed in the last 20 years, are adequate for detecting phytoplasmas in both plant material and insect vectors. They are helping to prevent the spread of these diseases and reduce their economic impact.

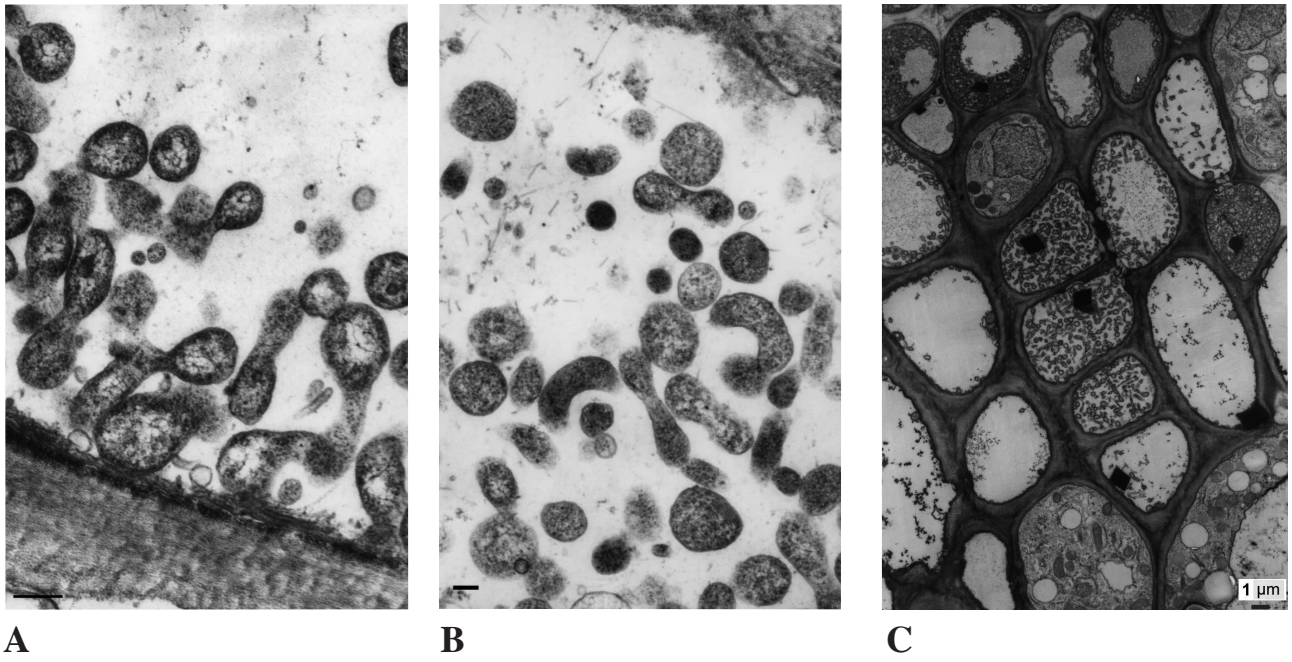
Diseases associated with phytoplasmas occur worldwide in many crops, although individual phytoplasmas may be limited in their host range or distribution. More than 300 distinct plant diseases have been attributed to phytoplasmas, affecting hundreds of plant genera (Hoshi *et al.*, 2007). Many of the economically important diseases are those affecting woody plants, including coconut lethal yellowing, peach X-disease, grapevine yellows, and apple proliferation. Herbaceous plants are also are severely affected by phytoplasma diseases, which in their epidemic phases can completely destroy cultivations of the hosts.

Phytoplasmas are obligate parasites occurring in plant phloem tissue and in several insects. They probably diverged from Gram-positive bacteria, and belong to the '*Candidatus* Phytoplasma' genus (IRPCM, 2004). They are pleomorphic (Figure 1), with diameters less than 1 micrometer, and they have very small genomes (680–1,600 kb).

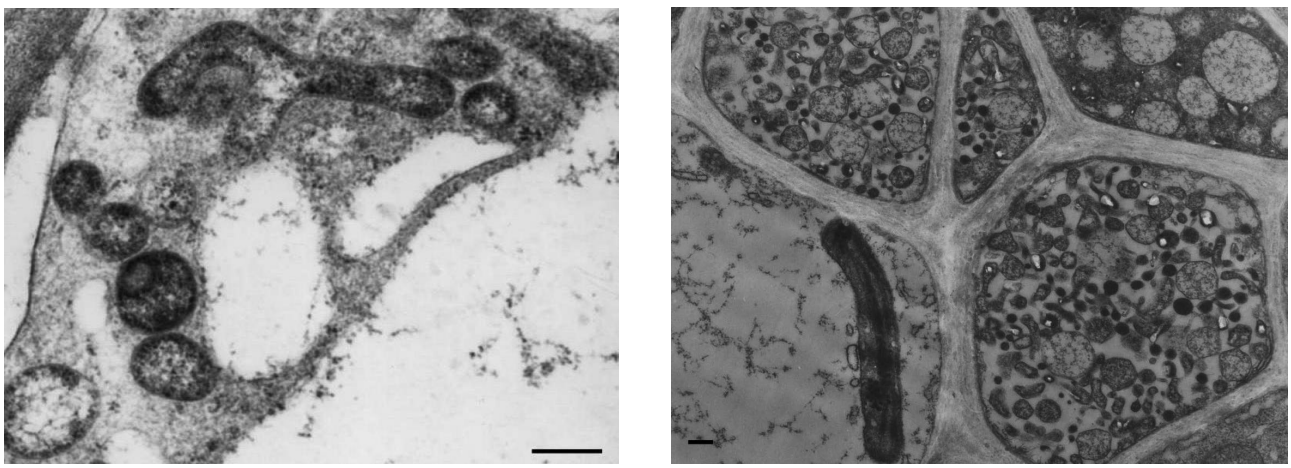
The genomes of phytoplasmas are very small when compared with those of their ancestors (walled bacteria in the *Bacillus*/*Clostridium* group) because they lack several pathways for the synthesis of compounds necessary for their survival. These substances must be obtained from host plants and insects (Bai *et al.*, 2006).

Phytoplasma diseases and their economic importance

Phytoplasmas are associated with plant diseases in several hundred plant species, including many important food, vegetable, and fruit crops; ornamental plants; and timber and shade trees. The list of diseases caused by phytoplasmas continues to grow; many newly emerging diseases, known diseases with an uncertain etiology, and diseases with diverse geographic distribution, have been identified in recent years as being associated with phytoplasmas. Examples are the citrus huanglongbing disease that is associated with aster yellows-related phytoplasmas in China (16SrI) (Teixeira *et al*., 2009) and with pigeon pea witches' broom-related phytoplasmas (16SrIX) in Brazil (Chen *et al*., 2008). These are only some of the most recently described diseases on the list of reported phytoplasma diseases, confirming the widespread occurrence of similar symptoms associated with diverse phytoplasmas. This situation requires molecular identification of the



A **B** **C**



D **E**

Figure 1. A-E, Electron micrographs of cross sections of sieve tubes showing variations in the size and shape of phytoplasmas infecting plants. Bar=200 nm unless specified.

pathogen in order to identify the respective phytoplasma and its vector: only in this way can the disease impact in the different ecosystems be reduced.

Many cultivated plants are subject to phytoplasma infection, both in countries where agriculture is not advanced and in countries where agriculture is highly industrialized. Phytoplasmas severely affect herbaceous and woody plants (Bertaccini, 2007), and are the primary limiting factors for many important crops all over the world. For example, the aster yellows phytoplasma causes major economic losses of vegetable crops (including lettuce, carrot, and celery) and ornamental plants (including gladiolus, hydrangea, China aster, and purple coneflower) in North America and Europe. During the 1990s, peach yellows and X-disease caused heavy losses in peach and cherry orchards in the United States. In several regions of the Middle East, citrus species are affected by phytoplasma diseases. For example, lime witches' broom has almost eliminated traditional lime production in the Sultanate of Oman and in Iran.

Rice yellow dwarf severely affects rice crops in several regions of southeastern Asia. Potato witches' broom and maize bushy stunt cause yield losses of potato and corn respectively in central and South America. Sweet potato witches' broom and related diseases cause the loss of sweet potato crops in Asia and Australia. Grapevine yellows severely affects grapevine production in Europe and in Australia, and possibly elsewhere. Pear decline, apple proliferation, European stone fruit yellows and other fruit declines associated with phytoplasmas reduce both production and quality of fresh fruit in Europe. Legume diseases such as peanut witches' broom, sesame and soybean phyllody cause considerable losses of these crops in Asia.

Forests are also severely damaged by diseases associated with phytoplasmas. Examples are paulownia witches' broom, coconut lethal yellowing, and mulberry dwarf, which kill forest trees in a number of continents. Elm yellows and witches' broom have almost eliminated historical and new elm plantations in Europe and in North America; specifically, elm trees surviving the severe epidemic of Dutch elm disease were killed by successive phytoplasma infections. Because of these diseases, the movement of many of the affected plant species should be internationally restricted by quarantine regulations (Lee *et al.*, 2000).

Symptoms and spreading of phytoplasma diseases

A common symptom resulting from phytoplasma infection is phyllody, a condition in which a plant produces leaf like structures instead of flowers. Evidence suggests that in stolbur-infected tomatoes, the phytoplasma deregulates a gene involved in flower formation (Pracros *et al.*, 2006). Leaf yellowing, one of the most common symptoms associated with the presence of these organisms, is thought to be caused by a modification in carbohydrate synthesis and transportation. Photosynthesis, especially photosystem II, is inhibited in many phytoplasma-infected plants and the breakdown of chlorophyll and carotenoids, together with inhibition of their biosynthesis, has been reported (Bertamini and Nedunchezhian, 2001). The induced expression of sucrose synthase and alcohol dehydrogenase I genes in phytoplasma-infected grapevine plants grown in the field has recently been demonstrated (Hren *et al.*, 2009). Phytoplasma-infected plants may also show virescence, the development of green flowers due to the loss of pigment in the petal cells (Lee *et al.*, 2000). Sometimes the flowers become sterile. Many phytoplasma-infected plants acquire a bushy or witches' broom appearance due to changes in their normal growth patterns. These are mainly related to the loss of apical dominance causing the proliferation of axillary shoots and decreased internode length (Figure 2). Phytoplasmas may cause many other aspecific symptoms resulting from stresses to which the host plants are subjected. In some cases a phytoplasma is beneficial in commercial production. An example is phytoplasma-infected poinsettia plants, which each have more than one flower and which are smaller, making it possible to grow them in pots (Bertaccini *et al.*, 1996; Lee *et al.*, 1997a).

Phytoplasmas are mainly spread by insects in the families Cicadellidae (leafhoppers), Fulgoridae (planthoppers), and Psyllidae (psyllids), which feed on the phloem tissues of infected plants. The host range of phytoplasmas is therefore strongly dependent upon the insect vectors. Phytoplasmas possess a major antigenic protein that makes up the majority of their cell surface proteins, and this has recently been shown to interact with microfilament complexes of the intestinal muscles of insects. This protein is believed to be important for both transmission and infection (Suzuki *et al.*, 2006; Hoshi *et al.*, 2007). Phytoplasmas may overwinter in insect vectors or in perennial plants and interact in various ways with insect hosts: examples of both reduced and enhanced fitness of the phytoplasmas while they are in the vectors have been reported (Christensen *et al.*, 2005). Phytoplasmas enter the insect through the stylet, then move through the intestine and are absorbed into the haemolymph. Subsequently the salivary glands are colonized, a process that can take up to several weeks. The time between a phytoplasma being taken up by the insect and its reaching an infectious titer

Figure 2. Symptoms of phytoplasmas in different plant species. A–F: A. periwinkle showing flower virescence from Colombia infected with 16SrIX-C phytoplasmas (Duduk *et al*., 2008); B. carrot infected with aster yellows phytoplasmas from Serbia (Duduk *et al*., 2009a); C. purple coneflower from Italy infected with 16SrIX-C phytoplasmas (Bertaccini *et al*., 2009); D and E. tomato infected with stolbur phytoplasmas from Italy; F and G. corn infected with stolbur phytoplasmas (Duduk and Bertaccini, 2006);

G

H

J

K

Figure 2. G–K: G. corn infected with stolbur phytoplasmas (Duduk and Bertaccini, 2006); H. lime witches' broom ('*Ca*. P. aurantifolia') on a lime tree in Oman; J. *Sophora japonica* from China infected with '*Ca*. P. japonicum' and stolbur phytoplasmas (Duduk *et al*., 2009b); K grapevine flavescence dorée symptoms in Serbia.

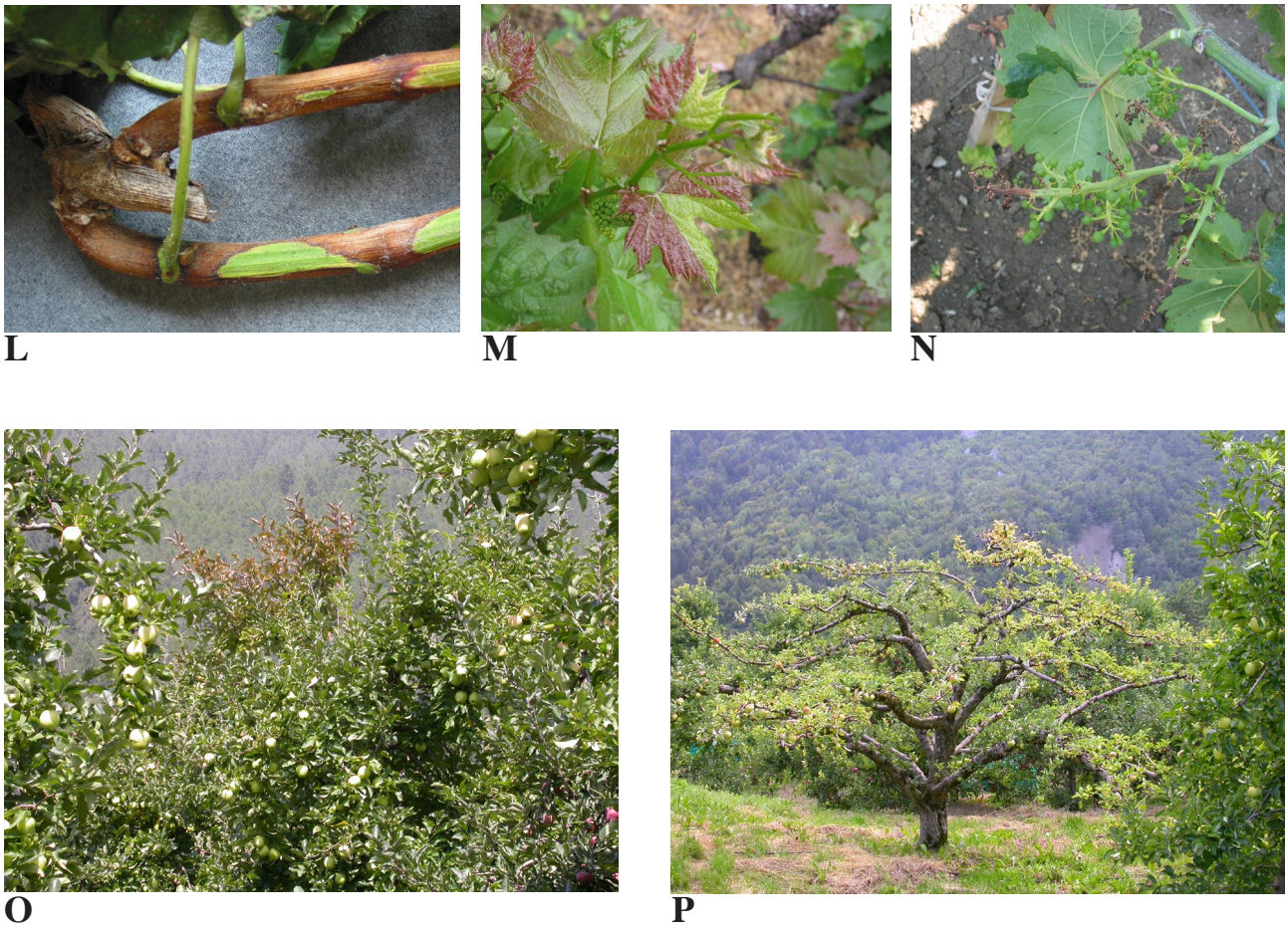


Figure 2. L–P: L, M, N, grapevine flavescence dorée symptoms in Serbia; O and P. apple proliferation disease symptoms in northern Italy.

in the salivary gland is defined as its latent period. Some phytoplasma transmissions in insects have been reported to be transovarial, such as the insect/disease combinations *Scaphoideus titanus*/aster yellows (Danielli *et al*., 1996; Alma *et al.*, 1997); *Hishimonoides sellatiformis/*mulberry dwarf (Kawakita *et al*., 2000)*, Matsumuratettix hiroglyphicus* (Matsumura)/sugarcane white leaf (Hanboonsong *et al.*, 2002), and *Cacopsylla melanoneura* apple (Tedeschi *et al.*, 2006).

Phytoplasmas may also be transmitted from infected to healthy plants through the parasitic plant dodder (*Cuscuta* sp.). Experimental transmission of a phytoplasma from infected to healthy dodder of the same or different species, is one of the main way by which experimental phytoplasma transmission is achieved. Recently the possibility that phytoplasmas were transmitted by seed has also been investigated. This type of transmission was first suspected in the spread of coconut lethal yellowing (Cordova *et al.*, 2003). Studies on alfalfa (*Medicago sativa*) in Oman cultivations severely affected by phytoplasmas further showed evidence of phytoplasma transmission by seed. Seeds from phytoplasma-infected lime (*Citrus aurantiaca*) and tomato (*Lycopersicum esculentum*) from Oman and Italy respectively were allowed to germinate under sterile conditions, and tested at several growth stages. Some of these seeds contained phytoplasmas belonging to ribosomal groups 16SrI, 16SrXII and 16SrII (Khan *et al*., 2002; Botti and Bertaccini, 2006). Phytoplasmas can also be spread via vegetative propagation such as the grafting of infected plants onto healthy plants, propagation through cuttings, micropropagation and any other method to multiply plant material that avoids sexual reproduction.

Phytoplasmas move within plants through the phloem from source to sink and they are able to pass through sieve tube elements in phloem tissues (Christensen *et al.*, 2004). Several studies have showed uneven phytoplasma distribution in host plants, and seasonal fluctuations of the pathogens in woody hosts. Generally, levels were low in the roots (sink organ) and moderate in the stems. The highest titer was found in source organs (mature leaves) (sometimes with a titer ≈40 times higher than that of the roots). In sink leaves, phytoplasma concentration is low or below the limit of detection. Variation in the amount of phytoplasma DNA between individual plants propagated from one infected parent plant has also been reported (Christensen *et al.*, 2004). For deciduous woody plants it has been suggested that phytoplasmas disappear from the aerial parts of trees during the winter and survive in the root system to re-colonize the stem and branches in spring. Waterworth and Mock (1999), however, detected phytoplasmas in the wood of dormant fruit tree scions collected during winter. Phytoplasmas have also been reported in the aerial parts of pear cultivars and *Prunus* spp. in winter (Jarausch *et al.*, 1999; Errea *et al.*, 2002).

Detection and identification

Before the application of molecular techniques, detection of phytoplasmas in diseased plants was difficult. The diagnostic techniques commonly used were therefore observation of symptoms, insect or dodder/graft transmission to host plants, and electron microscopy for observation of ultra-thin sections of the phloem tissue.

Serological diagnostic techniques for the detection of phytoplasma began to emerge in the 1980s with ELISA based methods. In the early 1990s, PCR coupled with RFLP analysis allowed accurate identification of different strains and species of phytoplasma. The disappearance of symptoms in some cases after antibiotic (tetracycline) treatment provided additional evidence that phytoplasmas are agents of several plant diseases (Ishie *et al.*, 1967). Phytoplasma strains were initially differentiated and identified by their biological properties, such as the similarity of the symptoms they caused in infected plants, plant hosts, and insect vector ranges. The determination of biological properties was laborious and time-consuming, and often the results were inconclusive.

Polyclonal and monoclonal antisera were produced to detect phytoplasmas. Some are still commercially available for economically important phytoplasma-associated diseases such as flavescence dorée and apple proliferation. Serological tools have also been used successfully to detect different phytoplasmas in leafhopper vectors or potential vectors. These tools include immunofluorescence, immunosorbent electron microscopy, dot blot and ELISA. In more recent years, antibodies to partial sequences of the major immunodominant proteins of some phytoplasmas have been prepared (Berg *et al.*, 1999; Blomquist *et al.*, 2001; Hong *et al.*, 2001; Mergenthaler *et al.*, 2001). These proteins have been expressed also as fusion proteins in *Escherichia coli* by cloning immunodominant membrane protein genes of phytoplasmas and obtaining their *in planta* expression (Kakizawa *et al*., 2009).

Molecular detection

In the 1990s, following the first cloning of phytoplasma DNA (Kirkpatrick *et al*., 1987), nucleic acid-based probes (randomly cloned DNA or its complementary RNA) were applied in assays to detect and differentiate phytoplasmas in plants and vectors. In the same years, probes based on cloned phytoplasma-specific chromosomal and extrachromosomal DNAs provided the first evidence of genetic differences in phytoplasma DNA between phytoplasma strains derived from different plant hosts and from different geographical locations. PCR assays using primers based on cloned DNA fragments (non-ribosomal DNAs), specific to a given phytoplasma, provided sensitive as well as specific means for phytoplasma detection. In contrast, PCR assays using generic or broad-spectrum primers based on conserved sequences (e.g. 16S rRNA, ribosomal protein, *tuf*, 16S-23S spacer) allowed detection of a wide array of phytoplasmas associated with plants and insects.

The success of PCR in detecting phytoplasma in field-collected samples largely depends on obtaining total nucleic acid preparations of good quality and enriched with phytoplasma DNA, but this has always been difficult (Firrao *et al.*, 2007). The amount of phytoplasma DNA is lower than 1% of total DNA extracted from tissue (Bertaccini, 2007). Different protocols for total DNA extraction have been reported for the detection of these plant pathogens. The main goal of each protocol has been to concentrate phytoplasma DNA while reducing enzyme-inhibitory plant polyphenolic and polysaccharide molecules. This is generally obtained by including a phytoplasma enrichment step in the nucleic acid extraction procedure. Nested-PCR assay, designed to increase both sensitivity and specificity, is however necessary for the amplification of phytoplasma DNA from samples having unusually low titers or inhibitors that may interfere with PCR efficacy (Gundersen *et al.*, 1994). Nested-PCR is performed by preliminary amplification using a universal primer pair followed by a second amplification using a second universal primer pair. By using a universal primer

pair followed by PCR using group-specific primer pairs, nested-PCR can detect phytoplasmas present in mixed infections (Lee *et al.*, 1994; 1995).

The design of primers based on conserved sequences such as the 16S rRNA gene, ribosomal protein gene operon, *tuf* and *SecY* genes has been a major breakthrough in the detection, identification, and classification of phytoplasmas (Gundersen *et al.*, 1996; Schneider *et al.*, 1997; Marcone *et al*., 2000; Martini *et al*., 2002, 2007; Wei *et al*., 2004a). Putative phytoplasmas are now routinely differentiated on the 16S rRNA gene by means of RFLP analysis of PCR-amplified DNA sequences using a number of endonuclease restriction enzymes (Lee *et al.*, 1998a; b). Because the RFLP patterns characteristic of each phytoplasmas are conserved, unknown phytoplasmas can be identified by comparing their patterns with the available RFLP patterns of known phytoplasmas without the need to analyse all the representative reference phytoplasmas.

The continuous effort to improve diagnostic procedures is aimed at devising quicker and more economic and robust methods. Sensitivity is not just an issue *per se*, as the current nested PCR protocols are extremely sensitive, but it is desirable to achieve high levels of sensitivity without the risk of false positive results, which can be associated with nested PCR. The recent introduction of diagnostic assays based on real-time PCR fulfils these requirements: due to its high sensitivity and the direct reading of its results, which reduces the risk of amplicon contamination and the need for a gel-based post PCR analysis.

Classification

No phytoplasma culture has yet been achieved in a cell-free medium. Phytoplasmas cannot be differentiated and classified using the classical biophysical and biochemical phenotypic criteria that are routinely used for cultivable micro-organisms. Reference phytoplasma strain collections maintained in periwinkle are however available for research and classification purposes (Bertaccini *et al*., 1992).

In the 1980s and early 1990s, improved serological and nucleic acid-based assay techniques provided new insights into the diversity and genetic interrelationships of phytoplasmas. With these techniques, several phytoplasma groups could be clearly distinguished by their genomic DNA sequences. In order to achieve a general and reliable system of phytoplasma detection and identification, molecular tools such as PCR/RFLP and nested-PCR on the conserved (16SrDNA) ribosomal phytoplasma region were developed and applied to general classification (Lee *et al.*, 1998a; Seemüller *et al.*, 1998).

The phytoplasma 16Sr groups (Table 1) have been shown to be consistent with the groups (clades) defined by phylogenetic analysis of near-full-length 16S rRNA gene sequences, indicating that the RFLP-based groups are phylogenetically valid. The approach using RFLP analyses of PCR amplified 16S rDNA provides a simple, reliable and rapid means to differentiate and identify known phytoplasmas.

For a finer differentiation of phytoplasmas, additional genetic markers such as ribosomal protein (rp) genes, *secY*, *tuf*, and the 16S-23S rRNA intergenic spacer region sequences have been used as supplementary tools (Smart *et al.*, 1996; Schneider *et al.*, 1997; Martini *et al.*, 2002; 2007; Lee *et al.*, 1994, 2004a, b, 2006a). Finer subgroup delineation could be achieved by combining RFLP analyses of 16S rRNA with the rp gene sequences: the subgroups recognized by this method were consistent with the subclusters identified by analysing the phytoplasma genomes with dot and Southern hybridizations using a number of cloned phytoplasma DNA probes (Lee *et al*., 1992, 1998a; Gundersen *et al*., 1996; Martini *et al*., 2007).

By means of RFLP or phylogenetic analysis of 16S rRNA gene sequences from a wide array of phytoplasma strains, 19 RFLP groups and more than 20 distinct ribosomal groups have been identified (Montano *et al*., 2001; Arocha *et al.*, 2005; Lee *et al.*, 2006b; Al Saady *et al*., 2008). A consensus for naming novel phytoplasmas was reached and recommended by the IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group (IRPCM, 2004). This Group concluded that "a '*Candidatus* (*Ca.*) Phytoplasma' species description should refer to a single, unique 16S rRNA gene sequence (>1200 bp)", and that "a strain can be recognized as a novel '*Ca.* Phytoplasma' species if its 16S rRNA gene sequence has <97.5% similarity to that of any previously described '*Ca.* Phytoplasma' species". So far, 33 members of '*Ca.* Phytoplasma' have been proposed (IRPCM, 2004; Arocha *et al.*, 2005, 2007; Schneider *et al.*, 2005; Lee *et al.*, 2006b; Valiunas *et al.*, 2006; Al-Saady *et al*., 2008; Zhao *et al.* 2009). Because of the highly conserved nature of the 16S rRNA gene, many biologically or ecologically distinct phytoplasma strains may warrant designation as new taxons, but these may fail to meet the requirement of sharing <97.5% sequence similarity with existing '*Ca.* Phytoplasma', and may not be readily differentiated and classified. In that case, additional unique biological properties such as antibody specificity, host range, and vector transmission specificity, as well as other molecular criteria (genes) need to be included for speciation (Seemüller and Schneider, 2004).

Table 1. Classification of phytoplasmas based on RFLP analyses of 16S rRNA gene (based on Lee *et al.*, 1998a).

16Sr group	Phytoplasma strain (acronym)	GenBank acc. No.	Reference
16SrI: Aster yellows			
I-A	Aster yellows witches' broom (AYWB)	NC_007716	Bai *et al.*, 2006
I-A	Tomato big bud (BB)	L33760	Lee *et al.*, 1992
I-B	Onion yellows mild strain (OY-M)	NC_005303	Oshima *et al.*, 2004
I-B	'*Ca.* P. asteris', aster yellows (MAY)	M30790	Lee *et al.*, 2004a
I-C	Clover phyllody (CPh)	AF222065	Lee *et al.*, 2004a
I-D	Paulownia witches' broom (PaWB)	AY265206	Lee *et al.*, 2004a
I-E	Blueberry stunt (BBS3)	AY265213	Lee *et al.*, 2004a
I-F	Aster yellows apricot - Spain (A-AY)	X68338 AY265211	Kison, 1992 * Lee *et al.*, 2004a
I-I	Strawberry witches' broom (STRAWB1)	U96614	Jomantiene *et al.*, 1998
I-K	Strawberry witches' broom (STRAWB2)	U96616	Jomantiene *et al.*, 1998
I-L	Aster yellows (AV2192)	AY180957	Lee *et al.*, 2003
I-M	Aster yellows (AVUT)	AY265209	Lee *et al.*, 2004a
I-N	Aster yellows (IoWB)	AY265205	Lee *et al.*, 2004a
I-O	Soybean purple stem (SPS)	AF268405	Lee *et al.*, 2002
I-P	Aster yellows from *Populus* (PopAY)	AF503568	Šeruga *et al.*, 2003
I-Q	Cherry little leaf (ChLL)	-	Valiunas *et al.*, 2006
I-R	Strawberry phylloid fruit (StrawbPhF)	AY102275	Jomantiene *et al.*, 2002a
16SrII: Peanut WB			
II-A	Peanut witches' broom (PnWB)	L33765	Gundersen *et al.*, 1994
II-B	'*Ca.* P. aurantifolia', witches' broom of lime (WBDL)	U15442	Zreik *et al.*, 1995
II-C	Faba bean phyllody (FBP)	X83432	Seemüller *et al.*, 1998
II-D	Sweet potato little leaf (SPLL)	AJ289193	Gibb *et al.*, 1995
II-E	*Pichris echioides* phyllody (PEY)	Y16393	Seemüller *et al.*, 1998
II-F	Cotton phyllody (CoP)	EF186827	Martini *et al.*, 2007
16SrIII: X-disease			
III-A	Western X-disease (WX)	AF533231	Liefting and Kirkpatrick, 2003
III-B	Clover yellow edge (CYE)	L33766 8	Gundersen *et al.*, 1994
III-C	Pecan bunch (PB)	EF186807	Martini *et al.*, 2007
III-D	Goldenrod yellows (GR1)	EF186810	Martini *et al.*, 2007
III-E	Spiraea stunt (SP1)	AF190228	Jomantiene and Davis, 1999*
III-F	Milkweed yellows (MW1)	AF510724	Davis and Dally, 2002*
III-G	Walnut witches' broom (WWB)	AF190226 AF190227	Jomantiene and Davis, 1999*
III-H	Poinsettia branch-inducing (PoiBI)	AF190223	Jomantiene and Davis, 1999*
III-I	Virginia grapevine yellows (VGYIII)	AF060875	Davis *et al.*, 1998

(continued on the next page)

16Sr group	Phytoplasma strain (acronym)	GenBank acc. No.	Reference
III-J	Chayote witches' broom (ChWBIII)	AF147706 AF1477067	Montano *et al*., 2000
III-K	Strawberry leafy fruit (SLF)	AF274876	Jomantiene and Davis, 2000*
III-L	Cassava frog skin disease (CFSD)	EU346761	Alvarez *et al*., 2009
III-P	Dandelion virescence (DanV)	AF370119 AF370120	Jomantiene and Davis, 2001*
III-Q	Black raspberry witches' broom (BRWB7)	AF302841	Davis *et al*., 2001
III-T	Sweet and sour cherry (ChD)	FJ231728	Valiunas *et al*., 2009
III-U	Cirsium white leaf (CWL)	AF373105 AF373106	Jomantiene *et al*., 2002b
16SrIV: Coconut lethal yellows			
IV-A	Coconut lethal yellowing (LYJ-C8)	AF498307	Harrison *et al*., 2002
IV-B	Yucatan coconut lethal decline (LDY)	U18753	Harrison *et al*., 1994
IV-C	Tanzanian coconut lethal decline (LDT)	X80117	Harrison *et al*., 1994
16SrV: Elm yellows			
V-A	'*Ca*. P. ulmi', elm yellows (EY)	AY197655	Lee *et al*., 2004b
V-B	'*Ca*. P. ziziphi', jujube witches' broom, (JWB-G1)	AB052876	Jung *et al*., 2003a
V-C	Alder yellows (ALY882)	AY197642	Lee *et al*., 2004b
V-C	Flavescence dorée (FD-C)	X76560	Daire *et al*., 1992
V-D	Flavescence dorée (FD-D)	AJ548787	Torres *et al*., 2005
V-E	Rubus stunt (RuS)	Y16395	Seemüller *et al*., 1998
16SrVI: Clover proliferation			
VI-A	'*Ca*. P. trifolii', clover proliferation (CP)	AY390261	Hiruki and Wang, 2004
VI-B	Strawberry multiplier disease (SMPD)	AF036354	Jomantiene *et al*., 1998
VI-C	Illinois elm yellows (ILEY)	AF268895 AF409069 AF409070	Jacobs *et al*., 2003
16SrVII: Ash yellows			
VII-A	'*Ca*. P. fraxini', ash yellows (AshY)	AF092209	Griffiths *et al*., 1999
VII-B	Erigeron witches' broom (ErWB)	AY034608	Barros *et al*., 2002
16SrVIII: Loofah witches' broom			
VIII-A	Loofah witches' broom (LufWB)	AF086621	Ho *et al*., 2001
16SrIX: Pigeon pea witches' broom			
IX-A	Pigeon pea witches'-broom (PPWB)	AF248957	Davis and Dally, 2000*
IX-B	'*Ca*. P. phoenicium', almond witches' broom (AlWB)	AF515636 AF515637	Verdin *et al*., 2002
IX-C	Naxos periwinkle virescence (NAXOS)	-	Duduk *et al*., 2008

(continued on the next page)

16Sr group	Phytoplasma strain (acronym)	GenBank acc. No.	Reference
16SrX: Apple proliferation			
X-A	'*Ca*. P. mali', apple proliferation (AP)	AJ542541	Seemüller and Schneider, 2004
X-B	'*Ca*. P. prunorum', European stone fruit yellows (ESFY)	AJ542544	Seemüller and Schneider, 2004
X-C	'*Ca*. P. pyri', pear decline (PD)	AJ542543	Seemüller and Schneider, 2004
X-D	'*Ca*. P. spartii', spartium witches' broom (SpaWB)	X92869	Marcone *et al*., 2004a
X-E	Black alder witches' broom (BAWB) or Buckthorn witches' broom (BWB)	X76431	Seemüller *et al*., 1994
16SrXI: Rice yellow dwarf			
XI-A	'*Ca*. P. oryzae', rice yellow dwarf (RYD)	AB052873	Jung *et al*., 2003b
XI-B	Sugarcane white leaf (SCWL)	X76432	Lee *et al*., 1997b
XI-C	Leafhopper-borne (BVK)	X76429	Seemüller *et al*., 1994
16SrXII: Stolbur			
XII-A	Stolbur (STOL)	X76427	Seemüller *et al*., 1994
XII-B	'*Ca*. P. australiense', Australian grapevine yellows (AUSGY)	L76865	Davis *et al*., 1997
16SrXIII: Mexican periwinkle virescence			
XIII-A	Mexican periwinkle virescence (MPV)	AF248960	Dally *et al*., 2000*
XIII-B	Strawberry green petal, Florida	U96616	Jomantiene *et al*., 1998
16SrXIV: Bermudagrass white leaf			
XIV-A	'*Ca*. P. cynodontis' bermudagrass white leaf (BGWL)	AJ550984	Marcone *et al*., 2004b
16SrXV: Hibiscus witches'-broom			
XV-A	'*Ca*. P. brasiliense', hibiscus witches' broom (HibWB)	AF147708	Montano *et al*., 2001
16SrXVI: Sugarcane yellow leaf syndrome			
XVI-A	'*Ca*. P. graminis'	AY725228	Arocha *et al*., 2005
16SrXVII: Papaya bunchy top			
XVII-A	'*Ca*. P. caricae'	AY725234	Arocha *et al*., 2005
16SrXVIII: American potato purple top wilt			
XVIII-A	'*Ca*. P. americanum'	DQ174122	Lee *et al*., 2006b
16SXIX: Cassia witches'-broom			
16SXIX-A	'*Ca*. P. omanense'	EF666051	Al-Saady *et al*., 2008

*, GenBank submission

Over the last few years, phytoplasmas have been discovered at an increasingly rapid pace in emerging diseases worldwide. These findings have raised expectations that the number of 16S rRNA RFLP groups (16Sr groups) and subgroups will rise considerably, warranting expansion of the existing phytoplasma classification scheme. A new computer-simulated RFLP (*in silico* restriction) analysis method has led to the identification of putative new phytoplasma groups, significantly expanding the existing 16S RNA gene based phytoplasma classification scheme (Wei *et al.*, 2007; 2008a). The mode of application of this system is still under discussion since it is necessary to strongly back up the virtual data with data obtained in from phytoplasma strains available in recognized collections.

More '*Candidatus* Phytoplasma' species have been published. These phytoplasmas are listed in Table 2.

Table 2. '*Candidatus* Phytoplasma' species.

'*Candidatus* Phytoplasma'	GenBank acc. No.	Associated diseases	Country	Reference
'*Ca*. P. japonicum'	AB010425	*Hydrangea* phyllody	Japan	Sawayanagi *et al*., 1999
'*Ca*. P. castaneae'	AB054986	Chestnut witches' broom	Korea	Jung *et al*., 2002
'*Ca*. P. pini'	AJ310849	*Pinus* decline	Germany	Schneider *et al*., 2005
'*Ca*. P. rhamni'	AJ583009	*Rhamnus* witches' broom	Italy	Marcone *et al*., 2004a
'*Ca*. P. allocasuarinae	AY135523 AY135524	Allocasuarina yellows	Australia	Marcone *et al*., 2004a
'*Ca*. P. fragariae'	DQ086423	Strawberry yellows	Lithuania	Valiunas *et al*., 2006
'*Ca*. P. lycopersici'	EF199549	'Brote grande' of tomato	Peru	Arocha *et al.,* 2007
'*Ca*. P. tamaricis'	FJ432664	Salt cedar witches' broom	China	Zhao *et al*., 2009

To these officially published designations, seven others should be added which were proposed (IRPCM, 2004) but have not yet been officially published. These are: '*Ca*. P. pruni', '*Ca*. P. vitis', '*Ca*. P. solani', '*Ca*. P. palmae', '*Ca*. P. luffae', '*Ca*. P. cocostanzianae' and '*Ca.* P. cocosnigeriae'. These '*Candidatus*' names were proposed at the Xth International Congress of the International Organization of Mycoplasmology, 1994, but have not yet been formally described; they are reported here as incidental citations that do not constitute prior citations according to rule 28b of the bacteriological code (Lapage *et al*., 1992).

Genes and genomics properties

Like other members of the *Mollicutes,* phytoplasma cells contain one circular double-stranded DNA chromosome. They also have the smallest genome of all bacteria. Phylogenetic studies suggest that the common ancestor for phytoplasmas is *Acholeplasma laidlawii,* in which the triplet coding for tryptophan (trp) is UGG. In the other prokaryotes, enclosing mycoplasmas and spiroplasmas, trp is coded by UGA, while phytoplasmas use UGA as a stop codon. Moreover phytoplasmas are genetically distinguishable from mycoplasmas because they have a spacer region (about 300 bp) between the 16S and 23S ribosomal regions, which codes for isoleucine tRNA ($tRNA^{Ile}$) and part of the sequences for alanine tRNA ($tRNA^{Ala}$).

Phytoplasmas have a genome with a low G+C content (sometimes as little as 23%, which is thought to be the threshold for a viable genome), a feature common to all members of the class *Mollicutes*. Despite their very small genomes, many predicted genes are present in multiple copies; they contain two rRNA operons, and the heterogeneity of these operons has been demonstrated for some phytoplasmas. Phytoplasma genomes contain large numbers of transposon genes and insertion sequences that are unique to these organisms. They are responsible for genome variability, to enable phytoplasmas to survive in diverse environments of plants and insects, and they produce the marked heterogeneity of phytoplasma genome sizes. These sequences were named as variable mosaics (SVM) (Jomantiene and Davis, 2006; Jomantiene *et al*., 2007) and as potential mobile units, (PMUs) (Bai *et al*., 2006). They also contain a unique family of repetitive palindromes (REPs) called PhREPS. The role of these is unknown, although it is suggested that the PhREPS (which form the stem loop structures) may play a role in transcription termination or genome stability (Jomantiene and Davis, 2006; Jomantiene

et al., 2007). These have been described as being at the roots of phytoplasma evolution (Wei *et al*., 2008b).

Phytoplasmas contain extrachromosomal DNA (EC-DNA) such as plasmids. Short circular extrachromosomal DNAs (1.7–7.4 kb) or plasmids were found in all members of the aster yellows group (16SrI) and stolbur groups (16SrXII) and in some members of X-disease (16SrIII) and clover proliferation (16SrVI) groups. Some small plasmids may be of viral origin; some of them share significant sequence similarity with genes in plant geminiviruses (Kuboyama *et al.*, 1998; Rekab *et al*., 1999; Nishigawa *et al.*, 2002a; b).

Genes encoded in EC-DNAs such as plasmids are known to play important roles in the pathogenicity and virulence of many plant pathogenic bacteria. In the '*Ca.* P. asteris', onion yellows strain (OY); a mildly pathogenic, insect-transmitted line (OY-M) contains two types of plasmids (EcOYM and pOYM), each of which possesses a gene encoding the putative transmembrane protein, ORF3. Likewise, a non-insect-transmissible line (OY-NIM) has the corresponding plasmids (EcOYNIM and pOYNIM), but pOYNIM lacks ORF3. It was recently shown that in OY-M, ORF3 was transcribed from two putative promoters while on EcOYNIM one of the promoter sequences was mutated and the other deleted. ORF3 was not expressed in the OY-NIM-infected plants by immuno-histochemical analysis. Moreover, ORF3 protein seemed more specifically expressed in OY-M-infected insects than in plants. These results suggest that ORF3 plays a role in the interactions of OY with the insect host of this phytoplasma (Ishii *et al*., 2009).

Differences in the chromosome size between phytoplasma species have been reported. '*Ca.* P. cynodontis' and a tomato strain of the stolbur phytoplasma that belong to different 16Sr groups have a chromosomal size of 530 and 1,350 kb respectively; also the rape virescence phytoplasma and the hydrangea phyllody phytoplasma from the same subgroup (16SrI-B) differ greatly in their respective genome sizes: 1,130 kb vs. 660 kb (Marcone *et al.*, 1999). These differences are usually due to the occurrence of gene duplication and redundancy. It was estimated that in the genome of the onion yellows (OY) phytoplasma (Oshima *et al.*, 2004), 18% of the total genes were multiple redundant copies of only five genes: *uvrD* (ATP-dependent DNA helicase, 3,117 nucleotides, 7 copies), *hflB* (ATP-dependent Zn protease, 1,551 nucleotides, 17 copies), *tmk* (thymidylate kinase, 624 nucleotides, 6 copies), *dam* (DNA methylase, 660 nucleotides, 4 copies), and *ssb* (single-stranded DNA-binding protein, 345 nucleotides, 15 copies). All of these are generally single copies (if they exist at all) in the other *Mollicutes* whose genomes have been sequenced. In addition, five genes encoding elements of transporter systems have multiple copies, presumably not all of these are functional. Multiple copies of insertion sequence-like elements also occur in the genome of the OY and other phytoplasma strains (Lee *et al.*, 2005) and the functional gene for thymidylate kinase (Miyata *et al*., 2003).

Phytoplasmas lack many genes for standard metabolic functions (reduction of biosynthetic genes) and have no functioning homologous recombination pathways, but they do have a *sec* transport pathway (a major route of protein translocation across various cell membranes) (Bai *et al.*, 2006). The phytoplasma genome encodes even fewer metabolic functions than do the mycoplasma genomes (Razin, 2007).

Analysis of the genome sequence of the OY phytoplasma gives some indication of the nutritional requirements of these micro-organisms. The genome of the OY phytoplasma is about 861 kb and contains 754 ORFs, corresponding to 73% coding capacity. Like other *Mollicutes*, the OY phytoplasma lacks genes for the biosynthesis of amino acids and fatty acids, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (production of ATP). Unlike the mycoplasmas, the phytoplasma genome also lacks genes for the phosphotransferase system and for metabolizing UDP-galactose to glucose 1-phosphate, suggesting that phytoplasmas possess a unique sugar intake and metabolic system (Oshima *et al.*, 2004). In addition, (unlike mycoplasmas) phytoplasmas lack the pentose phosphate cycle (normally used to synthesize NADPH and to supply the ribose 5-phosphate necessary to synthesize nucleotides), the arginine dehydrolase pathway, and, more unexpectedly, ATP-synthase subunits, which are thought to be essential for life. This suggests that phytoplasmas import host ATP by unknown mechanisms; alternatively, ATP synthesis in phytoplasmas may be strongly dependent on glycolysis. Lack of these important functions in phytoplasmas may be the result of a reductive evolution as the consequence of life as intracellular parasites in nutrient-rich environments.

Phytoplasmas also lack the phosphotransferase system to import sugars essential for glycolysis. Most bacteria use this system as an energy-efficient way of simultaneously importing and phosphorylating sugars such as sucrose, glucose and fructose. However, phytoplasmas have a maltose ABC transport system. The maltose binding protein may have an affinity to maltose, trehalose, sucrose and palatinose, and therefore import these to phytoplasma cells. Trehalose is a major sugar of the insect haemolymph.

However, enzymes for converting these sugars to glucose-6-phosphate (available for glycolysis) were not found in phytoplasmas (Christensen *et al.*, 2005; Razin, 2007). The possibility that the phytoplasmas take up phosphorylated hexoses from their hosts (Bai *et al.*, 2006) and other possibilities remain to be investigated.

Although metabolic genes are scarce, phytoplasma genomes contain many genes encoding transporter systems, such as malate, metal-ion and amino-acid transporters, some of which have multiple copies. This suggests an aggressive import of many metabolites from the host cell, and this probably disturbs the metabolic balance and causes disease symptoms. Also, the phytoplasma genome encodes folate synthesis genes, which may allow phytoplasmas to adapt to very different environments in plants and insects (Oshima *et al.*, 2004).

Interaction with hosts

At present, exploration of the interactions of phytopathogenic *Mollicutes* with their plant and insect hosts is a fascinating and very active field of research. Several lines of investigation are under way to determine the interactions of phytopathogenic *Mollicutes* with both plants and insects.

Membrane proteins

Serological studies have recognized one or two abundant immuno-dominant proteins (highly antigenic membrane proteins at the surface of phytoplasmas) with trans-membrane domains. Major surface proteins may play roles in *Mollicutes* recognition, adherence to plant or insect host cells (a prerequisite for colonization and infection), pathogenicity and the triggering of host resistance responses. The occurrence of major surface epitopes that are unique to each phytoplasma species suggests that these proteins are key participants in specific interactions with the host cells. The immunodominant membrane protein is a major portion of the total cellular membrane proteins in most phytoplasmas, and genes encoding immunodominant membrane proteins were isolated from several phytoplasma groups. They are classified into three distinct types: (1) immunodominant membrane protein (Imp), in the phytoplasmas causing sweet potato witches' broom (SPWB) (Yu *et al*., 1998), apple proliferation (AP) (Berg *et al.*, 1999), European stone fruit yellows (ESFY), pear decline (PD), and peach yellow leaf roll (PYLR) (Morton *et al*., 2003); (2) immunodominant membrane protein A (IdpA), in the phytoplasma causing western X-disease (WX) (Blomquist *et al*., 2001); and (3) antigenic membrane protein (Amp), in the phytoplasmas causing aster yellows (AY), clover phyllody (CPh) (Barbara *et al*., 2002), and onion yellows (OY) (Kakizawa *et al*., 2004). They are not orthologues of each other; nonhomologous proteins play the role of 'immunodominant', the major portion of the total cellular membrane proteins, in various phytoplasmas. A high expression of the Amp protein was confirmed in AY, CPh, and OY phytoplasmas (Barbara *et al*., 2002; Kakizawa *et al.*, 2004) and this protein was shown to be exported via the Sec protein secretion system, accompanied by the cleavage of its N-terminal signal sequence (Kakizawa *et al.*, 2004).

Cloning Amp genes from several strains in the AY-group phytoplasma revealed that Amp proteins were under positive selection, and positively selected amino acids were encoded in the central hydrophilic domain of the Amp (Kakizawa *et al*., 2006). Recently, it has been reported that the Amp of the OY phytoplasma forms a complex with an insect microfilament. In addition, the formation of Amp–microfilament complexes was correlated with the phytoplasma-transmitting capacity of leafhoppers, suggesting that the interaction between Amp and insect microfilament complexes plays a major role in determining the transmissibility of phytoplasmas (Suzuki *et al*., 2006).

Other types of immunodominant membrane proteins have also been analysed. Morton *et al*. (2003) isolated genes encoding Imps from several phytoplasma strains and found that the sequence identities of Imps in several phytoplasmas were not correlated with that of the 16S rRNA gene; this suggests that the variability of immunodominant membrane proteins reflects some factors other than evolutionary time. As the information on other types of immunodominant membrane protein is limited, however, analyses of several more types of immunodominant membrane protein would shed light on the biological diversity and evolution of phytoplasmas (Kakizawa *et al*., 2009).

Although the gene encoding the immunodominant membrane protein of the WX phytoplasma is idpA, Liefting and Kirkpatrick (2003) reported that a gene homologous to Imp was also encoded in the WX genomic fragment. It is not known, however, if Imp was expressed and found as a protein on the phytoplasma surface membrane. This observation suggests that Imp may be a common ancestor of phytoplasma immunodominant membrane proteins. The sequence identity of Imp was found to be low among the different groups, and a BLAST search did not detect any similarity between Imp genes from different groups. However, the gene organizations around Imp were well conserved in most phytoplasmas, and all Imps had a transmembrane region in their N terminus. Therefore, despite dissimilarities in sequence identity, the Imp genes studied

till now have been orthologous because of their similar gene organization and conserved transmembrane structure (Kakizawa *et al*., 2009).

In contrast, neither the orthologue of idpA, which is the immunodominant membrane protein of WX, in the complete genomic sequences of OY-M (Oshima *et al*., 2004), AY-WB (Bai *et al.*, 2006), '*Ca*. P. australiense' (Tran-Nguyen *et al*., 2008), or '*Ca*. P. mali' (Kube *et al*., 2008) nor the orthologue of Amp, in the complete genomic sequence of '*Ca*. P. mali' were found. It has been suggested that the ancestral type of immunodominant membrane protein had been Imp, and that subsequently expression levels of Amp in the AYgroup and IdpA in the WX-group respectively, increased (Kakizawa *et al*., 2009). Further sequence analyses of immunodominant membrane protein genes from many phytoplasma strains would contribute to a better understanding of the biological and evolutionary roles of the immunodominant membrane protein. Imp may have an important role in host–phytoplasma interactions, like many positively selected proteins; the accumulation of Amp was calculated as about 10-fold greater than that of Imp and this level was consistent with the 'immunodominant' property of Amp in AY-group phytoplasmas.

Detection of Imp in phytoplasma-infected plants is also possible, however, and therefore the protein amount of Imp must also be high. Blot analysis of Imp from OY-W suggested that the signal sequence of Imp was uncleaved and that Imp was retained in the phytoplasma cell membrane. This result agrees with previous reports that Imp is an immunodominant membrane protein in several phytoplasmas, including AP and SPWB (Yu *et al*., 1998; Berg *et al.*, 1999). Thus, as far as the prediction of phytoplasma secretor proteins is concerned, it may be important to consider the amino acid length between transmembrane regions and cleavage motifs. Elucidation of the function of Imp is important to understand the biology and the evolution of phytoplasmas.

In addition, as Imp is an immunodominant membrane protein in AP and SPWB phytoplasmas, and expression of Imp in OY-W is clearly shown, antibodies against Imp could be useful for the detection of phytoplasmas generally. Cloning of Imp and subsequent production of antibodies against Imp would provide a good tool to detect phytoplasmas (Kakizawa *et al*., 2009) as was recently shown for Amp (Arashida *et al*., 2008).

Titer and multiplication in plants

The phytoplasma titer varies greatly from plant to plant. Recently, different competitive PCR procedures have made it possible to estimate the phytoplasma titer in different host plants. Periwinkle was confirmed as a high-concentration species irrespective of the phytoplasma taxonomic affiliation, while other species should be considered medium- or even low-titer hosts (Berges *et al.*, 2000).

Plant species have been reported to differ in their susceptibility to phytoplasma infection. Using different approaches, a reduction/suppression of phytoplasma multiplication has been seen when susceptible cultivars are grafted on resistant or tolerant rootstocks. More recently, the disappearance of phytoplasmas from the canopy but not from the roots of recovered apple proliferation-infected trees has been reported (Musetti *et al.*, 2005). This suggests that some components of the oxidant-scavenging system in recovered leaves are not very active, leading to an overproduction of H_2O_2 and, possibly, to membrane lipid peroxidation. The production of H_2O_2 was reported as being involved in counteracting pathogen virulence (Musetti *et al.*, 2005).

Host susceptibility is not the only factor relevant in triggering pathogen concentration. Different strains of the same phytoplasma may occur at different concentrations in infected hosts, as reported by Sinclair and Griffiths (2000) for ash yellows. In a co-inoculation experiment, they showed that the aggressive strain rDNA restriction profile of the ash yellows phytoplasma was detected by PCR sooner and more frequently than restriction profile of a less aggressive strain.

Translocation in plants

Phytoplasmas are transferred by insects to plant sieve elements, from which they spread systemically through the plants using the sieve tube system, but they never settle in the meristems. Phytoplasmas are pleomorphic and sufficiently small to pass freely through the sieve pores, so they may be swept along with the assimilate flow from leaves to sugar-consuming plant organs. Accordingly, phytoplasmas have been found in sink tissues such as immature leaves and roots, whereas source leaves remain uninfected. By contrast, other researchers reported a high titer of phytoplasma in source leaves and low colonization in sink tissue (Christensen *et al.*, 2004). Studies on the translocation of phytoplasmas after localized inoculation (Wei *et al.*, 2004b) or the recolonization of trees (Garcia-Chapa *et al.*, 2003) provide evidence that the translocation of phytoplasmas cannot be explained only by assimilate flow. Active movement by the phytoplasmas seems unlikely, considering the lack of genes coding for cytoskeleton elements or flagella (Christensen *et al.*, 2005).

Pathogenicity and virulence

Despite their economic importance and unique biological features, phytoplasmas remain the most poorly characterized plant pathogens, primarily because efforts at *in vitro* culture, gene delivery and mutagenesis have been unsuccessful. It is important to identify the factors involved in the pathogenicity of these organisms to find effective measures for controlling the diseases they cause.

Energy metabolism is certainly a key topic for understanding phytoplasma biology and pathogenesis. It has been reported that altered levels of oxygen and carbon dioxide affect phytoplasma abundance in *Oenothera* leaf tip cultures (Sears *et al.*, 1997). Lepka *et al.* (1999) and Maust *et al.* (2003) reported that concentrations of carbohydrates in the phloem, roots and leaves of phytoplasma-infected plants differ from those in healthy plants. Because of the large array of symptoms in diseased plants, nutrient depletion is probably not restricted to sugars, but also includes other compounds. A reduction in the concentration of photosynthetic pigments (Bertamini *et al.*, 2001) and of total soluble proteins (Musetti *et al.*, 2000, 2005; Bertamini *et al.*, 2001), as well as alterations in the hormone balance (Maust *et al.*, 2003; Jagoueix-Eveillard *et al.*, 2001), amino-acid transport (Lepka *et al.*, 1999) and the occurrence of folate and endopetidase gene homologues in phytoplasma genomes (Davis *et al.*, 2003) were described as potentially affecting host plants infected with different phytoplasmas.

Impaired photosynthesis, the accumulation of carbohydrates in mature leaves and a lower starch content in sink tissues such as roots are often described for phytoplasma infection, but they seem to be only secondary effects and likely to be related to the inhibition of phloem transport. Higher levels of carbohydrates in source leaves are generally thought to produce feedback inhibition of photosynthesis, causing chlorosis (Lepka *et al.*, 1999; Bertamini *et al.*, 2001; Maust *et al.*, 2003;). A considerable reduction in phloem translocation can be expected when phytoplasmas multiply to such a level that the lumen of the sieve elements becomes clogged. However, a lower translocation has also been seen in plants with a low phytoplasma titer, suggesting that the parasite has a more indirect influence on host metabolism and phloem function.

Very little is known about phytoplasma virulence. Sequenced phytoplasmas possess none of the known virulence genes (such as *hrp*) found in other phytopathogenic bacteria (Oshima *et al.*, 2004). Because they lack most of the common metabolic pathways, it has been speculated that they must assimilate a wide range of materials from the host cells, probably with detrimental effects to the hosts. In onion yellows (OY) phytoplasma, two types of strains have been described: mild (OY-M, causing mild proliferation and yellowing) and severe (OY-W, causing yellowing, stunting, proliferation and witches' broom). Oshima *et al.* (2001) reported that the OY-W chromosome (1000 kbp) was larger than the OY-M chromosome (860 kbp). Further analysis showed that five glycolytic genes were duplicated in the severe strain. This implies that it is advantageous for the phytoplasma to retain these genes, and also that these genes may influence glycolytic activity. It was previously reported that the phytoplasma population of OY-W had a higher titer than OY-M (Oshima *et al.*, 2001). In view of these findings, the higher consumption of the carbon source (because of the duplication of the glycolytic genes) may affect the growth rate of the phytoplasma and may also directly or indirectly produce more severe symptoms (Oshima *et al.*, 2007). Very recently it was shown that a single virulence factor, "tengu-su" inducer (TENGU), induces witches' broom and dwarfism when expressed in transgenic plants of *Nicotiana benthamiana* and *Arabidopsis thaliana*. Although the localization of phytoplasma is restricted to the phloem, TENGU protein was detected in the apical buds by immunohistochemical analysis, suggesting that TENGU was transported from the phloem to other cells. Microarray analysis detected that auxin-responsive genes were down-regulated in the "tengu"-transgenic plants as compared with GUS-transgenic control plants. This suggests that TENGU inhibits auxin-related pathways, thereby affecting plant development (Hoshi *et al*., 2009).

Putative genes involved in phytoplasma-host interactions

Several recently developed molecular techniques, such as differential display and arbitrarily primed PCR, which facilitate the detection of differential gene expression, are being applied in several laboratories to investigate the biology of phytopathogenic *Mollicutes* in different environments, such as plant and insect hosts.

Phytoplasma infection can lead to the production of defense proteins, an increase in phenolic compounds and an overproduction of hydrogen peroxide in host plants (Musetti *et al.*, 2000, 2005; Junqueira *et al.*, 2004). Using the messenger RNA differential display method, Smart *et al.* (1996) recognized *Arabidopsis* genes regulated following infection with the aster yellows phytoplasma. Also, Jagoueix-Eveillard *et al.* (2001) isolated several up- and down-regulated genes from periwinkle plants infected with either *Spiroplasma citri*, the stolbur phytoplasma or '*Ca.* P. aurantifolia'. Eight of these genes had homologies with genes coding for proteins involved in photosynthesis,

sugar transport, response to stress, or pathways of phytosterol synthesis. These researchers showed that a gene coding for a pathogen-induced protein, a wall-associated kinase, was activated by *S. citri* infection. In *Arabidopsis* this gene is also induced by salicylic acid, and is involved in the plant defense response. In *A. thaliana*, mutation of the transketolase gene affects the expression of several photosynthetic genes and pigment production. Therefore, inhibition of the transketolase gene might cause repression of the genes involved in photosynthesis. The presence of specific genes for translocation has been reported for several phytoplasmas (Kakizawa *et al*., 2001).

A homologue of an amino acid transporter was found to be down-regulated after *Prunus armeniaca* was infected with '*Ca.* P. prunorum', suggesting that phytoplasmas affect amino acid transport. Three genes were also found to be up-regulated in host plants infected with this phytoplasma, including genes coding for a heat-shock protein (HSP70) and a metallothionein, and a homologue of the expressed sequence tag 673 clone of *P. armeniaca*. Metallothioneins are proteins that have potent metal binding and redox properties, and that are produced in response to heavy metal stress. This can also lead to the production of HSP70, as does growth under extreme temperatures (Carginale *et al.*, 2004).

Phytoplasmas have been detected in floral tissue. Infection of tomato plants by the stolbur phytoplasma causes typical flower abnormalities, including sepal hypertrophy, virescence, phyllody, and big buds. Flower malformations of stolbur phytoplasma-infected tomatoes are associated with early deregulations in the expression of key flower development genes (including *LeDEF*, *LeWUS* and *TAG1*). As phytoplasmas never settle in the meristems, they probably change floral development by long-distance signals, such as those impairing the translocation of sugars in phloem, or the hypermethylation of plant genomes through the activation of plant defense mechanisms (Pracros *et al.*, 2006). In plants and animals, the cytosine methylation process is known to regulate gene expression in an epigenetic manner. Studies have demonstrated that genes can be accidentally down-regulated when the number of methylated cytosines is greater than the normal methylation status of the genes.

Prospects for the control of phytoplasma diseases

Outbreaks of phytoplasma disease epidemics can be controlled either by controlling the vectors, or by eliminating the pathogens from infected plants by meristem tip culture, by antibiotics or by other chemicals (Bertaccini, 2007).

At present, insect vector control using pesticides is the method of choice for limiting outbreaks of phytoplasma diseases. Even apart from environmental considerations, however, the efficacy of chemical control is far from complete, and phytoplasma diseases continue to be severe in several areas of the world, despite extensive use of insecticides (Firrao *et al.*, 2007). On the other hand, removal of sources of inoculum is efficient for reducing mollicute diseases spread by monophagous vectors feeding on infected plants. Examples are the disease/vector systems of flavescence dorée/*Scaphoideus titanus* and pear decline/*Cacopsylla pyri*. It is difficult to achieve results when wild reservoir plants are sources of contamination for polyphagous leafhoppers such as stolbur/*Hyalesthes obsoletus*/bindweed/nettle, or when reservoirs and/or vectors are unknown. Similarly, it is easier to control monophagous insects reproducing on affected crops than insects that are also able to live on wild plants.

Curing infected plants with antibiotics or by stimulating the production of specific antibodies is not practicable because antibiotics are too costly, prohibited in several countries, and do not always provide long-time control. Moreover the production of transgenic plants producing antibodies or resistant to these pathogens is still a long way off (Ishiie *et al*., 1967; Chen and Chen, 1998; Le Gall *et al.*, 1998).

Recent research is elucidating several aspects of the biology and host relationships of phytoplasmas.

Interference with the colonization of insects by phytoplasmas, or with the phytoplasma nutrient uptake in the plant phloem are primary targets for plant protection without using to pesticides.

To reduce the infectivity of vector populations it is first necessary to identify the barriers that limit phytoplasmas from colonizing such vectors. Alternatively, phytoplasma nutrient uptake from host plant phloem may be targeted to reduce phytoplasma multiplication, and symptom expression in the host. Hopefully these approaches will lead to the protection of plants without the need for pesticides (Firrao *et al.*, 2007). However, the most promising approach to control phytoplasma infection and prevent outbreaks is by producing clean plant material or by finding phytoplasma-resistant varieties.

Our understanding of the mechanisms that make host plant resistant to phytoplasmas is also insufficient, but the paucity of effective disease management strategies for these diseases gives a high priority to this question. Efforts continue to identify germplasm coding for natural

resistance to *Mollicutes*, and to incorporate the appropriate genes into various crops and fruit and forest trees *via* selection and breeding programs. The resistance thus bred may include resistance to either the pathogens or to the insect vectors. Plant defense related proteins, known to be active in responding to invasion by other types of pathogens, may also be effective in responding to mollicute infection. Confirmation of this would require a demonstration that the compounds are in the right place at the right time, and at the right concentration (Garnier *et al.*, 2001).

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