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# MOLECULAR MECHANISM UNDERLYING SYMPTOM DEVELOPMENT IN PHYTOPLASMA ASSOCIATED DISEASES - THE KEY PLAYERS AND THEIR ROLE#

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**ABSTRACT**

**Phytoplasma, plant pathogenic Mollicutes that have a trans-kingdom life cycle, are insect transmitted and have been found to be associated with yield affecting traits in a large number of taxa. Many peculiar symptoms observed in the host taxa are often results of dramatic alterations in the normal development program that are generally controlled at the meristematic regions located in the shoot apices. Phytoplasma being vasculature limited are thus able to bring about genetic reprogramming in the regions of host plant that are far removed from their natural niche namely sieve elements of the phloem. Several proteins secreted by phytoplasma in the host plants have been identified and termed as effector molecules namely SAP54, SAP11, TENGU, SAP21 etc. that enable the colonisation, survival, spread of phytoplasma and also bring about dramatic alterations in the host plant. Nevertheless, the mechanisms underlying these peculiar phenomena are far from understood and remain a challenging area for the phytoplasma biologists. A thorough understanding of the processes involved is needed to provide platforms for developing control measures for phytoplasma associated diseases that will also enhance basic understanding on the plant developmental programs affected.**

**Key words:** Pathogens, effector molecules, phyllogeny, miRNA

Phytoplasmas are insect-transmitted bacterial plant pathogens belonging to the class Mollicutes that infect broad range of plants including an array of annual crop plants, several perennials and their associated wild and weedy taxa. Presence of phytoplasma in plants is associated with a wide variety of symptoms viz., stunting, development of numerous tiny branched shoots having small leaves (witches' broom), yellowing, greening of floral organs (virescence), phyllody, reddening of leaves and stems (purple top), stem flattening (fasciation), phloem necrosis etc. (Maejima et al., 2014).

This wall-less bacteria cause considerable yield loss in different crops worldwide. Symptoms induced in infected plants suggest that phytoplasma interfere with the developmental processes within the plant host via a variety of effector molecules to manipulate the host phenotype for their own benefit (Hogenhout et al., 2008; Hogenhout and Loria, 2008; Bai et al., 2009; Sugio et al., 2011). Phytoplasma also modulate immune response of the sap-feeding hemipteran (including plant hoppers, leafhoppers and psyllids) insect vectors in such a way that they spread these bacteria to other plants.

Since phytoplasmas have no cell wall and reside inside the host cells, their membrane proteins and secreted proteins (effectors) can function in the cytoplasm of the host plant or insect cells, and are predicted to have important roles in the interplay between pathogen and host (Oshima et al., 2013; Sugio et al., 2011, 2014).

Further, vasculature limited niche of phytoplasma necessitates the secretion of low molecular weight substances and their movement across the cells to access the meristmatic tissue localised in the apices in order to alter the normal developmental program of the host plant.

As phytoplasmas are obligate parasites, the movement of phytoplasmas is dependent upon their phloem sap-sucking insect vectors which get attracted to plants aggressively producing young and green vegetative tissues. Production of these effectors may stimulate leafhopper feeding, and thereby increase the frequency of phytoplasma acquisition by its vector. Prolonged availability of sap due to reversion of flowering phase to vegetative phase is yet another prospect particularly in case of annual plants.

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The precise mechanism by which these alterations take place has become an active area of research during the last two decades. Several effector molecules have been identified by various workers that have been proposed to genetically reprogram the host plants enabling the colonisation, survival and spread of phytoplasma.

**SAP54**

One such novel AY-WB (aster yellow phytoplasma strain witches Broom) effector protein, SAP54, that causes symptoms characteristic to phytoplasma infection, alters floral development and persuades production of leaf-like flowers was first identified from an array of 52 genes encoding Secreted AY-WB Proteins (SAPs) (Maclean et al., 2011).

Maclean et al. (2014) for the first time elucidated the mechanism by which phytoplasma alters floral development to convert flowers into vegetative tissues by producing a novel effector protein (SAP54) that interacts with members of the MADS-domain transcription factor (MTF) family, such as SEPALLATA3 and APETALA1 and occupy central positions in the regulation of floral development. SAP54 mediates degradation of MTFs by interacting with proteins of the RADIATION SENSITIVE23 (RAD23) family- eukaryotic proteins that transport substrates to the proteasome as the conversion of flowers into leaf-like tissues diminished in Arabidopsis rad23 mutants in the presence of SAP54. Remarkably, plants with SAP54- induced leaf-like flowers are more attractive for colonization by phytoplasma leafhopper vectors and this colonization preference is dependent on RAD23. Thus, SAP54 has been proposed to act as a link between two key pathways of the host to alter development resulting in sterile flowers/inflorescences (MacLean et al., 2014; Maejima et al., 2014).

The interaction between SAP54 and the K-domain is not well understood, however it depends on the sequence and structural similarities ascribed to convergent evolution during which SAP54 achieved molecular mimicry of K-domain of MIKC-TYPE MTFs. (Rumpler et al., 2015).

More studies on the sequence and structural aspects of SAP54-like proteins in a phylogenetic perspective are required in the future to clarify the case, but are potentially loaded by frequent horizontal transfer of SAP54 genes (Rumpler et al., 2015).

Replacement of leucine or isoleucine residues near the centre of α-helices by proline, α-helical content got massively reduced and mutant protein was unable to interact with any investigated MIKC-type MTFs and did not result in disease phenotype. However, when same residues were substituted with alanines, which did not break helices, SAP54 proteins were still able to interact with their targets and were capable of causing disease symptoms (Aurin et al., 2020). These findings suggest a strong link between the α-helical structures of SAP54 and its ability to interact with some MIKC-type MTFs. Further, removing either of the alpha-helices, SAP54 completely lost its ability to interaction specificity. Deletion of a part of C-terminal alpha helix was sufficient to abolish this interaction whereas deletion of turn of the protein did not alter any sort of interaction suggesting that the distance between the helices is of less importance for interaction (Aurin et al., 2020).

According to the results of dynamic light scattering and electrophoretic mobility shift assay (EMSA), it now seems likely that SAP54 preferentially targets multisets of MIKC-type MTFs (Aurin et al., 2020). SAP54 targets SEP1, SEP2, SEP3 and SEP4 which forms DNA-bound homotetramers (Jetha et al., 2014; Melzer et al., 2009; Rumpler et al., 2018), whereas not with AP3 and PI that do not form homotetramers (Melzer and Theißen, 2009; MacLean et al., 2014;Rumpler et al., 2018).

Furthermore, SAP54 binds to RAD23C and RAD23D (Iwabuchi et al., 2019; MacLean et al., 2014). RAD23 is known to transfer ubiquitylated substrates to the 26S proteasome (Farmer et al., 2010), thus SAP54 could be functioning as an adapter that connects MIKC-type MTFs with RAD23 proteins and delivers them to the proteasome (MacLean et al., 2014). The exact place and mode of RAD23 binding to SAP54 is unknown, however several models have been proposed in which MIKC-type MTFs and RAD23 bind to SAP54 (MacLean et al., 2014).

While it was shown that phyllogens target some MADS TFs, including SEP3 for degradation, it remains largely unknown whether the other SEPs (SEP1, SEP2, and SEP4) of *Arabidopsis* are also degraded by them. Maejima et al., (2015) examined whether phyllogen (PHYL1) of OY-W phytoplasma degrades SEP1, SEP2, and SEP4 of *Arabidopsis* which are highly conserved among various angiosperms. They transiently expressed SEP1, SEP2, SEP3, SEP4, or bZIP63, in *Nicotiana benthamiana* and reported that all SEP proteins of *Arabidopsis* were degraded in the presence of phyllogens *in-vivo*.

Induction of phyllody by phyllogen is not just restricted to *Arabidopsis* as phyllogen causes phyllody phenotypes in several eudicots. Moreover, phyllogen can interact with MTFs of not only angiosperm species including eudicots and monocots but also gymnosperms and a fern, and can induce their degradation (Kitazawa et al., 2017).

Initially *in-silico* predictions had shown that phytoplasma effector protein SAP54, an ortholog of PHYL1 comprises two α-helices connected by a short inter helical region in which a conserved proline breaks the α-helix (Rumpler et al., 2015). Crystal structures and 3D structure predictions further confirmed that SAP54 folds into at least two coiled-coils that are separated by inter helical region. Liao et al. (2019) published the crystal structure $PHYL1_{PnWB}$ from '*Candidatus* phytoplasma strain Peanut Witches' Broom. Similarly, Iwabuchi et al. (2020) for the first time determined the crystal structure of $PHYL1_{OY}$, a phyllogen from OY phytoplasma. The crystal structures of both proteins are very similar, comprising two α-helices connected by a central linker. The structure of $PHYL1_{OY}$ resembled a well-studied bacterial effector AvrRps4C as both of them have a highlighted coiled coil structure that is important for protein-protein interaction.

Using dynamic light scattering, Aurin et al. (2020) found complexes with an apparent molecular mass of ~20 kDa suggesting that SAP54 predominantly formed homodimers under experimental conditions.

Detailed analyses indicated that both α-helices of $PHYL1_{OY}$ contain a repeating series of hydrophobic residues that are highly conserved (Aurin et al., 2020). Remarkably, similar repeating series are found in K-domain of MTFs required for oligomerization.

The N64 residue exposed on the conserved surface of the phyllogen plays a key role in α-helix formation as N64R substitution disrupts the structural integrity supported by the fact that substitutions with glutamine, having similar properties to asparagines did not induce phyllody as glutamine generates more steric hindrance due to its larger size compared to asparagine (Bogan and Thorn, 1998). Thus the steric property of N64 may be crucial for the stabilization of phyllogen- MTF interaction (Iwabuchi et al., 2020). These results suggest that surface structures of phyllogens formed by these hydrophobic and hydrophilic residues are important for phyllogen- MTF interactions.

Recently, for detection of phyllogens in diverse phytoplasma groups' pair of degenerate primers were developed and were used to identify phyllogens from 25 different phytoplasma strains related to nine species (Iwabuchi et al., 2020). The phyllogen family was categorized in four groups: phyl-A,-B,-C and -D. Members of phyl-A,-C,-D, induced phyllody, decreased the amount of SEP1-4 and AP1*in planta*, and interacted with floral MTFs and RAD23C/D. In contrast, phyl-B group phyllogens lacking the ability to induce phyllody still interacted with host factors, but the interaction specificity was different from that of phyllody-inducing phyllogens.

The loss of phyllody inducing activity in the phyl-B group was ascribed to a polymorphic residue at position 64 that regulates the MTF binding and degrading activity of the effector in cooperation with another polymorphic residue at position 30. Auxiliary functional elucidation of the non-phyllody-inducing phyl-B group may reveal unresolved roles of the effector family that improve phytoplasma fitness.

Singh and Lakhanpaul (2020) identified an orthologs of SAP54 as S54LP of SP (SAP54 like Protein of Sesame Phyllody), from *Sesamum indicum* (L.) infected with phytoplasma. Non synonymous substitutions were detected in the SAP54 ortholog sequences from phytoplasmas belonging to same (sub) group using Fixed Effects Likelihood (FEL) approach. The ortholog was found to be under purifying (negative) selection, a total of three amino acid sites were found to be under pervasive purifying (negative) selection and one under pervasive diversifying (positive) selection. Asparagine residues at position 68 and 84 were inferred to be of much importance in the effector protein as they were under pervasive purifying selection. It was also concluded that the signal peptide evolved at a rate higher than mature protein.

The phylogenetic analyses have revealed that of phyllogen family evolved independently in '*Ca.* phytoplasma' species as compared to 16SrRNA phylogeny. Closely related phytoplasmas ('*Ca.* P. asteris' and *'Ca*. P. pruni') were separated into several different phylogenetic groups. It is reasonable to hypothesize that phyllogens were transferred horizontally among phylogenetically distinct phytoplasmas (Iwabuchi et al., 2020). Most of the phyllogens were found to be associated with potential mobile units (PMUs) (Jomantiene et al., 2007; Sugio and Hogenhout, 2012; Maejima et al., 2014). These findings suggest that horizontal gene transfer of phyllogens by PMUs has contributed to the acquisition and sharing of phyllody-

inducing activity among phytoplasmas. Interestingly, it is still unclear how these phyllogens originated as they do not have a potential homologous ancestral protein sequences.

Iwabuchi et al. (2019) found tetramers of SAP54 in the crystals but since SAP54 did not interact with itself in Y2H experiments, they concluded that SAP54 exists as monomer. However, well known bias of Y2H experiments against the detection of homodimer formation (Smirnova et al., 1999; Newman et al., 2000), this conclusion equivocal. Retaining dynamic light scattering, Aurin et al. (2020) found complexes with an apparent molecular mass of ~20 kDa suggesting that SAP54 predominantly formed homodimers under experimental conditions.

Detailed analyses indicated that both α-helices of $PHYL1_{OY}$ contain a repeating series of hydrophobic residues that are highly conserved (Aurin et al., 2020). Remarkably, similar repeating series are found in K-domain of MTFs required for oligomerization.

**SAP11**

The phytoplasma effector SAP11 results in witches' broom symptoms by inducing degradation of *A. thaliana* TCP (TEOSINTE-BRANCHED, CYCLOIDEA, PROLIFERATION FACTOR 1 and 2) transcription factors (TFs). The TCP TFs are known to repress branching and mediate leaf development. Thus, SAP11 results in induction of axillary branches with crinkled leaves (Sugio et al., 2014). Homologs of SAP11 have also been reported to interact with TCP homologs in other economically important plants such as apple, maize, wheat and coconut where phytoplasma leads to symptoms of witches' broom and alterations in leaf morphology (Janik et al., 2017; Pecher et al., 2019).

AY-WB SAP11 homologs have been reported from '*Ca.* P. solani' (strain SA-1) (Music et al., 2019), '*Ca.* P. asteris' OY-M strain (Chang et al., 2018), Maize bushy stunt phytoplasma, MBSP (strain M3) (Orlovskis et al., 2017), PnWB (Peanut witches' broom) phytoplasma (Chang et al., 2018) and '*Ca.* P. mali' strains AT, STAA and PM19 (Siewart et al., 2014, Janik et al., 2017, Strohmayer et al., 2020), but not from '*Ca.* P. australiense'.

The first report for the presence of the gene for SAP11 in a PMU region along with other genes such as *sigF*, *ssb*, *himA* and *tra5* and a sequence repeat of 331-bp was given by Bai et al. (2009). It was further suggested that SAP11 is a part of a larger transcript of ~ 2,800 bp which contains genes for four other SAPs and one membrane protein, thus indicating their simultaneous transcription *in planta*. Thus, SAP11 has been suggested to exist in an operon and it may function alone or in association with other secreted proteins present in the PMU-like pathogenicity island (Bai et al., 2009).

SAP11 was reported to contain a eukaryotic nuclear localization signal (NLS) which is crucial for its localization exclusively in nuclei of plants infected with AY-WB as confirmed by deletion experiments. Apart from NLS, a host factor, namely import in α assists in nuclear targeting of SAP11 in *Nicotiana benthamiana* confirmed by Virus Induced Gene Silencing (VIGS) assay. SAP11 has also been suggested to alter gene regulation at the transcriptional level in tomato plants (Bai et al., 2009).

SAP11 gets unloaded from the phloem tissues to access nuclei of mesophyll cells and other tissues as confirmed by immunofluorescence microscopic experiments. Moreover, most of the SAPs (51 out of 56) are smaller than 40 kDa. Since, the size exclusion limits (SELs) of plasmodesmata in the sink tissue varies between 10-40 kDa, the majority of the SAPs can unload from the phloem to target other developing tissues in plants (Bai et al., 2009). Initial reports on the presence of SAP11 in the leaf hopper *Macrosteles quadrilineatus* showed its accumulation in their midgut and salivary glands suggesting its transfer into plant cells when AY-WB infected insects feed upon plants (Bai et al., 2007).

SAP11 has a modular structure with distinct amino acids in different regions involved in its targeting to nucleus, binding with TCP and degradation of TCP (Sugio et al., 2014). The N-terminal of SAP11 has been reported to be crucial for its nuclear localization in the plant cell. The coiled-coil domain (91- 106 amino acids) at the C-terminal of SAP11 is required for its interaction with TCP13 (Sugio et al., 2014).

Alteration in the root structure has also been reported in *Arabidopsis* plants expressing AY-WB SAP11 leading to the formation of hairy roots, along with accumulation of cellular phosphate. The transgenic plants also showed upregulation of starvation-induced genes and microRNAs and downregulation of salicylic acid-mediated defense responses (Lu et al., 2014) leading to enhanced susceptibility to bacterial pathogens. In plantaproteolytic cleavage and *in vitro* self-interaction of SAP11 have also been reported (Lu et al., 2014).

SAP11 homolog from '*Candidatus* phytoplasma

mali' has also been reported to induce morphological alterations in the development of glandular trichomes and downregulate the expression of *NbOMT1* (encodes for *O*-methyl transferase) in transgenic *N. benthamiana* leading to reduced 3-isobutyl-2-methoxypyrazine (IBMP) synthesis. These changes result in alteration of aroma phenotype in transgenic *N. benthamiana* (Tan et al., 2016).

Chang et al. (2018) characterized the homologs of SAP11 from '*Candidatus* phytoplasma mali' (*Ca* PM), peanut witches' broom (PnWB) phytoplasma, AY-WB phytoplasma and OY-M phytoplasma ($SAP11_{CaPM}$, $SAP11_{PnWB}$, $SAP11_{AYWB}$ and $SAP11_{OYM}$). Alterations in the architecture of leaf and shoot and changes in flowering time were observed in *Arabidopsis* expressing these SAP11 homologs. SAP11 effectors are also involved in destabilization of class II TB/CYC-TCP TFs, thus inducing witches' broom (Chang et al., 2018). Alteration in miR156/SPLs module by $SAP11_{AYWB}$ has been reported to delay flowering (Chang et al., 2018).

SAP11 homologs from Maize bushy-stunt phytoplasma (MBSP) and AYWB differ in their interaction specificities with class II TCP TFs. Both $SAP11_{AYWB}$ and $SAP11_{MBSP}$ interact with CYC/TB1 BRC1 and BRC2 transcription factors, but only AYWB SAP11 interacts with CIN-TCP TFs. Thus, a smaller subset of class II TCP TFs has been reported to be destabilized by $SAP11_{MBSP}$ as compared to $SAP11_{AYWB}$ (Pecher et al., 2019).

A homolog of SAP11 has been identified from *Malusxdomestica* infected with '*Candidatus* phytoplasma mali' strain STAA (Janik et al., 2017). This effector, namely ATP_00189 is 41 per cent homologous to AY-WB SAP11 at the amino acid level and interacts with CIN-TCP group II proteins MdTCP25 (homologue of *Arabidopsis* TCP4) and MdTCP24 (homologue of TCP13/PTF1). Binding of ATP_00189 to MdTCP25 speculates its role in the downregulation of jasmonic acid (JA) biosynthesis, thus resulting in enhanced ABA response. The interaction of ATP_00189 with MdTCP24 is suggestive of its role in regulating plastid genes expression. Altered root growth and reddening are the symptoms commonly observed in apple proliferation which is postulated to be induced by SAP-11 mediated degradation of TCP13/PTF1 in '*Candidatus* phytoplasma mali' infected apple tree (Janik et al., 2017).

Anabestani et al. (2017) identified SAP11 homologs from three different strains of 16SrII group phytoplasma, namely Crotolaria Phyllody (CrP), Witches' Broom Disease of Lime (WBDL) and Faba Bean Phyllody (FBP).

A total of 37 putative effectors identified in Wheat Blue Dwarf (WBD) phytoplasma (Chen et al., 2014) were expressed in *N. benthamiana.* A SAP11 like protein, SWP1 was observed to induce distinct symptoms of witches' broom and was speculated to be the virulence factor. Wang et al. (2018) reported that apart from inducing witches' broom symptoms SWP1 also induces typical proliferation phenotype when expressed in *Arabidopsis.* On expressing deletion mutants of SWP1 in *N. benthamiana*, it was found that nuclear localization and the presence of typical coiled-coil domain are the two important requirements for SWP1 induction of witches' broom. Y2H and BiFC assays confirmed the interaction of SWP1 with BRC1 and BRC2, which are shoot-branching integrators. Further, *in planta* co-expression showed SWP1 mediated degradation of BRC1 involving a proteasome system. SWP11 induced HR like cell death, $H_2O_2$ accumulation and deposition of callose in *N. benthamiana.* SWP12 and SWP21 were reported to suppress defense related PCD in *N. benthamiana*.

Recently, Strohmayer et al. (2020) reported that the SAP 11- like protein of '*Ca.* P. mali' strain PM19, which is 41 per cent homologous to AY-WB SAP11 at amino acid level, results in the production of crinkled leaves and siliques along with symptoms of witches' broom when expressed in *Arabidopsis thaliana.* Yeast two-hybrid interaction studies revealed that Apple Proliferation (AP) SAP 11 -like protein interacts with a total of six members belonging to class 1 TCP TFs and all the members of class II TCP TFs in *A. thaliana.* It was further demonstrated that the AP_SAP11- like protein does not require the Nuclear Localization Signal (NLS) for its targeting or localization into the nucleus. In fact, the 17 amino acid long NLS, which is involved in targeting AY-WB SAP11 into the nucleus, has been reported to bind with few TCPs in case of '*Ca.*P.mali' SAP 11-like protein leading to crinkled leaf and silique phenotype in *A.thaliana.* Moreover, the presence of AP_SAP 11-like protein in the cytoplasm itself is sufficient for the induction of symptoms.

## TENGU

TENGU is a 70 amino acid protein (4.5kDa) known to cause witches' broom and dwarfism in the infected plant. It also induces plant sterility. TENGU gene is restricted to 16SrI group and shows high level

of sequence conservation. It is located near ABC transporter genes in OY-M and MBSP and near PMU-like genome elements in AY-WB genome. (Sugio et al., 2012)

Historically, Paulwonia witches' broom disease caused by phytoplasma was first described more than 140 years ago in Japan. The symptoms include witches' broom and dwarfism. Infected plants produce large number of auxiliary shoots and bear small flowers and leaves with shortened internodes (dwarfism) resulting in broom like appearance and is referred to as Tengu-su (Tengu's nest) disease in Japan because of its resemblance to the nest of Tengu (mythical Japanese goblin).

Hoshi et al. (2009) first characterized virulence factor, tengu-su inducer (TENGU) by expressing it in *N. Benthamiana* plants. They screened 30 putative secretory proteins from OY and each protein was expressed in *N. benthamiana*. PAM765 (one of the secreted protein) gene was transformed into *N. Benthamiana* using *A. tumefaciens*. These transgenic plants expressing PAM765 developed clear symptoms of witches' broom and dwarfism. Apart from this, these plants also had malformed phyllotaxis. This was further confirmed by inoculating *A. thaliana* with OY phytoplasma strain which exhibited the same symptoms. Therefore, PAM765 was termed as Tengu, a virulence factor that induces phytoplasma related witches' broom, dwarfism and abnormal reproductive organogenesis.

TENGU protein travels to the plant phloem via Sec dependent pathway where N terminal signal peptide (32aa) is cleaved. This produces the mature protein (secretory protein) that is only 38 amino acids long. The 11 amino acids of the N-terminal in mature protein make up the functional domain of TENGU and are sufficient to cause symptom development and are conserved. On the other hand, C-terminal is not necessary for symptom induction but it is still conserved. It was found that TENGU is processed by host plant factor to generate functional peptides. The leucine residue at the 11$^{th}$ position is significant for TENGU function. (Sugawara et al., 2013)

Minato et al. (2014) demonstrated that transgenic expression of TENGU induces abnormalities in flower development in *A. thaliana* and that both ARF6 and ARF8 transcripts are significantly diminished in *tengu*-transgenic and OY-infected plants. Other floral maturation genes like LOX2, MYB21 and MYB24 levels were also significantly lower in *tengu*-transgenic plants in comparison to wild-type plants. A significant decrease in total levels of cis-JA and active JA-Ile in tengu-transgenic buds shows that TENGU expression reduces JA biosynthesis. Microarray analysis showed that phytoplasma infection in grapevine induces production of *pathogenesis-related proteins 1* (PR-1) which is a marker gene for SA signalling pathway. This suggests that SA is involved in phytoplasmal infection response but PR-1 expression was not seen in *tengu*-transgenic plants. This indicates that antagonism between SA and JA does not lead to repression of JA synthesis in *tengu*-transgenic flowers.

Transcription profiles of *tengu*-transgenic and GUS-transgenic *Arabidopsis* plants showed 373 genes to be upregulated and 575 genes to be downregulated in tengu-transgenic plants. A large number of auxin related genes downregulated due to expression of TENGU belonged to early auxin responsive genes i.e *AUX/IAA* family genes, *small auxin induced RNA* (*SAUR*) family genes and *GH3* family genes. Moreover, expression of *Dormancy associated protein* (*DRM1*) was found many folds lower in tengu-transgenic plants, along with *2-pin formed* (*PIN*) genes, when compared with GUS-transgenic plants. These results suggest that TENGU interferes with signalling and biosynthesis pathways of auxin (Hoshi et al., 2009).

Thus, TENGU is an effector protein with pleiotropic effect on at least two phytohormones, namely auxin and JA and eventually leads to sterility in plants.

In addition to the proteinaceous effector molecules, role of small RNAs such as microRNA has been proposed in the phytoplasma associated dramatic alterations in the plants.

**miRNA**

microRNAs (miRNAs) are one of the category of small noncoding RNAs which in case of plants are predominantly 21 nucleotides in length (Chen et al., 2010). They are derived from genomic segments via transcription of RNA polymerase II. This primary miRNA after processing yields mature miRNA that is a duplex which is a result of RNase-III type enzyme DICER-LIKE1 activity (Bartel, 2004). The miRNAs show the feature of perfect complementarity with their target sequences and show transcript repression by processes such as mRNA slicing through ARGONAUTE endonuclease activity. Fifteen different miRNA families are reported in plants (Bartel and Bartel, 2003) which are known to play regulatory role both at transcriptional and post transcriptional level (Yu et al., 2017).

Studies on phytoplasma host plant interaction have revealed that the modulation of miRNAs is one of the factors behind disease development. Following is a brief overview of such reports.

Alterations of leaf morphology in phytoplasma diseased plants involve symptoms like size reduction, leaf yellowing, leaf curling and development of new leaves with short plastochron lengths. In phytoplasma infected paulownia plants such symptoms are shown to be associated with miR156-*SPL9* (Squamosa-promoter-binding-like-protein) interaction (Fan et al., 2015; Cao et al., 2018). Similar studies where over expression of miR156 and its subsequent regulation of SBP-box transcripts has been observed is other phytoplasma infected plants (Ehya et al., 2013; Gai et al., 2014; Shao et al., 2016). RNA sequencing studies have indicated that miR156 might function by degrading long non-coding RNAs (Fan et al., 2018). Symptoms such as internode shortening, smaller inflorescences have also been linked to miR156/*SPL* in *flavescence doree* (FDp) infected grapevine (Chitarra et al., 2018). In addition to miR156, role of miR172 has also been indicated with respect to flower abortion in aster yellows infected grapevine (Synman et al., 2017) and flower sterility in jujube witches' broom disease (Shao et al., 2016).

Deformation of leaves in grapevine seems to involve modulation of multiple miRNAs as altered expression of miR159 and miR319 has also been detected (Synman et al., 2017). These miRNAs target MYB transcriptional factors (R2R3 family) that are responsible for several key functions in plants (Dubos et al., 2010). Symptoms such as leaf roll and shortened petioles in phytoplasma infected jujube plants has been associated with downregulated miR159 and coincident over expression of its target gene *MYB33* (Rubio-Somoza and Weigel, 2013).

During phytoplasma infection of host plant, regulation of miRNAs involved in hormone signalling is shown to play role in development of few disease symptoms. Expression analysis of miRNAs in yellowing disease affected mulberry and grapevine showed high levels of miR160, which is a miRNA that targets ARF (Auxin Response factor) mRNAs for degradation (Gai et al., 2014; Synman et al., 2017). miR393 that targets auxin receptor mRNAs was also upregulated in phytoplama affected lime trees showing symptoms of witches' broom (Ehya et al., 2013). Studies on phytoplasma infected mulberry have shown that regulation by miR393 is more streamlined by production of secondary siRNAs (Gai et al., 2014). Further, studies on phytoplasma infected mulberry phloem sap detected altered miRNAs (miR1233e, miR529b, miR157a, miR849) to function in different hormonal pathways (Gai et al., 2018). *TCP*/miR319 was shown to control biosynthesis of jasmonic acid via regulation of lipoxygenase genes in FDp infected grapevine (Chitarra et al., 2018). Increased level of auxin and alteration in several iso-miRNAs involved in auxin signaling (of miR160, miR166 and miR167) was observed in lime trees suffering from phytoplasma caused witches' broom (Synman et al., 2017). In contrast, jujube plants with phyllody symptoms showed lower auxin accumulation during flower development phase (Ma et al., 2020). In continuation, significant downregulation of *ARF4* was observed before and during floral development in phytoplasma-infected jujube plants, indicating role of hormone signalling in onset of phytoplasma disease symptoms.

Some phytoplasma responsive miRNAs have also been associated with nutrient homeostasis, which can influence appearance of the disease symptoms. Increase of miR395 has been observed in phytoplasma infected grapevine and lime trees (Ehya et al., 2013). miR395 targets the *ATP-sulphurylase* (*ATPS*) gene family and a low-affinity sulphate transporter gene, that play important role in regulating sulphate homeostasis (Mattewman et al., 2012). Its relation to sulphur accumulation and allocation has been well documented (Liang et al., 2010). Significant downregulation of miR399, a miRNA known for maintaining phosphate homoeostasis was also reported in phytoplasma infected host plants (Gai et al., 2014; Synman et al., 2017). The target gene of miR399 is *PHO2* (code for an ubiquitin-conjugating E2 enzyme, UBC24) that positively regulate Pi uptake, translocation (Chiou et al., 2006; Hseih et al., 2009). It is suggested that such fluctuations in nutrient might prove favourable for pathogen growth or lead to some physiological imbalance causing symptom production.

Downregulation of miR166 that targets class III HD-Zip protein was associated with defects in meristem and dwarfing in phytoplasma infected mulberry (Gai et al., 2014). In FDp Infected grapevine, the existence of a regulatory system of AGO10-miR166-HD-ZIPIII was proposed as *AGO10* was found to be over expressed in infected plants and suggested to be responsible for reduced post transcriptional activity of miR166. This also supported the up regulation observed for class III HD-Zip genes *Phabulosa* and *Revoluta* genes in

affected grapevine (Chitarra et al., 2018). Interestingly, reduced lignification observed in phytoplasma infected grapevine was associated with up regulation of a novel miRNA (vvi_mi64) which coincided with down regulation of its target, the polyphenol oxidase II transcript (Chitarra et al., 2018).

Excessive branching and reduction in leaf size was associated with over expression of miR157 in infected lime trees (Ehya et al., 2013), as previously observed in case of torenia plants over expressing *Arabidopsis* miR157 (Shikata et al., 2012). Changes in plant architecture in phytoplasma infected paulownia plants was associated with the miR160-miR172-miR397 cluster and their target genes, which function by regulating the cytokinin pathway (Cao et al., 2018).

Yellowing symptoms in phytoplasma affected mulberry plants has shown accumulation of miRNA29b and miR160a-5p which are involved in downregulation of chlorophyll synthase gene and beta amylase gene, respectively (Gai et al., 2014). miRNA and degradome sequencing in phytoplasma infected paulownia plants showed role of the miR156-miR160-miR5368 cluster in leaf yellowing (Cao et al., 2018). miR156 targets PIF3 (a helix-loop-helix transcriptional factor) that interacts with phyA, phyB, phytochrome responsive genes and CHL1 that synthesizes an enzyme for chlorophyll synthesis. Downregulation of *PIF3* and *CHL1* in phytoplasma infected plants indicates reduced chlorophyll production. Downregulation of miR160a and upregulation of its target gene *RAV* was also observed in such plants (Cao et al., 2018). Over expresssion of RAV (a transcriptional factor with AP2/ERF and B3 domain) in transgenic tobacco showed reduction in chlorophyll content and photosynthesis rate (Zhao et al., 2008). Target of miR5368, the *ZEP*gene which is responsible for accumulation of violazanthin and antheraxanthin was shown to be upregulated in phytoplasma infected plants (Cao et al., 2018). Further, decreased abundance of chlorophyll a-b binding protein and photosystem II 10kDa polypeptide was observed in paulownia plants infected with phytoplasma (Mou et al., 2013; Wang et al., 2017). This reduction has been associated with long non-coding RNAs in addition to miRNAs (Fan et al., 2018).

Overall, the host miRNAs modulation by phytoplasma plays an important role in development of disease symptoms ranging from yellowing, leaf deformation, flower abortion, sterility to changes in plant architecture such as dwarfing and meristem defects including loss of apical dominance, excessive branching.

In summary, targeted research on phytoplasma effector proteins such as TENGU, SAP54, SAP11 and host plant microRNAs have thrown light on the intricate mechanisms underlying symptom development in phytoplasma infection. TENGU induced dwarfism and sterility in plants has been linked with alteration in auxin and jasmonic acid biosynthesis. Analysis of structural motifs and interacting partners of a phyllody causing effector, SAP 54 has revealed its role in degradation of transcriptional factors involved in flower development. Role of another effector molecule SAP11 has emerged in axillary shoot proliferation and hairy root symptoms. Differential interaction of SAP11 with transcriptional factors responsible for cell proliferation is known to mediate such morphological changes. In addition, alteration in microRNAs mediated gene regulation has been proposed for appearance of varied symptoms. Further progress with respect to various aspects of molecular studies on phytoplasma effector molecules and phytoplasma responsive miRNAs of host plant pertaining to different phytoplasma groups and their host taxa presented here will provide effective platform for comprehensive understanding of symptom development and their control in case of phytoplasma associated diseases.

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