1 **Geminivirus C4 proteins inhibit GA signaling via prevention**
2 **of NbGAI degradation, to promote viral infection and**
3 **symptom development in *N. benthamiana***

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Abstract

Phytohormone gibberellin (GA) is an important plant signaling molecule that regulates plant growth and defense against abiotic and biotic stresses. To date, the molecular mechanism of the plant responses to viral infection mediated by GA is still undetermined. DELLA is a repressor of GA signaling and is recognized by the F-box protein, a component of the SCF^{SLY1/GID2} complex. The recognized DELLA is degraded by the ubiquitin-26S proteasome, leading to the activation of the GA signaling. Here, we report that ageratum leaf curl Sichuan virus (ALCScV)-infected *N. benthamiana* plants showed dwarfing symptom and abnormal flower development. The infection of ALCScV alters the expressions of GA pathway-related genes and decreases the content of endogenous GA significantly in *N. benthamiana*. Further, ALCScV-encoded C4 protein interacts with the DELLA protein NbGAI, and interferes with the interaction between NbGAI and NbGID2 to prevent the degradation of NbGAI, leading to the inhibition of the GA signaling pathway. Silencing of *NbGAI* or exogenous GA₃ treatment significantly reduces viral accumulation and disease symptoms in *N. benthamiana* plants. The same results were proved by the experiments with C4 protein encoded by tobacco curly shoot virus (TbCSV). Therefore, we propose a novel mechanism of geminivirus C4 proteins controlling virus infection and disease symptom development through interfering GA signaling pathway.

Author Summary

Gibberellins (GAs) are plant hormones that are essential for many developmental

processes in plants. It has indicated that plant virus infection can induce abnormal flower development and influence GA pathway resulting the plant dwarfing symptom, but the underlying mechanisms is still not well described. Here, we demonstrate that geminivirus - encoded C4 protein regulates the GA signaling pathway to promote viral accumulation and disease symptom development. Through directly interacting with NbGAI, the C4 protein interferes with the interaction between NbGAI and NbGID2, which inhibits the degradation of NbGAI. As a result, the GA signaling pathway is blocked, and the infected plants display symptoms of typical dwarfing and delayed flowering. Our results reveal a novel mechanism by which geminivirus C4 proteins influence viral pathogenicity via interfering the GA signaling pathway, and provide new insights into the interaction between virus and host.

Introduction

Geminiviruses are a class of DNA viruses with circular single-stranded genomes and encode limited numbers proteins [1-4]. Geminiviruses are known to be capable of recruiting host factors to ensure their genome replications, virion assembly, movement, insect transmission, and other processes [5]. Plants infected with geminiviruses often develop disease symptoms such as yellow vein, leaf curling, enation, and curly shoots [4,6-8]. During virus-plant arms races, host plants have evolved specific proteins that can target various viral infection steps. On the other hand, viruses, including geminiviruses, have evolved specific strategies to hijack specific host factors to interfere host defense responses [9].

C4 proteins (also known as AC4 proteins of bipartite geminiviruses), encoded by monopartite geminiviruses in the genus *Curtovirus*, *Topocuvirus*, *Turncurtovirus*, and *Begomovirus*, are multifunctional proteins [10,11]. To date, multiple C4 proteins have been reported as disease symptom determinates that can disrupt host cell development to cause symptom formation [12-14]. Mutational analyses of C4/AC4 proteins have shown that the mutations can alleviate disease symptoms and suppress virus accumulation in infected plants [15]. It has shown that overexpression of *C4/AC4* in plants induces hyperplasia, callus-like tissues, and curly leaves, due to the changes of cell cycle-related gene expressions [13,16]. It was reported that C4 protein encoded by tomato leaf curl Yunnan virus (TLCYNV) can interact with NbSK η to reduce its nuclear accumulation. The cytoplasmic NbSK η is less phosphorylated and has a reduced ability to degrade cell cycle regulator NbCycD1;1 [17]. sweet potato leaf curl virus (SPLCV) was reported to interact with brassinosteroid-insensitive 2 (AtBIN2) to re-locate AtBIN2-interacting proteins, AtBES1/AtBZR1, into nucleus to induce abnormal plant development, via the activation of the BR signaling pathway [18]. Recently, C4 has been proved to suppress RNA silencing through its interaction with receptor-like kinase BARELY ANY MERISTEM 1 (BAM1) and BAM2. These interactions inhibit the functions of BAM1 and BAM2 to spread RNAi signal to adjacent cells [19]. cotton leaf curl Multan virus (CLCuMuV) C4 can interact with S-adenosyl methionine synthetase (SAMS) to inhibit its ability to suppress transcriptional and post-transcriptional gene silencing, resulting in a stronger CLCuMuV infection [20]. Geminivirus C4 can also regulate virus systemic infection in plants. For example, introduction of mutations into

beet severe curly top virus (BSCTV) C4 resulted in a systemic movement defective virus [21].

Gibberellins (GAs) are plant hormones that plays crucial roles in plant growth and development, and defense responses against abiotic and biotic stresses [22-24]. For example, GA defective mutant plants are stunted, and this stunted phenotype can be restored by spraying the plants with exogenous GA. Although GA-insensitive mutant plants also exhibit a dwarfing phenotype, similar to the GA defective mutant plants, this phenotype is not reversible through application of exogenous GA [25]. In contrast, mutant plants that produce constitutively activate GA showed elongated stems than the wild-type plants. The phenotype of this plant is, however, correlated with the content of GA. In the past two decades, the main elements involved in the GA signaling pathway, including GA receptor protein GID1 (GIBBERELLIN INSENSITIVE DWARF1), DELLA (aspartic acid–glutamic acid–leucine–leucine–alanine), F-box protein SLY1 (SLEEPY1), and SNZ (SNEEZY), have been identified through genetic screening of rice and *Arabidopsis* mutant lines [26].

DELLA is a member of the GRAS family and an inhibitor of plant growth and development, which is considered to function at the nexus in the GA signaling pathway to negatively regulate GA biosynthesis [27,28]. Like other GRAS family proteins, DELLA has a conserved C-terminal GRAS domain, necessary for the protein-protein interaction and transcriptional regulation, and an N-terminal DELLA domain. Deletion of the DELLA domain affects its binding to the GA receptor GID1 and results in a semi-dominant GA-insensitive dwarfing phenotype [29]. DELLA proteins of different

plant species, including *Arabidopsis*, wheat, corn, rice and barley, are highly conserved. *Arabidopsis* is known to encode five DELLA proteins (e.g., GAI, RGA, RGL1, RGL2 and RGL3) playing different, but redundant, functions in the inhibition of GA-dependent responses [30]. Whereas, tomato has only one DELLA gene, called *PROCERA* (*PRC*) [31].

Based on the transmission mode, GA can be divided into two major groups: the group utilizing the F-box protein-dependent transmission mode and the other group utilizing the F-box protein-independent transmission mode. After receiving the signal, the receptor protein GID1 binds the active GA and interacts with the negative regulatory DELLA to form a GA-GID1-DELLA complex. The DELLA is then recognized by the F-box protein in the SCF^{SLY1/GID2} complex and then degraded by the ubiquitin-26S proteasome, leading to the removal of the repressive effect of DELLA [32-36]. For those plants that lack of functional F-box proteins, large amount of DELLA will accumulate in cells, leading to an abnormal plant growth and development [37].

Ageratum leaf curl Sichuan virus (ALCScV) is a member in the genus *Begomovirus*, family *Geminiviridae*. ALCScV was first reported in *Ageratum conyzoides* in the Sichuan Province, China in 2018, and can cause severe symptoms on multiple plants [8,38]. Although ALCScV C4 has been discovered as a disease symptom determinant, the molecular mechanism underpinning this action is unclear. In this study, we have now determined that upon ALCScV infection, the expressions of GA pathway-related genes as well as the content of endogenous GA were significantly affected. Further analyses have revealed that ALCScV C4 can interact with NbGAI in

vitro and *in vivo*, which thwarts the interaction of NbGAI and NbGID2, thereby preventing degradation of NbGAI. In addition, silencing of *NbGAI* or spraying plants with exogenous GA₃ greatly enhanced *N. benthamiana* resistance to ALCSv infection. Meanwhile, tobacco curly shoot virus (TbCSV)-encoded C4 can also interact with NbGAI and silencing of *NbGAI* expression can inhibit TbCSV infection. In summary, we propose that one of the specific function of geminivirus C4 proteins is to interfere GA signaling pathway to promote virus infection and disease symptom development, a mechanism that has not been reported previously.

Results

ALCSv infection affects the expressions of GA pathway-associated genes and biosynthesis of endogenous GA

The ALCSv-infected *N. benthamiana* plants showed strong dwarfing and abnormal flower development, which were not observed in the mock-inoculated plants or the plants inoculated with ALCSv-mC4 (Fig 1A). The result of statistical analysis supported the symptom observations (S1 Fig). Because previous studies have demonstrated that GA can regulate plant height and flower development [39], therefore, the effect of ALCSv infection on GA biosynthesis and the expressions of GA signaling pathway-associated genes were investigated through qRT-PCR. The results showed that the relative expression levels of *NbGA20*, *NbGA-3β*, *NbPIF3* and *NbPIF4* were significantly down-regulated in the ALCSv-inoculated *N. benthamiana* plants (Fig 1B). Similarly, at 14 days post agroinfiltration (dpi), the content of endogenous

GA was also significantly decreased in the ALCScV-inoculated *N. benthamiana* plant (2.19 $\mu\text{g g}^{-1}$ FW) leaves compared to that in the mock- (3.04 $\mu\text{g g}^{-1}$ FW) or the ALCScV-mC4-inoculated *N. benthamiana* plant (2.77 $\mu\text{g g}^{-1}$ FW) leaves (Fig 1C). In a separate study, the plants inoculated with PVX-C4 also display significant dwarfing and delayed flowering (S1 Fig). As expected, the content of endogenous GA in the PVX-C4-inoculated plants (0.63 $\mu\text{g g}^{-1}$ FW) was significantly decreased compared to that in the PVX-inoculated plants (1.05 $\mu\text{g g}^{-1}$ FW) (Fig 1D). Consequently, we speculated that the C4 protein is a regulator of the GA pathway-associated genes as well as the biosynthesis of endogenous GA.

Fig 1. ALCScV infection influences gibberellin (GA) pathway in *N. benthamiana* plants. (A) Photographs of the mock-, ALCScV- and ALCScV-mC4-inoculated *N. benthamiana* plants, taken at 28 dpi. (B) qRT-PCR analyses of relative expressions of the GA pathway-associated genes in the mock- or ALCScV-inoculated *N. benthamiana* plants. ** indicates a significant difference between the two treatments at the $P < 0.01$ level, determined by the Student's *t*-test; * indicates a significant difference between the two treatments at the $P < 0.05$ level. This experiment had at least three plants per treatment. (C) and (D) Results of HPLC analyses showing the contents of endogenous GA in the PVX-, PVX-C4-, mock-, ALCScV-, and ALCScV-mC4-inoculated *N. benthamiana* plants at 14 dpi, respectively. Different letters above the bars indicate the significant differences ($p < 0.05$).

ALCScV C4 directly interact with NbGAI

To understand how ALCScV C4 regulates the GA pathway, Y2H assays was performed to screen host protein(s) that can interact with ALCScV C4. The result showed that a GA signaling pathway repressor, NbGAI, interacted with the C4 in yeast cells (Fig 2A). The GAI proteins belong to the family of GRAS transcription factors, of which only one member has been identified in Solanaceae plants [31]. To verify this interaction in *planta*, BiFC assay were performed, co-expressed of cYFP-C4 and nYFP-

NbGAI resulted in a YFP fluorescence signal in both nucleus and cytoplasm at 48 hours post incubation (hpi). No YFP fluorescence was observed in the leaves co-infiltrated with cYFP-C4 and nYFP or cYFP and nYFP-NbGAI (Fig 2B). Then the interaction between C4 and NbGAI was further confirmed by a Co-IP assays after transient co-expression of C4-His and NbGAI-GFP in *N. benthamiana* leaves (Fig 2C). Taken together, these results demonstrate that the ALCScV C4 protein directly interacts with NbGAI protein both *in vitro* and *in vivo*.

Fig 2. ALCScV C4 interacts with NbGAI *in vitro* and *in vivo*. (A) Result of Y2H assay showing the interaction between ALCScV C4 and NbGAI in yeast cells. Recombinant plasmids were co-transformed, in various combinations, into *S. cerevisiae* strain AH109 cells using the lithium acetate method. The transformants were 10-fold serially diluted and then grown on the SD/-Trp/-Leu or the SD/-Trp/-Leu/-His/-Ade selective medium. Images were taken at 72 hpi. (B) Results of BiFC assay showing the interaction between ALCScV C4 and NbGAI *in vivo*. Leaves co-expressing cYFP-C4 and nYFP, cYFP and nYFP-NbGAI, or cYFP-C4 and nYFP-NbGAI were examined and imaged under a confocal microscope equipped with an FITC filter. Bars = 50 μ m. (C) Result of Co-IP assay showing the interaction between ALCScV C4 and NbGAI *in vivo*. C4-His and NbGAI-GFP or C4-His and GFP (control) were co-expressed in *N. benthamiana* leaves. Total protein was extracted from the infiltrated leaf samples at 2 dpi. The input and the co-immunoprecipitated proteins were analyzed through Western blot analyses using an anti-GFP or an anti-His antibody.

The central region of NbGAI is responsible for the interaction with C4

The coding sequence of *NbGAI* contains 1701 nucleotides, and is predicted to encode a hydrophilic protein with 567 amino acids and of a molecular mass of about 62 kDa (<https://web.expasy.org/cgi-bin/protparam/protparam>). Amino acid sequence alignment using GAI sequences from *S. lycopersicum* (NP_001234365), *C. baccatum* (PHT30960), *C. maxima* (XP_022998067), *I. triloba* (XP_031108300), *N. benthamiana* (AMO02501), and *N. sylvestris* (XP_009796771) showed that like other

GAIs, NbGAI also contains two conserved domains (e.g., the N-terminal DELLA domain and the C-terminal GRAS domain) (S2A Fig). The phylogenetic tree also revealed that NbGAI is more closely related to the GAIs from other Solanaceae plants (S2B Fig).

To explore which region in NbGAI is responsible for the interaction with C4, five mutant plasmids were constructed based on the conserved domain prediction (Fig 3A). The Y2H assays showed that NbGAI-M2 (aa residue 98–566), but not the other four fragments, interacted with C4 (Fig 3B). This interaction was also confirmed *in planta* through BiFC assays (Fig 3C). These results suggest that the 98-566 amino acid positions of NbGAI is responsible for the interaction with ALCScV C4.

Fig 3. The middle region of NbGAI that is responsible for the interaction with ALCScV C4. (A) Schematic representations of NbGAI constructs. The deletion mutants were constructed based on the predictions of the conserved domains in NbGAI. (B) Result of Y2H assay showing the positive interaction between the C4 and NbGAI or C4 and NbGAI-M2. (C) Result of BiFC assay showing the interaction between the NbGAI-M2 and C4 *in planta*. Procedures of the Y2H and BiFC assays are similar to those described in Figure 2.

The interaction between NbGAI and NbGID2 causes the degradation of NbGAI via the ubiquitin-26S proteasome

Previous studies have shown that GAI and GID2 interaction results in the degradation of GAI and activation of GA signaling pathway [22]. In this study, the interaction between NbGAI and NbGID2 were analyzed via Y2H and BiFC assays. The result demonstrated that NbGAI did interact with NbGID2 in yeast cells (Fig 4A) and in *N. benthamiana* leaf cells (Fig 4B).

Because treatment of plants with exogenous GA₃ can promote the degradation of

GAI via the ubiquitin-26S proteasome [33,40], we investigated whether NbGAI degradation was also ubiquitin-26S proteasome dependent. cYFP-NbGID2 and nYFP-NbGAI was co-expressed transiently in *N. benthamiana* leaves, at 48 hpi, the green fluorescence signal was observed in the nuclei of the *N. benthamiana* leaf cells (Fig 4C). As expected, after the infiltrated leaves were treated with 50 μ M MG132 solution (MG132, a specific inhibitor of the 26S proteasome), the result showed that the YFP fluorescence intensity in the MG132-treated leaves was significantly stronger than that in the 0.8% ethanol solution (mock) treated leaves (Fig 4C and Fig 4D). In contrast, the YFP fluorescence intensity in the GA₃-treated leaves was significantly decreased compared to the mock-treated leaves (Fig 4C and Fig 4D). Together, these results indicated that the degradation pathway of NbGAI protein in *N. benthamiana* depends on the ubiquitin-26S proteasome.

Further analyses using various NbGAI deletion constructs indicated that the middle region of NbGAI, encompassing amino acid 98-566, is responsible for the interaction between NbGAI and NbGID2 (Fig 4E and Fig 4F).

Fig 4. The interaction between NbGAI and NbGID2 enhances the ubiquitin-26S proteasome-dependent degradation of NbGAI. (A) Result of Y2H assay showing the interaction between NbGAI and NbGID2. (B) Result of BiFC assay showing the interaction between NbGAI and NbGID2 in *N. benthamiana* leaf cells. Leaves co-expressing cYFP-NbGAI and nYFP, nYFP-NbGID2 and cYFP or cYFP-NbGAI and nYFP-NbGID2 were examined and imaged under a confocal microscope at 48 hpi. (C) Degradation of NbGAI is ubiquitin-26S proteasome-dependent. Fusion cYFP-NbGAI and nYFP-NbGID2 were transiently co-expressed in *N. benthamiana* leaves. The infiltrated leaves were sprayed with a solution containing 50 μ M MG132 at 12 h before Confocal Microscopy or with a solution containing 50 μ M GA₃ or 0.8% ethanol (control) at 2 h before Confocal Microscopy. (D) Fluorescence intensities from the BiFC assays were quantified based on the numbers of nuclei with GFP fluorescence. Different letters above the bars indicate the significant differences among the treatments at the $P < 0.05$ level. This experiment had at least three plants per treatment. (E) Result of Y2H assay showing the positive interaction between NbGAI-M2

and NbGID2. (F) Result of BiFC assay showing the positive interaction between NbGAI-M2 and NbGID2 *in planta*. Procedures of the Y2H and BiFC assays are similar to those described in Figure 2.

ALCScV C4 interferes the interaction between NbGAI and NbGID2

Because both ALCScV C4 and NbGID2 interacted with NbGAI-M2, but C4 and NbGID2 did not interact with each other (S3 Fig), we speculated that ALCScV C4 and NbGID2 may be competitively combined with NbGAI. To test this hypothesis, a competitive BiFC assay was performed. cYFP-NbGID2, nYFP-NbGAI and C4-His or cYFP-NbGID2, nYFP-NbGAI and pCV empty vector were co-expressed in *N. benthamiana* leaves through agroinfiltration. At 48 hpi, the result showed that the YFP fluorescence intensity was significantly decreased in the leaves co-expressing cYFP-NbGID2, nYFP-NbGAI and C4-His compared to that in the leaves co-expressing cYFP-NbGID2, nYFP-NbGAI and pCV empty vector (Fig 5A and Fig 5B). Meanwhile, the Western blot assay confirmed that C4-His was in deed expressed in the infiltrated leaves (Fig 5C). To further verify that ALCScV C4 can interfere the interaction between NbGAI and NbGID2, a competitive pull-down assay was conducted, and the result indicated that, in the presence of C4-His, the amount of NbGAI-GFP pulled down by NbGID2-Flag was significantly reduced (Fig 5D). In addition, as the amount of C4-His was gradually reduced, the amount of NbGAI-GFP pulled down by NbGID2-Flag was gradually increased, indicating the negative effect of C4 on the interaction between NbGAI and NbGID2.

Fig 5. ALCScV C4 can inhibit the interaction between NbGAI and NbGID2. (A) Result of

competitive BiFC assay showing the inhibitory effect of ALCSv C4 on the interaction between NbGAI and NbGID2. Fusion cYFP-NbGID2, nYFP-NbGAI and C4-His or cYFP-NbGID2, nYFP-NbGAI and empty vector were co-expressed in *N. benthamiana* leaves through agroinfiltration. The infiltrated leaves were harvested at 48 hpi and examined under the confocal microscope. (B) Result of statistical analyses showing the number of nuclei with YFP fluorescence per examined area. ** indicates the significant difference between the two treatments at the $P < 0.01$ level, determined using the Student's *t*-test. This experiment had at least three plants per treatment. (C) Result of Western blot analysis showing the expression level of ALCSv C4. After electrophoresis, the blot was probed with an anti-His antibody. The coomassie brilliant blue (CBB)-stained RuBisCo large subunit gel was used to showing sample loadings. (D) Result of competitive pull-down assay showing the interaction between NbGAI-GFP and NbGID2-Flag *in vitro*. In this assay, the purified NbGAI-GFP (4 µg) was mixed with 0, 2, 4, or 8 µg purified C4-His, respectively, and then incubated with 4 µg immobilized NbGID2-Flag.

ALCSv C4 can inhibit NbGAI degradation

It is known that the interaction between GAI and GID2 can enhance GAI degradation via the ubiquitin-26S proteasome [41]. In this study, to test whether ALCSv C4 could also affect the stability of NbGAI, NbGAI-GFP was transiently expressed in *N. benthamiana* leaves through agro-infiltration, the green fluorescence from NbGAI-GFP was found predominantly in nuclei, with small amount of fluorescence in the cytoplasm at 48 hpi. Meanwhile, NbGAI-GFP fusion protein was confirmed by the Western blot experiment (Fig S4). Then, NbGAI-GFP was co-expressed with C4-His or the empty vector in *N. benthamiana* leaf cells, the stronger green fluorescence was observed in the leaves co-expressing NbGAI-GFP and C4-His compared to the leaves expressing NbGAI-GFP alone (e.g., NbGAI-GFP and empty vector) (Fig. 6A, left two images). However, whether plants were treated with 50 µM GA₃ or MG132, the green fluorescence was enhanced when co-expressed with pCV-C4-His (Fig 6A). Then, the relative accumulation levels of NbGAI-GFP protein were analyzed by Western blot assay. The NbGAI-GFP protein accumulated to significantly

higher levels in *N. benthamiana* leaves co-infiltration with NbGAI-GFP and pCV-C4-His compared to co-infiltration with NbGAI-GFP and pCV empty vector. These results proved that C4 protein inhibits the degradation of NbGAI (Fig 6B). Furthermore, qRT-PCR and semi-qRT-PCR results indicated that transient expression of C4 did not affect the mRNA expression level of *NbGAI* (Fig 6C and 6D). Meanwhile, *N. benthamiana* expressing GFP alone was used as a control, and the results showed that C4 protein does not affect the stability of the GFP protein (Fig 6E - 6H). Taken together, these results suggest that the ALCScV C4 protein inhibited the degradation of NbGAI.

Fig 6. ALCScV C4 can inhibit NbGAI degradation *in vivo*. (A) NbGAI-GFP was transiently co-expressed with empty vector or C4-His in *N. benthamiana* leaves. GA₃ (50 μM) and MG132 (50 μM) were applied into the infiltrated leaves 2 h and 12 h before observation, respectively. At 48 hpi, the assayed leaves were harvested and examined under the confocal microscope. (B) Result of Western blot analysis showing the relative accumulation level of NbGAI-GFP. The blots were probed with an anti-GFP or an anti-His antibody. The CBB-stained RuBisCo large subunit gel was used to showing sample loadings. (C) and (D) Results of qRT-PCR and semi-qRT-PCR analyses showing the relative expression level of *NbGAI*. The expression level of *NbActin* was used as an internal control. The result of qRT-PCR was analyzed using the $2^{-\Delta\Delta C_t}$ method. (E) Result showing that ALCScV C4 does not affect stability of GFP *in vivo*. (F) Result of Western blot analysis showing the relative accumulation level of GFP in the mock-, GA₃- or MG132-treated plant leaves. (G) and (H) Results of qRT-PCR and semi-qRT-PCR showing the relative expression level of *GFP* gene. ns indicates not significant according to Student's *t*-test.

To further confirm the ALCScV C4-mediated inhibition of NbGAI degradation, agrobacterium culture carrying pNbGAI-GFP was co-infiltrated with the pC4-His, at various concentrations, into *N. benthamiana* leaves. At 2 dpi, Western blot analyses showed that the expression level of NbGAI-GFP increased when the concentration of agrobacterium culture expressing C4-His was increased, suggesting that the C4-mediated stabilization of NbGAI is C4 dosage dependent (Fig 7A). As expected, C4-His had no clear effect on the expression of GFP (Fig 7B).

Fig 7. Inhibition of NbGAI degradation is C4 concentration-dependent. (A) Agrobacterium culture carrying pGAI-GFP and agrobacterium culture expressing C4-His (diluted to OD600 = 0, 0.25, 0.5 and 1.0, respectively) were co-infiltrated into *N. benthamiana* leaves. Two days later, total protein was extracted from different tissue samples and analyzed through Western blot analyses using an anti-GFP or an anti-His antibody. The CBB-stained RuBisCo large subunit gel was used to show sample loadings. (B) Result of Western blot analysis showing that ALCSv C4 does not affect the stability of GFP *in vivo*.

Silencing of *NbGAI* expression in *N. benthamiana* inhibits ALCSv infection

To determine the role of NbGAI in ALCSv infection, *NbGAI* expression was silenced in *N. benthamiana* using a TRV-based VIGS vector, and then inoculated with TRV-PDS or TRV-GUS as controls. By 7 dpi, the TRV-PDS-inoculated plants showed photobleaching phenotypes, whereas no obvious symptoms were observed in TRV-GUS-inoculated plants (Fig. 8A). As expected, the TRV-NbGAI-inoculated plants resulted in excessive growth compared to TRV-GUS-inoculated plants, this is consistent with a GA-sensitive growth phenotype. qRT-PCR analysis revealed that the mean transcription level of *NbGAI* in the TRV-NbGAI-inoculated plants was down-regulated by about 80% compared to that in the TRV-GUS-inoculated control plants (Fig 8B). In the TRV-NbGAI-inoculated plants, the GA content increased significantly ($3.01 \mu\text{g g}^{-1}$ FW) compared to that in the TRV-GUS-inoculated plants ($2.14 \mu\text{g g}^{-1}$ FW) (Fig 8C). At 14 dpi, the mean height of the TRV-NbGAI-inoculated plants was significantly greater than the TRV-GUS-inoculated plants (Fig 8D). Furthermore, the NbGAI-silenced plants showed a significantly earlier flowering than that of the control plants (Fig 8E and S5). Then, the effect of NbGAI on ALCSv

accumulation and symptom induction were investigated. By 14 dpi, the TRV-GUS and ALCSv inoculated *N. benthamiana* plants exhibited severe leaf curling and plant dwarfing symptoms, whereas only mild leaf curling symptoms were observed on the TRV-NbGAI and ALCSv inoculated *N. benthamiana* plants (Fig 8F). The result of qRT-PCR showed that, compared to that in the control plants, the accumulation level of ALCSv DNA was significantly reduced in the NbGAI-silenced plants (Fig 8G). Similarly, silencing *NbGAI* can alleviate the ALCSv-induced dwarfing symptom and promote early flowering (Fig S6). Taken together, these results indicate that NbGAI plays important roles in the viral accumulation and disease symptoms development in the infected plants.

Fig 8. Silencing of *NbGAI* expression in *N. benthamiana* plants through VIGS inhibits ALCSv infection. (A) Symptoms shown by the *N. benthamiana* plants inoculated with TRV-PDS, TRV-GUS or TRV-NbGAI at 7 dpi. (B) Result of qRT-PCR analysis showing the relative expression level of *NbGAI* in the TRV-GUS- or TRV-NbGAI-inoculated *N. benthamiana* plants at 7 dpi. The relative expression level of *NbActin* was used as the internal control. Each treatment had three biological replicates with three technical replicates each. (C) Result of HPLC analysis showing the GA content in the TRV-GUS- and TRV-NbGAI-inoculated *N. benthamiana* plants, respectively, at 7 dpi. (D) Measurement of the height of the TRV-GUS- or TRV-NbGAI-inoculated *N. benthamiana* plants at 14 dpi. ** indicates a significant difference between the two treatments at the $P < 0.01$ level, determined by the Student's *t*-test. (E) Statistical analysis of flowering time of the TRV-GUS- or TRV-NbGAI-inoculated *N. benthamiana* plants. (F) The TRV-GUS- and ALCSv-inoculated *N. benthamiana* plants showing stronger symptoms of leaf curling and plant dwarfing than the TRV-NbGAI- and ALCSv-inoculated *N. benthamiana* plants at 14 dpi. (G) Result of qPCR analysis showing that the accumulation of ALCSv DNA in the TRV-NbGAI- and ALCSv-inoculated plants was significantly reduced compared to the TRV-GUS- and ALCSv-inoculated plants. * indicates a significant difference between the two treatments at the $P < 0.05$ level. This experiment had at least three plants.

Application of exogenous GA₃ enhances *N. benthamiana* resistance to ALCSv infection

To investigate how GA₃ affects ALCSv infection in plant, *N. benthamiana* plants was inoculated with ALCSv at 12 hours post 50 μM GA₃ or 0.8 % ethanol treatment (control treatment). By 14 dpi, the control plants showed severe leaf curling and dwarfing symptoms, while the GA₃-treated and ALCSv-inoculated plants exhibited only mild leaf curling symptoms (Fig 9A). To determine ALCSv accumulation in these plants, the systemic leaf tissues was harvested at 7, 14, and 21 dpi, and analyzed individually through qPCR. The result showed that the accumulation level of ALCSv DNA in the GA₃-treated and ALCSv-inoculated plants was significantly reduced than that in the control plants (Fig 9B). In addition, the ALCSv-induced dwarfing symptom and delayed flowering were significantly reduced by the application of exogenous GA₃ (Fig S7).

Fig 9. Application of exogenous GA₃ enhances *N. benthamiana* plant resistance to ALCSv infection. (A) *N. benthamiana* plants were treated with exogenous GA₃ or 0.8% ethanol (control), and then inoculated with ALCSv. After ALCSv inoculation, the plants were treated again with GA₃ or 0.8% ethanol (once every other day). The plants were photographed at 14 dpi. (B) Result of qPCR showing the relative accumulation level of ALCSv DNA in the GA₃ or 0.8% ethanol treated plants. ** indicates a significant difference between the two treatment at the $P < 0.01$ level, determined by the Student's *t*-test. This experiment had at least three biological replicates (plants) per treatment.

NbGAI can also inhibits the infection of tobacco curly shoot virus (TbCSV) in *N. benthamiana*

To further examine whether other begomovirus-encoded C4s can also interact with NbGAI, TbCSV, also a member in the genus *Begomovirus*, was selected as the test virus. Y2H and BiFC assays demonstrated that the TbCSV-encoded C4 also interacted with NbGAI and NbGAI-M2 (Fig 10A, 10B and S8). To investigate the effect of

NbGAI on TbCSV infection, the NbGAI-silenced (TRV-NbGAI) or non-silenced (TRV-GUS) *N. benthamiana* plants was inoculated with TbCSV. The results showed that the disease symptoms as well as TbCSV DNA level in the NbGAI-silenced *N. benthamiana* plants were clearly suppressed compared to those in the non-silenced control plants (Fig 10C and 10D). Similarly, TbCSV C4 could also inhibit the degradation of NbGAI (S9 Fig). Consequently, we propose that the begomovirus-encoded C4 proteins are inhibitors of NbGAI degradation, and NbGAI is an important regulator of begomovirus infection and disease symptom development in plants.

Fig 10. TbCSV C4 interacts with NbGAI *in vitro* and *in vivo*. (A) Result of Y2H assay showing the interaction between TbCSV C4 and NbGAI in yeast cells. (B) Result of BiFC assay showing the interaction between TbCSV C4 and NbGAI in *N. benthamiana* leaf cells. The agroinfiltrated leaves were harvested at 48 hpi and examined under the confocal microscope equipped with an FITC filter. Bars = 50 μ m. (C) *N. benthamiana* plants were inoculated with TRV-GUS and TbCSV or TRV-NbGAI and TbCSV. The TRV-GUS and TbCSV-inoculated plants showed severe leaf curling symptom, while the TRV-NbGAI and TbCSV-inoculated *N. benthamiana* plants showed mild leaf curling symptom at 7 dpi. (D) Result of qPCR analysis showing the accumulation level of TbCSV DNA. ** indicates a significant difference between the two treatments at the $P < 0.01$ level, determined by the Student's *t*-test. This experiment had at least three biological replicates (plants) per treatment.

Discussion

As the smallest protein of ALCSv, C4 plays an important role in disease symptom development [38]. To elucidate the molecular mechanism underlying the C4-dependent pathogenesis, we analyzed the function of ALCSv C4 in virus infection and symptom development in *N. benthamiana*, and found that ALCSv C4 is a regulator of GA signaling pathway. Further analyses demonstrated that C4 can directly interact with the negative regulator NbGAI of GA signaling pathway *in vitro* and *in*

vivo, and this interaction prevents the degradation of NbGAI, resulting in the block of GA signaling pathway, and causing infected plants typical dwarfing and delayed flowering (Fig 11). Meanwhile, the similar role of C4 protein encoded by *Tobacco curly shoot virus* (TbCSV), another species of *Begomovirus* [42], was revealed in this study. It is speculated that the C4-mediated induction of delayed flowering and plant dwarfing via the suppression of GA signaling pathway are probably common for begomoviruses.

Fig 11. Proposed model. In mock *N. benthamiana* plants, the interaction between NbGAI and NbGID2 leads to the ubiquitination and the degradation of NbGAI by 26S proteasome, releasing the specific transcription factors. Then genes of gibberellin (GA) signaling pathway were adequately regulated by corresponding transcription factors, promoting normal growth of *N. benthamiana* plants. Under ALCSeV infection condition, C4 directly interacts with NbGAI, which interferes the interaction between NbGAI and NbGID2 to prevent the degradation of NbGAI and block the GA signaling pathway, ultimately leading to the symptoms of plant dwarfing and abnormal flower development.

Virus infection in plants causes significant changes in physiological processes, leading to the onset of various disease symptoms [43]. Numerous reports have indicated that GA plays important roles in plant growth and flower development, plants defective in GA production often show severe plant dwarfing and development of abnormal flowering [22]. For example, the infection of barley yellow dwarf virus (BYDV) in barley causes significant reduction of endogenous GA content, leading to plant dwarfing symptom [44]. Similarly, rice plants infected with rice tungro spherical virus (RTSV) produces significantly less GA, which was considered as the main reason of plant dwarfing [45]. rice dwarf virus (RDV) P2 protein interacted with rice kauri oxidase to suppress GA biosynthesis, which caused rice dwarfing symptom. In contrast, treatment of rice plants with exogenous GA alleviated rice dwarfing symptom [39]. In

this study, we found that ALCSv infection also causes dwarfing symptom and delayed flowering, and the infected plants produced significantly less GA. Meanwhile, the ALCSv-induced dwarfing symptom and delayed flowering were significantly reduced by the application of exogenous GA₃, suggesting a direct correlation between the dwarfing symptom and delayed flowering with the reduced endogenous GA content.

DELLA is a key negative regulator of GA signaling pathway [28]. The GA-mediated plant responses are usually regulated by the SCF^{SLY1/GID2} complex, which is known to sense the GA signal and to recruit DELLA followed by ubiquitination and then degradation of DELLA, through the ubiquitin-26S proteasome, to activate the GA pathway [22,46]. Sequences of DELLA proteins from different plant species are relatively conserved, suggesting that their functions are similar [47,48]. DELLA proteins contain two conserved domains. The C-terminal GRAS domain is important for DELLA stability [49]. In this study, we have determined that the ALCSv C4 can directly interact with NbGAI, which interferes the interaction between NbGAI and NbGID2, leading to the prevention of NbGAI degradation and suppression of GA signaling pathway. Previous studies have demonstrated that to achieve infection in plants, viruses have evolved multiple mechanisms to defeat plant defense machinery. For example, RDV P2 protein hijacks OsIAA10 to block the interaction between OsIAA10 and OsTIR1, thereby prevents the ubiquitin-26S proteasome-mediated OsIAA10 degradation [50]. cotton leaf curl Multan betasatellite-encoded βC1 has been proved to interact with NbSKP1, which interferes the interaction between SKP1 and CUL1 and the function of SCF complex, resulting in the prevention of GAI degradation

and repression of plant response to GA [51]. rice black-streaked dwarf virus (RBSDV)-
 encoded P5-1 can regulate the ubiquitination activity of SCF E3 ligases and inhibit the
 jasmonate signaling pathway to benefit its infection in rice [52]. turnip mosaic virus
 (TuMV) P1 protein interacted with cpSRP54 and mediated its degradation via the 26S
 proteasome and autophagy pathways, thereby inhibiting the cpSRP54-facilitated
 delivery of AOCs to the thylakoid membrane and manipulation of JA-mediated defense
 [53]. tobacco mosaic virus (TMV) replication-associated protein has been demonstrated
 to affect the localization and stability of auxin/indole-3-acetic acid (IAA) proteins in
Arabidopsis to promote its infection [54,55]. The interaction of cucumber mosaic virus
 (CMV) 2b protein with JAZ can prevent the recruitment of JAZ by COI1 and the
 degradation of JAZ, resulting in an inhibition of the JA pathway [56]. DELLA not only
 plays role directly in the GA signaling pathways, but also in other hormone signal
 transduction pathways in plants. Whether C4 protein, through inhibiting the
 degradation of DELLA protein, impacts other hormone pathways remains to be further
 studied.

Previous studies have reported that TbCSV infection can also cause plants a
 certain degree of dwarf symptom [7]. In this study, we also found that the TbCSV C4
 protein interacts with the NbGAI protein and inhibits its protein degradation, which
 probably explained why the presence of TbCSV causes plant dwarf phenotype.

Phytohormones are not only significant signaling molecules that regulate plant
 growth and development, but also regulate plant abiotic and biotic stress responses
 [57,58]. For example, application of exogenous GA to *Arabidopsis* plants can increase

SA biosynthesis as well as the expression of *NPR1* gene to enhance plant resistance to abiotic stresses [59]. It was reported that auxin signaling plays a positive role in rice defense against RBSDV infection [60]. In addition, application of methyl jasmonate (MeJA) or brassinazole (BRZ) to rice leaves causes significant reductions in RBSDV incidence and virus accumulation [61]. In this study, we found that silencing of *NbGAI* expression or application of exogenous GA₃ greatly enhanced *N. benthamiana* resistance to ALCSv infection, which further confirms the role of plant hormone in plant anti-virus resistance. However, the molecular mechanism of how GA defense against ALCSv infection is still unknown, and which needs to be investigated next.

In summary, the results presented here reveal that geminivirus - encoded C4 proteins can directly interact with NbGAI, which interrupts the interaction between NbGAI and NbGID2, leading to the inhibition of NbGAI degradation and the block of the GA signaling pathway, therefore the infected plants display symptoms of typical dwarfing and delayed flowering. This is a novel mechanism by which geminivirus C4 proteins influence viral pathogenicity through interfering GA signaling pathway.

Materials and methods

Plant growth and virus inoculation

N. benthamiana plants were grown inside a growth chamber set at 26 °C and 16 h/8 h (light/dark) photoperiod. ageratum leaf curl Sichuan virus (ALCSv), ALCSv-mC4 (e.g., ALCSv lacks the *C4* gene), potato virus X (PVX) and PVX-C4 (PVX with an inserted ALCSv *C4* gene) were from a previously published source and maintained

inside the laboratory [38]. These viruses were inoculated to the leaves of 4-6-leaf-old *N. benthamiana* plants through agroinfiltration method.

Plasmid construction

For yeast two-hybrid (Y2H) assays, the full-length ALCScV *C4* gene sequence was PCR-amplified using gene specific primers (S1 Table), and then cloned into the pGBKT7 vector to produce pBK-C4. The full-length NbGID2, NbGAI, NbGAI deletion derivatives (refer to as NbGAI-M1, NbGAI-M2, NbGAI-M3, NbGAI-M4, and NbGAI-M5, these mutants encode NbGAI amino acid 1-97, 98–566, 191–566, 1–190, and 98–190, respectively) were PCR-amplified and cloned into the pGAD-T7 vector to produce pAD-NbGID2, pAD-NbGAI, pAD-NbGAI-M1, pAD-NbGAI-M2, pAD-NbGAI-M3, pAD-NbGAI-M4, and pAD-NbGAI-M5, respectively.

For bimolecular fluorescence complementation (BiFC) assays, the full length sequence of *C4* was amplified using the primer pair cYFP-C4-F/cYFP-C4-R, and then inserted into the pCV-nYFP vector to generate pcYFP-C4. The full-length *NbGAI*, *NbGAI-M2*, and *NbGID2* sequences were also amplified and inserted individually into the pCV-nYFP vector to generate pnYFP-NbGAI, pnYFP-NbGAI-M2, and pcYFP-NbGID2, respectively.

For Co-immunoprecipitation (Co-IP) assays, DNA fragments of *C4*-His, GFP, and NbGAI-GFP were amplified, individually, double digested with restriction enzymes, and cloned into the pCV vector to generate pC4-His, pGFP, and pNbGAI-GFP, respectively.

To silence *NbGAI* expression in *N. benthamiana*, a fragment of *NbGAI* sequence

437 bp was amplified and cloned into the pTRV2 vector (kindly provided by Professor Fei Yan, Ningbo University, Zhejiang, China) to generate pTRV2-NbGAI. Plasmid pTRV1, pTRV2-GUS, and pTRV2-PDS were also from the same source.

Yeast two-hybrid assay

The recombinant plasmids were co-transformed into *Saccharomyces cerevisiae* strain AH109 cells as indicated in the legends of Figure 2, 3 and 4 using the lithium acetate method. The transformed cells were grown on the SD/-Trp-Leu medium. After 2-3 days, positive colonies were selected and cultured on the SD/-Leu-His-Trp-Ade medium plate after being diluted to the concentration of 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} , respectively.

BiFC assay

Plasmid pcYFP-C4 and pnYFP-NbGAI were individually transformed into *Agrobacterium tumefaciens* strain EHA105 cells, and cultured overnight in a LB medium supplemented with kanamycin (50 $\mu\text{g/mL}$) and rifampicin (20 $\mu\text{g/mL}$). The cultures were pelleted, resuspended in an induction buffer (10 mM MgCl_2 , 100 mM MES, pH 5.7, and 2 mM acetosyringone, in distilled water) till $\text{OD}_{600} = 0.8\text{-}1.0$ followed a 3 h incubation at room temperature. The two agrobacterium cultures were mixed at a 1:1 (v/v) ratio, and then infiltrated into *N. benthamiana* leaves with needleless syringes. Mixed agrobacterium cultures carrying pcYFP-C4 and pnYFP (empty vector) or pcYFP (empty vector) and pnYFP-NbGAI were served as controls. At 36-48 hpi, the leaves were collected and examined under a confocal microscope (LEICA, Germany) equipped with an FITC filter.

Co-IP assay

For Co-IP assays, mixed agrobacterium cultures carrying plasmid pC4-His and pNbGAI-GFP, or pC4-His and pGFP were infiltrated into *N. benthamiana* leaves. At 2 dpi, the infiltrated leaves were harvested for total protein extraction in an extraction buffer (50 mM Tris-HCl, pH 8.0, 0.5 M sucrose, 1 mM MgCl₂, 10 mM EDTA, 5 mM dithionthreitol). The resulting protein samples were individually incubated with anti-GFP agarose beads (Sigma) for 4 h at 4°C followed by five rinses in an ice-cold PBS buffer. The input and co-immunoprecipitated protein samples were analyzed through Western blot assays using an anti-His or an anti-GFP antibody.

In *vitro* competitive pull-down assay

For competitive pull-down assay *in vitro*, C4-His, NbGAI-GFP, and NbGID2-Flag fusion proteins were purified by in *vitro* translation system (The TNT® Wheat Germ Extract Systems), respectively. The purified NbGAI-GFP (4 µg) was mixed with 0, 2, 4, or 8 µg purified C4-His, and then incubated with 4 µg immobilized NbGID2-Flag for 2 h at 4°C. After centrifugation and several rinses, the bead-bound proteins and the proteins in the supernatants from different treatments were analyzed through Western blot assays using an anti-Flag, anti-His, or an anti-GFP antibody.

VIGS and transient gene expression assays

For VIGS, plasmid pTRV1, pTRV2, pTRV2-NbGAI, pTRV2-GUS, and pTRV2-PDS were individually transformed into *A. tumefaciens* strain EHA105 cells. After overnight growth in the liquid LB medium, the cultures were pelleted individually, resuspended in an induction buffer followed by 3 h incubation at room temperature.

The agrobacterium culture harboring pTRV1 was diluted to $OD_{600}=1.0$ and mixed with an equal volume of diluted agrobacterium culture harboring pTRV2 (refers to as TRV), pTRV2-NbGAI (TRV-NbGAI), pTRV2-GUS (TRV-GUS) or pTRV2-PDS (TRV-PDS). The mixed agrobacterium cultures were individually infiltrated into the leaves of *N. benthamiana* plants at the 3-5-leaf-stage using needleless syringes. The plants inoculated with TRV-GUS or TRV-PDS were used as the controls. At 7 dpi, systemic leaves of the assayed plants were harvested for total RNA isolation followed by qRT-PCR analyses. The expression level of *NbActin* in individually tissue samples was used as the internal control. This experiment was repeated three times.

For transient gene expression assays, agrobacterium cultures harboring specific expression vectors were individually grown, pelleted and induced as described above. Each agrobacterium culture was diluted to $OD_{600}=1.0$ before infiltration into leaves of *N. benthamiana* plants at 4-6-leaf-stage. This experiment was repeated three times with 15 plants per treatment.

Hormone level analysis

Leaves of the TRV-GUS- and TRV-NbGAI-inoculated *N. benthamiana* plants were collected at 7 dpi, and the leaves of the mock-, ALCSsV-, ALCSsV-mC4-, PVX-, and PVX-C4-inoculated *N. benthamiana* plants were collected at 14 dpi. The harvested leaf tissues were weighed, stored at -80°C , and analyzed for endogenous hormone contents by Suzhou Grace Biotechnolgy Co., Ltd through high performance liquid chromatography (HPLC).

GA₃ treatment and ALCSsV inoculation

To test the effect of GA₃ on ALCSv infection, *N. benthamiana* plants at the 4-6-leaf stage were sprayed with a solution containing 50 μM GA₃ or 0.8% ethanol (mock treatment). After 12 h, the sprayed plants were inoculated with ALCSv through agroinfiltration method. The inoculated plants were grown inside the growth chamber and sprayed with the 50 μM GA₃ solution or the 0.8% ethanol solution once every other day till 7 dpi. Leaves of these plants were harvested and analyzed for ALCSv accumulation through qPCR assays.

Protein sequence alignment

Sequences of GAI proteins from *Solanum lycopersicum* (GenBank No. NP_001234883.1), *N. tabacum* (GenBank No. BAG68655.1), *Ipomoea batatas* (GenBank No. XP_031118124.1), *Capsicum annuum* (GenBank No. XP_016540318.1), *Camellia sinensis* (GenBank No. ANB66339.1) and *A. thaliana* (GenBank No. NP_001319041.1) were downloaded from the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>) in the US. Alignment of these sequences was performed using the Clustal V software in the MEGA7.0 and the phylogenetic tree was constructed using the MEGA7.0 software. Conserved domains in these GAI proteins are then identified.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the harvested leaf tissues using the RNAiso Plus reagent (TaKaRa, Dalian, China). Complementary DNA was synthesized using the PrimeScript™ RT reagent Kit supplemented with a gDNA Eraser (TaKaRa, Dalian,

China) and random primers. Quantitative PCR was conducted using the NovoStart SYBR qPCR SuperMix Plus kit (Novoprotein, Shanghai, China). Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [62]. The expression level of *NbActin* gene was used as an internal control during the assays. For each treatment, three technical replicates were used and each treatment was repeated three times. The final result was presented as the mean of three experiments.

DNA extraction and viral DNA accumulation analysis

Total DNA was extracted using the CTAB method. The relative accumulation levels of viral DNA were measured using the qPCR assays as described previously [38].

Western blot analysis

Total protein was extracted from leaf samples in a lysis buffer (Biotime, Shanghai, China), separated in 12% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels through electrophoresis, and transferred to PVDF membranes. The membranes were probed individually with a specific rabbit antibody followed by a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody. The detection signal was visualized through incubation of the membranes in an ECL solution.

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Supporting information

S1 Fig. ALCScV C4 influences plant height and flower development. (A) Photographs of the mock-, ALCScV-, and ALCScV-mC4-inoculated *N. benthamiana* plants at 28 dpi. (B) Result of statistical analysis showing the height of the mock-, ALCScV-, and ALCScV-mC4-inoculated *N. benthamiana* plants at 28 dpi. Different letters above the bars indicate significant differences at the $P < 0.05$ level. (C) Result of statistical analysis showing the flowering time of the mock-, ALCScV-, and ALCScV-mC4-inoculated *N. benthamiana* plants. (D) Photographs of the PVX- and PVX-C4-infected *N. benthamiana* plants at 14 dpi. (E) Result of statistical analysis showing the height of the PVX- and PVX-C4-inoculated *N. benthamiana* plants at 14 dpi. (F) Result of statistical analysis showing the flowering time of the PVX- and PVX-C4-inoculated *N. benthamiana* plants. This experiment had at least three plants per treatment.

S2 Fig. Sequence alignment and phylogenetic analysis of GAI proteins from different plant species. (A) Analysis of conserved domains in the GAI proteins from different plant species. The selected plant species and gene accession numbers are *Solanum lycopersicum* (NP_001234365), *Capsicum baccatum* (PHT30960), *Cucurbita maxima* (XP_022998067), *Ipomoea triloba* (XP_031108300), *Nicotiana benthamiana* (AMO02501), and *N. sylvestris* (XP_009796771). (B) Phylogenetic tree was constructed using the GAI protein sequences from *N. benthamiana* (AMO02501), *N. attenuata* (XP_019232709), *N. tomentosiformis* (XP_009590329), *N. sylvestris* (XP_009796771), *S. tuberosum* (NP_001305514), *S. lycopersicum* (NP_001234365), *C. baccatum* (PHT30960), *I. triloba* (XP_031108300), *Sesamum indicum* (XP_011099949), *Actinidia deliciosa* (AHB17746), *A. thaliana* (CAA75492), *Glycine soja* (XP_028212758), *C. maxima* (XP_022998067), *Zea mays* (NP_001306661), and *Oryza sativa* (AAR31213). The red triangle indicates the GAI protein of *N. benthamiana* obtained in this study.

S3 Fig. ALCScV C4 does not interact with NbGID2. The recombinant plasmids were co-transformed, in various combinations, into *S. cerevisiae* strain AH109 cells using the lithium acetate method. The transformants were 10-fold serially diluted and then grown on the SD/-Trp/-Leu or the SD/-Trp/-Leu/-His/-Ade medium plates for 3 days. The result showing that the C4 does not interact with NbGID2.

S4 Fig. Analysis of NbGAI subcellular localization. (A) Leaf tissues expressing GFP (top image) or NbGAI-GFP (bottom image) area shown in the left column. Images in the left column were captured under the UV light. The images in the middle column were captured under the bright field. The images in the right column are merged images. Scale bar = 50 μ m. (B) Result of Western blot analysis shows the expression level of GFP and NbGAI-GFP fusion protein. The blot was probed with an anti-GFP antibody. The CBB-stained RuBisCo large subunit gel was used to show sample loadings.

S5 Fig. Photographs of the TRV-GUS- and TRV-NbGAI-inoculated *N. benthamiana* plants at 28 dpi. The result showing that silencing *NbGAI* expression in *N. benthamiana* plants through VIGS significantly increases plant height, but early flowering.

S6 Fig. Silencing *NbGAI* can alleviate the ALCScV-induced dwarfing symptom and delayed flowering. (A) Statistical analysis of plant height of mock-inoculated and ALCScV-inoculated *N. benthamiana* plants at 14 dpi. (B) Statistical analysis of flowering time of mock-inoculated and ALCScV-inoculated *N. benthamiana* plants.

S7 Fig. Application of exogenous GA₃ increases plant height, but early flowering. (A) The mock- or ALCScV-inoculated *N. benthamiana* plants were treated with GA₃ or 0.8% ethanol (control), respectively. The plants were photographed at 14 dpi. (B) Result of statistical analysis showing the height of the mock- and ALCScV-inoculated *N. benthamiana* plants, respectively, at 14 dpi. (C) Result of statistical analysis showing the flowering time of the mock- and ALCScV-inoculated *N. benthamiana* plants, respectively.

S8 Fig. *NbGAI*-M2 is responsible for the interaction with TbCSV C4. (A) Result of Y2H assay showing that *NbGAI*-M2 is responsible for the interaction with TbCSV C4. (B) Result of BiFC assay agrees with the Y2H assay result.

S9 Fig. TbCSV C4 inhibits the degradation of *NbGAI*.

S1 Table. Sequence of primers used in this study.

A **B** **C** **D**

Figure

Figure

C

Figure

Figure

Figure

Figure

A **B**

Figure

Figure

A **Ethonal+ALCScV****50 μ M GA₃+ALCScV****B** **Figure**

A **B** **C** **D**

Figure

Figure