BIOLOGIA PLANTARUM 46 (3): 375-381, 2003

Valerophenone synthase-like chalcone synthase homologues in *Humulus lupulus*

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Abstract

Valerophenone synthase homologue of chalcone synthase (CHS) is the first key enzyme described to be involved in the biosynthesis of bitter acids, the compounds produced in hop lupulin glands valuable for the taste of beer. The complete sequence of a novel homologue of CHS *chs* 4 was isolated from hop. Protein predicted from *chs* 4 cDNA has 43.45 kDa and length 395 amino acids. It was found by the analysis of *chs* 4 flanking sequences that this gene is in the cluster with other CHS homologues – *chs* 3 and *vps*. The intron identified in *chs* 4 has been found to be homological to *vps* and *chs* 3 introns. Expression of *chs* 4 was partially characterized using reverse transcription polymerase chain reaction and it was found that *chs* 4 is specifically expressed in the glandular tissue of hop cones likewise *vps*. The predicted protein sequence CHS 4 was compared with other CHS-like proteins.

Additional key words: bitter acids, flavonoids, hop, prenylated flavonoids.

Introduction

The cones of hop plants (*Humulus lupulus* L.) are used in beer-brewing process as a source of lupulin components, which provide the characteristic taste of beer. The typical bitterness of beer comes from the izomerisation of the α-acids, valuable compounds of hop resins. Recently, valerophenone synthase (VPS), key enzyme and corresponding gene of hop resins biosynthetic pathway has been described (Paniego *et al.* 1999, Okada and Ito 2001). VPS is a homologue of chalcone synthase (CHS). These enzymes share the same reaction specificity but differ in substrate specificity. CHS catalyses condensation of three molecules of malonyl-CoA with coumaroyl-CoA. On the other hand, VPS accepts isovaleryl-CoA or isobutyryl-CoA instead of coumaroyl-CoA (Paniego *et al.* 1999).

The true chalcone synthase catalyses the key step of flavonoids biosynthesis. Flavonoids act, for instance, as UV protectants (Dawar *et al.* 1998) or phytoalexines (Ziouti *at al.* 1996). The valuable compounds in hop cones, prenylated flavonoids, are intensively studied for their antiproliferative and anti-cancer effects (Stevens *et al.* 1997, Miranda *et al.* 1999). The presence of prenyl-flavonoids in hop cones suggests the key role of enzyme with chalcone synthase activity. The corresponding gene *chs*_H1 and protein we have recently described (Matoušek *et al.* 2002b).

To date four genes encoding CHS homologues from hop are described in the GenBank: *vps* (AB047593) and *chs*_H1 (AJ304877) with characterized enzymatic activity (Okada and Ito 2001, Okada *et al.* 2001, Matoušek *et al.* 2002a) and two other CHS homologues, *chs* 2 and *chs* 3 (AB061020, AB061022) which substrate specificity has not been yet verified experimentally.

Received 7 March 2002, *accepted* 17 June 2002.

Abbreviations: CHS - chalcone synthase; VPS - valerophenone synthase.

Acknowledgments: The authors thank Dr. Josef Patzak from Hop Research Institute Žatec (Czech Republic) for his help with RNA isolation. We thank Ing. Lidmila Orctová and Helena Matoušková (Institute of Plant Molecular Biology, Academy of Sciences of CR, České Budějovice, Czech Republic) for their excellent technical assistance. The work was supported by grant 521/99/1591 from the Grant Agency of the Czech Republic.

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In this paper we have characterized the sequence of a novel *chs* homologue – *chs* 4 and its expression in hop. According to the protein structure comparisons and analysis of genomic organization of CHS homologues CHS 2, CHS 3 and CHS 4 we discussed their close relationship to previously described VPS.

Materials and methods

Plants: Hop (*Humulus lupulus* L.) plants were maintained in the breeding hopgarden in the Hop Research Institute, Žatec. Czech semi-early red-bine hop cv. Osvald clone 72 and the following comprehensive hop cultivars Magnum, Southern Brewer, Northern Brewer, Wye Target, Galena, Taurus and Sládek were analyzed in our experiments. For some comparisons we collected samples from field grown *Humulus neomexicanus* species.

DNA isolation and Southern blots: Genomic DNA from leaves was isolated as described by Tai and Tanksley (1991). Southern analyses were performed by using the protocol for *Qiabrane Nylon Plus* membrane (*Qiagen*, Hilden, Germany). Hybridization was conducted according to Church and Gilbert (1984) at 65 °C in 0.4 M phosphate buffer pH 7.2 containing 7 % SDS, 1 % bovine serum albumin, 1 mM EDTA pH 8.3 and probe labeled with *Redivue*™ [α-^{32}P]dCTP, 111 MBq mmol^{-1} random prime labeling kit *Rediprime*™ (*Amersham Pharmacia Biotech*, Freiburg, Germany). After overnight hybridization washing at 65 °C three-times for 30 min with 100 mM phosphate buffer pH 7.2, 1 % SDS, 1 mM EDTA pH 8.3 was performed. If necessary, the membranes were stripped by washing in 0.4 M NaOH at 42 °C for 15 min and then in 0.1×SSC, 0.1 % SDS, 0.2 M Tris HCl pH 7.5 at 42 °C for 30 min.

Three DNA probes were prepared - *vps* specific, *chs* 2 specific and *chs* 4 specific using primers VPS5end (5'ATATATCAGGTAATGGCGTC 3'), VPS3end (5' ATATATTTGATCATTTTATT 3'), CHS2NdeI (5'CATATGACCTCCATGACTGTGGAC3'), CHS2BamHI (5'GGATCCGAATTTATTGGCCACGCTGTG3'), CHS4NdeI (5'CATATGGCATCTGTAACTATGGAGCAA3') and CHS4BamHI (5'GGATCCTTAAACCTTGTTTGCAACGC3'), respectively. These probes covered genes from start to stop codone. Base changes to create *Nde*I and *Bam*HI sites for the cloning purposes are underlined.

PCR amplifications, cloning and sequencing: For amplification *chs* specific sequences we used degenerate primers CHScons1 (5'ATHAARGARTGGGGNCARCC3') and CHScons2 (5'CANCCYTGYTGRTACATCAT3'). For amplification flanking sequences were used primers INV52I (5'GCCAGGCATGTCGACACC'3) and INV52II (5'ACCAATGCGCCAAGCTTCTC3'). For amplification of *chs* 3 CHS3NdeI (5'CATATGTCCTCCTCCATAACTGT3') and CHS3BamHI (5'GGATCCAGTCTCACTTTTGACCTTGTT3') were used. For amplification of long products *DyNazyme*™ *EXT PCR kit* (*Finnzymes*, Espoo, Finland) was used.

Prior to sequencing, all PCR products have been cloned in the vector pGEM-T (*pGEM-T* vector system, *Promega*, Madison, USA). For sequencing *CEQ DTCS* quick start kit (*Beckmen Coulter*, Foster City, USA) was used.

RNA isolation and RT PCR: For RNA isolation we used *RNeasy Plant Mini Kit* (*Qiagen*, Hilden, Germany) with modification according to Gehrig *et al.* (2000). RNA has been isolated from leaves and from glandular tissue of hop cones in the same way as Okada and Ito (2001).

For amplification cDNA of CHS 4 primers CHS4NdeI and $GT_{24}W$ were used. RT PCR has been done with *Titan One Tube RT-PCR* kit (*Roche Molecular Biochemicals*, Mannheim, Germany). Quantitative RT PCR reactions were performed in standard conditions but 0.1 mm^3 of [α-^{32}P]dCTP (370 MBq cm^{-3} *ICN Pharmaceuticals*, Irvine, USA) was added to 50 mm^3 reaction volume and number of cycles were reduced to 17. Quantitative reactions were performed with CHS4A1 (5'ATCACAGCTTGTATCTTTCGCG3') and CHS4A2 (5'AGATCAACCCTGGCACGC3') primers.

GeneBank database sequences, computer analyses and other methods: For comparisons we used protein sequence of CHS 2 from alfalfa (AB061021), and nucleotide sequences from hop *vps*, *chs* 2, *chs* 3, *chs*_H1 (AB047593, AB061020, AB061022, AJ304877). Sequence of *chs* 4 is under accession number AJ430353.

Sequence data comparisons were carried out with *OMIGA* software (*Oxford Molecular Ltd.*, Oxford, United Kingdom). All autoradiograms were scanned and quantified using *STORM* device and *ImageQuaNT* software (*Molecular Dynamics*, Sunnyvale, USA).

Results

The identification of sequence and analysis of expression of *chs* 4 gene: We used PCR with degenerate primers CHScons1 and CHScons2 to fish for novel *chs* homologues. The PCR reaction resulted in amplification of 150 bp genomic fragment that was identified by sequencing as part of novel *chs* homologue and

designated *chs* 4. Specific primers for this sequence INV52I and INV52II were designed for inverse PCR amplification. Although the inverse PCR with *Bst*YI-digested and circularized DNA was unsuccessful, we gained direct PCR 4.3 kb product amplified from undigested DNA. The sequencing of the product revealed 5' end of *chs* 4 sequence on the one end of the fragment while on the other end the sequence was identical to 3' end of *vps* (Fig. 1). The complete coding region of *chs* 4 we obtained by RT PCR with primers CHS4NdeI and $GT_{24}W$. We also investigated sequence downstream the 3' end of *chs* 4 gene using INV52II primer. From this single primer reaction we got PCR product having 18 kbp, which was sequenced from both ends. It was found that terminal sequences of product are identical to 3' end of *chs* 4 and 3' end *chs* 3 (Fig. 1). These results show that homological genes are organized in clusters; *chs* 4 gene is surrounded with *vps* at its 5' end in the same orientation and with *chs* 3 sequence at 3' end in inverted orientation (Fig. 1).

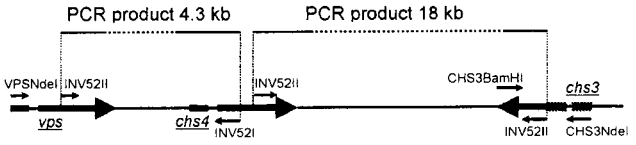

Fig. 1. Scheme of PCR amplification of *chs* 4 flanking sequences. *Black arrows* show position of primers used for PCR amplification. PCR products are shown as *full lines* (sequenced region) and *dotted lines* (non-sequenced). Deduced gene organization is depicted. *Arrows* represent *chs*-like genes and their orientation; introns are represented by the *thin lines interrupting the arrows*. *Doted part of arrows* represents undetermined (non-sequenced) part of *chs* 3 (see text). Scheme is not in scale.

We tried to confirm this gene organization by amplifying full genomic fragment by PCR with CHS3NdeI and VPSNdeI primers, but no PCR product was obtained from this reaction (see the positions of primers in Fig. 1). Surprisingly, we did not gain any PCR product with primers specific to 3' end and 5' end of the coding region of *chs* 3 (primers CHS3BamHI and CHS3NdeI). Because the previous experiments have been done only on Czech hop cultivar Osvald 72, in the next analyses we tested the presence of a complete *chs* 3 sequence by PCR using other cultivars. Positive reactions were detected only in cvs. Magnum and Taurus (Fig. 2), suggesting that *chs* 3 forms pseudogenes in the majority of genotypes.

In order to deduce a possible function of *chs* 4, tissue specific expression of this gene was investigated. *chs* 4 expression was tested by quantitative RT PCR by amplifying a short fragment of chs 4 mRNA with primers CHS4A1 and CHS4A2. It followed from our results that *chs* 4 is specifically expressed in the glandular tissue of hop cones, while no mRNA of chs 4 has been detected in leaves (Fig. 4). Different mRNA concentrations were found in three tested cultivars, suggesting that the expression was influenced during the breeding of hops for high concentrations of bitter acids.

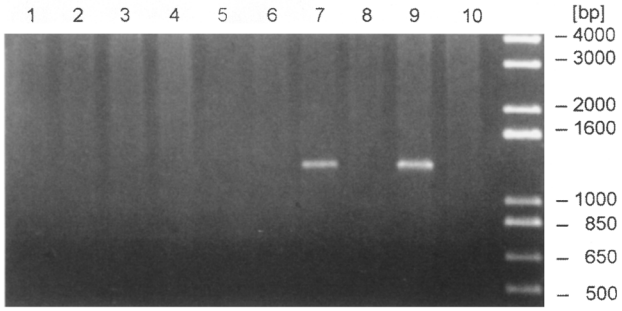

Fig. 2. PCR amplification of *chs* 3 with CHS3NdeI and CHS3BamHI primers from genomic DNA: 1 - Galena, 2 - Target, 3 - Northern Brewer, 4 - Southern Brewer, 5 - *Humulus neomexicanus*, 6 - Sládek, 7 - Magnum, 8 - Premiant, 9 - Taurus, 10 - Osvald 72. 1290 bp product is seen in cvs. Magnum and Taurus.

Because there are no data about the expression of *chs* 2 and chs 3, we tested the expression of these genes in glandular tissue of hop cones for the comparison using RT PCR primer combinations CHS2NdeI × CHS2BamHI and CHS3NdeI × CHS3BamHI for chs 2 and chs 3, respectively. It was found that *chs* 2 is expressed in the glandular tissue too, but this expression was not quantified. We did not detect any *chs* 3 messenger in Osvald 72 (not shown).

Genomic organisation of *chs* homologues: To estimate the amount of *chs* related genes and their genomic variability, the Southern blots with *chs*_H1, *vps*, *chs* 2 and *chs* 4 probes were performed (Fig. 3). The hybridization procedure clearly discriminated between *chs*_H1 and *vps*. Probe for true chalcone synthase gene *chs*_H1 did not cross hybridize with other homologues as can be judged from entirely different hybridization pattern. This sequence hybridized probably only with other homologues of true chalcone synthases, because the Southern blot pattern was quite specific and differed from all other, *vps*, *chs* 2 and *chs* 4 patterns (Fig. 3). Hybridization of *chs* 2 and *chs* 4 is not so clear. Probes *chs* 2 and *chs* 4 did not hybridize with *chs*_H1, but these probes hybridized with fragments at position of *vps* genes, suggesting significant homology of *chs* 2 and *chs* 4 to *vps*. Moreover, some additional genomic fragments appeared on the hybridizing patterns of *chs* 2 and *chs* 4 in comparison to *vps* pattern, this suggest that there are several additional non-identified members of *vps* homological genes subfamily. It is clear that genomic arrangement of *chs*-related genes, namely *chs* 2 and *chs* 4 have been changed during breeding. For instance, only minor differences were found between closely related

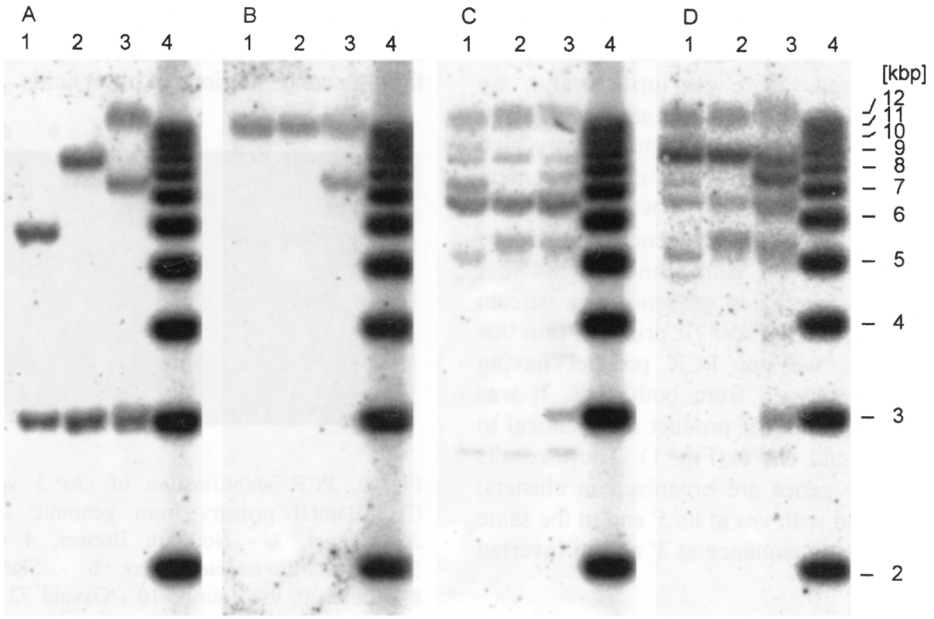

Fig. 3. Southern blot of genomic DNA from hop. *Lanes*: 1 - Osvald 72, 2 - Sládek, 3 - Taurus, 4 - 1 kb ladder (GibcoBRL). Used specific probes: A - *chs*_H1, B - *vps*, C - *chs* 2, D - *chs* 4. DNA was digested with *Eco*RI.

cultivars Osvald 72 and Sládek, whereas more distinctions were found when compared *e.g.* cvs. Taurus and Osvald 72 (Fig. 3).

The comparison of CHS homologues from hop: There is a high homology among all known CHS homologues from hop. From the percentage of identity at nucleotide and amino acid levels (Table 1) is clear that *chs*_H1 is quite different from the rest of homologues. In addition, CHS_H1 is the most identical to CHS 2 from alfalfa characterized by the crystallography (84 % identity at aa level) versus other homologues (67 - 73 % identity). All *chs*-like genes contain short intron in position interrupting predicted codone for cysteine characteristic for chalcone synthase genes. Introns of genes *chs* 3, *chs* 4 and *vps* are 90, 80 and 80 bp long, respectively and share high degree of similarity (48 - 62 % identity) in contrast to introns of *chs*_H1 and *chs* 2 having 187 bp and 115 bp, respectively. This intron similarity indicates common origin of *chs* 3, *chs* 4 and *vps* and suggests that they have been generated by a relatively recent gene duplication event.

Table 1. Homology between *chs* homologues from hop. Identity on the nucleotide level/identity on the amino acid level. Nucleotide sequences were compared from start codone to stop codone.

	CHS H1	CHS 2	CHS 3	CHS4
CHS 2	66 / 67			
CHS 3	69 / 72	82 / 76		
CHS4	69 / 70	81 / 76	83 / 81	
VPS	68 / 73	78 / 72	79 / 77	80 / 77

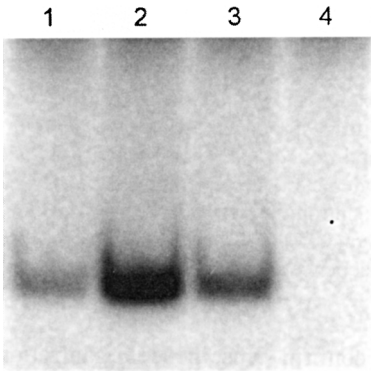

Fig. 4. An example of quantitative RT PCR of RNA from hop(s) cultivars. RNA was isolated from glandular tissue of hop cones. *Lanes*: 1 - Osvald 72, 2 - Sládek, 3 - Taurus, 4 - RNA from leaves of Osvald 72.

In order to predict enzymatic activity, we analyzed protein sequences derived from *chs* genes with respect to amino acid residues that contribute to enzymatic properties of chalcone synthase. Alignment of CHS 2 from alfalfa to CHS homologues is shown in Fig. 5. Except CHS_H1, all other CHS homologues differ in amino acids that play a role in enzymatic action of chalcone synthase. In addition in CHS 2, CHS 3 and VPS we found one insertion D231, D232, D231 respectively and in CHS 4 two insertions S199 and D232 were found in contrast to CHS 2 alfalfa and CHS_H1.

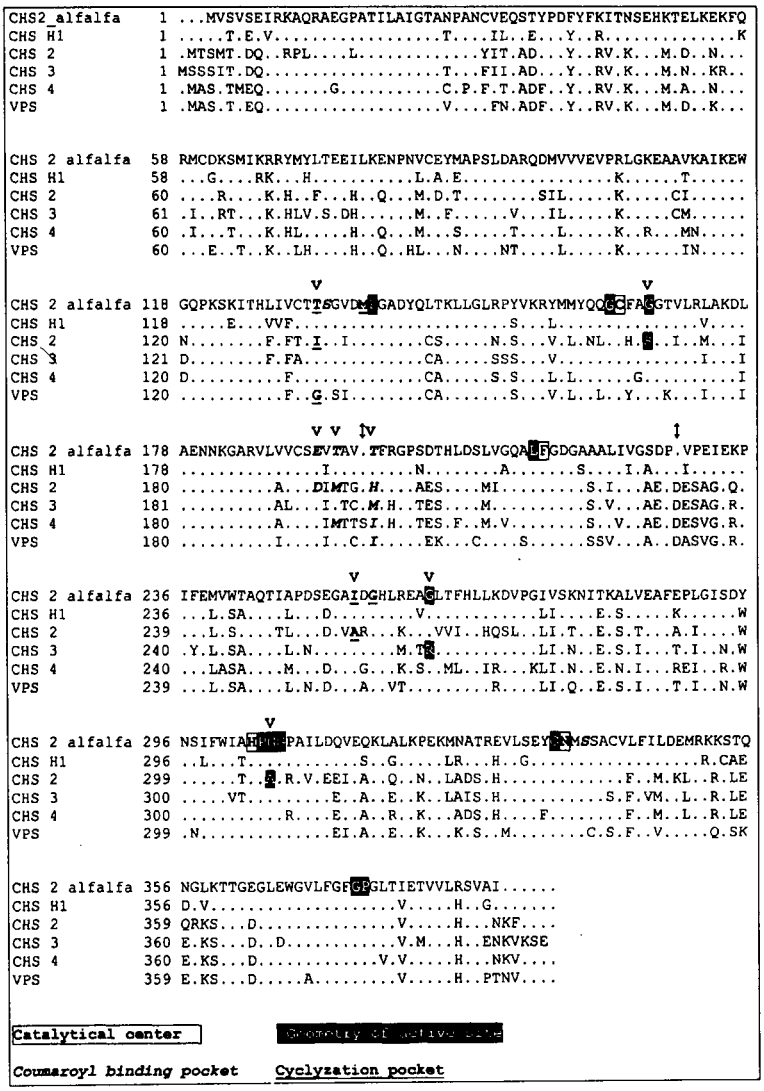

Fig. 5. An alignment of CHS homologue amino acid sequences from hop to alfalfa CHS 2. Only differences to alfalfa CHS 2 are shown. Residues, which participate in the reaction mechanism and the geometry of active site in CHS 2 (alfalfa) are labeled. The marks V indicate changed amino acids that play important role in enzymatic action. The marks ↕ identify insertion(s) of amino acids in comparison with CHS 2 alfalfa.

Discussion

We identified a novel homologue of chalcone synthase-*chs* 4 in hop genome. It was found that this gene is organized in one gene cluster together with *vps* and *chs* 3. Similar gene clusters encoding chalcone synthase have been previously reported only in leguminous plants namely in *Glycine max* (Akada and Dube 1995) in *Pisum sativum* (An *et al.* 1993), in *Trifolium subterraneum* (Arioli *et al.* 1994) and in *Phaseolus vulgaris* (Ryder *et al.* 1987).

In this identified cluster, only *vps* and *chs* 4 was found to be expressed in hop cv. Osvald clone 72 in spite of *chs* 3 sequence was also present in this cluster. The absence of *chs* 3 expression is consistent with the finding that *chs* 3 gene is disrupted in most of the tested hop cultivars. Thus, *chs* 3 forms probably pseudogenes in these genotypes. We found that *chs* 4 is specifically expressed in the glandular tissue of hop cones similarly to expression pattern reported for *vps* (Okada *et al.* 2001). This suggests the possibility that the expression of *vps* and other homologues organized in the same cluster are driven from some common promoter elements. For instance, cluster-specific gene expression has been observed previously by Ito *et al.* (1997) for pea *chs* homologues.

Number of *chs*-like genes forming multifamily in hop can be deduced from the Southern blots of different cultivars. It is clear that the multigene family consist of more then five homologous genes which sequences were identified so far. For instance, Matoušek *et al.* (2002a) deduced at least six genes hybridizing strongly to *chs*_H1 specific probe. From the number of weakly hybridizing bands in this study we can estimate at least six additional non-identified *vps*-like genes probably members of homological subfamily. It is not known whether or not all homologous genes are organized in some gene clusters. In addition, the Southern blots document some impact of hop breeding to genomic organization of *chs* homologues.

The crucial question deals with the possible function of described CHS homologues. Although CHS 2, CHS 3 and CHS 4 have not yet been enzymaticaly characterized one can only roughly predict a possible character of substrate specificity of these enzymes from known configuration of catalytical center. Owing to characterized three-dimensional structure of chalcone synthase from alfalfa (Ferrer *et al.* 1999) we were able to identify some important aa changes in CHS homologues that may differ their substrate specificities from the classical chalcone synthase (EC 2.3.1.7). It has been described recently that differential substrate specificity of CHS homologues might be attributed to the relative volume of the active site cavities; tree point mutant (T197L, G256L, S338I) of alfalfa CHS 2 alter activity from CHS to pyrone synthase (Jez *et al.* 2000). Similarly, triple mutant of acridone synthase yielded to enzyme with chalcone synthase activity (Lukačin *et al.* 2001). From this point of view, in enzyme with valerophenone synthase activity VPS only two point mutation T132/G134 and T197/I199 are localized in important residues, which form the geometry of the active site, active center, coumaroyl binding site and cyclization pocket (Ferrer *et al.* 1999). In CHS homologues we found also mutated residues. CHS 2, CHS 3, and CHS 4 have mutated residues H199, M200, and I199, respectively at position of T197 of chalcone synthase and in addition, in CHS 4 an amino acid insertion (S198) was found at position neighbouring T197. Residue T197 in alfalfa is localized in coumaroyl binding pocket and influences its volume cavity (Jez *et al.* 2000). Taking together, we can assume that the substrate specificity of recently described homologues CHS 2 and CHS 3 including CHS 4 is more close to valerophenone synthase-like homologue than to true chalcone synthase. If so, then one can assume the involvement of *chs* 2, 3 and 4 genes in synthesis of bitter acids or other homologous compounds of lupulin. Although an additional experiments are needed to confirm this hypothesis, the described sequences can serve as a molecular marker for breeding of hop or as a basis for genetic modifications to improve properties of hop plant.

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