

RESEARCH PAPER

# Identification of target genes for a MYB-type anthocyanin regulator in *Gerbera hybrida*

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## Abstract

Genetic modification of the flavonoid pathway has been used to produce novel colours and colour patterns in ornamental plants as well as to modify the nutritional and pharmaceutical properties of food crops. It has been suggested that co-ordinate control of multiple steps of the pathway with the help of regulatory genes would lead to a more predictable control of metabolic flux. Regulation of anthocyanin biosynthesis has been studied in a common ornamental plant, *Gerbera hybrida* (Asteraceae). An R2R3-type MYB factor, GMYB10, shares high sequence similarity and is phylogenetically grouped together with previously characterized regulators of anthocyanin pigmentation. Ectopic expression of *GMYB10* leads to strongly enhanced accumulation of anthocyanin pigments as well as to an altered pigmentation pattern in transgenic gerbera plants. Anthocyanin analysis indicates that GMYB10 specifically induces cyanidin biosynthesis in undifferentiated callus and in vegetative tissues. Furthermore, in floral tissues enhanced pelargonidin production is detected. Microarray analysis using the gerbera 9K cDNA array revealed a highly predicted set of putative target genes for GMYB10 including new gene family members of both early and late biosynthetic genes of the flavonoid pathway. However, completely new candidate targets, such as a serine carboxypeptidase-like gene as well, as two new MYB domain factors, GMYB11 and GMYB12, whose exact function in phenylpropanoid biosynthesis is not clear yet, were also identified.

Key words: Anthocyanin, Asteraceae, flavonoid, genetic modification, *Gerbera*, MYB.

## Introduction

Biosynthesis of flavonoids and especially anthocyanins, the most important compounds contributing to orange, red, and blue coloration of higher plants, have been intensively studied for decades (for reviews see Winkel-Shirley, 2001; Koes *et al.*, 2005). Flavonoid biosynthesis branches off from the general phenylpropanoid pathway that also leads to the biosynthesis of other important secondary metabolites such as lignans, lignin, and coumarins as well as to phytoalexins. Modification of the flavonoid pathway is an attractive target for metabolic engineering. In addition to the possibilities of producing novel colours and colour patterns in ornamentals (Teeri and Elomaa, 2003; Tanaka *et al.*, 2005), modification of flavonoid biosynthesis by increasing the levels or by altering the composition of different phenolic compounds can provide health benefits due to the nutritional and pharmaceutical properties of flavonoids. For example, in tomato, transformation of a single petunia gene encoding chalcone isomerase (CHI) led to a 78-fold increase in fruit peel flavonols and contributed to increased antioxidant capacity (Muir *et al.*, 2001; Verhoeven *et al.*, 2002). Instead of modifying single enzymatic steps, it has been suggested that co-ordinate control of multiple steps with the help of regulatory genes would lead to an even more predictable control of metabolic flux (Broun, 2004; Capell and Christou, 2004). Designing strategies for controlled metabolic engineering is challenging and requires detailed characterization of candidate regulatory proteins and their target genes.

Anthocyanin biosynthetic genes as well as the regulatory proteins involved in activation (or repression) of pigmentation are highly conserved in higher plants (Winkel-Shirley, 2001; Koes *et al.*, 2005). However, recent studies especially in *Petunia hybrida*, *Zea mays*, and *Arabidopsis thaliana* have revealed the complexity of the regulatory networks

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which, in addition to anthocyanin and proanthocyanidin production, control multiple developmental processes including the development of trichomes, root hairs, and seed coat mucilage in *Arabidopsis* (Schiefelbein, 2003; Zhang *et al.*, 2003; Broun, 2005; Serna and Martin, 2006) as well as the morphology of seed coat epidermal cells and the vacuolar pH of petals in *Petunia* (Spelt *et al.*, 2002; Quattrocchio *et al.*, 2006). Regulatory complexes in these processes have been shown to involve WD40, bHLH, and MYB proteins. The WD40 proteins and bHLH factors are more ubiquitously expressed while the regulation of these distinct developmental processes are determined by physical interaction of WD40 and bHLH proteins with different and functionally more specific MYB proteins (reviewed in Broun, 2005; Koes *et al.*, 2005; Ramsay and Glover, 2005).

The accumulation of anthocyanin pigments occurs via co-ordinated transcriptional activation of the structural biosynthetic genes. The MYB domain factors have been shown to bind to the promoters of the biosynthetic genes directly (Solano *et al.*, 1995; Sainz *et al.*, 1997; Lesnick and Chandler, 1998) or to activate genes encoding bHLH regulators (Spelt *et al.*, 2002). Moreover, MYB proteins can also act as repressors, either by competing for binding sites in target gene promoters or by interacting with bHLH factors and sequestering them into inactive complexes (Tamagnone *et al.*, 1998; Jin *et al.*, 2000; Aharoni *et al.*, 2001). Ectopic expression of maize regulators in cultured cell lines indicate that distinct MYB factors activate different sets of target genes (Grotewold *et al.*, 1998; Bruce *et al.*, 2000). The maize MYB factor C1, together with its bHLH partner R, activate the accumulation of cyanidin derivatives which are the common pigments found in differentiated maize tissues. In contrast, in the cell lines that just overexpress the maize MYB domain factor P, the accumulation of various 3-deoxy flavonoids (e.g. phlobaphenes), as well as several additional phenylpropanoid compounds, were observed. C1/R activated the whole late anthocyanin pathway in maize (*F3H*, *A2*, *Bz1*, and *Bz2*) while P affected a different branch of the pathway and activated the transcription of only *C2* and *A1* encoding chalcone synthase and dihydroflavonol-4-reductase, respectively (Grotewold *et al.*, 1998). More detailed mRNA profiling revealed >800 differentially expressed genes in maize suspension culture lines overexpressing estradiol-induced versions of the maize regulators (Bruce *et al.*, 2000). Similarly to the maize P factor, the *Arabidopsis* MYB12 has been shown to activate a subset of genes, in this case, those required for flavonol biosynthesis (*CHS*, *CHI*, *F3H*, and *FLS*) independently of a bHLH coactivator (Mehrtens *et al.*, 2005).

Activation tagging in *Arabidopsis* and tomato has revealed purple coloured lines accumulating large amounts of anthocyanin pigments (Borevitz *et al.*, 2000; Mathews *et al.*, 2003). In both species, the lines overexpress single MYB factors, PAP1 and ANT1, respec-

tively, which share high sequence similarity with the petunia AN2 (Quattrocchio *et al.*, 1999). The tomato lines accumulated delphinidin-, petunidin-, and malvidin-type anthocyanidins due to up-regulation of both early and late biosynthetic genes of the anthocyanin pathway. In addition, up-regulation of a homologue of homeodomain *GLABRA2* and a putative permease similar to *Arabidopsis* *TT12* encoding a vacuolar transporter of proanthocyanidins was observed (Mathews *et al.*, 2003). Comprehensive metabolic analysis of *Arabidopsis* lines overexpressing *PAP1* revealed the specific accumulation of cyanidin-type anthocyanins and quercetin-type flavonols (Tohge *et al.*, 2005). Moreover, microarray analysis revealed the induction of 38 genes including almost all genes encoding anthocyanin biosynthetic enzymes. Three transcription factor genes encoding a bHLH (*TT8*), a WRKY (*TTG2*), and an AP2 domain factor (*At5g61600*) were up-regulated.

An R2R3-type MYB domain gene, *GMYB10*, has been identified from *Gerbera hybrida*. *GMYB10* is phylogenetically grouped together with previously isolated anthocyanin regulatory genes of *Petunia* and *Arabidopsis* (Elomaa *et al.*, 2003). Ectopic expression of *GMYB10* strongly activated anthocyanin production in transgenic tobacco and in undifferentiated callus of gerbera (Elomaa *et al.*, 2003). In this paper, the analysis of mature transgenic gerbera plants that show altered anthocyanin pigmentation levels and pattern is reported. Transcriptome analysis was used to identify putative target genes affected by *GMYB10* overexpression. These data revealed a relatively limited set of genes including several gene family members of known biosynthetic genes of the flavonoid pathway but also new candidate genes with putative functions in phenylpropanoid biosynthesis or regulation.

## Materials and methods

### Transformation of gerbera

*Agrobacterium*-mediated transformation of the 35S-*GMYB10* gene construct to gerbera was performed as previously reported (Elomaa and Teeri, 2001). Mock-transformation was done with pHTT602 (Elomaa and Teeri, 2001) lacking the transgene insert. In addition, particle bombardment using Bio-Rad PDS-1000/He equipment (Bio-Rad Laboratories, Hercules, CA) was used. For bombardment, 5 mg of gold particles (0.6 µm in diameter) were precipitated with 12.5 µg of plasmid pHEP22 DNA (full-length *GMYB10* insert cloned into pHTT602 under the CaMV 35S promoter) using CaCl<sub>2</sub> and spermidine, and were finally suspended in 120 µl of ethanol. Pieces of petioles from *in vitro*-grown gerbera plantlets were placed on agar plates and were bombarded using 10 µl of particles for each shot and 900 psi or 1350 psi pressure plates. Regeneration of explants was done as in the *Agrobacterium* method (Elomaa and Teeri, 2001). Integration of the transgene was verified using standard DNA hybridization.

### Determination of total anthocyanin content

Plant material was ground in liquid nitrogen and freeze-dried for 2 d. 10 mg of the powder was extracted with 1 ml of extraction

solvent (70 ml methanol+20 ml water+10 ml 1 M HCl) at 4 °C for 20 h, and centrifuged (10 000 rpm for 20 min at 4 °C). There were three replicates for each sample. The absorbance of the supernatants was measured with Agilent 8453 UV-visible Spectroscopy System at 530 nm (Agilent Technologies, Waldbronn, Germany). The anthocyanin content (mg g<sup>-1</sup> freeze-dried plant material) was calculated as an average of the three replicates based on cyanidin chloride (Extrasynthese, France) as a standard.

#### HPLC analysis of flavonoids

Plant material was ground in liquid nitrogen. 500 mg of frozen powder was weighed into a centrifuge tube, 0.8 ml of methanol was added, and the tube was placed into a ultrasonic bath for 10 min. The sample was centrifuged (1500 rpm for 10 min), and the clear supernatant was collected. The procedure was repeated two times. Supernatants were combined and evaporated to dryness. Extracted anthocyanins were hydrolysed into their corresponding anthocyanidins as described in Zhang *et al.* (2004). Dried samples were dispersed in 1:1 v/v water/methanol solution containing 2 N HCl (50 ml methanol+33 ml water+17 ml 37% HCl) at 10 mg ml<sup>-1</sup>, and hydrolysed at 100 °C for 60 min. Hydrolysed samples were filtered through a 0.45 µm PTFE filter. HPLC analyses were conducted on Agilent 1100 Series HPLC System. Analytical separation of flavonoids was carried out on a Zorbax Rc-C18 column (4.6×250 mm, 5 µm; Agilent) at a flow rate of 1 ml min<sup>-1</sup>. Mobile phases were 0.4% TFA in water (solvent A) and 0.4% TFA in acetonitrile (solvent B; Zhang *et al.*, 2004). The elution conditions were from 0 min 18% B to 35 min 40% B using a linear gradient between the time points. Other chromatographic conditions were as follows: column temperature 40 °C; detection at 210, 280, 365, and 520 nm; injection 20 µl; and post-run time 15 min. Every sample had two biological replicates, each with two technical repeats. Delphinidin, cyanidin, pelargonidin, luteolin, apigenin (TransMIT, Marburg, Germany), quercetin, kaempferol, myricetin, naringenin (Sigma), eriodictyol (TransMIT, Marburg, Germany), dihydroquercetin (Roth), and *p*-coumaric acid were used as external standards.

#### cDNA microarray analyses

For the transcriptome analysis the gerbera cDNA microarray was used (Laitinen *et al.*, 2005). This array contains approximately 9000 cDNA probes originating from altogether nine sequenced cDNA libraries of both vegetative and floral tissues of varieties Terra Regina and Terra Nero (Laitinen *et al.*, 2005). In order to reveal changes in gene expression affected by *GMYP10* overexpression, callus tissues, petals at stages 4 (Helariutta *et al.*, 1993), and stamens at stage 8 from wild-type Terra Regina and transgenic line Tr14 were compared against each other. Total RNA was extracted using the Trizol reagent. For callus and stamen samples, total RNA was used for RNA amplification and labelling. 2 µg of total RNA was amplified using Ambion Aminoallyl Message Amp kit. 7.5 µg of labelled aRNA was used for hybridization. For petal samples, mRNA was extracted using a Nucleotrap mRNA isolation kit and was used directly for labelling. Labelling of the samples and hybridizations of the cDNA microarrays were done according to Laitinen *et al.* (2005).

The callus samples were collected from several independent transgenic lines and pooled for RNA isolation. Four technical replicates were performed. For petal and stamen samples, two biological replicates each with three technical replicates were performed. After hybridization, raw data were quantified using GenePix Pro whereafter further analysis was done using GeneSpring 7.2. In all cases, data was LOWESS normalized. In the data analysis, a one-sample *t* test was used and *P*-values were corrected for multiple testing using Benjamin–Hochberg False Discovery

Rate (FDR). FDR controls the number of false positives among differentially expressed genes. The FDR adjusted *P*-value <0.005 was used as a criterion for differential expression.

#### Real-time RT-PCR

The expression for 21 transcripts was verified using real-time RT-PCR as previously reported by Laitinen *et al.* (2005). Ubiquitin was used as an internal standard in callus and stamen samples and, due to variability of ubiquitin expression in petal tissues, *GGLO1* encoding the gerbera GLOBOSA1 MADS box protein in petal samples. The primers used are listed in Supplementary Table S4 at JXB online.

#### Isolation of full-length GMYB11 and GMYB12

Based on the EST sequence of *GMYB11* (G0000100029F05) and *GMYB12* (G0000300014D01), primers for standard 5' Race were designed. Ray flower petal cDNA synthesis was performed using poly(A)<sup>+</sup> RNA as the template. Developmental stages 2 and 3 and stages 6–8 (Helariutta *et al.*, 1993) were used for *GMYB12* and *GMYB11*, respectively. cDNA synthesis and amplification of cDNA ends was performed using a Smart Race cDNA Amplification Kit (Clontech Laboratories Inc.). To ensure that the fragments obtained originated from the same transcript, full-length cDNAs were amplified once more. The full-length sequence for *GMYB11* was amplified using 3' Race protocol (Smart Race kit) with a forward primer 5'-ACGCAGAGTACGCGGGGGTGGCTGATAT-3' and for *GMYB12* regular RT-PCR was used with a forward primer 5'-AACCAAAGACCTCCGTTAGCGA-3' and a reverse primer 5'-ACGCCATTAATAATCCCTCCAAACA-3'. Both cDNAs were cloned into pGEMTeasy vector and sequenced in both directions.

#### RNA blot analysis

The expression analysis for *GMYB11* and *GMYB12* using RNA blots was done as previously reported by Elomaa *et al.* (2003). A gene-specific probe (224 bp) for *GMYB11* was amplified from the plasmid G0000100029D05 with PCR using primers CGCATC-GAAAATCTCTCCTC and CATAAATAAAAGTGAACTAT-CATGG. For *GMYB12*, a 339 bp fragment corresponding to the 3' end of the gene was digested from the plasmid G0000300014D01 with *Bam*H1 and *Asp*718.

#### Particle bombardment of gerbera leaf tissue and luciferase assay

Particle bombardment using 35S-*GMYB10*, 35S-*GMYB11*, 35S-*GMYB12*, and 35S-*GMYC1* activator constructs was performed as in Elomaa *et al.* (1998).

#### Accession numbers

The information for the microarray design and the experimental data have been submitted to ArrayExpress (www.ebi.ac.uk/arrayexpress) under accession numbers A-MEXP-628 and E-MEXP-959, respectively. The full-length sequences for *GMYB11* and *GMYB12* have been deposited in GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers EU684238 and EU684239.

## Results

### Phenotypes of transgenic plants overexpressing GMYB10

For functional analysis of gerbera *GMYB10*, the full-length cDNA under the CaMV35S promoter was transformed into

*Gerbera hybrida* variety Terra Regina (Elomaa *et al.*, 2003). As previously reported, high levels of anthocyanin pigmentation were detected in the young developing transgenic calli (Elomaa *et al.*, 2003; Fig. 1C, D). Most of the calli never formed shoots, probably due to the toxic effects of excess anthocyanins on regeneration. However, eventually, eight transgenic lines were regenerated of which five overexpressed *GMYB10* (see Supplementary Fig. S1 at *JXB*

online). The presence of the transgene was verified with DNA blot hybridization (data not shown).

Three different lines (Tr1, Tr8, and Tr14) showed enhanced pigmentation levels as well as altered anthocyanin accumulation patterns which were similar in all of them. In accordance with the expression level of the transgene, the anthocyanin levels were most dramatically enhanced in Tr1 and Tr14 compared with Tr8 (see



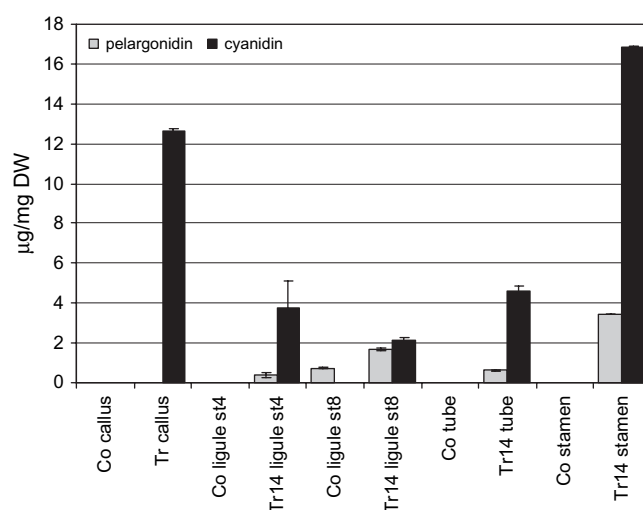
**Fig. 1.** Phenotype of the transgenic gerbera inflorescence of Tr14 that overexpresses *GMYB10* (A) compared to the inflorescence of the non-transgenic variety Terra Regina (B). Regenerating callus transformed with an empty vector (C) compared with transgenic callus tissue that accumulates anthocyanin pigments (D). Anthocyanin pigmentation was induced in leaf petioles (E) and flower scapes (F) of Tr14 (left) and wild type (right). Overexpression of *GMYB10* converts stamens (st) and tubular parts of petals (tu, pointed by arrow) to an intense red (G). Petal phenotypes of Tr14 (left) and wild type (right) at developmental stage 4 (H) and at stage 8 (I). Cross-sections of the petals at stage 8, Tr14 (left) and wild type (right) (J). Scale bars are 1 mm (G, J) and 1 cm (H, I).

Supplementary Table S1 at *JXB* online). Figure 1 demonstrates the characteristic phenotypic changes observed in line Tr14 which was selected for a more detailed analysis. In fully grown plants, enhanced pigmentation was observed in both vegetative and floral tissues (Fig. 1). Clear phenotypic changes were observed in petioles, leaf veins and flower scapes (Fig. 1E, F). In the central disc flowers, stamen pigmentation was converted from yellow to an intense red (Fig. 1G). The temporal accumulation pattern of anthocyanins was altered and the petals showed very intense coloration in young developing inflorescences at stage 2, while in the wild-type, anthocyanin accumulation is first visible at stage 5 (Helariutta *et al.*, 1993). Figure 1H shows developmental stage 4 when the petals of the control variety Terra Regina are still non-pigmented (right) and the transgenic line Tr14 shows very intense pigmentation (left). In addition, the visible shade of pigmentation in petals was altered (Fig. 1I), especially in the abaxial sides of the petals compared with the non-transgenic control plants. This was also seen in the tubes of the petals of Tr14 that showed deep purple and a more intense shade of colour compared with those of the wild-type Regina that are pale yellow (Fig. 1G; arrow). Moreover, cross-sections of the petals revealed that, in transgenic line Tr14, the mesophyll and the vascular tissues especially accumulated high levels of anthocyanins (Fig. 1J).

#### Flavonoid analysis of transgenic line TR14

Total anthocyanin content and anthocyanidin profiles of control and transgenic samples were compared using spectrophotometric analysis and with HPLC. Both quantitative and qualitative changes were observed in transgenic tissues. For the callus samples, several independent cell lines were pooled for flavonoid extraction. Non-pigmented control calli, transformed with an empty vector, were completely devoid of anthocyanin pigments ( $<0.07 \text{ mg g}^{-1}$  dry weight) while the red calli overexpressing *GMYB10* accumulated, on average  $4.7 \text{ mg g}^{-1}$  DW. Thus, at least a 66-fold increase of pigment levels was observed. In floral tissues, Tr14 showed the most severe phenotype with at least a 75-fold increase in total anthocyanin concentration in petal tubes, a 4-fold increase in ligules of the petals, and an almost 30-fold increase in whole disc flowers (which locate the differentially pigmented stamens). In Tr1 and Tr8 the changes were more modest although clearly enhanced pigmentation could be measured (see Supplementary Table S1 at *JXB* online).

HPLC analysis of the hydrolysed anthocyanin extracts showed that the major difference between the control (Co) and the transgenic (Tr14) tissues was in the accumulation of the core anthocyanidins pelargonidin and cyanidin. In green tissues such as leaves and callus, overexpression of *GMYB10* led to specific activation of cyanidin biosynthesis (Fig. 2). In non-transgenic plants, corresponding with



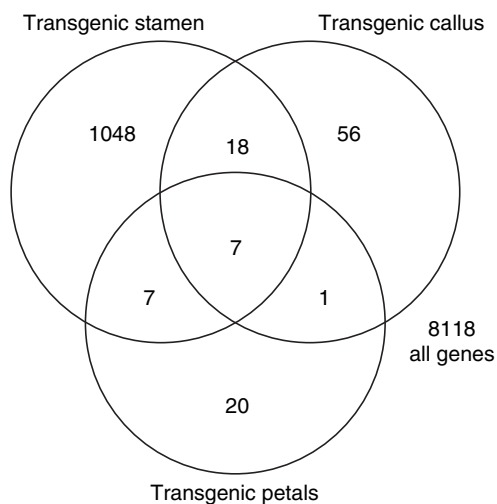
**Fig. 2.** Pelargonidin and cyanidin concentrations ( $\mu\text{g mg}^{-1}$  dry weight) of various tissues in wild-type Regina (Co) and the transgenic line Tr14 overexpressing *GMYB10*.

the visible phenotypes, petal ligules at stage 4, petal tubes (stage 8), and stamens (stage 8) completely lacked anthocyanidins (Fig. 2). Only the petal ligules at the later developmental stage 8 showed the accumulation of pelargonidin pigments. In Tr14, cyanidin was the major pigment in the corresponding floral tissues but enhanced pelargonidin levels were also detected (Fig. 2). The HPLC profiles of these tissue extracts monitored at 365 nm to detect flavonols and flavones did not show any major differences (data not shown).

#### Microarray comparison of transgenic lines with control lines

The gerbera 9K cDNA microarray (Laitinen *et al.*, 2005) was used to compare gene expression profiles of transgenic tissues against the corresponding control tissues to reveal putative target genes for *GMYB10* regulatory protein. Callus, stamen, and petal samples (containing both ligule and tube tissues) were included in the analysis. In untransformed Terra Regina, calli and stamens (at developmental stage 8) are non-pigmented in contrast to transgenic lines which show a strong accumulation of anthocyanins. For petals, developmental stage 4, that showed most dramatic change between the control and the transgenic sample, (Fig. 1H) were selected.

After statistical analysis, the largest number of differentially expressed genes were observed in stamen and callus samples (Fig. 3). 1080 cDNAs up-regulated in transgenic stamens were identified compared with control stamens and 82 cDNAs in transgenic calli, respectively. In transgenic petals, 35 cDNAs were up-regulated. In order to strengthen our analysis, the gene lists were combined and identified genes that were



**Fig. 3.** Venn diagram indicating the number of probes (cDNAs) identified in microarray analyses as up-regulated in a given transgenic tissue in comparison to the corresponding control tissue.

shared between different organs as true candidate targets responding to changes in *GMYB10* expression. In addition, the analysis revealed a large number of genes (972 in stamens, 190 in callus, and 32 in petals) that were down-regulated in transgenic tissues compared with wild-type tissues. Those shared between the various tissues are shown as supplementary data (see Supplementary Table S2 at *JXB* online). Most of them are encoding unknown proteins or components of the photosynthetic machinery and are not discussed here.

#### Transcripts up-regulated in the cyanidin accumulating calli and stamens

The microarray analysis revealed 25 transcripts that were up-regulated both in transgenic calli and in transgenic stamens (Fig. 3; Table 1). As expected, up-regulation of the transgene itself (*GMYB10*, *G0000200009F04*) was observed as well as several cDNAs homologous with well-known biosynthetic genes of the flavonoid pathway. These include homologues of phenylalanine ammonia-lyase (*PAL*), cinnamate-4-hydroxylase (*C4H*), chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H*), and flavonoid 3' hydroxylase (*F3'H*). All sequences except *F3H* represent new, functionally unidentified gene family members in gerbera. The EST encoding *F3H* corresponds to the previously isolated PCR fragment shown to be regulated as an early biosynthetic gene in gerbera (P Elomaa, unpublished results). One of the most highly up-regulated cDNA (*G0000100035C05*) corresponds to a glutathione *S*-transferase (*GST*) gene highly homologous with the *Petunia hybrida* *AN9* that encodes an enzyme needed for transport of toxic anthocyanins from cytoplasm into the vacuoles (Marrs *et al.*, 1995; Alfenito *et al.*, 1998; Mueller *et al.*, 2000). Strong up-regulation of a cDNA

**Table 1.** Transcripts up-regulated in transgenic callus and stamen tissues of *Tr14* overexpressing *GMYB10*

Adjusted *P*-value with FDR <0.005 was used as a criteria for differential expression.

EST code	Annotation
G0000800014E02	Caffeoyl-CoA <i>O</i> -methyltransferase (EC 2.1.1.104) (CCOMT)
G0000100035C05	Glutathione <i>S</i> -transferase ( <i>GST</i> )
G0000900005E02	Novel unknown protein 4104
G0000100004B03	Chalcone synthase (EC 2.3.1.74)
G0000600013G10	Novel unknown protein 3178
G0000300002G07	Flavonone-3-hydroxylase (EC 1.14.11.9) ( <i>F3H</i> ) ( <i>FHT</i> )
G0000800007E02	Phenylalanine ammonia lyase (EC 4.3.1.5) ( <i>PAL</i> )
G0000200025E09	Cinnamate-4-hydroxylase ( <i>C4H</i> )
G0000800009A10	Serine carboxypeptidase
G0000200047B04	Novel unknown protein 1247
G0000100028H02	Flavonoid 3' hydroxylase ( <i>F3'H</i> )
G0000700015H08	Novel unknown protein 1074
G0000100009F04	<i>Gerbera hybrida</i> <i>GMYB10</i>
G0000700014B10	Phenylalanine ammonia lyase (EC 4.3.1.5) ( <i>PAL</i> )
G0000200045C03	Putative 60S ribosomal protein L15
G0000700010F02	Glutathione transferase (EC 2.5.1.18) ( <i>GST</i> )
G0000100029D05	MYB-like transcription factor
G0000800014D03	Unknown protein 0906
G0000200014A07	<i>Gerbera hybrida</i> , MADS-box protein <i>GRCD3</i>
G0000700014H10	Putative citrate lyase
G0000300002B07	Ribosomal protein L11 homologue
G0000200020B01	18S rRNA gene
G0000200033F08	Arginyl-tRNA synthetase

similar to caffeoyl-CoA *O*-methyltransferases was also observed. Five of the up-regulated genes encode unknown proteins which do not find any hits in BLAST searches and in re-annotation, two sequences turned out to be contaminations.

Two transcription factor genes were up-regulated in both transgenic calli and in transgenic stamens (Table 1). *G0000100029D05* encodes an R2R3-type MYB factor that, in BLAST searches, shares the highest sequence similarity with the R2R3 MYB transcription factor C2 repressor motif protein of *Vitis vinifera*, *Gossypium hirsutum* MYB factor 6 and 5 (Cedroni *et al.*, 2003), and *Dendrobium* sp. MYB8 (Wu *et al.*, 2003). The precise functions for all these genes are currently unknown. In addition, modest up-regulation of a MADS domain transcription factor, *GRCD3*, was observed. *GRCD3* belongs to the *SEPALLATA*-like gene family in gerbera and is grouped phylogenetically close to *AGL6* of *Arabidopsis* (Kotilainen *et al.*, 2000).

#### Transcripts up-regulated in petals

Microarray analysis comparing petal samples at developmental stage 4 (Fig. 1H) identified 35 transcripts that were up-regulated in transgenic petals (Table 2). From these, seven transcripts are shared with both

**Table 2.** Up-regulated transcripts that were shared between various tissues overexpressing GMYB10 and transcripts up-regulated only in ray flower petal tissues of transgenic line Tr14

EST code	Annotation
In petals, callus and stamens	
G0000900005E02	Novel unknown protein 4104
G0000300002G07	Flavanone-3-hydroxylase (F3H, FHT) (EC 1.14.11.9)
G0000100029D05	MYB-like transcription factor
G0000100035C05	Glutathione S-transferase (GST)
G0000100009F04	<i>Gerbera hybrida</i> , GMYB10
G0000800009A10	Serine carboxypeptidase
G0000200025E09	Cinnamate-4-hydroxylase (C4H)
In petals and in callus	
G0000900010H03	Putative nucleoside triphosphatase
In petals and in stamens	
G0000900005C08	Putative tonneau 2 ( <i>Oryza sativa</i> )
G0000500018A02	Phenylalanine ammonia lyase (PAL) (EC 4.3.1.5)
G0000300003F07	UDP-galactose:flavonol 3-O-galactosyltransferase
G0000500004H11	Serine carboxypeptidase, putative
G0000700002A05	Caffeic acid O-methyltransferase (EC 2.1.1.6) (COMT)
G0000500014A11	Lipoxygenase (fragment)
G0000800001B11	Novel unknown protein 3512
Only in petals	
PGANS1H4	<i>Gerbera hybrida</i> , anthocyanidin synthase (ANS)
PGF3H1F4	<i>Gerbera hybrida</i> , flavanone-3-hydroxylase (F3H)
G0000300001C02	Putative protein, antiporter/drug transporter
G0000700001E06	Dicarboxylate/tricarboxylate carrier
G0000300014D01	MYB-related transcription factor, phantastica
G0000300006F02	Hypothetical protein
G0000400004C06	<i>Gerbera hybrida</i> , Gty37 protein
G0000300004C10	Chalcone isomerase (CHI)
G0000400019A08	Novel unknown protein 2444
G0000200049C08	Serine protease, SBT1
G0000100015A10	Putative cytosolic factor protein, patellin 1
G0000800006E06	Similar to cleavage stimulation factor, 77 kDa
G0000800008H06	Novel unknown protein 3733
G0000500018D09	Unknown protein 0168
G0000100010H03	Hypothetical protein
G0000100017H12	Polygalacturonase inhibitor-like protein
G0000600010G06	Probable ubiquitin-fold modifier 1 precursor
G0000100036A06	Serine carboxypeptidase
G0000500002E10	Novel unknown protein 0262
G0000100029H08	Putative histone H2A protein

transgenic callus and stamen samples, seven with only stamens, and one with only the callus sample (Fig. 3; Table 2).

The cDNAs up-regulated only in transgenic petal tissues include known biosynthetic genes of the flavonoid pathway such as those encoding chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), and anthocyanidin synthase (ANS). Both F3H and ANS probes represent previously identified gene family members that were

isolated by PCR from petal tissues (P Elomaa, unpublished results). The F3H probe (PGF3H1F4; Table 2) shares a short region (189 bp) that is identical with the EST G0000300002G07 that was detected to be up-regulated in all tissues (calli, stamens, and petals). However, the PGF3H1F4 probe represents the 5' end of the gene while the EST probe corresponds to the 3' end. It is thus possible that it detects a different gene family member specifically up-regulated in petals. Three up-regulated cDNAs encode putative transport activity related to membrane trafficking. G0000300001C02 has low sequence similarity with functionally unknown *Arabidopsis* transporter proteins while G0000700001E06 shares high sequence similarity (E-value 5e-43) with *Nicotiana tabacum* dicarboxylate/tricarboxylate carrier proteins which represent mitochondrial carriers capable of transporting di- or tricarbonylates (e.g. malate, oxaloacetate, oxoglutarate, citrate, isocitrate, *cis/trans*-aconitate) to or from the mitochondria (Picault *et al.*, 2002). Their transport activities are required in several metabolic processes including primary amino acid synthesis, photorespiration, fatty acid metabolism, and isoprenoid biosynthesis (Picault *et al.*, 2002). G0000100015A10 encodes a homologue of PATELLIN1 or Sec14p-related proteins (putative cytosolic factor protein) which are associated with membrane trafficking during plant cytokinesis in cell-plate expansion or maturation (Peterman *et al.*, 2006). Moreover, up-regulation of the gerbera *GTY37* cDNA was detected. *GTY37* has been isolated earlier as a petal abundant transcript but whose function during petal organogenesis is currently not known (M Kotilainen, unpublished results), as well as another MYB-related transcription factor encoded by G0000300014D01. In BLASTx search G0000300014D01 shares similarity with PHANTASTICA-like MYB domain transcription factors from several plant species. A sequence search against EST databases revealed several highly similar (>85% identical) genes from other Asteraceous species (e.g. *Lactuca* sp. and *Cichorium intybus*).

Excluding the transgene itself, six genes that were up-regulated in petals were also up-regulated in callus and stamen tissues overexpressing *GMYB10* (Table 2). These include genes encoding cinnamate-4-hydroxylase (C4H), flavanone-3-hydroxylase (F3H), and glutathione S-transferase (GST) of the flavonoid pathway. The cDNA encoding a MYB factor (G0000100029D05) was also up-regulated in petals as well as a novel unknown gene 4104 originally isolated from the anthocyanin pigmented pappus bristle library of variety Terra Nero (Laitinen *et al.*, 2005). Furthermore, a cDNA encoding serine carboxypeptidase-like protein, caffeic acid O-methyltransferase (COMT), phenylalanine ammonia-lyase (PAL), and UDP-galactose:flavonol 3-O-galactosyltransferase was identified.

### Verification of the up-regulated target genes using real-time RT-PCR

The expression of 16 genes identified as up-regulated in microarrays were verified using real-time RT-PCR (Table 3). In accordance with the microarray results, up-regulation of the known biosynthetic genes, *PAL*, *C4H*, *CHS4*, *F3H*, *F3'H*, and *GST*, as well as a serine carboxypeptidase-like gene, could be confirmed in transgenic tissues. Expression of *CHI*, that was only up-regulated in petals in the microarray experiments, was also observed in transgenic callus and stamen tissues most probably due to the high sensitivity of real-time RT-PCR. Similar results were obtained for UDP-galactose:flavonol 3-*O*-galactosyltransferase that showed up-regulation in all transgenic tissues. However, neither real-time RT-PCR nor northern hybridization could verify up-regulation of the MADS domain factor, *GRCD3*. The three novel gerbera sequences (4104, 4191, and 1483) which showed very high up-regulation in microarrays, were also tested. Their expression patterns could not be confirmed (data not shown). These ESTs were fully sequenced and found to encode relatively short 3' sequences (data not shown). Instead, both MYB factor genes *G0000100029D05* and *G0000300014D01* were highly up-regulated in all transgenic tissue accumulating anthocyanins (Table 3).

Also included in the analysis were five previously isolated genes from gerbera encoding different members of the chalcone synthase gene family (*GCHS1* and *GCHS3*; Helariutta *et al.*, 1995), chalcone synthase-related gene 2-pyrone synthase (*G2PS1*; Eckermann *et al.*, 1998), and the late flavonoid biosynthetic genes dihydroflavonol-

4-reductase (*GDRF1*; Helariutta *et al.*, 1993) and anthocyanidin synthase (*GANS1*; P Elomaa, unpublished data). *GCHS1* and *GCHS3* encode typical CHS enzymes and their expression in gerbera petals is temporally correlated with late anthocyanin and early flavonol biosynthesis, respectively (Helariutta *et al.*, 1995). *2PS1* shares 78% nucleic acid sequence identity with *GCHS1* and *GCHS3* but is functionally involved in the biosynthesis of 2-pyrone compounds contributing to disease resistance in gerbera (Eckermann *et al.*, 1998). Interestingly, *GCHS3* was specifically up-regulated in transgenic stamen tissues while no clear changes in *GCHS1* or *G2PS1* expression could be detected in any tissue. Both late pathway genes, *GDRF1* and *GANS1* were highly up-regulated in transgenic callus and stamens as well as in petals (Table 3).

### Sequence properties and expression pattern of GMYB11 and GMYB12

The microarray analysis revealed two previously unidentified gerbera genes encoding MYB domain factors, named here as *GMYB11* (*G0000100029D05*) and *GMYB12* (*G0000300014D01*). *GMYB11* was highly up-regulated in all pigmented tissues overexpressing *GMYB10* while *GMYB12* was specifically up-regulated in transgenic petal tissues. Both cDNAs were isolated as full length. BLAST searches (tBLASTx) verified sequence similarity of *GMYB11* with putative repressor proteins such as *Vitis vinifera* C2 repressor motif protein (EU181425) and *Arabidopsis* MYB3 (NM\_102111) annotated as a repressor of phenylpropanoid biosynthesis gene expression as well as *Arabidopsis* MYB4 (NM\_120023)

**Table 3.** The expression of 17 up-regulated genes and five known flavonoid pathway genes were verified with real time RT-PCR

The fold-change in expression (up- or down-regulation) for each gene in callus, Tr1 and Tr14 was obtained by comparing to corresponding control tissues of variety Terra Regina (ne, no expression; nd, not done).

EST code	Annotation	Callus	Stamen (stage 8)		Petal (stage 4)		
			Tr1	Tr14	Tr1	Tr14	
G8-7E02	PAL	2.86	12.82	9.08	4.28	22.42	
G2-25E09	C4H	nd	5.46	8.98	1.27	6.01	
G1-4B03	CHS4	709.18	30.48	113.12	35.34	70.69	
G3-4C10	CHI	1.97	2.89	6.16	2.24	2.12	
G3-2G07	F3H	4.41	9.17	46.42	3.03	12.13	
G1-28H02	F3'H	253.65	nd	187.84	10.85	39.31	
G1-35C05	GST	150.12	29.65	38.12	5.72	33.75	
G1-9F04	GMYB10	274.37	12.99	112.20	5.50	163.14	
G1-29D05	GMYB11	564.17	11.88	38.50	11.79	64.30	
G3-14D01	GMYB12	58.21	1.23	3.54	2.21	1.94	
G3-3F07	UDPgalactose:flavonol...	8.11	9.25	16.34	1.69	7.00	
G2-14A07	GRCD3 (MADS)	ne	0.64	0.53	1.69	0.90	
G8-9A10	Serine carboxypeptidase	1.44	8.22	4.26	0.79	0.93	
Known flavonoid pathway genes:							
GCHS1		ne	0.99	1.49	1.25	2.11	
GCHS3		ne	7.13	70.36	0.72	3.24	
2PS		0.18	1.15	1.03	1.31	0.15	
GDRF1		883.24	nd	136.24	25.81	85.83	
GANS1		3623.9	13.93	70.68	16.19	54.95	

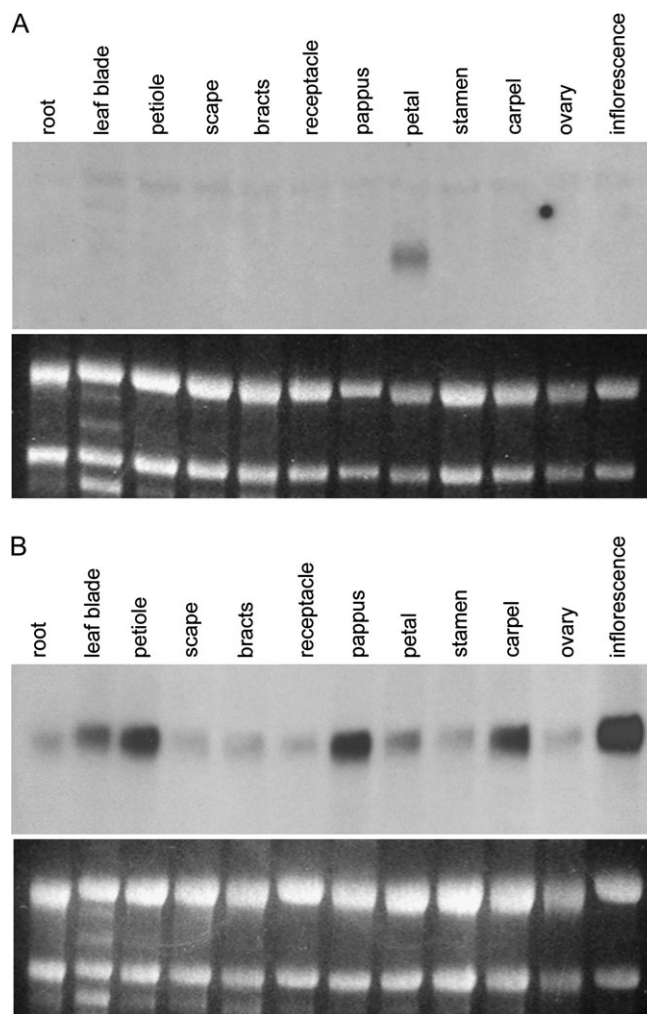


which acts as a repressor protein controlling the production of UV-protecting sunscreens by affecting cinnamate-4-hydroxylase expression (Jin *et al.*, 2000). GMYB12 shared the highest homology with *Morus alba* leaf dorsal-ventral development protein (MAPHAN1, EF408927) and functionally unknown *Malus domestica* MYB92 (DQ074474). Alignment of the amino acid sequences with previously identified anthocyanin regulators showed that GMYB11 did not contain the highly conserved KPRPR(S/T)F motif typical for anthocyanin regulators (Stracke *et al.*, 2001) (see Supplementary Fig. S2 at *JXB* online). GMYB11 still contained the amino acid residues identified to be important for interaction with bHLH regulatory proteins (Grotewold *et al.*, 2000; Zimmermann *et al.*, 2004). Furthermore, GMYB11 contained a conserved repressor motif, pdLNL(D/E)Lxi(G/S), in its C-terminal similar to that found in R2R3 MYB proteins of subgroup 4 such as AtMYB4 and AtMYB3 (Kranz *et al.*, 1998; Jin *et al.*, 2000). Neither of these domains could be identified in the GMYB12 sequence which was much longer and diverged even within the conserved MYB domain from the selected, functionally known anthocyanin regulators (data not shown).

Northern analysis of wild-type Terra Regina showed that *GMYB11* is petal specific and not detected in other organs (Fig. 4). Its expression starts at stage 5 during petal development correlating with the accumulation of anthocyanin pigments and continues until stage 8 when petals are fully expanded and opening of the inflorescence occurs (see Supplementary Fig. S3 at *JXB* online). This pattern was also confirmed by our previous microarray analyses covering the gerbera petal development from stages 2 to 9 (Laitinen *et al.*, 2007; data not shown). *GMYB12* was more ubiquitously expressed. The highest expression of *GMYB12* was detected in leaves and petioles as well as in all floral organs and especially in young developing inflorescences (Fig. 4). During petal development, its expression showed an opposite pattern to *GMYB11* as it was most highly expressed at the early stages before visible anthocyanin accumulation (see Supplementary Fig. S3 at *JXB* online).

#### Transient expression analysis of *GMYB11* and *GMYB12*

For functional analysis *GMYB11* and *GMYB12* cDNAs were bombarded under the CaMV 35S promoter into gerbera leaf tissues alone and together with the previously isolated bHLH factor, GMYC1 (Elomaa *et al.*, 1998). The gerbera *PGDFR2* promoter corresponding to a late biosynthetic gene (encoding dihydroflavonol-4-reductase) fused with the firefly luciferase gene (*LUC*), was used as a reporter construct. As observed earlier, the gerbera *GMYB10* in combination with GMYC1 showed activation of the target promoter (Elomaa *et al.*, 2003). However, neither *GMYB11* nor *GMYB12* alone or in



**Fig. 4.** Expression analysis of the two MYB domain factor encoding genes, *GMYB11* and *GMYB12* identified as up-regulated by *GMYB10* overexpression. *GMYB11* is specifically expressed in anthocyanin pigmented petal tissue (A) while *GMYB12* shows more ubiquitous expression pattern in various tissues (B).

combination with GMYC1 was able to activate the reporter construct (see Supplementary Table S3 at *JXB* online).

## Discussion

### Overexpression of *GMYB10* alters pigmentation pattern and colour intensity in transgenic gerbera

It has previously been shown that the gerbera R2R3-type MYB domain gene, *GMYB10*, is expressed in gerbera leaves, flower scapes, and at a very low level in petals (stages 3–7) of wild-type variety Terra Regina (Elomaa *et al.*, 2003). *In situ* analysis revealed that the transcript was localized in epidermal cells of adaxial sides of petals in variety Terra Regina in accordance with the anthocyanin accumulation pattern. Clear expression of *GMYB10* in green tissues that may accumulate anthocyanins (e.g.

under stress) suggested that activation of anthocyanin biosynthesis would require the presence of an (inducible) bHLH factor (Elomaa *et al.*, 2003). The transgenic lines analysed in this study showed that ectopic expression of 35S-*GMYP10* alone is able to induce extensive accumulation of cyanidin in vegetative organs suggesting that *GMYP10* itself might be induced. Further comparison of healthy green leaves from plants grown under standard greenhouse conditions to anthocyanin accumulating, visibly reddish leaves showed that, in fact, *GMYP10* expression correlated with the accumulation of cyanidin pigments (data not shown). It is concluded that in wild-type gerbera, vegetative *GMYP10* expression is most likely induced under stress although further studies are still needed to uncover the factor that elicits its expression. Activation tagging in *Arabidopsis* and tomato has also identified MYB-type regulatory genes, *PAP1* and *ANT1*, respectively, which both share similarity with *GMYP10* (43–45% amino acid sequence identity). Similarly to *GMYP10* in gerbera, constitutive overexpression of these genes in *Arabidopsis* and tomato leads to the formation of an intense purple coloration in vegetative organs (Borevitz *et al.*, 2000; Mathews *et al.*, 2003). All these genes, as well as *MdMYB10* that controls apple fruit pigmentation (Espley *et al.*, 2007) induced an intense purple coloration (cyanidin) in transgenic tobacco, indicating functional orthology.

In contrast to vegetative tissues, overexpression of *GMYP10* induced both cyanidin and pelargonidin accumulation in all floral tissues except in pappus bristles (modified sepals). This suggests that these floral tissues express an additional factor or factors that are lacking from vegetative organs and thus make pelargonidin formation possible. A most likely candidate for this factor would be a substrate specific dihydroflavonol-4-reductase that is expressed in floral but not in green tissues. This would imply that *GMYP10* induces a distinct, yet uncharacterized, cyanidin-specific *DFR* in gerbera. In fact, a discrepancy was found in our microarray analyses which failed to detect activation of the *GDFR1* expression in transgenic gerbera tissues. It is therefore suggested that the highly activated *DFR* expression detected using real-time RT-PCR may correspond to a different gene family member. Thereby, it is hypothesized that *GMYP10* encodes a regulator that specifically activates cyanidin production and that the enhanced pelargonidin production is an indirect consequence due to enhancement of the early pathway.

#### *GMYP10* affects the expression of several known genes involved in anthocyanin biosynthesis

Based on the microarray analysis, a relatively limited number of putative target genes was identified for *GMYP10*. As also observed with *ANT1* in tomato and *PAP1* in *Arabidopsis* these included several known, both

early and late biosynthetic genes of the flavonoid pathway (Borevitz *et al.*, 2000; Mathews *et al.*, 2003; Tohge *et al.*, 2005). The expression of the *F3'H* gene was up-regulated in all tissues analysed (callus, stamens, petals) suggesting that it is the key target gene for *GMYP10* required for opening of the cyanidin pathway in gerbera tissues. Interestingly, extremely strong up-regulation of an early gene of the anthocyanin pathway, corresponding to a previously uncharacterized member of the chalcone synthase gene family, *GCHS4*, was also observed. In wild-type variety Terra Regina *GCHS4* is most highly expressed in petals and carpels. It encodes a typical chalcone synthase enzyme that uses 4-coumaroyl-CoA as a substrate to produce naringenin chalcone (M Ainasoja *et al.*, unpublished results). Two chalcone synthase genes active during petal development, *GCHS1* and *GCHS3*, whose expression in gerbera petals is temporally correlated with late anthocyanin (pelargonidin) and early flavonol/flavone biosynthesis, respectively (Helariutta *et al.*, 1995) have previously been identified. The expression of *GCHS1* was not altered in transgenic tissues while *GCHS3* was up-regulated only in stamens. Strong activation of *GCHS4* in cyanidin-accumulating tissues suggests that, in addition to its putative role in pelargonidin biosynthesis, it might also function in a metabolon or multienzyme complex needed for cyanidin production (Winkel, 2004). Recent studies in *Arabidopsis* show that early gene expression is not reduced in TTG1(WD40)-dependent MYB mutants affecting anthocyanin pigmentation and suggest that up-regulation of early flavonoid genes by an excess of regulatory proteins (overexpression of *PAP1*) might be due to secondary effects by metabolite feedback resulting from strong up-regulation of late genes and increased flux through the pathway (Gonzalez *et al.*, 2008). In gerbera, the fact that other early genes except *GCHS4* (i.e. *PAL*, *C4H*, *CHI*, *F3H*) were relatively modestly up-regulated in contrast with very highly up-regulated late genes (*DFR*, *ANS*, *GST*) supports this view. However, whether *GCHS4* is a direct target for *GMYP10* or for the up-regulated new MYB domain factors (*GMYP11* or *GMYP12*) or affected by the increased metabolic flux remains to be studied.

In addition to the anthocyanin pathway genes, two up-regulated genes putatively functioning in the lignin pathway that branches off from the general phenylpropanoid pathway could be identified. Caffeoyl-CoA *O*-methyltransferase expression was induced in transgenic callus and stamens. *CCoAOMT* encodes an enzyme involved in monolignol biosynthesis by catalysing the methylation of caffeoyl-CoA ester. Moreover, in transgenic petals and stamens, the up-regulation of caffeic acid *O*-methyltransferase (*COMT*) was observed. MYB domain factors have also previously been shown to affect monolignol biosynthesis. *Arabidopsis* plants that overexpressed *PAP1*, showed increased amounts of guaiacyl and syringyl monomers that was reflected in increased lignin

content. However, this was connected to highly increased PAL activity while lignin *O*-methyltransferase (CCoAMT, COMT) activities barely differed (Borevitz *et al.*, 2000). In *Vitis vinifera*, the MYB domain factor, *VvMYB5a* was shown to suppress *CCoAMT* gene expression in transgenic tobacco plants leading to reduced lignification in anther walls (Deluc *et al.*, 2006).

#### *Serine carboxypeptidase, a potential new target for MYB-type anthocyanin regulators*

These data suggest a serine carboxypeptidase-like (*SCPL*) gene as a putative target gene for *GMYP10*, at least in stamen tissues. A serine carboxypeptidase gene was also up-regulated by the *PAP1* anthocyanin regulator in *Arabidopsis* (Tohge *et al.*, 2005). In general, serine carboxypeptidases are involved in protein degradation and processing in various organisms. The identified gerbera ESTs are most similar to *Medicago truncatula* genes and to *Arabidopsis SCPL6* and *SNG1*. The *sng1* mutant in *Arabidopsis* is defective in the synthesis of sinapoylmalate which is one of the major phenylpropanoid secondary metabolites in *Arabidopsis* and in some species in Brassicaceae (Lehfeldt *et al.*, 2000). The *SNG1* locus encodes sinapoylglucose:malate sinapoyltransferase (SMT) that is localized in the vacuole and catalyses a transacylation reaction needed to generate sinapoylmalate. Lehfeldt *et al.* (2000) discuss that the catalytic properties of serine carboxypeptidases suggest that they could also function in other secondary metabolic pathways, for example, in vacuolar targeting of anthocyanins. For example, acylation of cyanidin with sinapic acid is a prerequisite for vacuolar localization in *Daucus carota* (Hopp and Seitz, 1987). Recent studies in *Diospyros kaki* (persimmon) identified SCPL protein that might function in the biosynthesis of proanthocyanidins during fruit development (Ikegami *et al.*, 2007). A serine carboxypeptidase gene was also among the 116 genes that were up-regulated by sucrose treatment that induced anthocyanin biosynthesis in regenerating shoots of *Torenia fournieri* (Nagira *et al.*, 2006). Biochemical studies on these proteins, as well as suppression of gene expression in transgenic plants, would bring more light to their putative roles in the modification of anthocyanin molecules.

#### *Regulatory genes activated by GMYB10 overexpression*

Interestingly, the microarray analysis revealed two new MYB domain factors that were activated by *GMYP10*. However, their sequence properties do not support their direct involvement in the activation of anthocyanin pigmentation. Neither of them contain the KPRPR(S/T)F motif typical for anthocyanin MYBs (Stracke *et al.*, 2001). Nevertheless, the *GMYP11* sequence aligns with other flavonoid MYBs and our previous phylogenetic analysis (Elomaa *et al.*, 2003) also suggests that it might

be an orthologue of *AtTT2* that is involved in the regulation of proanthocyanidin accumulation (Nesi *et al.*, 2001). However, *AtTT2* functions as an bHLH-dependent activator of *DFR* and *BAN* expression while *GMYP11* shares the highest sequence similarity with putative phenylpropanoid biosynthesis repressors. A 64% amino acid sequence similarity with *Vitis vinifera* C2 repressor motif protein and 50% similarity with *Arabidopsis AtMYB4* (Jin *et al.*, 2000) was observed. The expression pattern of *GMYP11* was restricted to petals and correlated with the anthocyanin accumulation during late petal development. The *GMYP12* sequence was highly diverged compared with anthocyanin regulators. Furthermore, its ubiquitous expression patterns does not suggest a direct role in the regulation of pigmentation. Further studies are necessary to verify the putative regulatory network (e.g. protein–DNA, protein–protein interactions) and function for the newly identified regulators and *GMYP10*.

## Conclusion

Transformation of *GMYP10*, an anthocyanin regulator identified from an ornamental plant *Gerbera hybrida*, indicated that regulatory genes can be used to modify secondary metabolic pathways in a highly predicted manner. cDNA microarray analysis of transgenic tissues overexpressing *GMYP10* revealed completely new candidate targets, such as a serine carboxypeptidase-like gene, as well as two new MYB domain factors whose exact function in phenylpropanoid biosynthesis is not yet clear. In addition, several new gene family members of the biosynthetic genes of the flavonoid pathway, which are likely to encode enzymes functioning in same metabolon or enzyme complexes could be identified (Winkel, 2004).

## Supplementary data

Supplementary files for this manuscript can be accessed at *JXB* online (<http://jxb.oxfordjournals.org/>).

**Fig. S1.** RNA blot analysis of transgenic plants overexpressing *GMYP10*.

**Fig. S2.** Amino acid alignment for a selected set of functionally characterized MYB-type anthocyanin regulators.

**Fig. S3.** Expression of *GMYP11* and *GMYP12* during gerbera petal development.

**Table S1.** Total anthocyanin content (mg g<sup>-1</sup> FW) of control and transgenic tissues overexpressing *GMYP10*.

**Table S2.** Transcripts up-regulated in wild-type tissues in comparison to the corresponding transgenic tissues of Tr14 overexpressing *GMYP10*.

**Table S3.** Particle bombardment of gerbera MYB domain transcription factors.

**Table S4.** Primers used for real time RT-PCR analysis.

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