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Viral effector protein manipulates host hormone signaling to attract insect vectors

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Some plant and animal pathogens can manipulate their hosts to cause them to release odors that are attractive to the pathogens' arthropod vectors. However, the molecular mechanism underlying this process is largely unexplored, and the specific effectors the pathogens employ as well as the pathways within the hosts they target are currently unknown. Here we reveal that the aphid-borne cucumber mosaic virus (CMV) employs its 2b protein, a well-characterized viral suppressor of host RNA interference (VSR), to target the host's jasmonate (JA) hormone pathway, thus acting as a viral inducer of host attractiveness to insect vectors (VIA). 2b inhibits JA signaling by directly interacting with and repressing JA-induced degradation of host jasmonate ZIM-domain proteins, instead of using its VSR activity. Our findings identify a previously defined VSR protein as a VIA and uncover a molecular mechanism CMV uses to manipulate host's attractiveness to insect vectors by targeting host hormone signaling.

Keywords: cucumber mosaic virus; jasmonate; VSR; VIA; 2b

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Introduction

Many important diseases in plants and animals are caused by pathogens that are naturally transmitted by arthropod vectors such as mosquitoes and aphids. The emergence and success of these pathogens are shaped by their molecular interactions with both the host and the vector [1-3]. Intriguingly, infection with diverse arthropod-borne pathogens in plants and mammals induces changes in the host that make the host more attractive to the disease vector [4-6]. Recent studies have shown that pathogen-induced vector attraction can be odor-depen-

dent [7-10], suggesting, presumably, the presence of a specific mechanism by which pathogens manipulate the host's ability to emit odors that could attract disease vector.

Cucumber mosaic virus (CMV) is probably one of the most successful plant pathogens as it can infect more than 1 200 plant species including the model plant *Arabidopsis thaliana* [11]. CMV is transmitted by aphids in a non-persistent manner [11], and infection of CMV can induce odor-dependent attractiveness to aphids in squash plants [9]. It is well known that the primary defense against CMV and other viruses in *A. thaliana* is directed by antiviral RNA interference (RNAi) guided by the virus-derived small interfering RNAs (siRNAs) [12]. As a common counter-defense strategy of plant and animal viruses, CMV encodes a viral suppressor of host RNAi (VSR), the 2b protein, which is essential for infection [12]. The 2b protein exerts its VSR activity predominantly by inhibiting the production of viral siRNAs as a double-stranded RNA (dsRNA)-binding protein, though it

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also inhibits the RNA-induced silencing complex activity by direct interaction with Argonaute proteins [13–16].

Many defense responses to insect pests in *A. thaliana* are regulated by the phytohormone jasmonate (JA) [17–19]. Upon JA perception, the F-box protein COI1 recruits jasmonate ZIM-domain proteins (JAZs), the key repressors of JA signaling, for ubiquitination and degradation by the 26S proteasome pathway [20–26]. The JAZ family of proteins is widely distributed among higher plants and includes 12 members in *A. thaliana* [23–25]. JAZs normally bind and repress transcription factors (such as MYC2, MYC3, MYC4, MYB21 and MYB24), which are released upon JA-induced degradation of JAZs to activate JA-responsive genes essential for host defense and development [27–29]. Some JA responsive genes mediate production of various plant secondary metabolites including plant volatiles to modulate plant-insect interactions [18, 30–32]. For example, MYC2 can directly activate the expression of both *TPS10*, which promotes the biosynthesis of plant terpenes that reduce plant attractiveness to whiteflies [33], and *CYP81D11*, which encodes a putative p450 cytochrome whose overexpression in transgenic plants decreases the attractiveness to aphids [34].

In addition to suppressing host's antiviral immunity for a successful infection, arthropod-borne viruses also induce host attractiveness to insect vectors for efficient viral transmission. However, both the viral effector that mediates the induction of host attractiveness and its target have not been defined. In this study, we show that protein 2b from aphid-borne virus CMV serves as a viral inducer of host attractiveness to insect vectors (VIA), and enhances *Arabidopsis*'s attractiveness to CMV's aphid vectors. Mechanistically, 2b suppresses *Arabidopsis*'s JA signaling pathway by directly interacting with JAZ proteins and preventing the degradation of these key repressors of JA signaling by 26S proteasome.

Results

VSR 2b controls the viral induction of host attractiveness to aphid vectors

We performed two feeding preference assays to determine if the CMV infection induces odor-dependent attraction to aphid vectors in *Arabidopsis*. In the circular-dish bioassay, leaves from wild-type *Arabidopsis* (Col-0) either systemically infected with a subgroup I strain of CMV (SD-CMV) or mock inoculated were arranged next to each other in a circle. Apterous aphids (*Myzus persicae*) were set free from the center of the dish and their crawling traces were recorded with a digital camera (Supplementary information, Figure S1A and

S1B). We found that significantly higher percentages of aphids approached the CMV-infected leaves compared with leaves from the mock-inoculated plants (Figure 1A), indicating that CMV infection increases the attractiveness of *Arabidopsis* to aphid vectors.

In the Y-tube olfactometer bioassay, two airstreams carrying volatiles emitted from soil-grown *Arabidopsis* plants either systemically infected with SD-CMV or mock inoculated flowed through the two arms of the Y-tube. Individual apterous aphids were released from the base of Y-tube stem and allowed to make the choice to crawl toward either arm of the Y-tube while the plants were invisible to the aphids (Supplementary information, Figure S1C). CMV-infected plants also attracted significantly more aphids than mock-inoculated plants (Figure 1B) in this assay. Thus CMV infection in *Arabidopsis* can induce odor-dependent attraction to aphid vectors.

Previous studies showed that 2b suppresses host antiviral RNAi with its VSR activity to ensure CMV infection [12]. To examine whether 2b has a role in viral induction of odor-dependent host attractiveness to aphid vectors, we compared feeding preferences of aphids between wild-type *Arabidopsis* plants and transgenic plants stably expressing 2b. Similar to CMV-infected plants, transgenic plants expressing 2b were significantly more attractive to aphids than wild-type plants (Figure 1B and 1C; Supplementary information, Figure S2). These data demonstrate that expression of 2b alone in *Arabidopsis* is sufficient to induce odor-dependent aphid attraction.

To verify if 2b protein is required to control viral induction of host attractiveness to aphid vectors, we examined aphid attraction induced by Fny-CMV (another subgroup I strain of CMV) and the 2b deletion mutant, Fny-CMVΔ2b [35]. As shown in Figure 1D, Fny-CMVΔ2b was able to successfully infect *Arabidopsis* double mutant *dcl2/dcl4* (*dcl2/4*), which is deficient in host antiviral RNAi and, therefore, is susceptible to mutant CMV virus without VSR activity [15, 36, 37], causing similar symptoms as Fny-CMV (Figure 1D). Consistent with what was observed in SD-CMV (Figure 1A and 1B), Fny-CMV infection significantly increased aphid attraction in *dcl2/4* plants compared with mock-inoculated *dcl2/4* plants in the Y-tube bioassay (Figure 1E). However, no significant difference in aphid attraction was observed between Fny-CMVΔ2b-infected and mock-inoculated *dcl2/4* plants (Figure 1E). Both mutant virus Fny-CMVΔ2b and wild-type virus Fny-CMV caused similar viral disease symptom in *dcl2/4* plants (Figure 1D); however, enhanced aphid attraction was detected in the *dcl2/4* plants only after infection with Fny-CMV, but not with Fny-CMVΔ2b (Figure 1E), suggesting that the 2b protein, but not other viral components, is responsible

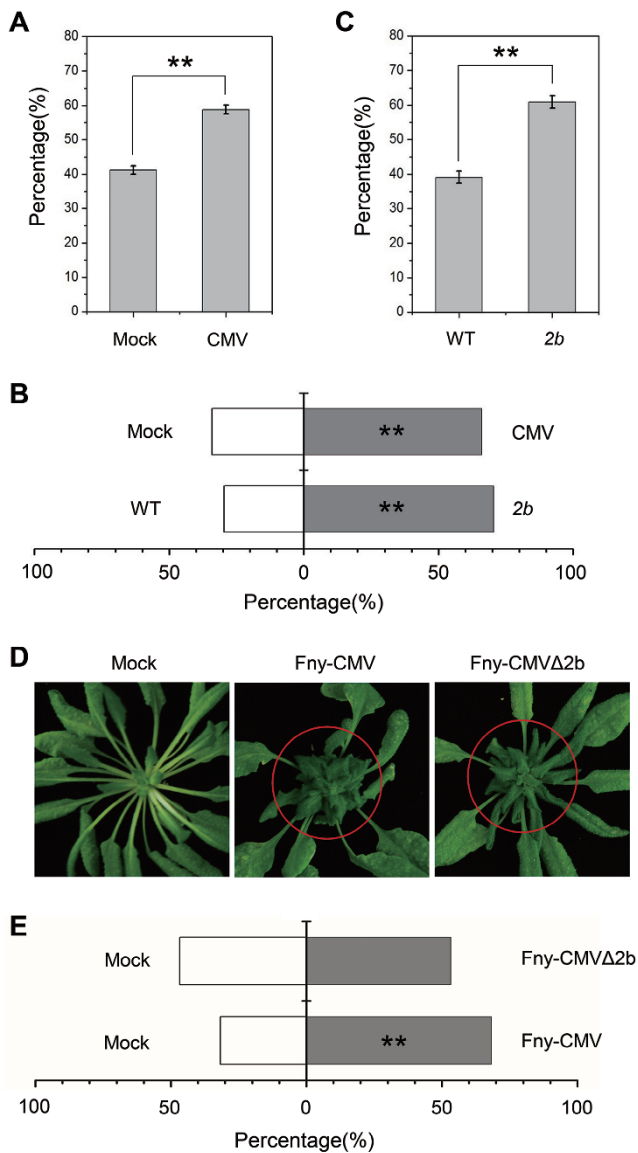


Figure 1 VSR 2b controls viral induction of host attractiveness to aphid vectors. **(A)** Leaves from CMV-infected plants exhibited higher attractiveness to aphids in circular-dish bioassay. Four-week-old soil-cultivated WT *Arabidopsis* plants (Col-0) were infected with SD-CMV (CMV) or mock inoculated with buffer (Mock). Upper non-inoculated leaves were used for aphid bioassay 14 days after inoculation. Data are mean percentages \pm SEM, $n = 5$. $**P < 0.01$, paired samples Student's t -test. **(B)** Plants infected by CMV or transgenic for 2b exhibited higher attractiveness to aphids in Y-tube olfactometer bioassay. Six-week-old soil-cultivated *Arabidopsis* plants transgenic for 2b (2b) and WT were used in the assay. The number of aphids that made a choice is 65 in the comparison of CMV and Mock, and 57 in the comparison of *Arabidopsis* plants transgenic for 2b (2b) and the wildtype (WT). Data are shown as percentages. $**P < 0.01$, χ^2 -test. **(C)** Plants transgenic for 2b exhibited higher attractiveness to aphids in circular-dish bioassay. Leaves of WT *Arabidopsis* plants or *Arabidopsis* plants transgenic for 2b (2b) grown in MS media for 3 weeks were used. Data shown are mean percentages \pm SEM, $n = 5$. $**P < 0.01$, paired samples Student's t -test. **(D)** Fny-CMVΔ2b caused similar symptom as Fny-CMV in *dcl2/4* plants. Four-week-old *dcl2/4* seedlings were inoculated with Fny-CMV, Fny-CMVΔ2b or buffer (Mock). Photos were taken 14 days post inoculation. Plant tissues that exhibited obvious disease symptoms were indicated within red circles. **(E)** Fny-CMV but not Fny-CMVΔ2b induced aphid attraction in *dcl2/4* plants. The number of aphids that made a choice is 60 in both the comparison of Fny-CMV and Mock, or Fny-CMVΔ2b and Mock. Data are shown as percentages. $**P < 0.01$, χ^2 -test.

for viral induction of host attractiveness to aphids.

Taken together, these data demonstrate that 2b is the viral effector responsible for CMV-induced odor-dependent aphid attraction, and suggest that 2b serves as a VIA in addition to its well-defined role as a VSR.

2b physically interacts with host JAZ proteins in the JA pathway

2b was previously found to alter the normal expression of many *Arabidopsis* genes regulated by abscisic acid [38], salicylic acid [39] or JA [40]. In particular, VSR 2b was found to inhibit normal responses of over 90% of *Arabidopsis* genes regulated by JA [40]. We thus determined whether 2b interacts with *Arabidopsis* proteins required in the early steps of JA signaling. Our

in vitro pull-down results showed that 2b specifically interacted with JAZ1, but not COI1 or MYC2 (Figure 2A and Supplementary information, Figure S3). Moreover, JAZ1 co-immunoprecipitated with 2b in the extract of *Arabidopsis* plants expressing both *flag-JAZ1* and *myc-2b* transgenes (Figure 2B). Notably, 2b interacted also with *Arabidopsis* JAZ3, JAZ6 and JAZ10 (Figure 2C), suggesting that the viral effector protein 2b can interact with multiple JAZ family proteins.

We used two independent approaches to determine if interaction of 2b with JAZ1 occurs *in vivo*. Luciferase (LUC) complementation imaging (LCI) assay showed that co-expression of JAZ1 fused with N-terminal half of LUC and 2b fused with C-terminal half of LUC in *Nicotiana benthamiana* leaves produced intense luminescence (Figure 2D). Similarly, yellow fluorescence was detected only when C-terminal half of yellow fluorescence protein (YFP) fused with 2b is co-expressed with N-terminal half of YFP fused with either JAZ1 or JAZ10 in the bi-molecular fluorescence complementation (BiFC) assay (Figure 2E and Supplementary information, Figure S4). Taken together, our findings demonstrate that 2b interacts with *Arabidopsis* JAZ proteins *in vitro* and *in vivo*.

As an overlapping gene thought to have emerged by a

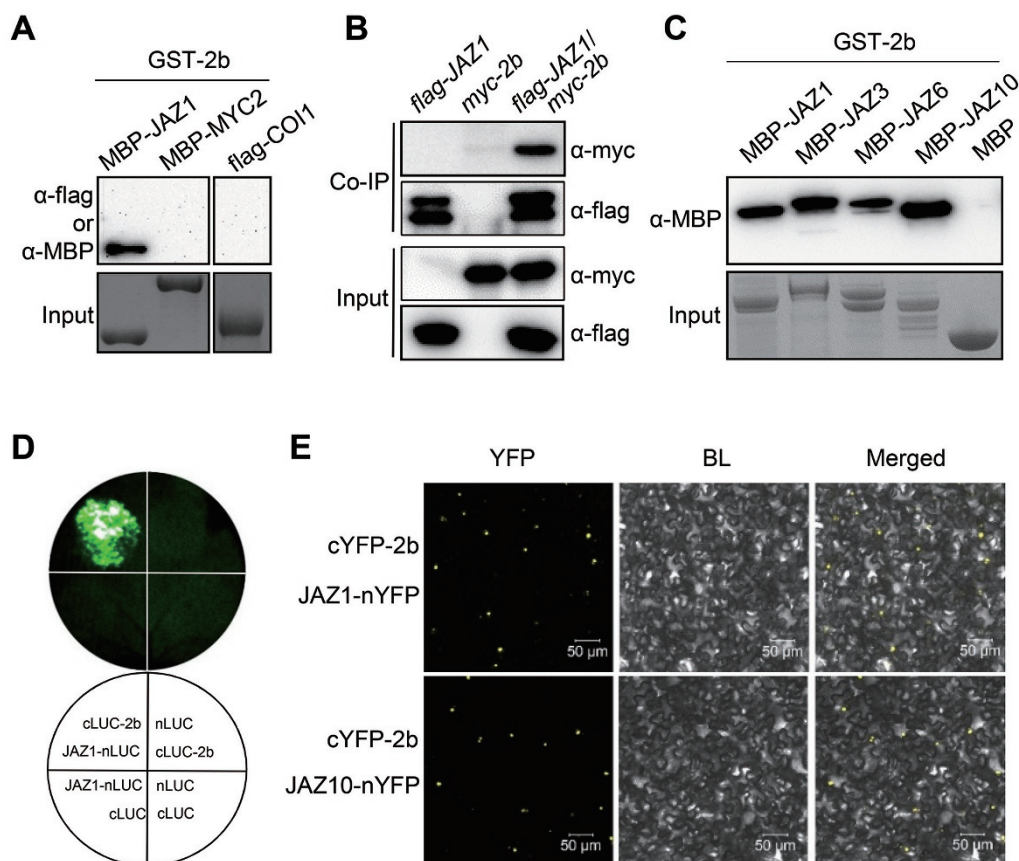


Figure 2 *2b* interacts with *Arabidopsis* JAZ proteins. **(A)** *2b* interacts with *Arabidopsis* JAZ1, but not with COI1 or MYC2. Precipitation of MBP-JAZ1, MBP-MYC2 and flag-COI1 with equal amount of immobilized GST-2b in the pull-down assay was detected by immunoblots using anti-MBP or anti-flag antibody. The input abundances of MBP-JAZ1, MBP-MYC2 and flag-COI1 were detected by Coomassie blue staining (Input). **(B)** *2b* interacts with JAZ1 in co-IP assay. Total proteins extracted from *Arabidopsis* seedlings expressing *flag-JAZ1*, *myc-2b* or both *flag-JAZ1* and *myc-2b* (*flag-JAZ1/myc-2b*) were incubated with anti-flag affinity resin. Flag-JAZ1 and *myc-2b* proteins before (Input) and after immunoprecipitation (co-IP) were detected by immunoblots using anti-flag or anti-myc antibody. The smaller-sized bands in the anti-flag antibody-detected co-IP samples were from partially degraded flag-JAZ1 protein. **(C)** *2b* interacts with various *Arabidopsis* JAZ proteins including JAZ3, JAZ6 and JAZ10. Precipitation of MBP-JAZs and MBP with equal amount of immobilized GST-2b was detected by immunoblots using anti-MBP antibody. Input abundances of MBP-JAZs and MBP were detected by Coomassie blue staining (Input). **(D)** *2b* interacts with JAZ1 in luciferase complementation imaging (LCI) assay. Luciferase activity was detected 50 h after co-expression of indicated construct pairs in leaves of *N. benthamiana*. **(E)** *2b* interacts with JAZ1 and JAZ10 in bimolecular fluorescence complementation (BiFC) assay. YFP signal was detected 50 h after the co-expression of indicated construct pairs in leaves of *N. benthamiana*.

mechanism known as evolutionary overprinting, *2b* is the most variable among the five viral genes encoded by different CMV strains [41]. Nevertheless, we found that *2b* encoded by Q-CMV, a subgroup II strain of CMV, also interacted with multiple *Arabidopsis* JAZ proteins (Figure 3A and Supplementary information, Figure S5).

Tobacco plant (*Nicotiana tabacum*), another natural host of CMV, encodes a large JAZ family of proteins, of which NtJAZ1 and NtJAZ2 show the highest similarity with *Arabidopsis t. JAZ1* (AtJAZ1) and AtJAZ3, respectively (Supplementary information, Figure S6). In the

LCI assay, both NtJAZ1 and NtJAZ2 interacted with the *2b* protein from CMV strains of both subgroup I and II (Figure 3B-3E). These results indicate that the interaction between the viral *2b* protein and host JAZ proteins is a conserved feature of CMV infection.

2b prevents JA-induced recruitment of JAZ1 by COI1

Previous studies showed that recruitment of JAZs by COI1 depends on the interaction of COI1 with the C-terminal Jas motif of JAZ proteins [24, 25, 42]. Consistently, recruitment of JAZ1 by COI1 was abolished when

the Jas motif was deleted or disrupted by point mutations (R205A and R206A) (Figure 4A). Furthermore, both the deletion and the point mutations in the Jas motif markedly reduced the interaction between 2b and JAZ1 (Figure 4B) as it reduced the interaction between COI1 and JAZ1 interaction (Figure 4A).

Coronatine is a JA mimic known to efficiently activate the association of COI1 with JAZ1 [22]. We found that 2b was unable to pull down COI1 through JAZ1 in the presence of coronatine (Supplementary information, Figure S7), suggesting that the interaction between JAZ1 and 2b is incompatible with JAZ1-COI1 interaction. Furthermore, competitive binding assay with COI1 as prey showed that the abundance of JAZ1, which was pulled down by COI1, decreased when 2b (Figure 4C) was increased. Taken together, these findings suggest that

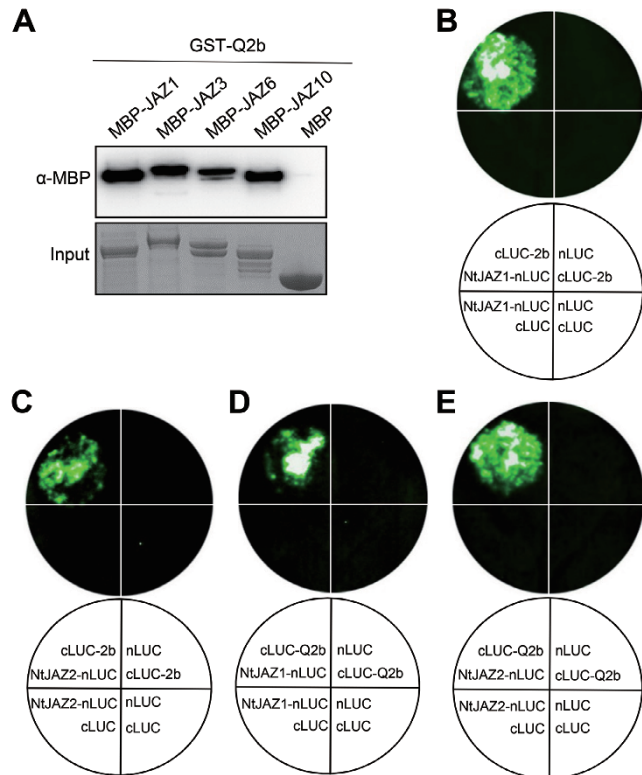


Figure 3 Interaction between 2b and host JAZ proteins is a conserved feature of CMV infection. **(A)** Q2b interacts with multiple *Arabidopsis* JAZ proteins. Precipitation of MBP-JAZs and MBP with equal amount of immobilized GST-Q2b (2b encoded by Q-CMV) was detected by immunoblots using anti-MBP antibody. Input abundances of MBP-JAZs were detected by Coomassie blue staining (Input). **(B-E)** 2b interacts with JAZ proteins of *Nicotiana tabacum*. Interactions of 2b proteins encoded by SD-CMV (2b) and Q-CMV (Q2b) with NtJAZ1 **(B, D)** and NtJAZ2 **(C, E)** were detected by LCI assays.

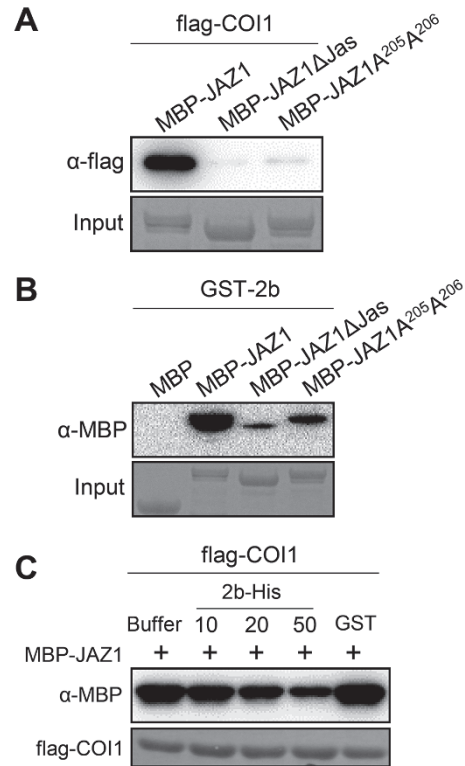


Figure 4 2b prevents JA-induced recruitment of JAZ1 by COI1. **(A, B)** The Jas motif is essential for the interaction of JAZ1 with COI1 and 2b. Precipitation of flag-COI1 (equal input) with the immobilized MBP-JAZ1, MBP-JAZ1ΔJas and MBP-JAZ1A^{205A206}, in the presence of 0.1 μM coronatine, was detected by immunoblots using anti-flag antibody **(A)**. Precipitation of MBP-JAZ1, MBP-JAZ1ΔJas, MBP-JAZ1A^{205A206} and MBP with equal amount of immobilized GST-2b was detected by immunoblots using anti-MBP antibody **(B)**. Input abundances of MBP and MBP-fused JAZ1 derivatives were detected by Coomassie blue staining (Input). 2b attenuates COI1-JAZ interaction **(C)**. Precipitation of MBP-JAZ1 with the immobilized flag-COI1 in the presence of 0.1 μM coronatine and the indicated amount (in μg) of 2b-His (or buffer and GST as controls) was detected by immunoblots using anti-MBP antibody. Equal amounts of flag-COI1 and MBP-JAZ1 were used for each reaction in this experiment. Abundances of flag-COI1 on the affinity resins after washes were detected with Memstain.

2b competes with COI1 in binding to the same domain of JAZ1, thereby preventing JA-induced recruitment of JAZ1 by COI1.

2b prevents JA-induced degradation of JAZ1 thereby suppressing JA signaling

JA-induced degradation of JAZ proteins depends on their interaction with the F-box protein COI1 [24, 25]. We then determined if the observed inhibition of the interaction between COI1 and JAZ1 by 2b attenuates the

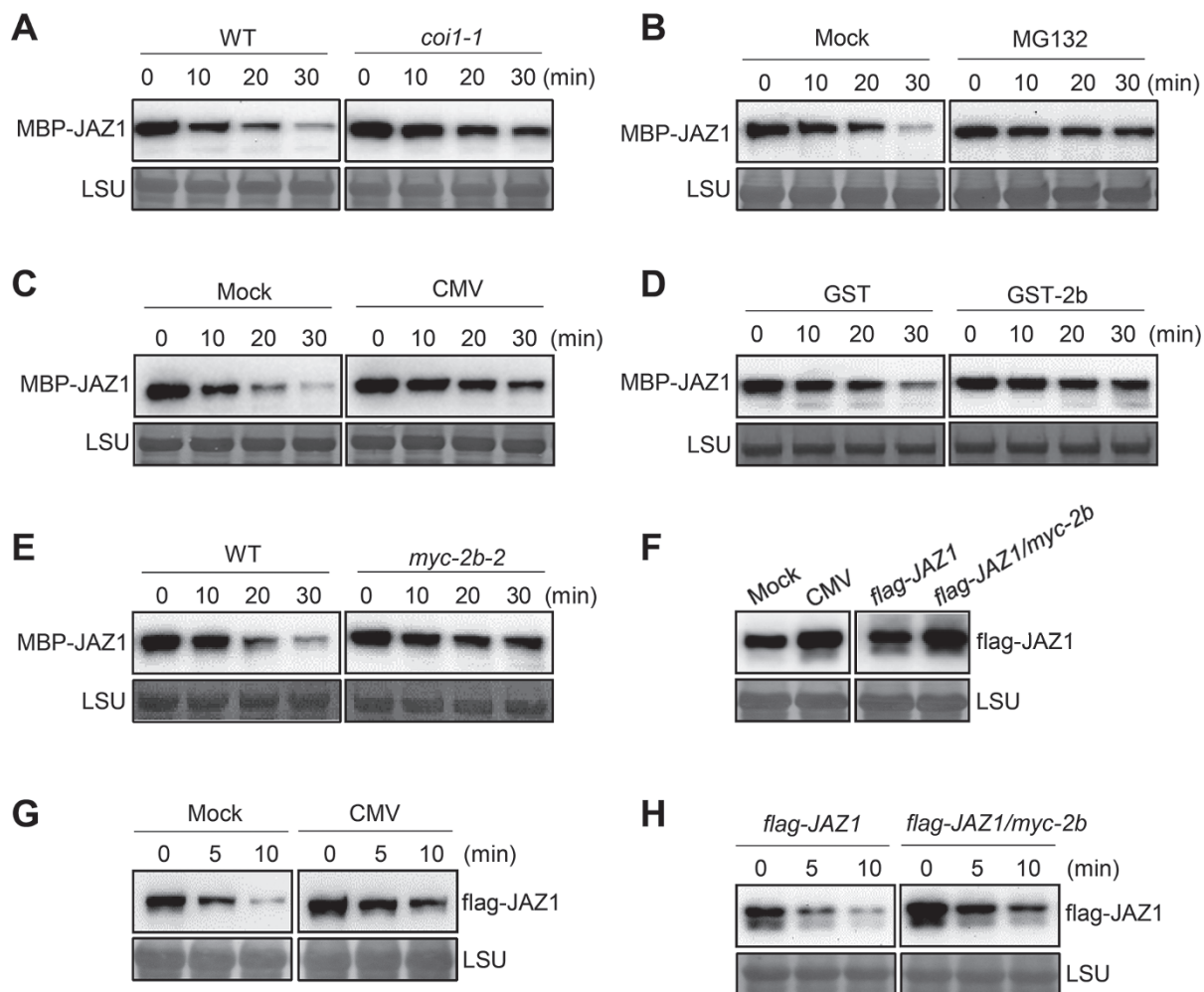


Figure 5 2b inhibits JA-induced degradation of JAZ1. **(A–D)** 2b suppresses JAZ1 degradation in the *in vitro* assays. Purified MBP-JAZ1 was incubated with total crude extract from WT plants and *coi1-1* plants **(A)**, total WT crude extract plus MG132 or ethanol (Mock) **(B)**, total crude extract from WT plants infected by CMV Shandong strain (CMV) or the Mock control **(C)**, total WT crude extract plus purified GST-2b or GST control **(D)**, total crude extract from WT plants and myc-tagged *2b* transgenic plants (*myc-2b*) **(E)**. MBP-JAZ1 was detected by immunoblots using anti-MBP antibody after the indicated incubation time. Large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (LSU) detected with Memstain was used as a control for equal loading. **(F–H)** 2b suppresses JA-induced JAZ1 degradation *in vivo*. Endogenous flag-JAZ1, before **(F)** or after MeJA treatment **(G, H)**, was detected by immunoblot using anti-flag antibody in paired plants, including CMV-infected (CMV) and mock-inoculated (Mock) plants transgenic for *flag-JAZ1* (*flag-JAZ1*) **(F, G)**, or *flag-JAZ1* plants and plants transgenic for both *flag-JAZ1* and *myc-2b* (*flag-JAZ1/myc-2b*) **(F, H)**.

COI1-dependent degradation of JAZ proteins. In our *in vitro* assay conditions, recombinant MBP-JAZ1 protein was rapidly degraded after the addition of crude protein extracts from wild-type *Arabidopsis*, but neither the extract from *coi1-1* mutant plants nor the extract from wild-type plant treated with MG132 was effective (Figure 5A and 5B; Supplementary information, Figure S8A and S8B), consistent with COI1-dependent degradation of JAZ1 by the 26S proteasome pathway as shown previously [24, 25].

Furthermore, we found that JAZ1 degradation in the crude extract from wild-type plants infected with CMV was markedly slower compared with the extract from mock-inoculated plants (Figure 5C and Supplementary information, Figure S8C). The degradation of JAZ1 was also inhibited either by adding recombinant 2b protein (GST-2b) to the crude extract from healthy wild-type plants (Figure 5D and Supplementary information, Figure S8D), or by the crude extract from transgenic plants expressing 2b (*myc-2b*) (Figure 5E and Supplementary

information, Figures S8E, S8F and S9).

Furthermore, our *in vivo* degradation assays showed that endogenous JAZ1, which is stably expressed in plants carrying *flag-JAZ1* transgene, accumulated to a higher level in plants infected by CMV or with transgenic expression of 2b, compared with the control plants (Figure 5F). Furthermore, rapid degradation of endogenous flag-JAZ1 protein, which was induced by exogenous JA treatment, was also suppressed by either CMV infection (Figure 5G) or transgenic expression of 2b (Figure 5H). Together, these findings demonstrate that both CMV infection and transgenic expression of 2b inhibit JA-induced, COI1-dependent degradation of JAZ proteins.

JA-induced degradation of JAZ proteins results in the activation of the JA pathway [18]. Consistent with the previous reports [40, 43], our quantitative RT-PCR analysis showed that either CMV infection or the transgenic expression of 2b in *Arabidopsis* plants significantly inhibited JA-dependent induction of many genes (Supplementary information, Figure S10A and S10B), including *LOX2*, *TAT1* and *VSP1*, as well as *CYP81D11*, *MYC2* and *TPS10*, the latter three genes encoding regulators of plant volatile biosynthesis. Furthermore, we generated *Arabidopsis* plants transgenic for β -glucuronidase reporter gene under the control of promoter of the JA-inducible *VSP1* gene (P_{VSP1} -GUS) either alone or together with a 2b transgene. Histochemical staining revealed that transgen-

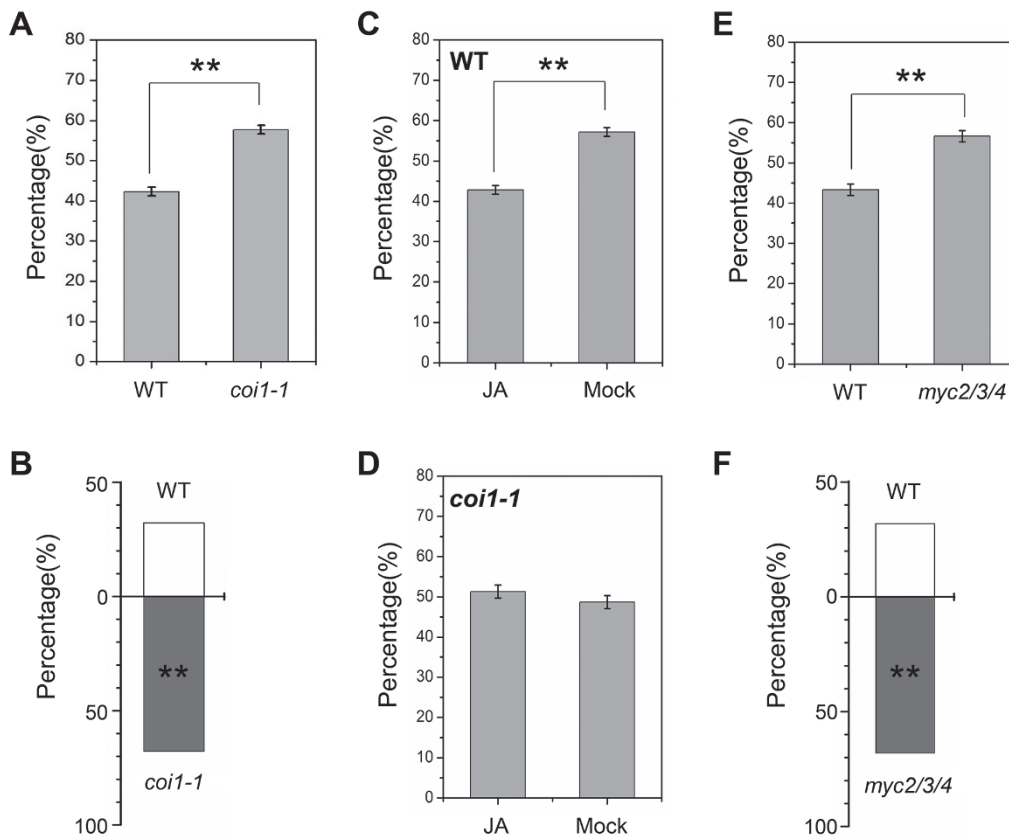


Figure 6 JA suppresses aphid attraction. **(A, B)** *coi1-1* mutant showed higher attractiveness to aphids than the WT in circular-dish bioassay **(A)** and Y-tube olfactometer bioassay **(B)**. For the circular-dish bioassay, data are mean percentages \pm SEM, $n = 5$. $**P < 0.01$, paired samples Student's *t*-test. For the Y-tube olfactometer bioassay, number of aphids that made a choice in this comparison was 56, data are shown as percentages. $**P < 0.01$, χ^2 -test. **(C, D)** JA treatment reduces attractiveness of WT leaves but not *coi1-1* leaves to aphids in circular-dish bioassay. Three-week-old WT or *coi1-1* plants grown on MS medium were pretreated with 100 μ M MeJA (JA) or equal concentration of ethanol (Mock) 8 h before the aphid bioassays. Data are mean percentages \pm SEM, $n = 5$. $**P < 0.01$, paired samples Student's *t*-test. **(E, F)** *myc2*, *myc3* and *myc4* (*myc2/3/4*) mutant plants showed higher attractiveness to aphids than WT plants in circular-dish bioassay **(E)** and Y-tube olfactometer bioassay **(F)**. For the circular-dish bioassay, data are mean percentages \pm SEM, $n = 5$. $**P < 0.01$, paired samples Student's *t*-test. For the Y-tube olfactometer bioassay, number of aphids that made a choice in this comparison was 66. Data are shown as percentages. $**P < 0.01$, χ^2 -test.

ic expression of 2b markedly inhibited JA-induced GUS activity in *Arabidopsis* plants (Supplementary information, Figure S10C), providing an independent verification of the inhibition of JA signaling by 2b.

2b targets the JA pathway for viral induction of host attractiveness to aphid vectors

Having shown that 2b interacts with and represses JA-induced degradation of host JAZ proteins thus suppressing JA signaling (Figures 2-5), we next examined the biological effect of 2b repression on JAZ degradation by comparing feeding preferences of aphids between wild-type *Arabidopsis* plants and *coil-1* mutant, which is defective in JA perception and accumulates higher amounts of JAZ proteins [18]. As shown in Figure 6A, leaves from *coil-1* mutant plants attracted significantly more aphids than those from wild-type plants in the circular dish bioassay (Figure 6A). Consistently, Y-tube olfactometer bioassay showed that less aphids approached wild-type plants than the *coil-1* mutant plants (Figure 6B). Furthermore, leaves of JA-treated wild-type plants attracted fewer aphids than leaves from the untreated wild-type plants in the circular dish bioassay (Figure 6C). On the contrary, JA treatment had no obvious effect on the aphid attraction of *coil-1* (Figure 6D). These results demonstrate that the genetic disruption of JA signaling induces the plant attractiveness to aphid vectors and that JA signaling represses the odor-dependent plant attractiveness to aphid vectors in a COI1-dependent manner.

Among JAZ-repressed downstream components, MYC2, MYC3 and MYC4 were previously found to have important roles in JA-regulated plant defense including plant volatile biosynthesis [18, 30, 33]. Thus, we next examined whether these MYCs were involved in JA-regulated odor-dependent plant attractiveness to aphids. As shown in Figure 6E and 6F, the *myc2/3/4* triple mutant plants exhibited higher aphid attraction than wild-type plants in both the circular dish bioassay and Y-tube olfactometer bioassay (Figure 6E and 6F). These results suggest that JA signaling represses the odor-dependent plant attractiveness to aphid vectors through MYC2, MYC3 and MYC4.

VIA function of 2b is independent of its VSR activity

Having uncovered a novel VIA function for 2b, we next investigated whether the VSR activity of 2b is required for the VIA function. Previous study showed that Fny2blm, a double mutant 2b (L15A and M18A) derived from Fny-CMV, is drastically compromised in VSR activity [44]. Fny2blm nevertheless was able to efficiently interact with JAZ1 (Figure 7A) and inhibit the JA-induced JAZ1 degradation (Figure 7B; Supplementa-

ry information, Figure S8G and S8H). Moreover, transgenic expression of Fny2blm effectively enhanced plant attractiveness to aphid vectors in both the circular dish and Y-tube olfactometer bioassays (Figure 7C-7E). These results demonstrate that Fny2blm, which lacks the VSR activity [44], still retains the VIA activity.

Further quantitative RT-PCR analysis of *Arabidopsis* plants transgenic for Fny2b or Fny2blm showed that JA-dependent induction of many genes was severely repressed in both Fny2b- and Fny2blm-transgenic plants compared with wild-type plants (Figure 7F). These data demonstrate that Fny2blm still maintains the ability to inhibit the expression of JA response genes, indicating that the 2b inhibition of JA response genes shown in this work (Figure 7F; Supplementary information, Figure S10) and in the previous studies [40, 43] may contribute largely to its VIA action (induction of host attractiveness to insect vectors) but not to the VSR activity (suppression of host defense against virus infection). In summary, our results demonstrate that VIA function of 2b, which inhibits JA signaling by stabilizing host JAZ proteins and suppressing MYC factors, is independent of its VSR activity.

Discussion

Recent studies have uncovered novel strategies arthropod-borne pathogens adopt to manipulate their hosts [45, 46]. In principle, these novel functions represent a core feature of arthropod-borne pathogens and are vital to their adaptive success during the co-evolution with their host and vector organisms. However, the molecular mechanisms involved in host manipulation by arthropod-borne pathogens are currently largely unclear. For example, it is unknown whether pathogen-induced host odor-dependent attractiveness to vectors is elicited by specific pathogen effectors and how these pathogen effectors target host pathways to effect host manipulation. In this study, we used an *Arabidopsis* model to uncover the mechanisms underlying odor-dependent vector attraction induced by CMV, an economically important aphid-borne plant pathogen [11]. We show that the CMV-induced vector attraction is under the control of the signaling pathway of the host hormone JA, which represses odor-dependent host attractiveness to aphids through MYC2 and its homologs MYC3 and MYC4. We define viral 2b protein, the previously known VSR of CMV, as a VIA. 2b physically interacts with host JAZ proteins and prevents JA-induced JAZ degradation, thus attenuating JA signaling to enhance host attractiveness to aphid vectors. 2b acts differently from the bacterial effectors HopZ1a and HopX1, which promote JAZ deg-

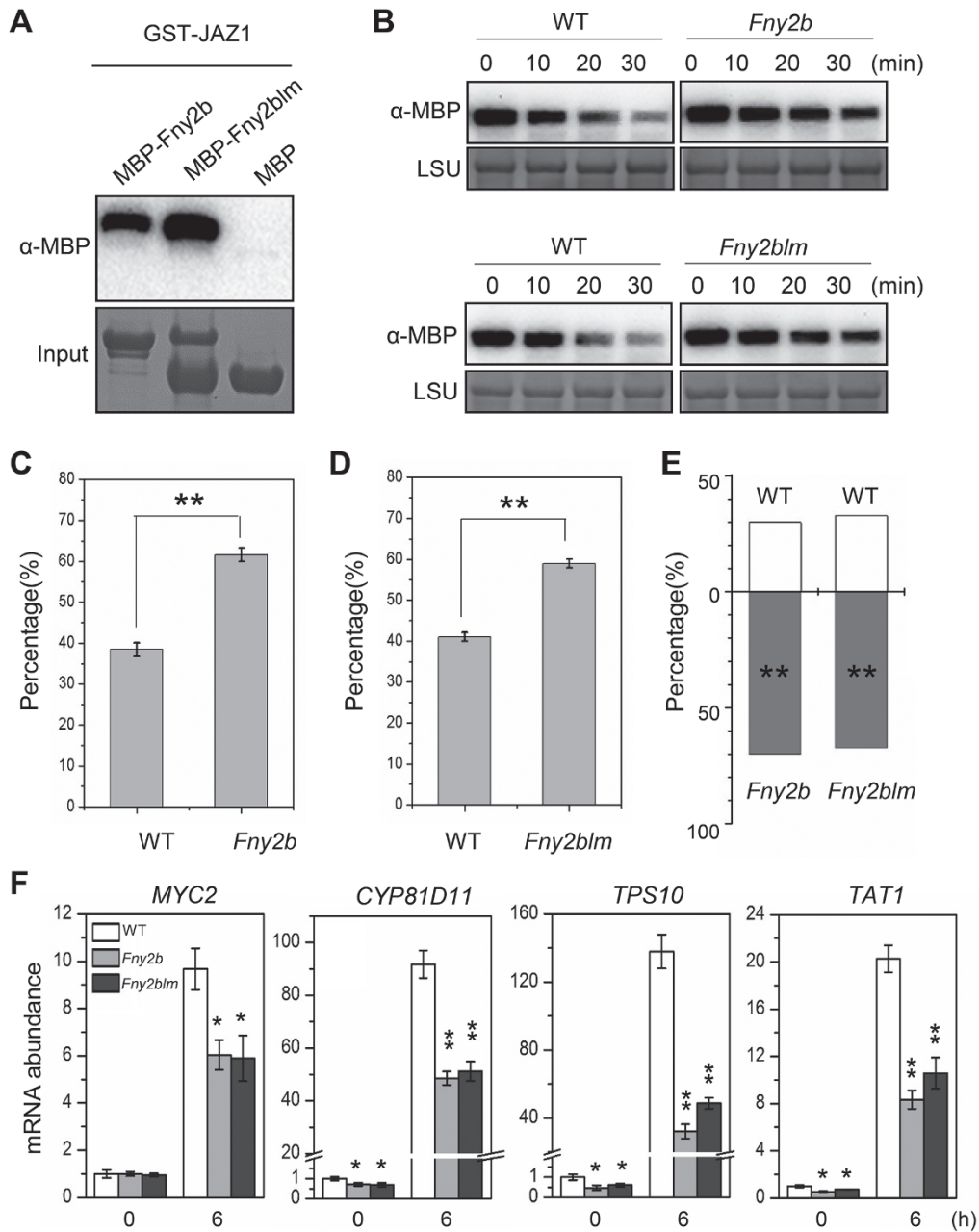


Figure 7 VIA function of 2b is independent of its VSR activity. **(A)** Both *Fny2b* and *Fny2blm* interact with JAZ1 in pull-down assay. Precipitation of MBP-*Fny2b*, MBP-*Fny2blm* and MBP with equal amount of immobilized GST-JAZ1 was detected by immunoblots using anti-MBP antibody. Input abundances of MBP-*Fny2b*, MBP-*Fny2blm* and MBP were detected by Coomassie blue staining (Input). **(B)** Both *Fny2b* and *Fny2blm* inhibit JAZ1 degradation in the *in vitro* degradation assay. Amount of purified MBP-JAZ1 in total crude protein extract from WT and *Fny2b* transgenic plants, or from WT and *Fny2blm* transgenic plants was detected by immunoblots using anti-MBP antibody after the indicated incubation time. LSU detected with Memstain was used as a control for equal loading. **(C-E)** Both *Arabidopsis* plants transgenic for *Fny2b* and *Fny2blm* showed higher attractiveness to aphids than WT in circular-dish bioassay **(C, D)** and Y-tube bioassay **(E)**. For the circular-dish bioassays, data are shown as mean percentages \pm SEM, $n = 5$. ** $P < 0.01$, paired samples Student's *t*-test. For the Y-tube bioassays, number of aphids that made a choice in the comparison of WT vs *Fny2b* and WT vs *Fny2blm* was 60 and 64, respectively. Data are shown as percentages. ** $P < 0.01$, χ^2 -test. **(F)** *Fny2blm* suppresses JA-inducible gene expression. The relative mRNA abundance of *MYC2*, *CYP81D11*, *TPS10*, *LOX2*, *TAT1* and *VSP1* was detected by quantitative RT-PCR in 14-day-old MS medium-cultivated WT and *Arabidopsis* plants transgenic for *Fny2b* or *Fny2blm*, treated with MeJA (100 μ M) for the indicated time periods. Data are means \pm SEM, $n = 3$. * $P < 0.05$, ** $P < 0.01$, Student's *t*-test significance compared with WT.

radation and activate JA signaling [47, 48].

2b protein from either of the two CMV subgroup strains interacts with JAZ proteins from both *Arabidopsis* and tobacco plants, indicating that targeting the repressor of JA signaling to inhibit JA-induced responses is an evolutionarily conserved function of the aphid-borne virus. Notably, odor-dependent vector attraction is suppressed by the induction of JA signaling and is enhanced in the *Arabidopsis coi1-1* mutant defective in JA signaling. Further studies are expected to identify the exact volatile(s) that mediates the JA-regulated and CMV-induced aphid attraction.

Interestingly, our study shows that 2b interacts with JAZ proteins known to bind both COI1 and MYCs (Figures 2-4) [18]. It has been reported that, in the resting stage, JAZs exist in complex with MYCs (JAZ-MYC complex) [27]. After perceiving JA, COI1 interacts with JAZ proteins and recruits them for degradation, thereby releasing MYCs to activate JA signaling [18, 27]. Upon CMV infection, 2b binds JAZs and inhibits COI1-mediated JAZ degradation, resulting in an elevated level of JAZs. As 2b-JAZ interaction is dynamic and reversible, an increase in JAZ proteins by 2b could conceivably cause an increase in the amount of JAZ-MYC complex in the cells by attenuating the dissociation of pre-existing JAZ-MYC complex and promoting the formation of new JAZ-MYC complex. This model is consistent with both the suppression of JA signaling by 2b and the observation that genetic suppression of MYC function in *myc2/3/4* triple mutant plants also induces aphid attraction.

Suppression of antiviral RNAi defense of the host is a widespread strategy in plant and invertebrate viruses [12]. 2b of CMV is a well-characterized VSR that effectively suppresses host RNAi-directed antiviral machinery [12]. In this study, we show that the VSR 2b also serves as a VIA to control viral induction of host attractiveness to insect vectors, and its VIA activity is independent of its VSR activity. Moreover, our study demonstrates how 2b interferes with the JA pathway and inhibits expression of many JA-regulated *Arabidopsis* genes that are essential for generation of both insect-repellent volatiles and feeding-deterrent/toxic products, thereby providing mechanistic understanding for our observation that CMV 2b induces odor-dependent host attractiveness to aphids, and also for the previously reported phenomenon that CMV 2b suppresses host resistance against insect feeding [49].

Our study shows that JA represses plant attractiveness to insect vectors (Figure 6), and the previously defined VSR 2b inhibits JA signaling to serve as a VIA, independent of its VSR activity, for inducing odor-dependent vector attraction (Figures 1-5 and 7). Interestingly, Carr and colleagues reported recently that four unrelated

VSRs encoded by both insect-transmissible and non-insect-transmissible plant viruses have a shared activity to suppress JA-induced host gene expression [43]. Future studies are required to experimentally verify whether all these VSRs, which repress JA signaling [43], induce odor-dependent host attractiveness to insects. Although some viruses may never be insect-transmissible due to their physiochemical properties including instability of virions inside the body of insects or inability to be acquired due to the lack of interaction with insect stylets [50, 51], we propose that such a virus-induced odor-dependent insect attraction has a role in the emergence and evolution of arthropod-borne pathogens. Pathogen-induced odor-dependent insect attraction will allow more insects to move toward the infected plants, thus providing more opportunity for viruses to interact with insects, which might be essential for some viruses to evolve and adapt to a vector-borne lifestyle.

Materials and Methods

CMV inoculation

For CMV infection, 4-week-old *Arabidopsis* plants that grown under a 10-h (23 °C)/14-h (19 °C) light/dark photoperiod were lightly dusted with silicon carbide and then rub-inoculated with purified virions at the concentration of 20 µg/ml. Mock-treated plants were inoculated with buffer (5 mM sodium borate, 0.5 mM disodium EDTA, pH = 9.0) only. The CMV-infected and mock-inoculated plants were used for aphid attraction bioassays, gene expression assays and protein degradation assays 2 weeks post inoculation.

Generation of transgenic plants

The full-length coding sequence of 2b was amplified from the cDNA of SD-CMV and inserted into modified pROK2 binary vector to generate the pMyc-2b construct. The full-length coding sequence of *JAZ1* was amplified from *Arabidopsis* cDNA and constructed into modified pCAMBIA1300 binary vector (driven by 35S promoter and fused with 3× flag tag) to generate the pCAMBIA1300-3flag-JAZ1 construct. The pMyc-2b and pCAMBIA1300-3flag-JAZ1 constructs were then transformed into *Arabidopsis* Col-0 wild-type plants using the *Agrobacterium*-mediated floral dip method to get the *myc-2b* and *flag-JAZ1* transgenic plants, respectively. Two individual transgenic lines of *myc-2b*, including *myc-2b-2* (*myc-2b*) and *myc-2b-4* were used in this study. *Arabidopsis* plants transgenic for both *myc-2b* and *flag-JAZ1* (*flag-JAZ1/myc-2b*) were obtained by crossing *myc-2b* plants with *flag-JAZ1* plants.

The *Fny2b* or *Fny2blm* constructs, the Fny-CMV or Fny-CMVΔ2b viruses, and the *Arabidopsis* plants transgenic for *Fny2b* or *Fny2blm* were described previously [35, 37, 44].

Aphid attraction bioassays

Wingless *M. persicae* aphids maintained on *Raphanus sativus* were used for aphid bioassays (as shown in Supplementary information, Figure S1A). Aphids were isolated from *R. sativus* and

starved for 6 h before testing.

For the circular dish bioassays, plants were cultivated on MS medium under a 16-h (23 °C)/8-h (19 °C) light/dark photoperiod for 3 weeks. As shown in the schematic diagram (Supplementary information, Figure S1B), in each pair-wised comparison, 12-15 leaves from each genotype were placed alternately in a circular dish, which was 15 cm in diameter and padded with three layers of water wet filter papers to prevent water loss of the leaves. In each time of the experiment, 60-100 aphids were released from the center of the dish for free walk, and their trace were recorded by a digital camera. An aphid was considered to have made its choice once it reached the leaf of either genotype and didn't walk directly away without a stop. The choices of aphids were counted according to the video records and the ratio between the numbers of aphids choosing each of the paired plants was calculated.

For the Y-tube olfactometer bioassays, plants grown in soil under a 10-h (23 °C)/14-h (19 °C) light/dark photoperiod for 5 weeks were used. As CMV infection or 2b transgenic expression causes morphological changes in *Arabidopsis*, such as dwarf and curled leaves (data not shown) [14, 37, 44], to ensure that equal weight of each kind of plants were used for the pair-wise comparisons in Y-tube olfactometer bioassays, we firstly weighed plants with CMV infection or 2b transgenic expression and the corresponding control plants, and then used the soil-grown plants (which grew at the same batch and had similar size with the above-weighed plants) for the pair-wise comparisons. As shown in the schematic diagram (Supplementary information, Figure S1C), plants that used for pair-wise comparison were placed into two odor source bottles, which connected to the two arms of the Y-tube. Under the action of the aspirator, air that was cleaned by activated charcoal and humidified by water flowed through the odor source bottles and flow meters into the arms of the Y-tube, which was placed in an enclosed incubator with diffused light. The rate of airflow in each arm was maintained at 200 ml/min. The inner diameter of the Y-tube was 1.0 cm. The stem and the arms of the Y-tube were 6 and 10 cm in length, respectively. In each test, an individual aphid was introduced at the end of the Y-tube stem and walked upwind toward the arms. The aphid was considered to have made its choice once it entered one arm and walked across the decision line that marked 3 cm away from the Y-junction. A "no choice" outcome was recorded and the aphid was replaced if it failed to make decision during the 5 min testing period. To compensate any unknown asymmetry in the setup, the odor sources were interchanged after every 6 tests, and the Y-tube and plants were replaced after every 12 tests. The used Y-tubes were cleaned and dried with 180 °C oven before reuse.

Preparation of recombinant proteins

To obtain recombinant GST-tagged 2b and JAZ1 proteins, the coding sequences of 2b from SD-CMV or Q-CMV and *Arabidopsis* JAZ1 were cloned into modified pGEX-4T-2 vector. To obtain recombinant MBP-JAZ proteins, the coding sequences of various JAZ genes were cloned into pMAL-C2X vector. These constructs were then introduced into *Escherichia coli* BL21 strain, and the recombinant proteins were purified using corresponding affinity resin followed by size exclusion chromatography.

Quantitative RT-PCR

Total RNAs were extracted from indicated plant materials using

Transzol reagent (Transgen) and reverse transcribed using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen). Quantitative RT-PCR was performed with ABI7500 real-time PCR system using SYBR Select Master Mix (Life Technologies). *ACTIN8* was used as the internal control.

GUS staining

For the GUS staining assay, homozygous P_{VSP1} -*GUS* (*GUS* reporter gene driven by *VSP1* promoter) transgenic plants were crossed with heterozygous *myc-2b* transgenic plant, and the P_{VSP1} -*GUS* plants in background of WT (P_{VSP1} -*GUS*/WT) and *myc-2b* (P_{VSP1} -*GUS*/*myc-2b*) were identified in the F1 population. Two-week-old P_{VSP1} -*GUS*/WT and P_{VSP1} -*GUS*/*myc-2b* seedlings were treated with 100 μM MeJA or equal amount of ethanol (Mock) for 8 h and then incubated in GUS staining buffer (1 mM X-glucuronide, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM pH 8.0 EDTA, 1% Triton X-100) at 37 °C overnight. The stained seedlings were cleared by washing with 70% ethanol.

Pull-down assay

For pull-down assays, about 50 μg of bait proteins were used to bind with corresponding resin (amylose resin for MBP-fused proteins, glutathione resin for GST-fused proteins and anti-flag resin for flag-fused proteins), and then were further incubated with about 10 μg prey proteins at 4 °C for 1 h in RB buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 0.1% (v/v) Tween 20, 10% (v/v) glycerol and 20 mM β-mercaptoethanol). Precipitated prey proteins on the resin were released by boiling at 100 °C for 5 min and detected by immunoblots using corresponding antibodies. The relative input abundances of the bait or prey proteins were detected by Coomassie blue staining.

For pull-down assay in Supplementary information, Figure S3, 50 μg of MBP and MBP-JAZ1 proteins were used to bind with amylose resin, and then further incubated with 1 ml concentrated total proteins extracted from 2 g *myc-2b* transgenic plants. Precipitated *myc-2b* on the beads was detected by immunoblots using anti-*myc* antibody.

For competitive pull-down assay in Figure 4C, 50 μg of flag-COII proteins were used to bind with anti-flag resin, and were further incubated with 10 μg MBP-JAZ1 plus increasing amount of 2b-His (10, 20 and 50 μg) or buffer and GST(50 μg) as controls, in the presence of 0.1 μM coronatine. Precipitated MBP-JAZ1 on the beads were detected by immunoblots using anti-MBP antibody.

Co-IP assay

For Co-IP assay, *Arabidopsis* plants transgenic for both *myc-2b* and *flag-JAZ1* (*flag-JAZ1*/*myc-2b*) or for *myc-2b*, *flag-JAZ1* alone, were used in the Co-IP assay. Total proteins were extracted from 2 g seedlings using 15 ml RB-plus buffer (RB buffer plus 1× EDTA-free complete mini protease inhibitor cocktail and 20 μM MG132) and concentrated to 1 ml using 10 kD ultrafiltration tubes (Millipore). About 50 μl anti-flag resin beads were added into 800 μl concentrated total proteins and incubated at 4 °C for 1 h. The beads were then washed with RB plus buffer for five times and resuspended with SDS loading buffer. The precipitated proteins on the beads were released by boiling at 100 °C for 5 min and detected by immunoblots using anti-*myc* and anti-flag antibody.

BiFC and LCI assays

For BiFC assays, the full-length coding sequence of 2b from SD-CMV was cloned into cYFP vector to get the cYFP-2b construct, and the full-length coding sequences of *Arabidopsis* JAZ1 and JAZ10 were cloned into the nYFP vector to get the JAZ1-nYFP and JAZ10-nYFP constructs. These constructs were then transformed into *Agrobacterium tumefaciens* strain GV3101 and transiently co-expressed with indicated combinations in the *N. benthamiana* leaves using *Agrobacterium* infiltration method. The YFP fluorescence signal for each combination was detected using confocal microscope (Zeiss LSM710) 50 h after infiltration.

For LCI assays, the full-length coding sequences of 2b from SD-CMV and Q-CMV were cloned into cLUC vector, and the full-length coding sequences of JAZ1 and JAZ2 from *Arabidopsis* and *N. tabacum* were cloned into nLUC vector. These constructs were transformed into *A. tumefaciens* strain GV3101 and co-expressed with indicated combinations in *N. benthamiana* leaves for 50 h. The LUC substrate (luciferin) was then sprayed onto the surface of the leaves and images were taken using a low-light cooled imaging apparatus (Andor iXon).

In vitro and *in vivo* protein degradation assay

For the *in vitro* degradation assays of JAZ1 in various conditions, 5 µg of purified MBP-JAZ1 protein was added to 600 µl crude total proteins (1 µg/µl) extracted from indicated plants using RB buffer. The mixture was incubated at 22 °C and sampled at indicated time points. The remained abundance of MBP-JAZ1 in these samples was detected by immunoblots using anti-MBP antibody. For the degradation assay in Figure 5B, 10 µM of MG132 or equal amount of ethanol (Mock) was added into the crude extracts from wild-type plants before adding MBP-JAZ1. For the degradation assay in Figure 5D, MBP-JAZ1 was incubated with 20 µg GST-2b or GST at 4 °C for 1 h before added to the crude extracts from wild-type plants. For quantitative analysis, the band densities of MBP-JAZ1 were quantified using ImageJ2x software.

For the *in vivo* degradation assay in Figure 5G, 4-week-old *flag-JAZ1* transgenic plants that are grown under a 10-h (23 °C)/14-h (19 °C) light/dark photoperiod were inoculated with SD-CMV (CMV) or buffer (Mock), and then treated with MeJA (30 µM) for indicated time periods 2 weeks after inoculation. The remained abundance of *flag-JAZ1* in these samples was detected by immunoblots using anti-*flag* antibody. For the *in vivo* degradation assay in Figure 5H, 3-week-old *flag-JAZ1* or *flag-JAZ1/myc-2b* plants that are grown on MS were treated with 10 µM MeJA for indicated time periods before immunoblot detection.

Accession numbers

Sequence data described in this article can be found in GenBank/EMBL or TAIR (www.Arabidopsis.org) under the following accession numbers: *COI1* (AT2G39940), *LOX2* (AT3G45140), *MYC2* (AT1G32640), *CYP81D11* (AT3G28740), *TPS10* (AT2G24210), *VSP1* (AT5G24780), *TAT1* (AT4G23600), *JAZ1* (AT1G19180), *JAZ3* (AT3G17860), *JAZ6* (AT1G72450), *JAZ10* (AT5G13220), *ACTIN8* (AT1G49240), *SD2b* (D86330), *Q2b* (Z21863), *Fny2b* (NC_002035), *NtJAZ1* (AB433896) and *NtJAZ2* (AB433897).

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Author Contributions

DX, SWD and LK designed the research; DW and TQ performed the experiments with the help of WL, HT, HG, JW, JG and RY; DX, SWD, LK, DW, TQ, CR, XW and YL analyzed the data; DX, DW, TQ and SWD wrote the manuscript with contributions of all the authors.

Competing Financial Interests

The authors declare no competing financial interests.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)