Plant Science 269 (2018) 32–46

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

The R2R3 transcription factor *Hl*MYB8 and its role in flavonoid biosynthesis in hop (*Humulus lupulus* L.)

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ARTICLE INFO

Keywords:
Bitter acids
Chalcone synthase
Humulus lupulus
Flavonoids
R2R3 myb
RNA sequencing

ABSTRACT

Hop is an important source of medicinally valuable secondary metabolites including bioactive prenylated chalcones. To gain in-depth knowledge of the regulatory mechanisms of hop flavonoids biosynthesis, full-length cDNA of *Hl*Myb8 transcription factor gene was isolated from lupulin glands. The deduced amino acid sequence of *Hl*Myb8 showed high similarity to a flavonol-specific regulator of phenylpropanoid biosynthesis *At*MYB12 from *Arabidopsis thaliana*. Transient expression studies and qRT-PCR analysis of transgenic hop plants overexpressing *Hl*Myb8 revealed that *Hl*MYB8 activates expression of chalcone synthase *Hl*CHS_H1 as well as other structural genes from the flavonoid pathway branch leading to the production of flavonols (*F3H*, *F'3H*, *FLS*) but not prenylflavonoids (*PT1*, *OMT1*) or bitter acids (*VPS*, *PT1*). *Hl*Myb8 could cross-activate *Arabidopsis* flavonol-specific genes but to a much lesser extent than *At*Myb12. Reciprocally, *At*Myb12 could cross-activate hop flavonol-specific genes. Transcriptome sequence analysis of hop leaf tissue overexpressing *Hl*Myb8 confirmed the modulation of several other genes related to flavonoid biosynthesis pathways (*PAL*, *4CL*, *ANR*, *DFR*, *LDOX*). Analysis of metabolites in hop female cones confirmed that overexpression of *Hl*Myb8 does not increase prenylflavonoid or bitter acids content in lupulin glands. It follows from our results that *Hl*MYB8 plays role in a competition between flavonol and prenylflavonoid or bitter acid pathways by diverting the flux of CHS_H1 gene product and thus, may influence the level of these metabolites in hop lupulin.

1. Introduction

Female hop (*Humulus lupulus* L.) cones are widely used in brewing industry to provide the beer bitterness and aroma and accredited to have potential pharmaceutical or biomedical applications. The commercial value of cones lies in lupulin glands, which contain several biologically active components, including essential oils, bitter acids, prenylflavonoids, having many different beneficial effects on human health [1–3]. Xanthohumol (XN) is the most abundant prenylflavonoid with its overall concentration in the cone dry mass varying from 0.2 and 1.1% depending on the genotype and the environmental conditions [4]. The other flavonoids, isoxanthohumol (IX) and 8-prenylnaringenin (8-PN) are present in ten to hundred-fold lower concentrations in comparison to XN level [2]. One of the recent efforts of hop breeders is to gain new hop cultivars with elevated levels of prenylflavonoids but met with limited success [5,6].

Flavonoids are generally synthesized via phenylpropanoid pathway which constitutes most detailed studied biosynthetic route among secondary metabolism in plants [7–9]. The phenylalanine serves as the precursor molecule for flavonoid synthesis, which is converted to cinnamic acid by phenylalanine ammonia lyase (PAL) (Fig. 1). One molecule of CoA-ester of cinnamic acid and three molecules of malonyl-CoA are condensed into the naringenin chalcone. This reaction is catalyzed by the enzyme chalcone synthase (CHS). The chalcone is isomerised to a flavanone by chalcone flavanone isomerase enzyme (CHI). From these central intermediates, the pathway splits into different

Abbreviations: 8PN, 8-prenylnaringenin; ANR, anthocyanin reductase; BA, bitter acids; CHI, chalcone isomerase; CHS, chalcone synthase; DEG, differentially expressed genes; DFR, dihydroflavonol reductase; F3'H, flavonoid 3-hydroxylase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, gene ontology; GUS, β-glucuronidase; IX, isoxanthohumol; LDOX, leucoanthocyanidin dioxygenase; MBW, a general MYB-bHLH-WDR transcription factor complex; MU, 4-methylumbelliferone; PA, proanthocyanidin; PAL, phenylalanine ammonia lyase; RNA-seq, RNA sequencing utilizing high-throughput deep sequencing of cDNA; qRT-PCR, quantitative real-time polymerase chain reaction; XN, Xanthohumol

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https://doi.org/10.1016/j.plantsci.2018.01.004
Received 3 November 2017; Received in revised form 12 January 2018; Accepted 14 January 2018

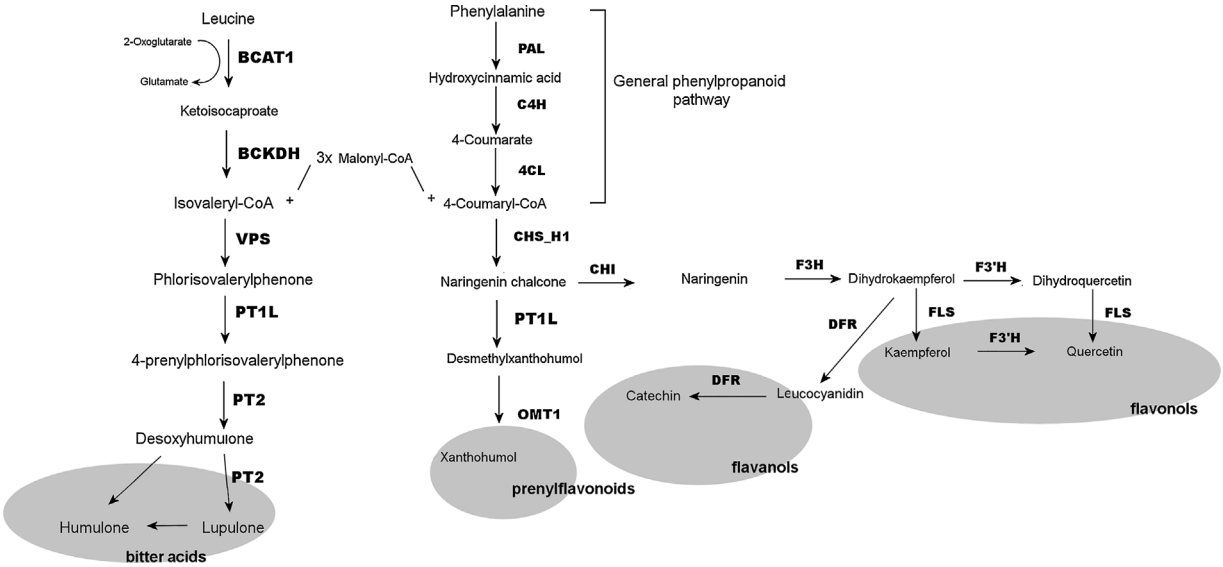

Fig 1. Simplified schematic representation of the phenylpropanoid pathway in hop lupulin glands. Abbreviations of the enzyme designations are 4CL, coumarate: CoA ligase; BCAT, branched-chain aminotransferase; BCKDH; branched-chain α-ketoacid dehydrogenase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3′H, flavonoid 3′-hydroxylase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; OMT1, O-methyltransferase 1; PAL, phenylalanine ammonia lyase; PT1L; Prenyltransferase; PT2, Prenyltransferase 2.

directions resulting in the formation of different class of flavonoids, including prenylflavonoids. The biosynthesis of the diverse groups of flavonoids (e.g. anthocyanins, flavonols, flavones and proanthocyanidins) share common precursors, dihydroflavonols, which serve as substrates for flavonol synthase (FLS) or dihydroflavonol 4-reductase enzymes and thus, these different metabolite pathways compete among each other leading to the varied compositions of secondary metabolites in plants [10]. In the hop glandular trichomes, naringenin chalcone is first prenylated by prenyltransferase (PT) and subsequently methylated by a substrate-specific o-methyltransferase (OMT), which is responsible for the stabilization of the xanthohumol [11].

The flavonoid biosynthesis is controlled by several transcription factors (TFs) belonging to the different families such as WD40 repeat (W), bHLH (B), MYB (M), WRKY, Zinc finger and MADS box as well as influenced by environmental factors such as light, temperature, biotic and abiotic stresses [8,12–15]. In monocotyledon plants, all genes of the flavonoid pathway are regulated simultaneously as a single unit [7,16]. The regulation of flavonoid biosynthesis pathway in dicotyledons occurs by different pattern. The early steps (leading to the flavonol and flavones) usually do not require MBW complex and are regulated by the independently acting MYB TFs which activate a certain set of the structural genes [e.g. *CHS*, *CHI*, flavanone 3-hydroxylase (*F3H*), *FLS*]. The final steps (leading to the anthocyanin or prenylflavonoids) require the activation by a ternary complex of MYB-bHLH-WD40 transcription factors (MBW complex) [13,14,17,18]. Single MYB TFs have been proposed to generally regulate usually only one branch of the flavonoid biosynthesis pathway [8]. For example, *At*Myb123 (TT2) TF regulates anthocyanin biosynthetic branch in *Arabidopsis* [19,20], whereas *At*MYB12, together with *At*MYB11 and *At*MYB111 are known to regulate flavonol biosynthesis [21]. However, some Myb TFs were found to regulate simultaneously several branches of flavonoid pathway such as grapevine TFs *Vv*MYB5a and *Vv*MYB5b regulating the biosynthesis of anthocyanins, proanthocyanidins (PAs), flavonols and lignins [22]. Increasing knowledge of the pathway key enzymes and revealing their function within the transcription regulatory network provides new tools for manipulation of phenylpropanoid biosynthetic route.

Hop prenylavonoids and bitter acids are unique compounds in the plant kingdom and elucidation of the regulatory mechanisms of their biosynthesis is very challenging. So far, their *de novo* chemical synthesis has not yet been succeeded. Several lupulin gland-specific genes encoding crucial enzymes of the phenylpropanoid pathway, like chalcone synthase (HlCHS_H1; GenBank accession: AJ304877) [23], o-methyltransferase (HlOMT-1; FM164641) [11] or prenyltransferase1 (HlPT-1; AB543053) [24] and their transcriptional regulators have been cloned and characterized. A combinatorial action of lupulin gland specific TFs from the R2R3MYB, bHLH and WD40 families in the regulation of the *HlCHS_*H1 gene has been described [14]. Recently, the function of the hop TF *Hl*WRKY1 as lupulin-specific regulator of biosynthesis of prenylated flavonoids and bitter acids was characterized. Transgenic hop plants overexpressing *Hl*WRKY1 have been successfully obtained and complex activation of series of pathway genes has been demonstrated [15].

To obtain new informative insight into the transcriptional regulation of the flavonoid biosynthesis pathways in hop, we have successfully isolated and cloned cDNA of homologue of *AtMyb12* gene (At2G47460) and designated as *HlMyb8*. The functional studies of *Hl*Myb8 were performed in transient expression system in *Nicotiana benthamiana* leaves as well as in transgenic hop plants overexpressing *Hl*Myb8. We identified potential targets of *Hl*Myb8 and compared its function with findings known for its *Arabidopsis* homologue *At*Myb12. Analysis of RNAseq data of *Hl*Myb8 overexpressing hop transgenic lines enabled us to gain more comprehensive overview about the modulated genes.

2. Material and methods

2.1. Plant material

Saaz semi-early red-bine hop (*Humulus lupulus* L.) Osvald's clone 72 was used for *Agrobacterium*-mediated plant transformation and subsequent gene expression analyses. The young leaves of *Nicotiana benthamiana* plants (2–4-week-old) were used for transient expression assays using agro-infiltrations of leaves. For transformation and gene expression studies, *Arabidopsis thaliana* ecotype Columbia was used. The mutant line (*myb12*-1f) of *A. thaliana* was obtained from the Nottingham Arabidopsis Stock Center (NASC) (number N9602). Plants were maintained in greenhouses at a temperature of 25 ± 3 °C and cultivated under natural light condition from March to September (2015–2017) with supplementary illumination [170 μmol m^{-2} s^{-1} PAR] to maintain a 16 h-day period. *Arabidopsis* seedlings were