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Overexpression analysis of bilberry (Vaccinium myrtillus L.) R2R3-MYB transcription factors

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Abbreviations

MYB	Myeloblastosis
PA	Proanthocyanidin
PAL	Phenylalanine ammonia-lyase
C4H	C_4H cinnamate 4-hydroxylase
4CL	4-coumarate: CoA ligase
CHS	Chalcone synthase
CHI	Chalcone isomerase
F3H	Flavanone 3-hydroxylase
DFR	Dihydroflavonol 4-reductase
ANS	Anthocyanidin synthase
F3'H	Flavonoid 3-hydroxylase
F3'5'H	Flavonoid-3,5-hydroxylase
LAR	Leucoanthocyanidin reductase
ANR	Anthocyanidin reductase
UFGT	UDP-glucose flavonoid 3-O-glucosyltransferase
FLS	Flavonol Synthase
KAN	Kanamycin
Rif	Rifampicin
TET	Tetracycline
TF	Transcription factors
PCR	Polymerase chain reaction

Abstract

Bilberry (*Vaccinium myrtillus* L.) is a dwarf shrub and its berries are rich source of nutrients, fibers and vitamins. Bilberry consumption has been shown to positively correlated with improvement of human health. Anthocyanins and proanthocyanidins (PAs) are important secondary metabolites that are synthetized in plants via flavonoid biosynthesis pathway. PAs also have anti-cancer, anti-inflammatory and antioxidant effect in humans. The main aim of this thesis was to identify role of bilberry *MYB* TFs in biosynthesis of anthocyanin and PAs. Eight R2R3-*VmMYB* transcription factors (TFs), *(VmMYBPA2.1, VmMYBPA2.2, VmMYBPA2.3, VmMYBPA2.4, VmMYBPA2.5, VmMYBPA3, VmMYB5, VmMYB7),* were cloned to *pGreen-62SK* plasmid. PCR amplification verified ligation of genes of interest into *pGreen* plasmid. Sanger sequencing further confirmed that the full-length sequences were ligated into plasmids. Agroinfiltration was used for overexpression analysis of *VmMYBs* together with *AtbHLH* into leaves of *Nicotiana benthamiana.* Spectrophotometry analysis confirmed that positive control, *VmMYBA1 and VmMYBPA2.2* accumulated anthocyanins and PAs. Furthermore, calluses from bilberry fruit explant, were induced in WPM media with hormones, naphthalene acetic acid (NAA), 6-benzylamino purine (BAP), and thidiazuron (TDZ). Appearance of reddish color in calluses after agroinfiltration of *VmMYBA1 and VmMYBA2* may indicate the accumulation of anthocyanin in calluses. In this study, it was shown that bilberry *VmMYBPA2.2* positively regulates biosynthesis of proanthocyanidins and *VmMYBA1* may be a key regulator of biosynthesis of both anthocyanins and proanthocyanidins in *Vaccinium myrtillus* L.

Keywords

MYB transcription factors, *Vaccinium myrtillus* L., overexpression, *Nicotiana benthamiana*, anthocyanins, PAs, regulation, callus

1 Introduction

1.1 Bilberry *(Vaccinium myrtillus* L.)

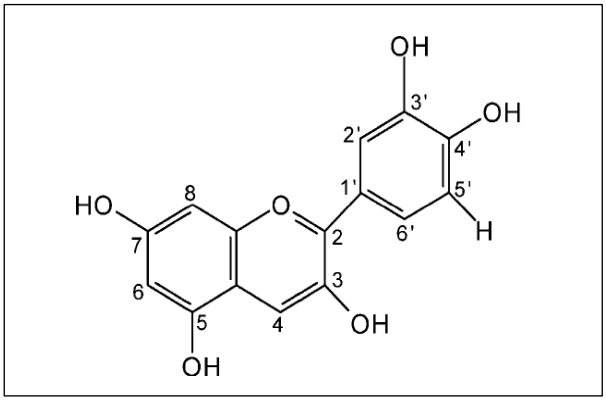
Genus *Vaccinium* contains economically important wild berries i.e. bilberry (*V. myrtillus* L*),* lingonberry (*V. vitis-idaea* L.*),* blueberry (e.g., *Vaccinium corymbosum* L., *V. angustifolium* Aiton) and cranberries (*V. macrocarpon* Aiton, *V. oxycoccos* L.). Berries of these species can be used fresh and also processed to derived products like yogurt, beverages, jellies, jam and canned fruits (Jaakola et al. 2009; Seeram et al. 2001).

Bilberry, also known as European blueberry, is low branched shrub belonging to family Ericaceae. Berries are rich source of phytonutrients, vitamins, fibers and metabolites and usually dark bluish in color. Also, they have strong health benefits for humans as their consumption can improve night vision and antioxidant potential or decrease glucose level in the blood (Karppinen et al. 2016). Bilberries also have shown antimicrobial and anti-inflammatory effects in humans by reducing risk of metabolic syndrome and other degenerative diseases (Nile & Park, 2014).

1.2 Anthocyanins and proanthocyanidins (PAs)

Bilberry fruit and leaves are rich sources of many phenolic compounds like anthocyanins, PAs, tannins, phenolic acids, flavanols (quercetin, myricetin or isorhamnetin) and ellagitannins (Chu, et al. 2011). Anthocyanins are water soluble vacuolar pigments that gives red, blue, purple or black color to plant flowers, seeds and fruits. They are usually produced in flowers during stage of petal expansion and in fruit they are synthesized during a phase (Lai et al. 2013). In bilberry, anthocyanins can be found in skin and flesh of fruit. Glycosides of anthocyanidins require addition of sugar group to form anthocyanin (Figure 1A). In bilberry, 15 major anthocyanin glycosides have been identified (Cassidy, 2018; Karppinen et al., 2016).

Proanthocyanidin (PAs) are polyphenolic oligomers of catechin and epicatechin. They are astringent molecules, mostly accumulated during early stage of berry development to aid against pathogenic or herbivore attack. PAs are synthesized from flavan-3-ols units (Figure 1B) and have important role as antioxidant, anti-inflammatory and anti-cancer. Astringent proanthocyanidins are colorless compounds (Jaakola et al., 2002).



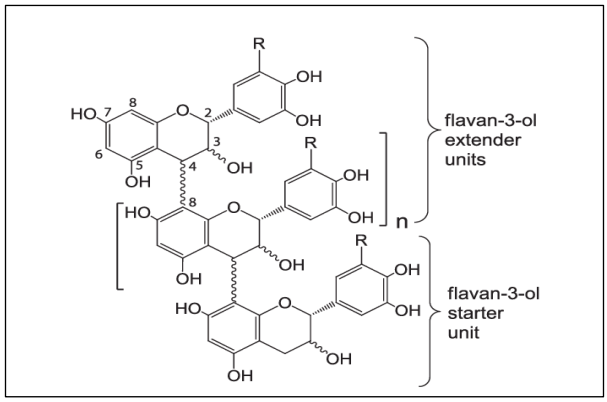


Figure1: General structure of Anthocyanidin and Proanthocyanidin (A) General structure of anthocyanidin that join with sugars to form anthocyanin.

(Pervaiz et al. 2017)

(B). General structure of proanthocyanidin (R = OH)

(Stringano et al. 2012)

During earlier stages of bilberry fruit development, PAs and flavonols appear as major phenolic compound mostly in fruit flesh but as fruit ripens, only anthocyanins contribute as major flavonoids (Figure 2). Concentration of PAs are usually higher in red colored *Vaccinium* species (cranberries, lingonberry) as compared to blue colored berries, such as blueberries and bilberry (Grace et al.2014; Zoratti et al., 2014).

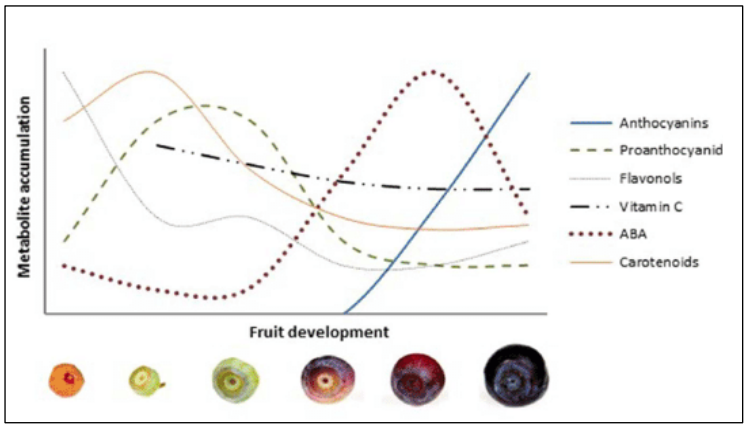


Figure2: Accumulation of anthocyanin and PAs during ripening of bilberry. Schematic representation of changes in concentration of anthocyanin and PAs during different developmental stages of bilberry. During early fruit development PAs are usually present in higher concentration but gradually decreases but in contrary, anthocyanin concentration increases with ripening of fruit. (Karppinen et al., 2016)

1.3 Flavonoid biosynthesis pathway

Plants synthesize a variety of aromatic metabolites such as phenolic compounds including tannins, prominently lignin and flavonoids, through the unique and complex phenylpropanoid pathway. Flavonoids have been studied extensively because of their major role in plant metabolism, defense, stress and against excessive UV light (Vogt, 2010). Chloroplasts harbor biosynthesis of amino acids i.e. tryptophan, tyrosine and phenyl alanine, through shikimate pathway (Iorizzo et al., 2019). Here, phenyl alanine acts as precursor of flavonoid biosynthesis pathway and is converted into 4-coumaroyl-CoA that leads to biosynthesis of flavonoids through many enzymatic steps (Czemmel et al. 2012).

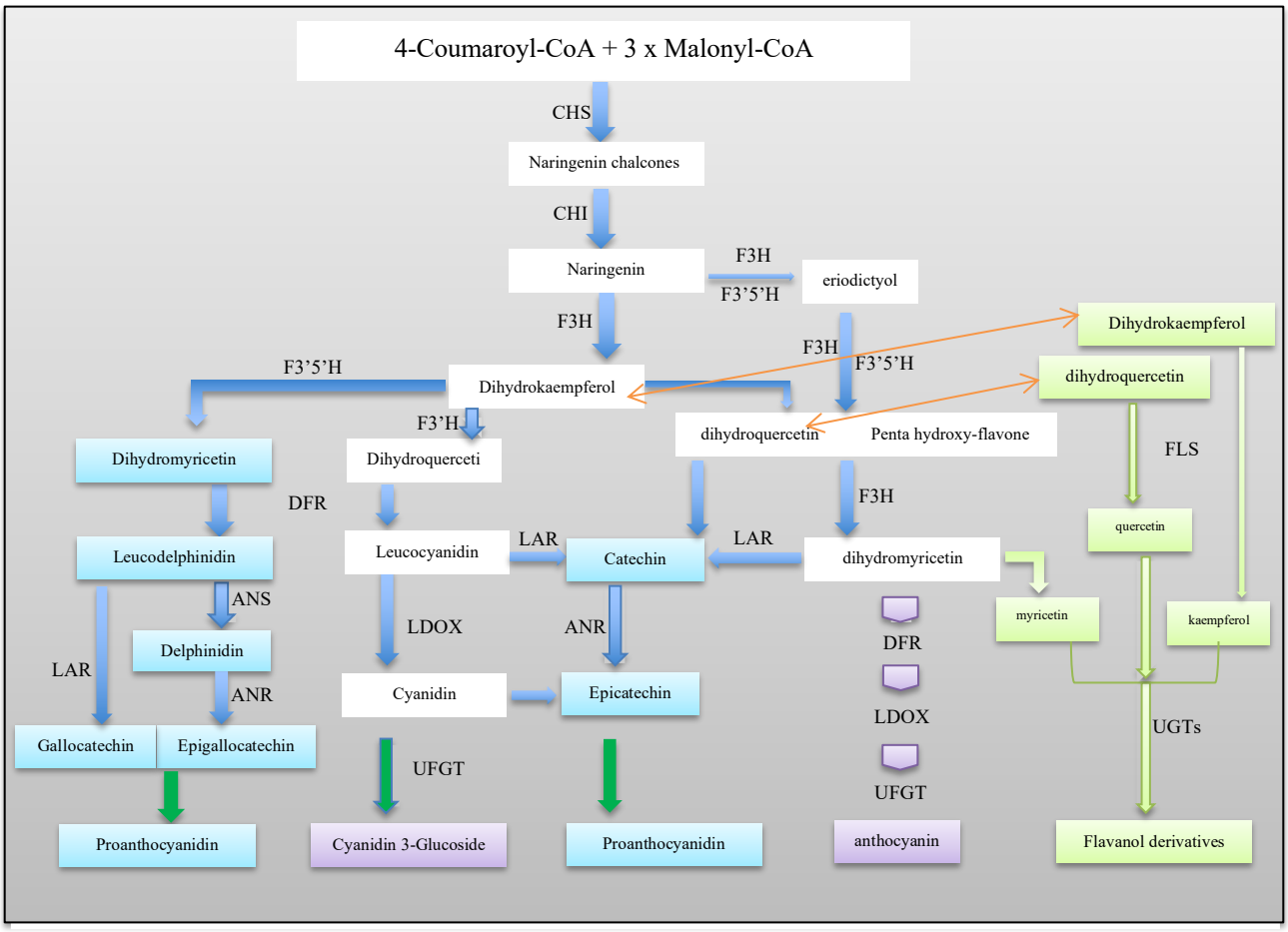


Figure 3: Flavonoid biosynthesis pathway. This pathway is showing biosynthesis of anthocyanins, PAs and flavonols. Blue boxes are representing biosynthesis of PA while purple boxes are indicating anthocyanin biosynthesis pathway. Flavonol biosynthesis is shown in light green boxes. Enzymes of early steps are CHS (chalcone synthase), CHI (chalcone isomerase), F3H (flavanone 3-hydroxylase) and F3'5'H (flavonoid-3,5-hydroxylase) are common to all. Enzymes for anthocyanin biosynthesis are LDOX (leucoanthocyanidin dioxygenase), UFGT (UDP-glucose flavonoid 3-O-glucosyltransferase) and DFR (dihydroflavonol 4-reductase). While, LAR (leucoanthocyanidin reductase), ANR (anthocyanidin reductase) are responsible for regulation of PAs biosynthesis. For flavonols biosynthesis, FLS (Flavonol Synthase) and UGT (UDP-glycosyltransferase) are responsible enzymes.

Figure 3 is illustrating the complete steps of flavonoid biosynthesis pathway and it was revised from Nakagawa et al., (2008) and Czemmel et al., (2012). In this pathway, both anthocyanins and proanthocyanidins are synthesized along with flavonols. *CHS, CHI*, and *F3H* play the major role during early steps to form dihydrokaempferol while other genes, *DFR* and *ANS* (anthocyanidin synthase) contribute for later steps of anthocyanin biosynthesis (Petroni and Tonelli, 2011). Initially, the pathway is common for both anthocyanin and PAs, that have shown by white boxes. From dihydrokaempferol, this pathway is divided into multiple branches and results in formation of anthocyanins, PAs and flavonols. They form PAs are formed through

delphinidin intermediates and enzymes *ANS* and *ANR* aid this process. Also, Leucoanthocyanidin reductase (*LAR)* converts leucocyanidin into flavan-3-ols catechin that results into PAs. Leucoanthocyanidin dioxygenase (*LDOX*) and *ANR* can work together and convert cyanidin to epicatechin and result into PAs (Zhou et al., 2015). Moreover, naringenin and dihydrokaempferol also make a separate pathway for flavonols, anthocyanins and PAs production. Similar to dihydrokaempferol, the dihydroquercetin and dihydromyricetin result into anthocyanins and PAs through catechin and epicatechin. In addition to these, green color is indicating formation of flavonols is facilitated by *FLS,* from dihydrokaempferol, dihydroquercetin and dihydromyricetin.

1.4 *MYB* transcription factors (TFs)

Myeloblastosis (MYB) proteins were identified in avian myeloblastosis virus but later they were discovered in all eukaryotic organisms (Lipsick, 1996). MYB transcription factors (TFs) are a large family in plant kingdom that is responsible for regulation of transcription of enzymes involved in various function, for instance biosynthetic pathways of secondary metabolites (Chen et al., 2019). MYB TFs act as either enhancer or suppressor by binding on promoter regions of genes (Ma & Constabel, 2019). They have conserved DNA binding domain of 53 amino acid long, that play role in secondary metabolism, development and signal transduction (Jin & Martin, 1999; Dubos et al., 2010). Based on conserved binding repeats, (R1, R2, R3), *MYBs* are classified into 1R-, R2R3-, 3R- and 4R-MYB proteins and with two repeats, R2R3-MYBs are most prominent among the family. They participate in regulation of secondary metabolism of terpenoid, phenylpropanoid, benzenoid and glucosinolate pathways (Ramya et al., 2017). More than 1548 candidates of R2R3- family have been identified from a diverse taxonomic groups resulted in 73 highly conserved subfamilies (Du et al., 2015). Most of the enzymes that participate flavonoid biosynthesis pathway have been isolated from variety of plant species, including apple, berries, grapes, petunia, arabidopsis, petunia (Winkel-Shirley, 2001; Jaakola, 2013). Three classes of transcription factors (TFs), R2R3-MYBs, basic helix-loop-helix (*bHLH*) and WD40 proteins join together to form a MBW-complex that regulate the structural genes of flavonoid biosynthetic pathway at transcriptional level (Grotewold, 2006; Ramsay and Glover, 2005).

1.5 Biotechnology approaches to study gene function

Genetic engineering is based on DNA manipulation by introducing genes into host organism through a competent vector (Jones et al., 2012). A number of techniques have been introduced

for plant transformation i.e. microprojectile method, microinjection method, silicon carbide-mediated transformation, electroporation mediated transformation and *agrobacterium* mediated transformation (Abdin et al. 2017).

1.5.1 Agrobacterium mediated gene transformation

Agrobacterium (*GV 3101*) mediated transformation is among one of the most commonly used transformation methods for plants. *Agrobacterium tumefaciens* is a soil borne gram negative bacteria which causes crown gall disease in plants. It has ability to transfer foreign T-DNA (transfer DNA or vector DNA containing gene of interest) into host organism efficiently through the help of its special transformation gene system that mainly consist of many *vir & VIP* proteins (Abdin et al. 2017).

1.5.2 Tissue Culturing

Plant cells are totipotent. By tissue culture technique cells or tissues (called explant) from donor organism can be grown *in vitro* into new plants/callus, in a growth medium, usually made up of broth or agar. Explants are cultivated on a culture medium containing essential nutrients, hormones and energy sources. This technique gives an *in vitro* tissue of particular plant that can be used for multiple research purposes (Carrel & Burrows, 1911).

1.5.3 Electroporation

Electroporation is a technique that do not directly used as studying gene function approach. Rather it is transformation of DNA into host cell through high voltage electric shocks. It usually requires fewer steps compared to other transformation approaches and give stable transformation and transient gene expression. Through this method, one can create new aqueous pathways in lipid bilayer of plasma membrane. Enhanced movement of molecules and ions is the base of electroporation and membrane recovery after the pulsing is also very important consideration to stop reversal of molecules (Weaver & Chizmadzhev, 1996). Electroporation referred for transfection of foreign molecule into host cell by electric pulse that create a pore across phospholipid bilayer and charged molecules like DNA can move across the membrane (Shigekawa & Dower, 1988).

1.6 Overexpression analysis of R2R3-MYBs

Genes can be overexpressed through expression system of other organisms. In molecular engineering, this approach can be used as a tool to study gene functions. By using a promoter, for example *CamV*, along with vector, it is possible to overexpress a gene and accumulate the

particular protein (Abdin et al., 2017). *Vitis vinifera* is the most studied fruit concerning the regulation of flavonoids biosynthesis by MYBs. *MYBF1* of *Vitis vinifera* regulates transcription of *FLS1* during flowering and induces high amount of flavonols. Afterwards, during early fruit developmental stages, *MYBF1* decreases while *MYBPA1, MYBPA2, MYB5a* and *MYB5b* induce PA production (Czemmel et al., 2012). These TFs regulate expression of enzymes i.e. CHS, CHI, DFR, LAR and ANR. Later, during fruit ripening, there is a switch from *PAMYBs* to anthocyanin biosynthesis regulators such as *MYBA* and *UFGT*. They regulate the accumulation of high concentration of anthocyanins in ripe fruit and also during ripening, *FLS1* is re-induced by *MYBF1* and produces flavanols (Bogs et al. 2007; Terrier et al., 2009). Similarly, *VvMYBA* of grapes can activate promoter of *VvUFGT* (Noda et al., 2004). Transcriptional regulation of the specific *R2R3-MYB* TFs of the flavonoid biosynthesis pathway can activates enzymes i.e. CHS, CHI, F3'H and DFR, and this regulation can enhance nutritional or medicinal aspects of fruits, vegetables and ornamental plants (Park et al., 2008). *GMYB10* of *Gerbera hybrida* activates genes of both early and late steps of flavonoid pathway (Laitinen et al., 2008) but *VvMYBA* of *Vitis vinifera* only regulates genes that are involved in later steps of anthocyanin biosynthesis pathway (Czemmel et al., 2012). *CsMYB5a* from *Camellia sinensis* downregulates the anthocyanin formation but enhances PA contents in tobacco flower while *CsMYB*5e also increased dimethylaminocinnamaldehyde stained PA concentration (Jiang et al., 2018).

Comparative analysis of *Vaccinium uliginosum* white mutant and wild type berries have shown that there was down regulation of structural genes i.e. *VuCHS, VuDFR, VuANS* and *MYBPA1* in mutant berries compared to wild type berries proving that there could be difference between regulation of anthocyanins and PAs synthesis in different plants (Primetta et al., 2015). A *R2R3-MYB* from blueberry having similarity with anthocyanin promoting *MYBs* of subgroup 6, was overexpressed transiently along with *bHLH* and resulted into activation of anthocyanin production in *Nictotiana benthamiana* (Plunkett et al., 2018). So according to their functional enzymatic profiles, *MYBs* can be classified in different subgroups based on their role in regulating flavonoids biosynthesis.

1.7 Aim of study

The main objectives of this study were to construct overexpression vectors with eight different bilberry *VmMYBs TFs* genes. To study specified functions of *VmMYBs* in regulation of proanthocyanidins and anthocyanins biosynthesis, the selected bilberry *R2R3MYBs* were overexpressed in leaves of the model plant *Nicotiana benthamiana*. Also, bilberry callus was developed and tested for the overexpression of the developed *R2R3MYB*.

2 Materials and Methods

2.1 Plant Materials

Nicotiana benthamiana seeds were sown on peat soil blended with limestone smack and Perlite. After germination, they were transferred in pots 12 x 12 (1 *liter*) in a controlled chamber at 21 ^{o}C, 24h light, 60% humidity. After 15 days of sowing seeds, N-fertilizer (mixed with P, K) was added into each pot. Plants were watered daily. Overexpression analysis was performed on newly grown leaves of tobacco.

2.2 Phylogenetic analysis

The phylogenetic analysis was carried out by using neighbor joining model in MEGAX 6.0 and data was screened with 1000 bootstrap. *MYB* genes of grapes and peach were obtained from NCBI. Grapevine *VvMYBPA1 (CAJ90831), VvMYBPA2 (ACK56131), MYB5a (AAS68190), MYB5b (AAX51291)* and in peach *PpMYB7* (Alo81018.1) were used.

2.3 Target genes and Primer designing

MYB genes have been isolated previously from bilberry and were stored in *pJet* plasmid. All studied *VmMYBs* were already sequenced, and their sequences were provided by Climate Lab for primer designing with restriction sites to facilitate cloning for pGreen 62-SK vector (Appendix I). "Primer 3" (http://bioinfo.ut.ee/primer3-0.4.0/) online software was used to design primers sequences. To avoid any restriction cutting site inside the sequence, they were checked online on "Web Cutter" (http://www.firstmarket.com/cutter/cut2.html) against all possible restriction enzymes. Primers were ordered from the Sigma-Aldrich, (Darmstadt, Germany) to develop primers. The designed primers are shown in Table 1. Primers were diluted to 10μM prior use.

Table 1: Sequences of designed primers used for cloning

MYB		Sequences	Restriction enzyme
2.1	F[1]	5'-CGGGATCCATGGGTAGAAGTCCTTGTTGT-3'	BamHI
	R[2]	5'-CGGGGTACCTTACTCTTGAAGCCAATCC-3'	KpnI
2.2	F	5'-CGAGCTCATGGGTAGGAGCCCTTGTTGT-3'	SacI
	R	5'-CGGGGTACCTTATTGATCTTGAAGCCAATCTATGC-3'	KpnI
2.3	F	5'-CGGGATCCATGGGGAGAAGCCCTTGTT-3'	BamHI
	R	5'-CCCGGGGTACCTTAATTATCTGCTAGCCAATCACC-3'	KpnI
2.4	F	5'-CGAGCTCATGGGGAGAAGCCCTTGTTGTGC-3'	SacI
	R	5'-CGGGGTACCCTAGCCAACCCCATTTGATTGA-3'	KpnI
2.5	F	5'-CGAGCTCATGGGAAGAAGACCATGCTGTG -3'	SacI
	R	5'-CGGGGTACCTCATCTAGTGATCCATTCCTCTG-3'	KpnI
3	F	5'-CGAGCTCATGGGTAGGAGTCCTTGTTGTG. -3'	SacI
	R	5'-CGGGGTACCCTATTCATCTTCTAACTCCAGAAA-3'	KpnI
MYB 5	F	5'-CGAGCTCATGAGGCAGCCATCAAGATCGT-3'	SacI
	R	5'-CGGGGTACCTCACTTGCAGAACTTCTTTTCAACA-3'	KpnI
MYB 7	F	5'-CGAGCTCATGGCAGAAGAGTCCATGAT -3'	SacI
	R	5'-GCGCGGGGTACCTTATGATAATTCACATAA-3'	KpnI

2.4 Overexpression Construct Development

2.4.1 PCR amplification of genes

Each reaction mix prepared consisted of 10µl Buffer 5xHF + 1µl dNTPs (10mM) + 0.3µl phusion polymerase + 36.2µl H_2O and 1 µl of *pJet* plasmid and primers each. Reactions were

[1] F= Forward primer

[2] R= Reverse primer

Yellow label sequences are restriction site attached with designed primers.

set up in PCR tubes and transferred to PCR machine (BIO RAD Mini Opticon real time PCR) and PCR was consisted of cycles shown in Table 2.

Table 2: Details of PCR cycle for amplification of genes

1	94 °C	5 min	
2	94 °C	1 min	Step 5=45 times
3	60 °C	2 min	
4	72 °C	2 min	
6	72 °C	10 min	

Visualization and separation of amplified *MYB* DNAs was done by gel electrophoresis using 1% agarose gel (composition in Appendix III) and ladder used was Thermo Scientific™ *Mass Ruler DNA Ladder* Mix (1kb) (Appendix IV). Amplified samples were mixed with 6x orange dye and run with BIO-RAD gel electrophoresis at 45V for more than one hour. DNA fragment/plasmids were visualized under UV light using a DigiDoc-It™ darkroom system and gel picture captured by using UVP Doc/It ® LS Image Acquisition software. The similar gel electrophoresis process and imaging software used for all electrophoresis in this study. Bands were cut under UV light.

Purification of DNA from gel was done by E.Z.N.A.® Gel Extraction kit-spin protocol by Omega BIO-TEK,(Norcross GA USA). To disslove gel, 250µl of XP2 binding buffer was added and incubated for 7 minutes at 60°C. *HiBind*® DNA Mini Column was placed in an 2ml eppendorf tube and 700µl of melted sample solution was added and centrifuged at 10,000g for 1 minute. After discarding the filtrate, 300µl of XP2 buffer was added, centrifuged and filtrate discarded and 700µl of SPW wash buffer was pipetted and centrifuged. At the end, *HiBind*® DNA Mini Columns were transferred to new eppendorf tubes, pellet was eluted with 30µl of deionized water, incubated for two minutes and centrifuged for 1 minute. Filtrate contained extracted DNA. Concentration of extracted *MYBs* fragments was quantified by nanodrop measurement *in* Thermo Scientific™ 2000/2000c spectrophotometer.

2.4.2 Vector Isolation, Restriction digestion and MYBs Ligation

pGreenII 62-SK (3342bp), a binary cloning vector that have *CamV* promoter along with enhancer 35S constructed by Hellens et al., (2005), was used for transient gene over expression in plant expression system. Complete structure and sequence of *pGreen* are shown in Appendix I and II. *pGreenII* contain kanamycin (kan) and rifampicin (rif) antibiotic resistance genes. *pSoup* was used as a helping plasmid for pGreen and it contains tetracycline (tet) resistance gene. Bacterial strain *DH5a E.coli* was utilized for cloning while to *Agrobacterium tumefaciens (GV3101)* was used to infiltrate the vectors into the host plant. In this study, this vector was used for transient overexpression into *Nicotiana benthamiana* leaves and bilberry calluses.

Colonies of *E.coli* containing *pGreen* and *pSoup* were grown in separate LB plates (composition in Appendix III) at 37 ^{o}C. From colonies, overnight cultures of both *pGreen* and *pSoup* containing bacteria were made with 6ml liquid LB and 2.4µl of tet for pSoup (5µg/ml) while 6µl of kan (50µg/ml) for *pGreen*. Plasmids were isolated by GenElute^{TM} plasmid miniprep kit (Sigma-Aldrich, Steinheim, Germany). Cultures were grown in shaker at 180rpm, 37^{0}C overnight. Next day, cultures were centrifuged at 12000g, 1 minute, supernatant discarded and 200µl resuspension solution was added into respective tube and pellet was homogenized by vortexing. Resulted mixtures were transferred to 1.5ml eppendorf tubes and 200µl of lysis solution was pipetted and tubes were inverted gently to lyse the bacteria. Amount of 350µl of neutralize solution was added, inverted and spun down at 13500 rpm, 10 minutes. GenElute Miniprep Binding column was inserted into microcentrifuge tube and clear lysate from above transferred into it. After centrifugation for 1 minute, 700 µl of diluted wash solution was pipetted into the columns and again centrifuged for 2 minutes, 13400 rpm. Columns were transferred to new 1.5ml eppendorf tubes and 50µl of molecular biology grade water added, waited for 7 minutes and centrifuged again for 1 minute. The elute contained DNA. Recovery and purity of isolated plasmids was analyzed by nanodrop.

In this study, 0.5µg of each gene and 2µg of *pGreen* plasmid was used for restriction cutting. For restriction digestion, protocol followed is shown in Table 3 and restriction cutting sites of genes have been shown in Table 1. *MYBs* PA 2.1 & 2.3 were digested with restriction enzyme *BamHI* and *KpnI (restriction site: 5'*GGATCC‘3 & *5'*GGTACC‘3, respectively) while all other PA *MYBs* were digested with *SacI* and *KpnI (*5’GAGCTC’3 & *5'*GGTACC‘3, respectively).

Total of 40µl reaction was used. The well mixed reactions were incubated in heat block at 37 ^{o}C for 50 minutes.

Table 3: Chemicals used for restriction digestion

10x buffer green (vortexed)	4µl
H_2O	According to calculations
DNA/plasmid	0.5µg (calculated)
Restriction enzyme	2 x 2 µl

Restriction reaction were run by gel electrophoresis and visualized under UV light. Both DNA and plasmid were extracted using E.Z.N.A.® Gel Extraction kit-spin protocol by Omega BIO-TEK and their purity and concentration was measured by nanodrop spectrophotometry.

Ligation of respective genes into plasmid was done according to Thermo Fisher Scientific T4 DNA Ligase- Sticky end Ligation. In PCR tubes, 2µl of 10X T4 DNA ligase buffer was added and vortexed. Around 100ng of vector was added while genes concentration was added with 100ng. Amount of 5µl of T4 DNA ligase was added in reaction mix. All reactions were incubated for 3 hours at room temperature.

2.4.3 Preparation of DH5*a E.coli* competent cells and pGreen Transformation:

DH5*a* were thawed from -80 ºC freezer, on ice and grown overnight in 5ml liquid LB at 37 ºC, 160 rpm. Next day, in two 1L flasks, 100ml LB media and 5ml of overnight grown culture was combined and grown continuously at 37 ºC until OD_{550} = 0.48 (2:50 h). Optimal density of bacteria was measured by Bio-RAD SmartSPec^{TM} plus spectrophotometer. Bacterial culture was then transferred into falcon tubes, incubated on ice for 15 minutes and centrifuged twice at 2000rpm, 5min, 4 ºC in centrifuge 5804R (Eppendorf) to obtain a pellet. Supernatant was discarded and 10ml TFB I buffer was used to suspend the pellet by turning the tubes. Centrifugation and resuspension were repeated with 33ml TFB I. Afterwards, tubes were centrifuged and 4ml of TFB II buffer was pipetted and pellet was suspended again in buffer. After keeping on ice for 20 minutes 200µl of bacterial suspension (competent cells) was pipetted into 2ml Eppendorf tubes and frozen in liquid nitrogen and stored at -80 ºC. Composition of TFBI & TFBII are given in appendix VIII.

Competent DH5*a E. coli* were thawed on ice for 15 minutes and 100ng of ligation reaction was added. Tubes were incubated for 40 minutes and then transferred for heat shock transformation

to 42 ºC water bath for 2 minutes and quickly transferred back on ice. 250µl of liquid LB media was added into each tube and shaken at 37 ºC, 150rpm for 1.5 hours. After shaking each insert was spread on three plates as 200µl, 100µl and 150µl. After drying, all plates were incubated at 37 ºC overnight to grow maximum number of colonies.

Transformation was confirmed by PCR and gel electrophoresis. Each reaction mixes for thermal cycler amplification was consisted of 5µl Buffer 5x (green Go taq Flexi), 1µl $MgCl_2$ (25mM), 0.5µl dNTP (10mM), 1µl pGreen forward & reverse primers (10mM), 0.15µl Go taq polymerase, Template DNA and H_2O. Half of the selected colony was used for PCR reaction while other half of same colony was spread in new LB + KAN plate to grow further and to keep bacteria colonies alive. Plates were incubated at 37 ºC, for 24 hours. Thermal cycler program consisted of steps that are shown in Table 4. After PCR, amplification reactions were run by gel electrophoresis and visualization of DNA bands was done under UV light and image was taken.

Table 4: Steps involved in PCR for trnasformation confirmation

1	95 ºC	3mintues	
2	94 ºC	0.30sec	Step #5
3	60 ºC	0.30sec	29 times
4	72 ºC	2.00 minutes	
6	72 ºC	10 minutes	

Replicates of each insert with clear bands were selected for plasmid isolation was done by GenElute™ plasmid miniprep kit from Sigma-Aldrich. Purity and quantification of isolated plasmid was measured by nanodrop spectrophotometry.

2.4.4 Confirmation of Inserts sequences by Sequencing:

Sanger sequencing was used to verify precise ligation of MYB sequences. For sequencing, PCR reaction was used to amplify inserts and for that template was diluted to 100ng. Master mix was consisting of 1µl RRM + 3.5µl Buffer + 1µl of *pGreen* forward (*ACTATCCTTCGCAAGACCCTTC)* and reverse (*CCCTTATCGGGAAACTACTCAC*) primers

(1 µM) and 100ng of template plasmid and H_2O to make each reaction 20µl. Steps of PCR are given in Table 5. Afterwards, the product was sent to Medical faculty of UiT, for sequencing of fragments.

Sanger sequences were viewed in a software "Chromas" to analyze the obtained full-length gene sequences. "The Bioweb" software was used to make complementary sequences by creating reverse strands. "Clustal Omega" was used to find overlapping sequences in forward and reverse strand to finalize sequences and to compare to the original sequences.

Table 5: Thermal cycler steps for Sequencing of Plasmids

1	96 ºC	30 sec	Step #4 29 times
2	55 ºC	15sec	
3	60 ºC	4 minutes	
5	4 ºC	End	

"ExPASy translate" (https://web.expasy.org/translate/) tool was used to obtain open reading frames (ORF) of respective MYBs insert and to translate amino acid (AA) sequences (Appendix XII). These AA sequences were also compared with original AA by "Clustal Omega" (https://www.ebi.ac.uk/Tools/msa/clustalo/). Correct restriction sites and presence of start codon in all inserts were also verified manually from sequences.

2.4.5 Preparation of competent *Agrobacterium* (GV3101) cells & Electroporation.

Agrobacterium Gv3101 were fetched from -80 ºC and melted on ice and cultured on already prepared agroLB + rif (Appendix III) containing petri plates. Overnight culture contained 5ml agroLB + 2µl rif and fresh cultured colony of Agrobacterium and incubated at 28 ºC, 180 rpm. Next day, 100ml of agroLB was added into conical flask along with 50 µl of rif. The flask was shaked at 28 ºC, 170 rpm until the required OD (0.5 - 1.0) at 600nm was obtained. Culture was transferred into two new falcon tubes and centrifuged at 4 ºC, 4000g, 15 minutes to get a clear pellet. Supernatant was discarded and 25ml of 10% glycerol was pipetted in each tube. Pellet was suspended slowly on ice, centrifuged again, supernatant discarded, and pellet was resuspended with 10ml of 10% glycerol. Again, pellet was suspended slowly, and this process

was repeated further with 10ml, 4ml and 2ml of 10% glycerol. At the end, 1ml of 10% glycerol was used to resuspend the pellet and 100µl of this culture was pipetted into 1.5ml eppendorf tubes, immediately frozen in liquid nitrogen and stored in -80 ºC freezer.

Electroporation was done by using BIO-RAD, GenePulser XCell electroporator. Amount of 1µg of *plasmid* & *pSoup* each was pipetted to same tube with GV3101 and mixture was transferred to a pre chilled 2mm gap cuvette carefully. On pulse-control unit of electroporator, resistance was set to 200 Ω (Ohm), capacitance 25 µFD and volts 2.5 kV. Cuvette was placed in cuvette holder and electroporated until the tone sound. Immediately, 1ml of LB media was added into cuvette and then transferred the suspension was transferred into culture tube. Electroporation time (mSec) was noted (Appendix XI) and same process was repeated for all other inserts. Bacterial suspension was grown in shaker at 28 ºC for 3 hours and then spread 100 µl, 10 µl, 1 µl, on agroLB with kan + rif plates for each gene. Plates were incubated at 28 ºC for 5-6 days until appearance of growing colonies.

After electroporation, transformation of vectors into agrobacterium was validated by PCR. Every reaction mixture used was consisted of 5µl Buffer HF + 1µl $MgCl_2$ (25mM) + 0.5µl dNTPs (10mM) + 1µl pGreen forward & reverse primers (10mM) + 0.15µl Phusion polymerase, template DNA and H_2O. The details of PCR reaction steps are similar to Table 4. For further confirmation, same process was repeated but with gene specific primers (Table 1) as well.

2.5 Development of bilberry callus culture

WPM media was prepared according to Appendix III. For callus growth, equal concentration of auxin and cytokinin was used. Naphthalene acetic acid (NAA) is an auxin, 6-Benzylamino purine (BAP) is cytokinin and Thidiazuron (TDZ) is cytokinin like hormone. Each hormone was added in 1ml/L concentration in WPM media. Three different hormonal combinations were used, 1) NAA+TDZ, 2) NAA+BAP, 3) NAA + BAP + TDZ.

Explants (bilberry unripen fruit and young leaves) were collected from Holt Arboretum. All fruits were cut half, seeds were removed while leaves were also cut into pieces. Explants were washed with sterile H_2O three times and with few drops of tween20 solution (Sigma Aldrich, Merck KGaA, Darmstadt, Germany). Explants were washed over with 70% ethanol and then 1% of bleach (NaOCl) was added for 5 minutes. Tissue samples were washed with water until all bleach had been removed. All explants were cultured on WPM media. All plates were sealed

with parafilm and incubated in growth chamber with 16 h light, 22 temperature, 60% humidity for callus growth. Total 56 plates were cultured. After 5-6 weeks, all newly developed calluses were subcultured into new media containing all three hormones (NAA+BAP+TDZ). Within 2-3 weeks calluses were subcultured again to maintain callus growth in greenish white color.

2.6 Agroinfiltration into *tobacco leaves* and bilberry calluses

Fresh colonies of Agrobacterium containing MYB genes were inoculated on agroLB with selective marker antibiotics kan + rif + tet. Agrobacterium colonies containing *bHLH* cofactor and *pGreen* empty vector (negative control) were also grown. One day prior to agroinfiltration, fresh colonies were again grown with only kan + rif antibiotics. Infiltration reaction was prepared by mixing fresh colonies with 10ml of infiltration buffer (0.5ml MES in 50ml H_2O, 0.5ml $MgCl_2$ in 50 ml H_2O and 100µl of acetosyringone in 50ml mixture). This buffer contained acetonsyringone that induces expression of *vir* genes serving helper agent for infiltration (Bundock et al. 1995). OD_{600} for each sample was confirmed between 0.4-0.7 and samples were incubated in dark for 2-3 hours. Mixture of each gene and AtbHLH (1:1) was made in a 2ml eppendorf tube. Then, by using 1ml of syringe without needle, the solution was infiltrated into lamina tissues of lower epidermis of selective leaves of *Nicotiana benthamiana*. For each gene, two plants were selected, and 2-3 leaves of each plant were infiltrated. *MYBA1* was infiltrated as a positive control for anthocyanin accumulation. Bilberry calluses were infiltrated with *MYBA1* and *MYBA2* and negative control. *MYBA1* and *MYBA2* constructs from bilberry were already prepared in an earlier study. All of the treated plants and calluses were grown in growth room with 21°C and around ~150 µmol, constant light for 7 days. Plants were watered daily, and pictures were taken before and after infiltration. Afterwards, infiltrated sites of tobacco leaves were cut and stored at -80 °C for further analysis.

2.7 Anthocyanin determination

Measurement of anthocyanins contents was done by methodology from H. Chu et al., (2013), with some modifications. Extraction was done with three replicates of each treatment. Frozen leaves were ground into fine powder by using liquid nitrogen. Approximately, 0.1g of finely ground plant samples were weighed to 2ml tubes. Samples were extracted with 1ml of methanol mixed with 1 % of HCl (v/v). Samples were then vortexed well and incubated for 18 hours at 100 rpm shaking, covered with aluminum foil in darkness. Pelleting of plant material was done by centrifugation for 10 minutes, 17000g at 23 °C. To remove the chlorophyll contents from extraction, 500µl of supernatant was mixed with 500µl of H_2O + 300µl of chloroform in 2ml

microcentrifuge tubes. Tubes were shaked for 30sec and centrifuged again for 5 minutes and 17000g. Supernatant was transferred into new tubes. Absorbance measurement of extracts was done by visible spectrophotometer (SmartSpec Plus, Bio-Rad) at wavelengths of 530nm and 657nm. Anthocyanin absorbance was estimated by using $Q_{anthocyanin} = [A_{530nm} - (0.25 \times A_{657nm})]$ while total anthocyanin contents were measured according to equation given in Appendix IX. Blanking of spectrometer was done by using 500µl of extraction solution + 500µl water.

2.8 PAs determination from infiltrated leaves

Total PA contents were determined by using modified protocol from Li et al. (1996). Tobacco leaf tissues were grounded in liquid nitrogen and approximately 0.05g of fine powder was weighed in 2ml tubes, 1ml methanol (80%) was added on the samples, and tubes were vortexed and incubated on moderate shaking for 18 hours in dark. All samples were centrifuged for 10 minutes, 17000g at 23 ^{o}C. Supernatant was collected and 550µl of liquid was transferred to 1.5ml microcentrifuge tubes containing 275µl of DMACA solution (4 – dimethylaminocinnamaldehyde). Samples were incubated for 30 minutes for color development and picture was taken. PA contents were measured by using visible spectrophotometer (SmartSpec Plus, Bio-Rad) at wavelengths of 643nm. Blanking was of spectrometer was done by using of 550µl 80% methanol + 275µl DMACA solution.

3 Results

3.1 Phylogenetic tree analysis of MYBs

Bilberry *R2R3MYBs* that were studied are closely homologous to responsible *R2R3MYBs* genes of *Vitis vinifera* and *Prunus persica*. Figure 4 demonstrates the phylogeny tree analysis between *MYB* family genes of bilberry, peach and grapes. It is likely that, all bilberry PA2 clade *MYBs* (*VmMYBPA* 2.1, 2.2, 2.3, 2.4, 2.5, PA3) are clustering with *VvMYBPA2*. *VmMYB5* has branched together with *VvMYB5b* so it is presumable that both of these genes share sequence similarity. *VmMYBA1* (positive control) sequence is close to *VvMYBA1 and VvMYBA2. VmMYB7* sequence have clustered with *MYB7* of *prunus persica.*

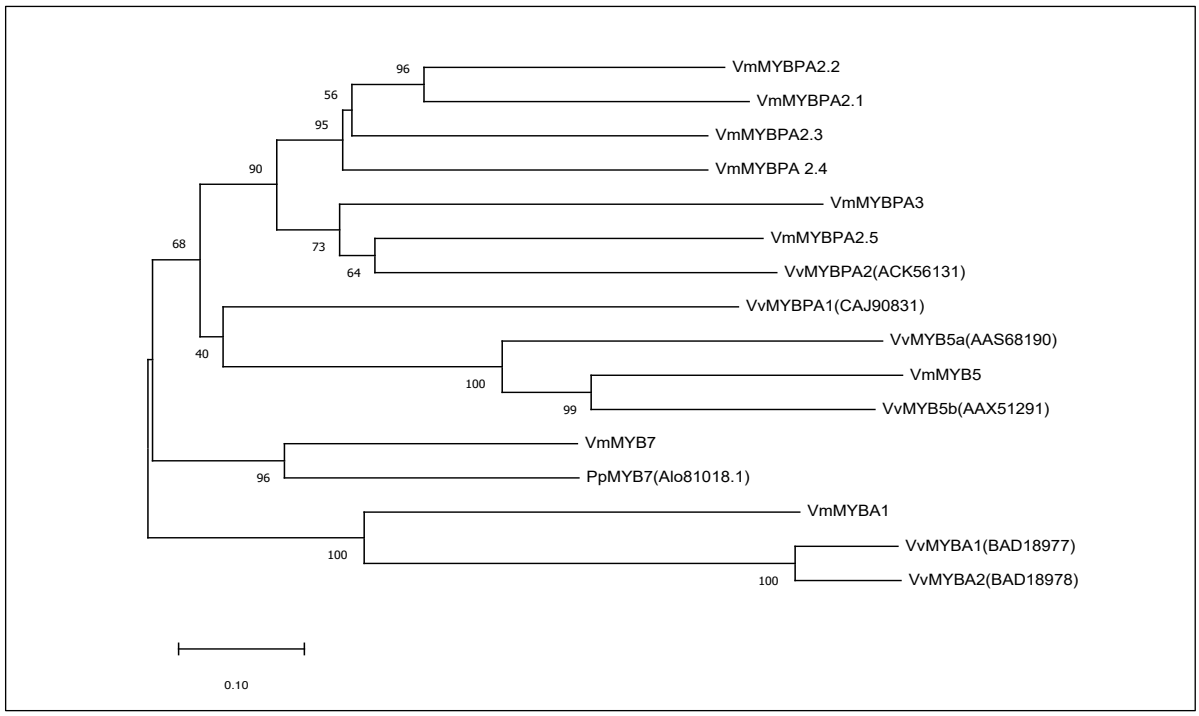


Figure 4: *Phylogenetic comparison of MYBs of bilberry, Prunus persica and Vitis vinifera.* This tree was constructed by using the Neighbor joining model in MEGAX version 6.0. All full-length amino acid sequences were aligned by using Muscle software. Number on branches are showing bootstrap value. The gene bank accession numbers of respective *MYB* genes of *Vitis vinifera & Prunus persica* has been shown in phylogenetic tree.

3.2 Vector Construction

In this study, *pGreenII 62-SK* vectors were successfully developed for eight bilberry *MYB* TF family genes. A dual-binary vector system *pGreen/pSoup* from Hellens et al., (2005), was tested for transient overexpression of bilberry *MYB* genes in to leaf lamella of *Nicotiana benthamiana*

and bilberry fruit callus. Functional overexpression analysis of these TFs in tobacco leaves indicates their role during biosynthesis of anthocyanin and PAs. All studied MYBs were already cloned during previous studies from *V. myrtillus*. Here, they were successfully ligated at right position in vector.

3.2.1 MYBs amplification

PCR assay was used to amplify genes of interest from *pJet* plasmid. Figure 5 shows the gel electrophoresis results of the PCR assay of *VmMYB* genes. Strong DNA fragments indicate successful primer binding and amplification of the genes compared to 1kb gene ruler. DNA fragments were extracted from the gel and their nanodrop concentrations are shown in Appendix V.

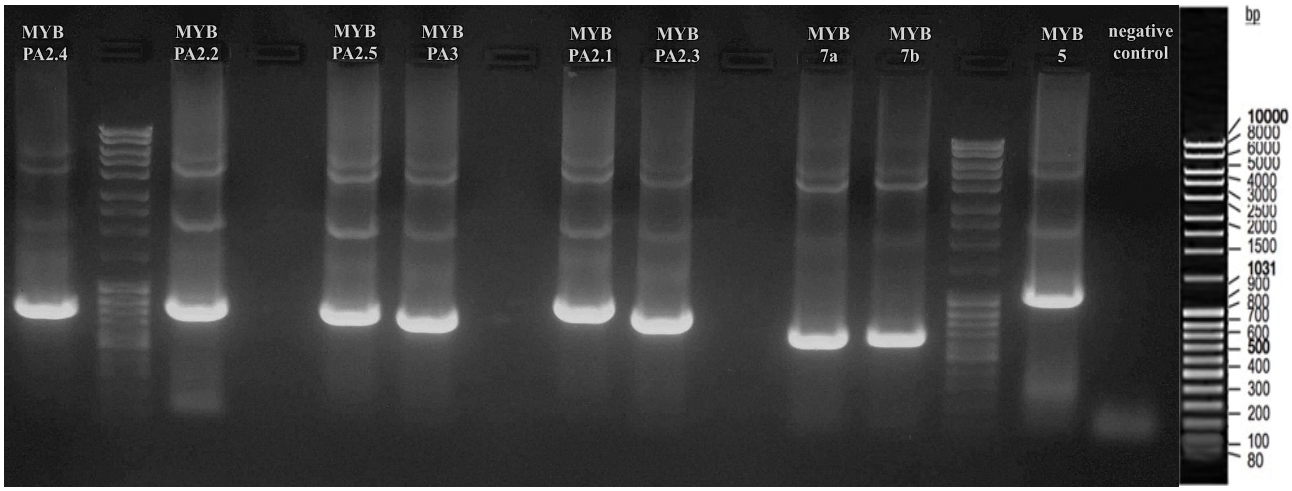


Figure 5: Gel electrophoresis of the PCR amplification of the target MYB genes. Clear fragments of all eight MYBs are visible and negative control without any DNA band. 1kb mass ruler was used to compare gene sizes.

3.2.2 Restriction digestion and ligation into linearized *pGreen* plasmid

pGreen and *pSoup* plasmid were isolated from *E.coli.* The DNA concentrations of the two samples of *pSoup* plasmids were 143.7 ng/µl and 154.8 ng/µl while for *pGreen* plasmids they were 647.8 ng/µl & 672.8 ng/µl. Restriction digestion of both *MYBs* and plasmids for sticky ends was done successfully using restriction sites that were present on 5' end of primers. *pGreen* plasmid was successfully linearized to 3.3kb sequence by both *SacI* and *KpnI* and also with *BamHI* and *KpnI.*

In the order to obtain full length fragments, restriction cutting was purified by agarose gel electrophoresis shown in Figure 6. Obtained DNA fragments were verified via known sizes of

MYBs and *pGreen*. *MYBs* genes and plasmid DNAs were extracted from the gel and their concentration was measured by nanodrop (Appendix VI). T4 DNA ligase successfully joint sticky ends of *MYB* genes at respective restricted site on *pGreen.*

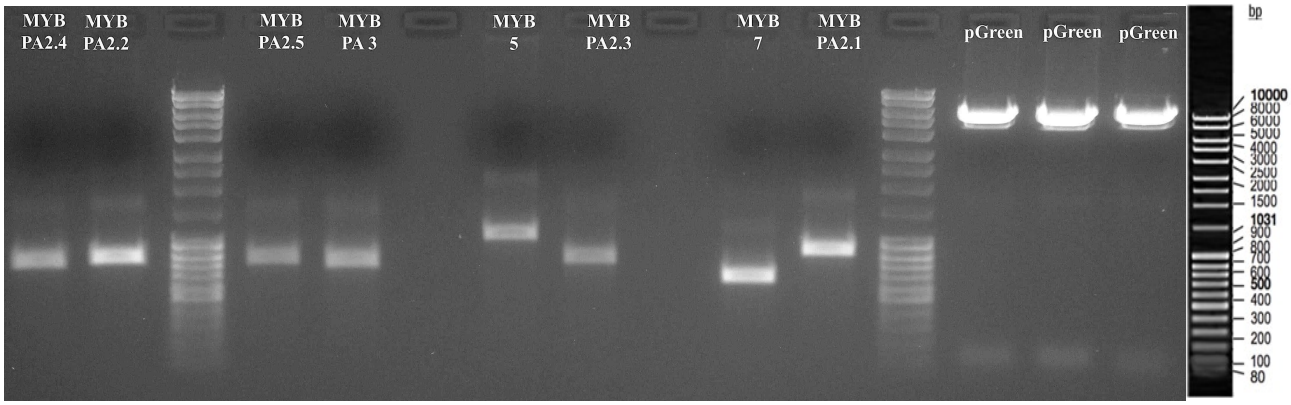


Figure 6: Gel electrophoresis assay for enzymatic digested MYBs. Clear fragments for all MYBs and plasmids illustrate that targeted digestion by restriction enzymes was successful and there is a clear difference in band sizes of gene fragments and plasmid DNA running along the Gel.

3.2.3 Confirmation of inserts into DH5*a E.coli*

The ligation and transformation processes were verified by *E.coli* colony growth with selective marker kan. After transformation, 24 hours incubation assay produced many bacterial colonies as shown in Figure 7 (example of one PA type *VmMYB* plasmid and similar colonies were grown for all other inserts).

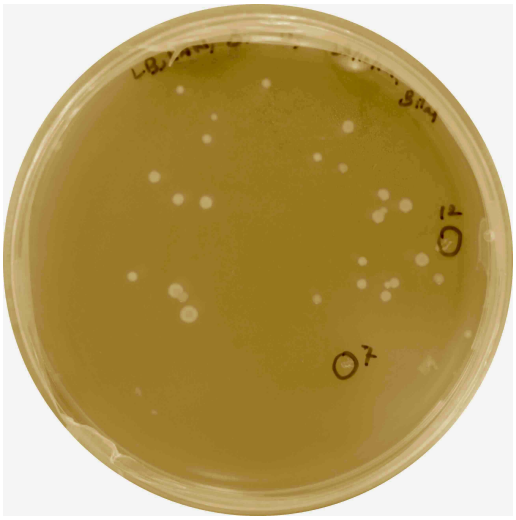
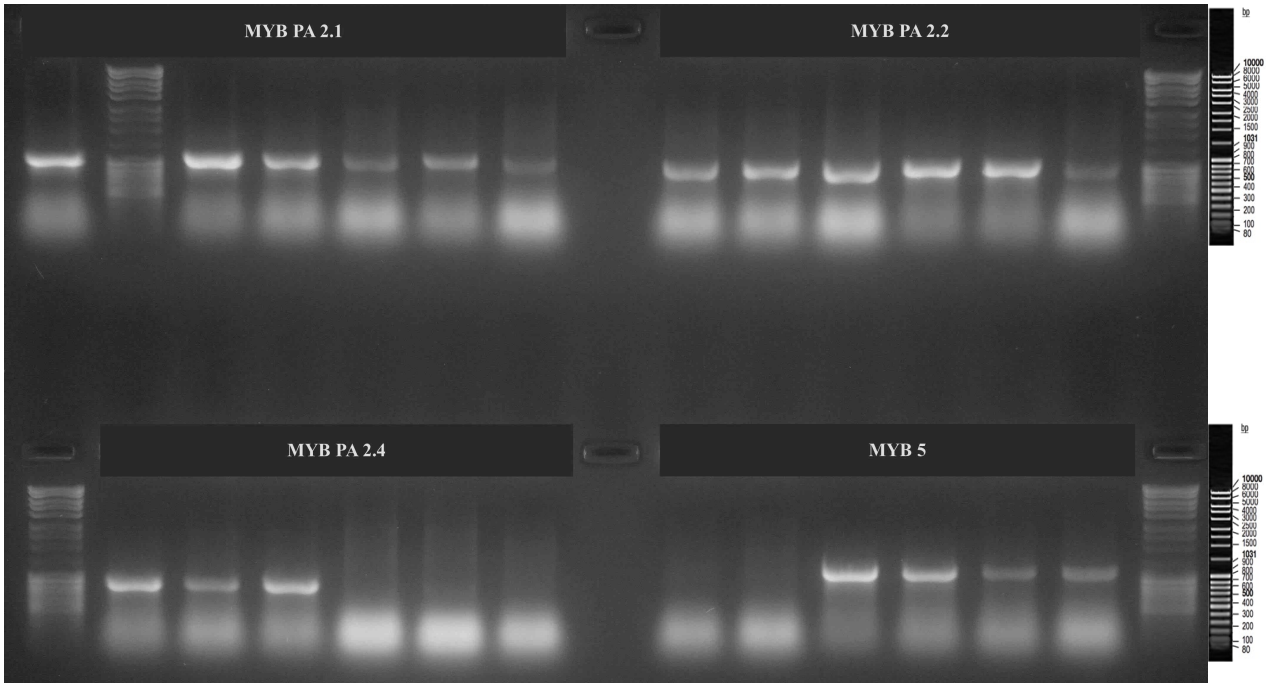


Figure 7: Growth of E.coli colonies for transformed bacterial clones. Colonies after transformation of recombinant pGreen with genes of interest. KAN was marker in LB media. Bacteria showing growth after 1-2 days.

PCR assay was further used to amplify selective inserts. The colony growth in selective marker and high amplification of inserts verified the successful ligation of *MYBs* into vector. Figures 8 is illustrating the gel electrophoresis results of PCR amplified product for all inserts. Visualized fragment lengths are larger than the original gene sequences because of used plasmid specific primers. Two samples of each gene with clearly visible DNA bands were selected. Plasmids were isolated from overnight culture of each genes and DNAs concentration was measured by nanodrop (Appendix VII).

(A)



(B)

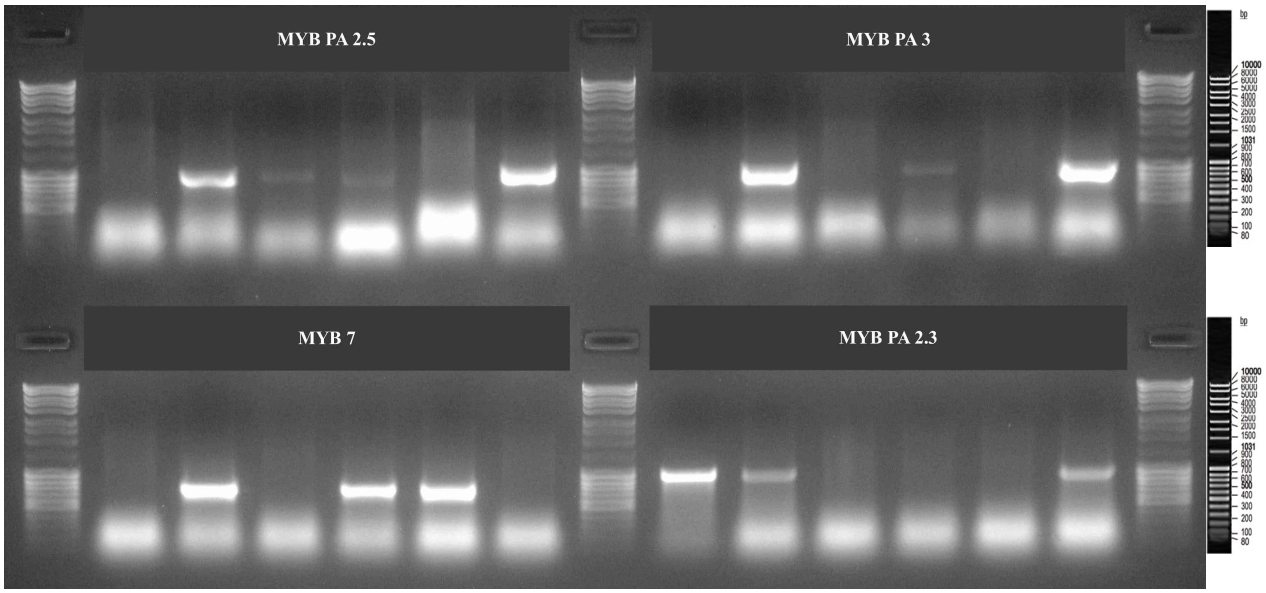


Figure 8: Visualization of the cloned Inserts for PA type MYBs (A) Colonies with high growth rate were analyzed by PCR and run in gel electrophoresis. Each gene had 6 replicates and most them have shown results. Only 3 samples of *MYBPA 2.4* and 2 of *MYBPA5* were not successful. *(B)* Most of samples are showing positive results for transformation.

Insert high copy number availability was necessary for electroporation transformation and that was obtained efficiently from all different samples of each target genes. Two samples of each gene with clearly visible DNA bands were selected. Afterwards, final verification of correct ligation was done by Sanger sequencing and sequencing output of inserts was compared with the original gene sequences. The results confirmed that all *VmMYB* genes were ligated correctly at projected site inside the *pGreen* plasmid. Additionally, predicted amino acid sequences were compared with amino acid sequences of the respected genes showing similarity with the original ORFs, confirming that constructs were ready to be transformed into agrobacterium and to continue with infiltration assay.

3.3 Electroporation transformation of construct into GV3101 *Agrobacterium*

Transformation of dual binary vectors (*pSoup* & recombinant *pGreen*) into *Agrobacterium tumefaciens* was successfully done by electrophoresis. Time (mSec) used for each electroporate reaction have given in Appendix X. Figure 9 presents example of colonies growth of *Agrobacterium* harboring developed construct *VmMYBPA2.4* under antibiotic selection markers. Transformants were selected on LB with kan and rif as selection marker where they showed distinctive growth and later their growth was also verified by using triple combination of antibiotics i.e. kan, rif, tet, as a selective marker. Growth of agrobacterium was slower than expected in tetracycline rich media and usually it took 5-6 days until fully grown colonies appeared. Only media that were plated with 100µl or higher electroporate filtrate, were able to grow clear colonies. Successful transformation of the constructs was validated by using PCR assay and through antibiotic selection markers.

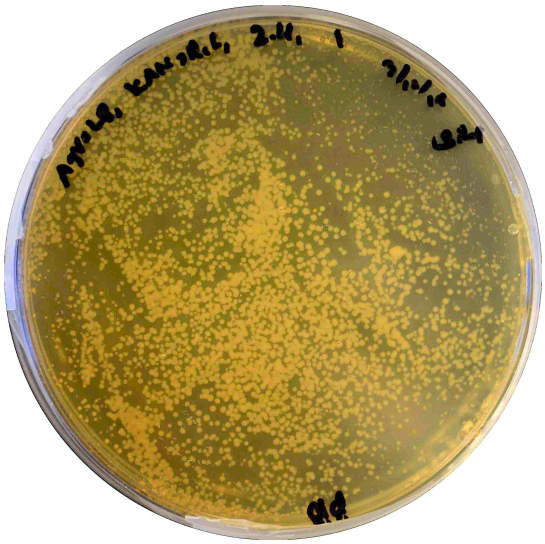
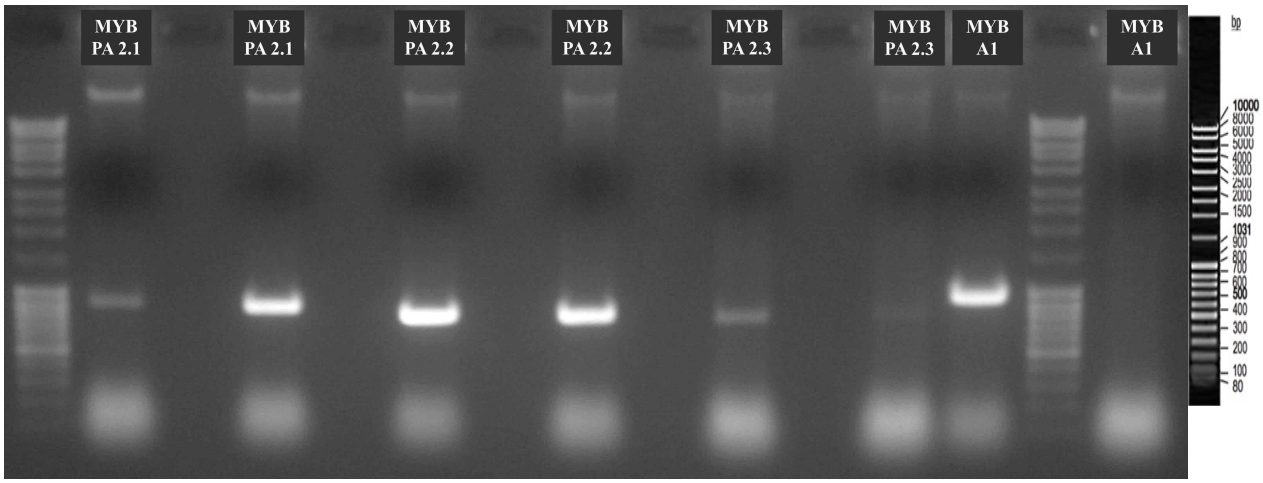


Figure 9: Agrobacterium colonies formation after electroporation. Transgenic agrobacterium containing recombinant *pGreen* and *pSoup* plasmids have grown colonies in agroLB with KAN + Rif selection marker medium.

3.3.1 Electroporation transformation was confirmed by PCR assay

Transformed constructs were verified by using gene specific primers and by plasmid specific primers. All the transformed constructs were successfully amplified in PCR. To validate the functional analysis with the target *MYB* genes, *VmMYBA1* from bilberry was also utilized for agroinfiltration as a positive control. This *MYB* in *pGreen* plasmid was also successfully grown with antibiotic selection marker. Figure 10 (A) highlights PCR validation done for three studied constructs (*VmMYBPA*, 2.1, 2.2, 2.3) by using gene specific primers while *VmMYBA1* was amplified by using *pGreen* primers. Clear DNA fragments can be seen in gel pictures that are confirming that *VmMYB* genes are correctly ligated in respective plasmids after electroporation transformation. Figure 9 (B) is showing results of transformation confirmation of five constructs through *pGreen* specific primers.

(A)



(B)

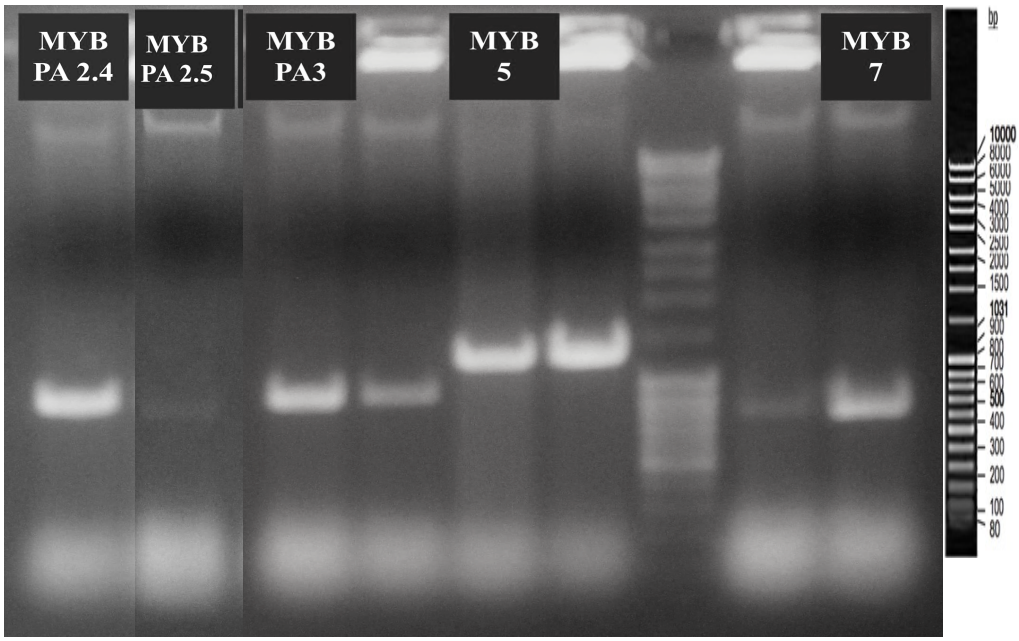


Figure 10: PCR validation of the constructs in agrobacterium. (A) There are clear DNA fragments for MYBPA 2.1, 2.2 and for MYBA1. MYBA1 was validated by using pGreen primers while all other MYBs were amplified by their specific primers. *(B) With plasmid specific primers MYBPA 2.4, 3, MYB5 & MYB 7 have visible DNA fragments.* MYBPA 2.5 did not show good fragments but as they were alternatively verified by the gene specific primers.

3.4 Transient overexpression analysis of *VmMYB* TFs in *tobacco* leaves

In order to validate agrobacterium infection protocol and to observe biosynthesis of anthocyanin and PAs, agrobacterium infiltrate was injected into of *Nicotiana benthamiana* leaf tissues. Figure 11 is illustrating the results in the injected zones of leaves seven days after infiltration. Figure 11A is showing a *Nicotiana* leaf that was introduced with empty vector and used as control. Positive control for this study, *VmMYBA1*, was clearly overexpressed throughout the infiltrated area and accumulation of anthocyanin pigments can be observed by the presence of reddish color (Figure 11B). In addition, the leaves injected with *VmMYBPA 2.2* construct, showed potential accumulation of flavonoids because of reddish color formation (Figure 10D). Furthermore, in case of *VmMYBPA*, 2.1, *2.3, 2.4, 2.5, 3, and MYB 5 & 7,* (Figure 11 C,E, F, G, H, I, J respectively), the infiltrated region on leaves is did not show any color change.

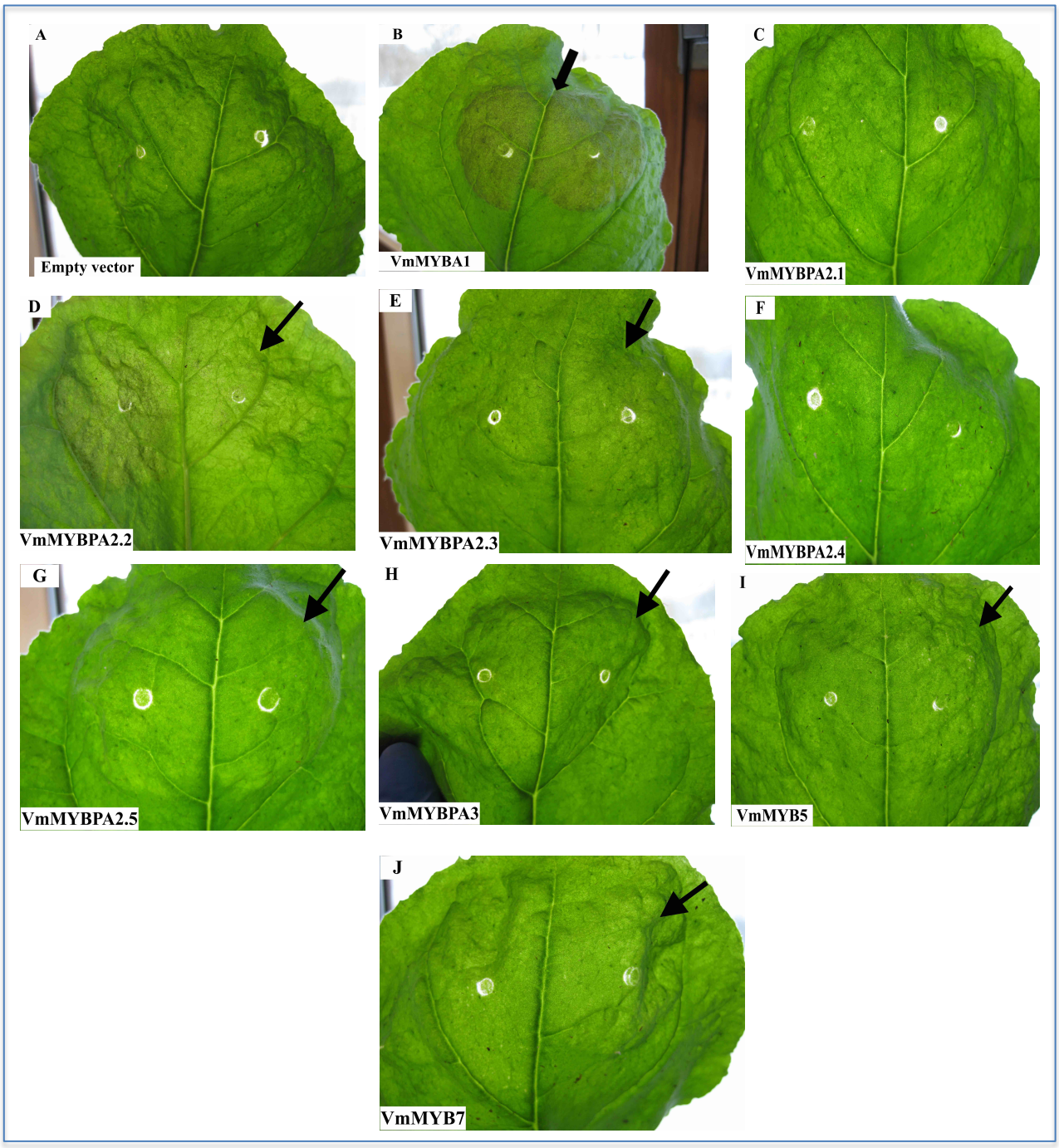


Figure 11: Transient overexpression analysis of the target genes in the Nicotiana benthamiana leaf. (A) Overexpression analysis of empty pGreen vector that was used as negative control. (B) MYBA1 is showing successful anthocyanin accumulation through the infiltrated area. But (C) MYBPA 2.1 (D) MYBPA (E) MYBPA2.3. (F) In MYB 2.4 (G) MYBPA 2.5 (H) MYBPA 3 (I) MYB 5 (J) MYB 7, did not show any significant color change.

3.5 Total PA determination from infiltrated leaves

DMACA-solution *(4-dimethylaminocinnamaldehyde*) is a histological dye that functions for PAs localization by staining in plants (Bogs et al., 2007). Figure 12 is showing DMACA-solution color development for PAs in samples of *VmMYBs* infiltrated in tobacco leaves. *VmMYBA1* and *MYBPA2.2* are more greenish in color compare to other treatments, considering that these two MYB samples have higher PAs concentration. Spectrophotometry analysis in Figure 13 is shows considerable higher level of PAs. *PAMYB 2.2 and MYBA1* (positive control) have highest absorbance for *PAs*. All other studied PA type *MYBs* have relatively low amount of PAs.

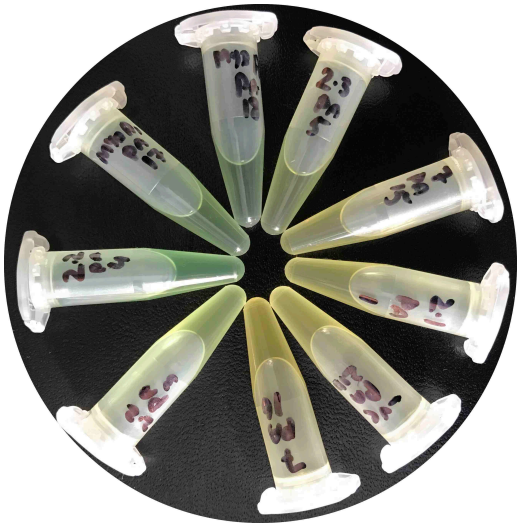


Figure 12. Color development in samples containing {As due to DMACA solution. VmMYBA1 & VmMYBPA 2.2 have distinctive colorization with DMACA solution. All other *MYBs* have relatively low PAs, resulting in less change in color.

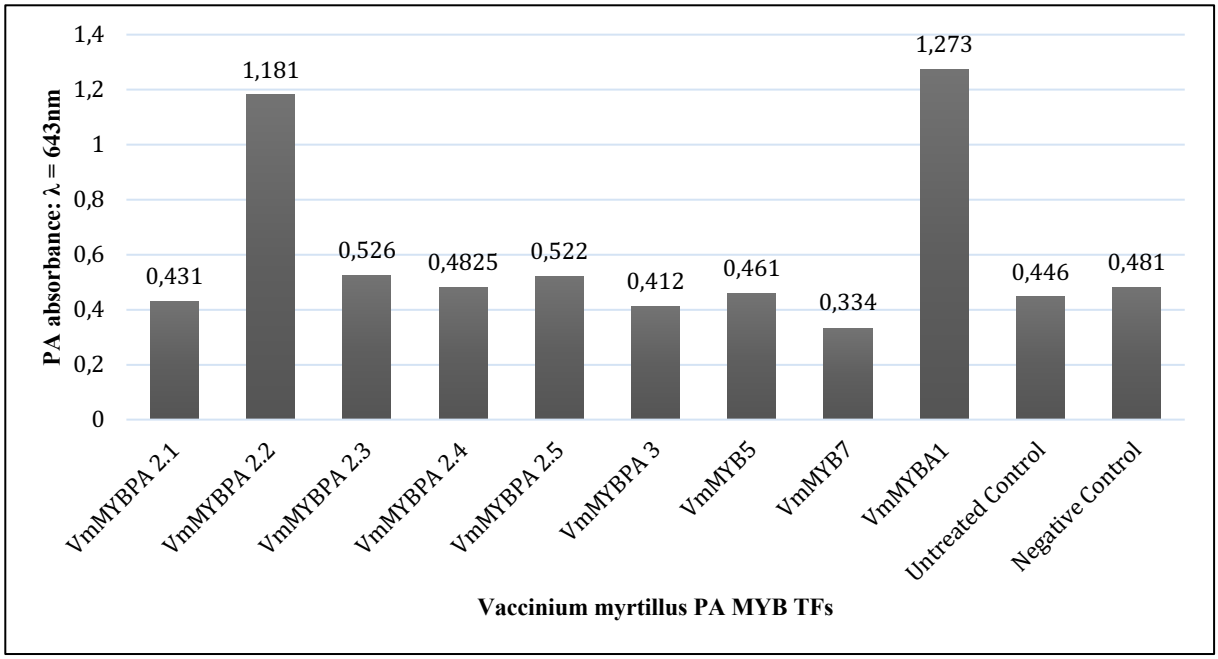


Figure 13: PA determination of PAs in Nicotiana benthamiana leaves by spectrophotometry analysis. Results of spectrophotometry absorbance analysis for PAs in *Nicotiana* leaves.

3.6 Anthocyanin determination from infiltrated leaves

Spectrophotometric absorbances were used to calculate total anthocyanin concentration (µg per gram) in leaves. Graph below is presenting average anthocyanin accumulation of three replicates of each gene sample. It is clear from Figure 14 that positive control *VmMYBA1* is regulating biosynthesis of anthocyanin. *VmMYBPA2.2* shows slight accumulation of anthocyanins Comparatively to controls, all other *VmMYBs* have low concentration of anthocyanin.

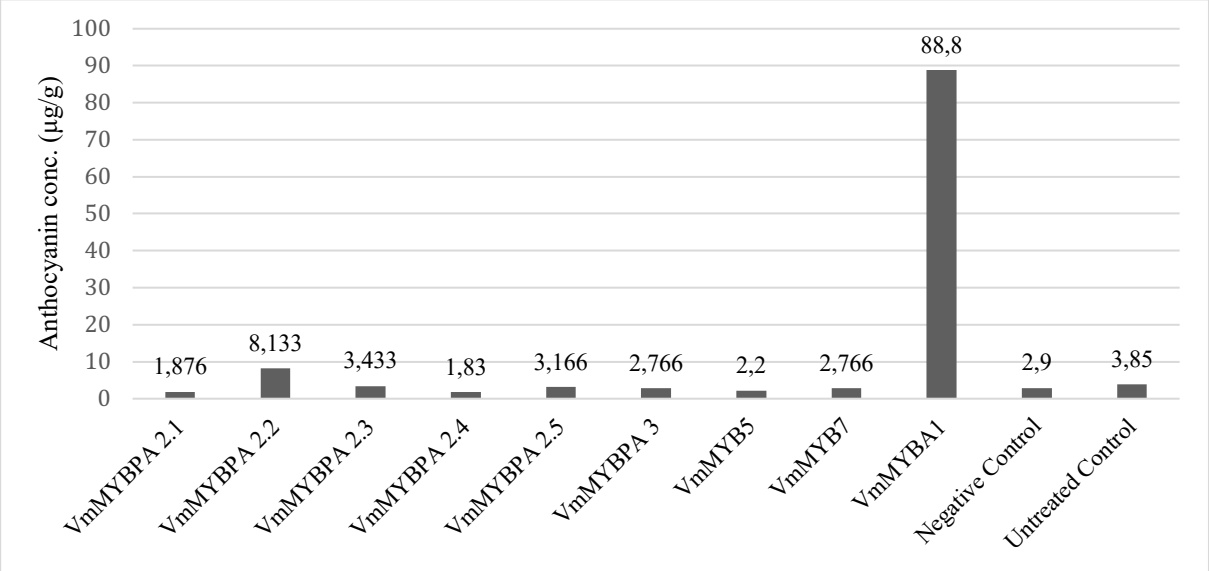


Figure 14: Spectrophotometric analysis for determine anthocyanin concentration in three replicates of each treatment.

3.7 Formation of bilberry calluses and overexpression

Bilberry fruits and leaves were used as explant for callus formation in woody plant media. Bilberry leaves were inoculated in all three combinations and they did not show any morphological differences rather they all turned brownish without callus production. Instead, bilberry fruit produced calluses after 6-8 weeks of first inoculation (Figure 15). Most of the callus were formed from fruit tissues that were treated with hormonal combination of either NAA + TDZ or NAA + TDZ + BAP. Afterwards, calluses were successfully grown in WPM media with NAA + TDZ + BAP. Calluses were ready for infiltration after 5-6 sub culturing. Figure 12C is showing the results of 6 days after the infection of bilberry callus cultures with *VmMYBA2* construct and Figure 12D with *VmMYBA1* constructs. The appearance of reddish-purple color may indicate production of anthocyanin by overexpression of *MYBA1* and *MYBA2.*

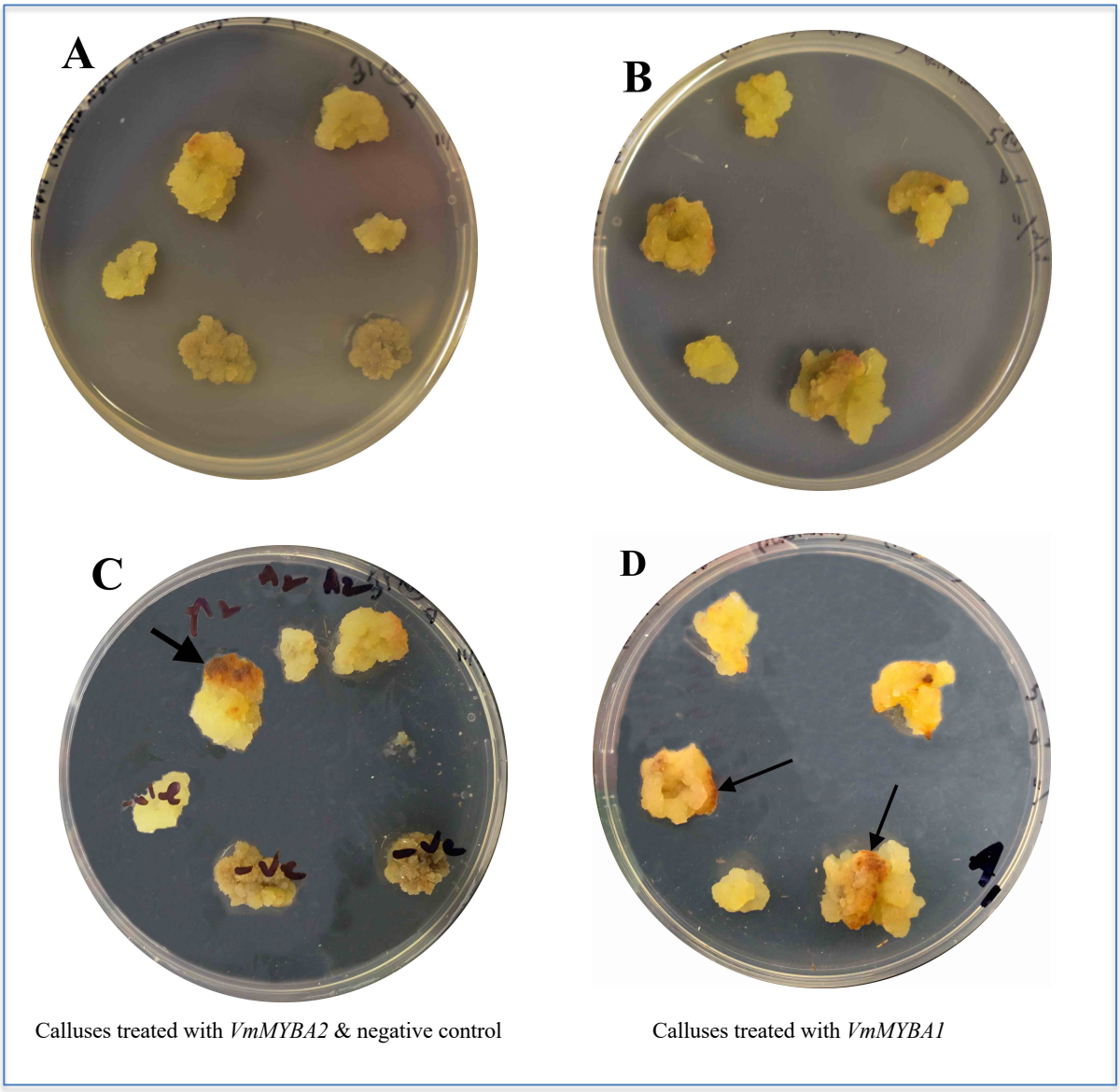


Figure 15: Bilberry callus formation and infiltration results in calluses by MYBA1 & A2.(A) Bilberry fruit calluses after 6 subcultures. These calluses were infiltrated with *MYBA2* and empty vector. *(B)* These calluses are also after 6 times subculturing and were injected by *MYBA1*. *(C)* All the calluses infected with *MYBA2,* accumulated anthocyanin that is visible at the edges of calluses while the calluses that were treated with empty pGreen vector are unchanged. *(D)* Changes in color of white calluses into red suggests overexpression of MYBA1.

4 Discussion

4.1 Predicted role of bilberry genes as *R2R3-MYBs* transcription factors

PAs are important phenolic compounds during early stages of fruit development while anthocyanin biosynthesis usually takes place during fruit ripening (Jaakola et al., 2002; Jaakola, 2013). In this study, tested *VmMYBs TF* were anticipated to be involved in the regulation of the structural genes of the flavonoid biosynthesis pathway. The sequences of these bilberry genes are closely resembled to that of *Vitis vinifera MYB TFs.* In grapes, *VvMYBPA1, VvMYBPA2 , MYB5a, MYB5b* and in peach *PpMYB7* have been shown to be involved in proanthocyanidin biosynthesis (Terrier et al., 2009; Czemmel et al.2012; Ravaglia et al., 2013). The phylogenetic tree in figure 4 is visualizing that all *Vaccinium myrtillus* PA2 type *MYBs* are clustered together with *VvMYBPA2* but *VmMYBPA2.5* sequence is most homologous. While *MYBPA3* sequence is also shares homology with *VvMYBPA2* because of having common branch point in the phylogeny tree. Similarly, *VmMYB5* is sharing the sequence similarity with *VvMYB5a & VvMYB5b.* These two grape *MYB* transcription factors have been reported to be involved in PA biosynthesis (Deluc et al., 2006; Deluc et al., 2008).

VvMYBA1 has been shown to be involved in anthocyanin biosynthesis in wine grapes (Walker et al., 2007), and shares sequence similarity with *VmMYBA1* (figure 4). In this study, *VmMYBA1* has been shown to be involved in anthocyanin & PA biosynthesis (Figure 11B & figure 13 ,14). *VmMYB7* sequence was closely assembled to *Prunus persica PpMYB7* (Zhou et al., 2015). Based on this phylogenetic tree analysis, the naming of *VmPAMYBs* was done according to *Vitis vinifera MYB TFs*. Plunkett et al., (2018) grouped *MYB TFs* in genus *Vaccinium* plants according to phylogenetic similarity that are involved in proanthocyanidin biosynthesis. *Vaccinium corymbosum VcMYB17 & VcPA1, Vaccinium myrtillus VmMYB2 and Vaccinium uliginosum VuMYBPA1* have categorized to belong in PAs biosynthesis *MYB TFs*. In bilberry, *VmMYBPA2.2* is involved in PAs biosynthesis, while *VmMYBA1* is related with production of both anthocyanins and PAs.

4.2 Preparation of *pGreenII 62-SK constructs*:

In this study, a dual-binary vector system *pGreen/pSoup* was used for building constructs for overexpression analysis of *MYB TFs* family genes. *pGreenII 62-SK* vectors have broad scope and primarily used for overexpression analysis in different plant species (Hellens et al., 2005).

In an earlier study, *FaMYB10* and *FvMYB10* cDNAs of *Fragaria* were ligated into *pGreen 62-SK* vector and infiltrated into *Nicotiana benthamiana* leaves through agrobacterium, resulting into high quantity of anthocyanin accumulation (Kui et al., 2014). Experimental agro-infiltration works well in *N. benthamiana* compared to other plants (Goodin et al., 2008). This plant can be genetically transformed and regenerate target genes efficiently, making itself ideal host for transient overexpression and gene silencing experiments (Liu et al., 2012). Agrobacterium GV3101 mediated transient system has a high transformation rate and reproducibility into targeted plant tissues. *McMYB12* and *McMYB12b* of *Malus crabapple* promoted accumulation of PAs and anthocyanins in *N. benthamiana* leaves (Tian et al., 2017). *pGreen II 62-SK* binary vector was used for transient expression of *MYB10* cDNA or gDNA from 10 species of Rosacea family, into leaves of *N. benthamiana* (Lin-Wang et al., 2010).

In the present study, cloning of all eight *VmMYB* genes in *pGreen* vector was successful, which was verified by selecting *E.coli* colonies in KAN antibiotics (figure 7) and by PCR assay (figure 8). Further confirmation of ligation of genes at correct site inside the plasmid was also successful, confirmed sequencing of the genes. Validation of the target genes by genes specific primers and by plasmid specific primers verified that the protocol used for building constructs had succeeded.

Interpretation of spectrophotometry absorbance results of *VmMYBPA2.2* and *VmMYBA1* in this study explains that, studied genes were susceptible to *N. benthamiana* expression system. The positive results of *VmMYBPA2.2* and *VmMYBA1* verifies that *pGreen 62-SK* is a robust vector for cloning of bilberry genes and its *CamV 35S* expression promoter has well adopted to handle overexpression analysis of *MYBs TF* family genes.

4.3 Functional analysis of R2R-MYB TFs in tobacco leaves

VmMYBA1 has been isolated and studied earlier for its role in anthocyanin biosynthesis during flavonoid biosynthesis pathway (Unpublished). In this study, the gene was applied as positive control for overexpression analysis in tobacco. Appearance of red color in tobacco leaf in Figure 11B and spectrophotometry absorbance concentration of anthocyanin and PAs (Figure 13, 14), is clearly indicating the accumulation of anthocyanins and PAs due to the overexpression of Vm*MYBA1*. Previously, role of *MYBA1 TF* in anthocyanin production has been extensively studied in different plants. *Vitis rotundifolia VrMYBA1* share sequence similarity with *Vitis vinifera MYBA1*. These TFs upregulated in *V. rotundifolia* red berries during veraison and

positively affected the biosynthesis of anthocyanins (Oglesby et al., 2016). *MYBA* of highbush blueberry also clustered with grapevine sequence and proved to be involved in anthocyanin production through biolistic transformation of *GFP-ER + MYBA* (Plunkett et al., 2018). Furthermore, transient infiltration of *VmMYBA* in *N. benthamiana* activated anthocyanin production in the present study. Based on previous literature and overexpression analysis of *MYBA1* in this study, it can be verified that *VmMYBA1* is involved in anthocyanin production in bilberry as well.

VvMYBPA2 activates LAR and ANR enzymes to regulate synthesis of proanthocyanin in grape skin (Terrier et al., 2009). In this study, the five genes from the clade *MYBPA2* were expected to be involved in proanthocyanidin production. By infiltration assay and spectrophotometry analysis (figure 13), *VmMYBPA2.2* can be predicted to be involved in regulation of PAs biosynthesis. Leaf lamella (Figure 11D) is showing appearance of reddish-brown color in infiltrated region due to *VmMYBPA2.2*. *VmMYBPA2.2* also appeared to accumulate low amounts of anthocyanins. There is lack of confidence of other *VmMYBPA2* genes for being involved in anthocyanins/PAs production in bilberry because they showed no accumulation of PAs or anthocyanins (figure 13 & 14). Could be that some other factors (bHLH, MYBs or cofactors) are needed in order to accumulate flavonoids. It is also good to note that function of the genes is not necessarily exactly the same in tobacco leaves as it is in bilberry.

VvMYB5a and *VvMYB5b* TFs have been shown to be responsible to control the expression of central steps of flavonoid biosynthesis pathway enzymes (CHS, CHI, DFR, LDOX) and also control the regulation of PA biosynthesis specific enzymes i.e. LAR & ANR (Czemmel et al. 2012). Moreover, Deluc et al. (2008) demonstrated the role of MYB5a and MYB5b from *Vitis vinifera* by its involvement in activating grapevine promoters in grape cells. Upon overexpression in *N. tabacum* these *VvMYBs* resulted in accumulation of anthocyanins and PA derived compounds. In this study, the leaf lamella infected with *VmMYB5* could not show any color change. Furthermore, spectrophotometry analysis of the leaves showed that anthocyanin and PAs concentrations were low, predicting requirement of different expression environment for this gene.

The tobacco leaves that were infiltrated by *MYBPA3* did not accumulate additional anthocyanins and PA. So far it has not been reported any PA3 MYB involved in proanthocyanidin biosynthesis in any plant species. Instead, MYB7 has been reported to be involved in PAs biosynthesis. In *Prunus persica, PpMYB7* with partners bHLH3 & bHLH33,

regulate PA biosynthesis in peach (Ravaglia et al., 2013). Alone infiltration of *PpMYB7* or bHLH factors resulted in low activity while combination of *PpMYB7* with either bHLH factor also resulted little activity on *PpANR* promoter. However, combination of all three genes resulted in significant increase in *DFR* and *LAR* promoter activity. As *VmMYB7* has sequence similarity with *PpMYB7,* it can be speculated that *VmMYB7* may require other *bHLH* gene to function. Based on phylogenetic tree, it can be presumed that *VmMYB7* is associated with proanthocyanidin formation in bilberry, but it also requires other transcription factors to express in tobacco expression system.

4.4 Agroinfiltration in bilberry callus accumulated with flavonoids.

In vitro callus cultures have their own usefulness in the study of functional gene analysis. The bilberry calluses are relatively difficult to grow *in vitro,* and usually takes longer time until fully white calluses are established. This is probably due to the presence of high concentration of phenolic compounds that oxidize and resulting into browning of calluses (He et al., 2009). White calluses are optimal for functional analyses due to lack of pigmentation and thus color produced by anthocyanin accumulation can be visible in such tissues.

Different hormonal combinations were tested for bilberry fruit and leaf explant i.e. I) NAA + BAP , II) NAA + TDZ, III) NAA + BAP + TDZ. From these combinations, the one with TDZ formed calluses effectively from bilberry fruit tissues. Thidiazurin (TDZ) along with 1-naphthaleneacetic acid (NAA) have previously been used for callus development of shoots of *Vaccinium pahalae* and *V. myrtillus* (Shibli & Smith, 1996) , shoot organogenesis in *V. vitis-idaea L.* (Debnath, 2003), and from nodal parts of blueberry and lingoberry (Meiners et al. 2007). In this study, calluses were infiltrated only with positive controls. i.e. *VmMYBA1 and VmMYBA2 + AtbHLH* to test their suitability as infiltration material. After 7 days, appearance of callus red pigmentation (figure 15) was detected which suggested that anthocyanin pathway genes could be functional in callus tissues.

5 Conclusion

In this thesis pGreen-62SK vectors were successfully inserted with eight VmMYB TFs family genes and correct construct sequences were verified by sanger sequencing. Moreover, the developed constructs were used in overexpression analysis of bilberry PA type R2R3-VmMYB TFs in *Nicotiana benthamiana* leaves and bilberry callus cultures. Based on the infiltration and spectrophotometry analysis results, it can be concluded that *VmMYBPA2.2* positively regulates biosynthesis of proanthocyanidins and slightly enhances anthocyanin biosynthesis. While *VmMYBA1* is the key regulator of biosynthesis of both anthocyanins and proanthocyanidins.

6 Future recommendations

In this study, *VmMYBPA* TFs genes have been overexpressed in the model plant tobacco leaves, which were further analyzed for anthocyanin and PAs concentration. To further assess the results, qRT-PCR analysis could be done in order to quantify the expression of the *VmMYB* transcripts and the downstream genes, and for detailed flavonoid profiles in the infiltrated tobacco leaves. In addition, these *MYBs* could be overexpressed together with different bHLH factors and those results could be compared to this study. In the present study, the calluses were infiltrated only with positive control but in the future, bilberry calluses can be injected with all developed *VmMYBs* constructs to study their function in bilberry tissues. This further study would help to understand the exact regulatory role of each of these MYBs in different steps of flavonoid biosynthesis pathway.

7 References

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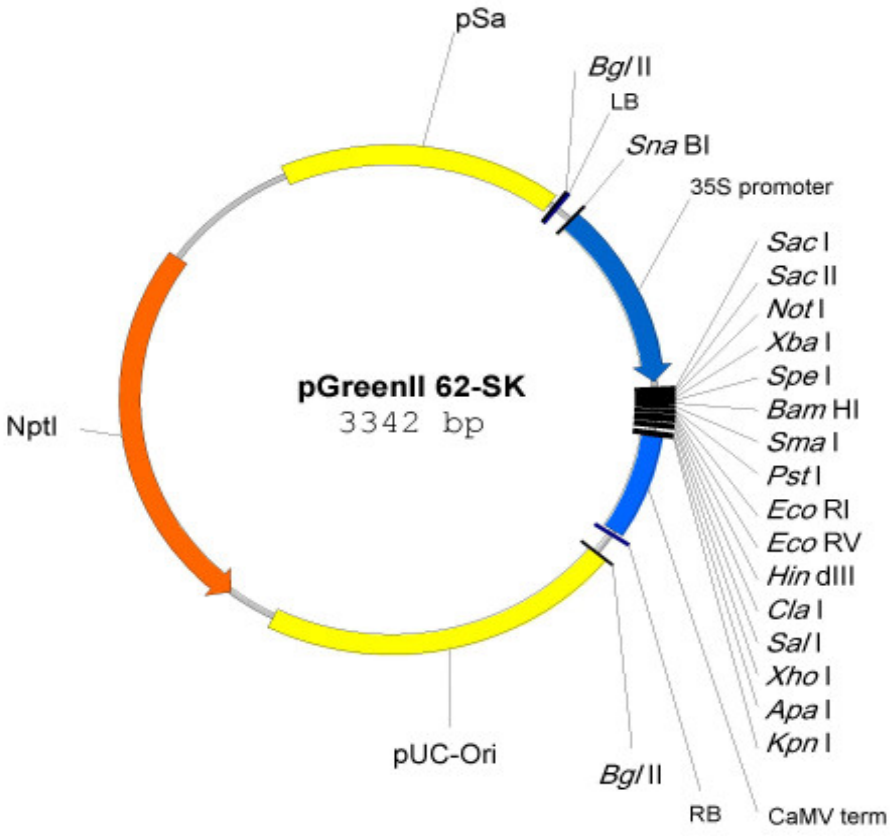
APPENDIX

Appendix I

pGreen 62-Sk plasmid (Hellens et al., 2005).



Appendix II

Full length nucleotide sequence of pGreen-62SK plasmid.

```
   1 agatcttggc aggatatatt gtggtgtaac gttatcagct tggtacagct tgcatgccgg
  61 tcgatctagt aacatagatg acaccgcgcg cgataattta tcctagtttg cgcgctatat
 121 tttgttttct atcgcgtatt aaatgtataa ttgcgggact ctaatcaaaa aacccatctc
 181 ataaataacg tcatgcatta catgttaatt attacatgct taacgtaatt caacagaaat
 241 tatatgataa tcatcgcaag accggcaaca ggattcaatc ttaagaaact ttattgccaa
 301 atgtttgaac gatctgcttg actctagcta gagtccgaac ccagagtcc cgctcagaag
 361 aactcgtcaa gaaggcgata gaaggcgatg cgctgcgaat cgggagcggc gataccgtaa
 421 agcacgagga agcggtcagc ccattcgccg ccaagctctt cagcaatatc acgggtagcc
 481 aacgctatgt cctgatagcg gtccgccaca cccagccggc cacagtcgat gaatccagaa
 541 aagcggccat tttccaccat gatattcggc aagcaggcat cgccctgggt cacgacgaga
 601 tcctcgccgt cgggcatccg cgccttgagc ctggcgaaca gttcggctgg cgcgagcccc
 661 tgatgctctt cgtccagatc atcctgatcg acaagaccgg cttccatccg agtacgtcct
 721 cgctcgatgc gatgtttcgc ttggtggtcg aatgggcagg tagccggatc aagcgtatgc
 781 agccgccgca ttgcatcagc catgatggat actttctcgg caggagcaag gtgagatgac
 841 aggagatcct gccccggcac ttcgcccaat agcagccagt cccttcccgc ttcagtgaca
 901 acgtcgagca cagctgcgca aggaacgccc gtcgtggcca gccacgatag ccgcgctgcc
 961 tcgtcttgga gttcattcag ggcaccggac aggtcggtct tgacaaaaag aaccgggcgc
1021 ccctgcgctg acagccggaa cacggcggca tcagagcagc cgattgtctg ttgtgcccag
1081 tcatagccga atagcctctc cacccaagcg gccggagaac ctgcgtgcaa tccatcttgt
1141 tcaatcatgc ctcgatcgag ttgagagtga atatgagact ctaattggat accgagggga
1201 atttatggaa cgtcagtgga gcatttttga caagaaatat ttgctagctg atagtgacct
1261 taggcgactt ttgaacgcgc aataatggtt tctgacgtat gtgcttagct cattaaactc
1321 cagaaacccg cggctgagtg gctccttcaa cgttgcggtt ctgtcagttc caaacgtaaa
1381 acggcttgtc ccgcgtcatc ggcgggggtc ataacgtgac tcccttaatt ctcatgtatc
1441 gtaccccccc actccaaaaa tgtcaaagat acagtctcag aagaccaaag ggctattgag
1501 acttttcaac aaagggtaat ttcgggaaac ctcctcggat tccattgccc agctatctgt
1561 cacttcatcg aaaggacagt agaaaaggaa ggtggctcct acaaatgcca tcattgcgat
1621 aaaggaaagg ctatcattca agatgcctct gccgacagtg gtcccaaaga tggacccccа
1681 cccacgagga gcatcgtgga aaaagaagac gttccaacca cgtcttcaaa gcaagtggat
1741 tgatgtgaca tctccactga cgtaagggat gacgcacaat cccactatcc ttcgcaagac
1801 ccttcctcta tataaggaag tcatttcatt tggagaggac agcccaagct gagctccacc
1861 gcggtggcgg ccgctctaga actagtggat cccccgggct gcaggaattc gatatcaagc
1921 ttatcgatac cgtcgacctc gaggggggggc ccggtaccaa ttcgctgaaa tcaccagtct
1981 ctctctacaa atctatctct ctctattttc tccataaata atgtgtgagt agtttcccga
2041 taagggaaat tagggttctt atagggtttc gctcatgtgt tgagcatata agaaacccttt
2101 agtatgtatt tgtatttgta aaatacttct atcaataaaa tttctaattc ctaaaaccaa
2161 aatccagtac taaaatccag atccactagc cttgacagga tatattggcg ggtaaactaa
2221 gtcgctgtat gtgtttgttt gagatct
```

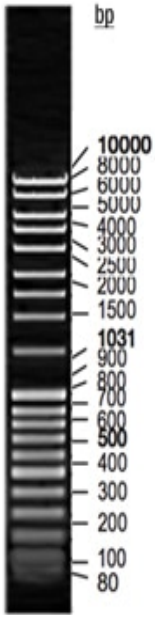
Appendix III

Different type of media and culture conditions were used in this study.

Media	Ingredients	H_2O Conc.
LB (Lysogeny broth)	10g tryptone + 5g yeast extract + 10g, NaCl + 15g bacto agar	1L H_2O
AgroLB	10g tryptone + 5g yeast extract + 5g NaCl +15g bactoagar 200µl KAN / 200ml LB 200µl Rif / 200ml LB 80µl TET / 200ml LB	1L H_2O
Agarose Gel	Agar 1.5g + TAE buffer 150ml + 2 drops of ethidium bromide (120ml)	
WPM media (Woody plant media)	30g Sucrose + 2.4g WPM + 8g Agar Hormones NAA + BAP + TDZ 1ml	1L H_2O

Appendix IV

1 kb MassRuler^{TM} ThermoScientific was used to relate sizes of DNA fragments in gel.



Appendix V

Nanodrop concentrations of amplified genes of interest after gel extraction.

VmMYB2.4	14.8 ng/µl
VmMYB2.2	41.3
VmMYB2.5	10.7
VmMYB3	13.0
VmMYB2.1	27.6
VmMYB2.3	9.2
VmMYB7	20.7
VmMYB5	14.4

Appendix VI

Nanodrop concentrations restriction digestion product extracted from gel after restriction.

VmMYB2.4	0.9 ng/µl
VmMYB2.2	8.8
VmMYB2.5	0.9
VmMYB3	4.0
VmMYB2.1	15.4
VmMYB2.3	2.0
VmMYB7	3.0
VmMYB5	1.8
pGreen (SacI + Kpn1)	381.1
pGreen (SacI + Kpn1)	23.4
pGreen (Bam + Kpn1)	33.5

Appendix VII

Nanodrop concentrations of *pGreen + MYB* Insert isolation after transformation in *E.coli*

VmMYB2.1	804.9 ng/µl
VmMYB2.1	465.2
VmMYB2.2	810.7
VmMYB2.2	792.2
VmMYB2.4	344.8
VmMYB2.4	485.0
VmMYB5	728.1
VmMYB5	455.8
VmMYB2.5	40.4
VmMYB2.5	770.7
VmMYB3	704.6
VmMYB3	705.2
VmMYB7	835.2
VmMYB7	830.1
VmMYB2.3	494.9
VmMYB2.3	795.6

Appendix VIII

TFB I

- [0.953g Na-acetate x $3H_2O$ (35mM)
- 0.294g $CaCl_2$ x $2 H_2O$ 10mM
- 30ml glycerol(15%v/v),
    - dissolved in 200ml ddH_2O,
    - Adjust pH 5.9 (acetic acid)
- 2.418g RbCl (100mM) +
- 1.979g $MnCl_2$(50mM)
    - Sterilize by filtering
    - Store -20°C

TFB II

- 0.2093g MOPS (10mM)
- 1.102g $CaCl_2$ x $2 H_2O$ (75mM)
- 15ml glycerol(15%v/v),
    - dissolved in 100 ml ddH_2O,
    - Adjust to pH 6.8 (KOH)
    - Add H_2O to volume 100ml
- 0.12g RbCl (10mM)
    - Sterilize by filtering
    - Store at -20°C

Appendix IX

Equation for determining Total anthocyanin concentration.

$$[\frac{AA}{\epsilon x1}\ x\ DF1\ x\ DF2\ X\ MW]\ /m$$

ϵ = 26.9 L $mmol^{-1}cm^{-1}$, molar extinction coefficient for cyanidin – 3 – glucoside.

1 = path length in cm

DF1 = 1: 4 extracts and buffer dilution factor.

DF2 = Volume of extraction solution

MW = 449.2 mg $mmol^{-1}$ molecular weight for cyaniding – 3 – glucoside

M = weight of plant material used for extraction.

Appendix X

Time used for each electroporation.

MYB PA Sample	Time taken
2.1	5.0 mSec
2.2	5.0 mSec
2.3	5.2 mSec
2.4	5.2 mSec
2.5	4.5 mSec
3	4.5 mSec
7	4.5 mSec
5	4.5 mSec