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Study of Molecular Markers for Xanthohumol and DMX Contents in Hop (*Humulus lupulus* L.) by QTLs Mapping Analysis

Recently, a medicinal usage of hop metabolic compounds is of increasing interest, mainly prenylflavonoids: xanthohumol (X) and desmethylxanthohumol (DMX). Quantitative trait loci (QTL) mapping is a good tool for finding reliable molecular markers for prospective breeding. We used STS, SSR, EST-SSR and AFLP molecular markers for analysing 116 individual female F1 hop genotypes. A total of 106 markers were used to construct the genetic linkage maps for female parent Taurus and male parent H06/14. In QTL analysis, 14 putative QTL molecular markers for xanthohumol content, 8 putative QTL molecular markers for DMX content and 8 putative QTL molecular for bitter acid content were detected. A QTL marker for an allele of the O-methyltransferase (omt1) gene confirmed the negative correlation between xanthohumol and DMX contents caused by final methylation. The other QTL molecular markers for alleles of chalcone synthase-like genes (vps, chs3, and chs4), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (ispF), geranylpyrophosphate synthase (gpps) and leucoanthocyanidin reductase 1 (lar1) genes showed the interconnection of bitter acids, terpenes and other polyphenols biosynthetic pathways. The reliability of QTL markers was proven by correlation analyses in 68 world hop cultivars.

Descriptors: humulus lupulus L., AFLP, EST-SSR, STS, SSR, correlation and linkage analyses, QTL mapping, polyphenols

1 Introduction

Hop (*Humulus lupulus* L.) is a dioecious, perennial and climbing plant. Only female plants are cultivated for commercial use, mainly in the brewing industry and to a smaller extent for pharmaceutical purposes. Female inflorescences, referred to as cones, contain hop bitter resins, essential oils, polyphenols and tannins. Hop bitter acids are the most important beer bittering agents, but they are also interesting from a medicinal perspective in view of their wide variety of reported biological activities, including antimicrobial, anti-inflammatory, and cancer chemopreventive activities [1].

Very perspective medicinal compounds are prenylflavonoids: desmethylxanthohumol (DMX) and xanthohumol (X), the most abundant in fresh and properly preserved hops [2]. Hop prenylchalcones show a broad spectrum of inhibitory mechanisms at the initiation, promotion and progression stages of carcinogenesis [3] and they are isomerized to isoxanthohumol (IX) and a mixture of the prenylflavanones, 8-prenylnaringenin (8-PN), and 6-prenylnaringenin (6-PN), respectively, in the brew kettle [4].

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Several structural genes of prenylflavonoids biosynthesis are known [5, 6], with hop O-methyltransferase (OMT1) performing the final reaction step (DMX is methylated to X) in the lupulin glands [7]. The expression of structural genes is specifically regulated by several transcription factors: MYB, bHLH, WDR and bZIP [8, 9, 10]. The content of prenylflavonoids is controlled by many genes and is a typical representative of quantitative inherited traits. The developed molecular markers, associated with prenylflavonoids accumulation trait, could help in the selection of superior progenies in breeding programs. Early stage selection is highly desirable, especially in perennial species, and quantitative trait loci (QTL) mapping is a good tool for this purpose. This method has already been used in hop to identify molecular markers for several quantitative traits [11, 12, 13].

In our study, we detected several potential molecular markers for xanthohumol and DMX contents by QTLs genetic linkage mapping analysis, which can be used for marker-assisted selection (MAS) in the future.

2 Materials and Methods

A total of 116 individual female hop genotypes from F1 progeny of crossing: Taurus x H06/14 (Sm06 H14 11/167) and 68 world hop cultivars [14, 15] were used for experiments. Hop plants were cultivated in nursery of the Hop Research Institute in Žatec for three years since 2007. In 2009, hop resins and polyphenols were

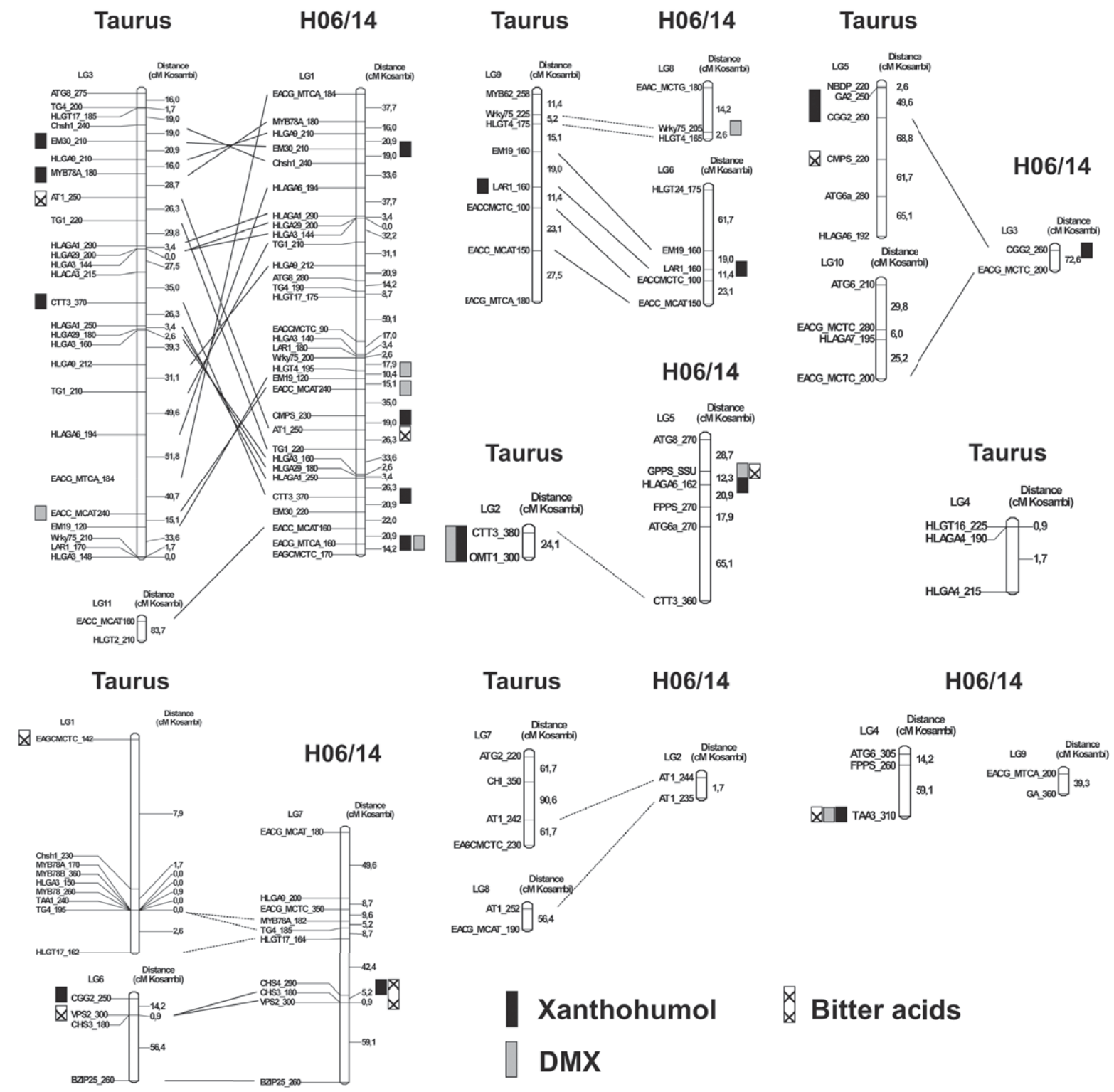

Fig. 1 Female and male genetic linkage maps for F1 progeny of Taurus x H06/14 crossing. Numbers on the right side of the linkage groups indicate genetic distance in centimorgans (cM) and designations on the left side are marker names. Bars indicate the QTL locations.

estimated from dry cones according to the EBC 7.7. method by liquid chromatography (HPLC) on the column Nucleosil RP C18 (Macherey-Nagel, Germany, 5 mm, 250 x 4 mm) using a SHIMADZU LC 20A chromatograph (Shimadzu, Japan) with diode array detectors (DAD) [16]. DNA was isolated from the young leaves of all samples according to Patzak [17] and it was used for molecular genetic analyses.

For molecular analyses, we used sixteen SSR [18, 19, 20, 21], thirty-two STS and EST-SSR [14, 22], loci and seven AFLP primer combinations [17, 23]. In a typical PCR reaction (Taq PCR master mix kit, Qiagen, Hilden, FRG) we used the following amplification conditions: 2 min at 94 °C, 35 cycles/ (30 s at 94 °C; 60 s at 54 °C, 90 s at 72 °C); 10 min at 72 °C for STS and EST-SSR molecular markers. Different annealing temperatures and number of cycles were used for SSR molecular markers according to literature [18, 19, 20, 21]. PCR was performed on a TGradient thermocycler (Biometra, Goettingen, FRG). AFLP reactions were carried out

according to Patzak [17, 23]. Amplification products were resolved via 5% denaturing (8M urea) polyacrylamide gel vertical electrophoresis and visualized by silver-staining [17]. The products were scored for the presence or absence in each sample, based on size measured with 20 bp DNA Marker (Bio-Rad, Hercules, CA, USA).

STATISTICA 8.0 CZ (StatSoft, Tulsa, OK, USA) was used for evaluation of chemical analyses data by basic statistic functions and for correlation analyses of detected molecular markers in world hop cultivars. Dominant polymorphic molecular markers were used to construct the genetic linkage map for female and male parents, using RIL Sib-mating strategy. Linkage analysis was performed using a Multipoint v2.1 (MultiQTL, Haifa, Israel). A recombination frequency of 0.3 and LOD score of 2.0 were set as the mapping thresholds. The Kosambi mapping function was used to transform the recombination frequency to genetic distances (cM). MultiQTL v2.6 (MultiQTL, Haifa, Israel) was used to identify and locate QTLs associated with xanthohumol, desmethylxanthohumol (DMX) and bitter acids. The association between phenotype and genotype was investigated by using marker analysis. The percent of variance explained by each QTL was calculated.

3 Results and discussion

3.1 Population genotyping and map construction

In molecular analyses of F1 progeny, 61 polymorphic STS and EST-SSR markers, 36 polymorphic SSR markers and 32 polymorphic AFLP markers were scored with segregation ratios similar to 3:1 or 1:1. From these 129 markers, only 106 markers fitted to the Mendelian segregation ratio. 41 (a0 × 00) and 29 (a0 × a0) markers were used for the construction of the genetic linkage map for female parent Taurus and 36 (00 × a0) and 29 (a0 × a0) markers were used for the construction of the genetic linkage map for male parent H06/14. Constructed genetic maps formed 11 and 9 merged linkage groups (LGs) for recombination frequency lower than 0.3, respectively (Fig. 1). Because the genome coverage by molecular markers was incomplete, we did not achieve identical number of linkage groups with number of hop chromosomes (n = 10). The problem is also that hop is an outcrossing high heterozygosity plant. In previous works, the number of linkage groups ranged around 9 [24, 11] and 10 [12] with limitations from 8 [11] to 14 [13] for female hop map and 7 [12] to 12 [13] for male hop map, respectively. Also compatibility of individual molecular markers and their linkage between different hop linkage maps were difficult to find due to the parental genotypes of the studied progenies: Wye Target [11, 24], Chinook [12] and Magnum [13]. Therefore, identical applied SSR and AFLP molecular markers were not always mapped similarly [11, 13, 24]. This distortion is caused by a segregation of molecular markers during breeding, which we found for SSR and STS markers in Magnum and Taurus [14] cultivars. However, three chalcone synthase-like genes (vps, chs3, and chs4) were mapped together on one linkage group (Fig. 1) similar to Magnum female map [13].

3.2 QTL analysis

The main purpose of genetic linkage mapping was QTL analysis of molecular markers for X and DMX contents in hop cones. The

Table 1 QTL analysis of statistically significant molecular markers for xanthohumol content by MultiQTL v2.6 (MultiQTL, Haifa, Israel).

Linkage group	Marker	Type	LOD	Average content	Phenotypic effect	%
Female map (Taurus)						
LG2	CTT3_380	EST-SSR	7.163	0.799	0.224	28.035
	OMT1_300	STS	2.757	0.772	0.146	18.912
LG3	MYB78A_180	EST-SSR	1.186	0.754	0.115	15.25
	CTT3_370	EST-SSR	1.786	0.841	–0.161	–19.14
	EM30_210	SSR	1.186	0.754	0.115	14.286
LG5	GA2_250	EST-SSR	1.883	0.795	–0.121	–15.22
	CGG2_260	EST-SSR	1.734	0.799	–0.118	–14.769
LG6	CGG2_250	EST-SSR	1.626	0.798	0.114	14.286
LG9	LAR1_160	EST-SSR	1.057	0.765	0.099	12.941
Male map (H06/14)						
LG1	CTT3_370	EST-SSR	1.786	0.841	–0.161	–19.14
	EACGMTCA_160	AFLP	1.695	0.789	–0.114	–14.449
	CMPS_230	EST-SSR	1.355	0.745	0.13	15.25
	EM30_210	SSR	1.186	0.754	0.115	14.286
LG3	CGG2_260	EST-SSR	1.734	0.799	–0.118	–14.769
LG4	TAA3_310	EST-SSR	1.937	0.777	0.123	15.83
LG5	HLAGA6_162	SSR	1.491	0.784	–0.107	–13.648
LG6	LAR1_160	EST-SSR	1.057	0.765	0.099	12.941
LG7	CHS4_290	EST-SSR	1.373	0.791	0.103	13.02

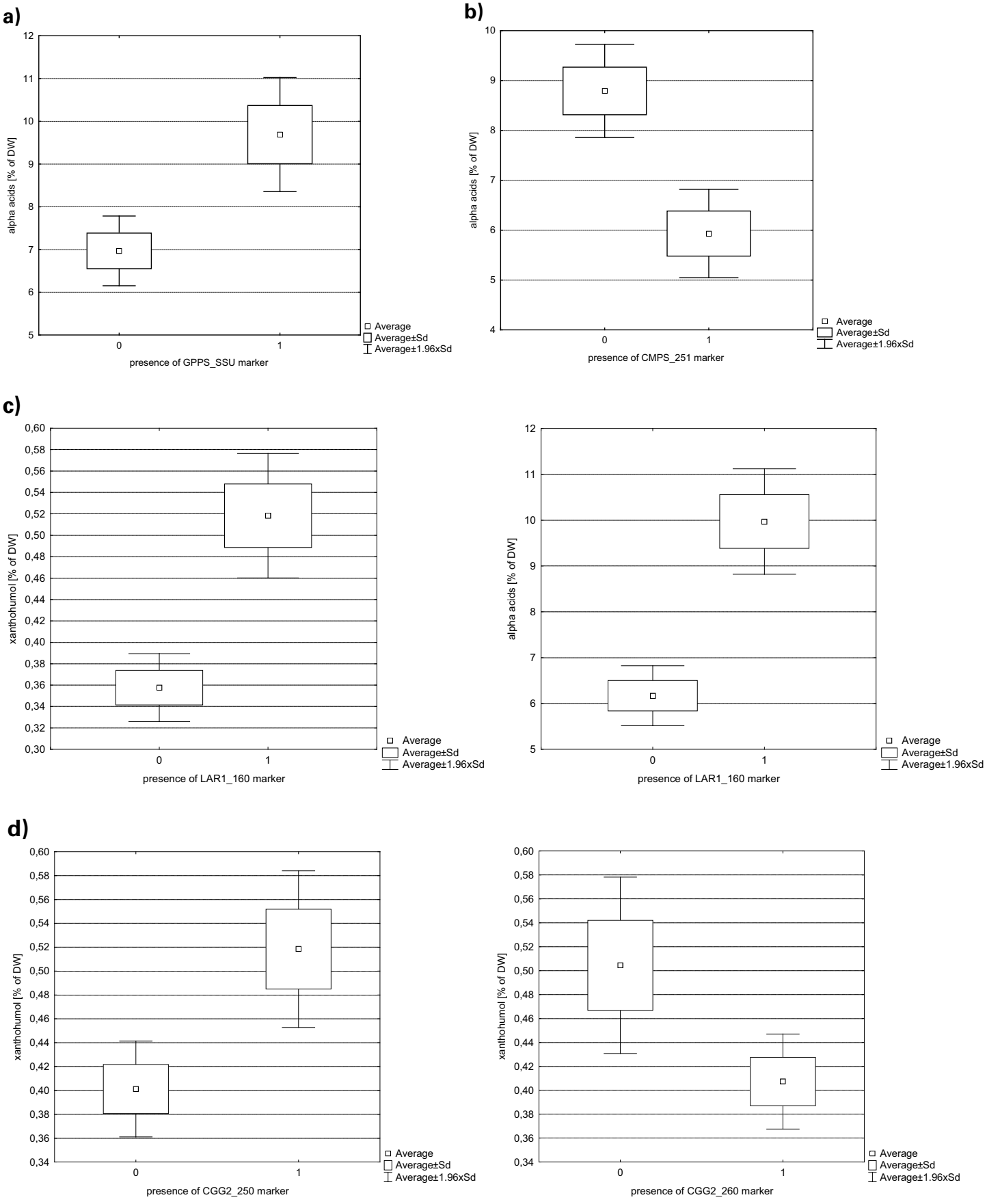

Fig. 2 Box graphs of t-test group statistical analyses: a) GPPS_SSU and bitter acids content, b) CMPS_251 and bitter acids content, c) LAR_160 and xanthohumol or bitter acids content, and d) CGG2_250 or CGG2_260 to xanthohumol content. 0 - absence of marker; 1 - presence of marker.

bitter acid and polyphenol content in hop cones in 116 used F1 progeny plants was used for analysis. The frequencies of observed contents of polyphenols (X and DMX), which ranged from 0.38 % to 1.38 % for X and from 0.02 % to 0.16 % for DMX, showed normal distributions. No correlations for bitter acids – polyphenols and X – DMX were obtained. The QTL analysis confirmed that the content of prenylflavonoids is controlled by many genes with a regulation network and no major genes. 14 putative QTL molecular markers for X content were identified on 5 and 6 LGs of female and male maps. Phenotypic effect of these markers varied from –13.648 to –19.14 % of decreasing and from 12.941 to 28.035 % of increasing, respectively (Table 1, Fig. 1). For DMX content, we found only 8 putative QTL molecular markers on 2 and 4 LGs of female and male maps, respectively (Table 2, Fig. 1). Four of them were similar to QTL molecular markers of X content. Phenotypic variations of QTLs were similar to previous accounted QTLs for alpha-bitter acid content in hop [12, 13]. We also identified 8 putative QTL molecular markers with high phenotypic effect (from –34.06 to 34.06 %) for bitter acid content on 4 LGs of female and male maps, respectively (Table 3, Fig. 1). The usage of gene specific molecular markers enabled us to better understand biosynthetic pathways of secondary metabolites. We confirmed that chalcone synthase-like genes (vps, chs3, and chs4, Table 3) are involved in synthesis of bitter acids as described earlier [25, 26]. *Čerenak* et al. [13], however, did not find any QTL molecular markers in this linkage group of the Magnum female map. The QTL marker for allele of the small subunit of geranylpyrophosphate synthase (GPPS-SSU) was the most positive phenotypic effect on bitter acid content (Table 3). Even though, the gpps gene is involved in the metabolic pathway of terpene synthesis [27], it can also be involved in bitter acids synthesis as prenyl transferase role as aIPT [6].

3.3 Correlation analysis of QTL markers

We have proven the reliability of gene specific QTL molecular markers by correlation analysis in 68 world hop cultivars [14, 15]. Only the results for the best correlated and informative QTL markers are mentioned. For example, the QTL marker GPPS-SSU for bitter acid content was presented in 23 genotypes with high correlation (r=0.38) to bitter acid content (Fig. 2a). In contrast, the QTL marker for allele 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (CMPS_220) with the most negative phenotypic effect on bitter acid content (Table 3) was presented only in 4 genotypes of 68 world hop cultivars. The ispF gene (HQ734721) is involved in the non-mevalonate (MVA) pathway of isopentenyl diphosphates synthesis, which is the main substrate of all biosynthesis in hop cones [7]. Therefore, the next more frequent allele in 68 world hop cultivars has similar phenotypic effect on bitter acid content with a high correlation (r = –0.40, Fig. 2b). The observed results showed that substrate competition in joined branched biosynthetic pathways influenced the content of secondary metabolites in hop cones and lupulin glands. Mainly, the final methylation of DMX to X by omt1 [7] causes a negative correlation of both prenylflavonoid contents. It was confirmed by two close QTL markers for allele of omt1 gene (OMT1_300) and unknown hypothetical gene (CTT3_380) (Table 1, 2). The utilization of these markers in MAS is limited, because they were present only in 14 and 4 genotypes of 68 world hop cultivars with correlations r = 0.2313 and 0.2358 to X content and r = –0.2 and -0.2233 to DMX content, respectively. The next interesting positive QTL marker of X content was the gene specific molecular marker for one allele of leucoanthocyanidin reductase 1 (LAR1_160). This gene (HQ734722) is involved in proanthocyanidin synthesis in polyphenol fractions [28], which follows a prenylflavonoid synthesis, but can be regulated by the same transcription factor complex [10]. We have shown that this molecular marker was presented in 26 genotypes of 68 world hop cultivars with a high correlation to X content (Fig. 2c). We also found a high correlation (r=0.56) to bitter acid content (Fig. 2c). Therefore, lar1 gene can also be involved in the synthesis of bitter acids as an oxidoreductase [25]. The other molecular markers for X content (CGG2_250, CGG2_260) suggested that this hypothetical gene [22] can also be involved in the regulation of X synthesis through its allelic effect (Table 1). CGG2_250 linked to CHS4_290 (Fig. 1), hence this hypothetical gene can be involved in chalcone synthesis an X precursor. These QTL markers had relatively good correlations with X content (r = 0,3129 and –0,2544) in 68 world hop cultivars (Fig. 2d).

4 Conclusions

In the present study, several potential molecular markers for X and DMX contents were detected by the analysis of QTLs genetic linka-

Table 2 QTL analysis of statistically significant molecular markers for DMX content by MultiQTL v2.6 (MultiQTL, Haifa, Israel).

Linkage group	Marker	Type	LOD	Average content	Phenotypic effect	%
Female map (Taurus)						
LG2	CTT3_380	EST-SSR	0.811	0.079	–0.01	–12.658
	OMT1_300	STS	1.118	0.081	–0.012	–14.815
LG3	EACCMCAT_240	AFLP	1.346	0.076	0.015	19.737
Male map (H06/14)						
LG1	EACGMTCA_160	AFLP	2.673	0.08	–0.018	–22.5
	EACCMCAT_240	AFLP	1.346	0.076	0.015	19.737
	HLGT4_195	SSR	1.116	0.076	0.014	18.421
LG4	TAA3_310	EST-SSR	1.274	0.079	0.013	16.456
LG5	GPPS_SSU	STS	0.844	0.08	0.01	12.5
LG8	WRKY75_205	EST-SSR	0.819	0.078	–0.01	–12.821

Table 3 QTL analysis of statistically significant molecular markers for bitter acids content by MultiQTL v2.6 (MultiQTL, Haifa, Israel).

Linkage group	Marker	Type	LOD	Average content	Phenotypic effect	%
Female map (Taurus)						
LG1	EAGCMCTC_142	AFLP	1.142	10.4	3.06	29.42
LG3	AT1_250	EST-SSR	0.762	8.98	2.9	32.3
LG5	CMPS_220	EST-SSR	1.993	10.13	–3.45	–34.06
LG6	VPS2_300	STS	0.858	9.94	2.27	22.8
Male map (H06/14)						
LG1	AT1_250	EST-SSR	0.762	8.98	2.9	32.3
LG4	TAA3_310	EST-SSR	0.513	9.49	1.99	20.9
LG5	GPPS_SSU	STS	2.056	10.08	3.49	34.6
LG7	CHS4_290	EST-SSR	0.919	10.08	2.36	23.4
	CHS3_180	EST-SSR	0.715	9.96	2.08	20.9
	VPS2_300	STS	0.858	9.94	2.27	22.8

ge map. The QTL marker for the allele of the O-methyltransferase (omt1) gene confirmed a negative correlation between the X versus DMX contents, but its usage is limited to Taurus progenies. The other gene specific QTL molecular markers confirmed that the content of prenylflavonoids is controlled by many genes within a regulation network and is combined with substrate competition with bitter acids, terpenes, and other polyphenol biosynthetic pathways. QTL molecular markers for alleles of 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (ispF), geranylpyrophosphate synthase (gpps), and leucoanthocyanidin reductase 1 (lar1) may be suitable for MAS of prenylflavonoids and bitter acid contents in breeding.

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