Review

Molecular and biological properties of phytoplasmas

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Abstract: Phytoplasmas, a large group of plant-pathogenic, phloem-inhabiting bacteria were discovered by Japanese scientists in 1967. They are transmitted from plant to plant by phloem-feeding insect hosts and cause a variety of symptoms and considerable damage in more than 1,000 plant species. In the first quarter century following the discovery of phytoplasmas, their tiny cell size and the difficulty in culturing them hampered their biological classification and restricted research to ecological studies such as detection by electron microscopy and identification of insect vectors. In the 1990s, however, tremendous advances in molecular biology and related technologies encouraged investigation of phytoplasmas at the molecular level. In the last quarter century, molecular biology has revealed important properties of phytoplasmas. This review summarizes the history and current status of phytoplasma research, focusing on their discovery, molecular classification, diagnosis of phytoplasma diseases, reductive evolution of their genomes, characteristic features of their plasmids, molecular mechanisms of insect transmission, virulence factors, and chemotherapy.

Keywords: phytoplasma, genome, host specificity, mycoplasma-like organism, pathogenicity, plant pathology

1. History of mysterious plant diseases

The earliest record of phytoplasma diseases dates back about 1,000 years.1) Phytoplasma-infected tree peonies exhibiting floral virescence (green flowers) were prized in the imperial court of Song China, not as exemplars of plant disease but as the most precious and beautiful variety of the plant (Fig. 1). The earliest scientific record of a phytoplasma disease in Japan is of mulberry dwarf disease,2) which was first observed about 200 years ago.3) This disorder is highly destructive to the production of mulberry leaves (Fig. 2A), which are the sole food source of silkworms. Mulberry dwarf disease thus causes considerable damage to the silk manufacturing industry, which was a major export industry and important in the modernization of Japan. In 1897, the National Diet set up the first national research committee to determine the cause of the disease, but the committee failed after researching the problem for 7 years. Later, the discovery of its transmissibility by insects and by grafting led to the hypothesis that mulberry dwarf disease was caused by a virus, although the pathogen remained undiscovered.3) Many similar diseases caused by unknown insect-transmissible agents had also long been considered to be caused by undiscovered viruses (Fig. 2B). These so-called “yellows diseases” included rice yellow dwarf (Fig. 2C) and sweet potato witches’ broom, both of which caused serious damage to agricultural production in Japan, as well as aster yellows (Fig. 2D). These diseases still devastate many plant species worldwide. For example, in 2001, an outbreak of witches’ broom disease in apple trees caused losses of about €100 million in Italy and €25 million in Germany.4) Lethal yellowing has killed millions of coconut palm trees in the Caribbean over the past 40 years.5) Aster yellows disease is probably the best-known of these virus-like diseases, affecting more than 300 species in 38 families of plants; the disease is transmitted by leafhoppers.6)

2. Discovery of mycoplasma-like organisms

In 1967, using electron microscopy, Doi et al. first described the consistent presence of small
The earliest record of a phytoplasma disease is evident in peonies, attributed to Zhao Chang, a court painter of the Song Dynasty of China. The two flowers on the middle-right exhibit floral virescence symptoms. Printed with permission of the Museum of the Imperial Collections, Sannomaru-Shozokan.

pleomorphic bodies (80–800 nm in diameter) resembling mycoplasmas (bacterial pathogens of humans and animals) in ultrathin sections of the phloem sieve elements of plants infected with mulberry dwarf, aster yellows, and other typical yellows diseases (Fig. 3A), but not in healthy plants. These microbes resembled mycoplasmas in their cell size, lack of a cell wall, and sensitivity to the antibiotic tetracycline (Fig. 3B), which inhibits protein synthesis by binding to the 30S ribosomal subunits of many prokaryotes. Given these similarities in morphological and biological properties, Doi et al. named them mycoplasma-like organisms (MLOs). The discovery of MLOs stimulated worldwide re-investigation of the etiology of numerous yellows diseases that had been assumed to be caused by viruses. Several hundred reports (initially employing electron microscopy) revealed the consistent presence of MLOs and their sensitivity to tetracycline, and confirmed the association between MLOs and yellows diseases.

3. Molecular detection and classification of MLOs

Although some researchers sought to differentiate yellows diseases based on symptomatology and host range, this was difficult because MLOs are associated with very similar symptoms in different plant species. Consequently, every time a new MLO disease was reported, the MLO was named using its original host plant name and symptom (e.g., mulberry dwarf, aster yellows). Eventually, more than 700 names were reported for MLO-associated diseases. Around 1990, advances in molecular biology enabled the direct detection of MLO DNA via DNA–DNA hybridization, polymerase chain reaction (PCR), and the cloning and sequencing of 16S ribosomal DNA (rDNA). A universal PCR amplification method for MLO 16S rDNA was then developed and has become the gold standard for the detection of MLOs. These rDNA sequences revealed that MLOs form a monophyletic group that is evolutionarily distinct from mycoplasmas and can be phylogenetically classified via sequence comparisons. In 1993, the first MLO-specific universal PCR primer set exploiting conserved regions of mollicute 16S rDNA was developed, which selectively amplified 16S MLO rDNA from infected plants. Moreover, direct sequencing of PCR-amplified 16S rDNA fragments was pioneered, which made cloning unnecessary and enabled simple phylogenetic analyses. At about the same time, other MLO classification approaches [PCR amplification of 16S rDNA followed by analysis of restriction fragment length polymorphisms (RFLPs) revealed by restriction enzymes] were reported. Taken together, these studies contributed to the establishment of the taxonomy of MLOs by the International Organization for Mycoplasmology. MLOs were renamed phytoplasmas (phyto-: Greek for “plant”; plasma: Greek for “a thing that is molded”), and classified as a new genus ‘Candidatus (Ca.) Phytoplasma’ with rules based on the 16S rDNA sequences for the description of organisms as novel taxa. Following these rules, 44 ‘Candidatus Phytoplasma’ species have been reported to date (Fig. 4). Each phytoplasma was also found to have two rDNA
Fig. 2. Various symptoms caused by yellows diseases. (A) Mulberry dwarf. (B) Paulownia witches’ broom. (C) Rice yellow dwarf. (D) Aster yellows. (E) Coconut lethal yellowing. (F) Poinsettia witches’ broom. (G) Hydrangea phyllody. (B, C, F, G) right side: infected plants; left side: healthy plants. The photographs were kindly provided by Drs. Akira Shirata (A), Norio Nishimura (B), and Akira Shinkai (C, D).

Fig. 3. The discovery of mycoplasma-like organisms (MLOs). (A) MLO cells observed via electron microscopy in an ultrathin section of a plant phloem cell. (B) Tetracycline-mediated recovery of mulberry from dwarf symptoms. Symptomatic mulberries infected with mulberry dwarf MLO (left panel) were treated with tetracycline (100 ppm) via foliage spraying (20 mL/pot) and soil drenching (200 mL/pot) at intervals of 2 or 3 days (12 applications). One month later, the plants had recovered (right panels). The photographs in (B) were originally published in ref. 8.
When closely related phytoplasmas within the same ‘Candidatus’ species are compared, housekeeping gene sequences are used in addition to those of 16S rDNA sequences for finer molecular characterization. These findings dramatically simplified the detection, diagnosis, and classification of MLOs, as well as encouraging phytoplasma research around the world.

4. Maintenance and mutation of phytoplasmas

Since the late nineteenth century, microbiology has relied on the ability to grow pure cultures of microorganisms on artificial media. Such culture enables the isolation, maintenance, propagation, and mutagenesis of microorganisms of interest, and plays central roles in many studies of microbial taxonomy and plant pathology. However, it is difficult to culture phytoplasmas, and maintenance requires periodic insect-vector-mediated transmission or grafting. The onion yellows phytoplasma (‘Ca. P. asteris’) OY-W (wild-type line) was selected for study because it has been stably maintained in a plant host [garland chrysanthemum (Glebionis coronaria)] using an insect vector (Macrosteles striifrons), and has been widely used as an experimental model in Japan. Garland chrysanthemum is a small long-lived plant that is suitable for propagation of both the phytoplasma and its insect vector. The isolation of phytoplasma mutant lines was attempted to identify the determinants of differences in their phenotypes. OY-M (mildly pathogenic line) was isolated from OY-W, which has been maintained with the aid of plant and insect hosts for 20 years (Fig. 5A); the mutant exhibits almost no plant pathogenicity. The genome size of OY-W is ca. 1 million base pairs (Mbp) but that of OY-M is ca. 0.86 Mbp. Furthermore, OY-NIM (non-insect-transmissible derivative of OY-M) was isolated after maintenance of OY-M in plants via periodic grafting onto healthy plants over 2 years (Fig. 5B). OY-NIM was not detected in the insect vector at any time after acquisition feeding on OY-NIM-infected plants, implying that the mutant cannot be acquired or multiply in the insect. The development of these first phytoplasma mutants (Fig. 5C) allowed us to take...
5. Phytoplasma genomics

Unlike common bacteria and many other organisms, including animals and plants, mycoplasmas use the UGA stop codon as a tryptophan-encoding codon.46) This unusual codon usage makes it difficult to study the functions of mycoplasma genes by expressing them in canonical hosts such as *Escherichia coli* and cultured eukaryotic cells; it is very burdensome to overcome this experimental barrier by replacing UGA with the general tryptophan codon UGG. However, when several phytoplasma operons were analyzed to determine the numbers of presumed tryptophan UGA codons, it was found that, unlike mycoplasmas, every UGA was a functional stop codon.47) Moreover, a gene encoding peptide chain release factor 2 that recognizes UGA as a termination codon is present in the phytoplasma genome.48) This, combined with several other genetic features shared between phytoplasmas and common bacteria such as *E. coli*,49),50) prompted us to commence advanced molecular and genetic analyses of phytoplasmas.

In 1994, phytoplasma whole-genome sequencing started in Japan. The onion yellows phytoplasma OY-W and mutant line OY-M were used, because these lines had been stably maintained in Japan, as described above, and because OY-M is a unique mutant lacking some determinants associated with pathogenicity. Finally, the draft genome of OY-W was reported in 200251) and the first complete genome sequence of 860,631 bp of the mutant OY-M was reported in 2004.52) The OY-M genome was circular with a G+C content of 28% and contained 754 open reading frames (ORFs) comprising 73% of the genome.

Gene annotation analysis revealed that although the genome encoded basic cellular functions including DNA replication, transcription, translation, and protein translocation, the genes required for amino acid and fatty acid biosynthesis, the tricarboxylic acid cycle, and electron transport/oxidative phosphorylation had been lost, as was also the case for *Mycoplasma genitalium* G-37 (580,070 bp), the smallest microbial genome known at that time (Fig. 6).53) The phytoplasma genome contained even fewer metabolic genes than the mycoplasma genome: the former genome lacked the phosphotransferase transport system, the pentose phosphate pathway, and (surprisingly) even adenosine triphosphate (ATP) synthase, which had previously been regarded as

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**Fig. 5.** Establishment of the first phytoplasma mutant lines. (A) The mildly pathogenic mutant OY-M was isolated after 20 years of maintenance of the wild-type OY-W line in plant and insect hosts. (B) The non-insect-transmissible mutant line OY-NIM was isolated via 2-year grafting maintenance of OY-M (in the absence of the insect host). (C) Healthy plant and plants infected with OY-W and the mutant lines OY-M and OY-NIM. This figure was reproduced with modifications based on the original literature.124)
essential for life. Rather, phytoplasma ATP synthesis may require the glycolytic\textsuperscript{54} and malate\textsuperscript{55} pathways. The genome was considered to reflect the reductive evolution often present in intracellular parasites and symbiotic bacteria\textsuperscript{56}–\textsuperscript{58}. Such organisms frequently exhibit reduced genomes because they do not need many metabolic genes, given that they live in host-created nutrient-rich environments.

Although metabolic genes were few in number, the OY-M genome contained many transporter genes absent from the mycoplasma genome. Phytoplasmas are obligate parasites of host cells, from which they obtain nutrients, instead of synthesizing essential substrates necessary for survival. The many transporters may play essential roles if an organism chooses “a little life without work”\textsuperscript{[7]} at https://www.newswise.com/articles/view/502315/). The phytoplasma genome is also rich in repeat regions with duplicated genes and transposon-like elements\textsuperscript{59} called potential mobile units;\textsuperscript{60} these feature similar genes organized in a conservative manner,\textsuperscript{59},\textsuperscript{61} and are thought to play roles in the regulation of gene expression\textsuperscript{62} and to serve as drivers of phytoplasma evolution in insect symbiosis and plant parasitism.\textsuperscript{54},\textsuperscript{63}

Phytoplasmas have also been discovered to possess plasmids\textsuperscript{64}–\textsuperscript{71}. Of note, these plasmids were found to have chimeric replication proteins (Reps) with characteristics of both bacterial plasmid Reps and DNA viral Reps (Fig. 7). Additionally, the membrane protein-encoding gene (ORF3) of OY-M plasmids was lacking in plasmids of the insect-transmission-deficient mutant OY-NIM, implying that the gene may be involved in insect-mediated transmission, as described below.
6. Mechanisms to infect plants and insects

Phytoplasmas parasitize plants and insects (Fig. 8). They reside endocellurally within the plant phloem and are spread between plants by phloem-feeding insects such as leafhoppers, planthoppers, and psyllids. Due to their wide range of plant hosts, phytoplasmas are often detected in various crops and wild plants on which insect vectors have fed. When phytoplasma-infected insects feed on plants, phytoplasmas are initially injected into the phloem sieve tubes and then propagate within and spread from phloem tissues of the infected leaf to the main stem, root, and leaves via the bipolar movement of the phloem fluid at night and in the day.72)

Because phytoplasmas have strict insect host ranges,73)–76) and are generally not transmitted transovarially (with a few exceptions),77) the presence or absence of insect hosts is a critical determinant of their survival in the natural environment. In insects, phytoplasmas establish systemic infection by spreading from the gut to hemocoel, then to the salivary gland, each of which presents a barrier to insect transmissibility (Fig. 8).45),78)–80)

When invading insects, extracellular membrane proteins of phytoplasmas may play important roles in terms of phytoplasma-host interactions. The phytoplasma membrane proteins are delivered to the cell surface by the Sec protein-translocation system (Fig. 9).48),81)–88) Notably, antigenic membrane pro-
tein (AMP) is a representative of phytoplasma membrane proteins that is predominantly detected on the phytoplasma cell surface.\(^8\)\(^5\) AMP was found to form a complex with host microfilaments (Fig. 10A) (Koinuma et al., unpublished data). The formation of such AMP-microfilament protein complexes determines whether an insect can transmit a phytoplasma (Fig. 10B).\(^8\)\(^9\),\(^9\)\(^0\) Additionally, the ATP synthase \(\beta\)-subunit of vector insects was found to be present in AMP-microfilament complexes.\(^9\)\(^1\) These reports contributed to elucidating the molecular mechanism by which pathogenic microorganisms of plants and animals are transmitted by specific insect vectors. Subsequently, immunodominant membrane protein (IMP), another phytoplasma membrane protein, was shown to bind to plant actin.\(^9\)\(^2\) Phytoplasmas lack movement genes, so this implies that actin-binding facilitates phytoplasma transport within sieve elements and through sieve plates, thus within the phloem, and ultimately ensures colonization of the plant host.\(^9\)\(^2\)

Furthermore, a highly sensitive phytoplasma microarray, an \textit{in vitro} transcription system, and an RNA sequencing method were developed to analyze gene expression patterns.\(^9\)\(^3\)\(^\text{-}\)\(^9\)\(^5\) These technical advances revealed that phytoplasmas dramatically alter the gene expression of approximately one-third of their genes using transcription factors to establish host switching between plants and insects.

### 7. Genetic factors determining symptom development

Phytoplasma-infected plants exhibit phloem necrosis and decline\(^9\)\(^6\) associated with a variety of unique morphological changes such as stunting, yellowing, witches’ broom (“tengu-su”, many tiny shoots with small leaves), phyllody (formation of leaf-like tissues instead of flowers), floral virecence; abnormal proliferation (growth of shoots from floral organs), and purple top (reddening of the upper leaves and apical parts).\(^9\)\(^7\) Some of these attributes have become successful gardening varieties worldwide. For example, all commercial poinsettias owe their economic value to their small bushy shape induced by grafting of a poinsettia branch-inducing strain of ‘\textit{Ca. P. pruni}’ (Fig. 2F).\(^8\)\(^7\) Moreover, hydrangeas exhibiting floral virecence and phyllody were very valuable until the plants were shown to host phytoplasmas (Fig. 2G). However, the mechanisms by which phytoplasmas induce these various symptoms were unknown until recently.

Some of the molecular mechanisms by which phytoplasmas induce their typical symptoms have gradually been elucidated. Comparing the genome sequences of OY-W and OY-M revealed duplication of glycolytic gene clusters in the OY-W genome. It has been suggested that this difference is responsible for the high consumption of carbon sources, resulting in the high growth rates and severe symptoms, such as yellowing, dwarfism, and decline (Fig. 5C), associated with OY-W phytoplasma.\(^5\)\(^4\) Furthermore, the mechanisms of purple-top symptoms have been revealed. Phytoplasma infection activates the anthocyanin biosynthetic pathway. Increased accumulation of anthocyanin not only changes the color of leaves to purple but also acts as an antioxidant that protects plant cells from damage caused by reactive oxygen species, which results in leaf cell death.\(^9\)\(^8\)

Many plant-pathogenic bacteria secrete proteins, termed effectors, into the cytoplasm of host cells for successful colonization. However, the phytoplasma genome does not contain any known effector-like genes.\(^5\)\(^2\) A comprehensive search was conducted for pathogenicity-related genes, in which phytoplas-
ma genes encoding secreted proteins were introduced into host plants with the aid of a potato virus X-based gene expression vector. However, because gene mapping focused mostly on large proteins comprising 100 amino acids or more, it took some time to focus on small proteins or peptides comprising several tens of amino acids. In 2009, the first phytoplasma effector protein, TENGU, a secreted peptide of 38 amino acids, was identified as an inducer of witches’ broom (“tengu-su” symptoms; Fig. 11). TENGU is conserved among various phytoplasma strains. Following secretion from the phytoplasma cell, TENGU is cleaved in planta to a peptide of 12 amino acids, which is then transported to the shoot apical meristem, wherein it inhibits the signaling pathway of the plant hormone auxin and induces witches’ broom symptoms. These results elucidated the molecular mechanism of “tengu-su” symptoms, and were important findings in plant physiology. They also opened the way to a new breeding technology using TENGU peptide, instead of traditional plant production by inoculating phytoplasmas into commercial horticultural plants such as poinsettias, which may spread phytoplasmas.

TENGU also induces the sterility of male and female flowers by inhibiting the signaling pathway of another plant hormone, jasmonic acid (JA). The reduction in endogenous JA levels is thought to contribute to attracting insect vectors. Similarly, another secreted protein, SAP11, downregulates JA synthesis and increases the fecundity of insect vectors.
Floral organs are modified leaves under the control of four combinations of floral homeotic proteins known as ABCE-class MADS-domain transcription factors (MTFs). In phytoplasma-infected plants, floral phyllody often affects sepals, but rarely stamens. Similarly, abnormal expression patterns of MTFs were found in all floral organs except stamens in phytoplasma-infected petunias. Recently, SAP54 and PHYL1 were found to be homologous proteins that induce phyllody in the floral organs of Arabidopsis thaliana. The proteins interact with and then degrade A- and E-class MTFs via the ubiquitin–proteasome pathway and are genetically and functionally conserved among phytoplasma strains and species. Therefore, these genetic homologs were placed in a phyllody-inducing gene/protein (phylogen) family; the proteins induce floral phyllody and related floral malformations (virescence and proliferation). Phylogens induce floral phyllody in various angiosperms (Fig. 13A), and MTF degradation in non-flowering plants (gymnosperms and a fern; Fig. 13B). These results indicated that phyllogen targets a conserved and functionally important region in MTFs. Phylogens induce virescence, phyllody, and proliferation symptoms, indicating that these floral symptoms are not independent symptoms induced by distinct effectors, but rather a series of gradually changing phenotypes that are all induced by a single effector, the phyllogen. In other words, floral virescence is a mild form of phyllody and loss of floral meristem determinacy is a severe form of phyllody. Thus, “phyllody” is a generic term that includes virescence, phyllody, and proliferation.

These findings elucidated the mechanisms of phyllody symptoms in plants at the molecular level, deepening the basic understanding of the mechanism.
of floral organogenesis and opening the way for new breeding technologies that use phyllogen to create high-value ornamental horticultural plants, such as hydrangeas with green flowers.

Why do phytoplasmas induce symptoms accompanied by unique morphological changes such as witches’ broom and phyllody? Both symptoms increase the prevalence of short branches and small young leaves, which are preferred by sap-feeding insects. Furthermore, the life of small young leaves and flowers with phyllody symptoms is prolonged. In particular, phyllody flowers remain green even when healthy flowers wither. These features are likely to enhance attraction of insect vectors and thus the spread of phytoplasmas. Such manipulations of the morphology of host plants appear to be a common strategy for the survival of phytoplasmas.

8. Detection and control of phytoplasmas

Because phytoplasmas are difficult to culture, electron microscopy observation using ultra-thin sections of sieve elements and plant recovery after tetracycline treatment were the only diagnostic methods available when phytoplasmas were discovered. Subsequently, several technologies to detect phytoplasmas have been developed, including autofluorescence when phloem-limited necrosis is present, fluorescent staining of phytoplasma DNA in phloem tissue, an antiserum-based method to detect the conserved membrane protein, and PCR to amplify conserved gene regions. However, these methods require expensive equipment or a complicated experimental technique, so diagnosing a phytoplasma disease is difficult in laboratories that lack modern facilities or with unstable infrastructure, such as unreliable power supply. Thus, easy, rapid, and sensitive diagnostic methods based on loop-mediated isothermal amplification (LAMP) were developed. The initial LAMP methods were species- or group-specific, but a universal LAMP kit for detecting all phytoplasma species in a single tube has recently been developed. Furthermore, to allow the kit to be carried, sent, or stored at room temperature, a method to dry the kit reagents was developed. Use of this dry kit is increasing in developing countries, where phytoplasma disease outbreaks are common.

In addition to the development of diagnostic methods, there has been an urgent requirement for agents/molecules to control phytoplasma diseases. To date, however, only tetracycline-class antibiotics have exhibited transient suppression of phytoplasma propagation and symptoms, no other chemical
control agents are currently available. This is because screening is difficult given that phytoplasmas cannot easily be cultured in vitro. Although treatment using tetracycline-class antibiotics also suppresses phytoplasma propagation in infected plants cultured in vitro, high concentrations of antibiotics (>100 ppm) damage plant tissues. Therefore, a comprehensive screening system was developed that uses a plant-phytoplasma co-culture system to evaluate antibiotics, using lower concentrations of antibiotics (<100–20 ppm), to reduce damage to plant tissues and sustain the defense response of plant cells to phytoplasma (Fig. 15). Using this system, more than 40 antibiotics were tested, and several decreased OY-W phytoplasma concentrations. Moreover, phytoplasmas were completely eliminated from infected plants not only by application of tetracycline but also by application of rifampicin. Rifampicin was the first antibiotic found by this method that specifically targets phytoplasmas, whereas tetracycline targets phytoplasmas and mycoplasmas. This co-culture system will contribute to the discovery of new anti-phytoplasma agents. Elimination of phytoplasma by in vitro culture using anti-phytoplasma agents will enable the production of phytoplasma-free nursery stocks, contributing to the preservation of mother trees and plants with cultural, biological, or religious significance; it will also support the international distribution of botanical resources.
Concluding remarks

Half a century ago, the discovery of phytoplasmas through Doi’s pioneering electron microscopy work led to a new research field on these novel pathogens, plant mycoplasmology, that was distinct from traditional virology and bacteriology. In the last quarter century, although there have been many barriers to the study of phytoplasmas, such as the difficulty of culturing and transforming them and the necessity of producing plant or insect hosts to maintain them, their molecular and biological properties have been elucidated. These advances have led to a new research field, phytoplasmology. Further developments, including functional definitions for all genes upon establishment of a transformation system, and the development of effective and eco-friendly agents to control phytoplasmas, will greatly contribute to both the understanding of phytoplasma biology and agricultural production over the next half-century.

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Profile

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