Stage-Specific Hormonal Control of Flowering in Kiwifruit

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ABSTRACT

The Chinese kiwifruit (*Actinidia chinensis*) is an important species with various cultivars that display differences in flesh color and ripening times. Effective management of flowering is essential for optimizing yield and fruit quality, involving complex hormonal signaling pathways. However, the molecular mechanisms regulating flowering time in kiwifruit are not well understood. In this study, we investigated the hormonal regulation of flowering in early flowering *'Hanhong'* and late flowering *'Guichang'* cultivars through integrated transcriptomic, proteomic, and functional genetic methods. Our findings indicate that gibberellin (GA) signaling promotes floral bud development but does not trigger the flowering transition, with stage-specific regulation of AdGID1 at protein level. Crucially, the DELLA protein AdRGA negatively regulates flowering by suppressing *FLOWERING LOCUS T (FT)* expression, as confirmed by delayed flowering in overexpression AdRGA in *Arabidopsis thaliana*. Conversely, cytokinin signaling promote flowering through AdAHP by up-regulating *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* levels. These results reveal the distinct roles of GA and cytokinin pathways in kiwifruit flowering, with AdRGA and AdAHP function as key antagonistic regulators of early flowering. Our study provides a framework for manipulating flowering time in kiwifruit breeding programs.

Key word: transcriptome; proteome; floral bud; flowering; GA; Cytokinin; kiwifruit

Highlights

1. GA signaling shows stage-specific regulation: promotes floral bud development through AdGID1 protein accumulation but not flowering transition

2. The DELLA protein AdRGA inhibits flowering by down-regulating *FT* expression.

3. Cytokinin signaling promotes flowering by activating *FT* and *SOC1* through AdAHP, contrasting with AdRGA's repressive role.

4. Transcriptomic and proteomic analyses reveal stage-specific hormonal regulation, with GA pathways dominant in flora budding and cytokinin pathways critical for flowering.

5. AdRGA and AdAHP represent conserved yet functionally divergent regulators, offering targets for kiwifruit breeding to control flowering time.

1. Introduction

The Chinese kiwifruit, known as *Actinidia chinensis*, is a deciduous climbing vine that belongs to the Actinidiaceae. This fruit is indigenous to China and is often referred to as the “king of fruits” due to its high economic importance[1]. Kiwifruit comes in various forms, with the color of the flesh being a significant distinguishing characteristic[2,3]. For instance, the *Actinidia ‘Hanhong’* variety, which has red flesh, hails from the Daba Mountain region in Sichuan Province and is classified as an early flowering cultivar[4,5]. In contrast, the *Actinidia 'Guichang'* kiwifruit (Actinidia deliciosa), a geographically protected cultivar from Xiuwen (Guizhou), produces green-fleshed fruits and exhibits delayed flowering compared to early-flowering varieties like *'Hanhong'*[6,7]. The timing of flowering is crucial for effective pollination and satisfactory fruit set rates. Consequently, understanding the regulatory mechanisms governing kiwifruit flowering is essential for enhancing both yield and quality[8,9]. Currently, the study of early and late flowering in kiwifruit remains[10-13].

The process of flowering in plants is complex, regulated by an array of hormonal signals. Recent studies have revealed the significant roles played by various plant hormones in this regulatory process, including cytokinin, gibberellin (GA), abscisic acid (ABA), auxin (IAA), salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) in the regulating of flowering[14-19]. These hormones establish a complex regulatory network that precisely determines the timing of floral induction in plants[20]. Among these key players, GA is a crucial hormone that promotes plant flowering[21]. Notably, GA displays its influence through interaction with the DELLA protein, facilitated by the GID1 receptor. This interaction triggers the degradation of the DELLA protein, thereby alleviating its inhibitory effect on GA signaling pathways and promoting flowering[22-26]. In *Arabidopsis thaliana*, five DELLA proteins have been identified : GAI, RGA, RGL1, RGL2, and RGL3[27,28].

RGA functions as a key repressor in GA signaling. When GA binds to the

GID1 receptor, it interacts with DELLA proteins like RGA, forming a GA-GID1-DELLA complex that promotes the ubiquitination and degradation of DELLA proteins, thus alleviating their repression on downstream target genes[29]. RGA RGA suppresses flowering by down-regulating integrator genes such as *Flowering Locus T (FT)* and MADS-box genes like *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* and *AGAMOUS-LIKE 8 (AGL8)*. Over-expressed RGA significantly reduce the expression of these genes in both short-day and long-day conditions, resulting in delayed flowering[30]. Additionally, RGA interacts with the photoperiod regulatory CONSTANS (CO) through a DELLA-CO complex, influencing the signaling of photoperiod signals to flowering genes[31].

In *Brassica rapa*, the BraRGL mutant displays early bolting and increased sensitivity to GA3, indicating its role in stem elongation and flowering timing[32]. In *Oryza sativa*, two DELLA proteins, SLENDER RICE 1 (SLR1) and OsGAI, are identified as key regulators of plant height, tillering, flowering time, and stress tolerance[33,34]. DELLA family members are conserved and exhibit both overlapping and distinct functions. These DELLA proteins interact with different proteins, leading to different roles. The phytochrome interacting factor (*PIFs*) play a crucial role in light signal transduction and may also be linked to circadian rhythms, integrating environmental signals like light intensity and temperature to regulate flowering[35-37]. DELLA proteins can bind to *PIFs*, inhibiting their activity and thereby affecting flowering time[38,39].

Cytokinin is an essential phytohormone that regulate plant growth, development, and flowering. The key regulators of the cytokinin signaling pathway include the receptor CRE1 (AHKs), Arabidopsis Histidine Phosphotransfer proteins (AHP), and *Arabidopsis thaliana* Response Regulators (ARRs)[40,41]. When cytokinin binds to AHK receptors, AHKs undergo autophosphorylation, leading to the transfer of a phosphate group to the AHP proteins. The phosphorylated AHPs then move to the nucleus, where they

phosphorylate type B ARRs, activating the transcription of downstream genes, including type A ARRs, which are involved in regulating cell division, differentiation, and organ development[42-44]. The AHP family contains five members (AHP1-5), which show significant functional redundancy. Mutants lacking a single *AHP* gene exhibit normal phenotypes, while mutants with deletions of multiple AHPs (*ahp1,2,3,4,5*) display significantly decreased sensitivity to cytokinin, negatively impacting plant development[45,46]. Interestingly, the function of AHP5 shows less similarity to the other AHPs, indicating a distinct function[47,48], although its role in flowering regulation remains unclear.

To investigate the mechanisms behind flowering in plants, we used transcriptome and proteome analysis, two key omics technologies[49-52], to facilitate a comprehensive understanding of the molecular regulatory networks. In this study, we revealed the molecular mechanism for the early flowering of '*Hanhong'* compared to '*Guichang'*.

2. Method

2.1. Plant Material Collection and Sample Preparation

The kiwifruit cultivars '*Hanhong'* and *'Guichang'* were grown in Guichang province, China. Small green floral buds were harvested from branches in March, and fully open flowers were collected in May at peak bloom when pollen was released for pollination.

2.2. Quantitative RT-PCR

Total RNA was extracted from floral buds and flowers using TRIzol Reagent (Invitrogen, USA) following the manufacturer's instructions. The total 1 μg RNA was utilized for gDNA removal and cDNA synthesis using the HiScript II Q RT SuperMix kit (Vazyme, China), also following the manufacturer's instructions. Quantitative PCR assays were performed on the CFX ConnecTM Real-Time PCR Detection System (Bio-Rad, USA) using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China), with *Adactin* or *actin* serving as the reference gene. The relative expression level was calculated by the $2^{-\Delta\Delta CT}$ method[53]. The primer sequences used for qPCR were listed in Table 1.

2.3. Vector constructions

The vectors used in this study were generated using a Lighting Cloning system (Biodragon Immunotechnology, China). The coding sequences (cDNA) of the AdRGA and AdAHP from kiwifruit were amplified and cloned into the pCambia2306 vector at the *Kpn I/Sal I* sites to generate *35S:AdRGA-GFP* plasmids or into pFGC5941 at the *Nco I/BamH I* sites to generate *35S:AdAHP-YFP* plasmids. For western blotting analysis, total protein of plants was extracted using lysis buffer that included 100 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM PMSF, 100 μM MG132 (MedChemExpress, USA) and a protease inhibitor cocktail (Sigma, USA). The supernatant was boiled in 5× SDS loading buffer at 100 °C for 8 min. The proteins were subjected to western blotting using anti-RGA (Agrisera, USA), anti-GFP (Sigma, USA) and anti-actin (Agrisera, USA) antibodies.

2.4. Protoplast transient transformation

Three-week-old leaves were selected and treated with cellulase and pectinase to isolate protoplasts. They were mixed with the *35S:RGA-GFP* and *35S:AHP-YFP* plasmids, followed by polyethylene glycol (PEG)-mediated transformation to incorporate the plasmids into the protoplasts. GFP and YFP fluorescence signal was detected by fluorescence microscopy[54].

2.5. Materials and Growth Conditions for *Arabidopsis thaliana*

The *Arabidopsis thaliana* mutants used in this study were derived from the Columbia (Col-0) background. The *rga-28* mutant (SALK_089146) and the *ahp5-2* mutant (SALK_079857) were obtained from Arashare (China) (https://www.arashare.cn/index/Product/index.html). Seeds were sterilized with 2% plant preservation mixture (Plant Cell Technology, USA), subjected to a 2-day stratification at 4 °C in the dark, and then plated on half-strength Murashige and Skoog (MS) medium containing 1% (w/v) sucrose and 0.4% (w/v) phytagel (pH 5.7). The plants were grown under long-day conditions (16 h of light and 8 h of dark) at 22°C in a growth chamber.

2.6. Endogenous GA content analysis

The floral buds and flowers of *'Hanhong'* and *'Guichang'* were collected to measure endogenous GA content, following the protocol outlined in the GA ELISA Kit (Biomatik, Canada) with three repetitions. 0.5g-1.0g of fresh samples were homogenized in ice-cold of PBS, then centrifuged at 10,000×g for 5 minutes. The supernatant was collected for ELISA analysis.

2.7. RNA-seq and Proteome analysis

Buds and flowers were collected from 10-year-old kiwifruit plants, with buds sampled in March and flowers in May. Three biological replicates were obtained from different individual plants. Transcriptome and proteome analyses and were performed by Novogene Co., Ltd. (Beijing, China). The data at https://www.ncbi.nlm.nih.gov/sra/PRJNA1271936 are available in NCBI BioProject.

3. Results

3.1 The GA signaling pathway is essential for promoting flowering in kiwifruit.

To investigate the mechanism underlying the early flowering of '*Hanhong'*, we examined the phenotype from floral bud emergence to flowering in both '*Hanhong'* and '*Guichang'*. Our results indicated that '*Hanhong'* requires eight weeks to fully flowerings, while '*Guichang'* requires ten weeks (Figure 1A), confirming '*Hanhong'* as an early flowering cultivar. It is widely recognized that GA signaling promotes flowering in plants[55-57]. Therefore, we assessed the GA levels in the floral bud and flowering stages. As shown in Figure 1B, there is a significant increase in GA content during the floral budding phase compared to the flowering phase for both cultivars, indicating that GA positively affects flowering in kiwifruit. Furthermore, we found that the GA content was more accumulated in the floral bud and flowering stages of '*Hanhong'* compared to that of '*Guichang'*, indicating '*Hanhong'* was an early-flowering cultivar.

To investigate how GA signaling regulates the early flowering of '*Hanhong'*, we collected samples from both stages, including the floral bud and flowering stages of '*Hanhong'* and '*Guichang'* (Figure S1A) and performed transcriptome and proteome sequencing, followed by data analysis. For '*Hanhong',* we identified 11,642 genes, including 6,217 down-regulated and 5,425 up-regulated genes. For '*Guichang'*, we identified 12,050 genes, including 5,847 down-regulated and 6,201 up-regulated genes (Figure S1B). KEGG pathway analysis of the differential expressed genes showed significant enrichment in the GA signaling pathway, when the floral bud stage compares to flowering stage (Figure S2A and S2B).

To confirm the transcriptome data, we carried out quantitative PCR (qPCR). As shown in Figure 1C and 1D, the transcription level of *AdPIF4* significantly increased from the floral bud stage to flowering in both varieties, indicating its role in enhancing flowering at the transcriptional level, consistent with the

KEGG pathway analysis (Figure S2A and S2B). In contrast, the transcription level of *AdGID1* in '*Hanhong'* decreased significantly, while in '*Guichang'*, *AdGID1* levels significantly increased from floral bud to flowering (Figure S3A and S3B), suggesting that *AdGID1* is not a key gene for regulating flowering transcriptionally.

RGA, a crucial member of the DELLA family, is known for its role in regulating stem elongation and flowering in *Arabidopsis thaliana*[58]. Although we found that the transcription levels of *AdRGA* remained relatively stable from the floral bud stage to flowering in both '*Hanhong'* and '*Guichang'* (Figure S3Cand S3D), there was a significant decrease in AdRGA protein levels (Figure 1D and 1E), whether in '*Hanhong'* or '*Guichang'*, suggesting that AdRGA negatively regulates flowering at the protein level.

3.2 Hormonal signal transduction pathways promote early flowering in '*Hanhong'* compared to '*Guichang'*.

A PCA 3D Plot was utilized to display the gene expression profiles during the floral bud of '*Hanhong'* and '*Guichang'*, revealing significant transcriptomic differences between the two varieties (Figure S4A). Analysis of the transcriptomic data from their floral bud stages revealed 20,265 genes associated with this phase, with 10,716 genes down-regulated and 9,549 genes up-regulated (Figure 2A).

KEGG pathway analysis of differentially expressed genes indicated a significant enrichment in hormonal transduction signaling during the floral bud stages of '*Hanhong'* compared to '*Guichang'* (Figure S4B). Within this is pathway, several GA-related genes showed notable enrichment and variability (Figure S4C). To further confirm these results, we carried out qPCR analysis of key genes in GA signaling pathway. We found out that the expression levels of *AdGID2* and *AdPIF4* were significantly higher in '*Hanhong'* than those of '*Guichang'* during the floral bud stage (Figure 2B and 2C), while *AdGID1* was significantly lower (Figure 2D), suggesting that *AdGID2* and *AdPIF4* positively

regulate floral bud development, but not *AdGID1*. We also used a PCA 3D Plot to examine gene expression profiles during the flowering stages of both varieties (Figure S5A). Comparative transcriptomic analysis revealed 9,575 flowering-related genes, with 5,727 down-regulated and 3,838 up-regulated (Figure 2A). KEGG analysis indicated a significant enrichment in hormone transduction pathways again (Figure S5B). Notably, only the DELLA gene linked to GA signaling showed a significant reduction in enrichment (Figure S5C). To confirm these results, we performed qPCR to analyze the expression levels of the DELLA gene (*AdRGA*). As shown in Figure 2E, transcription levels of *AdRGA* significantly decreased '*Hanhong'* compared to '*Guichang'*, indicating that RGA down-regulation may contribute to early flowering of '*Hanhong'*. Additionally, qPCR analysis demonstrated that transcript levels of *AdGID2* and *AdPIF4* did not significantly differences between flowering stages of the two varieties, implying they are not critical factors for the early flowering in '*Hanhong'* (Figure 2F and 2G). However, the mechanism by which AdRGA regulates early flowering in '*Hanhong'* remains unclear.

3.3 AdRGA negative regulates flowering in *Arabidopsis thaliana.*

To investigate the mechanism of how AdRGA regulates early flowering in '*Hanhong'*, we cloned the coding sequencing of *AdRGA* and constructed *35S:AdRGA-GFP* plasmid, which introduced into the *rga-28* mutant of *Arabidopsis thaliana*, resulting in *35S:AdRGA-GFP/rga-28* plants. The T-DNA insertion in the *rga-28* mutant was confirmed (Figure S6A), and we analyzed the protein levels in *35S:AdRGA-GFP/rga-28* through western blotting (Figure S6B). We expressed the *35S:AdRGA-GFP* plasmid into *rga-28* protoplasts to test the GFP fluorescence signal. The results showed that AdRGA-GFP fluorescence signal was observed in nucleus (Figure S6C). Compared to the Wild Type (WT), the transcript levels of *AdRGA* significantly increased in two independent lines of *35S:AdRGA-GFP/rga-28*, while they decreased in the *rga-28* mutant, as shown by qPCR analysis (Figure 3A).

Next, we conducted genotype analysis on WT, *rga-28* mutant, and *35S:AdRGA-GFP/rga-28* plants to explore the role of AdRGA in flowering. As shown in Figure 3B, *35S:AdRGA-GFP/rga-28* plants displayed lately flowering than WT and *rga-28* mutant. Moreover, the *rga-28* mutant displayed a similar phenotype to WT. We also calculated the number of rosette leaves, finding that *35S:AdRGA-GFP/rga-28* had more leaves than both WT and *rga-28* mutant (Figure 3C). To further investigate the role of *AdRGA* in flowering stage, we performed qRT-PCR to test the transcript expression level of *FT* and *SOC1* in WT, *rga-28*, and *35S:AdRGA-GFP/rga-28* plants. As shown in Figure 3D, the transcription level of *FT* was significantly decreased in *35S:AdRGA-GFP/rga-28* plants but increased in both WT and *rga-28* mutants. Interestingly, the expression of *SOC1* was similar in WT, *rga-28* mutant, and *35S:AdRGA-GFP/rga-28* plants (Figure 3E). These results suggests that *AdRGA* negatively regulates flowering by inhibiting *FT* expression in *Arabidopsis thaliana.* However, the *rga-28* mutant displays no distinct phenotype due to functional redundancy with other DELLA proteins. The inhibiting function of *AdRGA* in flowering was also in Figure 2E.

3.4 AdGID1 mediates floral bud development but not flowering in Kiwifruit.

To further clarify the regulatory mechanisms in kiwifruit development, we performed proteomics analysis of '*Guichang'* compared to '*Hanhong'* at the floral bud and flowering stages. We primarily used the coefficient of variation (CV) to test the reproducibility of the proteomics analysis data. The curve demonstrated a pronounced growth trend, suggesting a consistency between the samples of both cultivars during floral bud and flowering stages (Figure 4A and 4D).

KEGG pathway analysis of the differentially expressed proteins showed a significant enrichment in hormone signal transduction pathways during both the floral bud and flowering stages when comparing the proteomics data of '*Hanhong'* to '*Guichang'* (Figure 4B and 4E). Specifically, only AdGID1, related

to GA signaling, displayed a significant accumulated in floral bud stage, aligning with the transcription analysis results (Figure 2D). This indicates that AdGID1 may play a regulatory role in floral bud development of kiwifruit at both transcription and protein levels (Figure 4C). Furthermore, the accumulated of AdGID1 was relative stability (Figure 4F) in flowering stages, aligning with the transcription analysis results (Figure 2F), implying that AdGID1 may be critical for floral bud development, but not essential for flowering regulation in kiwifruit.

3.5 AdAHP promotes the transition from floral bud to flowering in Kiwifruit.

Our analysis unexpectedly revealed a significant enrichment of the cytokinin signaling pathway in the transcriptome and proteome data of '*Hanhong'* during the floral bud stages and flowering stages, compared to '*Guichang'*. We found a strong significant regulation of AdAHP at both transcriptional and protein levels (Figure S7A-S7D). To confirm the data analysis, we performed qPCR on *AdAHP* and found that its relative expression level markedly increased during the floral budding and flowering phases of '*Hanhong'* when compared to '*Guichang'* (Figure 5A and 5B).

To further investigate how AdAHP in regulating floral bud and flowering processes in kiwifruit, we performed qPCR to test the transcript expression level of *AdFT* and *AdSOC1.* As shown in Figure 5C-5E, the transcription levels of *AdFT* and *AdSOC1* in '*Hanhong'* significantly increased during flowering stages, while they remained stable during the floral bud stage, in contrast to '*Guichang'*. These results imply that *AdAHP* could promote the expression level of *AdFT* and *AdSOC1* during flowering stages, but not during the floral bud stage.

3.6 AdAHP plays a role in promoting early flowering in *Arabidopsis thaliana.*

To investigate the function of AdAHP in flowering, we cloned the coding sequence of *AdAHP* and constructed a 3*5S:AdAHP-YFP* plasmid, which was introduced into the *ahp5-2* mutant of *Arabidopsis thaliana,* generating *35S:AdAHP-YFP/ahp5-2* plants. T-DNA insertion in the *ahp5-2* mutant was

confirmed (Figure S8A), and qPCR confirmed the expression level of *AdAHP* in the *35S:AdAHP-YFP/ahp5-2* plants (Figure S8B). The protein levels of AdAHP in these plants were analyzed by western blotting (Figure S8C). Additionally, we expressed the *35S:AdAHP-YFP* plasmid into *ahp5-2* protoplasts to test the YFP fluorescence signal. The results showed that AdAHP-YFP fluorescence signal was observed in both the nucleus and cytoplasm (Figure S8D), which was further supported in the *35S:AdAHP-YFP/ahp5-2* plants, confirming that AdAHP indeed localizes to both in nucleus and cytoplasm (Figure S8E).

Next, we performed a genotype analysis in WT, the *ahp5-2* mutant and *35S:AdAHP-YFP/ahp5-2* plants. As shown in Figure 6A, the *35S:AdAHP-YFP/ahp5-2* plants exhibited an early flowering phenotype compared to both WT and *ahp5-2* mutants. Meanwhile, there was no significant differences in flowering time between WT and *ahp5-2*. We also calculated the number of rosette leaves and found a significantly decreased in *35S:AdAHP-YFP/ahp5-2* plants when compared to WT and *ahp5-2* (Figure 6B), indicating that AdAHP is responsible for the early flowering in *Arabidopsis thaliana*.

To further investigate how AdAHP regulates the flowering process, we performed qRT-PCR on WT, *ahp5-2* and *35S:AdAHP-YFP/ahp5-2* plants to analyze the expression levels of *FT* and *SOC1* during flowering. As shown in Figures 6C and 6D, the expression levels of *FT* and *SOC1* were significantly increased in *35S:AdAHP-YFP/ahp5-2* plants, while no significant differences were tested in the WT and *ahp5-2*, likely due to functional redundancy among other AdAHPs proteins. These results implying that AdAHP is essential for promoting early flowering in '*Hanhong'* at both the transcriptional and protein levels.

4. Discussion

Investigating the molecular mechanisms of flowering is crucial for improving kiwifruit yield. The early flowering cultivar of '*Hanhong'* and the late flowering cultivar of '*Guichang'* serve as two excellent resources for studying the floral bud and flowering development in kiwifruit. In this study, we found that hormone signaling pathways, especially GA and cytokinin, is crucial for the transition from floral bud to flowering. AdRGA functions as a negative regulator in flowering development by inhibiting the expression of FT, while AdAHP functions as a positive regulator by promoting the expression of FT and SOC1 (Fig 6E).

GA is essential for plant growth and development, particularly in kiwifruit during its transition phase (Figure 1B). In the GA signaling pathway, GID2 is a key factor that interacts with DELLA proteins to affect growth[59]. In rice, OsGID2 has been identified to promote plant growth[60,61]. PIF4, a factor that interacts with phytochromes, plays a role in photomorphogenesis, shade avoidance, and flowering regulation[62,63]. In this study, we found that *AdGID1* and *AdPIF4* are essential for floral bud development (Figure 2C and 2D), however, they are not response for the early flowering of '*Hanhong'* (Figure 2F and 2G).

DELLA proteins function as negative regulators of GA signaling, and their degradation initiates GA responses[64-66]. In *Arabidopsis thaliana*, there are five DELLA members (RGA, GAI, RGL1, RGL2, RGL3), with RGA being a key regulator in inhibiting GA-mediated responses, thus influencing plant growth and development. When RGA is degraded, it releases the suppression on GA-responsive genes, promoting flowering[67,68]. Previous studies have shown that RGA enhances the expression of the *FT,* further activating the flowering process[69]. In contrast, our findings show that overexpressing AdRGA in *Arabidopsis thaliana* inhibits flowering (Figure 3E-3G), indicating that the role of RGA is not conserved among eukaryotic. Furthermore, in *35S:AdRGA-GFP/rga-28* plants, the expression level of the *FT* was down-regulated,

suggesting that AdRGA regulates flowering by modulating FT expression levels. Additionally, further investigation is required to clarify whether AdRGA directly or indirectly represses *FT* expression levels.

AHP5 functions as a positive regulator in the cytokinin signaling pathway, facilitating plant development and stress responses[70]. In this study, we found that overexpressing AdAHP in *Arabidopsis thaliana* promotes flowering (Figure 6A and 6B), indicating its positive role in this process. Additionally, we found that in *35S:AdAHP-YFP/ahp5-2* plants, the expression levels of *FT* and *SOC1* were increased, implying that AdAHP regulates flowering by modulating the expression of these genes, which are crucial in the flowering regulatory network. *FT* and *SOC1* are critical factors in the regulatory network of flowering in plants. The activation of FT and *SOC1* by AdAHP may result from direct interaction with their promoter regions or through engagement with other transcription factors.[71,72]. This positive influence of AdAHP contrasts with the inhibitory role of AdRGA in GA signaling, highlighting the complex interactions among different hormonal signals governing flowering in kiwifruit. However, the direct relationship between AdAHP and *FT/SOC1* requires further investigated.

In summary, GA and cytokinin signaling pathways are crucial in regulating the transition from floral bud to flower in kiwifruit, with AdRGA and AdAHP being key factors that regulate the transcription expression level of *FT* and *SOC1*. Future study needs to be investigated the mechanisms how AdRGA and AdAHP influence kiwifruit flowering, as well as their interactions with other hormone signals to enhance understanding for kiwifruit cultivation and breeding.

5. Conclusions

This study elucidates the molecular mechanisms by which GA and cytokinin signaling pathways coordinately regulate flowering in kiwifruit, using the early flowering *'Hanhong'* and late flowering *'Guichang'* cultivars as models.

Key findings include: (1) **GA signaling promotes floral bud development but does not trigger the transition to flowering in kiwifruit.** While GA levels were elevated during floral budding in *'Hanhong'*, the GA receptor gene AdGID1 showed stage-specific protein accumulation without changes in transcription, suggesting post-translational regulation. (2) **AdRGA functions as a conserved repressor of flowering by inhibiting *FT* expression.** Although its transcript levels remain stable, the degradation of AdRGA protein during flowering aligns with the early flowering of *'Hanhong'*. When expressed in *Arabidopsis*, AdRGA delayed flowering and suppressed *FT*, confirming its negative regulatory role. (3) **Cytokinin signaling through AdAHP promotes flowering by up-regulating *FT* and *SOC1*.** AdAHP is more highly expressed and accumulated in *'Hanhong'*, and its overexpression in *Arabidopsis* leads to early flowering. These results provide a framework for manipulating flowering time in kiwifruit breeding.

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Declaration of competing interest

The authors affirm that they have no competing financial interests or personal relationships that could have influenced the research presented in this study.

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Figures

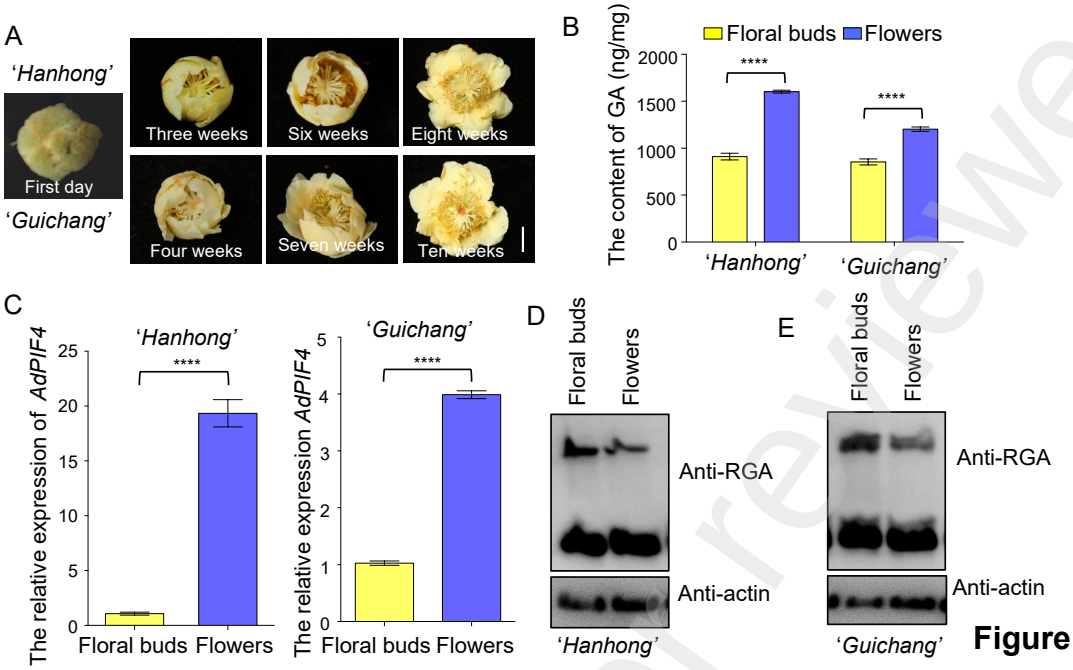

Figure 1. GA promotes flowering in kiwifruit. A. The phenotype was analyzed for transition from floral bud to flowering in both '*Hanhong*' and '*Guichang*'. Scale Bar, 1 cm. **B.** GA content was analyzed in the floral bud and flowering stages. Floral buds in March and flowers in May were collected for analyzing. The experiment was repeated three times. **C.** The relative expression of *AdPIF4* in the floral bud and flowering stages of both '*Hanhong*' and '*Guichang*'. The relative expression level of *AdPIF4* was determined by qPCR, with *Adactin* as the reference gene. Results are presented as means ± SD (n=3). **D.** Total protein from the floral buds and flowers of '*Hanhong*' was extracted using lysis buffer for western blotting with anti-RGA antibody. **E.** Total protein from the floral buds and flowers of '*Guichang*' was extracted using lysis buffer for western blotting with anti-RGA antibody.

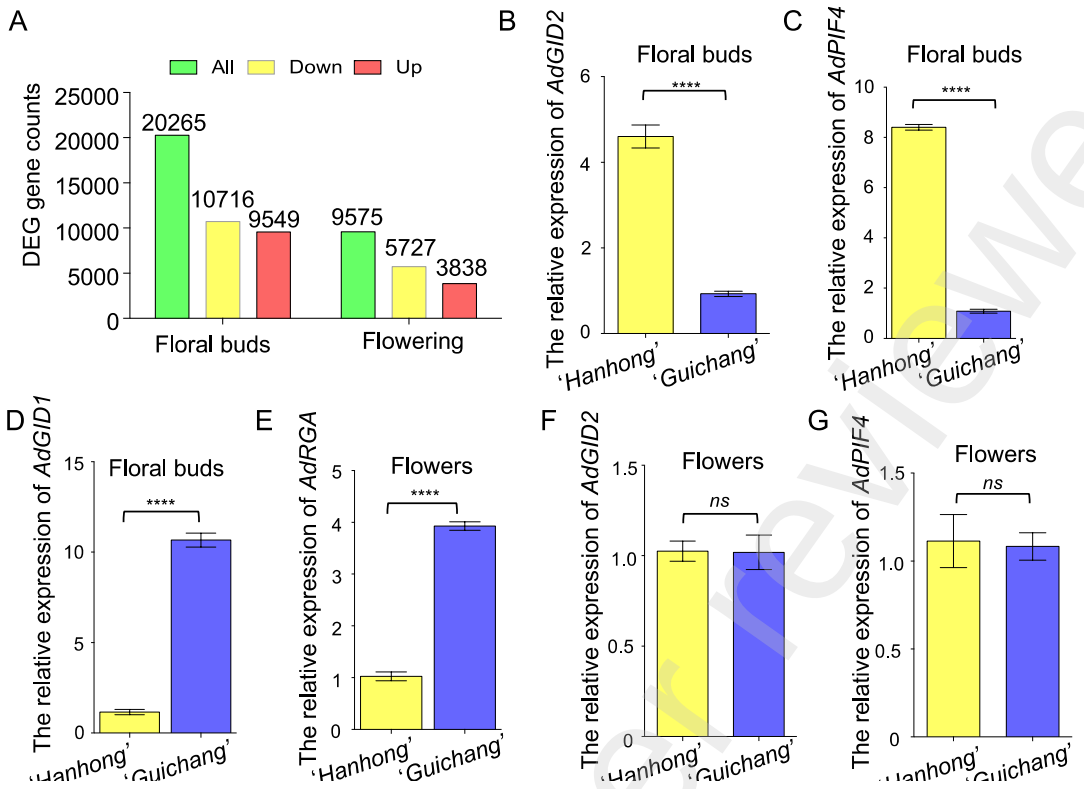

Figure 2. Hormonal signaling is responsible for the early flowering of '*Hanhong*'. A. Summary of the relative expression level of differentially genes by analyzing the transcriptomic data from the floral bud and flowering stages of both '*Hanhong'* and '*Guichang'*. **B-D.** The relative expression level of *AdGID2*、*AdPIF4 and AdGID1* in the floral bud stage of both '*Hanhong'* and '*Guichang'*. The relative expression level of genes were determined by qPCR using *Adactin* as a reference gene. The data are represented as means ± SD (n=3). ****p< 0.0001. **E-G.** The relative expression level of *AdRGA*、*AdGID2* and *AdPIF4* in the flowering stage of both '*Hanhong'* and '*Guichang'.* The relative expression level of genes were determined by qPCR using *Adactin* as a reference gene. The data are represented as means ± SD (n=3). ****p< 0.0001.

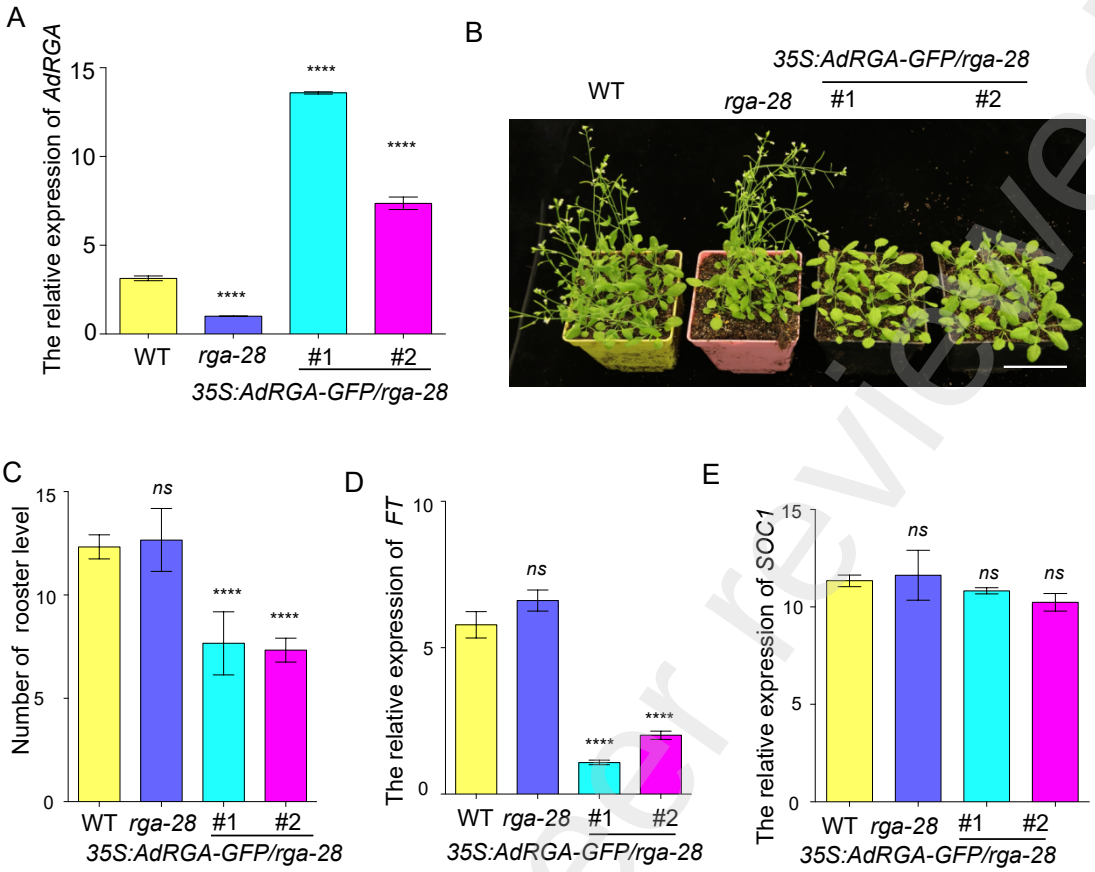

Figure 3. AdRGA negative regulates flowering in *Arabidopsis thaliana*. A. The relative expression level of *AdRGA* in WT, *rga-28* and *35S:AdRGA-GFP/rga-28* plants. The relative expression level of AdRGA determined by qPCR using *actin* as a reference gene. The data are represented as means ± SD (n=3). **B.** The phenotype of flowering in WT, *rga-28* and *35S:AdRGA-GFP/rga-28* plants. Four-weeks old seedlings were grown in natural soil. The photos (B) of plants were shown. Scar bar, 1 cm. **C.** The number of roosters in WT, *rga-28* and *35S:AdRGA-GFP/rga-28* plants. The statistical significance between WT and other genotype was determined using one-way ANOVA analysis (n=10). **D-E.** The relative expression of *FT* and *SOC1* in WT, *rga-28* and *35S:AdRGA-GFP/rga-28* plants. The relative expression level of *FT* and *SOC1* determined by qPCR using *actin* as a reference gene. The data are represented as means ± SD (n=3). ****p< 0.0001.

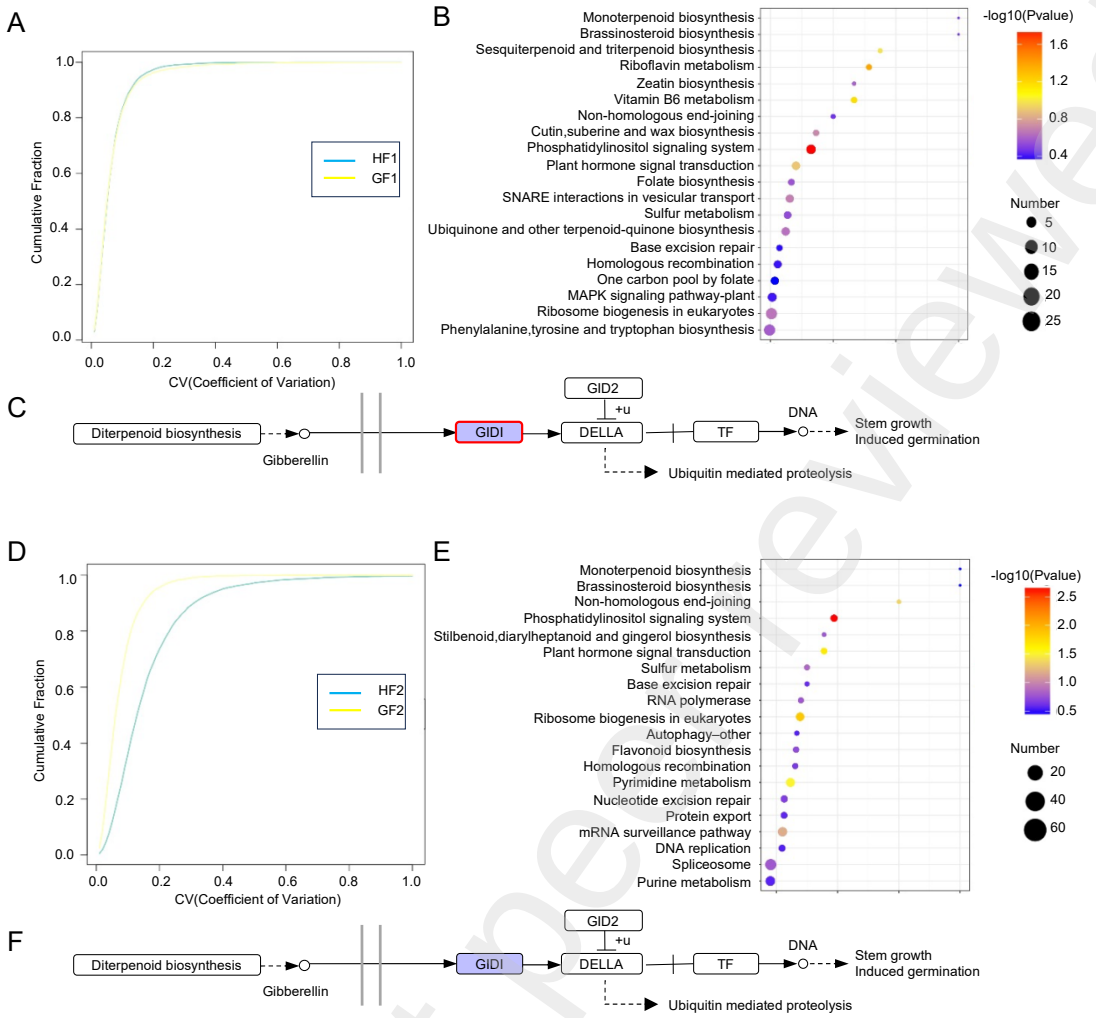

Figure 4. AdGID1 promotes floral bud development in Kiwifruit. A. The proteomics analysis of floral buds from both '*Hanhong'* and '*Guichang'* shows strong repeatability. The CV curve represents the cumulative protein values for each samples, with a steeper curve indicating higher repeatability. HF1 represents the floral bud stage of '*Hanhong'*. GF1 represents the floral bud stage of '*Guichang'*. **B.** KEGG pathway enrichment analysis was performed on all proteins from the floral bud stage in '*Hanhong'* compared with those in '*Guichang'*. **C.** By analyzing the proteomics data, the Diterpenoid biosynthesis pathway was significantly enriched in '*Hanhong*' compared to the floral bud stage of '*Guichang*'. AdGID1 was accumulated more in '*Hanhong*' than in 'Guichang'. AdGID1 is marked in the red boxes. **D.** The proteomics analysis of flowers from both '*Hanhong'* and '*Guichang'* shows strong repeatability. HF2 represents the flowering stage of '*Hanhong'*. GF2 represents the flowering

stage of '*Guichang'*. **E.** KEGG pathway enrichment analysis was performed on all proteins from the flowering stage in '*Hanhong'* compared with those in '*Guichang'*. **F.** By analyzing the proteomics data, the Diterpenoid biosynthesis pathway was significantly enriched in '*Hanhong*' compared to the flowering stage of '*Guichang*'. AdGID1 remains stability in '*Hanhong*' than in '*Guichang*'.

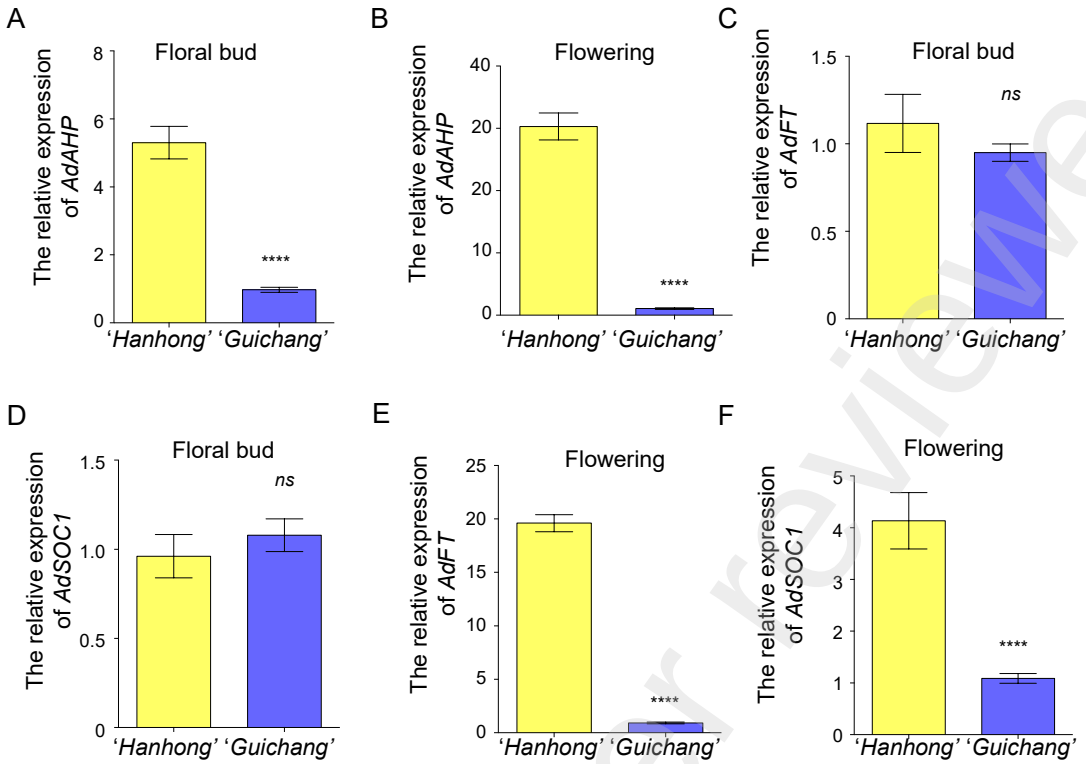

Figure 5. AdAHP is essential for early flowering in '*Hanhong*'. A and B. The relative expression level of *AdAHP* in the floral bud stage and flowering stage of '*Hanhong*' and '*Guichang*'. **C-D.** The relative expression level of *AdFT* and *AdSOC1* in the floral bud stage of '*Hanhong*' and '*Guichang*'. **E-F.** The relative expression level of *AdFT* and *AdSOC1* in the flowering stage of '*Hanhong*' and '*Guichang*'. The relative expression level of all genes determined by qPCR using *Adactin* as a reference gene. The data are represented as means ± SD (n=3). ****p< 0.0001.

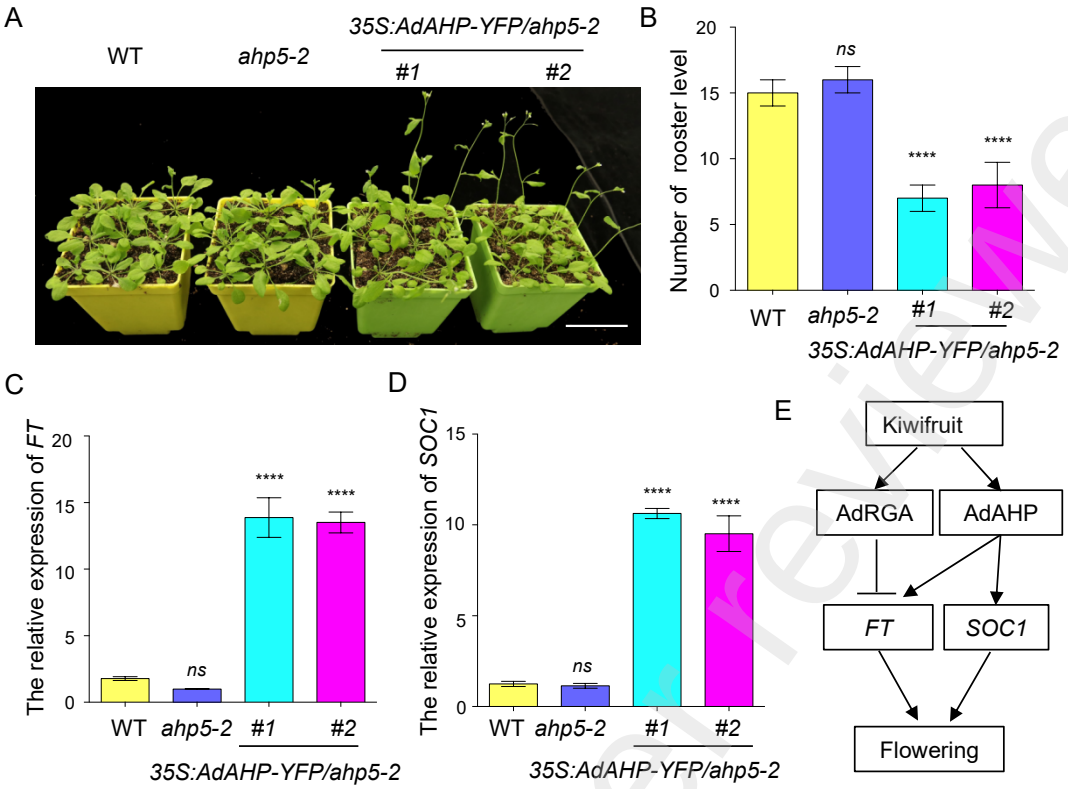

Fig 6. *Ad*AHP promotes early flowering in *Arabidopsis thaliana.* A. The phenotype of flowering in WT, *ahp5-2* and *35S:AdAHP-YFP/ahp5-2* plants. Four-weeks old seedlings were grown on soil naturally. The photos (A) of plants were shown. Scar bar, 1 cm. **B.** The number of rooster in WT, *ahp5-2* and *35S:AdAHP-YFP/ahp5-2* plants. The statistical significance between WT and other genotype were determined using one-way ANOVA analysis (n=10). ****p< 0.0001. **C and D.** The relative expression of *FT* and *SOC1* in the flowering stage of WT, *ahp5-2* and *35S:AdAHP-YFP/ahp5-2* plants. The relative expression level of *FT* determined by qPCR using *actin* as a reference gene. The data are represented as means ± SD (n=3). ****p< 0.0001. **E.** Model. AdRGA functions as a negative regulator in flowering by down-regulating the expression of *FT* gene, while AdAHP functions as a positive regulator in flowering by up-regulating the expression of *FT* and *SOC1* genes.

Stage-Specific Hormonal Control of Flowering in Kiwifruit

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