Plant Mol Biol
DOI 10.1007/s11103-016-0510-7

The "putative" role of transcription factors from *Hl*WRKY family in the regulation of the final steps of prenylflavonid and bitter acids biosynthesis in hop (*Humulus lupulus* L.)

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Received: 1 December 2015 / Accepted: 2 July 2016

Abstract Lupulin glands localized in female hop (*Humulus lupulus* L.) cones are valuable source of bitter acids, essential oils and polyphenols. These compounds are used in brewing industry and are important for biomedical applications. In this study we describe the potential effect of transcription factors from WRKY family in the activation of the final steps of lupulin biosynthesis. In particular, lupulin gland-specific transcription factor *Hl*WRKY1 that shows significant similarity to *At*WRKY75, has ability to activate the set of promoters driving key genes of xanthohumol and bitter acids biosynthesis such as chalcone synthase H1, valerophenone synthase, prenyltransferase 1, 1L and 2 and O-methyltransferase-1. When combined with co-factor *Hl*WDR1 and silencing suppressor p19, *Hl*WRKY1 is able to enhance transient expression of *gus* gene driven by *Omt*1 and *Chs*_H1 promoters to significant level as compared to 35S promoter of CaMV in *Nicotiana benthamiana*. Transformation of hop with dual *Agrobacterium* vector bearing *Hl*WRKY1/*Hl*WDR1 led to ectopic overexpression of these transgenes and further activation of lupulin-specific genes expression in hop leaves. It was further showed that (1) *Hl*WRKY1 is endowed with promoter autoactivation; (2) It is regulated by post-transcriptional gene silencing (PTGS) mechanism; (3) It is stimulated by kinase co-expression. Since *Hl*WRKY1 promotes expression of lupulin-specific *Hl*Myb3 gene therefore it can constitute a significant component in hop lupulin regulation network. Putative involvement of *Hl*WRKY1 in the regulation of lupulin biosynthesis may suggest the original physiological function of lupulin components in hop as flower and seed protective compounds.

Keywords Lupulin biosynthesis · Transcription factors · 5′ RNA degradome · Plant promoter activation · Hop transformation · WRKY oligofamily

Introduction

Hop (*Humulus lupulus* L.) plants are traditionally cultivated mainly for the brewing industry as a source of flavor-active secondary metabolites and bitter acids, and recently hop metabolome is of interest for medicinal purposes as a source of prenylated bioactive compounds. Hop female inflorescences (hop cones) contain glandular trichomes (lupulin glands) that accumulate these specific metabolites during its phased maturation (Oliveira and Pais 1990; Sugiyama et al. 2006; Patzak et al. 2015). The main constituents of lupulin are known as humulones (α-acids) and lupulones (β-acids), valuable flavor-active ingredients for beer brewing. Prenylated flavonoids, such as xanthohumol (X; up to 1.3 %, m/m, of the dry weight of a hop cone) and desmethylxanthohumol

Electronic supplementary material The online version of this article (doi:10.1007/s11103-016-0510-7) contains supplementary material, which is available to authorized users.

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Published online: 08 July 2016

(DMX; up to 0.2 %) are the principal prenylated chalcones, which are intensively studied for their potent medicinal activities (Stevens and Page 2004; Gerhäuser 2005; Zanoli and Zavatti 2008; Van Cleemput 2009; Bolca et al. 2009). The total yield of valuable compounds in hops depends on the composition of the lupulin metabolome, as well as on the number, size of hop cones and the density of the glandular trichomes (Patzak et al. 2015).

Numerous structural key genes are involved in the final steps of bitter acids and prenylflavonoid biosynthetic pathways (Fig. 1). While an oligofamily of chs_H1 genes encoding "true" chalcone synthase (CHS, E.C. 2.3.1.74) enzymes co-determines the biosynthesis of X and possibly bitter acids with low rate (Matoušek et al. 2006; Novák et al. 2006), valerophenone synthase (VPS, E.C. 2.3.1.156) has been characterized as a key enzyme involved in the biosynthesis of bitter acids (Okada et al. 2001). Recently, specific prenyltransferases (PRT, E.C. 2.5.1) (Tsurumaru et al. 2010, 2012; Clark et al. 2013; Li et al. 2015) were identified and an oligofamily of hop O-methyltransferase (OMT) genes has been described (Nagel et al. 2008). Especially, OMT1 (E.C. 2.1.1) which is specifically expressed in lupulin glands performs the final reaction step in the biosynthesis of X by methylating DMX (Nagel et al. 2008).

The lupulin biosynthetic pathways are regulated in high complexity manner with the involvement of the *chs*_H1 gene as shown in our previous studies (Matoušek et al. 2005, 2006, 2007, 2010, 2012). Previously, we described the parallel activation of *chs*_H1 promoter not only by ternary MBW (*Hl*s-Myb3/*Hl*bHLH2/*Hl*WDR1 or *Hl*MYB2/*Hl*bHLH2/*Hl*WDR1) complexes but also by binary (*Hl*bHLH2/*Hl*WDR1) combination, while *HlChs*4 promoter was preferably activated by the binary combination *Hls*-MYB3/HlWDR1 or by direct action of the *Hls-Myb3* gene (Matoušek et al. 2012). Independent or combinatorial activity of TFs (Singh 1998) in the regulation of flavonoid biosynthetic pathways has been also described in several previous studies (Ramsay and Glover 2005; Feller et al. 2011; Albert et al. 2014). Recently, the common regulatory network that governs complex anthocyanin pathway regulation was analyzed by bioinformatics tools (Zhu et al. 2015). Common regulation or promoter architecture specificity driving the whole xanthohumol or bitter acids pathways (Fig. 1) in hop has not been analyzed yet.

WRKY transcription factors belong to one of the largest TF families, involved in regulation of many physiological processes unique to plants, such as biotic and abiotic stress responses, senescence and trichome development (Rushton et al. 2010). WRKY proteins form a transcriptional network consisting of positive and negative feedback loops which modulate the complex signaling network in plant defense system against pathogen infection (Eulgem and Somssich 2007). WRKY TFs may be involved in small RNA-mediated pathways, which play an important role in plant development and immune responses (Pandey and Somssich 2009). Some WRKY TFs were described as regulators of metabolic processes, such as alkaloid pathways (Kato et al. 2007; Suttipanta et al. 2011; Schluttenhofer and Yuan 2015).

WRKY TFs act through direct interaction of the WRKY domain to the W box [(C/T) TGAC(C/T)] cis-regulatory elements in target promoter sequences (revised in Eulgem

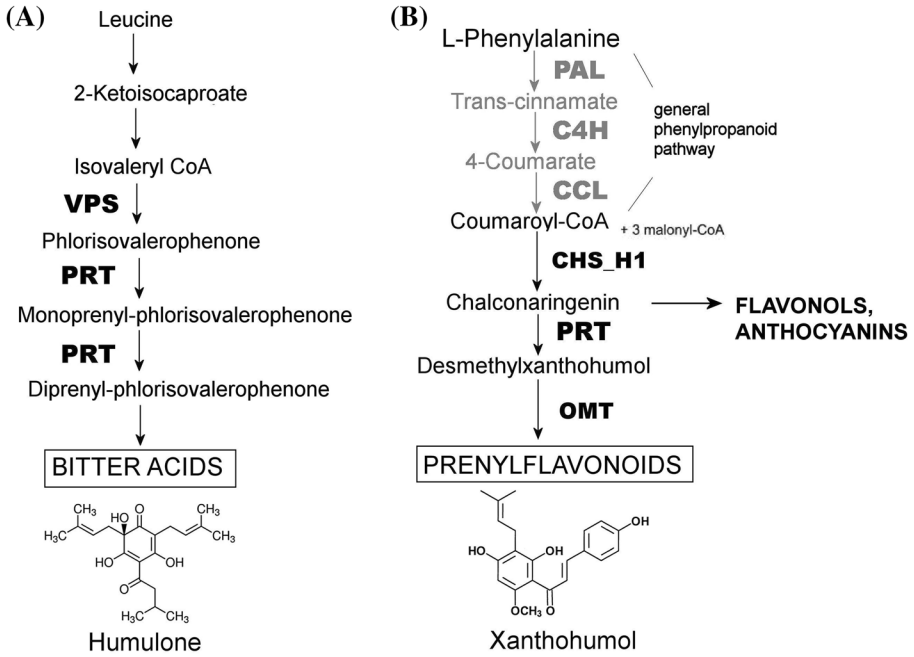

Fig. 1 Scheme of the bitter acids (**a**) and xanthohumol (**b**) biosynthetic pathways. The bitter acid pathway in hop lupulin glands. (**a**) The biosynthesis of humulone, the main α-acid found in hop trichomes, is shown. **b** Xanthohumol is the major prenylflavonoid of hop. *Bold* are indicated enzymes catalyzing different steps: *PAL* phenylalanine ammonia lyase, *C4H* cinnamate 4-hydroxylase, *CCL* 4-coumarate CoA ligase, *CHS*_H1 chalcone synthase, *OMT* o-methyltransferase, *PRT* prenyltransferase

and Somssich 2007; Ciolkowski et al. 2008). However, the binding selectivity of WRKY TFs to DNA is dependent on the flanking sequence outside the W box motif (Agarwal et al. 2011; Yamasaki et al. 2013). The characteristic structural feature of WRKY proteins is the highly conserved WRKY domain which contains WRKYGQK sequence at the N terminus followed by a Cx4-5 Cx22-23 HxH or Cx7Cx23HxC zinc-finger motif (Rushton et al. 2010). WRKY TFs can be auto-regulated (Turck et al. 2004; Eulgem and Somssich 2007) or cross-regulated (Hu et al. 2012; Xu et al. 2006). Important feature of some of these factors for their regulatory role is an ability to form dimers or to interact with some other proteins (Cheng et al. 2012; Chi et al. 2013).

More than 70 representatives of WRKYs were found in the plant model *Arabidopsis thaliana* (Eulgem et al. 2000). They have been arranged into three groups, depending on the number and type of their WRKY domains and features of the zinc-finger-like motif. WRKY proteins with two WRKY domains belong to group I, while those with one WRKY domain belong to groups II and III. These three WRKY groups can be further clustered into subgroups. For instance, group II has been clustered into five subgroups (a–e) based on the additional amino acid motifs outside the WRKY domain and their phylogenetic distance (Zhang and Wang 2005; Agarwal et al. 2011; Yamasaki et al. 2013).

In the present work we analyzed four hop WRKY TFs cloned from lupulin gland-specific cDNA library. We showed that lupulin gland-specific transcription factor *Hl*WRKY1, close homologue of *At*WRKY75, has ability to activate promoters of genes involved in the final steps of xanthohumol and bitter acids biosynthesis. This activation is significantly higher in combinatorial action with WD40 repeat protein1 (*Hl*WDR1) cloned previously from lupulin glands. Our results demonstrate for the first time that *Hl*WRKY1 is regulated by PTGS. Moreover, its expression can be activated by a protein kinase and modulated by autoactivation.

Materials and methods

Plant material and cultivation conditions

Czech semi-early red-bine hop (*Humulus lupulus* L.) Osvald's clone 72 was used for plant transformation and biolistic experiments. *Nicotiana benthamiana* plants were used for transient expression assays using agrobacterial infiltrations. Plants were maintained in greenhouses at a temperature of 25 ± 3 °C and cultivated under natural light from March to July with supplementary illumination [170 µmol m^{-2} s^{-1} PAR] to maintain a 16 h-day period.

Cloning of TFs WRKY cDNAs, isolation and cloning of promoter sequences, preparation of plant expression vectors, and hop transformation

Hop WRKY genes were amplified from a lupulin-specific library constructed previously (Matoušek et al. 2012). Amplification and cloning primers as well as GenBank numbers of the newly cloned TFs are listed in Supplementary Table S1 and Supplementary Table S2. For the cloning, cDNA fragments of hop TFs were re-amplified to generate appropriate ends as shown in Supplementary Table S1, treated with corresponding restriction enzymes and ligated into the unique sites of the derivative of vector pRT-100 (Töpfer et al. 1987) and designated pLV-68. From this intermediate vector, 35S:*Hl* TFs expression cassettes were excised using *Asc*I and *Pac*I restriction enzymes, further ligated into the plant expression vector pLV07 (Vrba and Matoušek 2005). The resulting plant vectors were introduced into *Agrobacterium tumefaciens* LBA4404 strain by the freeze–thaw method (Holsters et al. 1978) for transient expression experiments.

To transform hop, a dual expression cassettes vector WWpPCV91 bearing *Hl*WRKY1 and *Hl*WDR1 cDNAs (Supplementary Fig. S7A) was constructed. The construction was performed based on pPCV91 plasmid (Strizhov et al. 1996) obtained from Csaba Koncz (Max Plant Institute for Plant Breeding Research Cologne, Germany). *Hl*WRKY1 cDNA was modified using primers with *Bam*HI restriction sites on its ends and cloned into cassette containing tetrameric enhancer of 35S promoter. Correspondingly, *Sal*I sites were added to *Hl*WDR1 cDNA, *Sal*I-treated fragment was then cloned in P*mas*-driving expression cassette. Final constructs were subsequently selected after setting the cDNA orientations using appropriate restriction enzymes. WWpPCV91 (Supplementary Fig. S7A) was transferred into *A. tumefaciens* GV3101 strain by electroporation.

For genetic transformation of hop cv. Osvald 72 in vitro internodal stem explants were used as recommended by Horlemann et al. (2003). *A tumefaciens* with the plasmid WWpPCV91 (Supplementary Fig. S7A) was cultured overnight in LK medium (Langley and Kado 1972) supplemented with 100 mg/l carbenicillin and 50 mg/l kanamycin and then diluted with fresh LK medium with 200 µM acetosyringone (AS) up to OD_{600} 0.5–0.6 and cultured again at 28 °C with 200 RPM for additional 4–5 h. Subsequently, the suspension was centrifuged at 6000*g* for 10 min at 4 °C and bacterial pellet was resuspended in MTA medium (10 mM $MgSO_4$, 0.1 % Tween 20, 200 µM AS) into final OD_{600} 1.0. 5–10 mm in length hop nodal explants were co-cultivated in 20 ml of the suspension for 20 min. Then the explants were wiped on sterile filter paper and transferred onto shoot regeneration R medium (Murashige and Skoog/MS/basal medium supplemented with 20 g/l glucose, 1 mg/l

zeatin, 0.25 mg/l IAA, 6 g/l plant agar) supplemented with 200 µM AS and maintained at 22–24 °C for 3 days. Finally, the explants were transferred onto R medium fortified with 250 mg/l Timentin and 1.5 mg/l hygromycin B and cultured at 22–24 °C with 16 h photoperiod for several (4–6) weeks, i.e. until shoots were formed. Regenerated shoots were transferred onto rooting S medium (MS basal medium supplemented with 20 g/l glucose, 250 mg/l Timentin, 1.5 mg/l hygromycin B, 6 g/l plant agar) fortified with IAA and IBA, each of the concentration of 1 mg/l according to Roy et al. (2001).

For cloning of *HlPrt*1 promoter, an inverse PCR (iPCR) was performed. Genomic DNA was isolated from in vitro hop leaves as described by Tai and Tanksley (1990). The iPCR was carried out as reported by Thomas et al. (1994). *Bgl*II and *Bst*YI endonucleases (New England Biolabs, UK) were used for restriction digestion of 2 µg gDNA followed by T4 DNA ligase mediated intramolecular ligation. The primary iPCR with PRT1-F1 and PRT-R1 primers and nested iPCR with PRT1-F2 and PRT-R2 primers (see Supplementary Table S1 for primer sequences) were performed and final PCR products were purified by *NucleoSpin*® Gel and PCR Clean-up kit (Macherey–Nagel, Germany). Both strands of amplicons were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (PE Biosystems, UK) by an ABI PRISM 310 sequencer (PE Applied Biosystems, USA) yielding 716 bp sequence upstream of start codon of *HlPrt*1. Finally, the authenticity of the sequence was confirmed by the comparison with rough sequencing data (Natsume et al. 2015). For extraction of promoters of genes P*Prt2*, P*Omt*1, extension of P*Chs*_H1 to 1000 bp from previously cloned 500 bp variant (Matoušek et al. 2006) as well as for promoters of *HlMyb*3 (Matoušek et al. 2007) and *HlWRKY*1 we used raw sequencing data of *H. lupulus* published by Natsume et al. (2015). Promoter sequences were amplified and cloned using primers listed in Supplementary Table S1. The promoter fragments were fused via *Eco*R1 and *Xba*I with *gfp* and *gus* genes arranged as a tandem in the vector pBFG-0 (Chytilová et al. 1999). The resulting promoter-bearing vectors were maintained in *A. tumefaciens* LBA 4404. Promoter construct of valerophenone synthase P*HlVps* was prepared earlier (Matoušek et al. 2010).

Combinatorial transient expression systems in *N. benthamiana* and in hop leaves and GUS assays

For analyses of promoter activation in *N. benthamiana* leaves, co-infiltrations with *A. tumefaciens* LBA4404 containing various plant expression vectors (see Supplementary Table S2) were performed. Besides *Hl*WRKY TFs and *Hl*CDKF1 kinase cloned in this work, plant vectors with *HlMyb1* (Matoušek et al. 2005), *Hls-Myb3* (Matoušek et al. 2007), *HlMyb2, HlWDR1* and *HlbHLH2* (Matoušek et al. 2012) were used for co-infiltration. Vectors containing silencing suppressors p19 and HC-Pro (Voinnet 2005) were obtained from O. Voinnet (Institut de Biologie Moléculaire des Plantes, Zürich). Infiltration into *N. benthamiana* leaves was performed as described earlier (Matoušek et al. 2010). The activity of ß-glucuronidase (GUS) was assayed in homogenized tissues of treated leaf sectors 3–4 days after the infiltration by a fluorometric assay (Jefferson et al. 1987) modified by Matoušek et al. (2010). The amount of the released fluorescent dye 4-methylumbelliferone (MU) was measured using the VersaFluor™ fluorometer (Bio-Rad) with excitation at 365 nm and emission at 455 nm. GUS activity was expressed in pmol MU mg^{-1} fresh tissue min^{-1}. At least three independent repeated experiments were performed in each experimental variant. The data were statistically analyzed using Microsoft Excel 2010 software.

To analyze the *Omt*1, *Prt*1 and *Prt*2 promoter activation in hop leaves, transient expression assay using biolistic delivery of expression vectors was carried out using particle delivery system PDS 1000/He (Bio-Rad, USA) and gold 1 µm microparticles coated with plasmid DNAs according to the PDS apparatus manufacturer's manual. The young leaf segment of in vivo (greenhouse) hop plant sized about 2 × 2 cm was placed bottom side up into 9 cm Petri dish with MS medium (Murashige and Skoog 1962) supplemented with 2% glucose and helium pressure 1100 psi (7584 MPa), target distance 9 cm, chamber pressure reduction by 28 Hg (0.095 MPa) and 0.5 mg golden microcarriers coated with 0.8 µg DNA per each shot were applied during bombardment. Thereafter, the segment was incubated under dimmed light 2.7 µmol m^{-2} s^{-1} (200 lux) at 24 °C for 16 h followed with 6 h fluorescent tube light exposure of 75 µmol m^{-2} s^{-1} (5500 lux). Following GUS histochemical assay was performed according to Jefferson (1987) with two 5 min vacuum infiltration of X-GlcA (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) buffer solution into leaf tissues and overnight dark incubation at 37 °C.

RNA isolation and real-time PCR analyses

Total RNA was isolated from 100 mg of plant leaf tissue using the Concert™ Plant RNA Purification Reagent (Invitrogen) following RNA purification and DNA cleavage on columns (RNeasy Plant Mini kit, Qiagen). Total mRNA was further treated with DNase (DNA-free™ kit, Invitrogen, USA). The absence of contaminating DNA in samples was verified by PCR. The second DNase treatment of the samples was performed only if it was necessary. The primer combinations for the qRT-PCR are listed in Supplementary Table S1. The method described previously (Matoušek et al. 2012) was followed with few modifications. Briefly, 1 µg of total RNA was reverse transcribed using oligo dT18 primer and Superscript™ III Reverse Transcriptase (Invitrogen) at

50 °C for 60 min. To analyze mRNA levels of *Hl*WRKY1, 2, 3, 5, *HlOmt1, HlVps* and *HlPRT1*L, cDNA was diluted five times. To amplify mRNA of reference genes (GAPDH and actin), GUS, 35S:*Hl*WRKY1, 35S:*Hl*WDR1 and *Hl*chs_H1, cDNA was diluted 25 times. The total volume of 13 µl PCR reaction mixture contained 3 µl diluted cDNA, 6 µl iQ™ SYBR® Green Supermix (Bio-Rad) and 0.9 µl of each 10 µM primers. All qRT-PCR reactions were carried out on a Bio-Rad iQ™5 cycler for 40 cycles (94 °C for 20 s, 59–60 °C for 30 s, 72 °C for 30 s) following an initial denaturation step (94 °C for 1 min). The product specificity size was confirmed by melting curve analysis. Data were analyzed and quantified with the Bio-Rad iQ™5 Optical System version 2.0 software. The abundance of a reference transcript, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Nagel et al. 2008; Wang et al. 2008; Maloukh et al. 2009) was estimated in parallel in each hop sample. mRNA in *N. benthamiana* samples was normalized to β-actin housekeeping gene as reported previously (Matoušek et al. 2010). The relative values were standardized to housekeeping genes with the "Delta-delta method" and normalized to the sample with highest expression (calibrator, set to 100 %) according to Pfaffl (2001). The control sample mRNA concentration was set to 100 % to analyze *HlWRKY*1, *HlWDR*1, *Hl*chs_H1, *HlOmt*1, *HlVps* and *HlPRT1*L mRNA level in hop transgenic plants. The data points display the means and ±SD of two replicates of each qRT-PCR reaction.

Analyses of HlWRKY1 5′ P degradome from infiltrated *N. benthamiana* leaves and hop roots and "microwrky" identification

Total RNA samples from *N. benthamiana* infiltrated leaf sectors or of hop roots were isolated as described above. The mRNA fraction (mRNA polyA) was purified using Dynabeads protocol (Dynal). The procedure for specific 5′ RACE profiles of HlWRKY1 is described in detail in Supplementary Fig. S9. Degradome analysis involved PCR amplification of samples immobilized on magnetic beads by biotin-streptavidin (Dynabeads M-280 Streptavidin, Invitrogen) according to manufacturer's protocol. PARE RNA and DNA sequences of adaptors (see Supplementary Table S1) were used as described previously (German et al. 2009). The procedure (Supplementary Fig. S9) includes ligation of RNA adaptors, a reverse transcription step using SuperScript® III Reverse Transcriptase (Invitrogen), PCR amplifications, electrophoretic analyses on native 6 % polyacrylamide gels and silver-staining of DNA fragments (Schumacher et al. 1986). In some experiments, electroblotting and hybridization with [α^{32}P] dCTP-labeled cDNA probes or sequencing were performed. Adaptor ligation was performed using miRCat™ RNA Cloning Kit (IDT) according to manufacturer's instructions. *Hl*WRKY1 degradome analysis included reverse transcriptions with 3′-specific primer WRKY ST. "Bi-nested" PCR reactions were performed using primer 5′ ada in combination with primers WRKY NST and WRKY N2ST (Supplementary Table S1).

To check for possible micro-RNAs corresponding to mapped 5′ P degradome, 45 nt target signatures covering mapped positions (24 nt upstream and 21 nt downstream 5′ P ends) as 't-signatures' were aligned with the identified miRNAs and all mature known miRNAs stored in miRBase17.0 using the CleaveLand pipeline.

The detailed analysis of hop small RNA sequencing reads is described in Supplementary Material and Methods section.

Stem-loop RT-PCR assay was used to validate predicted miRNA. The cDNAs were synthesized from total RNAs using miRNA specific stem–loop RT primer (see Supplementary Table S1) according to criteria described previously (Kramer 2011). qRT-PCR was performed with a Bio-Rad iQ™5 cycler. The reactions were incubated in a 96-well plate at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. After the completion of the real-time reactions, the threshold was manually set and the threshold cycle (CT) was recorded. All reactions were conducted in triplicate.

Bioinformatic analyses and other methods

WRKY sequence comparisons were carried out with DNASIS v. 2.6 (Hitachi). Degradome mapping was performed using Visual Cloning 2000 (Redasoft). Alignment of the amino acid sequences of the WRKY domains was performed using CLUSTALW with default settings. Protein domain sequence analyses were performed using InterProScan module of Geneious v. 5.5.6. Subsequently, MEGA 6.0 software (Tamura et al. 2013) was employed to construct an unrooted phylogenetic tree based on alignments using the Neighbor-Joining (NJ) method with the following parameters: JTT model, pairwise gap deletion and 1000 bootstraps. Furthermore, Maximum likelihood, Minimal Evolution and PhyML methods were also applied in the tree construction to validate the results from the NJ method.

For electrophoretic mobility shift assay (EMSA) analysis we amplified 210 bp fragment of *HlOmt*1 promoter containing W-box using primers 5′ POMT_Eco564 and 3OMT_EMSA (Supplementary Table S1). EMSA was performed according to modified protocol described by Maliga et al. (1995). Activating combinatorial protein complex was assayed as crude protein extract (75 µg) from infiltrated *N. benthamiana* leaves after 3 days of transient expression of *Hl*WRKY1 co-infiltrated with *Hl*WDR1 and p19. Protein samples were extracted in buffer containing 83 mM Tris–HCl pH 7.5, 66 mM KCl, 100 mM

NaCl, 0.8 mM $MgCl_2$, 2 mM β-mercaptoethanol and 1 mM PMSF. Radiolabeled 200 bp fragment of P*HlOmt*1 was used as a probe. Protein extracts were mixed with 20,000 cpm of probe in the presence of 1 µg dsDNA as competitor and 10x binding buffer (100 mM Tris–HCl pH 7.5, 400 mM NaCl, 40 % glycerol, 10 mM EDTA, 1 mM β-mercaptoethanol) and incubated on ice for 30 min. Samples were run in 4 % acrylamide gel. Dried gel was exposed over-night and signal was detected by a Typhoon 9200 Imager (GE Healthcare Life Sciences).

Results

HlWRKYs cloning, characterization and tissue specific expression

In our previous work we described complex regulation of hop *chs*_H1 by lupulin gland-specific transcription factors from Myb, bHLH and WD40 families (Matoušek et al. 2012). Later we identified another abundant lupulin gland-specific transcription factor *Hl*WRKY1 that activates significantly *chs*_H1 promoter. This finding stimulated our work to isolate other WRKYs from the hop TrichOME database to study their possible impact on xanthohumol and bitter acids metabolome pathways (Fig. 1). We identified four WRKY genes from the database and subsequently cloned them from lupulin-gland expression library (clone Osvald's 72). Their comparison based on amino acid levels showed clustering in different groups (see Supplementary Fig. S1). While *Hl*WRKY1 and *Hl*WRKY5 were positioned in a wide cluster including previously described WRKY75 from *A. thaliana* (Devaiah et al. 2007; Encinas-Villarejo et al. 2009; Rishmawi et al. 2014) belonging to group IIc according to the classification of WRKY factors (Rushton et al. 2010), other two factors are clustered independently in group IIa (*Hl*WRKY2) or IIe (*Hl*WRKY3). The comparative analysis of amino acids (aa) sequences of *Hl*WRKY1 and *Hl*WRKY5 revealed 54 % degree of homology between them. This homology was found to be 71 % if only conserved region covering WRKY DNA binding domain is considered (Supplementary Fig. S2A,B). In this conserved part of aa sequence *Hl*WRKY1 and *Hl*WRKY5 share 85 and 63 % of identity respectively with *At*WRKY75, while the similarity was found to be low among all these TFs in N-terminal regions. Putative protein predicted from *Hl*WRKY1 cDNA is a basic protein having pI 9.75 and molecular mass of 16.9 kDa, while predicted *Hl*WRKY5 protein has molecular mass of 18.2 kDa and isoleletric point of 9.62. The molecular mass and pI values of both, *Hl*WRKY1 and *Hl*WRKY5 are comparable to *At*WRKY75 (16.8 kDa and pI 9.51).

To examine the tissue-specific expression of the cloned hop WRKY factors by qRT-PCR we isolated RNA extracts from seven different hop tissues. The results are summarized and depicted in Fig. 2. *Hl*WRKY1 showed the

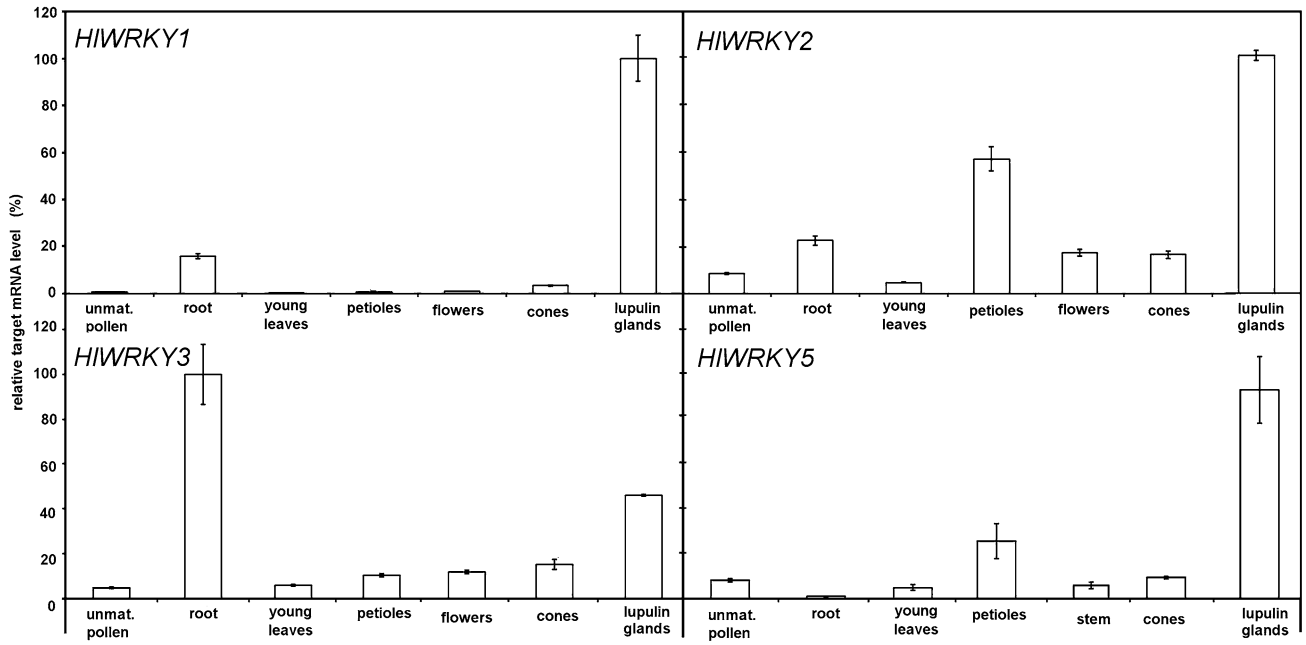

Fig. 2 Expression pattern of the newly cloned WRKY genes from *H. lupulus* Osvald's clone 72 in different tissues determined by quantitative real-time PCR. Expression levels are normalized relative to those of GAPDH, which was used as an internal control. Data are means ± SD of three replicates

highest specificity for lupulin glands, whereas in other tissues, except for hop roots (18 % of the level in lupulin glands), the expression was negligible. Although the highest level of the *Hl*WRKY5 transcript was reached also in lupulin, the expression profile in other tissues was different in comparison to *Hl*WRKY1. Apparent relative expression of *Hl*WRKY5 was exceeded also in petioles and immature pollen or cones. Expression of *Hl*WRKY2 showed the lowest tissue specificity among cloned WRKYs. The maximum expression of *Hl*WRKY3 was observed in roots. Different tissue specificity of analyzed WRKY TFs could reflect their different function.

Analyses of combinatorial promoter activation by *Hl*WRKYs discriminates *Hl*WRKY1 as a main activator in transient expression systems and in hop transformants

In order to assay the capability of newly cloned WRKY TFs to activate genes connected to xanthohumol biosynthetic pathway, we performed comparative analyses in the transient expression system using promoter of *Omt*1 gene (Supplementary Fig. S3A), which drives the final step of xanthohumol biosynthesis.

Promoter region of the *HlOmt*1 gene containing 564 bp of sequence from translation start site was isolated and sequenced in our previous work (Matoušek et al. 2010). One consensus WRKY binding box (W-box) [TTGACT] was identified on the plus strand at the position−394 bp upstream of the start codon ATG. Transient expression assays of GUS reporter gene showed that among analyzed WRKY factors, only *Hl*WRKY1 and its homologue *At*WRKY75 exhibited significant *Omt*1 promoter activation (Fig. 3a).

Besides the dominant activation by *Hl*WRKY1, this lupulin-specific promoter was found to be activated significantly by two Myb TFs, *Hl*Myb1 and s-*Hl*Myb3 (Fig. 3b). These two Myb TFs were characterized in our previous work (Matoušek et al. 2005, 2007). In all activation events a significant increase was observed in the presence of *Hl*WDR1 (after co-infiltration of *Hl*WDR1/*Hl*WRKY1 or *Hl*WDR1/*Hl*Myb1 or *Hl*WDR1/s-*Hl*Myb3) as shown in Fig. 3b. In the case of *Hl*WRKY1, the promoter activation effect was practically doubled after co-infiltration with *Hl*WDR1, while negligible increase was observed when *Hl*WDR1 was applied alone in the control experiments. On the contrary, the *HlOmt*1 promoter was not activated significantly by *Hl*MYB2, as well as by specific ternary complexes (s-*Hl*Myb3/*Hl*bHLH2/*Hl*WDR1 or *Hl*Myb2/*Hl*bHLH2/*Hl*WDR1) known as major activators of promoter of *chs*_H1 gene (Matoušek et al. 2012). This suggests that *Hl*WRKY1 has specific effect on P*HlOmt*1 activation especially in binary combination with *Hl*WDR1 (W/W)

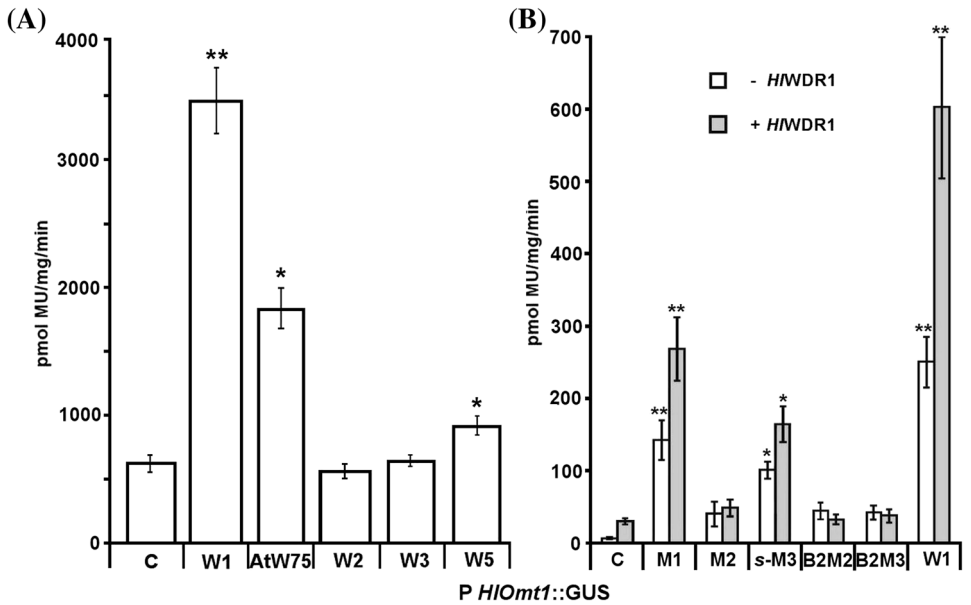

Fig. 3 Activity of the β-glucuronidase (GUS) driven under the control of *HlOmt*1 promoter (564 bp) co-infiltrated into *Nicotiana benthamiana* leaves together with different constitutively expressed hop TFs 72–76 h after co-infiltration. **a** Comparison of activation of *HlOmt*1 promoter by different WRKY TFs, co-infiltrated with *Hl*WDR1 and p19 silencing suppressor (under the control of 35S promoter). C-control sample containing no WRKY TF, W1—*Hl*WRKY1, W2—*Hl*WRKY2, W3 -*Hl*WRKY3, W5—*Hl*WRKY5, AtW75—*At*WRKY75, included as nearest homologue of *Hl*WRKY1. **b** Effect of constitutively expressed HlWDR1 on the activation of *HlOmt*1 promoter by different TFs. C-control-*A. tumefaciens* LBA 4404 strain; M1-*Hl*Myb1; M2-*Hl*Myb2; s-M3-s-*Hl*Myb3; B2M2-*Hl*bHLH2 coinfiltrated with *Hl*Myb2; B2M3-*Hl*bHLH2 co-infiltrated with s-*Hl*Myb3, W1, *Hl*WRKY1. The data were obtained from three independent experiment; *bars* show ± SD. *Statistically significant differences ($P < 0.05$); **significant at $P < 0.01$

that was considered as the basis for further combinatorial assays of *Hl*WRKY1 in our experiments.

*Hl*WRKY1 activation of P*HlOmt*1 was further increased by various PTGS silencing suppressors that are able to enhance levels of transient expression (Voinnet et al. 2005). Especially p19 and HC-Pro led to significant increase of the activation of P*HlOmt*1 by W/W by the factor of about 6 and 7, respectively (Supplementary Fig. S4). Combinations of *Hl*WRKY1/*Hl*WDR1 and p19 were resulted in high promoter activation levels (Supplementary Fig. S4B). In assays of both, GUS enzymatic activity and GUS mRNA levels, combinations of *Hl*WRKY1/*Hl*WDR1 and p19 resulted in strong activation of the *HlOmt*1 promoter (Fig. 4). The protein extracts from *N. benthamiana* leaf sectors infiltrated by these factor combinations caused shift of W-box containing P*HlOmt*1 promoter fragments in electrophoretic mobility shift assay (EMSA) (Supplementary Fig. S5), suggesting the existence of physical interaction between induced plant proteins and radioactively labeled promoter fragments.

To assay possible impact of *Hl*WRKY1 on regulation of genes involved in the final steps of the xanthohumol and bitter acids biosynthesis pathways in lupulin, we newly isolated and cloned upstream sequences and promoter regions for hop prenyltransferases 1 and 1L (designated P*Prt*1 for the simplicity) and 2 (P*Prt*2) and extended promoter of *Chs*_H1 (Matoušek et al. 2006) into the reference vector pBGF-0 (Chytilová et al. 1999) (Supplementary Fig. S3b–e) to perform GUS quantification assay (Fig. 5). The earlier prepared promoter construct of velerophenonsynthase (P*Vps*) was used in this study (Matoušek et al. 2010).

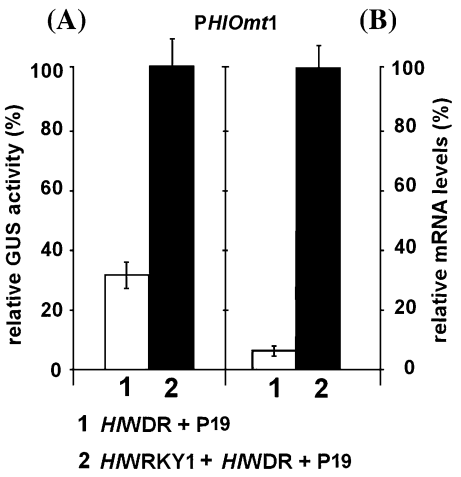

Fig. 4 Comparison of GUS enzymatic activity and *uid*A (*gus*) mRNA levels in the *N. benthamiana* leaf sectors coinfiltrated with P*Hl*Omt1:GUS fusion construct together with constitutively expressed *Hl*WDR1 and p19 silencing suppressor and/or *Hl*WRKY1. Two independent experiments were evaluated. mRNA data were normalized using β-actin gene (GenBank: JQ256516) as shown in "Materials and Methods". *Bars* represent ± SD. *Statistically significant differences ($P<0.05$); **significant at $P<0.01$

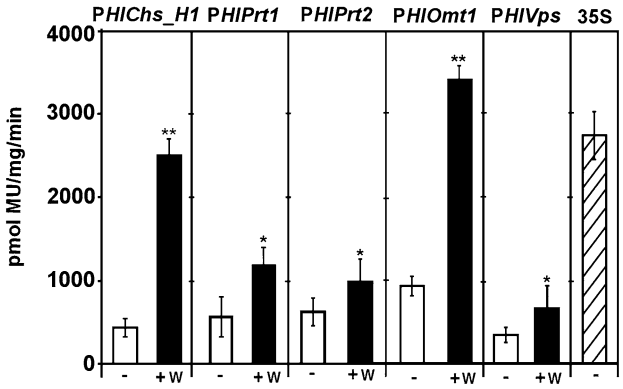

Fig. 5 Activity of GUS driven by five different promoters of hop genes involved in lupulin metabolism after co-infiltration into *Nicotiana benthamiana* leaves together with 35S:*Hl*WDR1 and p19 silencing suppressor in the absence/presence of the *Hl*WRKY1 TF. The activity was measured 68–76 h after infiltration. Single infiltration of 35S:GUS served as a control. At least three independent experiments were performed. The *numbers* indicate the pmol level of MU per 1 mg of fresh tissue per minute ± SD. *Statistically significant differences ($P<0.05$); **significant at $p<0.01$

The strength of hop promoters and co-activating transcription factors was compared with the GUS expression driven by 35S CaMV promoter (pBGF-35S). The highest activation, quite comparable with the signal from the pBGF-35S variant, was achieved for *HlOmt*1 and *HlChs*_H1 promoters. Apparent activation was also detected for promoters of *HlPrt*1, 2 and *HlVps*. These results suggest that *Hl*WRKY1 has ability to activate promoter elements of all crucial structural genes important for the final steps of xanthohumol and bitter acid biosynthetic pathways in the heterologous transient expression system. In silico analysis of *cis*-acting regulatory elements in 5′ upstream regions of the cloned promoters revealed the presence of single W-boxes (P*Omt1*, P*Vps*) or W-box clusters (P*Chs*_H1, P*Prt*1, P*Prt*2) (Supplementary Fig. S3a–g).

To confirm the ability of *Hl*WRKY1 to activate promoters of structural genes in hop tissue, we selected prenyltransferase and O-methyltransferase-1 promoter constructs for biolistic delivery as the equimolar mixtures of purified plasmids. In these samples *Hl*WRKY1, *Hl*WDR1 and p19 expression vectors were combined with the corresponding promoter vectors and shot to leaf tissues of hop. While in the negative controls (without *Hl*WRKY1) no GUS signals developed, P*Prt*1 exhibited on average 4.5, P*Prt*2 6.3 and P*Omt*1 16 blue spots per leaf (4–6 replications), positive control (pBGF-35 S) reached 96 spots per leaf (Supplementary Fig. S6). Moreover, in order to prove essential role of co-expression of *HlWRKY1* and *HlWDR1* genes in hop transient expression system, we shot hop leaf tissue without silencer p19, separately either with vector combinations *Hl*WRKY1/P*Omt*1; *Hl*WDR1/P*Omt*1 or *Hl*WRKY1/*Hl*WDR1/P*Omt*1 (Supplementary Table S3). As

expected, no signal was observed for *Hl*WDR1 alone, low frequency of blue spots was observed for *Hl*WRKY1, while the frequency was higher for *Hl*WRKY1/*Hl*WDR1 combination (Supplementary Table S3).

Finally, a plant expression vector bearing dual expression cassettes was constructed based on pCV91 plasmid (Strizhov et al. 1999) (Supplementary Fig. S7A) to transform hop explants by a combination of *Hl*WRKY1/*Hl*WDR1 cDNAs. From these experiments four hygromycin resistant transgenic lines (no. 9, 10, 11, 20) were obtained (Supplementary Fig. S7B). Expression level of *Hl*WRKY1 and *Hl*WDR1 was determined by qRT-PCR in young leaves. Although the expression of *Hl*WRKY1 was extremely variable (probably due to position effect of the T-DNA insertion), it reached significantly high levels from 4318 to 25,704 % compared to controls (100 %) (Fig. 6a). The fold change level of mRNA of *Hl*WDR1 in three lines (9, 10, 11) was found to be in the range from 1.3 to 1.9 compared to untransformed control plant (Fig. 6b). In all of these transgenic lines, significantly increased level of *Omt1, Chs_H1, Vps* and *Prt1L* transcripts was observed, while as expected lowest level of corresponding transcripts were observed in control leaves (Fig. 6c). These results confirmed activation of genes involved in the final steps of prenylflavonoid or bitter acid biosynthetic pathways by W/W transgenes suggesting the important role of *Hl*WRKY1 in their regulation.

Analysis of some regulatory features of *Hl*WRKY1: promoter autoactivation, involvement of PTGS in the regulation of its expression and modulation of *Hl*WRKY1 activity with kinase

High tissue-specificity of *Hl*WRKY1 expression for lupulin glands, as well as high expression variability of *Hl*WRKY1 in hop W/W transformants including its high stimulation by PTGS suppressors led us to study some regulatory features of this TF along with characterization of regulatory elements of *Hl*WRKY1 gene (Supplementary Fig. S3b). One intron was identified within the genomic sequence of *HlWRKY1* gene (5383 bp). This genomic organization corresponds to IIc group of *WRKY* genes including *AtWRKY75* gene from *A. thaliana* (classified according to Eulgem et al. 2000). Thanks to the hop draft genome we were able to retrieve 2438 bp upstream of the ATG start codon (Supplementary Fig. S3B) and 1588 bp promoter fragment was cloned for the analysis of possible regulatory effect of cloned TFs on *Hl*WRKY1 expression in heterologous transient expression system (Supplementary Fig. S8). *In silico* analysis of cloned *Hl*WRKY1 promoter fragment revealed three W-binding boxes and several potential Myb-binding sites (Supplementary Fig. S3f). The strongest activation was achieved by *Hl*WRKY1 suggesting its autoregulation. Significant activation of *HlWRKY1* promoter was also

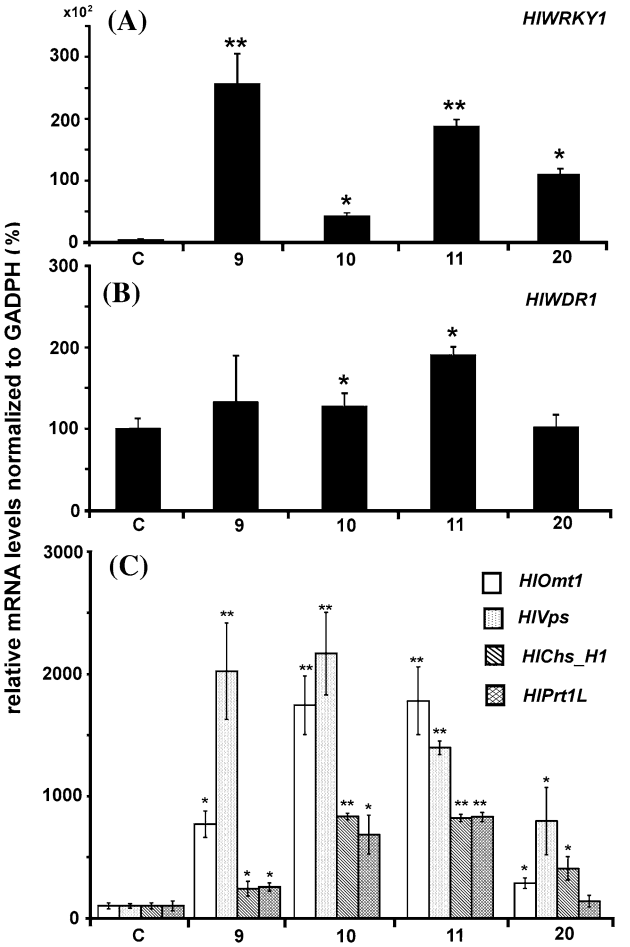

Fig. 6 Relative levels of TF transgenes *HlWRKY1* (**a**), *HlWDR1* (**b**) and their putative targets *HlOmt1, HlVps, HlChs_H1* and *HlPRT1L* (**c**) mRNAs in the leaves of three independent lines of hop transformed with *Hl*WRKY1 and *Hl*WDR1 genes using vector WWpPCV91. RNA was extracted and cleaved subsequently with DNAse RNAse-free as described in "Materials and Methods" to remove any traces of genomic DNA and transgene cDNAs. RT-qPCR analyses were normalized using GAPDH as a house-keeping gene. *Statistically significant differences ($P<0.05$); **significant at $p<0.01$

reached by its homologue from *A. thaliana At*WRKY75 and by *Hl*WRKY5, while no cross-activation was found for *Hl*WRKY2 and *Hl*WRKY3 (Fig. 7).

According to the additional comparative results (Supplementary Fig. S8), P*HlWRKY1* was stimulated by *Hl*Myb1, but not by *Hl*Myb3 or other cloned hop TFs. On the contrary, we found that *Hl*WRKY1 was able to activate promoter of *Hl*Myb3, which is supposed to be a crucial TF for hop prenylflavonoid pathway, as it has ability to activate P*Chs*_H1 either without complexing or in binary *Hl*bHLH2/*Hl*s-Myb3 or ternary *Hl*bHLH2/*Hl*s-Myb3/*Hl*WDR1 combinations (Matoušek et al. 2012). In P*HlMyb3* we identified three W-boxes (Supplementary Fig. S3g).

A significant increase of promoter activation by *Hl*WRKY1 with PTGS suppressors suggests that this

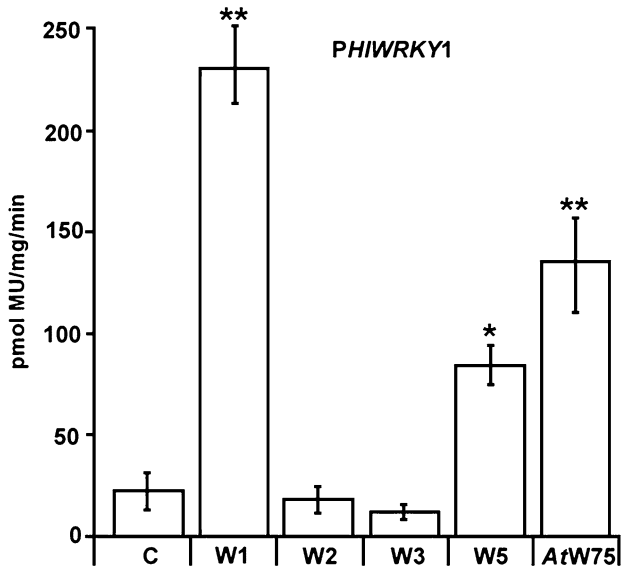

Fig. 7 Combinatorial activation of *HlWRKY1* promoter in transient expression system. Activity of the GUS enzyme was measured in extracts from *N. benthamiana* leaves 72–76 h after co-infiltration with the *Agrobacterium* vectors containing P*HlWRKY1*:GUS fusion construct and *Hl*WRKY vectors together with *Hl*WDR1 and P19 silencing suppressor. C-control sample containing no WRKY TF, W1—*Hl*WRKY1, W2—*Hl*WRKY2, W3—*Hl*WRKY3, W5—*Hl*WRKY5, AtW75—*At*WRKY75, included as nearest homologue of *Hl*WRKY1 from *A. thaliana*. The data were obtained from three independent experiments, bars represent ± SD. *Statistically significant differences ($P < 0.05$); **significant at $p < 0.01$

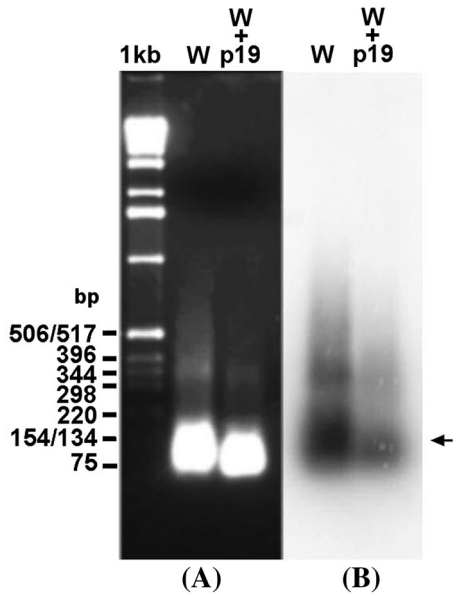

Fig. 8 The Southern blot analysis of "microwrky" amplified using stem-loop RT-PCR from *N. benthamiana* leaf sectors infiltrated either with *Hl*WRKY1-bearing *A. tumefaciens* (W) or co-infiltrated with *Hl*WRKY1 and p19 vectors (W + p19). In panel A PCR products were stained with ethidium bromide, in panel B PCR products were blotted on nylon membrane and screened using ^{32}P[dCTP]-labelled *Hl*WRKY1 cDNA probe. Low size bands of 1 kb DNA Ladder from Invitrogen are indicated by the numbers on the left side of the gel

TF may be regulated by PTGS i.e. its mRNA may be degraded by some of post-transcriptional silencing pathway. In order to study this possibility we performed 5′ degradome analysis of *Hl*WRKY1 using bi-nested PCR reactions (Matoušek et al. 2015) (Supplementary Fig. S9). *Hl*WRKY mRNA samples were purified either from infiltrated *N. benthamiana* leaf sectors or from hop roots, where *Hl*WRKY1 was moderately expressed. Purification of mRNA, adaptor ligation to free 5′ P ends followed by reverse transcription and PCR amplification yielded fragments that were analyzed in polyacrylamide gel and mapped according to calculated 5′ P end positions on corresponding cDNA sequences (Supplementary Fig. S9). While from infiltrated leaf tissue several minor and three dominant 5′ P positions were mapped at positions 322, 349 and 364, within the spectrum of fragments specific for hop roots, only one dominant 5′ P position 322 was observed (Supplementary Fig. S10). Predicted complementary sequence flanking that position was then searched in small RNA NGS data obtained for hop (Natsume et al. 2015). Indeed, we found fragment corresponding to small siRNA complementary to analyzed sequence predicted from the 5′ degradome of *Hl*WRKY1. This small complementary RNA was designated as "microwrky" for the simplicity.

Subsequently, microwrky was amplified also from RNA isolated from *N. benthamiana* infiltrated leaf sectors by stem-loop RT-PCR as strong band hybridizing to *Hl*WRKY1 cDNA (Fig. 8). The hybridizing signal was significantly weaker when RNA was isolated from leaf sectors co-infiltrated with the PTGS silencing suppressor p19 (Fig. 7).

The microwrky sequence was detected by the stem-loop RT-PCR in hop tissues (Supplementary Fig. S11). While weak bands were detected on polyacrylamide gel in the samples from hop cones and lupulin glands, much stronger bands were observed using RNA extracts from hop leaves (Supplementary Fig. S11). Our results suggest that the levels of *Hl*WRKY1 mRNAs are regulated in both, transient expression system and in native hop tissues by small RNAs like "microwrky".

*Hl*WRKY1 can be classified to group IIc. For some members of this group it is known that their activity is regulated by phosphorylation (e.g. Chi et al. 2013; Encinas-Villarejo et al. 2009) and that physiological responses they mediate are regulated via MAPK (Lei et al. 2014). Up to now, it is not known, which protein kinases or kinase cascades in hop lupulin glands regulate *Hl*WRKY1 action. In order to analyze this possibility, we isolated homologue of *At*CDKF serine protein kinase (Hajheidari et al. 2012) from lupulin gland-specific cDNA library. *Hl*CDKF1 from hop (Supplementary Table S2) was cloned into plant expression vector and used for co-expression in transient system of *N.*

benthamiana. Transient expression analysis of P*HlOmt*1 activation showed significant increase (43.7 ± 15.2 %) of GUS activity driven by this promoter upon co-expression of this HlWRKY1 and HlCDKF1 in comparison to controls (without kinase) (Supplementary Fig. S12). This suggests that phosphorylation leads to activation of *Hl*WRKY1.

Discussion

In this work, we amplified and cloned four WRKY TFs from hop expression library derived from purified lupulin glands and described a putative role of highly lupulin-specific *Hl*WRKY1 TF in the regulation of the final steps of xanthohumol and biter acids biosynthesis pathway. According to the phylogenetic comparisons and tissue specific expression data, two of the cloned WRKYs (*Hl*WRKY1 and *Hl*WRKY5) showed significant similarity to well described *At*WRKY75 (At5g13080), a nucleus-localized WRKY protein classified within the IIc subgroup (Eulgem et al. 2000; Devaiah et al. 2007; Eulgem and Somssich 2007). *At*WRKY75 or its orthologs were described to be involved in various processes like pathogen defense responses (Encinas-Villarejo et al. 2009; Choi et al. 2014) and senescence (Guo and Gan 2004; Li et al. 2012). Suppression of WRKY75 expression through RNAi silencing resulted in early accumulation of anthocyanin, indicating that the RNAi plants were more susceptible to Pi stress (Devaiah et al. 2007). Lei et al. (2014) found that *At*WRKY75 is required for MPK3/MPK6 kinases-induced alteration of Pi responses.

Other two TFs that we cloned, *Hl*WRKY2 and *Hl*WRKY3 are quite unrelated to *At*WRKY75. The expression analysis of *Hl*WRKY1, 2 and 5 showed the highest leves in lupulin glands, while the maximal expression of *Hl*WRKY3 was observed in the root tissue. The most lupulin-specific TF *Hl*WRKY1 also showed low expression level in root and negligible expression in other tissues. *Hl*WRKY1 expression in root is consistent with functional studies of *At*WRKY75 describing its involvement in regulation of root architecture (Devaiah et al. 2007) and root hairs development (Rishmawi et al. 2014). In addition to high expression level in lupulin, more distant TF from *At*WRKY75, *Hl*WRKY5 showed significant expression level in leaf petioles, roots, flowers and even low expression in leaves and immature pollen. Although similarity between *Hl*WRKY1 and *Hl*WRKY5 reached 71 % within the conserved region covering DNA binding domain, significant differences in expression pattern of this TF and other cloned *Hl*WRKYs suggest different functional specialization of these TFs in comparison to *Hl*WRKY1.

Functional studies in heterologous transient expression system as well as qRT-PCR of *Hl*WRKY1 overexpressing transgenic hop plants revealed that *Hl*WRKY1 has ability to activate lupulin–specific genes *HlOmt*1, *HlChs_H1, HlPrt1(2) and HlVps*, whereas other three WRKYs showed very low effects on the promoters of these structural genes involved in the prenylflavonoid or bitter acid pathway (Fig. 1). In silico analysis revealed the presence of W-boxes in the upstream sequences of all these structural genes.

Hop lupulin metabolome is composed of various bioactive prenylated substances with antimicrobial and antifungal activities (e.g. Hough et al. 1957; Larson et al. 1996; Schmalreck et al. 1975; Mizobuchi and Sato 1985; Bhattacharya et al. 2003; Blanco et al. 2007). However, based on the studies in animal systems (Ceremuga et al. 2013) and also analogy with other flavonoid producing plants, we assume that lupulin had originally flower and seed protective role in hop cones. Consistent with this physiological function is the distribution of lupulin glands in hop female flowers in the inner surface of bracts surrounding seeds and the fact that hop is wind-pollinated plant species (Patzak et al. 2015). Connection of some phenylpropanoid compounds and anthocyanin metabolome to plant resistance to pathogens, herbivores, and environmental stress was reviewed by Treutter (2006). Long-term selection of hop genotypes according to brewing industry demand changed the origin lupulin metabolome composition.

Although there are some similar steps for biosynthesis pathways leading to xanthohumol or hop bitter acids (Fig. 1) and anthocyanin biosynthesis, anthocyanins were not detected in lupulin glands, even after transformation of hop with *AtPap*1 gene, which is known to activate anthocyanin biosynthesis in our previous experiments (Gatica-Arias et al. 2012). In these experiments anthocyanins were observed in all parts of hop cones that turned red except lupulin glands. Transient overexpression of lupulin-specific transcription factors like *Hl*Myb3 or binary *Hl*Myb3/*Hl*bHLH2 or ternary MBW complexes that activated *chs*_H1 gene led to metabolome changes in petunia leaves, but not to anthocyanin production (Matoušek et al. 2007, 2012). The *Agrobacterium*-mediated transformation of hop with *HlMyb3* gene also did not lead to the accumulation of anthocyanins in hop tissues, although the expression of most of flavonoid and phloroglucinol biosynthetic genes increased considerably (Gatica-Arias et al. 2013).

According to our recent results there were several metabolome changes induced in the leaf sectors of transgenic petunia overexpressing PAP1 infiltrated either with *Hl*WRKY1 or with combination of *Hl*WRKY1 and *Hl*WDR1 (WW) vectors. PAP1 is known as anthocyanin pathway-inducing transcription factor (e.g. Tohge et al. 2005) and its overexpression causes intensive blue spots on petunia leaves (Matoušek et al. 2006). However on the leaves infiltrated by *Hl*WRKY or W/W no anthocyanin pigments were visible and although levels of some metabolites increased, most of PAP1-induced peaks were suppressed, as confirmed by HPLC analysis (Supplementary Table S4).

In addition, in the present study we found that promoter of *Hl*Myb3 was significantly activated by *Hl*WRKY1 in the transient expression system. These results altogether suggest that there might be some molecular switch, possibly specific network in lupulin glands that leads to accumulation of prenylated compounds including xanthohumol, but not to accumulation of anthocyanin pigments. We assume that the putative function of *Hl*WRKY1 in lupulin biosynthesis may be essential for this lupulin-specific regulation network.

Our results also suggest that *Hl*WRKY1 is endowed with several important features that make its expression very flexible. Its ability to activate promoters of our interest was higher in combinatorial action with lupulin-specific *Hl*WDR1 described by Matoušek et al. (2012) in *N. benthamiana* and hop leaves. Although in this work we did not study the concrete mechanism of this combinatorial action, it is known that members of WRKY superfamily are able to interact with various proteins as reviewed by Chi et al. (2013). Proteins from WDR family are capable of adapting and interacting with the appropriate partner in entirely different functional contexts (e.g. from RNA processing to cytoskeletal rearrangements) providing several sites for interaction with many different peptide motifs, thus serving as ideal platforms for recruiting different proteins and aiding the formation of transient complexes (Stirnimann et al.2010). Pesch et al. (2014) found that TTG2 (AtWRKY44), a specific regulator of proanthocyanidin synthesis is able to activate R3 Myb TRIPTYCHON (TRY) through the interaction with TTG1 from WD40 family, resulting in enhanced activity of GL3, the member of trichome activator MBW (GL1-GL3-TTG1) complex. Interaction of WRKY and WD40 forming complexes was also observed by Verweij et al. (2016), who found in petunia that WRKY protein PH3, which is highly similar to *At*WRKY44 (TTG2) and regulates hair development, tannin accumulation, and mucilage production, can bind to AN11 (WD40) and also to Arabidopsis homolog TTG1 (WD40). They concluded that the specificity of MBW complexes leading to flavonoid production is in part determined by interacting proteins, such as WRKY family members.

According to the transient expression assays, *Hl*WRKY1 can be self-activated and alternatively, *Hl*WRKY1 is strongly regulated by PTGS, as shown by degradome analyses. Although these features cause the rapid accumulation but also rapid *Hl*WRKY1 mRNA degradation depending on the physiological state, making presumably its steady state and turnover very flexible. Its high and variable expression in hop transgenic lines which we observed in our experiments supports this assumption. The high level of "microWRKY" in hop leaves correlated with negligible levels of *Hl*WRKY1 in control plants. Simultaneously, ectopic *Hl*WRKY1 expression could lead to activation of endogenous *HlWRKY1* gene. These two possibilities remain to be elucidated. The autoregulation of WRKY genes was described in previous studies (Turck et al. 2004; Eulgem and Somssich 2007).

The microWRKY RNA does not correspond to genome-encoded (classical) microRNA, as we did not find any specific "microWRKY" gene in the hop genome i.e. sequence that could serve as template for pre-microRNA transcription and subsequent microRNA maturation. Therefore, it seems that this small RNA is generated by some secondary or TASI-like process (reviewed in Aravin and Tuschl 2005) and derived directly via Dicer cleavage of dsRNA that could be generated from *Hl*WRKY1 mRNA by RDR6 polymerase (e.g. Garcia-Ruiz et al. 2010; Bai et al. 2012). Such polymerase we detected by qRT-PCR in different hop tissues including lupulin glands (unpublished). Interestingly, *Hl*WRKY1 level can increase significantly in hop leaves also due to pathogenesis induced by the grapevine variant of hop stunt viroid (HSVdg) (Füssy et al. 2013). HSVdg pathogenesis is accompanied by visible symptoms like leaf malformations but also by the disappearance of anthocyanins from hop leaf petioles (Füssy et al. 2013).

In conclusion, *Hl*WRKY1 similarly to *At*WRKY75 can be activated by protein kinases. MAPK cascades can be activated depending on various stresses or grow conditions like Pi starvation as described by Lei et al. (2014). In our experiments we cloned protein kinase *Hl*CDKF1 and showed stimulation of *Hl*WRKY1 in the transient expression system. At present it is not known which hop protein kinase(s) could be essential for possible regulation of *Hl*WRKY1 and lupulin pathways. Taking these features altogether, the simplified scheme of putative regulation of *Hl*WRKY1 is presented as Supplementary Fig. S13. In this scheme all dependencies of *Hl*WRKY1 activation on other regulatory factors are shown as observed in the present study. We showed clear capability of *Hl*WRKY1 to activate essential structural genes in the last step of the xanthohumol and bitter acids pathways. However, our present study doesn't reflect the extent of the putative contribution of the WRKY TF family in the final accumulation of lupulin, which is obviously determined by the polygenic background.

Acknowledgments The authors would like to thank Helena Matoušková, Olga Horáková, Lidmila Orctová and Jana Jehlíková from Biology Centre of the CAS v.v.i., Institute of Plant Molecular Biology (IPMB) for excellent technical assistance. The authors would like to acknowledge prof. Csaba Koncz from the Max Planck Institute for Plant Breeding, Cologne (Germany) for valuable discussions and for providing the vector plasmid for *Agrobacterium* transformation. This work was supported by the Czech Science Foundation GACR 13-03037S, by the cooperative project FP7-REGPOT-2012-2013-1 MODBIOLIN No. 316304, GAJU 143/2013/P and by institutional support RVO:60077344.

Author contributions JM conceived and coordinated the project, isolated and cloned the genes into *Agrobacterium* vectors, performed

degradome analysis, TK was responsible for transient expression experiments, JP screened ESTs, JB did the hop transformation and particle bombardment assays, participated on the promoters cloning, KS did the EMSA and RT-qPCR of transgenic hop, AKM performed bioinformatics analyses, GSD participated on the gene cloning, AT did the RT-qPCR of tissue specificity expression analysis, EO provided the genomic sequences of hop, KK did the HPLC analysis of lupulin metabolites. JM wrote the manuscript, TK and AKM edited the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest

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