Genetic and Environmental Regulation of Anthocyanin Biosynthesis in Winged Bean (Psophocarpus tetragonolobus L.)

D Jamshida
ICAR - Indian Institute of Agricultural Biotechnology
Devendra Kumar Singh
ICAR - Indian Institute of Agricultural Biotechnology
Danish Jawed
ICAR - Indian Institute of Agricultural Biotechnology
Chinta Sravani
ICAR - Indian Institute of Agricultural Biotechnology
Bidipta Ghosh
ICAR - Indian Institute of Agricultural Biotechnology
Sujit Kumar Bishi
ICAR - Indian Institute of Agricultural Biotechnology
Biplab Sarkar
ICAR - Indian Institute of Agricultural Biotechnology
Avinash Pandey
ICAR - Indian Institute of Agricultural Biotechnology
Sudhir Kumar
ICAR - Indian Institute of Agricultural Biotechnology
V. P. Bhadana
ICAR - Indian Institute of Agricultural Biotechnology
Binay K. Singh
ICAR - Indian Institute of Agricultural Biotechnology
Kishor U. Tribhuvan
kish.tribhuwan@gmail.com

ICAR - Indian Institute of Agricultural Biotechnology

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Abstract

The winged bean (*Psophocarpus tetragonolobus* L.) is a nutrient-rich yet underutilized legume with protein and oil contents comparable to those of soybean. Some genotypes exhibit distinct anthocyanin pigmentation in various plant parts, which helps the plant cope with biotic and abiotic stresses. These anthocyanin pigments have traditionally been used as natural food colorants and are also potential pharmaceutical ingredients, offering various health benefits to humans and animals. This study aimed to identify and quantify major anthocyanins in different tissues of winged bean, analyze their developmental and temperature-dependent accumulation patterns, identify candidate biosynthetic genes, and validate their expression under low-temperature conditions using RT-qPCR. Ultra-Performance Liquid Chromatography (UPLC) analysis revealed the presence of delphinidin-3-glucoside and cyanidin-3-glucoside as the two major anthocyanins in winged bean, with their contents increasing in leaves and pods—particularly in mature leaves and young pods—under declining temperature conditions. Candidate gene mining within QTL-mapped genomic regions identified two putative regulators of anthocyanin biosynthesis: PT04_g20719 (*AP2 ethylene-responsive transcription factor BBM2*) and PT04_g20862 (*gibberellin 3-beta-dioxygenase 4*). RT-qPCR analysis confirmed their differential expression, with significantly higher transcript levels in the high-anthocyanin-accumulating genotype WB Purple-3 compared to WB CR-2 in tissues harvested in November - a period characterized by cold climatic conditions. These findings suggest that anthocyanin biosynthesis in winged bean is strongly influenced by temperature and regulated by hormone signaling. This insight provides a valuable foundation for breeding programs aimed at enhancing anthocyanin accumulation in winged bean for increased industrial and nutraceutical value.

1. Introduction

Anthocyanins are a major class of water-soluble flavonoids responsible for the red, purple, and blue pigmentation in plant tissues. They primarily accumulate in vacuoles, where their hue is influenced by tissue pH (Glover & Martin, 2012). These pigments play essential roles in plant physiology, including fruit and flower pigmentation, pollinator attraction, and protection against environmental stresses (Wrolstad, 2004). Anthocyanins are classified based on variations in hydroxylation and methoxylation patterns. The major anthocyanidins include cyanidin, delphinidin, pelargonidin, peonidin, malvidin, and petunidin (Fang, 2015). Their biosynthesis follows the phenylpropanoid pathway, initiated by chalcone synthase, which catalyzes the condensation of malonyl-CoA and *p*-coumaroyl-CoA to form chalcone. Chalcone is then converted to naringenin by chalcone isomerase, followed by hydroxylation by flavanone 3-hydroxylase to produce dihydroflavonols. Subsequent modifications by flavonoid 3',5'-hydroxylase and anthocyanidin synthase lead to anthocyanin production (Deng et al., 2014). The expression of biosynthetic genes is regulated by transcription factors, including MYB, bHLH, and WD40, which form the MBW regulatory complex (Gonzalez et al., 2008). Environmental factors such as light, temperature, nutrient availability, and drought stress significantly influence anthocyanin accumulation (Spencer & Ksander, 1990). UV radiation enhances anthocyanin biosynthesis as a photoprotective mechanism, while cooler temperatures and drought stress promote anthocyanin accumulation for mitigating oxidative damage (An et al., 2020).

Anthocyanins function as antioxidants in plants, scavenging reactive oxygen species (ROS) generated under environmental stress conditions, thereby enhancing plant resilience (Gabrielska et al., 1999). They also provide significant health benefits to humans, exhibiting potent antioxidant, anti-inflammatory, and neuroprotective properties. Studies suggest their role in reducing the risk of cardiovascular diseases, diabetes, and certain cancers (Ereminas et al., 2017). Dietary anthocyanins, found in berries, red cabbage, and eggplants, contribute to vascular health, cognitive function, and gut microbiota regulation (Wang & Stoner, 2008). Additionally, specific anthocyanins, such as delphinidin-3-glucoside and cyanidin-3-glucoside, have shown promise in cancer immunotherapy by inhibiting immune checkpoints PD-1 and PD-L1 (Yang et al., 2012). Cyanidin-3-glucoside has also been reported to improve insulin sensitivity and reduce hyperglycaemia in diabetic mice via the downregulation of retinol-binding protein 4 expression (Sasaki et al.,

2007). Anthocyanins are widely used as natural food colorants, offering a viable alternative to synthetic dyes. Their ability to impart red, purple, and blue hues enhances the visual appeal and nutritional value of food products (Francis & Markakis, 1989). Their stability and solubility are influenced by glycosylation, where sugar moieties such as glucose, rhamnose, or galactose modify pigment properties (Pervaiz et al., 2017).

Winged bean (*P. tetragonolobus* L.) is a highly nutritious yet underutilized legume with protein (34.3–40.7%) and oil (16.4–21.3%) levels comparable to soybean (Hou et al., 2009). Some genotypes exhibit distinct anthocyanin pigmentation in the flowers, leaves, pods, and seeds. This study aimed to identify and quantify the major anthocyanins in different winged bean tissues and analyze their differential accumulation across developmental stages under progressively declining temperature conditions. Additionally, it sought to identify candidate genes associated with anthocyanin biosynthesis by leveraging QTL-mapped genomic regions and validate their expression through RT-qPCR using tissues harvested in November—a period characterized by cold climatic conditions—to elucidate the role of these genes in anthocyanin biosynthesis, particularly in response to low temperatures.

2. Materials and methods

2.1. Plant materials

We used two winged bean genotypes, WB CR-2 and WB Purple-3, which exhibit contrasting anthocyanin pigmentation patterns, for this study. WB Purple-3 is characterized by purple pigmentation in the flowers, leaves, pods, and seeds, whereas WB CR-2 is a non-pigmented genotype that lacks significant anthocyanin accumulation, producing white flowers, green leaves, green pods, and white seeds **(Fig. 1)**. These genotypes were originally collected from the Latehar district of Jharkhand, India. To ensure genetic homozygosity, the genotypes were cultivated under controlled field conditions over three consecutive cropping seasons (2021–2024) at the experimental farm of the ICAR-Indian Institute of Agricultural Biotechnology, Ranchi 834 003, Jharkhand, India.

2.2. Extraction of anthocyanins from winged bean tissues

In this study, we identified and quantified the major anthocyanins and analyzed their differential accumulation in various plant parts under different temperature regimes. To achieve this, total anthocyanins were extracted from various plant tissues, including 3-day-old flower buds, fully opened flowers, calyces, mature leaves at 30 days after emergence (DAE), and young pods at 5 days after anthesis (DAA). To examine the differential accumulation of anthocyanins under varying temperature conditions, leaf samples were collected at 5 and 30 DAE, and pod samples were harvested at 5 and 30 DAA at the end of October (28°C day/21°C night), November (22°C day/14°C night), and December (18°C day/8°C night). Total anthocyanins were extracted following a modified protocol based on the method described by Hosseinian et al. (2008). Briefly, approximately 1 g of lyophilized plant tissue was suspended in 10 mL of acidified 80% methanol (1% HCl) and vortexed vigorously for 2 minutes at room temperature. The suspension was then centrifuged at 10,000 rpm for 5 minutes at 25°C using a refrigerated centrifuge (5430 R, Eppendorf, Hamburg, Germany). The extraction process was repeated three times, and the pooled extracts were evaporated to dryness at 40°C. The resulting dried residue was reconstituted in 10 mL of acidified methanol and stored at -20°C until further analysis. Prior to analysis, the reconstituted samples were filtered through 0.22 µm syringe filters to remove particulates.

2.3. Identification and quantification of major classes of anthocyanins

The identification and quantification of major anthocyanins in winged bean tissues were carried out using Ultra-Performance Liquid Chromatography (UPLC) with a newly developed protocol, adapted from Ha et al. (2021) with necessary modifications. Anthocyanin standards, including delphinidin-3-glucoside chloride (CAS# 28500-00-7),

cyanidin-3-glucoside chloride (CAS# 7084-24-4), petunidin-3-glucoside chloride (CAS# 6988-81-4), peonidin-3-glucoside chloride (CAS# 6906-39-4), and pelargonidin-3-glucoside chloride (CAS# 18466-51-8) were procured from PhytoLab GmbH & Co. KG, Germany. The standards were dissolved in acidified methanol (1 N HCl, 85:15, v/v) to prepare stock solutions at a concentration of 1 mg/mL. UPLC-grade solvents, including methanol, acetonitrile, formic acid, and water, were obtained from Sigma-Aldrich, Darmstadt, Germany, and 0.22 µm syringe filters (SF9) were sourced from Himedia Pvt. Ltd., Mumbai, India. The UPLC analysis was performed using a Shimadzu Nexera X2 system (Kyoto, Japan) equipped with a degassing unit (DGU-20A5R), a quaternary pump (LC-30AD), an autosampler (SIL-30AC), a column oven (CTO-20AC), and a photodiode array detector (PDA, SPD-M20A) controlled by LabSolutions® software. Chromatographic separation was achieved on a Shim-pack GIST-HP AQ-C18 column (3.0 × 250 mm, 3 µm particle size). The column oven temperature was maintained at 50°C, with a flow rate of 0.6 mL/min, and the PDA detector was set at 520 nm for optimal detection of anthocyanins. The mobile phase consisted of solvent A (1% formic acid in water) and solvent B (0.5% formic acid in 50% acetonitrile). A gradient elution program was optimized to achieve complete separation of anthocyanins within 20 minutes. The injection volume for each sample was 5 µL. All samples were filtered through 0.22 µm syringe filters prior to analysis to ensure clarity and remove particulates.

2.4. Identification of candidate genes for anthocyanin biosynthesis from mapped QTL regions

Candidate genes involved in anthocyanin biosynthesis were identified using QTLs mapped for anthocyanin content and related traits, as reported by Chankaew et al. (2022). Flanking marker sequences of these QTLs were aligned to the winged bean genome assembly (BioProject: PRJNA1169650) using BLASTN to extract QTL-specific genomic regions. Genes within these regions were retrieved from the General Feature Format (GFF) file, and their functional annotations were verified through literature searches to identify those associated with anthocyanin biosynthesis. Selected candidate genes were validated using RT-qPCR expression analysis.

2.5. RT-qPCR-based validation of candidate genes for anthocyanin biosynthesis

The anthocyanin biosynthesis candidate genes were validated through RT-qPCR using RNA samples extracted from leaf and pod tissues of WB CR-2 (anthocyanin-deficient) and WB Purple-3 (anthocyanin-rich) winged bean genotypes, harvested in November—a period characterized by cold climatic conditions. Leaf tissues were harvested at 5-, 15-, and 30-days DAE, while pod tissues were collected at 5, 15, and 30 DAA. Gene-specific primers for the candidate genes were designed using the PrimerQuest™ Tool (Integrated DNA Technologies, IDT) and synthesized by Eurofins IT Solutions India Pvt Ltd., India. Total RNA was extracted in three biological replicates from each tissue type and time point using the RNeasy Plant Mini Kit (Qiagen, Germany). RNA quality was assessed by 1.2% agarose gel electrophoresis, and RNA concentrations were determined using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA). First-strand cDNA synthesis was performed with 1 µg of total RNA using the SuperScript™ III First-Strand Synthesis Kit (Invitrogen, USA). RT-qPCR was performed using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, USA) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The amplification protocol consisted of an initial denaturation step at 94°C for 3 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 20 seconds. Melting curve analysis was performed to confirm the specificity of amplification, starting at 56°C and increasing incrementally by 0.5°C until 95°C. The winged bean *Actin* gene was used as the internal reference for normalization. Relative gene expression levels were calculated using the 2^−ΔΔCt method (Chandna et al., 2012). Expression profiles of the candidate genes were compared across tissue types, developmental stages, and genotypes to validate their role in anthocyanin biosynthesis. Primer sequences used in the study are provided in Table 1.

Table 1
List of primers used for RT-qPCR expression analysis of candidate genes.

S. No.	Gene Id	Gene Name	Primer Id	Primer Sequence (5'-3')	Tm (ºC)	GC (%)	Amplicon length (bp)
1.	WB_Actin	*Actin* (reference)	WB_Actin (F)	CCGTTCTATCCCTGTATG	53.69	50.00	154
			WB_Actin (R)	GATCACGTCCTGCTAAG	52.77	52.94	
3.	PT04_g20719	*AP2-like ethylene-responsive transcription factor BBM2*	AP2ER (F)	CTCAAGAACACCCTTCTAGTC	57.87	48.00	129
			AP2ER (F)	GAGGAGTTGAGTCAGAAGTAAG	58.39	45.00	
5.	PT04_g20862	*Gibberellin 3-beta-dioxygenase 4*	G20 (F)	CACGGTCACTTGAGGATAAAC	57.87	48.00	134
			G20 (R)	CTCACTTGAAGCCCTCCTAT	57.30	50.00	

2.6. Statistical analysis

Statistical analysis of anthocyanin content across different tissue types and temperature regimes in the WB CR-2 and WB Purple-3 winged bean genotypes was performed using a three-way analysis of variance (ANOVA). This analysis assessed the effects of genotype, tissue type, and temperature on anthocyanin content. The analysis was conducted using DSAASTAT 1.1 (Onofri, Italy), an add-in program for Microsoft Office Excel. A significance level of $p < 0.05$ was considered to indicate statistically significant differences between groups. Appropriate data transformations were applied when necessary to meet the assumptions of normality and homogeneity of variance.

3. Results

3.1. Identification of major classes of anthocyanins in Winged bean

Ultra-Performance Liquid Chromatography (UPLC) analysis was performed to identify and quantify the anthocyanin content in 3-day-old flower buds, open flowers, calyxes, 30 DAE leaves, and 30 DAA pods of the WB Purple-3 winged bean genotype. The anthocyanins in the samples were identified by comparing their retention times to those of standard compounds: delphinidin-3-glucoside chloride, cyanidin-3-glucoside chloride, petunidin-3-glucoside chloride, peonidin-3-glucoside chloride, and pelargonidin-3-glucoside chloride. Further confirmation of identification was achieved by matching the UV absorption spectra of the sample peaks with the corresponding standards. Among the five anthocyanin classes evaluated, delphinidin-3-glucoside and cyanidin-3-glucoside were detected in winged bean tissues. The relative abundance of these two anthocyanins varied significantly across the different tissue types, as summarized in Table 2 and **Supplementary Fig. 1.**

Table 2
HPLC-based identification and quantification of major classes of anthocyanins in winged bean.

S. No.	Tissue	Anthocyanin				
		Delphinidin-3-glucoside (µg/g)	Cyanidin-3-glucoside (µg/g)	Petunidin-3-glucoside (µg/g)	Peonidin-3-glucoside (µg/g)	Pelargonidin-3-glucoside (µg/g)
1.	**Flower bud**	62.71 ± 1.33	44.48 ± 1.55	0.00	0.00	0.00
2.	**Flower**	91.38 ± 1.40	106.59 ± 1.13	0.00	0.00	0.00
3.	**Calyx**	20.54 ± 1.37	163.22 ± 3.65	0.00	0.00	0.00
4.	**Leaf**	28.00 ± 1.67	656.08 ± 29.03	0.00	0.00	0.00
5.	**Pod**	18.22 ± 0.71	260.89 ± 8.41	0.00	0.00	0.00

Values are mean of three replications ± SE; 0 is considered non-detected.

3.2. Low temperature induced differential accumulation of anthocyanins in various plant parts

The accumulation of anthocyanins in winged bean was analyzed under declining temperatures across different tissue types and developmental stages. The quantification of two major anthocyanins, delphinidin-3-glucoside and cyanidin-3-glucoside, was performed in leaves (5 and 30 DAE) and pods (5 and 30 DAA) from the winged bean genotype WB Purple-3. Samples were harvested at the end of October, November, and December to assess the influence of declining temperatures on anthocyanin biosynthesis. The anthocyanin content (µg/g dry weight) in leaf and pod tissues at various developmental stages and temperature regimes is detailed in Table 3. In October, anthocyanin accumulation was negligible in both young and mature leaves. However, in November, a significant increase in anthocyanin content was observed, particularly in mature leaves. The concentration of delphinidin-3-glucoside in mature leaves reached 27.79 ± 1.46 µg/g dry weight, whereas young leaves contained only 2.04 ± 0.11 µg/g dry weight. Similarly, cyanidin-3-glucoside accumulated to a greater extent in mature leaves (642.39 ± 8.96 µg/g dry weight) compared to young leaves (6.30 ± 0.34 µg/g dry weight). In December, the concentration of delphinidin-3-glucoside further increased in both young and mature leaves, with young leaves containing 9.77 ± 0.05 µg/g dry weight and mature leaves accumulating 80.55 ± 0.33 µg/g dry weight. A similar trend was observed for cyanidin-3-glucoside in young leaves, where its concentration reached 80.55 ± 0.33 µg/g dry weight. However, in mature leaves, cyanidin-3-glucoside concentration declined to 347.18 ± 6.14 µg/g dry weight compared to November levels. A different trend was observed in pods, where anthocyanin accumulation was negligible in October except for a minimal concentration of delphinidin-3-glucoside in young (0.23 ± 0.01 µg/g dry weight) and mature (1.998 ± 0.24 µg/g dry weight) pods. Unlike leaves, where mature tissues accumulated higher anthocyanin levels, young pods exhibited significantly greater anthocyanin concentrations compared to mature pods in both November and December. In November, delphinidin-3-glucoside and cyanidin-3-glucoside concentrations in young pods were 18.22 ± 0.41 µg/g dry weight and 260.89 ± 0.26 µg/g dry weight, respectively, whereas mature pods contained 13.18 ± 0.56 µg/g dry weight and 179.78 ± 10.29 µg/g dry weight. In December, the concentration of anthocyanins in young pods further increased, with delphinidin-3-glucoside reaching 34.13 ± 0.26 µg/g dry weight and cyanidin-3-glucoside reaching 300.45 ± 34.76 µg/g dry weight. Conversely, mature pods showed a slight decline in anthocyanin accumulation, with delphinidin-3-glucoside decreasing to 11.49 ± 0.60 µg/g dry weight and cyanidin-3-glucoside to 156.46 ± 10.52 µg/g dry weight.

Table 3
Anthocyanin content (µg/g dry weight) in leaf and pod tissues of winged bean genotype WB Purple-3 at various developmental stages and temperature regimes.

		Delphinidin-3-glucoside		Cyanidin-3-glucoside	
Tissue	**Month**	**Mature**	**Young**	**Mature**	**Young**
Leaf	Oct	0.00	0.00	0.00	0.00
	Nov	27.79 ± 1.46	2.04 ± 0.11	642.39 ± 8.96	6.30 ± 0.34
	Dec	80.55 ± 0.33	9.77 ± 0.05	347.18 ± 6.14	80.55 ± 0.33
Pod	Oct	1.998 ± 0.24	0.23 ± 0.01	0.00	0.00
	Nov	13.18 ± 0.56	18.22 ± 0.41	179.78 ± 10.29	260.89 ± 0.26
	Dec	11.49 ± 0.60	34.13 ± 0.26	156.46 ± 10.52	300.45 ± 34.76

Young leaf- Leaf 5 days after anthesis (DAE); **Mature leaf**- Leaf 30 days after emergence (DAE); **Young Pod**: Pod 5 DAA; **Mature Pod**: Pod 30 DAA; Values are mean of three replications ± SE; 0 is considered non-detected.

3.3. Mining candidate genes regulating anthocyanin accumulation in winged bean

We aligned the sequences of the markers flanking the QTLs *qAntho1.1a*, *qAntho5.1*, and *qAntho1.1b*, which are associated with anthocyanin accumulation in winged bean (Chankaew et al., 2022), to the winged bean reference genome using BLASTN. The QTL *qAntho1.1a* is flanked by the markers 90362_173 and 86339_105, *qAntho5.1* by 129210_17 and 74111_70, and *qAntho1.1b* by 59413_160 and 49233_195. The sequence information for these flanking markers is provided in **Supplementary Table S1**. This analysis positioned *qAntho1.1a* on chromosome 4 between 7,303,963 bp and 7,502,244 bp, *qAntho5.1* on chromosome 7 between 37,324,470 bp and 37,523,869 bp, and *qAntho1.1b* on chromosome 4 between 9,041,435 bp and 9,104,792 bp **(Supplementary Table S2)**. The total sequence coverage for these loci was 198 kbp for *qAntho1.1a*, 199 kbp for *qAntho5.1*, and 63 kbp for *qAntho1.1b* **(Supplementary Table S3)**. Sequence analysis within the identified QTL regions led to the identification of 39 genes, with 20 genes residing in *qAntho1.1a*, 13 in *qAntho5.1*, and six in *qAntho1.1b* **(Supplementary Table S4)**. Functional annotation and literature review facilitated the identification of two potential candidate genes regulating anthocyanin biosynthesis. These candidate genes include *PT04_g20719*, encoding an AP2 ethylene-responsive transcription factor BBM2 and *PT04_g20862*, encoding gibberellin 3-beta-dioxygenase 4 **(**Table 1**)**.

3.4. Functional validation of candidate genes for anthocyanin biosynthesis through RT-qPCR

To validate the involvement of candidate genes in anthocyanin biosynthesis, RT-qPCR expression analysis was conducted on *PT04_g20719* and *PT04_g20862* using leaf and pod tissues from winged bean genotypes, WB Purple-3 and WB CR-2, harvested at the end of November. The expression levels were assessed in 5, 15, and 30 DAE leaves and 5, 15, and 30 DAA pods, with 5 DAE leaves of WB CR-2 serving as the reference for relative gene expression quantification. The expression analysis revealed distinct differential gene expression patterns between the two genotypes. *PT04_g20719* exhibited minimal expression in WB CR-2, with relative expression levels of 1.4-, 1.0-, and 1.0-fold at 5, 15, and 30 DAE in leaves, and 2.0-, 2.8-, and 2.8-fold at 5, 15, and 30 DAA in pods. In contrast, WB Purple-3 demonstrated significantly elevated expression levels of *PT04_g20719*, with 11.3-, 6.9-, and 6.6-fold upregulation at 5, 15, and 30 DAE in leaves, and 6.64-, 7.2-, and 13.8-fold upregulation at 5, 15, and 30 DAA in pods (**Fig. 2a**). *PT04_g20862* followed a similar expression pattern. In WB CR-2, its expression remained low, with a minimal upregulation of 1.63-, 0.79-, and 1.0-fold at 5, 15, and 30 DAE in leaves, and 1.27-, 1.19-, and 2.04-fold at 5, 15, and 30 DAA in pods. However, in WB Purple-3,

PT04_g20862 expression was upregulated by 4.59-, 2.54-, and 2.50-fold at 5, 15, and 30 DAE in leaves, and 6.60-, 5.64-, and 7.88-fold at 5, 15, and 30 DAA in pods (**Fig. 2b**).

4. Discussion

Winged bean, a nutritionally rich legume, is a valuable source of bioactive compounds, particularly anthocyanins, which contribute to its characteristic purple pigmentation. Anthocyanins, a subclass of flavonoids, play a crucial role in human health by mitigating oxidative stress and inflammation, thereby reducing the risk of chronic diseases such as cancer, cardiovascular disorders, and diabetes (Esan et al., 2020). The biosynthesis and accumulation of anthocyanins in plants are influenced by environmental factors, including temperature fluctuations and light intensity (Lu et al., 2024). However, such information is limited for winged bean. Therefore, precise quantification of anthocyanin levels and identification of regulatory genes associated with their synthesis under varying environmental conditions are essential for understanding their regulation and optimizing their accumulation in winged bean.

4.1. Major classes of anthocyanins in winged bean and their differential accumulation under low temperature

The present study identified delphinidin-3-glucoside and cyanidin-3-glucoside as the two major anthocyanins present in winged bean and analyzed their accumulation across different tissue types (young and mature leaves, and pods) harvested at the end of October, November, and December to assess the influence of declining temperatures on their biosynthesis. The observed increase in anthocyanin accumulation in mature leaves under declining temperatures (particularly in November and December) aligns with previous studies that have shown cold stress to induce anthocyanin biosynthesis in a variety of plant species (Catala, 2011). The progressive increase in delphinidin-3-glucoside in mature leaves from November to December, alongside stable levels of cyanidin-3-glucoside, suggests that the biosynthesis of delphinidin-3-glucoside may be more strongly induced by cold stress. However, the observed decrease in cyanidin-3-glucoside in mature leaves during the same period may reflect metabolic regulation, including degradation or redistribution processes influenced by developmental cues or the catabolism of anthocyanins under prolonged cold exposure. In contrast to mature leaves, young leaves exhibited negligible anthocyanin accumulation in October, with a significant increase in November. Although the levels of both anthocyanins were lower than those in mature leaves, these findings suggest that young tissues may be less responsive to declining temperatures. This differential response could be due to the prioritization of other physiological processes over secondary metabolite production in young tissues. Pod tissues demonstrated a distinct pattern of anthocyanin accumulation. In October, anthocyanin levels were minimal, with only a slight presence of delphinidin-3-glucoside in both young and mature pods, suggesting that anthocyanin biosynthesis is not actively occurring in pods during early development. However, by November, the anthocyanin content in young pods surpassed that of mature pods, contrary to the trend observed in leaves. In December, young pods continued to accumulate anthocyanins, while mature pods exhibited a slight decline in both anthocyanins. This age-dependent discrepancy suggests that younger pods may be more responsive to cold-induced anthocyanin biosynthesis than mature pods, possibly due to their higher metabolic activity or distinct regulatory mechanisms. The greater accumulation of anthocyanins in young pods, particularly cyanidin-3-glucoside, may reflect a developmental strategy where younger tissues accumulate protective compounds in response to environmental stress, such as temperature fluctuations.

Overall, the data presented in this study indicate that anthocyanin accumulation in winged bean is strongly influenced by both temperature and developmental cues. The higher anthocyanin content in mature leaves and young pods compared to young leaves and mature pods aligns with the fact that mature leaves and young pods are more photosynthetically active (Garrido et al., 2023). As a result, they are likely to produce more reactive oxygen species (ROS), necessitating a higher accumulation of anthocyanins for detoxification. These findings suggest that anthocyanin metabolism in winged

bean is dynamically regulated, with temperature and developmental factors influencing its biosynthesis and accumulation across different tissues. This dynamic regulation warrants further investigation into the molecular mechanisms underlying these responses, particularly regarding the degradation or redistribution of anthocyanins in mature tissues under prolonged cold exposure. The temperature-induced changes in anthocyanin accumulation observed in this study are consistent with findings from other plant species (Gaiotti et al., 2018) and corroborate our findings and further reinforce the role of temperature in regulating anthocyanin biosynthesis across diverse plant species.

4.2. Regulatory roles of candidate genes in anthocyanin biosynthesis

For the identification of candidate genes potentially involved in anthocyanin biosynthesis in winged bean, we utilized QTL mapping information from Chankaew et al. (2022) and the high-quality reference genome of winged bean developed by ICAR - Indian Institute of Agricultural Biotechnology (IIAB), Ranchi 834 003, Jharkhand, India (BioProject: PRJNA1169650). Anthocyanin QTL markers were mapped onto the winged bean reference genome to determine their genomic locations and associated genes. A total of 39 genes related to anthocyanin biosynthesis were identified within the three mapped QTLs. After annotation and a literature survey, we identified two putative candidate genes, *PT04_g20719* (AP2 ethylene-responsive transcription factor BBM2) and *PT04_g20862* (Gibberellin 3-beta-dioxygenase 4), both of which have been implicated in regulating anthocyanin production in various plant species (Sun et al., 2021). RT-qPCR-based expression analysis revealed that both *PT04_g20719* and *PT04_g20862* exhibited distinct expression patterns in colourless (WB CR-2) and purple (WB Purple-3) genotypes across leaf tissues at 5-, 15-, and 30-days DAE and pod tissues at 5, 15, and 30 DAA, harvested at the end of November. The observed expression patterns suggest that these genes may be associated with anthocyanin biosynthesis in winged bean, particularly in response to cold stress.

4.2.1. AP2 ethylene-responsive transcription factor BBM2

BABY BOOM 2 (BBM2), a member of the AP2 ethylene-responsive transcription factor (ERF) family, is primarily known for its role in embryogenesis and cell proliferation. It has also been implicated to ethylene-mediated regulation of flavonoid biosynthesis, including anthocyanins. AP2/ERF transcription factors play a crucial role in regulating ethylene biosynthesis by binding to cis-regulatory elements, such as the GCC-box, in the promoters of ethylene biosynthetic genes (Wang et al., 2023).

Ethylene is synthesized through the sequential enzymatic actions of 1-aminocyclopropane-1-carboxylate synthase and 1-aminocyclopropane-1-carboxylate oxidase, converting S-adenosyl methionine into 1-Aminocyclopropane-1-carboxylic acid and subsequently into ethylene. The ethylene binds to ethylene receptors (ETRs), leading to the inactivation of the negative regulator CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) and the activation of ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE 1 (EIL1) transcription factors. These EIN3/EIL1 proteins, in turn, induce the expression of AP2/ERF transcription factors, which further modulate ethylene biosynthesis (Shin et al., 2023).

EIN3/EIL1 suppress sucrose-induced MYB activators, thereby reducing the expression of chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, dihydroflavonol 4-reductase, and anthocyanidin synthase, leading to decreased anthocyanin accumulation (Ni et al., 2023). However, the role of ethylene in anthocyanin regulation varies across species and environmental conditions. In *Arabidopsis*, ERF4 and ERF8 mutations reduce anthocyanin accumulation under high light stress, suggesting a context-dependent positive role of ethylene (Shin et al., 2023). Contrarily, in apple (*Malus domestica*), MdEIL1 directly activates MdMYB1, promoting anthocyanin biosynthesis (Wang et al., 2022). These contrasting findings highlight the complexity of ethylene-mediated anthocyanin regulation, where ethylene signalling can either suppress or enhance anthocyanin biosynthesis depending on species-specific transcriptional networks and environmental factors.

4.2.2. Gibberellin 3-beta-dioxygenase 4

Gibberellic acids (GAs) are diterpenoid plant hormones that regulate key physiological processes throughout the plant life cycle. They are synthesized in three distinct stages: (1) Geranylgeranyl diphosphate undergoes sequential enzymatic conversion by ent-copalyl diphosphate synthase and ent-kaurene synthase to form ent-kaurene, (2) ent-kaurene is oxidized to ent-kaurenoic acid by ent-kaurene oxidase, which is subsequently converted to GA_{12} via ent-kaurenoic acid oxidase, and (3) bioactive GAs, such as GA_1, GA_3, GA_4, and GA_7, are formed from GA_{12} through a process catalyzed by gibberellin 20-oxidase (GA20ox) and gibberellin 3-oxidase (GA3ox), with GA3ox1 and GA3ox4 playing crucial roles in converting GA_9 and GA_{20} into GA_4 and GA_1, respectively (Yamaguchi, 2008).

Studies on *ga3ox1* mutants show increased expression of anthocyanin-related genes, including the key transcriptional regulators *PAP1* and *PAP2*. Conversely, *AACT*, an enzyme involved in anthocyanin acetylation, exhibits reduced expression in these mutants. These findings suggest that GA3ox1 negatively regulates anthocyanin accumulation, possibly through GA-mediated DELLA degradation via the ubiquitin-proteasome pathway. Since DELLA proteins enhance the transcriptional activation of anthocyanin biosynthetic genes by interacting with *PAP1* and *PAP2*, their degradation suppresses anthocyanin production. Under environmental stresses, such as low temperature, DELLA proteins are stabilized, promoting anthocyanin biosynthesis. However, GA3ox1 counteracts this effect by facilitating DELLA degradation (Zhang et al., 2017).

Overall, both candidate genes appear to negatively regulate anthocyanin biosynthesis, particularly under cold stress conditions. This is consistent with our RT-qPCR-based expression analysis conducted on young and mature leaf and pod tissues harvested at the end of November, a period characterized by low temperatures at the experimental site. The observed expression patterns suggest a potential role for these genes in modulating anthocyanin accumulation in response to cold stress, highlighting their involvement in the complex regulatory network governing flavonoid biosynthesis in winged bean.

5. Conclusion

In this study, two anthocyanins, delphinidin-3-glucoside and cyanidin-3-glucoside, were identified in winged bean, with their accumulation being temperature-dependent, increasing under lower temperature conditions. Through the dissection of mapped QTLs, two candidate genes, PT04_g20719 (AP2 ethylene-responsive transcription factor BBM2) and PT04_g20862 (Gibberellin 3-beta-dioxygenase 4), were identified as potential regulators of anthocyanin biosynthesis under cold stress. RT-qPCR-based expression analysis demonstrated a strong correlation between the expression of these genes and anthocyanin accumulation, particularly in the pigmented WB Purple-3 genotype, where their expression was significantly higher compared to the colourless WB CR-2 genotype. These findings suggest that PT04_g20719 and PT04_g20862 play key roles in modulating anthocyanin biosynthesis in response to cold stress, contributing to the pigmentation observed in winged bean.

Declarations

Availability of data and material

The data used in the present study are available at NCBI under BioProject accession number PRJNA1169650 (will be release on October 02, 2025, data link will be available after the release date). The sequences of the genes identified in this study are provided in the supplementary material.

Permission to collect plant/ Plant parts

ICAR – IIAB, Ranchi is a research institute with the mandate to explore and collect novel germplasm of field crops available in the local region.

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Conflicts of interest/Competing interests

The authors of this manuscript have no conflicts/competing interests.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for Publication

All the authors read and approved the manuscript for publication in Journal of Food Science and Technology.

Code availability

Not Applicable

Authors' contributions

JD, KUT, and BKS designed the experiments and wrote the manuscript. JD, DKS, and BG performed the experiment. DJ and CS performed UPLC, SKB, and BS data analysis and wrote the manuscript. AP and SK performed data analysis, and VP and SK Coordinated the project and edited the manuscript. All the authors read and approved the manuscript.

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Figures

Figure 1

Different stages of leaf and pod development in winged bean genotypes WB Purple-3 and WB CR-2. **(a-c)** Leaf development at 5, 15, and 30 days after emergence (DAE) in WB Purple-3; **(d-f)** Pod development at 5, 15, and 30 days after anthesis (DAA) in WB Purple-3; **(g-i)** Leaf development at 5, 15, and 30 DAE in WB CR-2; **(j-l)** Pod development at 5, 15, and 30 DAA in WB CR-2.

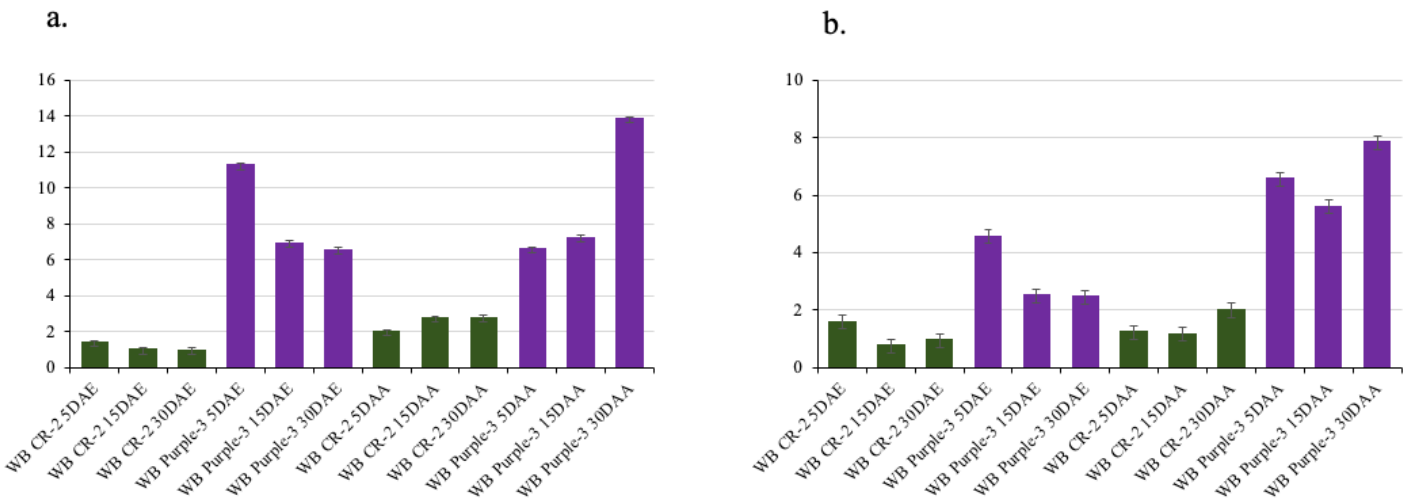

Figure 2

RT-qPCR-based expression analysis of putative anthocyanin biosynthesis candidate genes in leaf and pod tissues of winged bean genotypes WR CR-2 and WB Purple-3. **(a)** Expression profile of *PT04_g20719*(AP2 ethylene-responsive transcription factor BBM2); **(b)** Expression profile of *PT04_g20862*(Gibberellin 3-beta-dioxygenase 4). **DAE**: Days after emergence; **DAA**: Days after anthesis.

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