

Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis

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Phytoplasmas are insect-transmitted phytopathogenic bacteria that can alter plant morphology and the longevity and reproduction rates and behavior of their insect vectors. There are various examples of animal and plant parasites that alter the host phenotype to attract insect vectors, but it is unclear how these parasites accomplish this. We hypothesized that phytoplasmas produce effectors that modulate specific targets in their hosts leading to the changes in plant development and insect performance. Previously, we sequenced and mined the genome of Aster Yellows phytoplasma strain Witches' Broom (AY-WB) and identified 56 candidate effectors. Here, we report that the secreted AY-WB protein 11 (SAP11) effector modulates plant defense responses to the advantage of the AY-WB insect vector *Macrostelus quadrilineatus*. SAP11 binds and destabilizes *Arabidopsis* CINCINNATA (CIN)-related TEOSINTE BRANCHED1, CYCLOIDEA, PROLIFERATING CELL FACTORS 1 and 2 (TCP) transcription factors, which control plant development and promote the expression of lipoxygenase (LOX) genes involved in jasmonate (JA) synthesis. Both the *Arabidopsis* SAP11 lines and AY-WB-infected plants produce less JA on wounding. Furthermore, the AY-WB insect vector produces more offspring on AY-WB-infected plants, SAP11 transgenic lines, and plants impaired in CIN-TCP and JA synthesis. Thus, SAP11-mediated destabilization of CIN-TCPs leads to the down-regulation of LOX2 expression and JA synthesis and an increase in *M. quadrilineatus* progeny. Phytoplasmas are obligate inhabitants of their plant host and insect vectors, in which the latter transmits AY-WB to a diverse range of plant species. This finding demonstrates that pathogen effectors can reach beyond the pathogen–host interface to modulate a third organism in the biological interaction.

Arabidopsis development | insect herbivore | jasmonic acid | symbiosis | Hemiptera

Many (insect) vector-borne pathogens present a continuous hazard to human health and food production. The dispersal of these pathogens in nature largely depends on the frequency of interactions between the pathogens' hosts and vectors, and the fitness of the pathogen is also linked to the reproductive success of its vectors. Accordingly, pathogens may manipulate their primary hosts to attract more insect vectors or to facilitate insect feeding on the host organisms. Indeed, there are spectacular examples of alterations of host phenotypic traits in response to vector-borne pathogen infection. These include malaria parasites, which make humans more attractive to mosquito vectors, particularly at the plasmodium transmissible stage (1); several vector-borne animal pathogens, which alter blood viscosity to facilitate blood ingestion by vectors (2); the fungus *Puccinia monoica*, which induces the formation of pseudoflowers to attract insects that enable the reproduction of the fungus (3); and phytoplasmas and viruses that improve plant host quality for their insect vectors (4, 5) and alter volatile emission of host plants to attract insect vectors (6, 7). It is well documented that pathogens produce effectors that modify specific processes in the host and alter the host–pathogen interface, often to the advantage of the

pathogen (8, 9). For example, the Gram-negative plant pathogen *Pseudomonas syringae* secretes effectors into host cells that suppress the induction of pathogen-associated molecular pattern-triggered immune responses that act as a defense mechanism against the invading pathogen (10, 11), and *Xanthomonas* spp. produce transcription activator-like (TAL) effectors that target plant cell nuclei and activate the expression of host genes to facilitate their growth and possible spread in the host plant (12, 13). However, pathogen effectors that target specific processes in one host to attract insect vectors have not yet been identified.

Phytoplasmas are unculturable bacterial pathogens that belong to the class Mollicutes and are restricted to the cytoplasm of sieve cells of the plant phloem that is responsible for transport of carbohydrates and nutrients to plant sink tissues (reviewed in 14). Phytoplasmas depend on phloem-feeding insect vectors of the order Hemiptera, mostly leafhoppers, planthoppers, and psyllids, for transmission to plants (15). Thus, phytoplasmas are obligate pathogens of plants and insects that depend on both of their hosts for dispersal in nature. Nonetheless, Aster Yellows (AY) phytoplasmas, such as AY phytoplasma strain Witches' Broom (AY-WB), have a wide plant host range affecting more than 80 dicot and monocot plant species worldwide (reviewed in 16), including numerous members of the Brassicaceae (17), such as *Arabidopsis thaliana*. In the United States and Canada, AY phytoplasmas are primarily vectored by *Macrostelus quadrilineatus*, which has a wide plant host range as well (18).

Phytoplasma infection can evoke dramatic changes in plant morphology, such as inducing the growth of multiple little leaves, the production and clustering of multiple stems and branches (witches' brooms), retrograde metamorphosis of floral organs into leafy structures (phyllody), green coloration of nongreen flower tissues (virescence), growth of elongated stalks (bolting), and formation of bunched fibrous secondary roots (19). These morphological changes in the plant may attract insect vectors that preferentially lay eggs in leaves and stems, and some in roots (14). Furthermore, *M. quadrilineatus* leafhoppers survive longer and produce more progeny on AY phytoplasma-infected plants (4), suggesting that the phytoplasmas down-regulate plant defense against the leafhopper. However, the two phytoplasma isolates (“bolt” and “severe”) involved in the study were field

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isolates that were not formally classified, and the mechanisms by which these bacteria impair host defenses are not yet understood.

We wished to elucidate the mechanism by which AY phytoplasma-infected plants positively affect the fecundity of *M. quadrilineatus* and have reasoned for some time that phytoplasmas induce phenotypic changes in plants through the production of effector proteins (14, 20–22). To identify the effectors involved in mediating these alterations in phenotype, we sequenced the AY-WB genome (20) and mined the genome for genes encoding candidate effector proteins based on the presence of an N-terminal signal peptide (22). Because phytoplasmas do not appear to possess type III secretion systems, it is likely that effector proteins are secreted through the Sec-dependent pathway similar to related Gram-positive firmicutes (23) and in line with the presence of a functional SecA-dependent secretion system in phytoplasmas (24, 25). We identified 56 secreted AY-WB proteins (SAPs) that are candidate effector proteins, including SAP11 as described here (22). The well-characterized AY phytoplasma strain AY-WB readily infects the model plant *Arabidopsis*. Infected plants exhibit many symptoms associated with exposure to this phytoplasma, including the production of green leaf-like flowers (phyllody) and witches' brooms (26, 27). In a recent screen, we generated transgenic *Arabidopsis* plants for the majority of AY-WB effector genes leading to the identification of SAP54, which induces leafy flowers resembling the phyllody symptoms of AY-WB-infected *Arabidopsis* (27).

Here, we report that *M. quadrilineatus* produces more offspring when reared on AY-WB-infected *Arabidopsis* and that the AY-WB effector SAP11 is, in part, responsible for this increase in leafhopper performance. The mechanism involves SAP11-mediated destabilization of CINCINNATA (CIN)-TEOSINTE BRANCHED1, CYCLOIDEA, PROLIFERATING CELL FACTORS 1 and 2 (TCP) transcription factors, which are regulated by miR319 (28). The destabilization of CIN-TCPs results in the down-regulation of *LIPOXYGENASE2* (*LOX2*) expression and jasmonate (JA) production, which are involved in the *Arabidopsis* defense response to *M. quadrilineatus*. Thus, SAP11 functions beyond the phytoplasma-plant interface to increase the progeny of the phytoplasma insect vector, which transmits the phytoplasma to other plants.

Results

Phytoplasma Infection of *Arabidopsis* Changes Plant Morphology and Increases Insect Performance. *M. quadrilineatus* readily uses *Arabidopsis* as a feeding and reproductive host and can also inoculate it with AY-WB. Infected plants show severe symptoms, including witches' brooms caused by an increased production of stems (Fig. 1 and *SI Appendix*, Fig. 1A), flowers that become leafy, and various other morphological changes indicating that AY-WB interferes with plant developmental pathways (27). Previously, it was shown that leafhopper fecundity was increased on AY bolt-infected plants but not AY severe-infected plants (4). We determined if AY-WB induces similar phenotypes in *Arabidopsis*. Indeed, *M. quadrilineatus* adults of various age ranges that were allowed access to the experimental *Arabidopsis* plants for a maximum of 4 d produced significantly more progeny on the AY-WB-infected plants compared with noninfected plants (Fig. 2A), with a cumulative increase of 61% in nymph numbers for all leafhopper age ranges combined [analysis of deviance (ANODE); $F_{1,106} = 254.17$, $P < 0.001$]. *M. quadrilineatus* female survival rates did not significantly differ between AY-WB-infected and healthy plants (ANODE; $F_{1,107} = 0.33$, $P = 0.57$; *SI Appendix*, Fig. 1B), indicating that the increased nymph numbers are likely caused by more fecund females. We also counted the number of eggs laid, hatch rate of eggs, and number of feeding/probing sites on *Arabidopsis* leaves. *M. quadrilineatus* laid more eggs on AY-WB-infected plants compared with healthy plants within each adult leafhopper age



Fig. 1. AY-WB infection alters *Arabidopsis* morphology. (A) AY-WB-infected *A. thaliana* Col-0 plants are smaller and bushier (Right) than similar aged healthy plants (Left). (B) Close-up view of AY-WB-infected plant in A illustrating the witches' broom symptoms. (C) Top view of the plants shown in A. Plants were grown in 10-h/14-h light/dark at 22 °C; at 4 wk of age, they were transferred to 16-h/8-h light/dark at 23 °C/20 °C and exposed to two noncarrier (healthy plants) or AY-WB carrier (infected plants) *M. quadrilineatus* for 1 wk. Photographs were taken when plants were 8.5 wk old. (Scale bars = 1 cm.)

range (Fig. 2B) and for all age ranges combined (ANODE; $F_{1,68} = 372.06$, $P < 0.001$), whereas egg-hatching rates did not significantly differ (ANODE; $F_{1,68} = 1.97$, $P = 0.165$; *SI Appendix*, Fig. 1C). The number of leafhopper-feeding/probing sites was slightly but significantly reduced on AY-WB-infected plants (ANODE; $F_{1,22} = 30.22$, $P < 0.001$; *SI Appendix*, Fig. 1D). Thus, an increase in leafhopper egg-laying activity is most likely responsible for the higher leafhopper nymph production.

Because *M. quadrilineatus* can acquire AY-WB from infected plants within the 4-d exposure time, we also determined whether the cause of the nymph production increase was attributable to AY-WB infection of the plant host or leafhopper vector. Leafhoppers continuously exposed to AY-WB-infected plants before transfer to the experimental healthy plants did not show an increase in cumulative nymph production on these healthy plants

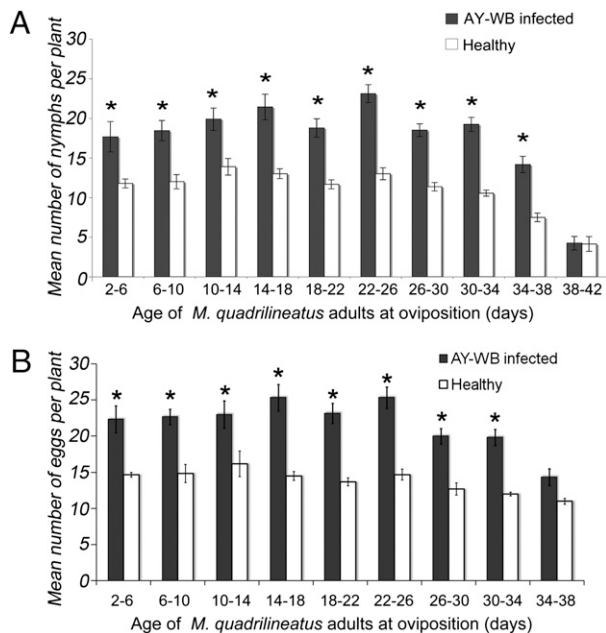


Fig. 2. *M. quadrilineatus* leafhoppers are more fecund on AY-WB-infected than healthy *Arabidopsis* Col-0 plants. Leafhoppers produce more nymphs (A) and lay more eggs (B) on AY-WB-infected plants. AY-WB-infected and healthy plants were obtained by exposing 3-wk-old plants to three AY-WB carrier and noncarrier *M. quadrilineatus* males, respectively, for 1 wk. Two weeks later, each plant received eight adult females and two adult males of different age ranges for 4 d (x axes). Numbers of eggs were counted 1 d after and numbers of nymphs were counted 15 d after removal of the adult insects (y axes). Each experiment included three plants per age range per treatment, and the experiments were repeated three times (A) or two times (B). Columns show the mean numbers of nymphs (A) and eggs (B) per plant of all biological replicates for each leafhopper age range. (Scale bars = SE.) * $P < 0.05$ when two treatments were compared at each adult age (t probabilities calculated within GLM).

for up to 6 d (ANODE; $F_{2,30} = 0.66$, $P = 0.526$; *SI Appendix, Fig. 1E*). Leafhoppers exposed to AY-WB-infected plants for 2 d before transfer to the experimental healthy plants increased nymph production on healthy plants from 10 d on (ANODE; $F_{2,84} = 10.68$, $P < 0.001$; t probability = 0.008; *SI Appendix, Fig. 1E*) at the time leafhoppers start to transmit AY-WB to plants. Together, these data indicate that the increased leafhopper nymph production and oviposition phenotype during the 4-d exposure of healthy insects to AY-WB-infected plants in the experiment shown in Fig. 2 are the result of changes that occur during AY-WB infection of the plant and are unlikely the result of AY-WB presence in the insect vector itself.

In summary, these results demonstrate that AY-WB infection induced various phenotypic alterations in *Arabidopsis* (i.e., it changes plant morphology and increases fecundity of its leafhopper vector *M. quadrilineatus*). Because *Arabidopsis* is a model organism in which plant development and defense responses against various pathogens and insects have been extensively investigated, we decided to use *Arabidopsis* to elucidate the molecular mechanisms by which AY-WB alters the *M. quadrilineatus*-plant interaction.

Expression of AY-WB Phytoplasma Effector SAP11 Changes *Arabidopsis* Morphology. Previously, we identified 56 SAPs, which are candidate effector proteins (22). To reveal the function of each effector candidate, we generated stable transgenic *Arabidopsis* lines that express the effector candidate genes under control of the *Cauliflower mosaic virus* 35S promoter and identified three effector proteins that induce clear morphological changes in *Arabidopsis* (27). Of these, SAP11 was previously shown to target

plant cell nuclei in transient expression assays and was detected beyond the phloem in nuclei of mesophyll and trichome cells in AY-WB-infected plants (22). Hence, we further investigated SAP11 action. *Arabidopsis* Col-0 lines expressing the SAP11 sequence (*SI Appendix, Fig. 2A*) corresponding to the 9-kDa mature protein (without signal peptide) had smaller rosettes (Fig. 3A), severely crinkled leaves (Fig. 3B), a bushy appearance (Fig. 3C), and crinkled siliques (Fig. 3D). The bushy phenotype of the 35S::SAP11 lines is likely derived from an increase in the number

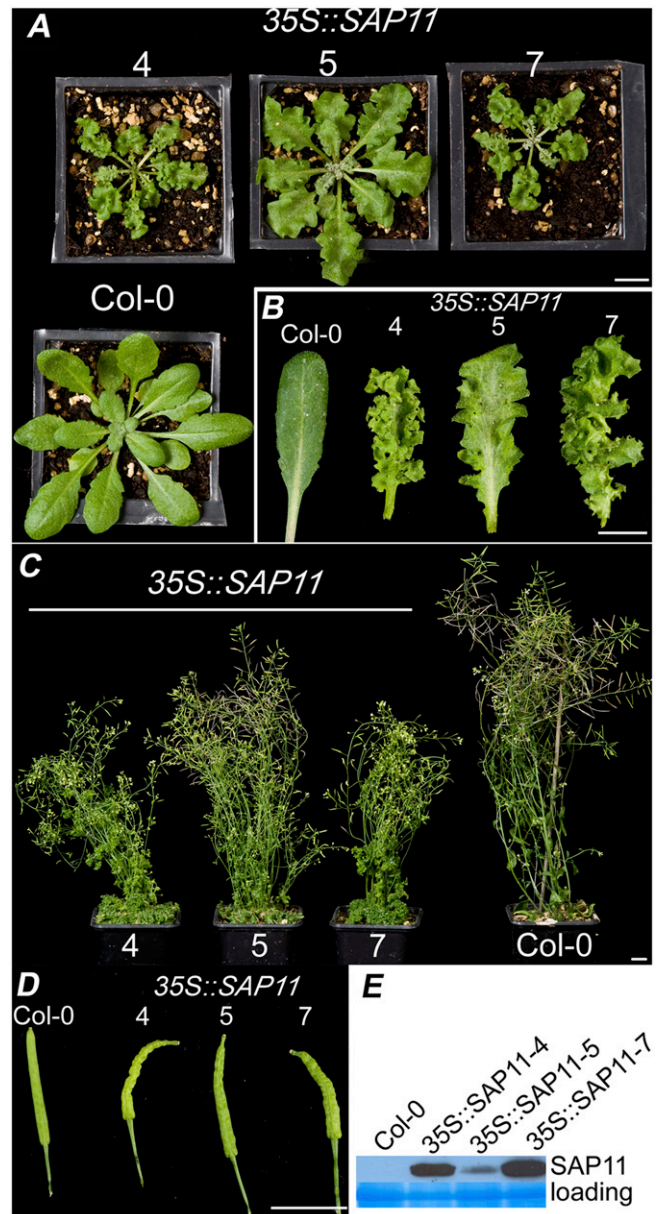


Fig. 3. SAP11 induces crinkled leaves and siliques, and it increases stem number in *Arabidopsis*. (A) Rosettes featuring crinkled leaves of 6-wk-old homozygous 35S::SAP11 lines 4, 5, and 7 grown in short-day (8-h/16-h light/dark) conditions. (B) Representative leaves taken from 10-wk-old plants shown in A. (C) Homozygous 10-wk-old 35S::SAP11 lines 4, 5, and 7 produce more stems (*SI Appendix, Fig. 2B–D*). Plants were grown in long-day (16-h/8-h light/dark) conditions. (D) SAP11 lines have crinkled siliques (taken from plants shown in C). (E) Western blot of protein extracts of 35S::SAP11 line 4, 5, and 7 transgenic plants probed with anti-SAP11. (Scale bars = 1 cm.) Coomassie-stained Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit in a duplicated gel serves as a loading control.

of stems compared with Col-0 (ANOVA; $F_{3,12} = 24.31$, $P < 0.001$; *SI Appendix*, Fig. 2B), because the 35S::SAP11 lines and Col-0 did not significantly differ in the lengths of stems (ANOVA; $F_{3,12} = 0.64$, $P = 0.604$; *SI Appendix*, Fig. 2C) and the number of branches on the main bolt (ANOVA; $F_{3,12} = 0.67$, $P = 0.5884$; *SI Appendix*, Fig. 2D) at the time when most of the siliques had matured. The 35S::SAP11 line 5 produced lower amounts of SAP11 than the two other lines (Fig. 3E), coinciding with the least severe phenotype exhibited by line 5 (Fig. 3A–D).

To reveal how SAP11 induces the morphological alterations in *Arabidopsis*, a yeast two-hybrid screen was conducted at Hybrigenics Services SAS using the company's ULTIMate Y2H service. This identified CIN-related TCP transcription factors 2 and 13 as SAP11 interactors (*SI Appendix*, Fig. 3A and Table 1). TCP transcription factors are conserved among plants (29, 30) and regulate various aspects of plant development (28, 31–33). *Arabidopsis* class I TCP members promote cell proliferation in leaves, whilst a subset of class II members, which are homologs of *Antirrhinum CINCINNATA* (31), negatively regulate leaf growth and positively regulate leaf maturation and senescence (32, 33). The balance between the two classes of TCPs determines leaf and plant morphology (32). We predicted that SAP11 may interact with multiple TCPs, because the 11 yeast two-hybrid screen prey fragments share the basic helix–loop–helix domain that is conserved among TCPs and is involved in TCP dimerization and DNA binding (29) (*SI Appendix*, Fig. 3A).

SAP11 Binds to and Destabilizes *Arabidopsis* TCP Transcription Factors. *pTRBO* is a *Tobacco mosaic virus*-based expression vector that allows for higher levels of *in planta* protein production compared with the 35S constructs (34), enabling the detection of interactions between unstable (low-abundance) proteins, such as TCPs. Therefore, we used the *pTRBO* vectors to conduct coimmunoprecipitation (co-IP) assays of SAP11 with TCP2, TCP13, TCP4, and TCP7. TCP4 is another *Arabidopsis* class II CIN-TCP, whereas TCP7 belongs to class I (29, 32). *Nicotiana benthamiana* leaves were infiltrated with pTRBO::FLAG-SAP11 or pTRBO::FLAG-RFP alongside one of the 35S::3myc-TCP2, TCP13, TCP4, and TCP7 constructs. FLAG-SAP11 coimmunoprecipitated with proteins corresponding to full-length 3× myc-TCP2, TCP4, and TCP7 (*SI Appendix*, Fig. 3B and C). We did not successfully coimmunoprecipitate 3× myc-TCP13 with FLAG-SAP11. This may have been caused by the complete disappearance of TCP13 in the presence of SAP11 (as discussed below). Thus, the co-IP experiments confirmed the yeast two-hybrid screen data that SAP11 interacts with TCP2 and revealed that SAP11 also interacts with another CIN-TCP, TCP4, and class I TCP7.

To investigate how SAP11 may affect the TCPs, we conducted transient coexpression analyses in *N. benthamiana* leaves of GFP-SAP11 and 3× myc-TCPs under control of the 35S promoter. All CIN-TCPs were absent or decreased in abundance in the presence of GFP-SAP11 compared with GFP alone, whereas the

abundance of class I TCP7 was similar between the GFP-SAP11 and GFP treatments (Fig. 4). Each experiment was repeated 9 times for TCP2, TCP4, TCP13, and TCP7 and twice for the other TCPs, and we constantly observed destabilization of CIN-TCPs but not TCP7. Similar results were achieved with the *pTRBO* expression system, in which 3× myc-TCP2 and TCP13 were less abundant in the presence of FLAG-SAP11 compared with FLAG-RFP, whereas 3× myc-TCP7 abundance was not visibly different between FLAG-SAP11 and FLAG-RFP presence (*SI Appendix*, Fig. 3D). The FLAG-SAP11-mediated reductions of 3× myc-TCP4 were more variable in the *pTRBO* experiments, possibly because of the high expression levels of TCPs in these experiments. Nonetheless, in the 12 times this experiment was repeated, 3× myc-TCP4 was less abundant 6 times, completely absent 1 time, and equally abundant 5 times in the FLAG-SAP11 treatment compared with the FLAG-RFP control. Thus, together, the coexpression analyses indicated that SAP11 destabilizes all the class II CIN-TCPs but not class I TCP7.

To obtain genetic evidence for TCP destabilization by SAP11, we made use of existing *miR319a*, *miR-3TCP*, and octuple *cin-tcp* (*miR319a* × *miR-3TCP*) *Arabidopsis* overexpression lines (32), in which *miR319* negatively regulates the expression of five CIN-TCP genes (*TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24*) (28) and the artificial *miR-3TCP* negatively regulates the other three CIN-TCP genes (*TCP5*, *TCP13*, and *TCP17*) (32). It was previously shown that the *miR-3TCP* lines and *Arabidopsis* mutants in individual *tcp* genes, such as *tcp2* and *tcp13*, have less obvious changes in leaf morphology compared with *miR319a* and the octuple *cin-tcp* lines, which exhibit deeply lobed and highly crinkled leaves, suggesting functional redundancy among the class II TCPs (32, 33). The 35S::SAP11, 35S::miR319a, and octuple *cin-tcp* plants had similar morphologies, and the 35S::SAP11-7 line resembled the octuple *cin-tcp* plants more closely than the 35S::SAP11-5 line did (Fig. 5A), consistent with higher SAP11 abundance in 35S::SAP11-7 plants compared with 35S::SAP11-5 plants (Fig. 3E). We also crossed 35S::SAP11-5 (Col-0) with a pBLS::rTCP4GFP (Ler) line, which overexpresses a *miR319a*-resistant *TCP4* (*rTCP4*) fused at the C terminus to GFP under control of the postmaturation phase promoter *BLS* (32). The crinkled leaf phenotypes of 35S::SAP11-5 (Col-0) and 35S::SAP11-5 (Col-0) × Ler (control) plants were visibly reduced in the 35S::SAP11-5 (Col-0) × pBLS::rTCP4GFP (Ler) plants (Fig. 5B–D), indicating that the SAP11-mediated destabilization of TCPs can be suppressed by overexpression of *rTCP4GFP*. Thus, the SAP11 transgenic lines phenocopy the octuple *cin-tcp* lines.

JA Synthesis Is Down-Regulated in SAP11 Transgenic Lines and AY-WB–Infected *Arabidopsis*. CIN-TCPs positively regulate plant senescence, and this is partly achieved through the up-regulation of *LOX2* by TCP4 binding to *LOX2* promoter sites (33). *LOX2* encodes a lipoxygenase that mediates the first step of the JA synthesis pathway by converting α -linolenic acid (α -LeA; 18:3) into 13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT) (35) and is induced by wounding and infestation of non-AY-WB carrier *M. quadrilineatus* (*SI Appendix*, Fig. 4A). We found that the expression levels of *LOX2* were considerably lower in the 35S::SAP11 lines vs. WT Col-0 before wounding (nontreated) and 60 min after wounding (Fig. 6A). The 35S::SAP11 plants also produce less JA 60 min after wounding than WT Col-0 plants (ANOVA; $F_{3,11} = 20.42$, $P < 0.001$; Fig. 6B). A 24-h time course of JA quantification on wounding indicated that 35S::SAP11 line 7 accumulates significantly less JA compared with Col-0 over time (Fig. 6C). Thus, the SAP11 transgenic lines have reduced *LOX2* expression and JA production.

We also assessed JA production in AY-WB–infected plants. JA accumulation was quantified in young developing leaves and fully expanded mature (old) leaves at 1, 2, and 3 wk after re-

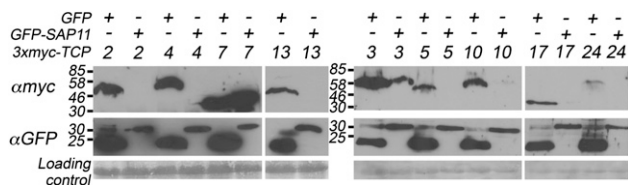


Fig. 4. SAP11 destabilizes CIN-TCP transcription factors. The abundance of the eight class II CIN-TCP members (TCP2, TCP4, TCP13, TCP3, TCP5, TCP10, TCP17, and TCP24) is reduced in the presence of GFP-SAP11 compared with GFP, whereas the abundance of class I TCP7 was not reduced in the presence of GFP-SAP11. Molecular weight markers (kDa) are indicated to the left of the blots.

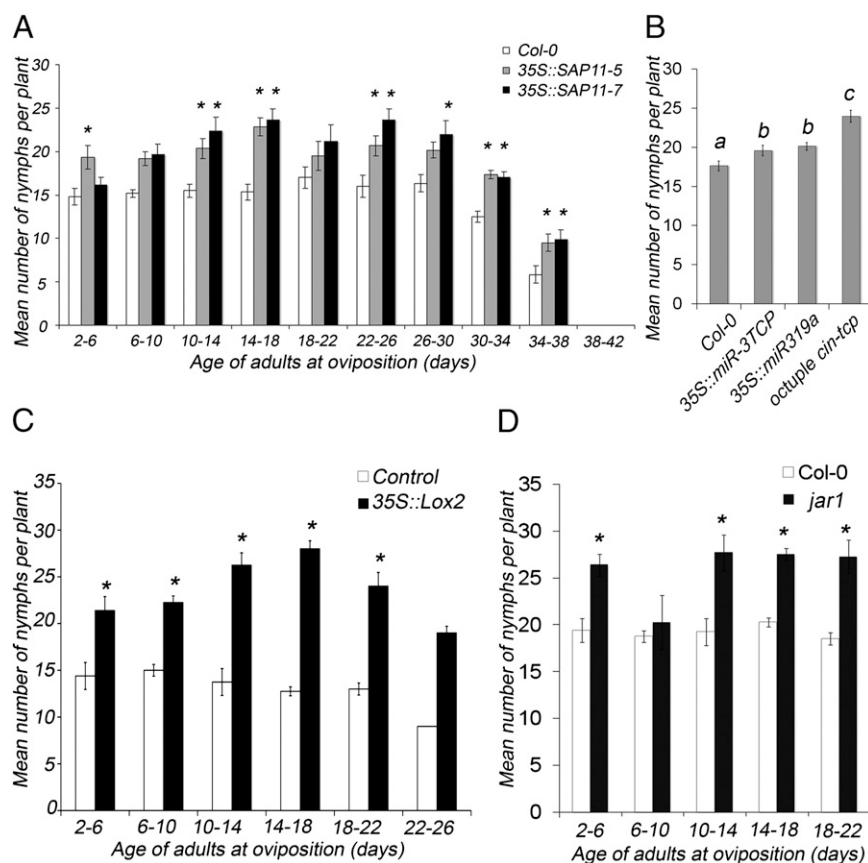


Fig. 7. *M. quadrilineatus* produces more progeny on SAP11 transgenic plants, TCP-silenced plants, and JA-deficient plants. (A–D) Seven-week-old plants were exposed to eight adult females and two adult males of different age ranges for 4 d (x axes). Numbers of nymphs on each plant were counted 15 d after removal of the adult insects. Each experiment included three plants per age range per treatment, and the experiments were repeated two times. (A, C, and D) Columns show the mean numbers of nymphs per plant of all biological replicates for each leafhopper age range. * $P < 0.05$ when lines were compared at each adult age (t probabilities calculated within GLM). (B) Columns show the mean numbers of nymphs per plant produced by adult leafhoppers of four different age ranges (2–6 d, 6–10 d, 10–14 d, and 14–18 d) obtained from two independent experiments. Different letters above the columns (a–c) indicate statistical difference ($P < 0.05$) shown by t probabilities calculated within the GLM. (Scale bars = SE.)

(ANODE; $F_{2,117} = 41.64$, $P < 0.001$) and no significant difference in nymph numbers between the two 35S::SAP11 lines (ANODE; $F_{1,79} = 1.31$, $P = 0.256$). Leafhoppers also laid more eggs on the two 35S::SAP11 lines (ANODE; $F_{2,106} = 76.09$, $P < 0.001$; *SI Appendix, Fig. 5A*). The hatch rates of the eggs were not significantly different (ANODE; $F_{2,107} = 0.89$, $P = 0.414$; *SI Appendix, Fig. 5B*), whereas the numbers of feeding/probing sites were reduced slightly in the two 35S::SAP11 lines compared with Col-0 (ANODE; $F_{2,25} = 14.1$, $P < 0.001$; *SI Appendix, Fig. 5C*). Although the *M. quadrilineatus* adult female survival rates were not significantly different for the majority of leafhopper age ranges (*SI Appendix, Fig. 5D*), the cumulative survival rates for all age ranges combined were significantly different between Col-0 and the SAP11 lines (ANODE; $F_{2,118} = 18.69$, $P < 0.001$). However, the female survival rates did not statistically influence leafhopper fecundity (ANODE; $F_{1,117} = 1.3$, $P = 0.256$) or oviposition (ANODE; $F_{1,106} = 1.11$, $P = 0.295$), indicating that an increase in leafhopper egg-laying activity is most likely responsible for the higher leafhopper oviposition and subsequent nymph production.

M. quadrilineatus also produced more nymphs on the 35S::miR319a, 35S::miR3TCP, and octuple cin-tcp lines (Fig. 7B). The leafhopper fecundity increase was lower on the 35S::miR319a and miR-3TCP plants than on the octuple cin-tcp line. Furthermore, the level of fecundity increase on the octuple cin-tcp line (36%) was comparable to that of the SAP11 lines (34–40%).

This is consistent with our finding that SAP11 destabilizes all eight class II CIN-TCPs.

Leafhopper adults produced 78% more progeny on the LOX2-silenced line (35S::LOX2, overexpression of LOX2 induces silencing of the endogenous LOX2) (38) compared with the control line, in which LOX2 is not silenced (antisense strand of LOX2 is expressed) (38) (ANODE; $F_{1,23} = 188.7$, $P < 0.001$; Fig. 7C) and laid more eggs on the LOX2-silenced line (ANODE; $F_{1,23} = 186.22$, $P < 0.001$; *SI Appendix, Fig. 6A*), whereas the egg-hatching rate increased only slightly (*SI Appendix, Fig. 6B*) on 35S::LOX2 plants (ANODE; $F_{1,24} = 21.91$, $P < 0.001$). There were no significant differences in number of feeding/probing sites (ANODE; $F_{1,14} = 0.87$, $P = 0.366$; *SI Appendix, Fig. 6C*) and the cumulative *M. quadrilineatus* adult female survival rates (ANODE; $F_{1,24} = 0.04$, $P = 0.853$; *SI Appendix, Fig. 6D*) between 35S::LOX2 and the control lines. The quantification of LOX2 expression and JA production of 35S::LOX2 and the control line after wounding confirmed silencing of LOX2 in the 35S::LOX2 line (*SI Appendix, Fig. 6E and F*).

LOX2 mediates the first step of the JA synthesis pathway. Hence, we determined if leafhopper reproduction is also affected on the *Arabidopsis* Col-0 JASMONATE RESISTANT 1 (*jar1*) mutant, because JAR1 acts further downstream of LOX2; it conjugates isoleucine (Ile) to JA producing JA-Ile, which is the active signaling molecule of the JA signaling pathway (39). Nymph numbers were increased at a cumulative 33% for all

adult age ranges combined on *Arabidopsis jar1* mutants compared with Col-0 (ANOVA; $F_{1,21} = 56.76$, $P < 0.001$; Fig. 7D). Although the overall *M. quadrilineatus* adult female survival rate was slightly but significantly increased in *jar1* compared with Col-0 (ANOVA; $F_{1,22} = 16.89$, $P < 0.001$; SI Appendix, Fig. 6G), it did not statistically influence the increase of fecundity observed (ANOVA; $F_{1,21} = 0.67$, $P = 0.422$).

Altogether, our results indicate that the SAP11-mediated destabilization of CIN-TCPs leads to the down-regulation of *LOX2* expression and JA synthesis and an increase in *M. quadrilineatus* progeny.

Discussion

AY-WB was originally isolated from infected lettuce fields in North America, and it induces characteristic symptoms, such as witches' broom, in this host and in China aster (40). Beanland et al. (4) previously demonstrated that the leafhopper *M. quadrilineatus* survives longer and produces more progeny on aster plants infected with the phytoplasma isolate bolt; however, this field isolate was not formally classified within *Candidatus* (*Ca.*) phytoplasma based on 16S rDNA sequence information, nor was a mechanism identified by which the phytoplasmas might positively influence leafhopper fitness. In this work, we report that *Arabidopsis* Col-0 plants infected with the well-characterized strain AY-WB show witches' broom symptoms and support the increased nymph production of *M. quadrilineatus*. In addition, *M. quadrilineatus* laid more eggs on AY-WB-infected plants than on noninfected plants, whereas egg-hatching rates and female survival rates were not affected, indicating that increased oviposition is predominantly responsible for the higher *M. quadrilineatus* progeny yield from infected plants. Numbers of *M. quadrilineatus* feeding/probing sites were reduced on AY-WB-infected plants compared with healthy plants. The leafhoppers may spend relatively more time feeding at the same location or overall spend less time feeding while they are laying eggs. Based on these data, we determined that the AY-WB *Arabidopsis* experimental system is an appropriate platform to enable further investigations into the mechanism(s) by which AY-WB effectors interfere with plant host development and leafhopper resistance.

An increase in leafhopper progeny on AY-WB-infected plants is likely to enhance AY-WB fitness in nature (SI Appendix, Fig. 7). *M. quadrilineatus* nymph production increased by 61% on AY-WB-infected vs. healthy plants (Fig. 2A), and this increased fecundity starts at the time of or shortly after AY-WB transmission to plants (SI Appendix, Fig. 1E). Following oviposition by the female, it takes ~10 d for the nymphs to hatch from the eggs, and this is also the approximate time required for AY-WB to spread systemically throughout the plant host (SI Appendix, Fig. 7). Hence, the newly hatched nymphs have a high chance of acquiring the phytoplasmas immediately on feeding. We have observed that *M. quadrilineatus* can acquire AY-WB during a 2-d acquisition period on infected plants and can transmit AY-WB to healthy plants 10 d later (SI Appendix, Fig. 1E). Thus, systemic AY-WB infection of the leafhoppers is achieved as the insects are either fourth or fifth instar nymphs or adults, all of which are capable of migrating to uninfected plant hosts and transmitting the phytoplasma (SI Appendix, Fig. 7). Hence, a 61% increase in nymph production on AY-WB-infected plants probably leads to a similar increase in AY-WB transmission to other plants if all leafhoppers migrate to uninfected plants (SI Appendix, Fig. 7). The latter is likely, because *M. quadrilineatus* is a highly mobile and opportunistic species; it migrates annually from South America to North America, including Canada (41), and both this leafhopper species and AY phytoplasmas use wide ranges of plant species as feeding and reproductive hosts (18). Based on the above, it is expected that phytoplasmas that can enhance leafhopper oviposition and nymph production rates will have a competitive advantage over those phytoplasmas that cannot.

Beanland et al. (4) found that isolate bolt increased leafhopper reproduction, whereas isolate severe did not. Hence, bolt would be expected to have a competitive advantage over severe. However, lettuce plants in the field can carry multiple AY phytoplasma strains (40); in this case, severe and probably other phytoplasmas would benefit from the increase in leafhopper reproduction rates induced by bolt.

We found that nymph production rates increased by 30–40% on 35S::SAP11 transgenic *Arabidopsis* plants. Because nymph production is increased by 61% on AY-WB-infected plants, it is likely that other AY-WB effectors in addition to SAP11 modulate leafhopper-plant interactions. SAP11 lies within a potential mobile unit (PMU)-like region in the AY-WB chromosome (22), consistent with the observation that virulence factors are often encoded on pathogenicity islands in the genomes of plant pathogens (42). Similarly, the SAP11 PMU-like region is predicted to encode additional effector proteins, such as SAP09, SAP56, SAP66, SAP67, and SAP68 (22), which may also affect plant-insect interactions.

The observation that the phytoplasma effector SAP11 targets JA synthesis via interaction with a plant transcription factor may be similar to the description of a viral protein produced by *Tomato yellow leaf curl China virus* (TYLCCNV). This geminivirus produces a protein (β C1) that acts as a molecular mimic of the plant regulatory protein AYSYMMETRIC LEAVES 2 (AS2) to suppress a subset of JA-responsive genes (43). In this manner, β C1 (and thus TYLCCNV) suppresses JA signaling, which is proposed to make infected plants into better hosts for the TYLCCNV whitefly vector, a phloem-feeding insect vector that acquires the plant virus while feeding on an infected plant (43). However, this has not been experimentally proven. Although the outcome would be similar in both cases (i.e., improvement of insect vector fitness), the mechanisms are different, because SAP11-mediated destabilization of CIN-TCPs causes a reduction in JA synthesis, whereas the β C1 complex formation with AS1 leads to a decrease in JA response. The 2b protein of the aphid-transmitted *Cucumber mosaic virus* is also reported to inhibit the induction of JA-responsive genes; however, the mechanism of the inhibition has not been revealed (44).

We found that AY-WB SAP11 interacts with a class I and a subset of class II TCP transcription factors and destabilizes all the class II CIN-TCPs, whereas at least one class I TCP (TCP7) appears to remain intact. This is consistent with the crinkly leaf phenotype of 35S::SAP11 transgenic lines. Class II CIN-TCPs positively regulate cell maturation, whereas class I TCPs regulate cell proliferation (32, 45). The two classes of TCPs are proposed to compete to occupy overlapping promoter sequences of downstream genes and to coordinately or competitively regulate the expression of genes (45). As seen in the 35S::miR319a and octuple *cin-tcp* plants, down-regulation of class II TCPs induces overproduction of immature plant cells and produces large crinkly leaves (32). Interestingly, however, 35S::miR319a and octuple *cin-tcp* plants did not produce an increased number of stems, indicating that the bushy appearance of 35S::SAP11 lines may not be caused by the destabilization of CIN-TCPs. Whereas SAP11 does not appear to destabilize the class I TCPs (based on the unchanged abundance of TCP7 in the presence of SAP11), it is possible that SAP11 affects three other class II CYC/TB1-like TCPs (TCP1, TCP12, and TCP18). TCP12 and TCP18 arrest the development of axillary buds (46), and SAP11-mediated degradation of these TCPs would explain the increase in stem numbers observed in SAP11 plants. That SAP11 interacts with TCPs is consistent with our previous observations that SAP11 targets nuclei in AY-WB-infected plants (22), because SAP11 is likely to encounter the transcription factors in plant cell nuclei. Furthermore, the similar level of insect fecundity increase on 35S::SAP11 and the octuple *cin-tcp* lines supports that SAP11 contributes to an increase in insect fecundity through the de-

stabilization of CIN-TCPs. The spatiotemporal destabilization of CIN-TCPs in the active AY-WB infection process and the mechanism by which SAP11 destabilizes class II but not class I TCPs remain to be investigated. SAP11 does not have any homology with already characterized proteins, such as proteases; therefore, we postulate that SAP11-mediated destabilization of TCPs is not attributable to direct protease activity of SAP11; rather, it requires host protein degradation systems.

LOX2 expression and JA synthesis are down-regulated in 35S::*SAP11* lines (vs. Col-0) following wounding. This is in agreement with the SAP11-mediated destabilization of the class II CIN-TCP transcription factor TCP4, which positively regulates plant senescence by up-regulating *LOX2* expression directly through binding to the *LOX2* promoter (33). *LOX2* is involved in the synthesis of JA (35, 38) because it encodes an enzyme that converts α -LeA into 13-HPOT, a substrate in the first step of the octadecanoid pathway for JA synthesis. The octadecanoid pathway plays an important role in the plant defense response to insect herbivores (47, 48) and is strongly up-regulated on wounding and insect feeding in *Arabidopsis* (49). *LOX2* expression is also up-regulated in response to *M. quadrilineatus* exposure, and the leafhopper has higher oviposition and nymph production rates on 35S::*SAP11*- and *LOX2*-silenced *Arabidopsis* lines, indicating that the SAP11-mediated down-regulation of *LOX2* is beneficial to the AY-WB insect vector.

Substrates generated by *LOX2* and other 13-HPOTs also feed into the hydroperoxide lyase pathway that produces green leaf volatiles (GLVs) in *Arabidopsis* (50), raising the possibility that the enhanced leafhopper fecundity may be related to the impaired synthesis of GLVs. However, the observation that *M. quadrilineatus* fecundity is also increased on the *Arabidopsis jar1* mutant indicates that disruption of JA synthesis is most likely responsible for the increase in *M. quadrilineatus* fecundity observed in SAP11 transgenic lines. JAR1 converts JA into JA-Ile, which is the biologically active molecule of the JA signaling pathway and acts much further downstream of *LOX2* in this pathway (39). The *M. quadrilineatus* egg hatch rate increased on 35S::*LOX2* plants, and overall female survival rate increased slightly on *jar1* mutants. Thus, the JA pathway has an impact on several aspects of *M. quadrilineatus* fitness, and as a consequence, we might observe clear increases in insect fecundity on JA pathway-deficient mutants.

Together, our data indicate that SAP11 interferes with the plant defense response against the insect vector through the destabilization of CIN-TCPs, thereby enhancing the reproductive success of the insect vector on which AY-WB is dependent for transmission and long-distance dispersal (model in *SI Appendix*, Fig. 7). At this time, it is not possible to generate AY-WB mutants that lack *SAP11*, because phytoplasmas cannot be cultured and transformed. Therefore, we cannot measure the SAP11 contribution to AY-WB-mediated increase in insect fecundity directly. Furthermore, we do not yet understand how SAP11 expression is regulated temporally or spatially during AY-WB colonization of the plant and if one or more of the remaining effectors (56 predicted so far) affect SAP11 expression or function. Nonetheless, we observed that on wounding, young leaves accumulate less JA in AY-WB-infected than noninfected *Arabidopsis* plants. These data are consistent with the detection of SAP11 in young leaves of AY-WB-infected plants (22) and observations that phytoplasmas and their effectors accumulate predominantly in sink tissues (26, 51). Taken together, we propose that SAP11 promotes AY-WB fitness by increasing insect progeny numbers (*SI Appendix*, Fig. 7).

It is interesting that SAP11 targets members of a transcription family that is highly conserved among plant species, an observation that is consistent with the wide host range of AY-WB. The observation that SAP11 targets class II CIN-TCPs for destabilization (and not class I regulators) is also intriguing, because CIN-TCPs

are important growth regulators that promote the differentiation of cells and maturation of leaves. The down-regulation of CIN-TCPs (as observed in the octuple *cin-tcp* plants) elicits the production of unusually large leaves that mature slowly and comprise a large population of small and immature cells (32). Thus, another consequence of SAP11 selectively destabilizing CIN-TCPs might be a delay in the maturation of developing vegetative tissues in infected plants. Because phytoplasmas are biotrophs, it is an advantage for the pathogen to reduce plant stress and delay death. Indeed, phytoplasmas are obligate inhabitants of the plant hosts and insect vectors; hence, too rapid a decline of their host would seriously impinge on phytoplasma survival and decrease their opportunity to be acquired by insect vectors that require relatively healthy plants for feeding and reproduction. Nevertheless, whereas the SAP11-CIN-TCP interaction might influence both developmental and defense pathways in infected plants, it is likely that the primary role of SAP11 comprises the down-regulation of plant defense mechanisms against insects, which involves the JA-mediated pathway, and possibly other unknown pathways regulated by CIN-TCPs, and that any developmental perturbations (e.g., witches' broom) arise as an indirect (albeit beneficial) consequence of destabilizing TCPs.

Whereas the function of some animal and plant pathogen effectors has been elucidated, effectors that reach beyond the pathogen–host interface to affect a third organism in the biological interaction have not yet been identified and functionally characterized. Our results show that *SAP11* has a phenotype beyond the organism in which it resides, affecting not only the AY-WB-infected plant host but the reproductive success of the leafhopper vector that transmits AY-WB to a diverse range of plant species and throughout North America (18, 41). Hence, *SAP11* is a vivid example of the extended phenotype of the gene, a concept put forward in Richard Dawkins' classic book (52).

Materials and Methods

AY-WB Maintenance and Insect Rearing and Fecundity Experiments. All insect rearing and maintenance of AY-WB-infected plant materials occurred in insect rearing cages in a growth room set at a 16-h/8-h light/dark period at 26 °C/20 °C. To obtain AY-WB-infected leafhoppers, ~50 female and male *M. quadrilineatus* leafhoppers were transferred to AY-WB-infected China aster (*Callistephus chinensis* Nees) for 2 wk to allow oviposition. Nymphs were kept on the infected plants until and during adulthood. The adults were used for experiments or were transferred to young China aster or *Arabidopsis* seedlings to generate new batches of AY-WB-infected plants and leafhoppers.

Insect fecundity experiments were conducted as explained in the figure legends at a 10-h/14-h light/dark period at 22 °C. All the *M. quadrilineatus* leafhoppers used for fecundity assay were anesthetized with CO₂ gas for sexing.

Statistical Analyses. All statistical analysis was completed in Genstat v.13 (International Ltd.). For all data points of insect fecundity and oviposition assay, we recorded the biological replicate, adult age, and plant treatment or line as explanatory variables and female survival as response variables. ANODE was used to determine differences in female survival rates, egg hatch rate, fecundity, oviposition, and number of feeding sites. All explanatory variables were described as factors and modeled to assess their contribution to the deviance in leafhopper survival, egg hatch rate, fecundity, oviposition rates, and number of feeding sites. Female survival data and egg hatch data were analyzed using a binomial distribution within a generalized linear model (GLM). Fecundity, oviposition, and feeding data were analyzed using a Poisson distribution within a GLM. When fecundity and oviposition data were analyzed, female survival in each experiment was added as a covariate. Means of nymph or egg numbers or female survival at each insect age were compared using *t* probabilities calculated within the GLM. For all the other experiments, datasets were first examined for normal distributions in Genstat and then compared using ANOVA or the Student *t* test (one-tailed).

Generation and Analyses of 35S::*SAP11 Arabidopsis* Lines. A codon-optimized version of the AY-WB SAP11 sequence (GenBank accession no. gi: 85057280) without the sequence corresponding to the signal peptide and attB1 and

attB2 adapters at the ends (*SI Appendix, Fig. 2A*) was synthesized (GenScript). This SAP11 fragment was amplified with attB1 and attB2 adapter primers (*SI Appendix, Table 2*) and was cloned into pDONOR207 (Invitrogen) using Clonase LR (Invitrogen). The SAP11 fragment in pDONOR207 was then Clonase LR-cloned into the Gateway-adapted vector pB7WG2 that has a 35S promoter fragment cloned at the 5' end of the clonase compatible insertion site (53). The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. *Arabidopsis* Col-0 was transformed by floral dip as described (54).

Detection of SAP11 in Transgenic Plants. Antibodies to SAP11 were raised against a partial peptide sequence of SAP11 (CEGSSSKQPDDSKK) in rabbits (GenScript). Four seedlings of 10-d-old *Arabidopsis* seedlings were ground in 200 μ L of extraction buffer [50 mM sodium phosphate (pH 7.0), 10 mM Triton X-100, 10 mM *N*-lauroylsarcosine, 1 mM 2-mercaptoethanol]. Twelve microliters of the extracts was mixed with 4 \times NuPAGE LDS sample buffer (Invitrogen), separated on 15% (wt/vol) SDS polyacrylamide gels (PAGE), and transferred to 0.45 μ m of Protran BA85 nitrocellulose membranes (Whatman) following standard procedures (55). The Western blots were incubated with α SAP11 and peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich), and detection of bound antibodies was conducted with Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Yeast Two-Hybrid Analysis. Yeast two-hybrid screening was performed by Hybrigenics Services SAS. The codon-optimized sequence of AY-WB SAP11 without signal peptide (*SI Appendix, Fig. 2A*) was PCR-amplified and cloned into pB27 as a C-terminal fusion to LexA (*N*-LexA-SAP11-C) and into pB66 as a C-terminal fusion to Gal4 DNA-binding domain (*N*-Gal4-SAP11-C). The constructs were used as bait to screen a random-primed *A. thaliana* seedlings cDNA library constructed into pP6. For the LexA bait construct, 130 million clones (13-fold the complexity of the library) were screened, using a mating approach with Y187 (mat α) and L40DGal4 (mat a) yeast strains as previously described (56). For the Gal4 construct, 74 million clones (7-fold the complexity of the library) were screened using the same mating approach with Y187 and CG1945 (mat a) yeast strains.

35S::miR319a, 35S::miR3TCP, BLS::rTCP4GFP, 35S::LOX2, and 35S::LOX2 Antisense Lines. Seed for the 35S::miR319a (Col-0), 35S::miR3TCP (Col-0), 35S::miR319a \times 35S::miR3TCP (Col-0), and pBLS::rTCP4GFP (Ler) lines was kindly provided by Idan Efroni (Weizmann Institute of Science, Rehovot, Israel), and the phenotypes of the transgenic lines were as described previously (32). The BLS promoter was selected for expression of rTCP4GFP, because 35S::rTCP4 plants show severe developmental phenotypes. Activity of the BLS promoter is initiated and maintained solely in young lamina (P3–P7 stages) (57). The 35S::LOX2 (NASC ID code N3748) and corresponding control line 35S::LOX2 antisense (NASC ID code N3749) were requested from the European *Arabidopsis* Stock Centre and were described previously (38).

Coexpression and Pull-Down Assays. The coexpression and pull-down assays were conducted in *N. benthamiana* leaves. The *A. tumefaciens* clones carrying the appropriate constructs were infiltrated into individual *N. benthamiana* leaves using a needleless syringe. After 3 d, 2 leaf disks 11 mm in diameter were collected (1 leaf disk per leaf). The two leaf disks were ground in liquid nitrogen in the presence of 50 μ L of 1 \times NuPage Sample Buffer (Invitrogen) and boiled for 5 min. Proteins were separated on 12.5% (wt/vol) SDS/PAGE gels. Remaining leaf samples were used for pull-down assays. The leaf was cross-linked with 30 mL of 10 mM Tris(hydroxymethyl) aminomethane (Tris)-HCl (pH 8.0), 400 mM sucrose, 3% (wt/vol) formaldehyde, 0.1 mM PMSF, and 5 mM β -mercaptoethanol using vacuum infiltration for 10 min. The reaction was then quenched with 2.5 mL 2-M glycine and vacuum-infiltrated for a further 5 min. Samples were ground into a fine powder in the presence of 2 mL of GTEN buffer [10% (vol/vol) glycerol, 25 mM

Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl], and centrifuged at 3,000 \times *g* for 10 min. The supernatant was transferred to a fresh tube and centrifuged at 14,000 \times *g* for 10 min. The FLAG-tagged proteins were extracted by combining 250 μ L of supernatant with 1.7 mL of GTEN buffer plus 0.1% Tween 20 (IP buffer) and 20 μ L of anti-FLAG resin (Sigma-Aldrich), which was prewashed with IP buffer. The bound proteins were eluted in 40 μ L of IP buffer containing 150 ng μ L⁻¹ 3 \times FLAG peptide (Sigma-Aldrich) for 30 min. On average, 15 μ L of the eluates was mixed with NuPAGE LDS sample buffer (Invitrogen), boiled for at least 30 min, and then separated on 12.5% (wt/vol) SDS/PAGE gels and analyzed on Western blots as described above.

Quantitative RT-PCR Experiments. For wounding experiments, mature leaves of 5-wk-old *Arabidopsis* were harvested carefully or wounded twice by forceps. Samples were harvested 60 min after wounding. For leafhopper infestation assays, mature leaves of 5-wk-old *Arabidopsis* Col-0 plants were singly equipped with a clip cage, each containing 10 *M. quadrilineatus* adults. The leafhoppers were anesthetized by CO₂ to facilitate handling. As nontreated controls, *Arabidopsis* leaves were clip-caged without *M. quadrilineatus*.

Arabidopsis leaves were snap-frozen and used for RNA extraction with TRI reagent (Sigma-Aldrich) and purified using Qiagen RNeasy columns (Qiagen Ltd.). cDNA was synthesized from 0.5 μ g of total RNA using M-MLV reverse transcriptase (Invitrogen), and subjected to real-time PCR using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in a DNA Engine Opticon 2 (BioRad, Life Sciences) using gene-specific primers (*SI Appendix, Table 2*).

Extraction and Quantification of Jasmonic Acid. *Arabidopsis* leaves were harvested carefully or wounded twice by forceps, and two to three leaves per treatment were collected at indicated time points and snap-frozen in liquid nitrogen. To generate AY-WB-infected plants, six 4-wk-old plants were placed in a perforated bag with 20 AY-WB carrier or noncarrier *M. quadrilineatus* males. The insects were removed 1 wk later, and the plants were wounded at specific time points as described. The samples were lyophilized and ground to powder with liquid nitrogen, and then extracted at 4 $^{\circ}$ C overnight with 5 mL of methanol containing 100 ng of deuterated JA (d₅-JA, C/D/N isotopes) as an internal standard. The total extracts were centrifuged in a swing-bucket rotor (Eppendorf 5810R; 4,000 rpm, 173-mm rotor radius, 5 min), and the supernatants were dried under N₂ gas. The pellets were dissolved in diethyl ether/methanol (60:9) and loaded onto a Strata NH2 solid phase extraction column (Phenomenex). After two washes with 0.8 mL of chloroform/isopropanol (2:1), JA was eluted twice by 1 mL of diethyl ether containing 4% (vol/vol) acetic acid. The combined eluates were dried with N₂ gas, and the pellets dissolved in methanol/water (1:4). Samples (10 μ L) were analyzed using an Agilent 1100 single-quadrupole liquid chromatography-MS system.

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