# University of Hohenheim

## Institute of Plant Breeding, Seed Science and Population Genetics

### Plant Breeding and Biotechnology

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# Metabolic engineering of flavonoid biosynthesis in hop (*Humulus lupulus* L.) for enhancing the production of pharmaceutically active secondary metabolites

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To my loving parents, Javier and Patricia, who supported me throughout my life

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# List of abbreviations

| | |
|---|---|
| 4CL | 4-coumarate-CoA-ligase |
| ANS | Anthocyanidin synthase |
| C4H | Cinnamate 4-hydroxylase |
| CaMV | Cauliflower Mosaic Virus |
| CHI | Chalcone isomerase |
| CHR | Chalcone reductase |
| CHS | Chalcone synthase |
| CHS_H1 | Chalcone synthase_H1 |
| CIM | Callus induction medium |
| DFR | Dihydroflavonol reductase |
| DMAPP | Dimethylallyldiphosphate |
| ESI | Electrospray ionisation |
| F3´5´H | Flavonoid 3´, 5´-hydroxylase |
| F3´H | Flavonoid 3´-hydroxylase |
| F3H | Flavanone-3-hydroxylase |
| FLS | Flavonol synthase |
| FNS | Flavone synthase |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GFP | Green fluorescent protein |
| GONC | Green organogenic nodule clusters |
| GPPS.LSU | Geranylgeranyldiphosphate synthase large subunit |
| HPLC | High performance liquid chromatography |
| IAA | Indole-3-acetic acid |
| LC-ESI | Liquid chromatography- electrospray ionisation |
| LC-MS | Liquid chromatography-mass spectrometry |
| LDOX | Leucoanthocyanidin dioxygenase |
| MS | Murashige and Skoog medium |
| $MS^n$ | Tandem mass spectrometry |
| t | Metric ton |
| NCBI | National Center for Biotechnology Information |
| nptII | Neomycin phosphotransferase II gene |
| OMT1 | O- methyltransferase 1 |
| PCA | Principal component analysis |
| PAL | Phenylalanine ammonia-lease |

| | |
|---|---|
| PAP1 | Production of anthocyanin pigment 1 |
| PDA | Photodiode array detection |
| qPCR | Quantitative real-time PCR |
| RITA® | Recipient for automated temporary immersion system |
| RNAi | RNA interference |
| RT-PCR | Reverse transcriptase-PCR |
| RT-qPCR | Reverse transcriptase-qPCR |
| STS | Stilbene synthase |
| TDZ | 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea |
| UF3GT | UDP-glucose flavonoid 3-glucosyltransferase |
| VPS | Valerophenone synthase |
| WT | Wildtype |

# Summary

For a long time, hop (*Humulus lupulus* L.) has been used in the brewing industry as flavoring and preserving agent. Moreover, the hop plant has been used for medicinal purposes. Recently, xanthohumol and desmethylxanthohumol have received special attention due to their potential cancer chemopreventive properties.

Hop breeding programs have been mainly focused on the development of new cultivars with a high content of *α*- and *β*-acids in order to satisfy the demand of the brewing industry. However, due to the medical and pharmaceutical importance of hops, new breeding efforts have been done to create new cultivars with a higher content of xanthohumol and desmethylxanthohumol. In order, to complement these efforts, metabolic engineering of flavonoid biosynthesis offers tremendous potential to modify the production of these compounds.

The flavonoid biosynthetic pathway has been intensively studied in plants. Often single target genes are regulated by several transcription factors. The R2R3 MYB transcription factor family plays an important role in the regulation of the biosynthesis of phenylpropanoids and flavonoids. Ectopic expression of these transcription factors in transgenic plants stimulated the production and enhanced the quantity of flavonoids.

The main objective of the research presented here was to modulate the production of pharmaceutical metabolites in hop through metabolic engineering of the flavonoid biosynthesis pathway.

Towards this goal, in a first approach, genetic engineering of hop cv. Tettnanger with the heterologous transcription factor PAP1/AtMYB75 from *Arabidopsis thaliana* L. was successfully accomplished. It was shown that *PAP1/AtMYB75* was stably incorporated and expressed in the hop genome. The transgenic events showed reddish to pink female flowers and cones. Moreover, compared to the wildtype plants, the expression of the structural genes *CHS_H1*, *CHI*, and *F3´H* was elevated in transgenic hop plants. In addition, the production of anthocyanins, rutin, isoquercitin, kaempferol-7-*O*-glucoside, kaempferol-7-*O*-glucoside-malonate, desmethylxanthohumol, xanthohumol, *α*-acids, and *β*-acids in transgenic hop plants was influenced by the PAP1/AtMYB75 transcription factor.

In a second approach, the homologous transcription factor HlMYB3 from *H. lupulus* L. was genetically introduced and expressed in the hop genome. The effect of the over-expression of the transgene on the expression rate of structural flavonoid and phloroglucinol biosynthetic genes, like *PAL*, *C4H*, *4CL*, *CHS_H1*, *CHI*, *F3H*, *F3´H*, *FLS*, *F3´5´H*, *OMT1*, *HlPT1*, and *VPS* was examined. Transgenic events with an elevated expression of genes of flavonoid and phloroglucinol biosynthesis were identified.

For quite some time successful plant tissue culture and *Agrobacterium tumefaciens*-mediated transformation procedures are available to genetically modify hop. However, these procedures are characterized by the low regeneration and transformation rates. Moreover, *A. tumefaciens*-mediated transformation is a laborious and time consuming process.

For that reason, in order to evaluate further homologous or heterologous transcription factors with respect to the regulation of flavonoid and phloroglucinol biosynthesis in hop was highly desirable to have a simple and fast transformation system. *A. rhizogenes*-mediated transformation represents an alternative to express genes in hairy roots. Therefore, hop explants were genetically transformed with *A. rhizogenes* strains K599 and 15834. Hairy roots were only induced by *A. rhizogenes* 15834. The transgenity of the obtained hairy roots was confirmed by histochemical GUS assay. The integration of *rolC* and *mgfp5* genes in transgenic hairy roots was confirmed by PCR.

Particle bombardment combined with the regeneration of plants in temporary immersion bioreactors could provide another alternative for hop genetic transformation. In this study, a protocol for the micropropagation and shoot induction from organogenic calli of hop cv. Tettnanger using the temporary immersion bioreactors was developed. Furthermore, a procedure for particle bombardment was established using the following parameters: helium pressure of 900 psi, and target distance of 6 cm.

The importance of hop relies on the secondary metabolites contain in the lupulin glands of the female cones. The present thesis demonstrated that the production of these secondary metabolites in transgenic hop plants could be influenced and enhanced by the expression of homologous or heterologous transcription factors. Moreover, the new developed transformation methods open the possibility for evaluating further genes that might influence the composition of secondary metabolites in the lupulin glands of hop.

# Zusammenfassung

Seit langer Zeit wird Hopfen (*Humulus lupulus* L.) von der Brauindustrie als Aroma- und Konservierungsmittel genutzt. Zudem wird Hopfen für medizinische Zwecke verwendet. In den letzten Jahren haben Xanthohumol und Desmethylxanthohumol aufgrund ihrer potenziellen krebsbekämpfenden Eigenschaften besondere Aufmerksamkeit erhalten.

In Hopfenzüchtungsprogrammen wurde das Hauptaugenmerk auf neue Sorten mit hohen Gehalten an *α*- und *β*-Säuren gelegt, um den Anforderung der Brauindustrie gerecht zu werden. Aufgrund der medizinischen und pharmazeutischen Bedeutung von Hopfen wurden jedoch neue Züchtungsanstrengungen unternommen, um Sorten mit einem hohen Gehalt von Xanthohumol und Desmethylxanthohumol zu erhalten. Im Hinblick darauf, bietet die Modifizierung der Flavonoidbiosynthese ein enormes Potential zur Steigerung der Produktion solcher Inhaltstoffe.

Der Flavonoidbiosyntheseweg ist in Pflanzen ausführlich erforscht worden. In der Regulation dieses Synthesewegs werden einzelne Gene durch verschiedene Transkriptionsfaktoren reguliert. Die Transkriptionsfaktorfamilie R2R3 MYB spielt eine wichtige Rolle in der Regulation der Biosynthese von Phenylpropanoiden und Flavonoiden. Durch eine ektopische Expression dieser Transkriptionsfaktoren in transgenen Pflanzen wurden die Produktionsrate und die Menge der Flavonoide erhöht.

Das Hauptaugenmerk dieser Forschungsarbeit war deshalb auf die Erhöhung der Produktion von pharmakologisch relevanten Inhaltsstoffen im Hopfen durch „*metabolic engineering*“ des Flavonoidbiosynthesewegs gerichtet.

So wurde in einem ersten Ansatz eine gentechnische Veränderung von Tettnanger Hopfen mit dem heterologen Transkriptionsfaktor PAP1/AtMYB75 aus *Arabidopsis thaliana* L. erfolgreich durchgeführt. Das Gen *PAP1/AtMYB75* wurde stabil im Hopfengenom integriert und exprimiert. Die Blüten und Dolden transgener Klone zeigten einen neuen Phänotyp, d.h. sie waren rosa bis rötlich gefärbt. Die Dolden des nicht transgenen Wildtyps haben eine grüne Farbe. Desweiteren war in transgenen Pflanzen, verglichen mit dem Wildtyp, die Expression der Strukturgene *CHS_Hl*, *CHI* und *F3'H* erhöht. Außerdem wurde die Produktion von Anthocyaninen, Kämpferol-7-*O*-Glykoside, Kämpferol-7-*O*-Glykoside-Malonate, Rutin, Isoquercitin, Desmethylxanthohumol, Xanthohumol**,** *α*-Säuren und *β*-Säuren in den transgenen Hopfenpflanzen durch den PAP1/AtMYB75 Transkriptionsfaktor beeinflusst.

In einem zweiten Ansatz wurde der homologe Transkriptionsfaktor HlMYB3 aus *H. lupulus* L. in das Hopfengenom integriert und exprimiert. Der Effekt einer Über-Expression des Transgens auf die Expressionsrate von Strukturgenen der Flavonoid- und Phloroglucinolbiosynthese, wie zum Beispiel *PAL*, *C4H*, *CHS_H1*, *CHI*, *F3H*, *F3'H*, *FLS*, *F3'5'H*, *OMT1*, *HlPT1*, und *VPS*, wurde untersucht. Transgene Events mit einer erhöhten Expression von Genen der Flavonoid- und Phloroglucinolbiosynthese wurden identifiziert.

Seit längerem gibt es Arbeitsverfahren für Hopfen in Pflanzengewebekultur und zur genetischen Transformation von Hopfen mittels *Agrobacterium tumefaciens*. Allerdings sind all diese Verfahren durch niedrige Regenerations- und Transformationsraten gekennzeichnet. Obwohl erfolgreiche genetische Transformationen von Hopfen durchgeführt werden kann, bleibt es ein arbeits- und zeitaufwändiger Prozess.

Aus diesem Grund, wäre es wichtig, ein einfacheres und schnelleres Transformationssystem zu entwickeln. Somit könnte der Einfluss einer Reihe homologer oder heterologer Transkriptionsfaktoren auf die Flavonoid- und Phloroglucinolbiosynthese im Hopfen abgeschätzt werden. Eine Alternative zum Gentransfer bietet *A. rhizogenes*. Hopfenexplantate wurden mit den *A. rhizogenes* Stämmen K599 und 15834 genetisch transformiert. Haarwurzeln konnten nur durch *A. rhizogenes* 15834 induziert werden. Die Transgenität dieser Haarwurzeln wurde durch histochemische GUS Tests nachgewiesen. Die Integration von *rolC* und *mgfp5* Genen in transgenen Haarwurzeln wurde durch PCR bestätigt.

Partikelbeschuss und anschließende Regeneration von Hopfepflanzen in „*temporary immersion bioreactors*" (z.B RITA®) könnte eine weitere alternative Methode für die genetische Transformation darstellen. In dieser Studie wurde ein Protokoll zur Mikropropagation und Sprossinduktion aus organogenen Kalli von Hopfen cv. Tettnanger in RITA-Gefäßen abwickelt. Weiterhin wurde ein Verfahren zum Partikelbeschuss erarbeitet. Unter folgenden Bedingungen wurde Hopfen behandelt: Heliumdruck von 900 psi und Entfernung des Ziels von 6 cm.

Die Bedeutung von Hopfen besteht im Gehalt der sekundären Metaboliten in den Lupulindrüsen der weiblichen Dolden. Durch diese Arbeit wurde gezeigt, dass die Produktion solcher Sekundärmetaboliten in transgenen Hopfenpflanzen durch die Expression homologer und heterologer Transkriptionsfaktoren beeinflusst und gesteigert werden kann. Zudem eröffnen die neu entwickelten Transformationsverfahren die Möglichkeit, die Bedeutung weiterer Transkriptionsfaktoren, welche die Zusammensetzung der sekundären Metabolite in den Lupulindrüsen der Hopfendolden beeinflussen könnten, abzuschätzen.

# Resumen

El lúpulo (*Humulus lupulus* L.) ha sido utilizado tradicionalmente en la elaboración de cerveza como saborizante y agente conservante. Además, ha sido empleado durante mucho tiempo con fines medicinales. Recientemente, el xanthohumol y el desmetilxantohumol han recibido gran atención debido a sus potenciales propiedades anti-cancerígenas.

Los programas de mejoramiento genético en lúpulo se han centrado principalmente en el desarrollo de nuevas variedades con alto contenido de *α*- ácidos y *β*-ácidos, con el fin de satisfacer la demanda de la industria cervecera. Debido a la importancia médica y farmacéutica del lúpulo, nuevos esfuerzos de mejoramiento genético se llevan a cabo con el fin de obtener nuevas variedades con alto contenido de xanthohumol y desmetilxantohumol. En este sentido, para complementar estos esfuerzos, la ingeniería metabólica de la biosíntesis de flavonoides ofrece un enorme potencial para modificar la producción de estos compuestos.

La biosíntesis de flavonoides ha sido estudiada intensamente en las plantas y ha sido demostrado que la actividad de los genes involucrados está regulada por varios factores de transcripción. La familia de factores de transcripción R2R3 MYB juega un papel importante en la regulación de la biosíntesis de los fenilpropanoides y flavonoides. La expresión ectópica de estos factores de transcripción en plantas transgénicas estimuló la producción y aumentó la cantidad de flavonoides.

Por lo tanto, el objetivo principal de esta investigación fue aumentar la producción de metabolitos secundarios de interés farmacológico en lúpulo a través de la ingeniería metabólica de la ruta de biosíntesis de flavonoides.

Para ello, en una primera aproximación, se modificó genéticamente la planta de lúpulo cv. Tettnanger con el factor de transcripción PAP1/AtMYB75 de *Arabidopsis thaliana* L. Se demostró que el transgen *PAP1/AtMYB75* fue incorporado y expresado en el genoma del lúpulo. Las plantas transgénicas mostraron flores femeninas y estróbilos de color rojizo a rosado. La expresión de los genes estructurales *CHS_H1*, *CHI*, y *F3'H* fue elevada en las plantas transgénicas de lúpulo en comparación con las plantas no transgénicas. Además, el factor de transcripción PAP1/AtMYB75 influenció la producción de antocianinas, rutina, isoquercitin, kaempferol-7-*O*-glucósido, kaempferol-7-*O*-glucósido-malonato, desmetilxantohumol, xantohumol, *α*-ácidos y *β*-ácidos en las plantas transgénicas de lúpulo.

En un segundo enfoque, el factor transcripción HlMYB3 de *H. lupulus* L. se introdujo genéticamente y se expresó en el genoma del lúpulo. Se evaluó el efecto de la sobre-expresión del transgen en la tasa de expresión de genes biosintéticos de flavonoides y floroglucinoles, tales como *PAL*, *C4H*, *4CL*, *CHS_H1*, *CHI*, *F3H*, *F3'H*, *FLS*, *F3'5'H*, *OMT1*, *HlPT1* y *VPS*. Se identificaron eventos transgénicos con una elevada expresión de los genes involucrados en la biosíntesis de flavonoides y floroglucinoles.

En lúpulo, desde hace varios años, se cuenta con protocolos de cultivo de tejidos y transformación genética mediada por *Agrobacterium tumefaciens*. Sin embargo, estos procedimientos se caracterizan por la baja regeneración y tasas de transformación. Asimismo, la transformación genética mediada por *A. tumefaciens* es un proceso laborioso y demanda mucho tiempo.

Por esa razón, con el fin de evaluar posibles genes candidatos con respecto a la regulación de la biosíntesis de flavonoides y floroglucinoles en lúpulo, es deseable disponer de un sistema de transformación simple y rápido.

En este sentido, la transformación genética mediada por *A. rhizogenes* constituye una alternativa para expresar genes en las raíces transgénicas. Por lo tanto, los explantes de lúpulo se transformaron genéticamente con *A. rhizogenes* K599 y 15834. Se obtuvieron solamente raíces transgénicas con *A. rhizogenes* 15834. La presencia del gen *gusA* se comprobó en las raíces transgénicas mediante el ensayo histoquímico de la enzima *β*-glucoronidasa. Asimismo, la integración de los genes *rolC* y *mgfp5* en las raíces transgénicas se confirmó mediante PCR.

La transformación genética mediante el sistema de biobalística en combinación con la regeneración de plantas en biorreactores de inmersión temporal podría proporcionar un método alternativo para la transformación genética en lúpulo. En este estudio, se estableció un protocolo para la micropropagación y la inducción de brotes a partir de callos de organogénicos de lúpulo cv. Tettnanger usando los biorreactores de inmersión temporal (RITA®). Asimismo, se estableció un procedimiento para la transformación genética mediante el sistema de biobalística. Las condiciones de biolística consisten en una presión de helio de 900 psi y una distancia de bombardeo de 6 cm.

El valor comercial del lúpulo radica en los metabolitos secundarios presentes en las glándulas de lupulina de los estróbilos femeninos. La presente tesis demostró que la producción de estos metabolitos secundarios en plantas de lúpulo transgénicas puede ser influenciada por la expresión de factores de transcripción. Por otra parte, los nuevos métodos desarrollados para la transformación genética de lúpulo abren la posibilidad para la evaluación de genes candidatos que pueden influir en la composición de metabolitos secundarios en las glándulas de lupulina.

# 1. Introduction[1]

## 1.1 General information about hop

The hop plant belongs to the order Rosales and to the *Cannabaceae* family, which comprises the genera *Humulus* L. and *Cannabis* L. (Chadwick et al. 2006; Bremer et al. 2009). Previously this family was placed in the order *Urticales*. The literature reported that the genus *Humulus* consists of three species: *H. lupulus* L., *H. japonicus* Sieb. & Zucc. and *H. yuannensis* Hu (Verzele and De Keukeleire 1991; Chadwick et al. 2006). Since, *H. yuannensis* Hu. has only been known from a herbarium collection, only *H. lupulus* L. and *H. japonicus* Sieb. & Zucc will be described in detail.

Based on geographical locations and leaf morphology the species of *H. lupulus* L. have been classified into five taxonomic varieties: *lupulus* Small for European hops, *cordifolius* Small for Japanese hops, *neomexicanus* Nelson & Cockerell, *pubescens* Small and *lupuloides* Small for hops of North America (Small 1978).

The common hop (*H. lupulus* L.) is a perennial, climbing, and herbaceous plant (Fig. 1.1.a). New shoots re-grow during spring from the rhizomes of an underground rootstock. The climbing dextrorse-twining herbaceous bines grow up to 6-9 m in length. In fall the plants dry up and only the perennial rootstock survives (Verzele and De Keukeleire 1991). The dark green colored leaves are heart-shaped with 3-5 lobes with serrate margins. They are distributed opposite one another in the stem. The abaxial surface of the leaves and the nodes of the stems of *H. lupulus* L. var *lupulus* have limited number of trichomes and lupulin glands (Small 1978).

As dioecious plant, male and female flowers are on separate plants; however monoecious plants have been found (Verzele and De Keukeleire 1991; Haunold et al. 1993). Male plants produce flowers in loose panicles (Fig. 1.1.b), whereas the female inflorescences develop to a strobile-like structure (Fig. 1.1.c). Female cones, known as hop cones or hops, are made up of overlapping bracts (Fig. 1.1.d). The base of each bract is covered with yellow lupulin glands (glandular trichomes) (Fig. 1.1.e, f).

Unlike *H. lupulus* L., the annual *H. japonicus* Sieb. & Zucc. is a very distinct plant, showing lobed and extremely pubescent leaves. The glands present in the leaves and the cones are smaller than those of *H. lupulus* L. Moreover, lupulin glands are absent in *H. japonicus* Sieb. & Zucc. (Small 1978).

[1] The citations included in this section are listed in general references.

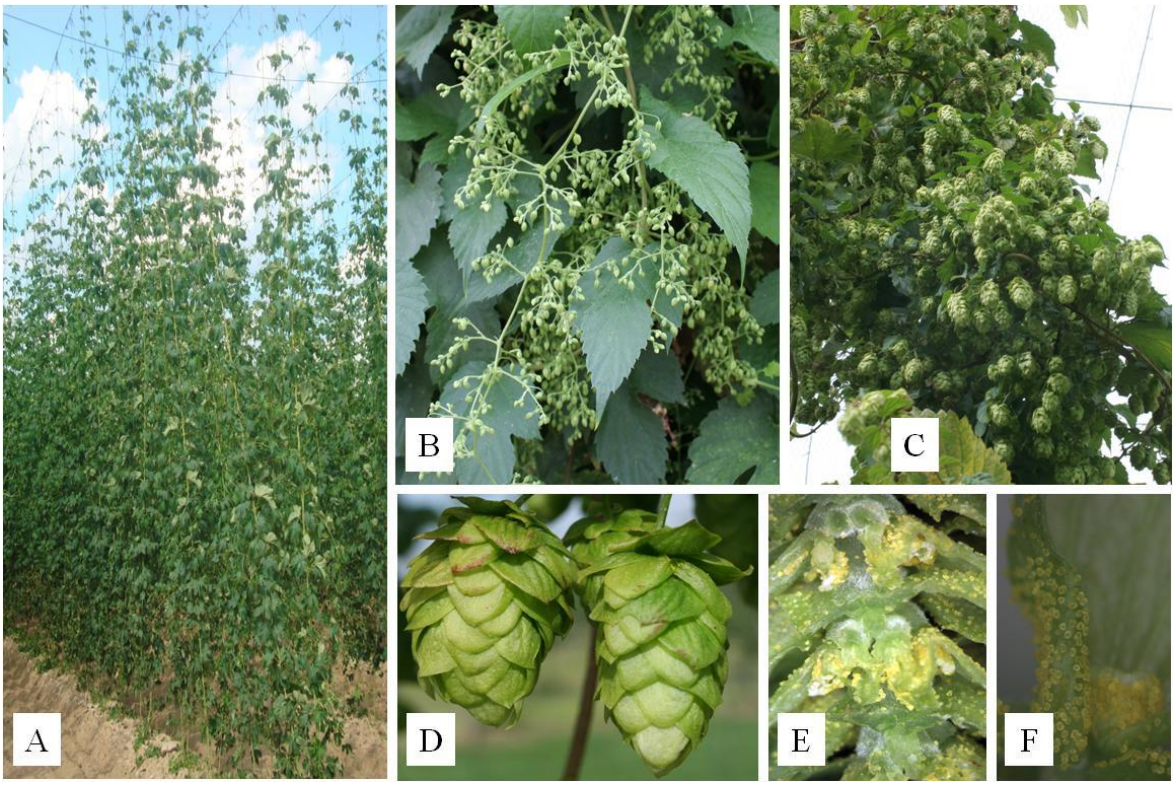



**Fig. 1.1 (A)** Female plants growing in a hop yard. A close-up view of a male **(B)** or female **(C)** plant. **(D)** Mature cones of hop as found on female plants. **(E)** Longitudinal section of a hop cone showing lupulin glands at the base of the bracts. **(F)** Light microscopic image of mature lupulin glands (Photos A. Gatica-Arias).

*H. lupulus* L. is a diploid (2n = 20) species with heteromorphic sex chromosomes. The chromosome system for hop plants of European origin is XX for females and XY for males. In *H. japonicus* Sieb. & Zucc. the chromosome system for females plants is 2n = 14+XX and for males is 2n = 14+$XY_1Y_2$.(Grabowska-Joachimiak et al. 2006).

## 1.2 Uses of hop

The economic value of hop relies on the secondary metabolites present in the lupulin glands of female cones. Hop is widely cultivated throughout the temperate zones of the world between latitudes 35º-55º north and south. Therefore, it is grown for commercial purpose in Europe, America, South Africa, Australia, and New Zealand. Hops are grown worldwide on 49,060 hectares. The largest hop growing areas are in Germany (37.1%), USA (25.0%), Czech Republic (9.6%), and China (8.9 %) (Hopsteiner 2011). In 2011 the total hop world production yielded approximately 96,672 t. From the total yield Germany produced 37,113 t (38.4 %), the USA 29,131 t (30.1 %), China 10,250 t (10.6 %), and Czech Republic 6,191 t (6.4 %) (Hopsteiner 2011).

Hops have been employed for a long time as ingredient for beer production confering aroma and flavor to beer, as well as for preserving it. In 2011, the beer production worldwide was approximately 1,854 hectoliter (Hopsteiner 2011). Moreover, hops are also used as flavoring ingredient in nonalcoholic beverages and foods. Female hop plants used for brewing purposes are derived from *H. lupulus* L., whereas male plants are essential to develop new varieties (Verzele and De Keukeleire 1991). *H. japonicus* Sieb. & Zucc. does not produce bitter substances nor xanthohumol and it is only cultivated for ornamental purposes (Verzele and De Keukeleire 1991; Stevens et al. 2000).

Hop plants are not only used in the brewing industry, they have also been utilized in the traditional medicine. For decades, the sedative effect of hop has been recognized contributing to the treatment of sleep disturbances and anxieties (Chadwick et al. 2006; Zanoli and Zavatti 2008). Besides these properties, anti-proliferative, anti-oxidative, anti-mycotic, anti-bacterial, and estrogenic effects have been reported in numerous studies (Stevens and Page 2004; Chadwick et al. 2006; Zanoli and Zavatti 2008). Therefore, hop was named as "Medical plant of the year 2007" by the Study Group for the Historical Development of Medicinal Plant Science at the University of Würzburg in Germany (Biendl 2008).

## 1.3 Composition of secondary metabolites in hop

Most of the important components of hop are produced in the glandular trichomes found at the base of the bracts, though, some of them are also synthesized in the trichomes on the underside of young leaves (Stevens and Page 2004). So far more than 1000 compounds have been identified in hop, including essential oils, bitter acids, and prenylflavonoids (Chadwick et al. 2006). The composition of these compounds is defined by the genotype and has been used for distinguishing different hop varieties (Wang et al. 2008).

### 1.3.1 Essential oils

Essential oils comprise 0.5% to 3% (v/w) of the weight of the female cones. The primary components of this fraction are the monoterpenes $\beta$-myrcene and the sesquiterpenes $\beta$-caryophyllene, and $\beta$-humulene (Fig. 1.2). The essential oil contributes to the aroma and flavor of beer (Chadwick et al. 2006; Wang et al. 2008). The content of the primary essential oils of some major German aroma and bitter hop varieties is shown in the Table 1.1.

**Table 1.1** Content of essential oils[a], *α*-acids[b], *β*-acids[b], xanthohumol[b], and isoxanthohumol of some major German aroma and bitter hops varieties

| | Variety | *β*-myrcene (%) | *β*-humulene (%) | *β*-caryophyllene (%) | *α*-acids (%) | *β*-acids (%) | Xanthohumol (%) | Isoxanthohumol ($mgl^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Aroma hops | Tettnanger | 20–35 | 22–32 | 6–11 | 2.5–5.5 | 3.0–5.0 | 0.3–0.4 | 0.73 |
| | Hallertauer Tradition | 17–32 | 35–50 | 10–15 | 4.0–7.0 | 3.0–6.0 | ~ 0.4 | 0.51 |
| | Perle | 20–35 | 35–55 | 10–20 | 4.0–9.0 | 2.5–4.5 | 0.4–0.5 | 0.66 |
| | Spalter | 20–35 | 20–30 | 8–13 | 2.5–5.5 | 3.0–5.0 | ~ 0.3 | 0.75 |
| | Saphir | 25–40 | 20–30 | 9–14 | 2.0–4.5 | 4.0–7.0 | 0.3–0.4 | 0.85 |
| Bitter hops | Hallertauer Magnum | 30–45 | 30–45 | 8–13 | 5.0–7.0 | 11.0–16.0 | 0.4–0.5 | 0.40 |
| | Hallertauer Taurus | 30–50 | 23–33 | 6–11 | 12.0–17.0 | 4.0–6.0 | 0.9–1.0 | 0.62 |
| | Hallertauer Merkur | 25–35 | 35–50 | 9–15 | 10.0–14.0 | 3.5–7.0 | ~ 0.3 | 0.37 |
| | Herkules | 30-50 | 30-45 | 7-12 | 12.0-17.0 | 4.0-5-5 | ~ 0.7 | 0.49 |
| | Nugget | 27–42 | 25–40 | 10–20 | 9.0–13.0 | 3.0–5.0 | 0.5–0.7 | 0.67 |

[a] % relative to the total oil content. [b] % weight (w/w)

Data obtained from The spirit of beer: hops from Germany (http://www.deutscher-hopfen.de/contentserv/hopfenpflanzerverband.de/data/media/2099/HM-eng-komplett-05.pdf) Accessed 22 August 2012

### 1.3.2 Bitter acids

The bitter acids comprise 5% to up 25% (v/w) of the weight of female hop cones (De Keukeleire 2000). They are di- or tri-prenylated phloroglucinol derivates and contain a 3-, 4-, 5-, or 6-carbon oxo-alkyl side chain. The bitter acids comprise *α*-acids (humulone, adhumulone, cohumulone, prehumulone, and posthumulone) and *β*-acids (lupulone, adlupulone, colupulone, prelupulone, and postlupulone) (De Keukeleire 2000; Chadwick et al. 2006; Zanoli and Zavatti 2008) (Fig. 1.2). The bitter acids are weak acids with poor solubility in water and no bitter taste (De Keukeleire 2000). The *α*-acids stabilize the beer foam and inhibit the growth of gram-positive bacteria. During the brewing of beer, *α*-acids are isomerized to *iso*- *α*-acids, which are responsible for the bitter taste of the beer (Verzele and De Keukeleire 1991; De Keukeleire 2000; Zanoli and Zavatti 2008). Moreover, bitter acids have sedative, anti-oxidative, anti-depressant, and anti-cancer properties (Tagashira et al. 1995; Chen and Lin 2004; Zanoli et al. 2005; Schiller et al. 2006; Lamy et al. 2007).

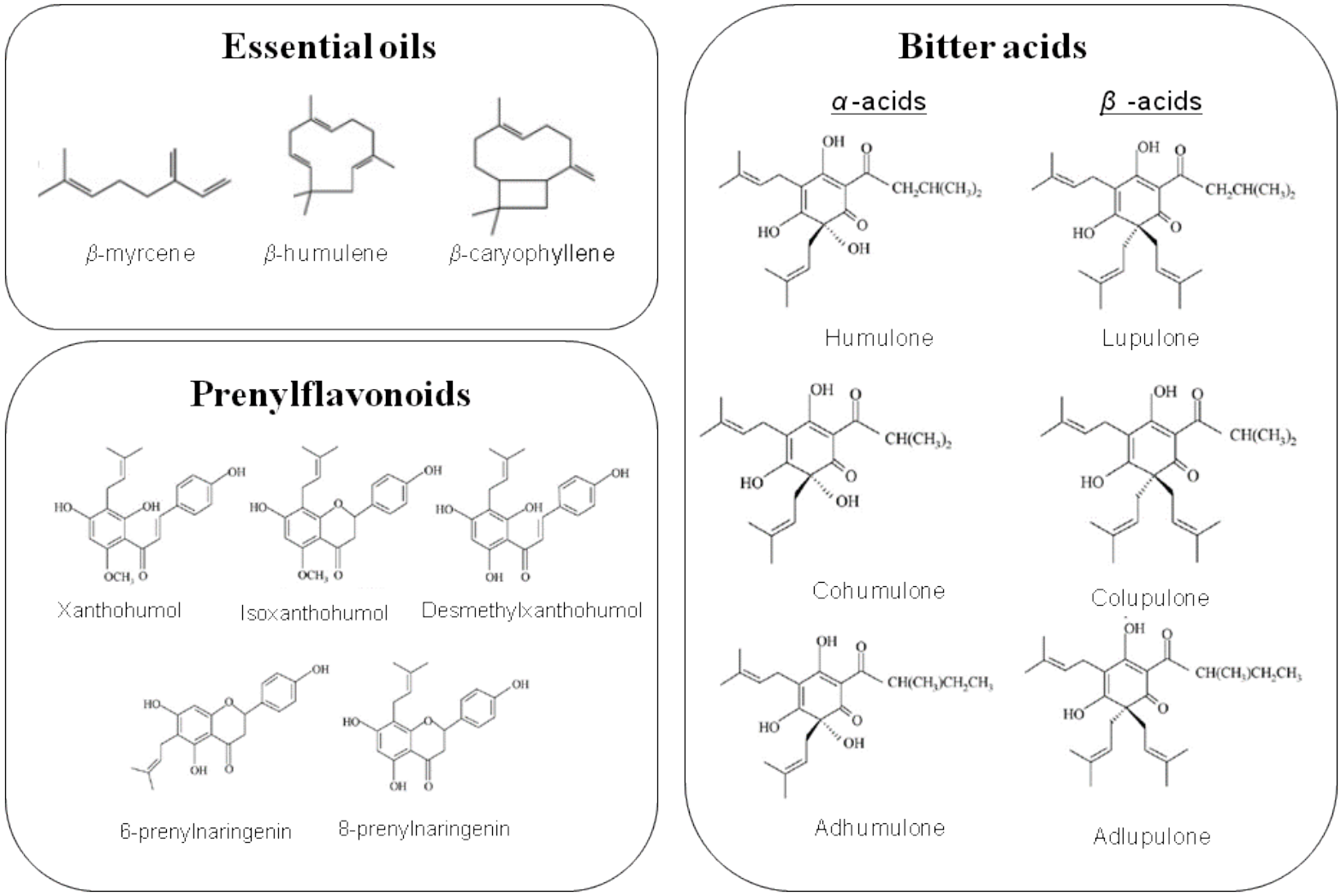



**Fig. 1.2** Chemical structure of essential oils, bitter acids, and prenylflavonoids present in hop. Figure adapted from Wang et al. (2008) and Zanoli and Zavatti (2008).

According to the content of $\alpha$- and $\beta$-acids hop varieties are grouped into aroma or bitter hops (Table 1.1). Aroma hops have a lower $\alpha$-acid content and but a higher essential oil profile associated with aroma, whereas bitter hops are characterized by a higher $\alpha$-acid content. German aroma varieties include Tettnanger, Hallertauer Tradition, Hallertauer Mittelfrüher, Perle, Spalter, Spalter Select, Opal, Smaragd, and Saphir. Tettnanger as a typical aroma cultivar originated in the Tettnang hop growing area of Germany as a land-race hop. On the other hand, Hallertauer Magnum, Hallertauer Taurus, Hallertauer Merkur, Herkules, Northern Brewer, and Nugget are representative varieties of bitter hops.

### 1.3.3 Prenylflavonoids

Prenylflavonoids comprise 4-14 % of the weight of female hop cones (Gerhäuser 2005). The primary components of this fraction are phenolic acids, prenylated chalcones, flavonoids, catechins, and proanthocyanidins (Stevens and Page 2004; Gerhäuser 2005) (Fig. 1.2). The principal prenylated flavonoid is the chalcone xanthohumol (3'-prenyl-6'-*O*-methylchalconaringenin). This compound comprises 0.1-1% of the dry weight of hop cones. Xanthohumol is only found in *H. lupulus* L and recently has received attention due to its cancer chemopreventive properties (Gerhäuser 2005). During the brewing process xanthohumol is converted into isoxanthohumol, which is the most abundant prenylflavonoid present in beer (Stevens and Page 2004) (Table 1.1). Another prenylated flavonoid named xanthogalenol (3'-prenyl-4'-*O*-methylchalconaringenin) has been isolated from *H. lupulus* L. cv. Galena and *H. cordifolius* Small (Stevens et al. 2000).

Most of the flavonoids present in the hop are derivated from the prenylated flavonoid desmethylxanthohumol (2′,4,4′,6′-tetrahydroxy-3′-prenylchalcone) (Chadwick et al. 2006). This compound comprises 0.1 % of the weight of female hop cones (Stevens et al. 1999). A chemical isomerization of desmethylxanthohumol gives rise to the flavanone 6-prenylnaringenin along with 8-prenylnaringenin. The former displayed antifungal activity, whereas the latter is the most potent phytoestrogen isolated to date (Mizobuchi and Sato 1984; Milligan et al. 2002).

## 1.4 Flavonoids

### 1.4.1 Structure of flavonoids

Flavonoids are a large family of low molecular weight polyphenolic secondary metabolites. They are accumulated in different types of plant tissues and organs. The basic skeleton of flavonoids consists of C6-C3-C6 structure forming the A, C, and B rings, respectively. Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of natural products includes three major subgroups: the flavonoids (2-phenylbenzopyrans), isoflavonoids (3-benzopyrans), and the neoflavonoids (4-benzopyrans) (Marais et al. 2006). Moreover, based on the modification of the C-ring, flavonoids can be subdivided in the following subclasses: flavanones, isoflavones, flavones, flavonols, flavanols, and anthocyanins (Fig. 1.3). The great variety of flavonoids within a class is the result of further modification such as hydroxylation, methylation, acylation, and glucosylation (Koes et al. 1994; Schijlen et al. 2004).

### 1.4.2 Functions of flavonoids

Although flavonoids are nonessential for plant growth and development, they play an important role in the survival and propagation of plants. For instance, they participate in the attraction of pollinators, and organisms for dispersing seeds. Additionally, they are involved in plant fertilization, and pollen germination. Other roles include the defense against insects, and microorganisms, and the protection against UV-radiation, and physical stress. As well, flavonoids are important for the pigmentation of flowers, fruits and seed (Koes et al. 1994; Schijlen et al. 2004; Buer et al. 2010). Moreover, flavonoids modulate the levels of reactive oxygen species (ROS) (Taylor and Grotewold 2005). Furthermore, they influence the transport of the phytohormone auxin (Peer and Murphy 2007).

Besides their function in plants, flavonoids have shown various benefits for the health of humans. Consumption of foods and beverages rich in flavonoids are beneficial to human health by preventing degenerative diseases associated with oxidative stress, coronary heart, and/or diseases related to aging (Ross and Kasum 2002; Schijlen et al. 2004). Moreover, it was demonstrated that flavonoids induce apoptosis, and inhibit the division of cancer cells (Khan et al. 2008). In addition, these secondary metabolites have shown to possess anti-inflammatory, neuroprotective, analgesic, bactericidal, fungicidal, and spasmolytic properties (Harbone and Williams 2000; Ross and Kasum 2002).

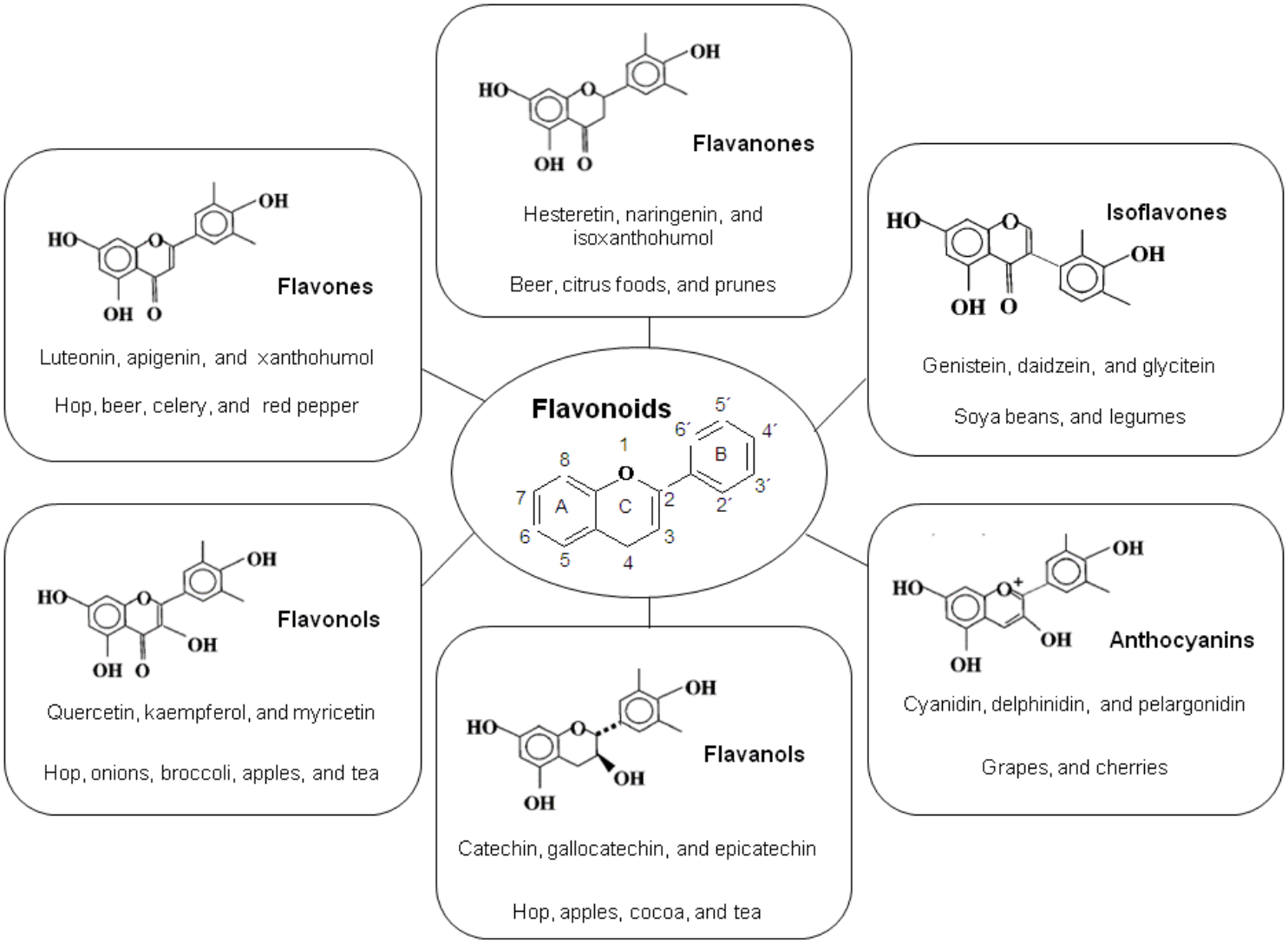



**Fig. 1.3** Chemical structure and food source of flavonoids. Figure adapted from Ross and Kasum (2002) and Marais et al. (2006).

### 1.4.3 Biosynthetic pathway of flavonoid biosynthesis

Flavonoids are synthesized in the cytosol and transported to the vacuole for storage. However, they are also present in the cell wall, in the nucleus, in the chloroplast, and in extracellular spaces (Zhao and Dixon 2009). The flavonoid biosynthetic pathway has been one of the most intensively studied metabolic systems in plants. Most of the genes and enzymes involved in flavonoid biosynthesis have been cloned and characterized in model plants such as *Arabidopsis thaliana* L., *Zea mays* L., *Petunia hybrida*, and *Vitis vinifera* L. (Lepiniec et al. 2006).

The first step in the flavonoid biosynthesis is the condensation of one molecule of 4-coumaroyl-CoA (or *p*-coumaroyl-CoA) with three molecules of malonyl-CoA to give naringenin chalcone. This reaction is catalyzed by the enzyme chalcone synthase (CHS, E.C. 2.3.1.74). Coumaroyl-CoA, a product of the phenylpropanoid pathway, is synthesized from the amino acid phenylalanine by the enzymes phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5), 4-coumarate-CoA ligase (C4H, E.C.1.14.13.11), and cinnamate 4-hydroxylase (4CL, E.C.6.2.1.12). Malonyl-CoA originates from the carboxylation of acetyl-CoA. In the next step of the biosynthesis of flavonoids, naringenin chalcone is isomerized by the enzyme chalcone isomerase (or chalcone-flavanone isomerase) (CHI, E.C.5.5.1.6) to yield the flavanone naringenin. A subsequent hydroxylation of naringenin in the C-3 position by the enzyme flavanone-3-hydroxylase (F3H, E.C.1.14.11.9) results in the formation of dihydrokaempferol, which is hydroxylated at the 3´ position of the B-ring to yield dihydroquercetin. The latter reaction is catalyzed by the enzyme flavonoid 3´-hydroxylase (F3´H, E.C.1.14.13.21). Moreover, the dihydrokaempferol is hydroxylated at the 3´ and 5´ positions of the B-ring by the enzyme flavonoid 3´, 5´-hydroxylase (F3´5´H, E.C.1.14.13.88) to yield dihydromyricetin. At this point, the pathway diverges into flavonols and anthocyanins. The enzyme flavonol synthase (FLS, E.C.1.14.11.23) catalyzes the conversion of dihydroflavonols (*i.e*. dihydrokaempferol, dihydroquercetin, and dihydromyricetin) into the corresponding flavonols (*i.e*. kaempferol, quercetin, and myricetin). The reduction of dihydroflavonols in position 4 by the enzyme dihydroflavonol 4-reductase (DFR, E.C. 1.1.1.219) leads to leucoanthocyanidins, which are converted into anthocyanidins by the enzyme anthocyanidins reductase (ANS, E.C. 1.14.11.19) (Schijlen et al. 2004; Ferrer et al. 2008). A generalized flavonoid biosynthetic pathway is shown in the Figure 1.4.

For the synthesis of prenylflavonoids in hop, the A-ring of naringenin chalcone is prenylated with dimethylallyldiphosphate (DMAPP) by an aromatic prenyltransferase to yield desmethylxanthohumol. A prenyltransferase, HlPT-1, has been cloned from hops and it has been implicated to catalyze this step (Tsurumaru et al. 2010, 2012). Subsequent desmethylxanthohumol is methylated at the 6'-hydroxylgroup by the enzyme *O*-methyltransferase-1 (OMT-1) to form xanthohumol (Nagel et al. 2008) (Fig. 1.4).

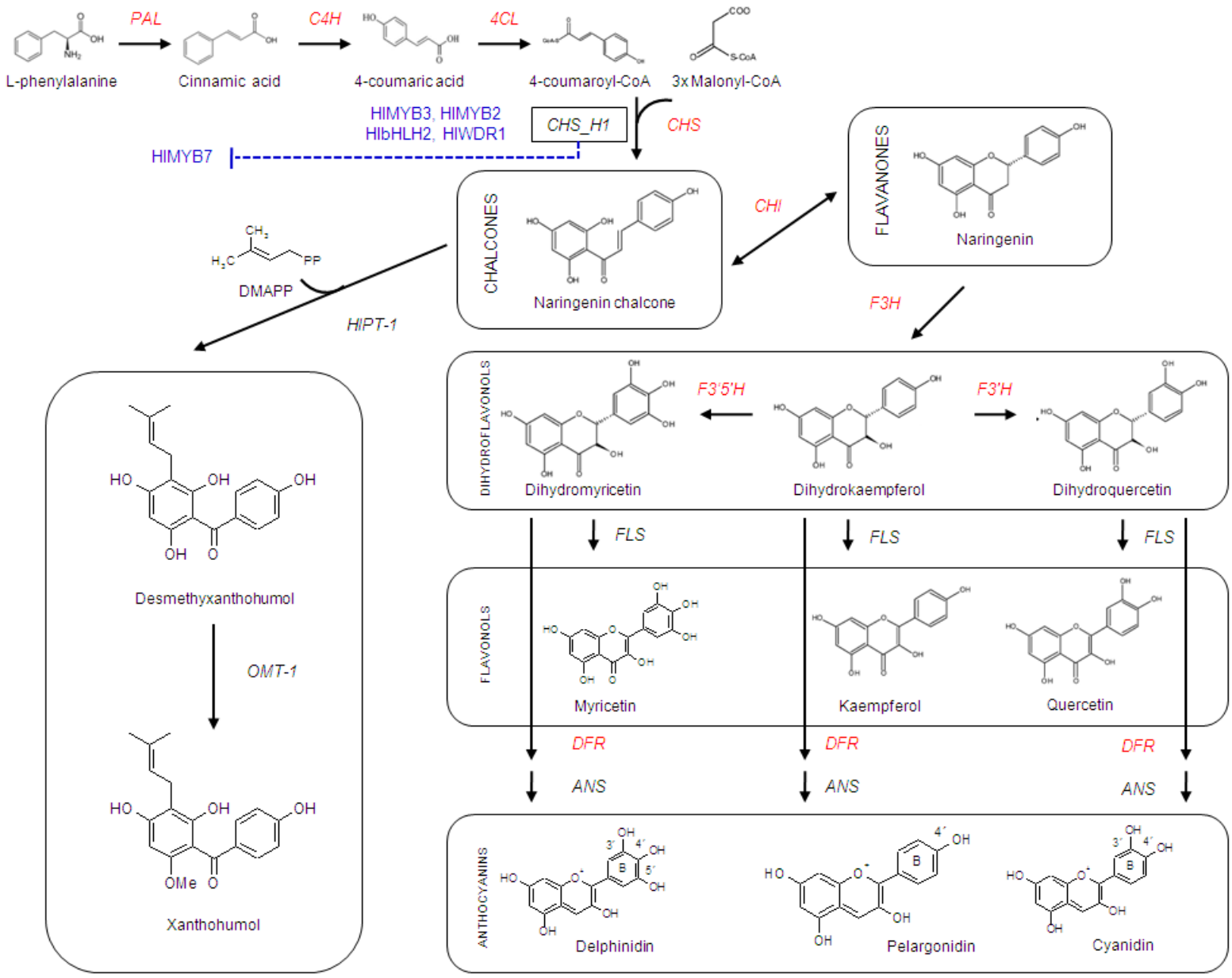



**Fig. 1.4** Schematic representation of the biosynthesis of flavonoids and its transcriptional regulation. Figure adapted from Stevens and Page 2004, Toghe et al. 2005, Lepiniec et al. 2006, Stracke et al 2007, Nagel et al. 2008, Zhang et al. 2010, Matoušek et al. 2012 and Tsurumaru et al. 2012. The genes regulated by PAP1/AtMYB75 are indicated in red, whereas the regulation of *CHS_H1* in hop by the transcription factors HlMYB3, HlMYB2, HlMYB7, HlbHLH2 and HlWDR1 is shown in blue.

## 1.5 Regulation of flavonoid biosynthesis

The activity of the genes involved in the biosynthesis of flavonoids is largely regulated at the transcriptional level by transcription factors (Winkel-Shirley 2001; Schijlen et al. 2004). Transcription factors are defined as proteins that recognise and bind to a specific DNA sequence and control the rate of transcription initiation of target genes. They can act as activators, repressors, or both (Stracke et al. 2001; Yanhui et al. 2006). R2R3 MYB and/or a basic helix-loop-helix (bHLH) transcription factors, together with WD40 or WDR1 proteins, are important for regulating the biosynthesis of flavonoids (Hichri et al. 2011).

### 1.5.1 MYB transcription factors

*MYB* genes are exclusive to eukaryotes and comprise one of the largest families of transcription factors in plants (Jiang et al. 2004). Plant *MYB* genes are characterized by the conserved N-terminal MYB domain, which consists of 1, 2, 3 or 4 imperfect repeats (named R) consisting of 52 residues. Each one of this repeats give rise to a helix-turn-helix secondary structure that binds to the major groove of a target DNA sequences. The N-terminal domain is involved in DNA binding and dimerization, whereas an activation or repression domain is located at the C-terminus (Dubos et al. 2010; Hichri et al. 2011). Plant MYB transcription factors are classified into four major groups: those with one (1R-MYB), with two (R2R3-MYB), with three (R1R2R3-MYB), or with four repeats (R1R2R2R1/2-MYB) (Dubos et al. 2010).

The R2R3 subfamily is the most abundant in plants. In *A. thaliana* L. 126 R2R3 members have been identified (Stracke et al. 2001). Moreover, R2R3 *MYB* genes have also been identified in *Populus trichocarpa* Hook. (192 members), *Vitis vinifera* L. (108 members), and *Oryza sativa* L. (109 members) (Yanhui et al. 2006; Matus et al. 2008; Wilkins et al. 2009). R2R3 *MYB* genes are involved in the regulation of several processes, including cell morphogenesis, meristem formation, floral and seed development, and the cell cycle (Stracke et al. 2001; Yanhui et al. 2006; Dubos et al. 2010; Hichri et al. 2011). They also participate in the control of the synthesis of flavonoids (Stracke et al. 2007), anthocyanins (Feng et al. 2010), and proanthocyanidins (Zifkin et al. 2012).

The first MYB transcription factor, C1 (Colorless 1), regulating the flavonoid biosynthesis was isolated from *Z. mays* L. (Paz-Ares et al. 1987). Since then several MYB transcription factor involved in the control of the flavonoid pathway have been identified and characterized in crop, ornamental, and model plants (Hichri et al. 2011).

For instance, the transcription factor PAP1/AtMYB75, which is a conserved member of the R2R3 gene family, stimulates the expression of genes of the biosynthesis of phenylpropanoids and flavonoids, such as *PAL*, *4CL*, *CHS*, *CHI*, *F3H*, *F´3H*, *ANS*, and *DFR* in *A. thaliana* L., *B. napus* L., and *S. lycopersicum* L. (Borevitz et al. 2000; Matsui et al. 2004; Tohge et al. 2005; Stracke et al 2007; Zuluaga et al. 2008; Li et al. 2010) (Fig 1.4). Furthermore, the transcription factors AtMYB11, AtMYB12, and AtMYB111 regulate the expression of the genes *CHS*, *CHI*, *F3H*, and *FLS* in *A. thaliana* L (Hartmann et al. 2005; Mehrtens et al. 2005; Stracke et al 2007).

By contrast, the transcription factor AtMYBL2 suppresses the expression of *DFR* and *TT8* (transparent testa 8 coding for a bHLH protein) and acts as a transcriptional repressor and negatively regulates the biosynthesis of anthocyanin in *Arabidopsis* (Matsui et al. 2008). In transgenic tobacco, over-expression of *FaMYB1*, a member of the strawberry R2R3 gene family, suppressed the expression of *ANS* affecting the biosynthesis of flavonoids (Aharoni et al. 2001). Similarly, the expression levels of *CHS*, *CHI*, *FLS*, *DFR*, *LDOX*, and *UF3GT* were lower in transgenic *Arabidopsis* plants over-expressing the transcription factor MdMYB6 from apples resulting in less anthocyanin accumulation (Gao et al. 2011).

### 1.5.2 Transcription factors in hop

Recently, a number of different transcription factors including members of the gene families *MYB* (Matoušek et al. 2005; Matoušek et al. 2007; Matoušek et al. 2012), *bHLH* (Matoušek et al. 2012), *bZip* (Matoušek et al. 2010), and *WDR* (Matoušek et al. 2012) have been identified and characterized in hop. These factors are expressed at a high rate in the lupulin glands of female cones. According to similarities of DNA sequences to known regulators of the flavonoid biosynthesis it has been suggested that these transcription factors are involved in the regulation of the synthesis of secondary metabolites in lupulin glands.

To date, seven R2R3 MYB transcription factors had been entered in the NCBI sequence database. Among them, positive regulators [(HlMYB2, Gen Bank: FN646081.1) and (HlMYB3, Gen Bank: AM501509.1)] and repressors (HlMYB7, Gen Bank: FR873650) of the flavonoid biosynthesis have been described. Furthermore, the identified bHLH, and WDR transcription factors correspond to HlbHLH2 (Gen Bank: FR751553.1) and HlWDR1 (Gen Bank: FN689721.1), respectively (Matoušek et al. 2012).

In hop, the chalcone synthase gene *CHS_H1* has been cloned and identified as part of a multiple gene family consisting of six members. This gene is specifically expressed in female cones and is responsible for the biosynthesis of naringenin chalcone (Matoušek et al. 2006). The analysis of the promoter of *CHS_H1* revealed various MYB and bHLH binding motifs suggesting its regulation by the complex MYB/bHLH/WDR. Consequently, transient expression analysis in *Nicotiana benthamiana* L. leaves showed that the complexes HlMYB2/HlbHLH2/HlWDR1 or HlMYB3/HlbHLH2/HlWDR1 strongly activated the *CHS_H1* gene expression. By contrast, the transcription factor HlMYB7 showed no ability to activate the promoter of *CHS_H1* (Matoušek et al. 2012).

## 1.6 Metabolic engineering of flavonoids

Plants as natural factories represent a promising system for the production of valuable pharmaceutical and industrial compounds. However, the yield is not always satisfactory because synthesis of many secondary metabolites is often limited to particular organs/tissues and triggered under specific climatic, stress, or nutrient conditions (Verpoorte et al. 2002). Moreover, because of the structural complexity of secondary metabolites, their *de novo* chemical synthesis is difficult (Tian and Dixon 2006).

As a result, efforts have been focused on the commercial production of plant metabolites using plant callus, cell suspension cultures, and differentiated cells. Nevertheless, these approaches have had limited success. Moreover, even if the proper metabolic pathway is activated, the production of certain compounds in cultured cells was very low (Verpoorte et al. 2002).

As an alternative, over the last years, plant metabolic engineering has become an attractive tool for the production of commercial important compounds in plants. The access to the complete genome sequences of several plants, the knowledge on the various biosynthetic pathways, the development of transformation methods, and the availability of plant tissue culture procedures have contributed for this (DellaPenna 2001; Wurtzel and Grotewold 2006).

Plant metabolic engineering involves the manipulation of one or more endogenous biosynthesis pathways to enhance the production of a desired compound, or to reduce the production of an undesired compound (DellaPenna 2001; Capell and Christou 2004). This can be achieved by manipulating a single step in a pathway or by manipulating multiple steps of the same pathway. The first strategy involves the over-expression or down-regulation of a single structural gene. The second strategy uses endogenous or heterologous transcription factors that control one or more genes in a given biosynthetic pathway (Capell and Christou 2004).

An increasing knowledge of flavonoid biosynthesis and their important function in plants and in human health have made flavonoids excellent targets for metabolic engineering. Metabolic engineering of flavonoids began in 1987 and has been successfully employed to improve flavonoid composition for nutritional purposes, to generate novel flower and fruit color, and to induce male sterility (Forkmann and Martens 2001).

Changes in flower color have been reported in several studies. For example, the over-expression of the *Viola spp. F3'5'H* gene in a *Rosa hybrida* resulted in violet-colored flowers (Katsumoto et al. 2007). In addition, *CHI*-suppression by RNAi led to reduced pigmentation and changed flavonoid components in *N. tabacum* L. flower petals (Nishihara et al. 2005). Transgenic hop plants expressing the transcription factor PAP1/AtMYB75 showed reddish to pink female flowers and cones (Gatica-Arias et al. 2012).

In tomato, over-expression of the structural genes *STS*, *CHS*, *CHR*, *CHI*, *FNS* in the fruit peel improved the flavonoid composition. These transgenic tomatoes contained high levels of stilbenes, deoxychalcones, flavones, and flavonols (Schijlen et al. 2006). Moreover, over-expression of the gene *CHI* in hairy roots of *Glycyrrhiza uralensis* Fisch enhanced the accumulation of flavonoids (Zhang et al. 2009). Another example is the RNAi silencing of the tomate *CHS* resulting in the reduction of total flavonoid levels and induction of parthenocarpic fruits (Schijlen et al. 2007).

The possibility of controlling a biosynthetic pathway by introducing transcription factors offers a novel approach for the metabolic engineering. Recent studies have demonstrated the role of MYB transcription factor in regulating the flavonoid biosynthesis. For example, over-expression of the *PyMYB10*, a R2R3 *MYB* gene from *Pyrus pyrifolia* L., induced anthocyanin accumulation in *A. thaliana* L. transgenic immature seeds (Feng et al. 2010). Similarly, over-expression of *MdMYB10* in transgenic apple enhanced anthocyanin production (Espley et al. 2007). Moreover, the transcription factor PAP1/AtMYB75 stimulated the production of flavonoids in *B. napus* L., and *S. lycopersicum* L. (Zuluaga et al. 2008; Li et al. 2010). In *S. miltiorrhiza* Bunge, expression of the transcription factor PAP1/AtMYB75 enhanced the production of flavonoids, anthocyanins, lignins, and phenolic acids (Zhang et al. 2010). In this regard, transcription factors have emerged as a potential tool for metabolic engineering of flavonoid biosynthesis.

## 1.7 Objectives of the thesis

The main objective of this research was to modulate the production of pharmaceutical metabolites in hop (*Humulus lupulus* L.) through the metabolic engineering of the flavonoid biosynthesis pathway. Furthermore, an efficient, simple and fast transformation protocol for functional validation of candidate genes, such as those involved in the biosynthesis of flavonoids was developed.

In order to accomplish the described objectives, the following specific objectives were proposed:

- To analyze at the molecular level the transgenic hop plants, which were transformed with the heterologous transcription factor PAP1/AtMYB75 from *Arabidopsis thaliana* L.
- To compare the level of gene expression of the flavonoid genes in transgenic hop plants, expressing the heterologous transcription factor PAP1/AtMYB75.
- To evaluate at the molecular level the transgenic hop plants, which were transformed with the homologous transcription factor HlMYB3 from *Humulus lupulus* L.
- To determine the expression level of flavonoid genes in transgenic hop plants, expressing the homologous transcription factor HlMYB3.
- To develop a transformation system through *Agrobacterium rhizogenes* or particle gun in order to test candidate genes that could modify the flavonoid pathway in hop.

## 1.8 Publications

The present cumulative doctoral thesis consists of five scientific articles[2], which have been published or submitted in peer reviewed academic journals. The publications II and IV are reproduced with the corresponding permission of Brewing Science, Fachverlag Hans Carl, Nürnberg, Germany. The full papers can be accessed through the presented links.

Publication I

Gatica-Arias A, Farag MA, Stanke M, Matoušek J, Wessjohann L, Weber G (2012) Flavonoid production in transgenic hop (*Humulus lupulus* L.) altered by *PAP1/MYB75* from *Arabidopsis thaliana* L. Plant Cell Rep 31: 111-119 (http://www.springerlink.com/content/130276n418p0u2l1/)

[2] Each one of the following five chapters represents one article. The reference system of each journal, to which the article was submitted, is maintained.

Publication II

Gatica-Arias A, Farag MA, Häntzschel KR, Matoušek J, Weber G (2012) The transcription factor AtMYB75/PAP1 regulates the expression of flavonoid biosynthesis genes in transgenic hop (*Humulus lupulus* L.). Brew Sci 65: 103-111 (http://www.brewingscience.de)

Publication III

Gatica-Arias A, Stanke M, Häntzschel KR, Matoušek J, Weber G (2012) Over-expression of the transcription factor HlMYB3 in transgenic hop (*Humulus lupulus* L. cv. Tettnanger) modulates the expression of genes involved in the biosynthesis of flavonoids and phloroglucinols (Submitted to Plant Cell Tiss Org Cult)

Publication IV

Gatica-Arias A, Amma N, Stanke M, Weber G (2012) *Agrobacterium rhizogenes*-mediated transformation of hop (*Humulus lupulus* L. cv. Tettnanger): establishment of a system for functional evaluation of genes. Brew Sci 65: 91-95 (http://www.brewingscience.de)

Publication V

Gatica-Arias A, Weber G (2012) Genetic transformation of hop (*Humulus lupulus* L. cv. Tettnanger) by particle bombardment and plant regeneration using temporary immersion system (Submitted to Plant Biotech Rep)

# 2. Flavonoid production in transgenic hop (*Humulus lupulus* L.) altered by *PAP1/MYB75* from *Arabidopsis thaliana* L.[3]

## 2.1 Abstract

Hop is an important source of secondary metabolites, such as flavonoids. Some of these are pharmacologically active. Nevertheless, the concentration of some classes as flavonoids in wildtype plants is rather low. To enhance the production in hop, it would be interesting to modify the regulation of genes in the flavonoid biosynthetic pathway. For this purpose, the regulatory factor PAP1/AtMYB75 from *Arabidopsis thaliana* L. was introduced into hop plants cv. Tettnanger by *Agrobacterium*-mediated genetic transformation. Twenty kanamycin-resistant transgenic plants were obtained. It was shown that *PAP1/AtMYB75* was stably incorporated and expressed in the hop genome. In comparison to the wildtype plants, the color of female flowers and cones of transgenic plants was reddish to pink. Chemical analysis revealed higher levels of anthocyanins, rutin, isoquercitin, kaempferol-glucoside, kaempferol-glucoside-malonate, desmethylxanthohumol, xanthohumol, $\alpha$-acids and $\beta$-acids in transgenic plants compared to wildtype plants.

**Keywords** Hop, plant transcriptional factors, genetic transformation, secondary metabolites, flavonol glycosides, anthocyanins

[3] This chapter consists of the correspondent article which was accepted by the journal Plant Cell Reports. Any reference to this chapter should be cited as: Gatica-Arias A, Farag M, Stanke M, Matoušek J, Wessjohann L, Weber G (2012) Flavonoid production in transgenic hop (*Humulus lupulus* L.) altered by *PAP1/MYB75* from *Arabidopsis thaliana* L. Plant Cell Rep 31: 111-119 http://www.springerlink.com/content/130276n418p0u2l1/)

## 2.2 Introduction

Hop (*Humulus lupulus* L.) has been cultivated for its commercial use in the brewing industry and as a medicinal plant for a long time (Verzele and De Keukeleire 1991). More than 1,000 compounds have been identified in hop, including volatile oils, $\alpha$-acids, $\beta$-acids and prenylated flavonoids (Chadwick et al. 2006). Xanthohumol and desmethylxanthohumol are important prenylated flavonoids in the lupulin glands (glandular trichomes) of the female cones (Matoušek et al. 2006). Because of their potential anti-cancer, anti-proliferative, anti-oxidative and estrogene-like properties, these compounds have received attention recently (Tagashira et al. 1995; Miranda et al. 2000; Milligan et al. 2002; Chen and Lin 2004; Gerhäuser 2005; Lamy et al. 2007). Due to these beneficial effects on the human health, there is a growing interest in the development of food crops with high levels and/or altered composition of flavonoids (Schijlen et al. 2004).

Toward this goal, metabolic engineering offers tremendous potential to modify, improve and enhance the production and quality of flavonoids (Tian and Dixon 2006; Sato et al. 2007). Flavonoid biosynthesis in plants appears to be complex and highly regulated. A tight correlation between the regulation of gene expression and flavonoid biosynthesis has been demonstrated in *Arabidopsis thaliana* L. (Yonekura-Sakakibara et al. 2008). Altering biosynthesis by introducing genes of enzymes of individual biosynthetic steps could be one approach for increasing the production of bioactive flavonoids. Another attractive way to improve yield could be the introduction of genes from heterologous sources known to up-regulate the production of flavonoids (Schijlen et al. 2004).

The activity of genes involved in flavonoid biosynthesis is largely regulated at the transcriptional level. Usually, regulation is facilitated by an R2R3 *MYB* and/or a basic helix–loop–helix (bHLH) transcription factor (Schijlen et al. 2004; Allan et al. 2008). For example, in *A. thaliana* L. as well as in *Petunia hybrida*, the transcription factor *PAP1/AtMYB75* (production of anthocyanin pigment 1) stimulated the expression of genes for enzymes of the biosynthesis of phenylpropanoids and flavonoids, such as phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*) and dihydroflavonol 4-reductase (*DFR*) (Borevitz et al. 2000; Matsui et al. 2004; Tohge et al. 2005; Matoušek et al. 2006). Ectopic expression of genes of *MYB* transcription factors in various plant species has confirmed that these regulatory elements are conserved among different species (Borevitz et al. 2000; Schijlen et al. 2004).

Here, we described the introduction of the gene of the regulatory factor *PAP1/AtMYB75* from *A. thaliana* L. into hop by genetic transformation. Moreover, transgenic plants were evaluated if and to which extent the transgene was expressed. The altered composition and quantity of secondary metabolites and in particular the anthocyanin content was determined and compared to wildtype plants.

## 2.3 Material and methods

### 2.3.1 Plant transformation, regeneration and acclimatization

Genetic transformation of hop (*H. lupulus* L. cv. Tettnanger) was performed with *Agrobacterium tumefaciens* EHA 101 harboring the plasmid pLV-65. The T-DNA contained the regulatory gene *PAP1/AtMYB75* (GenBank accession: AT1G56650.1) under the control of the 35S CaMV promoter and the selection marker *nptII* under the control of the nopaline synthase promoter (Matoušek et al. 2006) (Fig. 2.1.a).

From *in vitro* grown hop plants, 1,000 stem segments (1 cm) were transformed and regenerated to plants (modified from Horlemann et al. 2003). Two days prior to transformation, the explants were pre-cultured on shoot induction medium [MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 2% (w/v) glucose, 1.43 μM IAA, 9.08 μM TDZ and 0.6% (w/v) agar]. Timentin (250 $mgl^{-1}$) (Duchefa, The Netherlands) was used to remove *A. tumefaciens* from plant cultures. Rooted single shoots (5–7 cm length) were acclimated in the greenhouse and then the plants were transferred to an outdoor containment facility.

### 2.3.2 Detection of transgenic plants

Genomic DNA of leaves of WT or transgenic plants was extracted according to Fleischer et al. (2004). Plasmid DNA containing *nptII* and *PAP1/AtMYB75* was used as positive control. All putative transgenic plants were analyzed by triplex PCR for the detection of hop chitinase, *nptII*, and *virG* genes (Horlemann et al. 2003). Twenty transformants were further analyzed to confirm the presence of the transgene *PAP1/AtMYB75* using the primers PAP29.2S (5'-GGAGGGTTCGTCCAAAGG-3') and PAP29.2AS (5'-AGGAATGGGCGTAATGTC-3') (Fig. 2.1.a).

The PCR was performed in a mixture (25 μl) containing 100 ng of genomic DNA, 1X Mango Taq PCR buffer, 0.25 mM of each dNTPs, 0.2 μM of each primer, 1.5 mM of $MgCl_2$ and 2.5 U Mango Taq™ polymerase (Bioline, Germany). The amplification conditions were 94ºC for 3 min followed by 35 cycles at 94ºC for 1 min, 60ºC for 1 min, 72ºC for 1 min and a final step of 72ºC 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.

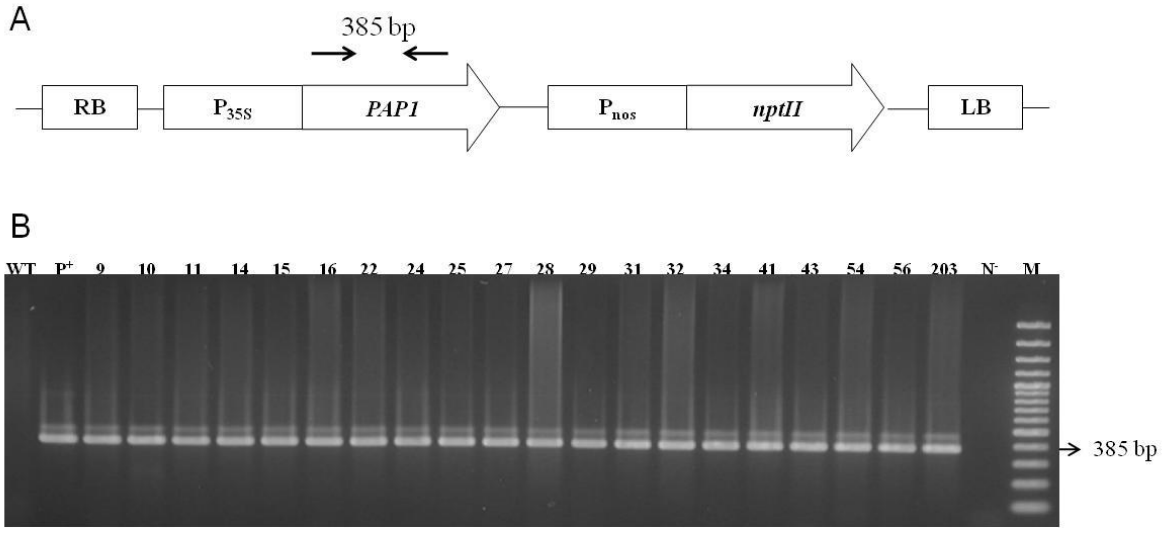



**Fig. 2.1 (A)** Schematic representation of the T-DNA of the pLV 65 vector used for transforming *H. lupulus* L. RB: Right border, P35S: 35S CaMV promoter, *PAP1* regulatory gene *PAP1/AtMYB75*, $P_{nos}$: nopaline synthase promoter, *nptII*: neomycin phosphotransferase II gene, LB: left border. **(B)** PCR analysis for the detection of *PAP1/AtMYB75* gene in transgenic plants 9, 10, 11, 14, 15, 16, 22, 24, 25, 27, 28, 29, 31, 32, 34, 41, 43, 54, 56 and 203. Lanes WT: wildtype, $P^+$: positive control (pLV 65), $N^-$: negative control (PCR reaction mix without template), M: molecular weight marker (100 bp DNA Ladder).

### 2.3.3 RNA isolation and RT-PCR

Total RNA was isolated from WT and transgenic plants using the RNeasy plant mini kit (Qiagen, Germany) with a user-developed lysis buffer (MacKenzie et al. 1997). After RNA extraction, DNA was removed by DNase I treatment (MBI Fermentas, St. Leon-Rot). RNA was purified by using the RNA clean-up protocol from the RNeasy plant mini kit. The total RNA was quantified using a spectrophotometer (NanoPhotometer™, Germany) at wavelengths of 260 and 280 nm, and RNA integrity was verified by analyzing samples on a 1.2% (w/v) denaturing agarose gel.

The transcript of the gene *PAP1/AtMYB75* was analyzed with the One Step RT-PCR Kit (Qiagen, Germany) using the primers PAPshortS (5'-TGGCACCAAGTTCCTGTA-3') and PAPshortAS (5'-AAAGACCACCTATTCCCT-3'). The reactions were carried out in 25 µl containing 1X One Step RT-PCR buffer, 10 mM of each dNTPs, 0.6 µM of each primer, 2 µl of One Step RT-PCR enzyme mix, 3 µl of total RNA (100 ngµl$^{-1}$), and 12 µl of RNase free water. As a positive control for the integrity of RNA, 18S RNA from each sample was amplified using the primers 18S_S: (5'-AGGTAGTGACAATAAATAACAA-3') and 18S_AS: (5'-TTTCGCAGTTGTTCGTCTTTC-3'). For detecting possible DNA contaminations in RNA preparations, each reaction mixture was divided into two aliquots. In one aliquot, the reverse transcriptase was activated at 50ºC for 30 min, and in the other sample the enzyme was inactivated for 15 min at 94ºC. A successive PCR with all the sample was carried out over 30 cycles under the following conditions: 95ºC for 15 min, 95ºC for 1 min, 53ºC for 30 s and 72ºC for 1.5 min, followed by a final elongation step at 72ºC for 10 min. The PCR products were evaluated on 1.5% (w/v) agarose gel, stained with ethidium bromide, visualized under UV and documented with a digital camera.

### 2.3.4 Gene expression analysis of *PAP1/AtMYB75*

Quantitative real-time PCR was used to determine the strength of *PAP1/AtMYB75* gene expression in cones from transgenic and WT plants. For cDNA synthesis, 1 µg of hop total RNA was reverse transcribed in 20 µl for 30 min at 42ºC using 1 µl RT primer mix, 4 µl Quantiscript RT Buffer and 1 µl Quantiscript RT. Then, the mixture was incubated for 3 min at 95ºC to inactivate the reverse transcriptase (Qiagen, Germany). Each PCR reaction was carried out in 20 µl of final volume containing 1X SensiMix$^{TM}$ SYBR Master Mix, 1 µM of each primer [(PAPshortS: 5'-TGGCACCAAGTTCCTGTA-3') and (PAPshortAS: 5'-AAAGACCACCTATTCCCT-3')], 2.5 µl cDNA (40 ngµl), and 3.5 µl RNase free water (Bioline, Germany). Real-time analysis was performed on an Step One$^{TM}$ System (Applied Biosystems, USA) with the following conditions: 95ºC for 10 min followed by 40 cycles at 95ºC for 15 s, 60ºC for 45 s, and 72ºC for 45 s. Melting curve analysis was used to verify PCR identity and single product formation. All samples were measured twice in two independent experiments. The difference between the cycle threshold (Ct) of the target gene and the Ct of GAPDH (ΔCt: $Ct_{Target}$ - $Ct_{GAPDH}$) was used to obtain the normalized expression of *PAP1/AtMYB75*, which corresponds to $2^{-\Delta Ct}$.

### 2.3.5 Extraction procedure and sample preparation

Transgenic and wildtype hop cones were harvested from plants grown outdoors in a containment facility during 2010. After harvest, they were dried at 65ºC and ca. 11% humidity for 24 h. Dried hop cones were ground in a mortar using liquid nitrogen. Then, the samples (40 mg) were homogenized five times for 20 s with 4 ml methanol containing 8 $\mu g ml^{-1}$ umbelliferone (used as internal standard for relative quantification); 1 min separated each mixing period. All extractions were carried out in triplicate. Then, the samples were vortexed vigorously and subsequently cleared by centrifugation for 30 min. Next, 500 µl was placed on a Chromoband C18 cartridge (500 mg) (Macherey & Nagel, Germany) preconditioned with methanol and water. Samples were eluted using 6 ml methanol and the eluent was evaporated under a nitrogen stream and the obtained dry residue was resuspended in 1.5 ml of methanol; 2 µl was used for LC–MS analysis.

### 2.3.6 LC-MS analysis

The LC–ESI and ESI-$MS^n$ mass spectra were obtained from an LCQ Deca XP MAX system (ThermoElectron, San Jose, USA) equipped with an ESI source (electrospray voltage 4.0 kV, sheath gas nitrogen, capillary temperature 275ºC). The Ion Trap MS system is coupled with a Surveyor micro-HPLC and equipped with a HYPERSIL GOLD RP18- column (5 mm, 150 x 1 mm, Thermo Scientific). For HPLC, a gradient system was used starting from $H_2O$:$CH_3CN$ = 90:10 (each of them containing 0.2% HOAc) to 100% $CH_3CN$ within 30 min, then isocratically for a further 10 min; the flow rate was 70 µlmin. All mass spectra were averaged and the background subtracted. The Xcalibur™ 2.0 software was used for data evaluation. The $MS^n$ spectra were recorded during the HPLC run by using the following conditions: MS/MS analysis with starting collision-induced dissociation energy of 20 eV and an isolation width of ±2 amu.

### 2.3.7 Anthocyanins quantification

For the analysis of anthocyanins, transgenic and wildtype hop cones (50 mg) were homogenized five times for 20 s with 5 ml of methanol (0.1% HCl); 1 min separated each mixing period. Samples were vortexed vigorously and subsequently cleared by centrifugation for 10 min. Then, 300 µl was aliquoted, and diluted with 200 µl of milliQ water and 500 µl chloroform. The mixture was vortexed vigorously for 1 min and centrifuged for 2 min. The upper aqueous phase was then aliquoted, diluted with an equal volume of methanol (0.1% HCl) and measured using UV spectrophotometer at 528 nm. Standard calibration curve of cyanidin standard prepared at 1, 10, 100 and 1,000 $\mu g ml^{-1}$ under the same conditions and measured at 528 nm was used to derive relative quantifications of anthocyanins in hops.

## 2.4 Results

### 2.4.1 PCR detection of transformed plants

The *A. thaliana* L. *PAP1/AtMYB75* gene was introduced into the hop genome via *A. tumefaciens*. A total of 20 kanamycin-resistant plants transformed with pLV-65 were obtained from 1,000 infected internodal segments. All the plants showed a signal for *nptII* (640 bp), whereas no signal was observed with the *virG* primer (data not shown). Every tested plant gave signals specific for hop chitinase, confirming the quality of extracted DNA (data not shown). Moreover, the *nptII* positive transgenic plants were tested for the integration of the *PAP1/AtMYB75* gene. When the primer set PAP29.2 was used for amplification, the 20 plants showed a signal for *PAP1/AtMYB75* (385 bp). No bands were observed in wildtype plants (Fig. 2.1.b). The overall transformation frequency [(transgenic plants confirmed by PCR/total internodal segments transformed) * 100] was 2%. The stability of the integrated *PAP1/AtMYB75* and *nptII* genes was assessed over the years 2008–2010. In all transgenic plants, the transgenes were present.

### 2.4.2 Analysis of transgene expression by RT-PCR

RT-PCR analysis was performed with seven transgenic plants (10, 11, 14, 15, 24, 29 and 56) as well as with WT plants. The primer pair PAPshortS and PAPshortAS was used to verify the expression of the gene *PAP1/AtMYB75*. A *PAP1* specific band (163 bp) was amplified from leaves (Fig. 2.2.a) and cones (Fig. 2.2.b) of transgenic plants. In WT plants, no signal was detected. As a positive control, a DNA sample from a transgenic plant was used. In all samples, the quality of RNA was verified by RT-PCR using the 18S primer (481 bp) (Fig. 2.2.c). In the controls where the reverse transcriptase was inactivated, no amplicons were detected (data not shown).

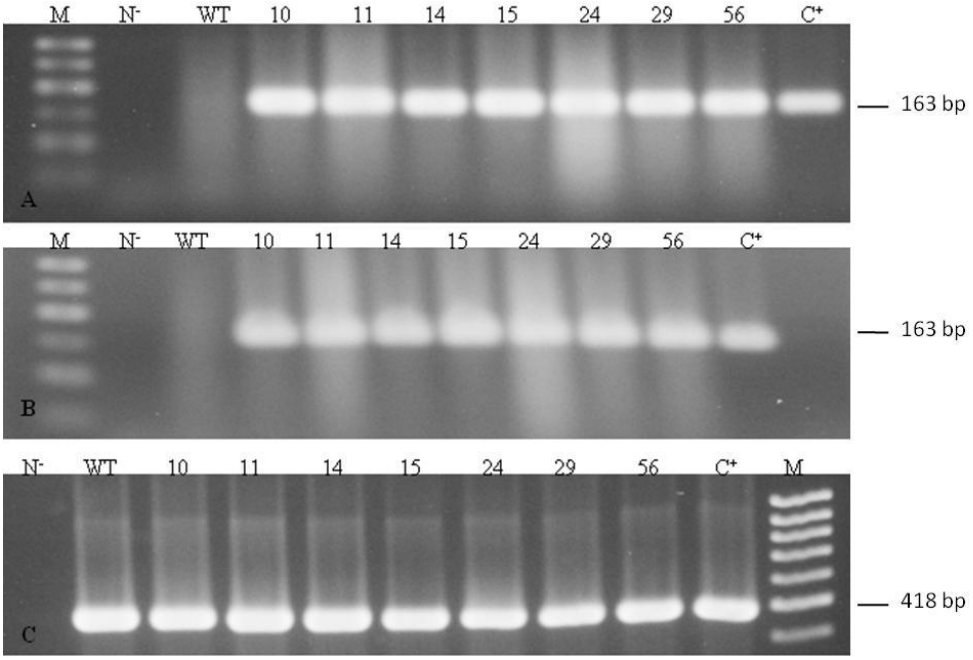



**Fig. 2.2** RT-PCR analysis demonstrating *PAP1/AtMYB75* in **(A)** leaves, **(B)** cones, and **(C)** *18S* expression in transgenic plants 10, 11, 14, 15, 24, 29 and 56. Lanes WT: wildtype, $C^+$: positive control (DNA transgenic plant), $N^-$: negative control (PCR reaction mix without template), M: molecular weight marker (50 bp DNA Ladder).

### 2.4.3 Quantitative analysis of transgene expression by real time PCR

The expression level of the transcription factor *PAP1/AtMYB75* in female cones was compared among seven transgenic hop plants (10, 11, 14, 15, 24, 29 and 56) and conspicuous differences were observed (Fig. 2.3). The highest expression level of *PAP1/AtMYB75* was observed in the transgenic plants no. 24, 29 and 56, in which the relative RNA level was ~1,089, ~614 and ~1,160, respectively. The *PAP1/AtMYB75* expression level of the other transgenic plants range from ~143 to~250 (Fig. 2.3). No *PAP1/AtMYB75* expression was detected in wildtype plants.

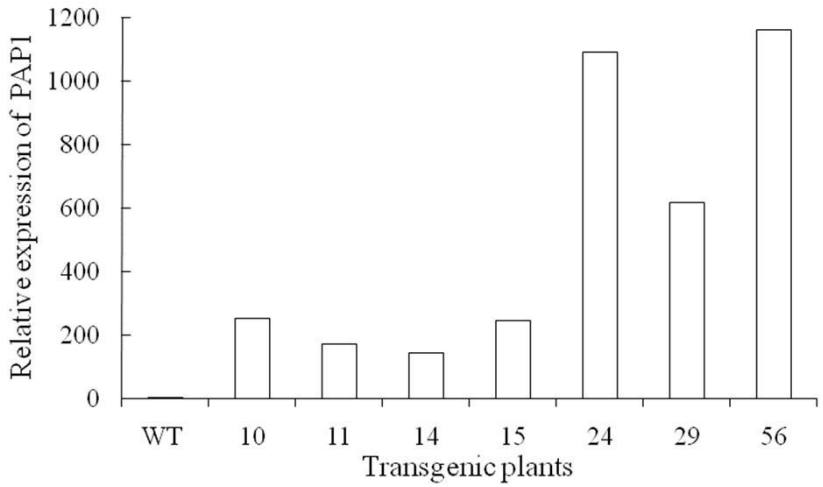



**Fig. 2.3** Relative expression of *PAP1/AtMYB75* in female cones of wildtype and transgenic plants. *GAPDH* was used as housekeeping gene to normalize the expression of the transcription factor *PAP1/AtMYB75*. Each experiment was performed with four replicates.

### 2.4.4 Morphology of transgenic plants and chemical analysis of secondary metabolites

The transgenic *PAP1/AtMYB75* hop plants showed no phenotypical difference to WT plants while growing under tissue culture conditions or in the greenhouse (data not shown). Seven transgenic plants (10, 11, 14, 15, 24, 29 and 56) as well as WT plants were grown under agronomic conditions in an outdoor containment facility. The plants were observed during the vegetation periods of 2008–2010 and at the end of the vegetation period all transgenic plants flowered and produced cones. However, compared to the wildtype plants, the *PAP1/AtMYB75* transgenic plants displayed an unusual coloration. Transgenic plants exhibited reddish to pink pigmentation of flowers and cones, whereas WT plants developed the usual green color of flowers and cones (Fig. 2.4.a, b, c, d). The phenotype of the *PAP1/AtMYB75* transgenic plants was maintained and consistently observed over the years 2008–2010.

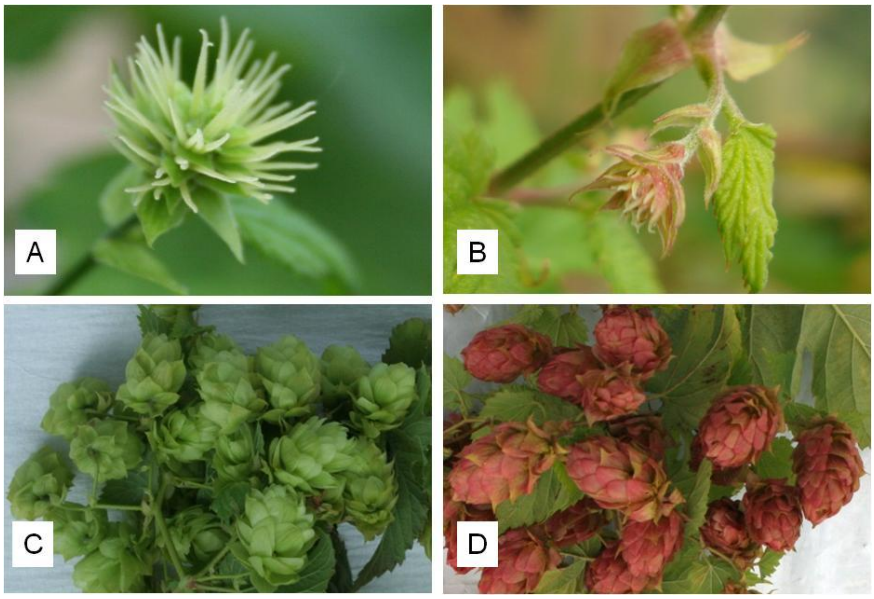



**Fig. 2.4** An example of the phenotype of transgenic hop plant 24 expressing the *PAP1/AtMYB75* gene. **(A)** Wildtype and **(B)** transgenic female flower **(C)** wildtype and **(D)** transgenic cones.

In addition, extracts prepared from hop cones revealed a significant increase in anthocyanins content in transgenic plants compared to WT as revealed by measuring the UV absorbance of extracts at 528 nm (Table 2.1). The expression of *PAP1/AtMYB75* in transgenic plants led to an increase in anthocyanin content. The highest yield of anthocyanins, calculated as cyanidin, was detected in transgenic plant 24 (4.2-fold), 15 (3.5-fold), 29 (3.3-fold), 56 (2.8-fold), 14 (1.8-fold), and 11 (1.5-fold) in comparison to WT plants. Transgenic plant 10 showed no difference compared to the WT (Table 2.1).

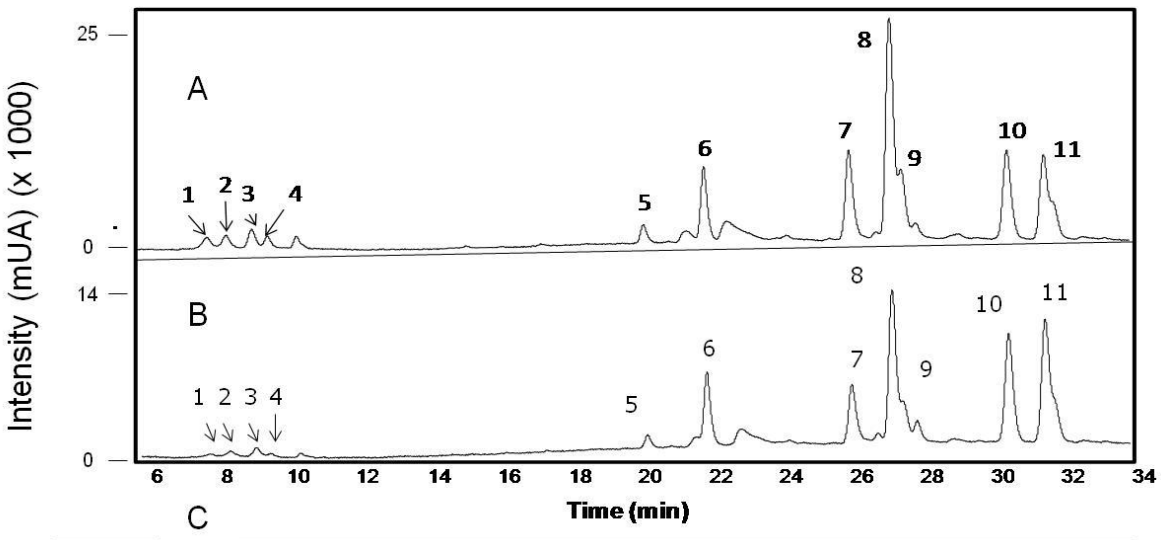
Moreover, the profile of secondary metabolites in both WT and transgenic *PAP1/AtMYB75* hop cones was determined using HPLC coupled to PDA and $MS^n$. Identified metabolites belonged to various classes including chalcones, flavanones, flavonols, and bitter acids with a total of 11 major peaks identified (Fig. 2.5.a, b). Flavonoids from female hop cone extracts were recognized by comparing retention time, UV/Vis and $MS^n$ spectra with compounds reported in the literature and with authentic standards when available (Fig. 2.5.c). The relative quantitative analysis of metabolites to that of spiked internal standard (umbelliferone) in the different transgenic *PAP1/AtMYB75* plants and WT is summarized in Table 2.1

**Table 2.1** Relative quantification (μgml$^{-1}$) of secondary metabolites identified in female cones from wildtype and transgenic *PAP1/AtMYB75* plants using LC-MS and normalized to the internal standard umbelliferone.

| | WT | 10 | 11 | 14 | 15 | 24 | 29 | 56 |
|---|---|---|---|---|---|---|---|---|
| Anthocyanins [a] | 2.8±0.7 | 2.8±0.9 | 4.1±0.2 | 4.9±0.3 | 9.8±2.4 | 11.8±0.9 | 9.2±0.3 | 7.8±1.6 |
| Rutin | 5.3±0.7 | 10.5±1.0 | 21.6±1.2 | 26.3±1.3 | 28.3±6.6 | 32.6±9.3 | 53.3±8.8 | 31.1±8.0 |
| Isoquercitin | 17.5±1.6 | 24.7±3.0 | 32.3±1.7 | 41.1±1.3 | 34.2±8.2 | 38.2±9.6 | 51.2±10.3 | 44.9±11.7 |
| Kaempferol-7-*O*-glucoside | 9.8±1.2 | 16.2±2.0 | 20.3±0.8 | 28.3±1.8 | 22.9±6.0 | 25.7±6.6 | 40.8±7.8 | 28.9±7.1 |
| Kaempferol-7-*O*-glucoside malonate | 16.8±1.5 | 17.3±1.6 | 27.8±3.4 | 29.5±3.8 | 22.8±5.2 | 29.0±7.9 | 50.7±6.7 | 33.1±8.9 |
| Desmethylxanthohumol | 50.5±5.8 | 34.7±2.8 | 48.7±7.2 | 57.5±8.3 | 43.6±11.5 | 49.1±17.1 | 85.5±17.6 | 78.9±23.0 |
| Xanthohumol | 344.2±32.0 | 200.9±7.2 | 246.3±28.0 | 305.7±16.8 | 293.7±80.6 | 313.0±112.7 | 424.7±81.5 | 417.5±106.5 |
| Cohumulone | 686.9±95.9 | 320.1±6.5 | 529.3±50.6 | 467.6±27.0 | 568.4±169.0 | 440.8±174.1 | 1185.3±244.4 | 893.2±246.9 |
| Humulone/adhumulone | 1640.8±225.5 | 758.6±19.1 | 1216.1±84.5 | 1017.8±50.2 | 1329.2±368.2 | 1054.3±403.3 | 2906.2±609.5 | 1978.1±496.1 |
| Colupulone | 1523.5±178.0 | 1181.2±53.9 | 1181.8±135.2 | 1750.8±189.6 | 1716.8±474.1 | 1656.9±592.3 | 1676.9±378.2 | 2516.4±690.6 |
| Lupulone/adlupulone | 1833.4±165.8 | 1495.0±80.5 | 1356.5±94.3 | 2027.7±159.8 | 2236.9±681.0 | 2005.4±663.1 | 1978.9±343.3 | 2888.9±735.4 |

Each value represents the mean (±SD) of 3 replicates. Transgenic and wildtype hop cones were harvested from plants grown outdoors in a containment facility during 2010.

[a] Quantification of total anthocyanins in extracts (μgml$^{-1}$) relative to that of cyanidin standard, measured using UV at 528 nm.




| Peak No. | Compound | Retention time (min) | UV max | [M-H]⁻ | MSⁿ product ions |
|---|---|---|---|---|---|
| 1 | Rutin | 7.76 | 265, 350 | 609 [M-H]⁻ | 301, 271, 255, 179 |
| 2 | Isoquercitin | 8.32 | 265, 350 | 463 [M-H]⁻ | 301, 271, 255, 179 |
| 3 | Isoquercitrin malonate | 9.04 | 285, 350 | 549 [M-H]⁻ | 505, 462, 301 |
| 4 | Astragalin | 9.56 | 265;285shd, 350 | 447 [M-H]⁻ | 285, 255 |
| 5 | Desmethylxanthohumol | 20.25 | 290, 365 | 339[M-H]⁻ | 220, 175, 151,133 |
| 6 | Xanthohumol | 21.89 | 235, 370 | 353[M-H]⁻ | 233, 218,189,165 |
| 7 | Cohumulone | 25.88 | 285, 323 | 347[M-H]⁻ | 278, 235, 223 |
| 8 | Humulone | 27.2 | 285, 325 | 361[M-H]⁻ | 361, 292 |
| 9 | Adhumulone | 27.64 | 285, 325 | 361[M-H]⁻ | 345, 292, 249,237 |
| 10 | Colupulone | 30.41 | 280,333 | 399[M-H]⁻ | 330, 287, 275, 262 |
| 11 | Lupulone/Adlupulone | 31.56/31.81 | 280,335 | 413[M-H]⁻ | 344, 301, 276, 289 |

**Fig. 2.5** Representative LC–MS chromatograms (total ion count) of secondary metabolites identified in **(A)** transgenic, **(B)** WT cones, and **(C)** major secondary metabolites identified in cones. Peak 1: rutin, 2: isoquercitin, 3: isoquercitin malonate, 4: astragalin, 5: desmethyl xanthohumol, 6: xanthohumol, 7: cohumulone, 8: humulone, 9: adhumulone, 10: colupulone, 11: lupulone/adlupulone.

Significant differences were found in flavonol glycosides levels especially that of rutin (quercetin-3-*O*-β-D-rutinoside) and isoquercetin (quercetin-3-*O*-β-D-glucoside) in all transgenic *PAP1/AtMYB75* plants relative to the WT (Table 2.1). In transgenic plant 29, rutin content was detected at 10.1 times that of the wildtype (Table 2.1). Moreover, the highest isoquercetin content was found in the transgenic plant 29 (2.9-fold that in the WT) (Table 2.1). Increase in other flavonol glycosides, i.e., kaempferol-7-*O*-glucoside (astragalin) and kaempferol-7-*O*-glucoside malonate levels, was observed in transgenic plants. Particularly, the highest kaempferol-7-*O*-glucoside and kaempferol-7-*O*-glucoside-malonate content was, respectively, 4.1 and 3.0 times higher in the transgenic plant 29 compared to WT.

The prenylated chalcones xanthohumol and desmethylxanthohumol are naturally present in hop. Nevertheless, the xanthohumol level was lower in most transgenic plants (10, 11, 14, 15 and 24) in comparison to the WT. Exceptions were found in the transgenic plants 29 and 56, which showed, respectively, 1.3 and 1.2 times more xanthohumol than the WT. Similarly, the higher concentration of desmethylxanthohumol was observed in the transgenic plants 29 and 56. Regarding bitter $\alpha$-acids, the transgenic plant 29 showed 1.7 times more cohumulone and 1.8 times more humulone/adhumulone than the WT plants. In contrast, a slight increase in quantity of the $\beta$-acids was observed in the transgenic plants 14, 15, 24, 29 and 56. Principally, in the transgenic plant 56, the colupulone content was 1.7 times and the lupulone/adlupulone content was 1.6 times that of the wild type (Table 2.1).

## 2.5 Discussion

In the present study, genetic engineering of hop (*H. lupulus* L. cv. Tettnanger) with the heterologous transcription factor *PAP1/AtMYB75* from *A. thaliana* L. was successfully accomplished. Previously, this regulatory element has been genetically transformed in other species (Borevitz et al. 2000; Tohge et al. 2005; Matoušek et al. 2006; Xie et al. 2006; Zhou et al. 2008; Zuluaga et al. 2008; Li et al. 2010).

*MYB* transcription factors represent a group of proteins that include the conserved *myb* DNA binding domain. This domain consists of three imperfect repeats (R1, R2, and R3), each forming a helix–turn–helix structure (Stracke et al. 2001). In plants, the *MYB* transcription factor family has been implicated in a range of functions, such as regulation of the flavonoid biosynthetic pathway and developmental steps (Stracke et al. 2001; Matoušek et al. 2007).

The *PAP1* gene encodes the *MYB75* transcription factor, which is a conserved member of the R2R3 gene family and is implicated in the regulation of the biosynthetic pathway of phenylpropanoids in *A. thaliana* L. (Allan et al. 2008; Rowan et al. 2009). When the expression of *MYB* transcription factors was up- or down-regulated, the color of plant organs changed (Allan et al. 2008). It is interesting to note that transgenic hop flower and cones displayed red color suggesting a tissue-specific regulation of flavonoid biosynthesis genes by *PAP1/AtMYB75*. The increase in red pigmentation among transgenic hop is likely to be attributed to anthocyanin compounds, considering that following standard procedure for anthocyanins extraction considerable increase in UV absorbance at 528 nm, typical UV maximum for anthocyanins, was observed in transgenic plants.

Over-expression of *PAP1* in *A. thaliana* L. resulted in red-colored leaves and pink roots and flowers (Borevitz et al. 2000). Moreover, it has been shown that transgenic tobacco plants showed purple leaves, stems, roots, and flowers (Borevitz et al. 2000; Xie et al. 2006), whereas transgenic calli were red or white (Zhou et al. 2008). Transgenic tomato plants expressing *PAP1* showed a red coloration in the veins, rachis, and petioles, as well as a reddish-purple pigmentation in stems, flowers, and fruits (Zuluaga et al. 2008). Furthermore, over-expression of PAP1 in canola resulted in purple plants (Li et al. 2010).

In the present study, it was demonstrated that the *PAP1/AtMYB75* transcription factor increased the production of anthocyanins, rutin, and isoquercitin in transgenic hop plants. This observation is consistent with the biochemical analysis of *PAP1* tomato plants, which showed higher anthocyanins level in transgenic plants than in the wild type (Zuluaga et al. 2008). Moreover, in *A. thaliana* L., the over-expression of *PAP1* resulted in increased biosynthesis of anthocyanins and quercetin glycosides (Borevitz et al. 2000). Furthermore, Zhou et al. (2008) have demonstrated that quercetin was dramatically increased by *PAP1* in transgenic tobacco plants. Recently, Li et al. (2010) reported that the level of cyanidin, pelagonidin, quercitin and sinapic acid increased in transgenic *Brassica napus* expressing the transcription factor *PAP1/AtMYB75*.

The economic value of hop relies on the important secondary metabolites present in the lupulin glands of the female cones, including $\alpha$-acids and $\beta$-acids, which are used as flavoring ingredients in beer production (De Keukeleire et al. 2003). Moreover, the lupulin glands contain important prenylated chalcones, which are of special interest to the pharmaceutical and medicinal field, due to their anti-cancer, anti-proliferative, anti-oxidative and estrogen-like activity (Tagashira et al. 1995; Miranda et al. 2000; Milligan et al. 2002; Chen and Lin 2004; Gerhäuser 2005; Lamy et al. 2007).

Several studies have demonstrated that the concentration of prenylated chalcones (xanthohumol and desmethylxanthohumol), $\alpha$-acids (humulone, cohumulone, and adhumulone) and $\beta$-acids (lupulone, colupulone, and adlupulone) differs among the developmental stages of hop cultivars and cones (Stevens et al. 1997; De Keukeleire et al. 2003; Kavalier et al. 2011). In current hop breeding programs, major focus is directed toward the development of new varieties with high $\alpha$-acids, $\beta$-acids and prenylated chalcones content (Nesvadba et al. 2011). In the last few years, research has been carried out to increase the production of these important secondary metabolites in hop through conventional breeding (Beatson et al. 2003; Nesvadba et al. 2011) or using plant suspension cultures (Pšenáková et al. 2009; Ürgeová et al. 2011).

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 of p-coumaroyl-CoA (Matoušek et al. 2006). The *PAP1/AtMYB75* was capable of activating the *CHS_H1* gene in tobacco and petunia, suggesting that this transcription factor is suitable for modifying the hop metabolome via activation of the *CHS_H1* gene (Matoušek et al. 2006).

In the present study, it was shown that the production of flavonol glycosides (i.e., kaempferol and quercetin conjugates), $\alpha$-acids, $\beta$-acids, xanthohumol, desmethylxanthohumol, kaempferol-glucoside and kaempferol-glucoside-malonate in transgenic hop plants was influenced by the *PAP1/AtMYB75* transcription factor.

Metabolic engineering of plants promises to create new opportunities in agriculture, environmental applications, production of chemicals, and even medicine (Lessard et al. 2002). Increasing the levels of flavonoids in food plants can provide health benefits, as these molecules often have antioxidant activity (Capell and Christou 2004). Several studies have demonstrated that the flavonoid biosynthesis can be engineered using transcription factors, such as the *MYB* genes. The present study demonstrates that the expression of the *PAP1/AtMYB75* transcription factor is a viable method for modifying flavonoid biosynthesis in hop.

## 2.6 Acknowledgements

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## 2.7 References


Allan A, Hellens R, Laing W (2008) *MYB* transcription factors that colour our fruit. Trends Plant Sci 13: 99-102

Beatson RA, Ansell KA, Graham LT (2003) Breeding, development, and characteristics of the hop (*Humulus lupulus*) cultivar 'Nelson Sauvin' New Zealand. J Crop Hortic Sci 31: 303-309

Borevitz J, Xia Y, Blount J, Dixon R, Lamb C. (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell 12: 2383-2394

Capell T, Christou P (2004) Progress in plant metabolic engineering. Curr Opin Biotech 15:148-154

Chadwick L, Pauli G, Farnsworth N (2006) The pharmacognosy of *Humulus lupulus* L. (hops) with an emphasis on estrogenic properties. Phytomedicine 13: 119-131

Chen W, Lin J (2004) Mechanisms of cancer chemoprevention by hop bitter acids (beer aroma) through induction of apoptosis mediated by Fas and caspase cascades. J Agric Food Chem 52: 55-64

De Keukeleire J, Ooms G, Heyerick A, Roldan-Ruiz I, Van Bockstaele, De Keukeleire D (2003) Formation and accumulation of *α*-acids, *β*-acids, desmethylxanthohumol, xanthohumol during flowering of hops (*Humulus lupulus* L.) J Agric Food Chem 51: 4436-4441

Fleischer R, Horlemann C, Schwekendiek A, Kling C, Weber G (2004) AFLP fingerprinting in hop: analysis of the genetic variability of the Tettnang variety. Genet Res Crop Evol 51: 211-220

Gamborg O, Miller R, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151-158

Gerhäuser C (2005) Beer constituents as potential cancer chemopreventive agents. Eur J Cancer 41: 1941-1954

Horlemann C, Schwekendiek A, Hohnle M, Weber G (2003) Regeneration and *Agrobacterium*-mediated transformation of hop (*Humulus lupulus* L.). Plant Cell Rep 22: 210-217

Kavalier AR, Litt A, Ma Ch, Pitra NJ, Coles MC, Kennelly EJ, Matthews PD (2011) Phytochemical and morphological characterization of hop (*Humulus lupulus* L.) cones over five developmental stages using high performance liquid chromatography coupled to time-of-flight mass spectrometry, ultrahigh performance liquid chromatography photodiode array detection, and light microscopy techniques. J Agric Food Chem 59: 4783-4793

Lamy V, Roussi S, Chaabi M, Gossé F, Schall N, Lobstein A, Raul F (2007) Chemopreventive effects of lupulone, a hop β-acid, on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. Carcinogenesis 28: 1575-1581

Lessard P, Kulaveerasingam H, York G, Strong A, Sinskey A (2002) Manipulating gene expression for the metabolic engineering of plants. Metab Eng 4:67-79

Li X, Gao MJ, Pan HY, Cui DJ, Gruber M (2010) Purple canola: *Arabidopsis PAP1* increases antioxidants and phenolic in *Brassica napus* leaves. J Agric Food Chem 58: 1639-1645

MacKenzie D, McLean M, Mukerji S, Green M (1997) Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription polymerase chain reaction. Plant Dis 81: 222-226

Matoušek J, Kocábek T, Patzak J, Škopek J, Maloukh L, Heyerick A, Fussy Z, Roldán-Ruiz I, Keukeleire D (2007) *HlMyb3*, a putative regulatory factor in hop (*Humulus lupulus* L.), shows a diverse biological effects in heterologous transgenotes. J Agric Food Chem 55: 7767-7776

Matoušek J, Vrba L, Škopek J, Orctová L, Pešina K, Heyerick A, Baulcombe D, De Keukeleire D (2006) Sequence analysis of a "true" chalcone synthase (chs_H1) oligofamily from hop (*Humulus lupulus* L.) and PAP1 activation of *chs*_H1 in heterologous systems. J Agric Food Chem 54: 7606-7615

Matsui K, Tanaka H, Ohme-Takagi M (2004) Suppression of the biosynthesis of proanthocyanidin in *Arabidopsis* by a chimeric PAP1 repressor. Plant Biotech J 2: 487-493

Milligan S, Kalita J, Pocock V, Heyerick A, De Cooman L, Rong H (2002) Oestrogenic activity of the hop phyto-oestrogen, 8-prenylnaringenin. Reproduction 123: 235-242

Miranda C, Yang Y, Henderson M, Stevens J, Santana-Rios G, Deinzer M (2000) Prenylflavonoids from hops inhibit the metabolic activation of the carcinogenic heterocyclic amine 2-amino-3-methylimidazol [4, 5-f] quinoline, mediated by cDNA-expressed human CYP1A2. Drug Metab Dispos 28: 1297-1302

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497

Nesvadba V, Krofta K, Poloncikova Z (2011) New knowledge in Czech hop breeding. In: Seigner E (ed) Proceedings of the Scientific Commission International Hop Grower's Convention. Scientific Commission Meeting. Lublin, Poland. http://www.lfl.bayern.de/ipz/hopfen/10585/sc_2011__proceedings.pdf. Accessed 31 July 2011.

Pšenáková I, Gašpárková L, Faragó J (2009) Polyphenol and flavonoid contents of hop callus and cell suspension cultures. In: Seigner E (ed) Proceedings of the Scientific Commission International Hop Grower's Convention. Scientific Commission Meeting. León, Spain. http://www.lfl.bayern.de/ipz/hopfen/10585/index.php#Tagung/Meeting%20in%20Le%C3%B3n,%20Spanien,%20Juni%202009 Accessed 31 July 2011

Rowan D, Cao M, Lin-Wang K, Cooney J, Jensen D, Austin P, Hunt M, Norling C, Hellens P, Schaffer R, Allan A (2009) Environmental regulation of leaf colour in red 35S:PAP1 *Arabidopsis thaliana*. New Phytol 182: 102-115

Sato F, Inui T, Takemura T (2007) Metabolic engineering in isoquinoline alkaloid biosynthesis. Curr Pharm Biotech 8: 211-218

Schijlen E, Ric de Vos C, van Tunen A, Bovy A (2004) Modification of flavonoid biosynthesis in crop plants. Phytochemistry 65: 2631-2648

Stevens JF, Ivancic M, Hsu VL, Deinzer ML (1997) Prenylflavonoids from *Humulus lupulus*. Phytochemistry 44: 1575-1585

Stracke R, Werber M, Weisshaar B (2001) The R2R3-MYB gene family in *Arabidopsis thaliana*. Curr Opin Plant Biol 4: 447-456

Tagashira M, Watanabe M, Uemitsu N (1995) Antioxidative activity of hop bitter acids and their analogues. Biosci Biotech Biochem 59: 740-742

Tian L, Dixon R (2006) Engineering isoflavone metabolism with an artificial bifunctional enzyme. Planta 224: 496-507

Tohge T, Nishiyama Y, Hirai M, Yano M, Nakajima J, Awazuhara M, Inoue E, Takahashi H, Goodenowe D, Kitayama M, Noji M, Yamazaki M, Saito K (2005) Functional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor. Plant J 42: 218-235

Ürgeová E, Polívka L, Faragó J, Vaverková S (2011) Study of the production of secondary metabolites in shoot and callus cultures and field grown plants of hop. In: Seigner E (ed) Proceedings of the Scientific Commission International Hop Grower's Convention Scientific Commission Meeting. Lublin, Poland. http://www.lfl.bayern.de/ipz/hopfen/10585/sc_2011__proceedings.pdf. Accessed 31 July 2011.

Verzele M, De Keukeleire D (1991) Chemistry and analysis of hop and beer bitter acids. Elsevier, Amsterdam p 417

Xie DY, Sharma S, Wright E, Wang ZY, Dixon R (2006) Metabolic engineering of proanthocyanidins through co-expression of anthocyanidin reductase and the PAP1 MYB transcription factor. Plant J 45: 895-907

Yonekura-Sakakibara K, Tohge T, Matsuda F, Nakabayashi R, Takayama H, Niida R, Watanabe-Takahashi A, Inoue E, Saito K (2008) Comprehensive flavonol profiling and transcriptome coexpression analysis leading to decoding gene – metabolite in *Arabidopsis*. Plant Cell 20: 2160-2176

Zhou L, Zeng N, Shi M, Xie D (2008) Development of tobacco callus cultures over expressing *Arabidopsis* PAP1/MYB75 transcription factor and characterization of anthocyanin biosynthesis. Planta 229: 37-51

Zuluaga L, Gonzali S, Loreti E, Pucciariello C, Degl'innocenti E, Guidi L, Alpi A, Perata P (2008) *Arabidopsis thaliana* MYB75/PAP1 transcription factor induces anthocyanin production in transgenic tomato plants. Funct Plant Biol 35: 606-618

# 3. The transcription factor AtMYB75/PAP1 regulates the expression of flavonoid biosynthesis genes in transgenic hop (*Humulus lupulus* L.) [4]

## 3.1 Abstract

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 the 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.

**Keywords** Hop, flavonoids, MYB transcription factor, quantitative real-time PCR, gene expression

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## 3.2 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*-, *ß*-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*-, *ß*-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].

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 *MYB* 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, $\alpha$-acids, and $\beta$-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.

## 3.3 Materials and methods

### 3.3.1 Plant material

Young leaves and mature female hop (*H. lupulus* L cv. Tettnanger) cones were collected (sampling date: 3$^{th}$ 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.

### 3.3.2 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 3.1. 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 50ºC for 30 min and ii) in the other sample the enzyme was inactivated for 15 min at 94ºC. The successive PCR of the sample and the respective control were performed over 30 cycles under the following conditions: 95ºC for 15 min, 95ºC for 1 min, 53ºC for 30 s, 72ºC for 1.5 min. Cycling was followed by a final elongation step at 72ºC 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.

**Table 3.1** Primer sequences used in this study and predicted length of the amplification products

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

### 3.3.3 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 $ng\mu l^{-1}$. The primer pairs PAPshort_S-PAPshort_AS and GPPS.LSU_S-GPPS.LSU_AS were employed for the quantitative real-time PCR (qPCR) analysis (Table 3.1). 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: 95ºC for 3 min followed by 40 cycles at 95ºC for 5 s, 60ºC for 10 s, and 72ºC 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 $2^{\Delta Ct}$ ($\Delta C_t$: $C_{t\ GPPS.LSU} - C_{t\ Target}$) [27].

### 3.3.4 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 70ºC 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. 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 42ºC using 1µl Quantiscript reverse transcriptase, 1 µl RT primer mix, and 4 µl Quantiscript RT Buffer. Then, the reverse transcriptase was inactivated at 95ºC 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 ng$\mu l^{-1}$) 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: 95ºC for 10 min followed by 40 cycles at 95ºC for 15 s, 60ºC for 45 s and 72ºC 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].

### 3.3.5 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 $MS^n$ (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 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.

## 3.4 Results and discussion

### 3.4.1 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. 3.1.a). 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. 3.1.b) [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. 3.1.b) [23]. The correlation between anthocyanin content and relative expression of *AtMYB75/PAP1* was calculated as $r^2$: 0.499 (Fig. 3.1.b).

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].

### 3.4.2 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].

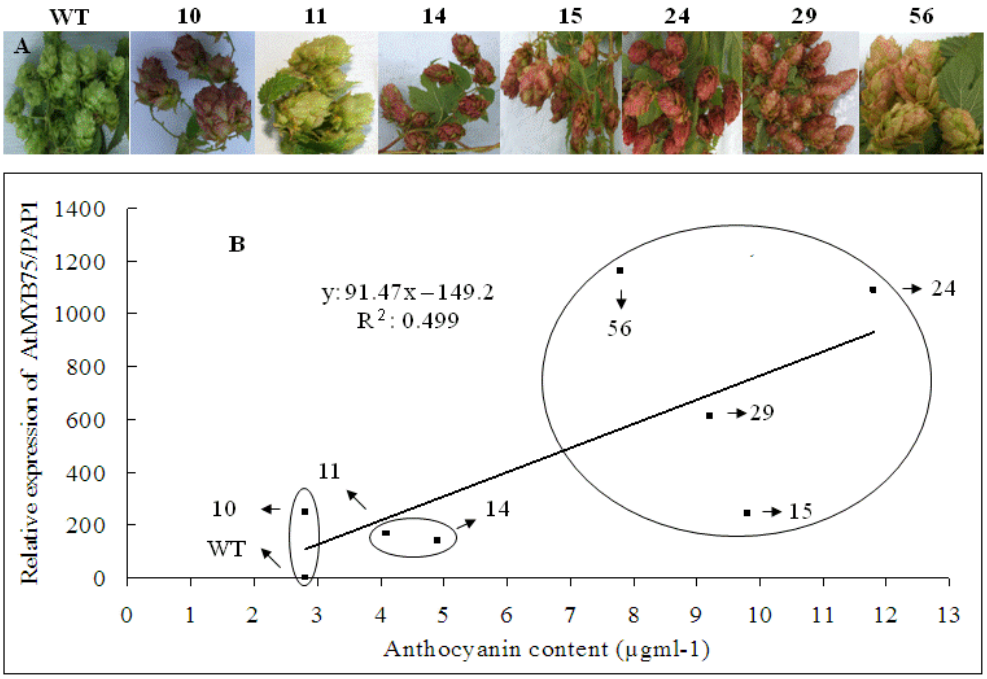


**Fig. 3.1 (A)** Phenotype of the wildtype and transgenic hop female cones expressing the transcription factor AtMYB75/PAP1. **(B)** Correlation of the *AtMYB75/PAP1* relative expression and the anthocyanin content (µg $ml^{-1}$) in wildtype and transgenic hop plants. Data correspond to the means from 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].

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$\mu l^{-1}$ 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 3.2). 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).

**Table 3.2** Estimated copy number of *AtMYB75/PAP1* in transgenic and wildtype hop plants

| Sample | $2^{\Delta CT}$ | | Estimated copy number |
|---|---|---|---|
| | First biological replication[a] | Second biological replication[a] | |
| 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: wild type.

An example of the *AtMYB75/PAP1* amplification plot is shown in the Fig. 3.2. 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. 3.2). 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, the non-specific product in the wildtype control exhibited a melting temperature of 85.56 °C (Fig. 3.2). 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].

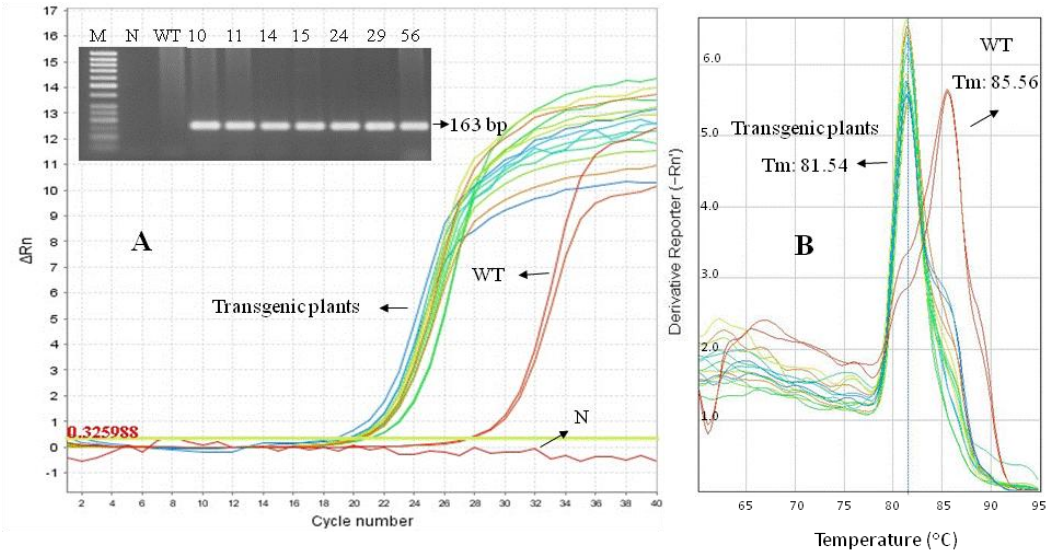


**Fig. 3.2 (A)** Quantitative real-time PCR amplification plot of *AtMYB75/PAP1* gene in transgenic and wildtype plants and resolution of the same PCR products in an agarose gel 1.5%. $C_T$ threshold cycle is indicated. N: negative control (reaction mix without template), WT: wildtype plants, M: molecular maker (50 bp DNA Ladder). **(B)** Melting curve corresponding to the amplification of the *AtMYB75/PAP1* gene in transgenic and wildtype plants.

### 3.3.3 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. 3.3). As positive control a DNA sample from a transgenic plant was used (Fig. 3.3). 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. 3.3). 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.

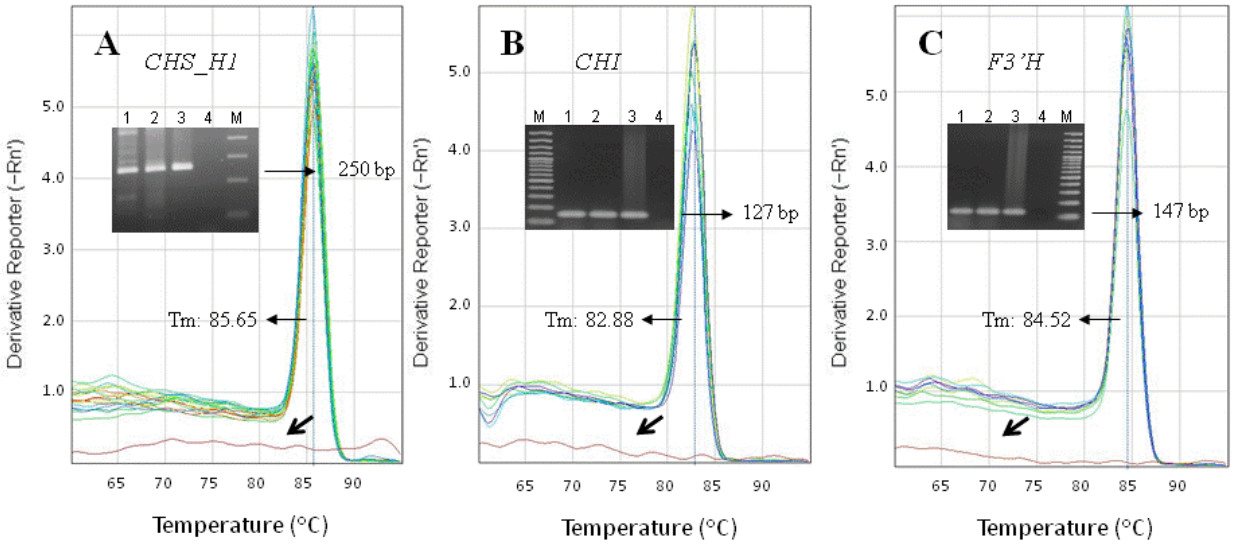


**Fig. 3.3** Specificity of primer pairs for quantitative real-time PCR amplification: 1.5% agarose gel showing a single RT-PCR product of the expected size and melt curve showing a single peak for **(A)** *CHS_H1*, **(B)** *CHI*, and **(C)** *F3´H*. The non template control is indicated by arrows. Lane 1: RNA from leaves, 2: RNA from cones, 3: DNA from leaves, 4: non template control (PCR reaction mix without template), M: molecular maker (1 Kb DNA Ladder).

### 3.4.4 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 was 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. 3.4.a). 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 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. 3.4.b). 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. 3.4.c). 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].

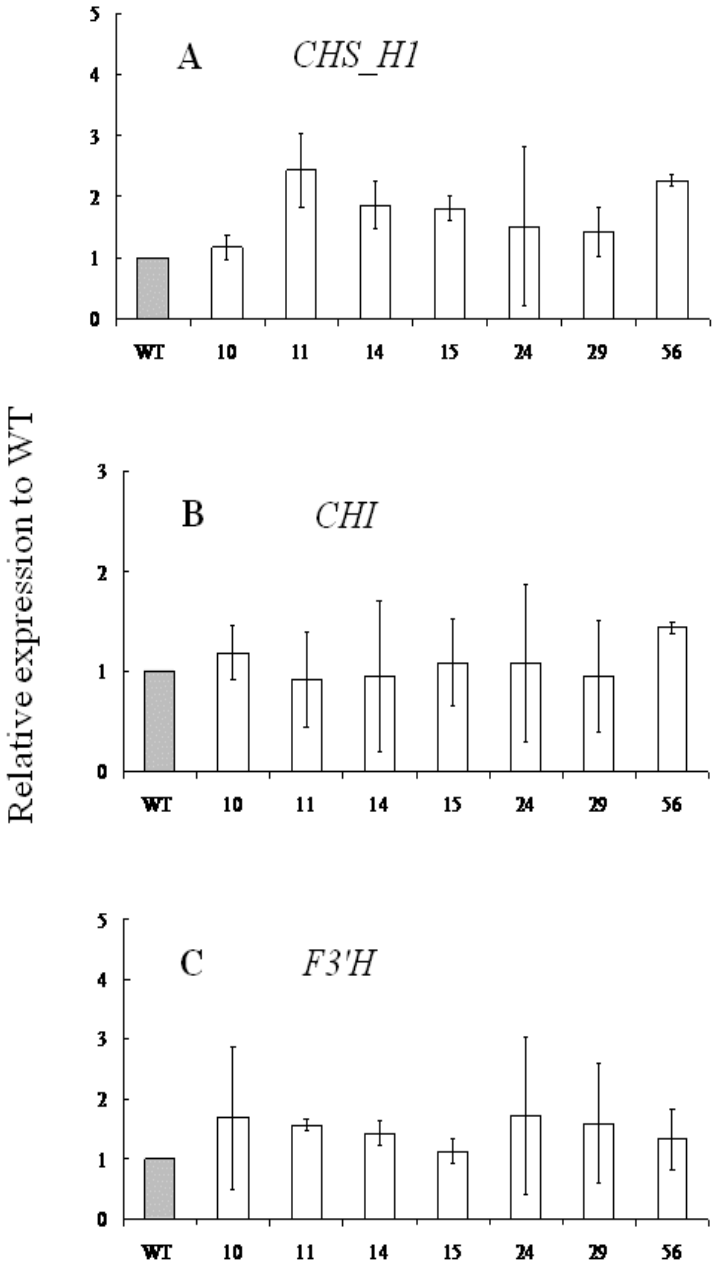


**Fig. 3.4** Normalized mRNA gene expression of **(A)** *CHS_H1*, **(B)** *CHI*, and **(C)** *F3´H* in transgenic (10, 11, 14, 15, 24, 29, and 56) and wildtype hop female cones determined by RT-qPCR. The relative expression is expressed as the fold increases relative to wildtype. WT: wildtype.

### 3.4.5 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 3.5.a, 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 $\alpha$-acids (humulone/adhumulone), and $\beta$-acids (lupulone/adlupulone) contributed to the separation of the transgenic plants (Fig 3.5.b).

In a second PCA analysis, in order to evaluate variation in other metabolites, $\alpha$-acids, and $\beta$-acids analogues were excluded from the data set (Fig 3.6.a). 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 3.6.a). 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 3.6.a), similar to the results derived from all metabolites shown in Fig 3.5.a. 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 3.6.b).

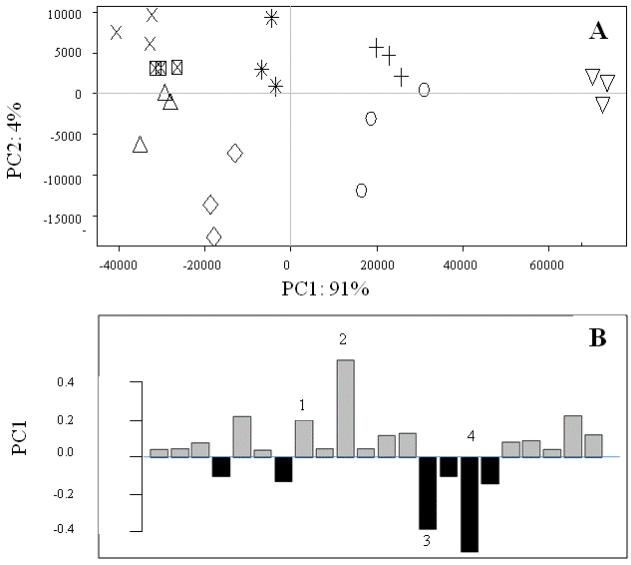


**Fig. 3.5** PCA model of metabolite profile in wildtype and transgenic hop female cones expressing the transcription factor AtMYB75/PAP1. **(A)** PCA characterization (PC1 vs PC2) using the biochemical profile as the analytical data. **(B)** Loading plot for PC1 contributing mass peaks. 1: cohumulone, 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 (✳), 14 (x), 15 (◊), 29 (▽), 24 (☒).

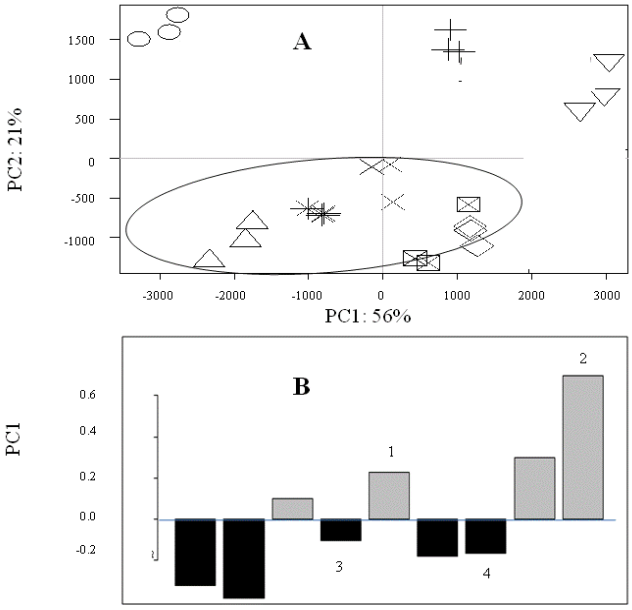


**Fig. 3.6** PCA model of metabolite profile in wildtype and transgenic hop female cones expressing the transcription factor AtMYB75/PAP1. **(A)** PCA characterization (PC1 vs PC2) using only the phenolics profile as the analytical data. **(B)** Loading plot for PC1 contributing mass peaks. 1: astragalin, 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 (✳), 14 (x), 15 (◊), 29 (▽), 24 (☒).

## 3.5 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.

## 3.6 Acknowledgments

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## 3.7 References

1. Martens, S.; Preuß, A. and Matern, U.: Multifunctional flavonoid dioxygenases: Flavonol and anthocyanins biosynthesis in *Arabidopsis thaliana* L., Phytochemistry, **71** (2010), no. 10, pp. 1040-1049.

2. Akagi, T.; Tsujimoto, T.; Ikegami, A.; Yonemori, K.: Effects of seasonal temperature changes on *DkMyb4* expression involved in proanthocyanidin regulation in two genotypes of persimmon (*Diospyros kaki* Thunb.) fruit, Planta, **233** (2011), no. 5, pp. 883-894.

3. Hirchri, I.; Barrieu, F.; Bogs, J.; Kappel, C.; Delrot, S. and Lauvergeat V.: Recent advances in the transcriptional regulation of the flavonoid biosynthesis pathway, Journal of Experimental Botany, **62** (2011), no. 8, pp. 2465-2483.

4. Schijlen, E.; Ric de Vos, C.; van Tunen, A. and Bovy, A.: Modification of flavonoid biosynthesis in crop plants, Phytochemistry, **65** (2004), no. 19, pp. 2631-2648.

5. Pourcel, L.; Routaboul, J.M.; Cheynier, V.; Lepiniec, L. and Debeaujon, I.: Flavonoid oxidation in plants: from biochemical properties to physiological functions, Trends in Plant Science, **12** (2007), no. 1, pp. 29-36.

6. De Keukeleire, J.; Ooms, G.; Heyerick, A.; Roldan-Ruiz, I.; Van Bockstaele, E. and De Keukeleire, D.: Formation and accumulation of α-acids, *β*-acids, desmethylxanthohumol, xanthohumol during flowering of hops (*Humulus lupulus* L.), Journal of Agricultural Food Chemistry, **51** (2003), no. 15, pp. 4436-4441.

7. Miranda, C.L.; Stevens, J.F.; Helmrich, A.; Henderson, M.C.; Rodriguez, R.J.; Yang, Y.H.; Deinzer, M.L.; Barnes, D.W. and Buhler, D.R.: Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (*Humulus lupulus*) in human cancer cell lines, Food and Chemical Toxicology, **37** (1999), no. 4, pp. 271-285.

8. Gerhäuser, C.: Beer constituents as potential cancer chemopreventive agents, European Journal of Cancer, **41** (2005), no. 13, pp. 1941-1954.

9. Lamy, V.; Roussi, S.; Chaabi, M.; Gossé, F.; Schall, N.; Lobstein, A. and Raul, F.: Chemopreventive effects of lupulone, a hop β-acid, on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis, Carcinogenesis, **28** (2007), no. 7, pp. 1575-1581.

10. Zuurbier, K.W.M.; Fung, S.; Sheffer, J.J.C. and Verpoorte, R.: Formation of aromatic intermediate in the biosynthesis of bitter acids in *Humulus lupulus*, Phytochemistry, **38** (1995), no. 1, pp. 77- 82.

11. Wang, G.; Tian, L.; Aziz, N.; Broun, P.; Dai, X.; He, J.; King, A.; Zhao, P.X. and Dixon, R.A.: Terpene biosynthesis in glandular trichomes of hop, Plant Physiology, 148 (2008), no. 3, pp. 1254-1266.

12. Matoušek, J.; Vrba, L.; Škopek, J.; Orctová, L.; Pešina, K.; Heyerick, A.; Baulcombe, D. and De Keukeleire, D.: Sequence analysis of a "true" chalcone synthase (*chs*_H1) oligofamily from hop (*Humulus lupulus* L.) and PAP1 activation of *chs*_H1 in heterologous systems, Journal of Agricultural and Food Chemistry, **54** (2006), no. 20, pp. 7606-7615.

13. Nagel, J.; Culley, L.K.; Lu, Y.; Liu, E.; Matthews, P.D.; Stevens, J.F.: EST analysis of hop glandular trichomes identifies an O-methyltransferase that catalyses the biosynthesis of xanthohumol, Plant Cell, **20** (2008), no. 1, pp. 186-200.

14. Matoušek, J.; Kocabek, T.; Patzak, J.; Fussy, Z.; Prochazkova, J. and Heyerick, A.: Combinatorial analysis of lupulin gland transcription factors from R2R3 Myb, bHLH and WDR families indicates a complex regulation of chs_H1 genes essential for prenylflavonoid biosynthesis in hop (*Humulus lupulus* L.), BMC Plant Biology, **12** (2012), pp. 27.

15. Okada, Y., Ito K.: Cloning and analysis of valerophenone synthase gene expressed specifically in lupulin gland of hop (*Humulus lupulus*). BioScience Biotechnology and Biochemistry, **65** (2001), no. 1, pp. 150- 155.

16. Tsurumaru, Y.; Sasaki, K.; Miyawaki, T.; Momma, T.; Umemoto, N. and Yazaki, K.: An aromatic prenyltransferase-like gene *HlPT-1* preferentially expressed in lupulin glands of hop, Plant Biotechnology, **27** (2010), no. 2, pp. 199-204.

17. Gao, J.J.; Shen, X.F.; Zhang, Z.; Peng, R.H.; Xiong, A.S.; Xu, J.; Zhu, B.; Zheng, J.L. and Yao, Q.H.: The myb transcription factor *MdMYB6* suppresses anthocyanin biosynthesis in transgenic *Arabidopsis*, Plant Cell Tissue and Organ Culture, **106** (2011), no. 2, pp. 235-242.

18. Allan, A.; Hellens, R. and Laing, W.: MYB transcription factors that colour our fruit, Trends Plant Science, **13** (2008), no. 3, pp. 99-102.

19. Capell, T.; Christou, P.: Progress in plant metabolic engineering, Current Opinion in Biotechnology, 15 (2004), no. 2, pp. 148-154.

20. Borevitz, J.; Xia, Y.; Blount, J.; Dixon, R. and Lamb, C.: Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis, Plant Cell, **12** (2000), no. 12, pp. 2383-2394.

21. Matsui, K.; Tanaka, H. and Ohme-Takagi, M.: Suppression of the biosynthesis of proanthocyanidin in *Arabidopsis* by a chimeric PAP1 repressor, Plant Biotechnology Journal, **2** (2004), no. 6, pp. 487-493.

22. Tohge, T.; Nishiyama, Y.; Hirai, M.; Yano, M.; Nakajima, J.; Awazuhara, M.; Inoue, E.; Takahashi, H.; Goodenowe, D.; Kitayama, M.; Noji, M.; Yamazaki, M. and Saito, K.: Functional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor, Plant Journal, **42** (2005), no. 2, pp. 218-235.

23. Gatica-Arias, A.; Farag, M.A.; Stanke, M.; Matoušek, J.; Wessjohann, L. and Weber, G.: Flavonoid production in transgenic hop (*Humulus lupulus* L.) altered by *PAP1/MYB75* from *Arabidopsis thaliana* L., Plant Cell Reports, **31** (2012), no.1, pp. 111-119.

24. Wang, G.; Dixon, R.A.: Heterodimeric geranyl (geranyl)diphosphate synthase from hop (*Humulus lupulus* L.) and the evolution of monoterpene biosynthesis, Proceedings of the National Academy of Sciences, **106** (2009), no. 24, pp. 9914-9919.

25. Udvardi, M.K.; Czechowski, T.; Scheible, W.R.: Eleven golden rules of quantitative RT-PCR, The Plant Cell, **20** (2008), no. 7, pp. 1736-1737.

26. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; Vandesompele, J.; Wittwer, C.T.: The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clinical Chemistry, **55** (2009), no. 4, pp. 611-622.

27. Gaines, T.A.; Zhang, W.; Wang, D.; Bukun, B.; Chisholm, S.T.; Shaner, D.L.; Nissen, S.J.; Patzoldt, W.L.; Tranel, P.J.; Culpepper, A.S.; Grey, T.L.; Webster, T.M.; Vencill, W.K.; Sammons, R.D.; Jiang, J.; Preston, C.; Leach, J.E. and Westra, P.: Gene amplification confers glyphosate resistance in *Amaranthus palmeri*, Proceedings of the National Academy of Sciences, **107** (2010), no. 3, pp. 1029-1034.

28. McKenzie, D.; McLean, M.; Mukerji, S. and Green, M.: Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription polymerase chain reaction, Plant Disease, **81** (1997), no. 2, pp. 222-226.

29. Livak, K.; Schmittgen, T.: Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta] CT method, Methods, **25** (2001), no. 4, pp. 402-408.

30. Xie, D.Y.; Sharma, S.; Wright, E.; Wang, Z.Y. and Dixon, R.: Metabolic engineering of proanthocyanidins through co-expression of anthocyanidin reductase and the PAP1 MYB transcription factor, Plant Journal, **45** (2006), no. 6, pp. 895-907.

31. Zhou, L.; Zeng, N.; Shi, M. and Xie, D.: Development of tobacco callus cultures over expressing *Arabidopsis* PAP1/MYB75 transcription factor and characterization of anthocyanin biosynthesis, Planta, **229** (2008), no. 1, pp. 37-51.

32. Zuluaga, L.; Gonzali, S.; Loreti, E.; Pucciariello, C.; Degl'innocenti, E.; Guidi, L.; Alpi, A. and Perata, P.: *Arabidopsis thaliana* MYB75/PAP1 transcription factor induces anthocyanin production in transgenic tomato plants, Functional Plant Biology, **35** (2008), no. 7, pp. 606-618.

33. Li, X.; Gao, M.J.; Pan, H.Y.; Cui, D.J. and Gruber, M.: Purple canola: *Arabidopsis PAP1* increases antioxidants and phenolic in *Brassica napus* leaves, Journal of Agricultural and Food Chemistry, **58** (2010), no. 3, pp. 1639-1645.

34. Beltrán, J.; Jaimes, H.; Echeverry, M.; Ladino, Y.; López, D.; Duque, M.C.; Chavarriaga, P. and Tohme, J.: Quantitative analysis of transgenes in cassava plants using real-time PCR technology, In Vitro Cellular & Developmental Biology - Plant, **45** (2009), no., 1, pp. 48-56.

35. Wen, L.; Tan, B. and Guo, W.W.: Estimating transgene copy number in precocious trifoliate orange by TaqMan real-time PCR, Plant Cell, Tissue and Organ Culture, **109** (2012), no., 2, pp. 363-371.

36. Yi, C.X.; Zhang, J.; Chan, K.M.; Liu, X.K.; Hong, Y.: Quantitativereal-timePCR assay to detect transgene copy number in cotton (*Gossypium hirsutum*), Analytical Biochemistry, 35 (2008), no. 1, pp. 150-152.

37. Song, P.; Cai, C.Q.; Skokut, M.; Kosegi, B.D. and Petolino, J.F.: Quantitative real-time PCR as a screening tool for estimating transgene copy number in WHISKERS™-derived transgenic maize, Plant Cell Reports, **20** (2002), no. 10, pp. 948-954.

38. Yang, L.; Ding, J.; Zhang, C.; Jia, J.; Weng, H.; Liu, W.; Zhang, D.: Estimating the copy number of transgenes in transformed rice by real-time quantitative PCR, Plant Cell Reports, **23** (2005), no. 10-11, pp. 759-763.

39. Casu, R.E.; Selivanova, A. and Perroux, J.M.: High-throughput assessment of transgene copy number in sugarcane using real-time quantitative PCR, Plant Cell Reports, **31** (2012), no. 1, pp. 167-177.

40. Giglio, S.; Monis, P.T. and Saint, C.P.: Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR, Nucleic Acids Research, 15 (2003), no. 22, pp. e136.

41. Karsai A, Muller S, Platz S, Hauser MT.: Evaluation of a homemade SYBR® Green I reaction mixture for real-time PCR quantification of gene expression, Biotechniques, 32 (2002), no. 4, pp. 790-796.

42. Mehrtens, F.; Kranz, H.; Bednarek, P. and Weisshaar, B.: The *Arabidopsis* transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis, Plant Physiology, **138** (2005), no. 2, pp. 1083-1096.

43. Stracke, R.; Ishihara, H.; Huep, G.; Barsch, A.; Mehrtens, F.; Niehaus, K. and Weisshaar, B.: Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling, The Plant Journal, **50** (2007), no. 4, pp. 660–677.

44. Butelli, E.; Titta, L., Giorgio, M.; Mock, H.P.; Matros, A.; Peterek, S.; Schijlen, E., Hall, R.D., Bovy, A.G., Luo, J. and Martin, C.: Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors, Nature Biotechnology, **26** (2008), no. 11, pp. 1301-1308.

45. Yu, O.; Shi, J.; Hession, A.O.; Maxwell, C.A.; McGonigle, B. and Odell, J.T.: Metabolic engineering to increase isoflavone biosynthesis in soybean seed, Phytochemistry, **63** (2003), no. 7, pp. 753-763.

46. Bovy, A.; de Vos, R.; Kemper, M.; Schijlen, E.; Almenar, M.; Muir, S.; Collins, G.; Robinson, S., Verhoeyen, M., Hughes, S., Santos-Buelga, C. and van Tunen, A.: High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes LC and C1, The Plant Cell, **14** (2002), no. 10, pp. 2509-2526.

47. Gonzalez, A.; Zhao, M.; Leavitt, J.M. and Lloyd, A.M.: Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings, The Plant Journal, **53** (2008), no. 5, pp. 814-827.

48. Goodacre, R.; Shann, B.; Gilbert, R.J.; Timmins, E.A; McGovern, A.C.; Alsberg, B.K.; Kell, D.B. and Logan, N.A.: Detection of the dipicolinic acid biomarker in *Bacillus* spores using Curie-point pyrolysis mass spectrometry and fourier transform infrared spectroscopy, Analytical Chemistry, **72** (2000), no. 1, pp. 119-127.

49. Granato, D.; Katayama, F.C.U. and Castro, I.A.: Assessing the association between phenolic compounds and the antioxidant activity of Brazilian red wines using chemometrics, LWT- Food Science and Technology, **43** (2010), no. 10, pp. 1542-1549.

50. Arvanitoyannis, I.S; Katsota, M.N.; Psarra, E.P.; Soufleros, E.H. and Kallithraka, S.: Application of quality control methods for assessing wine authenticity: use of multivariate analysis (chemometrics), Trends in Food Science and Technology, **10** (1999), no. 10, pp. 321-336.

51. Choi, H.K.; Choi, Y.H.; Verberne, M.; Lefeber, A.W.M.; Erkelens, C. and Robert, V.: Metabolic fingerprinting of wild type and transgenic tobacco plants by 1H NMR and multivariate analysis technique, Phytochemistry, **65** (2004), no. 7, pp. 857-864.

52. Watson, D.G.; Peyfoon, E.; Zheng, L.; Lu, D.; Seidel, V.; Johnston, B.; Parkinson, J. and Fearnley, J.: Application of principal components analysis to 1H-NMR data obtained from propolis samples of different geographical origin, Phytochemistry Analysis, **17** (2006), no. 5, pp. 323-331.

53. Pichichero, E.; Canuti, L. and Canini, A.: Characterisation of the phenolic and flavonoid fractions and antioxidant power of Italian honeys of different botanical origin, Journal of the Science of Food and Agriculture **89** (2009), no. 4, pp. 609-616.

54. Jiao, Z.; Si, X.X.; Li, G.K.; Zhang, Z.M. and Xu, X.P.: Unintended compositional changes in transgenic rice seeds (*Oryza sativa* L.) studied by spectral and chromatographic analysis coupled with chemometrics methods, Journal of Agricultural Food and Chemistry, **58** (2010), no. 3, pp. 1746-1754.

55. Picone, G.; Mezzetti, B.; Babini, E.; Capocasa, F.; Placucci, G. and Capozzi, F.: Unsupervised principal component analysis of NMR metabolic profiles for the assessment of substantial equivalence of transgenic grapes (*Vitis vinifera*), Journal of Agricultural Food and Chemistry, **59** (2011), no. 17, pp. 9271–9279.

56. Chang, Y.; Zhao, C.; Zhu, Z.; Wu, Z.; Zhou, J.; Zhao, Y.; Lu, X. and Xu, G.: Metabolic profiling based on LC/MS to evaluate unintended effects of transgenic rice with cry1Ac and sck genes, Plant Molecular Biology, **78** (2012), no. 4-5, pp. 477-487.

# 4. Over-expression of the transcription factor HlMYB3 in transgenic hop (*Humulus lupulus* L. cv. Tettnanger) modulates the expression of genes involved in the biosynthesis of flavonoids and phloroglucinols[5]

## 4.1 Abstract

Genes belonging to the R2R3 MYB transcription factor family play an important role in the regulation of the expression of structural genes involved in the biosynthesis of flavonoids. In this study, the regulatory gene s-*HlMYB3* under the control of the CaMV 35S promoter was genetically transformed into hop cv. Tettnanger in order to investigate its effect on the expression of genes involved in the biosynthesis of flavonoids and phloroglucinols. The transgene s-*HlMYB3* was stably introduced and expressed in the transgenic plants. Quantitative real-time PCR indicated that *HlMYB3* was highly over-expressed in transgenic events compared to wildtype plants. The effect of the over-expression of the transgene on the expression rate of structural flavonoid and phloroglucinol biosynthetic genes, like *PAL*, *C4H*, *4CL*, *CHS_H1*, *CHI*, *F3H*, *F3´H*, *FLS*, *F3´5´H*, *OMT1*, *HlPT1*, and *VPS*, as well as the hop regulatory genes *HlMYB1*, *HlMYB2*, *HlMYB7*, *HlbZip1*, *HlbZip2*, *HlbHLH2*, and *WDR1* was examined. Transgenic events with an elevated expression of genes of flavonoid and phloroglucinol biosynthesis were identified. In hop flavonoids and phloroglucinols play an important role as flavoring compounds in beer brewing. Moreover, prenylated chalcones (e.g. xanthohumol and desmethylxanthohumol) have received considerable attention due to their pharmacological properties. Metabolic engineering using genes of homologous MYB transcription factors may open the possibility for increasing the production of important secondary metabolites in hop.

**Keywords** Hop, *HlMYB3*, R2R3 MYB transcription factors, genetic transformation, flavonoid biosynthesis, phloroglucinol biosynthesis

[5] This chapter consists of an unpublished manuscript that has been submitted to Plant Cell Tissue and Organ Culture

## 4.2 Introduction

Hop (*Humulus lupulus* L.) has been mainly cultivated as material for the brewing industry. The secondary metabolites contained in the lupulin glands are responsible for flavoring and preserving beer. In addition, female cones of hop have been used for medicinal purposes (Zanoli and Zavatti 2008). Particularly, pharmacologically relevant compounds, such as xanthohumol, and desmethylxanthohumol have received considerable attention due to their potential anti-cancer, anti-proliferative, and anti-oxidative properties (Stevens and Page 2004).

In hop, research has been focused on the development of new cultivars with a high content of important secondary metabolites (Lutz et al. 2012). In this sense, metabolic engineering offers tremendous potential to modify, improve, and enhance the production and quality of flavonoids. Toward this goal, the flavonoid production in plants could be enhanced through the genetic manipulation of the structural genes or by the introduction of homologous or heterologous regulatory elements (Schijlen et al. 2004).

In plants, the biosynthesis of flavonoids has been intensively studied. It has been demonstrated that the activity of the genes involved is largely regulated at the transcriptional level (Schijlen et al. 2004). The R2R3 MYB and/or a basic helix-loop-helix (bHLH) transcription factors, together with WD40 or WDR1 proteins, are important for regulating this pathway (Hichri et al. 2011).

Ectopic expression of MYB transcription factors in transgenic plants has confirmed that these regulatory elements stimulated the production and enhanced the quantity of anthocyanins and flavonoids (Gonzalez et al. 2008; Zhou et al. 2008; Zuluaga et al. 2008; Li et al. 2010). Furthermore, the transcription factor AtMYB75/PAP1 (production of anthocyanin pigment 1) was introduced into *H. lupulus* L. cv. Tettnanger and the production of flavonoids in transgenic clones was enhanced (Gatica-Arias et al. 2012a).

In the present study, we described the introduction of the homologous transcription factor s-HlMYB3 from *H. lupulus* L. into hop by *Agrobacterium*-mediated genetic transformation. The gene s-*HlMYB3* was originally characterized by Matoušek et al. (2007). It was interesting to investigate how the over-expression of s-*HlMYB3* affects the expression of structural flavonoid and phloroglucinol genes, as well as hop regulatory genes of the phenylpropanoid pathway.

## 4.3 Material and methods

### 4.3.1 Plant transformation procedure

Genetic transformation of hop (*H. lupulus* L. cv. Tettnanger) and transgenic plant regeneration was performed following the procedure described by Gatica-Arias et al. (2012a). The T-DNA of the plasmid pLV-71 harbors the regulatory gene s-*HlMYB3* under the control of the CaMV 35S promoter, as well as the selection marker *nptII* (neomycin phosphotransferase II) under the control of the nopaline synthase promoter (Matoušek et al. 2007). The integrity of the construct was verified by sequencing (commercial service, GATC Biotec AG, Konstanz, Germany). The plasmid pLV-71 was introduced by the freeze-thaw method into *Agrobacterium tumefaciens* strain EHA 101, which was subsequently used for the stable transformation of hop.

### 4.3.2 Primer design and assessment of specificity

Primers were designed for the identification of the transgene, genes involved in flavonoid and phloroglucinols biosynthesis, as well as regulatory elements. Primers used are listed in the Table 4.1. The primer sequences of *HlMYB3* (AM501509.1), *CHS_H1* (AM263201.1), *CHI*, *F3´H*, *OMT1* (EU309725.1), *HlMYB2* (FN646081.1), *HlMYB7* (FR873650), *HlbZip1* (FN395065.1), *HlbZip2* (AM998490.1), and *WDR1* (FN689721.1) were obtained from previous studies (Matoušek et al. 2006; Matoušek et al. 2007; Nagel et al. 2008; Matoušek et al. 2010; Gatica-Arias et al. 2012b; Matoušek et al. 2012). Specific primers for *4CL* (NM101899.3), *F3H* (NM114983.3), *FLS* (NM120951.2), and *F3´5´H* (AY093085) were designed based on *A. thaliana* L. sequences. Development of primers for *PAL* (EF624245.1), *C4H* (FJ617541.1), *HlMYB1* (AJ876882.1), *HlPT1* (AB543053.1), *VPS* (AB015430.1), and *HlbHLH2* (FR751553.1) were based on hop sequences. The primers were developed using Primer Premiers (Premier Biosoft International) and synthesized by Biomers (Ulm, Germany). Primer specificity was confirmed by blasting each primer sequence against the nucleotide collection available at National Center for Biotechnology Information. Moreover, PCR products were sequenced to confirm the amplification of specific genes (commercial service, GATC Biotec AG, Konstanz, Germany).

### 4.3.3 Verification of transgenic plants and estimation of *HlMYB3* copy number

Genomic DNA of leaves (sampling date: 5th August 2011) of transgenic and wildtype plants were extracted according to Gatica-Arias et al. (2012a). Plasmid DNA, containing s-*HlMYB3* and *nptII* genes, was used as positive control. All putative transgenic plants were analyzed by triplex PCR for the detection of hop *chitinase*, *nptII*, and *virG* genes (Horlemann et al. 2003). Six transgenic plants, which belongs to 3 different events [(event 1: 2, and 209), (event 2: 137, and 233), (event 3: 129, and 224)], were further analyzed to confirm the presence of the transgene s-*HlMYB3* using the primers HlMYB3-2-F and HlMYB3-2-R (Table 4.1). PCR reactions were performed in a volume of 50 µl containing 100 ng of genomic DNA, 1X Mango *Taq* PCR buffer, 0.25 mM of each dNTPs, 0.3 µM of each primer, 1.5 mM of $MgCl_2$ and 0.5 U Mango *Taq*™ polymerase (Bioline, Germany).

Amplification conditions were 94ºC for 3 min followed by 35 cycles at 94ºC for 1 min, 60ºC for 1 min, 72ºC for 1 min and a final step of 72ºC for 10 min. After staining with DNA Stain G (Serva, Germany), the PCR products were evaluated on 1.5% (w/v) agarose gel, visualized under UV, and documented with a digital camera. Moreover, PCR fragments were recovered from gel, purified using the NucleoSpin ® Extract II Kit (Macherey-Nagel GmbH & Co, Germany) sequenced (commercial service, GATC Biotec AG, Konstanz, Germany) and the nucleotides sequences were compared with those deposited in the National Center for Biotechnology Information.

For the estimation of *HlMYB3* copy number, the primer set HlMYB3-1-F/ HlMYB3-1-R and GPPS.LSU-S/ GPPS.LSU-AS were employed for the quantitative real-time PCR analysis (Table 4.1). The *GPPS.LSU* gene was used as endogenous one copy gene (Wang and Dixon 2009). To obtain a standard curve for *HlMYB3*, and *GPPS.LSU* genomic DNA was diluted to final concentration of 250, 50, 10, and 2 $ng\mu l^{-1}$. The reactions were carried out in 15 µl containing 1X SensiFAST SYBR Hi-ROX buffer, 0.5 µM of each primer, 5 µl of total DNA (20 $ng\mu l^{-1}$), and 6 µl of RNase free water (Bioline, Germany). Quantitative real-time PCR analysis was performed on a StepOne™ System (Applied Biosystems, USA) following the requirements described by Bustin et al. (2009). Cycling conditions were: 95ºC for 3 min followed by 40 cycles at 95ºC for 5 s, 60ºC for 10 s and 72ºC for 5 s. Melting curve analysis and agarose gel electrophoresis were used to verify single PCR product formation. *HlMYB3* copy number was calculated as $2^{-\Delta\Delta Ct}$ (Ingham et al. 2001). Values are the mean ± SD of two biological replications in which each sample was measured in triplicate.

**Table 4.1** Primer sequences and predicted length of the amplification products used in this study

| Gen | Primer | Sequence (5' - 3') | PCR product size (bp)[1] | Annealing temperature (ºC) | Primer sequences obtained from |
|---|---|---|---|---|---|
| *s-HlMYB3* | HlMYB3-1-F<br>HlMYB3-1-R | gacgtcaacagcaagcaattc<br>ggcctctgacgtgtctgatg | 201 | 60 | Matoušek et al. 2007 |
| | HlMYB3-2-F<br>HlMYB3-2-R | catggacggttgaggaagat<br>ttaccccaccgagaatgaag | 214 | 60 | This study |
| *HlMYB1* | HlMYB1-1-F<br>HlMYB1-1-R | ctcaacttggctcggttctc<br>ctggtgttcccatttgttcc | 209 | 60 | This study |
| *HlMYB2* | HlMYB2-1-F<br>HlMYB2-1-R | tagtgggtcagagtacagtgctcat<br>caacctcgagaagctgctgata | 233 | 60 | Matoušek et al. 2012 |
| *HlMYB7* | HlMYB7-1-F<br>HlMYB7-1-R | accaacacgaccaccacaat<br>tgcggataaggagggctaat | n.a | 60 | Matoušek et al. 2012 |
| *HlbZip1* | Hl-bZip1-1-F<br>Hl-bZip1-1-R | agtggtacttcgggcagagg<br>cgtgctttctcatcctccag | 182 | 60 | Matoušek et al. 2010 |
| *HlbZip2* | HlbZip2-1-F<br>HlbZip2-1-R | tcactctgatcgacccgac<br>aagcagaaagtcctcgagc | 200 | 60 | Matoušek et al. 2010 |
| *HlbHLH2* | HlbHLH2-1-F<br>HlbHLH2-1-R | gaccagcggagcgggttgac<br>ctctggccgagttgacggcg | 156 | 60 | This study |
| *HlWDR1* | HlWDR1-1-F<br>HlWDR1-1-R | ttgcttagattggcttggaataa<br>ccggctgagcagatatgtctat | 188 | 60 | Matoušek et al. 2012 |
| *PAL* | HlPAL-1-F<br>HlPAL-1-R | ccgaagtcttgtcagccatt<br>tggggtgatgtcctaagagc | 226 | 60 | This study |
| *C4H* | HlC4H-1-F<br>HlC4H-1-R | ccactggaagaagccagaag<br>tctgcaccaaacgtccaata | 176 | 60 | This study |
| *4CL* | Hl4CL-1-F<br>Hl4CL-1-R | tccgatagccttaacggttg<br>ccatagccctgtccaagtgt | 156 | 60 | This study |
| *CHS_H1* | CHS-H1short-S<br>CHS-H1short-AS | atcactgccgtcactttc<br>aaataagcccaggaacatc | 250 | 55 | Matoušek et al. 2006 |
| *CHI* | CHIshort-S<br>CHIshort-AS | caactgccctcaactcaa<br>tttcttcctcaagccaac | 127 | 56 | Gatica-Arias et al. 2012b |
| *F3H* | HlF3H-2F<br>HlF3H-2R | cacctgaaacagtccccaat<br>gggagaaaactctccgatcc | 185 | 60 | This study |
| *F3´H* | F3´Hshort-S<br>F3´Hshort-AS | tcaggtccacgatgccaatt<br>gccggagaaaagatgaacagaa | 147 | 60 | Gatica-Arias et al. 2012b |
| *FLS* | HlFLS-2-F<br>HlFLS-2-R | atcactggggaagcttgttg<br>gtaaggatgtcgtggccagt | 154 | 60 | This study |
| *F3´5´H* | HlF3´5´H-1-F<br>HlF3´5´H-1-R | ggaaacttttcaggcaccaa<br>tgcactcgtttgagtggaag | 181 | 60 | This study |
| *OMT1* | OMT1-S<br>OMT1-AS | taaaggaacagtggtggacgttg<br>accgcatcagcactaggaattga | 186 | 60 | Nagel et al. 2008 |
| *HlPT1* | HlPT1-1-F<br>HlPT1-1-R | cgacctcctgaatctggaaa<br>cattcccaagagtgccctaa | 192 | 60 | This study |
| *VPS* | VPS-S<br>VPS-AS | gttatgccggtggaaaa<br>ccggcttccgttacg | 298 | 55 | Matoušek (personal communication) |
| *GPPS.LSU* | GPPS.LSU-S<br>GPPS.LSU-AS | cattccaaaccccaaaacaaa<br>gactgcggaaatggatgaaaa | n.a | 60 | Wang and Dixon 2009 |
| *7SL-RNA* | Hl-7SL-RNA-F<br>Hl-7SL-RNA-R | tgtaacccaagtgggggg<br>gcaccggcccgttatcc | 160 | 60 | Maloukh et al. (2009) |
| *GAPDH* | GAPDH_S<br>GAPDH_AS | accggagccgactttgttgttgaa<br>tcgtactctggcttgtattccttc | 165 | 60 | Nagel et al. 2008 |

n.a: Data not available

[1] The tool PCR Products from the Sequence Manipulation Site was used to determinate the product sizes (http://www.bioinformatics.org/sms2/pcr_products.html)

### 4.3.4. RNA isolation and RT-PCR

Mature female hop cones (consisting of bracts and lupulin glands) and leaves from one wildtype plant and from one of each *HlMYB3* transgenic plants (2, 209, 137, 233, 129 and 224) were collected during the vegetation period 2011 (sampling date: 8$^{th}$ September 2011) and immediately immersed in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was isolated using the ISOLATE Plant RNA Mini kit (Bioline, Germany) following the procedure described by Gatica-Arias et al. (2012b). After RNA extraction, DNA was removed by DNase I treatment (MBI Fermentas, St. Leon-Rot). The total RNA was quantified using a spectrophotometer (NanoPhotometer$^{TM}$, Germany) at wavelengths of 260 and 280 nm. The integrity of RNA was verified by analyzing samples on a 1.2 % (w/v) denaturing agarose gel.

The transcript of the gene *HlMYB3* was analyzed using MyTaq™ One-Step RT-PCR Kit (Bioline, Germany) with the primers HlMYB3-1-F and HlMYB3-1-R (Table 4.1). The reactions were carried out in a volume of 20 µl containing 1X MyTaq One-Step mix, 0.5 µM of each primer, 0.5 µl RiboSafe RNase inhibitor, 0.25 µl reverse transcriptase, 5 µl of total RNA (20 ngµl$^{-1}$), and 2.25 µl of RNase free water. For detecting possible DNA contaminations in RNA preparations, a negative control was included in which the reverse transcriptase was eliminated from the reaction. The reverse transcriptase-PCR was performed under the following conditions: 45ºC for 20 min, 95ºC for 1 min followed by 40 cycles at 95ºC for 10 s, 60ºC for 10 s, 72ºC for 30 s. The PCR products were evaluated on 1.5% (w/v) agarose gel, stained with DNA Stain G (Serva, Germany), visualized under UV, and documented with a digital camera.

### 4.3.5 Gene expression analysis

Reverse transcriptase quantitative real-time PCR was used to determine the level of *PAL*, *C4H*, *4CL*, *CHS_H1*, *CHI*, *F3H*, *F3´H*, *FLS*, *F3´5´H*, *OMT1*, *HlPT1*, *VPS*, *HlMYB1*, *HlMYB2*, *HlMYB7*, *HlbZip1*, *HlbZip2*, *HlbHLH2*, and *WDR1* gene expression in cones from transgenic and wildtype plants. Each PCR reaction was carried out in 20 µl final volume containing 1X SensiMix™ SYBR master mix, 0.5 µM of each primer, 0.2 reverse transcriptase, 0.4 RiboSafe RNase inhibitor, 2 µl RNA, and 5.4 µl RNase free water (Bioline, Germany). Reverse transcriptase quantitative real-time PCR was performed on a StepOne$^{TM}$ System (Applied Biosystems, USA) following the requirements described by Bustin et al. (2009). Cycling conditions were: 45ºC for 10 min, 95ºC for 2 min followed by 40 cycles at 95ºC for 5 s, and 60ºC for 20 s. Melting curve analysis and agarose gel electrophoresis were used to verify single PCR product formation.

The reference genes *7SL-RNA* or *GAPDH* (Nagel et al. 2008; Maloukh et al. 2009) were used to obtain the normalized expression of target genes, which was determined as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). RT-qPCR values are the mean ± SD of two biological replications in which each sample was measured in triplicate.

## 4.4 Results

### 4.4.1 Verification of transformed plants and estimation of *HlMYB3* copy number

The *H. lupulus* L. endogenous gene s-*HlMYB3* was introduced into the hop genome using *A. tumefaciens*. Putative transgenic events were screened by triplex PCR for the presence of the hop *chitinase* endogenous gene (290 and 330 bp), and the transgene *nptII* (640 bp), as well as for the absence of *virG* (390 bp). Of 1,000 transformed stem segments, a total of 6 kanamycin-resistant plants were obtained. These plants belonged to 3 different events (event 1: plant 2, and 209; event 2: plant 137, and 233; and event 3: plant 129, and 224) (Fig. 4.1.a). All the transgenic plants showed a signal for *nptII* whereas no signal was observed for *virG*, indicating no contamination with *A. tumefaciens*. Every tested plant gave signals specific for the hop *chitinase* gen, confirming the quality of extracted DNA (Fig. 4.1.a). Moreover, the presence of the transgene s-*HlMYB3* and the endogenous *HlMYB3* was confirmed in all previously identified transgenic plants by detecting a 214 bp and 400 bp fragment, respectively. The native *HlMYB3* was only detected in the wildtype plants (non-transformed controls) (Fig. 4.1.b). The partial sequences of the PCR amplification products obtained using the primer set HlMYB3-2-F/ HlMYB3-2-R showed high similarity to the sequence of the transcription factor HlMYB3 (GenBank: AM501509.1) (data not shown). The stability of the integrated s-*HlMYB3* and *nptII* genes was assessed over the years 2010, 2011, and 2012. In all transgenic plants the transgenes were present (data not shown).

In order to determine the copy number of *HlMYB3* in transgenic hop events, it was necessary to determine the efficiency of the quantitative real-time PCR reaction. Therefore, standard curves for *HlMYB3* and *GPPS.LSU* were generated using 250, 50, 10, and 2 $ng\mu l^{-1}$ of genomic DNA. A slope of -3.222 for *GPPS.LSU* showed that the PCR efficiency was 104.3%. PCR efficiency and slope were 150.7% and -2.506 for *HlMYB3*. The correlation coefficients for *HlMYB3* and *GPPS.LSU* were 0.998 and 0.993, respectively. The results indicated that each transgenic plant (2, 209, 137, 233, 129, and 224) harbored two copies of the gene *HlMYB3*, whereas wildtype plants contained one copy of *HlMYB3* (Table 4.2).

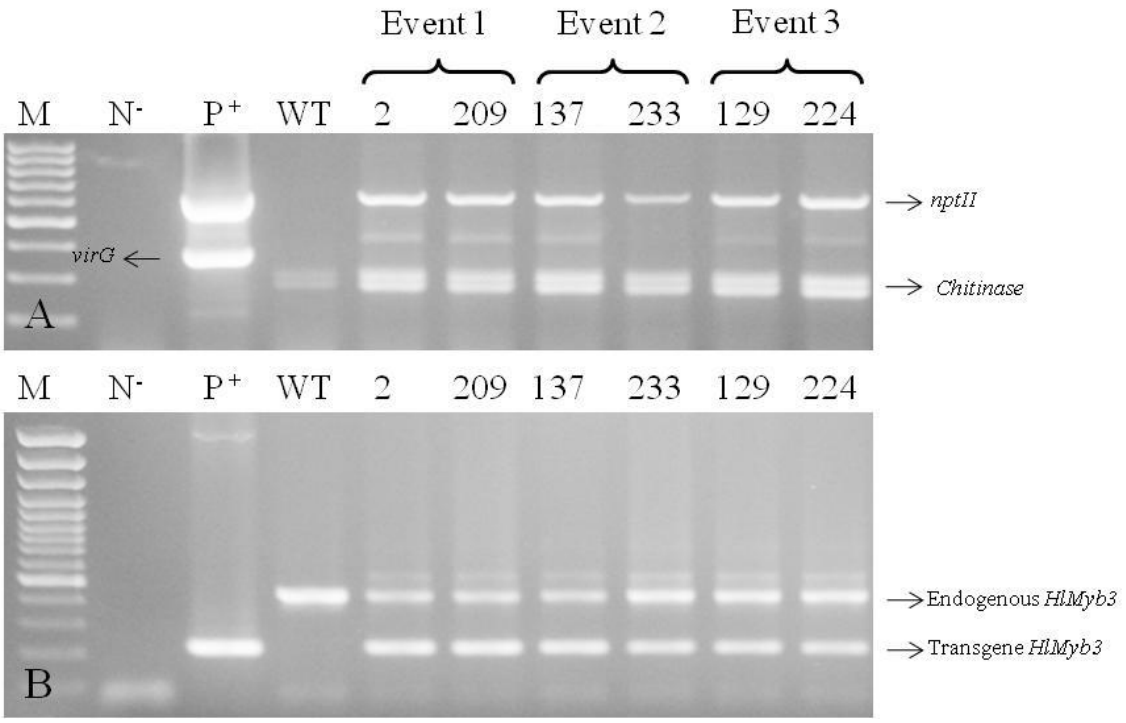


**Fig. 4.1** Detection of hop transgenic plants. **(A)** Triplex PCR showing *nptII* (640 bp), *virG* (390 bp) and hop *chitinase* gene (290 and 330 bp). **(B)** PCR analysis for the detection of the s-*HlMYB3* gene. M: molecular weight marker (100 bp DNA, MBI Fermentas, St. Leon-Rot), $N^-$: negative control (PCR reaction mix without template), $P^+$: positive control (plasmid pLV-71 DNA), WT: wildtype.

**Table 4.2** Estimated copy number of the transcription factor HlMYB3 in transgenic events and wildtype hop plants

| Event | Plant | $2^{-\Delta\Delta Ct}$ | | Average | Estimated copy number |
|---|---|---|---|---|---|
| | | First experiment [a] | Second experiment [a] | | |
| - | WT | 1.0 | 1.0 | 1.0±0.00 | 1 |
| 1 | 2 | 2.2 | 2.3 | 2.2±0.05 | 2 |
| | 209 | 1.8 | 1.8 | 1.8±0.00 | 2 |
| 2 | 137 | 1.5 | 1.7 | 1.6±0.15 | 2 |
| | 233 | 1.6 | 1.4 | 1.5±0.16 | 2 |
| 3 | 129 | 1.7 | 2.0 | 1.9±0.18 | 2 |
| | 224 | 1.4 | 2.6 | 2.0±0.89 | 2 |

[a] Values are the mean ± SD of two biological replications in which each sample was measured in triplicate. WT: wildtype

### 4.4.2 Morphology of transgenic events

Since May 2011, transgenic plants (2, 209, 137, 233, 129, and 224) as well as wildtype plants were grown under agronomic conditions in an outdoor containment facility. Throughout the growing season the phenotype of the transgenic plants was the same as that of wildtype plants (Fig. 4.2).


**Fig. 4.2** Phenotype of **(A)** transgenic and **(B)** wildtype hop female cones. Plants were photographed in September 2011.

### 4.4.3 Gene expression analysis of *HlMYB3*

Reverse transcriptase-PCR analysis was performed with six transgenic plants (2, 209, 137, 233, 129, and 224) and with wildtype plants to verify the expression of the gene *HlMYB3*. A 201 bp specific band was amplified from leaves (Fig. 4.3.a) and female cones (Fig. 4.3.b) of transgenic and wildtype plants. In the controls where the reverse transcriptase was omitted no signal was detected (data not shown).

In wildtype plants, the expression of the endogenous transcription factor HlMYB3 was assayed in leaves, roots and female cones by RT-qPCR. Relative expression of the endogenous gene *HlMYB3* in wildtype female cones was five and twelvefold higher than in roots and leaves, respectively (Fig. 4.4). For further analysis, the expression level of the endogenous *HlMYB3* in wildtype female cones was assigned as 1. The expression level of the transcription factor HlMYB3 in female cones was compared among the transgenic hop plants 209, 137, 233, and 224, as well as in wildtype plants. The levels of *HlMYB3* were higher in transgenic plant no. 209 (18.3 fold), no. 137 (14.3 fold), no. 233 (16.8 fold), and no. 224 (17.7 fold) than in wildtype plants (Fig. 4.3.c).

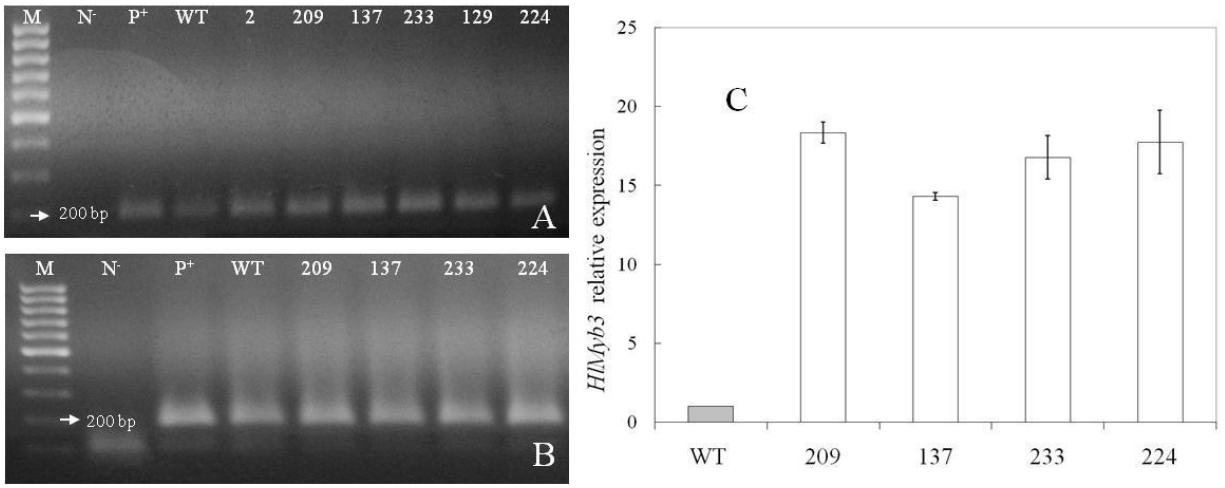


**Fig. 4.3** RT-PCR analysis demonstrating *HlMYB3* expression in **(A)** leaves and in **(B)** cones of wildtype and transgenic plants. **(C)** Relative expression of *HlMYB3* in female cones of wildtype and transgenic plants. *7SL-RNA* was used as housekeeping gene to normalize the expression of the transcription factor *HlMYB3*. RT-qPCR data represent the mean ± SD of two biological repeats with three replicates each one. M: molecular weight marker (100 bp DNA, MBI Fermentas, St. Leon-Rot), $N^-$: negative control (PCR reaction mix without template), $P^+$: positive control (plasmid pLV-71 DNA), WT: wildtype.

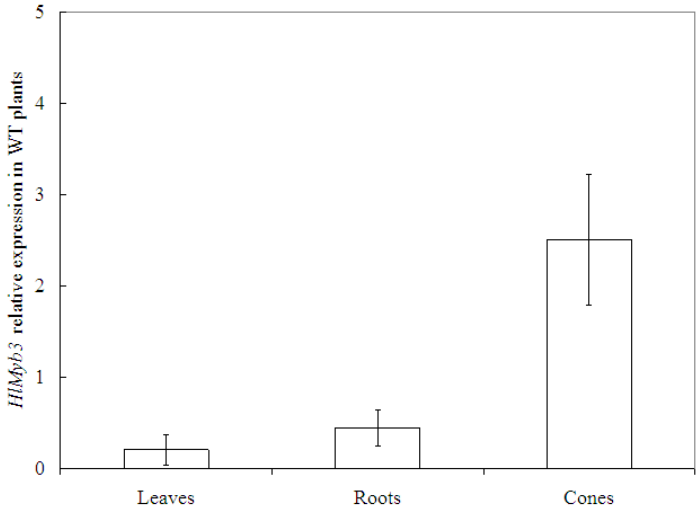


**Fig. 4.4** Expression of *HlMYB3* determined in leaves, roots, and female cones of wildtype hop plants. *GAPDH* was used as housekeeping gene to normalize the expression of the transcription factor *HlMYB3*. RT-qPCR data represent the mean ± SD of three replicates.

### 4.4.4 Expression analysis of genes of flavonoid and phloroglucinol biosynthesis

It was interesting to learn the effect of the over-expression of the regulatory gene *HlMYB3* on the expression of the structural genes involved in the biosynthesis of flavonoids and phloroglucinols. Therefore, the respective expression levels of *PAL, 4CL, CHS_H1, CHI, F3H, F3´H, F3´5´H, OMT1, HlPT1,* and *VPS* were investigated. The expression rate of the flavonoid biosynthetic genes *PAL, 4CL, CHS_H1, CHI, F3H, F3´H,* and *F3´5´H* was generally higher in transgenic plants compared to wildtype plants (Fig 4.5). Notably, transgenic plants no. 209, and no. 224 showed increased levels of *4CL, CHS_H1, CHI, F3H, F3´H,* and *F3´5´H.* Interestingly, the transgenic plant no. 137 showed lower levels of *CHS_H1, CHI, F3H, F3´H,* and *F3´5´H* in comparison to other transgenic or wildtype plants, respectively. No dramatically increased levels of expression of *C4H* were observed in transgenic plants. However, low transcript levels of *FLS* were observed, except in the transgenic plant no. 224, in comparison with wildtype plants.

Surprisingly, the expression level of the phloroglucinols biosynthetic genes *OMT1, HlPT1,* and *VPS* was up-regulated in hop transgenic plants compared to wildtype plants. *OMT1* transcripts levels were increased in transgenic plant no. 209 (1.4 fold), and no. 224 (3.9 fold). Similarly, the transcript levels of *HlPT1* and *VPS* were increased in the transgenic plant no. 209 (1.7 fold and 38 fold, respectively), no. 224 (3.1 fold and 15.2 fold, respectively) (Fig 4.5).

### 4.4.5 Expression analysis of regulatory genes

The effect of the over-expression of *HlMYB3* on the expression of other hop regulatory genes *HlMYB1, HlMYB2, HlMYB7, HlbZip1, HlbZip2, HlbHLH2,* and *WDR1* was examined (Fig. 4.6). The transcription factors HlMYB1, HlMYB2 and HlMYB7 were up-regulated in transgenic plant no. 137 in comparison with wildtype. The expression levels of HlbZip1 and HlbZip2 were slightly higher in the transgenic plant no. 137, no. 233, and no. 224 in comparison with wildtype plants. The expression level of HlbHLH2 was reduced in transgenic plants in comparison with wildtype plants. The transcript levels of HlWDR1 were slightly higher in the transgenic plant no. 209, no. 137 and no. 233 in comparison with wildtype plants.

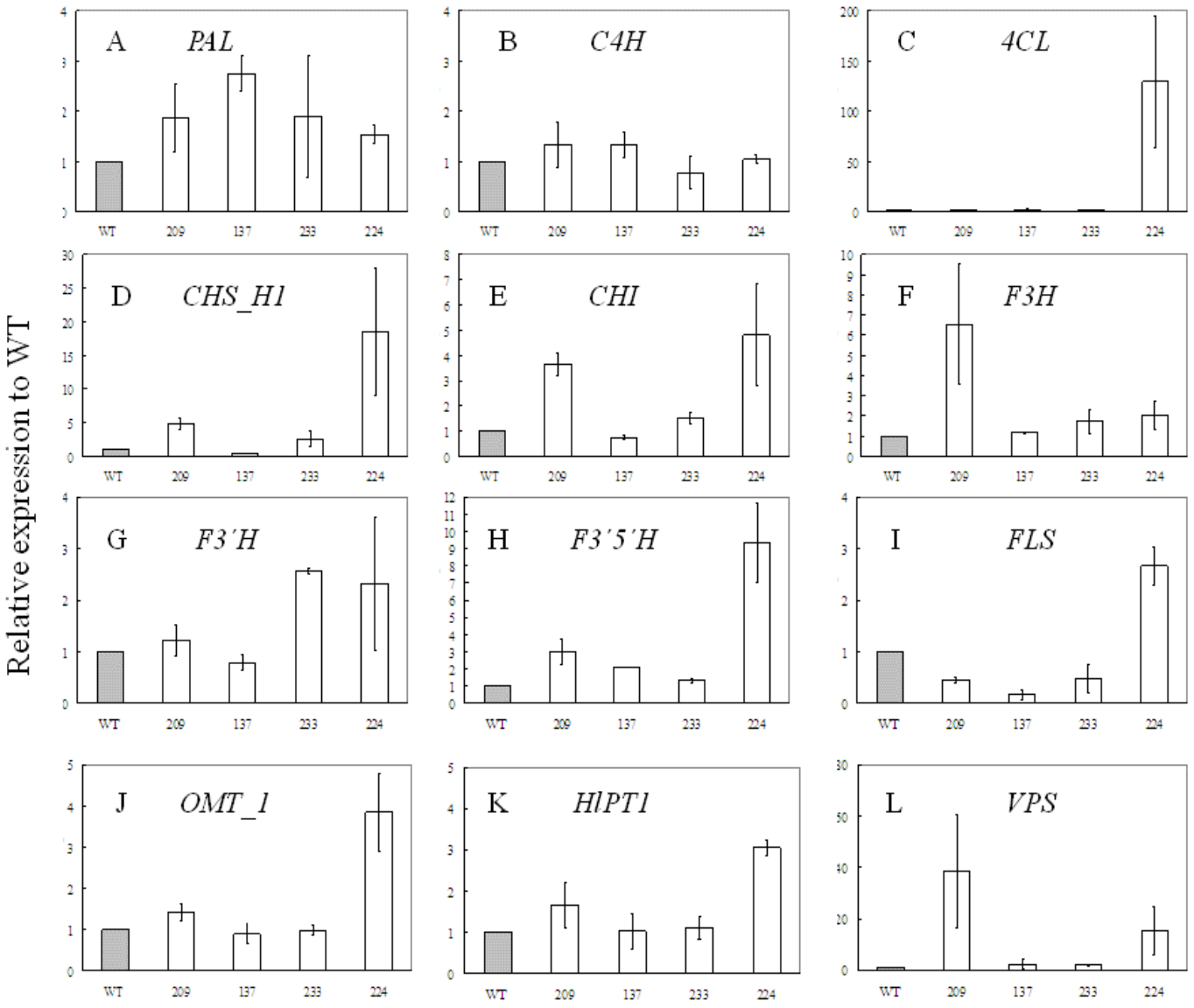


**Fig. 4.5** Normalized mRNA gene expression of **(A)** *PAL*, **(B)** *C4H*, **(C)** *4CL*, **(D)** *CHS_H1*, **(E)** *CHI*, **(F)** *F3H*, **(G)** *F3´H*, **(H)** *F3´5´H*, **(I)** *FLS*, **(J)** *OMT1*, **(K)** *HlPT1*, and **(L)** *VPS* in wildtype and transgenic hop female cones determined by RT-qPCR. *7SL-RNA* was used as housekeeping gene to normalize the expression of the target genes. RT-qPCR data represent the mean ± SD of two biological repeats with three replicates each one

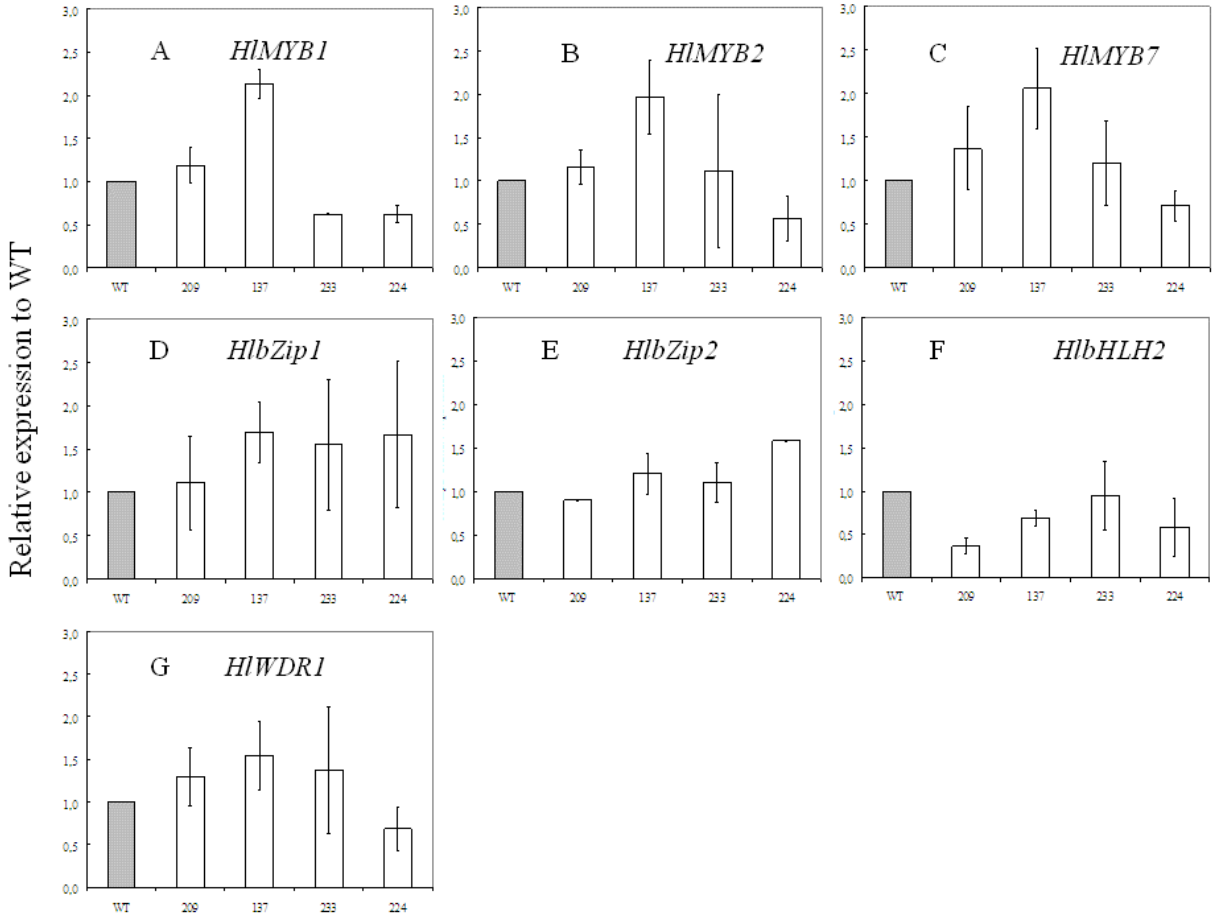


**Fig. 4.6** Relative expression of **(A)** *HlMYB1*, **(B)** *HlMYB2*, **(C)** *HlMYB7*, **(D)** *HlbZip1*, **(E)** *HlbZip2*, **(F)** *HlbHLH2*, and **(G)** *WDR1* in female cones of wildtype and transgenic plants. *GAPDH* was used as housekeeping gene to normalize the expression of the regulatory genes. RT-qPCR data represent the mean ± SD of three replicates

## 4.5 Discussion

In the present study, genetic engineering of hop cv. Tettnanger with the homologous transcription factor HlMYB3 under the control of the CaMV 35S promoter was successfully accomplished. Previously, this promoter has been employed to generate hop transgenic plants and all tissues showed *uidA* activity (Horlemann et al. 2003). The transcription factor HlMYB3 was characterized and ectopically introduced into *A. thaliana* L., *N. benthamiana* L. and *Petunia hybrida*. The gene *HlMYB3* encodes a typical R2R3 protein showing similarity to known regulators of the flavonoid biosynthesis, such as the *Gossypium hirsutum* L. MYB factor GhMYB5 (Matoušek et al. 2007).

Metabolic engineering has been used to introduce the heterologous transcription factor AtMYB75/PAP1 into hop cv. Tettnanger (Gatica-Arias et al. 2012a). Genetic transformation of MYB transcription factors has enhanced the production of flavonoids in *A. thaliana* L., *B. napus* L., *M. domestica* L., *N. tabacum* L., *Petunia hybrida*, *S. lycopersicum* L., and *H. lupulus* L. (Matoušek et al. 2007; Espley et al. 2007; Zhou et al. 2008; Zuluaga et al. 2008; Li et al. 2010; Gatica-Arias et al. 2012a). Moreover, constitutive or transient expression of MYB transcription factors in transgenic plants resulted in a change of the color of leaves, stems, roots, flowers, and fruits (Xie et al. 2006; Espley et al. 2007; Zuluaga et al. 2008; Li et al. 2010). Interesting, the over-expression of the regulatory gene *AtMYB75/PAP1* in transgenic tobacco produced red and anthocyanin free white callus (Zhou et al. 2008). Ectopic expression of the transcription factor AtMYB75/PAP1 in *H. lupulus* L. produced red flowers and female cones with red bracteoles (Gatica-Arias et al. 2012a). However, over-expression of s-*HlMYB3* in hop did not produce a visible change of the phenotype of all transgenic events. In contrast, the expression of the HlMYB3 transcription factor in *A. thaliana* L., *N. benthamiana* L. and *Petunia hybrida* influenced plant morphogenesis depending on the *HlMYB3* variant analyzed (Matoušek et al. 2007).

The expression of the transcription factor HlMYB3 in wildtype hop plants demonstrated that the transcript levels were higher in female cones than in roots and leaves. These observations were in accordance with previous studies in hop (Matoušek et al. 2010). These authors demonstrated that the HlMYB3 transcription factor is highly expressed in the lupulin glands and roots than in other tissues. Moreover, Matoušek et al. (2007) reported that in different hop genotypes the expression of *HlMYB3* increased during cone maturation.

In the present study, over-expression of the transgene s-*HlMYB3* in *H. lupulus* L. activated the genes involved in the flavonoid biosynthesis pathway. This is consistent with Matoušek et al. (2007) who hypothesized that *HlMYB3* is a positive regulator of the biosynthesis of flavonoids. Moreover, it has been demonstrated that transient expression of *GtMYB3* together with *GtbHLH1* enhanced the promoter activities of late anthocyanin biosynthesis genes in tobacco (Nakatsuka et al. 2008).

In *A. thaliana* L., and *B. napus* L. over-expression of MYB transcription factors, such as AtMYB75/PAP1, stimulated the expression of genes related to the biosynthesis of phenylpropanoids (Gonzalez et al. 2008; Li et al. 2010). Moreover, in our previous study, we have demonstrated that the AtMYB75/PAP1 transcription factor increased the expression rate of *CHS_H1*, *CHI*, and *F3´H* in transgenic hop in comparison to the wildtype plants (Gatica-Arias et al. 2012b).

In higher plants, the regulation of the flavonoid biosynthetic pathway is mediated by the R2R3 MYB and a basic helix-loop-helix (bHLH) transcription factors, together with WD40 or WDR1 proteins (Hichri et al. 2011). *A. thaliana* L. over-expressing *AtMYB75/PAP1* transgenic plants have elevated transcript levels of the *TT8* gene that encodes a bHLH protein involved in regulating anthocyanin biosynthesis (Nesi et al. 2000). *MYB*, *bHLH*, and *WDR1* genes have been recently identified in *H. lupulus* L. and they have been related to the regulation of the biosynthesis of flavonoid (Matoušek et al. 2012). Interesting, the HlMYB7 transcription factor has been described as an inhibitor of the complex MYB/bHLH/WDR1 (Matoušek et al. 2012). Our results showed that the transgenic plant no. 137 had the highest *HlMYB7* and at the same time the lowest *CHS_H1*, *CHI*, *F3H*, *F3´H*, *FLS*, and *OMT1* relative expression.

The present study demonstrated that the homologous transcription factor HlMYB3 modulated the expression of the structural genes involved in the biosynthesis of flavonoids in transgenic hop events. Moreover, the expression of phloroglucinol biosynthetic genes was up-regulated by the over-expression of the transcription factor HlMYB3. Therefore, metabolic engineering using homologous MYB transcription factors may open the possibility for modifying the lupulin metabolome in hop.

## 4.6 Acknowledgments

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## 4.7 References

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622

Espley RV, Hellens RP, Putterill J, Stevenson DE, Kutty-Amma S, Allan AC (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMyb10. The Plant J 49: 414-427

Gatica-Arias A, Farag MA, Häntzschel KR, Matoušek J, Weber G (2012b) The transcription factor AtMYB75/PAP1 regulates the expression of flavonoid biosynthesis genes in transgenic hop (*Humulus lupulus* L.). Brew Sci 65: 103-111

Gatica-Arias A, Farag MA, Stanke M, Matoušek J, Wessjohann L, Weber G (2012a) Flavonoid production in transgenic hop (*Humulus lupulus* L.) altered by PAP1/MYB75 from *Arabidopsis thaliana* L. Plant Cell Rep 31: 111-119

Gonzalez A, Zhao M, Leavitt JM, Lloyd AM (2008) Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/MYB transcriptional complex in *Arabidopsis* seedlings. The Plant J 53: 814-827

Hirchri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V (2011) Recent advances in the transcriptional regulation of the flavonoid biosynthesis pathway. J Exp Bot 62: 2465-2483

Horlemann C, Schwekendiek A, Hohnle M, Weber G (2003) Regeneration and *Agrobacterium*-mediated transformation of hop (*Humulus lupulus* L.). Plant Cell Rep 22: 210-217

Ingham DJ, Beer S, Money S, Hansen G (2001) Quantitative real-time PCR assay for determining transgene copy number in transformed plants. BioTechniques 31: 132-140

Li X, Gao MJ, Pan HY, Cui DJ, Gruber M (2010) Purple canola: *Arabidopsis* PAP1 increases antioxidants and phenolic in *Brassica napus* leaves. J Agric Food Chem 58: 1639-1645

Livak K, Schmittgen T (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta] CT method. Methods 25: 402-408

Lutz A, Kammhuber K, Seigner E (2012) New trend in hop breeding at the Hop Research Center Huell. Brew Sci 65: 24-32

Maloukh L, Matoušek J, Bockstaele EV, Roldán-Ruiz I (2009) Housekeeping gene selection for real time-PCR normalization in female hop (*Humulus lupulus* L.) tissues. J Plant Biochem Biotech 18: 53-58

Matoušek J, Kocábek T, Patzak J, Füssy Z, Prochazkova J, Heyerick A (2012) Combinatorial analysis of lupulin gland transcription factors from R2R3MYB, bHLH and WDR families indicates a complex regulation of *chs_H1* genes essential for prenylflavonoid biosynthesis in hop (*Humulus lupulus* L.) BMC Plant Biol 12: 27

Matoušek J, Rocábek T, Patzak J, Škopek J, Maloukh L, Heyerick A, Füssy Z, Roldán-Ruiz I, Keukeleire D (2007) HlMYB3, a putative regulatory factor in hop (*Humulus lupulus* L.), shows a diverse biological effects in heterologous transgenotes. J Agric Food Chem 55: 7767-7776

Matoušek J, Rocábek T, Patzak J, Sthelík J, Füssy Z, Krofta K; Heyerick A, Roldán-Ruiz I, Maloukh L, Keukeleire D (2010) Cloning and molecular analysis of HlbZip1 and HlbZip2 transcription factors putatively involved in the regulation of the lupulin metabolome in hop (*Humulus lupulus* L.). J Agric Food Chem 58: 902-912

Matoušek J, Vrba L, Škopek J, Orctová L, Pešina K, Heyerick A, Baulcombe D, De Keukeleire D (2006) Sequence analysis of a "true" chalcone synthase (chs_H1) oligofamily from hop (*Humulus lupulus* L.) and PAP1 activation of chs_H1 in heterologous systems. J Agric Food Chem 54: 7606-7615

Nagel J, Culley LK, Lu Y, Liu E, Matthews PD, Stevens JF (2008). EST analysis of hop glandular trichomes identifies an O-methyltransferase that catalyses the biosynthesis of xanthohumol. Plant Cell 20: 186-200

Nakatsuka T, Haruta KS, Pitaksutheepong C, Abe Y, Kakizaki Y, Yamamoto K, Shimada N, Yamamura S, Nishihara M (2008) Identification and characterization of R2R3-MYB and bHLH transcription factors regulating anthocyanin biosynthesis in Gentian flowers. Plant Cell Physiol 49: 1818-1829

Nesi N, Debeaujon I, Jond C, Pelletier G, Caboche M, Lepiniec, L. (2000) The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in Arabidopsis siliques. Plant Cell 12: 1863-1878

Schijlen E, Ric de Vos C, van Tunen A, Bovy A (2004) Modification of flavonoid biosynthesis in crop plants. Phytochemistry 65: 2631-2648

Stevens JF, Page JE (2004) Xantohumol and related prenylflavonoids from hops and beer to your good health! Phytochemistry 65: 1317-1330

Wang G, Dixon RA (2009) Heterodimeric geranyl (geranyl)diphosphate synthase from hop (*Humulus lupulus* L.) and the evolution of monoterpene biosynthesis. PNAS 106: 9914-9919

Xie DY, Sharma S, Wright E, Wang ZY, Dixon R (2006) Metabolic engineering of proanthocyanidins through co-expression of anthocyanidin reductase and the PAP1 MYB transcription factor. Plant J 45: 895-907

Zanoli P and Zavatti M (2008) Pharmacognostic and pharmacological profile of *Humulus lupulus* L. J Enthnopharmacol 116: 383-396

Zhou L, Zeng N, Shi M, Xie D (2008) Development of tobacco callus cultures over expressing *Arabidopsis* PAP1/MYB75 transcription factor and characterization of anthocyanin biosynthesis. Planta 229: 37-51

Zuluaga L, Gonzali S, Loreti E, Pucciariello C, Degl'Iinnocenti E, Guidi L, Alpi A, Perata P (2008) *Arabidopsis thaliana* MYB75/PAP1 transcription factor induces anthocyanin production in transgenic tomato plants. Funct Plant Biol 35: 606-618

# 5. *Agrobacterium rhizogenes*-mediated transformation of hop (*Humulus lupulus* L. cv. Tettnanger): establishment of a system for functional evaluation of genes[6]

## 5.1 Abstract

Secondary metabolites produced in the lupulin glands (glandular trichomes) of hop cones are important for beer production, and for the pharmaceutical and medicinal industry. However, in wildtype plants the concentration of these compounds is rather low. To enhance their production, it would be interesting to modify by genetic transformation the regulation of genes involved in the biosynthesis of flavonoids and phloroglucinols. Although, genetic transformation of hop has been used successfully for some time, it is a laborious and time consuming process. Therefore, the development of a simple and fast assay for functional validation of candidate genes is very desirable. As an alternative *A. rhizogenes*-mediated transformation could be employed for stably expressing genes in hairy roots. The latter being morphologically related to trichomes. *A. rhizogenes* K599 and 15834 were tested for their ability to induce the formation of hairy roots on hop explants. Only *A. rhizogenes* 15834 was able to induce hairy roots after 12-27 days of culture. The highest percentage of explants with roots as well as the total number of roots was obtained when infected leaf segments were cultivated with the adaxial surface in contact with the culture medium. Transgenic hairy roots were verified by GUS staining. The integration of *rolC* and *mgfp5* genes in transgenic hairy roots was confirmed by PCR. We developed a system to investigate the role of genes and transcription factors involved in the biosynthesis of flavonoids and phloroglucinols.

**Keywords** Hop, *Humulus lupulus*, *Agrobacterium rhizogenes*, genetic transformation

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## 5.2 Introduction

Hop (*Humulus lupulus* L., Cannabaceae) has been used in the brewing industry and as a medicinal plant. The lupulin glands (glandular trichomes) of the female cones contain *α*-, *ß*-acids, and essential oils. They are used in beer production as flavoring components, and as preservative agents [1, 2]. Moreover, the lupulin glands contain secondary metabolites (like desmethylxanthohumol, and xanthohumol) with a potential use as pharmaceuticals and in human medicine, due to their anti-cancer, anti-proliferative, anti-oxidative, and estrogene activity [3, 4, 5, 6, 7, 8]. Therefore, it is of growing interest to develop new hop cultivars with higher content of these important secondary metabolites.

The flavonoid production in plants could be enhanced through genetic transformation with structural genes or by the introduction of genes of homologous or heterologous regulatory elements [9, 10]. For the first time the transcription factor AtMYB75/PAP1 (production of anthocyanin pigment 1), which is a conserved member of the R2R3 gene family, was introduced in *H. lupulus* L. cv. Tettnanger and consequently the production of flavonoids in transgenic clones was elevated [11]. This transcription factor stimulates the expression of genes related to the biosynthesis of phenylpropanoids and flavonoids [12, 13, 14].

Successful genetic transformation of hop has been reported using *Agrobacterium tumefaciens*. However, it is a laborious, and time consuming process [11, 15, 16]. Consequently, using *A. tumefaciens*-mediated transformation in hop for functional validation of candidate genes has this major drawback.

Therefore, the development of an efficient, simple and fast transformation protocol for testing gene function in hop is very desirable. Particle bombardment [17] or *A. rhizogenes*-mediated transformation represents alternatives for generating transgenic hop plants. *A. rhizogenes* is a phytopathogenic gram negative soil bacterium responsible for the induction of transgenic hairy roots in plants [18]. This agrobacteria transfers a DNA segment (T-DNA) of the root-inducing (Ri) plasmid into the genome of the plant. The T-DNA carries a set of genes involved in the induction of hairy roots (*rolA*, *rolB*, *rolC*, *rolD*), and for the biosynthesis of auxins, and opines (*iaaM, iaaH, mas1'*, *mas2'*, and *ags*) [18].

Hairy root genetic transformation has been employed for studying the function of genes [19, 20, 21] or for producing secondary metabolites [22, 23, 24, 25]. This approach provides a major advantage over other methods because of its rapidity and technical simplicity. Moreover, hairy roots are morphologically similar to trichomes [40] making them a suitable system to study candidate genes which are normally functional in similar structures, like trichomes or lupulin glands, respectively.

The present study describes the induction of hairy roots in hop (*H. lupulus* L. cv. Tettnanger) through *A. rhizogenes*-mediated transformation and a method for screening and molecular analysis of putative transgenic hairy roots.

## 5.3 Materials and methods

### 5.3.1 Plant material

*In vitro* hop plants (*H. lupulus* L. cv. Tettnanger) were cultured in 500 ml polystyrene vessels with 20 ml of elongation medium under a 16 h light photoperiod at 21 ± 1ºC according to *Horlemann* et al. [15]. Leaf segments, internodes, petioles, and shoots with two nodes from *in vitro* grown plants were used as explants for genetic transformation.

### 5.3.2 Bacterial strains and binary plasmids

The following *Agrobacterium* strains were used: *A. rhizogenes* K599 (pRi 2659) [26] harboring the binary plasmid pCAMGUSGFP [the T-DNA contained the reporter genes *uidA* (which codes for *β*-glucuronidase) and *gfp* (which codes for green fluorescent protein) under the control of the cauliflower mosaic virus (CaMV) 35S promoter], *A. rhizogenes* 15834 (pRi 15834) [27] carrying the plasmid pCAMGFP-CvMV-GWox-Stisy [the T-DNA contained the reporter gene *gfp* under the control of the cassava vein mosaic virus (CvMV) promoter], and *A. rhizogenes* 15834 (pRi 15834) containing the plasmid pCAMBIA 1303 [the T-DNA contained the reporter genes *mgfp5*, and *uidA*, and the selectable marker gene *hptII* (which codes for hygromycin phosphotransferase) under the control of the CAMV 35S promoter]. The plasmids were introduced into the agrobacteria by the freeze- thaw method [28]. Their presence was corroborated by restriction analysis, as well as by PCR (Polymerase chain reaction) using specific primers for the gene *rolC*.

Prior to transformation experiments, a single agrobacteria colony was grown overnight in liquid YEB medium supplemented with $MgSO_4$ (2 mM), rifampicin (50 mg/l) and kanamycin (100 mg/l) on an orbital shaker with 250 rpm at 26 ºC. The bacterial suspensions were centrifuged at 3500 rpm for 15 min and then the pellets were resuspended in MS (Murashige and Skoog) medium supplemented with 10 mM MES to an $OD_{600}$ nm between 0.5 and 1.0.

### 5.3.3 Transformation procedure

In a first experiment, internodes, leaf segments, and double node shoots were co-cultured for 30 min with a solution of *A. rhizogenes* K599::pCAMGUSGFP. Afterwards, the explants were cultivated on co-culture medium (MS-B5 basal medium + 1.43 µM IAA + 9.08 µM TDZ + 2 % sucrose + 0.7 % agar) for 2 days. Then, the explants were collected, washed with distilled water, and cultivated on co-culture medium supplemented with 250 $mgl^{-1}$ cefotaxime [15]. Different co-culture media composition (with or without TDZ and IAA), light conditions (light, and darkness), and co-cultivation time (0, 2, 5, 7, 9, and 11 days) were evaluated.

In a second experiment, leaf segments, internodes, and petioles were co-cultivated with *A. rhizogenes* 15834::pCAMGFP-CvMV-GWox-Stisy following the protocol described by *Horlemann* et al. [15] with some modifications. Briefly, *Agrobacterium*-infected explants were cultivated on hormone-free co-culture medium supplemented with 250 $mgl^{-1}$ cefotaxime.

In a third experiment, leaf segments and internodes were transformed with *A. rhizogenes* 15834::pCAMBIA 1303 following the protocol described above. In a fourth experiment, the effect of the pre-culture of leaf segments prior to the transformation with *A. rhizogenes* 15834::pCAMBIA 1303 was evaluated. For this purpose, 500 leaf segments were cut two days prior to transformation and cultivated on hormone-free co-culture medium. Moreover, another 500 leaf segments, without pre-culture, were inoculated with the agrobacteria following the protocol described above. In all experiments, explants which had not been exposed to *A. rhizogenes* were included as controls.

### 5.3.4 Histochemical GUS assays

GUS assays were performed by immersing tissues in the staining buffer [50mM sodium phosphate (pH 7.0), 0,5 mM potassium ferrocyanide, 0,5 mM potassium ferricyanide, 10 mM EDTA, 0,1% Triton, 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide)] for 24 h at 37 °C in the dark [29]. The GUS stained tissue was observed under 10-fold magnification using a stereomicroscope (Stemi SV6, Zeiss, Germany) equipped with a digital camera (EOS 450D, Canon, Japan).

### 5.3.5 DNA isolation and PCR analysis

Genomic DNA from putative transgenic hairy roots was extracted using the CTAB method [30]. The PCR was performed in a mixture (25 µl) containing 1X Mango *Taq* PCR buffer, 0.25 mM of each dNTPs, 0.2 µM of each primer, 1.5 mM of $MgCl_2$, 0.5 U Mango *Taq*$^{TM}$ polymerase (Bioline, Germany), and 1 µl DNA. Three different specific primers were used: *mgfp5* [gfp-f: (5'-atgagtaaaggagaagaacttttcactgg-3') and gfp-r: (5'-ttatttgtatagttcatccatgccatgtg-3'); expected fragment length: 790 bp], *rolC* [rolC-f: (5'-tgtgacaagcagcgatgagc-3') and rolC-r: (5'-gattgcaaacttgcactcgc-3'); expected fragment length: 487 bp], and *virG* [virG-1f: (5'-ttatctgagtgaagtcgtctcagg-3') and virG-1r: (5'-cgtcgcctgagattaagtgtc-3'); expected fragment length: 900 bp]. Primers for *rolC* (X03433.1) and *gfp* (U87624.1) were designed based on sequences available in the GenBank. The primer sequences for *virG* were obtained from *Lee* et al. [41]. The amplification conditions for *mgfp5* and *rolC* were as follows: 94 ºC for 3 min followed by 35 cycles at 94 ºC for 1 min, 60 ºC for 1 min, 72 ºC for 1 min and a final step of 72 ºC for 10 min. For *virG* the amplification conditions were as follows: 96 ºC for 5 min followed by 36 cycles at 94 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 1 min and a final step of 72 ºC for 10 min [41]. PCR products were evaluated on 1.5% (w/v) agarose gel and stained with DNA Stain G (Serva, Germany). The plasmid pCAMBIA 1303 was used as positive control to test the presence of the genes *mgfp5*, and *rolC*, whereas DNA isolated from *A. rhizogenes* was used as positive control for *virG*.

## 5.4 Results and discussion

*A. rhizogenes* strain K599 (pRi 2659) and *A. rhizogenes* strain 15834 (pRi 15834) were tested for their ability to induce the formation of hairy roots on hop explants. Using *A. rhizogenes* strain K599 with different organ types, co-culture media composition, light conditions, and co-cultivation times, no hairy root formation was observed in any of the treatments (data not shown). It has been reported that *A. rhizogenes* strains, which differ in their virulence, determines the successful induction of hairy roots [20, 31, 32, 33]. *A. rhizogenes* K599 (pRi 2659) is a low virulence strain and has been used for genetic transformation of *Glycine max*, *Lotus corniculatus*, *Phaseolus vulgaris*, and *Cucumis melo* [34, 35, 36, 37].

As an alternative *A. rhizogenes* strain 15834 (pRi 15834) was used for the transformation of hop explants. The different response of leaf segments, internodes, and petioles after inoculation with *A. rhizogenes* 15834::pCAMGFP-CvMV-GWox-Stisy is shown in Table 5.1. The highest percentage of explants with roots and total number of roots were obtained when infected leaf segments were cultivated with the adaxial surface in contact with the co-culture medium (Table 5.1). No hairy root formation was observed in non-transformed controls.

Furthermore, this confirmed previous reports that the type of inoculated organ is an important factor affecting *A. rhizogenes*-mediated transformation [20, 21, 24, 33]. Only *A. rhizogenes* strain 15834 was capable of inducing hairy root formation. This difference could be due to genotypic differences as well as different Ri plasmid they were harboring. Plasmid pCAMGFP-CvMV-GWox-Stisy contains the reporter gene *gfp*, which has been used for screening of transgenic roots in *Daucus carota*, *Capsicum annuum*, *Saponaria vaccaria*, and *Cucumis melo* [24, 32, 37, 38]. However, in *Humulus lupulus* the fluorescence of *gfp* cannot be used to unambiguously differentiate transgenic from non-transgenic roots (data not shown). This could be attributed to hop secondary metabolites fluorescing at a similar wavelength as *gfp* [39].

**Table 5.1** Root induction in hop (*Humulus lupulus* L. cv. Tettnanger) after *Agrobacterium rhizogenes* 15834 inoculation of different type of organs.

| Type of inoculated organ | Number of inoculated explants | Explants that produced roots (%) | Total number of roots |
|---|---|---|---|
| Petioles | 100 | 8 | 10 |
| Internodes | 100 | 17 | 40 |
| Leaves-abaxial [a] | 100 | 0 | 0 |
| Leaves-adaxial [a] | 100 | 55 | 126 |

[a] The leaf segments were cultured after the transformation with *A. rhizogenes* 15834::pCAMGFP-CvMV-GWox-Stisy with the abaxial or adaxial surface in contact with the co-culture medium. Data correspond to the experiment 2 (see Material and Methods) and were collected 4 weeks after infection with *A. rhizogenes.*

After the conditions for the transformation were optimized, in a third experiment, leaf segments and internodes were transformed with *A. rhizogenes* 15834 harboring the plasmid pCAMBIA 1303, which contains the reporter genes *uidA* and *mgfp5*. Twelve days after co-culture with the agrobacteria, leaf segments (Fig. 5.1.a) and internodes (Fig. 5.1.b) showed blue staining, from the enzymatic activity of $\beta$-glucuronidase. Non-transformed controls (leaf segments and internodes) were not stained under similar reaction conditions (data not shown).

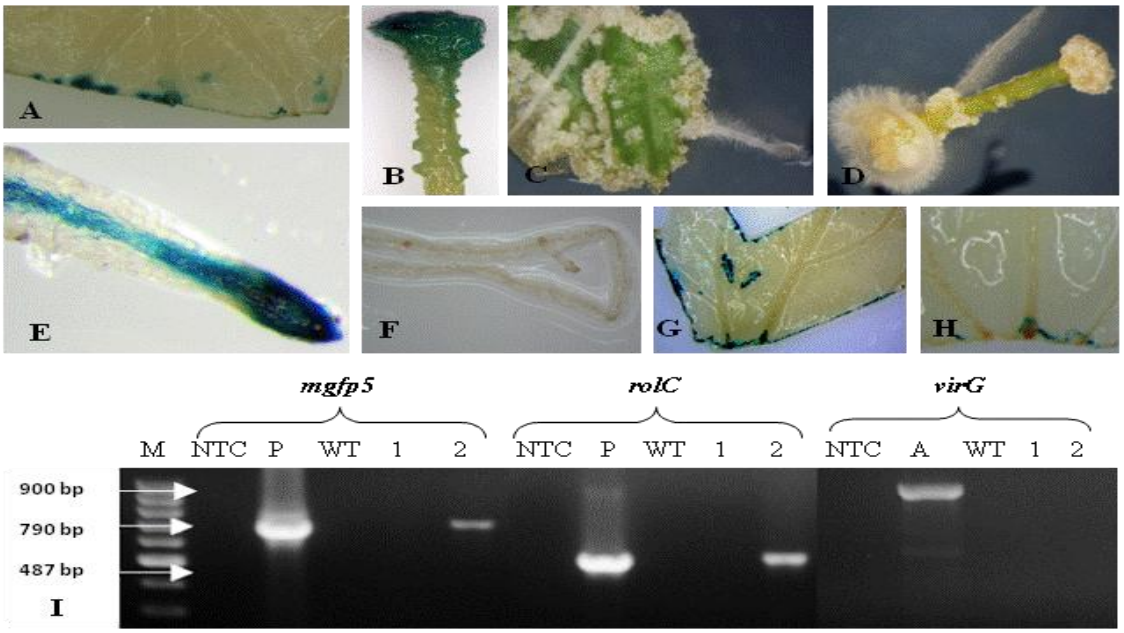


**Fig. 5.1** *Agrobacterium rhizogenes*-mediated transformation of *Humulus lupulus* L. cv. Tettnanger. Transient *uidA* expression in **(A)** leaf segments and **(B)** internodes. Hairy roots induction in **(C)** leaf segments and **(D)** internodes. **(E)** *uidA* expression in transgenic roots. **(F)** Wildtype root showing no *uidA* activity. Transient *uidA* expression in leaf segments **(G)** without pre-culture and with **(H)** pre-culture. **(I)** PCR analysis showing the presence of the genes *mgfp5* and *rolC* and absence of the gene *virG* in transgenic hop hairy roots. M: molecular weight marker (100 bp), NTC: negative control (PCR reaction mix without template), P: positive control (pCAMBIA 1303 DNA), WT: wildtype roots (non-transformed control), A: *A. rhizogenes* 15834 DNA, 1-2: putative transgenic hairy root.

Previously, hop transformation had been carried out using *uidA* as reporter gene [15]. Sixteen days after inoculation, of 207 inoculated leaf segments 3 % yielded hairy roots (Fig. 5.1.c). In this case, a GUS assay was not performed because of the insufficient quantity of roots. On the other hand, of 219 inoculated internodes, 35 % yielded hairy roots 12 days after inoculation (Fig. 5.1.d), of which 5 % (3 of 60) showed blue staining. The *uidA* expression was observed in the root tip and central cylinder of the putative transgenic hairy roots (Fig. 5.1.e), whereas non-transformed controls did not exhibit *uidA* activity (Fig. 5.1.f). Root induction was not observed in non-transformed controls (data not shown).

Regarding the effect of the pre-culture of explants prior to the transformation with *A. rhizogenes* 15834::pCAMBIA 1303, it was demonstrated that leaf segments without pre-culture (Fig. 5.1.g) showed a stronger *uidA* expression than leaf segments with pre-culture (Fig. 5.1.h). Moreover, of 500 leaf segments without pre-culture, 23 % yielded hairy roots 27 days after inoculation. On the other hand, of 500 leaf segments pre-cultured on hormone-free co-culture medium prior to the transformation, 11 % yielded hairy roots 27 days after inoculation.

Putative transgenic hairy roots (8-weeks-old) were pooled and tested by PCR for the presence of the T-DNA genes transferred from the binary vector (*mgfp5*), and the Ri plasmid (*rolC*), as well as for false positives caused by *A. rhizogenes* contamination (indicated by the presence of *virG* gene). The specific *mgfp5* (790 bp) and *rolC* (487 bp) fragments were amplified in 1 of 2 pooled putative transgenic hairy roots (Fig. 5.1.i). No DNA fragments were amplified by PCR in wildtype plants (Fig. 5.1.i). Moreover, the *virG* gene was not detected in transgenic hairy roots, indicating no contamination with *A. rhizogenes* (Fig. 5.1.i).

## 5.5 Conclusions

According to the best of our knowledge this is the first report for induction of hairy roots in hop (*Humulus lupulus* L. cv. Tettnanger). Instead of the 6-9 months-time required for regenerating hop transgenic plants following *A. tumefaciens* transformation [11,15,16], in the present study, production of hairy roots takes only 4 weeks. Therefore, prior to producing hop transgenic plants using *A. tumefaciens,* transgenic hairy roots provide a fast and easy tool for screening and evaluating a larger number of candidate genes, for instance, from the flavonoid and/or phloroglucinol biosynthetic pathways. In order to improve the transformation efficiency different strategies including sonication assisted transformation (SAAT) and co-cultivation with two *Agrobacterium* strains will be evaluated. Moreover, a liquid culture system for root growth will be established.

## 5.6 Acknowledgements

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## 5.7 References


1. De Keukeleire, J.; Ooms, G.; Heyerick, A.; Roldan-Ruiz, I.; Van Bockstaele, E. and De Keukeleire D.: Formation and accumulation of α-acids, β-acids, desmethylxanthohumol, xanthohumol during flowering of hops (*Humulus lupulus* L.), Journal of Agricultural Food and Chemistry, **51** (2003), no. 15, pp. 4436-4441.

2. Zanoli P. and Zavatti M.: Pharmacognostic and pharmacological profile of *Humulus lupulus* L. Journal of Enthnopharmacology, **116** (2008), no. 3, pp. 383-396.

3. Tagashira, M.; Watanabe, M. and Uemitsu, N.: Antioxidative activity of hop bitter acids and their analogues, Bioscience Biotechology and Biochemistry, **59** (1995), no. 4, pp. 740-742.

4. Miranda, C.; Yang, Y.; Henderson, M.; Stevens, J.; Santana-Rios, G. and Deinzer, M.: Prenylflavonoids from hops inhibit the metabolic activation of the carcinogenic heterocyclic amine 2-amino-3-methylimidazol [4, 5-f] quinoline, mediated by cDNA-expressed human CYP1A2, Drug Metabolism and Disposition, **28** (2000), no. 11, pp. 1297-1302.

5. Milligan, S.; Kalita, J.; Pocock, V.; Heyerick, A.; De Cooman, L. and Rong, H.: Oestrogenic activity of the hop phyto-oestrogen, 8-prenylnaringenin, Reproduction, **123** (2002), no. 2, pp. 235-242.

6. Chen, W. and Lin, J.: Mechanisms of cancer chemoprevention by hop bitter acids (beer aroma) through induction of apoptosis mediated by Fas and Caspase Cascades, Journal of Agricultural Food and Chemistry**, 52** (2004), no. 1, pp. 55-64.

7. Gerhäuser, C.: Beer constituents as potential cancer chemopreventive agents, European Journal of Cancer, **41** (2005), no. 13, pp. 1941-1954.

8. Lamy, V.; Roussi, S.; Chaabi, M.; Gossé, F.; Schall, N.; Lobstein, A. and Raul F.: Chemopreventive effects of lupulone, a hop β-acid, on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis, Carcinogenesis, **28** (2007), no. 7, pp. 1575-1581.

9. Dixon, R.A. and Steele, C.L.: Flavonoids and isoflavonoids- a gold mine for metabolic engineering, Trends Plant. Sci., **4** (1999), pp. 394-400.

10. Capell, T.; Christou, P.: Progress in plant metabolic engineering, Current Opinion in Biotechnology, **15** (2004), no. 2, pp. 148-154.

11. Gatica-Arias, A.; Farag, M.A.; Stanke, M.; Matoušek, J.; Wessjohann, L. and Weber, G.: Flavonoid production in transgenic hop (*Humulus lupulus* L.) altered by *PAP1/MYB75* from *Arabidopsis thaliana* L., Plant Cell Reports, **31** (2012), no.1, pp. 111-119.

12. Borevitz, J.; Xia, Y.; Blount, J.; Dixon, R. and Lamb, C.: Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis, Plant Cell, **12** (2000), no. 12, pp. 2383-2394.

13. Tohge, T.; Nishiyama, Y.; Hirai, M.; Yano, M.; Nakajima, J.; Awazuhara, M.; Inoue, E.; Takahashi, H.; Goodenowe, D.; Kitayama, M.; Noji, M.; Yamazaki, M. and Saito, K.: Functional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor, Plant Journal, **42** (2005), no. 2, pp. 218-235.

14. Matoušek, J.; Vrba, L.; Škopek, J.; Orctová, L.; Pešina, K.; Heyerick, A.; Baulcombe, D. and De Keukeleire, D.: Sequence analysis of a "true" chalcone synthase (*chs*_H1) oligofamily from hop (*Humulus lupulus* L.) and PAP1 activation of *chs*_H1 in heterologous systems, Journal of Agricultural and Food Chemistry, **54** (2006), no. 20, pp. 7606-7615.

15. Horlemann, C.; Schwekendiek, A.; Höhnle, M. and Weber, G.: Regeneration and *Agrobacterium*-mediated transformation of hop (*Humulus lupulus* L.), Plant Cell Reports, **22** (2003), no. 3, pp. 210-217.

16. Schwekendiek, A.; Spring, O.; Heyerick, A.; Pickel, B.; Pitsch, N.; Peschke, F.; De Keukeleire, D. and Weber, G.: Constitutive expression of a grapevine stilbene synthase gene in transgenic hop (*Humulus lupulus* L.) yields resveratrol and its derivatives in substantial quantities, Journal of Agricultural Food and Chemistry, **55** (2007), no. 17, pp.7002-7009.

17. Batista, D.; Fonseca, S.; Serrazina, S.; Figueiredo, A. and Salomé Pais, M.: Efficient and stable transformation of hop (*Humulus lupulus* L.) var. Eroica by particle bombardment, Plant Cell Reports, **27** (2008), no. 7, pp. 1185-1196.

18. Georgiev, M.I; Pavlov, A.I. and Bley T.: Hairy root type plant *in vitro* system as sources of bioactive substances, Applied Microbiology and Biotechnology, **74** (2007), no. 6, pp. 1175-1185.

19. Bosselut, N.; Van Ghelder C.; Claverie M.; Voisin R.; Onesto J.P.; Rosso M.N.; Esmenjaud D.: *Agrobacterium rhizogenes*-mediated transformation of *Prunus* as an alternative for gene functional analysis in hairy-roots and composite plants, Plant Cell Reports, **30** (2011), no. 7, pp. 1313-1326.

20. Alpizar, E.; Dechamp, E.; Espeout, S.; Royer, M.; Lecouls, A.C.; Nicole, M.; Bertrand, B.; Lashermes, P. and Etienne, H.: Efficient production of *Agrobacterium rhizogenes*-transformed roots and composite plants for studying gene expression in coffee roots, Plant Cell Reports, **25** (2006), no. 9, pp. 959-967.

21. Kifle, S.; Shao, M.; Jung, C. and Cai D.: An improved transformation protocol for studying gene expression in hairy roots of sugar beet (*Beta vulgaris* L.), Plant Cell Reports **18** (1999), no. 6, pp. 514-519.

22. Kim, J.A.; Kim Y.S. and Choi, Y.E.: Triterpenoid production and phenotypic changes in hairy roots of *Codonopsis lanceolata* and the plants regenerated from them, Plant Biotechnology Reports, **5** (2011), no. 3, pp. 255-263.

23. Zaraté, R.: Tropane alkaloid production by *Agrobacterium rhizogenes* transformed hairy root cultures of *Atropa baetica* Willk. (Solanaceae), Plant Cell Reports, **18** (1999), no. 5, pp. 418-423.

24. Aarrouf, J.; Castro-Quezada, P.; Mallard, S.; Caromel, B.; Lizzi, Y. and Lefebvre, V.: *Agrobacterium rhizogenes*-dependent production of transformed roots from foliar explants of pepper (*Capsicum annuum*): a new and efficient tool for functional analysis of genes, Plant Cell Reports, **31** (2012), no. 2, pp. 391-401.

25. Georgiev, M.I.; Ludwig-Müller J.; Alipieva K.; Lippert A.: Sonication-assisted *Agrobacterium rhizogenes*-mediated transformation of *Verbascum xanthophoeniceum* Griseb. for bioactive metabolite accumulation, Plant Cell Reports, **30** (2011), no. 5, pp. 859-866.

26. Savka, M.A.; Ravillion, B.; Noel, G.R. and Farrand, S.K.: Induction of hairy roots on cultivated soybean genotypes and their use to propagate the soybean cyst nematode, Phytopathology, **80** (1990), no. 5, pp. 503-508.

27. Lippincott, J.A.; Beiderbeck, R. and Lippincott, B.B.: Utilization of octopine and nopaline by Agrobacterium, Journal of Bacteriology, **116** (1973), no. 1, pp. 378-383.

28. Sambrook, J.; Fritsch, E.F. and Maniatis, T.: Molecular cloning: a laboratory edition, 2nd ed.; Cold Spring Harbour Laboratory Press, New York, 1989.

29. Jefferson, R.A.: Assaying chimeric genes in plants: the GUS gene fusion system, Plant Molecular Biology Reporter, **5** (1987), no. 4, pp. 387-405.

30. Fleischer, R.; Horlemann, C.; Schwekendiek, A.; Kling, C. and Weber, G.: AFLP fingerprinting in hop: analysis of the genetic variability of the Tettnang variety, Genetic Resources and Crop Evolution, **51** (2004), no. 2, pp. 211-220.

31. Akramian, M.; Tabatabaei S.M.F. and Mirmasoumi, M.: Virulence of different strains of *Agrobacterium rhizogenes* on genetic transformation of four *Hyoscyamus* species, American-Eurasian Journal of Agriculture and Environmental Science, **3** (2008), no. 5, pp. 759-763.

32. Baranski, R.; Klocke, E. and Schumann, G.: Green fluorescent protein as an efficient selection marker for *Agrobacterium rhizogenes* mediated carrot transformation, Plant Cell Reports, **25** (2006), no. 3, pp. 190-197.

33. Kajikawa, M.; Morikawa, K.; Abe, Y.; Yokota, A. and Akashi, K.: Establishment of a transgenic hairy root system in wild and domesticated watermelon (*Citrullus lanatus*) for studying root vigor under drought, Plant Cell Reports, **29** (2010), no. 7, pp.771-8.

34. Mankin, S.L.; Hill, D.S.; Olhoft, P.M.; Toren, E.; Wenck, A.R.; Nea, L.; Xing, L.; Brown, J.A.; Fu, H.; Ireland, L.; Jia, H.; Hillebrand, H.; Jones, T. and Song, H.S.: Disarming and sequencing of *Agrobacterium rhizogenes* strain K599 (NCPPB2659) plasmid pRi2659, In Vitro Cellular Developmental Biology-Plant, **43** (2007), no. 6, pp. 521-535.

35. Jian, B.; Hou W.; Wu C.; Liu, B.; Liu, W.; Song, S.; Bi, Y. and Han, T.: *Agrobacterium rhizogenes*-mediated transformation of *Superroot*-derived *Lotus corniculatus* plants: a valuable tool for functional genomics, BMC Plant Biology, **9** (2009), pp. 78.

36. Estrada-Navarrete, G.; Alvarado-Affantranger, X.; Olivares, J.E.; Guillén, G.; Díaz-Camino, C.; Campos, F.; Quinto, C.; Gresshoff, P.M.; Sanchez, F.: Fast, efficient and reproducible genetic transformation of *Phaseolus* spp. by *Agrobacterium rhizogenes*, Nature Protocols, **2** (2007), pp. 1819 – 1824.

37. Pak, H.K.; Sim, J.S.; Rhee, Y.; Ko, H.R.; Ha, S.H.; Yoon, M.S.; Kang, C.H.; Lee, S.; Kim, Y.H. and Hahn, B.S.: Hairy root induction in Oriental melon (*Cucumis melo*) by *Agrobacterium rhizogenes* and reproduction of the root-knot nematode (*Meloidogyne incognita*), Plant Cell Tissue and Organ Culture, **98** (2009), no. 2, pp. 219-228.

38. Schmidt, J.F.; Moore, M.D.; Pelcher, L.E.; and Covello, P.S.: High efficiency *Agrobacterium rhizogenes*-mediated transformation of *Saponaria vaccaria* L. (Caryophyllaceae) using fluorescence selection, Plant Cell Reports, **26** (2007), no. 9, pp.1547-1554.

39. Lin, M.H.; Gresshoff1, P.M.; Indrasumunar, A. and Ferguson, B.J.: pHairyRed: A novel binary vector containing the DsRed2 reporter gene for visual selection of transgenic hairy roots, Molecular Plant, **4** (2011), no. 3, pp. 537-545.

40. Wagner, G.J.: Secreting glandular trichomes: more than just hairs, Plant Physiology, **96** (1991), no. 3, pp. 675-679.

41. Lee, M.H.; Yoon, E.S.; Jeong, J.H. and Choi, Y.E.: *Agrobacterium rhizogenes*-mediated transformation of *Taraxacum platycarpum* and changes of morphological characters, Plant Cell Reports, **22** (2004), no. 11, pp. 822-827.

# 6. Genetic transformation of hop (*Humulus lupulus* L. cv. Tettnanger) by particle bombardment and plant regeneration using temporary immersion system[7]

## 6.1 Abstract

The efficiency of micropropagation of double-node shoots of hop (*Humulus lupulus* L. cv Tettnanger) was evaluated using semi-solid and liquid culture medium in RITA® bioreactors. The highest fresh and dry weight of shoots, the average number of shoots, and multiplication rate were obtained using the RITA® system. In contrast, the highest length of shoots was obtained on semi-solid medium. Moreover, the length of shoots was affected significantly by the density of the inoculum of double-node shoots in RITA® vessels. In addition, the RITA® bioreactors were suitable for shoot induction from organogenic calli. The percentage of shoot induction, fresh and dry weight of shoots was significantly higher in RITA® than in semi-solid medium. The age of organogenic calli and inoculum density significantly affected the induction of shoots from organogenic calli. The optimum conditions for DNA delivery into hop organogenic calli using the biolistic particle delivery system were determined. Organogenic calli were bombarded with the plasmid pSR5-2 (*gusA*, and *nptII*) using the following conditions: helium pressure (900, 1100, or 1350 psi), and target distance (6, 9, or 12 cm). The highest *gusA* transient activity was obtained using 900 psi and 6 cm. For stable genetic transformation, three-week-old organogenic calli were bombarded with the plasmid pCAMBIA 1301 (*gusA*, *mgpf5*, and *hpt*) using those conditions. Stable *gusA* expression was observed in organogenic calli and shoots after 4 weeks of culture on selection medium containing 2.5 $mgl^{-1}$ of hygromycin. The integration of the *gusA* and *mgfp5* genes into the hop genome was confirmed by PCR.

**Keywords** Hop, particle bombardment, temporary immersion, liquid culture, *gusA*, *mgfp5*, *hpt*

[7] This chapter consists of an unpublished manuscript that has been submitted to Plant Biotechology Report

## 6.2 Introduction

Hop (*Humulus lupulus* L., Cannabaceae) is a perennial, dioecious, and climbing plant cultivated mainly in Europe, North America, South Africa, Australia, and New Zealand. Secondary metabolites produced in the lupulin glands of the female cones are used as flavoring compounds in beer. Furthermore, they have important pharmaceutical and medicinal properties (Zanoli and Zavatti 2008).

Protocols for the micropropagation of hop and shoot induction from calli using semi-solid medium have been available for some time. However, these protocols are characterized by a low regeneration rates (Gurriarán et al. 1999, Roy et al. 2001; Horlemann et al. 2003). Moreover, taking into consideration that only a small number of cells integrate successfully a transgene, the development of any transformation system requires the establishment of efficient plant regeneration protocols. The use of liquid medium for the regeneration of cells provides an alternative to overcome this drawback. For instance, regeneration of shoots from a single GONC (green organogenic nodule clusters) of hop cv. US Tettnanger was improved using temporary immersion bioreactors (Schwekendiek et al. 2009).

Although successful genetic transformation of hop has been reported using *Agrobacterium tumefaciens*, it is laborious, time consuming and limited by the low transformation efficiency (approx. 1%) (Horlemann et al. 2003). Direct gene transfer via biolistic delivery offers advantages in relation to *Agrobacterium* transformation (Sharma et al. 2005). Genetic transformation of hop cv. Eroica using particle bombardment has been reported (Batista et al. 2008). However, the development of a biolistic-mediated transformation system required the optimization of physical, chemical, and biological parameters (Rasco-Gaunt et al. 1999). Therefore, the establishment of the best parameters for biolistic genetic transformation is necessary for transient and stable expression of transgenes in hop cv. Tettnanger.

Consequently, in hop the functional validation of a large number of candidate genes, such as those involved in the biosynthesis of flavonoids, with *A. tumefaciens*-mediated transformation and successive plant regeneration on semi-solid medium has major limitations. Therefore, the regeneration of plants in temporary immersion bioreactors combined with particle bombardment could provide an alternative method for genetic transformation of hop.

In the present study, a protocol for micropropagation, shoot induction, and plant regeneration in temporary immersion bioreactors was established. Additionally, biolistic delivery parameters were evaluated with the aim of optimizing DNA integration into the genome of hop (*Humulus lupulus* L. cv. Tettnanger).

## 6.3 Materials and methods

### 6.3.1 Plant material and organogenic calli induction

Hop plants (*H. lupulus* L. cv. Tettnanger) were cultured under *in vitro* conditions using 500 ml polystyrene vessels with 20 ml of elongation medium [MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 2 % (w/v) glucose, and 0.6% (w/v) agar] under a 16 h light photoperiod at 21 ± 1ºC. For organogenic calli induction, aseptically isolated shoot internodes were cultured on callus induction medium (CIM) [MS salts, B5 vitamins, 2 % (w/v) glucose, 1.43 µM IAA, 9.08 µM TDZ, 0.6% agar] (Horlemann et al. 2003).

### 6.3.2 Hop micropropagation using a temporary immersion system

In order to compare the growth and multiplication of double-node shoots on semi-solid and liquid medium in the RITA® system, two experiments were performed. All experiments consisted of five replications for each treatment. Cultures were maintained under a 16 h light photoperiod at 21 ± 1ºC. For treatments in semi-solid medium, 10 explants were cultured in each 500 ml polystyrene vessels containing 20 ml of elongation medium. The first experiment was aimed at optimizing the duration of daily immersion cycles. Fifty double-node shoots (~1 cm in length) were cultured in each RITA® vessel with 200 ml of liquid elongation medium. Immersion time consisted of four daily immersions of 1 min or 4 min each. Once the optimal immersion duration was determined, in a second experiment, 25 or 50 double-node shoots were cultured in each RITA® vessels with 200 ml of liquid elongation medium using an immersion duration of 1 min every 6 h. After 4 weeks of culture, the shoot length (cm), fresh and dry weight (g), the total number of shoots regenerated per explant, and the multiplication rate [(total number of shoots regenerated per explant/initial number of explant) x 100] were determined. Dry weight was measured after drying the shoots for 6 h at 160°C. Data are presented as mean ± SD, and differences between treatment means were considered using the Duncan test at the significance level of $P \leq 0.05$.

### 6.3.3 Shoot regeneration from organogenic calli using a temporary immersion system

The effect of culture system, age of explant, and density of culture on shoot regeneration from organogenic calli was evaluated. In a first experiment, 50 pieces of four-week-old organogenic calli were cultured in each RITA® vessel with 200 ml of liquid CIM. Immersion time consisted of four daily immersions of 1 min or 4 min each. As control, 10 organogenic calli were cultured per Petri plate (92x16 mm) containing 20 ml of semi-solid CIM. Once the best immersion period was determined, 50 pieces of three or four-week-old organogenic calli were cultured in each RITA® vessel with 200 ml of liquid CIM using an immersion period of 1 min every 6 h.

In a third experiment, 25 or 50 pieces of four-week-old organogenic calli were cultured in each RITA® vessel with 200 ml of liquid CIM using an immersion period of 1 min every 6 h. All experiments consisted of five replications of each treatment. All cultures were incubated under a 16 h light photoperiod at 21 ± 1ºC. After 4 weeks of culture, the percentage of organogenic calli with shoots, fresh and dry weight (g) of shoots was determined. Dry weight was measured after drying the shoots for 6 h at 160 °C. Data are presented as mean ± SD, and differences between treatment means were considered using the Duncan test at the significance level of $P \leq 0.05$.

### 6.3.4 Plasmid DNA

The plasmid pSR5-2 containing the reporter gene *gusA* under the control of the CaMV 35S promoter and the selection marker *nptII* under the control of the nopaline synthase promoter was used for biolistic delivery optimization (Horlemann et al. 2003). On the other hand, the plasmid pCAMBIA 1303 (Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia) containing the reporter genes *gusA*, and *mgfp5*, and the selection marker *hpt* under control of the CaMV 35S promoter was used for stable expression experiments. A volume of 20 µl of DNA (1 $\mu g \mu l^{-1}$) was precipitated onto 50 µl sterile gold particles (1 µM, Bio-Rad Laboratories, Inc) according to the protocol described by Russell (1993).

### 6.3.5 Optimization of DNA delivery into organogenic calli

Transient transformation of organogenic calli was performed with the PDS 1000/ He Biolistic Particle Delivery System (Bio-Rad) using a vacuum of 25 inches of Hg. Twenty four hours before particle bombardment, 25 pieces of four-week-old organogenic calli were cultured in a circular area (4 cm) in the center of a Petri plate (92x16 mm) containing 20 ml of CIM. Organogenic calli were bombarded once with the plasmid pSR5-2. In a first experiment, organogenic calli were bombarded using a helium pressure of 900, 1100 or 1350 psi respectively, with a target distance of 6 cm. In a second experiment, organogenic calli were bombarded using 900 psi with a target distance of 6, 9 or 12 cm. Each treatment was performed three times. As control, non- bombarded organogenic calli were used. Explants were cultured under a 16 h light photoperiod at 21 ± 1ºC. The average and the standard error of the transformation efficiency [(number of explants with *gusA* activity/total number of explants bombarded) x 100] were determined. Data were analyzed using one-way ANOVA and the differences between means were contrasted using the Duncan test at the significance level of $P \leq 0.05$.

### 6.3.6 Stable genetic transformation and selection of transformants

Following the establishment of an optimized protocol for transient transformation, three-week-old organogenic calli were cultured for 24 h in a circular area (4 cm) in the center of a Petri plate (92x16 mm) containing 20 ml of CIM. Particle bombardment was performed with the PDS 1000/ He Biolistic Particle Delivery System (Bio-Rad) using the following conditions: 900 psi, 6 cm distance, and 25 inches of Hg. Organogenic calli were bombarded once with the plasmid pCAMBIA 1303. After particle bombardment, the organogenic calli were cultured for one week without any selection agent on fresh CIM. Then, the bombarded organogenic calli were transferred to liquid (selection method I, Fig 6.1) or semi-solid/liquid selection medium (CIM supplemented with 2.5 $mgl^{-1}$ hygromycin) (selection method II, Fig 6.1). The appropriate concentration of hygromycin for the selection of transformants was previously determined by Batista et al. (2008). The cultures were maintained with 16 h light photoperiod at 21 ± 1°C. Four weeks after initiation of the selection, hygromycin-resistant calli and shoots were assayed for *gusA*, and *mgfp5* activity.

### 6.3.7 GUS and GFP assays

GUS assays were performed 48 hours after particle bombardment by incubating of tissue in assay buffer [50mM sodium phosphate (pH 7.0), 0,5 mM potassium ferrocyanide, 0,5 mM potassium ferricyanide, 10 mM EDTA, 0,1% Triton, 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide)] at 37 °C in the dark for 24 h (Jefferson 1987). Moreover, GFP activity was visualized using a fluorescence stereomicroscope (Stemi SV6, Zeiss, Germany).

### 6.3.8 Characterization of the putative transformants

In order to verify the presence of the genes *gusA*, and *mgfp5*, genomic DNA from four-week-old putative transgenic or non-transgenic shoots respectively, was isolated according to Fleischer et al. (2004). The PCR was performed in a volume of 25 µl containing 100 ng of genomic DNA, 1X Mango *Taq* PCR buffer, 0.25 mM of each dNTPs, 0.2 µM of each primer, 1.5 mM of $MgCl_2$ and 0.5 U Mango *Taq*™ polymerase (Bioline, Germany). Three different specific primers pairs were used: *mgfp5* [gfp-f: (5'-atgagtaaaggagaagaacttttcactgg-3') and gfp-r: (5'-ttatttgtatagttcatccatgccatgtg-3'); expected fragment length: 790 bp], *gusA* [gusA-f: (5'-gcagcccggctaacgtatccac-3') and gusA-r: (5'-gttctgcgacgctcacaccgat-3'); expected fragment length: 200 bp; Batista et al. (2008)], and *18S* [18S-S: (5'-aggtagtgacaataaataacaa-3') and 18S-AS: (5'-tttcgcagttgttcgtctttca-3'); expected fragment length: 481 bp]. The plasmid pCAMBIA 1303 was used as positive control for the PCR reaction.

The amplification conditions for *mgfp5* were: 94ºC for 3 min followed by 35 cycles at 94ºC for 1 min, 60ºC for 1 min, 72ºC for 1 min and a final step of 72ºC for 10 min. For *gusA* and *18S* the same conditions were used except that the annealing temperature was 80ºC and 53ºC, respectively. After staining with DNA Stain G (Serva, Germany), the PCR products were separated on 1.8 % (w/v) agarose gel, visualized under UV, and documented with a digital camera.

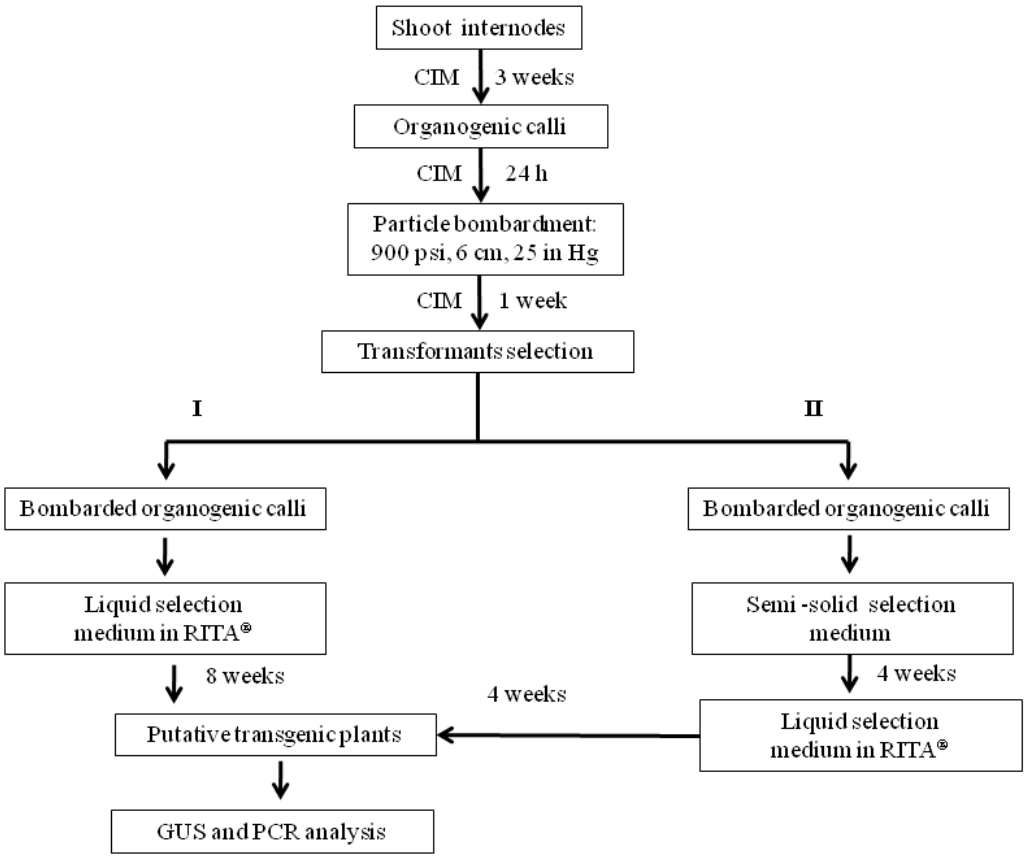


**Fig. 6.1** Schematic representation of hop (*Humulus lupulus* L. cv. Tettnanger) genetic transformation and selection of transformants. The basic stages, culture media, and the time required are indicated.

## 6.4 Results

### 6.4.1 Hop micropropagation using a temporary immersion system

Micropropagation of double-node shoots in semi-solid culture medium was compared to the micropropagation in bioreactors. The culture system, as well as the immersion duration in RITA® significantly affected the growth and development of double-node shoots. The highest shoot length was obtained using semi-solid medium followed by the RITA® system with an immersion duration of 4 min and 1 min every 6 h (Table 6.1). On the other hand, the highest fresh weight of shoots was obtained when liquid medium was used in RITA® vessels with an immersion duration of 4 min every 6 h (Table 6.1). In contrast, the highest dry weight of shoots, the highest average number of shoots, and the highest multiplication rate were obtained using immersion duration of 1 min every 6 h (Table 6.1). Therefore, the latter culture condition was chosen for further experiments.

Moreover, the density of the inoculum significantly affected the growth as well as the development of double-node shoots. The highest shoot length, fresh weight, dry weight, average number of shoots, and multiplication rate were obtained using 50 explants per RITA® (Table 6.2).

**Table 6.1** Effect of the culture system on the growth and development of double-node shoots of hop (*Humulus lupulus* L. cv. Tettnanger) after 4 weeks of culture

| Culture system | Plantlets | | | | |
|---|---|---|---|---|---|
| | Length (cm) | Fresh weight (g) | Dry weight (g) | Average number | Multiplication rate[a] |
| Semi-solid medium | 6.2±2.5 a | 1.8±0.8 c | 0.4±0.6 b | 12.8±2.0 c | 1.3 c |
| RITA® (1 min every 6 h) | 3.1±1.1 c | 8.8±2.2 b | 1.5±0.2 a | 197.8±37.6 a | 3.9 a |
| RITA® (4 min every 6 h) | 4.9±2.1 b | 11.6±0.8 a | 1.4±0.1 a | 149.6±37.7 b | 2.5 b |

Each value represents the mean ± SD of 5 replicates for each treatment. The same letter within columns denotes statistically equal means with the Duncan test at $P \leq 0.05$.

[a] Multiplication rate: [(total number of shoots regenerated per explant/initial number of explant) x 100]

**Table 6.2** Effect of inoculum density on the growth and development of double-node shoots of hop (*Humulus lupulus* L. cv. Tettnanger) after 4 weeks of culture in RITA®

| Inoculum density | Plantlets | | | | |
|---|---|---|---|---|---|
| | Length (cm) | Fresh weight (g) | Dry weight (g) | Average number | Multiplication rate[a] |
| 25 (8 ml medium/explant) | 2.0±0.9 b | 1.5 ±0.4 b | 0.4±0.3 a | 57.8±6.6 b | 1.3 b |
| 50 (4 ml medium/explant) | 2.8±1.0 a | 2.8±0.4 a | 0.5±0.1 a | 80.0±15.8 a | 1.6 a |

Each value represents the mean ± SD of 5 replicates. 25 or 50 double-node shoots were cultured per RITA® vessels using a 1 min of immersion every 6 h. The same letter within columns denotes statistically equal means with the Duncan test at $P < 0.05$.

[a] Multiplication rate: [(total number of shoots regenerated per explant/initial number of explant) x 100]

The micropropagation of double-node shoots using semi-solid medium takes approximately 8 weeks and the plantlets showed well broad leaves and roots (Fig. 6.2.a). Whereas, using the RITA® system in 4 weeks the double-node shoots developed plantlets also showing well formed leaves and roots (Fig. 6.2.b). The development and growth of double-node shoots was asynchronous and some micropropagated double-node shoots developed small plantlets with thin leaves that were curled, but without symptoms of hyperhydricity (Fig. 6.2.c).

### 6.4.2 Shoot regeneration from organogenic calli in the temporary immersion system

Induction of shoots from organogenic calli in semi-solid culture medium was compared to those developed in RITA® bioreactors. Over a period of 4 weeks, the highest induction rate of shoots, the highest fresh and dry weight of shoots were obtained using the RITA® system with an immersion duration of 1 min every 6 h (Table 6.3).

Moreover, the influence of the age of the organogenic calli on the shoot induction was evaluated. After 4 weeks of culture, the highest induction rate of shoots, the highest fresh weight and dry weight of shoots were obtained using four-week-old calli as explants (Table 6.4).

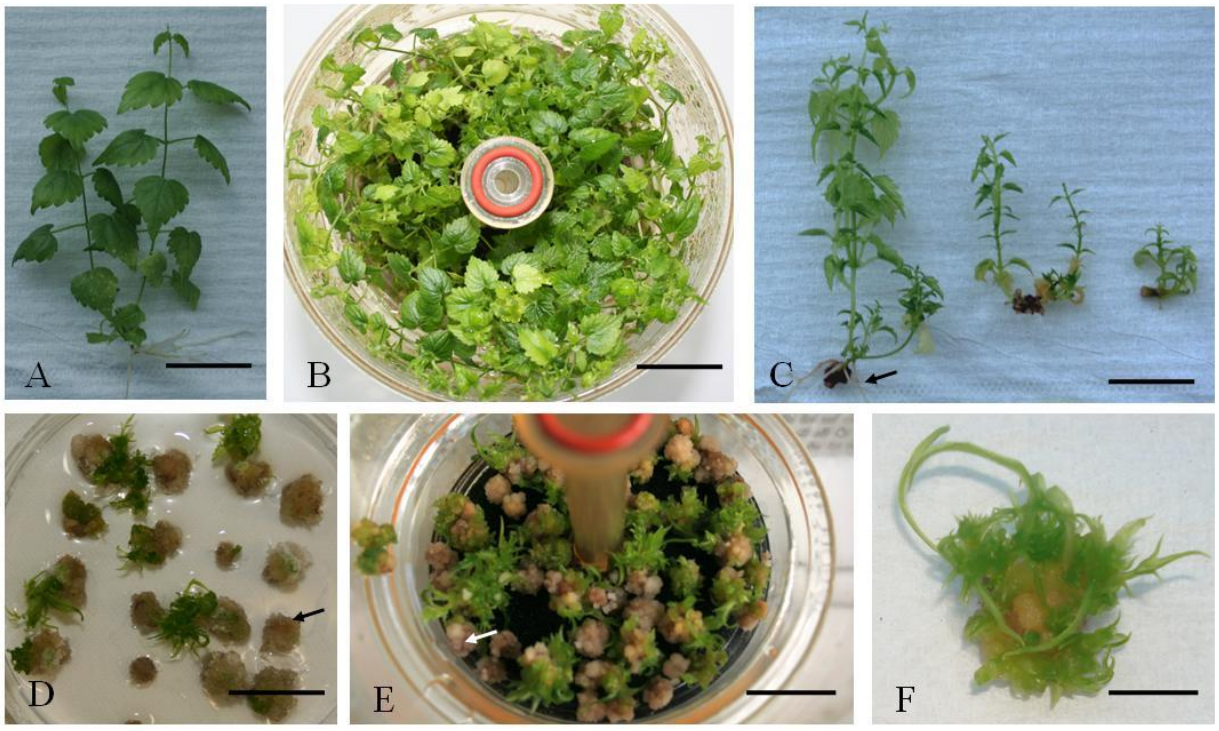


**Fig. 6.2** Micropropagation and shoot regeneration of hop (*Humulus lupulus* L. cv. Tettnanger) **(A)** plantlet propagated using semi-solid medium **(B)** plantlets grown in RITA® bioreactor using 1 min immersion every 6 h **(C)** close-up view of plantlets with roots (indicated by an arrow) obtained using the RITA® system **(D)** shoot regeneration from organogenic calli of hop on semi-solid medium and **(E)** temporary immersion bioreactor system using 1 min immersion every 6 h **(F)** close-up view of shoots from **(E)**. Non-regenerative calli is indicated by an arrow (Scale bar: 1 cm).

**Table 6.3** Effect of the culture system on shoot regeneration from organogenic calli of hop (*Humulus lupulus* L. cv. Tettnanger) after 4 weeks of culture

| Culture system | Shoot induction (%) | Shoot fresh weight (g) | Shoot dry weight (g) |
|---|---|---|---|
| Semi-solid medium | 40.0±49.1 b | 1.0±0.9 c | 0.3±0.2 c |
| RITA® (1 min every 6 h) | 68.3±46.6 a | 18.0±2.3 a | 1.3±0.1 a |
| RITA® (4 min every 6 h) | 18.9±39.2 c | 4.2±1.8 b | 0.7±0.2 b |

Four-week-old organogenic calli were used as explants. Each value represents the mean ± SE of 5 replicates for each treatment. The same letter within columns denotes statistically equal means with the Duncan test at $P< 0.05$.

**Table 6.4** Effect of explant age on shoot regeneration from organogenic calli of hop (*Humulus lupulus* L. cv. Tettnanger) after 4 weeks of culture in RITA®

| Explant age | Shoot induction (%) | Shoot fresh weight (g) | Shoot dry weight (g) |
|---|---|---|---|
| Three-week-old calli | 24.0±43.0 b | 1.8±1.6 b | 0.1±0.1 b |
| Four-week-old calli | 69.0±46.5 a | 14.6±1.4 a | 1.3±0.2 a |

The culture conditions in the RITA® vessels included an immersion duration of 1 min every 6 h. Each value represents the mean ± SD of 5 replicates. The same letter within columns denotes statistically equal means with the Duncan test at $P< 0.05$.

Furthermore, the initial inoculum density affected the shoot regeneration from organogenic calli. The highest shoot induction, fresh weight and dry weight of shoots were obtained when 50 pieces of calli of four-week-old were cultured per RITA® vessel (Table 6.5). Shoot regeneration from organogenic calli was observed either using semi-solid medium (Fig. 6.2.d) or RITA® bioreactors (Fig. 6.2.e). Adventitious shoots regenerated in the RITA® bioreactors are shown in the Fig. 6.2.f.

**Table 6.5** Effect of the number of explants in each RITA® vessel on shoot regeneration from organogenic calli of hop (*Humulus lupulus* L. cv. Tettnanger) after 4 weeks of culture

| Culture density | Shoot induction (%) | Shoot fresh weight (g) | Shoot dry weight (g) |
|---|---|---|---|
| 25 (8 ml medium/explant) | 33.0±47.3 a | 4.9±0.9 b | 0.4±0.2 b |
| 50 (4 ml medium/explant) | 40.8±49.2 a | 9.2±0.8 a | 0.7±0.1 a |

Four-week-old organogenic calli were used as explants and the culture conditions in the RITA® vessels included immersion duration of 1 min every 6 h. Each value represents the mean ± SE of 5 replicates. The same letter within columns denotes statistically equal means with the Duncan test at $P< 0.05$.

### 6.4.3 Optimization of DNA delivery into organogenic callus

The percentage of explants with transient *gusA* expression was significantly affected by the helium pressure, and the target distance. A 900 psi rupture disk pressure resulted in the highest *gusA* activity (80.5±5.2) in organogenic calli bombarded with pSR5-2, compared to 1,100 (38.3±6.3) or 1,350 psi (16.7±4.9) (Fig. 6.3.a). Therefore, in subsequent experiments for assessing target distance, bombardments were performed using 900 psi. Significant differences in the transient expression of *gusA* were obtained using 6 cm (48.0 ± 5.0), 9 cm (35.0 ± 4.8) or 12 cm (26.0 ± 4.4) (Fig. 6.3.b). The Figure 6.3.c shows transient *gusA* expression on bombarded hop organogenic calli using 900 psi and 6 cm. Non-bombarded calli did not exhibit activity of *gusA* (Fig. 6.3.d) but exhibited green fluorescence under UV light (Fig. 6.3.e). For this reason, the observation of transient *mgfp5* expression and confirmation of transgenic hop organogenic calli was ambiguous (Fig. 6.3.f).

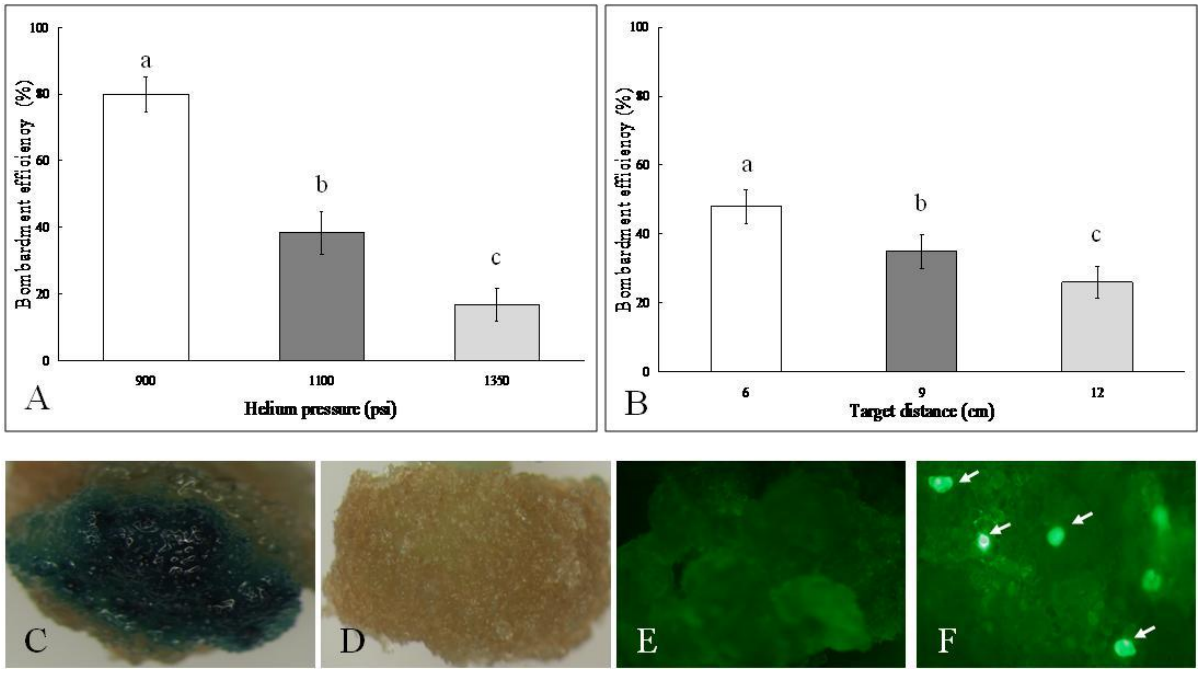


**Fig. 6.3** Effect of **(A)** helium pressure and **(B)** target distance on transient *gusA* expression in hop (*Humulus lupulus* cv. Tettnanger) organogenic calli bombarded with the plasmid pSR5-2 **(C)** organogenic calli showing transient *gusA* expression **(D)** non-bombarded tissue **(E)** non-bombarded tissue showing green auto-fluorescense **(F)** transient *mgfp5* expression on organogenic calli (indicated by an arrow). Different letters indicate values are significantly different by Duncan test at P< 0.05

### 6.4.4 Selection and regeneration of bombarded organogenic calli

A total of 800 three-week-old calli were bombarded in two different experiments. Eight weeks after bombardment, the transformation efficiency was estimated to be 14 % or 21 % when the bombarded organogenic calli were cultured in liquid (selection method I, Fig 6.1) or semi-solid/liquid selection medium (selection method II, Fig 6.1), respectively (Table 6.6). During selection on liquid CIM supplemented with 2.5 $mgl^{-1}$ of hygromycin, bombarded organogenic calli formed putative transgenic shoots (Fig. 6.4.a), whereas non-bombarded organogenic calli turned brown and shoot induction was inhibited within 30 days of culture (Fig. 6.4.b). Hygromycin-resistant shoots were subjected to histochemical GUS analysis and stable *gusA* expression was observed (Fig. 6.4.c), whereas non-transformed calli did not show *gusA* expression (Fig. 6.4.d).

**Table 6.6** Effect of the selection method on the transformation efficiency of hop (*Humulus lupulus* L. cv. Tettnanger)

| Selection method | Number of calli bombarded | Time of selection (weeks post-bombardment) | | Hygromycin-resistant calli with shoots[a] | | Transformation efficiency (%) [b] |
|---|---|---|---|---|---|---|
| | | Semi-solid medium | Liquid medium (RITA®) | After 4 weeks | After 8 weeks | |
| I | 300 | - | 8 | 0.2±0.4 (60) | 0.1±0.4 (43) | 14 |
| II | 500 | 4 | 4 | 0.3±0.5 (141) | 0.2±0.4 (103) | 21 |

[a] Mean ± SD of five repetitions. The number of hygromycin-resistant calli with shoots is indicated in parenthesis.

[b] Transformation efficiency (%): [(Number of hygromycin-resistant calli with shoots/ total number of calli bombarded) x 100]

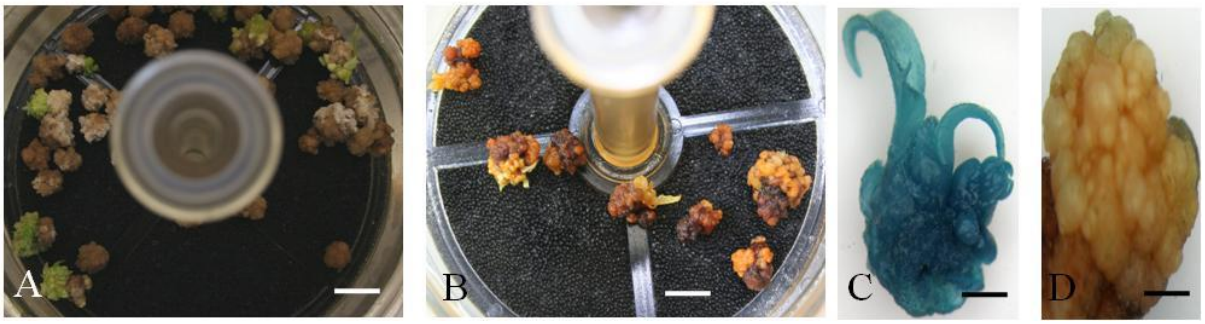


**Fig. 6.4** Selection of transgenic organogenic calli of hop (*Humulus lupulus* L. cv. Tettnanger) in a temporary immersion system **(A)** putative transgenic shoots developed in liquid selection medium using the RITA® system **(B)** complete growth inhibition of non-transformed organogenic calli in selection medium containing 2.5 mg $l^{-1}$ hygromycin **(C)** stable *gusA* expression on organogenic calli bombarded with pCAMBIA 1303 **(D)** non-bombarded organogenic calli showing no *gusA* activity

### 6.4.5 Characterization of the putative transformants

In addition to histochemical GUS staining, randomly selected four-week-old hygromycin resistant shoots were tested for the presence of the transgenes *mgfp5* (790 bp) and *gusA* (200 bp). The expected bands for the *mgfp5* or *gusA* genes were detected in transformed shoots after PCR amplification of genomic DNA (Fig. 6.5.a), but not from the shoots developed from non-bombarded organogenic calli (Fig. 6.5.b). To confirm the quality of extracted DNA, the 18S gene was also amplified in all samples (Fig. 6.5.c).

## 6.5 Discussion

A protocol for the micropropagation and shoot induction from organogenic calli of hop (*H. lupulus* L. cv. Tettnanger) using the RITA® system was established. Furthermore, a bombardment protocol for DNA delivery into hop organogenic calli was optimized.

In hop, existing protocols for micropropagation and shoot induction from calli usually used semi-solid media (Gurriarán et al. 1999, Roy et al. 2001; Horlemann et al. 2003). It is well known that the growth rate and the uptake of water, mineral nutrients, and plant growth regulators are reduced by gelling agents. Moreover, inhibitory and toxic exudates from explants do not dissipate as quickly as in liquid culture media (Gupta and Timmis 2005). Therefore, the use of liquid media in temporary immersion bioreactors (RITA®) should improve *in vitro* growth and morphogenesis. This technique allows a direct contact of the medium with the plant material and provides adequate gas exchange. In addition, temporary immersion bioreactors allow automation and scaled-up reducing costs and labor (Etienne and Berthouly 2002).

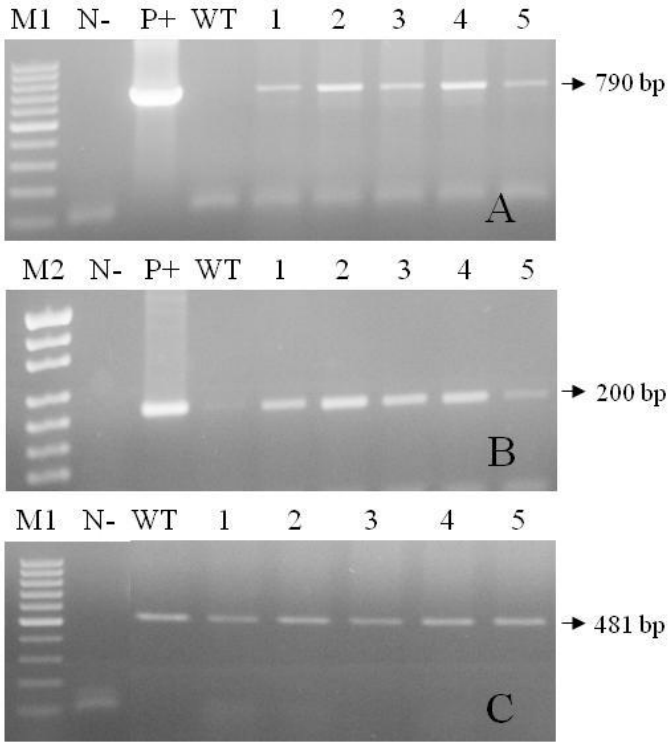


**Fig. 6.5** Detection of the genes **(A)** *mgfp5* (790 bp), **(B)** *gusA* (200 bp) and **(C)** *18S* (481 bp) in transgenic shoots by PCR. M1: molecular weight marker (100 bp DNA, MBI Fermentas, St. Leon-Rot), M2: molecular weight marker (pUC19 DNA/*Msp*I, MBI Fermentas, St. Leon-Rot), $N^-$: negative control (PCR reaction mix without template), $P^+$: positive control (DNA of plasmid pCAMBIA 1303), WT: wildtype DNA (non-bombarded shoots), 1-5: transgenic shoots.

For numerous plant species, successful *in vitro* plant regeneration though micropropagation, organogenesis, and somatic embryogenesis in temporary immersion has been reported (McAlister et al. 2005; Roels et al. 2005; Zhu et al. 2005; Niemenak et al. 2008; Shaik et al. 2010). Moreover, a temporary immersion system was used for the regeneration of transgenic strawberry and pineapple plants (Espinosa et al. 2002; Hanhineva et al. 2007).

In culture systems using bioreactors and liquid medium, the duration of the temporary immersion is crucial. It determines availability of nutrient and plant growth regulators and may influence hyperhydricity (Etienne and Berthouly 2002). In the present study, it was shown that short immersions times (1 min every 6 h) promoted hop micropropagation and induction of shoots from organogenic calli. Similar observations were made in *Coffea*, and *Theobroma* (Albarrán et al. 2005; Niemenak et al. 2008). Moreover, inoculum density affected micropropagation and organogenesis of hop; as shown in the present study. It has been reported that culture density regulated morphogenesis in *Coffea*, and *Chrysanthemum* cultures (van Boxtel and Berthouly 1996; Hahn and Peak 2005).

For genetic transformation, the process of DNA delivery needs to be optimized for each species. Previously, Batista et al. (2008) evaluated the interaction between helium pressure and target distance for the bombardment of petioles, calli and GONC of *H. lupulus* L. cv. Eroica. These authors concluded that higher *gusA* transient expression was obtained in petioles and GONC. In contrast, calli were not appropriate for transient and stable transformation due to their irregular surface which restricted particle penetration (Batista et al. 2008).

Nevertheless, Gurriarán et al. (1999) indicated that calli were suitable explants for transformation mediated by *Agrobacterium* or DNA bombardment. In the present study, it was shown that a lower helium pressure (900 psi) and shorter target distance (6 cm) may improve transient *gusA* expression in organogenic calli of hop. The combination of lower pressures with lower target distances improved the transient *gusA* expression in petioles of hop (Batista et al. 2008), in zygotic embryos of *Larix gmelinii* (Lin et al. 2005), and in pollen tubes of *Pinus sp*. (Fernando et al. 2000).

Parallel to the optimization of physical parameters affecting the efficiency of particle bombardment, suitable reporter genes have to be considered. To the best of our knowledge, this is the first report for hop genetic transformation using *mgfp5* as reporter gene. Previously, hop transformation has been carried out using *gusA* as reporter gene (Horlemann et al. 2003). Often, GFP has emerged as a vital reporter gene for the detection of transient and stable gene expression in transgenic plants. GFP has been expressed in several organisms, and it has several advantages over other reporter genes (Stewart 2001). Despite the auto-fluorescence of non-bombarded hop organogenic calli, green fluorescence of *mgfp5* expressing tissues were detected. This result suggested that this reporter gene can be used for monitoring transgene expression in early developmental stages. Auto-fluorescense of non-transformed tissues has been observed in *Citrus* sp., and *M. truncatula* (Ghordel et al. 1999; Duque et al. 2007). The auto-fluorescence could be attributed to secondary metabolites of hop cells, like phenolic compounds or chlorophyll, fluorescing at the same wavelength of *gfp* (Roshchina 2003).

The conditions for stable gene expression and plant regeneration in *H. lupulus* L. cv. Tettnanger using the RITA® system have been established. For genetic transformation with the particle gun the conditions consisted of a helium pressure of 900 psi and target distance of 6 cm. Furthermore, the optimized protocol developed in this study could be used for the validation of candidate genes with potential for modifying the production of secondary metabolites in hop.

## 6.6 Acknowledgments

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## 6.7 References

Albarrán JG, Bertrand B, Lartaud M, Etienne E (2005) Cycle characteristics in temporary immersion bioreactor affect regeneration, morphology, water and mineral status of coffee (*Coffea arabica* L.) somatic embryos. Plant Cell Tiss Org Cult 81: 27-36

Batista D, Fonseca S, Serrazina S, Figueiredo A, Salomé Pais M (2008) Efficient and stable transformation of hop ( *Humulus lupulus* L.) var. Eroica by particle bombardment. Plant Cell Rep 27: 1185-1196

Duque AS, Araújo S, Cordeiro MA, Santos DM, Fevereiro MP (2007) Use of fused *gfp* and *gus* reporters for the recovery of transformed *Medicago truncatula* somatic embryos without selective pressure. Plant Cell Tiss Organ Cult 90:325-330

Espinosa P, Lorenzo JC, Iglesias A, Yabor L, Menéndez E, Borroto J, Hernández L, Arencibia AD (2002) Production of pineapple transgenic plants assisted by temporary immersion bioreactors. Plant Cell Rep 21:136-140

Etienne H, Berthouly M (2002) Temporary immersion system in plant micropropagation. Plant Cell Tiss Org Cult 69: 215- 231

Fernando DD, Owens JN, Misra S (2000) Transient gene expression in pine pollen tubes following particle bombardment. Plant Cell Rep 19:224-228

Fleischer R, Horlemann C, Schwekendiek A, Kling C, Weber G (2004) AFLP fingerprinting in hop: analysis of the genetic variability of the Tettnang variety. Genet Res Crop Evol 51: 211-220

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151– 158

Ghordel R, Juarez J, Navarro, Peña L (1999) Green fluorescent protein as a screenable marker to increase the efficiency of generating transgenic woody fruits plants. Theor Appl Genet 99: 350-358

Gupta PK, Timmis R (2005) Mass propagation of conifer tress in liquid cultures - progress towards commercialization. Plant Cell Tiss Org Cult 81: 339- 346

Gurriarán MJ, Revilla MA, Tamés RS (1999) Adventitious shoot regeneration in cultures of *Humulus lupulus* L. (Hop) cvs. Brewers Gold and Nugget. Plant Cell Rep 18: 1007-1011

Hahn EJ, Peak KY (2005) Multiplication of *Chrysanthemum* shoots in bioreactors as affected by culture method and inoculation density of single node stems. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation. Springer, Dordrecht

Hanhineva K, Kokko H, Kärenlampi S (2005) Shoot regeneration from leaf explants of five strawberry (*Fragaria* × *Ananassa*) cultivars in temporary immersion bioreactor system. *In Vitro* Cell Dev Biol Plant 41: 826- 831

Horlemann C, Schwekendiek A, Hohnle M, Weber G (2003) Regeneration and *Agrobacterium*-mediated transformation of hop (*Humulus lupulus* L.). Plant Cell Rep 22: 210-217

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5: 387-405

Lin X, Zhang W, Takechi K, Takio S, Ono K, Takano H (2005) Stable genetic transformation of *Larix gmelinii* L. by particle bombardment of zygotic embryos. Plant Cell Rep 24: 418–425

McAlister B, Finnie J,. Watt MP, Blakeway F (2005) Use of the temporary immersion bioreactor system (RITA®) for production of commercial Eucalyptus clones in Mondi Forests (SA). Plant Cell Tiss Org Cult 81: 347-358

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497

Niemenak N, Saare-Surminski K, Rohsius C, Ndoumou DO, Lieberei R (2008) Regeneration of somatic embryos in *Theobroma cacao* L. in temporary immersion bioreactor and analyses of free amino acids in different tissues. Plant Cell Rep 27:667-676

Rasco- Gaunt S, Riley A, Barcelo P, Lazzeri PA (1999) Analysis of particle bombardment parameters to optimise DNA delivery into wheat tissues. Plant Cell Rep 19: 118-127

Roels S, Escalona M, Cejas I, Noceda C, Rodriguez R, Canal MJ, Sandoval J, Debergh P (2005) Optimization of plantain (*Musa* AAB) micropropagation by temporary immersion system. Plant Cell Tiss Org Cult 82: 57–66

Roshchina V (2003) Autofluorescense of plant secreting cells as biosensor and bioindicator reaction. J Fluoresc 13: 403-419

Roy AT, Leggett G, Koutoulis A (2001) Development of a shoot multiplication system for hop (*Humulus lupulus* L.). *In Vitro* Cell Dev Biol Plant 37: 79-83

Russell, JA (1993) The Biolistic® PDS-100/He device. Plant Cell Tiss Org Cult 33:221-226

Schwekendiek A, Hanson ST, Crain M (2009) A temporary immersion system for the effective shoot regeneration of hop. Acta Hort 848: 149-156

Shaik S, Dewir YH, Singh N, Nicholas A (2010) Micropropagation and bioreactor studies of the medicinally important plant *Lessertia (Sutherlandia) frutescens* L. South Afr J Bot 76: 180-186

Sharma KK, Bhatnagar-Mathur P, Thorpe TA (2005) Genetic transformation technology: status and problems. *In Vitro* Cell Dev Biol Plant 41: 102-112

Stewart C (2001) The utility of green fluorescent protein in transgenic plants. Plant Cell Rep 20:376-382

van Boxtel J, Berthouly M (1996) High frequency somatic embryogenesis from coffee leaves. Plant Cell Tissue Organ Cult 44:7-17

Zanoli P, Zavatti M (2008) Pharmacognostic and pharmacological profile of *Humulus lupulus* L. J Enthnopharmacol 116: 383-396

Zhu L, Li XY, Welander M (2005) Optimisation of growing conditions for the apple rootstock M26 grown in RITA containers using temporary immersion principle. Plant Cell Tiss Org Cult 81: 313–318

# 7. General discussion[8]

## 7.1 Genetic transformation of hop with the regulatory genes *PAP1/AtMYB75* and *HlMYB3*

### 7.1.1 Biological effects of *PAP1/AtMYB75* and *HlMYB3* in transgenic plants

Metabolic engineering of hop (*H. lupulus* L.) was successfully accomplished with the *PAP1/AtMYB75* or *HlMYB3* regulatory genes. The heterologous transcription factor PAP1/AtMYB75 from *A. thaliana* L. has been earlier genetically transformed into *A. thaliana* L., *N. benthamiania* L., *N. tabacum* L., *Petunia hybrida, S. lycopersicum* L., and *B. napus* L. (Borevitz et al. 2000; Tohge et al. 2005; Matoušek et al. 2006; Xie et al. 2006; Zhou et al. 2008; Zuluaga et al. 2008; Li et al. 2010). Previously, the homologous transcription factor HlMYB3 from *H. lupulus* L. has only been introduced in the model plants *A. thaliana* L., *N. benthamiania* L., and *Petunia hybrida* (Matoušek et al. 2007).

Heterologous expression of *PAP1/AtMYB75* in transgenic hop resulted in reddish female flowers and cones. This increase in red pigmentation among transgenic hop is likely to be attributed to the accumulation of anthocyanin compounds, which was correlated to the expression level of *PAP1/AtMYB75* (Gatica-Arias et al. 2012). Though, the regulatory gene *PAP1/AtMYB75* was under the control of the constitutive CaMV 35S promoter accumulation of anthocyanins was not observed in vegetative tissues. These results suggested that in hop additional flower-specific mechanisms regulate the production of anthocyanins. Unlike, transgenic *Arabidopsis*, tobacco, tomato, and canola plants over-expressing *PAP1/AtMYB75* had red, or purple-coloration (Borevitz et al. 2000; Xie et al. 2006; Zhou et al. 2008; Zuluaga et al. 2008; Li et al. 2010). In a similar approach, ectopic expression of the maize transcription factors Lc (Leaf color) and Pl (Purple leaf) resulted in purple-colored transgenic creeping bentgrass (*Agrostis stolonifera* L.) plants (Han et al. 2009).

In contrast, the constitutive over-expression in transgenic hop plants of the homologous regulatory gene *HlMYB3*, under the control of the CaMV 35S promoter, resulted in a normal phenotype, i.e. green female cones. It was shown that the expression of the HlMYB3 transcription factor in *A. thaliana* L., *N. benthamiana* L., and *Petunia hybrida* influenced plant growth and morphogenesis but not the color of organs (Matoušek et al. 2007). Moreover, a recent study demonstrated that the hop transcription factor HlMYB3 was unable to induce the accumulation of anthocyanin in petunia leaves (Matoušek et al. 2012).

[8] The citations included in this section are listed in general references.

The over-expression of a MYB transcription factor might not always change the color of plant organs and its role might vary among plant species. For instance, transgenic *S. miltiorrhiza* plants expressing *PAP1/AtMYB75* were characterized by a higher content of anthocyanins; however, the phenotype was similar to that of wildtype plants (Zhang et al. 2010). Moreover, tobacco calli expressing similar levels of *PAP1/AtMYB75* led to two types of calli: anthocyanin-producing and non- anthocyanin-producing calli (Zhou et al. 2008).

### 7.1.2 Composition of secondary metabolites in transgenic plants

The ectopic expression of *PAP1/AtMYB75* increased the production of anthocyanins, rutin, isoquercitin, kaempferol-7-*O*-glucoside, and kaempferol-7-*O*-glucoside-malonate in transgenic hop plants. Anthocyanins are more than plant colorants and benefits to human health have been reported. For instance, the consumption of anthocyanin-rich fruits and vegetables may prevent cancer, diabetes, cardiovascular and neurological diseases (Konczak and Zhang 2004). Rutin and kaempferol, two abundant flavonols in the human diet, have antioxidant, anti-inflammatory, antimicrobial, and anti-cancer properties (Lamson and Brignall 2000; Calderon-Montano et al. 2011). In hop, quercetin and kaempferol are present as glycosides. The content of these polyphenols in aroma hops is generally greater than in bitter hops (Kammhuber 2006/2007).

Xanthohumol, the most abundant prenylated chalcone in hop cones, has received much attention as cancer chemopreventive (Gerhäuser 2005). The concentration of the important health-promoting prenylated chalcones desmethylxanthohumol, and xanthohumol was also increased in hop transgenic plants expressing the *PAP1/AtMYB75* regulatory gene.

Furthermore, transgenic hop plants expressing *PAP1/AtMYB75* had enhanced $\alpha$-acids (humulone, cohumulone, and adhumulone), and $\beta$-acids (lupulone, colupulone, and adlupulone). This is the first study showing a possible role of *PAP1/AtMYB75* as transcriptional regulator of the biosynthesis of $\alpha$-acids, and $\beta$-acids in transgenic hop. It is well-known that the transcription factor PAP1/AtMYB75 regulates the biosynthesis of flavonoids; however, an additional unknown role as transcriptional regulator of the biosynthesis of phenolic acids has been reported (Zhang et al. 2010).

Previous studies have demonstrated the positive influence of the MYB transcription factors on the composition of secondary metabolites. For example, transgenic tomato plants expressing the *PAP1/AtMYB75* gene showed higher anthocyanins level than the wild type plants (Zuluaga et al. 2008). Moreover, the over-expression of *PAP1/AtMYB75* in *A. thaliana* L. resulted in increased biosynthesis of anthocyanins and quercetin glycosides (Borevitz et al. 2000). Additionally, the quercetin content was dramatically increased by *PAP1/AtMYB75* in transgenic tobacco plants (Zhou et al. 2008).

Recently, Li et al. (2010) reported that the level of cyanidin, pelagonidin, quercitin and sinapic acid increased in transgenic *B. napus* L. expressing the transcription factor PAP1/AtMYB75. The expression of *PAP1/AtMYB75* in *S. miltiorrhiza* transgenic plants enhanced the total content of phenolic, flavonoids, anthocyanins, and lignins (Zhang et al. 2010). Transgenic tobacco callus expressing the transcription factor, AtMYB12, displayed enhanced expression of flavonoid biosynthetic genes leading to increased accumulation of rutin (Pandey et al. 2012).

Biopesticide action of rutin against *Spodoptera litura* and *Helicoverpa armigera* larvae has been recently demonstrated (Pandey et al. 2012). Accordingly, an elevated tolerance against insect pests would be of great interest in transgenic hop expressing *PAP1/AtMYB75*, which had an elevated concentration of rutin.

Earlier, callus cultures of hop have been used for the production of flavonoids and polyphenols (Pšenáková et al. 2009; Ürgeová et al. 2011). Consequently, it would be attractive to analyze the production of secondary metabolites in transgenic callus culture expressing the transcription factor PAP1/AtMYB75. Callus cultures generally produce lower level of secondary metabolites in comparison to a whole plant. Nevertheless, due to rapid growth and biomass accumulation in callus cultures, they have been used for large-scale production of important secondary metabolites (Pandey et al. 2012).

It has been demonstrated that *N. benthamiania* L. and *Petunia hybrida* transgenic plants expressing the transcription factor HlMYB3 accumulated more phenolic acids and flavonol glycosides than the wildtype plants (Matoušek et al. 2007). These findings suggested the potential of *HlMYB3* as regulator of the metabolome composition in hop. Therefore, it could be interesting to investigate the chemical composition of the secondary metabolites in transgenic hop plants expressing the HlMYB3 transcription factor. Chemical analyses are planned, however, due to time restraints could not be incorporated in this thesis.

### 7.1.3 Expression of flavonoid and phloroglucinol biosynthetic genes in transgenic plants

In the present study, the transcript levels of *CHS_H1*, *CHI*, and *F3´H* were up-regulated by *PAP1/AtMYB75* in transgenic hop plants. Several studies have demonstrated that the *MYB* genes were implicated in the regulation of the flavonoid production (Borevitz et al. 2000; Espley et al. 2007; Gonzalez et al. 2008; Cutanda-Perez et al. 2009; Jung et al. 2009; Ballester et al. 2010; Feng et al. 2010).

The PAP1/AtMYB75 transcription factor activates a broad spectrum of genes of the biosynthetic pathway of phenylpropanoids and flavonoids in *A. thaliana* L., like *PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *F3H*, *F3´H*, *F3´5´H*, and *DFR* (Borevitz et al. 2000; Matsui et al. 2004; Tohge et al. 2005; Stacke et al. 2007; Zhang et al. 2010). It has been demonstrated that *CHS* gene expression could be induced by the transcription factors AtMYB75/PAP1, PFG1/MYB12, PFG2/MYB11, and PFG3/MYB111 (Borevitz et al. 2000, Mehrtens et al. 2005; Stacke et al. 2007). In contrast, in *S. miltiorrhiza* transgenic plants the *CHS* gene was not induced by AtMYB75/PAP1 suggesting that its role may vary among plant species (Zhang et al. 2010).

The enhanced production of desmethylxanthohumol, and xanthohumol in transgenic hop plants could be attributed to the expression of the *PAP1/AtMYB75* regulatory gene. The biosynthesis of prenylated chalcones in hop cones is mediated by CHS_H1, an enzyme with chalcone synthase activity. The regulatory gene *PAP1/AtMYB75* was capable of activating the *CHS_H1* gene in tobacco and petunia. As a result, the increased pool of naringenin chalcone may stimulate downstream biosynthesis and accumulation of prenylated chalcones (Matoušek et al. 2006; Matoušek et al. 2012). On the other hand, an additional role of *PAP1/AtMYB75* as transcriptional regulator of the biosynthesis of $\alpha$-acids, and $\beta$-acids should be determined and the expression level of the phloroglucinols biosynthetic genes *VPS*, and *HlPT1* must be analyzed.

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 (Butelli et al. 2008). Similarly, in transgenic soybean (*Glycine max* L. Merr) the expression of *CHI* was up-regulated by the transcription factors *C1* and *R* from maize (Yu et al. 2003).

Similarly, over-expression of the gene s-*HlMYB3* in transgenic plants activated the genes involved in flavonoid biosynthesis. The regulatory gene *HlMYB3* has been characterized and it encodes a typical R2R3 protein. The high specific expression of *HlMYB3* in lupulin glands of hop and the similarity to known regulators of the flavonoid biosynthesis proposed a positive role as regulator of the biosynthesis of hop specific flavonoids (Matoušek et al. 2007; Matoušek et al. 2012). However, transient expression analysis showed that HlMYB3 was not able to activate the *CHS_H1* promoter in tobacco leaves (Matoušek et al. 2012). By contrast, our results showed that over-expression of HlMYB3 strongly induced the expression of *CHS_H1* in transgenic hop plants suggesting a species-specific role.

The transcription factor HlMYB7 showed no ability to activate the promoter of *CHS_H1* (Matoušek et al. 2012). Interestingly, our results showed that the transgenic plant no. 137 had the highest *HlMYB7* and at the same time the lowest *CHS_H1*, *CHI*, *F3H*, *F3´H*, *FLS*, and *OMT1* relative expression. Earlier studies have demonstrated that the activity of anthocyanin biosynthetic genes in transgenic *Arabidopsis* and tobacco is negatively regulated by MYB transcription factor, like AtMYBL2, FaMYB1, and MdMYB6 (Aharoni et al. 2001; Matsui et al. 2008; Gao et al. 2011).

The regulation of the flavonoid biosynthetic pathway is mediated by the R2R3 MYB and bHLH transcription factors, together with WD40 or WDR1 proteins (Hichri et al. 2011). In tobacco, transient expression of GtMYB3 together with GtbHLH1 enhanced the promoter activities of late anthocyanin biosynthesis genes (Nakatsuka et al. 2008). Moreover, analysis of transient expression in tobacco leaves showed that the complex HlMYB2/HlbHLH2/HlWDR1 and HlMYB3/HlbHLH2/HlWDR1 strongly activated the *CHS_H1* gene but not *VPS*, and *OMT1* (Matoušek et al. 2012). In contrast, our results showed that in hop the expression of phloroglucinol biosynthetic genes *VPS*, and *OMT1* was up-regulated by the over-expression of the transcription factor HlMYB3.

## 7. 2 Alternative genetic transformation system in hop

The health-promoting effects of flavonoids have encouraged the development of food crops with high levels and/or altered composition of flavonoids (Schijlen et al. 2004). The flavonoid production in plants could be enhanced through genetic transformation with one or more structural genes or by the introduction of genes of homologous or heterologous regulatory elements. However, the first strategy is time consuming and has limited value (Capell and Christou 2004). Usually, metabolic pathways are regulated by one or more transcriptions factors that bind to the promoter of the structural genes in a given pathway activating their transcription. Thus, the second strategy offers a more attractive approach to activate an entire pathway (Wurtzel and Grotewold 2006). Therefore, the development of an efficient, simple and fast transformation protocol to analyze the function of several transcription factors is highly desirable.

Although genetic transformation of hop has been reported using *A. tumefaciens*, it is a laborious, and time consuming process (Horlemann et al. 2003). As a result, genetic transformation using *A. tumefaciens* is hindering large-scale screening and functional validation of candidate genes in hop. As alternative methods particle bombardment or *A. rhizogenes*-mediated transformation could be employed for testing gene function in transgenic hop plants.

The present study describes the induction of hairy roots in hop (*H. lupulus* L. cv. Tettnanger) through *A. rhizogenes*-mediated transformation. Moreover, an optimized bombardment protocol for DNA delivery into hop tissues is reported.

*A. rhizogenes* is a phytopathogenic gram negative soil bacterium responsible for the induction of transgenic hairy roots in plants (Georgiev et al. 2007). Transgenic hairy roots have been employed for studying the function of genes in *Beta vulgaris* L., *Coffea arabica* L., *Prunus* sp., and *Capsicum annuum* L. (Kifle et al. 1999; Alpizar et al 2006; Bosselut et al. 2011; Aarrouf et al. 2012). The rapidity and technical simplicity of this approach provides a major advantage over other methods. In the present study, hop tissues infected with *A. rhizogenes* produced hairy roots in only 4 weeks, which is rapid in comparison with the 6-9 months time required for the generation of hop transgenic plants following *A. tumefaciens* transformation (Horlemann et al. 2003).

Moreover, in comparison with *A. tumefaciens*-mediated transformation, hairy roots induction though *A. rhizogenes* does not require the regeneration of whole plants making them a suitable system to study large number of candidate genes (Bosselut et al. 2011). Additionally, the morphological similarity between hairy roots and trichomes (Wagner 1991) might allow the use of this system for studying genes which are normally active in the lupulin glands of the female cones of hop. In the last years, the culture of hairy roots in semi-solid or liquid medium has become an alternative method for the production and accumulation of secondary metabolites (Kim et al. 2011; Georgiev et al. 2011).

Among the direct gene transfer methods for plant transformation, particle bombardment has been widely used for the generation of transgenic plants and transient studies of gene expression. This technique consists in the acceleration of DNA coated microcarriers thorough cell wall/membrane delivering the foreign DNA inside the cell. This technique offers several advantages compared to *Agrobacterium*-mediated transformation. For instance, it is not limited to cell type, species or genotype. It eliminates the necessity of using a biological vector, and requires only the expression cassette. Also, it allows the transfer of multiple genes or large constructs (Altpeter et al. 2005). Previously, Batista et al. (2008) reported a genetic transformation protocol for hop cv. Eroica using particle bombardment. In the present study, two of the most important physical factors, helium pressure and target distance, were optimized for the delivery of foreign DNA into hop cv. Tettnager. The integration of the transgenes *gusA*, and *mgfp5* into transgenic shoots were corroborated by PCR after culture on selection medium for 4 weeks. This optimized protocol could be used for the validation of genes with a potential for modifying the production of secondary metabolites in hop, as well as for the improvement of hop cultivars with better resistance to biotic and abiotic stress, and for modifying plant architecture.

Taking into consideration that only a small number of cells integrate successfully the transgene, the development of any transformation system requires the establishment of efficient plant regeneration protocols. Existing protocols for the micropropagation of hop and shoot induction from callus using semi-solid medium are characterized by a low regeneration rates (Roy et al. 2001; Horlemann et al. 2003). Therefore, this fact contributes to the overall low transformation efficiency in hop. It has been reported that gelling agents limit the uptake of nutrients, plant growth regulators, and water. The use of liquid medium for the regeneration of transformed cells provides an alternative to overcome this drawback. The permanent contact of the medium with the plant improves *in vitro* growth and morphogenesis. Recently, Schwekendiek et al. (2009) developed a shoot regeneration protocol from GONC (green organogenic nodule clusters) of hop cv. US Tettnanger using temporary immersion bioreactors.

Particle bombardment coupled with the regeneration of plants in temporary immersion bioreactors could improve the efficiency of hop genetic transformation. Earlier studies have reported the use of temporary immersion system for the regeneration of transgenic strawberry and pineapple plants (Espinosa et al. 2002; Hanhineva et al. 2007).

## 7.3 Opportunities for metabolic engineering in hop

The hop plant is prominently known worldwide for the secondary metabolites present in the lupulin glands of the female cones, including essential oils, $\alpha$-acids, $\beta$-acids, xanthohumol, and desmethylxanthohumol. In the brewing industry, hops are responsible for conferring flavor, aroma, and bitterness to the beer. In addition, the secondary metabolites of hops have pharmaceutical value (Chadwick et al. 2006; Zanoli Zavatti 2008).

Current hop breeding programs have been directed toward the development of varieties with novel characteristics, such as exotic aromas and flavors, high $\alpha$-acids, and $\beta$-acids content, improved agronomic performance, and diseases resistance. Until now, hop breeding programs have been focused to satisfy the demand of the brewing industry (Lutz et al. 2011; Srečev et al. 2012).

A large quantity of research has demonstrated the health-promoting effects of xanthohumol, and desmethylxanthohumol (Stevens and Page 2004; Srečev et al. 2012). The addition of xanthohumol in beer or non-alcoholic beverages represents an alternative to increase the consumption of this compound. Since, wildtype hop contains only small quantities this approach requires extraction and purification of large amounts of xanthohumol (Stevens and Page 2004). Additionally, only few drugs or nutritional supplements containing xanthohumol are available in the market (Srečev et al. 2012).

Therefore, given the medical and pharmaceutical importance of xanthohumol and desmethylxanthohumol it is a strong argument for the redirection of breeding programs towards the creation of new cultivars with high content of these prenylated chalcones. Previously, hop varieties with enhanced content of xanthohumol have been obtained through conventional breeding (Nesvadba et al. 2011).

To complement these efforts, metabolic engineering of flavonoid biosynthesis offers tremendous potential for modifying the production of these compounds in hops. Several strategies have been proposed to accomplish this goal. One of them is the over-expression of one or more structural flavonoid biosynthetic genes. This approach has been used to increase the flavonoid content in tomato (Schijlen et al. 2006).

In a second approach, introduction and expression of homologous or heterologous transcription factors could improve the biosynthesis of flavonoids. As demonstrated in this study, the PAP1/AtMYB75 transcription factor influences the biosynthesis of flavonoids and prenylflavonoids. The synergistic action of MYB and bHLH hop transcription factors increased the expression of *CHS_H1* in tobacco, a key gene for the synthesis of naringenin chalcone (Matoušek et al. 2012). Consequently, further genetic transformation experiments using hop MYB and bHLH transcription factors, like HlMYB2 and HlbHLH2, could improve the synthesis of prenylflavonoids in hop.

Furthermore, higher amounts of prenylflavonoids could be obtained through the down-regulation or knockout of a specific enzymatic step (Stevens and Page 2004). Although several approaches are available for the down-regulation or knockout of a specific enzymatic step, RNAi represents an attractive alternative for regulation of gene expression and enhanced secondary metabolite biosynthesis (Jagtap et al. 2011). In hop, the biosynthesis of secondary metabolites has been studied and key genes, such as *CHS_H1*, *VPS*, *HlPT1*, and *OMT-1* has been described (Okada and Ito 2001; Matoušek et al. 2002; Nagel et al. 2008; Tsurumaru et al. 2010; Tsurumaru et al. 2012). For example, high xanthohumol/low bitter acids hops could be obtained through the down-regulation or knockout by RNAi of genes leading to bitter acids, like *VPS* and *HlPT1*. Similarly, knockout of *OMT-1* may possibly result in an accumulation of desmethylxanthohumol, which could be isomerized into pharmaceutical important 6- or 8-prenylnaringenin (Stevens and Page 2004).

An alternative to obtain hops with high levels of prenylated chalcones and bitter acids would be to increase the number of lupulin glands (glandular trichomes) of the female cones (Stevens and Page 2004). Several transcription factors, including members of the *MYB* and *bHLH* gene family, are implicated in the development of trichomes (Kirik et al. 2005; Gruber et al. 2006; Matoušek et al. 2012).

The *GLABRA3*, a member of *bHLH* gene family from *Arabidopsis*, induced trichomes in transgenic *B. napus* L. plants (Gruber et al. 2006). Similarly, the transcription factor MIXTA from *A. majus* L., modified the trichome density in the epidermis of petals and cotyledons in transgenic *N. tabacum* L. (Kirik et al. 2005). This engineering strategy offers an attractive alternative to enhance trichome production in hop and therefore to increase the production of valuable secondary metabolites.

Considering beer production in view of the German Purity Law from 1516, which is still applied, and the attitudes from some consumers to genetically modified food, non-transgenic approaches should also be considered. Mutation induction could be induced to modify the production of secondary metabolites in hop (Stevens and Page 2004). However, it is a laborious and time consuming process, which could also change many chemical characteristics of this cultivar.

In conclusion, metabolic engineering of flavonoids provides a chance for generating new economic opportunities for hop farmers. The pharmaceutical and medicinal industry could take advantage and pay attention to possible alternative applications of bioactive substances from hop.

# 8. General references


Aarrouf J, Castro-Quezada P, Mallard S, Caromel B, Lizzi Y, Lefebvre V (2012) *Agrobacterium rhizogenes*-dependent production of transformed roots from foliar explants of pepper (*Capsicum annuum*): a new and efficient tool for functional analysis of genes. Plant Cell Rep 31: 391-401

Aharoni A, De Vos CH, Wein M, Sun Z, Greco R, Kroon A, Mol JN, O'Connell AP (2001) The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. The Plant J 28: 319-332

Allan A, Hellens R, Laing W (2008) MYB transcription factors that colour our fruit. Trends Plant Sci 13: 99-102

Alpizar E, Dechamp E, Espeout S, Royer M, Lecouls AC, Nicole M, Bertrand B, Lashermes P, Etienne H (2006) Efficient production of *Agrobacterium rhizogenes*-transformed roots and composite plants for studying gene expression in coffee roots. Plant Cell Rep 25: 959-967

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TT, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N, Visser R (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. Mol Breed 15: 305-327

Ballester AR, Molthoff J, de Vos R, Hekkert BL, Orzaez D, Fernández-Moreno JP, Tripodi P, Grandillo S, Martin C, Heldens J, Ykema M, Granell A, Bovy A. (2010) Biochemical and molecular analysis of pink tomatoes: deregulated expression of the gene encoding transcription factor SlMYB12 leads to pink tomato fruit color. The Plant Cell 152: 71-84

Batista D, Fonseca S, Serrazina S, Figueiredo A, Salomé Pais M (2008) Efficient and stable transformation of hop ( *Humulus lupulus* L.) var. Eroica by particle bombardment. Plant Cell Rep 27: 1185-1196

Biendl M (2008) Hops: medicinal plant of the year 2007. Brauwelt International http://www.brauweltinternational.com/pdf/BRAUWELTInternational_5_2008_hopsteiner.pdf Accessed 1. August 2012

Borevitz JO, Xia Y, Blount J, Dixon RA, Lamb C (2000) Activation tagging identified a conserved MYB regulator of phenylpropanoid biosynthesis. The Plant Cell 12: 2383-2393

Bosselut N, Van Ghelder C, Claverie M, Voisin R, Onesto JP, Rosso MN, Esmenjaud D (2011) *Agrobacterium rhizogenes*-mediated transformation of *Prunus* as an alternative for gene functional analysis in hairy-roots and composite plants. Plant Cell Rep 30: 1313-1326

Bremer B, Bremer K, Chase MW, Fay MF, Reveal JL, Soltis DE, Soltis PS, Stevens PF, Anderberg AA, Moore MJ, Olmstead RG, Rudall PJ, Sytsma KJ, Tank DC, Wurdack K, Xiang JQY, Zmarzty S (2009) An update of the angiosperm phylogeny group classification for the orders and families of flowering plants: APG III. Bot J Linn Soc 161: 105-121

Buer CS, Imin N, Djordjevic MA (2010) Flavonoids: New roles for old molecules. J Integr Plant Biol 2010 52: 98-111

Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen E, Hall RD, Bovy AG, Luo J, Martin C (2008) Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nature Biotechnol 26: 1301-1308

Calderon-Montano J, Burgos-Moron E, Perez-Guerrero C, Lopez-Lazaro M (2011) A review on the dietary flavonoid Kaempferol. Mini Rev Med Chem 11: 298-344

Capell T, Christou P (2004) Progress in plant metabolic engineering. Curr Opin Biotech 15:148-154

Chadwick LR, Pauli GF, Farnsworth NR (2006) The pharmacognosy of *Humulus lupulus* L. (hops) with an emphasis on estrogenic properties. Phytomedicine 13: 119-131

Chen W, Lin J (2004) Mechanisms of cancer chemoprevention by hop bitter acids (beer aroma) through induction of apoptosis mediated by Fas and caspase cascades. J Agric Food Chem 52: 55-64

Cutanda-Perez MC, Ageorges A, Gomez C, Vialet S, Terrier N, Romieu C, Torregrosa L (2009) Ectopic expression of *VlmybA1* in grapevine activates a narrow set of genes involved in anthocyanin synthesis and transport. Plant Mol Biol 69: 633-648

De Keukeleire D (2000) Fundamentals of beer and hop chemistry. Química Nova 23: 108-112

DellaPenna D (2001) Plant metabolic engineering. Plant Physiol 125: 160-163

Dubos C, Stracke R, Grotewold E, Weisshar B, Martin C, Lepeinec L (2010) MYB transcription factors in *Arabidopsis*. Trends Plant Sci 15: 573-581

Espinosa P, Lorenzo JC, Iglesias A, Yabor L, Menéndez E, Borroto J, Hernández L, Arencibia AD (2002) Production of pineapple transgenic plants assisted by temporary immersion bioreactors. Plant Cell Rep 21: 136-140

Espley RV, Hellens RP, Putterill J, Stevenson DE, Kutty-Amma S, Allan AC (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMyb10. The Plant J 49: 414-427

Feng S, Wang Y, Yang S, Xu Y, Chen X (2010) Anthocyanin biosynthesis in pears is regulated by a R2R3-MYB transcription factor *PyMYB10*. Planta 232: 245-255

Ferrer JL, Austin MB, Stewart C, Noel JP (2008) Structure and function of enzymes involved in the biosynthesis of prenylpropanoids. Plant Physiol Biochem 46: 356-370

Forkmann G, Martens S (2001) Metabolic engineering and applications of flavonoids. Curr Opin Biotechnol 12: 155-160

Gao JJ, Shen XF, Zhang Z, Peng RH, Xiong AS, Xu J, Zhu B, Zheng JL, Yao QH (2011) The myb transcription factor MdMYB6 suppresses anthocyanin biosynthesis in transgenic *Arabidopsis*. Plant Cell Tiss Organ Cult 106: 235-242

Gatica-Arias A, Farag MA, Stanke M, Matoušek J, Wessjohann L, Weber G (2012) Flavonoid production in transgenic hop (*Humulus lupulus* L.) altered by PAP1/MYB75 from *Arabidopsis thaliana* L. Plant Cell Rep 31: 111-119

Georgiev MI, Ludwig-Müller J, Alipieva K, Lippert A (2011) Sonication-assisted *Agrobacterium rhizogenes*-mediated transformation of *Verbascum xanthophoeniceum* Griseb for bioactive metabolite accumulation. Plant Cell Rep 30: 859-866

Georgiev MI, Pavlov AI, Bley T (2007) Hairy root type plant *in vitro* system as sources of bioactive substances. Appl Microbiol Biotechnol 74: 1175-1185

Gerhäuser C (2005) Beer constituents as potential cancer chemopreventive agents. Eur J Cancer 41: 1941-1954

Gonzalez A, Zhao M, Leavitt JM, Llyod AM (2008) Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. The Plant Journal 53: 814-827

Grabowska-Joachimiak A, Sliwinska E, Pigula M, Skomra U, Joachimiak AJ (2006) Genome size in *Humulus lupulus* L. and *Humulus japonicus* Siebold & Zucc. (*Cannabaceae*). Acta Societatis Botanicorum Poloniae 75: 207-214

Gruber MY, Wang S, Ethier S, Holowachuk J, Bonham-Smith PC, Soroka J, Lloyd A (2006) "HAIRY CANOLA"-*Arabidopsis* GL3 induces a dense covering of trichomes on *Brassica napus* seedlings. Plant Mol Biol. 60: 679-98

Han YJ, Kim YM, Lee JY, Kim SJ, Cho KC, Chandrasekhar T, Song PS, Woo YM, Kim JI (2009) Production of purple-colored creeping bentgrass using maize transcription factor genes Pl and Lc through *Agrobacterium*-mediated transformation. Plant Cell Rep 28: 397-406

Hanhineva K, Kokko H, Kärenlampi S (2005) Shoot regeneration from leaf explants of five strawberry (*Fragaria* × *Ananassa*) cultivars in temporary immersion bioreactor system. *In Vitro* Cell Dev Biol Plant 41: 826- 831

Harborne JB, Williams CA (2000) Advances in flavonoid research since 1992. Phytochemistry 55: 481-504

Hartmann U, Sagasser M, Mehrtens F, Stracke R, Weisshaar B (2005) Differential combinatorial interactions of cis acting elements recognized by R2R3-MYB, BZIP, and BHLH factors control light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes. Plant Mol Biol 57: 155-171

Haunold A, Nickerson GB, Gampert U, Whitney PA, Hampton RO (1993) Agronomic and quality characteristics of native North American hops. J Am Soc Brew Chem 51: 133-137

Hirchri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V (2011) Recent advances in the transcriptional regulation of the flavonoid biosynthesis pathway. J Exp Bot 62: 2465-2483

Hopsteiner (2011) Entscheidungsdaten für den Hopfeneinkauf. http://www.hopsteiner.de/index.php?option=com_content&view=article&id=62&Itemid=66 Accessed 1. August 2012

Horlemann C, Schwekendiek A, Hohnle M, Weber G (2003) Regeneration and *Agrobacterium*-mediated transformation of hop (*Humulus lupulus* L.). Plant Cell Rep 22: 210-217

Jagtap UB, Gurav RG, Bapat VA (2011) Role of RNA interference in plant improvement. Naturwissenschaften 98:473-492

Jiang C, Gu J, Chopra S, Gu X, Peterson T (2004) Ordered origin of the typical two and three repeat *MYB* genes. Gene 326: 13-22

Jung CS, Griffiths HM, De Jong DM, Cheng S, Bodis M, Kim TS, De Jong WS (2009) The potato developer (D) locus encodes an R2R3 MYB transcription factor that regulates expression of multiple anthocyanin structural genes in tuber skin. Theor Appl Gen 120: 45-57

Kammhuber K (2006/2007) Quercetin and Kaempferol. Hopfenrundschau international. 52-55. http://www.deutscher-hopfen.de/ Accessed 30 August 2012

Katsumoto Y, Fukuchi-Mizutani M, Fukui Y, Brugliera F, Holton TA, Karan M, Nakamura N, Yonekura-Sakakibara K, Togami J, Pigeaire A, Tao GQ, Nehra NS, Lu CY, Dyson BK, Tsuda S, Ashikari T, Kusumi T, Mason JG, Tanaka Y (2007) Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. Plant Cell Physiol 48: 1589-1600

Khan N, Afaq F, Syed DN, Mukhtar H (2008) Fisetin, a novel dietary flavonoid, causes apoptosis and cell cycle arrest in human prostate cancer LNCaP cells. Carcinogenesis 29: 1049-1056

Kifle S, Shao M, Jung C, Cai D (1999) An improved transformation protocol for studying gene expression in hairy roots of sugar beet (*Beta vulgaris* L.). Plant Cell Rep 18: 514-519

Kim JA, Kim YS, Choi YE (2011) Triterpenoid production and phenotypic changes in hairy roots of *Codonopsis lanceolata* and the plants regenerated from them. Plant Biotechnol Rep 5: 255-263

Kirik V, Lee MM, Wester K, Herrmann U, Zheng Z, Oppenheimer D, Schiefelbein J, Hulskamp M (2005) Functional diversification of MYB23 and GL1 genes in trichome morphogenesis and initiation. Development 132: 1477-1485

Koes RE, Quattrocchio F, Mol JN (1994) The flavonoid biosynthetic pathway in plants: function and evolution. BioEssays 16: 123-132

Konczak I, Zhang W (2004) Anthocyanins-more than nature's colours. J Biomed Biotechnol 5: 239-240

Lamson DW, Brignall MS (2000) Antioxidants and cancer III: quercetin. Altern Med Rev 5: 196-208

Lamy V, Roussi S, Chaabi M, Gossé F, Schall N, Lobstein A, Raul F (2007) Chemopreventive effects of lupulone, a hop β-acid, on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. Carcinogenesis 28: 1575-1581

Lepiniec L, Debeaujon I, Routaboul JM, Baudry A, Pourcel L, Nesi N, Caboche M (2006) Genetics and biochemistry of seed flavonoids. Annu Rev Plant Biol 57: 405-430

Li X, Gao MJ, Pan HY, Cui DJ, Gruber M (2010) Purple canola: *Arabidopsis* PAP1 increases antioxidants and phenolic in *Brassica napus* leaves. J Agric Food Chem 58: 1639-1645

Lutz A, Kammhuber K, Seigner E (2012) New trend in hop breeding at the Hop Research Center Huell. Brew Sci 65: 24-32

Marais JPJ, Deavours B, Dixon RA, Ferreira D (2006) The stereochemistry of flavonoids. In: Grotewold E (ed) The science of flavonoids, Springer, Berlin, pp 1-46

Matoušek J, Kocabek T, Patzak J, Fussy Z, Prochazkova J, Heyerick A (2012) Combinatorial analysis of lupulin gland transcription factors from R2R3Myb, bHLH and WDR families indicates a complex regulation of *chs_H1* genes essential for prenylflavonoid biosynthesis in hop (*Humulus lupulus* L.) BMC Plant Biol 12: 27

, Patzak J, Niedermeierová H (2002) Cloning and characterisation of chs-specific DNA and cDNA sequences from hop (*Humulus lupulus* L.) Plant Sci 162: 1007-1018

Matoušek J, Rocabek T, Patzak J, Škopek J, Maloukh L, Heyerick A, Fussy Z, Roldán-Ruiz I, Keukeleire D (2007) *HlMYB3*, a putative regulatory factor in hop (*Humulus lupulus* L.), shows a diverse biological effects in heterologous transgenotes. J Agric Food Chem 55: 7767-7776

Matoušek J, Rocabek T, Patzak J, Sthelik J, Fussy Z, Krofta K; Heyerick A, Roldán-Ruiz I, Maloukh L, Keukeleire D (2010) Cloning and molecular analysis of HlbZip1 and HlbZip2 transcription factors putatively involved in the regulation of the lupulin metabolome in hop (*Humulus lupulus* L.). J Agric Food Chem 58: 902-912

Matoušek J, Vrba L, Novák P, Patzak J, De Keukeleire J, Škopek J, Heyerick A, Roldán-Ruiz I, De Keukeleire D (2005) Cloning and molecular analysis of the regulatory factor HlMyb1 in hop (*Humulus lupulus* L.) and the potential of hop to produce bioactive prenylated flavonoids J Agric Food Chem 53: 4793-4798

Matoušek J, Vrba L, Škopek J, Orctová L, Pešina K, Heyerick A, Baulcombe D, De Keukeleire D (2006) Sequence analysis of a "true" chalcone synthase (chs_H1) oligofamily from hop (*Humulus lupulus* L.) and PAP1 activation of chs_H1 in heterologous systems. J Agric Food Chem 54: 7606-7615

Matsui K, Tanaka H, Ohme-Takagi M (2004) Suppression of the biosynthesis of proanthocyanidin in *Arabidopsis* by a chimeric PAP1 repressor. Plant Biotech J 2: 487-493

Matsui K, Umemura Y, Ohme-Takagi M (2008) AtMYBL2, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in *Arabidopsis*. The Plant J 55: 954-967

Matus JT, Aquea F, Arce-Johnson P (2008) Analysis of the grape *MYB R2R3* subfamily reveals expanded wine quality-related clades and conserved gene structure organization across *Vitis* and *Arabidopsis* genomes. BMC Plant Biol 8:83

Mehrtens F, Kranz H, Bednarek P, Weisshaar B (2005) The *Arabidopsis* transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis. Plant Physiol 138: 1083-1096

Milligan S, Kalita J, Pocock V, Heyerick A, De Cooman L, Rong H (2002) Oestrogenic activity of the hop phyto-oestrogen, 8-prenylnaringenin. Reproduction 123: 235-242

Mizobuchi S, Sato Y (1984) A new flavanone with antifungal activity isolated from hops. Agric Biol Chem 48: 2771-2775

Nagel J, Culley LK, Lu Y, Liu E, Matthews PD, Stevens JF (2008). EST analysis of hop glandular trichomes identifies an O-methyltransferase that catalyses the biosynthesis of xanthohumol. Plant Cell 20: 186-200

Nakatsuka T, Haruta KS, Pitaksutheepong C, Abe Y, Kakizaki Y, Yamamoto K, Shimada N, Yamamura S, Nishihara M (2008) Identification and characterization of R2R3-MYB and bHLH transcription factors regulating anthocyanin biosynthesis in Gentian flowers. Plant Cell Physiol 49: 1818-1829

Nesvadba V, Krofta K, Poloncikova Z (2011) New knowledge in Czech hop breeding. In: Seigner E (ed) Proceedings of the Scientific Commission International Hop Grower´s Convention. Scientific Commission Meeting. Lublin, Poland. http://www.lfl.bayern.de/ipz/hopfen/10585/sc_2011__proceedings.pdf. Accessed 31 July 2011.

Nishihara M, Nakatsuka T, Yamamura S (2005) Flavonoid components and flower color change in transgenic tobacco plants by suppression of chalcone isomerase gene. FEBS Letters 579: 6074-6078

Okada Y, Ito K (2001) Cloning and analysis of valerophenone synthase gene expressed specifically in lupulin gland of hop (*Humulus lupulus* L.) Biosci Biotechnol Biochem 65: 150-155

Pandey A, Misra P, Chandrashekar K, Trivedi PK (2012) Development of AtMYB12-expressing transgenic tobacco callus culture for production of rutin with biopesticidal potential. Plant Cell Rep DOI: 10.1007/s00299-012-1300-6

Paz-Ares J, Ghosal D, Wienand U, Peterson P, Saedler H (1987) The regulatory *c1* locus of *Zea mays* encodes a protein with homology to *myb* oncogene products and with structural similarities to transcriptional activators. EMBO J 6: 3553-3558

Peer WA, Murphy AS (2007) Flavonoids and auxin transport: modulators or regulators? Trends Plant Sci 12: 556-563

Pšenáková I, Gašpárková L, Faragó J (2009) Polyphenol and flavonoid contents of hop callus and cell suspension cultures. In: Seigner E (ed) Proceedings of the Scientific Commission International Hop Grower´s Convention. Scientific Commission Meeting. León, Spain. http://www.lfl.bayern.de/ipz/hopfen/10585/index.php#Tagung/Meeting%20in%20Le%C3%B3n,%20Spanien,%20Juni%202009 Accessed 31 July 2011

Ross J, Kasum CM (2000) Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr 22: 19-34

Roy AT, Leggett G, Koutoulis A (2001) Development of a shoot multiplication system for hop (*Humulus lupulus* L.). In Vitro Cell Dev Biol Plant 37: 79-83

Schijlen E, de Vos C, Martens S, Jonker H, Rosin F, Molthoff J, Tikunov Y, Angenent G, van Tunen A, Bovy, A (2007). RNAi silencing of chalcone synthase, the first step in the flavonoid biosynthesis pathway, leads to parthenocarpic tomato fruits. Plant Physiol 144: 1520-1530

Schijlen E, Ric de Vos C, Jonker H, van der Broeck H, Molthoff J, van Tunen A, Martens S, Bovy A (2006) Pathway engineering for healthy phytochemicals leading to the production of novel flavonoids in tomato fruits. Plant Biotechnol J 4: 433-444

Schijlen E, Ric de Vos C, van Tunen A, Bovy A (2004) Modification of flavonoid biosynthesis in crop plants. Phytochemistry 65: 2631-2648

Schiller KH, Forster A, Vonhoff C, Hegger M, Biller A, Winterhoff H (2006) Sedating effects of *Humulus lupulus* L. extracts. Phytomedicine 13: 535-541

Schwekendiek A, Hanson ST, Crain M (2009) A temporary immersion system for the effective shoot regeneration of hop. Acta Hort 848: 149-156

Small E (1978) A numerical and nomenclatural analysis of morpho-geographic taxa of *Humulus*. Syst Bot 3: 37-76

Stevens JF, Page JE (2004) Xantohumol and related prenylflavonoids from hops and beer to your good health! Phytochemistry 65: 1317-1330

Stevens JF, Taylor AW, Deinzer ML (1999) Quantitative analysis of xanthohumol and related prenylflavonoids in hops and beer by liquid chromatography-tandem mass spectrometry. J Chromatogr A 832: 97-107

Stevens JF, Taylor AW, Nickerson GB, Ivancic M, Henning J, Haunold A, Deinzer ML (2000) Prenylflavonoid variation in *Humulus lupulus*: distribution and taxonomic significance of xanthogalenol and 4′-Omethylxanthohumol. Phytochemistry 53: 759

Stracke R, Ishihara H, Huep G, Barsch A, Mehrtens F, Niehaus K (2007) Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. The Plant J 50: 660-677

Stracke R, Werber M, Weisshar B (2001) The R2R3-Myb gene family in *Arabidopsis thaliana*. Curr Opin Plant Biol 4: 447-456

Sreček S, Zechner-Krpan V, Petravić-Tominac V, Košir I, Čerenak A (2012) Importance of medical effects of Xanthohumol, hop (*Humulus lupulus* L.) bioflavonoids in restructuring of world hop industry. Agriculturae Conspectus Scientificus 77: 61-67

Tagashira M, Watanabe M, Uemitsu N (1995) Antioxidative activity of hop bitter acids and their analogues. Biosci Biotech Biochem 59: 740-742

Taylor LP, Grotewold E (2005) Flavonoids as developmental regulators. Curr Opin Plant Biol 8: 317-323

Tian L, Dixon RA (2006) Engineering isoflavone metabolism with an artificial bifunctional enzyme. Planta 224: 496-507

Tohge T, Nishiyama Y, Hirai MY, Yano M, Nakajima J, Awazuhara M (2005). Fuctional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor. The Plant J 42: 218-235

Tsurumaru Y, Sasaki K, Miyawaki T, Momma T, Umemoto N, Yazaki K (2010) An aromatic prenyltransferase-like gene HlPT-1 preferentially expressed in lupulin glands of hop. Plant Biotechnol 27: 199-204

Tsurumaru Y, Sasaki K, Miyawaki T, Uto Y, Momma T, Umemoto N, Momose M, Yazaki K (2012) HlPT-1, a membrane-bound prenyltransferase responsable for the biosynthesis of bitter acids in hop. Biochem Biophys Res Com 417: 393-398

Ürgeová E, Polívka L, Faragó J, Vaverková S (2011) Study of the production of secondary metabolites in shoot and callus cultures and field grown plants of hop. In: Seigner E (ed) Proceedings of the Scientific Commission International Hop Grower´s Convention Scientific Commission Meeting. Lublin, Poland. http://www.lfl.bayern.de/ipz/hopfen/10585/sc_2011__proceedings.pdf. Accessed 31 July 2011.

Verpoorte R, Contin A, Memelink J (2002) Biotechnology for the production of plant secondary metabolites. Phytochemistry 1: 13-25

Verzele M, de Keukeleire D (1991) Chemistry and analysis of hop and beer bitter acids. Elsevier, Amsterdam.

Wagner GJ (1991) Secreting glandular trichomes: more than just hairs. Plant Physiol 96: 675-679

Wang G, Tian L, Aziz N, Broun P, Dai X, He J, King A, Zhao P, Dixon R (2008) Terpene bipsynthesis in glandular trichomes of hop. Plant Physiol 148: 1254-1266

Wilkins O, Nahal H, Foong J, Provart NJ, Campbell MM (2009) Expansion and diversification of the *Populus R2R3-MYB* family of transcription factors. Plant Physiol 149: 981-993

Winkel-Shirley B (2001) Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol 126: 485-493

Wurtzel ET, Grotewold E (2006) Plant metabolic engineering. In Lee S (Ed) Encyclopedia of Chemical Processing, Taylor and Francis, New York, pp 2191-2200

Xie DY, Sharma S, Wright E, Wang ZY, Dixon R (2006) Metabolic engineering of proanthocyanidins through co-expression of anthocyanidin reductase and the PAP1 MYB transcription factor. Plant J 45: 895-907

Yanhui C, Xiaoyuan Y, Kun H, Meihua L, Jigang L, Zhaofeng G, Zhiqiang L, Yunfei Z, Xiaoxiao W, Xiaoming Q, Yunping S, Li Z, Xiaohui D, Jingchu L, Xing-Wang D, Zhangliang C, Hongya G, Li-Jia Q (2006) The MYB transcription factor superfamily of *Arabidopsis*: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol Biol 60: 107-124

Yu O, Shi J, Hession AO, Maxwell CA, McGonigle B, Odell JT (2003) Metabolic engineering to increase isoflavone biosynthesis in soybean seed. Phytochemistry 63: 753-763

Zanoli P, Rivasi M, Zavatti M, Brusiani F, Baraldi M (2005) New insight in the neuropharmacological activity of *Humulus lupulus* L. J Ethnopharmacol 102: 102-106

Zanoli P, Zavatti M (2008) Pharmacognostic and pharmacological profile of *Humulus lupulus* L. J Enthnopharmacol 116: 383-396

Zhang HC, Liu JM, Lu HY, Gao SL (2009) Enhanced flavonoid production in hairy root cultures of *Glycyrrhiza uralensis* Fisch by combining the over-expression of chalcone isomerase gene with the elicitation treatment. Plant Cell Rep 28:1205-1213

Zhang Y, Yan YP, Wang ZZ (2010) The *Arabidopsis* PAP1 transcription factor plays an important role in the enrichment of phenolic acids in *Salvia miltiorrhiza*. J Agric Food Chem 58: 12168-12175

Zhao J, Dixon R (2009) The 'ins' and 'outs' of flavonoid transport. Trends Plant Sci 15: 72-80

Zifkin M, Jin A, Ozga JA, Zaharia I, Schernthaner JP, Gesell A, Abrams S, Kennedy JA, Constabel CP (2012) Gene expression and metabolite profiling of developing high bush blueberry (*Vaccinium corymbosum* L.) fruit indicates transcriptional regulation of flavonoid metabolism and activation of abscisic acid metabolism. Plant Physiol 158: 200-224

Zhou L, Zeng N, Shi M, Xie D (2008) Development of tobacco callus cultures over expressing *Arabidopsis* PAP1/MYB75 transcription factor and characterization of anthocyanin biosynthesis. Planta 229: 37-51

Zuluaga L, Gonzali S, Loreti E, Pucciariello C, Degl'innocenti E, Guidi L, Alpi A, Perata P (2008) *Arabidopsis thaliana* MYB75/PAP1 transcription factor induces anthocyanin production in transgenic tomato plants. Funct Plant Biol 35: 606-618

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# 10. Curriculum vitae

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# 11. Declaration (Erklärung)

Hiermit erkläre ich, Andrés Mauricio Gatica Arias, dass die vorliegende Dissertation Ergebnis meiner eigenständigen wissenschaftlichen Arbeit ist und ich zur Fertigstellung dieser keine weiteren als die angegebenen Quellen und Hilfsmittel verwendet habe. Die verwendeten Quellen und Hilfsmittel sowie wörtlich oder inhaltlich übernommene Stellen sind als solche gekennzeichnet.

Andrés Mauricio Gatica Arias

Stuttgart, den 26. September 2012