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MYBA and MYBPA transcription factors co-regulate anthocyanin biosynthesis in blue-coloured berries

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Summary

- The regulatory network of R2R3 MYB transcription factors in anthocyanin biosynthesis is not fully understood in blue-coloured berries containing delphinidin compounds.
- We used blue berries of bilberry (*Vaccinium myrtillus*) to comprehensively characterise flavonoid-regulating R2R3 MYBs, which revealed a new type of co-regulation in anthocyanin biosynthesis between members of MYBA-, MYBPA1- and MYBPA2-subgroups.
- *VmMYBA1*, *VmMYBPA1.1* and *VmMYBPA2.2* expression was elevated at berry ripening and by abscisic acid treatment. Additionally, *VmMYBA1* and *VmMYBPA1.1* expression was strongly downregulated in a white berry mutant. Complementation and transient overexpression assays confirmed VmMYBA1 and VmMYBA2 to induce anthocyanin accumulation. Promoter activation assays showed that VmMYBA1, VmMYBPA1.1 and VmMYBPA2.2 had similar activity towards dihydroflavonol 4-reductase (*DFR*) and anthocyanidin synthase (*ANS*), but differential regulation activity for UDP-glucose flavonoid 3-*O*-glucosyltransferase (*UFGT*) and flavonoid 3′5′-hydroxylase (*F3′5′H*) promoters. Silencing of *VmMYBPA1.1* in berries led to the downregulation of key anthocyanin and delphinidin biosynthesis genes. Functional analyses of other MYBPA regulators, and a member of novel MYBPA3 subgroup, associated them with proanthocyanidin biosynthesis and *F3′5′H* expression.
- The existence of 18 flavonoid-regulating *MYBs* indicated gene duplication, which may have enabled functional diversification among MYBA, MYBPA1 and MYBPA2 subgroups. Our results provide new insights into the intricate regulation of the complex anthocyanin profile found in blue-coloured berries involving regulation of both cyanidin and delphinidin branches.

Introduction

Flavonoids are a large group of polyphenols in plants. Anthocyanins, proanthocyanidins (PAs) and flavonols are the major classes of flavonoids found in almost all higher plants. PAs as astringent compounds are considered to provide defence against herbivory and pathogens in leaves and unripe fruits, while concentrations are low in ripe fruits (Czemmel *et al.*, 2012). Anthocyanins contribute to the red and blue colours in flowers and ripe fruits facilitating pollination and seed dispersal but they also have a role in protecting plants against stress (Saigo *et al.*, 2020). Fruits and berries are recognised as rich sources of anthocyanins, of which especially delphinidins and malvidins of blue-coloured berries have been recently linked to biological and health-beneficial activities (Overall *et al.*, 2017; Nagaoka *et al.*, 2019; Heysieattalab & Sadeghi, 2020).

The flavonoid biosynthetic pathway is well elucidated in plants, and consists of enzymatic steps leading to the different flavonoid classes (Tohge *et al.*, 2017). Chalcone synthase (CHS), chalcone isomerase (CHI) and flavanone 3-hydroxylase (F3H) are responsible for producing dihydroflavonol precursors for all flavonoid branches. At the branch point of flavonoid biosynthesis, flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′5′-hydroxylase (F3′5′H) direct the dihydroflavonol precursors to either the cyanidin or delphinidin branch, respectively. Flavonol synthase (FLS) directs the dihydroflavonol precursors to the flavonol route, while the action of dihydroflavonol 4-reductase (DFR) following anthocyanidin synthase (ANS) and its homologue leucoanthocyanidin dioxygenase (LDOX) contributes to both anthocyanin and PA synthesis (Jun *et al.*, 2018). The pathway to PAs involves leucoanthocyanidin reductase (LAR) producing 2,3-*trans*-2R,3S-flavan-3-ols (e.g. (+)-catechin, (+)-gallocatechin) and anthocyanidin reductase (ANR) for production of 2,3-*cis*-2R,3R-flavan-3-ols (e.g. (−)-epicatechin), 2,3-*cis*-2S,3S-flavan-3-ol and 2,3-*trans*-2S,3R-flavan-3-ols (Xie *et al.*, 2003; Gargouri *et al.*, 2010; Peng *et al.*, 2012). In the final

step in the pathway to anthocyanins, UDP-glucose flavonoid 3-*O*-glucosyltransferase (UFGT) glycosylates anthocyanidins to anthocyanins.

The transcription of the flavonoid structural genes is directly controlled by the MBW regulatory complex, consisting of R2R3 MYB transcription factors (TFs), basic helix–loop–helix (bHLH) TFs and WD40 proteins (Jaakola, 2013; Zhang *et al.*, 2019). The MYB component is considered as the main regulator in the complex to specify the target gene (Allan & Espley, 2018). This specificity is facilitated by N-terminal R2 and R3 DNA-binding domains conserved in all R2R3 MYBs, while motifs responsible for transcriptional activation or repression are usually located at the C-terminus (Dubos *et al.*, 2010; Heppel *et al.*, 2013). The expression pattern and the DNA-binding specificity of MYBs and, to some extent, bHLH proteins determine the activation of flavonoid pathway genes in plant tissues (Jaakola, 2013).

R2R3 MYBs comprise a large gene family in plants divided in 23 subgroups (SGs) regulating various metabolic pathways (Stracke *et al.*, 2001; Dubos *et al.*, 2010; Jiang & Rao, 2020). An increasing number of flavonoid-regulating *R2R3 MYBs* have been identified from various plant species and the gene homologues are generally considered to regulate the same pathways (Dubos *et al.*, 2010; Feller *et al.*, 2011; Saigo *et al.*, 2020) although in recent years there has been indication of some variation in regulation in fruit and berry bearing species (Uematsu *et al.*, 2014; Zhai *et al.*, 2016; Peng *et al.*, 2020). Some of the R2R3 MYBs specifically regulate expression of only one gene, while others have impacts on various genes and branches of the flavonoid pathway. Members of MYBA/SG6, such as AtMYB75/PAP1 and AtMYB90/PAP2 in Arabidopsis and VvMYBA1/2 in grapevine (*Vitis vinifera*) specifically contribute to anthocyanin biosynthesis by regulating *UFGT* and *DFR* expression (Takos *et al.*, 2006; Walker *et al.*, 2007; Heppel *et al.*, 2013; Ravaglia *et al.*, 2013). PA1-type and TT2/SG5/PA2-type MYBs, including VvMYBPA1 and VvMYBPA2, respectively, are generally considered as activators of PA biosynthesis (Bogs *et al.*, 2007; Terrier *et al.*, 2009). MYBF/SG7 members are positive regulators of flavonol biosynthesis (Mehrtens *et al.*, 2005; Czemmel *et al.*, 2009) while MYB5 members, including VvMYB5a/b, have been reported to modulate the biosynthesis of all flavonoid classes (Deluc *et al.*, 2006, 2008). The R2R3 MYB family also includes C2 repressors (SG4), shown to inhibit various branches in the flavonoid pathway (Dubos *et al.*, 2010; Albert *et al.*, 2014; Cavallini *et al.*, 2015). However, the overall orchestration of these key players in the coordination of different branches of flavonoid biosynthesis is not completely understood, especially in fruits and berries, which usually show complex flavonoid and anthocyanin profiles.

To deepen our understanding of the regulatory role of R2R3 MYBs in flavonoid biosynthesis in blue-coloured berries, we focused on wild European bilberry (*Vaccinium myrtillus*), which has an active flavonoid and anthocyanin metabolism. Anthocyanins accumulate in both peel and flesh at berry ripening stage, while vegetative parts are rich with PAs, which also accumulate in the berry at early developmental stages (Jaakola *et al.*, 2002; Karppinen *et al.*, 2016; Suvanto *et al.*, 2020). Due to the similar complex anthocyanin profile in both peel and flesh, with a total of 33 different anthocyanin compounds belonging to the delphinidin, cyanidin, petunidin, peonidin and malvidin classes (Jaakola *et al.*, 2002; Riihinen *et al.*, 2008; Zoratti *et al.*, 2014), bilberry has in recent years become an attractive fruit species for studying regulation of anthocyanin biosynthesis. The ripening-related anthocyanin biosynthesis of the nonclimacteric bilberry is positively regulated by abscisic acid (ABA) (Karppinen *et al.*, 2013, 2018). However, a comprehensive functional characterisation of the R2R3 MYB regulators is lacking in commercially important berries of genus *Vaccinium*. In blueberries, MYBA-type TFs of highbush blueberry (*Vaccinium corymbosum*) and rabbiteye blueberry (*Vaccinium virgatum* syn. *ashei*) were shown recently as activators of anthocyanin accumulation (Plunkett *et al.*, 2018; Die *et al.*, 2020), while VcMYBPA1 has earlier been indicated in PA biosynthesis (Zifkin *et al.*, 2012). Our earlier studies on *Vaccinium* species have suggested a role for MYBPA1-type TF in anthocyanin biosynthesis in *V. uliginosum* (Primetta *et al.*, 2015), *V. myrtillus* (Jaakola *et al.*, 2010) and in blueberry skin (Günther *et al.*, 2020) where *MYBPA1* expression is positively correlated with anthocyanin accumulation.

In this study, 18 *R2R3 MYB* genes, including a large number of PA-type members, were isolated in full length from bilberry and characterised for their roles in flavonoid biosynthesis to obtain a comprehensive overview of their regulatory function, with a particular focus on berry anthocyanin biosynthesis. Functional analyses demonstrated that two MYBA-type TFs control anthocyanin biosynthesis, VmMYBA1 in berries and VmMYBA2 in vegetative tissues. A regulatory role for two members from MYBPA1 and MYBPA2 subgroups in berry anthocyanin biosynthesis and control of delphinidin branch was identified. Our results suggest a new type of ABA-induced co-regulation among MYBA, MYBPA1 and MYBPA2 TFs in ripening-associated anthocyanin biosynthesis and provide functional evidence that MYBPA1-type TF contribute to anthocyanin biosynthesis during berry ripening by directly activating key biosynthetic genes. The findings offer new insights into the regulatory mechanism of anthocyanin biosynthesis in blue-coloured berries.

Materials and Methods

Bilberry plant material

Bilberry (*V. myrtillus* L.) plants originated from a natural forest stand in Oulu (65°01′N, 25°28′E) and Tromsø (69°42′N, 18°51′E). The developmental stages of bilberry fruit as well as samples from vegetative parts were collected as described previously (Karppinen *et al.*, 2013). White fruits of the bilberry mutant were collected from a natural forest stand in Utajärvi, Finland. The mutant berries lacked anthocyanins but showed a few small red spots on their surface indicating a mutation in the regulatory pathway. ABA treatments (0.5 mM ABA, 2 mM ABA, water) of bilberry fruits were conducted in Petri dishes as described earlier (Karppinen *et al.*, 2018) and collected after 2 and 4 d of treatment. Immediately after collection, all samples were frozen in liquid nitrogen and stored at −80°C.

Isolation of *R2R3 MYB* genes

Total RNA was extracted from bilberry tissues, white mutant berries and ABA-treated berries and cDNA synthesised as described previously (Karppinen *et al.*, 2018). Full-length coding sequences of *R2R3 MYBs* were amplified from cDNA of blue-coloured bilberries by PCR, using primers designed to gene sequences identified in publicly available *Vaccinium* transcriptomes (Rowland *et al.*, 2012; Polashock *et al.*, 2014; Nguyen *et al.*, 2018). PCR products were ligated into pJET-1.2/blunt cloning vector using CloneJET PCR Cloning Kit (Thermo Fischer Scientific, Waltham, MA, USA). Sequencing of the genes was performed using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and deposited in GenBank.

Sequence alignment and phylogenetic analysis

The full-length deduced amino acid sequences of bilberry R2R3 MYBs were aligned using the CLUSTAL OMEGA (https://www.ebi.ac.uk/Tools/msa/clustalo/) and visualised using GENEDOC software (PSC, Pittsburgh, PA, USA). To functionally classify the bilberry MYBs by phylogenetic tree analysis, the amino acid sequences of previously characterised eudicot R2R3 MYBs were obtained from GenBank (Supporting Information Table S1), covering all known flavonoid-regulating MYB subgroups especially including studied fruit species. Full-length protein sequences were aligned with CLUSTALW, and a phylogenetic tree was constructed according to Hall (2013) using the maximum likelihood method with JTT+G+I model in MEGA v.6.06 (Tamura *et al.*, 2013) with 1000 bootstrap replicates.

Gene expression analysis in bilberry tissues

Real-time quantitative reverse transcription PCR (qRT-PCR) analyses were performed as described previously (Karppinen *et al.*, 2018). The gene-specific primer sequences are listed in Table S2. Glyceraldehyde-3-phosphate dehydrogenase (*VmGAPDH*) was used as a reference gene. For hierarchical clustering analysis, the normalised relative gene expression data were converted to $\log_2$ values, and clustering performed by Pearson correlation using EXPANDER software (http://acgt.cs.tau.ac.il/expander/).

Transient overexpression assays

For functional characterisation of MYBs, the full-length coding regions of *VmMYBA*-, *VmMYB7*- and all the *VmMYBPA*-type genes were amplified by PCR with gene-specific primers (Table S3). *AtbHLH2* (GenBank accession no. AF251687) was amplified from Arabidopsis cDNA by PCR with gene-specific primers (Table S3). The amplified PCR products were digested using restriction enzymes (Thermo Fischer Scientific) as described in Table S3, and ligated into cloning site of expression vector pGreenII 0029 62-SK under the control of *CaMV35S* promoter constructed earlier by Hellens *et al.* (2005).

The constructed vectors were transformed into electrocompetent *Agrobacterium tumefaciens* (GV3101) cells, followed by growth on LB agar medium supplemented with selective antibiotics at 28°C. Harvested cells were resuspended in infiltration buffer (10 mM MES (pH 5.6), 10 mM $MgCl_2$, 200 μM acetosyringone) to reach an OD_{600} of 0.5. After incubation at room temperature for 2–3 h, the *Agrobacterium* solution was infiltrated into the abaxial side of leaves of 5-wk-old *Nicotiana benthamiana* (lab strain) using a syringe. *Agrobacterium* cells containing *MYB* constructs were introduced to leaves alone or with an equivalent dose of *Agrobacterium* cells containing the constructs with *AtbHLH2* from Arabidopsis (Feng *et al.*, 2015). Empty vector or vector containing only *AtbHLH2* served as a negative control to reveal the effect of *MYB* gene expression. Infiltration sites were collected 6 d after infiltration and stored at −80°C until they were used for qPCR analyses and measurement of flavonoids. At least three plants were transformed with each construct, and each transformation was repeated at least twice. The overexpression of *VmMYBA1* and *VmMYBA2* was repeated using pNWA101 or pHEX expression vectors with peach (*Prunus persica*) *PpbHLH3* construct with similar results.

For qRT-PCR analyses, total RNA was extracted from infiltrated sites of *N. benthamiana* leaves using a Spectrum™ Plant Total RNA kit (Sigma, St Louis, MO, USA) with on-column DNase I (Sigma) digestion followed by cDNA synthesis using SuperScript IV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). A MiniOpticon instrument and CFX MANAGER software 2.0 (Bio-Rad, Hercules, CA, USA) with SsoFast™ EvaGreen Supermix (Bio-Rad) was utilised with gene-specific primers (Table S4). The qRT-PCR conditions included an initial incubation at 95°C for 30 s followed by 40 cycles of 95°C for 5 s, and 60°C for 10 s. The relative expression was normalised to the expression of *NbActin*. The amplification of only one product in qRT-PCR analyses was confirmed by a melting curve analysis.

Biolistic complementation assays

To verify that *VmMYBA1* and *VmMYBA2* genes can complement anthocyanin mutation, the coding sequences of the genes were amplified by PCR using gene-specific primers (Table S3), cloned into pENTR-D-TOPO and recombined into the binary vector pNWA101 under the control of a *CaMV35S* promoter by LR clonase II (Life Technologies, Carlsbad, CA, USA). Biolistic experiments were performed as described earlier for *Antirrhinum majus rosea*^{dorsea} (*myb*$^{-}$) plants (Schwinn *et al.*, 2016), which lacks anthocyanin pigmentation in petals. The lack of pigmentation is due to a mutation in the *MYBA*-type gene providing an effective tool to assess the ability of *MYBA* genes to complement the mutation (Schwinn *et al.*, 2006). *Antirrhinum* plants were grown under standard growth conditions without supplemental light in a glasshouse that was heated at 15°C and ventilated at 25°C. *35S:GFP-ER* construct, which localises the GFP signal to the endoplasmic reticulum (Haseloff *et al.*, 1997), was co-transformed as an internal control. *35S:GFP* alone served as the negative control. At least three flowers were transformed with each construct and each transformation was repeated at least twice.

Promoter activation assays

To confirm MYB interaction with promoters of anthocyanin biosynthetic genes of *Vaccinium* origin, the vectors above for biolistic complementation assays were used for VmMYBA1 and VmMYBA2. The coding sequences of *VmMYBPA1.1* and *VmMYBPA2.2* were amplified by PCR using gene-specific primers (Table S3) and cloned as described above. The promoter fragment for *UFGT* was isolated from *V. virgatum* 'Velluto Blue' genomic DNA and promoters of *F3′5′H* and *ANS* from *V. corymbosum* and inserted into the pGreenII 0800-Luc vector (Table S3). The *DFR* promoter of *V. virgatum* described by Plunkett *et al.* (2018) was also used. Dual luciferase assays were performed on leaves of 5-wk-old *N. benthamiana* by *Agrobacterium* infiltration as previously described (Hellens *et al.*, 2005), with at least three independent plants. *VmMYB* constructs were tested in combination with *PpbHLH3* (Zhou *et al.*, 2015b). The reporter gene for β-glucuronidase (GUS) under the control of the *35S* promoter, or *PpbHLH3* alone were used as negative controls.

Virus-induced gene silencing

To analyse the effect of virus-induced gene silencing (VIGS) on berries, bilberry plants with their roots were harvested at the stage when fruits were small unripe green and were placed in boxes (50 cm × 70 cm) with forest peat soil. The VIGS experiment was performed according to the protocol described in Karppinen *et al.* (2018). A fragment of *VmMYBPA1.1* (243 bp) was PCR-amplified with gene-specific primers (Table S3) and introduced into the pTV00 vector which was subsequently transformed into *A. tumefaciens* cells (GV3101). At least 150 unripe green bilberries in six individual bushes/boxes were injected and the experiment was repeated twice. As a control, only *Agrobacterium* with pBINTRA6-vector was injected into the berry. Both silenced and control plants were grown at 18°C with 60% humidity and 125 μmol m^{-2} s^{-1} light intensity as described previously (Karppinen *et al.*, 2018) before berries were collected after *c.* 10 d of injection, and stored at −80°C until used for RNA extraction. RNA extraction and qRT-PCR were performed similarly as described above for *N. benthamiana* leaves using gene-specific primers (Table S2). The relative expression was normalised to the expression of *VmGAPDH*.

Determination of flavonoids

For berries, frozen tissues were ground to a fine powder with a mortar and pestle under liquid nitrogen and 0.1 g tissue powder was extracted and analysed for total anthocyanins as described previously (Karppinen *et al.*, 2018). Anthocyanins, PAs and flavonols from *N. benthamiana* leaves were analysed by liquid chromatography–high resolution accurate mass–mass spectrometry (LC–HRAM–MS). Freeze-dried, ground leaf samples (24 mg) were extracted in 1 ml ethanol/water/formic acid (80 : 20 : 1, v/v/v) and diluted (2×) with methanol, prior analysis by LC–HRAM–MS as described in Methods S1. All chemical analyses were performed with at least three biological replicates.

Accession numbers

The sequence data in this article have been deposited into GenBank under accession numbers indicated in Table S5.

Results

MYBPA-type TFs possess a large group among flavonoid-regulating R2R3 MYBs

Many important fruit and berry crops have been identified with multiple R2R3 MYB TFs regulating their flavonoid pathways (Czemmel *et al.*, 2012; Ravaglia *et al.*, 2013; Schaart *et al.*, 2013; Zhai *et al.*, 2016; Zhou *et al.*, 2016; Wang *et al.*, 2017). To study the regulatory network of MYBs in flavonoid biosynthesis in blue-coloured berries, we isolated full-length coding sequences of 18 putative flavonoid-regulating *R2R3 MYB* genes from bilberry. Some of the sequences had close identity with one another but clearly represented separate genes (Table S5). The phylogenic clustering and the alignment of the amino acid sequences, showing the presence of N-terminal R2 and R3 DNA-binding domains (Figs 1, 2a), confirmed the bilberry sequences as members of R2R3 MYB regulators. With the exception of VmMYBF, the bilberry R2R3 MYBs are predicted to interact with a bHLH partner (Fig. 2). Unlike MYBs controlling anthocyanin and PA biosynthesis, flavonol biosynthesis regulating MYBs (MYBF, SG7) act independently of a bHLH partner (Mehrtens *et al.*, 2005).

Analysis of signature sequence motifs and phylogenetic comparison with previously characterised eudicot flavonoid-regulating R2R3 MYBs were used to functionally classify the bilberry MYBs. VmMYBA1 and VmMYBA2 clustered in a phylogenetic tree close to the blueberry VcMYBA within the R2R3 MYB subgroup 6 (SG6; Stracke *et al.*, 2001) which regulates anthocyanin biosynthesis (Fig. 1). Both sequences contained an [A/S]NDV motif, an Arg residue in the R2 domain, a Val residue in the R3 domain and motif 6 (Figs 2a,b, S1), all described to be conserved among eudicot anthocyanin-regulating MYBs (Table S6), suggesting related function for VmMYBA1 and VmMYBA2. In all other MYBs, the [A/S]NDV motif was changed to NDEI or DNEV (Fig. 2a), as commonly found in PA-type MYBs and C2 repressors (Table S6).

Our analysis identified a large group of PA-type MYBs. Five of them fell into a PA2/TT2 clade (SG5) in the phylogenetic tree (Fig. 1) and contained a signature TT2-box motif (Figs 2b, S1; Table S6). VmMYBPA1.1 and VmMYBPA1.2 were identified as being similar to PA1-type MYBs (Fig. 1) and containing C1, PA1 and G-28 motifs (Figs 2b, S1; Table S6). Instead, VmMYBPA3 formed its own group with *Medicago truncatula* MtPAR (Fig. 1) and contained no previously described C-terminal motifs in its sequence (Figs 2b, S1). Two R2R3 MYBs were designated as VmMYB5a and VmMYB5b based on their close phylogenetic relationship with other MYB5 activators (Fig. 1) and presence of C1 and C3 motifs (Figs 2b, S1; Table S6). VmMYBF was identified as a flavonol-specific MYB (SG7) based on phylogenetic analysis (Fig. 1) and the presence of

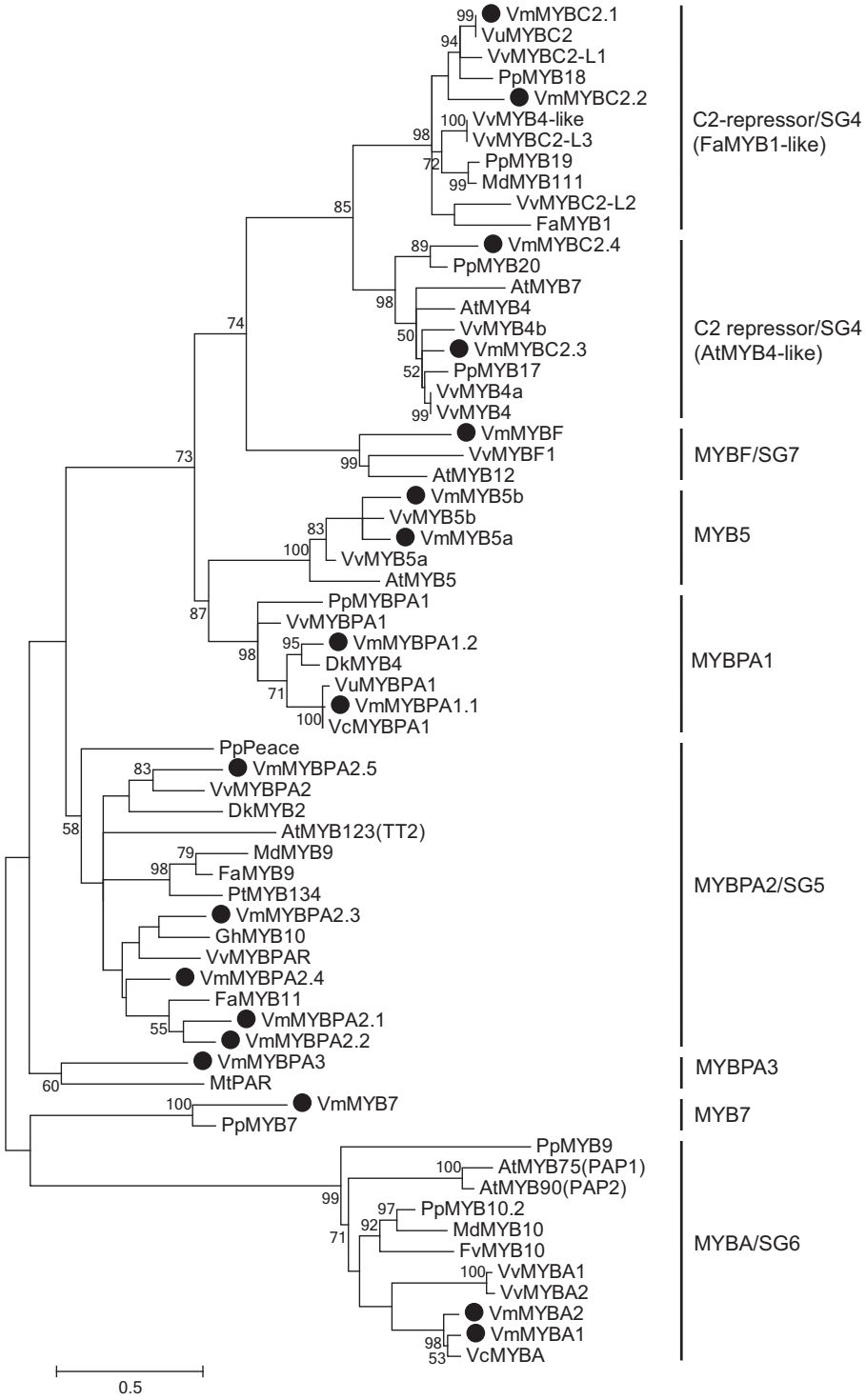

Fig. 1 Phylogenetic analysis of flavonoid-related R2R3 MYBs. The bilberry R2R3 MYBs are indicated with black circles. The numbers near branches indicate bootstrap estimates for 1000 replicates (only values > 50% are shown). Bar, 0.5 substitutions per site. The R2R3 MYB sequences were classified into nine flavonoid-related MYB subclades, including MYBA/SG6 associated with anthocyanin biosynthesis, PA1-type and PA2-type/TT2/SG5 MYBs generally associated with proanthocyanidin biosynthesis, MYBF/SG7 associated with flavonol biosynthesis, MYB5 associated with general flavonoid biosynthesis, C2 repressor group (SG4) under two subclades (FaMYB1-like and AtMYB4-like), MYB7 subclade and new MYBPA3 subclade described in this study. At, *Arabidopsis thaliana*; Dk, *Diospyros kaki*; Fa, *Fragaria* × *ananassa*; Fv, *Fragaria vesca*; Gh, *Gossypium hirsutum*; Md, *Malus* × *domestica*; Mt, *Medicago truncatula*; Pp, *Prunus persica*; Pt, *Populus tremuloides*; Vc, *Vaccinium corymbosum*; Vm, *Vaccinium myrtillus*; Vu, *Vaccinium uliginosum*; Vv, *Vitis vinifera*.

SG7 and SG7-2 motifs (Figs 2b, S1; Table S6). VmMYB7 grouped together with peach PpMYB7 showing no previously described motifs (Figs 1, 2b, S1).

In addition, four MYBs were identified as C2 repressors based on the presence of C1 and C2 motifs (Figs 2b, S1; Table S6), and clustered in a phylogenetic tree into the C2 repressor clade (SG4) under two subclades (Fig. 1), which have been described previously (Chen *et al.*, 2019). VmMYBC2.1 and VmMYBC2.2 were identified as members of the subclade D2 of C2 repressors by showing an additional TLLLFR/C5 repression motif (Figs 2b, S1; Table S6) and a characteristic amino acid substitution from DNEI to DNEV (Cavallini *et al.*, 2015; Chen *et al.*, 2019). Instead, VmMYBC2.3 was directed to subclade A of C2 repressors, due to the presence of the C4 motif (Figs 2b, S1; Table S6).

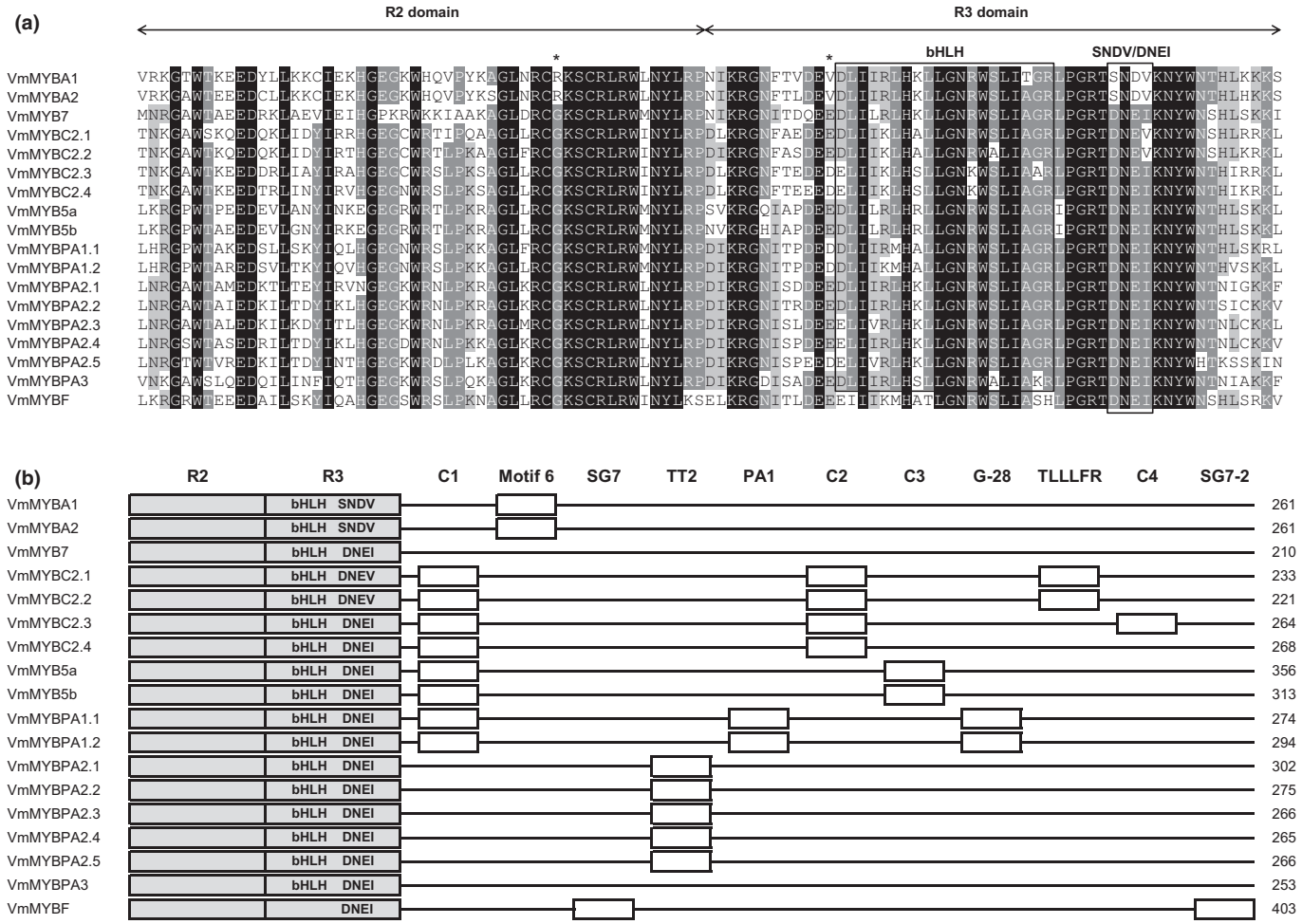

Fig. 2 Identification of conserved domains in bilberry R2R3 MYBs. (a) Amino acid sequence alignment of R2 and R3 domains of VmMYBs. Conserved residues are highlighted in black, and partial conservation is indicated in grey. The conserved regions of the bHLH interacting motif and 'SNDV/DNEI' motif are shown in boxes. Asterisks indicate the Arg residue in R2 domain and the Val residue in R3 domain, conserved among anthocyanin-regulating MYBs. (b) Schematic diagram of organisation of conserved domains and motifs in VmMYB sequences. Numbers on the right indicate length in amino acids. Motif features are described in Supporting Information Table S6.

The bilberry MYBs were named according to the sequence analysis. Generally, the naming followed the style used in grapevine, reflecting their proposed functions. The earlier described bilberry *VmMYB1* and *VmMYB2* genes (Jaakola *et al.*, 2010) were renamed as *VmMYBC2.1* and *VmMYBPA1.1*, respectively. As VmMYBPA3 did not show any signature motifs of PA1 or PA2 subgroups, it was named under a new subgroup, MYBPA3, together with *M. truncatula* MtPAR, which also does not contain any previously described C-terminal motifs but has been shown to regulate PA biosynthesis (Verdier *et al.*, 2012).

*R2R3 MYB*s show differential expression profiles with *VmMYBA1* and *VmMYBPA1.1* expression similar to anthocyanin biosynthetic genes

To investigate the spatial and temporal expression patterns of the *VmMYB* genes and to correlate the expression to their target genes for providing clues into their function, the measurements of transcript abundance followed by hierarchical clustering analysis were performed for various tissues (berry, leaf, stem, rhizome) as well as for different stages of berry development. In bilberry, PAs accumulate at the early stages of berry development while anthocyanins begin to accumulate at fruit ripening (Jaakola *et al.*, 2002; Karppinen *et al.*, 2016; Suvanto *et al.*, 2020). Our results revealed that the transcripts of *VmMYBA1* and *VmMYBPA1.1* were most highly associated with ripening fruit, showing a similar expression pattern to the structural genes related to anthocyanin biosynthesis (Figs 3, S2, S3), suggesting the involvement in regulation of anthocyanin accumulation in berry. The pattern of *VmMYBPA1.1* expression most closely resembled that of *VmANS* and *VmCHS*, while *VmMYBA1* correlated most closely with *VmF3H* and *VmUFGT* expression (Fig. 3), which might reflect their regulatory targets.

For all the other *VmMYB* genes, the expression was found to be highest in tissues other than berry, although transcripts of *VmMYBC2.1*, *VmMYBC2.2*, *VmMYB5a* and *VmMYB5b* were also detected at relatively high levels in berries (Figs 3, S2), indicating a role both in reproductive and vegetative tissues.

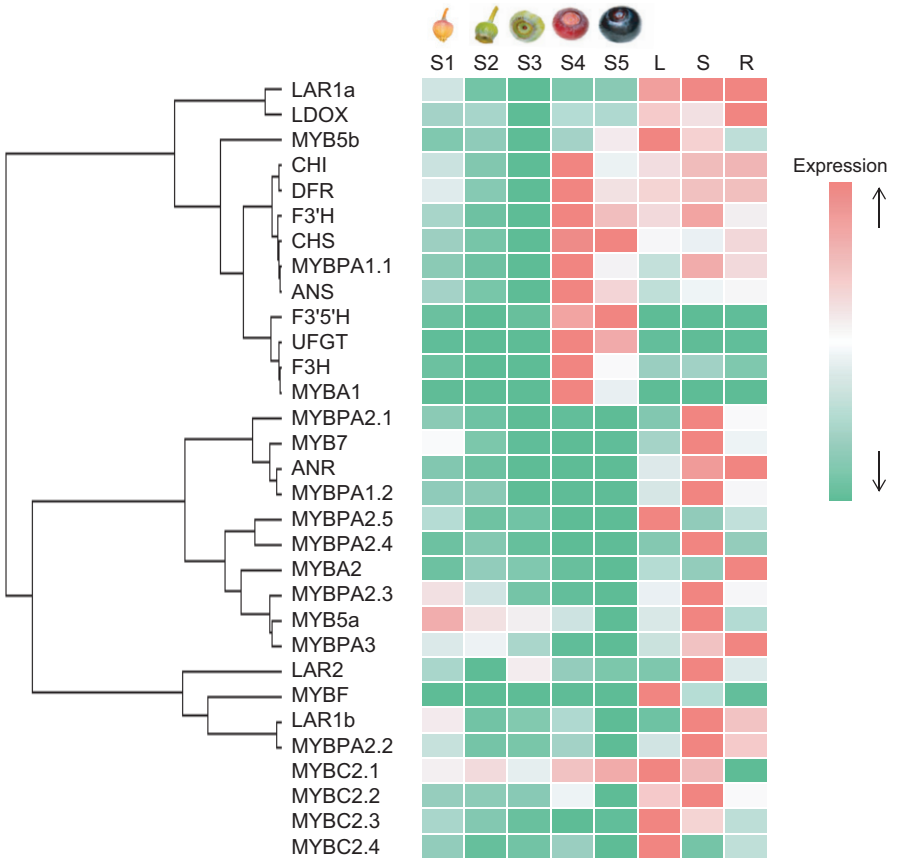

Fig. 3 Expression profiles of *VmMYBs* compared with flavonoid structural genes in bilberry tissues. Red and green boxes indicate high and low relative expression levels, respectively, of four biological replicates. Hierarchical clustering analysis was performed based on the expression levels during berry development and only included positive *VmMYBs*. S1, flower; S2, small unripe green berry; S3, large unripe green berry; S4, ripening purple berry; S5, fully ripe blue berry; L, leaf; S, stem; R, rhizome.

Furthermore, the upregulated expression of *VmMYBC2.1*, *VmMYBC2.2*, *VmMYBC2.4*, *VmMYB5b* and *VmMYBPA2.2* at the stage of berry ripening may imply an association with berry anthocyanin biosynthesis. Conversely, the transcript levels of *VmMYBA2*, *VmMYB7*, *VmMYBC2.3*, *VmMYB5a*, *VmMYBPA1.2*, *VmMYBPA2.1*, *VmMYBPA2.3*, *VmMYBPA2.4*, *VmMYBPA2.5* and *VmMYBPA3* were highest in flower or at the early berry developmental stage accompanied by a decreasing trend towards berry ripening (Figs 3, S2), suggesting that some of them might be associated with the regulation of PA biosynthesis in unripe berries or flavonoid biosynthesis in flowers. Association with PA biosynthesis was also supported by their expression resembling most closely that of *VmANR* expression (Fig. 3). However, the high transcript abundance of *VmMYBA2*, *VmMYBC2.3*, *VmMYBPA1.2*, *VmMYBPA2.1*, *VmMYBPA2.4*, *VmMYBPA2.5* and *VmMYBF* in stem and/or rhizome and/or green leaves (Figs 3, S2) suggest roles mainly in vegetative tissues.

Expression of *VmMYBA1* and *VmMYBPA1.1* is upregulated by ABA and downregulated in a white berry mutant

To investigate the gene expression of the *VmMYBs* more closely in berries, transcript levels were measured in berries with accelerated or suppressed anthocyanin biosynthesis. ABA has been recognised as a major positive regulator and accelerator of ripening and anthocyanin biosynthesis in nonclimacteric fruit, such as bilberry (Karppinen *et al.*, 2013, 2018; Chen *et al.*, 2020). Therefore, we hypothesised that *R2R3 MYB* genes that have a role in berry anthocyanin biosynthesis would be upregulated in berries under ABA treatment. Our results demonstrated that exogenous ABA applied to unripe berries upregulated the expression of especially *VmMYBA1* and *VmMYBPA1.1*, even at the lower ABA concentration (Fig. 4a), suggesting that these genes are under the hormonal control of ABA and are able to react sensitively to the ABA signal at the time of berry ripening. Also, the expression of *VmMYBC2.1*, *VmMYBC2.2*, *VmMYB5b*, *VmMYBPA1.2*, *VmMYBPA2.1* and *VmMYBPA2.2* was significantly induced by ABA (Fig. 4a). Conversely, the transcript levels of *VmMYBA2*, *VmMYB7*, *VmMYBC2.3*, *VmMYBPA2.3*, *VmMYBPA2.4* and *VmMYBPA3* were significantly downregulated by ABA, indicating that they may regulate pathways that are not induced at the time of berry ripening, such as the PA pathway, or have a functional role in tissues other than berries.

In addition, *VmMYB* transcript levels compared with those of flavonoid structural genes were quantified in the naturally occurring white mutant of bilberry lacking anthocyanins (Fig. 4b). The expression of anthocyanin and PA biosynthetic genes was generally downregulated in mutant berries, with the exception of *VmLAR2* (Fig. 4c). Also, the expression of most of the *VmMYBs* predicted to be associated with anthocyanin or PA regulation was decreased. In particular, the expression of *VmMYBA1* and *VmMYBPA1.1*, along with *VmCHS*, *VmANS* and *VmUFGT*, was strongly downregulated and barely detectable in the mutant

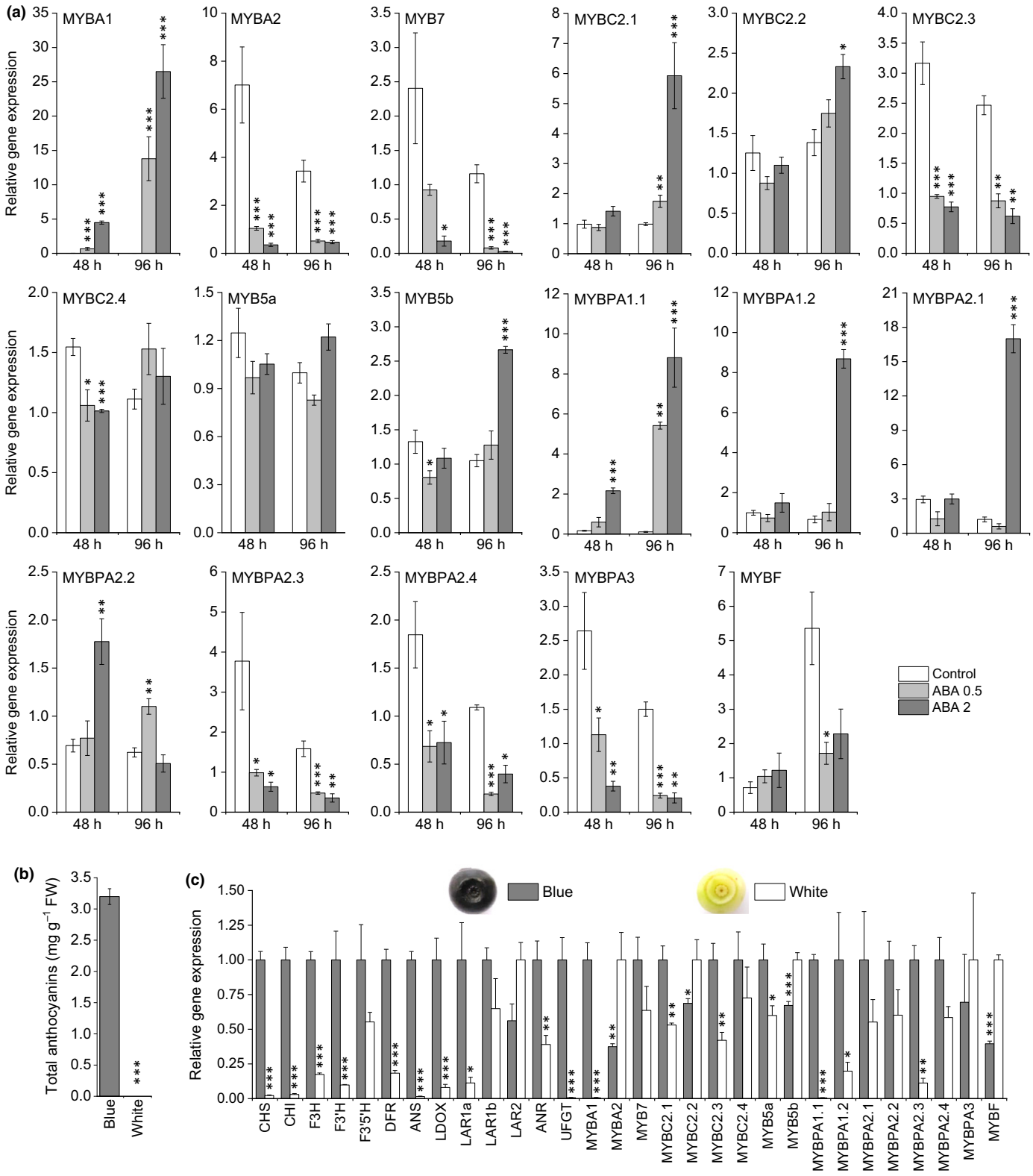

Fig. 4 Expression analysis of *VmMYBs* in berries with accelerated and suppressed anthocyanin biosynthesis. (a) Expression of *VmMYBs* in abscisic acid (ABA)-treated bilberries. The relative expression of the genes was quantified after 48 and 96 h from the beginning of the treatment (0.5 mM ABA, 2 mM ABA or water as negative control). Values represent the means ± SEs of three biological replicates. (b) Total anthocyanin content (expressed as mg of cyanidin-3-glucoside equivalents g^{-1} FW) in blue wild-type bilberry and naturally occurring white mutant bilberry. (c) Expression of *VmMYBs* and flavonoid structural genes in white mutant bilberry compared with blue bilberry. Values represent means ± SEs of four biological replicates. Asterisks indicate significant differences from control according to Student's *t*-test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) on log-transformed data. *VmMYBPA2.5* was not expressed in ripening/ripe berry.

berries (Fig. 4c), suggesting their essential roles in the white berry phenotype. The expression of the positive regulators *VmMYBA2*, *VmMYB5b* and *VmMYBF*, was upregulated in the white mutant berries, indicating that their transcript levels are not determining the lack of berry anthocyanins.

Members from MYBA and MYBPA regulators upregulate *F3′5′H* and genes associated with both anthocyanin and PA biosynthesis

To functionally characterise VmMYBs predicted in PA or anthocyanin biosynthesis, *Agrobacterium*-mediated transient overexpression assays in *N. benthamiana* leaves were performed. Accumulation of delphinidin 3-rutinoside and small amounts of flavan-3-ols were detected in leaves infiltrated with constructs containing *VmMYBA1* and *VmMYBA2* genes (Figs 5, S4, S5). The addition of a *bHLH* partner construct had no benefit for the accumulation of delphinidin 3-rutinoside, but was required for the production of gallocatechin. The pigmentation on leaves was more intense with *VmMYBA1* than with the *VmMYBA2* construct (Figs 5a, S4). All PA-type *VmMYBs* overexpressed with the *bHLH* partner enabled the accumulation of gallocatechin (Figs 5c, S5) and some of the PA2-type and PA3-type members in addition small amounts of other flavan-3-ols (Figs 5d, S5). Overexpression of *VmMYBs* generally decreased the content of flavonols, with the exception of some *VmMYBs* leading to the accumulation of myricetin glycosides from delphinidin branch (Fig. S6). Therefore, our results indicated dihydroflavonol precursor direction to delphinidin branch instead of cyanidin and flavonol branches by VmMYB TFs. No anthocyanins or PAs was detected in leaves infiltrated with *bHLH* construct alone or empty vector.

The infiltration sites of *N. benthamiana* leaves were confirmed for the presence of transgene expression (Fig. S7) followed by analyses of flavonoid structural gene expression to reveal the specific regulatory impacts on flavonoid biosynthesis. *VmMYBA1* and *VmMYBA2* overexpression induced the expression of all the anthocyanin and PA biosynthetic genes (with the exception of *NbLAR* and *NbF3′H* for *VmMYBA2*) in *N. benthamiana* leaves (Fig. 6a), in accordance with our chemical analyses. The expression of *NbF3′5′H*, *NbDFR*, *NbANS* and *NbUFGT* was most strongly induced by *VmMYBA1* and *VmMYBA2* overexpression, suggesting a role as a regulator of the anthocyanin pathway and delphinidin branch. As the expression of *N. benthamiana* endogenous TF *NbAN1*, a bHLH involved in anthocyanin biosynthesis (Montefiori *et al.*, 2015), also showed induction by *VmMYBA1* and *VmMYBA2* overexpression, we can assume that the VmMYBA1 and VmMYBA2 action in *N. benthamiana* is most likely mediated through the activation of *NbAN1*. Such hierarchical regulation of the *bHLH* genes by anthocyanin MBW complexes is well established (Albert *et al.*, 2014; Montefiori *et al.*, 2015) and our data showed that VmMYBA1 and VmMYBA2 TFs are capable of operating within these in *N. benthamiana*. This is also supported by our findings that both *VmMYBA1* and *VmMYBA2* constructs were able to induce anthocyanin accumulation without the addition of a *bHLH* partner construct (Fig. 5), while the activity of MYBA-type TFs has earlier been shown to be dependent on the interaction with a bHLH (Walker *et al.*, 2007; Huang *et al.*, 2013; Liu *et al.*, 2016). The addition of a construct containing the *AtbHLH2* partner, the TT8-type bHLH necessary for PA production in most groups of angiosperms (Zhang *et al.*, 2020), enabled the production of PAs.

For the PA-type MYBs infiltrated with *bHLH* partner, *VmMYBPA1.1* overexpression was shown to induce the expression of *NbF3′5′H*, *NbDFR*, *NbANS* and *NbLAR*, while *VmMYBPA1.2* overexpression additionally induced the expression of *NbANR* and higher rate of *NbDFR* expression (Fig. 6b), demonstrating the functional divergence between the two MYBPA1 regulators. Overexpression with the *VmMYBPA2*-type genes or *VmMYBPA3* led to the upregulation of *NbF3′5′H*, *NbDFR* and *NbANS* expression, but also showed divergence in gene induction (Fig. 6a,c). Additionally, *VmMYBPA2.2* upregulated *NbF3′H*, *NbLAR* and *NbANR* expression (Fig. 6a), *VmMYBPA2.1* and *VmMYBPA3* upregulated *NbLAR* expression, *VmMYBPA2.3* *NbLAR* and *NbANR* expression, and *VmMYBPA2.5* *NbUFGT*, *NbLAR* and *NbANR* expression (Fig. 6c), suggesting a subfunctionalisation among the PA2-type TFs. Similar to all MYBPA-type regulators, *VmMYB7* overexpression induced *NbF3′5′H* and *NbDFR* expression, indicating the importance of the *F3′5′H* regulation of delphinidin branch in blue-coloured berries.

Members from MYBA and MYBPA regulators activate promoters of *F3′5′H* and genes associated in anthocyanin biosynthesis

The regulatory role of VmMYBA1 and VmMYBA2 in anthocyanin biosynthesis was further confirmed by transient biolistic complementation assays using the *Antirrhinum rosea*^{dorsea} (Schwinn *et al.*, 2006), which lacks anthocyanin pigmentation in its petals due to mutation in the *MYB* gene, *Rosea1*. VmMYBA1 and VmMYBA2 both complemented *rosea*^{dorsea}, restoring anthocyanin pigmentation to bombarded cells (Fig. 7a), confirming VmMYBA1 and VmMYBA2 as anthocyanin regulators.

The ability of VmMYBA1, VmMYBA2, VmMYBPA1.1 and VmMYBPA2.2 to directly activate key structural genes in the flavonoid biosynthetic pathway was evaluated by promoter activation assays. VmMYBA1, VmMYBA2, VmMYBPA1.1 and VmMYBPA2.2 were all capable of strongly activating the promoters of *DFR* and *ANS* (Fig. 7b). However, differential activity was observed upon the *F3′5′H* and *UFGT* promoters. *F3′5′H* was more strongly activated by VmMYBPA1.1 and VmMYBPA2.2 than the MYBA TFs, indicating their key role in directing precursors toward the delphinidin branch. By contrast, the promoter of *UFGT* was strongly activated by VmMYBA1 and VmMYBA2, but also weakly by VmMYBPA1.1 (Fig. 7b). These findings suggested that overlapping regulation occurs for some common biosynthetic steps of anthocyanin and PA biosynthesis. The constructs only with *GUS* or *PpbHLH3* could not activate the promoters.

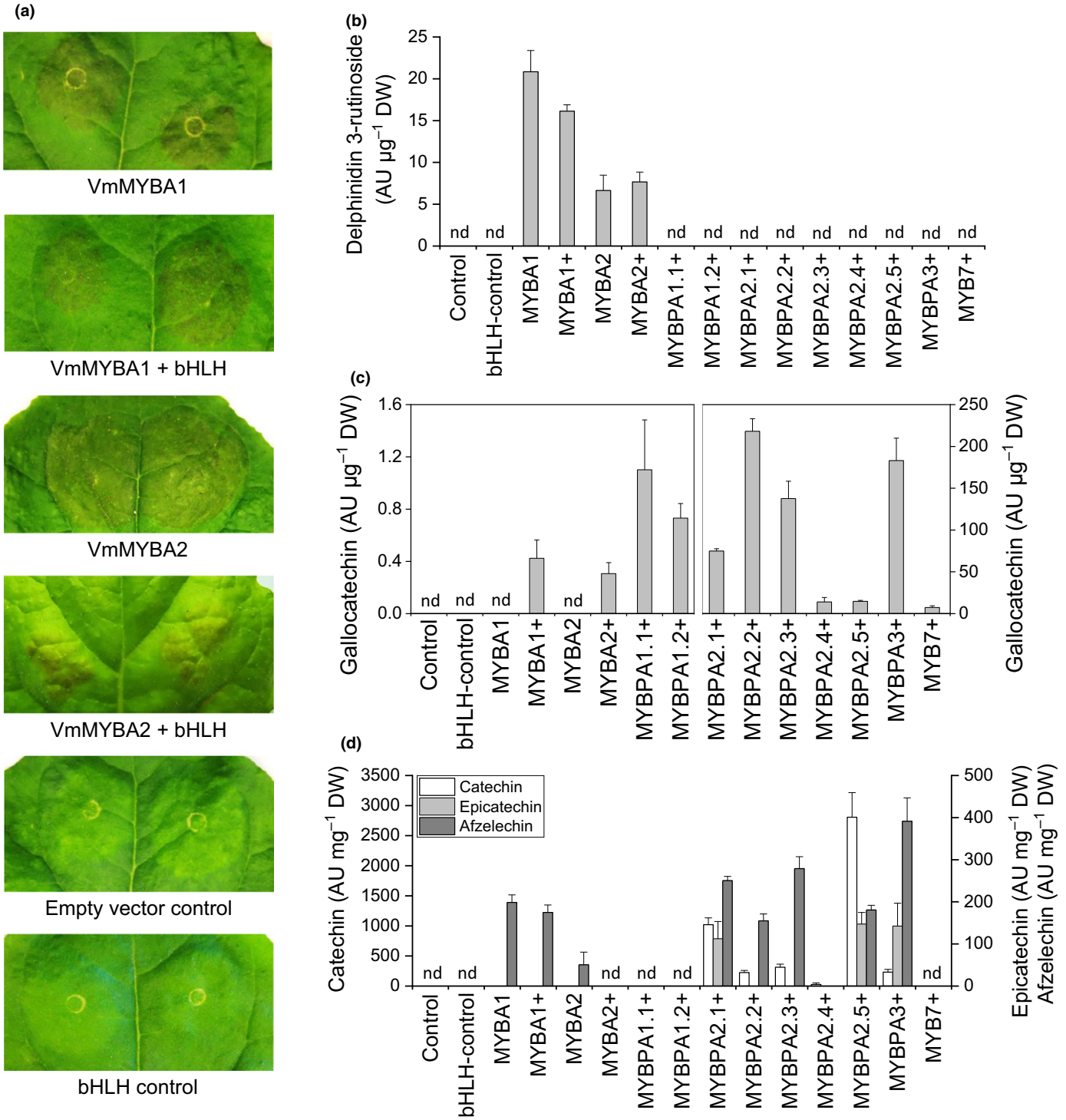

Fig. 5 Effect of transient overexpression of *VmMYBs* on anthocyanin and proanthocyanidin accumulation in *Nicotiana benthamiana* leaves. (a) Pigmentation in *N. benthamiana* leaves after 6 d of infiltration with *Agrobacterium* harbouring expression vector with *VmMYBA1* and *VmMYBA2*. Empty vector or *bHLH* alone served as negative control. (b) Content of delphinidin 3-rutinoside, (c) gallocatechin, (d) catechin, epicatechin and afzelechin in infiltration site of *N. benthamiana* leaves after 6 d of infiltration. Values represent means ± SEs of at least three biological replicates. '+' after a gene indicates overexpression with the bHLH partner, *AtbHLH2*. AU, absorbance unit; DW, dry weight; nd, not detected.

Suppression of *VmMYBPA1.1* represses anthocyanin biosynthesis and delphinidin branch in berries

To clarify the role of VmMYBPA1.1 in berry anthocyanin biosynthesis, the VIGS method was used to suppress *VmMYBPA1.1* expression during bilberry fruit ripening. After *c.* 10 d of injection of the *VmMYBPA1.1*-VIGS vector, chimeric fruits with green sectors at the site of injections were found (Fig. 8a), demonstrating reduced anthocyanin accumulation. The transcript levels of *VmMYBPA1.1* were confirmed to be

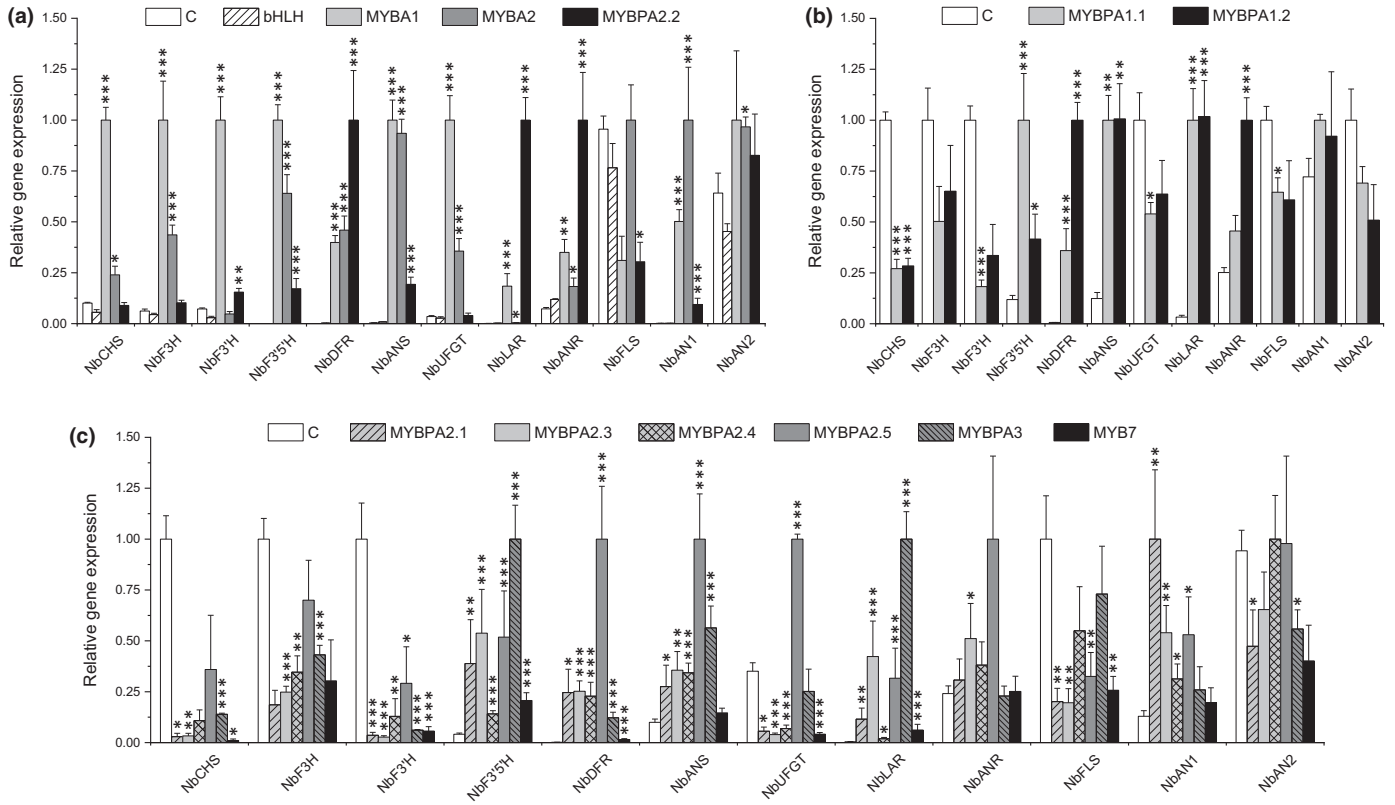

Fig. 6 Effect of transient overexpression of *VmMYBs* on the expression of flavonoid biosynthetic genes in *Nicotiana benthamiana* leaves. (a) Gene expression after transient overexpression with *VmMYBA1*, *VmMYBA2* and *VmMYBPA2.2*. (b) Gene expression after transient overexpression with *VmMYBPA1.1* and *VmMYBPA1.2*. (c) Gene expression after transient overexpression with *VmMYBPA2.1*, *VmMYBPA2.3*, *VmMYBPA2.4*, *VmMYBPA2.5*, *VmMYBPA3* and *VmMYB7*. Relative expression of the genes was quantified from infiltration sites after 6 d of infiltration. Empty vector or *bHLH* alone served as the negative control. Values represent means ± SEs of at least three biological replicates. Asterisks indicate significant differences from control (C) according to Student's *t*-test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) on log-transformed data.

suppressed in these berries compared with control berries (Fig. 8b) accompanied by the significant downregulation of *VmCHS*, *VmF3′5′H*, *VmANS* and *VmLAR1a* expression. This suggests that VmMYBPA1.1 is an important regulator of berry anthocyanin biosynthesis and delphinidin branch genes (Suvanto *et al.*, 2020). We also found that *VmUFGT* expression was downregulated, but not significantly, by contrast with significant upregulation of *VmLDOX*, and slight but not significant upregulation of *VmLAR2* and *VmANR* (Fig. 8b). These results are likely to indicate a positive effect of VmMYBPA1.1 on specific anthocyanin biosynthesis pathway gene but negative for the competing PA pathway specific genes at the time of berry ripening.

Discussion

MYBA-type R2R3 TFs are well known as positive regulators of anthocyanin biosynthesis and are usually considered responsible for controlling anthocyanin accumulation (reviewed in Jaakola, 2013; Allan & Espley, 2018). The current knowledge of transcriptional regulation of anthocyanin and PA biosynthesis is largely based on studies that have been performed in model species and tissues such as for *Arabidopsis thaliana*, *Petunia hybrida*, *Antirrhinum majus* and red fruits of the Rosaceae family, which exhibit simpler PA and anthocyanin profiles compared with blue-coloured berries. The present study was undertaken in bilberry, the berries of which show complex anthocyanin and flavonoid profiles, including compounds produced from both cyanidin and delphinidin branches (Jaakola *et al.*, 2002; Zoratti *et al.*, 2014). Our study demonstrated, in total, 18 flavonoid pathway-regulating R2R3 MYBs from bilberry (Fig. 9), a number comparable with that found earlier in grapevine, the widest flavonoid-specific R2R3 MYB family characterised so far (Czemmel *et al.*, 2012; Table S1). A majority of the characterised bilberry MYBs was identified by sequence analysis as PA-regulating MYBs. This raised the question whether some of these PA-type MYB TFs have a regulatory role in berries beyond driving PA production. Here, we showed evidence that, in addition to the MYBA-type regulator, also two members from MYBPA1 and MYBPA2 subgroups have an essential role in berry anthocyanin biosynthesis.

From the two bilberry MYBA-type TFs showing the direct regulation of anthocyanin biosynthesis, only VmMYBA1 seems to have this role in berries, corroborating earlier results of VcMYBA in blueberry (Plunkett *et al.*, 2018; Die *et al.*, 2020). The newly identified gene family member, *VmMYBA2*, was mainly expressed in unripe berries and other plant tissues. Furthermore, its expression was suppressed by ABA in berries and upregulated in the white bilberry mutant. This demonstrates that VmMYBA2

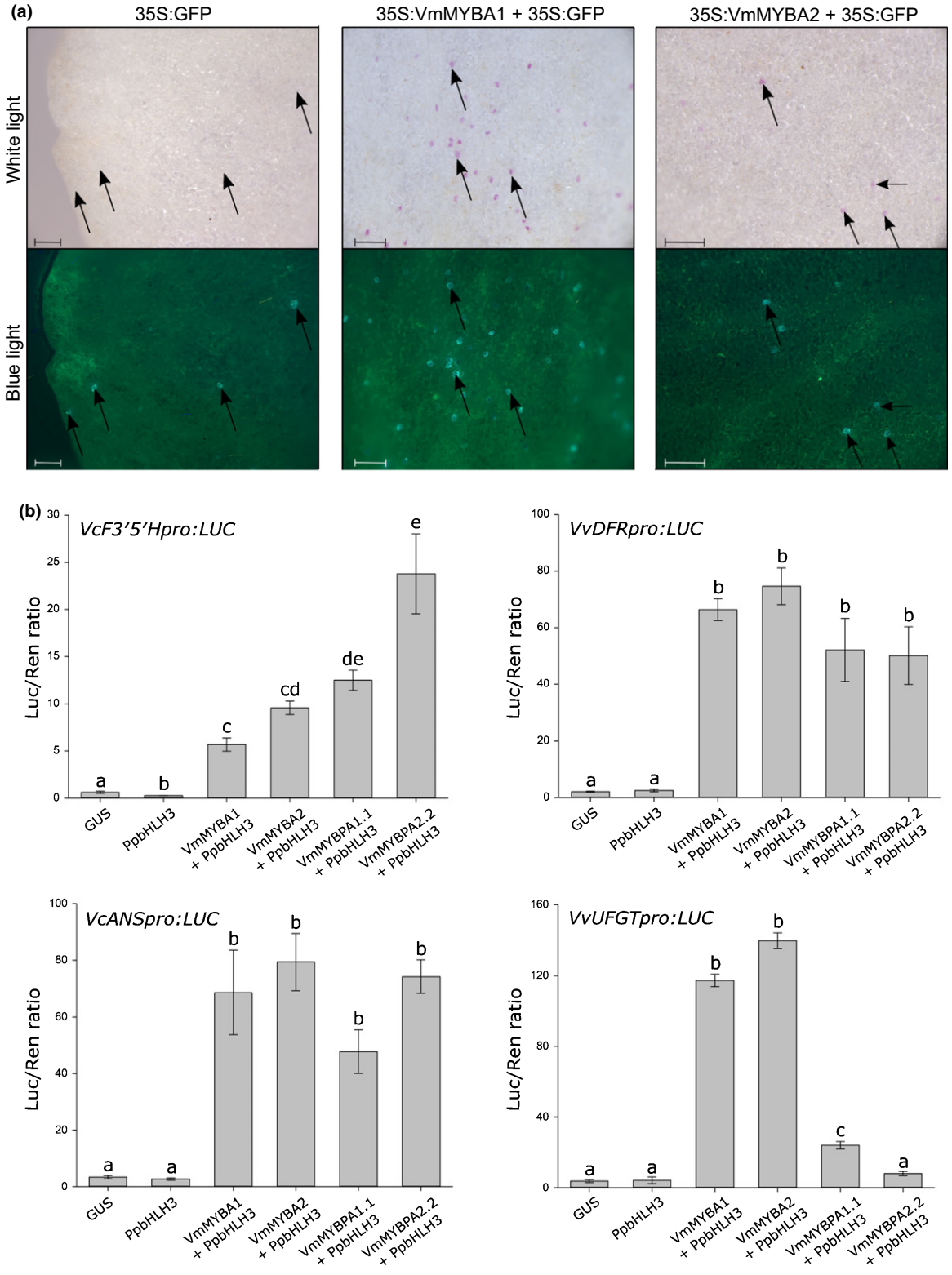

Fig. 7 Complementation and promoter activation analysis of VmMYB TFs. (a) Complementation of anthocyanin biosynthesis in *Antirrhinum majus rosea*^{dorsea} (*myb*$^{-}$) petals by VmMYBA1 and VmMYBA2. Petals were biolistically transformed with plasmid DNA containing *35S:VmMYBA1* or *35S:VmMYBA2* with *35S:GFP* (internal control) or *35S:GFP* alone (negative control). Fluorescence by GFP can be seen under blue light while anthocyanins are visible under white light. Anthocyanin pigmentation in bombarded cells is indicated by arrows. Bars, 200 μm. (b) VmMYBA1, VmMYBA2, VmMYBPA1.1 and VmMYBPA2.2 mediated activation of *F3′5′H*, *DFR*, *ANS* and *UFGT* promoters. VmMYBs were tested in combination with the bHLH partner, PpbHLH3. The constructs containing *GUS* or *bHLH* alone served as negative controls. Firefly luciferase (Luc) values are reported relative to Renilla luciferase (Ren) control. Values represent means ± SEs of at least three biological replicates. Letters indicate significant differences assessed by one-way ANOVA and Tukey's test ($P < 0.05$) on log-transformed data.

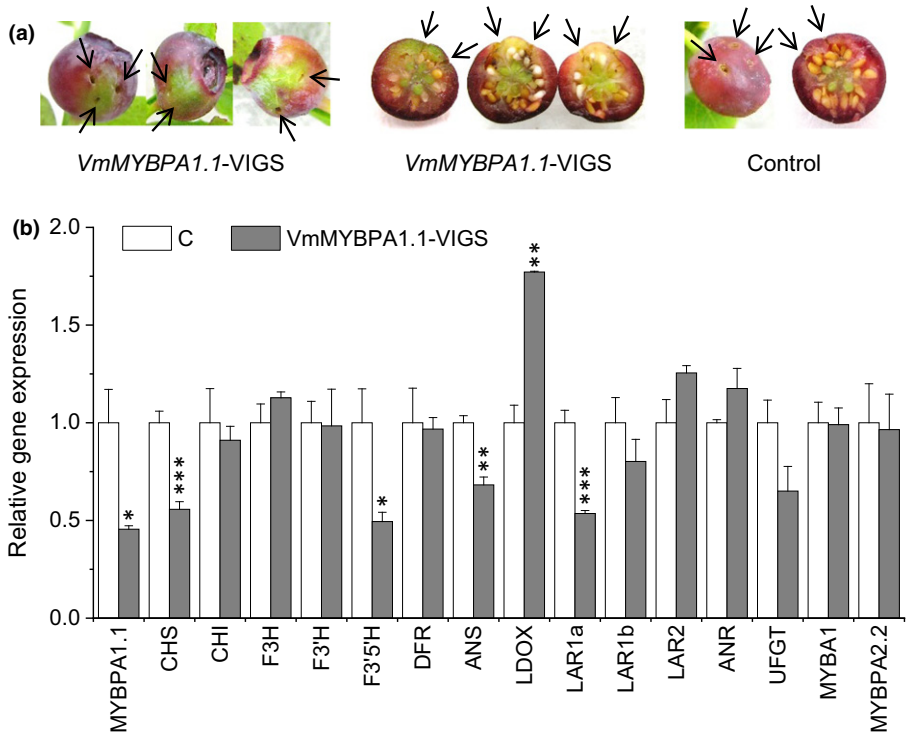

Fig. 8 Effect of suppression of *VmMYBPA1.1* expression by virus-induced gene silencing (VIGS) on the flavonoid biosynthesis in ripening bilberry fruit. (a) Pigmentation of fruits after *c.* 10 d of injection. Unripe green berries attached to the bilberry plants were injected with *VmMYBPA1.1*-VIGS vector or pBINTRA6 vector only (negative control). Arrows indicate injection sites. (b) The expression of *VmMYBPA1.1* and the key genes of flavonoid biosynthesis after *VmMYBPA1.1* suppression by VIGS. Values represent means ± SEs of three biological replicates. Asterisks indicate significant differences from control (C) according to Student's *t*-test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) on log-transformed data.

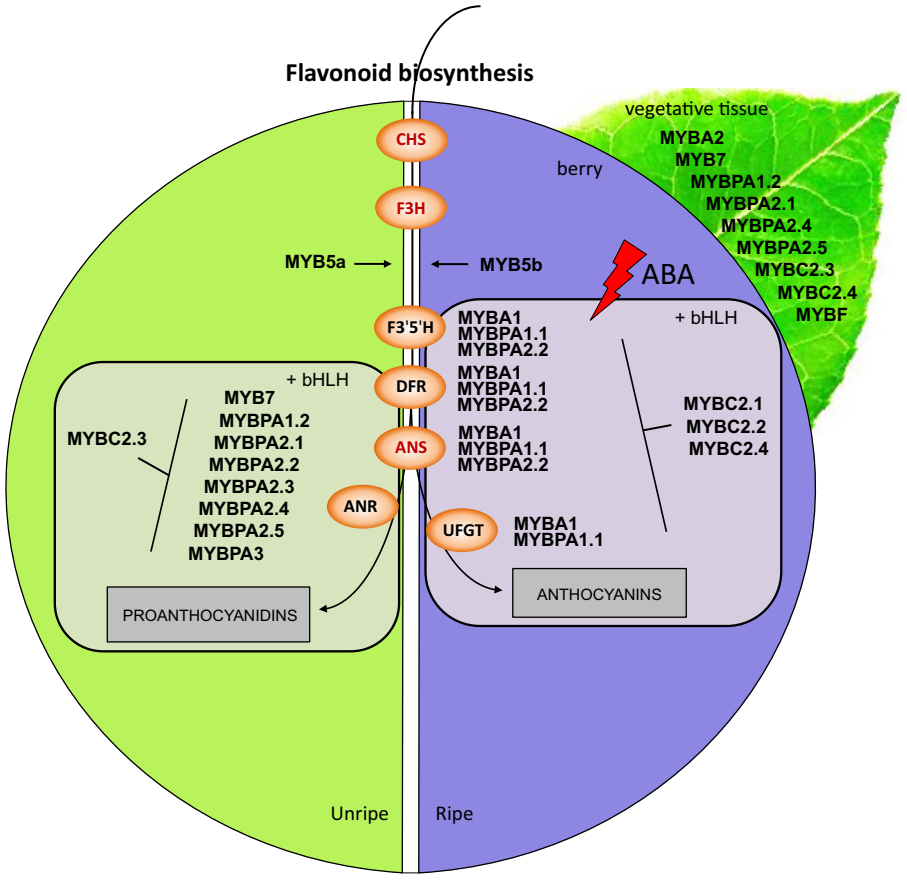

Fig. 9 Proposed model of central role of MYBPA1 and MYBA co-regulation in the control of abscisic acid (ABA)-induced anthocyanin biosynthesis in ripening blue-coloured berry. The important flavonoid pathway bottleneck genes are indicated in red. Arrows, promotion; bars, inhibition.

is not responsible for ripening-associated berry pigmentation but may be responsible for regulating anthocyanin accumulation in vegetative tissues.

We detected that, along with *VmMYBA1*, the transcripts of *VmMYBPA1.1* were highly induced at the initiation of berry ripening, significantly induced in ABA-treated berries, and

strongly downregulated in the white berry mutant lacking anthocyanin biosynthesis. These results are in line with our earlier gene expression level studies in *Vaccinium* species showing the correlation of *MYBPA1.1* expression with anthocyanin accumulation (Jaakola *et al.*, 2010; Primetta *et al.*, 2015; Günther *et al.*, 2020). In the current study, overexpression of *VmMYBPA1.1* in *N. benthamiana* upregulated *NbF3′5′H*, *NbDFR* and *NbANS* expression. *VmMYBPA1.1*-suppressed berries showed significant downregulation of flavonoid biosynthetic genes *VmCHS*, *VmF3′5′H* and *VmANS* with a slight impact also on *UFGT* expression, while the promoter activation assays confirmed VmMYBPA1.1 to activate promoters of *F3′5′H*, *DFR*, *ANS* and *UFGT*. Based on the overall data from our study, we propose that VmMYBPA1.1 contributes to berry anthocyanin biosynthesis and directs dihydroflavonol precursors to delphinidin branch. In particular, *VmF3′5′H* and *VmANS* seem to be the key regulatory targets to facilitate this role in bilberry fruit. The similar expression pattern between *VmMYBPA1.1* and *VmANS* in bilberry tissues may imply that VmMYBPA1.1 helps to overcome the important enzymatic bottleneck in anthocyanin biosynthesis described in *Vaccinium* fruits (Primetta *et al.*, 2015; Zorenc *et al.*, 2017; Günther *et al.*, 2020).

We also found that *VmANR* was not downregulated together with *VmANS* in *VmMYBPA1.1*-suppressed berries. This indicates that recruitment of MYBPA1-type regulator, which is able to strongly induce *ANS*, but importantly not to simultaneously upregulate *ANR*, may be crucial to overcome the anthocyanin biosynthesis bottleneck in ripening berry. It should be noted that PAs and anthocyanins share the same biosynthetic pathway from phenylalanine to leucocyanidin/leucodelphinidin and compete from common precursors. Decoupling *ANS* and *ANR* regulation should theoretically result in an increase in precursor flow to the anthocyanin branch over the PA branch when coupled with upregulated *DFR* and *UFGT* expression by co-expressed *MYBA*-type and *bHLH* TFs. It has recently been shown that the 'Black' peel variety of pomegranate (*Punica granatum* L.), with exceptionally high anthocyanin content, has a mutation in the *ANR* gene (Trainin *et al.*, 2021). Also, tobacco plants overexpressing *AtPAP1* accumulated large amounts of anthocyanins, but co-expression with *ANR* directed the precursors to PA biosynthesis, with a reduction in anthocyanin content (Xie *et al.*, 2006).

The spatial and temporal expression patterns of *MYB* genes *in vivo* need to be addressed when considering their function and deriving the final distribution of the substrates between the PA and anthocyanin pathways, as well as directing precursors between cyanidin and delphinidin branch. Our results implied that, at the time of berry ripening with the presence of ABA, *VmMYBPA2.2* expression is also upregulated together with *VmMYBA1* and *VmMYBPA1.1*, to further boost the production of anthocyanins in the delphinidin branch. VmMYBPA2.2 was shown to activate *DFR*, *ANS* and especially the *F3′5′H* promoter. MYBPA-type TFs have not generally been considered as central regulators of anthocyanin biosynthesis, although a PA1-type MYB was previously shown to be associated with anthocyanin biosynthesis at low temperatures in red-fleshed apples (Wang *et al.*, 2018). Also, PA2-type MYBs, peach PpPeace, PbMYB9 from pear (*Pyrus bretschneideri*) and MYBC1 from kiwifruit (*Actinidia purpurea*) have been shown to activate anthocyanin biosynthesis (as well as flavonol biosynthesis for PbMYB9) in addition to PA biosynthesis (Uematsu *et al.*, 2014; Zhai *et al.*, 2016; Peng *et al.*, 2020), demonstrating diversification in roles among PA-type MYBs. The large number of PA-type *MYB* genes in bilberry found in this study indicates the considerable gene duplication events inside the group and may have allowed the diversification of their function. We demonstrated that bilberry contained a second PA1-type MYB gene, *VmMYBPA1.2*, with a very different expression pattern to that of *VmMYBPA1.1*, resembling the expression pattern of *VmANR* and PA accumulation in bilberry (Suvanto *et al.*, 2020). Overexpression of *VmMYBPA1.2* in *N. benthamiana* demonstrated the differentiation from *VmMYBPA1.1* by its ability to strongly upregulate *NbANR*. Our results suggested that VmMYBPA1.2 has the conventional role of MYBPA1 TFs in the regulation of PA biosynthesis at the early stages of berry development and in vegetative tissues.

Proanthocyanidin biosynthesis was shown to be contributed also by VmMYBPA2.1, VmMYBPA2.3, VmMYBPA2.4, VmMYBPA2.5 and VmMYBPA3, representing a novel MYBPA3 subgroup that includes MtPAR from *M. truncatula* (Verdier *et al.*, 2012), as well as VmMYB7 that has a sequence similarity with peach PpMYB7, reported as the first characterised member of MYB7 clade regulating PA biosynthesis (Zhou *et al.*, 2015a). All could induce the accumulation of PAs in *N. benthamiana* leaves, especially gallocatechin of delphinidin branch (Fig. 5) by regulating flavonoid biosynthetic genes, including *F3′5′H* (Fig. 6). These results are in agreement with our expression data in bilberry.

Concerning the other MYB regulators, we identified two MYB5 members that are considered to provide common precursors for the flavonoid pathway (Li *et al.*, 2019). The gene expression pattern of *VmMYB5a* during berry development resembled that of grapevine *VvMYB5a*, and the expression of *VmMYB5b* that of *VvMYB5b*, suggesting that they may be homologues (Deluc *et al.*, 2006, 2008). Also, four C2 repressors were identified in our study. As *VmMYBC2.1*, *VmMYBC2.2* and *VmMYBC2.4* expression increased at the bilberry fruit ripening and upon ABA treatment, it is possible that these repressors provide feedback inhibition in the MBW complexes associated with anthocyanin biosynthesis (Fig. 9), as described by Albert *et al.* (2014). Interestingly, *VmMYBC2.3* expression was associated with tissues that had high PA content (Suvanto *et al.*, 2020) such as unripe fruit and vegetative tissues, and was strongly inhibited by ABA. This suggests that bilberry C2 repressors may also display a degree of subfunctionalisation for regulating PA or anthocyanin biosynthesis, as described earlier (Huang *et al.*, 2014; Albert, 2015; Cavallini *et al.*, 2015; Jun *et al.*, 2015), indicating the importance of the repression mechanisms for controlling distinct branches of the flavonoid pathway.

To conclude, our study reveals a new type of regulatory network of co-expressed members of *MYBPA1* and *MYBPA2* subgroups along with *MYBA* in the control of ripening-associated anthocyanin biosynthesis in berries. Based on the present and earlier data, we suggest that the co-regulation of MYBPA1 and

MYBA TFs is the key mechanism in anthocyanin biosynthesis, particularly among blue-coloured berries (Fig. 9). The increased expression of *MYBPA1* at the initiation of berry ripening, which correlates with anthocyanin accumulation, has been reported earlier in blue-coloured berries of bog bilberry, highbush blueberry, rabbiteye blueberry (*V. virgatum*), Chinese bayberry (*Myrica rubra*) and grapevine (Zifkin *et al.*, 2012; Primetta *et al.*, 2015; Shi *et al.*, 2018; Yang *et al.*, 2018; Günther *et al.*, 2020). However, so far, MYBPA1 TFs have generally been connected to PA biosynthesis, even if the grapevine VvMYBPA1 has been speculated to also have a role in anthocyanin biosynthesis (Bogs *et al.*, 2007; Czemmel *et al.*, 2012). Interestingly, blueberry VcMYBPA1, but not VcMYBA, was recently revealed to be the target of *VcSPL12* repressing anthocyanin biosynthesis in a microRNA156-SPL module (Li *et al.*, 2020). The differential regulatory role of MYBPA1 in these blue-coloured berries may derive from the presence of delphinidin branch/substrates and substrate preference by flavonoid biosynthetic gene isoforms, such as *ANS* and *LDOX*, which is not fully understood. Furthermore, it should be noted that all the functionally characterised VmMYBs of this study demonstrated the upregulation of *F3′5′H* expression, indicating that involving the delphinidin branch of anthocyanin biosynthesis brings new elements to the regulatory network of MYBs. Therefore, our results provide important new insight into the regulation of anthocyanin biosynthesis and fruit ripening in blue-coloured berries containing an active delphinidin branch.

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Author contributions

KK, NWA, RVE and LJ designed the experiments. KK and NM isolated the *MYB* genes and conducted the qPCR analyses. KK with contribution of BMA conducted the overexpression experiments. KK performed ABA and VIGS experiments. DJL conducted the biolistic and promoter activation experiments. TM conducted HPLC analyses. KK was responsible for the data analysis as well as writing the manuscript with contribution and comments from DJL, NWA, ACA, HH, RVE and LJ.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Alignment of the full-length deduced amino acid sequences of bilberry R2R3 MYBs.

Fig. S2 Expression profiles of *VmMYBs* in bilberry tissues.

Fig. S3 Expression profiles of flavonoid structural genes in bilberry tissues.

Fig. S4 Effect of transient overexpression of *VmMYBA1* and *VmMYBA2* on anthocyanin accumulation in *Nicotiana benthamiana* leaves.

Fig. S5 LC-MS profiles of analysed polyphenols.

Fig. S6 Effect of transient overexpression of *VmMYBs* on flavonol glucosides content in *Nicotiana benthamiana* leaves.

Fig. S7 Transcript levels of *VmMYBs* after overexpression in *Nicotiana benthamiana* leaves.

Methods S1 LC–HRAM–MS analysis of flavonoids from *Nicotiana benthamiana* leaves.

Table S1 R2R3 MYB protein sequences utilised for phylogenetic analysis.

Table S2 Bilberry gene-specific primers used for qRT-PCR analyses.

Table S3 Primers used for construction of vectors for functional analyses.

Table S4 *Nicotiana benthamiana* gene-specific primers used for qRT-PCR analyses.

Table S5 Identities of the *VmMYB* sequences to each other.

Table S6 Conserved motifs found in R2R3 MYB TFs.

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