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ARTICLE

Cloning and Molecular Analysis of *HlbZip1* and *HlbZip2* Transcription Factors Putatively Involved in the Regulation of the Lupulin Metabolome in Hop (*Humulus lupulus* L.)

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Hop (*Humulus lupulus* L.), the essential source of beer flavor is of interest from a medicinal perspective in view of its high content in health-beneficial terpenophenolics including prenylflavonoids. The dissection of biosynthetic pathway(s) of these compounds in lupulin glands, as well as its regulation by transcription factors (TFs), is important for efficient biotechnological manipulation of the hop metabolome. TFs of the bZIP class were preselected from the hop transcriptome using a cDNA-AFLP approach and cloned from a cDNA library based on glandular tissue-enriched hop cones. The cloned TFs *Hl*bZIP1A and *Hl*bZIP2 have predicted molecular masses of 27.4 and 34.2 kDa, respectively, and both are similar to the group A3 bZIP TFs according to the composition of characteristic domains. While *Hl*bZIP1A is rather neutral (pI 6.42), *Hl*bZIP2 is strongly basic (pI 8.51). A truncated variant of *Hl*bZIP1 (*Hl*bZIP1B), which is strongly basic but lacks the leucine zipper domain, has also been cloned from hop. Similar to the previously cloned *HlMyb3* from hop, both bZIP TFs show a highly specific expression in lupulin glands, although low expression was observed also in other tissues including roots and immature pollen. Comparative functional analyses of *HlbZip1A*, *HlbZip2*, and subvariants of *HlMyb3* were performed in a transient expression system using *Nicotiana benthamiana* leaf coinfiltration with *Agrobacterium tumefaciens* strains bearing hop TFs and selected promoters fused to the GUS reference gene. Both hop bZIP TFs and *HlMyb3* mainly activated the promoters of chalcone synthase *chs*_H1 and the newly cloned *O*-methyl transferase 1 genes, while the response of the valerophenone synthase promoter to the cloned hop TFs was very low. These analyses also showed that the cloned bZIP TFs are not strictly G-box-specific. HPLC analysis of secondary metabolites in infiltrated *Petunia hybrida* showed that both hop bZIP TFs interfere with the accumulation and the composition of flavonol glycosides, phenolic acids, and anthocyanins, suggesting the possibility of coregulating flavonoid biosynthetic pathways in hop glandular tissue.

KEYWORDS: Transcriptional factors; cDNA-AFLP analysis; hop cDNA library screening; transient expression; *Nicotiana benthamiana*; *Petunia hybrida*; secondary metabolites

INTRODUCTION

Hop (*Humulus lupulus* L.) plants are predominantly cultivated for the brewing industry as a source of flavor-active secondary metabolites contained in lupulin glands that develop in hop female inflorescences (cones). Lupulin glands are composed of biosynthetic cells secreting a specific but complex metabolome consisting mainly of terpenophenolics (hop bitter acids and prenylflavonoids) and terpenoids (essential oil components). Hop bitter acids include humulones (α-acids), which are converted in the brew kettle into isohumulones (iso-α-acids), the most important beer bittering agents, and lupulones (β-acids) (*1*). In addition to their brewing related properties, these hop-derived bitter acids also arouse interest from a medicinal perspective in view of their wide variety of reported biological activities, including antimicrobial, anti-inflammatory, and cancer chemopreventive activities (*2*). The prenylflavonoids, the other subclass of hop terpenophenolics, are competing strongly with hop bitter acids for their recognition as highly omnipotent bioactives. The principal hop prenylchalcones are desmethylxanthohumol

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(DMX) and xanthohumol (X). X, the most abundant prenylflavonoid in fresh and properly preserved hops (*3*), shows a broad spectrum of inhibition mechanisms at the initiation, promotion, and progression stages of carcinogenesis (e.g., refs *4* and *5*). Isomerization of X and DMX in the brew kettle yields isoxanthohumol (IX) and a mixture of the prenylflavanones, 8-prenylnaringenin (8-PN), and 6-prenylnaringenin (6-PN), respectively. 8-PN (also known as hopein) is the most potent phytoestrogen known to date (*6*, *7*). A food supplement based on the estrogenic properties of 8-PN was introduced in 2003 for alleviation of menopausal symptoms (*8*).

The lupulin metabolome appears to be a rather stable genetic trait having a characteristic composition in different hop cultivars (*9*). This suggests a possibility of efficiently manipulating the lupulin metabolome in the future by changing structural and regulatory genes within the metabolome signaling network using molecular genetic techniques such as transcription factor (TF) biotechnology (*10*). This approach would enable to improve the yield of valuable hop products, on the condition that more insight is gained regarding their biosynthesis and the overall hop metabolome regulation.

Several structural genes coding for hop chalcone synthases (EC 2.3.1.74) (CHS) or CHS-like enzymes expressed in lupulin glands have been described. While an oligofamily of *chs*_H1 genes encoding "true" CHS enzymes (*11*) codetermine the biosynthesis of X, valerophenone synthase (VPS) has been characterized as a key enzyme involved in the biosynthesis of bitter acids (*12*). Recently, a family of hop *O*-methyltransferase (OMT) genes has been identified (*13*). Especially, OMT1 that is highly expressed in lupulin glands performs the final reaction step in the biosynthesis of X by methylating DMX (*13*).

The expression of structural genes involved in lupulin biosynthesis must be specifically regulated by several transcription factors. For instance, Myb-like boxes, P-, H-box, and G-TF binding boxes have been predicted in the *chs*_H1 promoter sequence, suggesting the potential regulation of these genes by MYB, bHLH, and bZIP TFs (*11*). This assumption is in accordance with the knowledge about the regulation of the flavonoid biosynthetic pathways (for reviews, see refs *10* and *14*). Strong *chs*_H1 activation in response to coexpression of the R2R3 PAP1 Myb factor from *A. thaliana* in a transient expression system has indeed been found in our previous work (*11*). The first hop R2R3 MYB factors were cloned from a cDNA library prepared from glandular tissue-enriched hop cones. These TFs showed a specific expression in hop and a pleiotropic action on the metabolome and plant morphogenesis in heterologous transgenotes (*15*, *16*). In addition, it has been reported that two subvariants of *HlMyb3* cause diverse morphological and physiological changes including shifts in the metabolomes of transformed model plants (*16*).

bZIP TFs could coactivate or modulate the expression of lupulin biosynthesis genes. A wide comparison of plant bZIP genes has recently been carried out by Corrêa et al. (*17*). In this study, bZIP TFs from angiosperms were divided into 13 homologous groups. In total, 34 possible groups of bZIP ortologues were suggested, and the corresponding basic functions of these TFs were summarized. Plant bZIP TFs regulate processes such as organ and tissue differentiation, cell elongation, nitrogen/carbon balance control, pathogen defense, energy metabolism, unfolded protein response, hormonal effects, sugar signaling, light response, osmotic control, and seed storage protein gene regulation (*17*). A more detailed analysis of the combinatorial action of bZIP and other plant TFs in the regulation of phenylpropanoid biosynthesis genes has been performed by Hartmann et al. (*18*).

In the present study, we characterized two partly homologous bZIP regulatory elements isolated from hop cones via cDNA-AFLP identification and cDNA library screening steps. We showed that these TFs, similarly to the previously cloned hop Mybs, have a highly specific expression in lupulin glands and selectively activate the promoters of *chs*_H1, *vps* and *omt*1 in a transient expression system. Furthermore, both bZIPs modulate the accumulation of flavonol glycosides, phenolic acids, and anthocyanin pigmentation in infiltrated *Petunia hybrida* leaves suggesting the possibility to activate genes related to biosynthesis of secondary metabolites.

MATERIALS AND METHODS

Plant Cultivation Conditions, Sampling, and Collection of Lupulin Glands. The hop (*Humulus lupulus* L.) cvs. Whitbread Golding (WG), Wye Challenger (WC), and Admiral (A), found to possess contrasting characteristics for the accumulation of prenylchalcones in a previous work (*9*), were chosen for cDNA-AFLP analysis. These plant materials were harvested at the hop farm of Joris Cambie (Poperinge, Belgium) during the growing season of 2003. Inflorescences were collected at three different dates, corresponding to three stages of flowering, from the appearance of inflorescences (stage 1, harvest date: 31 July 2003) to the formation of small hop cones (stage 2, harvest date: 11 August 2003) and mature hop cones (stage 3, harvest date: 1 September 2003).

Czech semiearly red-bine hop Osvald's clone 72, other hop varieties, cvs. Target, Taurus, Admiral, Columbus, and *H. lupulus* var. *lupuloides* were grown under natural field conditions in the world hop collection of the Hop Research Institute in Žatec, Czech Republic. *H. japonicus* was grown from seeds in the greenhouse, and *Cannabis sativa* was collected from plants grown in natural field conditions near the village of Chbany (Northern Bohemia). Osvald's clone 72, *Petunia hybrida* cv. Andrea and *Nicotiana benthamiana* plants were maintained in the green house at a temperature of 25 ± 3 °C. Plants were grown under natural light conditions with supplementary illumination [170 μmol m^{-2} s^{-1} PAR] to maintain a 16 h-day period. Samples were collected from hop plants for RNA isolation during the period from the end of March to the beginning of September of 2008. All samples were immediately immersed in liquid nitrogen and stored at −80 °C until RNA extraction or biochemical analysis was carried out. Lupulin glands were isolated from Osvald's clone 72 cones using the procedure according to Nagel et al. (*13*).

cDNA-AFLP, TDFs Identification, and cDNA Library Screening. Double-stranded cDNA was synthesized using a standard protocol (*19*) with a biotinylated oligo(dT)25-primer. The cDNA was extracted with a chloroform−isoamyl alcohol mixture, precipitated with ethanol, and redissolved in a final volume of 20 μL of water. Following digestion with *Xho*II, 3′-ends were captured with streptavidin beads and digested with *Mse*I. Site-specific adapters were then ligated to the restriction fragments. The next step involved PCR amplification (30 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C for 25 cycles) with adapter-specific primers (preamplification). This pool was used as a template for selective amplification involving primers extended with two selective bases each. For the selective amplifications, all 16 possible primer pairs of two base extensions were tested, thereby rendering a total of 256 primer combinations. Radioactive labeling (^{33}P) of the *Xho*II primer was carried out according to Vos et al. (*20*). Selective amplification involved 35 cycles, including a 12-cycle touchdown (20 s at 94 °C, 30 s at 65 °C, the annealing temperature reduced from 65 to 56 °C in 0.7 °C steps for 12 cycles, 55 s at 72 °C), 23 cycles with the annealing temperature maintained at 56 °C, and 15 min at 72 °C. Fragment separation was carried out in 50 cm, 5% polyacrylamide gels, with a 10-bp ladder (Sequamark, Genetic Research Huntsville, AL, USA) as the length marker. Gels were dried on Whatman 3 M paper using a slab gel dryer, scanned with a PhosphoImager (BAS-2500, Fujifilm, Japan), and exposed to autoradiography films (Biomax MR films, Kodak, NY, USA). Gels and films were positionally marked prior to development.

To quantify differences in gene expression, scanned gel images were analyzed with AFLP-QuantarPro image analysis software (Keygene, Wageningen, The Netherlands), and individual band intensities were estimated. The raw intensity data were used to estimate the quantitative expression of each transcript in each sample, after correction for lane to lane differences and standardization, as described by Breyne et al. (*21*).

Transcript-derived fragments (TDFs) of interest were extracted from the polyacrylamide gels reamplified with the primers used in the selective amplification but using the PCR conditions for the preamplification (see above). PCR products were directly sequenced with the *Xho*II or *Mse*I primer. In some cases, when the sequencing reaction was not satisfactory, the PCR product was cloned in the TOPO cloning vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and resequenced. At least eight colonies were picked for each TDF, and the PCR products of five colonies were sequenced. Sequencing reactions were carried out with the M13 forward or reverse primer.

A hop cDNA library which had been established earlier (*15*) was screened by molecular hybridization as described previously (*11*) using radioactively-labeled bZip TDF selected from the cDNA-AFLP analysis. This TDF fragment probe designated cdb9−244b was reamplified with primers 9−244 R (5′-GAGAATCTGCTGCCAGGTCAA-3′) and 9−244 F (5′-TCTTGGAGTTTTCTTCTTTTCCTCC-3′) and then labeled using the Redivue [α-^{32}P] dCTP 3000 Ci·mmol^{-1} Rediprime II random prime labeling system (Amersham Pharmacia Biotech, Freiburg, Germany). The autoradiograms were scanned using a *STORM* device and *ImageQuaNT* software (Molecular Dynamics, Sunnyvale, CA, USA). Selected phage clones were converted to plasmids and sequenced.

Isolation of DNA and Genomic Blots. Hop genomic DNA was prepared by the CTAB method (*22*) from about 1 g of frozen young leaves. Genomic DNA (5 μg) from each plant was separately digested with 50 units *Pvu*I or *Bam*HI, subjected to electrophoresis, and transblotted to 0.45 μm Nylon Positively Charged membranes (Sigma Aldrich, Gillingham, UK) as described previously (*16*). The membranes were then cross-linked (15 s at 70 000 μJ/cm^2) and oven-dried (20 min at 80 °C). Prehybridization (2 h at 65 °C in 30 mL) and hybridization (overnight at 65 °C in 20 mL) were carried out in buffer according to Church and Gilbert (*23*) using a hybridization oven. The membranes were hybridized to *HlbZip1* and *HlbZip2* cDNA probes having a specific activity of 5 × 10^7 cpm/μg DNA. PCR products were blotted using alkaline blots.

RNA Isolation and Real-Time PCR Analyses. For the reverse transcription−polymerase chain reaction (RT-PCR) and real-time PCR, total RNA was isolated from 100 mg of plant tissue using CONCERT (Plant RNA Purification Reagent, Invitrogen, Carlsbad, CA, USA) following RNA purification and DNA cleavage on columns (RNeasy Plant Total RNA kit, Qiagen, Hilden, Germany). Prior to cDNA synthesis, each RNA sample was checked up on the presence of gDNA contamination by PCR with the GAPDH-specific primers (*13*) described below. If contaminated, samples were supplementarily treated with DNase I (DNA-freeTM Kit, Ambion, Austin, Texas, USA). Real-time PCR quantifications of the hop TFs under analysis were performed using the following primer combinations: R-HlbZip-PCR (5′-AGTGGTACTTCGGGCAGAGG-3′) and R-HlbZip-RT (5′-CGTGCTTTCTCATCCTCCAG-3′) for *HlbZip1* amplification; R-bZip2-PCR (5′-TCACTCTGATCGACCCGAC-3′) and R-bZip2-RT (5′-AAGCAGAAAGTCCTCGAGC-3′) for *HlbZip2* amplification and C5′M3PCR (5′-GACGTCAACAGCAAGCAATTC-3′) in combination with C3′M3RT (5′-GGCCTCTGACGTGTCTGATG-3′) for *Hl*bMyb3 as described previously (*16*). All these primers had an annealing temperature of 58 °C and amplified unique, nonconserved regions of the TFs. Four micrograms of total RNA were reverse transcribed using oligo dT^{18} primer and SuperscriptII reverse transcriptase (Invitrogen, Carlsbad, CA, USA) at 42 °C for 60 min. A total of 5 μL of 50× diluted cDNA was used for a 20 μL PCR reaction with 0.6 units of Hot Start Ex Taq polymerase (TaKaRa Bio Inc. Otsu, Shiga, Japan), Taq buffer 1×, dNTPs 200 μM each, Syber green 1:20000 (Molecular Probes, Leiden, The Netherlands) and primers 375 nM each. All amplifications were carried out on a Bio-Rad (Alfred Nobel Drive, Hercules, CA, USA) IQ5 cycler for 40 cycles (94 °C for 20 s, 59 °C for 30 s, and 72 °C for 30 s) following an initial denaturation/Taq activation step (94 °C for 5 min). Product size was confirmed by melting analysis and 2% agarose gel electrophoresis. Data were analyzed and quantified with the Bio-Rad IQ5 Optical System, version 2.0, software. The abundance of a reference transcript, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*13*, *24*), was estimated in parallel in each sample. GAPDH was amplified using the primer combination HL-GAP-F1 (5′-ACCGGAGCCGACTTTGTTGTT-G-3′) and HL-GAP-R1 (5′-TCGTACTCTGGCTTGTATTCCTTC-3′). GAPDH as the reference gene was previously used by Nagel et al. (*13*) and Wang et al. (*24*). In our study, we used primers originally described by Nagel et al. (*13*); however, in this study the primer HL-GAP-F1 was shortened for two AA bases on its 3′-end to achieve a more balanced annealing of the primer pair at 60 °C. The relative values were standardized to GAPDH according to Pfaffl (*25*). Efficiencies of primer pairs (bZIP1 = 1.78, bZIP2 = 1.86, Myb3 = 1.93, and GAPDH = 1.80) were taken from standard curves. The samples with the highest expression were set to 100%. Each PCR reaction was performed and run in duplicate to verify consistency. RNA was analyzed from five independent samples. In some cases, the 7SL RNA product was used as a supplementary constitutive control. Approximately 301 bp of 7SL cDNA were amplified using the primers α (5′- TGTAACC-CAAGTGGGGG-3′) and anti-*β* (5′-GCACCGGCCCGTTATCC-3′) as described previously (*16*, *26*).

Amplification of *omt1* and *vps* Promoter Regions and Preparation of Plant Vectors. To determine the genomic sequences of the *omt*1 and *vps* promoter regions, we have used plant DNA from hop cv. Osvald's 72 leaf tissue isolated by the CTAB method as performed for the genomic blots (*22*). Two to 3 μg of total plant DNA was digested with 50 U of *Eco*RI, for at least 2 h at 37 °C in a volume of 100 μL. After testing 10 μL aliquots by agarose gel electrophoresis, the samples were extracted with a phenol/chloroform mixture and precipitated with isopropanol (*19*). After self-circularization by ligation, 0.5 μg of plant DNA was subjected to PCR amplification using the OMTRi primer (5′-GAAATCTCATGATGCGA-GAGAG-3′) facing the start codon and OMTFi (5′-TGATGAGGT-GATCTCATTGTACG-3′) at the *Eco*RI site (217 bp from the start codon). The primers were designed from the authentic *omt1* sequence that we amplified from Osvald's 72 hop (AC: FM164641). OMT inverse PCR (iPCR) reactions were performed in 25 μL using LA (long and acurate) PCR polymerase (Top-Bio, Prague, Czech Republic) as recommended by the supplier. DNA samples were denatured at 95 °C for 2 min, and amplified using 35 cycles (94 °C for 30 s, 56 °C for 45 s, and 72 °C for 2 min) followed by elongation at 72 °C for 5 min. PCR was confirmed by a nested reaction performed using 1 μL from a 500-fold diluted first PCR reaction mixture in combination with the nested primers OMTRine (5′-CAT-GATGCGAGAGAGGTAAGG-3′) and OMTFine (5′-GGTGATCT-CAT-TGTACGGGC- 3′). These reactions yielded a 1300 bp PCR product strongly hybridizing to *omt*1 probe. This product was isolated from agarose gel using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into pCR-Script SK(+) vector (Stratagene, La Jolla, CA, USA). Before cloning the promoter into the plant vector, the sequence was reamplified with the primers EcoOMTp (5′-aatGAATTCGTGATCTCA-TTGTACGGGCTAAG-3′) and XbaOMTp (5′-attTCTAGAATTGG-TCTTTCTTAGTTATTCTTTGG-3′) using high fidelity *Pwo* polymerase (Roche Molecular Biochemicals, Basel, Switzerland). The promoter of the *vps* gene (*27*) was amplified from Osvald's 72 hop using the primers Eco-VPS (5′-aatGAATTCCTTGCTTATGAGAACTGCTC-3′) and XbaVPS (5′-attTCTAGATACCT-GATATATATATAGCTAG-3′ (*Eco*RI and *Xba*I restriction sites are underlined, and additional nucleotides are in lower case)). The *chs*_H1 promoter sequence was cloned previously (*28*).

The v*ps* and *omt1* promoter regions were fused to GFP/GUS genes by cloning in the unique *Eco*RI and *Xba*I restriction sites of the plant vector pBGF-0 (*29*) as described earlier (*11*). TFs *HlbZip1A* and *HlbZip2* CDS were reamplified with the high fidelity *Pwo* polymerase using the primer pair combinations XhoHLbZIP1A (5′- gacCTCGAGGGCCCATGG-CGTTGTCTAACGTGG-3′) x SacHLbZIP1A (5′-taaGAGCTC-AAA-GAGATCTAACTCTTCG-3′) and XhoHlbZIP2 (5′-gacCTCGAGG-GCCCATGAGCG-AAGAAGATTCTAGAAAC-3′) x XbaHLbZIP2 (5′-aatTCTAGATCAAAGAGATCTAAC-TCTTCGAAG-3′), respectively. *HlbZip1A* and *HlbZip2* CDS were then treated with *Xho*I and *Sac*I, and *Xho*I and *Xba*I, respectively. Restriction sites in the primers are underlined. A gel-purified fragment of *HlbZip1A* was then fused to the 35S CaMV promoter by cloning into the unique *Xho*I, *Sac*I sites of the intermediary vector pLV68 (*11*), while *HlbZip2* was cloned in this vector after cleavage with *Xho*I and *Xba*I. The whole TF cassettes containing the 35S CaMV promoter were excised using restrictases *Asc*I and *Pac*I and cloned in the plant vector pLV07 as described by Matoušek et al. (*30*). This vector was introduced in the *A. tumefaciens* strain LBA 4404 by the freeze−thaw method (*31*) and used for leaf infiltration of *N. benthamiana* or *P. hybrida* leaves as described previously (*11*). The *HlMyb3*-bearing plant vectors and *A. tumefaciens* strains were described previously (*16*), and the plant vector bearing the native *chs*_H1 gene tandemly arranged with the *pap*1 gene (pLV67) has been described earlier (*11*).

Transient Expression Systems and GUS Assays. For coinfiltration, agrobacteria suspensions were prepared at an OD_{600} = 0.7, and equal volumes of each suspension were mixed prior to infiltration. *N. benthamiana* seedlings were sown in flats and grown under green house conditions (24−30 °C) for 21 days. Agrobacteria carrying the appropriate gene constructs were activated by adding 150 μm acetosyringone[11] and were injected into leaf blades with a syringe. In an attempt to reduce the influence of different physiological conditions in the plant tissues, at least two plants and two leaves per plant were infiltrated with agrobacteria.

The activity of β-glucuronidase (GUS) was assayed in excized and pooled leaf sectors after 5 days following the infiltration using a fluorometric assay (*32*). One hundred milligrams of leaf tissue was homogenized in 500 μL of extraction/incubation buffer (E/I buffer). Fifty microliters of extract was applied to 500 μL of substrate solution and 4-methylumbelliferyl-β-D-glucuronide (MUG) in E/I buffer. The samples were then incubated at 37 °C for varying time intervals (0, 5, 10, 30 min) after which the enzymatic reaction was blocked with a stop solution (0.2 M Na_2CO_3). The amount of released fluorescent dye 4-methylumbelliferone (MU) was measured using DyNA Quant 200 Fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA, USA) with the wavelengths for excitation and emission set at 365 and 455 nm, respectively. GUS activity was expressed in pmol MU × mg^{-1} fresh leaf tissue × min^{-1}. Data obtained for bacteria-treated plants were compared with those found in parallel control experiments performed in the absence of bacteria. Three independently repeated experiments were performed for each experimental variant. Statistical significance of the treatment differences was assessed using Student's *t*-test. All data collected were analyzed using Microsoft Excel and Minitab version 13 software. Along with the quantitative fluorometric assays, histochemical assays on leaf discs were also performed. The discs were cut and immersed for 10 min in a fixation solution (1% glutaraldehyde in PBS buffer). Then the samples were put in a 1 mM X-gluc (5-bromo-4-choloro-3-indolyl-β-glucuronic acid) solution, subjected to 20 min of infiltration, and incubated at 37 °C overnight for blue color development. Finally, the leaf discs were washed repeatedly with 70% ethanol to remove chlorophyll.

Analysis of Secondary Metabolites. Leaves of *P. hybrida* were lyophilized prior to analysis of secondary metabolites. After grinding up lyophilized plant material in a mortar, samples of 10−20 mg were extracted in Eppendorf tubes using 1 mL of methanol/water (1/1, v/v), and after sonication, the extraction mixtures were kept for 12 h at 4 °C. After centrifugation at 18000 rpm for 10 min, the supernatant was analyzed by high performance liquid chromatography (HPLC) using a SHIMADZU LC 20A (Shimadzu, Nakagyo-ku, Japan) chromatograph with diode array detectors (DAD). The column was Nucleosil RP C_{18} (Macherey-Nagel, Düren, Germany, 5 μm, 250 × 4 mm), while the injection volume was 50 μL. Gradient elution over 60 min was applied from 15% of solvent B (methanol/acetonitrile, 1/1, v/v, with 0.025% formic acid) in solvent A (water with 0.025% formic acid) to 95% of solvent B in A. Chromatograms at 350 nm were extracted from the 3D data, and peaks were characterized on the basis of their UV spectra and retention times.

To additionally identify phenolic compounds, high-performance liquid chromatography−mass spectroscopy (HPLC-MS) was used as described previously (*16*). Peak integrations were carried out using standard parameters, and normalized peak areas were calculated by dividing peak areas by the sample weight and the area of the corresponding peak in the reference sample. Analyses were carried out in 5-fold in order to check the consistency of the peak areas.

Other Methods. For cluster analysis of bZIP TFs, sequences from the GeneBank database were used, as indicated on the individual figures. Sequence analyses were carried out with DNASIS for Windows, version 2.5 (Hitachi Software Engineering Company, Tokyo, Japan). The trees were generated using the ClustalW option of the DNASIS for Windows, version 2.5 software. Amino acid classes described by Bork et al. (*33*) were used for protein comparisons. Plant Cis-DNA elements database (PLACE) (*34*) was used to screen for TF binding sites.

RESULTS

Cloning and Sequencing and Genomic Analyses of the Transcription Factors *HlbZip1* and *HlbZip2* in Hop. The objective of the cDNA-AFLP screen was to preselect probes for the structural genes and regulatory elements involved in the phenylpropanoid pathway leading to bitter acids and prenylchalcones in hop. Originally, samples from three hop varieties, Whitbread Golding, Wye Challenger, and Admiral, found to possess contrasting characteristics for the accumulation of prenylchalcones in a previous work (*9*), were collected at different developmental stages and analyzed for differential cDNA expression. A set of 231 transcript-derived fragments were isolated, and finally, 294 unique nucleotide sequences were obtained and compared with publicly available sequence data. From this data set, the probe specific for bZip TF was selected. In the initial cDNA-AFLP screen, this TDF displayed a significantly higher expression level in fully developed hop cones than in leaves or young cones. After the elimination of adapter sequences, this 205 bp bZip-specific fragment fully corresponded to bZip cDNA clone 2330 (AC: FN395065) at positions 542−746.

The bZIP probe was used to screen a cDNA library from lupulin glandular tissue-enriched hop cones, which had been established previously (*15*). The sequencing of the positive clones revealed two sequence variants of bZip cDNA, comprising in addition to clone no. 2330 also clone no. 2327, which was concurrently deposited with EMBL under accession number AM998490. The cDNA of clone no. 2330 encodes for a TF, designated *HlbZip1A*. Via further PCR checking of the cDNA library using primers derived from the 5′ (primer 5′-start) and the 3′-ends of the *HlbZip1A* coding region (CDS) (3′-stop), we discovered a truncated variant of this TF and designated it *HlbZip1B*. *HlbZip1A* encoded for a typical G-box bZIP protein, having 246 amino acids (aa) (**Figure 1**) with an apparent molecular weight of 27.4 kDa and a pI of 6.42. *HlbZip1B* CDS is identical to *HlbZip1A* except for a 72bp deletion in the 3′ teminal part of the CDS. This truncated but in-frame sequence encoded for a shortened variant of *Hl*bZIP1, having 222 aa, an apparent molecular weight of 24.6 kDa, and a pI of 9.52. Clone no. 2327, designated *HlbZip2*, encoded for a related bZIP TF (**Figure 1**). The corresponding protein (314 aa) has a molecular weight of 34.2 kDa and a pI of 8.51. The bZIP TFs from hop were 53 to 66% homologous to the G-box specific group A bZIP TFs from *A. thaliana* (*35*). They contained an identical DNA-binding domain (MIKNRESAARSRERKQ) and a short central conserved element (MTLED) (**Figure 1**). A strongly basic, truncated variant of *Hl*bZIP1 (*Hl*bZIP1B) is lacking the leucine zipper domain (**Figure 1**). Both *Hl*bZIP1 variants and *Hl*bZIP2 contain A1 domains and motifs of A2 domains characteristic for group A bZIP TFs (*17*). The DFL motif of the *Hl*bZIP2 A2 domain is presumably important for casein kinase phosphorylation. Finally, unlike GBF4 from *A. thaliana*, hop bZIP TFs contain a partial LRRT[SL][ST] motif in their C terminus (**Figure 1**), which is characteristic for some bZIP TFs of the A3 group (*15*).

Homology comparisons of *Hlb*ZIP1 *and Hlb*ZIP2 at amino acid levels were performed with selected related bZIP TFs isolated from other plants listed in the NCBI using Blast 2 (*36*). Search results as provided by the SIB BLAST network service showed significant probabilities (E-value in the range from 1.5e-46 to 7e-34). These comparisons show that *Hlb*ZIP1 and *Hlb*ZIP2 are clustering in separate clusters (**Figure 2**). According to these trees, some bZIP variants isolated from *Vitis vinifera* and *Gossypium hirsutum* are more related to the hop bZIP TFs than the model G-box bZIP GBF4 from *A. thaliana* (**Figure 2**).

Analysis of hop genomic DNA for the variability of cloned hop bZip sequences was performed with the aim of roughly estimating differences among various hop cultivars and the presence of hybridizing sequences in species closely related to hop, especially *H. japonicus* and *C. sativa* (**Figure 3**). DNA samples were cleaved with *Bam*HI and *Pvu*I, and screened using the probes derived

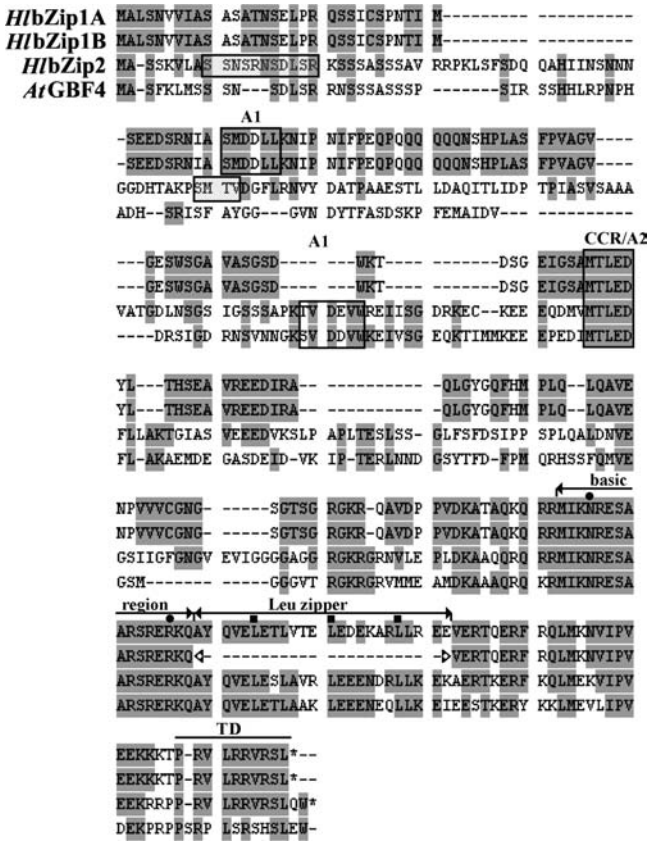

Figure 1. Alignment of deduced amino acid sequences of *Hlb*ZIP1A, *Hlb*ZIP1B, *Hlb*ZIP2, and AtbZIP40/GBF4. On the basis of primary amino acid sequences, several putative domains identified are marked. *Hlb*ZIP1A and *Hlb*ZIP1B are identical except for the absence of the leucine zipper in *Hlb*ZIP1B (deletion indicated by hollow arrows). Domains A1 and A2 with consensus sequences [NRST] [AIMV] [ADEG] [DEQ] [ILMV] [LW] and [ST] [IL] [EF] [DEQ] [DFL] [FL] [AFILSV] [KNQR], respectively (*17*), are marked and outlined. The A2 domain overlaps with the central conserved region (CCR) MTLED and is only partially present in hop (*Humulus lupulus* L.) bZIPs. In the highly conserved basic region (also the DNA-binding domain), the residues N and R are marked by a dot. According to the consensus N-x_7-[RK]-x_6-L-x_6-L-x_6-L (*35*), leucines of the leucine zipper follow the basic region, marked by a square. Additionally, a conserved terminal domain (TD) was found in hop bZIPs corresponding to the consensus [LI]-x-R-x_2-[ST] (*35*); matching amino acids are marked by an asterisk. A similar sequence was also found in GBF4, but all these sequences are only partially identical to the A3 domain (LRRT[SL] [ST]) described by Corra et al. (*17*).

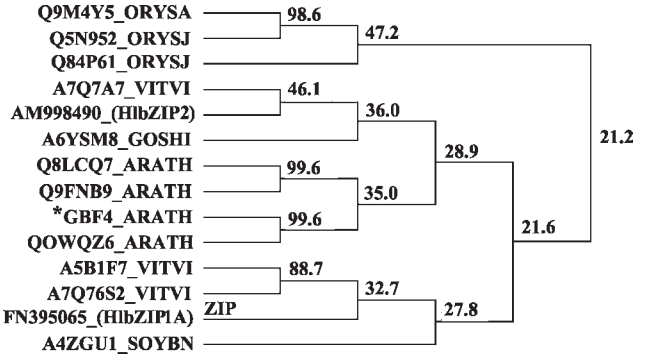

Figure 2. Cluster analysis for deduced amino acid sequences of selected plant bZIP TFs designated by the ID numbers from the EMBL database (see: Materials and Methods). The positions of *Hlb*ZIP1A *and Hlb*ZIP2 are shown in brackets. Unrooted trees were generated with the Clustal W option of DNASIS (Hitachi). The position of the G-box *Atb*ZIP transcription factor from *Arabidopsis thaliana* (ID: GBF4_ARATH), which was chosen for alignments in **Figure 1**, is designated by an asterisk. Percentages of amino acid identity are included in the phylogenetic tree.

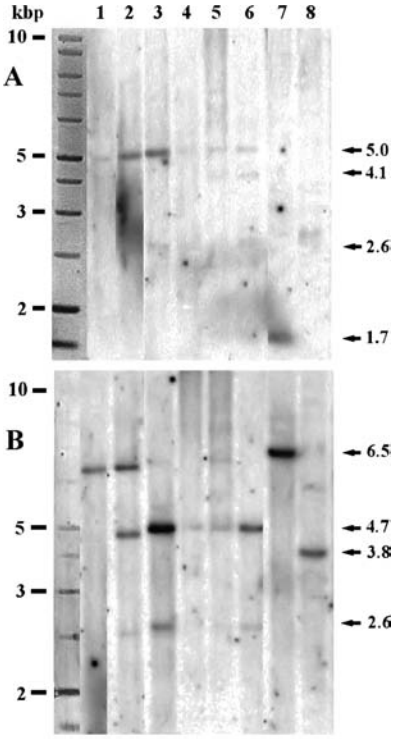

Figure 3. Southern analysis of genomic DNA from hop and related plant species with *HlbZip1* (**A**)- and *HlbZip2* (*B*)-specific cDNA probes. Genomic DNA was doublecleaved with *Bam*HI and *Pvu*I. Lane 1, DNA from cv. Osvald's 72; lane 2, cv. Target; lane 3, cv. Taurus; lane 4, cv. Admiral; lane 5, cv. Columbus; lane 6, *Humulus lupulus* var. *lupuloides*; lane 7, *Humulus japonicus*; lane 8, *Cannabis sativa*. DNA markers are aligned on the left side of the autoradiogram; arrows designate the positions of visible bands; and their approximate lengths in kbp are indicated by numbers on the right side.

from the 5′-end of CDS. In the case of the hop variety Osvald's 72, a single fragment corresponding to 5 kbp was detected using an *HlbZip1*-specific probe. Similar fragments were observed in all *H. lupulus* species investigated. For cv. Columbus and the American wild hop *H. lupuloides*, an additional distinct band of 4.1 kbp was visible (**Figure 3**). A different pattern of *HlbZip1*-hybridizing sequences was observed for *H. japonicus* and *C. sativa* (**Figure 3**), where the 5 kbp fragment was not visible. Instead, a 1.7 kbp band was found for *H. japonicus*, while only a weakly hybridizing band of 2.6 kbp was discerned for *C. sativa*. Hybridization of genomic DNA to a *HlbZip2*-specific probe revealed a single band (about 6.5 kbp) for cv. Osvald's 72. A similar single band was observed for *H. japonicus*. For cv. Admiral, a single band of about 4.7 kbp was detected, and for *C. sativa* (**Figure 3**), a strongly hybridizing single band having 3.8 kbp was detected. Other samples showed more complex patterns including a fragment of about 2.6 kbp. In view of the important differences among hop varieties and related species, it can be concluded that the genomic arrangements of the cloned bZIP genes are not conserved.

Analyses of Expression of Transcription Factors *HlbZip1* and *HlbZip2* in Hop Tissues and Comparative Analysis of Hop TFs in Lupulin Glands. Two cloning steps, cDNA-AFLP and specific hop cDNA library screening, in fact preselected *HlbZip1* and *HlbZip2* sequences specifically expressed in hop cones and lupulin glands. In

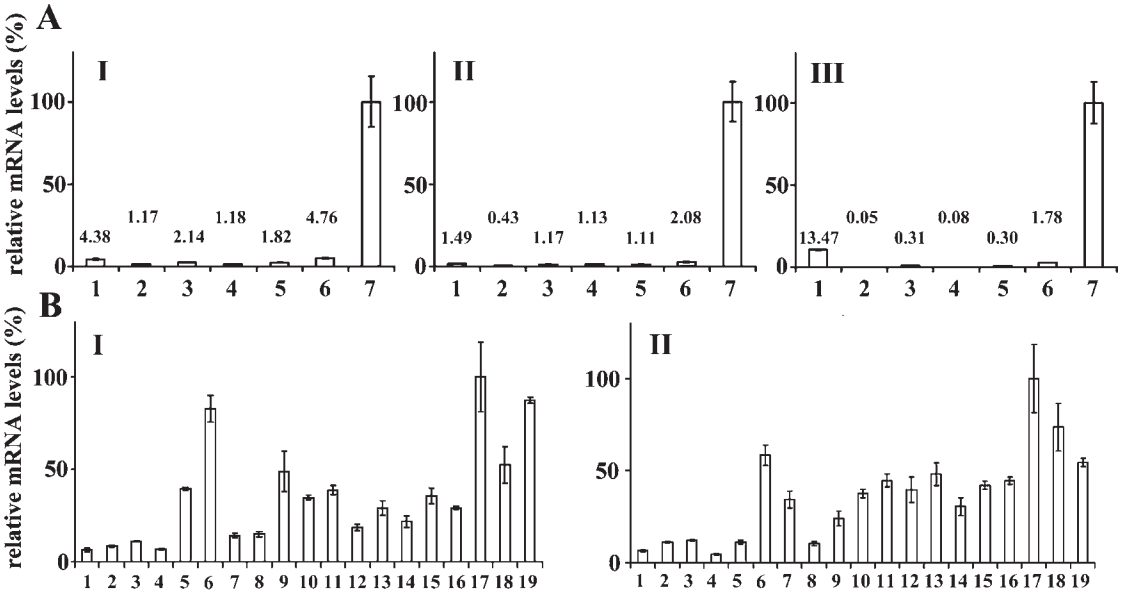

Figure 4. Real-time quantitative PCR analysis of hop transcription factors. (**A**) Relative mRNA levels of *HlbZip1* (panel I), *HlbZip2* (panel II), and *HlMyb3* (panel III) in isolated lupulin glands compared to other tissues. Description of samples: 1, root; 2, young leaves; 3, petioles; 4, flowers; 5, young cones; 6, unmatured pollen; 7, lupulin glands. (**B**) Expression of analyzed *HlbZip1* (panel I) and *HlbZip2* (panel II) in different developmental stages and different tissues of cv. Osvald's 72. Descriptions of the samples: 1, dormant buds, plant of 1 m height, start of rapid growth; 2, shoot tip; 3, leaves; 4, stem; 5, root, plant of 6 m height, growth stopped, blooming; 6, root; 7, shoot tip; 8, young leaves; 9, old leaves; 10, stem; 11, petioles; 12, flowers; several sizes of young maturing cones, 13, cones <1 cm; 14, cones 1−2 cm; 15, cones 2−3 cm; 16, cones >3 cm; immature pollen obtained from several sizes of male flower buds, 17, buds 2−2.4 mm; 18, buds 3−3.4 mm; 19, buds 4−4.4 mm. GAPDH was used as an internal reference transcript. Bars represent the confidence intervals at level $\alpha = 0.05$.

order to verify the tissue-specific expression of the cloned hop TFs *HlbZip1 and HlbZip2*, real-time quantitative PCR (RT qPCR) was used. Precise normalization to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (*13*, *24*), was performed and confirmed by sample normalizing against 7SL RNA (*26*). Lupulin glands of clone Osvald's 72 were separated and purified as described by Nagel et al. (*13*) to avoid RNA contaminations from hop cone bracts. Our GAPDH normalized results show that both bZip TFs, as well as the previously characterized *HlMyb3*, have a relatively high specific expression in lupulin glands. RT qPCR confirmed approximately 200× higher levels of *HlbZip1* mRNA, 500× higher levels of *HlbZip2* mRNA, and 5000× higher levels of *HlMyb3* mRNA when compared to their respective levels in young leaves (**Figure 4A**).

The tissue specificity of *HlbZip1* and *HlbZip2* was further examined by RT qPCR in several different developmental stages and in tissues other than lupulin glands where the specific expression was much lower (**Figure 4B**, compare panels I and II). This method showed up to approximately 20-fold differences in actual mRNA levels among 19 selected hop tissues from the hop variety Osvald's 72. The highest levels of *HlbZip1* and *HlbZip2* mRNAs were reached in the roots of plants of 6 m height (growth stopped, blooming) and in immature pollen samples. The lowest levels of *HlbZip1* and *HlbZip2* mRNAs were detected in dormant buds and also in the stems of plants of 1 m height (start of rapid growth) (**Figure 4B**).

Comparative Promoter Activation Analyses of Hop TFs in a Transient Expression System. According to our results, both hop bZip TFs and also *HlMyb3* TFs show high relative expression in the glandular trichomes of maturating hop cones, and therefore, they may be involved in the coactivation of genes directly related to the biosynthesis of secondary metabolites in lupulin glands (*9*, *37*). In the search for further support of this statement, we investigated the potential of the hop cloned TFs to activate the promoters of the biosynthesis genes chalcone synthase *chs*_H1 (P*chs*_H1), valerophenone synthase (P*vps*), and *O*-metyltransferase1 (P*omt1*) in a transient expression system that we had developed previously (*11*). It has been shown that PAP1 (AtMyb75) from *A. thaliana* is able to activate the *chs*_H1 promoter (**Figure 5A**, I) fused to the *gus* gene, when *A. tumefaciens* bearing P*chs*_H1 and *pap1* vectors were coinfiltrated into *N. benthamiana* leaves. In the present study, we constructed new *gus* reference vectors (**Figure 5B**, I) containing either P*vps* or P*omt1* and expression vectors (**Figure 5B**, II) bearing a newly cloned hop bZip TF sequences. Approximately 100-fold higher amounts of TF mRNAs were detected by RT qPCR in *N. benthamiana* leaves infiltrated with these vectors when compared to their levels in hop leaves (data not shown). The promoter region for *vps*, 660 bp upstream of the start codon, was cloned from Osvald's 72 genomic DNA according to the known sequence (AC: AB047593) (*27*). P*omt*1 was cloned from Osvald's genomic DNA using inverse PCR and the authentic *omt*1 clone from Osvald's 72 (AC: FM164641) was used for the preparation of the hybridization probe. The upstream *omt1* region of 573 bp was amplified in these reactions. The sequence has been deposited in the EMBL database under AC: FN395066. Comparisons of the P*chs*_H1, P*vps*, and P*omt1* promoter regions are shown in **Figure 5A**. While P*chs*_H1 (**Figure 5A**, I) contains two typical bZIP-binding G-boxes (CACGTG) at positions −412−407 and −232−227, as described earlier by Matoušek et al. (*11*, *28*), neither P*vps* nor P*omt1* contained these boxes. In P*vps*, a G-box A (TACGTA) was predicted (**Figure 5B**, II) using a database of Plant Cis-acting Regulatory DNA elements (PLACE) (*34*). In the P*omt1*, a box characteristic for ATB2, a subgroup of bZIP (ACTCAT) was found at position −487−482 (**Figure 5B**, I). In all three sequences, various MYB-binding boxes were detected in the region −500−400 bp upstream of the start codon, and characteristic TATA boxes were found at similar positions, around the −150−100 upstream of the coding region (**Figure 5A**).

Despite some positional similarities among the analyzed promoters as to the potential binding sites, the promoters responded highly selectively to the overexpression of the hop bZip TFs and

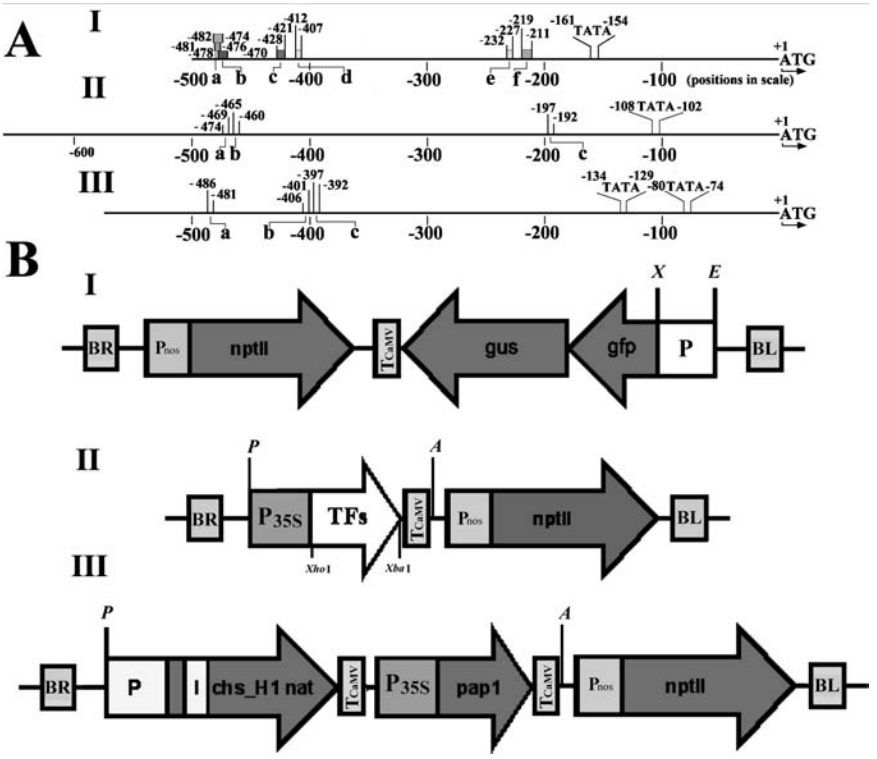

Figure 5. Schematic drawing of analyzed promoters (**A**) and expression cassettes within the T-DNA parts of the plant vectors used for leaf infiltration (**B**). Promoter schemes (**A**) are in scale. I: P*chs*_H1 summarized as described previously (*11*, *28*). a, P-Myb box/H box; b, Myb-like box; c, Myb box; d, G-box, e, G-box; d, P-Myb box. II: P*vps* region (*27*) of 660 bp as analyzed by the PLACE database (*34*), only boxes upstream of the TATA4 signal are shown: a, Myb consensus box; b, Myb2 consensus box; c, G-box A. III: P*omt1* sequence of 573 bp as cloned in this work (AC: FN395066) and analyzed by the PLACE database. a, AtbZip group B box; b, Myb core; c, Myb consensus box. TATA boxes 5 and 4 are shown at positions −134−129 and −80−74, respectively. Plant vector schemes (**B**) are not in scale. I: general scheme of vector pBGF-0 containing analyzed promoters (P). II: general cassette of vector pLV-07 bearing the 35S-driven transcription factor (TF) from hop. Cassette III shows the native *chs*_H1 gene head-to-tail arranged to the 35S-driven *pap*1 gene as a tandem (vector pLV-67). Coding sequences are in dark gray, promoters are marked by P, and the intron is marked by I. BR and BL are the right and left T-DNA borders, respectively. NptII designates the neomycin phosphotransferase gene for resistance to kanamycin. This gene is driven by the nopalin synthase promoter (Pnos). Terminators of CaMV (TCaMV) are shown. Restriction sites *Xba*I (X), *Eco*RI (E), *Asc*I (A), and *Pac*I (P) were used for the integration promoters and cassettes in the plant vector.

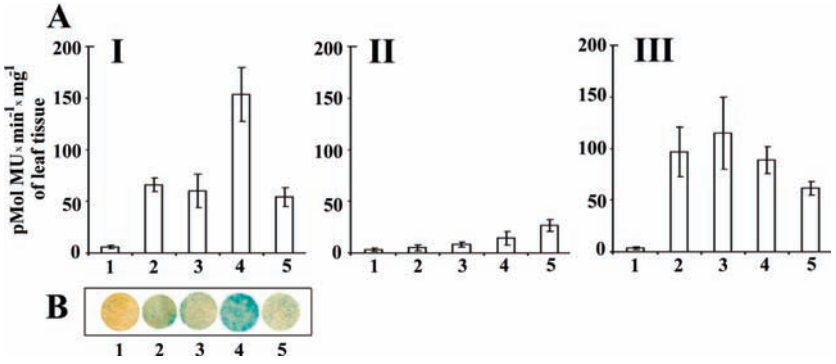

Figure 6. Analysis of *chs*_H1 (I), *vps* (II), and *omt*1 (III) promoter activation by *Hlb*Zip TFs and by *HlMyb3* variants using the GUS transient assay (**A**) and parallel histochemical analysis of activation of P*chs*_H1 (**B**). For infiltrations, activated *Agrobacterium tumefaciens* strains bearing hop TFs and promoter/GUS plant vectors, as shown in **Figure 5B**, were mixed at a 1:1 ratio. TFs samples: 1, LBA 4404-background control; 2, l-*HlMyb3*; 3, s-*HlMyb3*; 4, *HlbZip1A*; 5, *HlbZip2*. Bars represent the confidence intervals at level $\alpha = 0.05$.

subvariants of *HlMyb3* (**Figure 6A**). While generally a very low response was detected for P*vps* after TF coinfiltration, with the highest signal for *HlbZip2* (**Figure 6A**, panel II), the activation of P*chs*_H1 and P*omt1* was clearly much stronger (**Figure 6B**, panels I and III). P*chs*_H1 was activated more intensely by *HlbZip1* than by *HlbZip2* and *HlMyb3* variants, whereas P*omt1* was more highly stimulated by *HlMyb3* variants and *HlbZip1* than by *HlbZip2* (**Figure 6A**, panel III). Histological analysis of infiltrated leaf discs made for the P*chs*_H1 variant showed a good signal correlation of both histological and fluorescence methods. It must be noted that the selective activation was not due to different expression levels of hop TFs because parallel RT qPCR revealed similar levels of the TFs overexpressed in infiltrated *N. benthamiana* leaves (not shown). The activation of P*omt1* lacking G-boxes with both cloned hop bZIP TFs suggests that these transcription factors are not strictly G-box specific.

Table 1. Normalized Peak Areas for Secondary Metabolites Extracted[a] from Infiltrated and Control *Petunia hybrida* Leaves

	TF application[b]								
t_R	control	1	2	3	4	5	6	7	8
9.3[c]	1	0.84	0.92	0.99	7.15■	2.85	13.34■	1.31	6.20■
11.7[c]	1	3.59■	3.87■	2.47■	6.51■	3.04■	5.95■	6.66■	4.87■
13.4[c]	1	0.86	0.67	0.68	5.02■	2.46	9.25■	2.56	3.76■
14.6[c]	1	1.31	1.06	0.04	0.55	0.82	0.95	1.11	0.92
15.1[c]	1	1.04	0.61	1.13	0.77	0.70	0.92	0.71	0.99
15.9[c]	1	0.34	0.30	0.99	0.34	0.29	0.31	0.33	0.35
18.3[d]	0	0.20	0.48■	0.00	0.61■	0.37	0.98■	1.00■	0.77■
18.9[d]	1	0.86	0.65	0.75	0.69	0.72	0.90	0.99	1.08
20.9[c]	1	1.00	0.76	0.70	1.04	0.77	1.07	1.42	0.93
21.7[c]	0	0.63	0.58	0	0.57	0.58	0.60	1.00	0.65
22[d]	1	0.97	0.55	0.92	0.66	0.74	1.06	1.14	1.06
23.6[d]	1	1.14	0.98	0.84	0.92	0.88	1.30	1.63	1.14

[a]See Material and Methods for the procedure of extraction. Analyses were carried out in 5-fold in order to check the consistency of the peak areas. The coefficient of variation was below 10% for all compounds with an average of 5.6%. Significant changes in metabolite levels are marked by a square. Note the conversion of the peak with t_R 15.9 min to the peak with t_R 21.7 min by several TFs (underlined). t_R, retention time. [b]*Petunia hybrida* samples infiltrated by 1, *HlbZip1A*; 2, *HlbZip2*; 3, l-*HlMyb3*; 4, s-*HlMyb3*; 5, *HlbZip1A*+l-*HlMyb3*; 6, *HlbZip1A*+s-*HlMyb3*; 7, *HlbZip2*+l-*HlMyb3*; 8, *HlbZip2*+s-*HlMyb3*. [c]Peaks identified as phenolic acids, according to their absorption spectra. Peaks with t_R 9.3 and 13.4 min could be tentatively identified by LC-MS as glycosides of caffeic and ferulic acid, respectively. Peaks with t_R 15.1 and 20.9 min could be tentatively identified by LC-MS as malic acid conjugates of caffeic acid and ferulic acid respectively. [d]Peaks identified as flavonoids, t_R 18.3 min, a putative flavonol glycoside; t_R 18.9 min, a putative flavonol derivate; t_R 22.0 min and t_R 23.6 min, putative flavone derivatives according to their UV-absorption spectra.

Modulation of the Accumulation of Secondary Metabolites in *P. hybrida* Leaves Co-Infiltrated with Hop TFs. In our previous work (*16*), *P. hybrida* transgenotes were used as a model to control possible changes in phenolic acids induced by the *HlMyb3* transgene. It was found that this TF induces wide variations in the petunia metabolome. We now used the leaf infiltration strategy to check for possible changes of the *P. hybrida* metabolome in infiltrated leaf sections after application of the cloned hop bZip TFs in combination with *HlMyb3* variants (**Table 1**).

Infiltrated leaf sections of *P. hybrida* were shown to accumulate some phenolic acids and flavonol glycosides at much higher levels than in the controls. For instance, the peak with retention time (t_R) of 11.7 min (ESI MS (m/z): 399.0911 $[M - H]^-$, not yet identified), representing a phenolic acid derivate belonging to the hydroxycinnamate group according to the absorbance spectrum, was independently increased by all transcription factors (**Table 1**). Peaks with t_R of 9.3 min (ESI MS (m/z): 341.0876 $[M - H]^-$, putatively identified as caffeoyl glycoside) and 13.4 min (ESI MS (m/z): 355.1013 $[M - H]^-$, putatively identified as feruloyl glycoside), both representing phenolic acid derivatives belonging to the caffeate/ferulate group according to the absorbance spectra, were increased by *s-HlMyb3*, in accordance with our previous work (*16*). Combinatorial experiments showed that the *HlbZip1A* strongly coactivated the accumulation of these compounds when coinfiltrated with s-*HlMyb3* (**Table 1**). Both TFs (*HlbZip1A* and *HlbZip2*) also coactivated the accumulation of compounds with t_R values of 9.3 and 13.4 min when coinfiltrated with l-*HlMyb3* (**Figure 7A**). However, peaks with t_R values of 15.1 min (ESI MS (m/z): 295.0459, putatively identified as caffeoyl malic acid) and 20.9 min (ESI MS (m/z): 309.0593, putatively identified as feruloyl malic acid) did not vary widely upon different treatments. All analyzed TFs, except for l-*HlMyb3*, also coactivated the accumulation of a flavonol glycoside with t_R of 18.3 min. The combinations of s-*HlMyb3*+*HlbZip1* and l-*HlMyb3*+*HlbZip2* led to the strongest activations (**Figure 7B**).

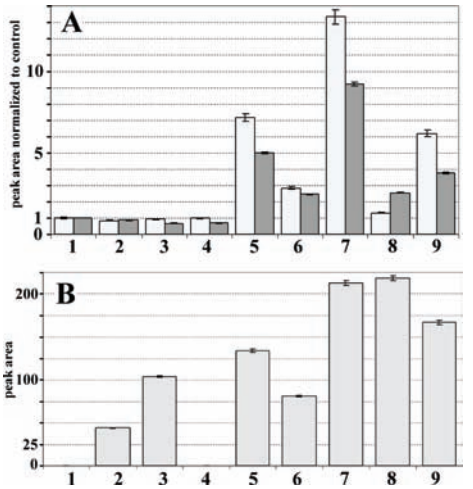

Figure 7. Analysis of accumulation of compounds of the ferulate type (**A**) and of the quercetin type (**B**) in *Petunia hybrida* leaves infiltrated with hop TFs. By the light and dark gray columns in graph **A**, we show the two phenolic acids of the caffeate/ferulate type having peaks at tR 9.3 and 13.4 min, respectively. Vector combinations: 1, LBA 4404-background control; 2, *HlbZip1A*; 3, *HlbZip2*; 4, l-*HlMyb3*; 5, s-*HlMyb3*; 6, *HlbZip1A*+l-*HlMyb3*; 7, *HlbZip1A*+s-*HlMyb3*; 8, *HlbZip2*+l-*HlMyb3*; 9, *HlbZip2*+s-*HlMyb3*. Bars represent the confidence intervals at level $\alpha = 0.05$.

In order to assay a quick responsiveness of the *P. hybrida* anthocyanin pathway to hop TF infiltration, we used the previously designed vector pLV-67 (*11*) bearing the "native" *chs*_H1 gene arranged in tandem with the 35S-driven *pap1*. Infiltration with this vector in combination with UV-A irradiation strongly stimulated a quick response of the anthocyanin biosynthesis pathway and a fast accumulation of blue anthocyanins in nontransformed petunia leaves, while no fast pigment accumulation occurred without UV-A treatment (*11*). We found in this study that coinfiltration of petunia leaves with the combinations of three vectors, pLV67, *HlMyb3*, and hop bZip TFs, led to quick pigment accumulation in leaves even in the absence of UV-A irradiation (**Figure 8**). These experiments suggest the involvement of *HlMyb3* and hop bZip TFs in the coactivation of the anthocyanin biosynthesis pathway in petunia. In conclusion, our results show that the investigated hop TFs interfere with the patterns of accumulation and in the composition of the phenolic metabolome in *P. hybrida* supporting their potential involvement in the phenolic biosynthetic pathways during the maturation of hop glandular tissue.

DISCUSSION

Structure and Expression of *HlbZip1* and *HlbZip2* in Hop. We aimed to preselect, clone, and characterize hop TFs potentially involved in the regulation of the lupulin gland metabolome. The biosynthesis of the class of terpenophenolics (including hop bitter acids and prenylflavonoids) receives the highest interest in perspective of the wide variety of reported biological activities. Despite the current difficulties with gene manipulations of hop such as the low efficiency of hop transformation (*38*) and the absence of the full genomic sequence of hop, dissection of the biochemical and regulatory networks driving terpenophenolic production in hop will result in a broader understanding and will stimulate the efforts to increase the yield of these valuable compounds via biotechnological manipulation. In our search for regulatory elements, a cDNA-AFLP approach was used to select short ESTs exhibiting some differential expression in hop varieties with varying contents of terpenophenolics. These short

Figure 8. An example of anthocyanin pathway activation in *Petunia hybrida* leaves after infiltration with hop TFs. The vector pLV-67 (see **Figure 5B**, III) was infiltrated in combination with l-*HlMyb3* and *HlbZip2* mixed in equal amounts. The anthocyanin spots developed two days postinfiltration. The sites of infiltration are indicated by arrows; i, infiltrated sector; ni, noninfiltrated leaf sector.

cDNAs were then used as probes to screen a hop cDNA library derived from glandular tissue-enriched hop cones. Combination of these two approaches led to the selection of two related bZIP TFs having the characteristics of G-box like bZIPs (*35*) that were recently classified in the A3 group of possible orthologues (*17*). The model representative of this group is GBF4 isolated from *A. thaliana* (*39*). However, alignment results showed that the hop bZIP TFs are more related to bZIP sequences cloned from *V. vinifera* and *G. hirsutum*. Via library screening, we discovered a truncated variant of *HlbZip1* (*HlbZip1B*) lacking the leucine zipper domain. Because this sequence was also present in the cDNA library and, except for the deletion, is quite identical to the *HlbZip1A* clone, it is possible that *HlbZip1B* is formed by an alternative splicing, as is known for other bZIP TFs (*35*). Genomic analyses for related (hybridizing) sequences in different hop varieties and two closely related species (*H. japonicus* and *C. sativa*) showed that the *HlbZip2* probe was strongly hybridizing to the genomic DNA of both hop-related species, while for *HlbZip1*, only weak signals were observed. This suggests that the *HlbZip2* sequence is more conserved than *HlbZip1*. Single fragments of about 6.5 and 5 kbp were present in genomic blots when hybridizing DNA from Osvald's clone 72 to the *HlbZip1* and *HlbZip2* probes, respectively. However, some differences in the hybridization patterns among various hop cultivars could be due to genomic changes introduced by selection and breeding processes (*40*) or due to the natural variation present in the species. Besides, we also cannot exclude that there is an oligofamily of related genes that are maybe tandemly arranged; at least three single-nucleotide variants of *HlbZip2* were detected by analysis of cDNA fragments using temperature-gradient-gel electrophoresis (*41*) in our experiments (unpublished work).

The RT qPCR technique revealed the highly specific expression of both hop bZip TFs in lupulin glands, although this expression was not restricted to this tissue. Expression was also detected in other somatic tissues including colored petioles and roots, and in generative organs, female flowers, and in immature pollen. This suggests on the one hand a strong tissue-specific regulation of these TFs in hop and on the other hand some polyfuncional character of both TFs. The relatively high specific expression of *HlbZip1* and *HlbZip2*, and also of the previously cloned *HlMyb3* (*16*) in lupulin glands supports the assumption about their involvement in the lupulin biosynthesis regulatory network.

Selective Activation of *chs*_H1, *vps*, and *omt1* Promoter Regions and the Potential of *Hl*bZIP1 and *Hl*bZIP2 to Interfere in the Metabolome Production. Among the known functions assigned to the A3 subgroup of bZIP TFs, ABA responsiveness, stress signaling, and seed development have been reported (*17*). Recent results show that the regulatory factors of this group are interconnected via a signaling network to bZIP TFs from other groups linked to plant morphogenesis and the light-dependent phenylpropanoid pathway regulation (*42*). Although some developmental changes in phytohormone profiles in hop were recently studied (*43*), the possible involvement of ABA networking in lupulin gland maturation is not known. In order to contribute to the study of possible function(s) of cloned hop bZIP TFs, we used a transient expression system based on leaf infiltration. Plant transient expression systems were successfully applied by others for similar purposes (*17*). Thus, combinatorial infiltrations with vectors containing the TFs under investigation and the promoters of three important hop genes, chalcone synthase *chs*_H1, valerophenone synthase, and *O*-methyl transferase 1, were performed. For these genes, highly specific expression in lupulin glands has been described (*12*, *13*, *28*). However, while *chs*_H1 is significantly expressed also in some other tissues such as colored petioles (*11*, *28*), valerophenone synthase is perhaps most specific for glandular and also leaf trichomes (*27*). Although promoter elements of these genes, including the newly cloned P*vps*, show some structural similarities in the distribution of putative binding boxes, they are totally sequence unrelated. While *chs*_H1 contains typical bZIP G-boxes, these boxes are absent in P*omt1*. In P*vps*, G-box A was present in the cloned region (*27*), which is sequence-unrelated to the G-box characteristic for P*chs*_H1. Significant transient activation of GUS by hop TFs was detected for P*chs*_H1 and P*omt1*, while the response of P*vps* was rather negligible. These results show that the transient expression response was sequence-specific and not an artifact due to unspecific binding. Furthermore, these findings are consistent with the fact that the *vps* promoter is not abundantly activated in tissues other than glandular hop tissue (*27*), suggesting a possible requirement of glandular-tissue specific cofactors that are currently still missing. Moreover, *HlZip1A* and *HlbZip2* lead to the activation of both P*chs*_H1 and P*omt1*, indicating that the activity of these bZIP TFs is not restricted to G-boxes. In P*omt*1, a binding box for group B of bZIP TFs was predicted; however, it remains to be determined which binding elements are responsible for the activation.

This study further demonstrated that l-*HlMyb3* and s-*HlMyb3* subvariants, which caused divergent phenotypic and morphogentic responses of heterologous plant transgenotes (*16*), did not result in significant differences in activation capacity with respect to the promoters P*chs*_H1 and P*omt1*. However, these *HlMyb3* subvariants caused differential changes of the metabolome in infiltrated leaf sectors of *P. hybrida*. These results are consistent with our previous work, in which we showed different accumulation of some classes of phenolic compounds and flavonol glycosides in *P. hybrida* transgenotes bearing either l-*HlMyb3* or s-*HlMyb3*. Consistent with all these facts, one can assume that the difference at the N-terminal domain of these two *Hl*MYB3 protein subvariants accounts for some protein−protein interactions and not for DNA binding. The hop bZIP TFs-mediated changes in metabolite and pigment accumulation in *P. hybrida* infiltrated leaves support the idea that these transcription factors, if significantly expressed, may selectively coactivate particular hop genes within the lupulin biosynthesis network.

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