

Metabolic and molecular analyses of white mutant *Vaccinium* berries show down-regulation of MYBPA1-type R2R3 MYB regulatory factor

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Abstract

Main conclusion MYBPA1-type R2R3 MYB transcription factor shows down-regulation in white mutant berries of *Vaccinium uliginosum* deficient in anthocyanins but not proanthocyanidins suggesting a role in the regulation of anthocyanin biosynthesis.

Berries of the genus *Vaccinium* are among the best natural sources of flavonoids. In this study, the expression of structural and regulatory flavonoid biosynthetic genes and the accumulation of flavonoids in white mutant and blue-colored wild-type bog bilberry (*V. uliginosum*) fruits were measured at different stages of berry development. In contrast to high contents of anthocyanins in ripe blue-colored berries, only traces were detected by HPLC–ESI–MS

in ripe white mutant berries. However, similar profile and high levels of flavonol glycosides and proanthocyanidins were quantified in both ripe white and ripe wild-type berries. Analysis with qRT-PCR showed strong down-regulation of structural genes chalcone synthase (*VuCHS*), dihydroflavonol 4-reductase (*VuDFR*) and anthocyanidin synthase (*VuANS*) as well as MYBPA1-type transcription factor *VuMYBPA1* in white berries during ripening compared to wild-type berries. The profiles of transcript accumulation of chalcone isomerase (*VuCHI*), anthocyanidin reductase (*VuANR*), leucoanthocyanidin reductase (*VuLAR*) and flavonoid 3'5' hydroxylase (*VuF3'5'H*) were more similar between the white and the wild-type berries during fruit development, while expression of UDP-glucose: flavonoid 3-*O*-glucosyltransferase (*VuUFGT*) showed similar trend but fourfold lower level in white mutant. *VuMYBPA1*, the R2R3 MYB family member, is a homologue of *VmMYB2* of *V. myrtillus* and *VcMYBPA1* of *V. corymbosum* and belongs to MYBPA1-type MYB family which members are shown in some species to be related with proanthocyanidin biosynthesis in fruits. Our results combined with earlier data of the role of *VmMYB2* in white mutant berries of *V. myrtillus* suggest that the regulation of anthocyanin biosynthesis in *Vaccinium* species could differ from other species studied.

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Abbreviations

ANR Anthocyanidin reductase
ANS Anthocyanidin synthase
CHI Chalcone isomerase
CHS Chalcone synthase

DFR	Dihydroflavonol 4-reductase
F3'5'H	Flavonoid 3'5' hydroxylase
LAR	Leucoanthocyanidin reductase
UFGT	UDP-glucose: flavonoid 3-O-glucosyltransferase

Introduction

The genus *Vaccinium* includes upwards of 450 deciduous or evergreen species, which occur in cool temperate regions and mountains of both the northern and southern hemispheres. The genus includes various economically important small fruit species such as cultivated blueberries (*Vaccinium* spp.) and large cranberry (*V. macrocarpon* Ait.) as well as wild growing lingonberry (*V. vitis-idaea* L.) and bilberry (*V. myrtillus* L.). The berries of *Vaccinium* plants are among the best sources of flavonoids, especially anthocyanins, giving distinctive dark blue color for bilberries and blueberries, but also rich with flavonols and proanthocyanidins (Määttä-Riihinen et al. 2004; Riihinen et al. 2008). These flavonoid groups are postulated to raise various health-promoting effects, for instance anti-inflammatory and anti-carcinogenic activity and prevention against cardiovascular and degenerative diseases (He and Giusti 2010; Schroeter et al. 2010).

Bog bilberry (*V. uliginosum* L., Fig. 1a) growing in circumboreal and circumarctic regions is less utilized in commercial berry products compared to bilberry and blueberries even though the berries contain abundantly anthocyanins as well as flavonols, especially myricetin and quercetin (Määttä-Riihinen et al. 2004; Lätti et al. 2010). Analyses of flavonoid contents have shown that anthocyanin and flavonol profiles differ between bilberries and bog bilberries in that delphinidin and malvidin are dominant anthocyanidins in bog bilberries, while delphinidin and cyanidin dominate in bilberries (Määttä-Riihinen et al. 2004; Lätti et al. 2008, 2010).

Flavonoids are biosynthesized via the phenylpropanoid pathway and the key enzymes leading to different flavonoid classes are well known (Suppl. Fig. S1). Understanding of the regulation of flavonoid biosynthesis in fruits has significantly increased during the recent years. The biosynthesis has shown to be highly controlled at a developmental level but also external factors, such as light conditions, temperature, and nutrition are involved in the regulation of fruit flavonoid biosynthesis (Koes et al. 2005; Azuma et al. 2012; Zoratti et al. 2014). At the molecular level, flavonoid biosynthesis is regulated via co-ordinated transcriptional control of the structural enzymes of the pathway by the interaction with DNA binding R2R3 MYB transcription factors, MYC-like basic helix-loop-helix (bHLH) and WD40-repeat proteins forming MBW complexes (Hichri et al. 2011; Jaakola 2013). Recent studies have also revealed upstream regulators of the pathway in genus *Vaccinium*; a SQUAMOSA class transcription factor (*VmTDR4*) was shown to have a role in the regulation of anthocyanin biosynthesis during bilberry fruit development (Jaakola et al. 2010).

Plants produce anthocyanin pigments to provide color to their flowers and fruits for attracting pollinators and seed dispersers. Sometimes white forms of berries lacking anthocyanin production are found in the nature. These types of mutants can provide new information on the regulation of anthocyanin biosynthesis in plants. Our previous studies have demonstrated a correlation between anthocyanin accumulation and expression of the flavonoid pathway genes during the ripening of bilberry fruits (Jaakola et al. 2002). We have also shown that in the white mutant bilberries, the expression of the structural flavonoid biosynthesis genes chalcone synthase (*VmCHS*), anthocyanidin synthase (*VmANS*), flavanone 3-hydroxylase (*VmF3H*), dihydroflavonol 4-reductase (*VmDFR*) as well as *VmMYB2*, a MYBPA1-type R2R3 MYB regulatory gene, was markedly lower compared to wild-type berries (Jaakola et al. 2002, 2010). The white bilberry mutant also



Fig. 1 Ripe blue-colored wild-type berries (a) and white mutant berries (b) of bog bilberry

lacked flavonol glycoside myricetin whereas both myricetin and quercetin were detected in the wild-type berries (Jaakola et al. 2002). The role of F3'5'H is to direct the biosynthesis of flavonoids to the branch in which myricetin, delphinidin type of anthocyanins and their derivatives (petunidin and malvidin glycosides) and prodelphinidins are biosynthesized.

The aim of the present study was to provide new information on the regulation of flavonoid biosynthesis in *Vaccinium* berries by analyzing biosynthesis of flavonoids in the white mutant and blue-colored wild-type bog bilberries, a less studied species of the genus. In this study, the fruits of wild-type (Fig. 1a) and white mutant (Fig. 1b) bog bilberries were collected during different stages of fruit development and the composition of anthocyanins, proanthocyanidins and flavonol glycosides in flowers and berries were analyzed by HPLC–DAD–ESI–MS. Transcript abundance of the specific structural and regulatory flavonoid pathway genes were measured from the different fruit developmental stages by qRT-PCR.

Materials and methods

Plant material

Fruits of wild-type bog bilberry (*Vaccinium uliginosum* L.) and of white mutant bog bilberry (*V. uliginosum* f. *leucocarpum*) were collected at different developmental stages in eastern Finland (Ruunaa 63°23'N, 30°21'E) during the summer 2009. The developmental stages of the wild-type berries were following: B1, flowers (collected May 29, anthesis); B2, small-sized unripe green fruits (21 days after anthesis); B3, middle-sized unripe green fruits just before coloring began (34 days after anthesis); B4, expanded fully red-colored fruits (59 days after anthesis); B5, fully ripe blue fruits (73 days after anthesis). Different developmental stages of white mutant berries (W1–W5) were collected at the same time with the wild-type berries.

Immediately after collection, the flower and berry samples were stored at –80 °C for RNA extraction. The samples for flavonoid analyses were stored at –25 °C before being freeze-dried within a month. The freeze-dried samples were further stored in a desiccator at –25 °C. Both flavonoid and gene expression analyses were performed with the wild-type berries at stages B1–B5. Due to the limited number of white mutant berries, the analyses of flavonoids were performed only from developmental stages W4 and W5 of white berries. Proanthocyanidins were analyzed at stages B3–B5 and W4–W5 due to the limited sample amounts. The water content of flowers was 86 %. In the wild-type and white mutant berries, the water content varied between 83–88 and 87–89 %, respectively.

Analyses of flavonoids

The extraction of flavonoids was performed in duplicate according to the method previously described (Lätti et al. 2008, 2011). Briefly, the freeze-dried berries and flowers were ground to a fine powder with mortar and pestle. The berry material (54 mg) was extracted with 0.8 mL of extraction solution. The extraction solution consisted of 10 % solvent A (acetonitrile:methanol, 85:15 v/v) and 90 % solvent B (8.5 % aqueous formic acid). The extraction was performed by sonication for 10 min followed by centrifugation (3645g for 5 min) at 4 °C. The obtained pellet was further re-extracted two times with extraction solution (1 × 0.8 and 1 × 0.4 mL). The supernatants were combined and the volume was adjusted to 2 mL with extraction solution. All extracts were filtered through a regenerated cellulose filter (Econofilter; Agilent Technologies, Palo Alto, CA, USA) equipped with a glass fiber prefilter (Agilent Technologies) prior to HPLC–DAD and HPLC–ESI–MS analyses.

Anthocyanins and flavonols of the samples were separated, tentatively identified and quantified by RP–HPLC–DAD/RP–HPLC–ESI–MS similarly as in the previous studies (Lätti et al. 2008, 2009, 2010, 2011). The berries of *V. myrtillus* were used as a comparison sample. Proanthocyanidins were analyzed as described previously (Määttä-Riihinen et al. 2005; Lätti et al. 2011). Tentative proanthocyanidin contents were analyzed directly from extracts as described by Lätti et al. (2011).

RNA extraction and cDNA preparation

Total RNA was isolated from bog bilberry flower and berry samples using the method described by Jaakola et al. (2001). The quality of the isolated RNA was verified by measuring the absorbance spectrum with a NanoDrop N-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and in 1 % (w/v) ethidium bromide-stained agarose gel. The RNA samples were reverse transcribed to cDNA with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNAs were purified from the genomic DNA using the method described by Jaakola et al. (2004).

Cloning and phylogenetic analysis

The fragments of bog bilberry structural flavonoid biosynthetic genes chalcone synthase (*VuCHS*; GenBank Accession No. KP218505), flavonoid 3'5' hydroxylase (*VuF3'5'H*; GenBank Accession No. KP218506), anthocyanidin synthase (*VuANS*; GenBank Accession No. KP218507), anthocyanidin reductase (*VuANR*; GenBank Accession No. KP218508) and UDP-glucose: flavonoid

3-*O*-glucosyltransferase (*VuUFGT*; GenBank Accession No. KP218512), as well as regulatory gene *VuTDR4* (GenBank Accession No. KP218511) were isolated according to the sequence homology with bilberry (*V. myrtillus*) (Jaakola et al. 2002, 2010; Koskimäki et al. 2009; unpublished bilberry EST-database). The nucleotide sequences of *VuMYBPA1*, *VuMYBC2* and *VuMYBR3* were isolated with complete coding region and submitted to GenBank under accession numbers KR106180, KT186104 and KT186105, respectively. The nucleotide sequences were determined using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with an ABI 3730 DNA sequencer (Applied Biosystems).

For phylogenetic analysis of *VuMYB* sequences, amino acid sequences of functionally characterized flavonoid biosynthesis related R2R3 and R3 MYB family proteins were obtained from GenBank and aligned using Clustal Omega program. A phylogenetic tree was constructed based on the alignment of the R3 domain by using the neighbor-joining method with the MEGA software version 6.06. The reliability of the tree was evaluated by bootstrap analysis with 1000 replicates.

Expression analysis of the flavonoid pathway genes

Transcript accumulation of the flavonoid biosynthetic genes during bog bilberry fruit development was detected using a LightCycler[®] SYBR Green I Master qPCR kit (Roche Applied Sciences, Indianapolis, IN, USA). qRT-PCR analyses were performed with a LightCycler[®] 480 instrument and software (Roche). The PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s (ramp rate 4.4 °C/s), 60 °C for 20 s (ramp rate 2.2 °C/s) and 72 °C for 10 s (ramp rate 4.4 °C/s). The primers used for qRT-PCR analyses are shown in Supplementary Table S1. The quantification of the PCR products was performed using glyceraldehyde-3-phosphate dehydrogenase (*VuGAPDH*; GenBank Accession No. KP218509) as a control gene. The results were calculated with the LightCycler[®] 480 software (Roche), using the calibrator-normalized PCR efficiency-corrected method (Technical Note No. LC 13/2001, Roche).

The specificities of the amplified qRT-PCR products were verified by melting curve analysis. The melting curve was measured by 95 °C for 0.5 s (ramp rate 4.4 °C/s), 57 °C for 15 s (ramp rate 2.2 °C/s) and 98 °C for 0 s (ramp rate 0.11 °C/s). The obtained PCR products were further subjected to agarose electrophoresis, followed by gel extraction using Montage[®] DNA Gel Extraction Kit (Millipore, Bedford, MA, USA) and sequenced to confirm the amplification of the desired product. The nucleotide sequences were determined as described above.

Statistical analyses

The significance of differences in gene expression between the wild-type and white mutant berries at the different stages of development was analyzed using the independent samples *t* test, SPSS Statistics 22 (IBM, New York, USA). The results of the pairwise comparisons are shown for last two stages of ripening, when the anthocyanin biosynthesis occurs, in three categories, $P < 0.05$, $P < 0.001$ and $P < 0.0001$.

Results

Accumulation of flavonoids during bog bilberry fruit development

Flavonols and proanthocyanidins dominated in the early stages of fruit development of wild-type bog bilberries (Fig. 2). Flavonol content increased after flowering and stayed relative stable level during the fruit development slightly decreasing at ripening. Proanthocyanidin content was measured at fruit developmental stages B3–B5 and showed decrease evenly in the course of berry ripening. All analyzed berry samples contained high yields of flavonols and proanthocyanidins and the levels did not markedly differ between white mutant and blue-colored wild-type bog bilberries (Fig. 2). The total flavonol content followed the same descending trend from the onset of ripening to fully ripe white mutant berries but was somewhat lower

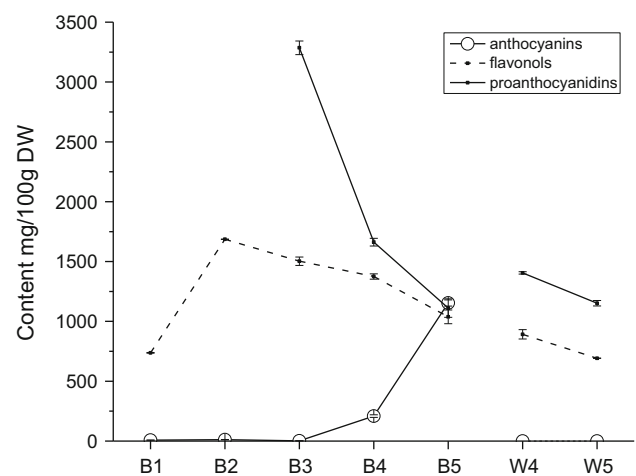


Fig. 2 Contents of anthocyanins, flavonols and proanthocyanidins at the different stages of berry development of blue-colored wild-type (B1–B5) and white mutant (W4–W5) bog bilberries. Mean \pm SD of two replicates. Berry developmental stages: B1, flowers; B2, small-sized unripe green fruits; B3, middle-sized unripe green fruits just before coloring began; B4 and W4, expanded fruits; B5 and W5, fully ripe fruits. *DW* dry weight

than in the blue-colored berries. The tentatively identified flavonol glycosides in the wild-type and white mutant bog bilberry fruits are shown in Table 1 and the contents of the aglycones in the Supplementary Table S2. The ripe blue-colored berries (B5) of *V. uliginosum* contained conjugates of myricetin, quercetin, laricitrin, isorhamnetin, syringetin and kaempferol similarly as shown in our previous studies using the same methods (Lätti et al. 2010). In the developmental stages B1 and B2, conjugates of myricetin-derived laricitrin and syringetin were not detected. In ripe berries of white mutant (W5), the profile of flavonols was similar compared to ripe blue-colored berries (Table 1). Also, the relative proportions of the conjugates of flavonols were at the same magnitude in ripe white mutant and ripe blue-colored berries: for quercetin (62 vs. 57 %), myricetin

(29 vs. 30 %), laricitrin (4 vs. 6 %), syringetin (4 vs. 5 %) and isorhamnetin (1 vs. 2 %), respectively. The tentatively identified proanthocyanidins are shown in Table 2. The number of the identified compounds was at the highest in the flowers (B1). The qualitative profile between blue-colored wild-type and white mutant berries at ripe stage did not differ remarkably. The difference in the proanthocyanidin contents between ripe white and ripe blue-colored berries was only 3.6 % (Fig. 2).

Anthocyanin content was low (below 12 mg/100 g DW) at the early stages of the wild-type bog bilberry fruit development (B2–B3) but started to increase after the ripening, when flavonol and proanthocyanidin contents decreased (Fig. 2, Suppl. Table S3). Anthocyanin content was at highest (1154 ± 26 mg/100 g DW) in ripe berry

Table 1 The tentatively identified flavonol glycosides in blue-colored wild-type and white mutant bog bilberries at different stages of fruit development

Compound	RT	[M + H] ⁺	MS ²	MS ³	Tentative identification ^{a,b}	Developmental stages			
						B1	B2	B5	W5
1	48.0–48.4	481	319	273, 301, 165, 245	Myricetin hexoside	x	x	x	x
2	49.6–49.8	495	319	273, 301, 165, 245	Myricetin glucuronide	x	x	x	x
3	52.4–53.0	451	319	273, 301, 165, 245	Myricetin pentoside	x	x ^{weak c}	x	x
4	53.9–54.5	465	303	257, 229, 285, 165, 303	Quercetin hexoside	x	x	x	x
5	55.2–55.4	495	333	318, 301, 277, 273, 139, 165	Laricitrin hexoside	ND	ND	x	x
6	54.7–55.1	479	303	257, 229, 285, 165, 303	Quercetin glucuronide	x	x	x	x
7	55.6–55.7	509	333	ND	Laricitrin glucuronide	ND	ND	x ^{weak}	x ^{weak}
8	55.6–55.8	435	303, 273	257, 229, 285, 165, 303	Quercetin xyloside	x	x	x	ND
9	56.1–56.3	435	303	257, 229, 165, 303	Quercetin arabinopyranoside	x	x	x	x
10	56.5–56.6	449	287	241, 165, 213, 287	Kaempferol hexoside	x	x ^{weak}	x	x ^{weak}
11	56.7–56.8	465	333	318, 277, 301, 273, 165	Laricitrin pentoside	ND	ND	x	x
12	56.8	435	303	257, 229, 285, 165, 303	Quercetin arabinofuranoside	x	x	ND	x
13	57.2–57.6	449	303	257, 229, 285, 165, 303	Quercetin rhamnoside	x	x ^{weak}	x ^{weak}	x ^{weak}
14	57.4–57.7	479	317	302, 285, 139	Isorhamnetin hexoside	ND	x	x	x
15	57.3	493	303	257, 229, 285, 165, 303	Quercetin derivative	x	x	x	x
16	57.7–57.9	509	347, 491	287, 332, 153	Syringetin hexoside (std) ^d	ND	ND	x	x
17	57.7	463	287	241, 213, 165, 287	Kaempferol glucuronide	x	x	x	x ^{weak}
18	58.0–58.1	419	287	241, 165, 213, 287, 258	Kaempferol pentoside	x	x	x	x ^{weak}
19	58.2–58.4	493	317	302, 285	Isorhamnetin glucuronide	x	x	x	x
20	58.6–58.7	449	317	302, 285	Isorhamnetin pentoside	x ^{weak}	x	x	x
21	58.8	479	347	291, 153, 287, 315, 332	Syringetin pentoside	ND	ND	x	x
22	58.9	537	347, 518	501, 486, 264, 245	Syringetin derivative	ND	ND	x	x

B1, flowers; B2, small-sized unripe green fruits; B5, W5, fully ripe fruits

ND not detected

^a Tentative identifications were based on our previous studies (Lätti et al. 2010, 2011) and the literature (Masuoka et al. 2007; Koponen et al. 2008)

^b The bilberry (*V. myrtillus*) samples were used as comparison samples

^c x^{weak} = only M⁺ ion was detected, not fragments

^d (std) = the identifications were confirmed by the respective standards (syringetin galactoside, syringetin glucoside). Under the RP-HPLC–DAD conditions galactoside and glucoside of syringetin coeluted

Table 2 The tentatively identified proanthocyanidins in blue-colored wild-type and white mutant bog bilberries at different stages of fruit development

Compound	RT	[M + H] ⁺	MS ²	MS ³	Tentative identification ^a	Developmental stages			
						B1	B2	B5	W5
1	5.3–5.6	307	139, 151, 289	139	(epi)Gallocatechin	x ^{weak b}	ND	x ^{weak}	x ^{weak}
2	5.7–6.3	595	443, 291, 425, 317	317, 425, 299, 275	Dimer B	x	x ^{weak}	x	x ^{weak}
3	6.0–6.4	577	425, 559, 409, 391, 437	407, 299	Dimer A	x ^{weak}	ND	ND	ND
4	6.1–6.5	595	443, 291, 425, 317	317, 425, 299, 275	Dimer B	x	ND	ND	ND
5	8.3–8.5	579	427, 409, 291, 301	409, 301, 275	Dimer B	x	x	ND	x ^{weak}
6	9.1–9.3	307	139, 151, 289	139	(epi)Gallocatechin	x	x ^{weak}	x	x
7	9.6–10.1	579	427, 409, 291, 301	409, 301, 275	Dimer B	x ^{weak}	ND	ND	x ^{weak}
8	11.8–11.9	291	123, 139, 165, 273	123	(+)-Catechin (std) ^c	x	x	ND	x ^{weak}
9	14.7–15.1	579	427, 291, 409	409, 301, 275	Dimer B	x ^{weak}	x ^{weak}	ND	x ^{weak}
10	16.9–17.3	579	427, 409, 291, 301	409, 301, 275	Dimer B	x	x	x	x
11	21.0–21.4	579	453, 409, 291, 301	435, 301, 273	Dimer B	x	x ^{weak}	ND	ND
12	22.5–22.7	291	123, 139, 165, 273	123	(-)-(epi)Catechin (std)	x	x	x	x
13	38.9	579	409, 427, 453, 291	287, 299	Dimer B	x ^{weak}	x ^{weak}	ND	ND
14	46.2–47.1	577	425, 437, 559	287, 299	Dimer A	x ^{weak}	x	x	x ^{weak}
15	49.6–49.7	577	425, 437, 287, 559	287, 299	Dimer A	x	x	x	ND
16	52.6–52.9	577	425, 437, 287, 559	287, 299	Dimer A	x	x	x	x
17	53.2	579	409, 427, 453, 291	287, 299	Dimer B	x	x	x	x

B1, flowers; B2, small-sized unripe green fruits; B5, W5, fully ripe fruits

ND, not detected

^a The tentative identifications were based on our previous study (Lätti et al. 2011) and the literature (Hokkanen et al. 2009)

^b x^{weak} = only M⁺ ion was detected, not fragments

^c (std) = the identifications were confirmed by the respective standards

indicated by the dark blue color of the berries. The tentatively identified anthocyanin compounds are shown in Table 3 and the contents of aglycones in the Suppl. Table S3. Ripe blue-colored berries (B5) contained 16 different anthocyanin compounds of delphinidin, cyanidin, petunidin, peonidin and malvidin classes (Table 3). The main anthocyanins were the glycosides of malvidin (40 %), delphinidin (27 %) and petunidin (18 %) and the minor ones were those of cyanidin (8 %) and peonidin (6 %) similarly to previous reports for bog bilberry (e.g. Lätti et al. 2010). The ripe berries of white mutant (W5) contained only traces of anthocyanins (Fig. 2). LC–ESI–MS analysis confirmed tentative identification of delphinidin glucoside, cyanidin galactoside, malvidin galactoside, malvidin glucoside and malvidin arabinoside in the white mutant berry (Table 3).

qRT-PCR analyses of flavonoid pathway genes

The transcript levels of the structural flavonoid pathway genes and candidate regulatory genes were examined at the different stages of development of both wild-type and white mutant bog bilberries by using qRT-PCR method. The

expression of the structural flavonoid biosynthetic genes *VuCHS*, *VuF3'5'H*, *VuDFR*, *VuANS* and *VuUFGT* increased in blue-colored bog bilberries during the ripening period reaching the highest level at the end of berry ripening (Fig. 3). In contrast, in the berries of the white mutant bog bilberry, the expression of *VuCHS*, *VuDFR* and *VuANS* was significantly down-regulated at the ripening period (Fig. 3a, d, g). Interestingly, the expression of *F3'5'H*, which catalyzes two hydroxylations in the 3' and 5'-positions of flavonoids, was up-regulated at the end of ripening both in the blue-colored and the white mutant berries (Fig. 3c). In bog bilberry, the *F3'5'H* appears to be fruit-specific, as on the contrary to other examined flavonoid pathway genes very low transcript level was detected in flowers. The expression of *VuLAR* and *VuANR*, the key enzymes of the proanthocyanidin biosynthesis, was both in wild-type and white mutant bog bilberries at highest level in flowers and relatively lower at the later steps of ripening similarly to *VuCHI* (Fig. 3b, e, f). Expression of *VuUFGT*, which binds the sugar moieties to anthocyanidins, increased both in blue-colored and in white mutant berries during the ripening stage, but the expression was fourfold lower in white mutant compared with the blue-colored berries (Fig. 3h).

Table 3 The tentatively identified anthocyanins in blue-colored wild-type and white mutant bog bilberries at different stages of fruit development

Compound	RT	M ⁺	MS ²	MS ³	Tentative identification ^a	<i>V. myrtillus</i>	Developmental stages			
							B1	B2	B5	W5
1	7.3–7.6	465	303	257, 303, 229	Delphinidin galactoside	x	ND	ND	x	ND
2	8.9–9.3	465	303	257, 303, 229	Delphinidin glucoside	x	ND	ND	x	x
3	10.4–11.1	449	287	287, 213, 231, 259	Cyanidin galactoside	x	x	x	x	x ^{weak b}
4	11.6–12.2	435	303	257, 303, 229	Delphinidin arabinoside	x	x	ND	x	ND
5	13.5–14.1	449	287	287, 213, 231, 259	Cyanidin glucoside (std) ^c	x	x	x ^{weak}	x	ND
6	15.3–16.0	479	317	302, 317	Petunidin galactoside	x	ND	ND	x	ND
7	17.1–17.6	419	287	287, 213, 231, 259	Cyanidin arabinoside	x	x	x ^{weak}	x	ND
8	19.1–20.0	479	317	302, 317	Petunidin glucoside	x	ND	ND	x	ND
9	21.8–22.5	463	301	286, 301	Peonidin galactoside	x	ND	ND	x	ND
10	24.5–24.8	449	317	302, 317	Petunidin arabinoside	x	ND	ND	x	ND
11	27.0–27.9	463	301	286, 301	Peonidin glucoside	x	ND	ND	x	ND
12	28.6–29.9	493	331	299, 315, 287, 270, 179	Malvidin galactoside	x	x ^{weak}	x ^{weak}	x	x
13	31.7–32.1	433	301	286, 301	Peonidin arabinoside	x	ND	ND	x	ND
14	33.8–34.7	493	331	299, 315, 287, 270, 179	Malvidin glucoside	x	ND	x ^{weak}	x	x
15	39.4–40.3	463	331	315, 299, 287, 270, 242, 331	Malvidin arabinoside	x	ND	ND	x	x ^{weak}
16	43.1–43.4	449	317	302, 317	Petunidin xyloside	x	ND	ND	x	ND
Total							5	5	16	5

B1, flowers; B2, small-sized unripe green fruits; B5, W5, fully ripe fruits

ND not detected

^a Ripe bilberries (*V. myrtillus*) corresponding ripening stage 5 were used as comparison sample

^b x^{weak} = only M⁺ ion was detected, not fragments

^c (std) = the identification was confirmed by the respective standard

The analysis of the candidate regulatory genes of flavonoid pathway showed strong down-regulation in the expression of *VuMYBPA1* at the ripening stages W4 and W5 in white mutant bog bilberry fruits (Fig. 4b). Instead, regulatory genes *VuMYBC2*, *VuMYBR3* and *VuTDR4* showed more similar expression patterns in white mutant berries compared to blue-colored wild-type berries, even though significant statistical differences were detected at single time points (Fig. 4). The expression of *VuMYBC2*, *VuMYBR3* and *VuTDR4* suggested that the ripe white mutant bog bilberry samples could have been slightly ahead in ripening compared with the blue-colored berry samples as the expression of these genes was already lowering at stage W5 (Fig. 4). During the berry development, the ripening-related changes in metabolism are typically fast. Even in the same plant, the berries can ripen slightly different rate even if they were flowering at the same time. This fact is important to consider when analyzing the gene expression data and, therefore, it is more essential to look the trends of gene expression than focus on differences in exact values at a single time point. In the present study, highly significant ($P < 0.0001$) differences, visible in the two last stages of ripening were emphasized

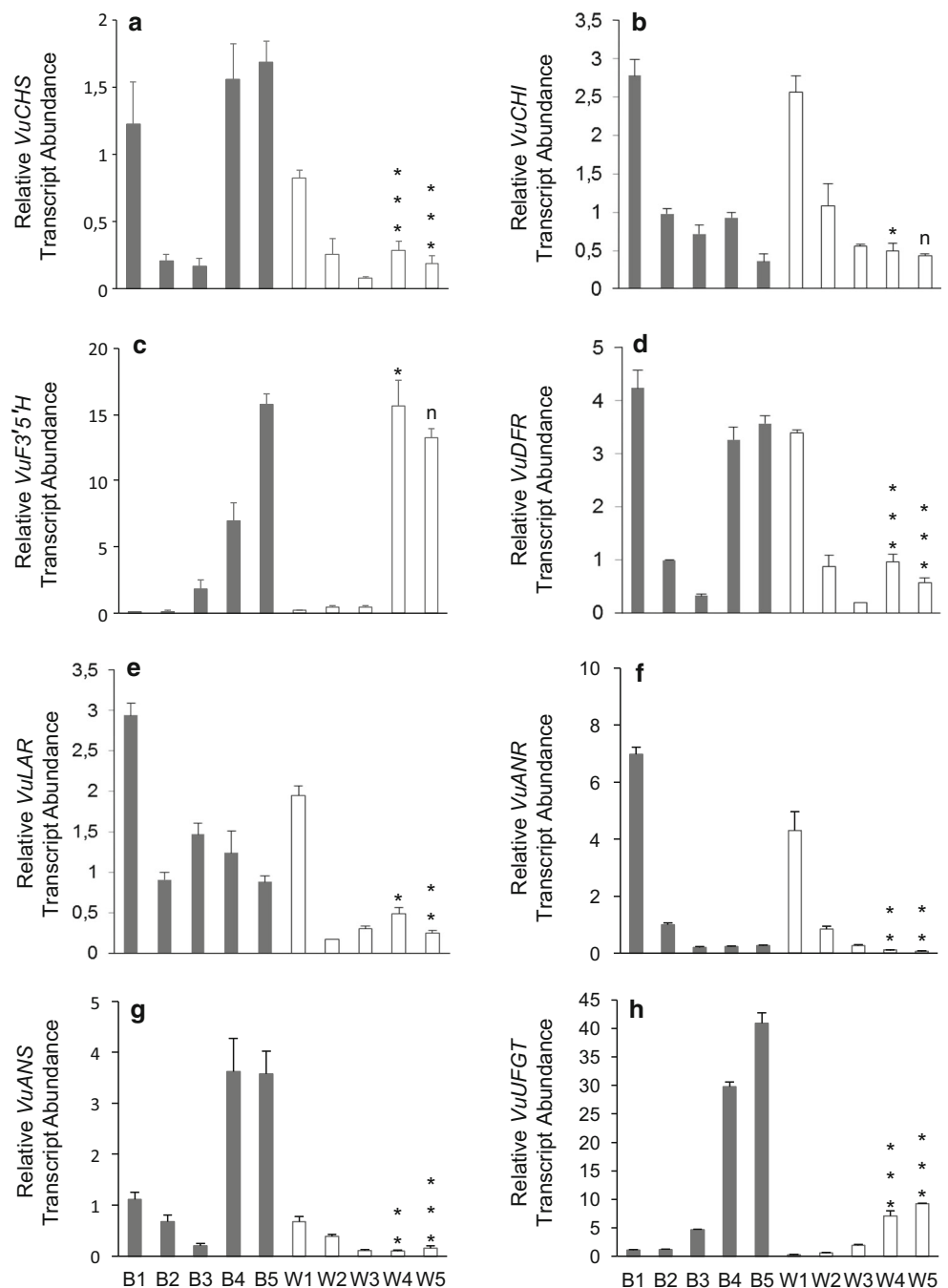
when comparing the gene expression results between the wild-type and the mutant samples.

To show the relationship between the target bog bilberry MYB proteins and other earlier identified MYB proteins related with flavonoid biosynthesis, the amino acid sequences of R3 region were aligned and a phylogenetic tree was constructed using the neighbor-joining method (Fig. 5). The phylogenetic tree shows that *VuMYBPA1* is clustered closely with *VmMYB2* of *V. myrtillus* and *VcMYBPA1* of *V. corymbosum*, and with members of MYBPA1-type regulatory factors *VvMYBPA1* and *DkMYB4*. The *VuMYBC2* has the closest homology in amino acid level with grapevine *VvMYBC2-L1* (71.2 %) and *VuMYBR3* with *PhMYBx* (60 %) from *Petunia hybrida*.

Discussion

Many species of genus *Vaccinium*, such as bilberries and blueberries, are well known for their high levels of flavonoids, especially health-promoting anthocyanins. Earlier studies have demonstrated a correlation between

Fig. 3 Transcript abundance of flavonoid structural genes *VuCHS* (a), *VuCHI* (b), *VuF3'5'H* (c), *VuDFR* (d), *VuLAR* (e), *VuANR* (f) *VuANS* (g) and *VuUFGT* (h) determined by qRT-PCR at the different stages of fruit development of blue-colored wild-type (B1–B5) and white mutant (W1–W5) bog bilberries. Values represent mean \pm SE of three replicates. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$; *n* no statistically significant difference (independent samples *t* test)



anthocyanin accumulation and the expression of the anthocyanin-related flavonoid pathway genes during the ripening of *Vaccinium* fruits (Jaakola et al. 2002, 2010; Li et al. 2012; Zifkin et al. 2012). Some regulatory genes of the flavonoid pathway have also been characterized but as a whole the regulation of anthocyanin biosynthesis in *Vaccinium* berries is still not clear (Jaakola et al. 2010; Zifkin et al. 2012). The existing anthocyanin mutant berries open possibilities to study the expression of the structural flavonoid genes and potential regulatory genes in comparison

with the changed levels of flavonoids during the fruit development. The white mutant of bog bilberry is described and characterized for the first time in the current study.

Analyses of flavonoids confirmed the presence of extremely low level of anthocyanins in ripe white mutant bog bilberries compared to the ripe blue-colored wild-type berries. This was consistent with the observed reduction in the expression of structural flavonoid biosynthetic genes of *VuCHS*, *VuDFR* and *VuANS* in the white mutant berries at the end of ripening. The expression of these genes, instead,

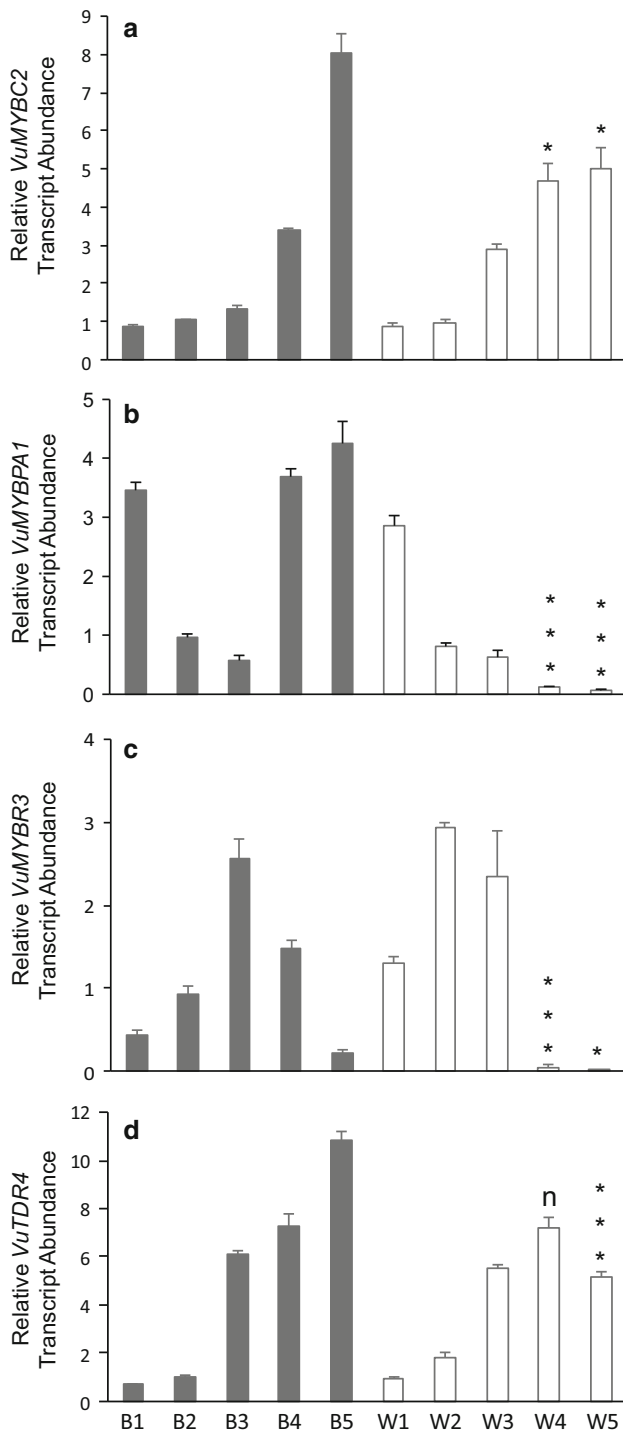


Fig. 4 Transcript abundance of transcription factors *VuMYBC2* (a), *VuMYBPA1* (b), *VuMYBR3* (c) and *VuTDR4* (d) determined by qRT-PCR at the different stages of fruit development of blue-colored wild-type (B1–B5) and white mutant (W1–W5) bog bilberries. Values represent mean ± SE of three replicates. *P < 0.05, **P < 0.001, ***P < 0.0001; n no statistically significant difference (independent samples *t* test)

increased in wild-type bog bilberries during the ripening along with the accumulation of anthocyanins. Expression of *VuUFGT*, which catalyzes the binding of the sugar

moieties to anthocyanidins, increased both in blue wild-type and in white mutant berries along with ripening, but the expression was fourfold lower in white mutant compared with the blue-colored berries.

In the white mutant berries, profiles and levels of proanthocyanidins and flavonol glycosides were not remarkably different from the blue-colored berries. Proanthocyanidins and flavonol glycosides were predominated flavonoids at the early stages of fruit development while the accumulation of anthocyanins began at the onset of ripening in wild-type berries. The expression of *VuLAR* and *VuANR*, catalyzing the conversion of leucoanthocyanidins and anthocyanidins into monomeric units of proanthocyanidins, were highest at the beginning of the berry development, which is consistent with observed decrease in proanthocyanidin content at the end of ripening.

Of the flavonols, many *Vaccinium* berries, including bilberries and bog bilberries, contain myricetin glycosides in addition to quercetin glycosides (Määttä-Riihinen et al. 2004). Our earlier studies revealed that the white mutant of bilberry contained quercetin but no myricetin nor anthocyanins and showed down-regulation of phenylalanine ammonia-lyase (*VmPAL*), *VmCHS*, *VmF3H*, *VmDFR* and *VmANS* during the ripening stages compared with blue-colored berries (Jaakola et al. 2002, 2010). Interestingly, the white bog bilberry mutant showed the similar qualitative and quantitative content of flavonols as the blue wild-type bog bilberry. In accordance with the detected flavonol profiles, the expression of *VuF3'5'H* was in white berries at the same level with wild-type berries. *F'3'5'H* catalyzes two hydroxylations in the 3' and 5'-positions of flavonoids which are needed in formation of myricetin and delphinidin derived anthocyanins. Earlier studies with flowers of various species and, e.g., grape berries have shown consistency in accumulation of 3,4,5,-hydroxylated anthocyanins and function of *F'3'5'H* (Shimada et al. 1999; Castellarin and Di Gaspero 2007; Tanaka and Brugliera 2013). Our results suggest that in bog bilberry, *VuF3'5'H* is regulated separately from the other flavonoid pathway genes analyzed in the current study. The expression of *VmF3'5'H* in white bilberry fruits was down-regulated compared to blue-colored bilberries, which is consistent with the absence of myricetin (unpublished data). These gene expression results confirm the observed differences in flavonol profiles between the white mutant berries of bog bilberry and bilberry.

Analysis on the expression of regulatory genes *VuMYBC2*, *VuMYBPA1*, *VuMYBR3* and *VuTDR4* revealed strong down-regulation of *VuMYBPA1* in the white mutant berries at the two last investigated developmental stages representing the ripening phase. This can implicate that the expression of *VuMYBPA1* is needed for the normal

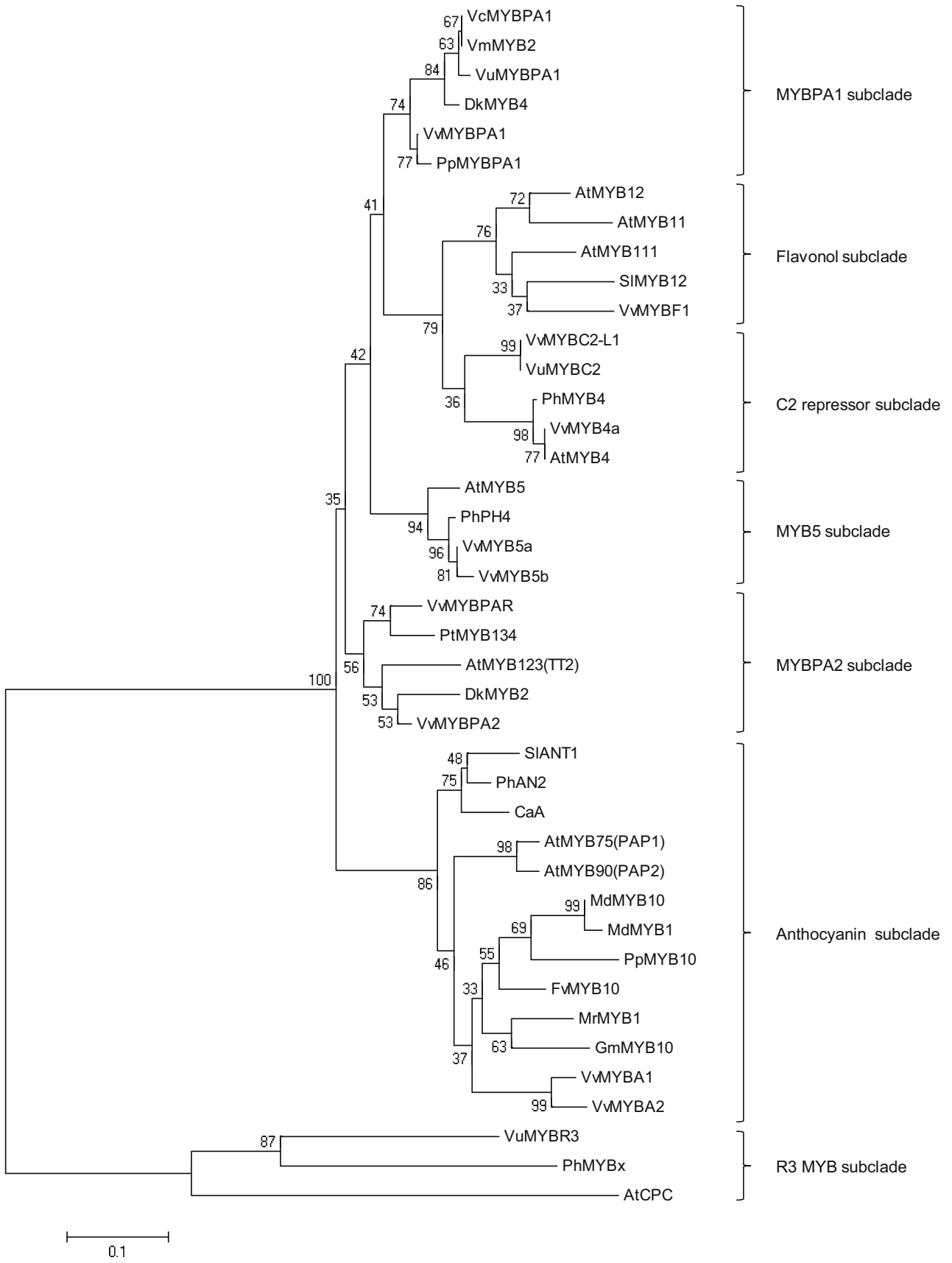


Fig. 5 Phylogeny for MYB transcription factors involved in flavonoid pathway. The phylogenetic tree was constructed using the neighbor-joining method in the MEGA software. The numbers next to the nodes are bootstrap values from 1000 replicates. The indicated scale represents amino acid substitutions per site. The GenBank accession numbers are as follows: AtCPC (NP_182164), AtMYB4 (AAC83582), AtMYB5 (NP_187963), AtMYB11 (NP_191820), AtMYB12 (NP_182268), AtMYB75 (PAP1, AAG42001), AtMYB90 (PAP2, NP_176813), AtMYB111 (NP_199744), AtMYB123 (TT2, Q9FJA2), CaA (CAE75745), DkMYB2 (BAI49719), DkMYB4 (BAI49721), FvMYB10 (ABX79948), GmMYB10 (ACM62751), MdMYB1 (ABK58136), MdMYB10 (ABB84753), MrMYB1 (ADG21957), PhAN2 (AAF66727), PhMYB4 (ADX33331), PhMYBx (AHX24371), PhPH4 (AAY51377), PpMYB10 (ABX79945), PpMYBPA1 (CV047374), PtMYB134 (ACR83705), SIAN1 (AAQ55181), SIMYB12 (ACB46530), VcMYBPA1 (AEV21970), VmMYB2 (ADK79068), VvMYB4a (ABL61515), VvMYB5a (AAS68190), VvMYB5b (AAX51291), VvMYBA1 (BAD18977), VvMYBA2 (BAD18978), VvMYBC2-L1 (AFX64995), VvMYBF1 (ACT88298), VvMYBPA1 (CAJ90831), VvMYBPA2 (ACK56131), and VvMYBPAR (BAP39802). The VuMYBPA1, VuMYBC2 and VuMYBR3 sequences have been deposited in GenBank under accession numbers KR106180, KT186104 and KT186105, respectively

expression of *VuCHS*, *VuDFR* and *VuANS* during the ripening stage and for the formation of anthocyanin pigments in bog bilberry.

VuMYBPA1 is a homologue of *VmMYB2*, a bilberry R2R3 MYB family member transcription factor, which was similarly detected to be strongly down-regulated in white mutant bilberries (Jaakola et al. 2010). These sequences have the closest identity with blueberry *VcMYBPA1* (Zifkin et al. 2012) and belongs to MYBPA1-type subclade of R2R3 MYB transcription factors along with grape berry *VvMYBPA1*. *VvMYBPA1* has been shown to be involved in the regulation of proanthocyanidin biosynthesis in grape berry and to control expression of *VvANR*, *VvLAR*, *VvANS* as well as *VvF3'5'H* (Bogs et al. 2007; Terrier et al. 2009; Kuhn et al. 2014). Expression of *VvMYBPA* genes is consistent with the accumulation of proanthocyanidins; higher at early stages of grape berry development and lower at the period of anthocyanin accumulation (Bogs et al. 2007; Terrier et al. 2009). On the contrary, in blueberry, the expression of *VcMYBPA1* was at the highest level at the end of berry ripening at the period of anthocyanin accumulation (Zifkin et al. 2012) similarly to *VmMYB2* in bilberry (Jaakola et al. 2010) and to our results on *VuMYBPA1* in bog bilberry in the present study. Since the levels of proanthocyanidins were high in both white mutant and blue-colored bog bilberries, despite the substantial difference in the expression of *VuMYBPA1* in the ripening stage, the results indicate that *VuMYBPA1* may have a different role in regulation of flavonoid biosynthesis in *Vaccinium* berries compared to other species. Noteworthy is also that the separate white mutant berries of two *Vaccinium*

species, bilberry and bog bilberry, show exactly same results on the expression of *VmMYB2/VuMYBPA1* gene. Sequencing of the white mutant *VuMYBPA1* coding region along with comparison to the wild-type bog bilberry *VuMYBPA1* sequence did not show differences that would explain the mutant phenotype. This suggests that down-regulated *VuMYBPA1* expression rather than aberrant *VuMYBPA1* function could lead to the mutant phenotype.

The *VuMYBC2* showed less notable difference in the expression profile between the wild-type and white mutant bog bilberries. The *VuMYBC2* has the closest homology at the amino acid level with the grapevine *VvMYBC2-L1*, which has recently characterized to act as a negative regulator of proanthocyanidin accumulation (Huang et al. 2014). Both of these genes have C1 and C2 motifs characteristic for C2 repressor type of R2R3 MYB transcription factors. For this reason, we also named the *VuMYBC2* gene accordingly. In grapevine, over-expression of *VvMYBC2-L1* in hairy root cultures reduced the expression of *VvMYBPA1*, *VvMYBPA2*, *VvDFR*, *VvLDOX* (*ANS*), *VvANR*, *VvLAR1* and *VvLAR2* along with the content of proanthocyanidins and flavan-3-ol monomers (Huang et al. 2014). In bog bilberry, the expression of *VuMYBC2* was lower at the beginning of the berry development and higher at the later stages, which could indicate similar function as *VvMYBC2-L1* has in grapevine.

The *VuMYBR3* has the closest homology with *PhMYBx*, a proposed R3 MYB repressor of anthocyanin biosynthesis in *Petunia hybrida*, which is closely related with *Arabidopsis CPC*, *TRY*, *ETC1*, *ETC2*, and *ETC3* (Albert et al. 2011, 2014). These genes belong to the single repeat R3 MYB family, members of which have been shown to interact with the MBW complexes (Albert et al. 2014). It has been suggested that *PhMYBx* could act as a part of feedback mechanism in anthocyanin production in *Petunia* flowers as transcripts of *PhMYBx* are reduced in anthocyanin lacking *an1* and *an11* *petunia* lines (Koes et al. 2005; Albert et al. 2011). In bog bilberry, the expression of *VuMYBR3* was significantly lower in the white mutant berries compared with the wild-type berries at the ripening stage 4, when the accumulation of anthocyanins begins (Fig. 4). However, also in blue wild-type berries, the expression lowered at the period of the highest anthocyanin accumulation (B4-B5).

In several berry producing species, MYBA/MYB10-type R2R3 MYB transcription factors have been shown to associate in anthocyanin biosynthesis (reviewed in Jaakola 2013). In grape berries, MYBA-type transcription factors have been shown to regulate the anthocyanin biosynthesis by controlling the expression of *VvUFGT* that catalyzes the glycosylation of anthocyanidins (Azuma et al. 2008; Kuhn et al. 2014). A tight association is detected between white-fruited grape cultivars and a mutation in the promoter

region of *VvMYBA1* or coding region of *VvMYBA2* (Kobayashi et al. 2004; Walker et al. 2007; Kuhn et al. 2014). MYBA/MYB10-type transcription factors are homologs of *Arabidopsis* AtMYB75, AtMYB90, AtMYB113, AtMYB114 and apple (*Malus domestica*) MdMYB10/MdMYB1/MdMYBA, and they have been isolated in many other members of Rosaceae family (Lin-Wang et al. 2010). In strawberry, silencing of MYBA-type *FaMYB10* transcription factor led to the down-regulation of several structural flavonoid pathway genes and inhibition of anthocyanin accumulation, suggesting a major role in regulation of anthocyanin biosynthesis (Medina-Puche et al. 2014). We have done numerous attempts for characterizing MYBA-type regulators involved in anthocyanin biosynthesis from the *Vaccinium* species starting with degenerated primers and going through cDNA libraries and different databases, but so far we have not been able to find potential candidates neither in our earlier bilberry EST nor recent bilberry transcriptome libraries (data not shown). Interestingly, the careful search by using sequence homology and available bioinformatics tools of the other currently available *Vaccinium* EST and transcriptome databases (Rowland et al. 2012; Zifkin et al. 2012), recently published genome database of American cranberry (*V. macrocarpon*) (Polashock et al. 2014) and draft genome of blueberry (Gupta et al. 2015) did either not reveal potential transcripts/sequences for anthocyanin biosynthesis associated MYBA-type R2R3 MYB genes. The difficulty to find MYBA-type R2R3 MYB transcripts in the *Vaccinium* transcriptome databases is noteworthy since *Vaccinium* species are among the best natural sources of anthocyanins. It could be assumed that the transcription of the key regulators would be active in berries at the onset of anthocyanin accumulation. Although further functional studies are needed, we suggest that the results with white mutant berries of *Vaccinium* species give indications on the differential regulation of anthocyanin and proanthocyanidin biosynthesis in *Vaccinium* berries compared with other fruits studied so far.

Author contribution statement AP and KR conducted chemical metabolite analyses. KK and LJ conducted gene expression and phylogenetic analyses. All authors attended writing of the manuscript. The manuscript has been read and approved by all authors.

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Compliance with ethical standards

Conflict of interest The authors declare that they do not have any competing interest.

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