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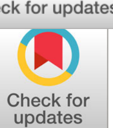

The transcriptional regulator JAZ8 interacts with the C2 protein from geminiviruses and limits the geminiviral infection in *Arabidopsis*°°

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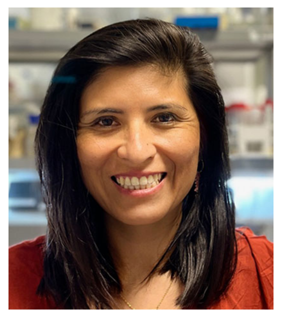
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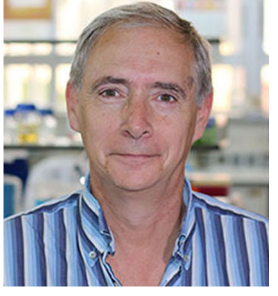
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ABSTRACT

Jasmonates (JAs) are phytohormones that finely regulate critical biological processes, including plant development and defense. JASMONATE ZIM-DOMAIN (JAZ) proteins are crucial transcriptional regulators that keep JA-responsive genes in a repressed state. In the presence of JA-Ile, JAZ repressors are ubiquitinated and targeted for degradation by the ubiquitin/proteasome system, allowing the activation of downstream transcription factors and, consequently, the induction of JA-responsive genes. A growing body of evidence has shown that JA signaling is crucial in defending against plant viruses and their insect vectors. Here, we describe the interaction of C2 proteins from two tomato-infecting geminiviruses from the genus Begomovirus, tomato yellow leaf curl virus (TYLCV) and tomato yellow curl Sardinia virus (TYLCSaV), with the transcriptional repressor JAZ8 from *Arabidopsis thaliana* and its closest orthologue in tomato, SlJAZ9. Both JAZ and C2 proteins colocalize in the nucleus, forming discrete nuclear speckles. Overexpression of *JAZ8* did not lead to altered responses to TYLCV infection in *Arabidopsis*; however, knock-down of *JAZ8* favors geminiviral infection. Low levels of *JAZ8* likely affect the viral infection specifically, since *JAZ8*-silenced plants neither display obvious developmental phenotypes nor present differences in their interaction with the viral insect vector. In summary, our results show that the geminivirus-encoded C2 interacts with JAZ8 in the nucleus, and suggest that this plant protein exerts an anti-geminiviral effect.

Keywords: C2 protein, geminivirus, jasmonates, JAZ8 transcriptional repressor, TYLCV

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INTRODUCTION

Phytohormones are chemical messengers essential to establish signaling networks to regulate plant growth and stress-related responses (Gimenez-Ibanez et al., 2017). Jasmonic acid (JA) and its methyl ester (MeJA) and isoleucine conjugate (JA-Ile) are formed from fatty acids and collectively known as jasmonates (JAs); jasmonates control critical biological processes in plants, including fertility, seedling development, response to wounding, and the growth-defense trade-off (Gimenez-Ibanez et al., 2017; Howe et al., 2018; Ruan et al., 2019). When encountering stress, such as those that result from insect feeding or the attack by necrotrophic fungi, eudicots rapidly biosynthesize JA and its bioactive form, JA-Ile (or dn-OPDA in bryophytes; Monte et al., 2018), to trigger signal transduction events, which ultimately lead to the onset of plant defense (reviewed in Campos et al., 2014; Yan and Xie, 2015; Howe et al., 2018). In basal conditions, JAs are maintained at low levels, and JA-mediated transcriptional responses are kept repressed by JASMONATE ZIM-DOMAIN (JAZ) proteins (Wasternack and Song, 2017). JAZ proteins repress the activity of crucial JA-inducible transcription factors (TFs) such as the basic helix–loop–helix (bHLH) MYC2 and its homologs MYC3/4/5. These TFs, in turn, regulate the expression of a large proportion of JA-responsive genes, including the expression of *JAZ* genes, to attenuate JA responses as part of a feedback loop pathway (Chico et al., 2008; Howe et al., 2018; Zander et al., 2020). The bioactive JA-Ile binds the receptor protein CORONATINE INSENSITIVE1 (COI1), adaptor subunit of the E3 ubiquitin ligase complex SKP1/Cullin1/F-box $(SCF)^{COI1}$, and acts as molecular glue, facilitating its interaction with JAZ proteins (Xie et al., 1998; Chini et al., 2007; Fonseca et al., 2009). The hormone-dependent interaction between COI1 and JAZ proteins leads to degradation of the latter through the ubiquitin/26 S proteasome pathway, allowing derepression of downstream TFs and, consequently, activation of JA-responsive genes (Howe et al., 2018).

There are 13 JAZ proteins (JAZ1-13) in *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*). Members of the JAZ family exhibit high sequence variability but generally possess two common domains: ZIM, which includes the conserved TIFY (TIFF/YXG) motif, and Jas (Thireault et al., 2015; Chini et al., 2016; Guo et al., 2018; Garrido-Bigotes et al., 2019). Within the Jas domain, a degron signal can be found, which is responsible for the degradation of JAZs in the presence of JA–Ile and may play a role in nuclear localization (Chini et al., 2007; Thines et al., 2007; Melotto et al., 2008; Grunewald et al., 2009).

The absence of solid phenotypes in most *jaz* single mutants and the analysis of *Arabidopsis* mutants defective in multiple *JAZ* indicated a relatively high level of redundancy among JAZ family members (Campos et al., 2016; Chini et al., 2016; Major et al., 2017; Guo et al., 2018), limited by tissue-specific expression patterns of some *JAZ* genes.

Some JAZ proteins, including *Arabidopsis* group IV (JAZ7, JAZ8, and JAZ13, and alternative splice variants of JAZ10), are recalcitrant to COI1 interaction since they harbor a non-canonical degron, and therefore present a greater stability and repressor activity in a jasmonate-stimulated context (Chung et al., 2009; Shyu et al., 2012; Thireault et al., 2015; Howe et al., 2018). JAZ8, when ectopically expressed in *Arabidopsis*, represses JA-regulated defense responses and senescence (Shyu et al., 2012; Jiang et al., 2014; Chen et al., 2021). Interestingly, JAZ8 has been described as a component of the so-called JJW (JAV1-JAZ8-WRKY51) complex, which finely controls JA biosynthesis genes to defend against insect attack (Yan et al., 2018). Besides, Chen and collaborators have determined that *JAZ8* overexpression repressed plant defense against *Botrytis cinerea* by interacting with the transcription factor WRKY75, which positively regulates JA-mediated plant defense against necrotrophic fungal pathogens (Chen et al., 2021). JAZ8 also interacts with VirE3 from *Agrobacterium tumefaciens* (hereafter referred to as *Agrobacterium*) to attenuate root tumorigenesis by antagonistically modulating salicylic acid- and JA-mediated plant defense signaling (Li et al., 2021b).

Geminiviruses represent a large family of insect-transmitted plant viruses with circular single-stranded DNA genomes (ssDNA, ~2.7–5.2 kb) packaged within geminate particles. Begomovirus, the largest genus within the *Geminiviridae* family, comprises viruses transmitted by whiteflies that can have either mono- or bipartite genomes, traditionally considered to encode six to eight proteins (Zerbini et al., 2017; Fiallo-Olivé et al., 2021). Despite their limiting coding capacity, the genome-encoded information is sufficient to complete all processes required for infection, such as viral replication, movement, and suppression or evasion of plant defense mechanisms (reviewed in Fondong, 2013; Hanley-Bowdoin et al., 2013; Ramesh et al., 2017; Aguilar et al., 2020).

It has been reported that geminiviruses have evolved strategies to subvert JA signaling, which plays an important role in the tripartite interaction between plants, geminiviruses, and their insect vectors (Sun et al., 2017; Wu and Ye, 2020; Pan et al., 2021). Repression of the JA pathway or JA-responsive genes has been demonstrated in *Arabidopsis*, tomato, and tobacco plants infected with begomoviruses as well as in transgenic plants expressing geminiviral pathogenicity factors like the C2 or the βC1 proteins (Ascencio-Ibáñez et al., 2008; Yang et al., 2008; Lozano-Durán et al., 2011a; Li et al., 2014; Rosas-Díaz et al., 2016; Shi et al., 2019; Li et al., 2019b; Guerrero et al., 2020). Notably, the exogenous application of MeJA negatively impacts geminiviral infection (Lozano-Durán et al., 2011a; Chakraborty and Basak, 2019).

C2 is an archetypical example of the multifunctionality of geminiviral proteins (Guerrero et al., 2020). This small viral protein (~15 kDa) localizes mainly in the nucleus and could act as a transcriptional factor for viral genes (Sunter and Bisaro, 1992). C2 is likewise able to trigger the transcription

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of host genes (Trinks et al., 2005; Oh et al., 2007; Lozano-Durán et al., 2011a; Rosas-Díaz et al., 2016; Li et al., 2019a), and suppresses transcriptional and post-transcriptional gene silencing (Dong et al., 2003; Wang et al., 2003; Vanitharani et al., 2004; Wang et al., 2005; Buchmann et al., 2009; Luna et al., 2012). Transgenic expression of the C2 protein from the begomoviruses tomato yellow curl Sardinia virus (TYLCSaV) and tomato yellow leaf curl virus (TYLCV) or the curtovirus beet curly top virus (BCTV) subverts the function of the COP9 signalosome complex (CSN), affecting cellular processes regulated by SCF complexes, including JA-signaling (Lozano-Durán et al., 2011a, 2011b). However, transcriptomic analyses show that plants expressing C2 from TYLCSaV are only affected in specific JA-induced responses, implying that C2 alters JA-dependent gene regulation by additional mechanisms conferring specificity (Rosas-Díaz et al., 2016). Moreover, C2 from TYLCV interacts with a ubiquitin-related protein, RPS27A, compromising the degradation of JAZ1 and resulting in the inhibition of JA-mediated plant defense (Li et al., 2019a).

While the role of C2 in suppressing JA-triggered responses has been demonstrated for several geminiviruses, whether other plant proteins besides RPS27A interact with C2 and contribute to its specific effect on JA responses remains to be determined. In this study, we found that the C2 proteins from the tomato-infecting begomoviruses TYLCV and TYLCSaV interact with JAZ8 from *Arabidopsis* and its closest orthologue in tomato, SlJAZ9, colocalizing in the nucleus. *Arabidopsis* plants with low sensitivity to JA due to overexpression of *JAZ8* did not show altered responses to TYLCV infection; however, knock-down of JAZ8 favors the viral infection. Finally, we observed that JAZ8 did not significantly contribute to the performance of the whitefly *Bemisia tabaci*, the insect vector for begomoviruses, in *Arabidopsis*. Our results show that JAZ8 interacts with C2 in the plant cell nucleus and suggest that JAZ8 exerts a specific anti-geminiviral effect.

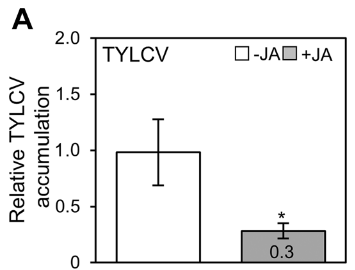
RESULTS

Exogenous application of jasmonates hampers TYLCV infection in *Arabidopsis*

It has been previously shown that exogenous MeJA (JA) treatment has a negative effect on the infection by BCTV in *Arabidopsis* (Lozano-Durán et al., 2011a). To determine if the application of JA also has an impact on the infection by begomoviruses, we inoculated JA- and mock-treated *Arabidopsis* plants with TYLCV. Four to 5-week-old plants inoculated with TYLCV were treated with 50 μM JA or mock solution every 2 d starting at 2 d post-inoculation (dpi). The transcript accumulation of JA-responsive genes and the amount of viral DNA were determined at 17 and 21 dpi, respectively. The results show that the application of exogenous JA reduced the accumulation of viral DNA (Figure 1A). The efficacy of the JA treatments was confirmed by the induction of JA-responsive genes (*AtAOS*, *AtJAZ8*, and *AtJAZ10*) in infected and uninfected plants treated with JA (Figure 1B). Interestingly, while in the non-treated plants, the presence of TYLCV does not alter the transcript accumulation of *AtAOS* and *AtJAZ10*, the expression of *AtJAZ8* is induced by the viral infection. However, the elevation in *JAZ8* transcripts triggered by TYLCV does not affect its induction by JA (Figure 1B).

C2 proteins from begomoviruses interact with the transcriptional repressor JAZ8 from *Arabidopsis* and its tomato orthologue

JAZ proteins have been identified as targets of plant pathogen effectors, including viruses (Wu et al., 2017; Li et al.,



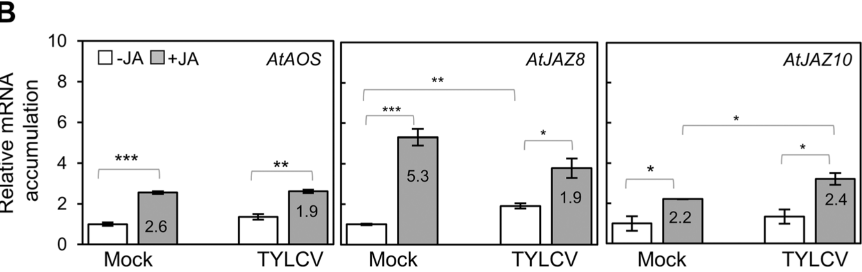


Figure 1. Exogenous methyl jasmonate (MeJA) application negatively impacts tomato yellow leaf curl virus (TYLCV) infection, while geminivirus infection alters Jasmonate (JA) signaling in *Arabidopsis*

(A) Relative TYLCV DNA accumulation was determined by real-time quantitative PCR (qPCR) of total DNA extracted from whole plants at 21 dpi. The average values of 12 infected plants are represented. Bars represent standard error. Asterisks indicate a statistically significant difference between untreated and treated samples with *p-value < 0.05, according to a Student's t-test. **(B)** Expression of JA-responsive genes upon exogenous MeJA treatment in *Arabidopsis* plants. Mock- (pBINX') and TYLCV-infected plants were treated with 50 µM MeJA (+JA) or mock solution (-JA); JA responsive genes *AtAOS* (At5g42650), *AtJAZ8* (At1g30135), and *AtJAZ10* (At5g13220) were quantified by real-time RT-qPCR at 17 d post-treatment. The average values of the three plants are represented. Bars represent standard error. Asterisks (between –JA vs. +JA or pBIN vs. TYLCV) indicate a statistically significant difference compared to the relevant control (***p-value < 0.005; **p-value < 0.01; *p-value < 0.05), according to a Student's t-test. Numbers inside the grey bars indicate mRNA fold change accumulation upon JA treatment (+JA) compared to mock control (-JA). Three independent experiments were performed in A and B with similar results; results from one representative replicate are shown. Four- to 5-week-old *Arabidopsis* plants were agroinoculated with TYLCV and, at 2 d post-inoculation (dpi), sprayed every other day with 50 µM MeJA (+JA) or mock solution (-JA) (0.5% ethanol in water (v/v)).

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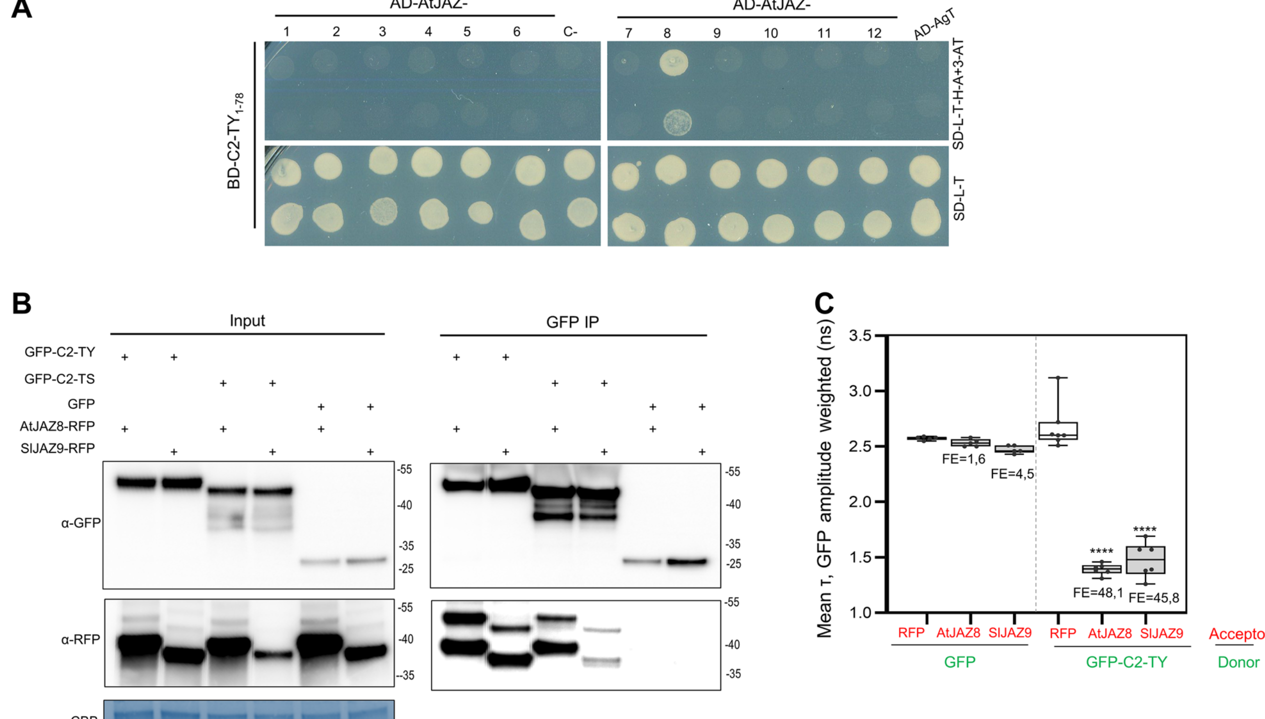

Figure 2. C2 from tomato yellow leaf curl virus (TYLCV) interacts with JAZ8 from *Arabidopsis* and JAZ9 from tomato
(A) Yeast two-hybrid assay to determine the interaction between C2 (amino acids from 1 to 78, fused to BDGal4) from TYLCV and JASMONATE ZIM-DOMAIN (JAZ) proteins (from JAZ1 to JAZ12, fused to ADGal4). Growth on plasmid-selective media (SD-L-T, minimal media lacking leucine (L) and tryptophan (T)) and interaction-selective media (lacking adenine (A), histidine (H) and with 3-AT, SD-L-T-H-A + 3-AT) are shown (10-fold dilution). The interaction of BD-C2 with a fragment of SV40 large T-antigen (AD-AgT) was used as negative control to discard the possible autoactivation of BD-C2. C(-) corresponds to yeast expressing two proteins that do not interact (BD-lamC and AD-AgT, negative control for interaction in Y2H assay, Clontech). **(B)** Co-immunoprecipitation analysis of C2 from tomato yellow leaf curl virus (C2-TY) and tomato yellow leaf curl Sardinia virus (C2-TS) with AtJAZ8 and SlJAZ9 from *Arabidopsis* and tomato, respectively, upon transient co-expression in *N. benthamiana* leaves. Molecular weight is indicated. CBB: Coomassie brilliant blue. **(C)** Interaction between 35S:GFP-C2-TY (donor) and 35S:AtJAZ8/SlJAZ9-RFP (acceptors) by FRET-FLIM upon transient co-expression in *N. benthamiana* leaves. 35S:GFP and 35S:RFP are used as a donor and acceptor controls, respectively. FE: FRET efficiency. Asterisks indicate statistically significant different samples from the control sample (*****p*-value < 0.0001; ***p*-value < 0.01 **p*-value < 0.05), according to a Student's *t*-test. FRET-FLIM analysis was performed using a Stellaris 8 Leica confocal microscope.

2019a; Oblessuc et al., 2020; Yang et al., 2020). To further investigate the mechanism conferring specificity to the C2-mediated interference with JA responses (Rosas-Díaz et al., 2016), we analyzed if C2 from TYLCV (C2-TY) was able to interact with JAZ proteins. For this purpose, we tested the interaction between $C2_{1-78}$, a truncated version of the C2 protein lacking the autoactivation domain (Lozano-Durán et al., 2011a), and 12 members of the JAZ family from *Arabidopsis* by yeast two-hybrid (Y2H). The results showed that $C2_{1-78}$ interacts with AtJAZ8 (Figure 2A). To confirm this interaction *in planta*, and determine if the interaction is conserved in the natural agriculturally relevant host, tomato, we included in the analyses the closest tomato orthologue of AtJAZ8, SlJAZ9 (Figure S2), and conducted co-immunoprecipitation (Co-IP, Figure 2B) and Förster resonance energy transfer-fluorescence lifetime imaging microscopy (FRET-FLIM, Figures 2C, S3) assays using transient expression in *Nicotiana benthamiana*.

For Co-IP, *N. benthamiana* leaves were co-infiltrated with constructs to express JAZ proteins (RFP-tagged at the C-terminus) and the geminiviral C2 protein (GFP-tagged at the N-terminus). As a negative control, leaves were co-infiltrated with a construct to express free GFP. As shown in Figure 2B, GFP-C2-TY associate with AtJAZ8-RFP and SlJAZ9-RFP, while free GFP does not. In the input, we observed one intense band corresponding to the predicted size of JAZ proteins fused to RFP (around 40 kDa) and a heavier but faint band (approximately 50 kDa) that could correspond to post-translational modifications by ubiquitin or SUMO, previously detected in JAZ proteins (Gough and Sadanandom, 2021). Notably, JAZ proteins co-immunoprecipitated with C2-TY showed these two bands with similar intensities. Similar results were obtained by Y2H (Figure S4A), Co-IP (Figure 2B), and FRET-FLIM (Figure S4B) using the C2 protein from TYLCSaV (C2-TS), which produces a suppression of jasmonate response when expressed in *Arabidopsis* plants (Rosas-Diaz et al., 2016) and presents a limited homology to C2-TY in the C-terminal region (Figure S1). FRET efficiency, used to quantify the strength of protein-protein interactions, shows that C2 from

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TYLCV interacts with JAZ proteins more strongly (Figures 2C, S3) than C2 from TYLCSaV (Figure S4B).

C2 from TYLCV co-localizes with the transcriptional repressor JAZ8 from *Arabidopsis* and its tomato orthologue in the nucleus

To evaluate the subcellular localization of the viral proteins and the transcriptional repressors to determine if their individual localization changes when both are present, we transiently expressed them, alone or in combination, fused to fluorescent proteins in *N. benthamiana* leaves (western blot for confirmed protein accumulation is available in Figure S5).

As previously shown, C2 from TYLCV (C2-TY) (GFP-tagged at the N-terminus) is localized in the nucleoplasm but excluded from the nucleolus (Figure 3; Wang et al., 2022). Like the viral protein, both JAZ proteins localize in the nucleoplasm. AtJAZ8 accumulates as aggregates in the nucleoplasm and is excluded from the nucleolus, while SlJAZ9 is homogenously distributed in the nucleoplasm and present in the nucleolus.

Interestingly, the sub-nuclear localization of C2-TY changes when it is co-expressed with either JAZ protein, colocalizing with the plant proteins in discrete nuclear speckles and forming aggregates all over the nucleoplasm (Figure 3).

The localization of AtJAZ8 is not altered by C2-TY, while the accumulation of SlJAZ9 is modified to form aggregates in the nucleoplasm similar to those observed for AtJAZ8 (Figure 3). To determine whether the presence of the virus alters JAZ subcellular localization, JAZ proteins were visualized in the context of a TYLCV infection (Figure S6). Similar to the results obtained upon co-expression with C2-TY, the localization of AtJAZ8 does not change in the presence of the virus. SlJAZ9, however, is redistributed, showing a more significant accumulation in the nucleolus compared to the nucleoplasm.

These results indicate that C2 from begomoviruses co-localizes with both AtJAZ8 and SlJAZ9 in the nucleoplasm, forming nuclear speckles of undetermined identity.

Changes in *JAZ8* expression do not lead to obvious developmental alterations in *Arabidopsis*

To study the relevance of AtJAZ8 in the repression of JA responses, we characterized *Arabidopsis* lines altered in *AtJAZ8* expression. To reach this aim, we used a previously described over-expressing transgenic line, *35S:AtJAZ8-GUS* (*JAZ8*-OX, Shyu et al., 2012), that accumulates 20 times more *JAZ8* transcript than the wild type (WT) (Figure 4B), and generated *AtJAZ8* knock-down lines expressing the precursor of an artificial miRNA targeting the *AtJAZ8* transcript (amiRNA*JAZ8*; hereafter referred to as amjaz8 plants) (Figure 4A). Two amjaz8 homozygous lines (Line 19 and 20) showing a drastic reduction of the *AtJAZ8* transcript accumulation (between 87% and 97% compared to the WT; Figure 4B) were selected and used for further analysis. As previously reported by Shyu et al. (2012), the increase in *AtJAZ8* expression does not significantly affect plant morphology in young plants. Similarly, no noticeable differences were observed in the transgenic plants showing lower accumulation of *AtJAZ8* either (Figure 4C). However, the amjaz8 lines seem to have a slightly shorter inflorescence stem than WT and *JAZ8*-OX plants (Figure S7).

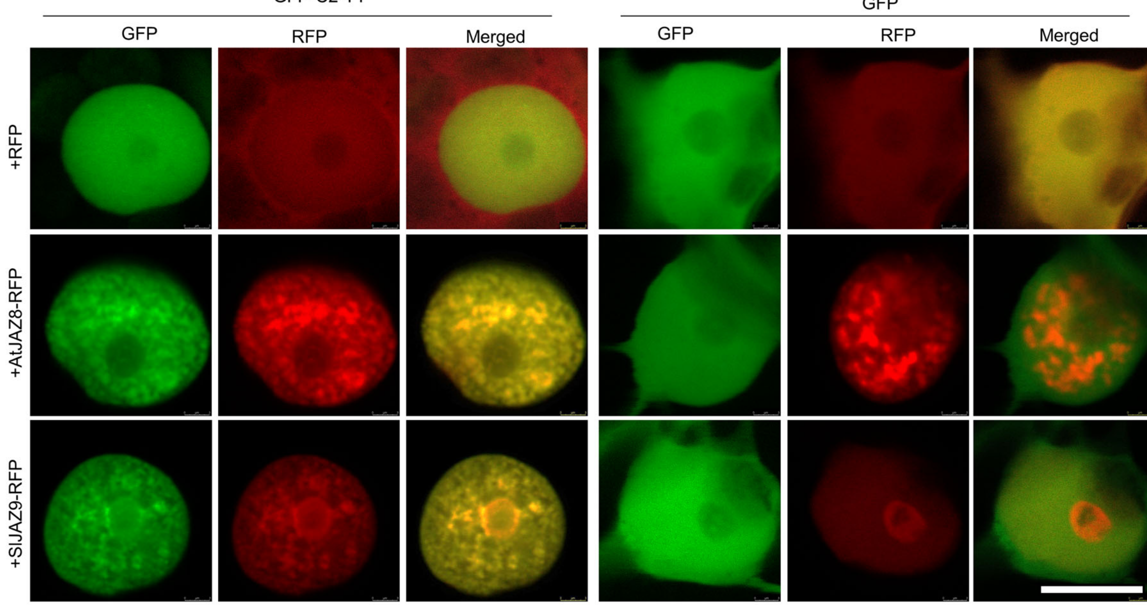

Figure 3. AtJAZ8 and SlJAZ9 co-localize with C2 from tomato yellow leaf curl virus (TYLCV) in the nucleus
Subcellular co-localization of 35S:GFP-C2-TY and 35S:AtJAZ8/SlJAZ9-RFP upon transient co-expression in *N. benthamiana* leaves at 2 d post-infiltration. Subcellular co-localization of 35S:GFP and 35S:AtJAZ8/SlJAZ9-RFP is used as a control. GFP signals are shown in green, RFP signals in red. Co-localization of GFP and RFP signals is visualized in yellow. Images were taken with Stellaris 8 Leica with a 40× objective confocal microscope. Bar represents 10 μm.

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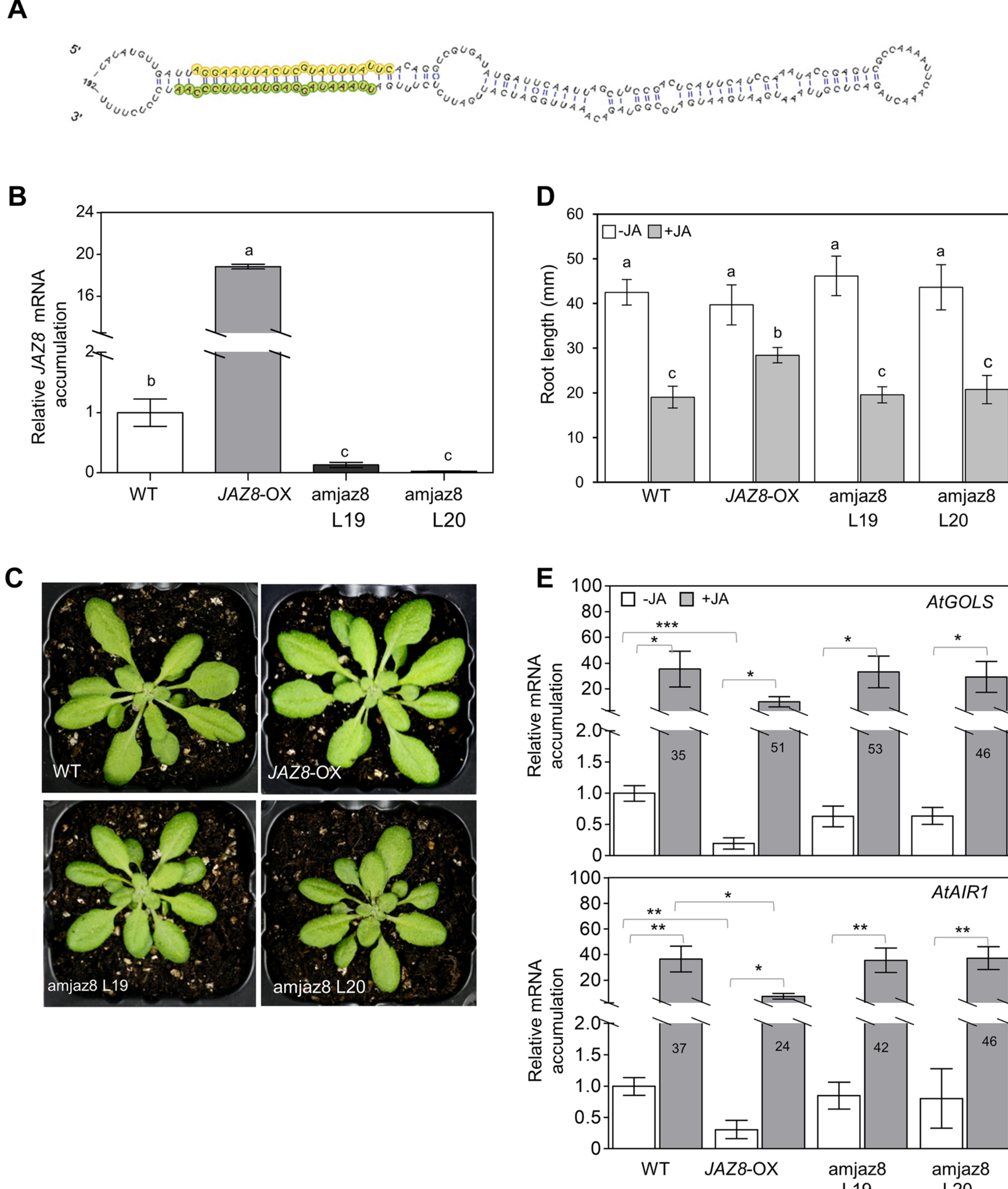

Figure 4. Changes in *JAZ8* expression do not lead to obvious developmental alterations in *Arabidopsis*

(A) Generation of *Arabidopsis JAZ8*-knockdown line using artificial microRNAs. Secondary structure of the artificial micro RNA precursor targeting *JAZ8* (amjaz8). The mature amjaz8 and amjaz8* sequences are highlighted in green and yellow. The secondary structure was predicted using Mfold (Zuker, 2003) and visualized using varna (http://varna.lri.fr/). **(B)** Relative *JAZ8* mRNA levels in wild type (WT), 35S:*JAZ8* (*JAZ8*-OX) and amjaz8 seedlings. Total RNA was extracted and subjected to real-time quantitative RT-PCR (RT-qPCR) analysis to measure the *JAZ8* mRNA levels normalized to *ACTIN 2*. Values are represented as the relative expression compared to WT. Bars represent the mean ± SD for three different pools of 8–10 seedlings. Lowercase letters indicate significant differences among columns according to one-way ANOVA followed by Dunnet's multiple comparison test (*p*-value < 0.05). Experiments were repeated twice with similar results; results from one representative experiment are shown. **(C)** Pictures of 4-week-old *Arabidopsis* plants 35S:JAZ8 (*JAZ8*-OX) and two independent amjaz8 homozygous lines (L19, L20) compared with wild type (WT) plants. **(D)** Root growth inhibition assays in 35S:JAZ8 (*JAZ8*-OX), amjaz8, and wild type (WT) *Arabidopsis* seedlings grown in 50 µM MeJA (+JA) or mock media (-JA) (0.5% ethanol in water (v/v)). **(E)** Relative expression levels of *AtGOLS* (At2g47180) and *AtAIR1* (At4g12550) genes in seedlings from (A), determined by real-time RT-qPCR. mRNA levels were normalized to *ACTIN. 2*. Values are represented as the relative expression compared to WT. Bars represent the mean ± SD for three different pools of 8–10 seedlings. Asterisks (between −JA vs. +JA, -JA vs. -JA or +JA vs. +JA) indicate a statistically significant difference compared to the relevant control (****p*-value < 0.005; ***p*-value < 0.01; **p*-valuev < 0.05), according to a Student's *t*-test. Numbers inside the grey bars indicate mRNA fold change accumulation upon JA treatment (+JA) compared to mock control (-JA). Experiments were repeated twice with similar results; results from one representative experiment are shown.

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Overexpression of *AtJAZ8* affects the sensitivity to exogenous JA (Shyu et al., 2012). To determine whether the reduction of *AtJAZ8* expression has a similar effect, we carried out a growth inhibition assay using amjaz8 plants. *JAZ8*-OX plants were used as control. The results showed that the reduction of *AtJAZ8* transcripts does not alter the root growth inhibition produced by exogenous JA application (Figures 4D, S8).

Our previous work indicated that the induction of several jasmonate-responsive genes was partially suppressed in transgenic plants expressing C2 from TYLCSaV (Rosas-Díaz et al., 2016). To determine whether changes in *AtJAZ8* expression affect the expression of two of those genes, *AtGOLS* and *AtAIR1*, we measured the accumulation of their transcripts in the *JAZ8*-OX and amjaz8 lines. No significant changes were observed in the amjaz8 lines, either JA-treated or untreated. However, *JAZ8*-OX plants showed an apparent reduction in the expression of these marker genes in both treated and untreated plants, suggesting that overexpression of *AtJAZ8* mimics the effect of *C2* expression (Figure 4E).

Of note, when analyzing an *AtJAZ8* line containing a T-DNA insertion in the gene promoter (*jaz8-1*), previously described as a *jaz8* knock-down line (Jiang et al., 2014) (Figure S9), we found that homozygous T-DNA plants over-accumulate *AtJAZ8* transcripts (Figure S9D), but their JA-induced inhibition of root growth is not altered (Figure S9E, F). Although we do not know the cause of the differences in the JA-response phenotype observed in both overexpressing lines (*JAZ8*-OX and *jaz8-1*), a dose-effect could be hypothesized since *AtJAZ8* transcript accumulation is comparatively four times higher in *JAZ8*-OX plants (Figures 4A, B, S9D).

JAZ8 limits TYLCV infection in *Arabidopsis*.

Under natural conditions, TYLCV is transmitted by the whitefly *Bemisia tabaci*. However, most of the viral infection experiments performed in laboratory conditions are carried out using *Agrobacterium*-mediated inoculation. Considering that alterations in the salicylic acid (SA) and jasmonate-mediated signaling pathways could affect the efficiency of *Agrobacterium*-mediated T-DNA transfer (Rosas-Díaz et al., 2017), we tested the competence of T-DNA transfer in WT, *JAZ8*-OX, and amjaz8 lines. For this purpose, leaves were infiltrated with *Agrobacterium* containing a binary plasmid with a GUS-intron construct (Zipfel et al., 2006), which allows expression of the reporter in plants but not in bacteria. We included the *Arabidopsis NahG* transgenic plants, which have enhanced susceptibility to *Agrobacterium*-mediated transient transformation (Rosas-Díaz et al., 2017), as control. As shown in Figure S10, in control WT leaves, only weak GUS staining was detectable at 4 d post-inoculation (dpi); *JAZ8*-OX and amjaz8 leaves showed similar GUS staining to WT plants, while *NahG* leaves exhibited stronger staining, as expected. This result reveals that neither overexpression nor lack of *JAZ8* alters *Agrobacterium*-mediated transient transformation in *Arabidopsis*. Next, to evaluate the relevance of AtJAZ8 to geminiviral infection, we agroinoculated *JAZ8*-OX and amjaz8 lines with a TYLCV infectious clone, since TYLCSaV is unable to infect this plant species (Cañizares et al., 2015). As shown in Figure 5, the viral accumulation in *JAZ8*-OX at 21 dpi was similar to that in WT plants, but, strikingly, viral accumulation was significantly higher in both amjaz8 lines (Figure 5). Therefore, knock-down of JAZ8 favors viral accumulation, suggesting that this transcriptional repressor limits the infection in natural conditions.

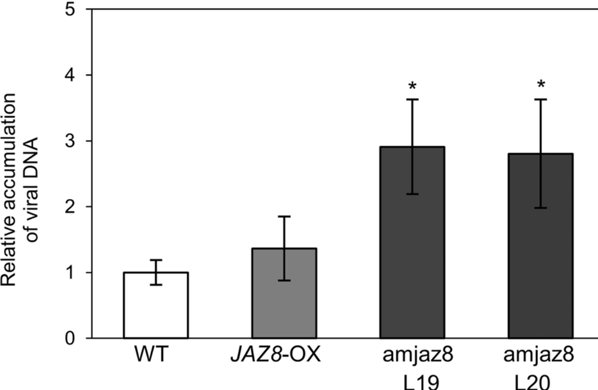

Figure 5. amjaz8 plants are more susceptible to tomato yellow leaf curl virus (TYLCV) infection

Four- to 5-week-old wild type (WT), 35S:*JAZ8* (*JAZ8*-OX), and amjaz8 lines *Arabidopsis* plants were agroinoculated with a TYLCV infectious clone. The relative accumulation of viral DNA was determined by quantitative real-time PCR of total DNA extracted from whole plants at 21 d post-inoculation (dpi). Average values of 8–10 infected plants are represented. Bars represent standard error. Asterisks indicate statistically different samples from the control sample (*p-value < 0.05) according to a Student's t-test. Three independent experiments were performed with similar results; results from one representative replicate are shown.

JAZ8 does not affect the insect vector's performance in the plant.

The whitefly *B. tabaci* suppresses jasmonate-mediated defenses in *Arabidopsis* (van de Ven et al., 2000; Kempema et al., 2007; Zarate et al., 2007; Walling, 2008). Moreover, *Arabidopsis* and tomato plants impaired in JA-defenses show increased oviposition and enhanced development of the whitefly nymphs (Sanchez-Hernandez et al., 2006; Zarate et al., 2007; Zhang et al., 2013; Zhang et al., 2018). Since a plethora of viral proteins have emerged as manipulators of JA-mediated responses (Westwood et al., 2014), a scenario where the impairment of this pathway improves the performance of viral insect vectors, hence viral propagation, has been proposed (reviewed in Csorba et al., 2015; Zhang et al., 2018; Wu and Ye, 2020). To test whether JAZ8 also plays a role in the *Arabidopsis* response to whiteflies, we examined the effect of JAZ8 on insect performance, determining whitefly survival and fecundity on *JAZ8*-OX, amjaz8, and WT plants. Per each assay, 10 adult whiteflies (five males and five females) were collected and released on the *Arabidopsis* plants; 7 d later, the survival of adults and the number of eggs on

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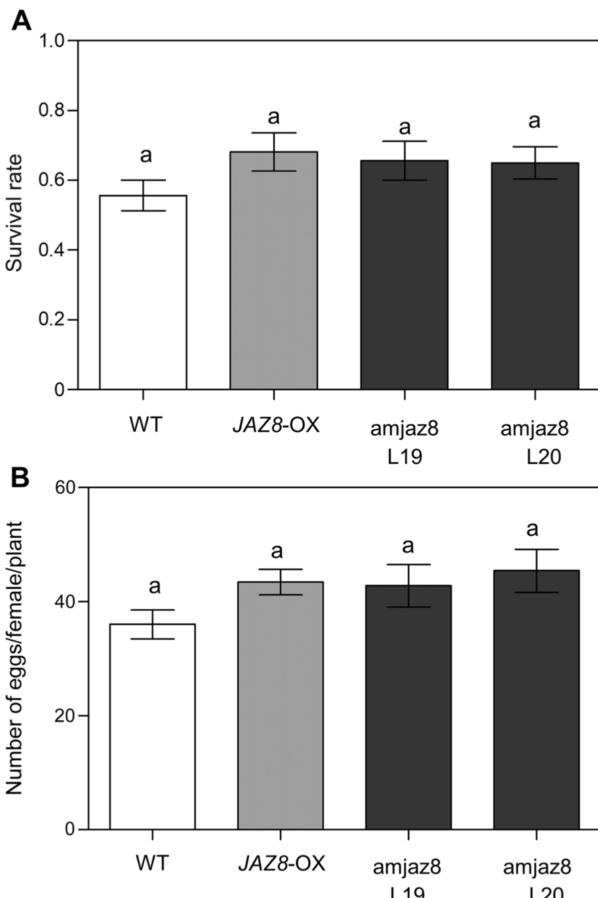

Figure 6. Changes in *JAZ8* expression have no effect on the performance of the virus' insect vector, the whitefly *Bemisia tabaci*, in *Arabidopsis*.
Performance of whitefly adults on the different *Arabidopsis* genotypes. Seven days after the release of whitefly adults, survival of adults **(A)** and number of eggs per female **(B)** on wild-type control (WT), 35S:*JAZ8* (*JAZ8*-OX), and amjaz8 plants were compared. Values are means ± SE, $n = 16$. Different letters denote significant differences (Student's t-test, p-value < 0.05).

all three genotypes were quantified. Our tests showed that neither enhanced accumulation nor depletion of the *AtJAZ8* transcript significantly affected the survival and fecundity of whiteflies (Figure 6).

DISCUSSION

Besides its impact on the begomovirus vector fitness, jasmonate responses also positively affect the plant antiviral defense. Our results with jasmonate-treated plants infected by TYLCV confirmed that induction of jasmonate-response negatively affects viral accumulation (Lozano-Durán et al., 2011a; Jia et al., 2016; Chakraborty and Basak, 2019). The relevance of this hormonal response against geminiviruses is further supported by the identification of viral proteins able to hinder it: at least two geminiviral proteins have evolved mechanisms to reduce the intensity of the jasmonate response. In begomoviruses associated with betasatellite, a satellite-encoded protein, βC1, interferes with this pathway at several levels: (i) reducing the JAZ degradation by impairing the integrity of SCF^{COI1} complex through its interaction with SKP1 (Jia et al., 2016); (ii) suppressing of JA-responsive genes involved in defense against insects by interaction with the ASYMMETRIC LEAVES 1 (AS1) (Yang et al., 2008); or (iii) blocking the activation of terpene synthase genes by binding the basic helix-loop-helix transcription factor MYC2 (Li et al., 2014). In geminiviruses not associated with betasatellite, the virus-encoded C2 protein may play a similar role in limiting the jasmonate-response. C2 impacts jasmonate signaling by at least two mechanisms: (i) obstructing the correct assembly and disassembly of SCF E3 ligases, which depend on the activity of the CSN complex, and, therefore, must affect the function of all hormone responses dependent on SCF function (Lozano-Durán et al., 2011a); (ii) indirectly compromising the degradation of JAZ1, and therefore impairing the JAZ1-dependent plant defense responses (Li et al., 2019a).

The JA signaling pathway regulates multiple plant processes, including development, growth, and defense. The F-box protein COI1 perceives jasmonates, but the complexity of the response is orchestrated by the degradation of JAZ repressors and the release of numerous TFs, including MYC2 and its homologues. Although JAZ proteins were initially assumed to be functionally redundant, differential expression patterns, protein interactions, sensitivity to COI1-dependent degradation, and altered JA responses in several loss- and gain-of-function mutants suggest that they also have specific functions (Chini et al., 2016; Goossens et al., 2016). The functional diversity of the JAZ proteins and the fact that other pathogen-derived proteins from RNA viruses (Wang et al., 2014; Wu et al., 2017; Yang et al., 2020; Li et al., 2021a), bacteria (reviewed in Schreiber et al., 2021), or oomycetes (Mukhtar et al., 2011) interact with JAZs, brought us to explore those transcriptional regulators as potential additional targets of the multifunctional C2 protein. Although C2 could indirectly compromise the degradation of AtJAZ1 (Li et al., 2019a), a direct interaction of C2 with the JAZ proteins has not been reported.

We show here that C2 interacts with one member of the group IV of JAZ proteins, AtJAZ8 from *Arabidopsis*, and its closest orthologue in tomato, SlJAZ9 (Figures 2, S3, S4). The fact that viral accumulation is higher in *AtJAZ8*-silenced plants indicates that the presence of JAZ8 limits the infection in natural conditions. Notably, overexpression of *JAZ8* does not alter viral accumulation, suggesting that JAZ8 is not rate-limiting for its antiviral effect, or that other rate-limiting factors are involved in this process. Like all JAZ proteins, JAZ8 modulates the expression of downstream genes by interacting with multiple transcription factors, including two members of the NAC (NAM, ATAF1/2, and CUC2) family of proteins (ANAC062 and ANAC091) (Altmann et al., 2020) that function as regulators of defense-related genes (Bian et al., 2021). ANAC091, which is involved in hypersensitive

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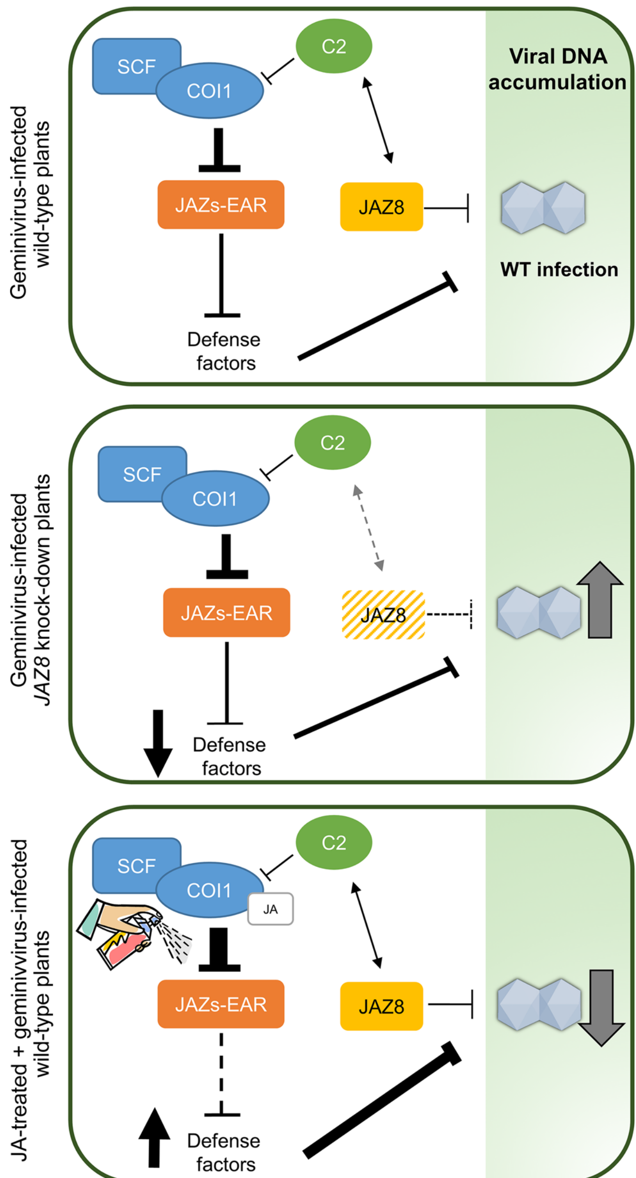

Figure 7. Tentative model to explain the impact of JA-induced responses on the geminivirus infection

(A) When plants are infected with geminiviruses, C2 interferes with the jasmonate-dependent response to the viral propagation through impairing the function of SCF complexes and so affecting all COI1-dependent responses, and it also physically interacts with the JAZ8 protein. The result is a level of viral accumulation in the plant that we consider a wild-type infection. **(B)** The fact that *JAZ8* silencing favors the viral infection indicates that JAZ8 is negatively impacting the viral ability to infect the plant. Whether this negative effect of JAZ8 depends on the expression of JAZ8-regulated plant genes or is rather a consequence of a direct inhibitory effect of JAZ8 over C2, which gets released when the former is silenced, remains to be determined. Finally, when JA is applied **(C)**, the C2-mediated suppression of SCF function is overridden, and there is a burst of expression of defense-related genes controlled by JA-sensitive JAZ proteins, while the expression of the JAZ8-dependent genes remains unaffected. This induction of defense-related genes negatively affects the viral infection, which explains the reduction in viral accumulation observed in JA-treated plants. Line width is proportional to the intensity of the effect. Dashed lines represent absence of effect.

response-mediated resistance to turnip crinkle virus (TCV) (Ren et al., 2000), functions through transcriptional activation to promote a basal level of resistance in the plant, while ANAC062 induces a group of *PR* genes, including *PR1*, *PR2*, and *PR5*, in an SA-independent manner. In accordance with this mechanism, overexpression of an active NAC062 form exhibited enhanced disease resistance to the plant pathogenic bacterium *Pseudomonas syringae* (Seo et al., 2010). Those NAC genes may participate in the plant response to geminivirus infection: infection with cabbage leaf curl virus induced the expression of *ANAC062* in *Arabidopsis* (Ascencio-Ibáñez et al., 2008), while TYLCV infection of tomato plants altered the expression of a large number of annotated *NAC* genes, including induction of *ANAC062/091* tomato homologues (*SlNAC25* and *SlNAC55*) (unpublished data and Huang et al., 2017).

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Considering these results, we propose a model to explain the impact of the JA-induced response on the geminivirus infection (Figure 7). Jasmonate-dependent genes involved in plant defense are mainly controlled by the JAZ proteins sensitive to degradation mediated by the COI1-JA complex. In contrast, JAZ8, which hampers the geminiviral infection, cannot associate strongly with COI1 in the presence of JA-Ile. When plants are infected with a geminivirus, the virus-encoded C2 protein interferes with jasmonate-dependent response to the viral propagation by impairing the function of SCF complexes, and so affecting all COI1-dependent responses, and at the same time physically interacts with JAZ8 homologues. The result is a level of viral accumulation in the plant that we consider a wild-type infection. The fact that *JAZ8* silencing favors the viral infection indicates that *JAZ8* is negatively impacting the virus' ability to infect the plant. Whether this negative effect of JAZ8 depends on the expression of JAZ8-regulated plant genes or is a consequence of a direct inhibitory effect of JAZ8 over C2, which gets released when the former is silenced, remains to be determined.

When JA is applied, the inhibitory effect of C2 on SCF complexes is overridden, and there is a burst of expression of defense-related genes controlled by JA-sensitive JAZ proteins, while the expression of the JAZ8-dependent genes remains unaffected. This induction of defense-related genes might negatively affect the viral infection, which would explain the reduction in viral accumulation observed in JA-treated plants. Interestingly, the JAZ8 impact on the geminiviral infection seems to be specific, because amjaz8 plants were not affected in development or whitefly's fitness (Figures 4, 6, S7).

In summary, the data presented here, revealing that JAZ8 has an antiviral function and that it physically interacts with the geminivirus-encoded C2, offer additional support to the relevance of JA-dependent responses in plant defense against geminiviruses. However, the fact that SA-dependent responses also participate in setting up a defense program against geminiviruses (Li et al., 2019b; Medina-Puche et al., 2020) draws a complex scenario; further work will be required to clarify the specific contribution of each of these hormones and their interplay in anti-geminiviral defense.

MATERIALS AND METHODS

Microorganisms and general methods

Manipulations of *Escherichia coli* and nucleic acids were performed according to standard methods (Ausubel et al., 1989; Sambrook and Russell, 2001). *Agrobacterium tumefaciens* (*Agrobacterium*) GV3101 strain was used for the agro-infiltration in *Nicotiana benthamiana* and *Arabidopsis thaliana* (*Arabidopsis*) and for geminiviral infections in *Arabidopsis*. *Saccharomyces cerevisiae* strain AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, MEL1 GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ) was used for the yeast two-hybrid experiments.

Plant DNA extraction using the CTAB method was performed as described in Lukowitz et al. (2000). Plant RNA was isolated using TRIzol reagent (Invitrogen). GUS staining was performed according to the protocol previously described by Rosas-Díaz et al. (2017).

Plasmids and cloning

Plasmids are summarized in Table S1. Vectors from the pGWB and ImpGWB series were described in Nakagawa et al. (2007a, 2007b). pB7RWG2.0 was described in Karimi et al. (2002). pBIN19-35S::GUS was described in Zipfel et al. (2006).

AtJAZ8 (At1g30135) and *SlJAZ9* (Solyc08g036640.2) genes were amplified by PCR (without stop codon) and cloned into pENTR/D-TOPO vector (Invitrogen) using the primers listed in Table S2 generating TOPO-*AtJAZ8* and TOPO-*SlJAZ9*, respectively. pENTR/D-TOPO containing the *C2* open reading frame from TYLCV (with stop codon) was described in Wang et al. (2017a). For yeast two-hybrid assays (Y2H), the Gal4-based pGBKT7 (BD-Gal4) and pGADT7 (AD-Gal4) vectors (Clontech Laboratories Inc.) were used. The interaction between a fragment of 1 to 78 aa of C2 proteins (C2 $_{1\text{-}78aa}$) from TYLCV and TYLCSaV (fused to BD-Gal4, pGKBKT7) and twelve *A. thaliana* JAZ genes (from JAZ1 to JAZ12, fused to AD-Gal4, pGADT7) was tested. The bait and prey constructs are listed in Table S1. Nucleotides from 1 to 234 of C2 from TYLCV were amplified by PCR (with stop codon) with primers listed in Table S2. pGBKT7 was digested with *Nde*I/*Sal*I and C2 PCR-amplified was digested with *Nde*I/*Sal*I and fused to the C-terminus of Gal4 DNA-binding domain.

Plant materials and growth conditions

Arabidopsis plants accession Columbia (Col-0) wild type (WT), and mutant or transgenic derivatives were grown in growth chambers with 8 h light: 16 h dark cycles at 21°C. The T-DNA insertion *jaz8-1* line mutant (WiscDsLox255G12) was provided by the Nottingham *Arabidopsis* Stock Centre (NASC; http://www.Arabidopsis.info), transgenic *35S:JAZ8 and NahG* plants are described in Shyu et al. (2012) and Lawton et al. (1995), respectively.

Root growth inhibition assays in *Arabidopsis* seedlings were performed as described in Rosas-Díaz et al. (2016). *N. benthamiana* plants were grown in soil at 24°C in long-day conditions with a 16 h light: 8 h dark photoperiod.

Transient expression assays

Transient expression was performed in 4-week-old *N. benthamiana* leaves through *Agrobacterium* infiltration ($OD_{600} = 0.5$) as described in Rosas-Díaz et al. (2018). For co-IP clones expressing 35S:JAZ-RFP and 35S:GFP-C2 or 35S:GFP (used as negative control) were co-infiltrated. For FRET-FLIM and subcellular co-localization assays, clones expressing 35S:GFP-C2 and 35S:RFP-C2 were co-

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infiltrated with clones to express 35S:JAZ-RFP and 35S:JAZ-GFP, respectively (see "Plasmids and cloning"). As controls for FRET-FLIM, 35S:RFP was co-expressed either with 35S:GFP-C2 or 35S:JAZ-GFP. Samples were taken 2 d after infiltration.

Agrobacterium-mediated expression in *Arabidopsis* was performed as described in Rosas-Díaz et al. (2017).

Yeast two-hybrid assay

The bait and prey plasmids were transformed into *S. cerevisiae* strain AH109 as previously described (Gietz and Schiestl, 1994) and transformants were grown on plasmid-selective media (SD/-Trp-Leu). Two proteins previously shown to interact (BDGal4-p53 and ADGal4-AgT) and two that do not (BDGal4-lamC and ADGal4-AgT) were used as positive and negative controls, respectively (MATCHMAKER Handbook PT3062-1, Clontech). Plates were incubated at 28°C for 4 d and independent colonies for each bait-prey combination were resuspended in 200 μL of sterile water. Ten-fold serial dilutions were made and 5 μL of each dilution were spotted onto three alternative interaction-selective medium (SD/-Trp-Leu-His+3-AT (3-amino-1, 2, 4-triazole, 2 mM), SD/-Trp-Leu-Ade, and SD/-Trp-Leu-Ade-His+3-AT). Plates were incubated at 28°C and photographed 4 or 7 d later.

Co-immunoprecipitation and immunoblot analysis

Agrobacterium clones expressing 35S:JAZ-RFP and 35S:GFP-C2 or 35S:GFP were co-infiltrated. Two days after infiltration, 0.5 g of infiltrated *N. benthamiana* leaves were harvested. Protein extraction and Co-immunoprecipitation (Co-IP) were performed as previously described in Rosas-Díaz et al. (2018). Immunoblot analysis was performed as described in Rosas-Díaz et al. (2018) except that 0.5% (v/v) Triton X-100 (CAS: 9036-19-5, Sigma-Aldrich) was used instead of 2% of Nonidet P-40. The antibodies used are as follows: anti-GFP (Abiocode M0802-3a, sc-9996, Santa Cruz Biotechnology), anti-RFP (Chromotek 5F8, G6G, Chromotek), anti-Rabbit IgG (Sigma A0545), and anti-Mouse IgG (Sigma A2554).

Confocal imaging

N. benthamiana plants were agroinfiltrated with clones to express GFP- or RFP-tagged proteins. Infiltrated tissues were imaged 2 or 3 d later using the confocal laser scanning microscope described in the figure's footnotes. Preset settings for GFP with Ex: 489 nm, Em: 500–550 nm, and for RFP with Ex: 554 nm, Em: 580–630 nm were used. Confocal images were processed using ImageJ software.

FRET-FLIM analysis

Leaf discs of *N. benthamiana* plants transiently coexpressing donor and acceptor, as indicated in the figures, were visualized 2 d after infiltration. Accumulation of the GFP- and RFP-tagged proteins was confirmed before measuring lifetime. Fluorescence confocal imaging and fluorescence lifetime imaging were conducted using a scanning confocal microscope Stellaris 8 FALCON Leica with a 40× objective lens (HC PL APO CS2 40x/1.30 OIL) except for the FRET-FLIM assay described in Figure S4B of which the analysis was performed as described previously (Rosas-Díaz et al., 2018).

A picosecond pulsed laser lines (489 nm) from a white light laser system were used as excitation sources. A hybrid photon detector (HyD S3) was used to collect emissions in wavelength for pinhole airy calculation of 580 nm and a frame average of 5. Fluorescence confocal and lifetime images consisted of 512 × 512 pixels were simultaneously recorded using a galvo-stage and time-correlated single-photon counting technique. All data manipulations were performed using the Leica suited software (LAS X Ver. 4.5.0.25531). Average lifetime values were calculated by the amplitude-weighted method using the data from the biexponential fit (GFP-fused donor protein with free RFP acceptor or with interacting RFP-fused acceptor protein). The region of interest (ROI) was set in the nuclei cells. The mean of donor lifetimes are presented as means ± *SD* based on 6–10 cells from two or three independent experiments. FRET efficiency was calculated according to the formula $E = 1 - \tau DA/\tau D$, where τDA is the average lifetime of the donor in the presence of the acceptor and τD is the average lifetime of the donor in the absence of the acceptor.

Geminivirus infection assays

TYLCV infection of *Arabidopsis* plants was performed by agroinoculation (Lozano-Durán et al., 2011a). Plants were agroinoculated with pBINX' (mock) (Sanchez-Duran et al., 2011) or TYLCV infectious clone (Navas-Castillo et al., 1999). Samples were taken at 21 dpi. Viral DNA accumulation was quantified by quantitative real-time PCR with primers listed in Table S2. JA treatments for geminiviral infection experiments were carried out as done by Lozano-Durán et al. (2011a).

For TYLCV local infection assays, fully expanded young leaves of 4-week-old *N. benthamiana* plants were infiltrated with *Agrobacterium* carrying a TYLCV infectious clone (Rosas-Díaz et al., 2018) or an empty binary vector as negative control. Samples were collected 3 d later to detect viral accumulation by PCR. To avoid the DNA originated from *Agrobacterium* we used primers to amplify episomal TYLCV replicons (see Table S2).

Quantitative Real-Time PCR (RT-qPCR)

cDNA preparation and RT-qPCR were performed as previously described by Rosas-Díaz et al. (2016). Primers are listed in Table S2.

Construction of *AtJAZ8* artificial miRNA and generation of transgenic plants

To generate the 21nt artificial microRNA (amiRNA) against *AtJAZ8* (amjaz8), we used Web MicoRNA Designer 3 (WMD3) (Ossowski et al., 2008). Using WMD3 we obtained the sequence of the presumably best amiRNA candidate and the primers used to amplify this sequence (Table S2). Using PCRs and overlapping PCRs as indicated in WMD3 protocol, we cloned the 21nt sequence of interest into the miR319 precursor backbone. The

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PCR fragment was gel-purified using Wizard SV gel and PCR Clean-up System (Promega), ligated into pGEM-T (Promega, USA), and checked by sequencing. Finally, this construct was subcloned into the pBINX' plasmid using *Sal*I (Takara, Japan) and *BamH*I (Takara, Japan). pBINX'-am*jaz8* or the empty vector (EV) were transformed into *Agrobacterium*. *Arabidopsis* plants were transformed using the floral dipping method (Clough and Bent, 1998). Transgenic seeds were selected on ½ standard Murashige and Skoog medium containing Kanamycin (50 mg/L). T3 antibiotic-resistant transformed plants were verified by sequencing. Repression of *JAZ8* was verified on T3 antibiotic-resistant transformed plants by RT-PCR and qPCR, using primers listed in Table S2.

Bemisia tabaci bioassays

The whitefly *B. tabaci* MEAM1 (mtCOI GenBank accession: GQ332577) was maintained on 6–7th true-leaf stage cotton (*Gossypium hirsutum*) in a climate-controlled chamber with 14 h light: 10 h dark cycles at 27 ± 1°C, 70% ± 10% RH. Three days post-emergence, 10 adult whiteflies (five males and five females) were collected, and then released into a modified Lock&Lock Box with a host *Arabidopsis* placed inside. Seven days later, the survival of adults was recorded, and all the eggs laid were counted to assess host plant suitability. Sixteen replicates of this experiment were conducted.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

T.R.-D., P.C.-Q., G.F.-B., M.W., A.P.-M., A.G.-C., and H.D. performed the experiments. R.S., R.L.-D., E.R.-B. have planned and directed the experimental design of the work. T.R.-D., R.L.-D. and E.R.-B wrote the paper. AC-G participated in the discussion and experimental design of the work. All authors participated in the critical reading of the manuscript and contributed to the article and approved the submitted version.

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SUPPORTING INFORMATION

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Figure S1. Sequence alignment of the C2 proteins from the begomoviruses TYLCV and TYLCSaV
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Table S1. List of plasmids used in this work
Table S2. List of primers for used in this work PCR and qPCR

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