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The Transcription Factor AtMYB75/PAP1 Regulates the Expression of Flavonoid Biosynthesis Genes in Transgenic Hop *(Humulus lupulus* L.)*

Metabolic engineering of the flavonoid biosynthesis pathway may be used for modifying nutritional and pharmaceutical properties of food crops as well as for producing ornamentals with novel color patterns. In plants, MYB transcription factors play a crucial role in regulating the biosynthesis of flavonoids. The AtMYB75/PAP1 is a member of the R2R3 MYB gene family and stimulates the expression of genes involved in the biosynthesis of flavonoids. Previously, AtMYB75/PAP1 from *Arabidopsis thaliana* L. was introduced into *Humulus lupulus* L. cv. Tettnanger plants by Agrobacterium-mediated genetic transformation. In this study, the copy number of AtMYB75/PAP1 was estimated in seven transgenic hop plants employing quantitative real-time PCR. Using this system it was demonstrated that each transgenic plant harbors only one copy of AtMYB75/PAP1. Moreover, the expression of genes CHS_H1, CHI, and F3'H in AtMYB75/PAP1 transgenic and wildtype hop plants was analyzed by reverse transcriptase quantitative real-time PCR. The expression of the structural genes CHS_H1, CHI, and F3'H was elevated in transgenic hop plants compared to the wildtype plants. Chemometric methods were successfully used to discriminate between wildtype and transgenic plants expressing the transcription factor AtMYB75/PAP1. These results revealed that the transcription factor AtMYB75/PAP1 activated the expression of these three genes essential for the biosynthesis of flavonoids in transgenic hop plants. Therefore, metabolic engineering using transcription factors, such as the MYB genes, may open the possibility for improving the content of pharmaceutically important secondary metabolites in hop.

Descriptors: hop, flavonoids, MYB transcription factor, quantitative real-time PCR, gene expression

1 Introduction

Flavonoids are a large family of secondary metabolites which are accumulated in different types of tissues and organs of plants. Flavonoids can be important for the survival of plants with roles in the attraction of pollinators, seed dispersal, and as part of a defense response against insects, diseases, UV light, and physical stress. Furthermore, flavonoids are essential for the pigmentation of flowers, fruits, and seeds [1, 2, 3]. Besides their functions in plants, flavonoids are important in the medicinal and pharmaceutical field. Flavonoids have been found to have a preventive capacity in terms of human degenerative diseases associated with oxidative stress, coronary heart, and age related diseases [3, 4, 5]. Moreover, it has been demonstrated that flavonoids have also neuroprotective, anti-inflammatory, analgesic, bactericidal, fungicidal, and spasmolytic properties [3].

The female cones of hop are mainly used in the brewing industry to provide flavor and taste to the beer [6]. Moreover, several secondary metabolites are of special interest for the pharmaceutical and medicinal field, due to their anti-cancer, and anti-proliferative activity [7, 8, 9]. The α-, β-acids, essential oils, and prenylated chalcones, which are synthesized and accumulated in the lupulin glands of female cones, are responsible for these traits. Therefore, research has been carried out to understand the biosynthesis of important secondary metabolites in hop and the biosynthetic pathway of α-, β-acids bitter acids, and essential oils have been reported [10, 11]. In order to improve the content and quality of these compounds, it is important to identify the genes responsible for their biosynthesis. Several structural genes and transcription factors, including CHS_H1 (chalcone synthase_H1), VPS (valerophenone synthase), OMT1 (O-methyltransferase-1), HlPT-1 (prenyltransferase), MYB, bHLH, and WDR have been cloned in *H. lupulus* L. ([12, 13, 14, 15, 16].

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The biosynthesis of flavonoids in plants has been intensively studied [4]. Two groups of genes are required: structural genes, i.e. enzymes for biosynthetic steps, as well as regulatory genes like transcription factors [17]. R2R3 MYB transcription factors play a critical role in the regulation of the biosynthesis of phenylpropanoids and flavonoids [18]. The flavonoid production in plants could be enhanced through the genetic transformation with structural genes or by the introduction of homologous or heterologous regulatory elements [19]. The transcription factor AtMYB75/PAP1 (Production of Anthocyanin Pigment 1) of *Arabidopsis thaliana* L. is a conserved member of the R2R3 gene family and stimulates the expression of genes related to the biosynthesis of phenylpropanoids and flavonoids [12, 20, 21, 22,]. For the first time, metabolic engineering was employed to increase the flavonoid content in *H. lupulus* L. cv. Tettnanger [23]. In those AtMYB75/PAP1 transgenic hop plants higher levels of anthocyanins, rutin, isoquercitin, kaempferol-glucoside, kaempferol-glucoside-malonate, desmethylxanthohumol, xanthohumol, α-acids, and β-acids in cones were observed compared to wildtype plants [23].

Here we have investigated how the level of expression of the genes involved in the flavonoid biosynthesis was modified in transgenic hop plants by the presence of AtMYB75/PAP1. The expression of the genes involved in the flavonoid biosynthesis was analyzed in transgenic hop plants using quantitative real-time PCR. Moreover, a principal component analysis (PCA) was used to discriminate between transgenic hop plants and wildtype plants.

2 Materials and methods

2.1 Plant material

Young leaves and mature female hop (*H. lupulus* L cv. Tettnanger) cones were collected (sampling date: 3th September 2010) from three-year-old wildtype and AtMYB75/PAP1 transgenic (10, 11, 14, 15, 24, 29, and 56) plants grown in an outdoor containment facility. For DNA and RNA extraction, the material was immediately immersed in liquid nitrogen and stored at –80 °C.

2.2 Primer design and assessment of specificity

The primer sequences of AtMYB75/PAP1 and hop CHS_H1 (chalcone synthase_H1) were obtained from previous studies [12, 23]. Specific primers were designed for CHI (chalcone isomerase) and F3'H (flavonoid 3'-hydroxylase) using available hop DNA sequences (unpublished data). Primers used are listed in table 1. Primer specificity was confirmed by blasting each primer sequence against the nucleotide collection available at NCBI (http://www.ncbi.nlm.nih.gov/). Moreover, the amplifications of specific genes were confirmed by sequencing of the PCR (polymerase chain reaction) products (commercial service, GATC Biotec AG, Germany).

RT-PCR (reverse transcriptase-PCR) was performed with RNA from leaves and cones of AtMYB75/PAP1 transgenic and wildtype plants and the newly designed primers using the procedure described by Gatica-Arias et al. [23]. Briefly, the reactions were carried out in 25 µl containing 1X OneStep RT-PCR buffer, 10 mM of each dNTPs, 0.6 µM of each primer, 2 µl of OneStep RT-PCR enzyme mix, 3 µl of total RNA (100 ngµl-1) and 12 µl of RNase free water (Qiagen, Germany). In order to test if RNA was existent, the 18S gene from each sample was amplified. An additional control was incorporated to detect DNA contaminations in RNA preparations. For this purpose each reaction mixture was divided into two aliquots: i) in one sample the reaction of the reverse transcriptase was carried out normally at 50 °C for 30 min and ii) in the other sample the enzyme was inactivated for 15 min at 94 °C. The successive PCR of the sample and the respective control were performed over 30 cycles under the following conditions: 95 °C for 15 min, 95 °C for 1 min, 53 °C for 30 s, 72 °C for 1.5 min. Cycling was followed by a final elongation step at 72 °C for 10 min. After staining with ethidium bromide, the PCR products were evaluated on 1.5 % (w/v) agarose gel, visualized under UV, and documented with a digital camera.

2.3 Estimation of AtMYB75/PAP1 copy number

Genomic DNA was extracted from leaves of transgenic and wildtype plants following the procedure described by Gatica-Arias et al. [23]. Total DNA was quantified using a spectrophotometer (NanoPhotometer™, Germany) and adjusted to a concentration of 20 ngµl^{-1}. The primer pair PAPshort_S-PAPshort_AS and GPPS.LSU_S-GPPS.LSU_AS were employed for the quantitative real-time PCR (qPCR) analysis (Table 1). The GPPS.LSU (geranyl geranyl diphosphate synthase large subunit) gene was used as an endogenous reference for a gene present in one copy in the genome [24]. To obtain a standard curve for AtMYB75/PAP1 and GPPS.LSU, genomic DNA was diluted to final concentration of 250, 50, 10, and 2 ngµl^{-1}. The reactions were carried out in 20 µl containing 1X SensiFAST SYBR Hi-ROX buffer, 0.5 µM of each primer, 5 µl of total DNA (20 ngµl^{-1}), and 6 µl of RNase free water (Bioline, Germany). qPCR analysis was performed in the StepOne™ System (Applied Biosystems, USA) following the requirements described by *Udvardi* et al. [25] and *Bustin* et al. [26]. Cycling conditions were: 95 °C for 3 min followed by 40 cycles at 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 5 s. Melting curve analysis and agarose gel electrophoresis were used to verify single PCR product formation. Two sets of DNA, each one corresponding to a biological replication were used. Every individual sample was measured as duplicates in two independent experiments. AtMYB75/PAP1 copy number was calculated to be $2^{\Delta Ct}$ (ΔC_t: $C_{t\ GPPS.LSU} - C_{t\ Target}$) [27].

2.4 Expression analysis of flavonoid biosynthesis genes

Total RNA was isolated from female cones of AtMYB75/PAP1 transgenic and wildtype plants using the RNeasy plant mini kit (Qiagen, Germany) following the procedure described by *McKenzie* et al [28]. Plant material was ground in a mortar to a fine powder using liquid nitrogen. The powder was transferred immediately to a tube containing 600 µl lysis buffer [4 M guanidinium thiocyanate, 0.2 M sodium acetate, 25 mM EDTA, 2.5 % (w/v) PVP-40, and 1 % (v/v) β-mercaptoethanol] and vortexed. Then, 60 µl of 20 % (w/v) sarkosyl were added to the mixture and it was incubated at 70 °C for 10 min with vigorous shaking. The lysate was pipetted directly onto a QIAshredder Spin Column (Qiagen, Germany) placed in a 2 ml tube and centrifuged for 2 min at maximum speed. All following steps including precipitation with ethanol, matrix binding, washing and drying were carried out according to the Qiagen user's manual.

Table 1 Primer sequences used in this study and predicted length of the amplification products

Gen	Primer	Sequence (5' – 3')	PCR size (bp)	Annealing temperature (°C)	Reference
AtMYB75/PAP1	PAPshort_S PAPshort_AS	tggcaccaagttcctgta aaagaccacctattccct	163	58	23
CHS_H1	CHS_H1short_S CHS_H1short_AS	atcactgccgtcactttc aaataagcccaggaacatc	250	55	12
CHI	CHIshort_S CHIshort_AS	caactgccctcaactcaa tttcttcctcaagccaac	127	56	this study
F3'H	F3'Hshort_S F3'Hshort_AS	tcaggtccacgatgccaatt gccggagaaaagatgaacagaa	147	60	this study
GAPDH	GAPDH_S GAPDH_AS	accggagccgactttgttgttgaa tcgtactctggcttgtattccttc	165	60	13
GPPS.LSU	GPPS.LSU_S GPPS.LSU_AS	cattccaaaccccaaaacaaa gactgcggaaatggatgaaaa	59	60	24
18S	18S_S 18S_AS	aggtagtgacaataaataacaa tttcgcagttgttcgtctttc	481	53	this study

After RNA extraction a digestion with DNase I (MBI Fermentas, St. Leon-Rot) was performed, and the RNA was cleaned up using the clean-up protocol from the RNeasy plant mini kit. The total RNA was quantified at wavelengths of 260 and 280 nm using a spectrophotometer (NanoPhotometer™, Germany) and the RNA integrity was verified by analyzing samples on a 1.2% (w/v) denaturing agarose gel.

For cDNA synthesis, 1 μg of total RNA was reverse transcribed in 20 μl for 30 min at 42 °C using 1μl Quantiscript reverse transcriptase, 1 μl RT primer mix, and 4 μl Quantiscript RT Buffer. Then, the reverse transcriptase was inactivated at 95 °C for 3 min (Qiagen, Germany).

RT-qPCR (reverse transcriptase-qPCR) was used to determine the level of gene expression of CHS_H1, CHI and F3'H in cones from AtMYB75/PAP1 transgenic and wildtype plants. Each PCR reaction was carried out in 20 μl containing 1X SensiMix™ SYBR Master Mix, 1 μM of each primer, 2.5 μl cDNA (40 ngμl-1) and 3.5 μl RNase free water (Bioline, Germany). RT-qPCR analysis was performed in the StepOne™ System (Applied Biosystems, USA) following the requirements described by *Udvardi* et al. [25] and *Bustin* et al. [26]. Cycling conditions were: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 45 s and 72 °C for 45 s. Melting curve analysis and agarose gel electrophoresis were used to verify single PCR product formation. Two experiments were carried out with one set of RNA. Each sample was analyzed twice in each experiment. Normalization was performed against GAPDH (glyceraldehyde-3-phosphate dehydrogenase), which has been reported as a suitable reference gene in hop [13]. The normalized expression of target genes was determined as $2^{-\Delta\Delta Ct}$ [29].

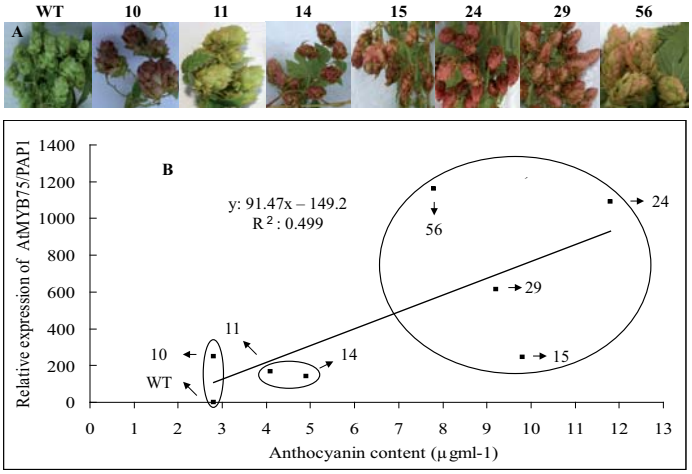

Fig. 1 (A) Phenotype of the wild type and transgenic hop female cones expressing the transcription factor AtMYB75/PAP1. (B) Correlation of the AtMYB75/PAP1 relative expression and the anthocyanin content (μg ml-1) in wildtype and transgenic hop plants. Data correspond to the means from two replicates in the case of AtMYB75/PAP1 and 3 measurements in the case of anthocyanin content. Data obtained from *Gatica-Arias* et al. [13]

2.5 Chemometrics analysis

A principal component analysis (PCA) was performed as an unsupervised statistical method to determine the differences caused in the metabolite profiles caused by the transcription factor PAP1/AtMYB75 in transgenic plants. The profile of secondary metabolites (rutin, isoquercitin, kaempferol-7-O-glucoside, kaempferol-7-O-glucoside malonate, desmethylxanthohumol, xanthohumol, humulone, cohumulone and adhumulone, and lupulone, colupulone, and adlupulone) in PAP1/AtMYB75 transgenic and wildtype hop female cones was determined using HPLC (high performance liquid chromatography) coupled to PDA (photodiode array detection) and MSn (tandem mass spectrometry) as described by *Gatica-Arias* et al. [23]. Relative quantification of hops metabolites profiles after LC/MS (liquid chromatography-mass spectrometry) was performed using XCMS data analysis software (http://137.131.20.83/download/). Native LC/MS files from Xcalibur 1.4 (Thermo Fisher Scientific, Inc., USA) were first converted into netCDF files and arranged in one folder that was set as the file source. Peaks were subsequently extracted using XCMS under R 2.9.2 environment with signal-to-noise ratio set at 4. After peak extraction and grouping, nonlinear retention time correction of peaks was accomplished in two iterative cycles with descending bandwidth. This was accomplished manually by decreasing the bandwidth parameter (from 30

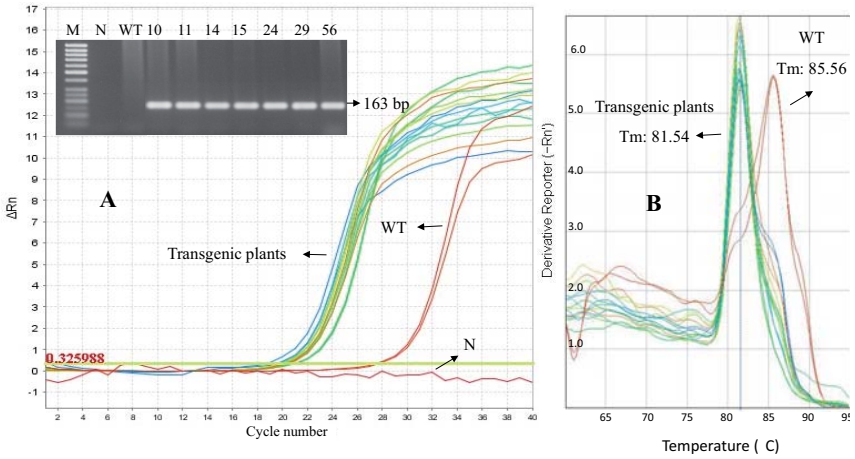

Fig. 2 (A) Quantitative real-time PCR amplification plot of AtMYB75/PAP1 gene in transgenic and wildtype plants and resolution of the same PCR products in an agarose gel 1.5%. CT threshold cycle is indicated. N: negative control (reaction mix without template); WT: wildtype plants; M: molecular maker (50 bp DNA Ladder)
(B) Melting curve corresponding to the amplification of the AtMYB75/PAP1 gene in transgenic and wildtype plants

to 10 s). The resulting peak list was further processed using the Microsoft Excel software (Microsoft, USA) where the ion features were normalized to the total integrated area (1,000) per sample and imported into the R 2.9.2 software package for PCA. Absolute peak area values were auto scaled (the mean area value of each feature throughout all samples was subtracted from each individual feature area and the result divided by the standard deviation) prior to PCA. This provides similar weights for all the variables. PCA was then performed on the MS-scaled data to visualize general clustering, trends, and outliers among all samples on the scores plot.

3 Results and discussion

3.1 Phenotypic characterization of AtMYB75/PAP1 transgenic plants

As described in our previous study [23], the transgenic hop plants expressing the heterologous transcription factor AtMYB75/PAP1 were characterized by the reddish to pink pigmentation of the female cones (Fig. 1A). The total amount of anthocyanins accumulated by the transgenic plants varied from 2.8 to 11.8 $\mu g ml^{-1}$. The transgenic plants were classified in three groups according to the levels of anthocyanins accumulated in the female cones. Low levels of anthocyanins were observed in the transgenic plant no.10, which was comparable to the wildtype plants. Transgenic plants no. 11 and no. 14 showed intermediate levels of anthocyanins, while high levels of anthocyanins were observed in the transgenic plants no. 15, no. 24, no. 29, and no. 56 (Fig. 1B) [23]. The accumulation of anthocyanins in transgenic hop plants was correlated to the expression level of AtMYB75/PAP1. Those transgenic plants with high levels of anthocyanins showed also elevated expression of AtMYB75/PAP1 (Fig. 1) [23]. The correlation between anthocyanin content and relative expression of AtMYB75/PAP1 was calculated as r^2: 0.499 (Fig. 1).

The heterologous AtMYB75/PAP1 transcription factor has been genetically transformed into *H. lupulus* L., *A. thaliana* L., *Nicotiana benthamiania* L., *N. tabacum* L., *Petunia hybrida*, *Solanum lycopersicum* L. and *Brassica napus* L. [12, 20, 22, 23, 30, 31, 32, 33]. It has been demonstrated that when the AtMYB75/PAP1 transcription factor was expressed in *A. thaliana* L., *N. tabacum* L., and *S. lycopersicum* L. the color of plant organs changed [20, 30, 31, 32].

3.2 Estimation of AtMYB75/PAP1 copy number

The estimation of the transgene copy number is an indispensable step after obtaining transgenic plants. The number of transgene copies has a great influence on the level of expression, as well as the stability of the exogenous gene in transgenic plants [34]. Conventionally, Southern blot analysis has been used to estimate the copy number. Nevertheless, it is a laborious and time-consuming method and large amounts of DNA are required. Recently, qPCR has become an alternative tool to determine the gene copy number in transgenic *Manihot esculenta* Mill., *Citrus sp.* L., *Gossypium hirsutum* L., *Zea mays* L., *Oryza sativa* L. and *Saccharum officinarum* L. [34, 35, 36, 37, 38, 39].

However, before using this method to determine the gene copy number in transgenic hop plants, it is necessary to demonstrate that the amplification efficiencies are approximately equal for the transgene and the reference gene [34, 38]. For this purpose, GPPS.LSU and AtMYB75/PAP1 standard curves were generated using 250, 50, 10, and 2 ng μl^{-1} of genomic DNA. A slope of –3.504 for GPPS.LSU showed that the PCR efficiency was 92.94%. PCR efficiency and slope were 98.92% and –3.348 for AtMYB75/PAP1. The correlation coefficients for GPPS.LSU and AtMYB75/PAP1 were 0.988 and 0.989, respectively.

The copy number of the transgene AtMYB75/PAP1 was determined relative to the endogenous one-copy-gene GPPS.LSU following the method described by *Gaines* et al. [27]. In this approach, when normalized to an endogenous one copy-gene, a one copy insert should have a ΔCt value of zero; a two copy insert should have a ΔCt value of one, etc. Therefore, a reference gene with low copy number and high conservation should be chosen [35]. Seven transgenic hop plants (10, 11, 14, 15, 24, 29, and 56) were tested and the results indicated that each transgenic plant carried only one copy of the transcription factor AtMYB75/PAP1 (Table 2). Variation in the copy number of the samples across different runs of PCR was minimal, except for the transgenic plant no.56. Nevertheless, in all cases the coefficient of variation [(standard deviation/ average C_T)*100] values were less than 5% (data not shown).

An example of the AtMYB75/PAP1 amplification plot is shown in the figure 2. Though there was a low level of amplification in the wildtype control, the resolution of the quantitative real-time PCR products indicated that the primer pair PAPshort_S-PAPshort_AS do not amplify any endogenous gene in hop, since no bands were observed in the wildtype plants (Fig. 2). The analysis of the dissociation curve showed the ratio between specific and non-specific products. Whereas the specific AtMYB75/PAP1 product in transgenic plants showed a melting temperature of 81.54 °C,

Table 2 Estimated copy number of AtMYB75/PAP1 in transgenic and wild type hop plants

	$2^{\Delta CT}$		
Sample	First biological replication[a]	Second biological replication[a]	Estimated copy number
WT	0.03±0.03	0.01±0.00	0
10	0.92±0.21	1.11±0.06	1
11	1.13±0.10	1.03±0.09	1
14	1.07±0.05	1.31±0.14	1
15	1.02±0.36	0.78±0.11	1
24	1.40±0.83	1.11±0.16	1
29	1.00±0.10	1.14±0.04	1
56	0.75±0.26	1.53±0.17	1

[a] Values are the mean (±SD) of two independent experiments in which each sample was measured in duplicate WT: wildtype

the non-specific product in the wildtype control exhibited a melting temperature of 85.56 °C (Fig. 2). One of the most commonly used qPCR chemistries is SYBR Green I Dye, which is an intercalating fluorescent dye and binds to any double-stranded DNA molecule, whether it is the specific or the non-specific product. The latter melts at temperatures above or below that of the desired product [40, 41]. However, it has been demonstrated that SYBR Green I Dye binds preferentially to specific DNA sequences [40, 41], in our case the AtMYB75/PAP1 product in transgenic plants. Moreover, the binding performance of SYBR Green I Dye could be altered by the additional components, which increase shelf life or enhance PCR, of commercial SYBR Green kits [41].

3.3 Assessment of primer specificity

RT-PCR analysis was performed with RNA from AtMYB75/PAP1 transgenic and wildtype plants. Primer pairs for CHS_H1 (250 bp), CHI (127 bp), and F3'H (147 bp) amplified a single PCR product with the expected size and no additional background bands were observed, indicating that the primers were specific to that one gene only (Fig. 3). As positive control a DNA sample from a transgenic plant was used (Fig. 3). In all samples a 481 bp PCR fragment was amplified using the 18S primer, indicating that RNA was existent (data not shown). In the controls where the reverse transcriptase was inactivated no amplicons were detected (data not shown).

In addition, single product formation for each primer pair was confirmed by the presence of a single peak in the melting curve obtained after 40 cycles of amplification (Fig. 3). The sequenced PCR fragments of CHS_H1, CHI, and F3'H showed similarity with the naringenin-chalcone synthase of *H. lupulus* L. (GenBank: AM263201.1), chalcone isomerase of *Cannabis sativa* L. (GenBank: JN679226), and flavonoid 3'-hydroxylase of *Fragaria* x *ananassa* (GenBank: AB665441), respectively.

3.4 Effect of gene modification on expression analysis of flavonoid biosynthesis genes

The effect of the heterologous transcription factor AtMYB75/PAP1 on the expression level of CHS_H1, CHI and F3'H in transgenic and wildtype hop plants were compared using quantitative real-time PCR. AtMYB75/PAP1, a member of the R2R3 MYB gene family, stimulated the expression of genes involved in the biosynthesis of flavonoids of *A. thaliana* L., *S. lycopersicum* L., and *B. napus* L. [21, 32, 33]. In the present study, the expression level of CHS_H1 was up-regulated in hop transgenic plants compared to wildtype plants. The CHS_H1 expression levels were increased in transgenic plant no. 10 (1.2 fold), no. 11 (2.4 fold), no. 14 (1.9 fold), no. 15 (1.8 fold), no. 24 (1.5 fold), no. 29 (1.4 fold), and no. 56 (2.3 fold) (Fig. 4A). The biosynthesis of prenylated chalcones in hop cones could be mediated by an enzyme with chalcone synthase activity, which catalyzes the condensation of three molecules of malonyl-CoA and one molecule p-coumaroyl-CoA [12]. It has been reported that CHS gene expression could be induced by transcription factors, such as AtMYB75/PAP1, PFG1/MYB12, PFG2/MYB11, and PFG3/MYB111 [20, 42, 43]. Moreover, in tobacco and petunia used as heterologous expression system, AtMYB75/PAP1 was capable of activating the CHS_H1 gene, suggesting that this transcription

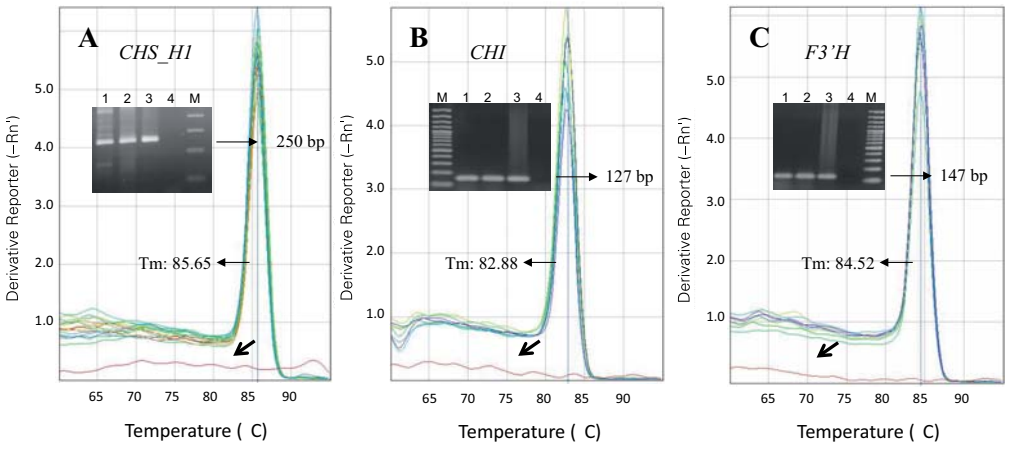

Fig. 3 Specificity of primer pairs for quantitative real-time PCR amplification: 1.5% agarose gel showing a single RT-PCR product of the expected size and melt curve showing a single peak for (A) CHS_H1, (B) CHI, and (C) F3'H. The non template control is indicated by arrows. Lane 1: RNA from leaves; 2: RNA from cones; 3: DNA from leaves; 4: non template control (PCR reaction mix without template); M: molecular marker (1 Kb DNA Ladder)

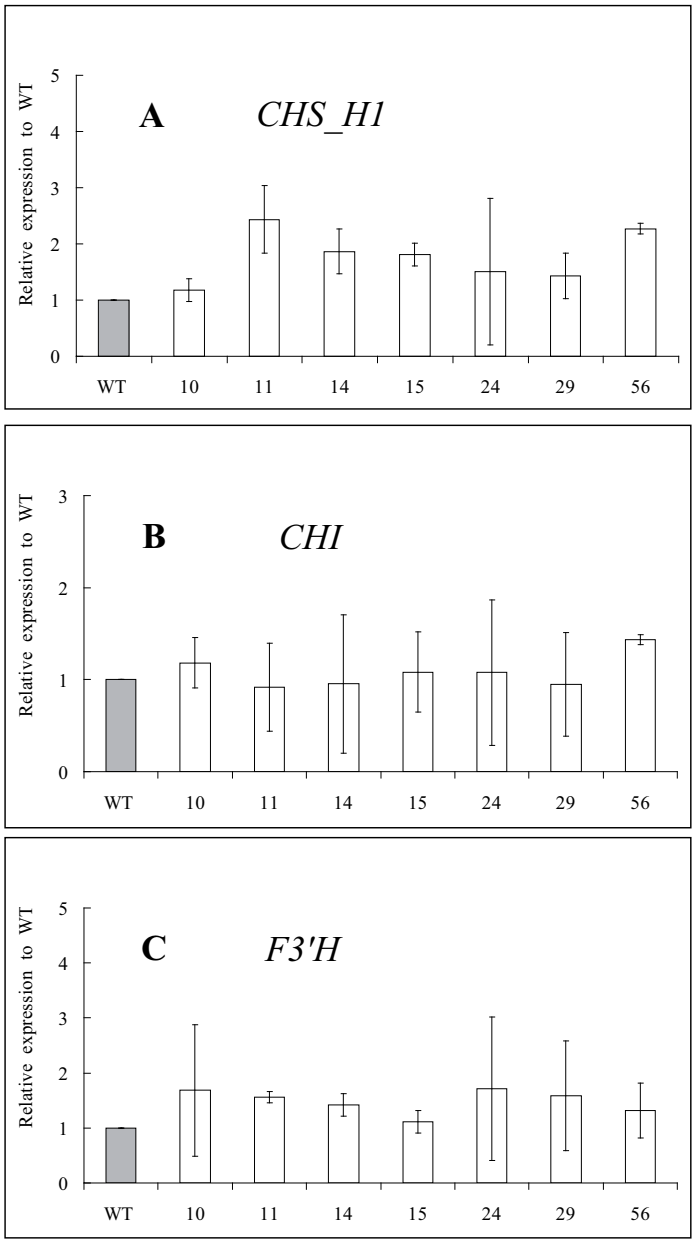

Fig. 4 Normalized mRNA gene expression of (A) CHS_H1; (B) CHI; and (C) F3'H in transgenic (10; 11; 14; 15; 24; 29; and 56) and wildtype hop female cones determined by RT-qPCR. The relative expression is expressed as the fold increases relative to wildtype WT: wildtype

factor is suitable for modifying the hop metabolome via activation of CHS_H1 gene [12].

CHI showed different expression levels among hop transgenic plants. The expression levels of CHI were slightly increased in the transgenic plant no. 10 (1.2 fold), no. 15 (1.1 fold), no. 24 (1.1 fold), and no. 56 (1.4 fold) in comparison with wildtype plants (Fig. 4B). Whereas the expression levels of CHI in transgenic plant no. 11 (0.9 fold), no. 14 (1.0 fold) and no. 29 (0.9 fold) were comparable to the wildtype. The expression of the *Delila* and *Rosea1* genes encoding transcription factors from snapdragon (*Antirrhinum majus* L.) resulted in a transient increase of CHI activity in transgenic tomato fruits [44]. In transgenic soybean (*Glycine max* L. Merr) seeds, which express the transcription factors C1 and R from maize, the expression of CHI was increased [45]. The expression of the maize LC and C1 transcription factors in the flesh and peel of transgenic tomatoes induced the flavonoid gene CHS; but not the genes CHI and F3'H [46].

The expression levels of F3'H were slightly higher in hop transgenic plants compared to wildtype plants. The expression of F3'H was 1.7, 1.6, 1.4, 1.1, 1.7, 1.6, and 1.3 times higher in the transgenic plant no. 10, 11, 14, 15, 24, 29, and 56 respectively (Fig. 4C). In *A. thaliana* L., the transcription factor AtMYB75/PAP1 was capable of regulating late anthocyanins biosynthetic genes, from F3'H onwards [47]. In transgenic *B. napus* L. expressing the transcription factor AtMYB75/PAP1, the gene F3'H was induced and the expression was increased (~50 fold) compared with wildtype plant [33].

3.5 Effect of gene modification on metabolic profiles

In this study, PCA was able to discriminate among hop transgenic plants and wildtype plants. PCA is a clustering method requiring no knowledge of the data and identifies patterns and allows highlighting similarities and differences in data [48, 49]. PCA has been used for the differentiation and classification of plants products according to geographical origin or for the chemotaxonomic approach to botanical classification, as well as for the determination of the substantial equivalence of transgenic plants [50, 51, 52, 53, 54, 55, 56].

The application of the PCA resulted in the PC plot shown in figure 5A, where the first principal component (PC1) describes 91% and the second one (PC2) 4% of the total variance. The transgenic plants were clearly separated from the wildtype plants. The PCA score plot showed that samples for wildtype and transgenic plants no. 11, and no. 29 were located in the positive region of PC1, while other samples (10, 14, 15, 24, and 56) were positioned in the negative region of PC1. A loading plot was constructed to determine the metabolites that were responsible for the separation and it was determined that α-acids (humulone/adhumulone) and β-acids (lupulone/adlupulone) contributed to the separation of the transgenic plants (Fig 5B).

In a second PCA analysis, in order to evaluate variation in other metabolites, α-acids and β-acids analogues were excluded from the data set (Fig 6A). The first two main PCs captured 77% of the variance. PC1 explained most of the variance observed (56%) and was related to quercetin and kaempferol glycosides, contributing for samples segregation along PC1 (Fig 6B). The plotting of samples was slightly different from that obtained when all analytical data were considered. The PCA score plot showed that samples for wildtype plants were situated on the top of the negative region of PC1, whereas samples for transgenic plants no. 11, and no. 29 were located on the top of the positive region of PC1 (Fig 6A), similar to the results derived from all metabolites shown in Fig 5A. The other samples for transgenic plants no.10, no.14, no.15, and no.56 were located in one group separated along PC2 (negative PC2 values). Examination of the loadings plot suggested that the variables referred to rutin contributed the most to the discrimination of samples (Fig 6B).

4 Conclusions

The flavonoid biosynthesis pathway appears to be conserved in plants. Efforts have been made to manipulate the structural

genes in order to increase or decrease the production of a desired compound [4]. Flavonoid biosynthesis is largely regulated at the transcriptional level and it is controlled at multiple levels. Therefore, the manipulation of single genes is of limited value and attention has been focused towards the simultaneous modification of several steps in a given pathway [19]. The present study demonstrates that the heterologous transcription factor AtMYB75/PAP1 influenced the expression of CHS_H1, CHI, and F3'H in transgenic hop plants. These results indicated that AtMYB75/PAP1 is a positive regulator of the biosynthesis of flavonoids in hop. Therefore, metabolic engineering using transcription factors, such as the MYB genes, may open the possibility for altering the content of important secondary metabolites in hop.

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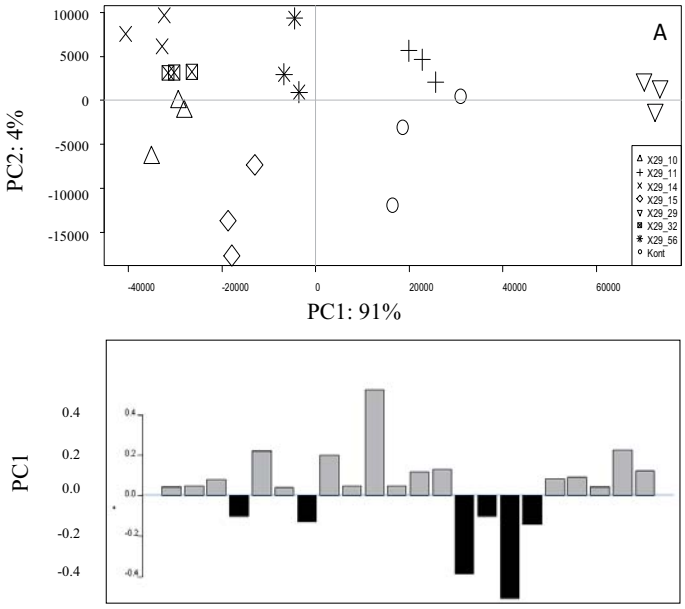

Fig. 5 **PCA model of metabolite profile in wildtype and transgenic hop female cones expressing the transcription factor AtMYB75/PAP1**
(A) PCA characterization (PC1 vs PC2) using the biochemical profile as the analytical data
(B) Loading plot for PC1 contributing mass peaks. 1: cohumulone, 2: humulone/adhumulone, 3: colupulone, 4: lupulone/adlupulone. Group discrimination in samples is related to qualitative and quantitative differences in bitter acids pattern. WT (○); 11 (+); 10 (Δ); 56 (á); 14 (x); 15 (◊); 29 (Ñ); 24 (T)

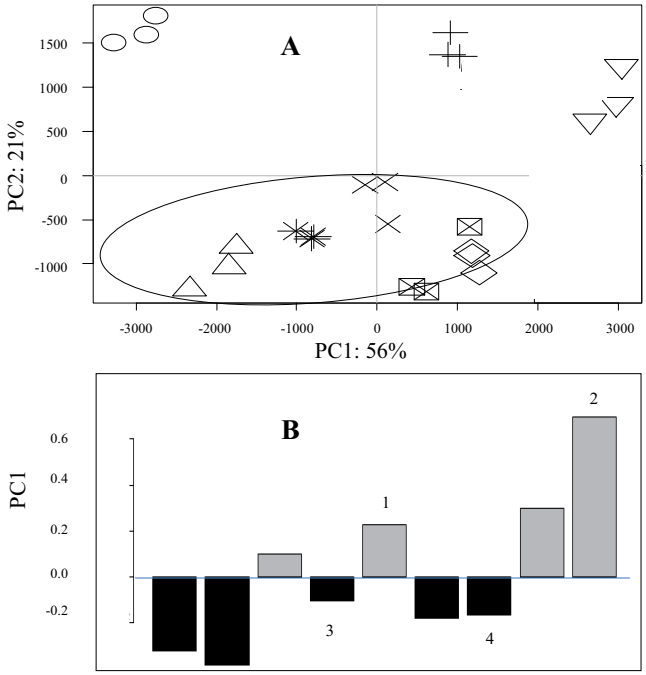

Fig. 6 **PCA model of metabolite profile in wildtype and transgenic hop female cones expressing the transcription factor AtMYB75/PAP1**
(A) PCA characterization (PC1 vs PC2) using only the phenolics profile as the analytical data
(B) Loading plot for PC1 contributing mass peaks. 1: astragalin, 2: rutin, 3: isoquercetin, 4: isoquercetin malonate. Group discrimination in samples is related to qualitative and quantitative differences in flavonoids pattern. Ellipses do not denote statistical significance. WT (○); 11 (+); 10 (Δ); 56 (á); 14 (x); 15 (◊); 29 (Ñ); 24 (T)

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