A multi-layered mechanistic modelling approach to understand how effector genes extend beyond phytoplasma to modulate plant hosts, insect vectors and the environment

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Members of the Candidatus genus Phytoplasma are small bacterial pathogens that hijack their plant hosts via the secretion of virulence proteins (effectors) leading to a fascinating array of plant phenotypes, such as witch’s brooms (stem proliferations) and phyllody (retrograde development of flowers into vegetative tissues). Phytoplasma depend on insect vectors for transmission, and interestingly, these insect vectors were found to be (in)directly attracted to plants with these phenotypes. Therefore, phytoplasma effectors appear to reprogram plant development and defence to lure insect vectors, similarly to social engineering malware, which employs tricks to lure people to infected computers and webpages. A multi-layered mechanistic modelling approach will enable a better understanding of how phytoplasma effector-mediated modulations of plant host development and insect vector behaviour contribute to phytoplasma spread, and ultimately to predict the long reach of phytoplasma effector genes.

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Introduction
Social engineering malware is designed to be spread by people via attracting them to infected websites, social media posts, or email links [1]. The ability to spread relies heavily on human psychology, something that cannot be deduced from studying the computer code alone. Similarly, the insect transmitted plant pathogenic bacteria, phytoplasma, use methods to modulate the behaviours of their insect vectors, which are sap-feeding hemipteran insects, enabling the spread of these bacteria in the environment. Phytoplasma secrete specific virulence proteins, named effectors, into their host plants to accomplish this. Intriguingly, two phytoplasma effector proteins, SAP11 and SAP54, influence leafhopper fecundity and plant preference. Here, we describe how these two effectors have a crucial role in re-programming plant cellular processes leading to the induction of specific plant phenotypes. We then summarise how these effectors modulate the behaviours of phytoplasma insect vectors. Finally, we argue that a modelling approach will enable the dissection of the mechanisms involved in phytoplasma epidemics. Indeed, using experimental data of how phytoplasma effectors change plant development and plant interactions with insect vectors, we can now model the impacts of phytoplasma effector genes on the wider environment, including how the changes in insect vector behaviours may contribute to spread of phytoplasma to other plants within the field, to neighbouring fields and across longer distances. This can be achieved through multi-layered mechanistic modelling. In mechanistic models, relationships between the variables in the data set are specified in terms of the biological processes that are thought to have given rise to the data, in contrast to phenomenological and statistical models, where the relationship seeks to best describe the data [2]. The interactions and feedbacks between different layers of organization calls for multilevel modelling, the layers allowing to include the modelling of: effector-mediated changes within the plant; effector-triggered modifications of leafhopper behaviours; the impact of leafhopper behaviours on phytoplasma acquisition and transmission; and the impact of spatial dimensions and plant species occurrences within a field and its neighbouring fields on phytoplasma spread.

Phytoplasma have small repeat-rich genomes
Phytoplasma possess the smallest genome of any plant pathogenic bacteria and have limited metabolic pathways [3–6] and hence are completely reliant on plant host cells for essential nutrients. While phytoplasma genomes sizes are only 600–900 kb [5,7], a large portion of their genomes consist of repeats that resemble composite transposable elements, called potential mobile units (PMUs),
sequence variable mosaics (SVMs) or mobile unit genes (MUGs) [3,8,9]. The repeats are pathogenicity islands, as these tend to cluster together in phytoplasma genomes, form extrachromosomal units, are horizontally transferred among phytoplasma and carry the majority of effector genes [3,10–12]. Genomic synteny between closely related phytoplasma is low, particularly in PMU-rich regions, indicating that these regions are prone to frequent recombination events in which effector genes may be lost or gained [3,13,14,15,16]. Comparing the complete genome sequences of three phytoplasmas to those of *Acholeplasma* species, the closest free-living relatives of phytoplasma, revealed that the former are enriched in genes for host-interactions and virulence proteins [17].

**Phytoplasma secrete effectors that migrate systemically in plants**

Phytoplasma-infected plants often show dramatic symptoms, such as extensive stem proliferations, creating a witch’s broom phenotype, the retrograde development of flowers into leaf-like flowers, called phyllody, and greening of flower organs, called virescence (Figure 1a). These symptoms are now known to be caused by effector proteins secreted by the phytoplasma into the cytoplasm of phloem sieve cells and that unload from the phloem to other cells and migrate systemically [18–21]. Phytoplasma have a functional Sec-dependent pathway [22] and candidate effectors were identified via the presence of cleavable signal peptides. For example, the genome of aster yellows phytoplasma (AYWB; *Ca. P. asteris*) phytoplasma encodes genes for 56 secreted AY-WB proteins (SAPs) that are candidate effectors [18], whereas fewer effectors genes were found in the genomes of maize bushy stunt phytoplasma (MBSP; *Ca. P. asteris*) and *Ca. P. aurantifolia* strains [14,23]. SAP11 and SAP54 of AYWB (Figure 1b,c) and TENGU from onion yellows phytoplasma (OY; *Ca. P. asteris*) and homologs of these effectors from other phytoplasmas were demonstrated to be genuine virulence factors. Here we describe how these effectors contribute to the symptoms induction of phytoplasma-infected plants.

**Phytoplasma effector SAP11 induces stem proliferations and alters leaf development**

Transgenic *Arabidopsis thaliana* plants that stably express *SAP11* under control of a 35S promoter produce more stems with leaves that have altered shapes, resembling the witch’s broom symptoms of infected plants (Figure 1a,b) [24–26]. *SAP11* transgenic plants also exhibit altered root architectures, higher expression of genes involved in phosphate (Pi) starvation and reduced defence responses mediated by suppression of jasmonic acid (JA) synthesis and JA and salicylic acid (SA) responses [24,26,27**]. SAP11 interacts with and destabilizes proteins of the plant-specific TIEOSINTE BRANCHED 1, CYCOIDEA, PCF1 (TCP) transcription factor family [25,26] (Figure 1c). This family consists of class I and II members that at the cellular level antagonistically regulate cell proliferation and senescence, including the JA signalling pathway [28–31]. SAP11 interacts and destabilizes class II TCPs specifically, thereby inhibiting senescence, including reducing JA synthesis, but promoting cell proliferation leading to leaf crinkling and stem proliferation phenotypes (Figure 1c) [25,26]. SAP11 targeting of plant cell nuclei, via it is nuclear localization signal (NLS), contributes to TCP destabilization [18,25] and in AYWB-infected plants, SAP11 unloads from the phloem to migrate to adjacent tissues and was detected in nuclei of trichomes [18]. Interestingly, SAP11 undergoes proteolytic cleavage in plants [32], but how this affects its function, including nuclear localization and interactions with TCPs, is not yet clear.

A SAP11 homolog identified from apple proliferation phytoplasma (AP; *Ca. P. mali*) [33] interacts with TCPs as well [34], and so does SWP11 of wheat blue dwarf phytoplasma [35]. *Nicotiana benthamiana* plants that express *SAP11* show defects in the development of glandular trichomes and reduced expression of a gene encoding an o-methyltransferase involved in the biosynthesis of the volatile organic compound (VOC) 3-isobutyl-2-methoxypyrazine (IBMP) [27**]. This is in agreement with the class II TCP4 being involved in the regulation of trichome branching [36] and AP-infected apple trees emitting altered VOCs resulting in the attraction of AP pylid vectors [37]. Therefore, SAP11 effectors of diverse phytoplasmas interact with plant TCP transcription factors and induce a range of phenotypes in plants.

**The phytoplasma effector TENGU induces transcriptional changes in plants**

Plants that stably produce TENGU display witches’ brooms, dwarfism and flower sterility [19,38]. TENGU is highly expressed during OY infection [19] and is cleaved into shorter peptides of 19-21 amino acid long, of which an 11 amino acid domain induces the changes in plant phenotype [39]. Plants stably expressing TENGU show misregulation of transcripts involved in auxin and JA signalling leading to the conclusion that TENGU suppresses the expression of auxin response factor 6 (ARF6) and ARF8 genes [38].

**Phytoplasma effector SAP54 induces the development of leaf-like flowers**

*Arabidopsis* plants that stably express *SAP54* phytoplasma under control of the ubiquitous 35S and the phloem-specific ActSu2 promoters produce leaf-like flowers, often with new stems emerging from within the flower, resembling phytoplasma-induced phyllody and virescence symptoms (Figure 1a,b) [20]. SAP54 and homologs of this effector, named phyllogen, degrade MADS-box transcription factors (MTFs), including those involved in flower development (Figure 1c) [40,41]. For SAP54, this degradation process requires the 26S
The long reach of phytoplasma effector genes

Figure 1

(a) Symptoms of phytoplasma-infected plants
Normal branch (left) and witch’s broom symptoms (right) in lime

(b) Phenotypes generated by SAP11 and SAP54 phytoplasma effectors in A. thaliana

(c) SAP11 and SAP54 mechanisms in plant cells

(a) Phytoplasma-infected plants often display an array of fascinating changes in plant development, including stem proliferations (witch’s brooms) and the retrograde development of flowers into leaves (phyllody). (b) Single phytoplasma virulence proteins (effectors) produced by phytoplasma and secreted into plants during infection alter Arabidopsis thaliana development; SAP11 of Aster Yellows Witches Broom phytoplasma (AY-WB) changes leaf development and the production of more branches that resemble witch’s broom symptoms and AY-WB SAP54 the formation of leaf-like indeterminate flower-like tissues that look like phyllody symptoms. (c) The molecular mechanisms of how these virulence factors induce plant developmental changes are known, that is: SAP11 destabilizes plant TEOSINTE BRANCHED 1, CYCLOIDEA, PCF1 (TCP) transcription factors, generating witch’s brooms and crinkled leaves; and SAP54 degrades plant MADS-box transcription factors (MTFs) by interacting with the plant proteasome shuttle factor RAD23 (susceptibility factor) and via 26S proteasome, generating leaf-like flowers.
proteasome shuttle factors RAD23, particularly RAD23C and D (Figure 1c) [40]. SAP54AYWB binds the MTF K-domains [40], which facilitates dimerization of MTFs and is characterized by a coiled-coil structure [42]. It was proposed that SAP54 may fold into a structure similar to that of the K-domain, and as such, SAP54 could be useful for studying the contributions of MTFs to flowering of genetically intractable plants [43*]. SAP54/phylogen homologs were identified in at least 17 diverse phytoplasma species, and, for those tested, interactions with MTFs were shown [20,41,44*,45**].

Phytoplasma colonize multiple organs within their insect vectors

Phytoplasma are transmitted to plants by sap-feeding insects of the order Hemiptera, primarily leafhoppers, planthoppers and psyllids [46]. Transmission of phytoplasma through insects involves invasion of gut cells, migration throughout the haemolymph, colonization of various organs, including the salivary glands, and passage to the saliva from where the phytoplasma are introduced into the phloem sieve cytoplasm when the insects feed [15]. Phytoplasma can have broad or narrow plant host ranges, often coinciding with the host ranges of their predominant insect vectors [47]. Phytoplasma adjust to their plant and insect hosts via differential regulation of gene expression, including effector genes [48,49]. For AYWB phytoplasma, approximately 30% of the 56 effector genes are upregulated in AYWB-infected leafhoppers, and 60% in AYWB-infected plants [40]. Specific sigma factors are likely to regulate this gene expression transition [48,49]. So far, only a few researchers have focused on dissection of the molecular mechanisms involved in phytoplasma–insect interactions. The abundant cell-surface phytoplasma membrane protein antigenic membrane protein (Amp) of Ca. P. asteris OY forms a complex with actin and myosin heavy and light chains in the guts of OY vector insects, but not in those of non-vectors, indicating that Amp binds specific gut receptors of insect vectors only [50]. The Amp proteins of another Ca. P. asteris also form complexes with actin and ATP synthases located in the gut and salivary glands of insect vectors only [51,52]. Moreover, the Amp proteins of stolbur phytoplasmas (named StAmp) show signatures of diversifying selection [53]. Therefore, Amp is likely to have a crucial role in determining the specificity of phytoplasma–vector interactions. Ca. P. asteris also produce Amp in the plant phloem [54] and this is likely important, as insect vectors feed from the phloem and acquire the phytoplasma from there. Interestingly, Ca. P. mali and related phytoplasmas produce a different type of immunodominant membrane protein, called immunodominant membrane protein (Imp), which are under strong diversifying selection [55]. By contrast, the Ca. P. mali immunodominant protein Idp specifically binds plant actin and appears to have a role in phytoplasma mobility within the plant [56].

Phytoplasma versus malware, an analogy

Predicting spread of vector-borne disease agents requires an understanding of the mechanisms by which these agents are transferred among hosts. Following up on our analogy, malware is unknowingly transmitted by computer users, often when visiting infected websites or opening infected email attachments (Figure 2a). Social engineering malware makes use of social disguises, cultural ploys and psychological tricks to lure users to infected servers [1], for example through the promise of prizes or financial gain, or by pretending to be sent by friends or family. Another characteristic of such malware is its ability to combat existing technical countermeasures, again typically involving the user voluntarily removing or turning off existing protection [1]. Once a new computer host becomes infected, the malicious software can spread rapidly, infecting other software or resources, or modifying core functions, potentially leaving the system open to further attacks. The spread of phytoplasma can be viewed in a comparable manner. Insect vectors, such as leafhoppers, are attracted to infected plants, contract the pathogen, and then transmit it to new host plants (Figure 2b). Similar to malware, effector proteins modify the physiology of infected plants triggering modulations in leafhopper behaviours, which in turn increase the number of infected vectors. The same core strategies are involved, namely luring in more insects and reducing countermeasures, together increasing both the number of leafhopper visits and the fecundity of those visiting leafhoppers. Note, however, also the limitation of such an analogy, because unlike computer users, leafhoppers get colonized by the phytoplasma and transmit these bacteria, adding an additional level of complexity. In the next sections, we will describe the mechanisms by which this impact on the leafhoppers is achieved.

How phytoplasma virulence effectors contribute to insect vector transmission

Phytoplasma is a biotrophic bacterial pathogen that depends on insect vectors for transmission and colonization of plants. In this regard, it is interesting that phytoplasma SAP11 suppresses JA synthesis via targeting class II TCPs, because plants compromised in JA synthesis and signalling are more susceptible to insects, including leafhoppers that vector phytoplasma [26,57]. Therefore, phytoplasma effectors appear to have evolved to promote insect vector colonization. By contrast, biotrophic pathogens that do not depend on insect vectors for transmission often produce effectors that activate the JA pathway leading, via phytohormone crosstalk, to SA pathway inhibition, thereby promoting colonization of these pathogens [58]. Consistent with this, P. syringae HopBB1 targets class I TCPs to de-repress JA signalling [59] and some effectors from biotrophic fungi and oomycetes were found to interact with class I TCPs too [60–62].
Insect vectors reproduce more on phytoplasma-infected plants in nature and the laboratory in choice and no-choice tests [26,40,63,64] and the SAP11 and SAP54 effectors are likely to play a role in this (Figure 3). SAP11AYWB promotes egg laying of the AYWB leafhopper vector Macrosteles quadrilineatus on A. thaliana plants in no-choice tests (Figure 3), in agreement with increased egg production of these insects on plants that produce less JA [26]. Leafhoppers also show a preference for colonization of Nicotiana attenuata plants that are compromised in JA synthesis, in both nature and the glasshouse [57]. Given that SAP11AP modulates VOCs of plants [27**], it is likely that SAP11 effectors also promote leafhopper vector attraction in choice tests, though this has not yet been examined.

SAP54AYWB promotes M. quadrilineatus colonization of A. thaliana in choice tests, but not in no-choice tests (Figure 3) [40]. The leafhoppers are also more attracted to AYWB-infected plants in a manner that is dependent on RAD23, which is involved in the SAP54-mediated degradation of MADS-box transcription factors (Figure 1c) [40]. Interestingly this attraction also occurs before flowering and to single leaves of the transgenic plants, indicating that the leaf-like flowers are not required for the leafhopper choice [63]. Nonetheless, inhibition of flower development may have other advantages for phytoplasma by, for instance, delaying plant senescence and death, which often occurs in herbaceous plants upon seed production.

**A multi-layered mechanistic approach for predicting phytoplasma epidemics**

We have described phytoplasma infection at two different levels: firstly, the mechanisms acting within the plant, and secondly, how the changes they induce modulate...
leafhopper behaviour. The final step is to develop an understanding of how leafhopper behaviour influences outbreaks of phytoplasma infection. The spread of social engineering malware cannot be understood by looking at the source code alone, but requires, at a completely different level, understanding of human psychology as well. Likewise, we argue that understanding the spread of phytoplasma requires a comparable multi-level mechanistic approach, designed to tackle some of the challenges of predicting plant disease propagation, as pointed out by [65], for example, capturing host spatial structure, realistic vector dispersal, vector preference and the inclusion of multiple pathogen strains, along with their evolution. We envision such a model to be designed to integrate each of the three levels we have described (Figure 4). The lowest layer comprises the phytoplasma and their effector genes driven processes acting within the plants, phytoplasma titre, effector protein-driven modifications in plant development and the consequential plant phenotypes and their impact on the insect vectors. The next layer captures the field setting, taking the spatial structure of both hosts and vectors into account [66,67]. It describes realistic leafhopper dispersal, including their natural movement as well as their preference for specific (modified) hosts, which can take the form of either directed movement towards specific plants or increased residence time [68]. One possible choice would be to use individual-based models (IBMs), to straightforwardly capture the individual behaviour of insect vectors under different conditions [69]. The top layer takes environmental conditions and crop and weed distribution into account, as well as their impact on dispersal, which can directly include geographical information systems (GIS)-based data [70,71]. Such an approach would allow us to link changes in external forcing, such as climate change, to phytoplasma outbreak. Moreover, through the modelling of competition between multiple phytoplasma effectors, as displayed in the figure, potentially including their evolution, one could address how impact propagates through the diverse biological scales in order to be able to answer how the presence or absence of a single effector gene in the genome of a phytoplasma population can contribute to large-scale changes in phytoplasma infection dynamics.

*M. quadrilineatus* colonizes AYWB phytoplasma-infected *A. thaliana* better in choice and no-choice tests that involves the actions of the AYWB effectors SAP11 and SAP54. (a) Leafhoppers are more attracted to (choice tests) and have increased fecundity on (no-choice tests) phytoplasma-infected plants. (b) Leafhoppers have increased fecundity on and are more attracted to SAP11\textsubscript{AYWB} transgenic plants. (c) Leafhoppers are more attracted to SAP54\textsubscript{AYWB} transgenic plants.
Conclusions

Two distinctive features of social engineering malware are its pervasiveness and persistence. It has been proposed that these cannot be combated by the pursuit of purely technical solutions alone, but require a multi-pronged attack [1]. Data discussed here suggests the same to be true of phytoplasma infections. Phytoplasma effector proteins not only modulate plant development, but also promote colonisations of insect vectors that are crucial for phytoplasma spread. However, it is not yet clear to what extent an increase in vector colonization helps phytoplasma spread, and whether some effectors are more advantageous to spread than others. A multi-layered modelling approach, with the ability to incorporate all levels of phytoplasma infection, would provide the missing link between insect vector behaviours and pathogen spread, and, in particular, how these are influenced by specific effector proteins.

Acknowledgements

This work was supported by the Human Frontiers Science Program [RGP0024/2015]; the Kohn Foundation and the Royal Society [NF161176]; and the UK Biological and Biotechnology Research Council (BBSRC) via grants BB/P012574/1 and BB/P013511/1 to the John Innes Centre.

We thank Allyson MacLean, Pascal Pecher and Andrew Davis for photographs of the SAP11 and SAP54 transgenic *A. thaliana* plants, JIC horticultural services and insectary for rearing the plants, Ali Al-Subhi and Abdullah Al-Saadi (Sultan Qaboos University, Muscat, Oman) for hosting us to take the photos of phytoplasma-infected lime and alfalfa plants in Oman, and Agnieszka Zwolińska (Institute of Plant Protection, National Research Institute, Poznań, Poland) for hosting us to take the photos of phytoplasma-infected oilseed rape plants in Poland.
References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

● of special interest
● of outstanding interest


This study shows that the effector SAP11 of MBSP is conserved in distinct isolates in Mexico and Brazil, and is one of the first whole genome comparisons among multiple phytoplasma strains with a Ca. species.


This publication shows that SAP11 is altering the expression of a gene involved in the production of volatiles.


The long reach of phytoplasma effector genes


This paper provides compelling evidence that the SAP54 effector could have a similar fold to that of the K-domain of MADS-box transcription factors.


This study builds on previous evidence that SAP54-like effectors interact with MADS-box transcription factors making it relevant for a broad range of phytoplasma–plant interactions.


This elegant study shows the mechanisms by which an effector of a biotrophic bacterial pathogen degrades a class I TCP transcription factor thereby derepressing jasmonate responsive genes leading to higher pathogen virulence.


