The transcription factor AtGLK1 acts upstream of MYBL2 to genetically regulate sucrose-induced anthocyanin biosynthesis in Arabidopsis

Dongming Zhao†, Yuxuan Zheng†, Lingjun Yang, Ziyu Yao, Jianfeng Cheng, Fang Zhang, Haiyan Jiang and Dong Liu*

Abstract

Background: The regulation of anthocyanin biosynthesis by various factors including sugars, light and abiotic stresses is mediated by numerous regulatory factors acting at the transcriptional level. Here experimental evidence was provided in order to demonstrate that the nuclear GARP transcription factor AtGLK1 plays an important role in regulating sucrose-induced anthocyanin biosynthesis in Arabidopsis.

Results: The results obtained using real-time quantitative PCR and GUS staining assays revealed that AtGLK1 was mainly expressed in the green tissues of Arabidopsis seedlings and could be induced by sucrose. The loss-of-function glk1 glk2 double mutant has lower anthocyanin levels than the glk2 single mutant, although it has been determined that loss of AtGLK1 alone does not affect anthocyanin accumulation. Overexpression of AtGLK1 enhances the accumulation of anthocyanin in transgenic Arabidopsis seedlings accompanied by increased expression of anthocyanin biosynthetic and regulatory genes. Moreover, we found that AtGLK1 also participates in plastid-signaling mediated anthocyanin accumulations. Genetic, physiological, and molecular biological approaches demonstrated that AtGLK1 acts upstream of MYBL2, which is a key negative regulator of anthocyanin biosynthesis, to genetically regulate sucrose-induced anthocyanin biosynthesis.

Conclusion: Our results indicated that AtGLK1 positively regulates sucrose-induced anthocyanin biosynthesis in Arabidopsis via MYBL2.

Keywords: Arabidopsis, AtGLK1, Anthocyanin biosynthesis, MYBL2
Background

Anthocyanins are a group of plant pigments known to be responsible for the purple coloration of plant parts at particular developmental stages, or under special environmental conditions. The presence of anthocyanin in flowers and fruits is required for attracting pollinators and seed-dispersing animals [1]. Anthocyanins are also an important class of polyphenols which are characterized with remarkable antioxidant activities. Such activities help to protect plants against different abiotic and biotic stress conditions [2–5].

The anthocyanin biosynthetic pathways have been extensively studied in various plant species. The gene encoding enzymes required for the anthocyanin biosynthetic pathways are conserved among different plants [6], and can be grouped into the following two classes [7, 8]. The early biosynthesis genes (EBGs) are involved in the common steps of the different flavonoid subpathways, and mainly include CHALCONE SYNTHASE (CHS), CHALCONE ISOMERASE (CHI), and FLAVANONE 3-HYDOXYLASE (F3H). The late biosynthesis genes (LBGs) primarily include FLAVONOID 3'-HYDOXYLASE (F3' H), DIHYDROFLAVONOL 4-REDUCTASE (DFR), LEUCOANTHOCYANIDIN OXYGENASE (LDOX), ANTHOCYANIDIN REDUCTASE (ANR), and UDP-GLUCOSE:FLAVONOID 3-O-GLUCOSYLTRANSFERASE (UF3GT). The expression levels of the aforementioned genes are regulated by positive and negative regulatory transcription factors. For example, it has been determined that the WD-repeat independent MYBs and MYBs/hHLH/WD-repeat complex regulates the expressions of EBGs and LBGs, respectively [9–11]. In Arabidopsis, the transcription factors PIF3 and HY5 positively regulate anthocyanin biosynthesis by directly binding to the promoters of the anthocyanin biosynthetic genes, including CHS, CHI, F3H, F3'H, DFR, and LDOX [12]. In contrast to the positive transcription factors mentioned above, the R3-MYB protein MYBL2 acts as a transcriptional repressor, and negatively regulates the biosynthesis of anthocyanin [13, 14]. Further studies have revealed that MYBL2 inhibits anthocyanin biosynthesis by interacting with TT8 protein to form a transcriptional inhibitory complex which has the ability to bind to the DFR promoter and inhibit the transcription of the DFR gene [14].

Sugars play essential roles in the growth and development of higher plants, serving as both energy sources and signaling molecules [15]. It has been well established that sucrose is a strong inducer of anthocyanin production in different organs of several plant species [16–19]. The application of exogenous sucrose can significantly increase in the transcript levels of DFR and LDOX [20, 21]. This sucrose-induced expression of anthocyanin biosynthetic genes may be attributed to the up-regulation expression of positive transcript factors such as PAP1, TT8, and GL3 [22]. The sucrose transporters (SUCs) may play an important role in sucrose-induced anthocyanin biosynthesis [18]. It has been found that AtSUC1 expression levels were higher in sucrose-grown plants when compared with those grown without sucrose. When cultured in sucrose-containing medium, Arabidopsis suc1 mutants were found to accumulate less anthocyanins. Global expression analyses have revealed reduced expression of many genes important for anthocyanin biosynthesis [23]. Interestingly, AtSUC1 is preferentially expressed in plant roots, while anthocyanin tends to mainly accumulate in the epidermal layers of the entire abaxial surface, as well as the edges of the adaxial surfaces of the cotyledons [23, 24]. Therefore, it has been indicated that AtSUC1 may play a role in sucrose uptake, rather than acting as a sugar sensor for anthocyanin production [25].

AtGLKs (GOLDEN2-LIKE) are the nuclear GARP transcription factors that have been extensively studied for their roles in regulating chloroplast development [26]. In Arabidopsis, AtGLK genes exist as a homologous pairs designated as AtGLK1 and AtGLK2. Although glk1 and glk2 single mutants showed no obvious phenotypes throughout the majority of the developmental processes, the glk1 glk2 double mutant is pale green with a severe reduction in chloroplast thylakoids, suggesting that the AtGLK genes are functionally redundant [26, 27]. Consistent with the rudimentary thylakoid lamellae, the transcript abundance of nuclear genes encoding photosynthesis-related proteins is down-regulated, especially those associated with chlorophyll biosynthesis and PSI [26, 28]. It has also been found that in addition to chloroplast development, AtGLK genes are involved in mediating chloroplast-to-nucleus retrograde signaling in response to the functional states of the chloroplast [29–31]. The ppi2 (plastid protein import2) mutant, which lacks the Toc159 chloroplast preproteins receptor, exhibits its repression of photosynthesis-related nuclear genes expression, altered chloroplast morphology, and a severe albino phenotype. Transcript analysis results have revealed that AtGLK1 expression was significantly down-regulated in the ppi2 mutant. Furthermore, the expression of some photosynthesis-related genes has been found to be partially restored in transgenic plants overexpressing AtGLK1 in a ppi2 background. These findings suggested that AtGLK1 acts as a positive regulator in a chloroplast-to-nucleus signaling pathway that regulates nuclear genes expression in response to the functional status of chloroplasts [29].
In the present research investigation, the identification of AtGLK1 as a positive regulator of sucrose-induced anthocyanin biosynthesis was verified. Our results showed that AtGLK1 was preferentially expressed in green tissues and it could be induced by exogenous sucrose. Loss-of-function glk1 glk2 double mutant seedlings were found to have accumulated less anthocyanins in response to sucrose, whereas AtGLK1-overexpressing Arabidopsis seedlings accumulated more anthocyanins in response to sucrose. Further investigations demonstrated that AtGLK1 acts upstream of MYBL2 to genetically regulate anthocyanin biosynthesis. Therefore, all of the above-mentioned results suggested that AtGLK1 is a key factor which positively regulates sucrose-induced anthocyanin accumulation via MYBL2.

Results

AtGLK1 is a sucrose-inducible gene in Arabidopsis

Sugars function as signal molecules to regulate growth, development, and gene expression in higher plants [15]. In order to investigate whether or not the Arabidopsis transcription factor AtGLK1 is involved in responses to sugar signalling, we examined the effects of exogenous sucrose on AtGLK1 expression levels. In addition, mannitol was included in the experiment as an osmotic control. The results of the real-time quantitative PCR analysis showed that the AtGLK1 transcript was significantly up-regulated by treatment with 2% sucrose. However, the mannitol treatment did not dramatically increase the AtGLK1 transcript level (Fig. 1a). In order to further examine the sucrose inductive expression patterns of AtGLK1, the AtGLK1 promoter-controlled GUS activities in response to exogenous sucrose were also analyzed. As shown in Fig. 1b, stronger GUS expression was detected in both the cotyledons and hypocotyls of transgenic AtGLK1::GUS Arabidopsis seedlings grown on 1/2 MS medium supplemented with 2% sucrose when compared with the control. Consistent with the qPCR data, it was observed that the expression of AtGLK1::GUS was not changed largely after the treatment with mannitol. The sucrose-induced expression of GUS indicated that AtGLK1 may be involved in plant responses to sugar signaling.

AtGLK1 and AtGLK2 exhibit functional redundancy in regulating sucrose-induced anthocyanin biosynthesis

It has been well established that sucrose is a strong inducer of anthocyanin production in Arabidopsis [18, 32]. The induction of AtGLK1 expression by sucrose in Arabidopsis suggested that it may be involved in regulating anthocyanin biosynthesis. In order to confirm this, the single mutants of glk1 and glk2 and the glk1 glk2
double mutant were investigated. These loss-of-function mutants had previously been demonstrated to impact chloroplast development in Arabidopsis [26]. Figure 2a illustrates that the \textit{AtGLK1} transcripts displayed very little accumulation in the \textit{glk1} mutant. However, they were present at normal levels in the \textit{glk2} mutant. Similarly, the \textit{AtGLK2} transcripts were observed to be very low in the \textit{glk2} mutant but were accumulated to normal levels in the \textit{glk1} mutant. The transcript levels of both the \textit{AtGLK1} and \textit{AtGLK2} genes were very low in the \textit{glk1 glk2} double mutant. Seeds of both the wild type and the \textit{glk} mutants (\textit{glk1}, \textit{glk2}, and \textit{glk1 glk2}) were germinated and grown vertically on 1/2 MS medium supplemented with 2% sucrose for 4 days following stratification. It was observed that the anthocyanin accumulations in the \textit{glk2} single mutant and the \textit{glk1 glk2} double mutant seedlings were significantly decreased in the upper part of hypocotyls, when compared with that of the corresponding wild-type seedlings. However, when the seedlings were germinated and grown on 1/2 MS medium without sucrose or with 58 mM mannitol, no significant differences could be observed among the wild-type, the single mutants of \textit{glk1} and \textit{glk2}, and the \textit{glk1 glk2} double mutant (Fig. 2b). Quantitative analysis showed that the anthocyanin contents of seedlings grown in the absence of sucrose were fairly low and there were no significant

![Figure 2](image-url)

**Fig. 2** Anthocyanin accumulation in wild-type, single mutants of \textit{glk1} and \textit{glk2}, and the \textit{glk1 glk2} double mutant. a Real-time quantitative PCR analysis of the \textit{AtGLK1} and \textit{AtGLK2} transcript accumulation in the wild type (Col), single mutants of \textit{glk1} and \textit{glk2}, and the \textit{glk1 glk2} double mutant seedlings. The total RNA was isolated from 4-d-old seedlings grown on 1/2 MS medium supplemented with 2% sucrose. b Images of representative seedlings of the wild-type (Col), single mutants of \textit{glk1} and \textit{glk2}, and the \textit{glk1 glk2} double mutant grown for 4 days on 1/2 MS medium supplemented without sucrose (-S), with 2% sucrose (+S), or with 58 mM mannitol (Man), respectively. The black arrows indicate the locations of the anthocyanin accumulation in different genotypic Arabidopsis seedlings. c Quantitative measurement of anthocyanins in 4-d-old seedlings (Col, \textit{glk1}, \textit{glk2} and \textit{glk1 glk2}) grown on 1/2 MS medium supplemented without sucrose (-S), with 2% sucrose (+S), or with 58 mM mannitol (Man), respectively. The asterisks indicate statistically significant differences compared with the corresponding wild-type (Student’s t test: *P<0.05)
differences observed between the glk mutants (glk1, glk2, and glk1 glk2) and the wild-type seedlings. However, there were marked inductions of anthocyanin accumulations in both the wild-type and glk mutants (glk1, glk2, and glk1 glk2) in the presence of sucrose. Although no significant differences were observed in the anthocyanin contents between the wild-type and glk1 mutant, the anthocyanin contents of glk2 single mutant and glk1 glk2 double mutant were found to be significantly lower than those of the wild-type seedlings. Furthermore, the glk1 glk2 double mutant was observed to be more defective in anthocyanin accumulation when compared with the glk2 single mutant. In order to determine if the differences in the anthocyanin accumulation levels in the wild-type and glk mutants (glk1, glk2, and glk1 glk2) seedlings were due to osmotic effects, the seedlings were also grown on equimolar concentrations of mannitol (58 mM = 2%), and the anthocyanin contents were assayed. The mannitol failed to induce anthocyanin accumulations in either the wild-type or the glk mutants (glk1, glk2, and glk1 glk2) seedlings, which suggested that the sucrose-induced anthocyanin accumulations could not be regarded as an osmotic effect (Fig. 2c).

Overexpression of AtGLK1 enhances sucrose-induced anthocyanin accumulation in Arabidopsis

To investigate whether or not the accumulation of anthocyanin was affected in AtGLK1-overexpressing lines, the AtGLK1 gene, driven by CaMV 35S promoter, was introduced into Arabidopsis. 9 independent 35S::AtGLK1 transgenic lines were obtained on a selection 1/2 MS medium with 50 μg ml−1 kanamycin. Through kanamycin-resistance assay and PCR analysis (data not shown), the homozygous transgenic progeny lines (T3 to T4 generations) were selected for further examination. The expression levels of two representative independent transgenic lines (OEGKL1-1 and OEGKL1-2) were examined using real-time quantitative PCR analysis with gene-specific primers. As expected, the transgenic lines OEGKL1-1 and OEGKL1-2 were found to have higher relative expression levels of AtGLK1 when compared with the wild type (Fig. 3a). We also detected the expression of AtGLK2, a homologous gene to AtGLK1, in the wild-type and 35S::AtGLK1 transgenic plants. It was interesting to note that the expression of AtGLK2 was found to be significantly impaired in the AtGLK1-overexpressing seedlings, when compared with the corresponding wild-type plants (Fig. 3b).

When grown on 1/2 MS medium in the absence of sucrose, the anthocyanin accumulation in the AtGLK1-overexpressing seedlings was indistinguishable from that in wild-type seedlings, a result similar to that observed in the seedlings grown on 1/2 MS medium in the presence of 58 mM mannitol. However, we observed an obvious difference in the anthocyanin pigmentation intensity in the upper part of the hypocotyls of these seedlings in the presence of sucrose. In comparison with wild-type seedlings, clear increases in the level of purple anthocyanin were observed in both of the selected AtGLK1 overexpression lines (Fig. 3c). Quantification of the anthocyanin level validated the phenotypic observations and confirmed the higher anthocyanin levels in both the selected AtGLK1 overexpression lines when compared with the wild-type seedlings (Fig. 3d). Taken together, the data obtained in this study revealed a positive correlation between the AtGLK1 expression and anthocyanin accumulation in the Arabidopsis seedlings.

Expression of the structural and regulatory genes of the anthocyanin biosynthetic pathway

The results described above indicated that AtGLK1 is involved in the regulation of anthocyanin synthesis. Therefore, in order to more clearly understand the molecular basis of the changes in anthocyanin levels, we first examined the expression of the early biosynthetic genes CHALCONE SYNTHASE (CHS) and CHALCONE ISOMERASE (CHI) using reverse transcription followed by real-time quantitative PCR. As detailed in Fig. 4, the transcript levels of CHS had not dramatically changed in the single mutants of glk1 and glk2. However, it was found that the CHI transcript levels were clearly decreased in the two mutants. In addition, when compared with the wild type, it was observed that expression levels of the CHS and CHI were not greatly changed in the AtGLK1 overexpression lines. However, the expression of both genes was majorly decreased in the glk1 glk2 double mutant. We then monitored the expression levels of the following late biosynthetic genes DIHYDROFLAVONOL 4-REDUCTASE (DFR), FLAVONOID 3’ HYDROXYLASE (F3’H), LELICOANTHOCYANIDIN OXYGENASE (LDOX), UDP-GLUCOSE:FLAVONOID 3-O-GLUCOSYL TRANSFERASE (UFGT), UDP-GLUCOSYL TRANSFERASE 75C1 (UGT75C1), and UDP-GLUCOSYL TRANSFERASE 78D2 (UGT78D2). The late biosynthetic genes showed the same expression patterns, in which the transcript levels of the genes were lower in the glk mutants (glk1, glk2, and glk1 glk2) than in the wild type but higher in the AtGLK1-overexpressing lines. Subsequently, the expression levels of several regulatory genes in the anthocyanin biosynthetic pathways were further examined, including PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1), PRODUCTION OF ANTHOCYANIN PIGMENT 2 (PAP2), TRANSPARENT TESTA 8 (TT8), and MYB11. As expected, the PAP1, TT8, and MYB11 expressions were found to be
consistently and substantially higher in the \textit{AtGLK1}-
overexpressing lines when compared with the wild type
but lower in the \textit{glk} mutants (\textit{glk1}, \textit{glk2}, and \textit{glk1 glk2}).
However, there were no significant differences observed
in the gene expression levels of the \textit{PAP2} between the
wild type and \textit{AtGLK1}-overexpressing transgenic lines,
while its expression was dramatically decreased in the
\textit{glk} mutants (Fig. 4). In summary, the results obtained in
this study suggested that \textit{AtGLK1} positively regulates
anthocyanin accumulations in \textit{Arabidopsis} seedlings
through modulating the expression levels of structural
and regulatory anthocyanin biosynthetic genes.

\textbf{AtGLK1 participates in the plastid retrograde
signal-mediated anthocyanin accumulation in \textit{Arabidopsis}}

Since \textit{AtGLK1} is an important component of the plastid
retrograde signal pathway [29], whether \textit{AtGLK1}
participates in the plastid retrograde signal-mediated
anthocyanin accumulation was further investigated.
Therefore, wild-type seedlings were treated with nor-
flurazon (NF) or lincomycin (Linc), which are two
drugs known to activate retrograde signaling by inhib-
itng chloroplast biogenesis [33, 34]. The results of the
real-time quantitative PCR analysis showed that the
\textit{AtGLK1} gene was strongly down-regulated by the NF
and Linc treatments at the transcription level (Fig. 5a). Next, NF or Linc were used to treat wild-type Arabidopsis, glk1 glk2 double mutant, and AtGLK1-overexpressing seedlings, and the anthocyanin contents of these samples were then determined. The results are shown in Fig. 5b-c and Fig. S1. For the wild-type seedlings, both NF and Linc were determined to have significantly induced anthocyanin accumulation and the expression of anthocyanin biosynthetic and regulatory genes. In the control group, the anthocyanin accumulation was observed to be lower in the glk1 glk2 double mutant but higher in AtGLK1-overexpressing lines when compared with the wild type. Treatments with NF and Linc significantly induced anthocyanin accumulation in wild-type and glk1 glk2 double mutant seedlings. However, no significant inductive effects were observed in either of the AtGLK1-overexpressing lines (Fig. 5c). These findings suggested that AtGLK1 participates in plastid retrograde signal-mediated anthocyanin accumulation in Arabidopsis.

AtGLK1 acts upstream of MYBL2 to genetically regulate anthocyanin accumulation in Arabidopsis

It has been previously reported that MYBL2 acts as a transcriptional repressor and negatively regulates the biosynthesis of anthocyanin in Arabidopsis [13, 14]. In the MYBL2 knockout line (mybl2), the expression of the anthocyanin biosynthetic and regulatory genes was enhanced and resulted in the ectopic accumulation of anthocyanin, while ectopic expression of MYBL2 or of a chimeric repressor that is a dominant negative form of MYBL2 suppressed the expression of anthocyanin biosynthetic and regulatory genes, and the biosynthesis of anthocyanin [13, 14]. To determine the genetic relationship between AtGLK1 and MYBL2,
the 35S::MYBL2 (OEMYBL2) was crossed with the AtGLK1 overexpression line (OEGLK1), and the double overexpressing line 35S::MYBL2/OEGLK1 (OEGLK1/OEMYBL2) was obtained (Fig. 6a). Our results showed that the overexpression of MYBL2 significantly suppressed the anthocyanin biosynthesis of AtGLK1-overexpressing seedlings, which indicated that MYBL2 was epistatic to AtGLK1 in anthocyanin biosynthesis (Fig. 6b-c). Consistency was observed in the transcript levels of the anthocyanin biosynthetic (DFR, F3’H, LDOX, UF3GT, UGT75C1, and UGT78D2) and regulatory (PAPI and TT8) genes, which were dramatically up-regulated in AtGLK1-overexpressing seedlings, all were down-regulated when the MYBL2 was overexpressed in the 35S::MYBL2/OEGLK1 (OEGLK1/OEMYBL2) double overexpressing line (Fig. 6d). Therefore, these results indicated that AtGLK1 acts upstream of MYBL2 to genetically regulate anthocyanin accumulation in Arabidopsis.

**Discussion**

In higher plants, the regulation of anthocyanin biosynthesis by various transcription factors [9–14, 32, 35]. The GLK transcription factors were originally identified in maize, and were subsequently found in Arabidopsis, maize, rice, tomato, and the moss *Physcomitrella patens* [26, 27, 36–39]. GLK transcription factors belong to the GARP transcription activator family, and the protein sequences are highly conserved among different species, with Myb-like DNA-binding domain and the C-terminal box [26, 39, 40]. In Arabidopsis, AtGLK genes exist as a pair of homologous genes, AtGLK1 and AtGLK2. The previous studies found that AtGLKs mainly regulate the chloroplast development in higher plants [26–28].
In recent years, more and more studies have shown that AtGLKs play important roles not only in responding to biotic and abiotic stresses, but also in regulating leaf senescence [41–45]. The current study found that AtGLKs have an important function in regulating the accumulation of anthocyanins in Arabidopsis.

Anthocyanins are water-soluble, vacuolar pigments in plants that belong to the family of flavonoid compounds [46]. Since sucrose is a strong inducer of flavonoid biosynthesis and is known to induce anthocyanin accumulation in a variety of plant species [16–18], we analyzed the expression patterns of AtGLK1 in response to exogenous sucrose treatment. Real-time quantitative PCR analyses revealed that the mRNA accumulation of AtGLK1 was significantly promoted by sucrose (Fig. 1a). The increased AtGLK1 transcript level in response to sucrose appeared to originate from its promoter activities since it was observed that exogenous sucrose treatments significantly increased GUS expression in the cotyledons and hypocotyl of AtGLK1::GLK1 transgenic seedlings (Fig. 1b). Such an expression pattern suggested that AtGLK1 may be involved in sucrose-induced anthocyanin accumulation during the early stages of Arabidopsis development.

Through the phenotypic, physiological, and molecular analyses conducted in this work, strong positive correlations were identified between AtGLK1 expression and anthocyanin accumulation to sucrose treatment. First, the loss-of-function glk1 glk2 double mutant was found to have lower anthocyanin levels than the glk2 single mutant, although loss of AtGLK1 alone had not affected the anthocyanin accumulation (Fig. 2). The absence of an anthocyanin-less phenotype for the glk1 mutant may have been due to functional redundancy or compensation between the AtGLK1 and AtGLK2. Similarly, the
AtGLK1 and AtGLK2 have been shown to be functionally redundant in the regulation of chloroplast development [26, 27]. During the early developmental stage of Arabidopsis seedlings, single glk mutants (glk1 and glk2) largely resemble wild-type, only the glk1 glk2 double mutant showed a chloroplast-defective phenotype, suggesting that the each of two AtGLK genes acts redundantly to direct monomorphic chloroplast development [26]. The AtGLK genes were found to exhibit partial redundancy since there was an anthocyanin-less phenotype specific to the glk2 mutant allele, but no phenotype specific to the glk1 allele (Fig. 2). The following two aspects of the experimental data may have reflected the fact that the two genes had different expression levels rather than different functions. On the one hand, overexpression of AtGLK1 significantly enhanced anthocyanin accumulation in the 35S::AtGLK1 transgenic Arabidopsis seedlings, even though the expression of AtGLK2 was dramatically impaired (Fig. 3). On the other hand, real-time quantitative PCR results showed that the mRNA accumulation of AtGLK1 was significantly lower than that of AtGLK2 in the wild-type Arabidopsis seedlings (Fig. S2). Second, when overexpressed in Arabidopsis, the 35S::AtGLK1 transgenic seedlings displayed enhanced anthocyanin accumulation (Fig. 3). We also detected the expression of AtGLK2 in the wild-type and 35S::AtGLK1 transgenic seedlings. It was interesting to find that the expression of AtGLK2 was significantly impaired in the AtGLK1-overexpressing plants when compared with the corresponding wild-type plants (Fig. 3b). There were two possible explanations. The first explanation was that the AtGLK1 has an additional function of regulating AtGLK2 expression. The second explanation is that the decreased transcription of the AtGLK2 in the AtGLK1-overexpressing plants were most likely for the purpose of maintaining a constant total mRNA amount of AtGLKs via expressional reprogramming between the two homologous genes. Third, We found that glk mutants (glk1, glk2 and glk1 glk2) seedlings had accumulated lower transcript levels of DFR, F3’H, LDOX, UF3GT, UGT75C1, and UGT75C2, which are known to be involved in the late step of anthocyanin biosynthesis, while the AtGLK1-overexpressing seedlings showed higher transcript levels than those observed in the wild-type seedlings (Fig. 4). In contrast, the transcript levels of the early biosynthesis genes, such as CHS and CHI, were not observed to be greatly altered in the AtGLK1-overexpressing plants (Fig. 4c). Another potential target of AtGLK1 action could be PAP1, which has been shown to trigger the activation of expression of late anthocyanin biosynthesis genes [18, 47]. PAP1 is an R2R3 MYB-type transcription factor that is capable of mediating ectopic activation of an array of genes involved in anthocyanin biosynthesis in several plant species, including Arabidopsis, tobacco, petunia and rose [47–50]. Indeed, our study found that the transcript level of PAP1 was lower in the glk mutants (glk1, glk2, and glk1 glk2) seedlings, but significantly higher in AtGLK1-overexpressing seedlings, when compared with the corresponding wild-type plants (Fig. 4). It therefore appeared that the AtGLK1 regulates sucrose-induced anthocyanin accumulation mainly through influencing the expression of late anthocyanin biosynthesis genes. Therefore, based on the results mentioned above, our study considered that AtGLK1 is potentially a positive regulator of anthocyanin accumulation in Arabidopsis.

The intracellular signaling from the chloroplast to the nucleus is referred to as plastid retrograde signaling. These signaling processes play essential roles in coordinating the expression of nuclear and plastid-encoded genes [51]. In the present study, it was found that norflurazon and lincomycin (two drugs known to block chloroplast biogenesis via different mechanisms), which induce retrograde signaling [33, 34], were found to enhance the anthocyanin accumulation of sucrose-treated Arabidopsis seedlings (Fig. 5; Fig. S1). These findings suggested that the anthocyanin biosynthesis is positively regulated by plastid retrograde signaling. If the positive signals from dysfunctional chloroplasts are transmitted exclusively via AtGLK1, then these signals should be abrogated in glk1 glk2 double mutants. However, the effects of norflurazon and lincomycin on the sucrose-induced anthocyanin accumulation were observed to be greater in the glk1 glk2 double mutants, but lower in AtGLK1-overexpressing seedlings, when compared with wild-type seedlings (Fig. 5c-d). These observations suggested the possibility that AtGLK1 acts as a negative regulator in plastid retrograde signal-mediated anthocyanin accumulation. Consistent with this speculation, the results of the real-time quantitative PCR analysis showed that the AtGLK1 had been strongly down-regulated by the norflurazon and lincomycin treatments at the transcription level (Fig. 5a). Despite this, further studies will be needed in order to unravel the detailed molecular mechanisms of AtGLK1-mediated plastid retrograde signaling pathways which regulate anthocyanin accumulation.

MYBL2 is a negative regulator of anthocyanin biosynthesis. The analyses of the expression patterns of the mybl2 mutant, or transgenic plants overexpressing MYBL2, have demonstrated that MYBL2 regulates the expression of anthocyanin biosynthesis-related genes [13, 14]. Similar expression patterns were observed in the structural and regulatory genes in the anthocyanin biosynthetic pathways in the AtGLK1-overexpressing plants and the glk1 glk2 double mutant in this study (Fig. 4), which raised the possibility that AtGLK1 regulates anthocyanin biosynthesis by modulating MYBL2
expression. However, the MYBL2 transcript levels showed no obvious changes in either the glk1 glk2 double mutant or AtGLK1-overexpressing plants when compared with the wild-type (data not shown). Therefore, it was hypothesized that AtGLK1 may regulate MYBL2 expression at the post-transcriptional level. To determine the genetic relationship between AtGLK1 and MYBL2, we generated transgenic lines overexpressing MYBL2 in AtGLK1-overexpressing plants. The results indicated that the overexpression of MYBL2 completely complemented the anthocyanin overaccumulation phenotype in the AtGLK1-overexpressing seedlings (Fig. 6b-c), which suggested that MYBL2 is epistatic to AtGLK1 in anthocyanin biosynthesis. Also, consistency was found in the transcript levels of the anthocyanin biosynthetic (DFR, F3′H, LDOX, UF3GT, UGT75C1, and UGT75C2) and regulatory (PAP1 and TT8) genes, which were up-regulated in the AtGLK1-overexpressing seedlings, and all down-regulated when MYBL2 was overexpressed (Fig. 6d).

Conclusion
In summary, the results obtained in this study indicated that in addition to regulating chloroplast development [26], abiotic and biotic stress responses [41, 42, 44, 45], and leaf senescence [43], AtGLK1 positively regulates sucrose-induced anthocyanin biosynthesis in Arabidopsis. Furthermore, it was determined that MYBL2 plays an important genetical role in the downstream of AtGLK1. It is believed that future research will clarify the exact molecular mechanisms of the AtGLK1-mediated plastid signaling pathways which regulate anthocyanin accumulation.

Methods
Plant material and growth conditions
The wild type and mutant lines of Arabidopsis thaliana were all in the Columbia ecotype (Col-0). Transfer DNA insertion mutants glk1 (CS9805), glk2 (CS9806), and glk1 glk2 (CS9807) were obtained from the Arabidopsis Biological Resource Center (ABRC), and the transgenic Arabidopsis plants overexpressing both the AtGLK1 and MYBL2 (OEGLK1/OEMYBL2) were produced by crossing transgenic homozygous lines overexpressing AtGLK1 and MYBL2. Following 3 days of stratification in the dark at 4 °C, the surface-sterilized seeds were germinated on 1/2 MS medium [0.8% (w/v) agar, 2% (w/v) sucrose, pH 5.8] at 22 °C with a 16-h-light/8-h-dark cycle unless otherwise stated. All phenotypic characterization experiments were conducted on multiple biological samples and repeated at least 3 times.

To examine the effects of norflurazon (NF) and lincomycin (Linc) on anthocyanin biosynthesis, the sterilized and cold-treated seeds were germinated and grown vertically on 1/2 MS medium without (Mock) or with 5 μM NF or with 0.5 mM Linc for 4 days (under continuous light conditions). The 4-day-old seedlings were then harvested for anthocyanin measurement.

Verification of dSpm insertions in glk mutants
The dSpm insertions in glk1 and glk2 mutants were confirmed by PCR using dSpm-specific primers, with spm5 for glk1 and spm8 for glk2; and AtGLK genespecific primers, with 2bg2s for glk1, and ara4 for glk2. PCR genotyping primers are listed in Table S1 and the results of PCR genotyping of the mutants are shown in Fig. S1.

Constructs and plant transformation
To construct the AtGLK1::GUS fusion gene, a 1,702-bp DNA fragment upstream of the ATG start codon of the AtGLK1 gene (At2g20570) was amplified from Arabidopsis thaliana genomic DNA by PCR. The pair of primers used in the PCR was PGLK1-F and PGLK1-R (BanHI and NcoI sites were introduced). The specific PCR fragment was then inserted into binary vector pCAMBIA 1301 between BanHI and NcoI sites, replacing the CaMV 35S promoter, to create the recombinant transcription unit AtGLK1::GUS. For the construction of 35S::AtGLK1 unit, the full-length coding sequence (CDS) corresponding to the AtGLK1 gene locus was cloned by using RT-PCR from Arabidopsis thaliana. The pair of primers used in the PCR was OEGGLK1-F and OEGGLK1-R (XbaI and SacI sites were introduced). The specific PCR fragment was then inserted into binary vector pBII 121 between XbaI and SacI sites, replacing the GLU gene, to create the recombinant transcription unit 35S::AtGLK1. For the construction of 35S::MYBL2 unit, the full-length coding sequence (CDS) corresponding to the AtGLK1 gene locus was cloned by using RT-PCR from Arabidopsis thaliana. The pair of primers used in the PCR was OEMYBL2-F and OEMYBL2-R (NcoI and BstE II sites were introduced). The specific PCR fragment was then inserted into binary vector pCAMBIA 1301 between NcoI and BstE II sites, replacing the GLUS gene, to create the recombinant transcription unit 35S::MYBL2. All primers used are listed in Table S1.

The recombinant plasmids were then introduced into Agrobacterium tumefaciens strain GV3101 and transformed into wild-type Arabidopsis (Col-0) using the floral dip method [52]. The transformants were then screened on 1/2 MS medium containing 50 μg ml⁻¹ Kanamycin (35S::AtGLK1) or 50 μg ml⁻¹ hygromycin (AtGLK1::GUS and 35S::MYBL2).
RNA extraction, cDNA synthesis, and gene expression analysis

RNA extraction and cDNA synthesis were performed according to the method reported in the previous work [53]. For real-time quantitative PCR analysis, the reaction was performed using SYBR Green Perfect mix (TaKaRa, Dalian, China) on a CFX96 (Bio-Rad), following the manufacturer’s instructions. The following standard thermal profile was used for all PCRs: 95 °C for 2 min; 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Gene expression was normalized to that of ACTIN2 by subtracting the C_T value of ACTIN2 from the C_T value of the gene of interest. Expression ratios were then obtained from the Eq. 2^ΔΔCT. Primers for genes of interest are listed in Table S1.

Anthocyanin measurement

Anthocyanin measurement was performed as previously described [54]. The seedlings were grown for 4 days after sowing on 1/2 MS medium, and then used for anthocyanin measurement. Seedlings of each genotype were incubated overnight in 0.6 mL of 1% HCl in methanol at 4 °C and extracted using an equal volume of chloroform after the addition of 0.4 mL of water. After centrifugation, the quantity of anthocyanins was determined by spectrophotometric measurement of the aqueous phase (A530-0.25A657) and normalized to the fresh weight of each sample. 3 independent biological samples were used to measure anthocyanin for each genotype.

Histochemical GUS staining

Histochemical GUS staining of homozygous T3 transgenic lines harboring AtGLK1::GUS fusion gene was done as previously described [55]. At least 5 individual lines were analyzed to give typical results shown here.

Statistical analysis

All experiments with each group were performed at least in triplicate. Error bar represents ± S.D. (n = 3). The significant differences between control and treatment of the samples or between wild-type and other genotypes were analysed by the Student’s t test. Significant differences from control are denoted by one star corresponding to P < 0.05.

Supplementary Information

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Authors’ contributions

DZ, YZ, LY, ZX, JC, FZ and HJ performed the experiments. DL helped in planning, interpretation, analysis and manuscript writing. All authors discussed the results, revised the manuscript and approved submission of this work.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. B Winkel-Shirley 2001 Flavonoid biosynthesis: a colorful model for genetics, biochemistry, cell biology, and biotechnology Plant Physiol. 126 2 485 493
2. K Gould J Mckelvie KR Markham 2002 Do anthocyanins function as antioxidants in leaves? Imaging of H2O2 in red and green leaves after mechanical injury Plant Cell Environ. 25 10 1261 1269
3. R Nakabayashi K Yonekura-Sakakibara K Utano M Suzuki Y Yamada T Nishizawa F Matsuda M Kojima H Sakakibara K Shinozaki A Itohge M Yamazaki K Saito 2014 Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids Plant J. 77 3 367 379
4. MJ Rao Y Xu Y Huang X Tang X Deng X Q Xu 2019 Ecopotic expression of citrus UDP-GLUCOSYL TRANSFERASE gene enhances anthocyanin and proanthocyanidins contents and confers high light tolerance in Arabidopsis BMC Plant Biol. 19 603
5. Y Zhang E Butelli R Stefano De A Maquin C Pacliari A N Wellner L Hill D Orzaez A Granell JDG Jones C Martin 2013 Anthocyanins double the shelf life of tomatoes by delaying overripening and reducing susceptibility to gray mold Curr Biol. 23 12 1094 1100
6. TA Holton EC Cornish 1995 Genetics and biochemistry of anthocyanin biosynthesis Plant Cell, 7 7 1071 1083
7. Pelletier MK, Murrell JR, Shirley BW. Characterization of flavonol synthase and leucanthocyanidin dioxygenase genes in Arabidopsis. Further evidence for differential regulation of “early” and “late” genes. Plant Physiol. 1997;113(4):1437–1445.
8. E Groterwold 2006 The genetics and biochemistry of floral pigments Annu Rev Plant Biol. 57 1 761 780
9. F Mehrtens H Kranz P Bednarek A Weisshaar 2005 The Arabidopsis transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis Plant Physiol. 138 2 1083 1096
10. A Gonzalez M Zhao JM Leavitt JM Lloyd 2008 Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings Plant J. 53 5 814 827
11. R Somovec H D’Ippolito H Egea A Barsch F Mehrtens K Niehaus B Weisshaar 2007 Differential regulation of closely related R2R3-MYB
transcription factors controls flavonol accumulation in different parts of the Arabidopsis thaliana seedling Plant J. 50 4 660 677

12 J Shin E Park G Choi 2007 PIF3 regulates anthocyanin biosynthesis in an HY5-dependent manner with both factors directly binding anthocyanin biosynthetic gene promoters in Arabidopsis Plant J. 49 6 981 994

13 C Dubos J Gourrieriec Le A Baudy G Hupel E Lanet I Debeaupin JM Routaboul A Alaboresi B Weisshaar L Lepiniec 2008 MYB2 is a new regulator of flavonoid biosynthesis in Arabidopsis thaliana Plant J. 55 6 940 953

14 K Matsui Y Umemura M Ohme-Takagi 2008 AtMYB2, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in Arabidopsis Plant J. 55 6 954 967

15 HB Saksena M Sharma D Singh A Laxmi 2020 The versatile role of glucose signalling in regulating growth, development and stress responses in plants Plant Biochem Biotechnol. 29 4 687 699

16 Y Nagira Y Ozeki 2004 A system in which anthocyanin synthesis is induced in regenerated torenia shoots J Plant Res. 117 5 377 383

17 Y Nagira K Ikegami T Koshiba Y Ozeki 2006 Effect of ABA upon anthocyanin synthesis in regenered torenia shoots J Plant Res. 119 2 137 144

18 C Soffanelli A Poggi E Loreti A Alpo P Pirato 2006 Sucrose-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis Plant Physiol. 140 2 637 646

19 VOE Wim SK El-Esawie 2014 Sucrose signaling pathways leading to fructan and anthocyanin accumulation: A dual function in abiotic and biotic stress responses? Environ Exp Bot. 108 4 13

20 R Gollop S Even V Colova-Tsoloval A Peri 2002 Expression of the grape dihydroflavonol reductase gene and analysis of its promoter region J Exp Bot. 53 373 1397 1409

21 R Gollop S Farhi A Peri 2001 Regulation of the leucoanthocyanidin dioxygenase gene expression in Vitis vinifera Plant J. 161 3 579 588

22 JW Dong PK Das SC Jeoung JH Song HK Lee YK Kim WJ Kim IP Yong SD You SB Choi 2010 Ethylene suppression of sugar-induced anthocyanin pigmentaton in Arabidopsis thaliana Plant Physiol. 154 1515 1531

23 AB Sivitz A Reinders JM Ward 2008 Arabidopsis sucrose transporter AtSUC1 is important for pollen germination and sucrose-induced anthocyanin accumulation Plant Physiol. 147 1 92 100

24 H Kubo AJM Peeters MGA Aerts Pereira M Koornneef 1999 ANTHOCYANINLESS2, a homeobox gene affecting anthocyanin distribution and ANTHOCYANIN accumulation Plant Physiol. 117 1217 1226

25 B Chaudhuri F Hormann S Lalonde SM Brady DA Orlando P Benfey WB 2008 Expression of nuclear photosynthesis genes in the dark and in roots of the Arabidopsis Bisa sp. mutant Plant Cell. 11 5 901 910

26 Y Zong X Zhu Z Liu X Xi G Li D Cao L Wei J Li B Liu 2019 Functional MYB transcription factorencoding gene AN2 is associated with anthocyanin biosynthesis in Lycium ruthenicum Murray BMC Plant Biol. 19 1 169

27 Y Liu T Li Z Dou Y 2004 Overexpression of the PAP1 transcription factor enhances production of phenylpropanoids in Arabidopsis J Exp Bot. 55 5 695 708

28 R Ahmad Y Liu TJ Wang Q Meng H Yin X Wang Y Wu N Nan B Liu ZY Xu 2019 GOLDEN2-LIKE transcription factors regulate WRKY10 expression in response to abscisic acid Plant Physiol. 179 4 1844 1860

29 JL Woodson J Chory 2008 Coordination of gene expression between organelar and nuclear genomes Nat Rev Genet. 9 5 383 395
S2  SJ Clough AF Bent 1998 Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana Plant J. 16 6 735 743
S3  D Liu W Li J Cheng 2016 The novel protein DELAYED PALE-GREENING1 is required for early chloroplast biogenesis in Arabidopsis thaliana Sci Rep. 6 1 25742
S4  M Wu X Lv Y Zhou Y Zeng D Liu 2019 High anthocyanin accumulation in an Arabidopsis mutant defective in chloroplast biogenesis Plant Growth Regul. 87 3 433 444
S5  J Yi D Zhao J Chu J Yan J Liu M Wu J Cheng H Jiang Y Zeng D Liu 2019 AtDPG1 is involved in the salt stress response of Arabidopsis seedling through ABI4 Plant Sci. 287 110180

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