Diverse Targets of Phytoplasma Effectors: From Plant Development to Defense Against Insects

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Abstract

Phytoplasma research begins to bloom (75). Indeed, this review shows that substantial progress has been made with the identification of phytoplasma effectors that alter flower development, induce witches' broom, affect leaf shape, and modify plant-insect interactions. Phytoplasmas have a unique life cycle among pathogens, as they invade organisms of two distinct kingdoms, namely plants (Plantae) and insects (Animalia), and replicate intracellularly in both. Phytoplasmas release effectors into host cells of plants and insects to target host molecules, and in plants these effectors unload from the phloem to access distal tissues and alter basic developmental processes. The effectors provide phytoplasmas with a fitness advantage by modulating their plant and insect hosts. We expect that further research on the functional characterization of phytoplasma effectors will generate new knowledge that is relevant to fundamental aspects of plant sciences and entomology, and for agriculture by improving yields of crops affected by phytoplasma diseases.

INTRODUCTION

Phytoplasmas Are Mollicutes

Plasmodesmata: the channels that traverse the cell walls of plant cells

Hemolymph: the circulating fluid in open tissue spaces of invertebrates

Salivary glands: organs that produce saliva

Phytoplasmas are mycoplasma-like bacterial pathogens that cause serious yield losses in a diverse range of economically important crops, including oilseed rape, wheat, many vegetables, and high-value perennial crops, such as coconut, grapes, and (stone) fruit trees. Phytoplasmas belong to the class Mollicutes, phylum Tenericutes along with mycoplasmas, spiroplasmas, and acholeplasmas (52). Mollicutes are believed to have diverged from the Firmicutes (*Bacilli*, *Clostridia*, *Erysipelotrichi*, and other low guanine (G) + cytosine (C) gram-positive bacteria) through loss of an outer cell wall and a reduction of genome size (88, 91). Whereas mycoplasmas are predominantly human and animal pathogens, all phytoplasmas and three *Spiroplasma* species (i.e., the corn stunting agent *Spiroplasma kunkelii*, the citrus stubborn disease agent *Spiroplasma citri*, and the periwinkle yellows agent *Spiroplasma phoeniceum*) are plant pathogens that require sap-feeding insect herbivores as vectors for transmission to plants (2, 32).

Although phytoplasmas and the three *Spiroplasma* spp. share similar habitats and common environmental niches, these bacteria are only distantly related in the class Mollicutes. Within this class, two clades diverged at an early stage of evolution to give rise to the AAA (*Asteroplasma*, *Anearoplasma*, and *Acholeplasma*) and the SEM (*Spiroplasma*, *Entomoplasma*, and *Mycoplasma*) branches of Mollicutes; the genera *Spiroplasma* and *Mycoplasma* fall within the SEM branch, whereas phytoplasmas fall within the AAA branch (47). The SEM and AAA branch mollicutes have different codon usage, i.e., UGA (uracil, guanine, and alanine) is used as a stop codon in AAA branch mollicutes, whereas it codes for tryptophan in the SEM branch mollicutes (69). Genome sequence comparisons also revealed that phytoplasmas and spiroplasmas have different metabolic pathways. Many *Spiroplasma* and *Mycoplasma* species have been cultured in artificial medium outside their hosts. In contrast, phytoplasmas have not been cultured and hence were assigned the genus name *Candidatus* Phytoplasma (80). This review will focus on phytoplasmas. The rationale for this will be further explained below.

Phytoplasmas Spread Systemically in Plants Via the Phloem Sieve Cells

In plants, phytoplasmas and the three *Spiroplasma* spp. remain predominantly restricted to the cytoplasm of phloem sieve cells of the vascular system (19, 89) (**Figure 1**). Phloem sieve cells are live anucleate cells that contain only limited organelles, such as ribosomes, and possess small vacuoles and large plasmodesmata. The latter connect the sieve cells to adjacent nucleate companion cells that nourish the sieve cells. Adjacent sieve cells are connected by sieve plates that have small pores to allow the systemic transport of sugars and other nutrients in the plant. Phytoplasmas systemically infect their plant hosts by moving through the pores of sieve plates, thereby spreading throughout the plant's vascular system.

Phytoplasmas Are Invasive Microbes of Insects

Sap-feeding insects become infected by phytoplasmas and spiroplasmas in a process called acquisition feeding (61) (**Figure 1**). These insect vectors feed from the nutrient-rich sieve cells containing the phytoplasmas and spiroplasmas. Once these bacteria have entered the intestinal lumen of the feeding insect, they invade and appear to replicate in the intestinal brush border epithelial cells and adjacent muscle cells, which line the intestinal cells at the hemolymph site (2, 24, 32, 64a). The bacteria are released into the hemolymph from which they infect various other insect organs and tissues, including the salivary glands. Salivary gland cells have large vacuoles that collect salivary proteins, such as enzymes, for secretion into the plant during insect feeding (84). Phytoplasmas and spiroplasmas were found to accumulate in these vacuoles (45, 49) from which they can access the plant

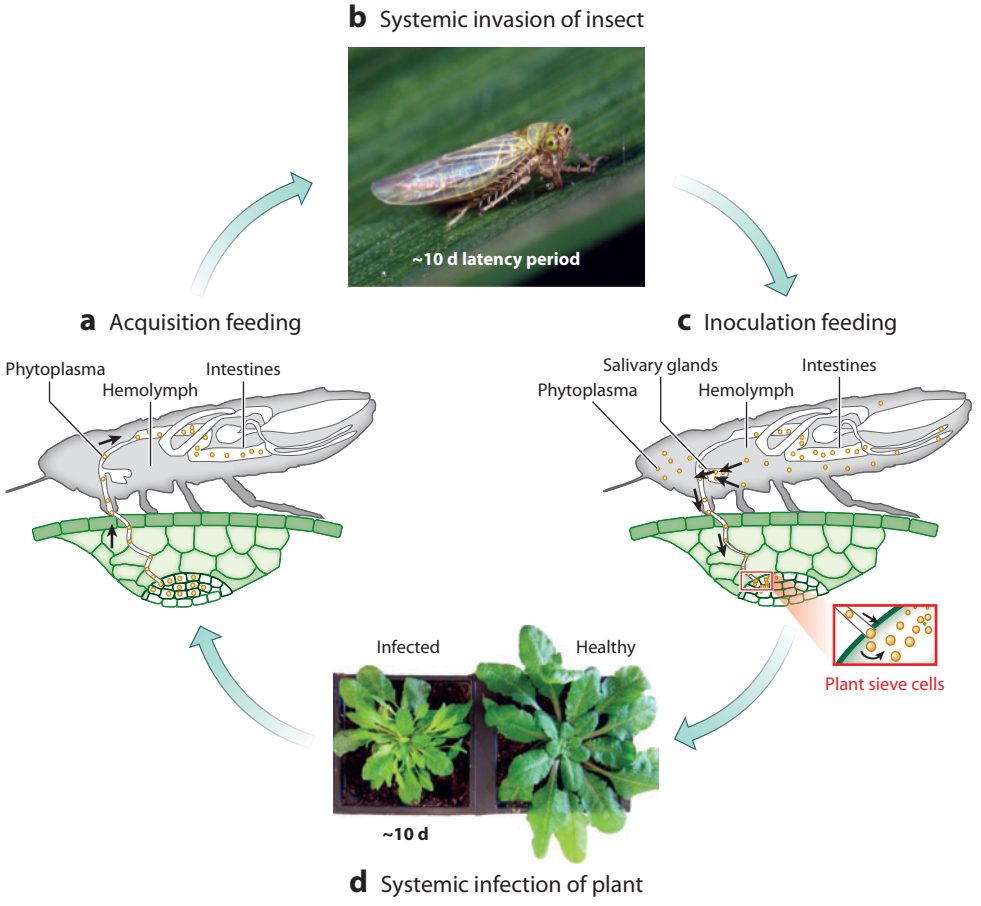

Figure 1

Phytoplasmas have a unique dual host life cycle. They are plant pathogenic bacteria that are transmitted to plants by an insect vector, such as the aster leafhopper *Macrosteles quadrilineatus*. This figure illustrates the following biological events: (*a*) A naïve leafhopper acquires the phytoplasmas (*yellow dots*) while feeding from the phloem of an infected plant, a process referred to as acquisition feeding. The bacteria are ingested into the midgut of the insect and invade the leafhopper gut cells and adjacent muscle cells that line the gut at the hemolymph site. (*b*) Phytoplasmas systemically infect the leafhopper over a period of 10 days or more. During this time, phytoplasmas appear to replicate in gut and muscle cells, and then escape to the hemolymph, from which they invade other leafhopper organs, such as the salivary glands. (*c*) When phytoplasmas have reached the salivary glands, the leafhoppers become competent to inject the bacteria into the phloem of a healthy plant, a process referred to as inoculation feeding. (*d*) The bacteria colonize the new plant host within approximately 10 days, at which point the infected plants show a variety of symptoms, such as yellowing and stunting.

sieve cells during insect feeding in a process referred to as inoculation feeding (61) (**Figure 1**). The time lapse between acquisition and inoculation is called the latency period, which is approximately 10 days but may be much longer (up to 12 weeks) depending on the bacterium, insect species, and temperature (58).

Insect vectors of phytoplasma and spiroplasma belong to specific phylogenetic groups

Fitness: the relative ability of an organism to survive and transmit its genes to the next generation

Type III secretion system (TTSS): a protein secretion system that directly transfers proteins from bacteria to eukaryotic host cytoplasm

of phloem-feeding leafhoppers, planthoppers, and psyllids within the order Hemiptera (54, 86). Other phylogenetically distinct groups of phloem (sieve cell)-feeding hemipterans, such as aphids, do not transmit phytoplasmas and spiroplasmas, indicating that there is specificity in the acquisition and transmission of these bacteria. Furthermore, phytoplasma and spiroplasma infections may decrease the fitness (measured as survival and fecundity rates) of the insect hosts, have a neutral effect, or in several cases exhibit a positive effect on the insect vector fitness (2, 6, 32, 41a, 53, 60, 68, 86). The likelihood of a positive interaction (one that benefits the insect) versus a negative interaction (one that impairs insect fitness) often reflects the evolutionary time of a given insect-microbe interaction: The longer the two organisms have coexisted and coevolved, the more likely the insect vector is to benefit from the interaction (60).

Phytoplasmas Are Intracellular Pathogens of Plants and Insects

It is evident that the phytoplasma and spiroplasma plant pathogens have an unusual life cycle compared with other plant pathogenic bacteria that have been the primary focus of molecular investigations. Indeed, gram-negative bacterial pathogens of the genera *Pseudomonas*, *Ralstonia*, and *Xanthomonas* inhabit the apoplast of infected plants and have evolved elaborate mechanisms, such as the type III secretion system (TTSS), to penetrate beyond the exterior surface of the plant cell to enable the pathogens to release virulence proteins (effectors) into host cells during infection (9). These effectors are required for the modulation of host cell processes to enable pathogen infection. In other words, these pathogens remain extracellular at all stages of plant colonization. In contrast, phytoplasmas and spiroplasmas inhabit the cytoplasm of living sieve cells. Because these bacteria reside intracellularly, they do not need elaborate protein secretion systems, such as a TTSS, to introduce the effectors (32). As further discussed below, the effector proteins may simply be secreted via the general SecA-dependent protein translocation system, which is known to be functional in phytoplasmas (39). Another interesting aspect of phytoplasmas is that their host range includes organisms of two distinct kingdoms, namely plants (Plantae) and insects (Animalia) (32). Finally, they are capable of invading and accumulating in cells of different insect tissues and organs. The ability of these bacterial pathogens to successfully invade and colonize two such dissimilar host environments and replicate intracellularly in both is remarkable and implies the evolution of mechanisms that enable the bacteria to modulate cellular processes in both eukaryotic hosts.

Phytoplasma Infection Disturbs Plant Developmental Processes

Although phytoplasmas and spiroplasmas both inhabit the phloem sieve cells of their plant hosts, their infections elicit different symptoms. The majority of plant-pathogenic mollicutes induce stunting and yellowing symptoms, and phytoplasmas also frequently induce witches' broom (proliferation of stems, branches, and leaves), virescence (non-green tissues of flowers turn green), and phyllody (conversion of flowers to leaves) (8) (**Figure 2**). We previously proposed that phytoplasmas produce effectors that modulate cellular processes in plant development and probably also those involved in plant defense (31). In recent years, experimental evidence for the existence of such effectors has accumulated. The identification and functional characterization of these effectors were facilitated by whole genome sequence information of various phytoplasma genomes, the use of model plants such as *Arabidopsis thaliana* in the analyses of phytoplasma symptom development and effector function, and the development of new functional genomics tools (33).

What Are Effectors?

Plants have evolved potent strategies to defend themselves against insect herbivory and pathogen invasion. These include physical barriers (trichomes that can act as sensors)

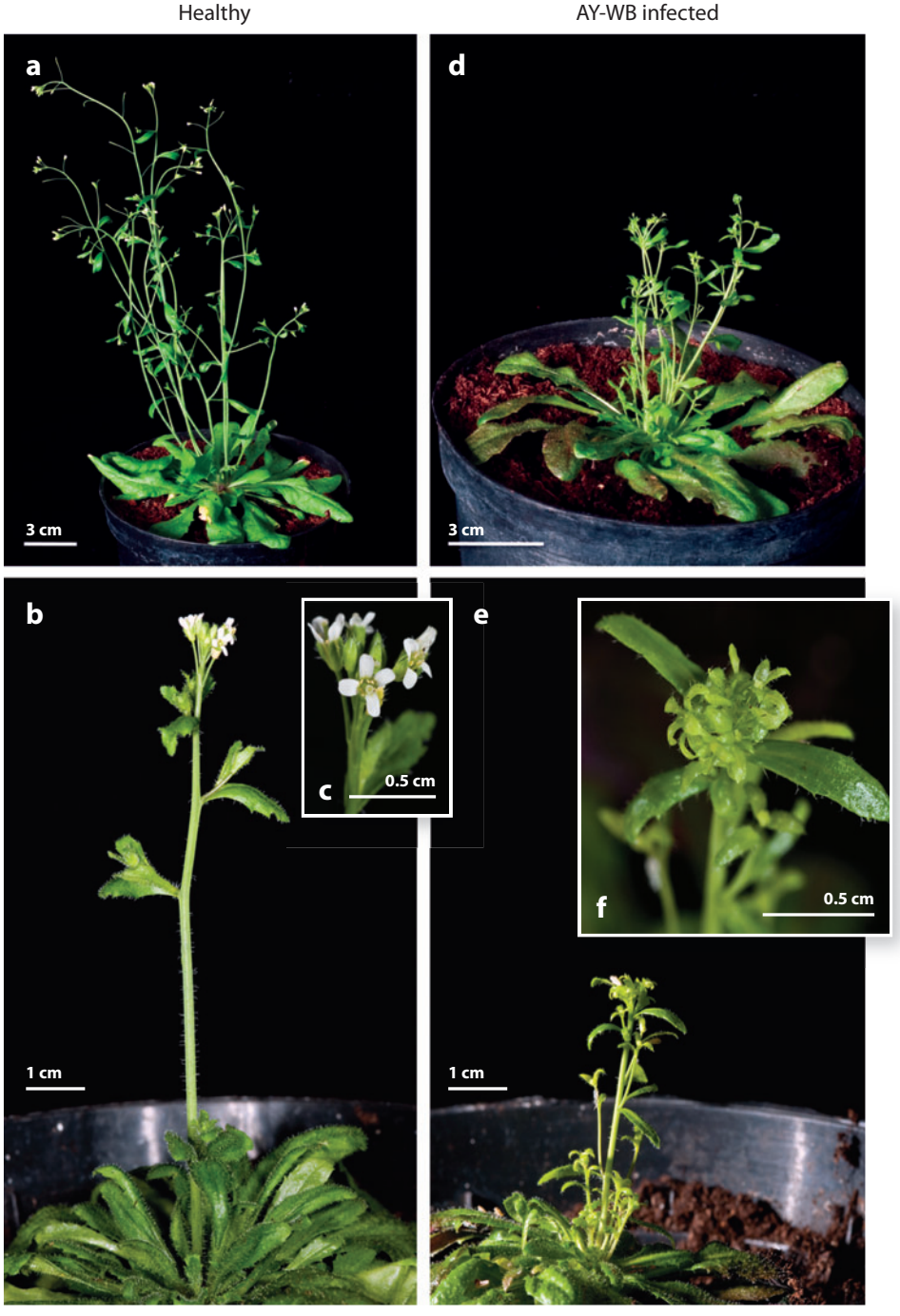

Figure 2

Arabidopsis thaliana plants infected with aster yellows–witches' broom (AY-WB) phytoplasma show various symptoms. (*a*,*b*) Healthy plants. (*c*) Close-up of flowers in (*b*). The infected plants are stunted (*d*,*e*), have increased stem production from the center of the rosette [witches' broom phenotype (*d*, *e*)], and produce leafy flowers (*e*). (*f*) Close-up of the flowers in (*e*). Petals are green and have trichomes.

JA: jasmonic acid

Sec-dependent protein translocation pathway: a protein secretion pathway that translocates proteins from cytosol across the cytoplasmic membrane

(81), cuticular wax (42) and cell walls (65), constitutive chemical defenses (toxic secondary chemicals) (40), and both direct and indirect inducible defenses (recently reviewed in 92). Plant pathogens and herbivores need to overcome these physical and biochemical defenses. Plant microbial pathogens and nematodes produce specific molecules, called effectors, that alter their hosts in order to successfully invade and multiply in plants, and there is increasing evidence that insect herbivores also produce such effectors (10, 92). The word effector typically denotes a protein that is secreted by a microbial pathogen or insect into a host cell to enhance colonization and facilitate multiplication of the pathogens/insects, but in a broader definition, effectors can also include elicitors, toxins, phytohormone analogs, cell wall–degradation enzymes, and other molecules that alter host plants (34).

Why Would Phytoplasma Produce Effectors?

A question of relevance is why would phytoplasmas produce effectors? It is generally supposed that effectors improve the pathogen fitness (34). Thus, one can speculate that phytoplasma effectors induce various physiological and morphological changes during infection of their hosts, and phytoplasmas gain fitness advantages from inducing such changes. One possibility is that phytoplasmas incur a competitive advantage through the increased generation of young vegetative tissues (witches' broom symptom and virescence), which are attractive to phytoplasma vectors. Indeed, phytoplasmas require insect vectors for transmission to other plants; hence, an increase in insect vector fitness would also result in an increase in phytoplasma fitness. Another possibility is that phytoplasmas have a competitive advantage by extending the lifespan of the plant host. Many herbaceous plants die upon completion of the reproductive phase and therefore reverting flower development (phyllody) may prolong the vegetative growth phase of the plant and delay plant death. As biotrophic pathogens, phytoplasmas require a living host to survive. A final possibility that we propose is that phytoplasmas interfere with fundamental processes in plant development in order to reduce or alter the production of phytohormones, such as jasmonic acid (JA; jasmonate), that in addition to regulating plant development also have fundamental roles in plant defense signaling.

Focus

This review focuses on the discovery of phytoplasma effector proteins and the mechanisms by which these effectors modulate plant development and increase phytoplasma fitness. The readers are referred to several other reviews (8, 11, 23, 28, 32) and a book (87) for information on phytoplasma and spiroplasma genome sequence contents, metabolism, and general biology.

IDENTIFICATION AND LOCALIZATION OF PHYTOPLASMA EFFECTORS

Phytoplasmas Secrete Effectors

Currently, four phytoplasma genomes have been sequenced to completion. These are Onion Yellows phytoplasma strain M (OY-M; *Candidatus* Phytoplasma asteris) (64), Aster Yellows phytoplasma strain Witches' Broom (AY-WB; *Ca.* P. asteris) (5), Australian Grapevine Yellows (AUSGY; *Ca.* P. Australiense) (83), and Apple Proliferation phytoplasma (AP; *Ca.* P. mali) (43). Sizes of the circular genomes of OY-M, AY-WB, and AUSGY are 700 kbp to 900 kbp, and the linear chromosome of AP is 600 kbp. In all four phytoplasma genomes, genes encoding membrane transporters for nutrients, amino acids, and peptides are relatively abundant compared with those encoding metabolic and biosynthetic proteins, indicating that phytoplasmas acquire most basic metabolites from their hosts (reviewed in 33). Phytoplasmas' genomes also harbor the minimal set of genes necessary for a functional Sec-dependent protein translocation pathway (i.e., SecA, SecE, and SecY), which transports bacterial proteins to the exterior of a

phytoplasma cell. Proteins secreted by the Sec-dependent pathway require a conserved N-terminal signal peptide that is recognized and cleaved during translocation across the membrane, resulting in the secretion of a mature protein lacking the signal peptide. Indeed, there is evidence that the N-terminal signal peptide sequence of the OY phytoplasma membrane protein Amp (antigenic membrane protein) is cleaved, indicating that the Sec-dependent transport system is functional in phytoplasmas (39). As phytoplasmas are located intracellularly for much of their life cycle, it is likely that phytoplasmas secrete their effectors via Sec-dependent translocation.

SecA-secreted proteins are candidate effectors, as they are likely to interact with host cell components upon secretion from the phytoplasma cell. Signal peptide sequences of secreted proteins may be identified by prediction programs such as SignalP (62) or PSORT (59). Using this software, 56 candidate effectors were identified and named secreted AY-WB proteins (SAPs) (4). Of the 56 SAPs, 49 are encoded on the chromosome, and 7 on the four plasmids that are part of the AY-WB genome (4, 5). A similar approach allowed the identification of 45 candidate effectors encoded on the chromosome of OY, 41 in AUSGY, 13 in AP, and 25 in maize bushy stunt phytoplasma (MBSP). MBSP, OY, and AY-WB are all members of the AY phytoplasma group classified as group I based on 16S rDNA sequence and restriction fragment length polymorphism data (group 16Sr1) (47, 95), indicating that effector content can vary even between closely related phytoplasmas. It is striking that the number of predicted effector genes in the specialist (restricted host range) phytoplasmas AP and MBSP are by far the lowest among the five phytoplasmas examined to date. The host ranges of AP and MBSP are restricted to apple trees (*Malus* spp.) and maize (*Zea mays* L.), respectively, whereas the host ranges of AY-WB, OY, and AUSGY include multiple plant species from several plant families. Thus, phytoplasmas with restricted plant host ranges (AP and MBSP) may have fewer effectors than those with broad plant host ranges (OY, AY-WB, and AUSGY). Whole genome sequence information of more phytoplasmas should determine whether this correlation between candidate effector gene number and plant host range is a common phenomenon.

Systemic Movement of Phytoplasma Effectors in Plants

Phytoplasmas are limited to the phloem sieve cells of their plant hosts. Thus, effectors are released into the cytoplasm of sieve cells upon secretion by phytoplasmas. The effectors may interact with plant components in the sieve cell or may unload from the phloem to interact with target molecules in companion, mesophyll, and other plant cells. The effector proteins SAP11 (9 kDa) of AY-WB and TENGU (<5 kDa) of OY phytoplasmas were detected in tissues beyond the phloem (4, 35). Furthermore, SAP11 contains a nuclear localization signal (NLS) that is required for protein targeting of cell nuclei (4), and nuclei are absent from phloem sieve cells, further supporting a hypothesis that SAP11 targets tissues beyond the phloem. TENGU of OY phytoplasma was detected at the tip of the stem, in the branching region of axillary buds and in the apical meristem, but does not appear to specifically target plant cell nuclei (35).

The finding that phytoplasma effectors can unload from the phloem leads to the question of how these proteins translocate out of a sieve cell and move between plant cells. It is most likely that the proteins are transported across the plasmodesmata that connect the plant cells. The size exclusion limits (SELs) of plasmodesmata that connect sieve cells and companion cells in the loading phloem are reported to be larger than 67 kDa (74), whereas the SELs of other cells vary from 10 kDa to 50 kDa (36) (**Figure 3**). Plasmodesmata SELs differ between source and sink tissues of the plant. Source tissues, which produce most of the carbohydrates that are transported in the phloem have SELs of approximately 10 kDa, whereas sink tissues (which require carbohydrates for

Size exclusion limit (SEL): the maximum size of a molecule that passes passively through plasmodesmata

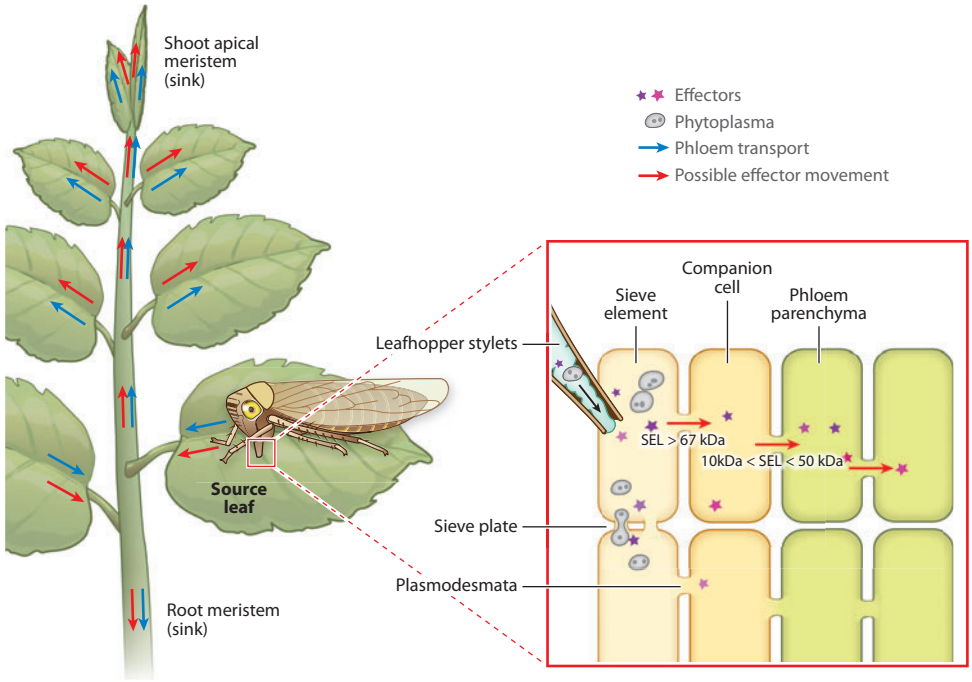

Figure 3

Schematic overview of the systemic movement of phytoplasmas and effectors in the plant and effector unloading from the phloem. Leafhoppers mostly inoculate phytoplasmas into the phloem of source leaves, which are predominantly responsible for the production of sugars and nutrients. Phytoplasmas and secreted effectors migrate systemically throughout the plant and reach sink tissues, which utilize sugars and nutrients for growth. The phytoplasma effectors unload from the phloem to target plant factors in neighboring tissues, particularly in sink tissues.

growth) have SELs of about 50 kDa (36). It was noted that most AY-WB effector candidates are less than 40 kDa (4). Hence, the majority of effector proteins may unload from the phloem through plasmodesmata, particularly in sink tissues (31) (**Figure 3**). This is in agreement with the symptomatology of phytoplasma-infected plants, as the symptoms are frequently observed in sink tissues, such as the shoot apical meristem and flowers (**Figure 2**). Alternatively, phytoplasmas may degrade plant cell walls or generate holes in plant cell membranes to facilitate effector translocation between plant cells. However, genes encoding proteins that may modify plant cell walls and membranes have yet to be identified in the phytoplasma genomes sequenced thus far, and it is also unclear how such proteins would travel alongside the effectors upon secretion into the sieve cell cytoplasm.

The Majority of AY-WB Effector Candidate Genes Lie on Mobile DNA Elements

Phytoplasmas have reduced genome sizes and restricted metabolic capabilities. Nonetheless, these genomes encode large repeat rich regions organized into units of up to approximately 20 kb that are referred to as potential mobile units (PMUs) and resemble large conjugative transposons (5, 82). Sequence-variable mosaics (SVMs) are genetic elements that have been described in phytoplasmas, with a gene content that is comparable to PMUs, and are proposed to originate from prophages (85). However, cornerstone genes typically associated in the identification of phages were not identified in the phytoplasma genomes (85), creating uncertainty about whether

the repeated regions originally derived from prophages (82). Interestingly, the majority of the 49 chromosomally encoded AY-WB effectors lie within PMU-like regions (82), thus PMUs may contribute towards phytoplasma virulence and enhance phytoplasma fitness. This is consistent with the general observation that genes involved in bacterial pathogenicity and symbiosis frequently lie on pathogenicity/symbiosis islands derived from transposon and phage integrations into the bacterial chromosome and plasmids (26).

The largest repeats in the AY-WB genome are PMU1 through PMU4, and the SAP11 pathogenicity island, which encodes AY-WB effector SAP11 (4, 5, 32), the function of which will be further discussed below. Of these elements, the approximately 20-kb PMU1 appears to contain the fewest pseudogenes and is flanked by 327-bp inverted repeats (5, 82). It was recently discovered that PMU1 exists in a linear form (L-PMU1) within the AY-WB chromosome and in a circular form (C-PMU1) that is extrachromosomal (82). The C-PMU1 copy number is consistently higher in AY-WB-infected insects compared with infected plants, and expression of the genes encoded in PMU1 is upregulated in insects compared with plants (82). PMU1 encodes at least one candidate effector protein (SAP36) and several proteins with predicted transmembrane domains (4, 5, 32, 82). Based on these data, it was proposed that PMU1 gene products compose part of a phase-variation mechanism that allows phytoplasma adaptation to the insect host. In this scenario, the PMU1 proteins may be involved in insect cell invasion and suppression of immune responses (82). Evidence that phytoplasmas require proteins to enable insect invasion is provided by studies on the OY protein Amp (also discussed above). This membrane-associated protein interacts with insect cell microfilament components actin, myosin heavy chain, and myosin light chain of leafhoppers capable of vectoring OY, but not of nonvector species, indicating that Amp may determine vector specificity (78). An alternative hypothesis that does not exclude the phase-variation hypothesis is that the insect environment initiates PMU1 mobilization and exchange of the extrachromosomal C-PMU1 among phytoplasma cells, in which case the PMU1-encoded proteins may generate a structure for conjugation. The next phase in this research is to investigate if PMU1-encoded membrane proteins are located on the surface of phytoplasma cells and to examine whether SAP36 modulates cellular processes in insect cells.

CIN-TCP: *CINCINNATA-TEOSINTE BRANCHED1, CYCLOIDEA, PROLIFERATING CELL FACTORS 1 and 2*

FUNCTIONAL CHARACTERIZATION OF PHYTOPLASMA EFFECTORS

Phytoplasma Effectors Induce Stem and Leaf Proliferation

Phytoplasma infection can induce stunting and stimulate the production of large numbers of axillary shoots, resulting in a witches' broom in infected plants (**Figure 2**). We found that one of the 56 AY-WB effectors, SAP11, may induce the witches' broom symptoms (76). Transgenic *Arabidopsis* plants that express SAP11 have curly leaves and an increased number of axillary stems that resemble the witches' broom symptoms exhibited by AY-WB infected plants. Yeast two-hybrid screening and immunoprecipitation studies revealed that SAP11 interacts with plant TCP (*TEOSINTE BRANCHED1*, *CYCLOIDEA*, *PROLIFERATING CELL FACTORS 1* and *2*) transcription factors. Plant TCP transcription factors are divided in two groups, class I and II. Class II TCPs are further divided into CIN (*CINCINNATA*)-TCPs and TB/CYC (*TEOSINTE BRANCHED1/CYCLOIDEA*)-TCPs. Class I TCPs are reported to control cell proliferation, whereas class II CIN-TCPs control cell maturation, and a fine balance between the two classes of TCPs controls plant development (55). Coexpression analyses demonstrated that SAP11 destabilizes CIN-TCPs but not the class I TCPs. This is in agreement with the finding that the SAP11 transgenic lines and a *CIN-TCP* knockdown line show similarities in leaf morphology. Thus, SAP11 is a

negative regulator of CIN-TCPs (76). Whereas CIN-TCP knockdown lines overproduce immature cells that lead to production of large and curly leaves (21), mutants of TB/CYC-TCPs overproduce axillary stems (1). Therefore, the increased stem numbers in SAP11 expression lines indicate that SAP11 may also destabilize class II TB/CYC-TCPs.

Hoshi et al. screened for OY effector proteins that induce such morphological changes (35). In this study, the transient expression of OY phytoplasma effector candidates in *Nicotiana benthamiana* identified a gene that induces witches' broom and dwarfism. The encoded protein is estimated to be 4.5 kDa in size, and a mature protein (lacking a signal peptide) is only 38 amino acids in length. The corresponding gene was named *tengu* (a class of supernatural creatures found in Japanese folklore), as witches' broom–like symptoms are called tengu-su (nest of Tengu) in Japanese. Transgenic *Arabidopsis* lines that express TENGU show a variety of morphological alterations, including witches' broom, dwarfism (i.e., short internodes), defects in phyllotaxis, and production of sterile flowers. Microarray analysis of *tengu*-expressing *Arabidopsis* lines revealed a downregulation of several auxin responsive genes and auxin efflux carrier genes. Although TENGU may directly interfere with auxin biosynthetic and signaling pathways, it is also possible that TENGU alters plant morphology by manipulating other molecular pathways, and auxin physiology is altered as an indirect consequence of this activity.

Phytoplasma Effectors Alter Plant-Insect Interactions

We also found that the SAP11-mediated destabilization of class II TCPs leads to a decreased synthesis of JA, a phytohormone that is involved in the defense response against the AY-WB leafhopper vector *Macrosteles quadrilineatus* (76). It was previously demonstrated that the survival and reproduction of leafhopper vectors are enhanced when reared upon plants infected with AY phytoplasmas (6, 41a, 53, 68). A recent continuation of this pioneering work revealed that healthy *M. quadrilineatus* produces about 60% more progeny on AY-WB-infected plants compared with noninfected plants, using the model plant *Arabidopsis* (76). The fecundity of AY-WB-infected *M. quadrilineatus* does not increase until approximately 10 days after acquisition of AY-WB, a point in time at which AY-WB migrates to the insect salivary glands, and leafhoppers become competent to inoculate plants (**Figure 1**). Thus, AY-WB infection of the plant, but not of the insect vector, alters leafhopper fecundity. Interestingly, *M. quadrilineatus* also produces more progeny on the SAP11 transgenic *Arabidopsis* lines. As discussed above, SAP11 destabilizes *Arabidopsis* CIN-TCPs and one of these, TCP4, positively regulates expression of *LIPOXYGENASE 2* (*LOX2*), which produces oxylipins that are precursors of JA synthesis (71). In agreement with these findings, we found that *LOX2* expression and JA accumulation are reduced in the SAP11 transgenic *Arabidopsis* lines (76). *M. quadrilineatus* produces more progeny on *Arabidopsis* lines in which *LOX2* expression is silenced or JA synthesis is impaired through the mutation of JAR1 (JASMONATE RESISTANT1) that converts JA into the biologically active JA-isoleucine (76). In addition, *LOX2* expression is upregulated in *Arabidopsis* plants, following exposure to *M. quadrilineatus*. Together, these data indicate that the JA signaling pathway in *Arabidopsis* is involved in the plant defense response to *M. quadrilineatus* herbivory and that AY-WB SAP11 interferes with this defense response through destabilization of TCPs that positively regulate JA synthesis, leading to increased leafhopper fitness (**Figure 4**).

In all experiments, the increase in nymph production is the result of an increased egg laying activity exhibited by the leafhoppers (76). As discussed above, effectors are expected to provide the pathogen with a competitive advantage compared with pathogens that do not have these effectors. We propose that SAP11 enhances the fitness of the phytoplasma AY-WB (**Figure 4**). Indeed, nymphs that hatch from the eggs will immediately commence

feeding from the AY-WB-infected plants and will thereby acquire phytoplasmas (**Figure 4**). The AY-WB-carrying leafhoppers will subsequently migrate to other plants as they age and will introduce the phytoplasma into naïve plant hosts. Thus, increasing leafhopper fecundity (mediated by the effector SAP11) is an effective strategy of increasing the dispersal of phytoplasmas in nature (**Figure 4**). Therefore, SAP11 is a vivid example of the extended phenotype of the gene, a concept put forward in Richard Dawkins's classic book *The Extended Phenotype: The Long Reach of the Gene* (16).

More Examples of Plant-Insect Interaction Alterations in Phytoplasma-Infected Plants

There are at least two other examples of alterations of plant interactions with insect vectors exhibited by phytoplasma-infected plants. Apple trees infected with the AP phytoplasma *Ca*. P. mali have an increased emission of E-β-caryophyllene, a sesquiterpene and a well-studied insect semiochemical that acts as an attractant for an AP insect vector, the psyllid *Cacopsylla picta*, that is a pest of apple trees (56, 57). The finding that (E)-β-caryophyllene

Semiochemical: a chemical that affects the behavior of an organism

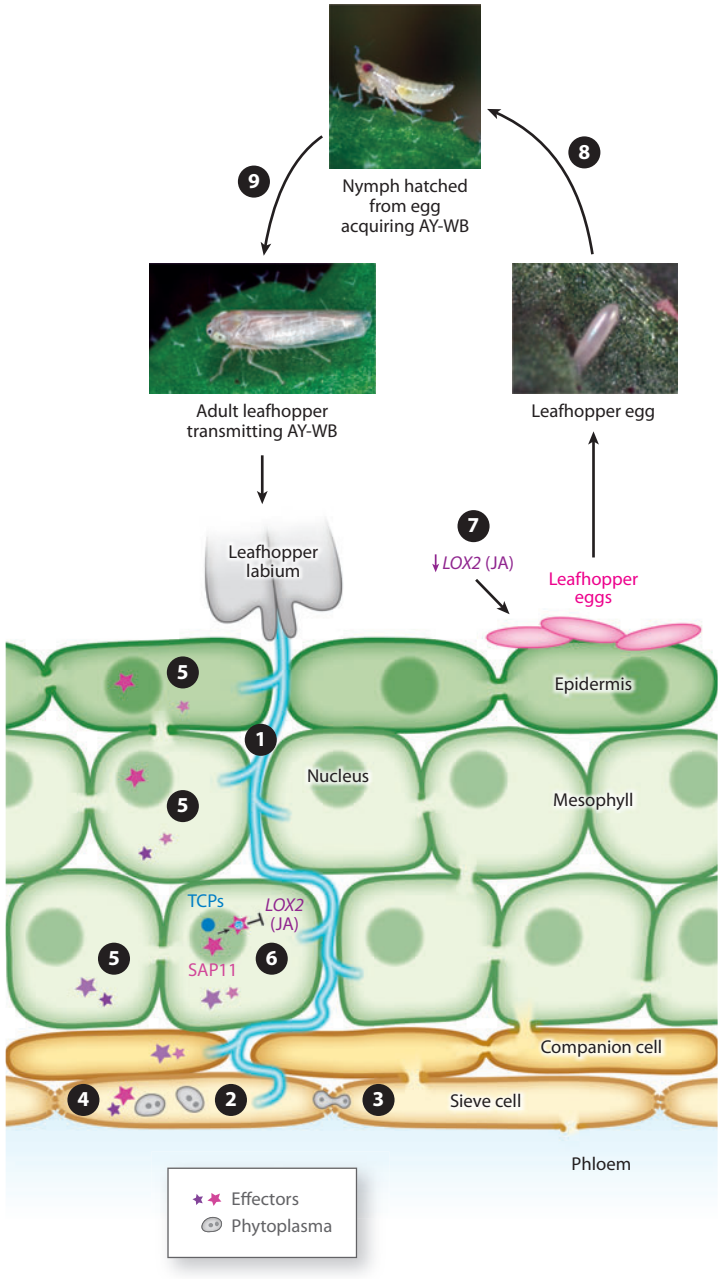

Figure 4

The phytoplasma aster yellows–witches' broom (AY-WB) effector protein SAP11 promotes the production of AY-WB leafhopper vector progeny, thereby improving the chance of AY-WB transmission to other plants. The numbers in this figure indicate biological events as follows: ① The leafhopper mouthparts (stylets) navigate between the plant cells to the phloem sieve cells; ② while feeding, leafhoppers salivate, introducing phytoplasmas into the sieve cells; ③ phytoplasmas systemically disperse throughout the plant by navigating through the pores of sieve plates connecting the sieve cells; ④ phytoplasmas secrete effectors that unload from the phloem into adjacent plant cells via plasmodesmata; ⑤ the phytoplasma effectors interact with various plant targets, inducing disease symptoms; ⑥ the AY-WB phytoplasma effector SAP11 destabilizes plant TCP transcription factors, leading to inhibition of *LOX2* expression that is essential for production of the phytohormone jasmonate (JA); ⑦ the reduction in JA promotes egg laying activity of the AY-WB leafhopper vector *M. quadrilineatus*; ⑧ upon hatching from the eggs, the leafhopper nymphs start feeding and acquire the phytoplasmas from the infected plant; ⑨ when the nymphs age, they migrate to other plants and inoculate these plants with phytoplasmas, starting the process anew.

production increases in AP phytoplasma-infected apple trees suggests that AP may produce effectors to manipulate the sesquiterpene synthesis pathway, as it is unlikely that apple trees voluntarily produce (E)-β-caryophyllene to attract plant herbivores that damage themselves.

AY phytoplasma infection alters plant-insect interactions in another way. *Arabidopsis* plants infected with AY phytoplasmas are not only more attractive to the insect vector *M. quadrilineatus* but also become feeding hosts for the maize-specialist leafhopper *Dalbulus maidis* (41a, 68). We recently found that *D. maidis* survives longer and reproduces (i.e., they lay eggs in the leaves and nymphs hatch from these eggs) on AY-WB-infected *Arabidopsis* plants (76, 41a). This leafhopper species is a true specialist of maize; adult *D. maidis* individuals confined to dicot plant species, such as *Arabidopsis*, lettuce, and China aster, 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. Thus, phytoplasma infection alters nonhost resistance of plants against leafhoppers. *D. maidis* does not acquire AY-WB and cannot vector it, and hence it is unclear how phytoplasma fitness is increased by supporting this leafhopper species. Nonetheless, the loss of nonhost resistance phenotype may extend to other leafhopper species that can vector AY phytoplasmas. We found that *D. maidis* longevity and fecundity were not significantly altered in SAP11 transgenic *Arabidopsis* plants, indicating that SAP11 activity is specific and does not alter nonhost resistance of *Arabidopsis*. Investigations into the AY-WB effectors that can convert *Arabidopsis* into a host for *D. maidis* are ongoing.

Phytoplasma Effectors Interfere With Flower Development

The most dramatic symptoms in phytoplasma-infected plants include alterations in flower morphology, such as virescence, phyllody, sepal hypertrophy, big bud symptoms, and the production of influorescence shoots from flowers (indeterminate growth of flower organs) (8). Virescence is a condition in which non-green floral organs (such as petals) remain green due to the abnormal presence of chlorophylls. In phyllody, the floral organs are converted into green leaf–like organs. Plants with big bud symptoms typically show enlarged calyces with aborted whorls (no petals, stamens, and carpels).

The development of determinate flowers involves four major stages: (*a*) transition from vegetative growth to reproductive growth; (*b*) establishment of a floral meristem and its maintenance; (*c*) activation of floral organ identity genes and formation of sepals, petals, stamens, and carpels; and (*d*) termination of the floral meristem. These steps have been genetically dissected in *Arabidopsis*, and we have highlighted those components that are likely to be altered by phytoplasma-infected plants (**Figure 5**). For more detailed information on flower development processes, readers are referred to several recent reviews (37, 50, 70).

A study by Pracros and colleagues (67) provided the first molecular insight into the flower malformation induced by stolbur phytoplasma infection in tomato. The flower organs of stolbur-infected tomato plants exhibit sepal hypertrophy, big bud symptoms, virescence, and phyllody. Semiquantitative reverse-transcription polymerase chain reaction experiments revealed downregulation of tomato homologs of *WUSCHEL* (*WUS*), *CLAVATA 1* (*CLV1*), *APETALA 3* (*AP3*), and *AGAMOUS* (*AG*), and upregulation of *LEAFY* (*LFY*) in flowers of infected tomatoes (**Figure 5**) (67). In agreement with Pracros et al., Himeno et al. (30) reported that homologs of *WUS* and some class B genes that regulate floral organ identity (**Figure 5**) are downregulated in OY phytoplasma-infected petunia (genus *Petunia*) flowers, showing virescence of petals and leaf-like carpels. Furthermore, Cettul & Firrao (13) reported that *SEPALLATA3* (*SEP3*) (**Figure 5**) is downregulated in Italian clover phyllody phytoplasma-infected *Arabidopsis*, exhibiting altered flowers.

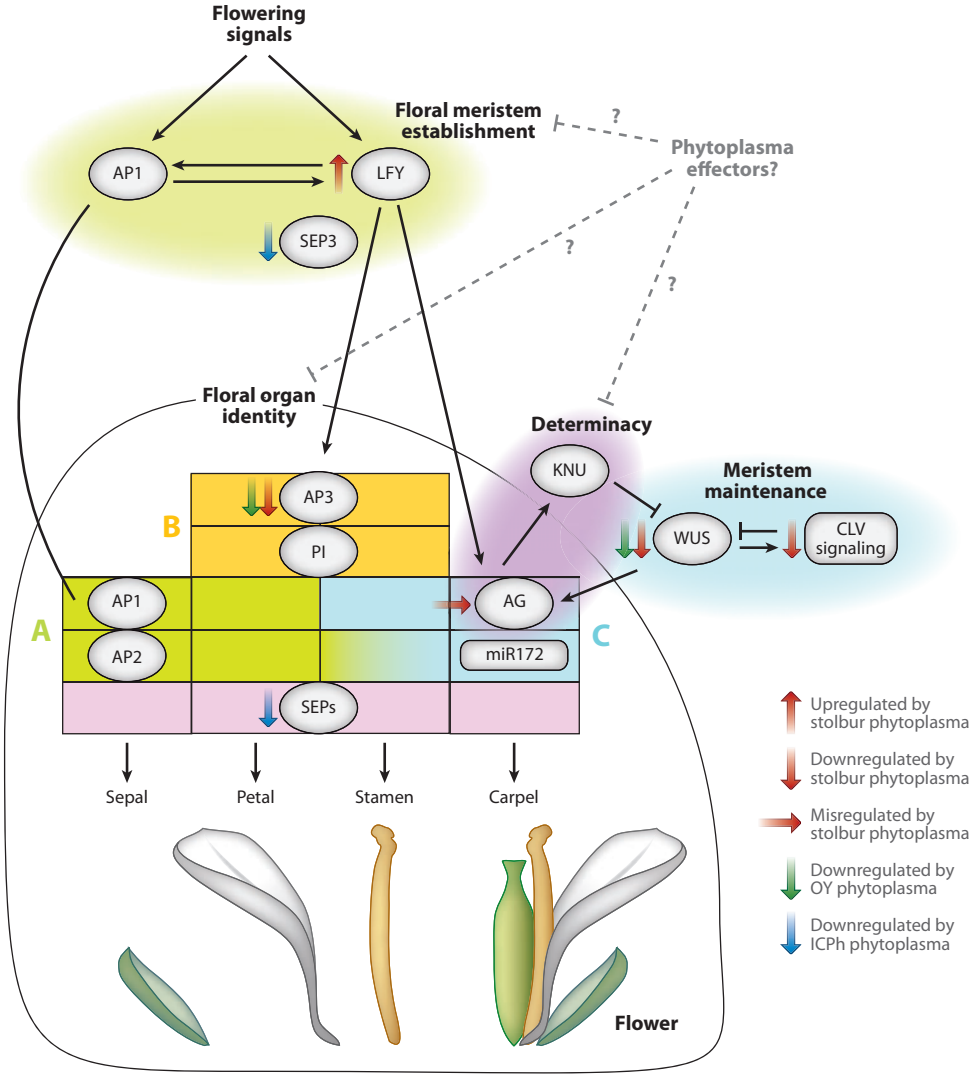

Figure 5

Simplified overview of the regulation of *Arabidopsis* flower development. Flowering signals are converged in the expression of AP1 and LFY, which establishes the floral meristem. AP1 and LFY, together with a cofactor SEP3, induce the expression of floral organ identity ABC genes that regulate the formation of flower organs in separate whorls (15a). AG specifies the identity of carpels but also terminates the floral meristem by suppressing WUS through the activation of KNUCKLES. In plants infected with stolbur, OY, and ICPh phytoplasmas, the expression of various flower developmental gene homologs are up- or downregulated or expressed at the wrong time (misregulated), as indicated in the figure.

Recently, we have identified an effector of AY-WB, named SAP54, that alters flower development when overexpressed in *Arabidopsis* (52a). The transgenic *Arabidopsis* lines exhibit an indeterminate flower phenotype of producing new flowers in lieu of carpels, and some flowers produce green leaf-like petals with trichomes. In addition, the transgenic plants show sepal hypertrophy. Interestingly, AY-WB-infected *Arabidopsis* plants produce

flowers with green petals with trichomes (**Figure 2**) and sometimes produce indeterminate flowers that resemble those produced by the SAP54 expressing transgenic plants. The flowers of AY-WB-infected plants occasionally produce a terminal flower at the apex of an inflorescence. In contrast, the SAP54 *Arabidopsis* lines do not produce terminal flowers but produce inflorescences that grow continuously. Investigations to identify the mechanisms by which AY-WB effectors alter flower development in *Arabidopsis* are ongoing.

The variety of developmental symptoms observed in phytoplasma-infected plants suggests that effectors interfere with floral meristem establishment/maintenance (e.g., phyllody), specification of organ identity (e.g., virescence and big bud), and termination of floral meristem (e.g., production of stems in flower axis). The effectors may induce these pleiotropic effects by altering the expression (e.g., by occupying a relevent promoter) and/or by interfering with protein function (e.g., by stimulating protein degradation) of key genes/proteins in floral developmental pathways. Because many flower development genes influence each other's expression patterns (**Figure 5**), it is difficult to determine at what level phytoplasmas interfere with flower development. For example, WUS expression is fine-tuned by the WUS/CLV regulatory loop in which the CLV-signaling pathway mediated by CLV3 suppresses WUS, and in turn WUS induces expression of CLV3 (**Figure 5**) (12, 25). WUS is required for maintenance of the vegetative, inflorescence, and floral meristems, and it regulates AG expression (**Figure 5**) (27, 46, 48). AG is required for the development of stamens and carpels, and also terminates floral meristem by suppressing WUS through the activation of KNUCKLES (77). Flowers of *AG* mutants are indeterminate and produce flowers with extra petals or a new flower bud within a flower (15, 51). Such indeterminate flower-like structures are sometimes seen in phytoplasma-infected plants (52a; G. Firrao, personal communication). Pracros et al. (66) suggested that phytoplasma infection may lead to the reduction of DNA methylation, leading to suppression of gene induction. This is an interesting finding that needs further investigation. Yet another hypothesis is that the phytoplasma effectors target flower developmental genes/proteins that belong to one family. *APETALA 1*(*AP1*), *AP3*, *PISTILLATA* (*PI*), *AG*, and SEPs are MADS (*MCM1*, *AGAMOUS*, *DEFICIENS* and *SRF*) domain transcription factors, and effectors, such as SAP54, may target all MADS domain transcription factors. The effector SAP11, which destabilizes all class II CIN-TCP transcription factors, exemplifies this scenario. The targeting of multiple proteins in a single family would also explain the pleiotropic phenotypes observed in flowers of infected plants.

Comparison of Phytoplasma and Other Microbial Effectors

Phytoplasma effector SAP11 targets plant nuclei and destabilizes plant CIN-TCP transcription factors. The destabilization of CIN-TCPs causes morphological changes in the plant and suppresses JA-mediated defense. There are several other examples of pathogen effectors that target plant nuclei or induce morphological changes. However, their modes-of-action are either distinct or unknown from that of SAP11.

Ralstonia solanacearum TTSS effector PopP2 contains a bipartite NLS. In plants resistant to *R. solanacearum*, PopP2 is an avirulence determinant and colocalizes with the corresponding resistance (R) gene product (RRS1) in plant nuclei and prevents proteasomal degradation of RRS1 (79). PopP2 displays acetyltransferase activity, and in susceptible plants *R. solanacearum* PopP2 may have a virulence function (17). *Xanthomonas* species secrete TTSS transcription activator-like (TAL) effectors that are targeted to plant nuclei. These transcription factors encode proteins with transcription activation and repeat rich domains that control the binding of the effector to specific promoter sequences of plant genes to activate expression (90). *Xanthomonas* cells are located in the xylem and need sugars and other nutrients for growth. Recently, it was demonstrated that *Xanthomonas*

oryzae pv. *oryzae* TAL effectors, PthXo1 and AvrXa7, target promoters of plant sugar transporters that seem to pump sugars into the plant apoplast and xylem (3, 14). Another group reported that the same gene upregulated by PthXo1 encodes a protein that interacts with copper transporters that reduces the xylem level of copper, which is toxic to the pathogen (94). Effectors that target plant cell nuclei have been identified from a number of other plant pathogens, such as the oomycetes (72).

Some pathogen effectors induce morphological changes in the host plants. The TAL effector AvrBs3 of *Xanthomonas campestris* pv. *vesicatoria* induces the expression of a pepper transcription factor named *UPA20* (41). UPA20 activates the expression of downstream genes to cause cell hypertrophy (enlargement), which induce pustule formation on the leaves of solanaceous plants (41). Similarly, a TAL effector of *Xanthomonas axonopodis* pv. *citri*, PthA, induces hypertrophy and hyperplasia (multiplication), and eventually ruptures the epidermis of citrus leaves. It is hypothesized that the bacteria can ooze out from the ruptures of the leaf surface and effectively disseminate in nature (20). In addition to some *Xanthomonas* strains, diverse plant-associated microbes, nematodes, and insect herbivores induce cell hypertrophy and/or hyperplasia, resulting in pustule and gall formation in plants. An example of a pathogen that induces galls is the gram-negative plant pathogen *Agrobacterium tumefaciens*. This bacterium uses a specialized type IV secretion system to transform host cells with bacterial DNA to enhance production of the plant growth hormones auxin (indole-3-acetic acid) and cytokinins, causing gall formation (98).

Phytoplasma effector SAP11 alters the interaction of plant and insect vectors by suppressing JA-mediated plant defense pathways against the insect. Another example of a pathogen effector that appears to improve the fitness of its insect vector is βC1 of the geminivirus *Tomato yellow leaf curl China virus* (TYLCCNV), which is transmitted by whiteflies (*Bemisia* spp.). βC1 induces the formation of upward curling leaves, a symptom typically observed in TYLCCNV-infected plants. In addition to the alteration of leaf morphology, βC1 suppresses induction of some JA-response genes (93) through the interference of *Arabidopsis* Myb transcription factor ASYMMETRIC LEAVES 1 (AS1). It was proposed that TYLCCNV domain protein βC1 suppresses JA-mediated plant resistance to promote the fitness of whitefly vectors (93).

Do Phytoplasma Effectors Suppress the Plant Defense Response?

In recent years, it has become evident that the inducible plant defense response to microbial pathogens (and probably insect herbivores and nematodes) is a multilayered process consisting of at least two phases (38, 63). Phase 1 is initiated with the recognition of microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) by the plant pattern recognition receptors (PRRs), resulting in PAMP-triggered immunity (PTI). PAMPs and MAMPS are defined as conserved epitopes within essential molecules that are recognized by a broad range of hosts (reviewed in 73). A successful pathogen may employ virulence proteins (effectors) that suppress PTI, resulting in a compatible interaction or effector-triggered susceptibility (ETS), unless plants recognize these effectors and trigger phase 2 of the plant defense response. Phase 2 is triggered upon recognition of the pathogen effectors or their activities by plant disease *R* genes, resulting in effector-triggered immunity (ETI). There are many bacterial effectors that have been shown to interfere with host immune responses such as PTI and ETI (18). For example, a systematic characterization of *P. syringae* strain DC3000 effectors revealed that the majority of the effectors suppress either PTI or ETI or both, indicating that suppression of both types of host defense reactions is a key for successful bacterial colonization (29). Therefore, it is relevant to ask whether phytoplasma effectors suppress PTI/ETI. PAMPs of gram-negative bacterial pathogens include lipopolysaccharides, peptidoglycans, and a

MAMP: microbe-associated molecular pattern

PAMP: pathogen-associated molecular pattern

Pattern recognition receptor (PRR): the proteins that recognize PAMPs and trigger immune responses

PTI: PAMP-triggered immunity

ETI: effector-triggered immunity

conserved domain of flagellin (flg22), which are generally located on the exterior of the bacterial cells. These PAMPs are recognized by a wide range of plants (73, 96). PAMPs also include intracellular proteins, such as cold shock proteins (CSPs) and the translation elongation factor Tu (EF-Tu). The conserved domains of CSP (csp15) and EF-Tu (elf18) are recognized by *Solanaceae* and *Brassicaceae*, respectively, and trigger PTI (22, 44, 97). Phytoplasmas have no outer cell wall and no flagella, and hence lack the peptidoglycans and flg22 PAMPs. However, phytoplasmas have genes encoding CSPs and the EF-Tu, and these gene products may induce PTI.

All the plant PRRs identified to date seem to receive the ligands in the extracellular space, whereas R-mediated ETI can be triggered by extracellular and intracellular effectors (7), but it is unclear whether sieve cells induce PTI/ETI. Because the intracellular phytoplasmas reside within the sieve cell cytoplasm, the bacteria may be hidden from the plant detection apparatus, resulting in the absence of PTI/ETI. Phytoplasmas may avoid detection by a plant host via an absence of both PAMPs and recognizable (avirulent) effectors or by secreting effectors that suppress PTI/ETI and/or by virtue of residence within nonresponsive phloem sieve cells.

SUMMARY POINTS

1. Phytoplasmas have a unique life cycle among pathogens, as they invade organisms of two distinct kingdoms, namely plants (Plantae) and insects (Animalia), and they replicate intracellularly in both plant and insect hosts.
2. Phytoplasmas have a functional Sec-dependent translocation pathway that enables these pathogens to secrete membrane-associated proteins, such as Amp, and effectors, such as SAP11 and TENGU, that are released into the host cells of plants and insects to target host cell molecules.
3. Phytoplasmas may employ a phase-variation mechanism that enables the differential regulation of effector genes during plant and insect infection.
4. In plants, phytoplasma effectors SAP11 and TENGU are released into the cytoplasm of phloem sieve cells and unload from the phloem to target plant cell (nuclei) beyond the phloem.
5. Effectors may provide phytoplasmas with fitness advantages by (*a*) generating more vegetative tissues in which phytoplasmas can replicate and that are attractive to the insect vectors on which phytoplasmas depend for dispersal in nature, (*b*) prolonging the vegetative growth phase of the plant to delay plant death, (*c*) altering signaling pathways and modulating the production of phytohormones that regulate plant defense responses against their insect vectors, (*d*) modulating plant volatile production or nonhost resistance to attract insect vectors, and (*e*) suppressing PTI/ETI or other inducible defense pathways to stimulate colonization of phytoplasmas and insect vectors.
6. SAP11 destabilizes *Arabidopsis* class II TCPs, which are involved in the regulation of leaf development and stem branching, consistent with the crinkled leaf phenotype and increased stem production of SAP11 transgenic *Arabidopsis* lines and the witches' broom phenotype exhibited by AY-WB-infected plants.
7. SAP11 positively affects AY-WB fitness as follows. SAP11-mediated destabilization of CIN-TCPs directly reduces *LOX2* expression and impaires the synthesis of JA, thereby increasing leafhopper production of nymphs, which transmit AY-WB to naïve plants.

FUTURE ISSUES

1. Functional investigations of phytoplasma effectors do not only provide information on bacterial virulence strategies but also are likely to expose novel plant defense mechanisms against bacteria and insect herbivores, and may reveal novel pathways affecting flower and vegetative growth of plants.
2. Phytoplasma effector studies may lead to a greater understanding of how sieve cells unload macromolecules for transport to other plant tissues, and how these plant cells detect pathogens and sap-sucking insect vectors.
3. Further studies on phytoplasma invasion and involvement of effectors in this process will advance our understanding of insect immune systems.
4. Phytoplasma may employ phase variation mechanisms to adapt to two different eukaryotic hosts. Functional characteriation of these mechanisms may contribute to develop strategies to suppress phytoplasma multiplication in crop plants.
5. The research on phytoplasma effectors and dissection of plant defense responses that these effectors may target is expected to generate knowledge for the design of novel benign control strategies for phytoplasmas and hemipteran insects.

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Errata

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