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REVIEW ARTICLE

Molecular biology and pathogenicity of phytoplasmas

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Abstract

Phytoplasmas are a large group of plant-pathogenic wall-less, non-helical, bacteria associated with diseases, collectively referred to as yellows diseases, in more than a thousand plant species worldwide. Many of these diseases are of great economic importance. Phytoplasmas are difficult to study, in particular because all attempts at culturing these plant pathogens under axenic conditions have failed. With the introduction of molecular methods into phytoplasmology about two decades ago, the genetic diversity of phytoplasmas could be elucidated and a system for their taxonomic classification based on phylogenetic traits established. In addition, a wealth of information was generated on phytoplasma ecology and genomics, phytoplasma–plant host interactions and phytoplasma–insect vector relationships. Taxonomically, phytoplasmas are placed in the class *Mollicutes*, closely related to acholeplasmas, and are currently classified within the provisional genus '*Candidatus* Phytoplasma' based primarily on 16S rDNA sequence analysis. Phytoplasmas are characterised by a small genome. The sizes vary considerably, ranging from 530 to 1350 kilobases (kb), with overlapping values between the various taxonomic groups and subgroups, resembling in this respect the culturable mollicutes. The smallest chromosome, about 530 kb, is known to occur in the Bermuda grass white leaf agent '*Ca.* Phytoplasma cynodontis'. This value represents the smallest mollicute chromosome reported to date. In diseased plants, phytoplasmas reside almost exclusively in the phloem sieve tube elements and are transmitted from plant to plant by phloem-feeding homopteran insects, mainly leafhoppers and planthoppers, and less frequently psyllids. Most of the phytoplasma host plants are angiosperms in which a wide range of specific and non-specific symptoms are induced. Phytoplasmas have a unique and complex life cycle that involves colonisation of different environments, the plant phloem and various organs of the insect vectors. Furthermore, many phytoplasmas have an extremely wide plant host range. The dynamic architecture of phytoplasma genomes, due to the occurrence of repetitive elements of various types, may account for variation in their genome size and adaptation of phytoplasmas to the diverse environments of their plant and insect hosts. The availability of five complete phytoplasma genome sequences has made it possible to identify a considerable number of genes that are likely to play major roles in phytoplasma–host interactions. Among these, there are genes encoding surface membrane proteins and effector proteins. Also, it has been shown that phytoplasmas dramatically alter their gene expression upon switching between plant and insect hosts.

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Introduction

Phytoplasmas are a large group of plant-pathogenic wall-less, non-helical, bacteria associated with diseases collectively referred to as yellows diseases. These diseases were thought to be caused by virus until a group of Japanese scientists recognised in the phloem sieve tube elements of yellows-diseased plants numerous wall-less, pleomorphic bodies which resembled morphologically

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and ultrastructurally mycoplasmas known to cause animal and human diseases. Owing to this resemblance, the structures detected in plants were called mycoplasma-like organisms (MLOs) (Doi *et al.*, 1967). In 1994, the cumbersome and rather vague name MLO was replaced by the trivial term phytoplasma (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*, 1993, 1997). Later, under the provisional taxonomic status '*Candidatus*', this term was adopted as the genus name of this group of plant pathogens (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004). Phytoplasmas are difficult to study, in particular because all attempts at culturing these plant pathogens under axenic conditions have failed. A new era in phytoplasma research began when molecular methods were introduced into phytoplasmology about two decades ago, following the development of procedures to isolate and enrich phytoplasmal DNA from infected plants and insects. Using these methods, the genetic diversity of phytoplasmas could be elucidated and a system for their taxonomic classification based on phylogenetic traits established. In addition, a wealth of data was generated on phytoplasmal diseases and their geographic distribution, phytoplasma ecology and genomics, phytoplasma–plant host interactions and phytoplasma–insect vector relationships. Hence, this review will focus on different aspects of molecular biology and pathogenicity of phytoplasmas.

Morphology and ultrastructure

As seen by transmission electron microscopy observations, phytoplasmas appear to be mostly spherical to ovoid in shape and their size varies from 80 to 900 nm, with an average of about 400 nm. They typically lack a firm cell wall but are surrounded by a uniform trilaminar membrane and possess, in their cytoplasm, dispersed strands resembling DNA and ribosome granules. Budding forms and spherical bodies, presumably undergoing binary fission or fragmentation, may be common. Also, pleomorphic structures passing through the narrow slits in the sieve plates and phytoplasma chain-like configurations are observed in some instances. Some sieve tube elements may be packed with phytoplasma bodies, whereas others are less populated, depending upon colonisation pattern and/or plant host species (McCoy *et al.*, 1989) (Fig 1A–D). Ultrastructural aspects of several genetically different phytoplasmas have also been studied using scanning electron microscopy (SEM), which is unrivaled for demonstration of topographical forms in three dimensions (for review see Marcone & Ragozzino, 1996). Scanning electron microscopy observations revealed that phytoplasmas were represented by polymorphic bodies such as spherical, budding, dimpled- and dumbbell-shaped cells, and filamentous branching forms present only in tube sieve elements of infected plants. The morphological differences observed most probably represent various developmental stages of phytoplasmas, depending on factors such as nutritional conditions and number and age of the bodies (Marcone & Ragozzino, 1996) (Fig 1E and F).

Taxonomy and phylogeny

Taxonomically, phytoplasmas are placed in the class *Mollicutes* (*mollis*, soft; *cutis*, skin, in Latin) of the phylum *Tenericutes* (Brown, 2010; Brown *et al.*, 2010). Members of the class *Mollicutes* (trivial names 'mycoplasmas' or 'mollicutes') are distinguished phenotypically from other eubacteria by their minute size and total lack of a cell wall. Mollicutes have evolved as a branch of *Firmicutes*, Gram-positive bacteria with a low guanine plus cytosine (G + C) content of the genome, through a process of reductive or degenerative evolution. During this process, the mollicutes lost significant genomic sequences but retained genes essential for life. Their closest walled eubacterial relatives are certain members of *Bacilli* and *Clostridia*. The G + C content of mollicutes is between 22% and 40% (Razin *et al.*, 1998; Brown *et al.*, 2007, 2010). Sequence analysis studies of 16S ribosomal DNA (rDNA) and other conserved genes suggest that phytoplasmas comprise a large, discrete, monophyletic clade that is most closely related to acholeplasmas within the class *Mollicutes* (Lee *et al.*, 2007, 2010). Like acholeplasmas, phytoplasmas use UGA in its conventional role, that is, as a stop codon, while in other mollicutes UGA is used as tryptophan codon, a feature found in mitochondria (Razin *et al.*, 1998). Formal classification of phytoplasmas, using the criteria and the traditional methods adopted for other mollicutes, has been constrained by the inability to culture them axenically, as mentioned above. Therefore, phytoplasmas are currently classified within the provisional genus '*Candidatus* Phytoplasma' based primarily on 16S rDNA sequence analysis (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004). The provisional taxonomic status '*Candidatus*' was introduced by Murray and Stackebrandt (1995) for incompletely described, uncultured prokaryotes for which specific molecular data, chiefly 16S rDNA sequences, were available. According to the classification scheme proposed by Martini *et al.* (2014), the genus '*Candidatus* Phytoplasma' along with the genus *Acholeplasma*, is assigned to the family Acholeplasmataceae within the order *Acholeplasmatales*. However, other classification schemes, relying on the assumption that the status '*Candidatus*' is not compatible with formal

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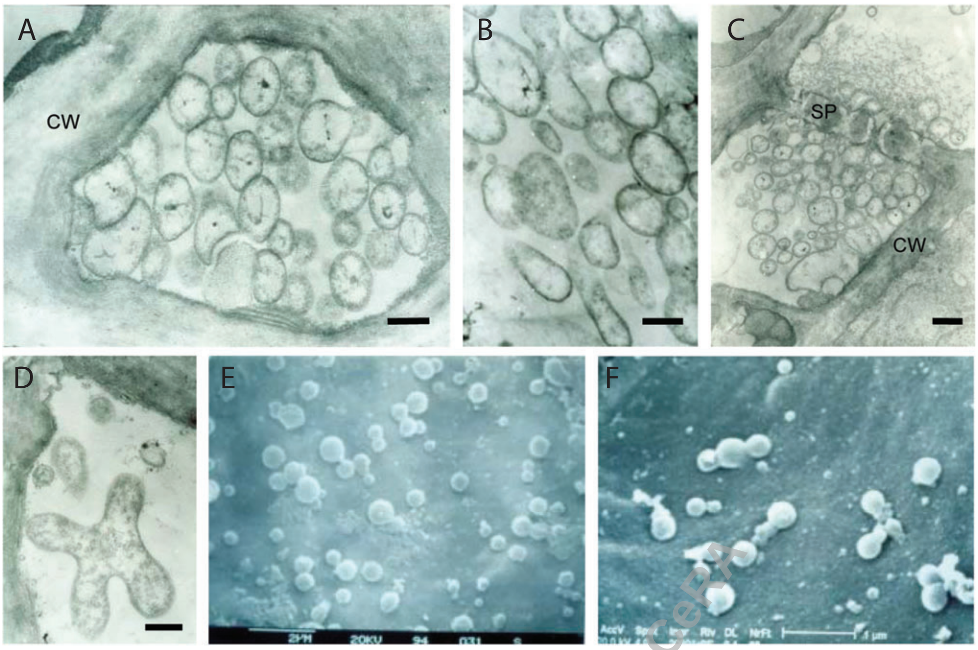

Figure 1 Transmission (A through D) and scanning (E and F) electron micrographs showing phytoplasmas in sieve tube elements of diseased plants. Elongated forms (B), chain-like configurations with bodies appearing to have completed division but still connected by a very fine cytoplasmic bridge (A and B), and an octopus-like structure of phytoplasmas (D) are present. In (C), phytoplasmas are crowded on one side of the sieve plate of contiguous sieve tubes, while a few bodies are present on the other side, suggesting that they pass through from the more crowded side to the less crowded element. CW = cell wall; SP = sieve plate; Bar = 800 nm in (A) and (B), 900 nm in (C) and 100 nm in (D). Dividing forms of phytoplasmas (E and F). Some bodies are budding, others undergoing binary fission. A triradiate body (E) and a short chain-like structure (F) are also present.

systematics, recognise the genus '*Candidatus* Phytoplasma' as *incertae sedis* (Brown *et al.*, 2010; Harrison *et al.*, 2010).

On the basis of phylogenetic analysis of 16S rDNA sequences, approximately 20 major phylogenetic groups or subclades have been identified within the phytoplasma clade. This number is generally in accordance with the phytoplasma groups or 16Sr groups established by restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified rDNA (Lee *et al.*, 1998, 2000; Seemüller *et al.*, 1998, 2002). Each phytoplasma subclade (or corresponding 16Sr group) is considered to represent at least one distinct species under the provisional taxonomic status '*Candidatus*' (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group, 2004). Also, within most of the phytoplasma groups, several distinct subgroups (16Sr subgroups) have been delineated on the basis of RFLP analysis of 16S rDNA sequences (Lee *et al.*, 2007). Recently, the number of 16Sr groups and subgroups was expanded to 32 and more than 100, respectively, through the use of a computer-simulated RFLP analysis method (Wei *et al.*, 2007, 2008*a*, 2011; Quaglino *et al.*, 2009; Zhao *et al.*, 2009*a,b*, 2013; Davis *et al.*, 2012, 2013; Wu *et al.*, 2012; Nejat *et al.*, 2013). To date, 37 '*Candidatus* Phytoplasma' species have been formally described in accordance with the guidelines established by the IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group (2004) (Table 1). Of these, 31 were solely based on 16S rDNA sequence dissimilarity with the standard threshold of 2.5% for assigning species rank to phytoplasmas, whereas the remaining species that did not fulfill this requirement were designated by examining specific biological and ecological properties and other molecular markers (Lee *et al.*, 2007, 2011; Zhao *et al.*, 2009*b*; Davis *et al.*, 2012, 2013; Martini *et al.*, 2012, 2014; Harrison *et al.*, 2014).

Over the last decade, several studies showed that many closely related phytoplasma strains cannot be readily differentiated by analysis of the highly conserved 16S rDNA sequences. Therefore, several less-conserved genes including *rpsV* (*rpl22*), *rpsC* (*rps3*), *rplP*, *rpmC*, *rpsQ*, *rplN*, *rplX*, *rplE*, *rpsN*, *rpsH*, *rplF*, *rplR*, *rpsE*, *rpmD*, *rplO*, *tuf*, *secA*, *secY*, *nus*, *vmp1*, *stamp*, *groEL*, *rpoB*, *potC* and 23S rRNA genes, and the 16S–23S rRNA spacer region sequences were employed as additional molecular markers for finer differentiation of closely related strains as well as derivative variants for a given strain (Lee *et al.*, 2007, 2010; Martini *et al.*, 2007; Hodgetts *et al.*, 2008, 2009; Cimerman *et al.*, 2009; Fabre *et al.*, 2011; Mitrović *et al.*, 2011; Durante *et al.*, 2012; Valiunas *et al.*, 2013; Manimekalai

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Table 1 Formally described '*Candidatus* Phytoplasma' species

Species name	16Sr group-subgroup[a]	Reference strain	GenBank accession no.	Reference
'*Ca*. Phytoplasma asteris'	16SrI-B	OAY	M30790	Lee *et al*. (2004*a*)
'*Ca*. Phytoplasma aurantifolia'	16SrII-B	WBDL	U15442	Zreik *et al*. (1995)
'*Ca*. Phytoplasma australasia'	16SrII-D	PpYC	Y10097	White *et al*. (1998)
'*Ca*. Phytoplasma pruni'	16SrIII-A	PX11CT1	JQ044393 (*rrnA*) JQ044392 (*rrnB*)	Davis *et al*. (2013)
'*Ca*. Phytoplasma ulmi'	16SrV-A	EY1	AY195655	Lee *et al*. (2004*b*)
'*Ca*. Phytoplasma ziziphi'	16SrV-B	JWB-G1	AB052876	Jung *et al*. (2003*a*)
'*Ca*. Phytoplasma rubi'	16SrV-D	Rus	AY197648	Malembic-Maher *et al*. (2011)
'*Ca*. Phytoplasma trifolii'	16SrVI-A	CP	AY390261	Hiruki and Wang (2004)
'*Ca*. Phytoplasma sudamericanum'	16SrVI-I	PassWB-Br3	GU292081	Davis *et al*. (2012)
'*Ca*. Phytoplasma fraxini'	16SrVII-A	AshY1	AF092209	Griffiths *et al*. (1999)
'*Ca*. Phytoplasma phoenicium'	16SrIX-D	A4	AF515636	Verdin *et al*. (2003)
'*Ca*. Phytoplasma mali'	16SrX-A	AP15	AJ542541	Seemüller and Schneider (2004)
'*Ca*. Phytoplasma pyri'	16SrX-C	PD1	AJ542543	Seemüller and Schneider (2004)
'*Ca*. Phytoplasma spartii'	16SrX-D	SpaWB	X92869	Marcone *et al*. (2004*a*)
'*Ca*. Phytoplasma prunorum'	16SrX-F	ESFY-G1	AJ542544	Seemüller and Schneider (2004)
'*Ca*. Phytoplasma oryzae'	16SrXI-A	RYD-J	AB052873	Jung *et al*. (2003*b*)
'*Ca*. Phytoplasma solani'	16SrXII-A	STOL11	AF248959	Quaglino *et al*. (2013)
'*Ca*. Phytoplasma australiense'	16SrXII-B	AUSGU	L76865	Davis *et al*. (1997)
'*Ca*. Phytoplasma japonicum'	16SrXII-D	JHP	AB010425	Sawayanagi *et al*. (1999)
'*Ca*. Phytoplasma fragariae'	16SrXII-E	StrawY	DQ086423	Valiunas *et al*. (2006)
'*Ca*. Phytoplasma cynodontis'	16SrXIV-A	BGWL-C1	AJ550984	Marcone *et al*. (2004*b*)
'*Ca*. Phytoplasma brasiliense'	16SrXV-A	HibWB26	AF147708	Montano *et al*. (2001)
'*Ca*. Phytoplasma graminis'	16SrXVI-A	SCYLP	AY725228	Arocha *et al*. (2005)
'*Ca*. Phytoplasma caricae'	16SrXVII-A	PAY	AY725234	Arocha *et al*. (2005)
'*Ca*. Phytoplasma americanum'	16SrXVIII-A	APPTW12-NE	DQ174122	Lee *et al*. (2006)
'*Ca*. Phytoplasma castaneae'	16SrXIX-A	CnWB	AB054986	Jung *et al*. (2002)
'*Ca*. Phytoplasma rhamni'	16SrXX-A	BWB	X76431	Marcone *et al*. (2004*a*)
'*Ca*. Phytoplasma pini'	16SrXXI-A	Pin127S	AJ632155	Schneider *et al*, (2005)
'*Ca*. Phytoplasma palmicola'	16SrXXII-A	LYDM-178	KF751387	Harrison *et al*. (2014)
'*Ca*. Phytoplasma omanense'	16SrXXIX-A	IM-1	EF666051	Al-Saady *et al*. (2008)
'*Ca*. Phytoplasma tamaricis'	16SrXXX-A	SCWB1	FJ432664	Zhao *et al*. (2009*b*)
'*Ca*. Phytoplasma costaricanum'	16SrXXXI-A	SoySTc1	HQ225630	Lee *et al*. (2011)
'*Ca*. Phytoplasma malaysianum'	16SrXXXII-A	MaPV	EU371934	Nejat *et al*. (2013)
'*Ca*. Phytoplasma allocasuarinae'	N.D.	AlloY	AY135523	Marcone *et al*. (2004*a*)
'*Ca*. Phytoplasma lycopersici'	N.D.	THP	EF199549	Arocha *et al*. (2007)
'*Ca*. Phytoplasma convolvuli'	N.D.	BY-S57/11	JN833705	Martini *et al*. (2012)
'*Ca*. Phytoplasma balanitae'	N.D.	BltWB	AB689678	Win *et al*. (2013)

N.D., not determined.

[a] 16Sr group and subgroup designation according to Davis *et al*. (2012, 2013), Nejat *et al*. (2013) and Harrison *et al*. (2014), based on computer-simulated RFLP analysis.

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et al., 2014). These studies revealed that phylogeny based on the mentioned less-conserved genes was nearly congruent with that inferred by 16S rDNA sequence analysis, indicating similar interrelatedness among phytoplasma taxa. However, less-conserved genes showed greater efficacy in resolving distinct phytoplasma strains than the 16S rRNA gene (Lee *et al*., 2007, 2010, 2012; Martini *et al*., 2007; Valiunas *et al*., 2013). Recently, multi-locus sequence analysis employing genes with varying degrees of genetic variability revealed new insights into the genetic diversity of some phytoplasmas that are relatively homogeneous at 16S rDNA sequence level. In particular, this analysis provided useful molecular tools for delineation of genetically closed but pathologically and/or ecologically distinct strains. Identification of these strains is essential and highly relevant for epidemiological studies and to better define '*Ca*. Phytoplasma' species (Lee *et al*., 2007, 2012; Cimerman *et al*., 2009; Duduk *et al*., 2009; Adkar-Purushothama *et al*., 2011; Casati *et al*., 2011; Wei *et al*., 2011; Durante *et al*., 2012; Davis *et al*., 2013).

Genome

Chromosome size

Chromosome size is a particularly significant character of mollicutes including phytoplasmas because the evolutionary history of these prokaryotes involved significant losses of their ancestors' chromosome portions

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(Razin *et al.*, 1998). The application of pulsed-field gel electrophoresis (PFGE) to mollicute genome size determinations has provided a wealth of genome size data. These data show a continuum of genome sizes among culturable mollicutes, ranging from less than 600 to over 2200 kilobases (kb), with overlapping values between the various genera (Razin *et al.*, 1998). A similar wide range has been observed for over 100 phytoplasmas, belonging to major phylogenetic groups and subgroups, whose genome sizes varied from 530 kb to 1350 kb (Neimark & Kirkpatrick, 1993; Marcone *et al.*, 1999, 2001; Fig. 2). In particular, considerable size variation, from 660 kb to 1130 kb, was observed among aster yellows (AY) phytoplasmas. Chromosome size heterogeneity was also observed in the stolbur phytoplasma group (range: 860–1350 kb). In this group, the tomato-infecting strain STOLF contains the largest chromosome found in a phytoplasma to date (Fig. 2B). A wide range of chromosome sizes, from 670 kb to 1075 kb, was also identified in the X-disease group. The other phytoplasmas examined, which included members of the apple proliferation (AP), peanut witches'-broom, pigeon pea witches'-broom, sugarcane white leaf, Bermuda grass white leaf, ash yellows, clover proliferation, and elm yellows groups, all have chromosomes smaller than 1000 kb, and the size range within each of these groups is narrower than in the AY, stolbur, and X-disease groups (Marcone *et al.*, 1999, 2001). The smallest chromosome, about 530 kb (Fig. 2A), was found in seven isolates of the Bermuda grass white leaf agent '*Ca*. Phytoplasma cynodontis', collected at different locations in southern Italy (Marcone *et al.*, 1999, 2004*b*). This value represents the smallest mollicute chromosome reported to date.

The genome sizes of many phytoplasmas are larger than those of many culturable mollicutes. Therefore, small genome size alone cannot explain the inability to culture phytoplasmas, although, a larger genome and consequently a larger number of genes would be expected to endow the organism with better adaptability to grow in axenic culture. In the work by Marcone *et al.* (1999), the AY phytoplasmas examined differed greatly in virulence for periwinkle and caused a wide variety of symptoms in this host. However, no relation between symptoms or virulence and genome sizes was observed. Also, in the stolbur phytoplasma group, the two strains with the largest genomes, namely MOL and STOLF, were much less virulent for periwinkle than the other stolbur strains. Similarly, in the work by Seemüller & Schneider (2007), two avirulent or mild strains of '*Ca*. Phytoplasma mali' showed larger chromosomes that three virulent strains of the same pathogen, when examined by PFGE analysis. In contrast, it has been shown that a derivative line of the onion yellows phytoplasma causes milder symptoms and has a smaller chromosome than the wild type (870 kb *versus* 1000 kb) (Oshima *et al.*, 2001). According to these authors, the reduced virulence of the derivative line may be due to the loss of genes responsible for pathogenicity. The considerable variation in genome sizes among phytoplasmas, including closely related strains of the same taxon, may result from chromosomal rearrangements due to the occurrence of repetitive elements of various types. These elements are known from phytoplasmas from which the complete genome sequence is available (see Section on 'Genome sequencing' below).

Physical mapping of phytoplasma chromosomes and sizing of restriction fragments as well as sequencing of the complete genomes have also served as an additional source of data on genome size of five phytoplasmas (see Sections on 'Physical and genetic maps' and 'Genome sequencing' below).

Physical and genetic maps

Pulsed-field gel electrophoresis has had a major impact on genomic studies of unculturable phytoplasmas, mainly due to its ability to separate physically full-length phytoplasma chromosomes from contaminating host plant nucleic acids. Mapping of phytoplasma chromosomes was done for '*Ca*. Phytoplasma pruni' (the X-disease agent), '*Ca*. Phytoplasma mali' (the AP agent), '*Ca*. Phytoplasma prunorum' (the European stone fruit yellows agent), sweet potato little leaf and flavescence dorée phytoplasmas using PFGE-purified full-length chromosomes from high-titre phytoplasma-infected herbaceous host plants (Firrao *et al.*, 1996; Lauer & Seemüller, 2000; Padovan *et al.*, 2000; Marcone & Seemüller, 2001; Malembic-Maher *et al.*, 2008). The physical maps were generated with single and double digestions using rare-cutting restriction endonucleases possessing GC-rich recognition sequences and resolving the fragments by PFGE. These studies revealed that different restriction endonucleases were required to obtain suitable macrorestriction fragments for map construction among the phytoplasmas examined. Also, frequency and location of restriction sites varied considerably. The distribution of the mapped restriction sites was relatively uniform in the chromosomes of the X-disease and sweet potato little leaf phytoplasmas, whereas a pronounced clustering was observed in the AP, European stone fruit yellows and flavescence dorée phytoplasma chromosomes. This clustering reflects differences in the overall base pair composition of the genomes. Several genetic loci were placed on the maps by Southern hybridisation of restriction fragments with various phytoplasma probes. It could be shown that the two rRNA operons, which seem to be present in all phytoplasmas, were not linked. However,

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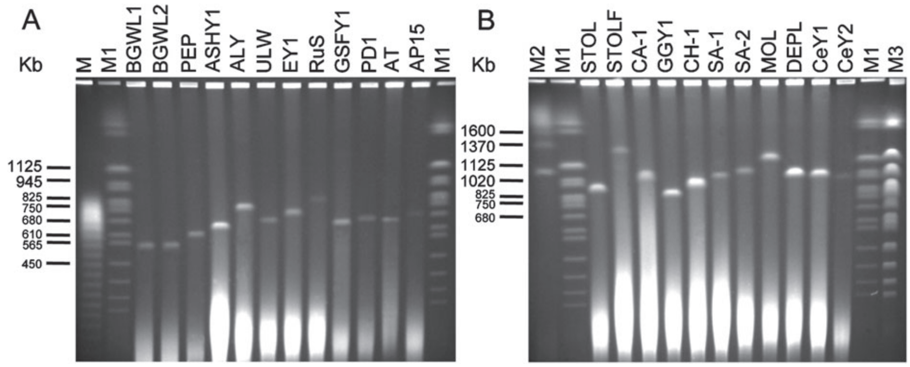

Figure 2 Full-length phytoplasma chromosomes isolated by pulsed-field gel electrophoresis (PFGE). The size markers were: M, concatameric lambda DNA ladder (Bio-Rad Laboratories, Hercules, CA, USA); M1, *Saccharomyces cerevisiae* chromosomes (Bio-Rad Laboratories); M2, *Hansenula wingei* chromosomes (Bio-Rad Laboratories); M3, *S. cerevisiae* chromosomes (Pharmacia Biotechnology, Inc., Uppsala, Sweden). (A) BGWL1 and BGWL2, Bermuda grass white leaf; PEP, picris echioides phyllody; ASHY1, ash yellows; ALY, alder yellows; ULW and EY1, elm yellows; RuS, rubus stunt; GSFY1, European stone fruit yellows; PD1, pear decline; AT and AP15, apple proliferation. (B) STOL, stolbur of pepper; STOLF, tomato stolbur; CA-1, GGY1, CH-1, SA-1 and SA-2, grapevine yellows; MOL, Molière disease of cherry; DEPL, lavender decline; CeY1 and CeY2, celery yellows.

in the sweet potato little leaf phytoplasma chromosome one of the rRNA operons had an opposite orientation (Padovan *et al.*, 2000). Although there were differences in restriction enzymes that were suitable for map construction and in frequency and location of restriction sites, the overall genetic arrangement of chromosomes of the closely related AP and European stone fruit yellows phytoplasmas was similar, as evidenced by gene positions on the maps (Marcone & Seemüller, 2001). Physical and genetic maps of phytoplasma chromosomes are useful for genome comparisons within the '*Candidatus* Phytoplasma' genus and assisting contig assembly in sequencing projects targeting either selected chromosomal fragments or entire chromosomes.

Restriction mapping of full-length phytoplasma chromosomes using a few rare-cutting enzymes also revealed that the pear decline agent '*Ca*. Phytoplasma pyri' had macrorestriction fragment patterns different from those of the closely related AP and European stone fruit yellows agents (Seemüller & Schneider, 2004). Similarly, the closely related sweet potato little leaf and tomato big bud phytoplasmas showed different macrorestriction fragment patterns, while six strains of the AP phytoplasma, which differed in virulence and genome size, all had distinctly different macrorestriction fragment patterns (Padovan *et al.*, 2000; Seemüller & Schneider, 2007). Macrorestriction mapping is a useful tool for typing phytoplasma strains. Work by Kube *et al.* (2008) has shown that '*Ca*. Phytoplasma mali', '*Ca*. Phytoplasma prunorum' and '*Ca*. Phytoplasma pyri' have linear chromosomes and not circular as is the case for almost all walled bacteria. To prove chromosome linearity, the PFGE-purified, agarose-embedded full-length chromosomes of the three mentioned phytoplasmas were digested with I-*Ceu*I, an intron-encoded endonuclease that cleaves specifically in the 23S rRNA gene of many bacteria. This digestion yielded three macrorestriction fragments, as expected for linear chromosomes having two rRNA operons whereas I-*Ceu*I restriction of the '*Ca*. Phytoplasma australiense' resulted in only two fragments, as expected for a circular chromosome with two rRNA operons.

Genome sequencing

The genomes of five phytoplasmas have been completely sequenced to date. These phytoplasmas include strains onion yellows M (OY-M) and witches'-broom (AY-WB) of '*Ca*. Phytoplasma asteris' (AY phytoplasmas), an Australian strain (PAa) and the New Zealand strain strawberry lethal yellows (SLY), both of '*Ca*. Phytoplasma australiense', and the AP agent '*Ca*. Phytoplasma mali' (strain AT) (Oshima *et al.*, 2004; Bai *et al.*, 2006; Kube *et al.*, 2008; Tran-Nguyen *et al.*, 2008; Andersen *et al.*, 2013). In addition, draft genome sequences were determined for a number of phytoplasmas including four strains of the X-disease phytoplasma group, two strains of the stolbur phytoplasma, and the peanut witches'-brooms and wheat blue dwarf agents (Saccardo *et al.*, 2012; Chung *et al.*, 2013; Chen *et al.*, 2014; Mitrović *et al.*, 2014).'*Ca*. Phytoplasma asteris' strains OY-M and AY-WB and '*Ca*. Phytoplasma australiense' strains have circular chromosomes, like those of all culturable mollicutes examined so far, whereas '*Ca*. Phytoplasma mali' has a linear chromosome as mentioned above (Table 2). In addition to the chromosome, the genomes of '*Ca*. Phytoplasma asteris' strains OY-M and AY-WB and '*Ca*. Phytoplasma australiense' strains contain extrachromosomal DNA elements (plasmids). These extrachromosomal elements allow the integration

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Table 2 General features of the five completely sequenced phytoplasma genomes[a]

'*Ca.* Phytoplasma' species Strain	asteris OY-M	asteris AY-WB	australiense PAa	australiense SLY	mali AT
Chromosome size (kb)	860 631	706 569	879 324	959 779	601 943
Chromosome organisation	Circular	Circular	Circular	Circular	Linear
G + C content (%)	28	27	27	27	21.4
Protein-coding regions (%)	73	72	74	78	78.9
Protein-coding genes with assigned function	446	450	502	528	338
Conserved hypothetical genes	51	149	214	249	72
Hypothetical genes	257	72	123	349	87
Total no. of genes	754	671	839	1126	497
rRNA operons	2	2	2	2	2
tRNA genes	32	31	35	35	32
Extrachromosomal DNAs	2	4	1	1	0
GenBank accession no.	AP006628	CP000061	AM422018	CP002548	CU469464

[a]Data were obtained from Oshima *et al.* (2004), Bai *et al.* (2006), Kube *et al.* (2008), Tran-Nguyen *et al.* (2008) and Andersen *et al.* (2013).

of their genetic material into the chromosome and may play an essential role in determining insect transmissibility of phytoplasmas, as has been shown for some strains (Nishigawa *et al.*, 2002; Ishii *et al.*, 2009). With a size of 960 kb '*Ca*. Phytoplasma australiense' strain SLY has the largest phytoplasma chromosome deciphered so far while '*Ca*. Phytoplasma mali' with a size of 602 kb stands at the opposite end. '*Ca*. Phytoplasma mali' has a G + C content of less than 22%. This is the lowest value among all mollicutes and most walled bacteria analysed to date. A similar G + C content of 27–28% is known for the other four phytoplasma genomes (Table 2). The number of predicted protein-coding genes ranges from 497 to 1126, comprising from 72% to 79% of the phytoplasma chromosomes. The highest number of strain-specific genes is present in '*Ca*. Phytoplasma australiense' strain SLY. Forty-six to sixty-eight per cent of the potentially protein-coding genes have assigned functions, while the remaining are described as hypothetical with unknown function. Chromosome of '*Ca*. Phytoplasma mali' is characterised by large terminal inverted repeats that have covalently closed hairpin ends protecting the chromosome (Kube *et al.*, 2008, 2012). This chromosome organisation is unique within the bacteria. Also, the '*Ca*. Phytoplasma mali' chromosome has a regular cumulative GC-skew pattern. In contrast, the other sequenced phytoplasma chromosomes have irregular GC-skew patterns, indicative of high genome instability (Bai *et al.*, 2006; Kube *et al.*, 2008, 2012).

Phytoplasma genomes harbour large clusters of repeated sequences which are mostly multicopy genes. These clusters, which are of up to approximately 20 kb in size, were termed potential mobile units (PMUs) because, at least some of them, have the characteristics of replicative composite transposons (Bai *et al.*, 2006; Hogenhout *et al.*, 2008). Potential mobile units tend to congregate as tandem or multiple repeats in certain regions of the phytoplasma chromosomes. They may also form extrachromosomal elements that may replicate independently and integrate again into phytoplasma chromosomes (Toruño *et al.*, 2010). Potential mobile units appear to be unique to phytoplasmas. Recent studies have shown that horizontal transfer of PMUs may occur among different phytoplasmas (Chung *et al.*, 2013; Ku *et al.*, 2013). Potential mobile unit-like regions carrying truncated copies of PMU genes and lacking various segments of full-length PMUs, are also present in the phytoplasma genomes. These sequences are supposed to be degenerated forms of PMUs. Genomes of '*Ca.* Phytoplasma asteris' strains OY-M and AY-WB and '*Ca*. Phytoplasma australiense' strains have many copies of PMUs and PMU-like regions. In contrast, '*Ca*. Phytoplasma mali' genome only has few copies. Thus, this pathogen has the most condensed genome among the five mentioned phytoplasmas (Kube *et al.*, 2008, 2012; Andersen *et al.*, 2013). Genetic elements with a gene content similar to that of PMUs have also been described in phytoplasma genomes. These elements have been named sequence-variable mosaics (SVMs) and are proposed to originate from attacks of phages of the order *Caudovirales* (Jomantiene & Davis, 2006; Wei *et al.*, 2008*b*). Therefore, the occurrence of PMUs, prophage-related sequences and plasmids in phytoplasma genomes may account for variation in genome size and adaptation of phytoplasmas to the diverse environments of their plant and insect hosts (Bai *et al.*, 2006; Jomantiene & Davis, 2006; Hogenhout *et al.*, 2008; Kube *et al.*, 2012).

Phytoplasmas have reduced biosynthetic capabilities. In all five complete phytoplasma genomes, many genes coding for the biosynthesis of amino acids and fatty acids, nucleotides, oxidative phosphorylation, tricarboxylic acid cycle, pentose phosphate pathway,

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and the phosphotransferase system (PTS), by which sugars are imported and phosphorylated to feed the glycolytic pathway, are lacking. Also, most pronounced in phytoplasma genomes is the lack of genes coding for F-type ATPases (ATP synthases) responsible for generating transmembrane potential and consequently ATP synthesis. In addition, '*Ca*. Phytoplasma mali' has a rudimentary glycolysis in which the energy-yielding part is completely missing (Kube *et al.*, 2008, 2012). Despite the missing PTS, glycolysis is considered to be the major energy-yielding pathway of other phytoplasmas (Oshima *et al.*, 2004; Bai *et al.*, 2006; Tran-Nguyen *et al.*, 2008). An alternative ATP-yielding pathway has recently been identified in phytoplasma genomes (Siewert *et al.*, 2014). In this pathway, malate or oxaloacetate is taken up by the symporter MleP (synonym CitS) and is oxidatively decarboxylated to pyruvate by the malate dehydrogenase. Then, pyruvate is converted to acetyl-CoA by the pyruvate dehydrogenase multienzyme complex. The PduL-like phosphotransacetylase forms acetyl-phosphate, which is transformed by the acetate kinase to acetate and ATP. Key enzymes in this pathway are malate dehydrogenase and acetate kinase. The malate dehydrogenase has not been identified in the genomes of culturable mollicutes including acholeplasmas but has been reported in all complete phytoplasma genomes and several phytoplasma draft genomes (Kube *et al.*, 2014; Siewert *et al.*, 2014). Phylogenetic analyses based on malate dehydrogenase and acetate kinase sequences also revealed that phytoplasmas are more closely related to *Bacilli* and *Clostridia* than to *Mycoplasma* (Kube *et al.*, 2014; Siewert *et al.*, 2014). This finding indicates an early divergence and independent evolution of *Acholeplasmatales* from other mollicutes. Phytoplasmas have a relatively large number of genes encoding efficient transporter systems for the uptake of the required nutrients. These include ATP-binding cassette (ABC) transporter systems for dipeptides/oligopeptides (DppCBADF), spermidine/putrescine (PotABCD), D-methionine (MetNQI), cobalt (CbiOQ), manganese/zinc (ZnuBCA), sugar (MalE) and malate (MleP). MalE may have affinity to maltose, trehalose, sucrose and palatinose. Sucrose and trehalose are major sugars in plant phloem and insect haemolymph, respectively. A putative thiamine transporter of the YuaJ-family is encoded in phytoplasma genomes. In addition, phytoplasma genomes encode multidrug efflux pumps (MdlA/B, NorM), P-type ATPases and the large-conductance mechanosensitive channel (MscL). The mentioned genomic features reflect the marked dependency of phytoplasmas on the supply of many metabolic compounds from their hosts (Kube, 2011; Kube *et al.*, 2012, 2014; Andersen *et al.*, 2013; Siewert *et al.*, 2014).

Ecology and habitats

Phytoplasmas are obligate parasites associated with diseases in more than a thousand plant species worldwide. Many of these diseases, especially those of woody plants, are of great economic importance. In diseased plants, phytoplasmas reside almost exclusively in the phloem sieve tube elements and are transmitted from plant to plant by phloem-feeding homopteran insects, mainly leafhoppers (Cicadellidae) and planthoppers (Fulgoromorpha), and less frequently psyllids (Psyllidae) (Weintraub & Beanland, 2006). A few species of heteropteran insects of the family Pentatomidae (stinkbugs) are also reported as phytoplasma vectors (Hiruki, 1999; Weintraub & Beanland, 2006). Once phytoplasmas have entered the phloem sieve tube elements, they spread systemically throughout the plant by passing through phloem sieve plate pores, with the sieve tube flow. Occasionally, a few phloem parenchyma and companion cells adjacent to sieve tubes are also invaded (Seemüller *et al.*, 2002). However, it is unknown how phytoplasmas gain access to these cells. It seems impossible that phytoplasmas squeeze through the pore-plasmodesmata units or plasmodesma because these structures, which are responsible for regulated exchange of compounds between sieve elements and companion cells, have a pore diameter of only 3–4 nm. Also, changes to the ultrastructures of pore-plasmodesmata units and plasmodesma have not been observed in phytoplasma infected plants (Christensen *et al.*, 2005). Another argument to be considered in questioning the occurrence of phytoplasmas in parenchyma or companion cells is the marked physiological difference between sieve tube elements and parenchyma cells. Sieve elements are largely free from cell organelles when mature forming thus a low resistance pathway for assimilates and facilitating the spread of phytoplasmas. The sieve element sap is under high hydrostatic (turgor) pressure and rich in nutrients. The content varies depending on the plant species but always includes large amounts of carbohydrates. The most abundant is sucrose that may have a concentration of 10–30% (Evert, 1977). Phloem parenchyma has a much lower osmotic pressure and a full complement of cell organelles. Companion cells also have a complete set of cell organelles. However, like sieve elements, they have a high sucrose content and a high osmotic pressure and may be a more appropriate environment than parenchyma cells for harbouring phytoplasmas. In their natural insect vectors, phytoplasmas must pass through a complex biological cycle in order to be transmitted to a plant. After being ingested with phloem sap from an infected plant, phytoplasmas must traverse the insect mid-gut lining, reach the organic compound-rich haemolymph, where they circulate and multiply, and invade various other

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insect organs and tissues, including the salivary glands, in which they multiply further. Then, phytoplasmas are introduced, along with saliva, into sieve tube elements of a new host plant during insect feeding (Hogenhout *et al.*, 2008; Gasparich, 2010). Hence, phytoplasmas are able to colonise hosts of two distinct kingdoms, that is, plants (Plantae) and insects (Animalia). The list of plants and insects known to harbour phytoplasmas is continuously increasing, as is the number of taxonomically characterised phytoplasma strains (McCoy *et al.*, 1989; Seemüller *et al.*, 1998, 2002; Lee *et al.*, 2000; Bertaccini, 2007; Bertaccini & Duduk, 2009; Marcone, 2012).

Although phytoplasma DNA has been detected in embryos of lethal yellowing diseased-coconut palms and seeds from phytoplasma-infected plants of lime, alfalfa, tomato, oilseed rape, maize and apricot, there is no clear-cut evidence that phytoplasmas are seed-borne pathogens (for reviews see Faghihi *et al.*, 2011; Dickinson *et al.*, 2013). Also, phytoplasmas are not sap-transmissible. However, they can be spread by the use of infected vegetative propagating material (Lee *et al.*, 2000; Dickinson *et al.*, 2013). Many phytoplasmas have been experimentally transmitted from naturally infected plants to periwinkle (*Catharanthus roseus*) via dodder (*Cuscuta* spp.) bridges. Periwinkle is the most commonly used experimental host in which phytoplasmas are routinely maintained by periodic grafting.

Most of the phytoplasma host plants are angiosperms in which a wide range of specific and non-specific symptoms are induced (Fig. 3). Symptoms of affected plants may vary with the phytoplasma strain, host plant, stage of the disease, age of the plant at the time of infection, phytoplasma concentration in infected tissues, strain interactions and environmental conditions (for reviews see McCoy *et al.*, 1989; Lee *et al.*, 2000; Seemüller *et al.*, 2002; Marcone, 2010). Specific symptoms include virescence, phyllody, big bud, flower proliferation and other flower abnormalities, all resulting in sterility, witches'-brooms, rosetting, internode elongation and aetiolation, shortened internodes, enlarged stipules, off-season growth and brown discoloration of phloem tissue. Less specific and non-specific symptoms which are most often common in woody plants, include foliar yellowing and reddening, small leaves, leaf roll, leaf curl, vein clearing, vein enlargement, vein necrosis, premature autumn coloration, premature defoliation, undersized fruits, poor terminal growth, sparse foliage, die-back, stunting of overall plant growth, and decline. In rare instances, phytoplasma-infected plants are fully non-symptomatic over their life span, whereas a temporary or permanent remission of symptoms may also occur. Fewer phytoplasmas have been detected in gymnosperms of which most hosts are from *Pinaceae* and *Cupressaceae* families. Infections usually result in yellowing symptoms, stunted growth, dwarfed needles and proliferation of shoots (Schneider *et al.*, 2005; Davis *et al.*, 2010; Kamińska *et al.*, 2011).

Phytoplasmas occur worldwide, but there are differences in the distribution of the various taxonomic groups and subgroups. For example, 16SrI-B subgroup phytoplasmas are distributed worldwide, whereas phytoplasmas of subgroups 16SrI-L and 16SrI-M appear to be restricted to Europe. Fruit tree phytoplasmas of the AP group (16SrX group) are known to occur in Europe with the exception of the peach yellow leaf roll and pear decline Taiwan agents, whereas rice yellow dwarf group phytoplasmas are only known from Asian countries. The geographic distribution of phytoplasmas appears to be correlated with that of their plant hosts and insect vectors (for reviews see Seemüller *et al.*, 1998, Lee *et al.*, 2000, 2004*a*). Phytoplasmas may differ considerably in their plant host specificity. As mentioned above, phytoplasmas of the subgroups 16SrI-A, 16SrI-B and 16SrI-C, have a wide plant host range which is composed of more than 80 plant species. In contrast, fruit tree phytoplasmas of the AP group preferentially infect only one host (for reviews see Seemüller *et al.*, 1998, 2002; Lee *et al.*, 2000). Plant host specificity is still poorly understood. Because most or all phytoplasmas grow in periwinkle and induce specific symptoms in this host, it seems that there is no strict plant host specificity in phytoplasmas. However, in nature the plant host range of a given phytoplasma largely depends on the three-way interaction between pathogen, plant host and insect vector. Over the last two decades, several studies have shown that a single plant can be doubly or multiply infected with different phytoplasmas. This phenomenon is common in perennial plants, whose long life spans provide vast opportunities to be visited and inoculated by vectors carrying various phytoplasmas. Furthermore, distinctly different phytoplasmas may induce similar symptoms in a given plant host. A well known example of distinct phytoplasmas inducing similar symptoms in the same plant is grapevine affected by grapevine yellows disorder, which can be caused by either the 16SrV group phytoplasmas including the flavescence dorée agent or by phytoplasmas from the 16SrI, 16SrII, 16SrIII and 16SrXII groups (Belli *et al.*, 2010). There are also indications that several phytoplasmas, including AP, European stone fruit yellows, ash yellows, alder yellows and AY agents, exist as strains which greatly differ in aggressiveness, ranging from being avirulent (or nearly avirulent) to highly virulent. Interactions between distinct strains of the same taxon have been described for a number of phytoplasma–plant host combinations (for review see Marcone, 2010). Recent work has shown that multiple infections by distinctly different strains of AP

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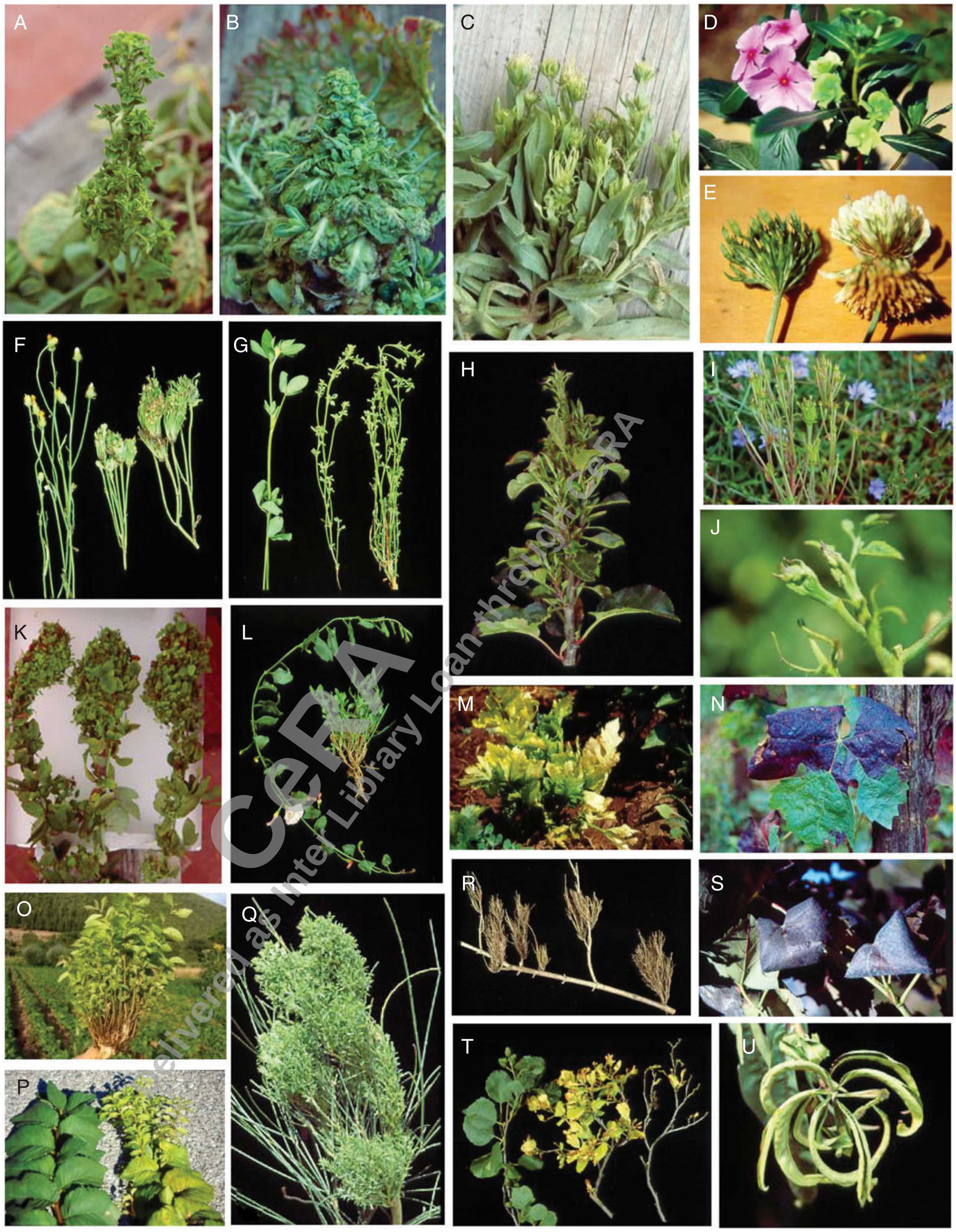

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phytoplasma are widespread in AP-affected apple trees (Seemüller *et al.*, 2010, 2011). These studies also revealed that multiple infections are of pathological relevance due to antagonistic strain interactions leading to shifts in the phytoplasma composition, that drastically alter virulence.

Phytoplasmas also differ in their insect vector specificity. Some phytoplasmas have a low insect vector specificity, being transmitted by several vector species, for example, subgroup 16SrI-B phytoplasmas, whereas others show a very high vector specificity, being transmitted by only one or a few vector species, for example, temperate fruit tree phytoplasmas of the AP group (Seemüller *et al.*, 1998, 2002; Lee *et al.*, 2000). Also, many insect vectors can transmit more than one phytoplasma. The number of insect vectors and their feeding behaviour play major roles in determining the plant host range of a given phytoplasma. For example, phytoplasmas of subgroups 16SrI-A, 16SrI-B and 16SrI-C, which are transmitted by numerous polyphagous leafhoppers including *Macrosteles* spp., *Euscelis* spp., *Scaphytopius* spp. and *Aphrodes* spp., are causing diseases in a wide range of plant species, whereas the flavescence dorée phytoplasma, which is transmitted by the monophagous vector *Scaphoideus titanus*, is known to infect only grapevine in nature (for review see Lee *et al.*, 2000). Although phytoplasmas were not believed to be transmitted vertically to the progeny of vector insects for many years, PCR-based and electron microscopical studies conducted over the last years, provided indications for transovarial transmission of AY phytoplasmas by the leafhoppers *S. titanus* and *Hishimonoides sellatiformis* (Alma *et al.*, 1997; Kawakita *et al.*, 2000), and sugarcane white leaf and European stone fruit yellows phytoplasmas by *Matsumuratettix hiroglyphicus* and *Cacopsylla pruni*, respectively (Hanboonsong *et al.*, 2002; Tedeschi *et al.*, 2006).

Interactions with plant hosts

As phytoplasmas live and multiply in functional phloem sieve tube elements, the main effect of phytoplasmal infections apparently is the impairment of the sieve tube function. Several studies have shown that inhibition of phloem transport occurs in phytoplasma-infected plants, which, in turn, leads to an accumulation of abnormal amounts of carbohydrates in source leaves, that is, mature leaves, and a marked reduction of these essential energy-storage compounds in sink organs, that is, young leaves, shaded leaves, flowers, growing fruits and roots (Catlin *et al.*, 1975; Braun & Sinclair, 1976, 1978; Kartte & Seemüller, 1991; Lepka *et al.*, 1999; Maust *et al.*, 2003). Changes in photosynthate translocation along with other impaired physiological functions including reduced photosynthesis, pigment content, stomatal conductance, transpiration and root respiration, and altered secondary metabolism and disturbed plant hormone balance, could account for symptoms exhibited by infected plants (Lepka *et al.*, 1999; Lo Gullo *et al.*, 2000; Tan & Whitlow, 2001; Bertamini *et al.*, 2003; Maust *et al.*, 2003; Choi *et al.*, 2004; Endeshaw *et al.*, 2012; Ding *et al.*, 2013; Vitale *et al.*, 2013; Gai *et al.*, 2014). However, the exact mechanisms by which phytoplasmas induce disease in plants and the reason for different reactions of the host plants to phytoplasmal infections are still poorly understood.

Recent studies have shown that symptoms of flower abnormalities occurring in phytoplasma-infected plants are associated with deregulations of key floral development genes. In tomato plants infected by the stolbur phytoplasma, *FALSIFLORA* (*FA*), an orthologue of the *Arabidopsis thaliana LEAFY* (*LFY*) gene, which controls the identity of the inflorescence meristem, was up-regulated, whereas *LeWUSCHEL* (*LeWUS*) and *LeCLAVATA1* (*LeCLV1*), orthologues of *A. thaliana* genes *WUS* and *CLV1*, respectively, regulating the meristem development, and *LeDEFICIENS* (*LeDEF*), orthologue of the *A. thaliana* gene *APETALA3* (*AP3*), responsible for petal and stamen identity, were down-regulated. In contrast, *TAG1*, orthologue of the *A. thaliana* gene *AGAMOUS* (*AG*), which regulates

Figure 3 (A through E) Symptoms of phyllody and virescence induced by aster yellows (16SrI) phytoplasmas in *Raphanus raphanistrum* (wild radish) (A), *Brassica oleracea* var. *capitata* (cabbage) (B), *Calendula officinale* (pot marigold) (C), *Catharanthus roseus* (periwinkle) (D) and *Trifolium repens* (white clover) (E). In (D) and (E), healthy control on the left and right, respectively. (F) Diseased plants of *Crepis setosa* (hawksbeard) showing abnormal flowers from which numerous shoots proliferated (left, healthy) associated with 16SrIX phytoplasma infections. (G) 16SrII-phytoplasma infected *Medicago sativa* (alfalfa) showing witches'-broom (left, healthy). (H) Witches'-broom on apple caused by the apple proliferation agent '*Ca*. Phytoplasma mali'. (I) *Cichorium intybus* (common chicory) showing phyllody and proliferation symptoms infected by a phytoplasma of the 16SrII group. Symptoms of flower abnormalities on tomato (J), stunting and proliferation on *Convolvulus arvensis* (field bindweed) (L), yellowing on *Apium graveolens* (celery) (M), and grapevine yellows (N and S), all associated with stolbur (16SrXII) phytoplasma infections. In (L), healthy control on the left. (K) Rubus stunt (16SrV) phytoplasma-affected *Rubus fruticosus* (wild blackberry) showing witches'-brooms. (O and P) Elm yellows (16SrV) phytoplasma-infected *Ulmus minor* (European field elm) with witches'-brooms arising from a root (O) and at the tip of a twig (P) (left, healthy). (Q) Witches'-brooms on *Spartium junceum* (Spanish broom) caused by '*Ca*. Phytoplasma spartii' and a 16SrV phytoplasma. (R) Witches'-brooms arising from a branch of *Rhamnus catharticus* (buckthorn) associated with '*Ca*. Phytoplasma rhamni'. (T) Dieback and yellowing of *Alnus glutinosa* (alder) caused by the alder yellows (16SrV phytoplasma). (U) Symptoms of leaf rolling and curling on peach associated with European stone fruit yellows. (R and T, courtesy E. Seemüller, Julius Kuehn Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Dossenheim, Germany).

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stamen and carpel identity and controls the terminal growth of a flower through repression of *LeWUS*, was up-regulated in the early stages of flower development and down-regulated in the late stages (Pracros *et al.*, 2006). In petunia (*Petunia hybrida*) plants infected by the OY agent '*Ca.* Phytoplasma asteris' (strain OY-W), the expression levels of several homeotic genes required for organ development, such as *PETUNIA FLOWERING GENE* (*PFG*), *P. hybrida GLOBOSA1* (*PhGLO1*) and *FLORAL BINDING PROTEIN7* (*FBP7*) were significantly reduced in sepals, petals and pistils, all affected by morphological changes, but not in stamens which proved to be symptomless. Moreover, the expression levels of genes which are known to be involved in floral meristem identity, such as *TERMINATOR* (*TER*), *ABERRANT LEAF AND FLOWER* (*ALF*) and *DOUBLE TOP* (*DOT*) were significantly reduced in infected petunia meristems. These findings imply that phytoplasmal infections affect an upstream signaling pathway of floral meristem identity (Himeno *et al.*, 2011). In hydrangea (*Hydrangea* spp.) plants affected by the Japanese hydrangea phyllody agent '*Ca.* Phytoplasma japonicum', down-regulation of the class-A, -B and -C genes, which specify floral organ identity, occurred only in the early stages of floral organ development (Kitamura *et al.*, 2009). Down-regulation of class-B and -C genes, and pigmentation genes was reported to occur in periwinkle plants following infection with either the periwinkle leaf yellowing (PLY) agent '*Ca.* Phytoplasma asteris' or the peanut witches'-broom phytoplasma. Among the genes examined, *CrSEP3*, an *A. thaliana SEPALLATA3* orthologue, showed the greatest suppression. As SEP proteins are important for both floral organ and floral meristem identities, the loss of SEP functions may play a major role in inducing flower malformations (Su *et al.*, 2011). Furthermore, Cettul and Firrao (2011) reported that *SEP* genes are down-regulated in Italian clover phyllody phytoplasma-infected *A. thaliana* plants, showing flower malformations.

Over the last few years, several other plant host genes, which are differentially expressed upon phytoplasmal infections, have been identified. These include genes involved in phytohormone activity, photosynthesis, carbohydrate and lipid metabolism, amino acid transport, phenylpropanoid biosynthesis, cell wall biosynthesis and degradation, and plant stress and/or defence response (Jagoueix-Eveillard *et al.*, 2001; Carginale *et al.*, 2004; Nicolaisen & Horvath, 2008; Albertazzi *et al.*, 2009; Hren *et al.*, 2009*a,b*; Chen & Lin, 2011; De Luca *et al.*, 2011; Zamharir *et al.*, 2011; Guerriero *et al.*, 2012; Ding *et al.*, 2013; Mou *et al.*, 2013). Furthermore, the availability of complete phytoplasma genome sequences has made it possible to identify a considerable number of genes that are likely to play major roles in phytoplasma–host interactions. Among these, there are genes encoding surface membrane proteins and effector (virulence) proteins (Bai *et al.*, 2009; Hoshi *et al.*, 2009; MacLean *et al.*, 2011; Sugio *et al.*, 2011*a,b*; Kube *et al.*, 2012).

Like other plant pathogens, phytoplasmas secrete effector proteins that interfere with host cell processes, leading to changes in plant development. These proteins are likely to be released into the host cell cytoplasm through the general SecA-dependent protein translocation system, which is known to be functional in phytoplasmas (Kakizawa *et al.*, 2004). Among the 56 candidate effector proteins (SAPs) identified by mining the genome sequence data of the AY agent '*Ca.* Phytoplasma asteris' strain AY-WB, SAP11 contains a nuclear localisation signal (NLS) that is functional in plant cells (Bai *et al.*, 2009). Nuclear localisation signal of SAP11 interacts with α-importin, a plant protein required for transporting proteins into plant nuclei. SAP11 is released directly inside phloem cells and then is transported through plasmodesmata to mesophyll and other cells of sink tissues where it targets nuclei of plant host cells to affect expression of various genes, including transcription factors. Transgenic *A. thaliana* plants that express the gene *SAP11*, showed symptoms resembling those exhibited by *A. thaliana* plants infected by AY-WB phytoplasma. These symptoms included phyllody and witches'-brooms (Sugio *et al.*, 2011*a*). It has been shown that SAP11 interferes with plant TCP (*TEOSINTE BRANCHED1*, *CYCLOIDEA*, *PROLIFERATING CELL FACTORS 1* and *2*) transcription factor family, known to control several traits in the plant development. In particular, SAP11 destabilises class II TCPs (=*CINCINNATA*[CIN]-TCPs), which control cell maturation and senescence, thereby inducing morphological changes (Sugio *et al.*, 2011*a,b*). Another AY-WB phytoplasma effector protein, SAP54, is known to alter flower development when overexpressed in *A. thaliana* plants (MacLean *et al.*, 2011). This protein induces in transgenic *A. thaliana* plants indeterminate leaf-like flowers which are similar to those observed in AY-WB phytoplasma-infected *A. thaliana* plants. Changes in flower development were also observed when SAP54 was expressed in the phloem tissues (MacLean *et al.*, 2011). SAP54 exerts its effect by promoting the degradation of members of the MADS-domain transcription factor (MTF) family, including key regulators SEPALLATA3 and APETALA1, which occupy central positions in the regulation of floral development (MacLean *et al.*, 2014). This degradation process is mediated by proteins of the RADIATION SENSITIVE23 (RAD23) family, which are eukaryotic proteins that shuttle the transcription factors to the protein degradation machinery. Also, plants with SAP54-induced leaf-like flowers are more attractive for colonisation by phytoplasma leafhopper

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vectors and this colonisation preference is dependent on RAD23 (MacLean *et al.*, 2014). Effector proteins may also be involved in DNA methylation which is responsible of specific down-regulation of certain floral development genes as has been shown for stolbur phytoplasma-infected tomato plants (Pracros *et al.*, 2007; Ahmad *et al.*, 2013). A different virulence protein, named TENGU, is secreted by the OY phytoplasma (Hoshi *et al.*, 2009). When *tengu* was expressed in *Nicotiana benthamiana* plants, these plants showed symptoms of witches'-broom and dwarfism, which are typical of phytoplasmal infection. Also, transgenic *A. thaliana* plants expressing *tengu*, showed a variety of symptoms, including witches'-broom, dwarfism, defects in phyllotaxis, and sterile flowers. Microarray analyses revealed that auxin-responsive genes, auxin efflux-related genes and dormancy-associated genes were down-regulated in *tengu*-expressing *A. thaliana* plants (Hoshi *et al.*, 2009). TENGU protein is transported through plasmodesmata from phloem tissue into parenchyma and meristem tissues, especially into tip region of the stem and axillary buds. However, unlike SAP11, TENGU does not target plant cell nuclei (Hoshi *et al.*, 2009). Work by Sugawara *et al.* (2013) showed that TENGU is processed to bioactive peptides in the plant, and it was suggested that these peptides regulate the plant development, by an unknown mechanism.

Interactions with insect vectors

Insect vectors of phytoplasmas are differently affected by the phytoplasmas they transmit. *Colladonus montanus* leafhoppers infected with the X-disease phytoplasma lived approximately half as long as uninfected leafhoppers. In the infected leafhoppers, pathological lesions of several organs, including salivary glands were reported to occur. Also, X-disease phytoplasma-infected *C. montanus* leafhoppers produced fewer offspring than did healthy leafhoppers, whereas increases in mortality were reported for six leafhopper species which transmit the maize bushy stunt phytoplasma (for review see Kirkpatrick, 1991). Work by Bressan *et al.* (2005*a,b*) showed that the flavescence dorée phytoplasma greatly reduced longevity and fecundity of its natural and experimental vectors, the leafhoppers *Scaphoideus titanus* and *Euscelidius variegatus*, respectively. A beneficial effect was observed when the aster leafhopper, *Macrosteles quadrilineatus*, fed on AY phytoplasma-infected plants of aster, lettuce, carrot and periwinkle. The exposed leafhoppers lived longer and produced more offspring than non-exposed leafhoppers (for review see Hogenhout *et al.*, 2008). Recent studies revealed that the reproduction of *M. quadrilineatus* increased considerably when this leafhopper was reared on either AY phytoplasma-infected *Arabidopsis thaliana* plants or transgenic *A. thaliana* plants expressing the gene *SAP11* (Sugio *et al.*, 2011*a,b*). As mentioned above, SAP11 destabilises class II TCPs, leading to a decreased synthesis of jasmonic acid (JA), a phytormone that is involved in the plant defence response against insect herbivores, including the AY-WB leafhopper vector *M. quadrilineatus*. Therefore, an increase in *M. quadrilineatus* population would also result in an increase in AY-WB phytoplasma spread in nature. Adults of *Dalbulus maidis*, a maize leafhopper, confined on aster, lettuce and *A. thaliana* plants do not attempt to lay eggs and die within a few days. However, when these plants are infected with AY phytoplasmas, adults live longer and lay eggs from which nymphs hatch approximately 15 days later (Purcell, 1988; Sugio *et al.*, 2011*a*). Thus, phytoplasma infections can manipulate plants to convert them from being non-hosts into hosts or better hosts for a given insect vector (Hogenhout *et al.*, 2008; MacLean *et al.*, 2014). Another example showing a positive effect of phytoplasma infections on the insect vector–plant interactions was observed on apple affected by the AP agent '*Ca*. Phytoplasma mali'. Diseased trees showed an increased emission of β-caryophyllene, a sesquiterpene that acts as an attractant for the AP vector, the psyllid *Cacopsylla picta*. These findings suggest that AP phytoplasma may produce effector proteins to alter the sesquiterpene synthesis pathway, thereby increasing the number of transmitting psyllids (Mayer *et al.*, 2008).

There is evidence that highly specific phytoplasma–insect interactions are involved in the transmission process. In particular, specific attachment reactions between phytoplasmas and insect receptors are required for penetration of the gut and salivary glands barriers of the vector. Work by Suzuki *et al.* (2006) revealed that an abundant surface membrane protein of the OY phytoplasma, designated as antigenic membrane protein (Amp), formed a complex with insect microfilaments, including actin, myosin heavy chain and myosin light chain proteins, of the visceral smooth muscle surrounding the intestinal tract, in all OY phytoplasma-transmitting leafhopper species but not in those of non-OY phytoplasma-vector species. Similar results were obtained by Galetto *et al.* (2011), who reported that Amp of the chrysanthemum yellows (CY) phytoplasma, which is a '*Ca*. Phytoplasma asteris'-related strain, selectively interacted with actin and α and β subunits of ATP synthase of CY phytoplasma-insect vector species including *Euscelidius variegatus* but not with those of non-CY phytoplasma-vector species. The ATP synthase β subunit was present on plasma membrane of midgut and salivary gland cells of the leafhopper vector *E. variegatus*. Therefore, interaction between Amp and specific

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insect vector proteins determines vector specificity of phytoplasmas.

Switching of phytoplasmas between plant and insect hosts

Switching between plant and insect hosts is associated with dramatic transcriptional changes in phytoplasmas. Work by Oshima *et al.* (2011) revealed that marked changes in gene expression occurred in '*Ca.* Phytoplasma asteris' strain OY-M upon host switching between *Chrysanthemum coronarium* (garland chrysanthemum) plants and *Macrosteles striifrons* insects. Approximately 33% of the genes (246 genes) in the genome were differentially expressed in a host-specific manner. Genes encoding the mechanosensitive channel, multidrug efflux pump and cobalt transporters were significantly up-regulated in the plant host, whereas those encoding zinc, sugar and oligopeptide transporters were significantly up-regulated in the insect host. In particular, the expression level of gene encoding the large-conductance mechanosensitive channel (gene *MscL*), in the plant host, was five times higher than in the insect host. MscL channel is known to open in response to stretch forces in the lipid bilayer and participate in the regulation of osmotic pressure within the cell. Therefore, MscL channel plays a crucial role in adaptation of phytoplasmas to the osmotic pressures of the plant-cell environment. Indeed, a partial suppression of the strain OY-M population growth in garland chrysanthemum plants treated with an MscL channel inhibitor, gadolinium chloride, was only recorded in the early stages of infections (Oshima *et al.*, 2011). This finding implies that MscL channel might play an important role in survival of phytoplasmas in the plant host. Also, the osmotic pressure of phloem sap differs between source leaves and sink organs. Thus, phytoplasmas might differentially express the *MscL* gene to adapt to diverse osmotic pressures in the plant host. '*Ca.* Phytoplasma asteris' strain OY-M genes, which encode zinc-uptake transporters, namely *znuA*, *znuB* and *znuC*, were expressed only in *M. striifrons* insects. Because most of the zinc present in eukaryotic cells is accumulated in vesicular sites or tightly bound to proteins, it is likely that only little of this element inside host cells is readily available to colonising agents. Therefore, phytoplasmas may strongly express genes coding for zinc-uptake transporters to adapt to an intracellular environment with a low zinc content (Oshima *et al.*, 2011). As mentioned above, phytoplasma genomes lack genes coding for F-type ATPases (ATP synthases) responsible for generating transmembrane potential. However, they have five genes encoding P-type ATPases. One of these, *mgtA1*, is a P2C ATPase similar to the animal cell Na^+/K^+ and H^+/K^+ pumps, and was the first P2C ATPase identified in a prokaryote (Christensen *et al.*, 2005). Thus, this P2C ATPase is the most likely candidate for generating the electrochemical gradient over the membrane (Christensen *et al.*, 2005). Among the P-type ATPase genes of strain OY-M, *mgtA3* was up-regulated in *M. striifrons* while *mgtA1* and *zntA* were up-regulated in garland chrysanthemum plants (Oshima *et al.*, 2011). These results suggest that phytoplasmas use P-type ATPases for adaptation to the two different environments. Two sigma factor genes, namely *rpoD* and *fliA* (*rpoF*) are known to occur in the OY-M genome. Of these genes, *rpoD* was up-regulated, four times, in *M. striifrons* whereas the expression signal of *fliA* was detected only in garland chrysanthemum. These findings imply that *rpoD* activates the transcription of genes expressed specifically in the insect host, while *fliA* likely serves as a transcription factor for genes expressed in the plant host. Marked changes in the expression profile between OY-M infecting garland chrysanthemum and OY-M occurring in *M. striifrons* were also observed for genes encoding metabolic enzymes and secreted proteins. For instance, the expression of the gene encoding the secreted protein PAM486 was highly up-regulated, 90 times, in the plant host compared to the insect host. This protein was also identified *in planta* by immunohistochemical methods. In contrast, expression of PAM486 was hardly detected in the insect host. Therefore, it is suggested that PAM486 protein functions mainly when '*Ca.* Phytoplasma asteris' strain OY-M occurs in the plant host (Oshima *et al.*, 2011).

Major changes in gene expression profiles of '*Ca.* Phytoplasma asteris' strain AY-WB upon switching between plant and insect hosts were reported by MacLean *et al.* (2011). In this study, 28 genes encoding SAPs were significantly up-regulated in *A. thaliana* plants, whereas 18 SAPs were predominantly up-regulated in the AY-WB leafhopper vector *M. quadrilineatus*. SAP27 was the most highly expressed protein in *A. thaliana,* whereas SAP36 was the most highly expressed in *M. quadrilineatus*. Genes encoding SAP11 and SAP54, which are know to induce changes in plant morphology and flower development, respectively, were up-regulated in the plant host compared with the insect host. Thus, it is likely that genes that are differentially expressed in a host-specific manner encode proteins with functions that are essential to colonisation of the respective, primary host. However, it cannot be excluded that these genes also have a function in the alternate host. For instance, SAP11 has also been detected in the salivary glands of *M. quadrilineatus* (MacLean *et al.*, 2011).

Studies conducted by Toruño *et al.* (2010) demonstrated that '*Ca.* Phytoplasma asteris' strain AY-WB has a linear chromosomal PMU1 (L-PMU1) and a circular

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extrachromosomal PMU1 (C-PMU1) in its genome. C-PMU1 copy number and PMU1 expression levels proved to be several times higher in AY-WB-infected *M. quadrilineatus* than in AY-WB-infected *A. thaliana* and *N. benthamiana* plants. Therefore, it is suggested that genes located within PMU1 encode proteins relevant for adaptation of phytoplasmas to an insect host. Among the genes located with PMU1, there is *SAP36*.

Conclusions

Molecular biology data on phytoplasmas have been accumulated at an exponential rate over the past years. These data, appeared in several leading microbiological and phytopathological journals including *Annals of Applied Biology*, have considerably advanced our knowledge about this recalcitrant group of plant pathogens. A most important contribution of molecular-based methods to phytoplasmology has been the proper classification of phytoplasmas as a distinct monophyletic clade within the class *Mollicutes*, closely related to acholeplasmas. Prior to the introduction of these methods, classification and differentiation of phytoplasmas relied primarily on their biological properties, such as symptoms induced in the affected plants, plant host range and insect vector transmission specificity. However, the determination of biological properties was time-consuming, laborious and very often unreliable. Because of the inability to culture phytoplasmas under axenic conditions and the paucity of accessible phenotypic properties, the system for their taxonomic classification is inevitably based largely on molecular criteria.

Pulsed-field gel electrophoresis has had a major impact on genomic studies of unculturable phytoplasmas, mainly due to its ability to separate physically full-length phytoplasma chromosomes from contaminating host plant nucleic acids. Using this technique, it could be shown that chromosome sizes of phytoplasmas vary considerably, ranging from 530 kb to 1350 kb, with overlapping values between the various taxonomic groups and subgroups. The smallest chromosome, about 530 kb, was found in the Bermuda grass white leaf agent '*Ca*. Phytoplasma cynodontis'. This value represents the smallest mollicute chromosome reported to date. The application of PFGE has also made possible studies on genome organisation and structure of phytoplasmas and the generation of genomic libraries suitable for sequencing projects targeting either selected chromosomal fragments or entire chromosomes.

Phytoplasmas have a unique and complex life cycle that involves colonisation of different environments, the plant phloem and various organs of the phloem-feeding homopteran insects. As phytoplasmas cannot be transmitted mechanically and are not transmitted vertically in plants, both plant hosts and insect vectors are necessary for their dispersal and long-term survival in nature. Phytoplasmas are known to occur in nature most frequently in angiosperms, which represent a relatively recently evolved class of potential hosts, less frequently in gymnosperms, whereas there is no indication on the occurrence of phytoplasmas in ferns. Thus, their origin is apparently relatively recent. It is hypothesised that phytoplasmas, and possibly other mollicutes, have arisen from insect-inhabiting organisms that gained access to the phloem during insect feeding, thereby bringing phytoplasmas to a novel, nutrient-rich environment. Alternatively, phytoplasmas could have gained access to the phloem by attaching to another bacterial or fungal pathogen that targets the phloem (Sears & Kirkpatrick, 1994). The predominant association of acholeplasmas with insects and their wide occurrence as saprophytes of plant surfaces, together with the monophyletic evolutionary pathway of phytoplasmas from an acholeplasma-like progenitor, provide support that phytoplasma progenitors may have first colonised insect hosts, and through them, may have been transmitted to plant hosts and may have developed parasitism of internal plant tissue. The evolutionary diversification of phytoplasmas may have paralleled evolutionary changes in their insect and/or plant hosts, or may have occurred more rapidly, allowing them to adapt to other extant hosts. To keep the parasitic mode of life, phytoplasmas have developed, during their evolution, rather sophisticated mechanisms that enable rapid responses to host chemical signals, successful evasion of host surveillance systems, and adaptations to different hosts. Adaptation is particularly important for phytoplasmas because their replication occurs in physico-chemically different environments. These include the intracellular habitats of plant sieve tube elements, and intra- and extracellular habitats of guts, salivary glands and several other organs and tissues of insect vectors. Furthermore, many phytoplasmas have an extremely wide plant host range. The dynamic architecture of phytoplasma genomes may account for the great adaptability of phytoplasmas, enabling them to parasitically colonise new ecological niches and hosts. The availability of complete phytoplasma genome sequences has made it possible to identify a considerable number of genes that are likely to play major roles in phytoplasma–host interactions. Among these, there are genes encoding surface membrane proteins and effector proteins. Such knowledge is of primary significance for future studies on pathogenicity of phytoplasmas on the molecular level, and for designing new control measures of phytoplasmal diseases based on biotechnological approaches. Furthermore, the completion of genome sequencing projects of additional phytoplasmas, which

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are currently in progress, should also provide a better understanding of genome organisation and evolution and coding capacity of phytoplasmas.

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