

## Molecular mechanisms of plant manipulation by secreting effectors of phytoplasmas

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### ABSTRACT

Phytoplasmas (genus '*Candidatus Phytoplasma*') are plant pathogenic bacteria that reside intracellularly within the plant phloem. Phytoplasmas induce a wide range of unique symptoms, including phyllody, yellowing, dwarfing, witches' broom and purple tops. Phytoplasma diseases cause great damage to agricultural production and forest trees. Although there is an agricultural need to develop effective methods to control phytoplasma diseases, phytoplasmas are still one of the most poorly characterized groups of plant pathogens. Despite their small genomes, phytoplasmas induce unique symptoms to host plants, thus how phytoplasmas manipulate plants has been of interest. Since phytoplasmas are cell wall-less and reside inside of host cells, their secreting proteins via Sec system function in the cytoplasm of the host plant cell and are predicted to have some important roles in host-parasite interactions and/or virulence as effector proteins. Although the molecular mechanisms behind the symptoms are not fully understood, several secreting effector proteins of phytoplasmas have been shown to induce the symptoms or manipulate the host factors. In this review, we summarize the molecular mechanisms by which phytoplasma effector proteins manipulate plants. Phytoplasma effectors have been found to have a wide variety of functions, such as regulating plant morphogenesis and attracting insect vectors. Since these plant manipulations are thought to be essential for phytoplasma survival strategies, the inhibition of effector proteins could lead to control of phytoplasma infection.

### 1. Introduction

Phytoplasmas (genus '*Candidatus Phytoplasma*') are plant pathogenic bacteria that reside intracellularly within the plant phloem [1,2]. Phytoplasma-infected plants exhibit a wide range of unique symptoms, including flower malformation, yellowing, dwarfing, witches' broom, purple tops, and phloem necrosis [3–5]. Phytoplasmas are transmitted by insect vectors such as leafhoppers and psyllids and can infect more than a thousand plant species worldwide [3,6,7]. Since the infected plants eventually decline and die in most cases, phytoplasmas cause great damage to agricultural production and forest trees [8]. For example, in 2001, a phytoplasma outbreak in apple trees caused losses of about €100 million in Italy and €25 million in Germany [9]. Lethal yellowing of palm has killed millions of coconut palm trees in the Caribbean over the past 40 years [10]. Although there is an agricultural need to develop effective methods to control phytoplasma diseases, phytoplasmas are still one of the most poorly characterized groups of

plant pathogens.

Genome analyses have been very useful for analyses of the feature of plant pathogens. The first complete genome sequence of a mildly pathogenic line of the '*Candidatus Phytoplasma asteris*' onion yellows strain (OY) was determined in 2004 [11]. Although phytoplasma genomes contain genes for basic cellular functions such as DNA replication, transcription, translation, and glycolysis [12–15], they lack genes for amino acid biosynthesis, fatty acid biosynthesis, the tricarboxylic acid cycle, and oxidative phosphorylation. The phytoplasma genome encodes even fewer metabolic functional proteins than mycoplasma genomes, which were previously thought to have the minimum possible gene set [16]. For example, phytoplasma genomes lack the pentose phosphate pathway genes and genes encoding F<sub>1</sub>F<sub>0</sub>-type ATP synthase [11]. Since ATP synthase had been thought to be essential for life, the loss of its genes in the phytoplasma genome provided opportunities for reconsidering the question "what is life?" [17]. ATP synthesis in phytoplasmas may be dependent on glycolysis or malate-related pathway

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instead of ATP synthase [14]. Interestingly, phytoplasmas harbor multiple copies of transporter-related genes not found in mycoplasmas. These genomic features suggest that phytoplasmas are highly dependent on metabolic compounds from their hosts [18].

Despite their small genomes, phytoplasmas induce unique symptoms to host plants such as phyllody and witches' broom, thus how phytoplasmas manipulate plants has been of interest. Many gram-negative pathogens of plants and animals possess Type III secretion systems (T3SSs) that can inject bacterial virulence effector proteins into host cells [19]. Although phytoplasmas doesn't have genes for T3SS, genes for protein translocation called Sec system in their genomes [20–22]. Since phytoplasmas are cell wall-less and reside inside of host cells, their secreting proteins via Sec system function in the cytoplasm of the host plant cell and are predicted to have some important roles in host-parasite interactions and/or virulence as effector proteins.

The phytoplasma Sec system utilizes recognition and cleavage of a signal sequence, as in other bacterial Sec systems [21]. This finding suggests that signal sequence prediction programs, such as SignalP [23, 24] or PSORT [25], are applicable to phytoplasma proteins and can be used to identify secreting effector proteins from the genomes. Many candidate effectors have been found in the phytoplasma genomes so far. For example, 56 candidate effectors for '*Ca. Phytoplasma asteris*' AY-WB strain and 45 for '*Ca. Phytoplasma asteris*' OY strain are predicted to be encoded in the genome [26]. Although the molecular mechanisms behind the symptoms are not fully understood, several effector proteins of phytoplasmas have been shown to induce the symptoms or manipulate the host factors (Table 1). Here we summarize the molecular mechanisms by which phytoplasma effector proteins manipulate plants.

## 2. TENGU

A small secreting peptide, TENGU, encoded by onion yellows phytoplasma, was the first phytoplasma pathogenic factor identified that affects plant morphology [27]. TENGU is predicted to be translated as a 70 amino-acid preprotein, with a 32 amino-acid signal peptide at its N-terminus. The C-terminal 38 amino acids of TENGU are secreted into plant cytoplasm via the Sec system. Transient or transgenic expression of this 38-amino-acid peptide, the putative secreted region of TENGU in *Nicotiana benthamiana* and *Arabidopsis thaliana*, results in a short and bushy phenotype similar to the symptoms of phytoplasma-infected plants [27,28]. Although phytoplasmas can infect both plant and insect hosts [7,29], the level of expression of TENGU in the plant host is approximately 5 times that in the insect host, suggesting TENGU may play an important role when phytoplasmas infect the plant host. Interestingly, the N-terminal 11 amino acids of the secreted region of TENGU are sufficient for its function [30]. Microarray analyses revealed that the expression of many auxin-related genes was significantly downregulated

in TENGU-transgenic plants, suggesting that TENGU suppresses auxin signaling or biosynthesis pathways [27]. It has been also reported that phytoplasma-infected periwinkles can show remission of disease symptoms when cultured in medium containing a high concentration of auxin [31,32]. Thus, auxin may have a great impact on the plant-phytoplasma interaction.

It has been also shown that TENGU acts as an inducer of sterility [33]. Transgenic expression of TENGU induced developmental defects in *A. thaliana* similar to those observed in auxin response factor 6 (ARF6) and ARF8 double mutants. The levels of the ARF6 and ARF8 genes were significantly decreased in TENGU-expressing transgenic plants, and jasmonic acid (JA) and auxin syntheses were decreased in TENGU-transgenic buds. These findings suggest that TENGU inhibits the JA and auxin biosynthesis pathways by repression of ARF6 and ARF8, resulting in impaired flower maturation [33].

## 3. SAP11

SAP11 (SWP1 in '*Ca. Phytoplasma tritici*') is a secretory protein that affects plant morphogenesis, which was firstly identified from aster yellows witches' broom (AY-WB) phytoplasma [44]. SAP11<sub>AYWB</sub>-expressing *A. thaliana* showed shoot morphology and bushy phenotype like the witches' broom symptom caused by phytoplasmas [34]. Transgenic *A. thaliana* expressing SAP11<sub>AYWB</sub> or SAP11<sub>CaPM</sub> (SAP11 of '*Ca. Phytoplasma mali*') shows crinkled leaves, but SAP11<sub>PnWB</sub> (SAP11 of '*Ca. Phytoplasma aurantifolia*' peanut witches' broom [PnWB] phytoplasma) or SAP11<sub>OYM</sub> (SAP11 of onion yellows phytoplasma) expressing transgenic lines shows very weakly altered leaf phenotypes [45]. SAP11 and its homologues interact with and destabilize class II CINCINNATA (CIN)-related TEOSINTE BRANCHED1, CYCLOIEDA, PROLIFERATING CELL FACTORS (TCPs) transcription factors [34,35,45–47]. SAP11 binds to and destabilizes class II CIN-TCPs (TCP2, TCP4, TCP13, TCP3, TCP5, TCP10, TCP17, and TCP24) [34]. SAP11 also destabilizes class II TB/CYC-TCPs such as TCP12, TCP18, and TB1 [45], but not class I PCF-TCPs (TCP7) [34]. SAP11<sub>AYWB</sub> exhibits a strong ability to destabilize most class II CIN-TCPs. On the other hand, SAP11<sub>CaPM</sub> is not able to destabilize TCP3, TCP4, TCP5, and TCP24, and SAP11<sub>PnWB</sub> and SAP11<sub>OYM</sub> only exhibit a weak ability to destabilize TCP2. These different abilities may be related to the results that severe crinkled leaves were observed in SAP11<sub>AYWB</sub> transgenic plants [45]. CIN-TCPs are involved in the up-regulation of *LOX2* that encodes a lipoxygenase that mediates the first step of the JA synthesis pathway [48]. Therefore, the destabilization of CIN-TCPs by SAP11 leads in turn to down-regulation of jasmonic acid biosynthesis [34].

Transgenic *Nicotiana benthamiana* lines expressing the secreted effector SAP11 exhibit an altered aroma phenotype, which is correlated with defects in the development of glandular trichomes and the

**Table 1**  
Target, host factor and function of secreting effectors of phytoplasmas.

Effector	Organism(s) <sup>a</sup>	Target(s)	Other host factors	Function	References
TENGU	' <i>Ca. P. asteris</i> ' <sup>b</sup> OY strain	Unknown	–	suppresses auxin signaling, increases production of stems and induces sterility	[27,33]
SAP11/SWP1	' <i>Ca. P. asteris</i> ' AY-WB strain ' <i>Ca. P. tritici</i> '	CIN-TCPs TB/CYC-TCPs	–	destabilizes TCPs, downregulates jasmonic acid biosynthesis and increases production of stems	[34,35]
Phylogen (PHYL/ SAP54)	' <i>Ca. P. asteris</i> ' OY strain ' <i>Ca. P. asteris</i> ' AY-WB strain	A- and E-class MADS domain transcription factors (MTFs)	RAD23	degrades A- and E-class MTFs via ubiquitin-independent pathway and induces phyllody symptom	[36–39]
SAP05	' <i>Ca. P. asteris</i> ' AY-WB strain	SPL and GATA transcription factors	RPN10	degrades SPL/GATA via ubiquitin-independent pathway and increases production of stems	[40]
SWP12	' <i>Ca. P. tritici</i> '	TaWRKY74	–	degrades TaWRKY74 via ubiquitin-dependent pathway and weakens plant resistance	[41]
SWP16 PM19_00185	' <i>Ca. P. tritici</i> ' ' <i>Ca. P. mali</i> '	Unknown E2 UBC09 E2 UBC10	–	inhibits RNA silencing in plant has E3 ligase activity and suppresses plant basal defense	[42] [43]

<sup>a</sup> Several other phytoplasma species have the effector homologues, but only representative ones are listed here.

<sup>b</sup> '*Ca. P. asteris*' = '*Candidatus Phytoplasma asteris*'.

biosynthesis of 3-isobutyl-2-methoxypyrazine [49]. The fecundity of insect vectors was increased on the SAP11-expressing plants compared to normal plants, which may be advantageous for the fitness of phytoplasmas and their insect vectors [34]. It has been also reported that SAP11 suppresses salicylic acid-mediated defense responses and enhances the growth of a bacterial pathogen [50].

#### 4. Phylogen

Flower malformation, such as phyllody (transformation of floral organs into leaf-like structures), virescence (greening of floral organs), and proliferation (shoot development from flowers) is a characteristic symptom of phytoplasma infection [51]. It has been reported that a novel gene family of phytoplasma effectors, designated phyllody-inducing genes or the phyllogen family, induces flower malformation in *Arabidopsis* plants [36,37,52]. Phyllogen was shown to cause phyllody phenotypes in several other eudicot species belonging to three different families [53]. Phyllogen genes have been found from a variety of ‘*Ca. Phytoplasma*’ species, and are suggested to be horizontally transferred among phytoplasmas [54]. These reports indicate that the phyllogen family is a universally effective phyllody-inducing effector common to phytoplasmas.

Phyllogen family proteins, such as SAP54 and PHYL, target the products of floral homeotic genes constituting the floral quartet model [37,39,55], which in turn encode MADS domain transcription factors (MTFs). Phyllogens degrade A- and E-class MTFs of angiosperms in a proteasome-dependent manner [37,39,53,56], suggesting that phyllogens recognize a structure highly conserved among these MTFs. A crystal structure analyses have revealed that the K domain of MTFs, a phyllogen-binding region [39], has two  $\alpha$ -helices with conserved hydrophobic residues which are important for the multimerization of MTFs [57]. These conserved residues in the K domain are important for the recognition by phyllogens [38]. This illustrates well why phyllogens can target MTFs of a wide range of plants. Two phyllogens (PHYL<sub>OY</sub> and PHYL<sub>PnWB</sub>, phyllogens of ‘*Ca. Phytoplasma asteris*’ OY and ‘*Ca. Phytoplasma aurantifolia*’ PnWB strains, respectively) also consist of two  $\alpha$ -helices which are important for phyllody-inducing activity [58,59]. Sequence comparison of phyllogens showed that their phyllody-inducing activity is determined by a single amino acid polymorphism on the second  $\alpha$ -helix [54].

Phyllogen-mediated phyllody induction requires Rad23 (RADIA-TION SENSITIVE23). Rad23, which was originally identified as an important factor involved in the recognition of DNA lesions, is one of the most studied shuttle proteins that translocate ubiquitinated proteins to the proteasome system [39]. Phyllogens translocate MTFs to the proteasome system by mediating the interaction between MTF and Rad23 to form a ternary complex [38]. This ternary complex is formed in the order MTF/phyllogen to MTF/phyllogen/Rad23 in a ubiquitin-independent manner (Fig. 1). This indicates that phyllogens functionally mimic the ubiquitin to translocate their targets into the proteasome system. Further structural insights into the interaction between phyllogen family proteins, MTFs, and Rad23s will help elucidate the molecular mechanisms how phytoplasmas manipulate the

morphology of plant hosts.

#### 5. SAP05

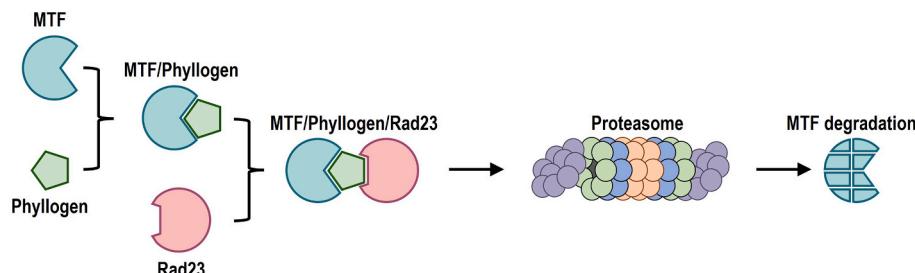
SAP05 is a secreting protein found from ‘*Ca. Phytoplasma asteris*’ AY-WB strain [40]. SAP05-expressing *A. thaliana* shows witches’ broom-like proliferations of leaf and sterile shoots. In addition, SAP05-expressing plants continue to grow over 12 weeks compared to GFP-expressing plant (a negative control), suggesting that SAP05 delays plant senescence. By yeast two hybrid screening, SAP05 has been shown to interact with GATA and SPL transcription factors of *A. thaliana*. SPL transcription factors have a conserved role in controlling developmental phase transitions of vascular plants [60], whereas GATA transcription factors regulate plant organ development, timing of flowering, and branching patterns [61]. SAP05 also interacts with 26S proteasome subunit RPN10 and destabilizes SPL and GATA transcription factors via the ubiquitin receptor RPN10. SAP05 seems to inhibit plant developmental phase transitions and cause delayed plant aging, witches’ broom-like excessive vegetative tissue by the degradation of SPL and GATA transcription factors. In general, RPN10 serves as one of the main ubiquitin receptors recruiting ubiquitinated proteins for proteasomal degradation. However, interestingly, SAP05 mediates SPL/GATA degradation in a ubiquitination-independent manner because it acts as a link between SPL/GATA and RPN10 [40]. Some phytoplasmas possess one or two SAP05 homologues, some of which have the property of degrading either SPL or GATA. *A. thaliana* with a two-amino-acid substituted RPN10 did not produce severely deformed leaves nor an increased number of lateral shoots by the phytoplasma infection. This suggests that the blocking SAP05 activities reduces host tolerance toward phytoplasmas.

#### 6. Other effectors of phytoplasmas

PM19\_00185 is a secreting protein found from ‘*Ca. Phytoplasma mali*’, a causal agent of apple proliferation [43]. Yeast-two hybrid screenings revealed that PM19\_00185 interacts with E2 ubiquitin-conjugating enzymes of *A. thaliana*. An in vitro ubiquitination assay showed that PM19\_00185 is enzymatically active as E3 ligase with E2 UBC09 of *A. thaliana* and E2 UBC10 of *Malus domestica*. A nonhost bacteria (*Pseudomonas syringae* pv. *tabaci*) can grow in PM19\_00185-expressing transgenic *A. thaliana*, suggesting that PM19\_00185 could suppress basal plant defense.

SWP16 is a secreting protein identified from ‘*Ca. Phytoplasma tritici*’ (wheat blue dwarf phytoplasma) as an RNA silencing suppressor (RSS) [42]. SWP16 inhibits accumulation of GFP siRNA and led to the accumulation of GFP mRNA in systemic *N. benthamiana*. The accumulation of SWP16 introduced potato virus X (PVX) is increased in *N. benthamiana* compared with PVX independent infection. Generally, RSSs are encoded in the genome of plant viruses, but some bacteria, oomycetes and fungi also have RSSs [62]. RSSs produced by *Phytophthora sojae* were shown to interrupt accumulation and biogenesis of small RNAs [63]. Similarly, SWP16 also inhibits production of miRNAs in *A. thaliana* [42].

SWP12 is a secreting protein found from ‘*Ca. Phytoplasma tritici*’.



**Fig. 1.** Phyllogen-mediated stepwise formation of ternary complex with MADS-box transcription factor (MTF) and Rad23 leads proteasomal degradation of MTF.

The expression of SWP12 in wheat plants (*Triticum aestivum*) promoted phytoplasma colonization [41]. Furthermore, SWP12 weakened the basal immunity of *Nicotiana benthamiana* and promoted leaf colonization by fungi (*Phytophthora parasitica* and *Sclerotinia sclerotiorum*) and a virus (tobacco mild green mosaic virus). TaWRKY74 (*T. aestivum*) and NbWRKY17 (*N. benthamiana*) were identified as host targets of SWP12 by yeast-two hybrid screening and GST pulldown analysis [41]. SWP12 degrades TaWRKY74 via the ubiquitin-dependent pathway, so that SWP12 may weaken plant resistance and promote phytoplasma colonization.

## 7. Perspectives

Phytoplasma effectors have been found to have a wide variety of functions, such as regulating plant morphogenesis and attracting insect vectors. Since these plant manipulations are thought to be essential for phytoplasma survival strategies, the inhibition of effector proteins could lead to control of phytoplasma infection [40,64]. In general, the phytoplasma genome encodes dozens of effector proteins. However, only the functions of the few effectors mentioned in this review were suggested, and the roles of most of the remaining effectors are unknown. Further advances in our understanding of the molecular mechanisms underlying host manipulation by effectors will enable us to devise control strategies for phytoplasma diseases that target these factors. Since whole genome sequences and draft genome sequences have been determined for several phytoplasma strains in the past decade [18, 65–70], more and more effectors will be discovered from these genomic data in the future, which will contribute to the understanding of phytoplasma infectivity and pathogenicity. In addition to effectors, phytoplasmas that are closely related but have different pathogenicity have been reported to have different genome and plasmid structures [14, 71–73], so the molecular mechanisms involved in pathogenicity will be clarified through comparative genomic analysis.

Phytoplasma genomes contain clusters of repeated gene sequences called potential mobile units (PMUs) [74], which consist of similar genes organized in a conserved order [75]. Many of the effector genes are encoded within PMU, and horizontal transfer of PMU may have occurred between divergent phytoplasma lineages [66,76], so that effector genes are also thought to have transferred horizontally between phytoplasmas. Phytoplasmas possess different effector genes even in phylogenetically close strains and species [40,54] probably because they have evolved to adapt to different hosts such as plants and insects. Since some effector genes are upregulated during insect infection [15,29], there should be effectors that manipulate insect hosts, but the role of effectors in insects is still unknown. Elucidation of the role of effectors whose function is not yet understood may lead to the elucidation of not only how phytoplasma manipulates the host but also the molecular mechanism of host determination. Furthermore, phytoplasma effectors regulate plants in unique ways not found in other plant pathogenic bacteria, such as Phyllogen and SAP05 degrading host factors in a ubiquitin-independent manner [38,40]. Phytoplasma effectors may be applied not only as a novel method to control plant morphogenesis and phytohormone balance, but also as a ubiquitin-independent method to control protein degradation.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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