

AcGLK2 involves anthocyanin biosynthesis via bidirectionally modulating expression of MYB transcription factors in *Actinidia chinensis*

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Abstract

The GOLDEN2-LIKE (GLK) gene family, known for its role in chloroplast development, has recently been implicated in involvement of anthocyanin biosynthesis in kiwifruit (*Actinidia* spp.), but the underlying regulatory mechanism remains unclear. Here we report the characterization of a kiwifruit GLK homolog AcGLK2 in regulating anthocyanin accumulation. We found that expression of *AcGLK2* is much higher exclusively in the red pigment-accumulated fruit tissue. Overexpression of *AcGLK2* in *Arabidopsis* and kiwifruit significantly enhanced anthocyanin content, whereas its RNAi-mediated silencing compromised anthocyanin accumulation. RNA-Seq analysis revealed significant upregulation of many structural genes and transcription factors (TFs) associated with the flavonoid pathway in *AcGLK2*-overexpressing kiwifruit. ChIP-Seq analysis indicated that *AcGLK2* directly binds to *AcMYB5* and *AcTRY*. We showed *AcMYB5*, an R2R3-MYB TF, promotes anthocyanin accumulation by interacting with the bHLH protein *AcbHLH42*, while *AcTRY*, an R3-MYB protein, competitively inhibits the interaction between key MYBs and *AcbHLH42*, thereby repressing anthocyanin biosynthesis. Transgenic and molecular assays in tobacco, tomato, and kiwifruit demonstrated that *AcGLK2* positively participates into regulation of anthocyanin accumulation through transcriptionally activating *AcMYB5* expression and concomitantly suppressing *AcTRY* expression. This study reveals the dual regulatory mechanism of *AcGLK2* in anthocyanin biosynthesis, broadening the understanding of GLK gene functions and providing a valuable genetic resource for molecular breeding of nutritional quality in kiwifruit and other crops.

Introduction

The regulation of plant growth and development is a complex process governed by numerous genetic and environmental factors. Transcription factors (TFs) play central roles in this process, modulating key physiological aspects such as photosynthesis, stress responses, and secondary metabolism. Among these, the GOLDEN2-LIKE (GLK) TFs have attracted considerable interest due to their established function in chloroplast development and photosynthetic regulation [1–3]. As members of the GARP superfamily of MYB TFs, GLK genes are widely distributed across the plant kingdom [4, 5]. Recent studies have further implicated GLK genes in the regulation of anthocyanin biosynthesis in *Arabidopsis* [6–8]. However, the molecular mechanisms underlying this regulatory role remain elusive.

Anthocyanins are water-soluble pigments belonging to the flavonoid family. They not only contribute to the aesthetic appeal of plants but also play crucial roles in protecting plants

against various environmental stresses, such as UV radiation, drought, and extreme temperatures [9, 10]. The biosynthesis of anthocyanins is a well-characterized pathway that involves a series of enzymes catalyzing the conversion of phenylalanine to anthocyanins through various intermediates. This pathway is tightly regulated at the transcriptional level by a complex network of genes, including structural genes encoding biosynthetic enzymes and regulatory genes encoding TFs [11–13]. Flavonoid accumulation is primarily controlled by ternary MBW complexes. In *Arabidopsis*, the model system for dicotyledon plant biology studies, the regulation of flavonoid biosynthesis has been extensively investigated. The pathway is orchestrated through a combinatorial logic where specific MYB factors (e.g. TT2 for PAs, PAP1/2, MYB113, MYB114 for anthocyanins, MYB11, MYB12, MYB111 for flavonols) partner with a set of bHLH (TT8, GL3, EGL3) and WD40 (TTG1) proteins to direct the spatial and temporal accumulation of distinct flavonoid end-products [13, 14]. Light quality, duration, and intensity critically regulate anthocyanin

accumulation [15, 16]. HY5, a bZIP transcription factor, acts as a master activator, requires BBX proteins as partners [17]. In the dark, the E3 ubiquitin ligase COP1 degrades HY5 and other activators, suppressing anthocyanin production. Light inactivates COP1, stabilizing HY5 to directly upregulate key biosynthetic genes (e.g. PAP1) and indirectly inhibiting the repressor MYBL2 (a single MYB domain protein) [18, 19]. Furthermore, microRNAs (miRNAs) are key regulators of anthocyanin. The miR156 module promotes anthocyanin by silencing repressive SPL TFs [20, 21], while miR828 and miR858 have complex, species-specific roles—either promoting or inhibiting accumulation by targeting different MYB regulators [22]. Additionally, previous studies have also found the regulatory role of other types of TFs in anthocyanin synthesis in fruit trees, including WRKY, RAV, and NAC play a dual role, functioning as both activators and repressors of anthocyanin accumulation [23–26].

The GLK gene family in Arabidopsis consists of two members, *AtGLK1* and *AtGLK2*, that share high sequence similarity and overlapping functions [27]. Many previous studies have primarily focused on the role of GLK genes in chloroplast development and photosynthesis. For instance, *AtGLK2* has been found to be involved in the biosynthesis of chlorophyll in Arabidopsis roots through combined action with AtHY5 [28]. Recently, emerging evidence has implicated GLK genes in the regulation of anthocyanin biosynthesis. *AtGLK2* was shown to play a positive regulatory role in sucrose-induced anthocyanin biosynthesis through HY5-mediated light signaling pathway [7]. Consistent with these findings, another investigation reported that *AtGLK1* also promotes anthocyanin biosynthesis via an MBW complex in Arabidopsis [8]. And *AtGLK2* transcriptionally activates anthocyanin late biosynthetic genes (LBGs; e.g. *DFR*, *LDOX* or *UF3GT*) and TTG1. Additionally, *AtGLK2* physically interacts with TT8 to co-regulate anthocyanin biosynthesis [6]. Despite the progress made in understanding the functions of GLK genes in Arabidopsis, it would be interesting to investigate whether the roles of GLK genes in chloroplast development and flavonoid biosynthesis can work the same way in other plant species. In tea plants, *CsGLK1* and *CsGLK2* have been shown to be involved in regulating chlorophyll biosynthesis and flavonoid (catechin) accumulation [29]. The expression of *NtGLK1*, a tobacco homolog of Arabidopsis GLK genes, was induced by PAP1 overexpression, suggesting a potential feedback loop between GLK genes and the flavonoid biosynthetic pathway [30].

In kiwifruit (*Actinidia* spp.), GLK genes exist in pairs, with *AcGLK1* predominantly expressed in leaves and *AcGLK2* in fruits, and have been shown to play conserved roles in promoting chloroplast division and development [31, 32]. Among diverse kiwifruit cultivars, red-fleshed varieties, which accumulate abundant anthocyanins, have garnered significant attention due to their enhanced nutritional profile and economic value. Meanwhile, advancements in genomics have facilitated the molecular dissection of this trait [33–36].

This study found that red-flesh kiwifruit exhibits a significantly higher abundance of *AcGLK2* expression compared to green-flesh varieties. Further analysis established that *AcGLK2* involves anthocyanin accumulation by influencing the expression of R2R3MYB and R3MYB TFs. This study has shed insights into understanding the coloring mechanism of red-flesh kiwifruit, offering novel gene resources for molecular breeding and facilitating quality enhancement in kiwifruit cultivation.

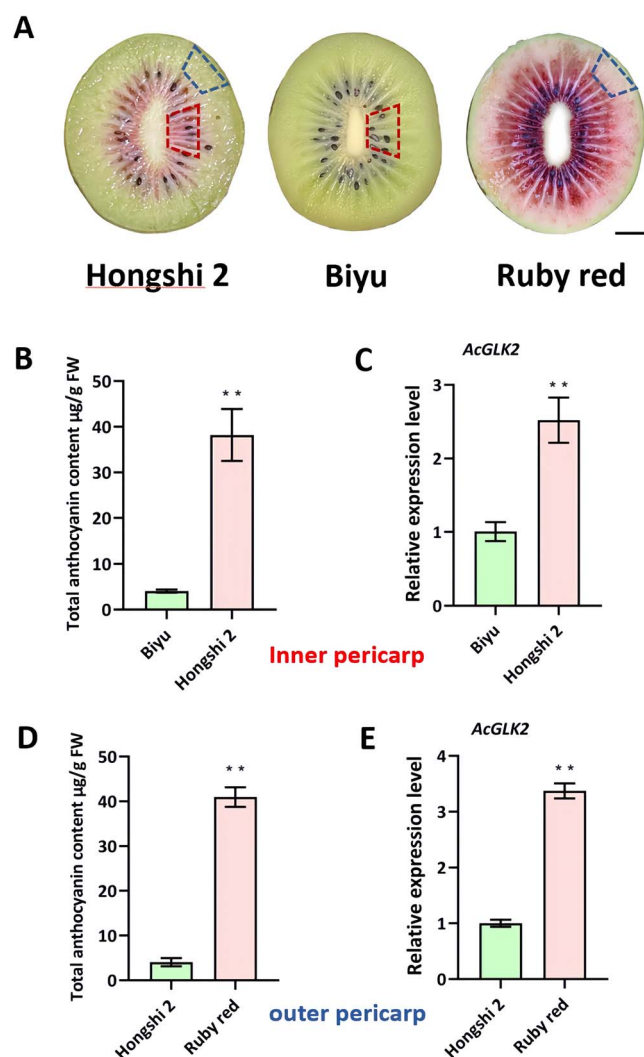


Figure 1 Role of *AcGLK2* is associated with the induced anthocyanin synthesis in *A. chinensis*. (A) Photos of red-centered kiwifruit 'Hongshi 2', green-fleshed kiwifruit 'Biyu' and red-fleshed kiwifruit 'Ruby red' at 120 DPA. The red box area represents the inner pericarps. The blue box area represents the outer pericarps. bar=1 cm. (B) Total anthocyanin content in inner pericarps of 'Hongshi 2' and 'Biyu'. (C) The expression level of *AcGLK2* differentially expressed in the inner pericarps of 'Hongshi 2' and 'Biyu'. (D) Total anthocyanin content in outer pericarps of 'Hongshi 2' and 'Ruby red'. (E) The expression level of *AcGLK2* differentially expressed in the outer pericarps of 'Hongshi 2' and 'Ruby red'.

Results

The relative expression abundance of *AcGLK2* is increased in red-fleshed kiwifruit

The diploid cv. 'Hongshi 2' (*Actinidia chinensis*) accumulates anthocyanins in the inner pericarp, while 'Biyu' does not (Fig. 1A and B). GLK pairs in kiwifruit, with *AcGLK2* predominantly expressed in fruits while *AcGLK1* was hardly detected in fruit tissues [31, 32]. Initially, we conducted quantitative PCR (qPCR) analysis on the inner pericarps of the red-fleshed cv. 'Hongshi 2' and the green-fleshed cv. 'Biyu' at 120 days post-anthesis (DPA),

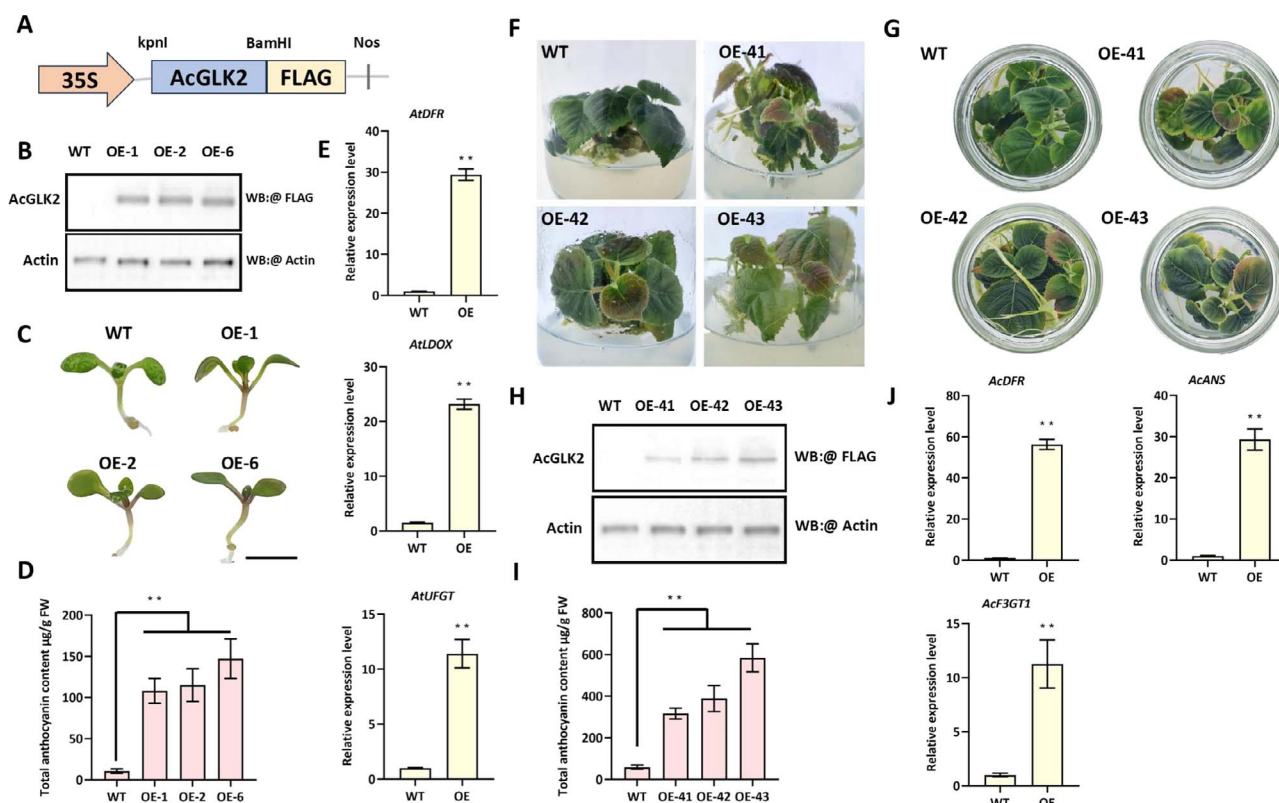


Figure 2 *AcGLK2* enhances anthocyanin biogenesis in *Arabidopsis* and kiwifruit. (A) Schematic diagram of the *AcGLK2* gene overexpression vector. (B) The protein expression levels of *AcGLK2* in WT and *AcGLK2* transgenic lines (OE-1, OE-2, and OE-6) by western blot. Actin was used as the internal control. (C) The phenotype of the *AcGLK2* transgenic *Arabidopsis* seedlings. Bar = 1 cm. (D) Total anthocyanin content in WT and transgenic *Arabidopsis* lines. (E) qPCR analysis of expression levels of LBGs (*AtDFR*, *AtANS*, and *AtUFGT*) in anthocyanin accumulation pathway. (F, G) The phenotype of the *AcGLK2* transgenic kiwifruit seedlings (side and above view). (H) The protein expression levels of *AcGLK2* in transgenic kiwifruit lines (OE-41, OE-42, and OE-43) by western blot. (I) Total anthocyanin content in WT and transgenic kiwifruit. (J) qPCR analysis of expression levels of LBGs (*AcDFR*, *AcANS*, and *AcF3GT1*) in anthocyanin accumulation pathway.

belonging to mature fruits (MF) (Fig. 1A). The results demonstrated that the expression of *AcGLK2* exhibited a concomitant increase and became much higher than that in the green-fleshed cultivar (Fig. 1C). These results suggest that the elevated expression level of *AcGLK2* in the inner pericarp of ‘Hongshi 2’ is highly correlated with the increased anthocyanin accumulation, thereby establishing a distinct expression gradient compared to ‘Biyu.’

In addition, we performed qPCR analysis to compare *AcGLK2* expression level in the outer pericarps of two distinct *A. chinensis* cultivars, ‘Ruby Red’ that accumulates anthocyanin in both inner and outer pericarps, but ‘Hongshi 2’, which maintains green coloration in its outer pericarps (Fig. 1A and D). Consistently, our analysis revealed significantly higher levels of *AcGLK2* expression in the anthocyanin-rich outer pericarps of ‘Ruby Red’ compared to its counterpart in ‘Hongshi 2’ (Fig. 1E).

Constitutive overexpression of *AcGLK2* increases anthocyanin content in *Arabidopsis* and kiwifruit

The *Agrobacterium* strain GV3101 harboring the overexpression vector 35S::*AcGLK2*-FLAG (Fig. 2A) was used for stable transformation in both *Arabidopsis thaliana* (Columbia) and *A. chinensis* cv. ‘Hongyang’ that accumulates anthocyanin specifically in the

inner pericarp [37]. Through molecular screening, we successfully obtained at least three independent overexpression transgenic lines for individual species.

In *Arabidopsis*, the transgenic lines #1, #2, and #6 were confirmed to be homozygous *AcGLK2*-overexpressing lines by target protein level (Fig. 2B). According to previous reports, sucrose and light are used to induce anthocyanin accumulation in plant [7, 8, 38]. To further investigate the role of *AcGLK2* in anthocyanin biosynthesis, we treated *Arabidopsis* seedling [wild-type (WT) and *AcGLK2*OE] with the suggested sucrose concentration and light intensity. Approximately five days after germination, we observed distinct purple pigmentation accumulation in the transgenic seedlings, particularly in the hypocotyls and the junction between cotyledons, whereas no visible purple pigment was detected in WT seedlings (Fig. 2C). Quantitative analysis showed that *AcGLK2*OE displays a noticeable difference in anthocyanin accumulation compared to WT, and the anthocyanin levels in *AcGLK2*OE #1, #2, and #6 are much higher than that in WT (Fig. 2D). We subsequently examined the transcript levels of genes (*AtDFR*, *AtLDOX* and *AtUFGT*) involved in anthocyanin biosynthesis by qPCR. Compared to WT, there were significant higher in the expression levels of these genes detected in *AcGLK2*OE lines (Fig. 2E).

We also created three transgenic lines (*AcGLK2*OE #41, #42, and #43) of ‘Hongyang’ (Fig. 2F and G) and *AcGLK2* protein expression level was confirmed by western blot (Fig. 2H). The treated

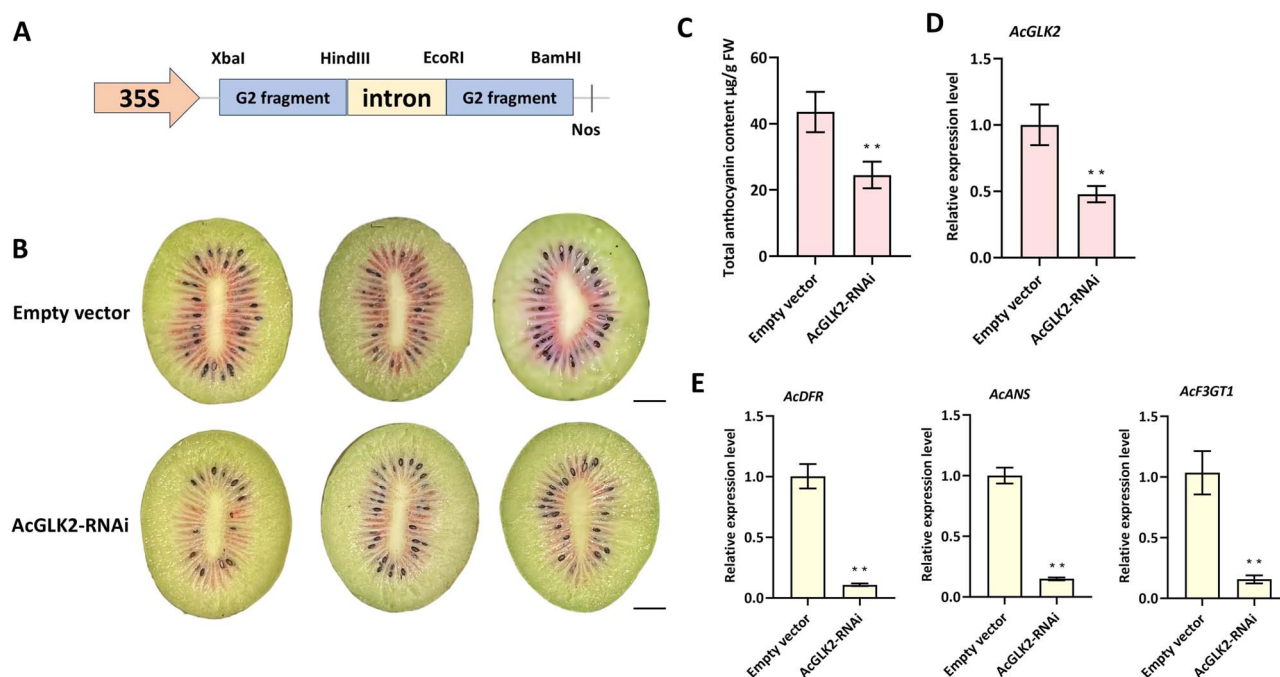


Figure 3 *AcGLK2-RNAi* reduces anthocyanin content in kiwifruit. (A) Schematic diagram of the *AcGLK2-RNAi* interference vector. (B) The phenotype of the transient expression *AcGLK2-RNAi* and empty vector in kiwifruits. Bar = 1 cm. (C) Total anthocyanin content in empty vector and *AcGLK2-RNAi* in fruits. (D) qPCR analysis of expression levels of *AcGLK2* in fruits of empty vector and *AcGLK2-RNAi*. (E) Expression levels of LBGs (*AcDFR*, *AcANS*, and *AcF3GT1*) in fruits of empty vector and *AcGLK2-RNAi*.

transgenic seedling plants were characterized with significantly increased red leaves (Fig. 2F and G), suggesting that *AcGLK2* overexpression can increase the anthocyanins accumulation in kiwifruit (Fig. 2I). The transcript accumulation patterns of anthocyanin synthetic pathway genes (*AcDFR*, *AcANS* and *AcF3GT1*) were examined. A comparative assessment between *AcGLK2*-overexpressing lines and ‘Hongyang’ WT plants demonstrated substantially elevated expression levels of these structural genes in the transgenic lines (Fig. 2J).

Transiently silencing *AcGLK2* inhibits anthocyanin accumulation in kiwifruit

Previous research has demonstrated that transient transformation through fruit injection in kiwifruit serves as an effective approach for monitoring changes in pigmentation [37]. The *Agrobacterium* strain harboring the 35S::*AcGLK2-RNAi* construct (Fig. 3A) was injected into pre-color change stage (70 DPA) fruits of the ‘Hongshi 2’ cultivar. The treated fruits were harvested at nearly maturing stage (120 DPA). Quantitative analysis revealed that RNA interference (RNAi)-mediated silencing of *AcGLK2* was effective and resulted in markedly reduced anthocyanin content relative to empty vector-transformed control fruits, establishing a clear correlation between *AcGLK2* expression and pigment accumulation (Fig. 3B–D). The expression abundance of structural genes (*AcDFR*, *AcANS*, and *AcF3GT1*) in the anthocyanin synthesis pathway was significantly downregulated (Fig. 3E). These findings indicate a positive correlation between the expression of *AcGLK2* and anthocyanin accumulation in kiwifruit, suggesting its potential regulatory role in anthocyanin biosynthesis.

AcGLK2 regulates the expression of genes involved in flavonoid-related pathway

Transcriptome analysis was conducted on WT plants and transgenic plants overexpressing 35S::*AcGLK2-FLAG* to investigate the role of *AcGLK2* in regulating the anthocyanin accumulation. Each sample included three biological replicates. Differentially expressed genes (DEGs) were identified between the WT and 35S::*AcGLK2-FLAG* groups using the criteria of a $|\log_2\text{fold change}| \geq 1$ and an FDR-adjusted P -value < 0.05 . In total, 13 421 DEGs were identified. There were 7612 upregulated genes and 5809 downregulated genes in the 35S::*AcGLK2-FLAG* transgenic plants compared to WT (Table S1, Fig. 4A). Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis demonstrated that these genes were significantly enriched in terms of flavonoid biosynthesis and phenylpropanoid biosynthesis (Fig. 4B). Heatmap analysis revealed that 35 structure genes (*PAL*, *4CL*, *C4H*, *CHS*, *F3H*, *F3'5'H*, *DFR*, *ANS*, *F3GT1*, and *F3GGT1*), 5 glutathione S-transferases (*GST*), and 6 TFs (*MYB* and *bHLH*) were significantly differentially expressed in the 35S::*AcGLK2-FLAG* transgenic plants (Fig. 4C).

Genome-wide binding profiles of *AcGLK2* in kiwifruit

Chromatin immunoprecipitation sequencing (ChIP-Seq) analysis was conducted on 35S::*AcGLK2-FLAG* transgenic plants to pinpoint *AcGLK2* binding sites across the genome. The overlapping peaks were assigned to the closest genes, and a large number of TFs such as ERF, bHLH, MYB, C2H2, NAC, and WRKY were identified as potential *AcGLK2*-bound targets (Table S2, Fig. S1A).

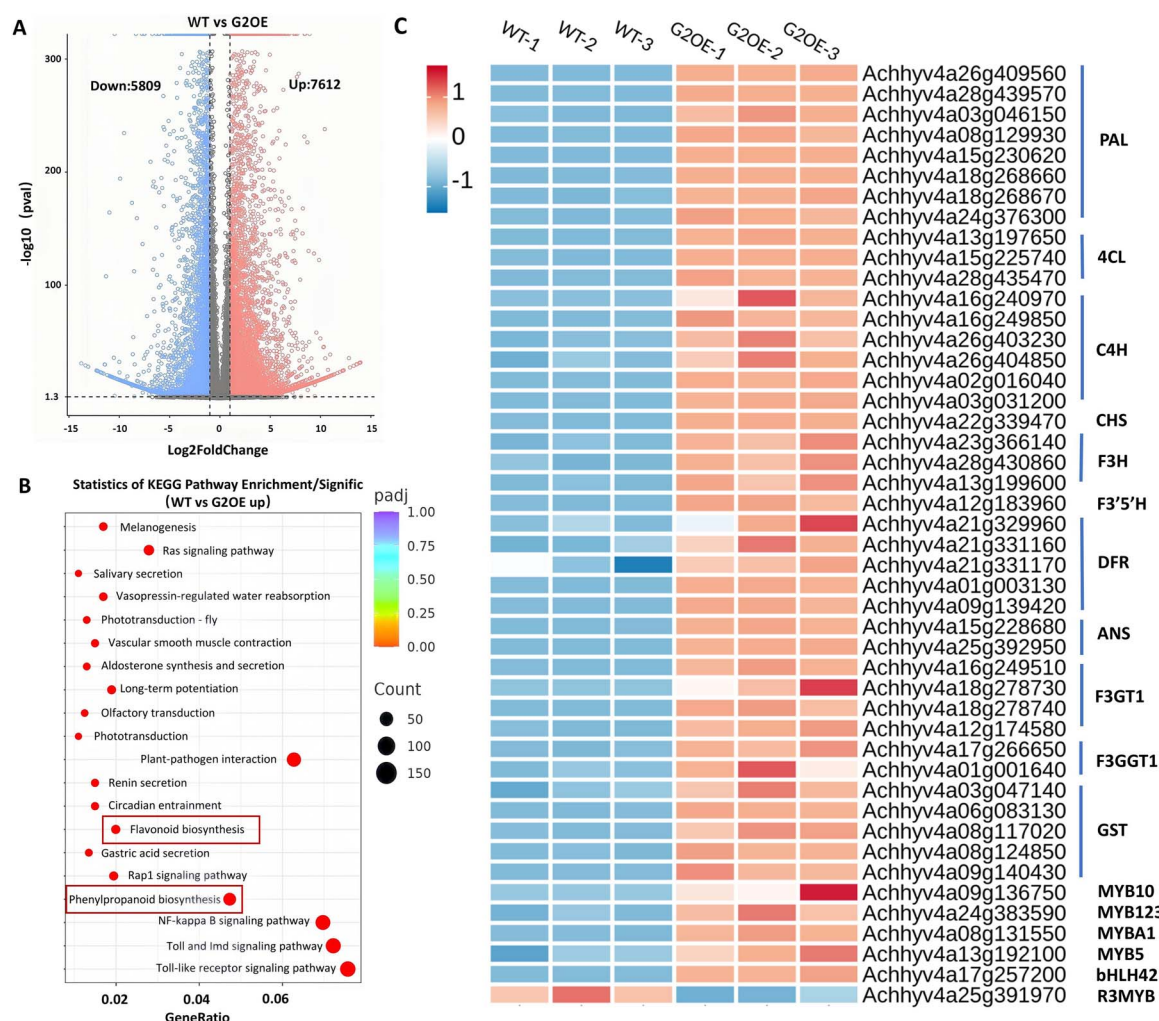


Figure 4 RNA-Seq analysis of DEGs between WT and *AcGLK2*-overexpression kiwifruit. (A) Volcano plots showing the number of DEGs regulated by *AcGLK2*. (B) KEGG enrichment results of peak-related genes. (C) Heatmap analysis of the *AcGLK2*-regulated DEGs.

Subsequently, we focused our investigation on MYB TFs that are intensively involved in regulation of flavonoid biosynthesis. By cross-referencing the MYB members identified from the aforementioned ChIP-seq screening with differential expression MYBs in the transcriptome analysis, we identified a subset of overlapping candidates. Seventy-eight upregulated and 18 downregulated MYBs appeared to be potential binding targets of *AcGLK2* (Fig. S1B). The ChIP-seq analysis showed that *AcGLK2* binding peaks were notably present in various genomic regions, such as introns, coding sequences (CDSs), promoters, and intergenic regions (Table S3, Fig. S1C). Further investigation into the *AcGLK2* binding profile in the promoter region revealed an enrichment of binding sites substantially in the proximal promoter region, with a peak occurring ~1 kb upstream of the transcription start site (TSS) (Fig. S1D). In this way, we gradually narrowed down the research scope and screened out the members of MYBs that only bind to the promoter 0–1 kb, resulting in 20 MYBs. Phylogenetic analysis incorporating known positive (e.g. *AtMYB75*, *AtMYB90*) [39] and negative (e.g. *AtMYBL2*, *AtMYB4*) [13,40] anthocyanin regulators from *A. thaliana* revealed two candidate members from 20 MYB candidates, *Achhyv4a13g192100* and *Achhyv4a25g391970*. *Achhyv4a13g192100* clustered closely with the positive regulators, while *Achhyv4a25g391970* grouped with the negative regulators,

respectively, suggesting their putative roles as activators and repressors in the regulatory network (Fig. S1E).

We isolated *Achhyv4a13g192100* and *Achhyv4a25g391970* genes and conducted amino acid sequence alignment and phylogenetic tree analysis. The results showed that *Achhyv4a13g192100* encodes an R2R3-MYB TF highly homologous to *AtMYB5* (Fig. S2A and B), whereas *Achhyv4a25g391970* encodes an R3-MYB TF closely homologous to *AtTRY* (Fig. S3A and B). Furthermore, subcellular localization analysis revealed that *AcMYB5* is localized exclusively in the nucleus (Fig. S2C), while *AcTRY* is predominantly targeted to nuclear but also exhibits minor cytoplasmic fluorescence signal (Fig. S3C).

AcGLK2 regulates anthocyanin biosynthesis through modulating expression of *AcMYB5* and *AcTRY*

To further elucidate the regulatory mechanism of *AcGLK2* in anthocyanin biosynthesis, we investigated its role in modulating downstream gene expression. Integrated analysis of RNA-Seq and ChIP-Seq data implicated that both *AcMYB5* and *AcTRY* are direct targets of *AcGLK2*. Subsequent qPCR assays showed

that the expression of *MYB5* was significantly upregulated in transgenic lines compared to the WT, whereas *TRY* was markedly downregulated in *AcGLK2*-overexpressing plants (Fig. S4A and B). Conversely, in *AcGLK2*-RNAi fruits, the expression of *AcMYB5* was suppressed, while that of *AcTRY* was elevated (Fig. S4C).

The promoters of *AcMYB5* (3158 bp) and *AcTRY* (2548 bp) were fused to the *GUS* and *LUC* reporter genes (Fig. 5A). Subsequently, β -glucuronidase (*GUS*) enzyme activity and dual-luciferase assays in transiently expressed tobacco leaves were performed to evaluate promoter activity. The results showed cotransfection with *AcGLK2* significantly enhanced the activity of the *AcMYB5* promoter, as evidenced by intensified *GUS* staining, increased *GUS* enzyme activity, and stronger *LUC* fluorescent signal (Fig. 5B–E). In contrast, *AcGLK2* suppressed the activity of the *AcTRY* promoter, with both *GUS* and *LUC* activities markedly reduced in coinfiltrated tobacco leaves (Fig. 5B–E). These results demonstrated that *AcGLK2* directly regulates the expression of both *AcMYB5* and *AcTRY*.

GGAATC, CACGTG, GATATC, and GATTCT have been previously reported in Arabidopsis as several conserved promoter sequences bound to GLK [41,42]. The promoters of *AcMYB5* and *AcTRY* contain some above motifs (Fig. S5). To examine the direct interaction between *AcGLK2* and the promoters of *AcMYB5* and *AcTRY*, yeast one-hybrid (Y1H) assays and electrophoretic mobility shift assays (EMSAs) were conducted. Promoter-derived DNA fragments (P1/P2) and corresponding DNA probes, which encompass the CACGTG and GATATC *cis*-elements, respectively, were employed for these analyses (Fig. S5). Y1H assay was initially conducted to evaluate the interaction between *AcGLK2* and promoters of *AcMYB5* and/or *AcTRY*. As shown in Fig. 5F, the yeast cells were selected on the SD/-Ura/-Trp medium and *AcGLK2* was shown to be directly bound to P1 and P2 promoter regions of *AcMYB5* and *AcTRY*, respectively. The EMSA results further demonstrated that recombinant *AcGLK2*-HIS protein was directly bound to labeled DNA probes containing the respective motifs, but not to corresponding mutant probes in which these *cis*-elements were disrupted (Fig. 5G).

***AcMYB5* positively regulates the anthocyanins accumulation**

AcMYB5 belongs to the R2R3-MYB (Fig. S2). The involvement of MYB5 in the regulation of anthocyanin biosynthesis has been reported in various plant species, including grape, strawberry, *Freesia hybrida* and *Dendrobium* [43–46]. However, the function of *AcMYB5* in kiwifruit remains to be validated. In this study, we generated stable transgenic tobacco plants expressing 35S::*AcMYB5* (Fig. 6A and B) and observed significantly enhanced pigment accumulation in the flowers of *AcMYB5* overexpression lines (Fig. 6C and D). The structural genes (*NtDFR*, *NtANS* and *NtUGT*) involved in the anthocyanin biosynthesis pathway were also significantly upregulated (Fig. 6E).

RNAi-mediated gene silencing of *AcMYB5* was performed (Fig. 6F) using the same injection method and treatment duration as previously described for *AcGLK2* silencing. The experimental results demonstrated that suppression of *AcMYB5* expression led to a reduction in anthocyanin accumulation in the kiwifruit fruits (Fig. 6G–I), accompanied by downregulation of downstream structural gene (*AcDFR*, *AcANS*, and *AcF3GT1*) expression (Fig. 6J).

However, transient expression of 35S::*AcMYB5* alone in tobacco leaves did not induce visible color changes (Fig. 7A). Previous studies have established that MYB TFs often function in conjunction with bHLH partners, and specifically *AcbHLH42* has been reported to interact with key MYB members such as *AcMYBF110* and *AcMYB123* in kiwifruit [37]. To explore this potential interaction, we coexpressed 35S::*AcMYB5* with 35S::*AcbHLH42* in tobacco leaves, which led to the production of anthocyanins in the infiltrated regions (Fig. 7A–C). To further validate the interaction between *AcMYB5* and *AcbHLH42*, we performed split-luciferase complementation imaging assay (LCI) and yeast two-hybrid (Y2H), both of which confirmed a direct interaction between these two proteins (Fig. 7D–F). Through *GUS* reporter activity assay, we demonstrated that *AcMYB5*–*AcbHLH42* complex can effectively activate the promoters of *AcANS* and *AcF3GT1* (Fig. 7G), thereby functioning as a positive regulator in the anthocyanin biosynthesis pathway.

***AcTRY* negatively regulates the anthocyanins biosynthesis**

TRY (TRIPTYCHON), also known as CPC or ETC1 [47–49], belongs to the R3-MYB family (Fig. S3). In *A. thaliana*, *AtTRY* has been demonstrated to suppress trichome formation [49]. We overexpressed the homologous gene *AcTRY* from kiwifruit in Arabidopsis and observed a similar phenotype, with transgenic plants showing significantly reduced trichome density compared to WT controls (Fig. S6). Furthermore, we introduced 35S::*AcTRY* (Fig. 8A) into purple tomato (*Indigo Rose*) and found that the transgenic tomatoes exhibited a substantial decrease in anthocyanin content, resulting in a greener fruit (Fig. 8B–D). The LBGs also significantly decrease (Fig. 8E). Similarly, we performed 35S::*AcTRY* (*AcTRY*-OE) in kiwifruit. The results demonstrated that infiltrated overexpression of *AcTRY* reduced anthocyanin accumulation in the fruit (Fig. 8F–H). Consistently, qPCR analysis showed the expression levels of structural genes involved in the anthocyanin biosynthesis pathway were markedly downregulated (Fig. 8I).

Previous studies have demonstrated that *AcbHLH42* interacts with several positive regulators (i.e. *AcMYBF110*/*AcMYB123*) of anthocyanin biosynthesis, thereby promoting anthocyanin accumulation [37]. Interestingly, *AcTRY* appears to compete with these positive regulators for binding to bHLH proteins, consequently inhibiting anthocyanin biosynthesis. Preliminary evidence from LCI assays in transiently transformed tobacco leaves and Y2H experiments confirmed that protein *AcTRY* and *AcbHLH42* can interact (Fig. 9A–C). In tobacco, the expression of *AcMYBF110* with empty vector resulted in substantial anthocyanin accumulation, coexpression of *AcTRY* with *AcMYBF110* (without altering *AcMYBF110* protein levels) led to a significant reduction in anthocyanin content. A similar trend was observed in leaves coexpressing *AcMYBF110*, *AcbHLH42*, and empty vector compared with those coexpressing *AcMYBF110*, *AcbHLH42*, and *AcTRY*. The anthocyanin content was significantly lower in regions coexpressing *AcMYBF110*–*AcbHLH42*–*AcTRY* (Fig. 9D and E). LCI assays revealed that the introduction of *AcTRY* protein reduced the interaction efficiency between *AcMYBF110* and *AcbHLH42*. Moreover, the presence of *AcMYBF110* protein effectively interfered with the interaction between *AcTRY* and *AcbHLH42* (Fig. 9F–H). These results demonstrate that *AcTRY* and *AcMYBF110* compete for binding with *AcbHLH42*.

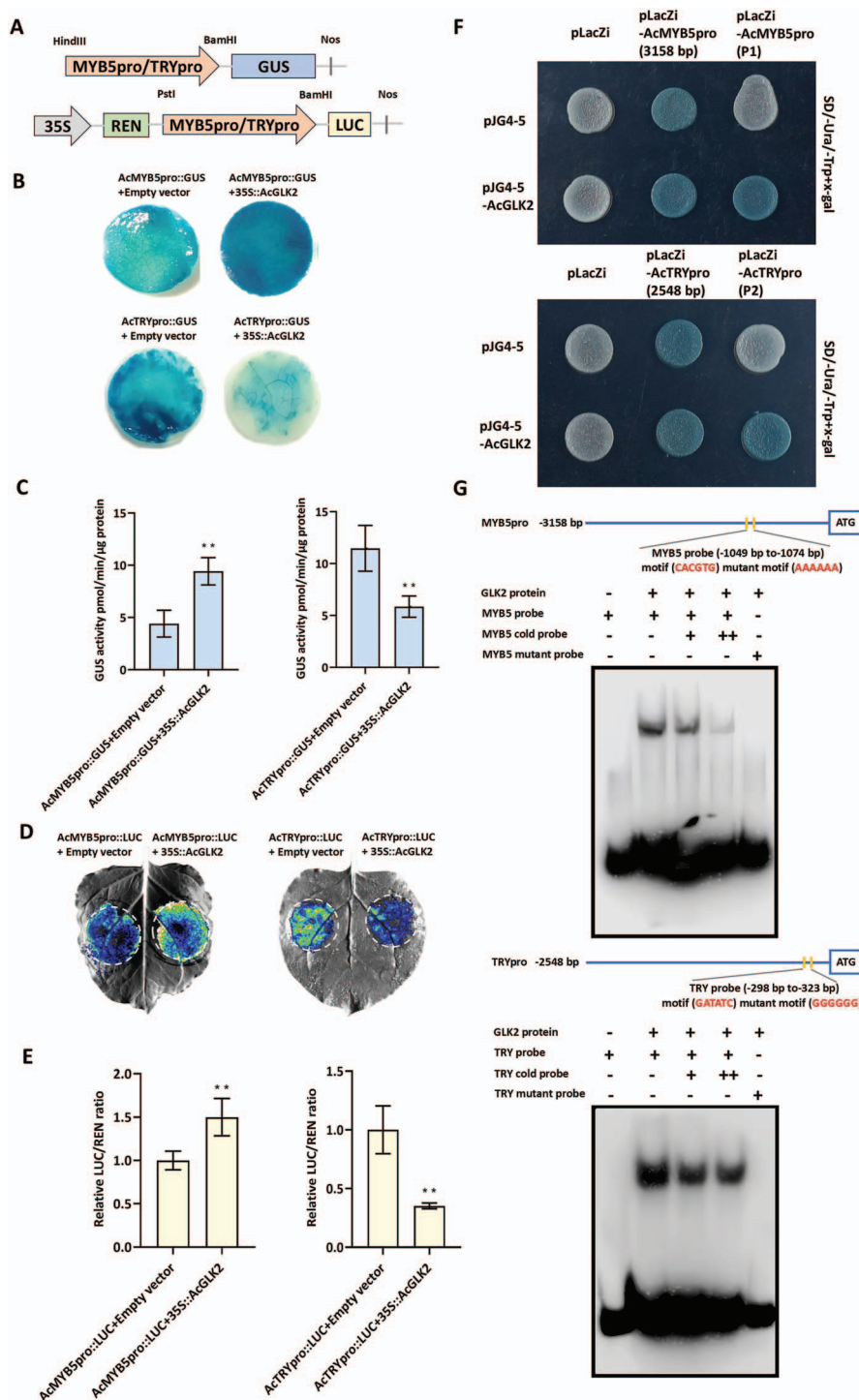


Figure 5 The transcription regulation of AcGLK2 on the expression of *AcMYB5* and *AcTRY* through binding two of promoter. (A) Schematic diagram of the *AcMYB5pro/AcTRYpro::GUS/LUC* vector. (B, C) GUS staining and GUS activities measurement in *N. benthamiana* leaves following transient co-transformation with 35S::AcGLK2 (or empty vector) and *AcMYB5pro/AcTRY::GUS*. (D, E) Transient expression assays shows that 35S::AcGLK2 activates *AcMYB5pro::LUC* and represses *AcTRYpro::LUC* expression. Representative images of *N. benthamiana* leaves were taken at 48 h after infiltration. (F) Y1H assays showed that AcGLK2 directly bound to the promoters of *AcMYB5* and *AcTRY*. (G) EMSA showing that AcGLK2 bound to the CACGTG and GATATC contained in P1/P2, respectively.

Discussion

GLKs are potent positive regulators of chloroplast development in the plant kingdom [1, 27]. In flowering plants, two GLK homologs typically exist, with GLK1 being minimally expressed in fruits

while GLK2 predominantly functions [31, 32, 50]. By effectively utilizing GLK, crop yields can be enhanced, thereby facilitating crop improvement. For instance, maize GLK gene expression can potentially convert C3 rice into C4-like plants to increase yield [51]. In tomatoes, GLK overexpression has been shown to enhance

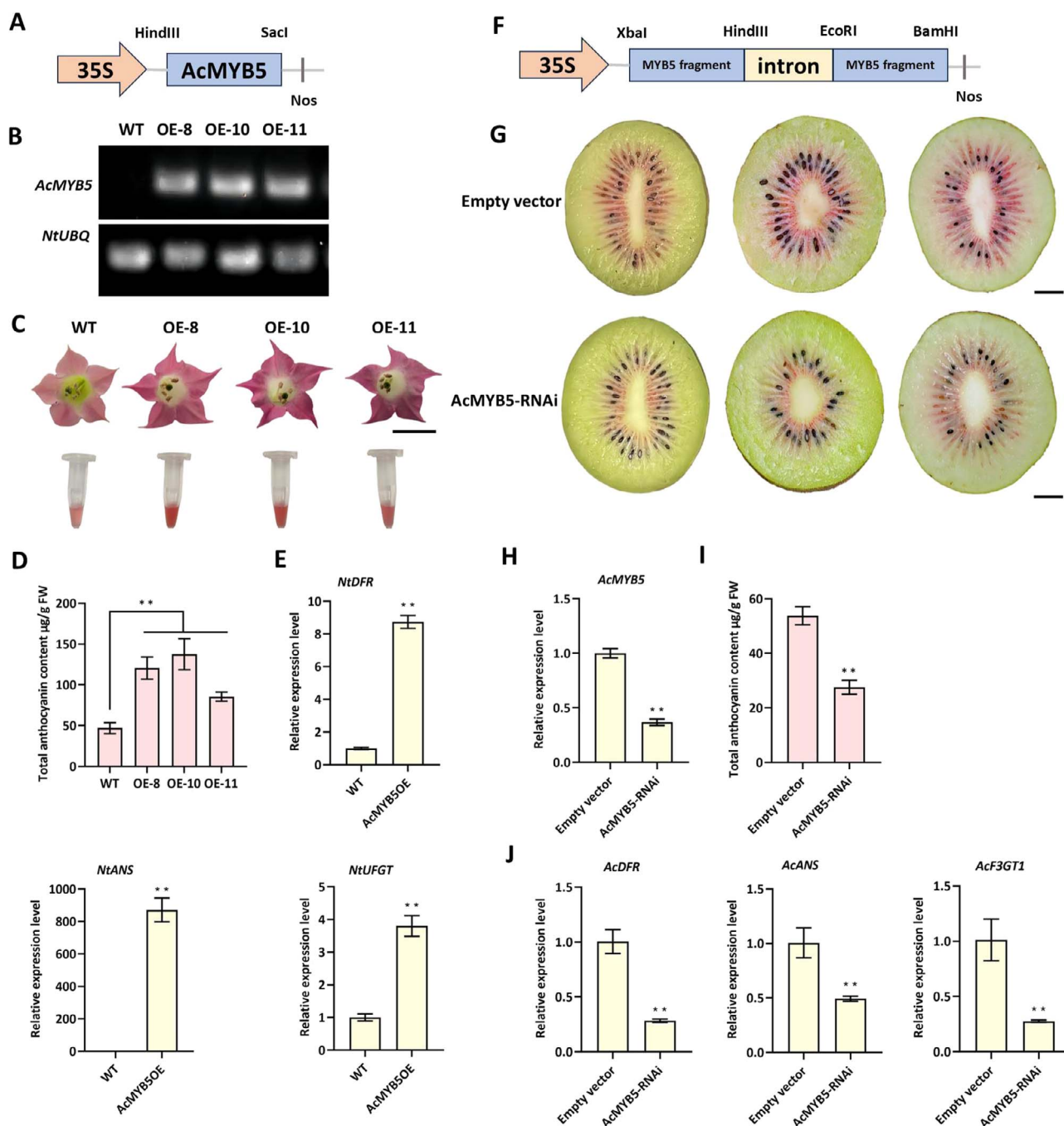


Figure 6 *AcMYB5* positively regulates anthocyanin accumulation in tobacco and kiwifruit. (A) Schematic diagram of the *AcMYB5* overexpression vector. (B) The transcript accumulation of *AcMYB5* in WT and *AcMYB5* transgenic tobacco (OE-8, OE-10, and OE-11) with RT-PCR analysis. *NtUBQ* was used to normalize the expression of the genes. (C) Appearance in flowers of tobacco plants transformed with 35S-driven *AcMYB5*. Bar = 1 cm. (D) Total anthocyanin content in WT and *AcMYB5* transgenic tobacco lines in petal. (E) The expression levels of endogenous tobacco genes (*NtDFR*, *NtANS*, and *NtUGT*) were investigated by qPCR using cDNA from petal limbs collected at anthesis. (F) Schematic diagram of the *AcMYB5*-RNAi interference vector. (G) The phenotype of the transient expression *AcMYB5*-RNAi and empty vector in kiwifruits. Bar = 1 cm. (H) The expression levels of *AcMYB5* in empty vector and *AcMYB5*-RNAi. Fruits were investigated by qPCR. (I) Detecting the total anthocyanin content of kiwifruit injected with empty vector and *AcMYB5*-RNAi. (J) qPCR analysis that the expression levels of kiwifruit genes (*AcDFR*, *AcANS*, and *AcF3GT1*).

fruit quality by increasing soluble solids content and lycopene accumulation [50, 52]. Additionally, GLK exhibits disease defense and biotic stress resistance [53–55]. In addition, recent studies have revealed novel functions of GLKs. Notably, in tea plants, both GLK homologs have been found to promote catechin synthesis, thereby improving tea quality [29, 56]. Interestingly, Arabidopsis

GLKs were shown to regulate anthocyanin biosynthesis [6–8]. Despite these advancements, research on GLK functions in fruit trees remains limited.

These findings prompt us to consider whether GLKs play a regulatory role in flavonoid metabolism in kiwifruit. In kiwifruit, two GLK members have been shown to function in regulation

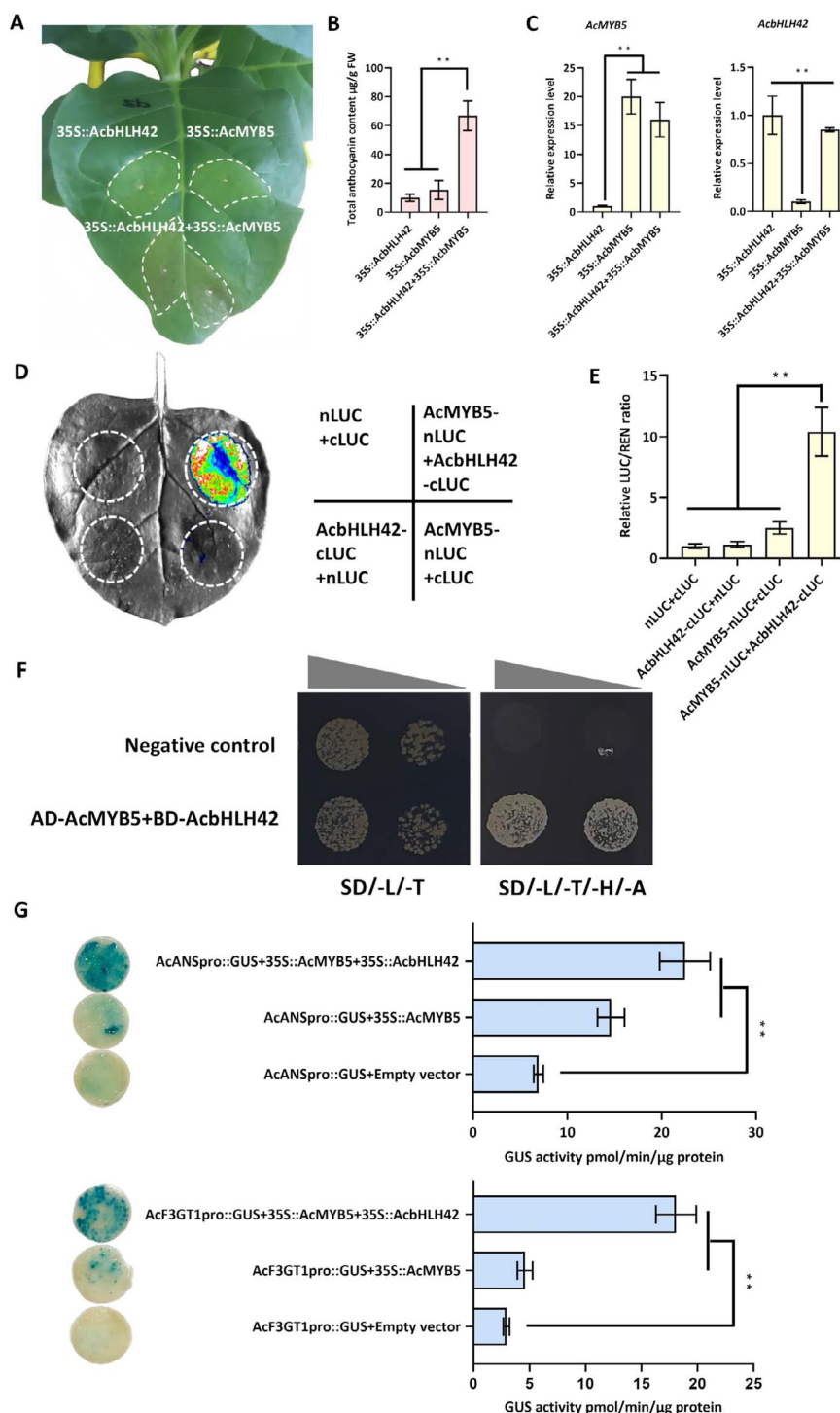


Figure 7 AcMYB5 and AcbHLH42 proteins interact to activate structural genes *AcANS* and *AcF3GT1* of anthocyanin synthesis pathway. (A) Phenotypic analysis of 35S::AcMYB5 and 35S::AcbHLH42 overexpress separately and co-injection and co-injection in tobacco leaves. (B) Determination of total anthocyanin content in injection area of tobacco leaves. (C) qPCR analysis of expression levels of *AcMYB5* and *AcbHLH42* in injection area of tobacco leaves. (D, E) Interaction of *AcMYB5* with *AcbHLH42* in LCI assays. *Agrobacterium* strains GV3101 harboring *AcMYB5*-nLUC and *AcbHLH42*-cLUC constructs were immersed into *N. benthamiana* leaves. After incubation for 48 h, the fluorescence signal was detected, with relative enzyme activity measured as LUC/REN. (F) *AcMYB5* interacts with *AcbHLH42*, verified by Y2H. The transformed yeasts were 10-fold diluted and dotted on SD/-L/-T and SD/-L/-T/-H/-A media to observe growth. Cotransformation of AD and BD into yeasts serves as a negative control. (G) GUS staining and GUS activities measurement in *N. benthamiana* leaves transiently expressing *AcF3GT1pro/AcANSpro::GUS* with Empty vector, *AcF3GT1pro/AcANS pro::GUS* with 35S::AcMYB5, and *AcF3GT1pro/AcANS pro::GUS* with 35S::AcMYB5 + 35S::AcbHLH42.

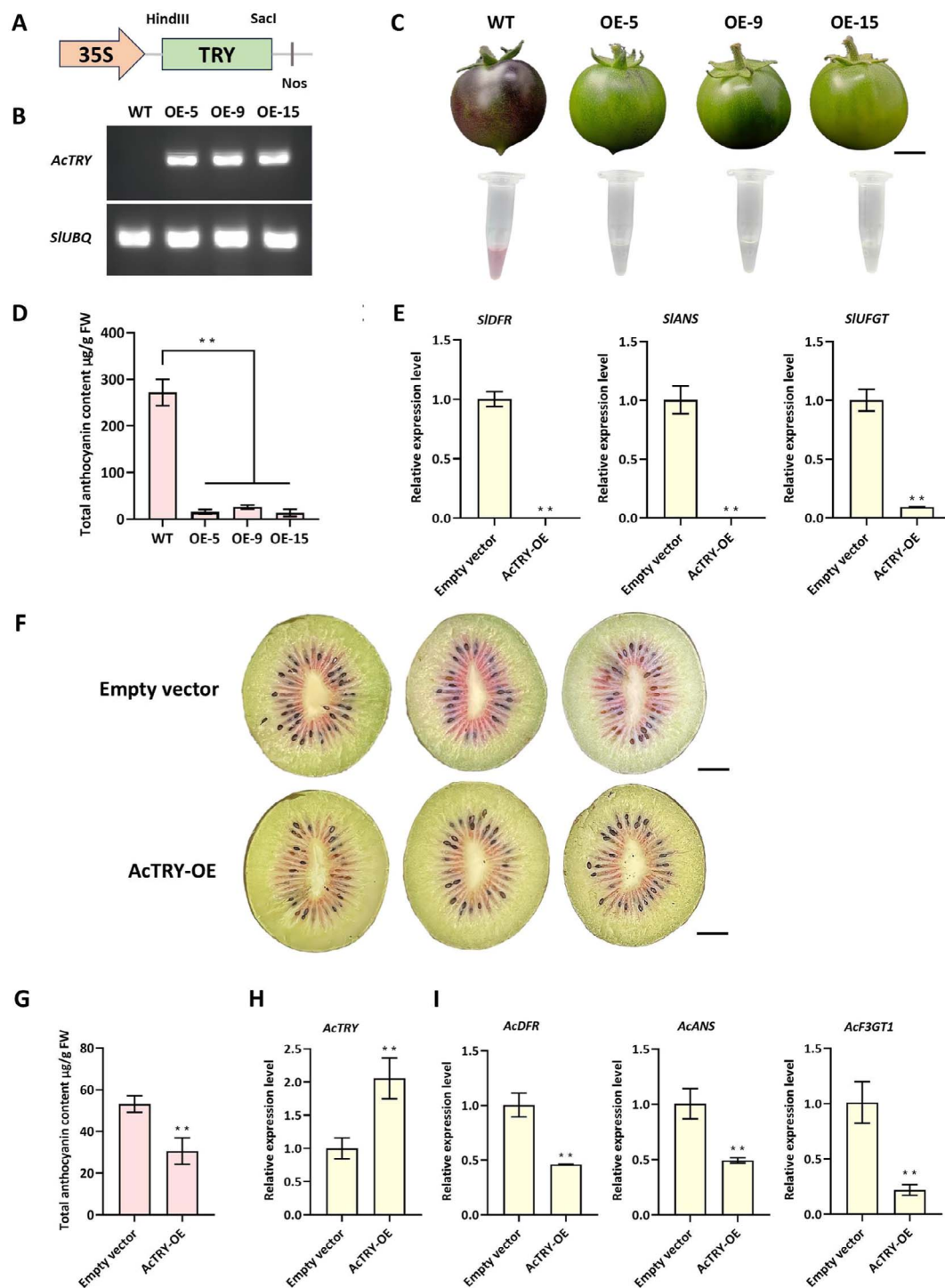


Figure 8 *AcTRY* negatively regulates anthocyanin biosynthesis in tomato and kiwifruit. (A) Schematic diagram of the *AcTRY* overexpression vector. (B) The transcript accumulation of *AcTRY* in WT and *AcTRY* transgenic tobacco (OE-5, OE-9, and OE-15) with RT-PCR analysis. *SIUBQ* was used to normalize the expression of the genes. (C) Appearance in green mature tomato fruits transformed with 35S::*AcTRY*. Bar = 1 cm. (D) Analysis of anthocyanin content in fruit skin of WT and transgenic tomatoes. (E) The expression levels of tomato genes (*SIDFR*, *SIANS*, and *SIUGT*) by qPCR. (F) The phenotype of the transient expression 35S::*AcTRY* and empty vector in kiwifruits. Bar = 1 cm. (G) Total anthocyanin content of kiwifruit injected with empty vector and 35S::*AcTRY*. (H) The expression levels of *AcTRY* in empty vector and *AcTRY*-OE fruits were investigated by qPCR. (I) qPCR analysis that the expression levels of kiwifruit genes (*AcDFR*, *AcANS*, and *AcF3GT1*).

of chloroplast development and carotenoids accumulation [31, 32]. Notably, *AcGLK2* is predominantly expressed in the edible fruit tissue [31]. Our analysis revealed significantly higher *AcGLK2* expression levels in the anthocyanin-rich fruit tissue compared to

green tissues or green-fleshed varieties. In addition to comparing the kiwifruit varieties of 'Hongshi-2' and 'Biyu', we also selected two other 'Hongyang' and 'Cuiyu' kiwifruit varieties with differences in anthocyanin accumulation for comparison of *AcGLK2*

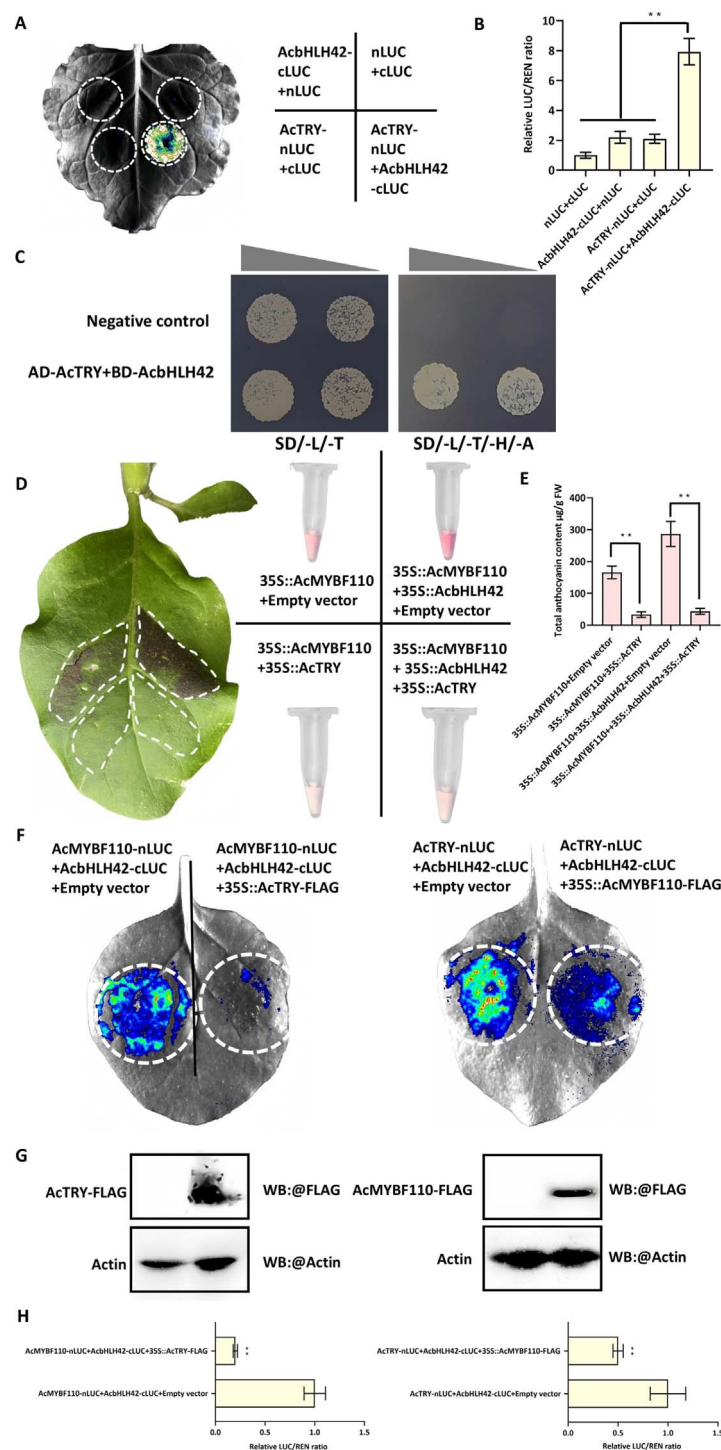


Figure 9 AcTRY, competition with AcMYBF110 and interact with AcbHLH42 affect the anthocyanin accumulation. (A, B) The interactions between AcTRY and AcbHLH42 in LCI assays. AcTRY-nLUC + AcbHLH42-cLUC, including AcTRY-nLUC + cLUC, nLUC + AcbHLH42-cLUC, and nLUC + cLUC together with the negative controls, were injected into different parts of the same *N. benthamiana* leaves. Luminescence was detected 48 h after infiltration, the fluorescence signal was detected, with relative enzyme activity measured as LUC/REN. (C) Interaction of AcTRY with AcbHLH42 in Y2H assays. The negative control (NC) was the cotransformation of AD and BD. The transformed yeasts were 10-fold diluted and dotted on SD/-L/-T and SD/-L/-T/-H/-A media to observe growth. (D, E) Phenotypic analysis of 35S::AcMYBF110 + Empty vector, 35S::AcMYBF110 + 35S::AcTRY, 35S::AcMYBF110 + 35S::AcbHLH42 + Empty vector, and 35S::AcMYBF110 + 35S::AcbHLH42 + 35S::AcTRY co-injection in tobacco leaves. Total anthocyanin content of tobacco leaves injected area. (F) Interaction of AcMYBF110-nLUC with AcbHLH42-cLUC, compete with AcTRY, and interaction of AcTRY-nLUC with AcbHLH42-cLUC, compete with AcMYBF110 in LCI assays. (G) Expression of competitive proteins AcTRY-FLAG and AcMYBF110-FLAG detected in tobacco leaves by western blot. (H) The fluorescence signal was detected, with relative enzyme activity measured as LUC/REN.

expression levels. The same expression difference trend results were shown (Fig. S7). Importantly, the outer pericarps of entirely red-fleshed cultivar showed even greater *AcGLK2* expression than the green outer pericarps of red-centered one. Interestingly, while green kiwifruit flesh is rich in chlorophyll, its *AcGLK2* expression levels are lower than in anthocyanin-containing flesh (Fig. 1), suggesting that anthocyanin biosynthesis requires substantial GLK activity. We also noted that prior work in *Actinidia arguta* [57] has shown that *GLK2* (Aar05572) expression is markedly elevated in the red-fleshed cultivar ‘Zhonghongbei’ (ZHB) relative to the green-fleshed cultivar ‘Zhonglvbei’ (ZLB) during the color transition stage (S2). Taken together, we hypothesized that the differential expression of *GLK2* between the red- and green-fleshed cultivars may arise from sequence variations in its promoter region. Therefore, by leveraging published genomes and comparative genomics approaches [34, 35, 58–62], we selected red-fleshed cultivars (‘Hongyang’, ‘Donghong’, ‘ZPS’, ‘Red5’) and green-fleshed cultivars (‘Biyu’, ‘Midao’, ‘Kuoye’, ‘White’, ‘Changye’, ‘Changguo’) for analysis of the –3000-bp promoter region upstream of *GLK2*. We identified a common insertion region in the green-fleshed cultivars, which was enriched with CAT-, CAAT- and TATA-box elements (Fig. S8A). Based on the two published *A. arguta* genomes (cultivars ‘Tianyuanhong’ and ‘Longcheng 2’), we analyzed their *GLK2* promoter sequences. Compared to the green-fleshed cultivar ‘Longcheng 2’, the red-fleshed cultivar ‘Tianyuanhong’ harbored two insertion regions within the promoter (Fig. S8B). Taken together, these structural variations in the promoter might be involved in the differential *GLK2* expression observed between red- and green-fleshed cultivars, although the precise molecular mechanisms require further investigation.

We constructed an *AcGLK2* overexpression vector and transformed it into both Arabidopsis and kiwifruit. Consistent with previous reports in Arabidopsis [6–8], the transgenic plants exhibited significant anthocyanin accumulation (Fig. 2). Furthermore, we performed a transient overexpression assay of *AcGLK2* in fruits of the green-fleshed *Actinidia deliciosa* cv. ‘Guichang’. The results showed that, compared to the empty vector control, the injection sites at the lower end of the fruits with 35S::*AcGLK2* exhibited discernible pigmentation accumulation. Concurrently, expression analysis revealed that *AcMYB5* was significantly upregulated, whereas *AcTRY* was downregulated in the corresponding areas, respectively (Fig. S9). Additionally, we observed a conserved function of *GLK2* in tomato, another model plant. *SLGLK2*-RNAi in purple tomato significantly impaired anthocyanin accumulation in the fruit of transgenic lines (Fig. S10). These results provide evidence that the role of GLK in modulating anthocyanin biosynthesis is functionally conserved across species.

To elucidate the molecular mechanism by which *AcGLK2* regulates anthocyanin biosynthesis, we sought to identify its downstream target genes. We generated stable transgenic kiwifruit lines expressing *AcGLK2* fused with a FLAG tag, which enabled chromatin immunoprecipitation followed by sequencing (ChIP-seq) to uncover potential targets. By association analysis between ChIP-seq and RNA-seq, several structural genes in the anthocyanin biosynthesis pathway (Fig. 4C) as well as other TFs (Fig. S1A) can also be identified as putative targets of *AcGLK2*, such as a glutathione S-transferase (*AcGST*, Achhyv4a09g140430), we have also discovered that *AcGLK2* can activate the promoter of *AcGST* (Fig. S11), with homologs of that allowing transportation of anthocyanins into vacuoles [63,64]. Previous reports have

demonstrated that *GLK2* can activate *DFR*, *LDOX*, and *UF3GT* gene promoter regions in Arabidopsis to achieve direct regulatory effects [6]. Although the regulatory network of GLK is likely broader and merits further investigation, this study focused on MYB-type TFs. By integrating ChIP-seq binding peaks with differentially expressed genes, we identified two key regulatory targets *AcMYB5* (a positive regulator) and *AcTRY* (a negative regulator), whose functions in kiwifruit were previously uncharacterized, prompting us to select them for further investigation. In addition, it is worth noting that transcriptome analysis indicated that *AcMYBF110* and *AcMYB123* were significantly upregulated in the *AcGLK2* overexpression lines (Fig. 4C), but combined ChIP-seq data and GUS activity assays excluded the possibility that these two genes are direct targets of *AcGLK2* (Fig. S12).

We showed the transcription factor *AcMYB5* is localized in the nucleus (Fig. S2C), whereas *AcTRY* is targeted to both the nucleus and cytoplasm (Fig. S3C). This type of localization has also been reported in strawberry R3MYB [44]. Notably, the expression pattern of *AcMYB5* exhibited substantial synchronization with that of *AcGLK2*, while *AcTRY* displayed an inverse expression trend relative to *AcGLK2* at different development stages of red- and green-fleshed kiwifruits (Fig. S13). In addition, we utilized a cultivar of *A. arguta* characterized by a green-to-red transition in the fruit skin during ripening, which was associated with increased anthocyanin accumulation. qPCR analysis confirmed that the red-skinned fruit exhibited upregulated transcript levels of *AcGLK2* and *AcMYB5*, concomitant with a downregulation of *AcTRY* expression (Fig. S14),

The evolutionary lineage of *AcMYB5* within the R2R3-MYB subfamily indirectly supports its function in anthocyanin biosynthesis (Fig. S2A and B). Phylogenetic analysis revealed that *AcMYB5* is closely clustered with *FaMYB5* and *VvMYB5*, both of which have been previously reported to be positively correlated with anthocyanin and proanthocyanidin biosynthesis [44,46]. Although stable expression of *AcMYB5* alone in tobacco did not induce significant changes in leaf pigmentation, we observed substantial anthocyanin accumulation in flowers (Fig. 6C and D). In transient expression assays using tobacco leaves, *AcMYB5* alone failed to induce visible anthocyanin production (Fig. 7A and B). However, considering that MYB TFs typically require bHLH partners for full functionality [14], we coexpressed *AcMYB5* with *AcbHLH42*, a key transcription factor in kiwifruit known to interact with multiple MYBs [37]. This coexpression resulted in a significant increase of anthocyanin accumulation in tobacco leaves (Fig. 7A and B). Furthermore, both Y2H and LCI assays confirmed the physical interaction between *AcMYB5* and *AcbHLH42* (Fig. 7D–F). The presence of both *AcMYB5* and *AcbHLH42* was shown to significantly enhance the activation of downstream structural genes (Fig. 7G).

TFs suppressing anthocyanin synthesis have been acknowledged [65]. Several of the MYB TFs are single R3-type MYB proteins. The Arabidopsis proteins TRIPTYCHON (TRY), CAPRICE (CPC), ENHANCER OF TRY AND CPC1 (ETC1), ETC2, and ETC3 are proteins with a single R3-MYB domain [66, 67]. They act redundantly as negative regulators in the determination of epidermal cell fate, in competition with R2R3-MYB proteins that act as positive regulators of the developments of trichomes [67, 68]. The sequence alignment indicates that the *AcTRY* gene of kiwifruit is highly homologous to the TRY, CPC, and ETC1 genes of *A. thaliana*. In Arabidopsis, TRY has been well characterized as a negative regulator of trichome formation [68]. We demonstrated that *AcTRY*, its homologous

replicates were prepared for each sample. *Actin* or *UBQ* were used for normalizing data. The $2^{-\Delta\Delta CT}$ method was used for data analysis [74]. The primers utilized are provided in Table S4.

Subcellular localization

The experiments were performed as previously described [32]. The CDS of *AcMYB5* and *AcTRY* were cloned into the pART27 vector and fused to green fluorescent protein (GFP), resulting in *35S::AcMYB5/AcTRY-GFP*, which were then transformed into *Agrobacterium* GV3101. *35S::AcMYB5/AcTRY-GFP* were transiently expressed in tobacco leaves by infiltration (expressed with nucleus-located mCherry). Fluorescent signals of the epidermis cell were detected with confocal laser-scanning microscope (LSM 800, Zeiss, Germany). The primers utilized are provided in Table S4.

Vector construction and generation of transgenic plants

Kiwifruit transformations were made in *A. chinensis* cv. 'Hongyang'. Overexpressed *AcGLK2* (*35S::AcGLK2-FLAG*) vector was constructed with the pCAMBIA2300-FLAG vector. The constructed vectors were transformed into *Agrobacterium* K599, then genetic transformation of kiwifruits was achieved using previously described methods [75, 76]. Transformed calli were screened on a kanamycin selective medium. The successfully transformed overexpression lines were identified by western blot. The above-mentioned vector also stably transformed into *A. thaliana*. Transgenic seedlings were obtained by inflorescence infection as described previously [77]. The mature seeds were collected for 50 mg·l⁻¹ kanamycin resistance screening, and the survivals were transferred to soil and cultivated in the greenhouse to confirm the expression of the exogenous *AcGLK2* protein by western blot analysis.

AcGLK2 was ligated into the plant expression pHB vector with 35S promoter and transformed into *Agrobacterium* GV3101. Transgenic tomato plants were performed according to the described procedure [78]. The positive transgenic callus was selected in medium containing hygromycin antibiotic. The gene expression of the plant was detected by reverse transcription PCR (RT-PCR). The above recombinant vector *35S::AcGLK2* was transformed into *N. tabacum* using the previously reported tobacco leaf disc transformation method [79].

RNA-Seq and ChIP-seq analysis

RNA-seq analysis was performed by Biotree Biomedical Tech Co., Ltd (Shanghai, China) and was carried out on leaves from the 'Hongyang' leaves, including both WT and *35S::AcGLK2-FLAG*. All plants were collected from three biological replicates with three plants per replicate. Libraries were constructed using total RNA and sequenced on an Illumina Novaseq PE150 platform. The reference genome HongyangV4.0 was acquired from the kiwifruit PanGenome Database [80, 81].

ChIP-seq was performed by Igenebook Biotech Co., Ltd. (Wuhan, China). *35S::AcGLK2-FLAG* leaves of 'Hongyang' were collected for the ChIP-seq experiment. The samples were cross-linked with 1% formaldehyde (v/v) and were pulverized into powder using liquid nitrogen. The DNA-protein soluble complex

was extracted, followed by sonication and immunoprecipitation with anti-FLAG antibody. The Illumina NovaSeq was employed for ChIP-seq analysis. The clean reads were subsequently mapped to the reference genome HongyangV4.

Transient transformation in fruits of *A. chinensis*

To construct RNAi vectors, the designated sequence fragments of *AcGLK2* and *AcMYB5* were inserted into the pSK plasmid. For transient transformation of kiwifruits, *Agrobacterium* carrying *AcGLK2-RNAi*-, *AcMYB5-RNAi*-, or *AcTRY*- overexpressing vectors was transformed into 70 DPA fruits using a needle injection. Until the mature stage, the fruits were collected. The color change of the fruits was observed, the seeds were removed, and the inner pericarps were separated for anthocyanin content determination and gene expression analysis. The primers used for constructing all the vectors are shown in Table S4.

B-Glucuronidase staining and activity assay

The promoter fragment of *AcMYB5* and *AcTRY* were inserted into pBI121-GUS as a reporter constructs. The full-length coding sequence of *AcGLK2* was inserted into pHB as an effector plasmid. The recombinant plasmids were transformed into tobacco leaves via *Agrobacterium*-mediated transient transformation. *GUS* were stained with a GUS staining kit (Coolaber). *GUS* activity in injection leaves area was measured with a fluorescence spectrophotometer (CYTATION1, BioTek) according to a previous report [29]. At least three biological replicates were performed for each combination of effectors and reporters. The specific primers used for plasmid construction are listed in Table S4.

Dual-luciferase reporter assay

AcMYB5 and *AcTRY* promoters were cloned upstream of the LUC reporter gene into vector pGreenII 0800-LUC to generate the *AcMYB5/AcTRYpro::LUC*. The constructed vectors were transformed into *Agrobacterium tumefaciens* GV3101 (pSoup). The empty pHB vector and the recombinant pHB-AcGLK2 plasmids was used as the effector. Combinations of effector and reporters were coexpressed in *N. benthamiana* leaves for 36-48 h. LUC image and LUC luminescence intensity were captured in Tanon 4600SF. The firefly (LUC) and *Renilla* (REN) activities were analyzed using a dual-luciferase assay kit (TransGen, China). Finally, relative enzyme activity was calculated as LUC/REN. At least three biological replicates were performed for each combination of effector and reporters. All the primer sequences used in this experiment are listed in Table S4.

Yeast one-hybrid assay

Y1H experiments were performed as previously described [82]. The CDS of *AcGLK2*, were cloned into the pJG4-5 prey vector, and the promoter fragments of the *AcMYB5* and *AcTRY* were integrated into the pLACZi bait vector. These constructs were transformed into the EGY48 yeast strain using indicated combinations. The yeast was cultured in SD/-Ura/-Trp media. Positive transformants

were inoculated onto SD/-Ura/-Trp medium supplemented with raffinose, galactose, and X-gal for blue development. All the primer sequences used in this experiment are listed in Table S4.

Electrophoretic mobility shift assay

The full-length open reading frame (ORF) of *AcGLK2* was ligated into the pET-28a vector obtain His-tagged. The *Escherichia coli* Rosetta cells carrying the recombinant vectors *AcGLK2*-HIS was induced at 16°C for 16 h. The recombinant protein purification was carried out as described [83]. Probes harboring specific *cis*-elements of *AcMYB5* and *AcTRY* were synthesized separately and biotinlabeled. All DNA probes were manufactured by General Biol (Anhui) Co., Ltd. The free probe and bound probe were separated by acrylamide gel electrophoresis. A competitor of unlabeled DNA fragment was used as the cold probe while a mutated sequence was used as the mutation probe. The EMSA Probe Biotin Labeling Kit and Chemiluminescent EMSA Kit (Beyotime, China) were used for the EMSA according to the manufacturer's instructions. Finally, the probe blot was performed using a imaging system (Azure biosystems c500, USA). The EMSA probes are listed in Table S4.

Firefly luciferase complementation imaging assays

Expression vectors JW771-nLUC and JW772-cLUC were used in bimolecular fluorescence complementation assays. The full-length coding sequence of *AcMYB5*, *AcTRY*, and *AcbHLH42* were cloned into JW771 and JW772, respectively. Four different mixtures, nLUC+cLUC, *AcTRY*/*AcMYB5*-nLUC+cLUC, nLUC+*AcbHLH42*-cLUC, and *AcTRY*/*AcMYB5*-nLUC+*AcbHLH42*-cLUC were infiltrated into different parts of *N. benthamiana* leaves. After 48 h, the fluorescence emitted on the surface of the leaves was detected by infiltrating the 0.2 mM substrate fluorescein potassium salt into the same part of the leaves in the dark for 5 min. Images were captured using imaging apparatus (Tanon 4600SF). Primer information is presented in Table S4.

Yeast two-hybrid assay

The *AcMYB5* and *AcTRY* coding region was ligated to the activation domain vector pGADT7, while the *AcbHLH42* coding region was inserted into the DNA-binding domain vector pGBKT7. The recombinant vectors *AcMYB5*/*AcTRY*-AD and *AcbHLH42*-BD were cotransferred into the AH109 yeast cells. The empty pGADT7 and pGBKT7 vectors served as negative controls. Transferred yeast strains were grown on SD selection medium (-Trp/-Leu or -Trp/-Leu/-His/-Ade medium) and cultured in a 30°C incubator for 3 days to investigate their interactions. The primers utilized are provided in Table S4.

Transient transformation and competition assays in tobacco leaves

Four different combinations, 35S::AcMYBF110 + EV, 35S::AcMYBF110 + 35S::AcTRY, 35S::AcMYBF110 + 35S::AcbHLH42 + EV, and 35S::AcMYBF110 + 35S::AcbHLH42 + 35S::AcTRY; were

injected into 4-week-old leaves in *N. tabacum*, and the anthocyanin content and expression genes in the corresponding and injection area of the same injection leaf were collected. Both of AcMYBF110-nLUC+*AcbHLH42*-cLUC and AcMYBF110-nLUC+*AcbHLH42*-cLUC+35S::AcTRY were infiltrated into *N. benthamiana* leaves. Similarly, another comparison group (AcTRY-nLUC + *AcbHLH42*-cLUC and AcTRY-nLUC + *AcbHLH42*-cLUC + 35S::AcMYBF110) was also presented using the same method. Enzyme activity was calculated as relative LUC/REN ratio. The experiment was conducted with three biological replicates.

Statistical analysis

All experiments with each group were performed at least in triplicate. The mean values and standard deviations (SDs) were calculated from three biological and three technical replicates. Statistical significance was determined using Student's *t*-test (the asterisks indicate statistically significant differences, **P* < 0.05 and ***P* < 0.01).

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Author contributions

L.W. conceived the ideas and designed the experiments. L.Z., M.T., and G.W. performed all experiments, analyzed the data, and drew the figures. P.W. prepared the materials. Z.C. completed genome analysis. G.W., Y.Z., and S.H. repeated the experiments and verify the data. L.W. and Y.L. wrote the manuscript. P.Z., J.Y., and S.W. edited the manuscript. All authors read and approved the final manuscript.

Data availability

All data generated from the study appear in the submitted article.

Conflicts of interest statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material is available at Horticulture Research online.

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