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1 **Running head:** A Bilberry MADS-box gene involved in anthocyanin biosynthesis

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Title:

A SQUAMOSA MADS-box gene involved in the regulation of anthocyanin accumulation in bilberry fruits

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Abstract

Anthocyanins are important health promoting phytochemicals that are abundant in many fleshy fruits. Bilberry (*Vaccinium myrtillus* L.) is one of the best sources of these compounds. Here we report on the expression pattern and functional analysis of a *SQUAMOSA* (*SQUA*) class MADS-box transcription factor, *VmTDR4,* associated with anthocyanin biosynthesis in bilberry. Levels of *VmTDR4* expression were spatially and temporally linked with colour development and anthocyanin-related gene expression. Virus induced gene silencing (VIGS) was used to suppress *VmTDR4* expression in bilberry resulting in substantial reduction in anthocyanin levels in fully ripe fruits. Chalcone synthase was used a positive control in the VIGS experiments. Additionally, in sectors of fruit tissue in which the expression of the *VmTDR4* gene was silenced, the expression of R2R3 MYB family transcription factors related to the biosynthesis of flavonoids were also altered. We conclude that *VmTDR4* plays an important role in the accumulation of anthocyanins during normal ripening in bilberry; probably through direct or indirect control of transcription factors belonging to the R2R3 MYB family.

Introduction

Naturally occurring polyphenols in plants are likely to make a major contribution to human health and therefore life expectancy (Renaud & Delorgeril 1992). Fruits are some of the most accessible dietary sources of polyphenols, of which anthocyanins are recognised for their high antioxidant capacity and reported positive effects on blood vessels (Kähkonen *et al*. 2003, Vinson *et al*. 2005, Dragsted *et al*. 2006). Furthermore, these secondary metabolites are thought to have positive anticancer effects (Butelli *et al*. 2008) and to promote brain function (Williams *et al*, 2008). Anthocyanins generate the characteristic red, blue and purple pigments in many flowers and in fruits such as bilberry, blackcurrant, blackberry and strawberry.

Bilberry (*Vaccinium myrtillus* L.) is one of the richest natural sources of anthocyanins, the ripe fruit typically containing 29 mg g^{-1} dry weight (Lätti *et al*., 2008). In many fruits these coloured compounds accumulate only in the skin, while in bilberry they occur throughout the fruit flesh. At least 15 different anthocyanidin glycosides contribute to the anthocyanin profile of the fruit, which also contains high levels of the flavonols quercetin and myricetin, in addition to hydroxycinnamic acids (Jaakola *et al*., 2002, 2004; Riihinen *et al*., 2008).

The biosynthetic pathway in plants leading to anthocyanins is well established and transcription factors that directly regulate multiple steps of the expression of genes in this metabolic pathway are known (Grotewold 2006). This information has recently been used to generate tomatoes containing high concentrations of anthocyanins (Butelli *et al*. 2008). In bilberry fruit, ripening-related gene expression has been studied in relation to flavonoid biosynthesis (Jaakola *et al*., 2002). Many berry species accumulate anthocyanins in their fruits only during the ripening phase, but while anthocyanins are markers of ripening, the molecular circuits connecting their accumulation to the ripening process are poorly understood. A major goal is to identify and understand how those molecular circuits involved with anthocyanin biosynthesis are interlinked with the control of ripening and then to harness this information to breed novel fruit varieties with enhanced nutritional and health benefits.

The regulation of ripening in fleshy fruits has been most extensively studied and is best understood in tomato (Giovannoni, 2007; Seymour *et al.,* 2008). Tomato and bilberry differ in their ripening behaviour. Tomatoes are climacteric fruits where ripening

is under the control of ethylene, while bilberry is a non-climacteric fruit where ethylene appears to have little influence on ripening. Both these fruits are, however, classified as berries and there is increasing evidence that fleshy fruit species may share common mechanisms of ripening control related to developmental cues that act above and beyond the influence of ethylene (Giovannoni, 2004). Information on the identity of these ripening-control genes has now become apparent through the study of non-ripening mutants of tomato. A small number of single gene mutants have been identified that almost completely abolish ripening, including *ripening inhibitor* (*rin*) and *Colourless non-ripening* (*Cnr*) (Giovannoni, 2007). The gene at the tomato *rin* locus is a member of the MADS-box *SEPALLATA* (*SEP*) sub-family; *LeMADS-RIN* (Vrebalov *et al*., 2002). MADS-box genes are normally associated with floral development, but *LeMADS-RIN* is necessary for ripening. The gene at the *Cnr* locus encodes a *SQUAMOSA* promoter binding protein (SBP-box) transcription factor (Manning *et al*., 2006), that is likely to interact with the promoters of the *SQUAMOSA* (*SQUA*) sub-family of MADS-box genes (Lännenpää *et al*., 2004). *SQUA* MADS-box genes are commonly associated with floral development and especially the regulation of floral meristem identity (Simpson *et al*., 1999, Theissen *et al*., 2000, Scott *et al.*, 2002). This class of transcription factors has also been shown, however, to have other functions. They have been implicated in the regulation of tuber formation in vegetative meristems of potato (Rosin *et al*., 2003a) and the *FRUITFULL* gene, *FUL*, in Arabidopsis mediates cell differentiation during fruit development (Dinneny *et al*, 2005) as well as floral meristem identity(Mandel & Yanofsky, 1995). In tomato, *TDR4,* which is a likely *FUL* orthologue, shows enhanced expression during fruit ripening (Eriksson *et al*., 2004), although it has yet to be assigned a function.

Elevated expression of *SEP* and *SQUA* class MADS-box genes during ripening has now been observed in a wide range of climacteric and non-climacteric fruits including strawberry, banana, and grape (Rosin *et al*. 2003b, Liu *et al*. 2009, Vrebalov *et al*, 2002). *LeMADS-RIN* and *LeSBP-CNR* are necessary for normal ripening in tomato, with the latter likely controlling the expression of the *SQUA* gene *TDR4*. These genes and other related transcription factors can therefore be seen as part of the high level regulatory network controlling the ripening process. Their links to downstream effectors which bring about changes in colour, texture and flavour are, however, poorly understood.

The aim of the present work was to investigate the regulatory mechanisms controlling anthocyanin accumulation in bilberry, and especially the higher level regulatory network linking anthocyanin biosynthesis to ripening. We have cloned a bilberry homologue of the tomato *TDR4* gene, which we name *VmTDR4*. Transcripts of this gene were found to have a strong ripening-related pattern of expression. In this paper we report on *VmTDR4* expression in ripening bilberry and how inhibition of VmTDR4 expression substantially reduces accumulation of anthocyanins in the fruit. Our data also suggest that the *VmTDR4* gene may act directly or indirectly on transcription factors belonging to the R2R3 MYB family, the known regulators of flavonoid biosynthesis.

Results

VmTDR4 is a bilberry homolog of the tomato *TDR4* and Arabidopsis *FRUITFULL* genes

The aim of this work was to better understand the molecular events controlling ripening in bilberry, a fruit which accumulates high levels of health promoting anthocyanins. In preliminary experiments we attempted to isolate bilberry orthologues of the tomato ripening regulatory genes *CNR* and *TDR4.* We were able to clone a *TDR4-like* gene, *VmTDR4,* (accession number FJ418852, Jaakola, unpublished). The full length *VmTDR4* cDNA is related by sequence homology to *FRUITFULL* (*FUL*) from Arabidopsis and *TDR4* / *FUL*-like genes from tomato; *VmTDR4* showed 72% identity to tomato *TDR4* at the nucleotide level and 70% identity at the amino acid level. A phylogenetic analysis (Fig. 1) indicated a close relationship between protein sequences from Arabidopsis, tomato and bilberry. In Arabidopsis, the *FRUITFULL* gene has pleiotrophic effects, being involved in regulating the formation of the lignified dehiscence zone in the fruit as well as floral meristem identity (Dinney *et al*., 2005). The role of *TDR4* in tomato fruits is unknown.

VmTDR4 is expressed in bilberry fruit tissues and the expression pattern is temporally and spatially related to anthocyanin biosynthesis

In situ hybridization experiments revealed that *VmTDR4* transcripts are present in various tissues of the unripe fruit (Fig 2A-G). These included pericarp cells, the skin and developing embryo tissues, and especially in the vascular regions of the placenta. Ripe

fruits were unsuitable for *in situ* experiments due to problems with effective fixation of the material. *VmTDR4* expression was investigated in ripe fruit and other organs by QRT-PCR (Fig. 2H and I). Transcripts were detected mainly in the fruit and floral tissues, with the highest levels in the ripe flesh of the berry (Fig. 2H). Limited expression was detected in leaves and none in the rhizome. The expression pattern of *VmTDR4* during berry development and ripening is shown in Fig. 2I. *VmTDR4* transcripts continued to accumulate until just before the fruit were fully ripe and just prior to anthocyanins reaching their highest levels (Jaakola *et al*., 2002). The expression patterns of several genes known to be involved in anthocyanin biosynthesis (Jaakola *et al*, 2002) are shown in Fig. 3 in addition to novel *MYB* transcription factors *VmMYB1* and *VmMYB2*. Chalcone synthase (*CHS*; AY123765), dihydroflavonol 4 reductase (*DFR*; AY123767), and anthocyanin synthase (*ANS*; AY123768) all showed a ripening-related pattern with similarities to that of *VmTDR4* (Fig. 3A-D). The levels of anthocyanin reductase (*ANR*; FJ666338) are low during the ripening phase, reflecting the flux toward coloured anthocyanin pigments and away from proanthocyanidins (Fig. 3D, also see pathway Fig. 4). We have recently cloned R2R3-MYB family transcription factors from ripening bilberry fruit, including *VmMYB1* (accession number GU904211) and *VmMYB2* (accession number GU227356). The expression profile of the *VmMYB2* gene was very similar to *VmTDR4* being strongly ripening-related and reaching its highest level before the fully ripe stage (Fig. 3E). In contrast VmMYB1 expression was at its highest level during early fruit development (Fig. 3F).

A natural bilberry fruit colour mutant, white bilberry, which ripens with a white fleshy pericarp has been described (Jaakola *et al*, 2002). The mutant fruits have greatly reduced levels of anthocyanins and decreased expression of anthocyanin biosynthetic genes compared to wild type (Jaakola *et al*., 2002). QRT-PCR analyses of *VmTDR4*, *VmMYB2* and *CHS* in white bilberry showed that at the same stage of ripening (ripe fruits) genes are down regulated in the fruits of the white mutant compared with wild type controls (Fig.5*A*-*C*). Collectively the expression data from wild type and mutant bilberry fruits are suggestive of a relationship between *VmTDR4* expression and anthocyanin biosynthesis, but they do not, of course, provide a functional link.

Functional analysis of VmTDR4 in bilberry using virus induced gene silencing

To further investigate the function of *VmTDR4* in relation to flavonoid biosynthesis in bilberry we used virus induced gene silencing (VIGS) with a *CHS*

construct as a positive control; following the lead of other studies involving fruits where anthocyanins are the principal pigments affecting fruit colour (Hoffman et al, 2006). This is the first report of VIGS in this species. Fragments of the *VmTDR4* and *CHS* genes were amplified by PCR and cloned into the VIGS vectors (see Materials and Methods). Inoculations with the *VmTDR4* sequence resulted in fruits with altered pigmentation which included green sectors (Fig. 6). Fruits were photographed during the ripening process at 10 (Fig. 6A), and 32 days (Fig.6B and C) after injections. A similar experiment was undertaken in a second year whereby fruits were collected 42 days after the injections when they were fully ripe (Fig. 6 D). Each gene construct was tested on 100 individual berries. In the *VmTDR4* and *CHS* treatments between 16 and 20 % of berries showed sectors with abnormal pericarp colour development (Fig.6A-C). Cross sections of fruits at the fully ripe stage revealed a substantial reduction in red pigmentation in the pericarp in fruit from both the *VmTDR4* and *CHS* treatments (Fig. 6D). All untreated fruits or those injected with the empty vector (negative control) ripened normally developing a dark red / black pigmentation in the pericarp and skin. Green sectors were completely absent.

Expression levels of *VmTDR4, CHS, DFR, ANS, ANR*, *VmMYB1* and VmMYB2 were examined in *VmTDR4* and *CHS* VIGS treated fruits by QRT-PCR (Fig. 7). RNA was isolated just prior to the fully ripe stage at 32 days post injections (Fig. 6B and C) as this is the point at which anthocyanin-related gene expression reaches a peak. In VIGS experiments the effects are often localised to small, tissue sectors and therefore gene expression was compared between the ‘green’ and ‘red’ sectors. It is difficult to compare absolute values to empty vector treated material directly because individual fruits on the same plant can ripen at slightly different rates. However, the expression of genes related to flavonoid biosynthesis in both untreated and empty vector treatments were consistent with transcript levels observed during normal ripening in bilberry.

In pTV00-VmTDR4 treated fruit the levels of *VmTDR4* expression were ~25% lower in the green than in red sectors (Fig. 7) and this difference was significant ($P \leq 0.005$). This initially suggested an uncoupling between *VmTDR4* expression and reduced pigment accumulation in the ‘green sectors’ where a more dramatic reduction in *VmTDR4* levels might have been expected. However, beneath the skin in the green sectors of the fruits partial colour development occurred in the fleshy pericarp tissue (Fig. 6C). While VIGS may lead to ‘patchy’ ripening effects and make data interpretation more

challenging, it is an effective method for functional analysis in a species where analysis of stable transformants could take several years. The small amount of material in the sectors was insufficient for both RNA and metabolomic analysis, so the latter was performed on whole fully ripe berries (Fig. 6D, see below) where the most dramatic effects of the VIGS was seen. In contrast with the *VmTDR4* injected fruits, *VmTDR4* expression was not suppressed in the pTV00-CHS treated material, although these fruits also developed green sectors. This is consistent with *VmTDR4* being upstream of *CHS* in a regulatory network. In pTV00-CHS treated fruits, *CHS* expression was markedly down-regulated in the 'green' sectors as might be predicted and indicates effective silencing of this gene with the *CHS* construct. However, *CHS* expression was also lower in green compared with red sectors from pTV00-VmTDR4 infected fruits. CHS expression in both treatments is relatively low and this may reflect some silencing of the target genes throughout the fruit by the VIGS treatment. Alternatively, as the VIGS method causes transient silencing, it is possible that at the time samples were collected (32 days after injections), the silencing effect is already weaker with respect to its effect on *VmTDR4*, but silencing of *VmTDR4* has had longer lasting effects on the suppression of *CHS* mRNA levels.

We also examined the expression of novel bilberry MYBs and several genes known to be involved in the anthocyanin pathway (Fig 7). *VmMYB2* expression was significantly ($P \leq 0.005$) lower in the pTV00-VmTDR4 'green' sectors as compared to red sectors indicating a link between *VmTDR4* and *VmMYB2* expression and development of the red anthocyanin pigmentation. There was, however, no significant difference in *VmMYB2* expression in the red or green sectors of the CHS VIGS fruit indicating that the effects of CHS on pigment accumulation is downstream of these two genes. Interestingly, the expression of *VmMYB1* was significantly higher ($P \leq 0.005$) in the pTV00-VmTDR4 'green' sectors as compared with red sectors. *VmMYB1* shares the closest sequence level homology with *FaMYB1*, which is a known suppressor of anthocyanin biosynthesis in strawberry fruit (Aharoni *et al.* 2001). The expression of *DFR*, *ANS*, and *ANR*, were also examined. *DFR* and *ANS*, which are below *CHS* in the pathway leading to anthocyanins (Fig. 4) showed significant ($P \leq 0.05$) reductions in their levels of expression in 'green' sectors in both pTV00-VmTDR4 and pTV00-CHS VIGS fruits. In contrast, *ANR* expression was higher in the 'green' sectors from both VIGS treatments. ANR catalyses the synthesis of flavan-3-ols such as (-)-epicatechins, a step

in the pathway prior to proanthocyanidins, which are abundant in bilberry fruit tissues prior to the accumulation of anthocyanins.

We wanted to obtain metabolite data along with the information from the transcriptomics, but this proved difficult with the small sectors from the VIGS fruits at 32 days after injection. However, we were able to repeat the VIGS experiments in a second season with similar results, and this time fully ripe bilberry samples were collected 42 days after injections where distinct effects on pigment accumulation could be seen throughout the fruit (Fig. 6D). This provided sufficient tissue for metabolite analysis. Furthermore, as the fruit were fully ripe and the effect was apparent throughout the fruits they could also then be compared with whole fully ripe empty vector or untreated fruits. The metabolite data (Table 1) demonstrated that the principal effect in *VmTDR4* and *CHS* silenced fruits was a two to three fold reduction in levels of anthocyanins compared to the empty vector or non-treated fruits. Levels of quercetin derivatives were low in all samples, and despite levels in the *VmTDR4* and *CHS* silenced fruits being elevated these values are within normal variation of these compounds in fully ripe bilberry fruits; unlike the anthocyanin fraction. The biosynthesis of quercetin is at highest in the early stages of fruit development and the quercetin contents are three to four folds lower in the ripe bilberries compared with the unripe fruits (Jaakola *et al*. 2002). The VIGS treatments were made during berry ripening and after the peak in quercetin content. It is possible that quercetin-derivatives seen in the VIGS fruit accumulated prior to this stage. The empty vector fruits showed evidence of an increase in the early parts of the phenylpropanoid pathway (e.g. *p*-coumaric acid) and in cyanidin that was not apparent in the *VmTDR4* and *CHS* treated fruits. This may be the result of defence related responses by the fruits, arising from the presence of the viral vector in the absence of constructs that modulate expression of genes in the flavonoid pathway. Collectively these data provide strong evidence for a role for *VmTDR4* in the regulation of flavonoid biosynthesis in bilberry fruits.

Discussion

Some of the key genes involved in the molecular regulation of fruit ripening have been revealed through the study of non-ripening mutants in tomato such as *rin*, *Cnr*, *Nr* and *nor* (Giovannoni, 2007; Seymour *et al*, 2008). However, the links between these

regulators and their down-stream effectors that bring about the changes in colour, texture and flavour are poorly understood.

In many fleshy fruit species, flavonoids including anthocyanins are the major coloured pigments synthesised during ripening. Regulation of flavonoid biosynthesis occurs *via* co-ordinated transcriptional control of enzymes in the biosynthetic pathway by the interaction of DNA binding R2R3 MYB transcription factors, MYC-like basic helix loop helix (bHLH) and WD40-repeat proteins (Stracke *et al*, 2001, Broun, 2005; Koes *et al*., 2005; Ramsay & Glover 2005; Grotewold, 2006). It is also known that other factors e.g. light, temperature, and nutrients play a role in flavonoid accumulation (Peng *et al.* 2009). A major missing part of the network is the association between the regulatory genes controlling fruit ripening and the down-stream pathways that lead to the synthesis of important secondary products such as anthocyanins. In this work we report a functional link between a *SQUA* MADS-box transcription factor and the control of flavonoid biosynthesis in bilberry fruit.

VmTDR4 **is a** ***SQUA*** **MADS-box gene involved in anthocyanin biosynthesis in ripening bilberry**

A role for MADS-box transcription factors in regulating anthocyanin biosynthesis in a range of plant tissues has been reported previously. A sweet potato *SQUA* transcription factor, *IbMADS10*, showed a pattern of expression that was tightly correlated with anthocyanin levels in pigmented tissues, especially in the red roots. Also when ectopically expressed in sweet potato callus it induced anthocyanin accumulation (Lalusin *et al*., 2006). *IbMADS10* shares significant sequence homology with *DEFH28* (from *Antirrhinum majus*) (Müller *et al*, 2001) a likely orthologue of *VmTDR4* from bilberry, *TDR4* from tomato and *FUL* from Arabidopsis (Fig. 1). Furthermore, a link between the expression of a MADS-box gene and accumulation of flavonoids has also been reported in Arabidopsis for seed coat pigmentation. The TT16 / ABS MADS domain protein is required for normal development and pigmentation of the seed coat and is postulated to modulate anthocyanin biosynthesis by interacting directly with an R2R3-MYB DNA binding domain protein (Nesi *et al*., 2001; Debeaujon *et al*., 2003).

In bilberry fruits, high levels of anthocyanins accumulate in the skin and flesh during ripening and these changes are correlated with the expression of the *SQUA*

MADS-box gene, *VmTDR4*. The *in situ* hybridisation experiments and QRT-PCR indicated that *VmTDR4* expression was associated with numerous different tissues in the unripe fruit including the vascular tissues and with anthocyanin biosynthesis in ripe fruits. Interestingly *VmTDR4* expression is low in a white mutant of bilberry which ripens normally. These observations are suggestive of a link between *VmTDR4* and flavonoid biosynthesis but do not establish any functional relationship.

Functional analysis of the *VmTDR4* gene in bilberry was undertaken by VIGS. Down regulation of *VmTDR4* in the fruit suppressed the expression of several flavonoid biosynthesis genes and inhibited anthocyanin biosynthesis. *VmTDR4* and *VmMYB2* were not down-regulated in the CHS VIGS fruits, indicating that their effects are up-stream of this gene. The down regulation of *VmTDR4* also suppressed the expression of *VmMYB2*, a bilberry R2R3-*MYB* family member transcription factor, the expression of which is closely associated with accumulation of anthocyanins during the fruit development. The partial sequence we obtained for *VmMYB2* shares 62-63% amino acid identity with *R2R3* region of Arabidopsis *AtPAP1* and *AtPAP2* and 61.5% identity with *VvMYBA1*, an orthologue of *AtPAP* genes of grapevine (*Vitis vinifera*) berry (Fig. 1S). However, the closest identity *VmMYB2* shares with *VvMYBPA1* (86%) and *VvMYB5b* (73%) (Bogs *et al*. 2007; Deluc *et al*. 2008). Moreover, another *R2R3 MYB* family member, *VmMYB1* was isolated from bilberry fruits recently. *VmMYB1* shares 78% identity with *FaMYB1*, a negative regulator and suppressor of anthocyanin biosynthesis from strawberry (Aharoni *et al*. 2001). The QRT-PCR analysis of *VmMYB1* shows that transcripts are more abundant at the early stages of the bilberry fruit development and in the green sectors of the *VmTDR4* VIGS berries. These results indicate that *VmTDR4* functions via known regulators of flavonoid biosynthesis including R2R3-*MYB* family members. The metabolic analyses of VIGS treated fruits revealed that the levels of anthocyanins were two to three-fold lower in *VmTDR4* and *CHS* silenced fruits, whereas the contents of hydroxycinnamic acids or flavonols were not affected. The lack of an effect on hydroxycinnamic acids may be because they are produced in an earlier part of the phenylpropanoid pathway and prior to the regulatory step affected by *VmTDR4*. Also flavonols such as quercetin are generated at the beginning of the bilberry fruit ripening (see Fig. 4), at a stage before the VIGS injections were made. It is still unclear if *VmTDR4* only regulates anthocyanin biosynthesis or if it also is involved in the regulation of other flavonoid sub-groups. From grapevine (*Vitis vinifera*) it is known that different R2R3-*MYB* family members can control separately the biosynthesis of

anthocyanins, flavonols and proanthocyanins (Bogs *et al*, 2005, Bogs *et al*., 2007; Azuma *et al*., 2008; Czemmel *et al*., 2009).

In the *VmTDR4* suppressed VIGS fruits transcript levels of *ANR*, leading to the synthesis of proanthocyanidins, were elevated. In many species proanthocyanidins are produced at the early phases of fruit development and it has been suggested that they act as defensive and astringent compounds to provide protection against fungal pathogens and predation of unripe fruits (Harborne, 1997; Jaakola *et al*., 2002; Bogs *et al*., 2005). In the skin of red grape the flavonoid pathway switches to the production of anthocyanins instead of proanthocyanidins at the onset of ripening, and this phenomenon is controlled by *MYB* transcription factors (Bogs *et al*., 2007). In bilberry, the content of proanthocyanidins and the flavonol quercetin are higher at the early stages of fruit development. At the onset of ripening the level of proanthocyanidins declines whereas quercetin remains at a constant, but low level, during the ripening phase. The flavonol myricetin is synthesised in bilberry skin and flesh at the same time as the accumulation of anthocyanins (Jaakola *et al*., 2002). Therefore, the higher levels of *ANR* transcripts in *VmTDR4* silenced bilberry fruit sections could suggest a delay in ripening in the sectors. However, QRT-PCR analysis of another ripening related MADS-box transcription factor *VmTAGL1*, which is expressed at higher level in earlier stages of bilberry fruit development, did not show differences between the green and red sectors of the *VmTDR4* or *CHS* silenced fruits (data not shown). An alternative metabolic explanation could be that lack of anthocyanidin intermediates in the *VmTDR4* silenced sectors stimulates *ANR* transcription.

Role of TDR4-like genes in other plant species

Elegant work in the model plant Arabidopsis has demonstrated that a range of MADS-box and other transcription factors are involved in regulating the development of their dry fruits. This includes expression of *FUL*, a *SQUA* gene which is primarily responsible for proper valve development (Dinneny *et al*., 2005). In the fleshy fruits of tomato the *TDR4* gene, which shares sequence homology with *FUL*, shows a strong ripening-related pattern of expression (Eriksson *et al*., 2004), but its function is not known. Studies we have undertaken to reveal the role of *TDR4* in tomato fruits were inconclusive with no obvious phenotypes where the gene was down-regulated in RNAi lines (Seymour and Poole, unpublished). This is probably due to the presence of other *TDR4-like* genes in the tomato genome (Hileman *et al*., 2006). However, we observed that tomato *TDR4*

induced anthocyanin biosynthesis when it was expressed ectopically in Arabidopsis siliques (Supplementary Fig. 2). These effects are consistent with those observed for *VmTDR4* in bilberry in that they indicate a link between *SQUA* genes and the regulation of the phenylpropanoid pathway. Furthermore, in Arabidopsis the endogenous *FUL* gene, which is strongly related to *TDR4* and *VmTDR4* by sequence homology, is necessary for the expression of the MYB transcription factor *PAP2* under condition of nitrogen starvation (Supplementary Fig. 3). This MYB is known to be involved in the regulation of anthocyanin biosynthesis (Borevitz *et al.*, 2000).

The data reported in this paper provides strong evidence that *VmTDR4* plays an important role in the control of anthocyanin biosynthesis in in bilberry, acting directly or indirectly through MYB transcription factors to control carbon flux through the phenylpropanoid pathway. Further work is required to determine the precise molecular links between the regulatory factors involved in this process. However, the current study provides the first evidence for a functional association between SQUA MADS-box genes and anthocyanin biosynthesis during ripening in a fleshy fruit species.

Materials and Methods

Plant Material

The flowers and fruits of wild bilberries, growing in the natural forest stand in Oulu (65°01' N, 25°28' E), Finland, were collected at six different ripening stages, from flower to ripe fruit. Samples were immediately frozen in liquid nitrogen and stored at -70°C until analysed. *Arabidopis thaliana* L*er* and Col-0 seeds were obtained from NASC (European Arabidopsis Stock Centre, University of Nottingham). *ful* mutant lines were donated by Prof. M. Yanofsky (UCSD, USA).

Cloning of VmTDR4 and TDR4

Total RNA was isolated from bilberry fruit samples with the method described by Jaakola *et al*. (2002). The quality of the isolated RNA was verified by measuring the absorbance spectrum with NanoDrop N-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and on 1% (w/v) ethidium bromide-stained agarose gel.

Degenerate primers VmTDR4f 5'-GTG ATG CWG AGG TTG STT TGA-3' and VmTDR4r 5'-AGC WGG TTR TTT TGC TCC TGC-3' for VmTDR4 were designed for isolating the VmTDR4 based on homology of the MADS-box genes from tomato (*TDR4*), and Arabidopsis (*FUL*). The full length sequence of *VmTDR4* was cloned using the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) with the gene specific primers gspTDRf 5'-TGGAACCTGGAATACCCGAAGCTCA-3' and gspTDRr 5'-TGAAGCTCTCTCAGGGTCAAGGTGTCA-3'. The full-length cDNA sequence was deposited in GenBank under accession number (FJ418852).

Phylogenetic estimation

Full length protein sequences were aligned using MAFFT version 5 (Katoh *et al*., 2005) and an average distance tree was constructed based on percentage identity between sequences in JALVIEW 2.4 (Clamp *et al*., 2004).

Quantification of transcript abundance

*T*ranscript accumulation of *VmTDR4*, *VmMYB2* , *VmMYB1* and the flavonoid pathway genes (*CHS*, *DFR*, *ANS*, *ANR*) was detected using DyNAmo™ Capillary SYBR® Green qPCR Kit (Finnzymes). *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, AY123769) was used as control gene for relative quantification. QPCR analyses were performed with LightCycler 2.0 instrument and software (Roche, Mannhaim, Germany). The PCR conditions were 95 °C for 10 min, followed by 45 cycles of 95 °C for 10s, 60 °C for 20s and 72 °C for 10s. Concerning the transcript abundance of *VmTDR4* and *VmMYB2*, a t-test was used to determine if the relative quantity was significantly lower ($p \leq 0.05$) in green versus red parts of the VIGS treated fruits based on data from 3-4 technical replicates. We separated those visible sectors of *VmTDR4* or *CHS* silenced fruits with a scalpel.

In situ hybridisation

Unripe bilberry fruits were cut into cubes of 5 mm^2, fixed in 4% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0) overnight at 4°C. The samples were rinsed in 0.1 M sodium phosphate buffer (pH 6.8) and dehydrated in a graded series of ethanol up to absolute. The ethanol was replaced by a series of xylene (25, 50, 75 and 100%, v/v), after which the samples were

gradually infiltrated with paraffin (Merc). Paraffin-embedded samples were sectioned to a thickness of 8 µm with a microtome (Microm HM 325, Walldorf, Germany). The sections were spread on glass slides previously coated with 2% (v/v) 3-aminopropyltriethoxysilane (Sigma) in acetone and dried overnight at 40°C. Two 20 minute incubations in xylene were used for removing paraffin from the samples. For in situ hybridizations, samples were rehydrated in a graded ethanol series up to water.

Using primers 5'-CACCTTGACCCTGAGAGAGC-3' and 5' GTC CAC CTT GGT TTT GTT GC-3', a 218-bp fragment from VmTDR4 was amplified from bilberry fruit cDNA by PCR with DyNazyme™ II DNA polymerase (Finnzymes) under standard PCR conditions. The PCR fragment was gel purified using Montage® DNA Gel Extraction KIT (Millipore) and ligated into pGEM-T Easy vector (Promega, Madison, WI, USA). DIG-labelled sense and antisense probes were prepared from the linearised plasmid by *in vitro* transcription with SP6 or T7 RNA polymerase, using DIG RNA Labelling Kit (Roche) according to manufacturer's instructions. Before hybridization, rehydrated tissue sections were treated with proteinase K (1µg mL^{-1} in Tris-HCl and 50 mM EDTA, pH 7.5) for 30 min at 37 °C followed by dehydration in a graded ethanol series up to absolute. For the hybridization, 100 µL of hybridization mixture (0.1 µg mL^{-1} of DIG-labelled antisense or sense probe, 50%(v/v) deionised formamide (Sigma), 0.3 M NaCl, 10 mM Tric-HCl (pH 7.5), 1mM EDTA, 1x Denhardt's solution (Sigma), 150 µg mL^{-1} tRNA (Roche) , 500 µg mL^{-1} polyadenylic acid (sigma), 10% dextran sulphate and 0.06 M dithiothreitol) was dispersed on the sections and the hybridization was carried out at 50 °C over night. After hybridization, slides were washed in 2x SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0), in 1x SSC at 37 °C and 0.5x SSC at 37 °C, for 10 min in each. Excess RNA probes were removed in RNase A treatment at 37 °C for 60 min following by 4x 15 min washes in 10 mM Tris-HCl, 500 mM NaCl and 1 mM EDTA and 1x 30 min incubation in 2x SSC. For localisation of the hybridized transcripts, slides were washed in 100 mM Tris-HCl, 150 mM NACl, 0.3% v/V Triton X-100 for 5 min and blocked with 2% (w/v) blocking reagent (Roche) for 30 min, followed by a 2 h incubation at RT with 1:750 dilution of anti-(DIG-AP) conjugate (Roche) and 4x 10 minute washes in the same buffer. For colour development, slides were washed 5 min in 100 mM Tris-HCl, pH 9.5, 100mM NaCl and 50 mM MgCl2 and immersed in 5-bromo-4chloro-3-indolylphosphate and blue tetrazolium chloride (Roche) over night in the same buffer. Next day slides were washed in water and dehydrated in a graded series of ethanol up to absolute and air-dried before sealed with coverslips.

Virus induced gene silencing

The pBINTRA6 and pTV00 vectors were obtained from David Baulcombe and PBL at the Sainsbury Laboratory, Norwich Research Park, Colney Lane, Norwich, UK. For pTV00-*VmTDR4* construction a 150-bp fragment of the *VmTDR4* gene was PCR amplified from bilberry fruit cDNA using primers (forward: 5'-CTC GGA TCC GGT GGA CAA AGT TCA TCC-3' and reverse: 5'-GCT AAG CTT CGG CGG CAT CAA AGT GTT-3'). The resulting PCR product was cloned in to pTV00 to form pTV00-VmTDR4. For *pTV00-VmCHS* construction a 100-bp fragment of the *VmCHS* (AY123765) gene was PCR amplified from bilberry fruit cDNA using primers (forward: 5'-CTC GGA TCC AAG ATC ACC CAC TCA GTC TTT TG-3' and reverse: 5'-GCT AAG CTT GCT TCA CGG AGG GAC GGA GCC-3'). The resulting PCR product was cloned in to pTV00 to form pTV00-VmCHS. The pTV00-VmTDR4 and pTV00-VmCHS vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 for the inoculations.

Plant infiltration was performed as described previously (Ratcliff *et al*., 2001). The *Agrobacterium* strains GV3101 containing pTV00-VmTDR4 or pTV00-VmCHS and C58c1 containing pBINTRA6 were grown at 28°C in liquid LB including antibiotics (50 ug/ml kanamycin, 5 ug/ml tetracylin, 50 ug/ml rifampicillin, pH 5.6). After 24 h, the cells were harvested by centrifugation and resuspended in the infiltration buffer (10 mM MgCl2 with 200µM acetosyringone and 10mM MES, pH 5.6) to a final OD_{600} of ~1 and shaken 2 hours (+28 °C) before mixing in a 1:1 ratio. The *Agrobacterium* mix containing either pBINTRA6 + pTV00-VmTDR4 or *VmCHS* vectors were injected in bilberry fruits that were in the middle of fruit development with 1 ml syringe and needle. As a control, only *Agrobacterium* with pBINTRA6 or infection buffer was injected in the fruits.

Metabolite analysis

Contents of anthocyanins, flavonols and hydroxycinnamic acids from fully ripe bilberry fruits 35-42 days following VIGS injections with *VmTDR4*, *CHS* or empty vector constructs were determined. Phenolics were extracted from homogenised freeze-dreid material (20 to 80mg) with methanol (1ml) containing Salicylic acid as an internal standard (10µg). The mixture was heated to 90^{o}C for 60 min after cooling the suspension centrifuged at 3000*g* for 5 min the supernatant removed and extracts passed through a 0.2µm syringe fitting filter before HPLC analysis. An Agilent 1100 HPLC solvent delivery system was used to separate phenolics on a reverse phase C_{18} column

(250mm x 4.6 mm; 5 µm; Hichrom, Reading, UK). The mobile phase consisted of: (A) 2% water in methanol acidified with 0.015% HCl by vol., and (B) acetonitrile. The initial gradient conditions used were 95% A, 5% B for 10 min, followed by a linear gradient to 50% B over 30 min. An on-line photo diode array detector enabled detection and identification from characteristic UV/Vis spectra. Authentic standards were used to confirm the identity of the phenolics. Relative quantification was carried by comparison of integrated peak areas with the internal standard at the λmax of the phenolics detected. Previously the identity of the phenolics had been confirmed with LC-ESI/MS using identical conditions except that formic acid was used insread of HCl.

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Footnotes

The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Phylogenetic analysis of the relationship between *SQUAMOSA* class MADS-box genes. Full length protein sequences were aligned using MAFFT version 5 (Katoh *et al.*, 2005) and an average distance tree was constructed based on percentage identity between sequences in JALVIEW 2.4 (Clamp *et al.,* 2004) of Arabidopsis (*AGAMOUS*; NP_567569, *AP1*; NP_177074, *CAULIFLOWER*; Q39081, *FRUITFULL*; NP_568929), tomato (*MADS-MC*; AAM15774, *TDR4*; AAM33098, *LeFUL2*; AY306156), *Anthirinhum* (*SQUAMOSA*; CAA45228, *DEFH28*; AAK72467) and bilberry (*VmTDR4*; FJ418852).

Fig.2. *In situ hybridisation* and expression profiles for VmTDR4 in bilberry. (A-G) *In situ hybridisation*, bluish colour indicates expression of *VmTDR4* in samples hybridised with the antisense probe. (A) Cross section of whole bilberry fruit. (B) Placenta and developing seed, antisense probe. (B) Close up of developing seed, antisense probe. (D) Vascular tissue, antisense probe. (E) Vascular tissue, sense probe. (F) Epidermis and pericarp, antisense probe. (G) Epidermis and pericarp, sense probe. Image A scale bar is 1 mm, B and C 10 µm and D-G 1 µm. Panels H and I, determination of *VmTDR4* transcript abundance by quantitative RT-PCR. (H) bilberry leaf L, flower F, ripe berry skin BS, ripe berry flesh BF, mature seed S and rhizome R. (I) *VmTDR4* transcript levels during bilberry fruit development and ripening in flowers F, immature green IG, mature green MG, turning T, ripe R and fully ripe FR fruits. Data are means ± SE (n=3).

Fig.3. Determination of transcript abundance of genes at different stages of bilberry fruit development and ripening. (A) chalcone synthase (*CHS*), (B) dihydroflavonol reductase (*DFR*), (C) anthocyanidin synthase (*ANS*), (D) anthocyanidin reductase (*ANR*), (E) *VmMYB2*, (F) *VmMYB1*. Stages are flower F, immature green fruit IG, mature green MG, turning T, ripe R and fully ripe FR fruit. Data are means ± SE (n=3).

Fig. 4. A schematic presentation of the flavonoid biosynthetic pathway. Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; UFGT, UDP glucose-flavonoid 3-o-glucosyl transferase RT, rhamnosyl transferase.

Fig. 5. Gene expression in wild type and white mutant bilberry. Determination of *VmTDR4* and *CHS* transcripts abundance by quantitative PCR in wild type (filled bars) and white mutant (clear bar) bilberry fruits. Data are means ± SE (n=3).

Fig.6. Images of bilberry fruits during development and ripening after injection of *VmTDR4* and chalcone synthase (*CHS*) silencing constructs. Bilberry fruit (A) 10, (B-C) 32 and (D) 42 days following injections with *VmTDR4* and *CHS* VIGS vectors in comparison with the Non-treated control NTC, empty vector control EV fruits. Fruit in panel D are fully ripe.

Fig.7. Effects of *VmTDR4* and chalcone synthase (*CHS*) silencing constructs on bilberry gene expression during ripening. Relative expression of *VmTDR4*, *VmMYB1*, *VmMYB2* and flavonoid biosynthetic genes *CHS*, *DFR*, *ANS* and *ANR* in green and red sectors of bilberry fruits 32 days after injection of VIGS vectors. *VmTDR4* silenced fruit green sector (TG), *VmTDR4* silenced fruit red sector (TR), *CHS* silenced fruit green sector (CG), *CHS* silenced fruit red sector (CR). Empty vector injected fruits. Data are means ± SE (n=3).

Table 1. Phenolic-related metabolite changes in bilberry VIGS fruits. Determination of phenolics present in ripe bilberry fruit 42 days after injection of VIGS *VmTDR4* and *CHS* constructs. HPLC analysis was undertaken on pooled samples of three individual fruits in each treatment. All values are provided in µg mg dry wt according to the protocol in the methods section. Three samples were analysed from each pool. Data are means ± SE.

Supplementary Figures

Fig. S1. Alignment of R2 and R3 DNA-binding regions of VmMYB2 with Arabidopsis PAP1, PAP2, ATMYB113 and ATMYB12, *Vitis vinifera* VvMYBA1, VvMYBPA1, VvMYB5b and Zea mays C1 anthocyanin regulator. The amino acid residues shown to be required for interaction with the Zea mays C1 with a bHLH cofactor R are marked with arrows.

Fig.S2. Effects of ectopic expression of tomato *TDR4* in Arabidopsis. (A) *TDR4* transgenic lines (a,b,g,k) display reduced stature in comparison to wild type (WT), k not shown. (B) *TDR4* siliques revealing differential accumulation of red pigmentation in light. (C) Quantification of anthocyanin accumulation in Arabidopsis siliques collected from lines a, b, g and k. (D) Quantification of *AtPAP2* transcript abundance in WT and *TDR4* lines (mean of a, g and k, error bars, SEM, n=3).

Fig. S3. Determination of *AtPAP2* transcript abundance by quantitative PCR of cDNA in WT and *fruitfull* (*ful*) mutant leaves in response to growth with and without nitrogen (N, error bars, SEM, n=3).

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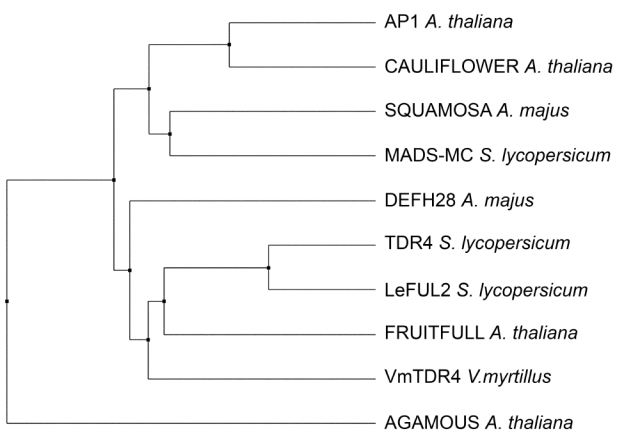
Tissue sample	Chlorogenic acid	p- Coumaric acid	Quercetin-derivatives	Delphinidin	Cyanidin
Untreated fruits	200 ± 38.0	**126 ± 10.0**	0.23 ± 0.02	78.7 ± 4.5	**55.0 ± 3.1**
Empty vector	280 ± 36.0	276 ± 12.0	0.2 ± 0.1	95.0 ± 10.6	67.7 ± 5.0
VmTDR4 VIGS	208 ± 22.0	**172 ± 10.0**	**0.3 ± 0.04**	**33.8 ± 2.7**	**14.8 ± 0.6**
CHS VIGS	196 ± 16.0	**136 ± 0.06**	**0.9 ± 0.20**	**42.5 ± 1.8**	**33.8 ± 2.7**



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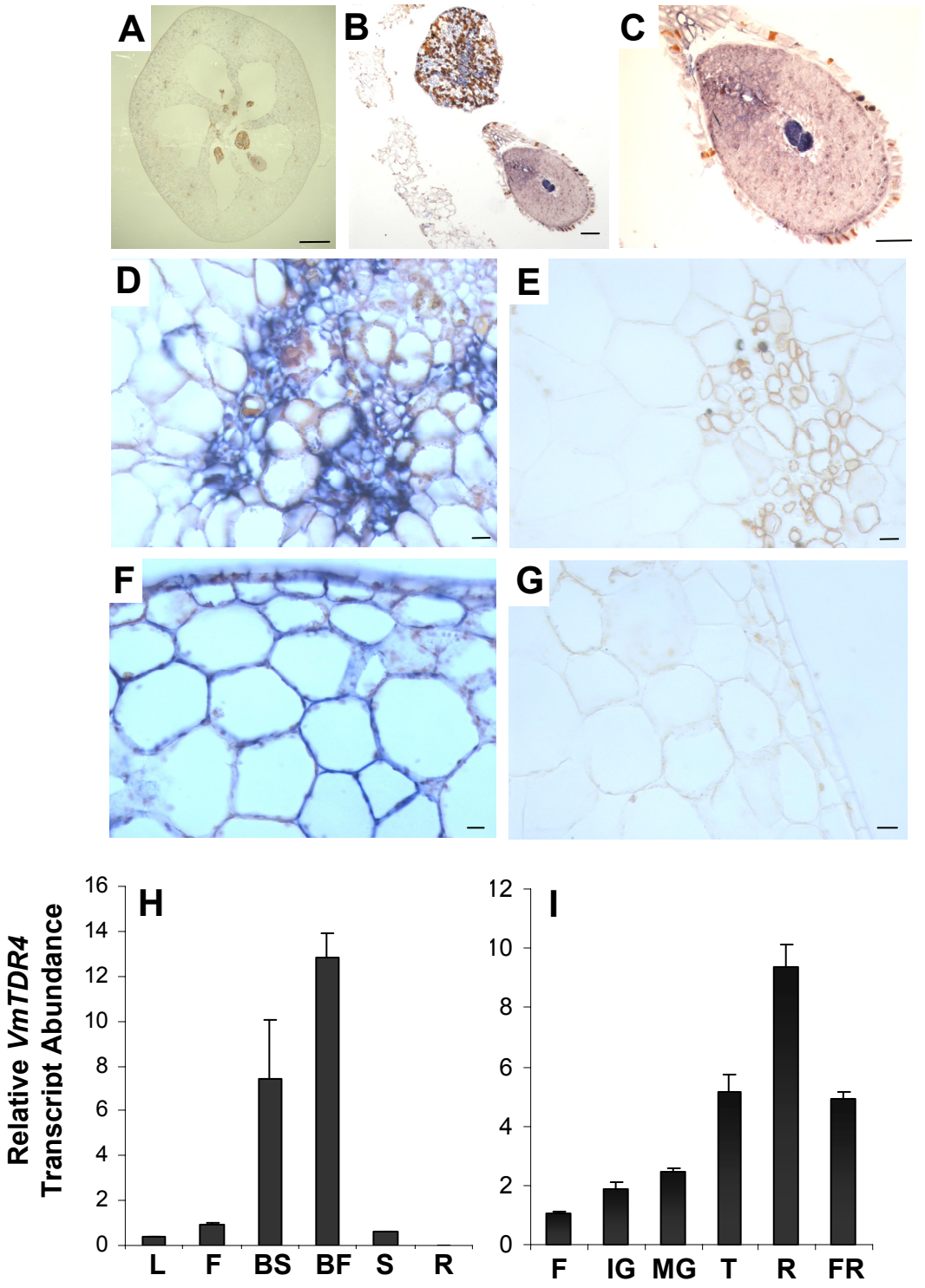

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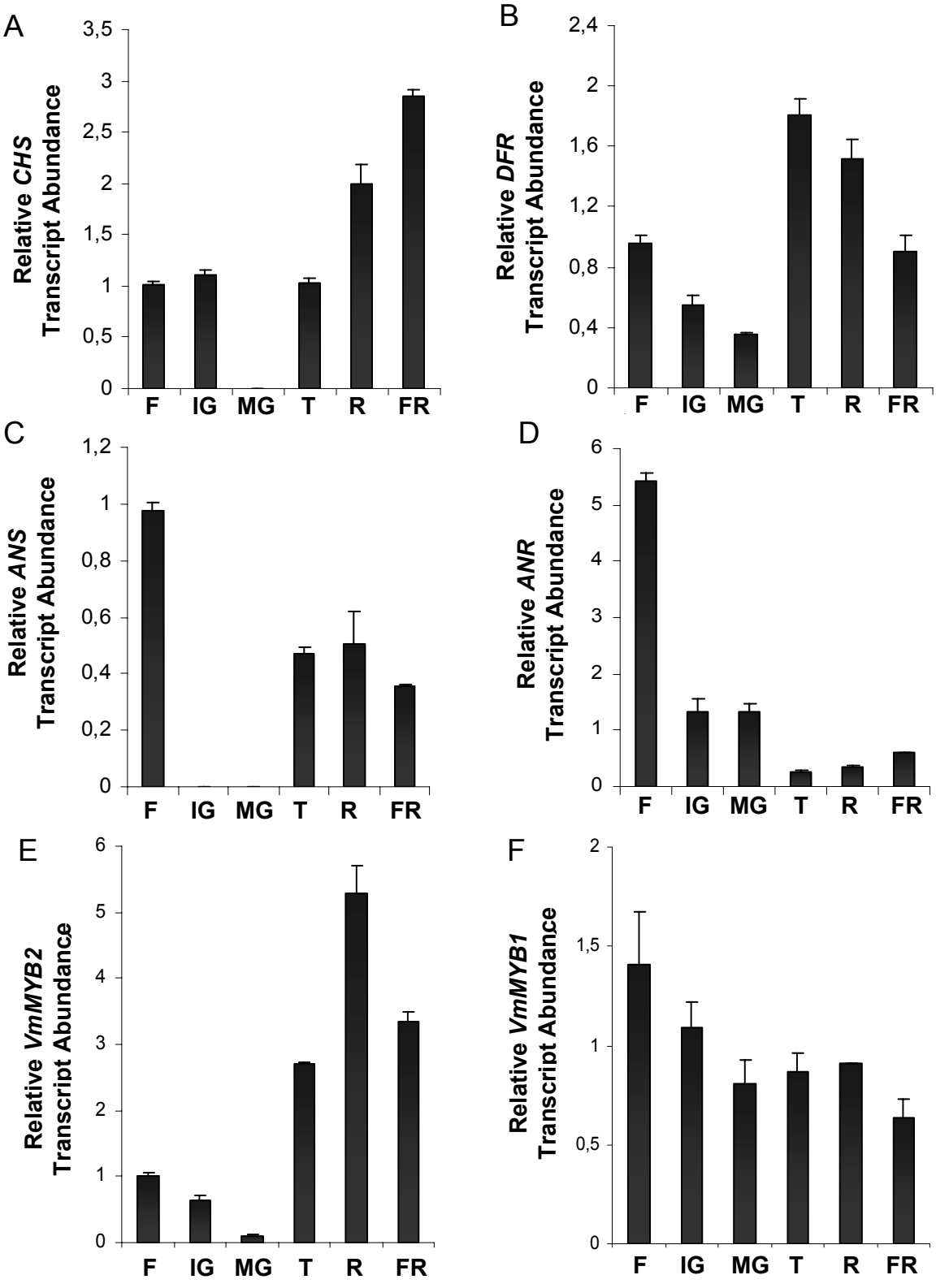

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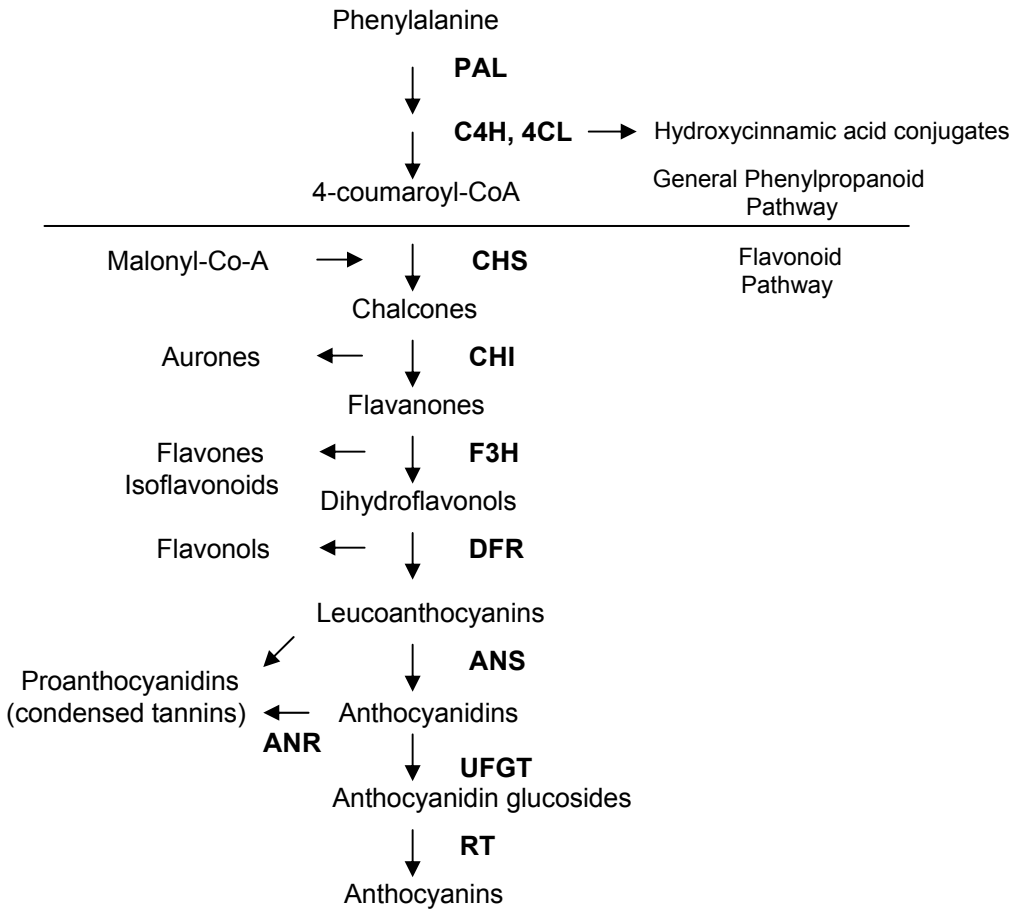

Fig. 4. A schematic presentation of the flavonoid biosynthetic pathway. Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; UFGT, UDP glucose-flavonoid 3-o-glucosyl transferase RT, rhamnosyl transferase.

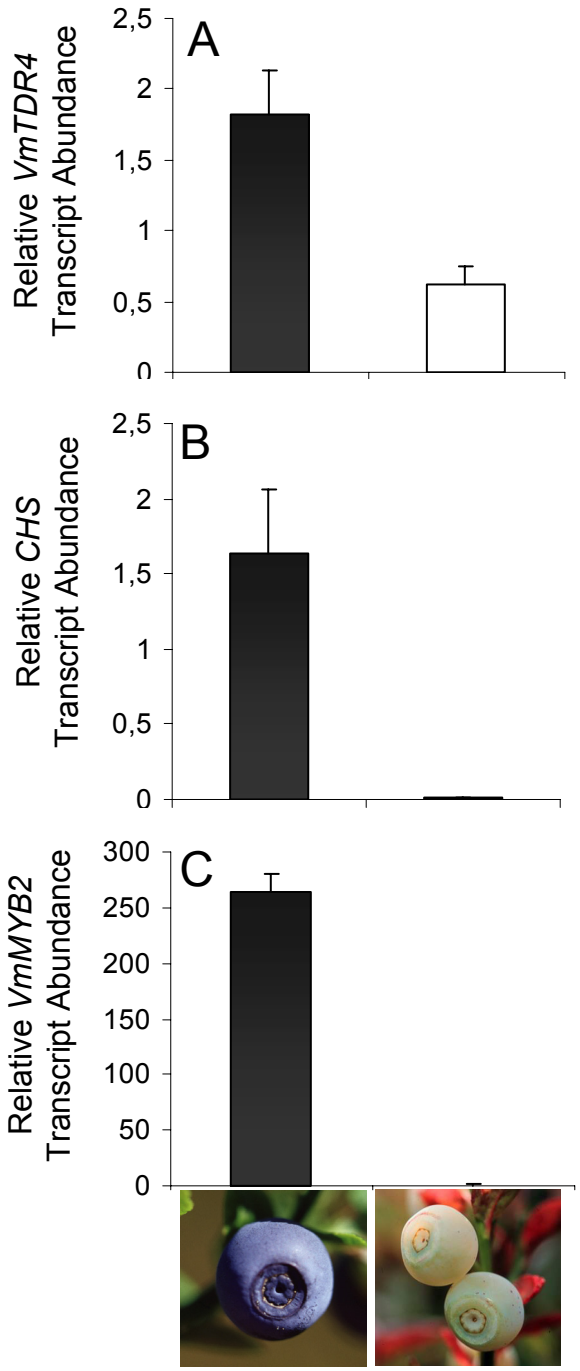

Fig. 5 Determination of *VmTDR4*, *CHS and VmMYB2* transcripts abundance by quantitative RT-PCR in wild type (filled bars) and white mutant (clear bar) bilberry fruits. Data are means ± SE (n=3).

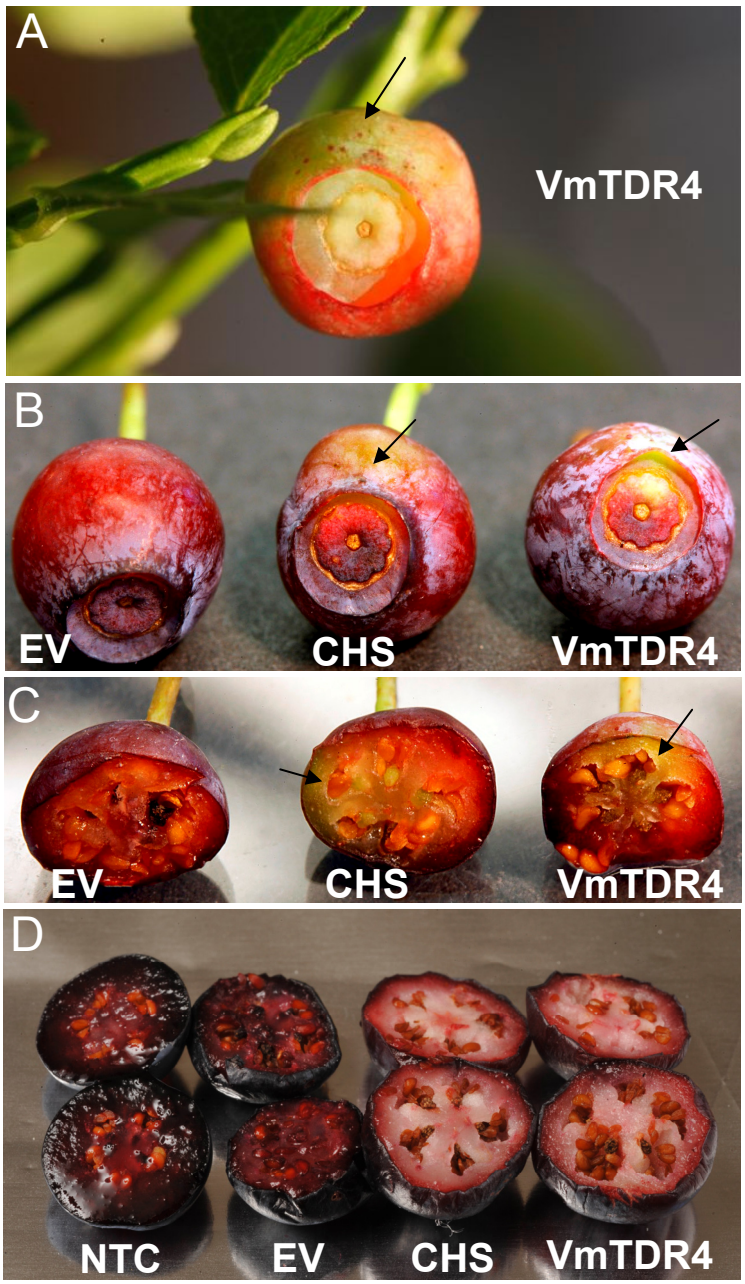

Fig.6. Images of bilberry fruits during development and ripening after injection of *VmTDR4* and chalcone synthase (*CHS*) silencing constructs. The arrows highlight the green sectors on the injected sites of the VIGS fruits. Bilberry fruit (A) 10, (B-C) 32 and (D) 42 days following injections with *VmTDR4* and *CHS* VIGS vectors in comparison with the Non-treated control NTC, empty vector control EV fruits. Fruit in panel D are fully ripe.

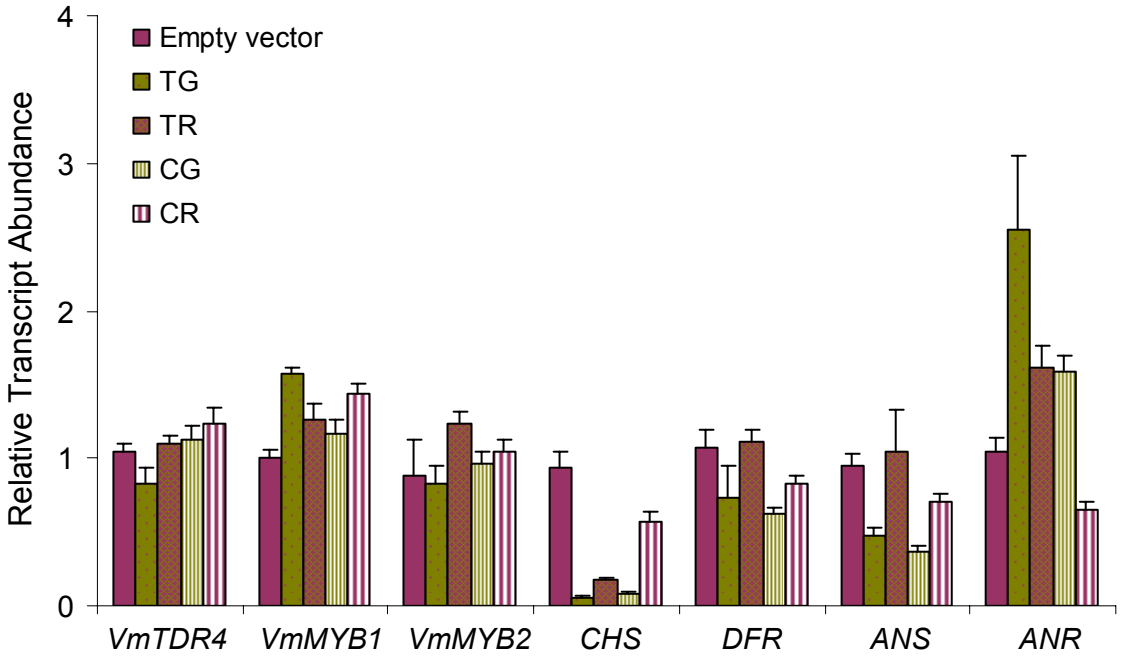

Fig.7. Effects of *VmTDR4* and chalcone synthase (*CHS*) silencing constructs on bilberry gene expression during ripening. Relative expression of *VmTDR4*, *VmMYB1*, *VmMYB2* and flavonoid biosynthetic genes *CHS*, *DFR*, *ANS* and *ANR* in green and red sectors of bilberry fruits 32 days after injection of VIGS vectors. *VmTDR4* silenced fruit green sector (TG), *VmTDR4* silenced fruit red sector (TR), *CHS* silenced fruit green sector (CG), *CHS* silenced fruit red sector (CR). Empty vector injected fruits. Data are means ± SE (n=3).