

Analysis of flowering-associated gene expressions and metabolic characteristics in adzuki bean (*Vigna angularis* L.) with different short-day induction

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Research Article

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Abstract

Background

Adzuki bean is an important miscellaneous grain crop and a kind of typical short-day crop that is used in a variety of foods because of its high nutritional and medicinal value. The flowering time of adzuki beans is affected by multiple environmental factors, particularly the photoperiod. Adzuki bean can meet at flowering period, accelerate breeding process and avoid natural disasters by adjusting the day-length. Therefore, RNA-seq analysis was used to determine the effects of different day-length on the expression and metabolic characteristics of genes related to flowering time in adzuki beans.

Results

In this study, ‘Tangshan Hongxiaodu’ was used as experimental material. Transcriptome sequencing was performed under SD-5d, SD-10d and SD-15d treatments, respectively. The results showed that a total of 5,939 differentially expressed genes (DEGs) were identified by sequencing. Among these common genes, 38.09% were upregulated and 23.81% were downregulated in three groups. Gene Ontology (GO) enrichment analysis was performed on the target genes to identify common functions related to photosystem I and II. Kyoto Encyclopedia of Genes and Genomes (KEGG) enriched analysis was performed to predict two pathways involved in antenna protein and circadian rhythm. And that the flowering of adzuki beans was promoted by downregulating genes in the circadian rhythm pathway through the blue light metabolic pathway, whereas the antenna protein promoted flowering by enhancing the reception of light signals and accelerating electron transport. In these two metabolic pathways, the number of DEGs was the greatest changes in SD-5d vs SD-15d comparison group. Real-time qRT-PCR validation of eight DEGs in these two metabolic pathways was consistent with the transcriptome results, indicating that the sequencing results were accurate and reliable and that these genes may be candidate genes affecting the regulation of short-day induction at the adzuki bean seedling stage.

Conclusion

The results indicated that short-day induction can downregulate the expression of genes related to adzuki bean flowering in the circadian rhythm and upregulate the expression of some genes in the antenna protein pathway. In addition, the results not only provide a theoretical reference for the molecular mechanism of adzuki bean flowering induced by short days, but also provide multi-level information on the next steps in exploring the functional verification of key genes regulating adzuki bean flowering.

Background

Adzuki bean, which originated in China, is an important grain crop. As a homologous medicinal food species, it plays an important role in the human diet with a perennial export volume of approximately 40,500 tons [1]. However, this species experiences serious shedding of flowers and pods, so the yield of adzuki beans is low and unstable [2]. Many recent studies [3–7] have found that sunshine duration could be set at the seedling stage to regulate flowering time, which has had a significant impact on improving the bean yield and quality in the later stage. To date, there have been few reports on the molecular mechanisms of short-day induction and regulation of adzuki bean flowering. RNA-Seq technology can comprehensively identify transcripts, conduct transcriptome analyses, and rapidly identify genes with high sensitivity and accuracy. Therefore, using this technology to identify the regulatory pathways and candidate genes involved in plant flowering is important for understanding the molecular mechanisms underlying plant flowering.

As an important phenological stage, the flowering phase initiates plant reproduction and involves complex transduction mechanisms of environmental signals, including light and temperature [8–10]. Among these, photoperiod is one of the most important environmental factors affecting flowering time in plants. The molecular mechanisms of *Arabidopsis thaliana* in response to photoperiod flowering have been extensively studied [11]. As a typical long-day plant, the flowering of *A. thaliana* can be triggered rapidly under 16 h of light, but is delayed under 8 h of light. The regulatory mechanism is related to the fact that CO protein levels are high in the long term and the mRNA level of FT is increased. Finally, the FT protein is transported to the meristematic tissue through the vascular bundle, promoting the differentiation of floral primordia into floral tissue [12–13]. Under short-day conditions, the COP1/SPA1 complex is formed to degrade the CO protein, thus, downregulating FT expression and inhibiting *Arabidopsis* flowering [14–15]. For soybean, flowering genes have been studied extensively, and 10 QTL loci (namely *E1–E9* and *EJ*) related to flowering time have been identified [16–17]. Among those, *E1–E4*, *E7*, and *E8* regulate the photoperiod flowering of soybeans, with *E1* contributing the most to soybean flowering [18]. *E5*, which does not exist separately, may have been caused by accidental outcrossing with pollen with the *E2* allele [19]. *E9* is a leaky allele of FT2a, the abundance of which is directly related to changes in soybean flowering time [20]. In addition to the E genes, the FT gene also plays an important role in the flowering process of soybeans [21]. In adzuki beans, three metabolic pathways related to flowering have been identified by transcriptome sequencing, including plant hormones, circadian rhythms, and antenna proteins, by comparing different short-day induction periods with their respective controls. Among them, the homologues of 13 verified genes are all related to flowering, indicating that these genes are key candidates for regulating the flowering of adzuki beans [22]. Other studies screened eight significantly different genes related to flowering by transcriptome analysis after short-day induction in kidney beans. With the function of these differential genes related to the regulation of the biological clock, TOC1 has been identified to play a key role in controlling the photoperiodic flowering response through clock function. Upregulation or downregulation of TOC1 leads to changes in downstream genes that regulate the flowering time of kidney beans [23].

With the deepening of transcriptome research on plant flowering, the metabolic pathways and key genes that regulate flowering through the photoperiod have been described in detail in *Arabidopsis* species, Adzuki bean, and *Phaseolus vulgaris*. Additionally, transcriptomics has increasingly been used to regulate flowering and flower development in diverse plants, including lute [24], tobacco [25], lily [26], cucumber [27], and hemp [28]. However, understanding of the effect of the photoperiod on plant flowering needs to be broadened through transcriptome analysis. In particular, transcript information and gene expression

profiles of adzuki bean under different short-day inductions have not yet been determined. Therefore, applying RNA-seq to identify candidate genes related to flowering would help to elucidate the regulatory mechanism of short-day-induced flowering in adzuki beans. In this study, three short-day induction treatments, SD-5d, SD-10d, and SD-15d, were performed to compare the transcriptome of adzuki bean leaves, explore differentially expressed transcripts, and identify candidate transcripts involved in the regulation of flowering. This is of great significance for revealing the molecular mechanisms underlying the regulation of flowering time in adzuki beans.

Result

Characteristics of growth and flowering

The effects of different short-day inducement periods on the growth, rate of apical flower bud differentiation, flowering time, and flowering promoting rate of adzuki beans were very different. The longer the short-day induction period, the greater the inhibitory effect on adzuki bean plants (Additional file 1:Figure S1). Leaf area, plant height, and stem diameter decreased with the prolongation of short-day induction, and plant height showed significant differences among the three treatments at the flowering and podding stages. At the seed-filling stage, the plant height of SD-15d significantly decreased by 25.87% and 18.45% compared to that of the SD-5d and SD-10d treatments, respectively. The stem diameter of SD-15d significantly decreased by 10.91% and 13.27% compared to that of SD-5d at the flowering and seed filling stages, respectively, whereas there was no significant difference between the SD-5d and SD-10d treatments. There was a significant difference in the stem diameter among the three treatments during the podding stage. Significant differences in leaf area were observed among the three treatments for all growth periods (Additional file 2:Table S1) .

The advanced flowering days and flowering promoting rate showed that the flowering date of plants in SD-15d was 12 and 7 days earlier than that in SD-5d and SD-10d (Additional file 1:Figure S1C), and the flowering promotion rates were 22.03% and 11.83%, respectively (Additional file 1:Figure S1D). The advanced flowering days of plants in SD-10d were 6 days compared with SD-5d, and the flowering promoting rate was 10.20%. These results indicated that short-day induction promoted the early flowering of adzuki beans by inhibiting the vegetative growth of plants. Short-day induction had a cumulative effect, showing that the longer the short-day inducement, the earlier the flowering time, and the greater the flowering promotion rate.

Functional analysis of differentially expressed genes

By comparing the data among the three groups, the total number of differentially expressed genes (DEGs) was 5,939, including 3,107 upregulated genes and 2,832 downregulated genes (Fig. 2). On comparing SD-10d and SD-5d, 2,044 DEGs were detected, including 961 upregulated and 1,083 downregulated genes. Comparing SD-5d and SD-15d, a total of 3,068 genes were detected, including 1,711 upregulated genes and 1,357 downregulated genes. Comparing SD-10d and SD-15d, a total of 827 genes were detected, including 435 upregulated and 392 downregulated genes (Additional file 3:Figure S2A). In addition, by comparing the three groups, 105 common genes were detected, of which 40 were upregulated and 25 were downregulated (Additional file 4,5,6:Figure S2B,C,D). In addition, it was observed that the number of DEGs between SD-5d and SD-15d was the highest, indicating that the longer the interval between the two short-day inducement treatments, the higher the number of DEGs detected.

Cluster analysis showed that the three treatments, SD-5d, SD-10d, and SD-15d, had a significant impact on gene expression (Additional file 7:Figure S2E). Among these, 40 genes were gradually upregulated with an extension of short-day time, whereas 25 genes were gradually downregulated. Another 40 genes did not show obvious expression rules, of which 25 genes showed up-down-up regulation and 15 genes showed down-up-down regulation in all treatments. The above results showed that 40 upregulated and 25 downregulated genes played key roles in the growth and development of adzuki beans, but the other genes had no obvious rules, indicating that the function of different genes varied in response to different short-day induction times.

Gene Ontology function enrichment analysis

GO terms (DEGs > 2) were screened separately for biological processes, cell components, and molecular functions. GO function enrichment analysis showed that the light-related function of DEGs was found among all three comparison groups, in which two types of cell components involved in DEGs between SD-5d and SD-10d were related to light function, that is, photosystem I (PSI) and II (PSII), both of which included five upregulated genes. In addition, there was a circadian function related to light in biological processes, which contained 14 genes, of which six were upregulated and eight were downregulated (Additional file 8:Figure S3A). The DEGs in the two comparison groups of SD-5d vs SD-15d and SD-10d vs SD-15d had light-driven functions as PSI and PSII, but the number of genes involved was different. For SD-5d vs SD-15d, PSI and PSII contained nine and twelve genes, respectively; the former nine genes were upregulated, eleven genes of the latter group were upregulated, and the remaining one gene was downregulated. Both SD-10d and SD-15d contained five upregulated genes (Additional file 9,10:Figure S3B,C). These results suggested that the three comparison groups all contained PSI and PSII functions related to light (Additional file 11:Figure S3D), and that photosystem genes in leaf cell components could be activated after different short-day induction times. These genes are key regulators of the growth and development of adzuki bean seedlings and are closely related to photoelectron transport under short-day-induced conditions.

Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of DEGs

KEGG pathway enrichment analysis of DEGs in the three groups was performed to select the top 20 signaling pathways with significant differences in each comparison group. Figure 4 shows that DEGs between SD-5d vs SD-10d and SD-10d vs SD-15d were enriched in two light-related pathways, that is, the antenna protein pathway and circadian pathway, respectively, with different numbers of genes involved in these pathways. For the SD-5d vs SD-10d groups, five genes were upregulated in the antenna protein pathway, and of sixteen genes in the circadian pathway, three were upregulated and thirteen were downregulated. For the SD-10d vs SD-15d group, there were seven genes in the circadian pathway, including four upregulated and three downregulated. Four light-related pathways were enriched in the SD-5d vs SD-15d group, including nine, seventeen, forty-five, and fourteen genes, respectively. The expression patterns of genes in different pathways were different, nine genes in the antenna protein pathway were upregulated; of seventeen genes in the circadian

pathway, three were upregulated and fourteen were downregulated; forty-five genes in the plant hormone signal transduction pathway consisted of twenty seven upregulated and eighteen downregulated genes; and all fourteen genes in the photosynthesis pathway were upregulated. The above results showed that the three comparison groups all contained two pathways related to light and circadian rhythms, of which SD-5d vs SD-10d and SD-5d vs SD-15d had the most significant enrichment degree in the circadian rhythm pathway. This suggests that different short-day inductions, on the one hand, activate gene expression in the antenna protein pathway and promote photosynthesis, and on the other hand, regulate photosynthesis, stomatal rhythmic opening and closing, leaf rhythmic opening and closing, and other important physiological processes, by regulating the biological clock. This could help plants optimize their energy utilization efficiency to the maximum extent and guarantee the early flowering of adzuki beans. Additionally, the circadian pathway plays a key role in the response to different short-day induction periods (Additional file 12,13,14,15:Figure S4A,B,C,D).

Gene set enrichment analysis in the KEGG pathway

It is generally believed that a positive ES value indicates that a certain functional gene set is enriched at the front of the ranked sequence and that the involved pathway is upregulated. A negative ES value indicates that a certain functional gene set is enriched at the rear of the ranked sequence and that the involved pathway is downregulated. The core genes that appeared in the ES map indicate that this functional gene set has biological significance. Here, gene set enrichment analysis revealed that the antenna proteins and circadian pathways in the three comparison groups had the same enrichment scores, as shown in Fig. 5. The antenna protein pathway was upregulated (Additional file 16:Figure S5A,B), the circadian pathway was downregulated (Additional file 17,18:Figure S5C,D,E,F), and the core genes were identified in both pathways. The core genes of the antenna protein pathway were enriched in the front of the sequencing sequence, whereas those of the circadian pathway were enriched in the rear of the sequencing sequence in the three comparison groups, indicating that these core genes play an important role in the response of adzuki beans to short-day induction.

Heatmap cluster analysis was performed on the core genes of antenna proteins and circadian metabolic pathways (Fig. 6). The antenna protein pathways of the three comparison groups, SD-5d vs SD-10d, SD-5d vs SD-15d, and SD-10d vs SD-15d, contained 13, 15, and 17 core genes, respectively, which were upregulated with an increase in the number of short-day induction days (Additional file 22,23,24:Figure S6D,E,F). The circadian rhythm pathways in the three comparison groups contained 12, 15, and 14 core genes, which were inversely downregulated as the number of short-day induction days increased (Additional file 19,20,21:Figure S6A,B,C). These results indicated that adzuki beans responded to different short-day induction periods by upregulating the key genes in the antenna protein pathway or downregulating the key genes in the circadian pathway, thus, promoting early flowering.

Analysis of circadian and antenna protein pathway maps

The circadian rhythm is a rhythmic change in plants in response to daylight length. Analysis of the circadian metabolic pathway showed that blue light promoted and far-red light inhibited flowering in adzuki beans. After short-day induction, PHYB, a far-red light receptor, interacted with PIF3, a phytochrome interaction factor, to promote the transcription of NDA and the formation of LHY, a circadian clock regulatory protein. The PRR7 protein of the central oscillator-controlled internal clock inhibited the transcription of PIF3 and formation of LHY. In addition, PRR7, a key gene in the photoperiodic flowering pathway, could inhibit the expression of the LHY protein, thus, inhibit the flowering of adzuki beans. After short-day induction, one pathway of flowering regulation was that a complex formed by morphogenesis regulator COP1 and signal sensing protein SPA1 was inhibited by the cryptochrome CRY-blue light receptor protein, and this complex was downregulated and ubiquitinated to inhibit the transcription of HY5, a negative regulator of photomorphogenesis, thus, indirectly, promoting photomorphogenesis. Another pathway of flowering regulation is that of CRY, a blue light receptor protein, which inhibits complex formation by COP1 and ELF3, thus, inhibits GI by ubiquitylation. As a negative regulator, GI downregulates the function of the complex formed by FKF1 and GI, inhibits CDF1 binding to the promoter of CO by ubiquitylation, thus, initiates the transcriptional activation of CO and upregulation of FT. GI can also directly promote DNA transcription into CO, which can promote FT upregulation, and indirectly promote adzuki bean flowering (Fig. 7A). Figure 7B shows that the changes in the expression levels of seven protein genes, PIF3, PRR7, SPA1, HY5, ELF3, GI, and FKF1 were different among SD-5d vs SD-10d, SD-5d vs SD-15d, and SD-10d vs SD-15d three comparison groups. Difference genes showed more significant changes in SD-5d vs SD-15d comparison group, indicating that the longer the short-day induction, the greater differential gene change and most genes were downregulated.

Chloroplasts in green plants are comprised of PS and PS. PS contains five light-trapping pigment protein complexes, named Lhca1, Lhca2, Lhca3, Lhca4, and Lhca5, while PS contains six, named Lhcb1, Lhcb2, Lhcb3, Lhcb4, Lhcb5, and Lhcb6, of which the first three (Lhcb1-3) play major roles. Different short-day inductions could affect the expression of genes encoding antenna proteins, allowing them to adapt to the growth and development of Adzuki beans. Figure 8A, B shows that, after a short-day induction period, the genes involved in the Lhca3, Lhcb1, Lhcb2, Lhcb3, Lhcb4, and Lhcb6 proteins were all upregulated, and there was a higher number of upregulated genes in the Lhcb1, Lhcb2, and Lhcb3 proteins than in the other proteins. Figure 8B shows that the largest differential genes were changed in the comparison group of SD-5d vs SD-15d, indicating that the light signal reception ability in the plants was enhanced with an increase in the number of short-day induction days; that is, the longer short-day induction period, the stronger reception ability. These results indicated that the adzuki beans responded to short-day induction by upregulating PS and PS genes. These genes may play key roles in regulating the growth and development of adzuki bean seedlings under short-day induction conditions, the longer the period of induction, the greater the differential genes change.

Identification of DEGs by qRT-PCR

To verify the reliability and accuracy of the transcriptomic data, new samples from the same treatment group were subjected to qRT-PCR analysis. Eight genes with significant variability were screened from the circadian rhythm and antenna protein pathways, all of which were closely related to light and associated with flowering by homologous sequence alignment (Additional file 25:Table S2). qRT-PCR was performed to verify these eight genes in SD-5d vs SD-10d, SD-5d vs SD-15d and SD-10d vs SD-15d three comparison groups.

The results showed that the LOC108331766 gene was significantly different in the SD-5d vs SD-10d and SD-5d vs SD-15d comparison groups, but was not significantly different in the SD-10d vs SD-15d group. The LOC108322606 gene was significantly different in the SD-5d vs SD-10d and SD-10d vs SD-15d groups, but was not significantly different in the SD-5d vs SD-15d groups. In the antenna protein pathway, LOC108345872 and LOC108344684 gene was significantly different among the three groups. Meanwhile, LOC108335068 gene was significantly different in the SD-5d vs SD-15d and SD-10d vs SD-15d groups, but was not significantly different in the SD-5d vs SD-10d comparison group. LOC108328079, LOC108333950, and LOC108338432 gene was significantly different in the SD-5d vs SD-10d, SD-5d vs SD-15d, and SD-10d vs SD-15d groups, respectively, but not in the other groups. These results suggest that the qRT-PCR results for the eight genes were consistent with the transcriptome sequencing results of RNA-seq (Additional file 26:Figure S9A1B1,A2B2,A3B3,A4B4). Eight genes were significantly different in circadian rhythm and antenna protein pathways, indicating that the transcriptome sequencing data were accurate and reliable.

Discussion

The life cycle of higher plants includes vegetative and reproductive growth, and flower initiation marks the transition between plant growth stages, which determines whether a plant can successfully reproduce [35]. As an important agronomic index, flowering time is a prerequisite for high yield and high harvest index of crops and is greatly affected by the photoperiod [36–37]. In this study, a short-day sensitive variety, 'Tangshan hong xiao dou', was used under SD-5d, SD-10d and SD-15d treatments, and it was found that the longer short-day induction period, the earlier flowering was initiated and the greater inhibitory effect on plants. These results indicated that adzuki bean plants promote flowering by inhibiting plant growth after short-day induction, which is consistent with the results of studies on chrysanthemums and soybeans [38–39].

The plant light-dependent signaling response is an extremely complex process involving the regulation of many related genes. To further explore the regulatory mechanism of the short-day induction of adzuki bean flowering and the expression of key genes, a cDNA library of adzuki bean leaves was constructed after different short-day induction periods and transcriptome sequencing was conducted using Illumina sequencing technology. A total of 5,939 differential genes were obtained in three comparison groups, among which the SD-5d vs SD-15d group had the maximum number of differential genes (3,068), which increased with an increasing number of interval days between the two short-day induction groups. GO functional enrichment results showed that PSI and PSII, which have two light-related functions, were both present in the three comparison groups, and the number of DEGs in each comparison group was different. There were nine and twelve genes corresponding to two light-related functions in the SD-5d vs SD-15d comparison group, respectively. All nine genes were upregulated, whereas among the twelve genes, the expression of eleven was upregulated and that of one was downregulated, indicating that the longer interval of short-day induction, the higher number of DEGs identified, and most of these genes were upregulated. PSI and PSII are related to plant light-trapping pigment protein complexes that receive solar energy and assimilate carbon dioxide to form chemical energy [40–41].

There are three main light-trapping pigment protein complexes encoded by Lhcb1, Lhcb2, and Lhcb3 in the peripheral light-trapping antenna of PSII. The main function of this complex is to capture and transfer light energy and balance the excitation energies of PSI and PSII. It also plays an important role in maintaining the membrane structure of thylakoids in response to changes in the external environment and in photoprotection [42–43]. From these results, it can be speculated that the photosynthetic capacity of adzuki bean plants was enhanced by the upregulation of genes in PSI and PS after short-day stress. In particular, the photocapture capacity of the peripheral photocapture antenna of PSII was enhanced. In addition, the electron transfer rate was accelerated to rapidly respond to changes in the external environment and maintain normal growth and development. The subsequent gene pathway map of antenna protein gene expression also showed that genes in the three comparison groups were upregulated, and the number of DEGs in the SD-5d vs SD-15d comparison group was the highest. Among these, five genes in the Lhcb1 protein were upregulated, suggesting that the Lhcb1 protein plays an important role in light energy absorption after short-day induction in adzuki beans.

There is a complex interaction between exogenous photoperiodic signals and the flowering biological clocks of plants when sensing changes in the lengths of day and night, which causes a series of activations or inhibitions of flowering genes, ultimately leading to changes in the flowering rhythm [44]. Different plant flowering rhythm and photoperiodic pathways are conservative in large part [45]. In this study, KEGG enrichment analysis showed that two light-related antenna proteins and circadian pathways were present in three comparison groups, and the degree of enrichment of the circadian pathways was most significant in the SD-5d vs SD-15d group. Further analysis of gene set enrichment revealed that adzuki beans can respond to short-day induction by upregulating antenna protein pathway and downregulating key genes in the circadian rhythm pathway, thus, promoting early flowering. This is similar to the findings of previous studies on different species of edible fungi and peaches under short-day induction using transcriptome sequencing [46–47].

Further analysis of the regulatory network showed that the antenna protein enhanced the absorption of light energy through gene upregulation; in the circadian rhythm pathway, early flowering of plants was promoted through the blue light metabolic pathway, while far-red light inhibited the flowering of adzuki beans. Early studies on Arabidopsis species found that the red light receptor PHYB and the blue light receptor CRY2 have antagonistic effects on the regulation of flowering time [48–49]. CRY1, CRY2, and PHYA stabilize CO, whereas PHYB promotes its degradation [50].

How does blue light regulate the CO protein in adzuki beans? One regulatory pathway is the far-red light signaling pathway; in the circadian rhythm system, PHYB binds to PIF3 to promote the transcription of NDA into LHY, and PRR7 inhibits the transcription of PIF3 into LHY protein, thus, inhibiting flowering. Another pathway is the blue light pathway, in which CRY inhibits the formation of the COP1-SPA1 complex, which is downregulated, and inhibits the transcription of HY5 by ubiquitination, thus, indirectly promoting light morphogenesis. In addition, CRY inhibits the formation of the COP1-ELF3 complex and GI by ubiquitination. As a negative regulator, GI downregulates the formation of the FKF1-GI complex, inhibits CDF1 binding to the CO promoter by ubiquitination, transcriptionally activates CO, and upregulates FT to indirectly promote adzuki bean flowering. GI can also directly promote DNA transcription into CO, which, in turn, promotes FT upregulation, thereby indirectly promoting adzuki bean flowering. Based on this analysis, it was found that light regulates CO activity through at least two processes: the biological clock regulates the expression of CO mRNA and the stability of the CO protein is regulated through the signal transduction of different light receptors.

In *A. thaliana*, CRY2 cannot interact with SPA1 during the dark period, when high CO gene expression occurs under short-day conditions, which leads to the degradation of the CO protein by the COP1–SPA1 complex and flowering [51–52]. Under long-day conditions, GI and FKF1 proteins at high levels form a complex and degrade the CDF1 protein under blue light, which eliminates the inhibitory effect of CDF1 on the CO gene. Subsequently, CO mRNA begins to accumulate, promoting the upregulation of FT genes and flowering [53–54]. It can be seen that the flowering regulation mechanism of adzuki beans is similar to that of *A. thaliana*. Substantial progress has been made in the study of cryptochrome genes in soybeans [55–57], *Arabidopsis* species [58–59], and rice [60]. Cryptochrome acts as a photoreceptor in various angiosperms, regulates the biological clock, and plays an important role in the induction of flowering. However, the number of upregulated or downregulated genes in flowering proteins varies between plants. Eight DEGs selected in circadian rhythms and antenna proteins were verified to be consistent with the RNA-seq results, and the functions of these eight genes were related to light and flowering. This study clarified the regulatory mechanism of the short-day induction of adzuki bean flowering, laying a foundation for further exploration of the functions of key genes that regulate adzuki bean flowering.

Conclusion

The transcriptional results showed that the number of DEGs in the SD-5d vs SD-15d comparison group was the highest (3,068 genes). The longer short-day induction time, the greater promotion of flowering. This regulatory mechanism is associated with the downregulation of key genes in the circadian rhythm pathway, thus, regulating the blue light metabolism pathway to promote flowering under short-day induction. In addition, the antenna protein pathway accelerates electron transfer through gene upregulation to promote the flowering of adzuki beans. Eight DEGs screened from these two metabolic pathways were mostly upregulated and were re-verified by RNA-seq as candidate genes for regulating adzuki bean flowering. These results further clarify the metabolic pathway of adzuki bean flowering under short-day induction and provide valuable information for future functional studies of flowering-related genes.

Methods

Experimental material

A late maturity variety ‘Tangshan Hongxiaodou’sensitive to short-day was selected as the experimental material, provided by the adzuki Bean Breeding Research Group of the Institute of Grain and Oil Crops, Hebei Academy of Agricultural and Forestry Sciences. The experiment had been conducted in the Teaching and experimental base of Hebei Agricultural University, Baoding (longitude: 115°47 ', latitude: 38°87 ') in 2021. As a typical short-day crop, adzuki bean is very sensitive to short-day induction, and thus the flowering time and maturity stage are significantly earlier than previously.

Soil Fertility

The soil type of this experimental field is loam soil. 400g compound fertilizer (N-P₂O₅-K₂O = 24:4:8) was applied to each plot (length 5m, width 1m) before sown, and the nutrient content of the experimental plot was measured after ploughing. The fertility of the cultivated soil layer (0-20cm) of the experimental plot was shown in Table 3 (Additional file 27:Table S3).

Table 1
Plant height, stem diameter and leaf area of adzuki bean under different short-day inducement times.

Determination index	Treatment	Flowering	Podding	Seed-fling
Plant height/cm	SD-5d	39.51 ± 1.01a	44.03 ± 0.63a	45.61 ± 0.64a
	SD-10d	35.00 ± 2.29b	39.04 ± 0.55b	41.46 ± 1.42a
	SD-15d	28.96 ± 3.57c	32.18 ± 0.92c	33.81 ± 1.12b
Stem diameter/cm	SD-5d	1.65 ± 0.08a	1.84 ± 0.04a	2.11 ± 0.12a
	SD-10d	1.59 ± 0.04ab	1.72 ± 0.03b	1.99 ± 0.11ab
	SD-15d	1.47 ± 0.06b	1.48 ± 0.04c	1.83 ± 0.14b
Leaf area/cm ²	SD-5d	28.62 ± 0.92a	30.55 ± 0.59a	36.04 ± 1.88a
	SD-10d	25.77 ± 0.49b	27.85 ± 0.28b	31.99 ± 1.26b
	SD-15d	22.92 ± 0.69c	22.58 ± 0.77c	26.78 ± 1.01c
Values are means ± S.E. The different small letters in the same column indicate statistical significance at 0.05 level by DMRT.				

Table 2
List of different genes associated with light in circadian rhythm and antenna proteins signaling pathways.

Gene ID	LOC108331766	LOC108322606	LOC108345872	LOC108328079	LOC108344684	LOC108335068	LOC108333950	LOC108333950
KEGG map	Circadian rhythm-plant (KEGG map)		Photosynthesis-antenna proteins (KEGG map)					
Gene symbol	F17H15.25	F11C10.3 /F11C10.4	CAB21	F27I1.2	CAB1B	CAB3	LHBC1	LHCE
Regulation	Up	Down	Up	Up	Up	Up	Up	Up
Location	Chromosome 4 NC_030640.1	Chromosome Un NW_016115133.1	Chromosome 10 NC_030646.1	Chromosome 3 NC_030639.1	Chromosome 10 NC_030646.1	Chromosome 6 NC_030642.1	Chromosome 5 NC_030641.1	Chromosome 5 NC_030641.1
Description	Protein early flowering 3-like	Protein suppressor of phyA-1051-like	Chlorophyll a-b binding protein of LHCII type 1-like	Chlorophyll a-b binding protein CP29.3, chloroplastic	Chlorophyll a-b binding protein of LHCII type 1-like	Chlorophyll a-b binding protein of LHCII type 1-like	Chlorophyll a-b binding protein 13, chloroplastic-like	Chlorophyll a-b binding protein 215, chloroplastic-like
Differential groups	SD-5d VS SD-10d; SD-5d VS SD-15d	SD-5d VS SD-10d;SD-10d VS SD-15d	SD-5d VS SD-10d;SD-5d VS SD-15d SD-10d VS SD-15d	SD-5d VS SD-10d	SD-5d VS SD-10d;SD-5d VS SD-15d SD-10d VS SD-15d	SD-5d VS SD-15d;SD-10d VS SD-15d	SD-5d VS SD-15d	SD-5d VS SD-15d
q-value	2.83E-03 6.78E-06	5.84E-33 1.44E-51	3.64E-08 4.27E-39 1.23E-05	8.03E-07	6.62E-14 1.19E-41 3.09E-14	4.91E-18 1.58E-12	2.32E-12	6.97E-12
Functional annotations of orthologs	Protein early flowering 3-like	WD40 repeat	Chlorophyll A-B binding protein	Chlorophyll A-B binding protein	Chlorophyll A-B binding protein	Chlorophyll A-B binding protein	Chlorophyll A-B binding protein	Chlorophyll A-B binding protein

Table 3
Determination of soil nutrients content in experiment field

Experimental site	Year	Determination index				
		Organic matter(%)	Total nitrogen(%)	Available nitrogen(ppm)	Available phosphorus(ppm)	Available potassium(ppm)
Teaching and experimental base of Hebei Agricultural University	2021	1.61	0.0969	85.88	66.761	189.2

Environmental characteristics in the community after shading

The environmental characteristics of the plot after shading treatment are shown in Table 4 (Additional file 28:Table S4). Compared with the atmospheric environment, the light intensity in the plot after shading is near zero, and the relative humidity is significantly increased by 21.41%, while the CO₂ concentration and temperature are slightly increased, but not reaching a significant level.

Table 4
Changes in field microclimate with shading treatment

Treatment	Illumination intensity (lux)	CO ₂ concentration (ppm)	Relative humidity (%)	Temperature (°C)
Ambient environment	58865.41 ± 1547.23a	478.46 ± 15.56a	75.35 ± 5.83b	25.88 ± 1.33a
Environment of Plots	11.67 ± 4.21b	534.54 ± 18.76a	95.88 ± 6.43a	26.87 ± 1.47a
Note: Values are means ± S.E, The different small letters in the same column indicate statistical significance at 0.05 level by DMRT. The same as below.				

Test Methods

Short-day treatment

Before sowing, the experimental field plot with 5m long and 1m wide was applied 400g compound fertilizer (N-P₂O₅-K₂O = 24:4:8). Then, ploughing was carried out and sowing was carried out on June 24. All plots were two-rows planting with a row spacing of 15cm×40cm. When the true leaf unfolded, short-day treatments was implemented by extending the night length (10h light/14 h dark) automatically, that realized by putting the stainless-steel shelf covered with opaque cloth on the upper side of the plots. The shading treatment is 10h-light and 14h-dark with shading 5d, 10d and 15d respectively. By adopting the

randomized-block arrangement, this experiment was initiated shading treatment with opaque cloth begun at 18:00 every day and finished at 8:00 the next morning. After the shading treatment, all plants were grown to maturity under natural light. 0.4% potassium dihydrogen phosphate was sprayed at the initial flowering stage and pod setting stage, respectively, when one-time irrigating and controlling of insects and diseases were performed. There were three shading treatments with three replicates and nine plots. After 5, 10 and 15 days of shading, the middle leaflet samples of top trifoliate leaves were uniformly taken at 9:00 am with 5–6 plants duplications. All samples were marked as SD-5d-1, SD-5d-2, SD-5d-3; SD-10d-1, SD-10d-2, SD-10d-3; SD-15d-1, SD-15d-2, SD-15d-3, respectively. After sampling, the samples were quick-frozen in liquid nitrogen and placed in a refrigerator at -80 ° C, and RNA extraction and sequencing were performed one week later.

Determination of meteorological factors

The light intensity was measured by TES1332 illuminometer (provided by College of Plant Protection, Agricultural University of Hebei, TES1332, Taiwan) at 20–30 cm above the canopy of adzuki bean community. CO₂ concentration was measured by Li-6400 portable photosynthetic meter (LI-COR, Lincoln, NE, USA). Temperature and humidity were measured by HOBO Pro V2 series (U23-002, produced in the United States), which counted and recorded data automatically every hour.

Determination of growth index

plant height, stem diameter and leaf area were measured with three representative plants selected from each plot at flowering, pod setting and grain filling stages, respectively. Plant height was the distance from the true leaf to the growing point of the plant. The diameter of the plant stem at the true leaf was measured with a vernier caliper, and calculated with the formula: circumference = 2*3.14* (diameter/2). Leaf area was measured by YMJ-B leaf area measuring instrument (Hangzhou Huier Instrument Equipment Co, LTD).

Investigation and statistics of flowering characteristics

At the flowering stage, 3 representative plants were selected from each treatment with 3 replicates. The advanced flowering days was recorded and the flowering promotion rate was calculated. The determination formula is as follows:

Advanced flowering days = days from emergence to flowering of plants in control-days from emergence to flowering of plants in different treatments

Flowering promoting rate (%) = [(days from emergence to flowering in control - days from emergence to flowering in different treatments) ×100]/days from emergence to flowering in control

RNA extraction and cDNA library construction

Total RNA was isolated from samples and DNA was digested by DNase. Eukaryotic mRNA was enriched using oligo (dT) coupled to magnetic beads, and the mRNA was broken down into short fragments by the addition of interrupting reagents. The interrupted mRNA was used as the template to synthesize one-strand cDNA with six-base random primers; and then a two-strand synthesis reaction system was prepared to synthesize two-strand cDNA, which was further purified by the kit and carried out end-to-end repair, A-tail addition and sequencing adapter connection, fragment size selection, and finally PCR amplification and library construction. After qualified by Agilent 2100 Bioanalyzer, the constructed libraries were sequenced using Illumina HiSeqTM2500 or Illumina HiSeq X Ten sequencer to produce 125bp or 150bp double-ended reads.

Screening of differentially expressed genes

FPKM[29], bowtie2[30] and express software [31] were used to analyze the transcript levels. With a reference genome of *Vigna angularis* (<https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/001/190/045/>)[32], the number of transcript (protein - coding) reads for all samples were obtained by express software. Genes with an average number of reads greater than 2 were screened, and related data was standardized by the estimateSizeFactors function of DESeq[33] R package. The p-value and fold-change values of difference comparison were calculated by nbinomTest function. Differentially expressed genes (DEGs) were screened based on Fold Change ≥ 2 and FDR < 0.05.

Functional annotation analysis of differentially expressed genes (DEGs)

Gene Ontology (GO) annotations and functional classification of differential genes (p < 0.05 and Fold Change > 2) were generated using Blast2 GO and WEGO. Further, BLAST was used to align gene sequences to KEGG (Kyoto Encyclopedia of Genes and Genomes) database for gene annotation of biochemical pathway and identification of the regulatory-metabolic network of organisms.

qRT-PCR validation

Under the same treatment, other samples of adzuki bean were used to conduct qRT-PCR on 8 selected DEGs to verify the accuracy of sequencing results. RNA was extracted using an RNA extraction kit followed by reverse transcription to produce cDNA (HiScript II Q RT SuperMix for qPCR). Primer 5.0 was used to design the primers, and the primer sequences are shown in Table 5 (Additional file 29:Table S5). The quantitative Kit (QuantiFast® SYBR® Green PCR Kit) was used to prepare the qRT-PCR reaction system and the transcripts were detected on the fluorescence quantitative PCR instrument. The qRT-PCR cycling conditions were as follows: 40 cycles of predenaturation at 95°C for 10 min, denaturation at 95°C for 10s, and annealing extension at 60°C for 30s. Gene expression was calculated using the method of 2-ΔΔCt [34].

Table 5
Primer design for qRT-PCR validation of eight differentially significant genes

Order number	Gene ID	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')
1	ACTIN	CTAAGGCTAATCGTGAGAA	CGTAAATAGGAACCGTGT
2	LOC108331766	AAAAGGGAGGACCAAGAGCAC	TGAGTGGCACAACACCTGAAT
3	LOC108322606	AAGCAAACAAGGAAGGGAAAG	TGAAACATGGCTGCAAAAGAT
4	LOC108345872	GAGGCTCCTTCTTACCTGACG	AGTTCACGGTTTCGAGCAAAT
5	LOC108328079	AAGAAACGCAGAACTTGACCC	GCTTGAATGGCAAAGATGAGG
6	LOC108344684	GAGGCTCCTTCTTACCTGACG	AGTTCAAGGTTCCGAGCAAAT
7	LOC108335068	GAGGTGACCGACCCAATTTAC	CACAATCGCCTGAACAAAGAA
8	LOC108333950	GGGTTCTTTGTTCAAGCCATT	ACCCAAGCATTGTTAGCCACT
9	LOC108338432	CAGGTTGTGCTTATGGGGTTT	AGCGACCATTCTTGAGTTCCT

Declarations

Authors' contributions

Weixin Dong Yuechen Zhang designed the experiment in advance, Lei Zhang prepared for the test and treatment of test materials. Peijun Tao designed and performed the experiment. Weixin Dong analyzed the data and wrote the manuscript. Dongxiao Li revised the paper and all authors read and agreed to the final manuscript.

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Availability of data and materials

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI-PRJNA817421.

Ethics approval and consent to participate

The seeds used in this study were licensed, and the collection of materials in field studies have complied with relevant institutional and national legislation.

Consent for publication

All authors agree for publication, and test data were detailed and accurate, 'Tangshanhongxiaodou' was used as material for this experimentis. It was a farmer species resources and used widely in scientific research.

Competing interests

The authors declare that they have no competing interests.

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Figures

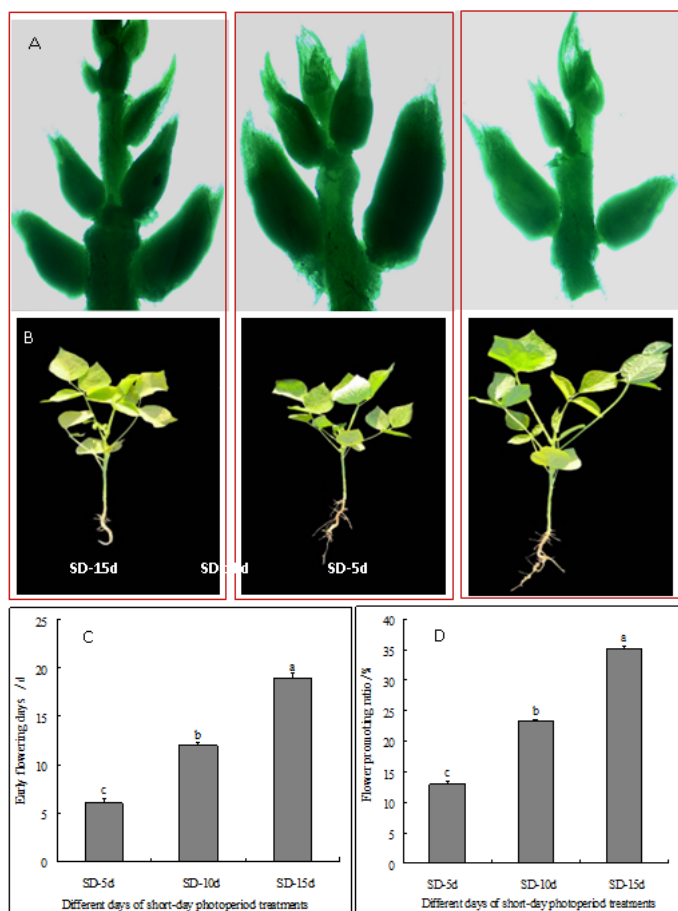


Figure 1

Effect of short-day induction on growth, apical inflorescence, flowering days and flowering promotion rate in adzuki bean

A Adzuki bean plant pictures of different short-day inducement times. There were three treatments (SD-5d, SD-10d, SD-15d); **B** Advanced flowering days of adzuki bean in three different treatment groups; **CD** Early flowering days and flowering promoting rate of adzuki bean in three different treatment groups.

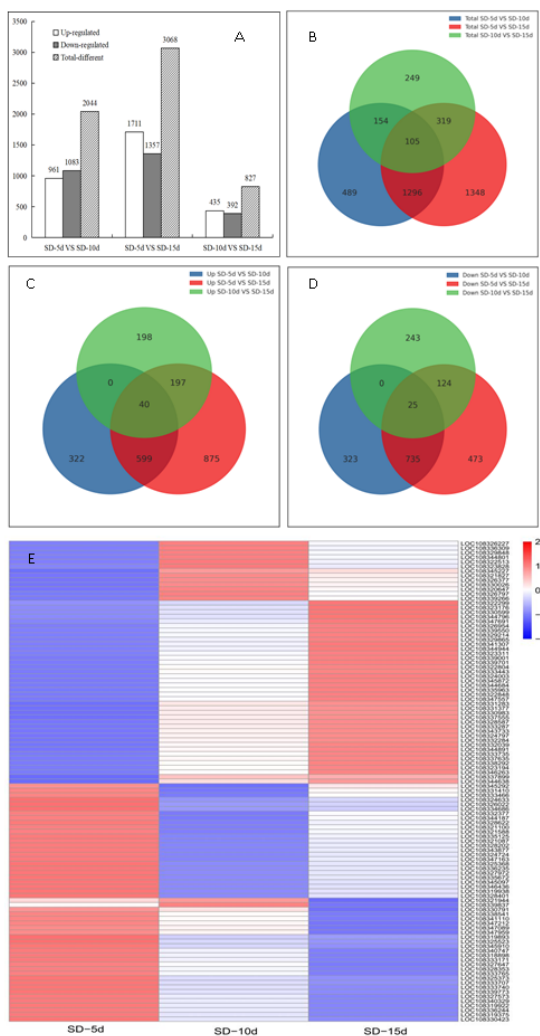


Figure 2

Analysis of effect on different genes number under different short-day induction

A Numbers of the significantly regulated genes under short-day induction treatment different days. The numbers on the horizontal axis reflect different treatment and the vertical axis represents numbers of total, up-and down-regulated genes, respectively. **BCD** Venn diagram analysis of significantly regulated genes under short-day induction treatment different days. **E** Heatmap clustering of global pattern of the strongly regulated genes conducted using Hierarchical Clustering (HCL) algorithm under short-day induction treatment different days. The color scale represents the values of lg FPKM (FPKM-Fragments Per Kilobase of transcript per Million fragments mapped).

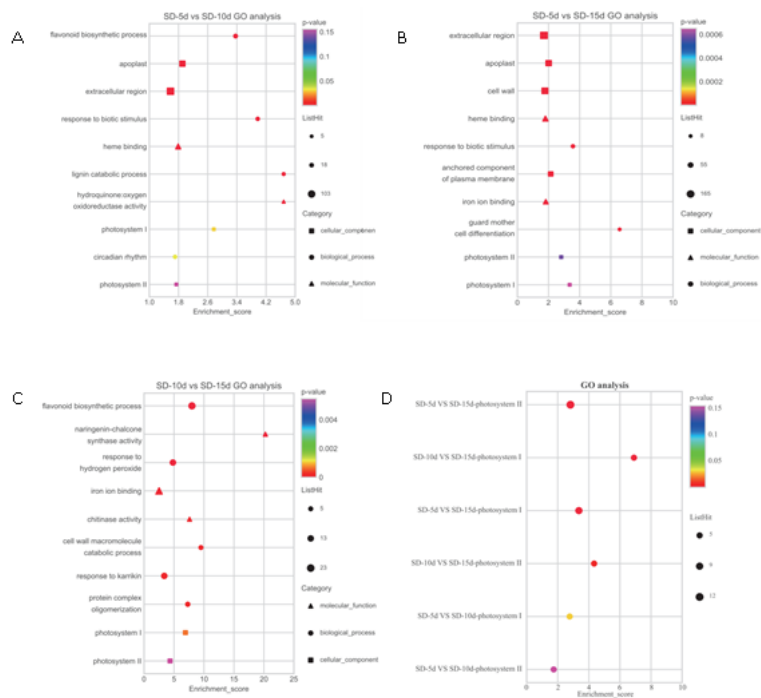


Figure 3

Results of the GO enrichment in three group comparing SD-5d vs SD-10d, SD-5d vs SD-15d, and SD-10d vs SD-15d

A SD-5d vs SD-10d GO analysis under short-day induction treatment different days. **B** SD-5d vs SD-15d GO analysis under short-day induction treatment different days. **C** SD-10d vs SD-15d GO analysis under short-day induction treatment different days. **D** The GO enrichment results are common to three comparison groups.

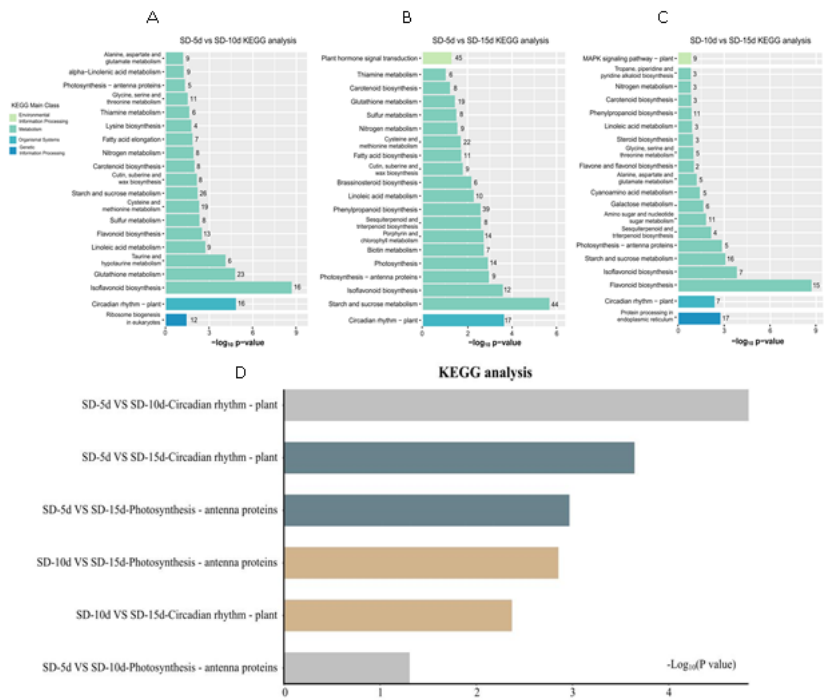


Figure 4

Results of the KEGG enrichment in three group comparing SD-5d vs SD-10d, SD-5d vs SD-15d, and SD-10d vs SD-15d

A SD-5d vs SD-10d KEGG analysis under short-day induction treatment different days. **B** SD-5d vs SD-15d KEGG analysis under short-day induction treatment different days. **C** SD-10d vs SD-15d KEGG analysis under short-day induction treatment different days. **D** The KEGG enrichment results are common to three comparison groups.

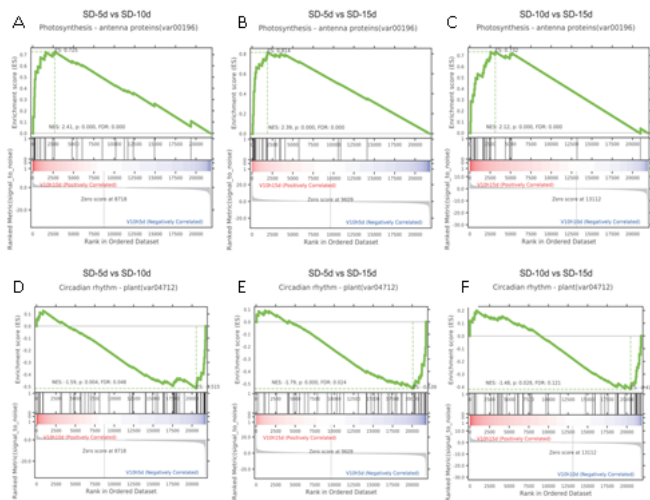


Figure 5

Gene set enrichment analysis of antenna proteins and circadian rhythm pathway

ABC Gene set enrichment analysis of antenna proteins in SD-5d vs SD-10d, SD-5d vs SD-15d and SD-10d vs SD-15d three comparison groups. **DEF** Gene set enrichment analysis of circadian rhythm pathway in SD-5d vs SD-10d, SD-5d vs SD-15d and SD-10d vs SD-15d three comparison groups.

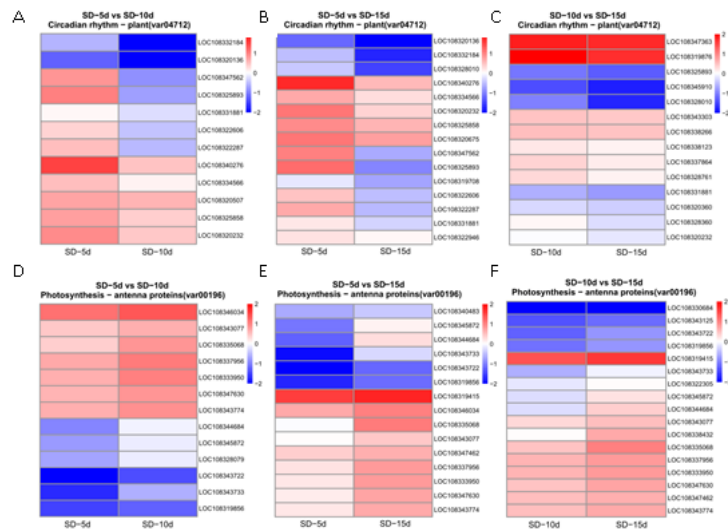


Figure 6

Heatmap cluster analysis of differential genes in antenna proteins and circadian rhythm metabolic pathways

ABC Enrichment analysis of circadian rhythm pathways in SD-5d vs SD-10d, SD-5d vs SD-15d and SD-10d vs SD-15d three comparison groups. **DEF** Enrichment analysis of antenna proteins pathways in SD-5d vs SD-10d, SD-5d vs SD-15d and SD-10d vs SD-15d three comparison groups.

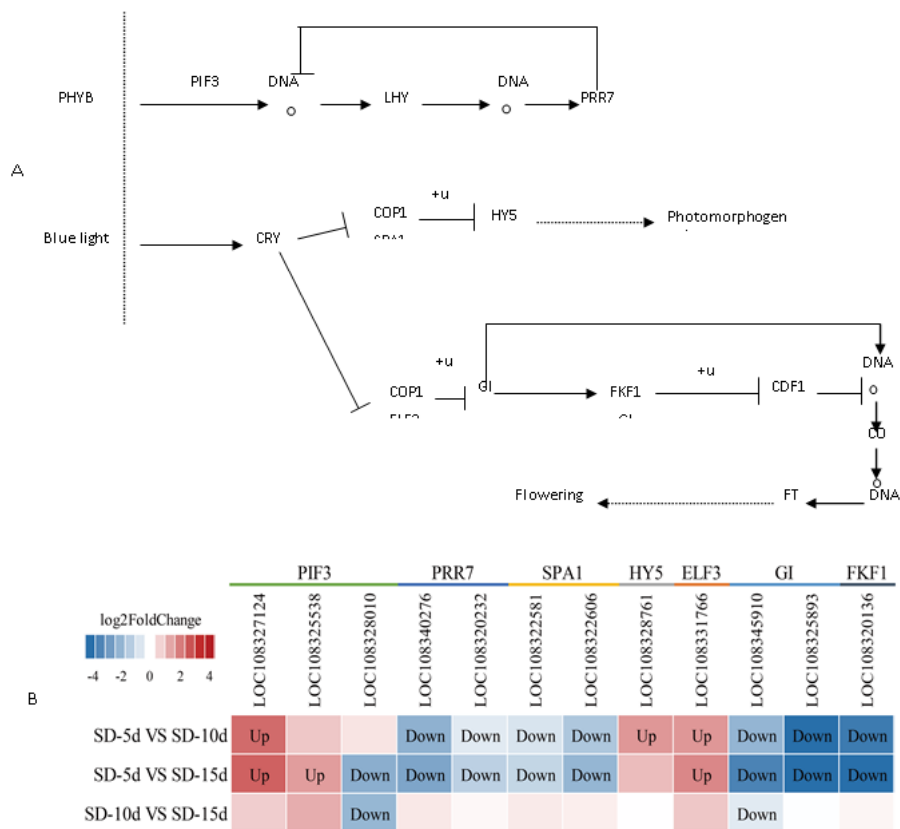


Figure 7

Circadian rhythm metabolic pathway and analysis of gene expression in this pathway

A Circadian rhythm metabolic pathway of adzuki bean. **B** Analysis of gene expression in SD-5d vs SD-10d, SD-5d vs SD-15d and SD-10d vs SD-15d three comparison groups of circadian rhythm metabolic pathway.

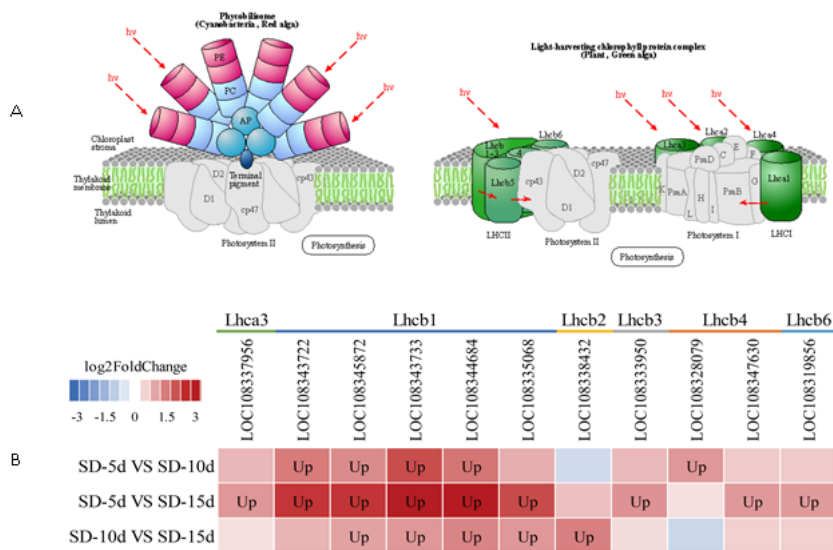


Figure 8

Antenna proteins metabolic pathway and analysis of gene expression in this pathway

A Antenna proteins metabolic pathway diagram of adzuki bean. **B** Analysis of gene expression in SD-5d vs SD-10d, SD-5d vs SD-15d and SD-10d vs SD-15d three comparison groups of antenna proteins metabolic pathway.

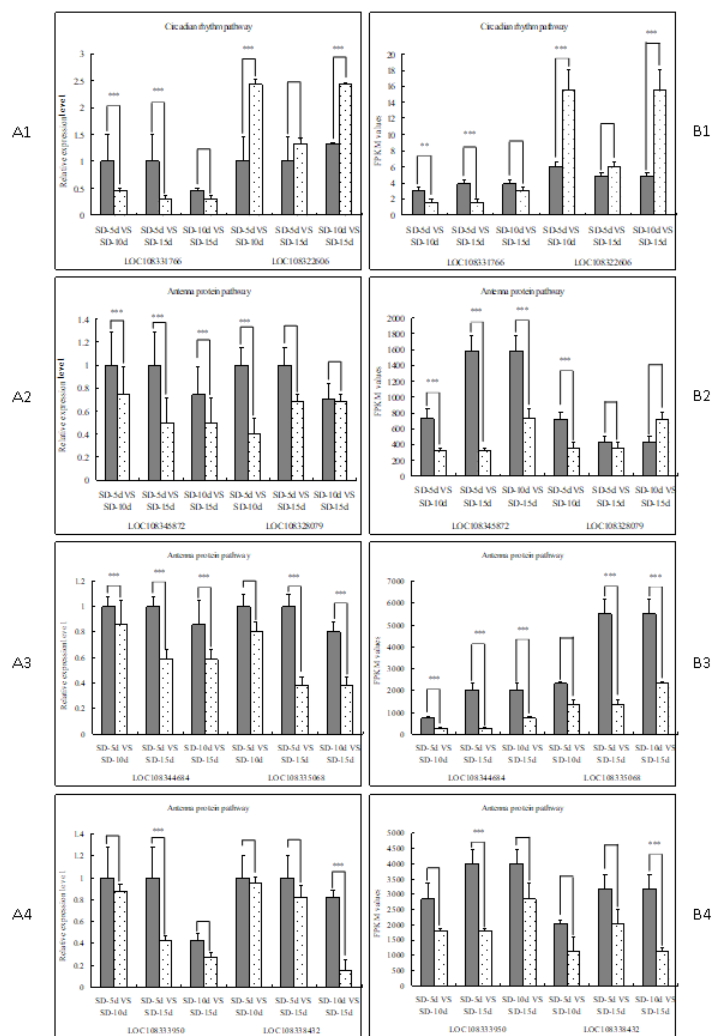


Figure 9

The qRT-PCR validation of 8 differentially related photoperiod expressed genes in circadian rhythm and antenna proteins pathways

*Indicates significant difference at 0.05 level, ** and *** indicate extremely significant difference at 0.01 and 0.001 level.

Supplementary Files

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- [File16figure.5AB.csv](#)
- [File17figure.5CD.csv](#)
- [File18figure.5EF.csv](#)
- [File19figure.6A.xlsx](#)
- [File20figure.6B.xlsx](#)
- [File21figure.6C.xlsx](#)
- [File22figure.6D.xlsx](#)
- [File23figure.6E.xlsx](#)
- [File24figure.6F.xlsx](#)
- [File25table2.xlsx](#)
- [File26figure.9.xlsx](#)
- [File27table3.xlsx](#)
- [File28table4.xlsx](#)
- [File29table5.xlsx](#)