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Functional Analyses of Lupulin Gland-Specific Regulatory Factors from WD40, bHLH and Myb Families of Hop (*Humulus lupulus* L.) show Formation of Crucial Complexes Activating *chs*_H1 Genes

Complex lupulin gland-specific cDNA library from Osvald's clone 72 hop was constructed to dissect regulation of biosynthetic pathway(s) leading to the accumulation of hop bitter acids and prenylflavonoids with promising health-beneficial activities. From this cDNA library we isolated first hop-specific allelic isoforms of transcription factors (TF) bHLH (*Hl*bHLH2, GeneBank AC:FR751553) and WD40 (*Hl*WD40_1, AC:NM_122360) which are involved in combinatorial control of light-responsive and tissue-specific activation of phenylpropanoid pathway. *Hl*bHLH2 and *Hl*WD40_1 are quite lupulin gland-specific and, according to our transient expression experiments, they form specific complexes with another novel hop TF *Hl*Myb2 (AC:FN646081) and previously isolated lupulin gland-specific *Hl*Myb3 (AC:AM501509). The complex formation leads to strong activation of chalconsynthase gene (chs_H1) promoter. The interplay and regulation of expression of these crucial TF complexes could co-determine the rate of accumulation of valuable metabolites of lupulin.

Descriptors: *Humulus lupulus* L., *N. benthamiana,* lupulin metabolome, transcription factors, protein complexes, transient expression assay

1 Introduction

Although hop (*Humulus lupulus* L.) plants are mainly cultivated for the brewing industry as a source of flavor-active secondary metabolites contained in lupulin glands, several compounds like prenylated chalcones (e.g. xanthohumol) are of particular interest due to their medicinal properties [1, 2]. Xanthohumol is a fascinating cancer-chemopreventive compound exhibiting a broad spectrum of inhibition mechanisms at all stages of carcinogenesis [3] and another lupulin-derived prenylflavonoid, 8-prenylnaringenin (8-PN), is one of the most potent phytoestrogens known to date [4, 5].

An important role in biosynthesis of these compounds is attributed to chalcone synthase-CHS_H1 (EC 2.3.1.74) [6] encoded by the oligofamily of chs_H1 genes that we previously characterized including promoter elements [7]. "True" chalcone synthase (EC 2.3.1.74) efficiently catalyzes the production of naringenin chalcone by condensation of three malonyl-CoA units and p-coumaroyl-CoA [6]. The complexity of promoter elements of chs_H1 genes suggests the regulation by several types of transcription factors (TFs), mainly from Myb, bHLH or bZip families. During last years we described several lupulin-gland specific transcription factors (TF) from Myb and bZip families putatively involved in hop metabolome regulation [8–10]. It is known, that some TFs form complexes by protein:protein interaction that specifies their function [11, 12]. Especially cooperation between MYB and bHLH proteins in so-called combinatorial TFs action has been studied [11–15]. The crucial binding sites in these interactions are located in the Myb R3 domain and N-terminal region of bHLH [16].

To date, two Myb TFs (*Hl*Myb1 and *Hl*Myb3) were suggested to be involved in the lupulin metabolome production. It was deduced from the protein sequence homology and specific expression in hop cones [8, 9]. It was shown, that these TFs are able to activate expression of chs_H1 genes, but the mechanism of this activation has not been investigated in detail [8, 9].

In the present work, we cloned novel lupulin-specific TFs *Hl*bHLH2, *Hl*WD40_1 and *Hl*Myb2 and showed that together with previously described *Hl*Myb3 [9] these TFs form crucial complexes, which efficiently activate *chs*_H1 promoter and thus, are able to regulate production of naringenine chalcone, precursor in the xanthohumol biosynthesis.

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Tables and figures see Appendix

2 Materials and Methods

Hop transcription factors (TFs) *Hl*Myb1 and *Hl*Myb3 were described previously [7–9, 17]. These genes were cloned from cDNA library of hop clone Osvald 72 using probes generated from conserved motifs or based on cDNA ESTs selected using cDNA AFLP method [18]. For the cloning of *Hl*bHLH2 and *Hl*WDR1 we combined screening of EST (Expressed Sequence Tags) and trichome databases (GenBank: www.ncbi.nlm.nih.gov/ and TrichOME: trichome.noble.org/trichomedb/) for specific motifs with PCR amplification of cDNA fragments from lupulin-specific cDNA library of Osvald's 72 hop. The cDNA library was prepared from isolated lupulin glands according to *Nagel* et al. [19]. In addition, 3'RACE [20] and inverse PCR approach [21] was used to complete *Hl*WD40_1 cDNA. Other genes like *Hl*Myb7, *Hl*Myb2 and MIXTA genes and sequences from *A. thaliana* were amplified using sequence information from EMBL database (www.ebi.ac.uk/embl/). Based on cloned cDNA sequences, plant expression vectors pLV07 were constructed and *A. tumefaciens* strains were prepared as described previously [10]. Promoter activity was evaluated using β-glucuronidase (GUS) activity assay [22]. Hop RNA was isolated from 100 mg of different tissues of Osvald's 72 clone using Concert™Plant RNA Reagent (Invitrogen) following RNA purification and DNA cleavage on columns (RNeasy Plant Total RNA kit, Qiagen). Real-time quantitative PCR (RT qPCR) mRNA quantifications were performed using specific primers as described previously [10].

3 Results and discussion

3.1 Cloning and characterization of novel hop TFs

In the present work, we cloned several transcription factors (TFs) from the cDNA library derived from lupulin glands of Czech hop Osvald's clone 72. This cDNA library was more specific and complex ($3.5.10^6$ pfu) than previously constructed cDNA library from hop flowers and cones [9, 10]. The high complexity and specificity of the library enabled us to amplify full-length clones of genes for *Hl*bHLH2, *Hl*WD40_1 and *Hl*Myb2 TFs. Homology comparisons showed that *Hl*bHLH2 with predicted molecular weight (MW) about 77.1 kDa and isoelectric point (pI) 5.1 shares significant homology with other bHLH type TFs from different species, e.g. AN1 from *Petunia hybrida* [23], TT8 from *Arabidopsis thaliana* [24] (Fig.1). *Hl*WD40_1 with predicted MW about 38 kDa and pI 4.79 is significantly homologous to transparent testa glabra (TTG) TFs (Fig.1). TTG proteins are able to regulate several developmental and biochemical pathways, including the development of trichomes or root hairs as well as production of anthocyanin pigment [25]. *Hl*Myb2 shares significant homology with TT2 like Myb from *Theobroma cacao* as well as with many other Mybs from *Vitis vinifera*, *Populus trichocarpa* etc.

3.2 Novel cloned hop TFs are specifically expressed in lupulin glands

Based on the protein alignment data it can be deduced, that all these newly cloned hop genes encode for proteins which might play role in the regulation of lupulin metabolome. To elucidate if the cloned regulatory factors are specific for lupulin glands, we performed real-time quantitative PCR analysis (RT qPCR). It was shown that *Hl*bHLH2, *Hl*WD40_1 and *Hl*Myb2 as well as previously cloned HlMyb3 [9] are highly specifically expressed in hop glandular tissue (Fig. 2, A–D).

3.3 Hop TFs form complexes capable to activate chs_H1 gene promoter

Recent genetic, biochemical and molecular data have shown that MYB, bHLH and WD40 factors participate in the regulation of genes in combinatorial regulatory complexes [15, 26, 28, 29]. To check if our cloned hop TFs form such complexes in the regulation of the chs_H1 genes expression, we performed "combinatorial" transient expression assay as described previously [10]. We used system of infiltration of *Agrobacterium tumefaciens* strains containing TF vectors and hop chs_H1 promoter (P*chs_H1*) fused to GUS reporter gene containing vector into *Nicotiana benthamiana* leaves. Transient expression system revealed that basically two activation TF complexes are formed after co-infiltration of *Hl*bHLH2, *Hl*WD40_1 and either *Hl*Myb2 (complex *Hl*M2W1H2) or *Hl*Myb3 (*Hl*M3W1H2), while individual TFs did not show any significant P*chs*_H1 activation (Fig. 2 E). Activation by *Hl*M3W1H2 usually reached about 30 % of *Hl*M2W1H2 activity (Fig. 2, Table 1). It was found that individual complex components can be partly substituted by other TFs from hop or *A. thaliana* (Table 1). For instance, Myb components can be partly substituted by *At*Myb12 and *At*Myb23; *Hl*WD40_1 component can be substituted by *ttg1* from *Arabidopsis* so that the heterologous complex reached 40 % of activity of homologous *Hl*M2W1H2 (Table 1). Our results show that homologous hop complexes are quite *Hl*Myb2 and *Hl*Myb3-specific, as no activity was observed with other hop Myb or Myb-like TFs (Fig. 4). In addition, according to our unpublished results, previously cloned *Hl*Myb7 (AC: FR873650) is a strong competitive inhibitor of both hop TF complexes. Simultaneously, our results revealed that there are clear differences between *Hl*M2W1H2 and *Hl*M3W1H2 complexes in dynamics of action (not shown), as well as in their dependency on various binding boxes within P*chs*_H1. It was found that the function of both complexes depends on the complexity of PMyb-like box CCWACC positioned on the 5' end of cloned P*chs*_H1 (not shown). While complex *Hl*M2W1H2 is quite specific for P*chs*_H1, *Hl*M3W1H2 has the ability to co-activate also promoter of another chalcone synthase P*chs*4. Non of these complexes has the ability to activate another promoters of genes involved in lupulin biosynthesis like o-methyltransferase (*omt*1) [19] or valerophenone synthase (*vps*) [27] promoters. The expression balance among various promoter activating complexes and complex inhibitors, as has been shown also in other studies [15], influences final mRNA steady state levels. Our study indicates that this TFs network is valid also for *chs*_H1 and it could co-determine the rate of accumulation of valuable metabolites of lupulin. Similar or identical functions of orthologous protein complexes encoded by flavonoid regulatory genes have been described also for other agronomic plants, such as maize, cotton or grape wine [16, 28, 29].

4 Conclusions

In the present study, new TF homologues corresponding to the plant R2R3Myb, bHLH and WD40 repeat families were cloned using a cDNA library derived from hop Osvald's clone 72 lupulin gland tissue. The cloned *Hl*Myb2, *Hl*bHLH2 and *Hl*WD40_1 TFs display a high similarity to known TFs regulating the flavonoid biosynthesis pathway and were found to be specifically expressed in lupulin glands. The functional activity of these TFs was investigated using a combinatorial transient expression system in infiltrated leaf sectors of *N. benthamiana*. These experiments provided new insight into the complex character of the regulation of chs_H1 genes, as well as into the differential activation of chs_H1 promoter. The regulation is mainly based of the action ternary complex of *Hl*WD40_1, *Hl*bHLH2 and *Hl*Myb2 or *Hl*Myb3 TFs (see Fig. 4). Complementation of hop and *A. thaliana* TFs was shown in particular combinations. *Hl*Myb7 was characterized as an R2R3 repressor that could act as a potential co-regulator of the lupulin metabolome biosynthesis. Elucidation of the gene regulatory networks in agronomically important plants, including hop, poses very significant challenges for the future.

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Appendix

Table 1 Substitution of activating complexes with hop and arabidopsis TFs

Substituting component			MWH complex activity %**
TF type	Gene symbol	GenBank	
Myb (M)[1]	*Hl*Myb2	FN646081	100.0
	*Hl*s-Myb3	AM501509	33.7
	*Hl*l-Myb3	AM501509	15.5
	*Hl*Myb1	AJ876882	1.4
	*Hl*Myb7	FR873650	0.3
	*At*Myb23	AT5G40330	23.3
	*At*Myb12	AT2G47460	35.3
Myb like[1]	*Hl*Mixta3	–	2.2
	*Hl*Mixta4	–	1.0
WD repeat[2] (W)	*ttg1*	AT5G24520	40.8
MW[3]	*Hl* s-Myb3 + *ttg1*	–	15.8
	*At*Myb23 + *ttg1*	–	10.8
	*At*Myb12 + *ttg1*	–	25.7
bHLH (H)[4]	*Hl*bHLH1	FN646080	1.8
WH[5]	*ttg1* + *Hl*bHLH1	–	6.7
MH[6]	*Hl*bHLH1 + *Hl* s-Myb3	–	6.4
	*Hl*bHLH1 + *Hl* Myb1	–	3.4

The following TFs formed the basis: [1]*Hl*bHLH2 and *Hl*WD40_1; [2]*Hl*Myb2 and *Hl*bHLH2; 3HlbHLH2; [4]*Hl*Myb2 and *Hl*WD40_1; [5]*Hl*Myb2; [6]*Hl*WD40_1.

**activity of *Hl*M2W1H2 with P*chs*_H1 is taken as 100 %; samples were collected at optimal interval post infiltration.

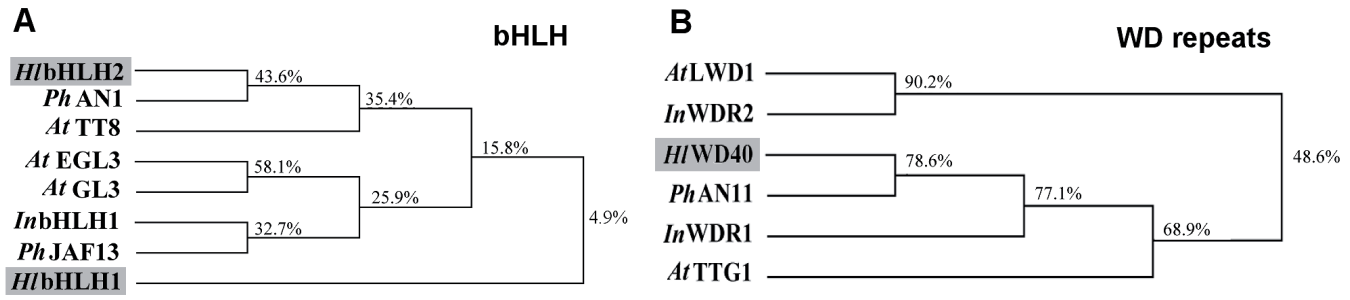

Fig. 1 **Homology of cloned hops bHLH (A) and WDR (B) TFs (their names are marked with gray background) to known regulators of phenylpropanoid pathways (amino acid identity % is shown)**

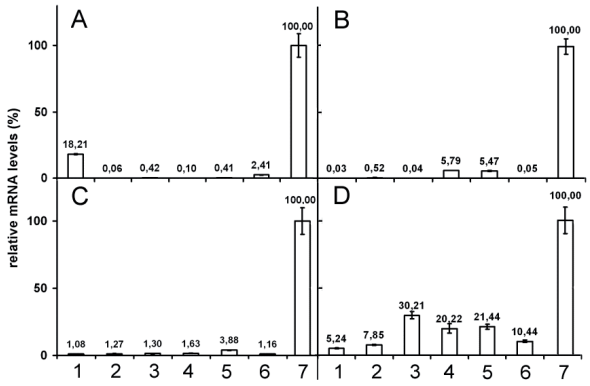

Fig.2 **Real-Time quantitative PCR (RT qPCR) analysis of expression of hop HlMyb3 (A), HlMyb2 (B), HlbHLH2 (C) and HlWD40_1 (D) in roots (1); young leaves (2); petioles (3); flowers (4); young cones (5); immature pollen (6) and lupulin glands (7). GAPDH was used as internal reference transcript**

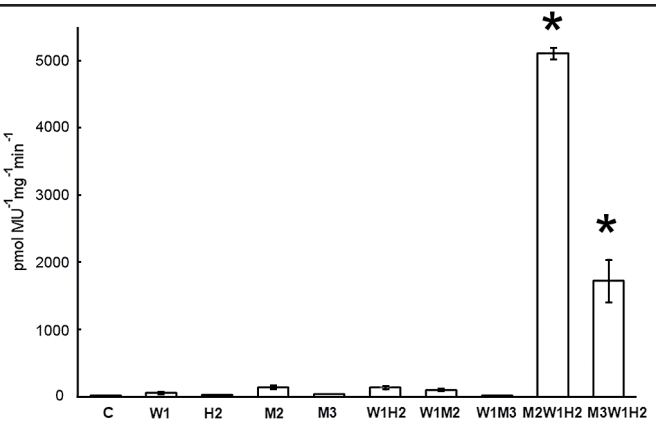

Fig.3 **Activation of P*chs*_H1 fused to *uidA (gus)* gene by hop TF and TFs complexes. Statistically significant activation by *Hl*M2W1H2 and *Hl*M3W1H2 complexes is marked by asterisks**

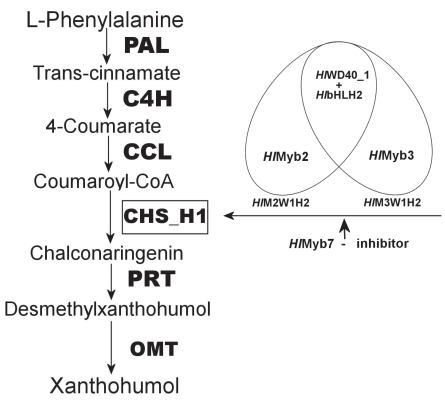

Fig.4 **Scheme of the xanthohumol biosynthesis pathway with presumed mechanism of *Hl*chs_H1 gene regulation. Bold are indicated enzymes catalyzing different steps: PAL- phenylalanine ammonia lyase; C4H- cinnamate 4-hydroxylase; CCL- 4-coumarate CoA ligase; CHS_H1 – chalcone synthase; PRT – aromatic prenyltransferase; OMT- o-methyltransferase**