



OPEN Three bilberry bHLHs of subgroup III_f play divergent roles in fruit anthocyanin and flavonoid biosynthesis

Katja Karppinen¹✉, Lauri Raami^{1,2}, Hilary Edema¹, Muhammad Furqan Ashraf¹, Heikki M. Salo², Richard V. Espley³ & Laura Jaakola^{1,4}

Bilberry (*Vaccinium myrtillus* L.) fruit are one of the best natural sources of anthocyanins. Anthocyanin and flavonoid biosynthesis are transcriptionally regulated by the conserved MBW complex, including R2R3 MYB, basic helix-loop-helix (bHLH) and WD40 proteins. In this study, homology-search in the bilberry genome identified three putative anthocyanin biosynthesis-regulating *bHLH* genes, all containing a MYB binding domain, indicating their possible interaction with R2R3 MYBs. Phylogenetic analysis assigned the *VmbHLH*s to three different clusters within the III_f subgroup of bHLHs. The expression of *VmbHLH2* elevated at berry ripening, while *VmbHLH1* and *VmbHLH3* showed decreasing expression towards berry maturation. When transiently overexpressed together with *MdMYB10* in *Nicotiana benthamiana* leaves, *VmbHLH2* and *VmbHLH3* were able to complement *MdbHLH3* and upregulate a full set of anthocyanin biosynthetic genes leading to anthocyanin accumulation. In contrast, *VmbHLH1* induced a reduced set of flavonoid pathway genes. Our results provide the first functional characterization of flavonoid-regulating bHLHs that contribute to the MBW complex in the economically important genus *Vaccinium*. We propose that the TT8-type *VmbHLH2* is involved in anthocyanin biosynthesis in ripening bilberry fruit, while the MYC1-type *VmbHLH1* and GL3/EGL3-type *VmbHLH3* are associated with anthocyanin and PA biosynthesis, mainly in tissues other than berries.

Keywords Anthocyanin biosynthesis, Basic helix-loop-helix transcription factor, Berry ripening, MBW regulatory complex, *Vaccinium myrtillus*

Basic helix-loop-helix (bHLH) transcription factors (TFs) comprise a large gene family in plants, with over 160 members identified in *Arabidopsis* and more than 180 in rice¹. As the second largest TF family in plants, bHLHs are involved in many growth and developmental processes, including regulation of hormone signaling, responses to biotic and abiotic stresses and regulation of flavonoid and anthocyanin biosynthesis². The bHLH proteins can activate or repress gene expression through binding DNA promoter regions, in the form of bHLH homo- or heterodimers, or in a complex associated with proteins from other TF families, such as MYBs³.

Anthocyanins contribute to the red and blue colors in many flowers and ripe fruits, such as berries, facilitating pollination and seed dispersal⁴. Many fruits and berries are recognized as rich sources of anthocyanins and are associated with various health benefits^{5–8}. Anthocyanins are produced from the well-elucidated flavonoid biosynthetic pathway which consists of several consecutive enzymatic steps leading to different flavonoid classes, namely anthocyanins, proanthocyanidins (PAs) and flavonols⁹. The transcription of flavonoid biosynthetic structural genes encoding these enzymes is controlled by the conserved MBW regulatory complex, consisting of R2R3 MYB, bHLH and WD40 proteins^{10–12}. In this complex, the bHLH physically interacts with a MYB partner to enable activation or repression of target genes. The expression pattern and the DNA-binding specificity of MYBs and bHLHs determine the activation of flavonoid pathway target genes in plants^{13,14}. Flavonoid regulation is considered to be the most ancient function of the MBW complex, pre-dating MBW roles in development of trichomes and root hairs and production of seed coat mucilage, which evolved after gene duplication and subsequent diversification¹⁰.

¹Department of Arctic and Marine Biology, UiT The Arctic University of Norway, Tromsø, Norway. ²Ecology and Genetics Research Unit, University of Oulu, Oulu, Finland. ³Plant and Food Research Group, Bioeconomy Science Institute, Auckland, New Zealand. ⁴Division of Food Production and Society, Norwegian Institute of Bioeconomy Research (NIBIO), Ås, Norway. ✉email: katja.karppinen@uit.no

Within the bHLH family, several members of subgroup IIIf have been associated with anthocyanin and flavonoid biosynthesis. In most species, more than one bHLH is generally found to operate within MBW complexes to regulate anthocyanin and PA biosynthesis^{3,15}. For example, in Arabidopsis, four members of the subgroup IIIf bHLHs, transparent testa 8 (TT8; AtbHLH042), AtMYC1 (AtbHLH012), glabra3 (GL3; AtbHLH001) and enhancer of glabra3 (EGL3; AtbHLH002), have all been associated with the control of anthocyanin and PA biosynthesis, in a partially redundant manner^{3,16,17}. In *Petunia* (*Petunia hybrida*), two well-characterized bHLHs, PhAN1 and PhJAF13, regulate anthocyanin biosynthesis in floral organs together with the R2R3 MYB partner, PhAN2, although PhAN1 also assists in controlling vacuolar pH with another R2R3 MYB, PhPH4^{18,19}. Similarly, two divergent bHLHs in snapdragon (*Antirrhinum majus*), AmDELILA and AmIncolorata I/Mutabilis, play partly overlapping roles with AmROSEA1 in anthocyanin-derived flower color^{20,21}.

Several members of subgroup IIIf bHLHs have also been identified in fruit and berry flavonoid biosynthesis. Two bHLHs exist in grape (*Vitis vinifera*), of which VvMYCA1 interacts with VvMYBA2 to induce anthocyanin biosynthesis, while VvMYC1 interacts with several R2R3 MYBs, to control both anthocyanin and PA biosynthesis in berry skin^{22,23}. Two homologous bHLH proteins in apple (*Malus domestica*), MdbHLH3 and MdbHLH33, regulate anthocyanin biosynthesis together with MdMYB10, allelic to MdMYB1, in the MBW complex^{24–26}. MdbHLH3 binds the promoters of anthocyanin biosynthesis genes *MdDFR* and *MdUGT* to regulate their expression²⁷. Two bHLH proteins also exist in Chinese bayberry (*Morella rubra*), of which MrbHLH1, but not MrbHLH2, is an essential partner for MrMYB1 in berry anthocyanin production²⁸. In black goji berry (*Lycium ruthenicum* Murray), either LrAN1b or LrJAF13 form MBW complex with R2R3 MYB and WD40 protein in regulating anthocyanin biosynthesis²⁹. In white goji berry variant, a deletion in the *LrAN1b* promoter region abolishes expression of the *LrAN1b*, leading to the absence of pigmentation²⁹. In woodland strawberry (*Fragaria vesca*), three distinct bHLHs have been demonstrated to interact with several R2R3 MYBs in regulating expression of flavonoid structural genes³⁰. The orchestration of these key factors in coordinating different branches of flavonoid biosynthesis is not completely understood across all fruit species, including berries which often show more complex anthocyanin and PA profiles.

To deepen our understanding of the regulatory role of MBW-associated bHLHs, we focused on the identification of anthocyanin-regulating bHLHs in bilberry (*Vaccinium myrtillus* L.), which has active flavonoid metabolism over the course of fruit development and ripening. While astringent PAs mainly accumulate in bilberry fruit in the early stages of development, anthocyanins accumulate at high concentrations in ripe fruit^{31–33} and make bilberry one of the best sources of anthocyanins. In bilberry, the ripening-related anthocyanin biosynthesis is positively regulated by the phytohormone abscisic acid (ABA)^{34,35}. ABA has a well-known regulatory role in promoting fleshy fruit maturation through many ripening-related processes, including accumulation of anthocyanin pigments by upregulating the expression of key biosynthetic genes and TFs regulating them³⁶. Fleshy fruit ripening is a tightly regulated developmental process. Especially in non-climacteric fruit, but also in climacteric fruit, ABA is acting as a ripening inducer by interacting with other plant hormones, including ethylene, auxin, gibberellic acid and brassinosteroids^{37,38}.

Recently, we provided a comprehensive report of bilberry flavonoid-related R2R3 MYBs, showing that VmMYBA1, VmMYBPA1.1, and VmMYBPA2.2 co-regulate ripening-related anthocyanin biosynthesis in bilberry fruit³⁹. However, the bHLHs associated with anthocyanin biosynthesis as part of the MBW complex have not yet been verified in bilberry, or the genus *Vaccinium*, which contains commercially important species, such as highbush blueberry (*V. corymbosum*) and American cranberry (*V. macrocarpon*). In highbush blueberry, over 50 bHLH family members have been identified^{40,41}. Gene expression patterns indicate that some of these bHLHs could be associated with flavonoid biosynthesis^{42–46}. However, the functional characterization of bHLH regulators within the MBW complex is still lacking in the genus *Vaccinium*. Since bHLHs are integral parts of the MBW complexes and protein-protein interactions with bHLHs are known to affect the DNA-binding properties of the R2R3 MYB components³⁰, identification of bHLH partners is important for further elucidation of the flavonoid and anthocyanin biosynthesis regulation in genus *Vaccinium*.

In this study, three potential anthocyanin-regulating bHLH genes were identified in the bilberry genome of the Norwegian ecotype⁴⁷. Their role in anthocyanin biosynthesis was studied by gene expression analysis in bilberry, after ABA treatment of berries, and by overexpression analyses in *Nicotiana benthamiana* leaves. VmbHLH2 and VmbHLH3 were found to regulate the complete anthocyanin biosynthesis pathway leading to anthocyanin accumulation. Further, VmbHLH1 induced specific genes in the flavonoid pathway. Our results suggest that these three bHLHs act as a part of the flavonoid-regulating MBW complex and exhibit divergent and partially overlapping roles in anthocyanin biosynthesis in bilberry tissues.

Results

Bilberry subgroup IIIf bHLH genes

To study the regulation of anthocyanin biosynthesis in bilberry by bHLH TFs, three putative anthocyanin-regulating bHLHs were identified in the bilberry genome based on sequence homology with Arabidopsis subgroup IIIf bHLH members (Table 1). When the three genes were cloned and fully sequenced, they showed some size variation: *VmbHLH1* was 1923 bp, *VmbHLH3* 1803 bp, while *VmbHLH2* was 2190 bp long. The nucleotide sequences clearly represented separate genes with *VmbHLH1* showing 54.42% similarity to *VmbHLH2* and 65.06% similarity to *VmbHLH3*, while *VmbHLH2* and *VmbHLH3* shared 53.29% identity (Table 1). When phylogenetic analysis was performed with 169 Arabidopsis bHLHs, the three VmbHLHs were clustered with known flavonoid biosynthesis-regulating AtbHLHs belonging to the subgroup IIIf described by Heim et al.¹⁵, namely AtbHLH001, AtbHLH002, AtbHLH012 and AtbHLH042 (Fig. S1).

Gene	Accession ID ¹	Characteristics			Sequence identity at nucleotide level (%) ²		
		CDS (bp)	Amino acids	Subgroup	VmbHLH1	VmbHLH2	VmbHLH3
VmbHLH1	Vmy12g484.t1	1923	640	IIIIf	100	54.42	65.06
VmbHLH2	Vmy06g33670.t1	2190	729	IIIIf		100	53.29
VmbHLH3	Vmy10g9347.t1	1803	600	IIIIf			100

Table 1. Characteristics of the sequences of Bilberry *bHLH* genes. ¹Retrieved from the Genome Database for *Vaccinium* (<https://www.vaccinium.org>). ²The values were obtained from sequence alignments on Clustal Omega. CDS, coding sequence; bp, base pair.

The bilberry bHLHs show sequence similarity to known flavonoid biosynthesis regulators

A closer phylogenetic examination with the subgroup IIIIf of flavonoid biosynthesis-regulating bHLHs showed three major clades which were strongly supported by bootstrap values $\geq 91\%$ (Fig. 1). The three bilberry bHLHs were clustered in different clades. VmbHLH1 grouped among MYC1-type bHLHs (bootstrap support 98%), which included grapevine VvMYCA1, apple MdbHLH33 and MrbHLH2 from Chinese bayberry, and showed 48.58% identity with Arabidopsis AtbHLH012 (MYC1). VmbHLH2 clustered in a large clade containing previously characterized TT8-type bHLHs in anthocyanin/PA biosynthesis regulation (bootstrap support 100%), including MdbHLH3, MrbHLH1, VvMYC1, PpbHLH3 and NtAN1a/b, and also showed 57.88% identity to AtbHLH042 (TT8). VmbHLH2 appears to be a homolog of the blueberry VcbHLH2 (VcbHLH042)^{42–46} with 98.35% similarity. Conversely, VmbHLH3 grouped with previously characterized GL3/EGL3-type bHLHs associated with anthocyanin biosynthesis regulation (bootstrap support 91%), including Antirrhinum AmDELILA and Gerbera GhMYC1, and with 52.74% identity to AtbHLH001 (GL3) and 53.18% to AtbHLH002 (EGL3) (Fig. 1). VmbHLH3 seems to be homologous to blueberry VcbHLH1-1 and VcbHLH1-2 proteins⁴⁴, with 97.00% and 96.67% similarity, respectively. The previously described blueberry VcbHLH004⁴⁸ showed no sequence homology with IIIIf subgroup members and lacked the MYB interaction region.

Alignment of the VmbHLH amino acid sequences with well-characterized subgroup IIIIf bHLHs in anthocyanin biosynthesis showed a strong sequence similarity and the presence of three conserved regions (Fig. 2). The N-terminus of all three VmbHLHs contained the MYB interaction region, including the characteristic boxes 11, 13 and 18 (Fig. 2A) conserved within the IIIIf subgroup, which enable the binding and interaction with suitable MYB partners in the MBW complex^{15,24,49}. All VmbHLH sequences also contained the signature basic helix-loop-helix (bHLH) domain although only VmbHLH2 and VmbHLH3 showed a conserved HER (His5-Glu9-Arg13) motif (Fig. 2B), which is found in anthocyanin biosynthesis-regulating bHLH TFs. All three VmbHLHs contained the C-terminal transactivation (ACT) domain (Fig. 2C) which enables transcription^{3,49}.

VmbHLHs show differential expression patterns in bilberry

To investigate the spatial and temporal expression patterns of the *VmbHLH* genes, transcript abundance was measured during berry ripening and in various bilberry tissues, including flower, ripening berry, leaf, stem and rhizome. Our real-time quantitative PCR (qPCR) analyses demonstrated the transcript levels of *VmbHLH1* and *VmbHLH3* to be highest in the unripe berry followed by a decrease in the later berry-ripening stages (Fig. 3A). Conversely, the expression of *VmbHLH2* was upregulated during berry ripening and expression peaked at stage 4.5, just before berries were fully ripe (Fig. 3A). This expression pattern of *VmbHLH2* follows anthocyanin accumulation³² and resembles the expression patterns of the key bilberry anthocyanin biosynthesis genes and R2R3 MYB regulators during bilberry fruit ripening (Fig. S2).

For all three *VmbHLH* genes, expression was found to be highest in the bilberry stem, although *VmbHLH2* transcripts were also detected at relatively high levels in leaves, followed by rhizome, ripening berries and flowers (Fig. 3B), indicating roles in both reproductive and vegetative tissues. In contrast, the expression of *VmbHLH1* and *VmbHLH3* was relatively low in reproductive tissues, especially berries, compared with those in vegetative tissues.

Since bilberry fruit ripening is mediated by ABA³⁵, we applied ABA treatment to unripe berries to test its impact on the expression of *VmbHLHs*. Both the 0.5 mM and 2 mM ABA treatments significantly downregulated *VmbHLH1* expression (Fig. 3C). Only slight concentration-dependent downregulation was observed with *VmbHLH3*, while *VmbHLH2* expression was unaffected by ABA (Fig. 3C), indicating that *VmbHLH2* expression is not regulated by the ABA signal.

VmbHLH2 and VmbHLH3 induce anthocyanin accumulation when co-expressed with MdMYB10

To functionally characterize bilberry bHLHs, *Agrobacterium*-mediated transient overexpression assays in *N. benthamiana* leaves were performed. The *VmbHLHs* were separately co-expressed with anthocyanin biosynthesis-regulating apple *MdMYB10*, which is known to be dependent on the co-expression of a suitable bHLH partner in *Nicotiana* leaves, such as MdbHLH3^{24,50}. This provides an excellent system for functional characterization of ectopic bHLHs since many A-type bHLHs, including VmMYBA1 and VmMYBA2 of bilberry³⁹, do not require the co-expression of a bHLH for anthocyanin accumulation in *Nicotiana* leaves. Anthocyanins were detected in *N. benthamiana* leaves after seven days following infiltration with either *VmbHLH2* or *VmbHLH3* or *MdbHLH3* (positive control) together with *MdMYB10* (Fig. 4A). Pigmentation was most visible with *VmbHLH2*. No pigmentation was observed when leaves were infiltrated either with *VmbHLH1* + *MdMYB10* or with any of the

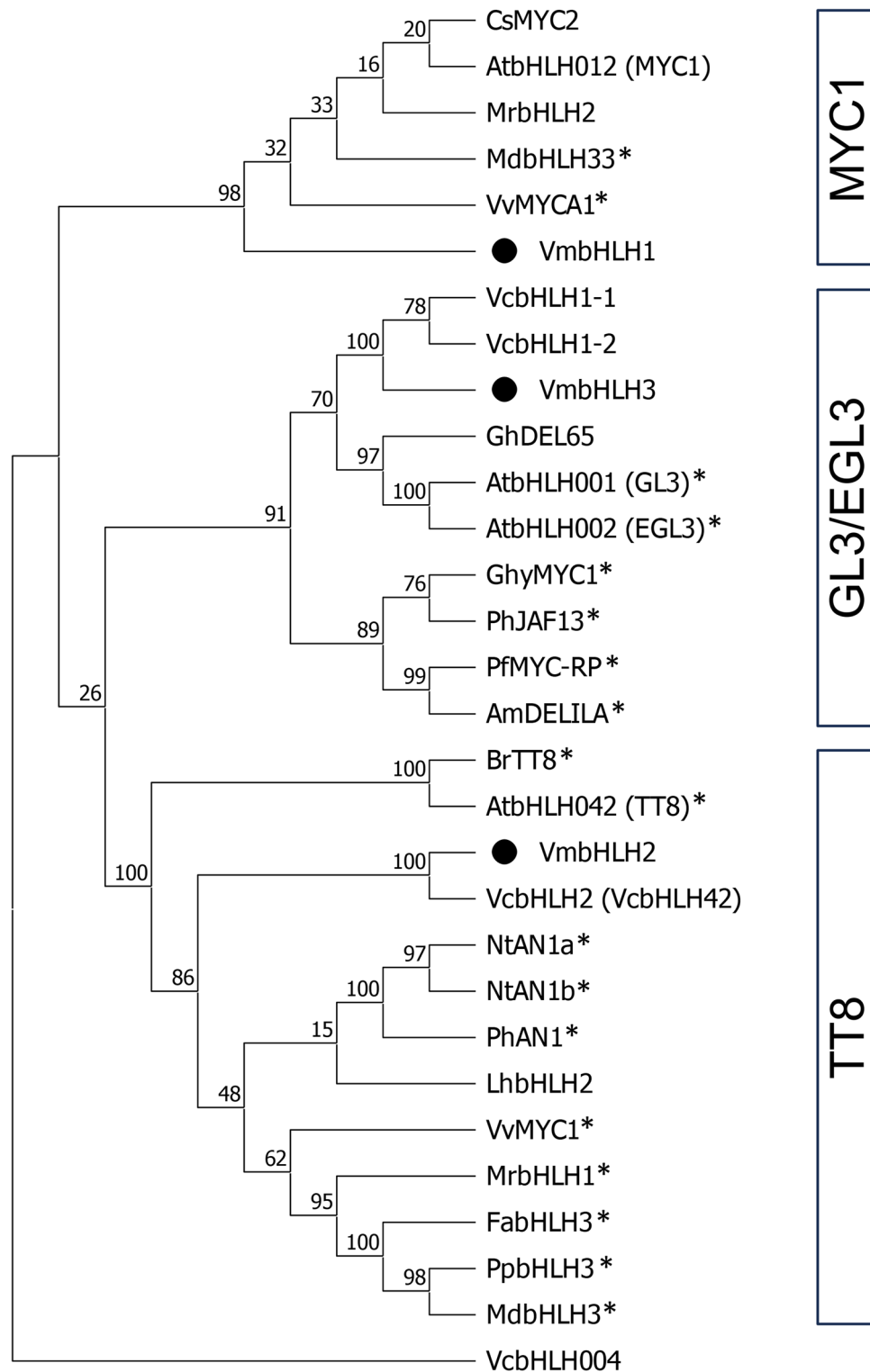


Fig. 1. Phylogenetic analysis of subgroup IIIb bHLHs. The bilberry bHLHs are indicated as black circles. Asterisks indicate functionally characterized flavonoid biosynthesis-regulating IIIb bHLHs in other plant species. The tree was constructed using MEGA 11 and numbers near branches indicate bootstrap values in percentage (1000 replicates). Am, *Antirrhinum majus*; At, *Arabidopsis thaliana*; Br, *Brassica rapa*; Cs, *Citrus sinensis*; Fa, *Fragaria x ananassa*; Gh, *Gossypium hirsutum*; Gh, *Gerbera hybrida*; Lh, *Lilium hybrid*; Md, *Malus domestica*; Mr, *Morella rubra*; Nt, *Nicotiana tabacum*; Pf, *Perilla frutescens*; Ph, *Petunia x hybrida*; Pp, *Prunus persica*; Vc, *Vaccinium corymbosum*; Vm, *Vaccinium myrtillus*; Vv, *Vitis vinifera*.

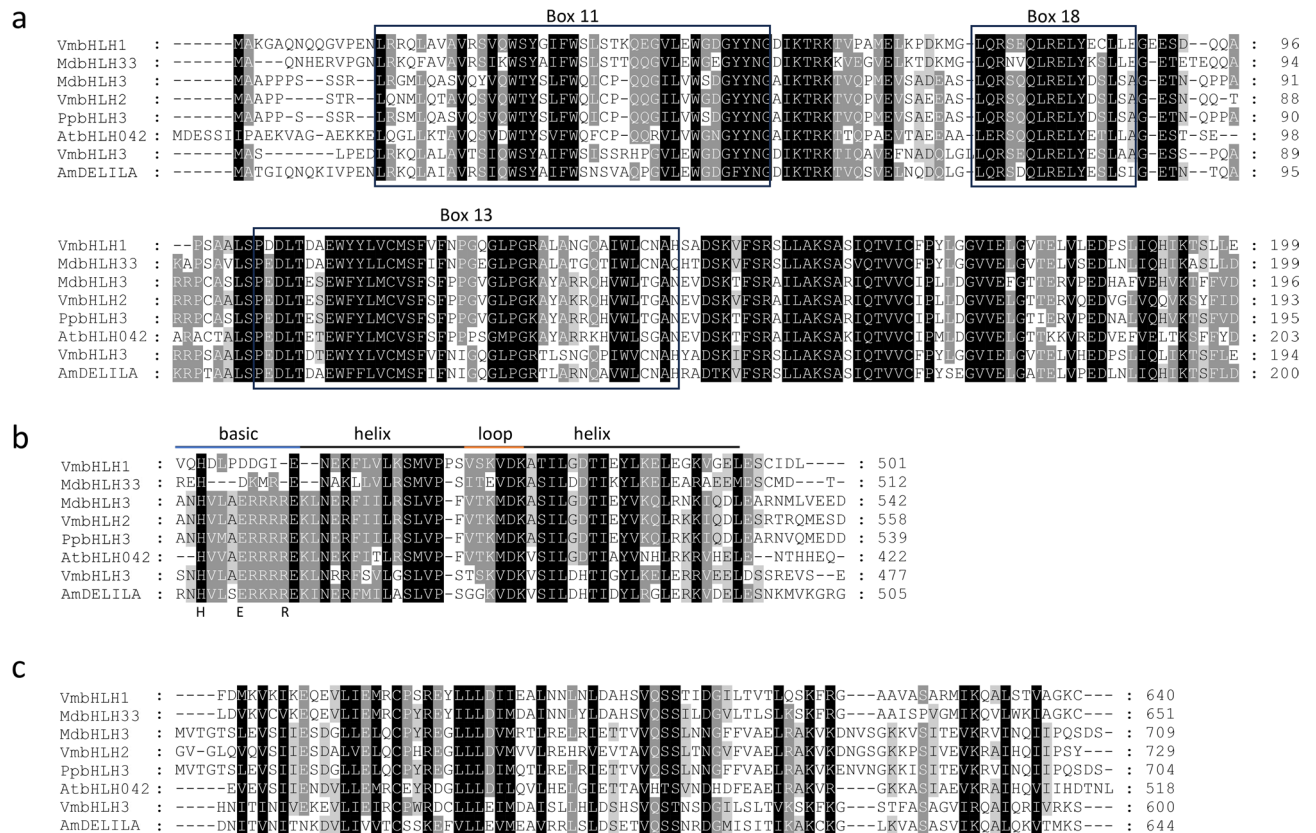


Fig. 2. Identification of conserved domains in bilberry bHLHs. Amino acid sequence alignment of three VmbHLH proteins and the known anthocyanin bHLH regulators in other species indicate presence of (A) MYB interaction region, (B) bHLH domain and (C) ACT-like domain. Conserved residues are highlighted in black, and partial conservation is indicated in grey. Am, *Antirrhinum majus*; At, *Arabidopsis thaliana*; Md, *Malus domestica*; Pp, *Prunus persica*; Vm, *Vaccinium myrtillus*.

VmbHLHs alone, or MdMYB10 alone, or with negative controls (empty vector or untreated) (Fig. 4A). The leaf infiltration sites were confirmed for the presence of VmbHLH expression (Fig. S3).

Anthocyanin content analysis at infiltration sites confirmed that VmbHLH2 + MdMYB10 induced the highest concentration of anthocyanin while VmbHLH3 together with MdMYB10 induced slightly lower amounts than VmbHLH2 + MdMYB10 or MdbHLH3 + MdMYB10 (Fig. 4B). Anthocyanin in *Nicotiana* leaves infiltrated either with VmbHLH1 + MdMYB10, VmbHLHs alone, or MdMYB10 alone, was in similar concentration to those with the empty vector or untreated controls (Fig. 4B).

VmbHLHs activate the expression of specific sets of anthocyanin biosynthetic genes

The regulatory role of VmbHLHs in anthocyanin biosynthesis was further analyzed for flavonoid biosynthetic structural gene expression. Results showed that, although VmbHLH1 did not induce accumulation of anthocyanins in *N. benthamiana* leaves when co-expressed with MdMYB10, it could upregulate a restricted set of structural genes in the flavonoid pathway. Significant upregulation of *NbF3H*, *NbF3'5'H*, *NbLAR* and *NbANR* expression was detected, while the expression of *NbANS* and *NbUGT* was also slightly enhanced when compared with infiltration with MdMYB10 alone (Fig. 5A). VmbHLH2, together with MdMYB10, significantly upregulated almost all the tested flavonoid and anthocyanin biosynthetic genes, while *NbF3H* expression was only slightly increased (Fig. 5B). This indicates that it can complement MdbHLH3 and induce a similar expression pattern among anthocyanin and flavonoid biosynthetic genes (Fig. 5D). VmbHLH3 also activated almost all the anthocyanin and flavonoid biosynthetic genes when co-expressed with MdMYB10, the exception of *NbF3H* (Fig. 5C). In addition, VmbHLH1 and VmbHLH3 upregulated the endogenous bHLH *NbAN1*. The expression of *NbFLS* was unchanged by bHLH infiltration (Fig. S4).

Discussion

We previously conducted a comprehensive study of bilberry flavonoid-regulating R2R3 MYB TFs³⁹, but their bHLH partners interacting in the MBW complex had not been functionally characterized in *Vaccinium* berries before the present study. In earlier reports, *VcbHLH2* and *VmbHLH2* sequences were found to be unstable in plasmids, possibly because of a microsatellite repeat, restricting functional analyses^{45,51}. The lack of bHLH partners poses a limitation to studies on the regulation by the MBW complexes and their regulatory targets in flavonoid biosynthesis in *Vaccinium* berries. Here, we identified, cloned and functionally characterized

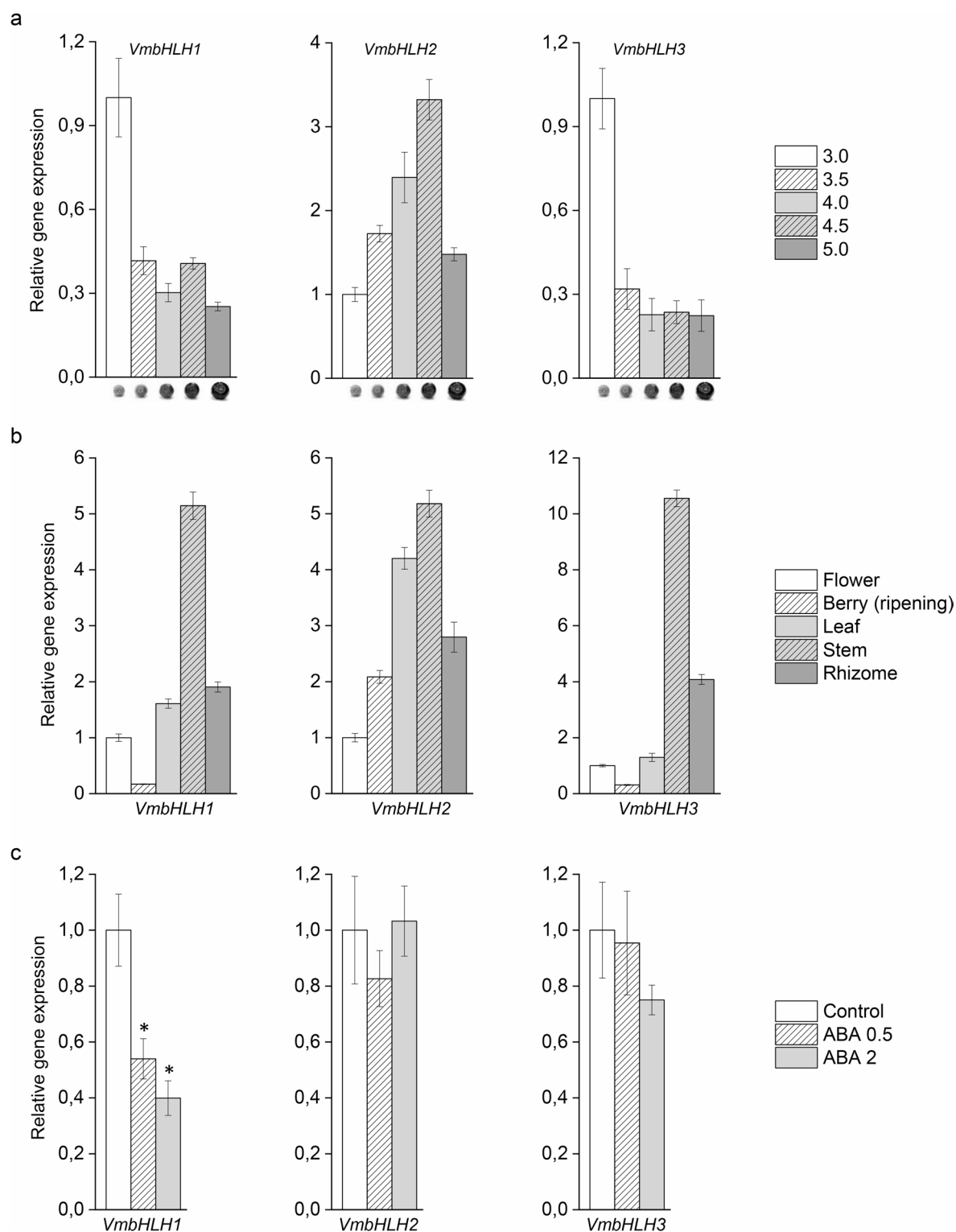


Fig. 3. The expression profiles of *VmbHLH* genes (A) during bilberry fruit ripening, (B) in various bilberry tissues and (C) after 48-h treatment of unripe fruit by 0.5 mM ABA, 2 mM ABA or water (control). The relative gene expression was quantified by qPCR and normalized to *VmGAPDH* and *VmActin*. Values represent means $\pm$ SEs of four replicates. Asterisks indicate significant differences from control in Student's *t*-test ($p \leq 0.05$). 3.0–5.0 represent fruit ripening stages from unripe to fully ripe berry.

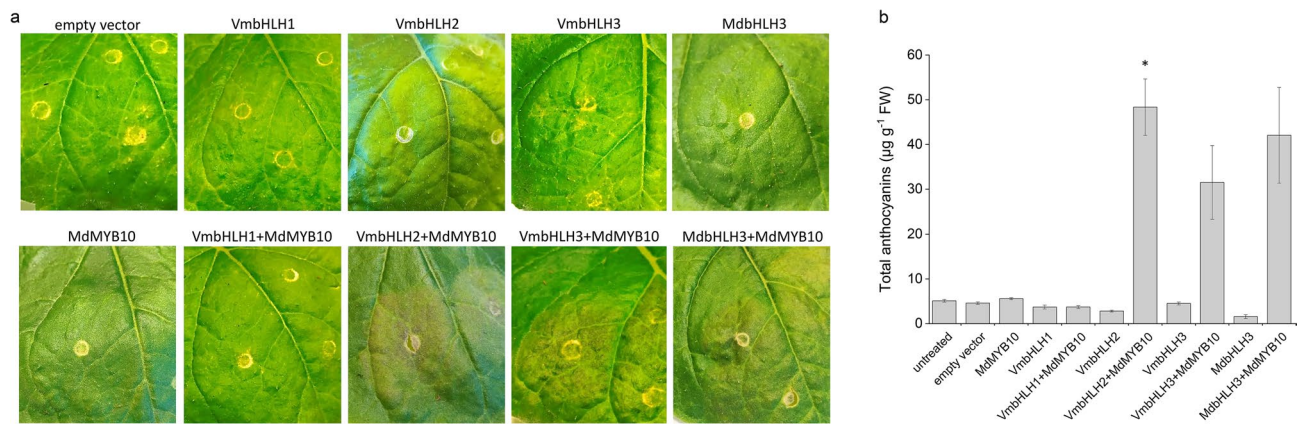


Fig. 4. Effect of transient overexpression of *VmbHLHs* on anthocyanin accumulation in *Nicotiana benthamiana* leaves. (A) Pigmentation in *N. benthamiana* leaves after 7 days of infiltration. Infiltration of *bHLHs* was conducted either alone or with *MdMYB10*. (B) Total anthocyanin content in *N. benthamiana* leaves after 7 days of infiltration. Untreated leaves, infiltration with empty vector and *VmbHLH* or *MdMYB10* alone served as negative controls. Values represent means $\pm$ SEs of at least three biological replicates. Asterisks indicate significant differences in anthocyanin concentration from infiltration with *MdMYB10* alone in Student's *t*-test ($p \leq 0.05$). FW, fresh weight.

three *bHLH* genes from bilberry which, based on our results, are associated with anthocyanin and flavonoid biosynthesis.

All three *VmbHLHs* showed high similarity to *bHLHs* of subgroup IIIf of Arabidopsis and other plant species, members of which are known to regulate flavonoid biosynthesis³. Phylogenetic analysis showed that the bilberry *bHLHs* were separated under three clades in the IIIf subgroup, which have been described previously by Starkevič et al.⁵².

The *VmbHLH1* sequence was clustered with MYC1-type *bHLHs*, including the anthocyanin biosynthesis-regulating apple *MdbHLH33* and grapevine *VvMYCA1*^{23,24}. *VmbHLH2* was found to be homologous to TT8-type *bHLHs*, which include *MdbHLH3*, *MrbHLH1*, *VvMYC1*, *PpbHLH3*, *PhAN1* and *AtTT8*, all of which have been reported to function in anthocyanin and PA biosynthesis regulation^{18,22,24,27,28,53,54}. For example, Arabidopsis *AtTT8* is involved in the regulation of both anthocyanin and PA biosynthesis in seeds and seedlings^{55–57}. Our results further indicated that *VmbHLH3* shared identity with GL3/EGL3-type *bHLHs*. In our phylogenetic tree, *VmbHLH3* clustered with Antirrhinum *AmDELILA*, Petunia *PhJAF13* and Gerbera *GhyMYC1*, as well as Arabidopsis *AtGL3* and *AtEGL3*, which are associated with the regulation of anthocyanin biosynthesis in flower petals and roots, respectively^{16–18,56,58,59}. Several studies have demonstrated that *AmDELILA* functions together with the R2R3 MYB, *AmROSEA1*, for the production of high anthocyanin concentration in different overexpression systems^{60–62}.

The role of conserved MBW complexes in regulating anthocyanin and PA biosynthesis is relatively well understood. The MYB interaction region in the *bHLH* sequence is generally considered indispensable for the interaction with the R2R3 MYBs that regulate anthocyanin and PA biosynthesis^{49,63}. All three *VmbHLHs* contained an intact MYB interaction area in their N-terminus, indicating a possible protein-protein interaction with R2R3 MYBs. In these complexes, the R2R3 MYB interacts with a *bHLH* to induce anthocyanin/flavonoid accumulation by activating promoters of flavonoid structural genes. Apple *MdMYB10* (an allele of *MdMYB1*) is an example of a MYBA-type R2R3 MYB, which is dependent on the presence of a suitable *bHLH* partner, such as *MdbHLH3*, *MdbHLH33* or *AtbHLH002*, for the induction of anthocyanin biosynthesis genes when overexpressed in *Nicotiana* leaves^{24,50,64}. Similarly, in our study, *MdMYB10* alone was not capable of inducing anthocyanin accumulation in *N. benthamiana* leaves, although it upregulated the expression of a restricted set of flavonoid structural genes. However, this was greatly enhanced when co-expressed with either *MdbHLH3*, *VmbHLH2* or *VmbHLH3* and led to anthocyanin accumulation. This indicates that the two bilberry *bHLHs* could co-operate with the MYBA-type *MdMYB10* to induce a complete set of flavonoid and anthocyanin biosynthetic genes (except for *NbF3'H*). Our results also show that *VmbHLH2* and *VmbHLH3* can form MBW complexes with *MdMYB10* in *N. benthamiana* leaves without addition of a bilberry WD40 protein construct.

It should also be noted that *MdbHLH3* and *VmbHLH2* induced similar expression patterns of the flavonoid structural genes, supporting the evidence for *VmbHLH2* being orthologous to the TT8-type *MdbHLH3*. Conversely, GL3/EGL3-type *VmbHLH3* induced slightly divergent expression patterns, particularly for *NbF3'H*, *NbLAR* and *NbAN1*, although it induced anthocyanin accumulation. The redundancy between *bHLHs* has been reported previously. In Arabidopsis, GL3/EGL3 can act redundantly with TT8 to control anthocyanin biosynthesis⁵⁶. Also, in Antirrhinum GL3/EGL3-type *AmDELILA* has been shown to complement the loss of a TT8-type *AmIncolorata I/Mutabilis* function in anthocyanin biosynthesis^{20,21}. In Petunia, both TT8-type *AN1* and GL3/EGL3-type *PhJAF13* are suggested in the regulation of anthocyanin biosynthesis but *PhJAF13* does not compensate for the loss of *AN1* in *an1* mutants and has been found to stimulate *PhAN1* expression during inductive conditions, such as fruit ripening, through a hierarchical mechanism^{18,19,65}. The same mechanism has

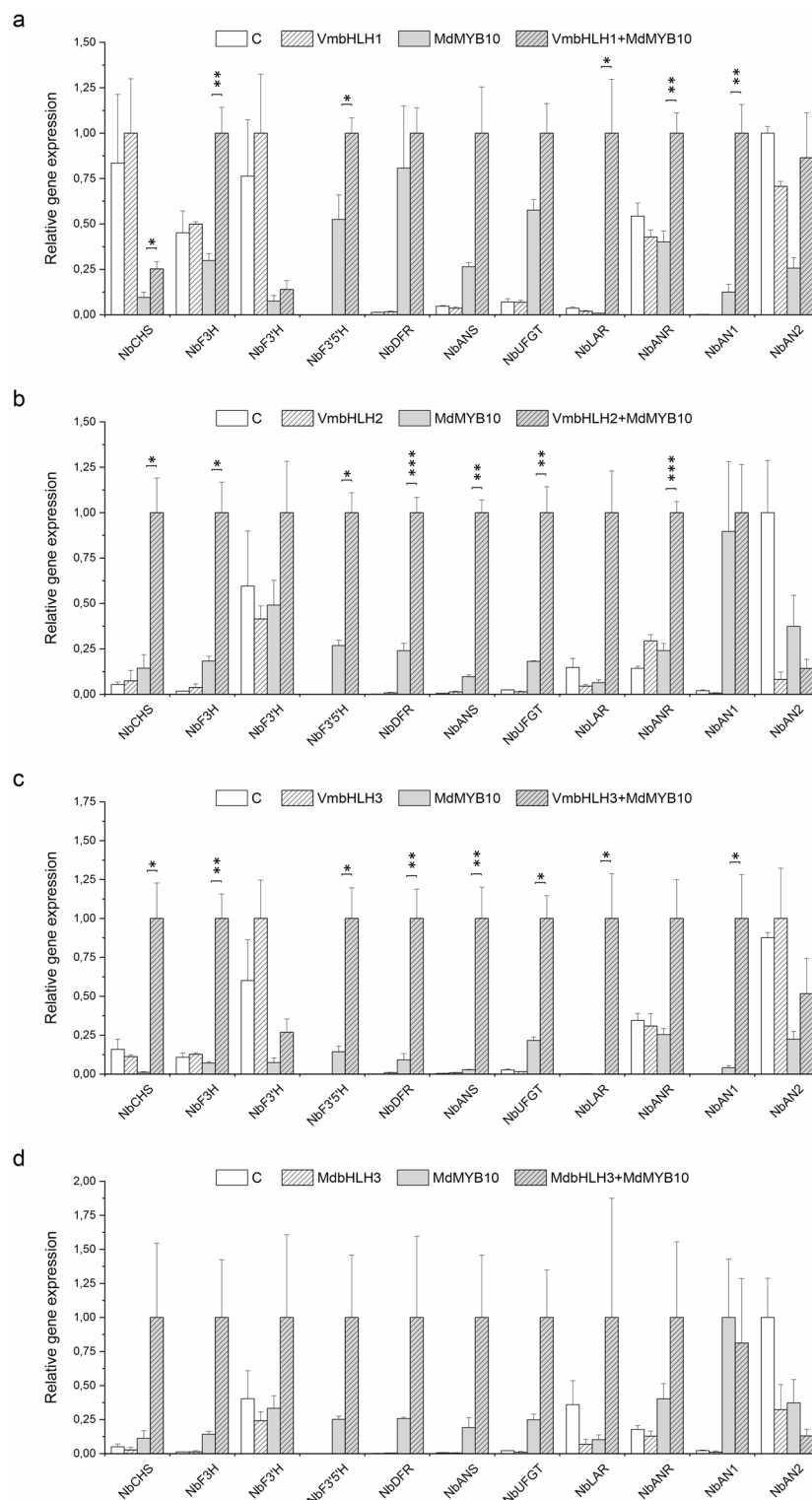


Fig. 5. Expression of flavonoid biosynthetic genes in *Nicotiana benthamiana* leaves after transient overexpression with (A) *VmbHLH1*, (B) *VmbHLH2*, (C) *VmbHLH3* and (D) *MdbHLH3* (positive control). Relative expression of the genes was quantified from infiltration sites after 7 days of infiltration by qPCR and normalized to *NbEF1* and *NbActin*. Values represent means $\pm$ SEs of at least three biological replicates. Asterisks indicate significant differences from infiltration with *MdMYB10* alone according to Student's *t*-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$). C, empty vector control.

been reported in *Ipomoea* sp., pea and *Dahlia*⁶⁵. The redundancy or hierarchical regulation between subgroup IIIb bHLHs have been studied less in berry anthocyanin biosynthesis. However, a recent study showed that LrJAF13 (PhJAF13 homolog) was not able to restore anthocyanin pigmentation in white goji berry variant caused by deletions in *LrAN1b* (PhAN1 homolog) promoter^{29,66}. In fact, hierarchical regulation was found between two MBW complexes. LrJAF13-LrAN11(WD40)-LrAN2-like MBW complex upregulated *LrAN1b* expression by binding its promoter, leading to formation of LrAN1b-LrAN11-LrAN2-like MBW complex, which activated transcription of anthocyanin biosynthetic genes²⁹.

VmbHLH1 did not induce anthocyanin accumulation in *N. benthamiana* leaves but did induce the expression of structural genes of the flavonoid pathway, including *NbF3H*, *NbF3'5'H*, *NbANS*, *NbUGT*, *NbLAR*, and *NbANR*. The inability to induce *NbCHS* and *NbDFR* expression is likely to explain the lack of anthocyanin accumulation in *N. benthamiana* leaves. This result is in accordance with the *VmbHLH1* sequence lacking the conserved HER motif, which is known to control binding to bHLH-binding sites (E-box) in promoter regions of anthocyanin biosynthetic genes^{3,67}. Our results are similar to that reported for Chinese bayberry bHLHs, as anthocyanin accumulation in tobacco leaves was demonstrated when anthocyanin-regulating *MrMYB1* was co-expressed with TT8-type *MrbHLH1*, but not when co-expressed with MYC1-type *MrbHLH2*²⁸. On the other hand, grape VvMYCA1 was demonstrated to interact with VvMYBA2r to induce anthocyanin biosynthesis in tobacco leaves²³. Thus, it seems that both MYC1-type and GL3/EGL3-type bHLHs of different species can demonstrate divergent functions and mechanisms in the regulation of flavonoid and anthocyanin pathway. This variability throughout the Eudicots is suggested to reflect functional specialization of the bHLH factor roles between genera⁶⁵. The ability of *VmbHLH1* and *VmbHLH3* to attend in regulation of anthocyanin biosynthesis remains to be further explored in bilberry, which shows complex anthocyanin and flavonoid metabolism. None of the *VmbHLHs* induced expression of *NbFLS*, indicating that these *VmbHLHs* were not able to induce flavonol biosynthesis and their regulation is restricted to anthocyanin and PA pathway genes. This is in accordance with the current knowledge that flavonol biosynthesis-regulating MYBs (MYBF, SG7) act independently of bHLHs^{49,63}.

When deciphering the role of *VmbHLHs* in bilberry, their spatial and temporal expression and that of their potential MYB partners should be considered. It has been shown that within the same species, MBW complexes can include different classes of MYBs and bHLHs to regulate different branches of the flavonoid pathway. For example, MYBA-type AtPAP1 or AtPAP2 together with AtTT8 and AtTTG1 regulate anthocyanin synthesis in Arabidopsis, whereas AtTT8 is also capable of acting with MYBPA2-type AtTT2 and AtTTG1 complexes to regulate PA biosynthesis via activation of BAN/ANR expression^{55,57}. VvMYC1 has also been shown to interact with several R2R3 MYBs, such as VvMYB5a, VvMYB5b, VvMYBA1/A2 and VvMYBPA1, to control both anthocyanin and PA biosynthesis²². In our study, *VmbHLH1* expression was higher in unripe bilberry fruit than in ripening fruit and was significantly downregulated by ABA, the known inducer of fruit ripening and anthocyanin biosynthesis in bilberry^{34,35}. These findings support our results from the overexpression experiments in that *VmbHLH1* may not associate with anthocyanin biosynthesis regulation and indicate that its role in berry may be associated with PA biosynthesis, especially in the early stages of bilberry fruit development³³. Our earlier study showed the expression of many of the PA-type R2R3 MYBs to be associated with early stages of bilberry fruit development and being downregulated by ABA³⁹. A similar role was previously suggested by Günther et al.⁴³, describing a partial sequence homolog of *VmbHLH1*, blueberry *VcbHLH1*, to be co-expressed with *VcMYBPA1* in a transcriptome of blueberry fruit flesh. This correlated with the accumulation of PAs and hydroxycinnamic acids. However, our study showed higher expression of *VmbHLH1* in bilberry tissues other than fruits, suggesting that the MYC1-type *VmbHLH1* could also have a role in non-fruit tissues.

In contrast, the TT8-type *VmbHLH2* showed increased expression during bilberry fruit ripening. This coincided with its potential target flavonoid structural genes and potential A-type R2R3 MYB partner in anthocyanin biosynthesis, VmMYBA1. Earlier studies have also found the expression of *VmbHLH2* homologs to correlate with MYBA1 and MYBPA1.1 expression in fruit skin and anthocyanin biosynthetic genes^{42,43,45,46}. Thus, our data suggests that *VmbHLH2* functions as a regulator of anthocyanin biosynthesis in ripening bilberry, probably in partnership with VmMYBA1. In contrast, although *VmbHLH3* could also complement *MdbHLH3* to induce anthocyanin accumulation in *N. benthamiana*, its expression in bilberry differs drastically from that of *VmbHLH2*, resembling that of *VmbHLH1* instead. Therefore, it is likely that *VmbHLH3* regulates PA biosynthesis in unripe berries and anthocyanin biosynthesis in tissues other than berries, which is supported by its apparent slight downregulation in response to ABA treatment. Anthocyanin regulation may be driven by interaction with the previously described VmMYBA2, which shows decreasing expression during fruit maturation and high expression in tissues other than fruits as well as being negatively regulated by ABA³⁹. Xu et al.³⁰ previously suggested a more extensive role for woodland strawberry GL3/EGL3-type bHLH than that of TT8- and MYC1-type bHLHs in flavonoid biosynthesis. The expression of this bHLH correlated with PA content and it was shown to interact with ten different R2R3 MYB TFs, including FvMYB10, and it was suggested to be a universal partner for FvMYB proteins³⁰. The interaction of *VmbHLHs* with the large group of PA-type TFs, found to exist in bilberry in our previous study³⁹, cannot be ruled out. For example, it is likely that TT8-type *VmbHLH2* also interacts with other MYBs since the expression of *VmMYBA1* is restricted to ripening berries³⁹. The high expression of the three *VmbHLHs* present in non-fruit bilberry tissues may indicate functions for all of them in both vegetative and reproductive tissues with their role being dependent on the presence of suitable R2R3 MYB partners. Since the *bHLH* expression patterns do not always correlate with R2R3 MYB partner expression or product accumulation^{22,24,29,68}, the suggested protein-protein interactions between *VmbHLHs* and their putative 18 R2R3 MYB partners in bilberry needs to be verified in future studies.

ABA treatment did not result in enhanced expression of any of the three *VmbHLHs*, but *VmbHLH1* expression was significantly downregulated and *VmbHLH3* slightly downregulated by ABA. We previously showed that VmMYBA1, VmMYBPA1.1 and VmMYBPA2.2, which are all associated with anthocyanin regulation in

bilberry fruit, were upregulated by ABA³⁹. This indicated that the bilberry fruit ripening-related anthocyanin biosynthesis induced by ABA is controlled by *MYB* transcription, not the *bHLH* expression. Similar results have been reported previously in strawberries indicating that ABA upregulates the expression of anthocyanin-related *FaMYB1* and *FaMYB10* but does not regulate TT8-type *FabHHLH3* while GL3/EGL3-type *FaMYC1* is negatively regulated by ABA^{69,70}. The R2R3 MYB is usually considered as the rate-limiting component of the MBW complex, and it can activate expression of *bHLH* and *WD40* genes through a conserved hierarchy⁶⁵. For example, overexpression of Arabidopsis *AtPAP1* was shown to upregulate the expression of *AtTT8*⁷¹. In black goji berry, *LrAN2*-like is known to regulate *LrAN1b* expression²⁹. Also in our earlier study, *VmMYBA1*, *VmMYBA2*, *VmMYBPA2.2*, and to a certain extent *VmMYBPA1.1*, were able to activate the expression of TT8-type *NbAN1* in *N. benthamiana* leaves³⁹. This hierarchy is supported by our current study, demonstrating that *MdMYB10* could activate *NtAN1* expression in *N. benthamiana* leaves, while *VmbHLHs* could not significantly activate *NtAN2* (MYB) expression. However, based on our study, such hierarchy does not exist in bilberry fruit as elevated *VmMYBA1*, *VmMYBPA1.1* and *VmMYBPA2.2* expression by ABA did not lead to upregulation in TT8-type *VmbHLH2* expression. Since *VmbHLH2* expression increased during bilberry fruit ripening, the induction in expression seems to be regulated by signals or phytohormones other than ABA and needs to be clarified by future studies. As discussed above, hierarchical regulation has also been described to exist between the different IIIf subgroup bHLH members in certain species, commonly MYC1/GL3/EGL3-type bHLHs regulating TT8-type bHLH expression during flowering or fruit ripening^{21,29,65,72}. However, it appears that the hierarchical control between bHLH components varies in Eudicots⁶⁵. Thus, this needs to be studied in the future between bilberry bHLHs, and the availability of bilberry genome provides possibilities for promoter activation studies. Similarly, the degree of redundancy among the different IIIf subgroup bHLH members seems to vary by species⁷² and remains to be studied in the genus *Vaccinium* in the future.

Conclusions

Our study shows, for the first time, the likely alternate functional roles for flavonoid-related bHLH TFs. The evidence suggests that the TT8-type *VmbHLH2* is directly involved as the bHLH component in the MBW complex controlling anthocyanin production in berries. Further, the MYC1-type *VmbHLH1* and GL3/EGL3-type *VmbHLH3* are associated with anthocyanin and PA regulation in unripe fruit and non-fruit tissues.

Materials and methods

Bilberry plant material

Bilberry (*Vaccinium myrtillus* L.) fruits at five ripening stages, ranging from unripe green to fully ripe blue (stages 3.0, 3.5, 4.0, 4.5, and 5.0), were collected from Tromsø, Norway (69°75' N, 19°03' E). Vegetative bilberry tissue samples were collected from forest stands in Oulu (65°01' N, 25°28' E). The formal identification and deposition of voucher specimens into the public herbarium were undertaken by the Department of Arctic and Marine Biology at UiT The Arctic University of Norway. Bilberry fruit treatments with 0.5 mM ABA [(±)-abscisic acid; Sigma-Aldrich, St. Louis, MO, USA], 2 mM ABA or water as negative control were conducted in Petri dishes under sterile conditions as described earlier³⁵. Three replicate Petri plates with approximately 50 unripe berries per plate were employed. The plates were kept at 18 °C under 30 μmol m⁻² s⁻¹ light. Berry samples were collected after 48 h from the beginning of the ABA treatment. Immediately after collection, all samples were frozen in liquid nitrogen and stored at -80 °C before RNA extraction.

Identification and sequence analysis of bilberry bHLH genes

Protein sequences of *Arabidopsis thaliana* bHLHs of subgroup IIIf were used to identify potential bilberry anthocyanin biosynthesis-related bHLH genes using blastn searches against bilberry genome representing the Tromsø ecotype⁴⁷, accessible in the Genome Database for *Vaccinium* (<https://www.vaccinium.org>). Full-length deduced amino acid sequences of bilberry bHLHs were aligned using the Clustal Omega program (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo>) and visualized using GeneDoc software⁷³. For phylogenetic tree analysis, the amino acid sequences of previously characterized plant bHLH subgroup IIIf proteins were obtained from GenBank (Supplementary Table S1). Arabidopsis bHLH sequences were retrieved from UniProt (<https://www.uniprot.org/>). Full-length protein sequences were aligned with ClustalW and a phylogenetic tree was constructed using the maximum likelihood method with the JTT + G model in MEGA software (v. 12).

RNA extraction and gene expression analysis in bilberry tissues

Frozen samples were homogenized into a fine powder using a mortar and pestle under liquid nitrogen. Total RNA was isolated from bilberry tissues by using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich) with on-column digestion using DNase I (Sigma-Aldrich). The quality of RNA was verified and quantified using a NanoDrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA) and RNA integrity was verified electrophoretically. Total RNA was converted to cDNA by using the SuperScript™ IV reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. From ABA-treated berries, total RNA was extracted and cDNA synthesized and purified as described previously³⁵.

Real-time quantitative PCR (qPCR) analyses were performed as described previously⁷⁴ using a Bio-Rad CFX96™ Real Time System (Bio-Rad, Hercules, CA, USA) and SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad). Gene-specific primer sequences used for the qPCR analyses are listed in Supplementary Table S2. All analyses were performed with at least three biological replicates. Relative expression levels of bHLH genes were calculated in CFX Maestro™ software (Bio-Rad) using glyceraldehyde-3-phosphate dehydrogenase (*VmGAPDH*) and *VmActin* as reference genes.

VmbHLH gene cloning and construction of expression vectors

Full-length coding sequences of three *VmbHLHs* were amplified from bilberry fruit cDNA by PCR using Phusion[®] High-Fidelity DNA Polymerase (Thermo Fischer Scientific) with gene-specific primers (Supplementary Table S3). The amplified PCR products were ligated into the expression vector pEAQ-HT-DEST3 under the control of *CaMV35S* promoter by Gateway cloning. The sequences of the constructed expression vectors were verified by sequencing using the BigDye[™] Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with a 3130xl Genetic Analyzer (Applied Biosystems) at the UiT The Arctic University of Norway sequencing facility. *MdMYB10* and *MdbHLH3* cloning into a binary expression vector pSAK277 was as previously described in Espley et al.²⁴.

Transient overexpression assays

The expression vectors were transformed into electrocompetent *Agrobacterium tumefaciens* (GV3101) cells, followed by growth on LB agar media supplemented with selective antibiotics at 28 °C. Harvested cells were resuspended in infiltration buffer [10 mM MES (pH 5.6), 10 mM MgCl₂, 200 µM acetosyringone] to reach an OD₆₀₀ of 0.6. After incubation at room temperature for 2–3 h, the *Agrobacterium* solution was infiltrated into the abaxial side of leaves of 5-weeks-old *Nicotiana benthamiana* using a syringe. *Agrobacterium* cells containing *bHLH*-constructs were introduced to leaves alone or with an equivalent dose of *Agrobacterium* cells containing the *MdMYB10* construct. An empty vector served as a negative control. At least three plants were transformed with each construct combination. Infiltration sites were collected 7 days after the infiltration and stored at –80 °C until they were used for qPCR analyses and measurement of anthocyanins.

For qPCR analyses, total RNA from the infiltrated sites of *N. benthamiana* leaves was extracted and cDNA prepared as described above. qPCR analyses were performed as described above using gene-specific primers listed in Supplementary Table S4. All analyses were performed with at least three biological replicates. The relative expression levels were calculated in CFX Maestro[™] software (Bio-Rad) by using elongation factor 1 (*NbEF1*) and *NbActin* as reference genes.

Measurement of anthocyanins

Frozen tissues were ground to fine powder with a mortar and pestle under liquid nitrogen. For analyses of anthocyanins from *N. benthamiana* leaves, tissue powder of 0.1 g was extracted and measured spectrophotometrically as described by Chu et al.⁷⁵. All the analyses were performed with three to four biological replicates. The results were expressed as µg (cyanidin-3-glucoside equivalent) g^{–1} fresh weight (FW).

Statistical analysis

The quantitative results of gene expression and anthocyanin determination were analyzed with Student's *t*-test using SPSS Statistics program, version 29.0.2.0 (IBM, New York, NY, USA).

Data availability

The data used to support the findings of this study are included within the manuscript and its supplementary information files.

Received: 10 May 2025; Accepted: 8 August 2025

Published online: 14 August 2025

References

1. Wei, K. & Chen, H. Comparative functional genomics analysis of bHLH gene family in rice, maize and wheat. *BMC Plant Biol.* **18**, 309 (2018).
2. Gao, F. & Dubos, C. The arabidopsis bHLH transcription factor family. *Trends Plant. Sci.* **29**, 668–680 (2024).
3. Feller, A., Machemer, K., Braun, E. L. & Grotewold, E. Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *Plant. J.* **66**, 94–116 (2011).
4. Saigo, T., Wang, T., Watanabe, M. & Tohge, T. Diversity of anthocyanin and proanthocyanin biosynthesis in land plants. *Curr. Opin. Plant. Biol.* **55**, 93–99 (2020).
5. Basu, A., Rhone, M. & Lyons, T. J. Berries: emerging impact on cardiovascular health. *Nutr. Rev.* **68**, 168–177 (2010).
6. Pojer, E., Mattivi, F., Johnson, D. & Stockley, C. S. The case for anthocyanin consumption to promote human health: A review. *Compr. Rev. Food Sci. Food Saf.* **12**, 483–508 (2013).
7. Cassidy, A. Berry anthocyanin intake and cardiovascular health. *Mol. Asp. Med.* **61**, 76–82 (2018).
8. Pires, T. C. S. P., Caleja, C., Santos-Buelga, C., Barros, L. & Ferreira, I. C. F. R. *Vaccinium myrtillus* L. fruits as a novel source of phenolic compounds with health benefits and industrial applications - a review. *Curr. Pharm. Des.* **26**, 1917–1928 (2020).
9. Tohge, T., de Souza, L. P. & Fernie, A. R. Current understanding of the pathways of flavonoid biosynthesis in model and crop plants. *J. Exp. Bot.* **68**, 4013–4028 (2017).
10. Ramsay, N. A. & Glover, B. J. MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. *Trends Plant. Sci.* **10**, 63–70 (2005).
11. Jaakola, L. New insights into the regulation of anthocyanin biosynthesis in fruits. *Trends Plant. Sci.* **18**, 477–483 (2013).
12. Zhang, B., Chopra, D., Schrader, A. & Hülskamp, M. Evolutionary comparison of competitive protein-complex formation of MYB, bHLH, and WDR proteins in plants. *J. Exp. Bot.* **70**, 3197–3209 (2019).
13. Pireyre, M. & Burow, M. Regulation of MYB and bHLH transcription factors: a glance at the protein level. *Mol. Plant.* **8**, 378–388 (2015).
14. Chen, C. et al. MYB transcription repressors regulate plant secondary metabolism. *Crit. Rev. Plant. Sci.* **38**, 159–170 (2019).
15. Heim, M. A. et al. The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. *Mol. Biol. Evol.* **20**, 735–747 (2003).
16. Gonzalez, A., Zhao, M., Leavitt, J. M. & Lloyd, A. M. Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. *Plant. J.* **53**, 814–827 (2008).
17. Feyissa, D. N., Løvdaal, T., Olsen, K. M., Slimestad, R. & Lillo, C. The endogenous *GL3*, but not *EGL3*, gene is necessary for anthocyanin accumulation as induced by nitrogen depletion in *Arabidopsis* rosette stage leaves. *Planta* **230**, 747–754 (2009).

18. Spelt, C., Quattrocchio, F., Mol, J. N. M. & Koes, R. *anthocyanin1* of petunia encodes a basic helix–loop–helix protein that directly activates transcription of structural anthocyanin genes. *Plant. Cell.* **12**, 1619–1631 (2000).
19. Quattrocchio, F. et al. PH4 of petunia is an R2R3 MYB protein that activates vacuolar acidification through interactions with basic-helix-loop-helix transcription factors of the anthocyanin pathway. *Plant. Cell.* **18**, 1274–1291 (2006).
20. Schwinn, K. et al. A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus *Antirrhinum*. *Plant. Cell.* **18**, 831–851 (2006).
21. Albert, N. W. et al. Discrete bHLH transcription factors play functionally overlapping roles in pigmentation patterning in flowers of *Antirrhinum majus*. *New. Phytol.* **231**, 849–863 (2021).
22. Hichri, I. et al. The basic helix-loop-helix transcription factor MYC1 is involved in the regulation of the flavonoid biosynthesis pathway in grapevine. *Mol. Plant.* **3**, 509–523 (2010).
23. Jiu, S. et al. The role of VvMYBA2r and VvMYBA2w alleles of the MYBA2 locus in the regulation of anthocyanin biosynthesis for molecular breeding of grape (*Vitis* spp.) skin coloration. *Plant. Biotechnol. J.* **19**, 1216–1239 (2021).
24. Espley, R. V. et al. Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. *Plant. J.* **49**, 414–427 (2007).
25. Wang, N. et al. MYB12 and MYB22 play essential roles in proanthocyanidin and flavonol synthesis in red-fleshed apple (*Malus sieversii* f. *niedzwetzkyana*). *Plant. J.* **90**, 276–292 (2017).
26. Yang, J. et al. Identification and expression analysis of the apple (*Malus × domestica*) basic helix-loop-helix transcription factor family. *Sci. Rep.* **7**, 28 (2017).
27. Xie, X. B. et al. The bHLH transcription factor MdbHLH3 promotes anthocyanin accumulation and fruit colouration in response to a low temperature in apples. *Plant. Cell. Environ.* **35**, 1884–1897 (2012).
28. Liu, X. F. et al. The role of MrbHLH1 and MrMYB1 in regulating anthocyanin biosynthetic genes in tobacco and Chinese bayberry (*Myrica rubra*) during anthocyanin biosynthesis. *Plant. Cell. Tiss. Organ. Cult.* **115**, 285–298 (2013).
29. Li, T. et al. Anthocyanin biosynthesis in goji berry is inactivated by deletion in a bHLH transcription factor *LrLAN1b* promoter. *Plant. Physiol.* **195**, 1461–1474 (2024).
30. Xu, P. et al. Identification of MBW complex components implicated in the biosynthesis of flavonoids in woodland strawberry. *Front. Plant. Sci.* **12**, 774943 (2021).
31. Jaakola, L. et al. Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonol levels during bilberry fruit development. *Plant. Physiol.* **130**, 729–739 (2002).
32. Karppinen, K., Zoratti, L., Nguyenquynh, N., Häggman, H. & Jaakola, L. On the developmental and environmental regulation of secondary metabolism in *Vaccinium* spp. berries. *Front. Plant. Sci.* **7**, 655 (2016).
33. Suvanto, J., Karppinen, K., Riihinen, K., Jaakola, L. & Salminen, J. P. Changes in the proanthocyanidin composition and related gene expression in bilberry (*Vaccinium myrtillus* L.) tissues. *J. Agric. Food Chem.* **68**, 7378–7386 (2020).
34. Karppinen, K. et al. Changes in the abscisic acid levels and related gene expression during fruit development and ripening in bilberry (*Vaccinium myrtillus* L.). *Phytochemistry* **95**, 127–134 (2013).
35. Karppinen, K., Tegelberg, P., Häggman, H. & Jaakola, L. Absciscic acid regulates anthocyanin biosynthesis and gene expression associated with cell wall modification in ripening bilberry (*Vaccinium myrtillus* L.) fruits. *Front. Plant. Sci.* **9**, 1259 (2018).
36. Bai, Q., Huang, Y. & Shen, Y. The physiological and molecular mechanism of abscisic acid in regulation of fleshy fruit ripening. *Front. Plant. Sci.* **11**, 619953 (2021).
37. Fenn, M. A. & Giovannoni, J. J. Phytohormones in fruit development and maturation. *Plant. J.* **105**, 446–458 (2021).
38. Perotti, M. F., Posé, D. & Martin-Pizarro, C. Non-climacteric fruit development and ripening regulation: 'the phytohormones show'. *J. Exp. Bot.* **74**, 6237–6253 (2023).
39. Karppinen, K. et al. MYBA and MYBPA transcription factors co-regulate anthocyanin biosynthesis in blue-coloured berries. *New. Phytol.* **232**, 1350–1367 (2021).
40. Li, X. et al. De novo sequencing and comparative analysis of the blueberry transcriptome to discover putative genes related to antioxidants. *Gene* **511**, 54–61 (2012).
41. Song, Y. et al. High-throughput sequencing of highbush blueberry transcriptome and analysis of basic helix-loop-helix transcription factors. *J. Integr. Agric.* **16**, 591–604 (2017).
42. Zhao, M. et al. Identification and characterization of MYB-bHLH-WD49 regulatory complex members controlling anthocyanidin biosynthesis in blueberry fruits development. *Genes* **10**, 496 (2019).
43. Günther, C. S. et al. Spatiotemporal modulation of flavonoid metabolism in blueberries. *Front. Plant. Sci.* **11**, 545 (2020).
44. Zhang, Y. et al. Identification, characterization and expression analysis of anthocyanin biosynthesis-related bHLH genes in blueberry (*Vaccinium corymbosum* L.). *Int. J. Mol. Sci.* **22**, 13274 (2021).
45. Lafferty, D. J. et al. The coordinated action of MYB activators and repressors controls proanthocyanidin and anthocyanin biosynthesis in *Vaccinium*. *Front. Plant. Sci.* **13**, 910155 (2022).
46. Lafferty, D. J. et al. Hierarchical regulation of MYBPA1 by anthocyanin- and proanthocyanidin-related MYB proteins is conserved in *Vaccinium* species. *J. Exp. Bot.* **73**, 1344–1356 (2022).
47. Wu, C. et al. A chromosome-scale assembly of the bilberry genome identifies a complex locus controlling berry anthocyanin composition. *Mol. Ecol. Res.* **22**, 345–360 (2022).
48. Guo, X. et al. Metabolome and transcriptome profiling unveil the mechanisms of light-induced anthocyanin synthesis in rabbiteye blueberry (*Vaccinium ashei*: Reade). *BMC Plant. Biol.* **22**, 223 (2022).
49. Pattanaik, S., Xie, C. H. & Yuan, L. The interaction domains of the plant Myc-like bHLH transcription factors can regulate the transactivation strength. *Planta* **227**, 707–715 (2008).
50. Niu, S. S. et al. Coordinated regulation of anthocyanin biosynthesis in Chinese bayberry (*Myrica rubra*) fruit by a R2R3 MYB transcription factor. *Planta* **231**, 887–899 (2010).
51. Zhang, Y. L. et al. Identification of a strong anthocyanin activator, VbMYBA, from berries of *Vaccinium bracteatum* Thunb. *Front. Plant. Sci.* **12**, 697212 (2021).
52. Starkevič, P. et al. Expression and anthocyanin biosynthesis-modulating potential of sweet cherry (*Prunus avium* L.) MYB10 and bHLH genes. *PLoS ONE*. **10**, e0126991 (2015).
53. Spelt, C., Quattrocchio, F., Mol, J. & Koes, R. ANTHOCYANIN1 of petunia controls pigment synthesis, vacuolar pH, and seed coat development by genetically distinct mechanisms. *Plant. Cell.* **14**, 2121–2135 (2002).
54. Rahim, M. A., Busatto, N. & Trainotti, L. Regulation of anthocyanin biosynthesis in peach fruits. *Planta* **240**, 913–929 (2014).
55. Nesi, N. et al. The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in *Arabidopsis* siliques. *Plant. Cell.* **12**, 1863–1878 (2000).
56. Zhang, F., Gonzalez, A., Zhao, M., Payne, C. T. & Lloyd, A. A network of redundant bHLH proteins functions in all TTG1-dependent pathways of *Arabidopsis*. *Development* **130**, 4859–4869 (2003).
57. Baudry, A. et al. TT2, TT8, and TTG1 synergistically specify the expression of BANYULS and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. *Plant. J.* **39**, 366–380 (2004).
58. Elomaa, P. et al. Activation of anthocyanin biosynthesis in *Gerbera hybrida* (Asteraceae) suggests conserved protein-protein and protein-promoter interactions between the anciently diverged monocots and eudicots. *Plant. Physiol.* **133**, 1831–1842 (2003).
59. Ramsay, N. A., Walker, A. R., Mooney, M. & Gray, J. C. Two basic-helix-loop-helix genes (*MYC-146* and *GL3*) from *Arabidopsis* can activate anthocyanin biosynthesis in a white-flowered *Matthiola incana* mutant. *Plant. Mol. Biol.* **52**, 679–688 (2003).

60. Butelli, E. et al. Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nat. Biotechnol.* **26**, 1301–1308 (2008).
61. Sharma, S. et al. Cyanidin based anthocyanin biosynthesis in orange carrot is restored by expression of *AmRosea1* and *AmDelila*, MYB and bHLH transcription factors. *Plant. Mol. Biol.* **103**, 443–456 (2020).
62. Piao, C., Wu, J. & Cui, M. L. The combination of R2R3-MYB gene *AmRosea1* and hairy root culture is a useful tool for rapidly induction and production of anthocyanins in *Antirrhinum majus* L. *AMB Expr.* **11**, 128 (2021).
63. Mehrrens, F., Kranz, H., Bednarek, P. & Weisshaar, B. The Arabidopsis transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis. *Plant. Physiol.* **138**, 1083–1096 (2005).
64. Takos, A. M. et al. Light-induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. *Plant. Physiol.* **142**, 1216–1232 (2006).
65. Albert, N. W. et al. A conserved network of transcriptional activators and repressors regulates anthocyanin pigmentation in eudicots. *Plant. Cell.* **26**, 962–980 (2014).
66. Li, T. et al. Transcriptome and flavonoids metabolomic analysis identifies regulatory networks and hub genes in black and white fruits of *Lycium ruthenicum* Murray. *Front. Plant. Sci.* **11**, 1256 (2020).
67. Qi, Y. et al. *PsbHHLH1*, a novel transcription factor involved in regulating anthocyanin biosynthesis in tree peony (*Paeonia suffruticosa*). *Plant. Physiol. Biochem.* **154**, 396–408 (2020).
68. Lai, B. et al. Two *LcbHLH* transcription factors interacting with *LcMYB1* in regulating late structural genes of anthocyanin biosynthesis in *Nicotiana* and *Litchi chinensis* during anthocyanin accumulation. *Front. Plant. Sci.* **7**, 166 (2016).
69. Medina-Puche, L. et al. Extensive transcriptomic studies on the roles played by abscisic acid and auxins in the development and ripening of strawberry fruits. *Funct. Integr. Genomics.* **16**, 671–692 (2016).
70. Sánchez-Gómez, C. & Posé, D. Martín-Pizarro, C. Insights into transcription factors controlling strawberry fruit development and ripening. *Front. Plant. Sci.* **13**, 1022369 (2022).
71. Tohge, T. et al. Functional genomics by integrated analysis of metabolome and transcriptome of Arabidopsis plants over-expressing an MYB transcription factor. *Plant. J.* **42**, 218–235 (2005).
72. Montefiori, M. et al. In the Solanaceae, a hierarchy of bHLHs confer distinct target specificity to the anthocyanin regulatory complex. *J. Exp. Bot.* **66**, 1427–1436 (2015).
73. Nicholas, K. B., Nicholas, H. B. Jr. & Deerfield, D. W. II. GeneDoc: analysis and visualization of genetic variation. *EMBnet News.* **4**, 1–4 (1997).
74. Edema, H., Ashraf, M. F., Samkumar, A., Jaakola, L. & Karppinen, K. Characterization of cellulases from softening fruit for enzymatic depolymerization of cellulose. *Carbohydr. Polym.* **343**, 122493 (2024).
75. Chu, H. et al. Expression of the sweetpotato R2R3-type *IbMYB1a* gene induces anthocyanin accumulation in *Arabidopsis*. *Physiol. Plant.* **148**, 189–199 (2013).

Acknowledgements

We thank Prof. Teemu H. Teeri for providing pEAQ-HT-DEST3 expression vector and Dr. Amos Samkumar for verifying VmbHLH sequences in bilberry transcriptome. Gardeners Leidulf Lund and Rigmor Reiersen are acknowledged for providing *N. benthamiana* plants.

Author contributions

KK, LR and LJ designed the experiments. KK, LR and HMS identified the *VmbHLH* genes. KK and LR with the contribution of MFA performed qPCR analyses. KK, LR and HE constructed expression vectors and conducted overexpression experiments. KK was responsible for the data analysis as well as writing the manuscript with contributions from RVE and LJ. All authors have read and approved the final manuscript.

Funding

Open access funding provided by UiT The Arctic University of Norway (incl University Hospital of North Norway)

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

Experimental research on plants, including the collection of plant material, was performed in accordance with the relevant institutional, national, and international guidelines and legislation.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-15557-w>.

Correspondence and requests for materials should be addressed to K.K.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2025