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Genetic basis of barley contributions to beer flavor☆

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ABSTRACT

Barley malt is critical for the malting, brewing, and distilling industries, as it is one of the main ingredients of beer and some types of spirits. There is growing evidence that barley genotype - via malt - can impact the flavors of beers and spirits. However, information on the barley genes involved in these flavors is lacking. Therefore, we used quantitative trait locus (QTL) mapping of malt quality traits, beer sensory descriptors and metabolic compounds on a biparental population of doubled haploids derived from the cross of the cultivars Golden Promise and Full Pint. Putative candidate genes for QTLs were identified by alignment with the reference barley genome sequence. There were thirty-seven QTLs across all chromosomes except 4H, with three QTL clusters located on 3H (1 cluster) and 5H (2 clusters: mid-5H and end-5H). Those "hotspots" contained QTLs for multiple phenotypes. Several candidate genes that regulate plant metabolism were identified within the QTLs and included *HvAlaAT*, *HvDep1*, *HvMKK3*, *HvGA20ox1* and *HvGA20ox2*. These genes are involved in seed dormancy and plant height. Alleles at these loci, and perhaps at physically linked loci, can have key downstream effects on malting quality, beer flavor, and abundance of volatile metabolites.

1. Introduction

Barley (*Hordeum vulgare* L.) is the main cereal grain used in the malting and brewing industries, as well as in distilling. Barley malt provides critical starches and enzymes to the brewing process, which in turn provides the necessary sugar and nutrients to yeast for fermentation in order create the end-products, which are typically beer (Paynter and Young, 1996) and/or spirits. Modifications to any step of the malting process can alter the overall malt flavor, with the largest driver being kilning, resulting in the wide range of base and specialty malts used to make different styles of beer. A growing body of evidence – based on base malts and pale lager/ale style malt-forward beers brewed from them – indicates that barley genotype can make significant contributions to beer flavor (Bettenhausen et al., 2018, 2020; Craine et al., 2021; Herb et al., 2017a, 2017a, 2017a; Kyraleou et al., 2021; Morrissy et al., 2021; Windes et al., 2020). The environment can modulate the effects of these genes – a source of barley variety "terroir". Kyraleou et al. (2021) for example, reported differences in flavor of spirits attributed to where the barley was grown.

The assessment of contributions of genotype and environment to flavor is an area of recent research; historically, the suitability of barley varieties for brewing has been based on a suite of malt quality parameters. Organizations around the world set the acceptable standards and approve new malting varieties. Notable examples include the American Malting Barley Association (AMBA), the Brewing and Malting Barley Research Institute (BMBRI), Barley Australia, the Canadian Malting

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Barley Technical Center (CMBTC), and the European Brewery Convention (EBC). These organizations have strict guidelines and lengthy testing requirements for barley varieties to be approved and recommended for malting and brewing. AMBA, for example, uses pilot malting evaluations followed by plant (commercial) scale malting and brewing trials. A barley variety is recommended only if ratings are satisfactory at all stages. However, a barley may have satisfactory malt specifications but fail at later stage, commercial-scale brewing trials due to negative flavors. Therefore, to date, selection for barley flavor has been a defect elimination process, rather than a process designed to identify and promote positive flavors. Negative attributes may be due to flavor components of barley grain, flavors developed during malting that are not associated with current malting quality attributes, and/or flavors developed in brewing as a result of interactions with hops, yeast and other beer ingredients. The defect elimination strategy does provide evidence for flavor contributions from the barley genotype and/or the production environment. If these factors can contribute negative flavor attributes, could they potentially contribute positive flavor attributes? Ultimately, all causes of differences in beer flavor - be they positive or negative - between barley genotypes, and production environments, will have a genetic basis. Identifying these genes will require systematic assessment of all possible phenotypes that could contribute to beer flavor. A starting point, for barley, is the suite of malting quality parameters, such as kernel weight and plumpness, malt extract, wort color and protein, barley protein, and various enzyme parameters.

Barley malting quality traits, as well as their regulatory genetics, have been the subjects of intense study (Fang et al., 2019; Han et al., 1997; Igartua et al., 2000; Mather et al., 1997; Mohammadi et al., 2015; Muñoz-Amatriaín et al., 2010; Potokina et al., 2004; Szűcs et al., 2009). Malt quality *per se* is a complex meta-phenotype, and target values for critical component attributes will vary depending on the intended use of the malt – all-malt brewing, adjunct brewing, or distilling. In general, well-modified malts with high levels of malt extract are desirable for all end uses. Modification is a term widely used in malting and brewing to describe a malt with the optimum balance of starch and protein-related factors for the intended end-product. Enzyme-related parameters – such as α-amylase activity, diastatic power and free amino nitrogen (FAN) - will vary between end uses. Lower enzyme levels are desired for all-malt brewing; higher enzyme levels are required for adjunct brewing; and even higher levels are targeted for grain distillers' malts. Genes encoding key enzymes have been cloned and the bases of allelic variation described (reviewed in Shewry and Darlington, 2002). These include loci encoding α-amylases, β-amylases, β-glucanases, β-glucosidases, and limit dextrinases (Bamforth, 2009; Evans et al., 2010; Knox et al., 1987; Muthukrishnan et al., 1984; Erkkilä et al., 1998; Clark et al., 2003; Kreis et al., 1988; Han et al., 1995; Litts et al., 1990; Tibbot and Skadsen, 1996; Burton et al., 1999). These enzymes are important in the breakdown of starches which are utilized during fermentation and brewing. However, known-function and candidate genes are lacking for many other important malting quality traits – and these are typically only reported as quantitative trait loci (QTLs) (Mohammadi et al., 2015).

The degree of modification, and resulting malting quality profile, is driven by the grain's ability to germinate, and to germinate uniformly. Germination characteristics - which can be further broken down as germinative energy, capacity, and water sensitivity - are key gateway characteristics that are assessed on a sample of prospective malting barley prior to malting (Briggs, 1978). Seed dormancy lies at one extreme of the germination continuum. Pre-harvest sprouting (PHS) susceptibility lies at the other end of this continuum. Between these extremes lie the degrees of dormancy, which may be key drivers of traits affecting malting. The hormones abscisic acid, gibberellin, ethylene, and auxin play key roles in seed dormancy (Corbineau et al., 2014; Ishibashi et al., 2017; Li et al., 2004; Liu et al., 2013). Briefly, abscisic acid induces and maintains dormancy (Corbineau et al., 2014; Ishibashi et al., 2017); gibberellins coordinate the release from dormancy so the seed can germinate (Corbineau et al., 2014; Ishibashi et al., 2017; Li et al., 2004); ethylene inhibits abscisic acid while also stimulating the biosynthesis of gibberellin (Corbineau et al., 2014); and auxin plays a role in seed dormancy by stimulating abscisic acid action, therefore promoting dormancy (Liu et al., 2013).

Dormancy, and the degree dormancy, have been areas of intensive and extensive research in barley, leading to the identification of qualitative and quantitative genetic determinants (Edney and Mather, 2004; Hori et al., 2007; Li et al., 2004; Nakamura et al., 2016, 2017; Prada et al., 2004). Two of the most important QTLs are *SD1* and *SD2*, located, respectively, in the centromeric region (mid) and long arm (end) of chromosome 5H (Nakamura et al., 2017). *Alanine aminotransferase* (*AlaAT*) has been identified as the causal gene for *SD1* (Sato et al., 2016). *Mitogen Activated Protein Kinase Kinase 3* (*MKK3*) was the first gene reported to be responsible for *SD2* (Nakamura et al., 2016), and this gene has been validated in subsequent reports (Mao et al., 2019; Shorinola et al., 2017; Vetch et al., 2019, 2020). Nagel et al. (2019) reported that a gibberellin oxidase gene involved in dormancy alleviation (*HvGA20ox1*) maps to the *SD2* region as well. *MKK3* and *HvGA20ox1* are located ~1600 kb apart based on the Morex V2 reference genome (Monat et al., 2019). The distal end of the long arm of chromosome 5H is one of the key "hotspots" for barley malting quality QTLs (Fang et al., 2019; Igartua et al., 2000; Mather et al., 1997; Mohammadi et al., 2015). Therefore, it is tempting to speculate that the candidate gene(s) for *SD2* are involved in dormancy, degree of dormancy, and PHS.

Whereas tremendous progress has been made in elucidating the genetic basis of both agronomic and malting quality traits, information on the genes determining the contributions of barley to beer flavor is lacking. There are strong anecdotal opinions in the malting and brewing community – both for and against the contributions of barley to beer flavor: a key piece of evidence in favor is the persistence of older varieties in the market due to their perceived unique contributions to beer flavor. These varieties range from heirlooms, such as Chevalier and Bere to more recent varieties that do not have competitive agronomic and malting quality profiles, such as Golden Promise, Maris Otter, and Klages (Mallet, 2014). Genetic analysis requires harder evidence than opinions and sales figures. Recent experimental work has established the necessary foundation. Herb et al. (2017b) provided the first clear evidence that there is a genetic basis for the barley contribution to beer flavor. Sensory descriptors were notably different between parental varieties (Golden Promise and Full Pint) and variation for sensory attributes was observed in a sample of 34 doubled haploids derived from the cross (referred to as the Oregon Promise population). The conclusion that there is a genetic basis to flavor was based on estimates of heritability and preliminary estimates of marker:trait relationships. The malts upon which this research was based were generally under-modified – an unavoidable confounding factor when experimental genotypes and varieties of historical interest are micro-malted in batches using protocols designed for assessing contemporary and future malting varieties. Therefore, Herb et al. (2017a) specifically addressed the impact of degree of modification on barley genotype contributions to beer flavor and concluded that even with intentional under-modification and over-modification, there is a genetic contribution of the barley to beer sensory attributes. Bettenhausen et al. (2020) extended this flavor assessment to larger-scale malting and brewing on a subset of three Oregon Promise doubled haploids, confirmed differences in flavor, and identified the top-rated doubled haploid for release as the variety "Oregon Promise" in 2020.

The Bettenhausen et al. (2020) work also included metabolomics, a powerful tool that is used to better understand the chemical composition of a sample. Since the focus is on the sensory attributes of the beer, in this case volatile metabolites are of the most interest. Volatiles are the aromatic compounds contributing to flavor perception. Bettenhausen et al. (2018) first applied metabolomics to answer questions about the effect of malt source on beer flavor and flavor stability. They demonstrated that malt sources (location grown, maltster) did have an impact on beer flavor/flavor stability and metabolite variation that could

account for flavor differences among beers. Subsequently, metabolomics has been applied to the assessment of beers made from Oregon Promise selections (Bettenhausen et al., 2020) as well as beers and hot steeps made from currently available winter malting varieties and experimental spring varieties (Windes et al., 2020). Morrissy et al. (2021) extended the analysis pipeline of pilot malting, brewing, sensory, and metabolomics of hot steeps and beer to assess contributions to beer flavor in doubled haploids derived from crosses of Maris Otter with contemporary varieties.

There is evidence, therefore, that (i) barley genotypes can differ in their contributions to beer flavor, (ii) there is a genetic basis to these contributions, and (iii) differences in sensory attributes and metabolite profiles are not simply due to the degree of modification of malt and/or differences in beer analytics. In this report, we build on these findings by providing an integrated and comprehensive analysis of the genetic basis of malting quality, beer sensory traits, and beer metabolites. Specifically, we expand the scope of inference on the Oregon Promise population (Herb et al., 2017b) via genetically characterizing 236 doubled haploids from the Oregon Promise population, using a high density genotyping array, (Barley 50k iSelect SNP array; Bayer et al., 2017) and integrating - via biparental QTL mapping - the genotype data with phenotype data on malting quality, beer sensory, and beer metabolomics. This integration of new findings with a review of the literature on the topic provides a platform for identifying next steps in this exciting area of research.

2. Materials and methods

2.1. Plant material and micro-malting

The development of the Oregon Promise population was described in detail by Herb et al. (2017b). Briefly, the entire population consists of 236 doubled haploids (DHs), developed using the anther culture protocol of Cistué et al. (2003), from the cross of Golden Promise (Foster, 2001) x Full Pint (Verhoeven et al., 2011). The full mapping population was grown at Corvallis, Oregon USA in 2013 and 2014. Grain samples from the 2013 crop were malted at the USDA Cereal Crops Research Unit (CCRU), Madison Wisconsin, USA, following the procedures described by Mahalingam et al. (2021). Analysis methods are per American Society of Brewing Chemists Methods, except for quality score and overall rank. Quality score is a weighted measure of all quality parameters (C. Martens, personal communication). The higher the value, the more suitable the malt for adjunct brewing. The overall rank is the inverse of quality score, where the top ranked sample (1) has the highest quality score. The malting quality data used for quantitative trait locus (QTL) mapping in this report trace to these samples. Sufficient malt from these samples was not available for nano-brewing (see section 2.2). Therefore, grain from the 2014 crop was malted at the CCRU but no malt analyses were conducted in order to have sufficient grain for nano-brewing. One hundred and sixty-two DH lines from the 2014 crop had sufficient grain for malting and subsequent brewing. Samples from the 2014 crop were submitted for malting in August 2015 – approximately one year post-harvest. For the parents, there was not sufficient residual grain from the Corvallis 2013 or 2014 crops for micro-malting and subsequent nano-brewing. Therefore, residual malt from a 2016 experiment grown at Lebanon, Oregon USA (described by Herb et al., 2017b) was used for nano-brewing of Golden Promise and Full Pint.

2.2. Nano-brewing

Nano-brewing was performed at Rahr Malting Co. (Shakopee, MN, USA) using a beer recipe developed by Rahr Malting Co. for the purpose of this project. The method was developed to accommodate the large number of samples and limited amount of malted barley available per sample. Each sample of micro-malt was milled according to the ASBC Coarse Grind Extract method (ASBC Extract Method, *Malt-4*) on a Bühler Universal Laboratory Disk Mill DLFU (Bühler AG, Uzwil, Switzerland). 150 g of milled micro-malt of each sample were divided equally into each of two mash cans, which were prepared to yield 0.47 L of beer per sample. The strike water was prepared with gypsum and CaCl using reverse osmosis (RO) water. 0.45 L of strike water, heated to 65 °C, were added to each mash can with malt. A single infusion mash was employed; the mash cans were maintained at 65 °C for 60 min and stirred using magnetic stir-bars. At the end of the 60-min mash, the mash cans were removed from the bath and weighed. Reverse osmosis water was added to each mash can to standardize the volume of all mashes to 0.45 L before filtering. The contents of each of the two mash cans per sample were poured over Goldtone Reusable Basket Coffee Filters (GoldTone, Pompano Beach, FL, USA) into a single beaker, to separate the wort from grist. Sparge water was prepared by heating two beakers of RO water at 82 °C. The sparge water was then cooled to 77 °C before 0.2 l was added to the grist. Approximately 1 L of wort was collected from each beaker, covered with parchment paper, and heated to 204 °C. Once boiling, the parchment paper was removed and 0.9 g of Fuggle hops, with 4.9% α-acid, were added to each beaker. The aim was ~20 IBUs in the wort post-boil, translating to 10–12 IBUs in the final beer. The beakers were boiled for approximately 60 min each. To clarify the beer, 0.1 g of Irish Moss were added during the final 7 min of boiling. Beakers were then removed from the hot plate, covered with a sterile aluminum foil lid, and transferred to an ice bath for 15–20 min. Samples were swirled periodically until the wort cooled to 18–20 °C. Beakers were then sanitized and placed in a biohood for hot trub settling. After the hot trub settled, wort was poured into autoclaved 1000 mL VWR media bottles (Avantor, Radnor, PA, USA). Specific gravity was recorded and adjusted to 11°P using autoclaved RO water. Yeast was pitched, using serological pipets, directly from White Labs *Pure Pitch* packets (White Labs, San Diego, CA, USA) with a goal of ~7–10 × 10^6 cells/mL. Pitched wort was mixed in the media bottle and incubated at 20 °C for 6–7 days until fermentation was complete. The media bottles of beer were incubated at 1 °C for 24 h and then the beer was poured into 1 L SodaStream bottles (SodaStream, Kefar Sava, Israel) and carbonated. These beers were then ready for laboratory analyses. 11 DH nano-brews were brewed each day, along with a Golden Promise control.

Laboratory analyses included specific gravity, pH, color, alcohol by volume (ABV), and international bitterness units (IBUs). Data were collected throughout the brewing process at mash (pH), pre-boil (specific gravity, pH, and color), post-boil (specific gravity, pH, and color), fermentation (specific gravity and pH), pre-bottle (specific gravity, pH, and color), and bottle (specific gravity, pH, color, ABV, GC-MS, and IBUs) steps. These data are available upon request. The GC-MS used was a Thermo Scientific GENESYS 10S UV–Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). All analyses, excluding sensory, were done following ASBC methods (American Society of Brewing Chemists, 1992).

2.3. Sensory evaluation

Sensory evaluations were conducted at Rahr Malting Co. using a trained sensory panel. Sensory panelist candidates were chosen based on robust brewing knowledge and previous sensory panel experience. These panelists were trained on 30 common beer flavors, including off-flavors, by beers spiked with ~3x the average threshold concentration for each flavor compound. Panelists that were able to correctly identify all the compounds more than 70% of the time were selected for the final panel. In total there were 20 panelists that participated in the sensory data collection for this project. Due to the limited and varying amounts of micro-malt available, 162 DH beers were tasted between 6 and 13 times each. On average, each beer was tasted 12 times.

Sensory assessment was based on the comparison to a reference method. Descriptors were provided for the research beers (beer color, sweet flavor, cereal flavor, malty flavor, honey flavor, caramel/toffee flavor, grassy flavor, fruity flavor, floral flavor, and toasted flavor). All

research beers along with a randomly distributed Golden Promise (brewing control) and Miller High Life (Miller Brewing Company, Milwaukee, WI, USA) (sensory control) were presented to panelists with a unique 3-digit code so they would not know which samples they were receiving. Miller High Life was used as the reference/control beer because the trained panel had the consensus that it contains a relatively neutral flavor profile within the style and has consistent quality control. Panelists were aware of the controls but not their identities. All beers brewed at Rahr were carbonated to similar levels using a SodaStream (SodaStream, Kefar Sava, Israel) and stored at 4 °C in a capped 5 oz cup prior to distribution to the sensory panel. The DH population research beers and the Golden Promise research beers were all tasted the same day they completed brewing. A maximum of 13 samples were tasted each day (11 DHs, 1 Golden Promise, and 1 Miller High Life).

Sensory descriptors were scored on a scale of +4 to −4, where positive numbers indicate more intensity than the sensory reference (Miller High Life) and negative numbers indicate less intensity than the reference, and 0 being the same as the reference. The sensory data were also transformed to a simplifed +1/0/-1 scale (more than/similar to/less than the reference) to check if additional QTLs could be detected. Using that scale, we were able to identify significant QTLs for malty, honey, and grassy flavors. The original +4 to −4 scale was used for QTL mapping of beer color, cereal flavor, and toasted flavor.

2.4. Metabolomics analysis and data processing

After sensory was completed, the remaining beers were shipped frozen in 50 mL Falcon Tubes from Rahr Malting Company (Shakopee, MN) to the Analytical Resource Core – Bioanalysis and Omics laboratory at Colorado State University (ARC-BIO, Fort Collins, CO). Of the 162 original beers, 155 had enough sample to conduct further testing, including the Golden Promise and Full Pint parents.

The contents were then pipetted to 20 mL vials. Headspace Solid Phase Microextraction gas chromatography mass spectrometry (HS/SPME-GC-MS) was used to detect volatile compounds such as ketones, aldehydes, and esters using methods previously described (Bettenhausen et al., 2018, 2020). For instrumental analysis, the samples were first incubated at 65 °C for 5 min, and then the headspace volatiles were extracted at the same temperature by a SPME fiber (DVB/PDMS/CAR 50/30 μm, Stableflex, Sigma-Aldrich) for 20 min, and injected into a DBWAXUI column (30 m × 0.25 mm x 0.25 μm, Agilent) in a Trace 1310GC (Thermo Scientific) coupled to an ISQLT MS (Thermo). The SPME fiber was desorbed at injection port (250 °C) for 3 min, and then at fiber conditioning port (270 °C) for 5 min. The GC inlet was operated under splitless mode during fiber desorption. The oven program started at 40 °C for 4 min, ramped to 240 °C at a rate of 5 °C/min, and a final hold at 240 °C for 0.5 min. Data were acquired under electron impact mode, with full scan of 40–500 amu at a rate of 5 scans/second. Transfer line and source temperatures were held at 250 °C. Samples were not provided in replicates. Pooled QC samples were run every six samples. Data were processed as described by Bettenhausen et al. (2018). Briefly, each sample resulted in a matrix of molecular features generated using XCMS software in R. Samples were normalized, relative abundances were calculated, mass spectra was deconvoluted using RamClust (Broeckling et al., 2014), and then metabolites were annotated by searching clustered features against in house and external libraries (NIST [http://www.nist.gov], Metlin [Tautenhahn et al., 2012; Zhu et al., 2013], the Human Metabolome Database [Wishart et al., 2013], and the Golm Metabolome Database [Hummel et al., 2013]).

2.5. SNP genotyping and linkage map construction

The entire population of 236 DH lines and the two parents were genotyped with the Barley 50k iSelect SNP array (Bayer et al., 2017). Genotyping was performed by the Neogen GeneSeek laboratory (Scotland, UK: https://www.neogen.com/). SNPs were called using the GenomeStudio 2.0 software (Illumina Inc, San Diego, CA, USA). SNPs with >20% missing and/or heterozygous calls were removed, together with monomorphic SNPs. We did not identify highly distorted SNPs in the dataset. Data for each DH line were inspected; seven duplicated individuals were identified and eliminated from the population as well as one line having a high percentage of missing and/or heterozygous calls (22.5%).

The resulting 228 DH lines and 12,458 SNPs were used for linkage mapping. MSTmap (Wu et al., 2008; http://www.mstmap.org/mstmap_online.html) was used, with the following parameters: grouping LOD criteria = 10; population type = DH; no mapping size threshold = 2; no mapping distance threshold: 15 cM; try to detect genotyping errors = no; and genetic mapping function = kosambi. Physical coordinates of iSelect SNPs on the barley reference genome (Morex v2; Monat et al., 2019) were retrieved from BARLEX (Colmsee et al., 2015; http://apex.ipk-gatersleben.de/) and used to name and orient linkage groups.

2.6. QTL analysis and candidate gene identification

QTL mapping of the malting quality, beer sensory, and metabolomics traits was conducted using the mixed model method of Xu (2013) that was implemented in R by Lo et al. (2018). -$\log_{10}$ (p-values) were generated for each SNP. A genome-wide significance cutoff value was calculated based on false discovery rate (FDR) correction (Benjamini and Hochberg, 1995) at $\alpha = 0.05$ and used to identify significant QTLs. The percentage of phenotypic variation attributed to each QTL was calculated as described in Lo et al. (2018). Genotype and malting quality phenotype data were used for QTL analysis of all 228 DH lines. QTL analysis of sensory data was based on 162/228 DH lines; those DH lines without phenotype data were treated as missing values. A subset 155/162 DH lines was used for metabolomics data QTL analysis. DH lines without phenotype data were treated as missing values. QTLs were displayed on linkage groups using MapChart version 2.32 software. QTL naming was based on the format described in Szűcs et al. (2009). Briefly, QTL names start with a "Q", followed by abbreviations for the trait and the population, as well as the chromosome number. Multiple QTLs on the same chromosome are indicated by a period and then the QTL number. Metabolite QTLs have the metabolite abbreviations following the "Q", and then follow the same format for population abbreviation and chromosome numbering.

The physical region of each QTL was determined based on the barley reference genome (Morex V2 [Monat et al., 2019]) and used to identify putative candidate genes in the QTL intervals. Physical positions in Morex V1 (Mascher et al., 2017) were also retrieved.

3. Results

3.1. Phenotypic variation for malting quality, sensory descriptors, and metabolites

All phenotypic data generated in the DH population and the parents are provided in Supplementary Tables 1 and a summary of phenotypes can be seen in Table 1. The malting quality traits barley color, wort β-glucan (BG) and overall rank - together with the sensory traits cereal flavor, malty flavor, and grassy flavor and the metabolites linalool, ethyl hexonate-like, and oxalic acid dibutyl ester - all had higher values in Golden Promise than in Full Pint. Conversely, Full Pint had higher values for the remaining malting quality traits (kernel weight, kernel plumpness, malt extract, wort color, barley protein, wort protein, the ratio of soluble to total protein (S/T), diastatic power (DP), α-amylase (AA), free amino acid (FAN) and quality score), the sensory traits beer color, honey flavor, and toasted flavor, and the metabolites 2-methoxy-4-vinylphenol, and acetic acid, 2-phenylethyl ester. Transgressive segregation was observed for all traits except kernel plumpness.

Overall, the malts are under-modified, as evidenced by the low malt

Table 1
Phenotypic information for the parental lines and the DH population. Mean, standard error, range, and skewness values are shown. Malt quality abbreviations: S/T = soluble/total protein, DP = diastatic power, AA = α-amylase, BG = β-glucan, FAN = free amino nitrogen. Unit abbreviations: mg = milligrams, % = percent, DU = dextrinizing units, ppm = parts per million, a.u. = arbitrary units.

Category	Trait	Golden Promise	Full Pint	DH population			
				Mean	SE	Range	Skewness
Malt quality	Kernel weight (mg)	44.00	49.70	42.58	0.33	32.75–52.47	0.12
	Kernel plumpness (%)	41.00	100.00	91.77	0.62	67–100	−1.16
	Barley color (Agtron)	96.00	43.00	46.53	0.56	31–65	0.00
	Malt extract (%)	77.80	78.10	77.51	0.13	73.67–80.88	−0.03
	Wort color	2.00	2.60	2.13	0.03	1.48–4	1.03
	Barley protein (%)	11.90	13.90	12.78	0.09	10.58–15.98	0.38
	Wort protein (%)	3.64	5.07	4.51	0.06	3.29–6.29	0.42
	S/T (%)	32.20	36.40	36.78	0.45	26.29–49.64	0.33
	DP (°ASBC)	98.00	204.00	137.02	2.08	87.70–221	0.68
	AA (20°DU)	52.10	122.40	77.09	1.77	43.23–126.53	0.32
	BG (ppm)	677.00	421.00	361.25	13.15	48.13–743.72	0.36
	FAN (ppm)	172.00	245.00	179.29	3.58	109.97–284.84	0.37
	Quality score	29.00	42.00	38.84	0.94	13–67	0.39
	Overall rank	199.00	53.00	75.95	3.70	1–156	0.03
Sensory	Beer color (−4 – +4 scale)	−1.13*	−0.50*	−0.59	0.06	−2.36–1.08	−0.11
	Cereal flavor (−4 – +4 scale)	0.88*	0.75*	0.49	0.03	−0.4–1.25	0.00
	Malty flavor (−4 – +4 scale)	0.38*	−0.13*	0.21	0.02	−0.45–1	0.09
	Honey flavor (−4 – +4 scale)	0.38*	0.57*	0.41	0.02	−0.38–1.13	0.13
	Grassy flavor (−4 – +4 scale)	0.75*	0.25*	0.80	0.03	0–1.69	0.27
	Toasted flavor (−4 – +4 scale)	−0.25*	0.00*	−0.04	0.02	−0.71–0.80	0.48
Metabolites	2-methoxy-4-vinylphenol (a.u.)	3,745,686.15*	4,826,833.96*	3,513,127.46	62,018.71	1,917,265–6,797,483	0.51
	Acetic acid, 2-phenylethyl ester (a.u.)	279,516,231.50*	643,562,567.60*	790,741,728.85	19,694,988.91	151,122,999–1,425,190,984	−0.06
	Linalool (a.u.)	6,103,479.69*	5,183,004.04*	4,152,971.83	94,007.80	1,919,898–7,788,532	0.60
	Ethyl hexonate-like (a.u.)	76,978.45*	21,639.55*	35,891.96	2214.40	0.005–104,310	0.34
	Oxalic acid dibutyl ester (a.u.)	23,780,778.51*	21,752,774.06*	23,260,970.58	448,241.33	11,862,902–39,044,261	0.54

* Data from samples grown in Lebanon, OR, USA (2016).

extract and S/T values and the high β-glucan values. There was limited variation for beer sensory descriptors, for which significant QTLs were detected, compared to the sensory reference (Miller High Life). Overall, Golden Promise was rated higher for cereal, malty, and grassy flavors. Full Pint was rated higher for beer color, and honey and toasted flavors.

A total of 543 volatile compounds were detected in the beer samples, 144 of which could be annotated as metabolites. The description of the beer flavor metabolome generated in this study is reported in Supplementary Table 1. Metabolite variation among the beers was detected and is visualized in Supplementary Fig. 1. Of the 144, only 5 metabolites could be associated to a QTL ($p < 0.05$, Table 1). Those metabolites are: 2-methoxy-4-vinylphenol (MVP), acetic acid, 2-phenylethyl ester (PEA), linalool (LOO), a compound with similar, but slightly modified structure as ethyl hexonate (ethyl hexonate-like, EHEXL), and oxalic acid dibutyl ester (DBOA).

All possible pairwise correlations (n = 325) among malt quality, sensory and metabolic traits as well as their respective p-values are shown in Supplementary Table 2. Correlations (positive and negative) between traits ranged from 0 to 0.9; of these, 119 were significant ($p \leq 0.05$). Most of the significant correlations were between malting quality traits. The highest positive correlation was found between FAN and wort protein (0.92; $p = 0.000$), while the highest negative correlation was between overall rank and quality score (−0.98; $p = 0.000$).

Focusing on correlations >0.5 or < -0.5, there were expected patterns of trait relationships for most malting quality traits. For example, barley protein was positively correlated with diastatic power and negatively correlated with malt extract; wort β-glucan was negatively correlated with wort protein and S/T; FAN, wort color, wort protein, S/T, AA, and quality score were all positively correlated with each other; malt exact, S/T, AA, and quality score were also all positively correlated with each other. Quality score was positively correlated with many of the traits listed above (malt extract, wort color, wort protein, S/T, AA, and FAN) and negatively correlated with overall rank. Conversely, overall rank was negatively correlated with the same traits listed for quality score. Of the sensory traits, there were no correlations >0.5 or < -0.5 between sensory traits. Of the five flavor metabolites, the content of DBOA was positively correlated with LOO content.

No correlations were found which included traits from each of the three categories, but beer color was positively correlated with wort protein, S/T, AA, FAN, quality score; and negatively correlated with overall rank. Also, PEA content was negatively correlated with BG (Supplementary Table 2).

3.2. Development of the Golden Promise genetic map

A total of 12,458 polymorphic SNPs and 228 DHs were used to generate a genetic map of the Oregon Promise population. MSTmap (Wu et al., 2008) was the software of choice for genetic map construction, which mapped 12,453 SNPs into 1073 bins across the seven linkage groups representing each of the seven barley chromosomes (Table 2; Supplementary Table 3). The genetic map spanned 1221.76 cM and had an average density of 1 bin per 0.88 cM. There were two large gaps of 31.1 and 30.5 cM on chromosomes 1H and 6H, respectively (Supplementary Table 3). Linkage groups ranged in size from 134.77 cM for chromosome 4H to 212.60 cM for chromosome 5H. The genetic map of the Oregon Promise population together with the SNP information used for its construction is available in Supplementary Table 3.

3.3. Identification of QTLs and putative candidate genes

QTLs were identified for malt quality, sensory, and metabolite traits using the mixed model for QTL mapping of Xu (2013) implemented in R (Lo et al., 2018). These QTLs were distributed across all chromosomes except 4H and include: 21 QTLs for 14 malting quality traits, eight QTLs for six sensory descriptors, and eight QTLs for five metabolic compounds (Fig. 1; Table 3; Supplementary Table 4). The percentage of phenotypic variation accounted for by individual QTLs ranged from low (6.25%) for one of the DP QTLs (*QDp.GpFp-1H*) to substantial (48.3%) for overall rank (*QOr.GpFp-5H*) (Table 3). QTL clustering for traits belonging to different categories were identified on chromosomes 3H and 5H

Table 2
Distribution of SNPs in the Oregon Promise genetic map.

Chr	1H	2H	3H	4H	5H	6H	7H	Total
Markers	1,512	2,284	1,487	1,310	2,145	1,492	2,223	12,453
Bins	143	174	151	128	194	102	181	1073
cM	170.63	190.12	193.94	134.77	212.60	135.92	183.79	1221.76

(mid-5H and end-5H), the end of 5H being the largest QTL hotspot (Fig. 1). Annotated barley genes that fell within each QTL region were identified for all QTLs and are provided in Supplementary Table 5. Details of QTLs and putative candidate genes within each phenotypic category are provided below.

3.3.1. Malt quality

Twenty-one QTLs for 14 malt quality traits were identified on chromosomes 1H, 2H, 3H, 5H, and 7H (Table 3; Fig. 1) and their $-\log_{10}$ (*P*-values) ranged from 3.40 for one of the kernel plumpness QTLs (*QKp.GpFp-3H*) to 16.00 for wort protein, S/T, quality score, and overall rank (Table 3). The percentage of phenotypic variation accounted for by each QTL ranged from 6.25% for a DP QTL (*QDp.GpFp-1H*) to 48.27% for overall rank (*QOr.GpFp-5H*). There were overlapping QTLs for kernel plumpness and barley protein on 3H, which were in close proximity to the kernel weight QTL (Fig. 1). Kernel plumpness, barley color, and barley protein QTLs overlapped at the mid-5H hotspot, while malt extract, wort color, wort protein, S/T, AA, BG, FAN, quality score, and overall rank QTLs clustered at the end-5H hotspot (Table 3; Fig. 1). It is expected that the quality score and overall rank would coincide, and that they would coincide with the QTLs for the traits used to calculate the score.

QTLs contained between 24 and 3903 annotated genes for *QKp.GpFp-3H* and *QBc.GpFp-5H*, respectively, with an average of 540 genes (Supplementary Table 5). Barley genes *HORVU.MOREX.r2.5HG0398940* and *HORVU.MOREX.r2.5HG0397930*, which correspond to *Alanine aminotransferase* (*HvAlaAT*; Sato et al., 2016) and *Dense and erect panicle 1* (*HvDep1*; Wendt et al., 2016), respectively, were identified within the kernel plumpness (*HvAlaAT* and *HvDep1*) and barley color (*HvDep1*) QTLs at the 5H-mid QTL cluster. *HvAlaAT* has been shown to control the length of dormancy, while *HvDep1* is involved in culm elongation and grain size in barley. Gene models *HORVU.MOREX.r2.5HG0447180* and *HORVU.MOREX.r2.5HG0446540*, corresponding to *Mitogen-Activated Kinase Kinase 3* (*HvMKK3*; Nakamura et al., 2016) and *Gibberellin 20-oxidase 1* (*HvGA20ox1*; Nagel et al., 2019), respectively, were contained within the overlapping region of all QTLs except BG at the 5H-end hotspot. Both *HvMKK3* and *HvGA20ox1* are reported to be involved in the regulation of seed dormancy in barley. The relationships of these genes with QTLs for malting quality, and the other two categories of data (sensory and metabolite), are explored in the Discussion. The *Sdw1/Denso* locus, where the determinant gene is *HvGA20ox2* (Xu et al., 2017; Jia et al., 2009), is on chromosome 3H and coincides with the barley grain protein QTL. Two amino acid permease genes (*HORVU.MOREX.r2.3HG0256690* and *HORVU.MOREX.r2.3HG0256700*), with roles in nitrogen remobilization (Kohl et al., 2012), were also identified among the annotated genes in the kernel plumpness and barley protein QTLs (Supplementary Table 5). Putative candidate genes for the singleton malt quality QTLs (Fig. 1; Table 3) were not explored in detail.

3.3.2. Sensory

A total of eight QTLs for six sensory traits were identified on chromosomes 2H, 3H, 5H, and 7H (Table 3; Fig. 1), with $-\log_{10}$ (*p*-values) ranging from 3.52 for cereal flavor to 14.00 for beer color (Table 3). The percentage of phenotypic variation accounted for by each QTL ranged from 6.88% for cereal flavor (*QCe.GpFp-7H*) to 21.36% for beer color (*QCo.GpFp-5H*). Overlapping QTLs for this category were located on chromosome 3H (malty flavor [*QMa.GpFp-3H*] and toasted flavor [*QTo.GpFp-3H*]), which also overlapped with the malt quality QTL for kernel weight (*QKw.GpFp-3H*), and chromosome 5H (beer color [*QCo.GpFp-5H*] and toasted flavor [*QTo.GpFp-5H*]), which were located on the 5H-end hotspot for malt quality and metabolic traits (Fig. 1). The QTL for honey flavor on 5H (*QHo.GpFp-5H*), which does not overlap with any other sensory QTL, is coincident with the malt quality QTLs for kernel plumpness, barley color, and barley protein at the mid-5H cluster (Fig. 1).

Sensory QTLs contained between 1 (*QCe.GpFp-7H*) and 5467 (*QHo.GpFp-5H*) genes, with an average of 792 genes (Supplementary Table 5). Putative candidate genes for the 5H QTLs included those mentioned above for malting quality: *HvAlaAT* (Sato et al., 2016) and *HvDep1* (Wendt et al., 2016) for the honey flavor QTL on the mid-5H cluster, and *HvMKK3* (Nakamura et al., 2016) and *HvGA20ox1* (Nagel et al., 2019) for the beer color QTL located on the 5H-end hotspot (Fig. 1). Among the genes located within the 3H hotspot we can highlight *HORVU.MOREX.r2.3HG0259410*, which encodes an ethylene-responsive transcription factor (ERF). ERFs play crucial roles in plant developmental processes and have been associated with kernel size (Zhang et al., 2020). This region is ~5000 kb from *HvGA20ox2* (Xu et al., 2017). Putative candidate genes for the remaining singleton sensory QTLs were not explored in detail.

3.3.3. Flavor metabolites

Eight QTLs for the accumulation of five flavor metabolites were identified on chromosomes 2H, 3H, 5H, 6H, and 7H (Table 3; Fig. 1). $-\log_{10}$ (*p*-values) ranged from 3.32 for one of the oxalic acid dibutyl ester QTLs (*QDBOA.GpFp-3H.2*) to 7.87 for a 2-methoxy-4-vinylphenol QTL (*QMVP.GpFp-5H*; Table 3). The percentage of phenotypic variation accounted for ranged from 6.71% for *QDBOA. GpFp-3H to* 17.50% for *QMVP. GpFp-5H*.

The metabolite QTLs for 2-methoxy-4-vinylphenol (MVP) and oxalic acid dibutyl ester (DBOA) located on 3H overlapped, but with no other QTL for any category of data (Fig. 1). QTLs for MVP (*QMVP.GpFp-5H*) and acetic acid, 2-phenylethyl ester (PEA) (*QPEA.GpFp-5H*) overlapped on the 5H-end hotspot, where many malt quality and sensory QTLs also colocalized (Fig. 1).

QTLs contained between 1 (*QDBOA.GpFp-3H.1*) and 4429 (*QPEA.GpFp-7H*) genes, with an average of 781 genes. *HvMKK3* (Nakamura et al., 2016) and *HvGA20ox1* (Nagel et al., 2019) are putative candidate genes for the 5H-end hotspot. It should be noted that in the 3H QTL for MVP (*QMVP.GpFp-3H*) there is also a gene (*HORVU.MOREX.r2.3HG0247750*) encoding a cytochrome P450 family cinnamate 4-hydroxylase, which is involved in the synthesis of precursors (cinnamic acids) to 2-methoxy-4-vinylphenol (Harakava, 2005; Gómez-López et al., 2019). Putative candidate genes for singleton metabolite QTLs were not explored further.

4. Discussion

This research generated multiple data sets on a large biparental mapping population, which allows for the first comprehensive look at the genetic basis of barley contributions to beer flavor, together with metabolomic compounds in beer. The sensory and metabolite data sets are anchored in the malting quality data set: malt precedes beer. Unmalted barleys do not display notable flavor or aroma differences: it is the malting process that leads to these differences. Therefore, an analysis of the contributions of barley genotype to beer flavor is inextricably confounded by the style of malt, and how each genotype responds to the

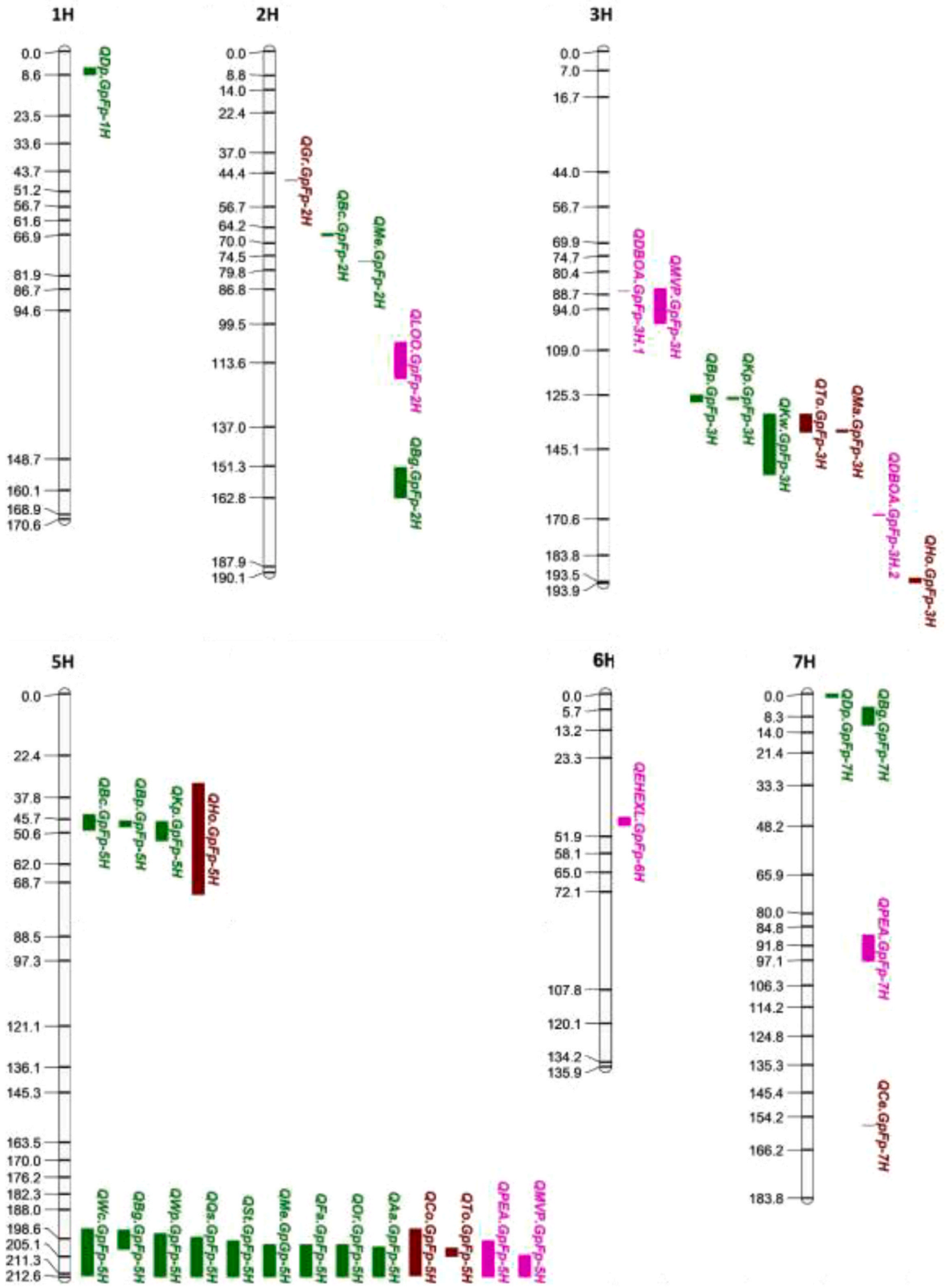

Figure 1. QTL regions shown by chromosome. cM positions are on the left, with only one every tenth position being shown. Malt quality QTLs are represented in green, sensory QTLs are represented in brown, and metabolite QTLs are shown in pink.

malting protocol that was used to make the malt. This focus on malt, of course, does not account for the significant flavor contributions of hops and yeast to the finished beer. In short, the analysis of the contributions to beer flavor made by barley genotypes (via their malt) is a challenging prospect. In the case of this research, it is important to bear in mind that the malting quality data, while based on the same barley germplasm (the Oregon Promise population) grown at the same location (Corvallis, Oregon) and malted at the same facility (the USDA-ARS Cereal Crops

Table 3
QTLs identified for malting quality, sensory, and metabolite traits. The genetic and physical positions are also given, with physical positions based on the barley reference genome Morex V2 (Monat et al., 2019). A positive effect value means the positive allele was contributed by Golden Promise, while a negative value indicates the positive allele was contributed by Full Pint.

Category	Trait	QTL	Peak SNP	Chr.	Position (kb) *	-Log$_{10}$(*P*)	QTL region (cM)	QTL region (kb)*	% Phenotypic variation	Effect	Known gene(s) in QTL
Malt quality	Kernel weight	*QKw.GpFp-3H*	JHI-Hv50k-2016-207525	3H	582,098	4.51	132.30–154.37	577,460–594,378	9.91	1.28	-
	Kernel plumpness	*QKp.GpFp-3H*	JHI-Hv50k-2016-205406	3H	572,529	3.40	126.15–127.03	572,529–573,139	6.64	0.11	-
		QKp.GpFp-5H	JHI-Hv50k-2016-307371	5H	435,709	4.48	46.61–53.63	374,134–446,936	11.06	−2.77	*HvAlaAT1, HvDep1*
	Barley color	*QBc.GpFp-2H*	JHI-Hv50k-2016-98501	2H	492,803	3.77	66.52–67.40	489,367–520,441	13.71	2.92	-
		QBc.GpFp-5H	JHI-Hv50k-2016-301330	5H	349,008	6.95	43.98–49.68	35,704–437,198	25.61	−3.82	*HvDep1*
	Malt extract	*QMe.GpFp-2H*	JHI-Hv50k-2016-103558	2H	563,603	3.52	76.69	563,603–565,975	10.46	0.59	-
		QMe.GpFp-5H	JHI-Hv50k-2016-365534	5H	594,137	12.82	200.76–212.60	588,682–598,994	28.92	−0.86	*HvMKK3, HvGAox1*
	Wort color	*QWc.GpFp-5H*	JHI-Hv50k-2016-362729	5H	590,865	16.00	195.05–212.60	586,795–598,994	32.73	−0.25	*HvMKK3, HvGAox1*
	Barley protein	*QBp.GpFp-3H*	SCRI_RS_103215	3H	572,324	5.16	125.27–127.91	571,521–573,139	10.01	0.36	*HvGA20ox2*
		QBp.GpFp-5H	JHI-Hv50k-2016-301330	5H	349,008	4.09	46.17–48.36	349,008–431,457	15.00	0.51	-
	Wort protein	*QWp.GpFp-5H*	JHI-Hv50k-2016-362943	5H	591,069	16.00	196.81–212.60	587,561–598,994	37.19	−0.42	*HvMKK3, HvGAox1*
	S/T	*QSt.GpFp-5H*	JHI-Hv50k-2016-363828	5H	592,162	16.00	199.44–212.60	588,466–598,994	46.00	−3.57	*HvMKK3, HvGAox1*
	DP	*QDp.GpFp-1H*	JHI-Hv50k-2016-4906	1H	4,865	3.60	5.97–8.60	4798–5592	6.25	6.65	-
		QDp.GpFp-7H	SCRI_RS_161101	7H	1,401	3.92	0–1.32	227 - 2619	7.43	−7.42	-
	AA	*QAa.GpFp-5H*	JHI-Hv50k-2016-363791	5H	592,087	15.00	201.66–212.60	590,717–598,994	42.14	−12.92	*HvMKK3, HvGAox1*
	BG	*QBg.GpFp-2H*	BOPA1_3608-2133	2H	648,278	5.01	151.81–162.78	644,545–655,253	9.56	−52.41	-
		QBg.GpFp-5H	JHI-Hv50k-2016-360298	5H	586,834	3.39	195.49–202.51	586,834–590,865	7.16	45.03	-
		QBg.GpFp-7H	JHI-Hv50k-2016-438742	7H	3,362	3.83	4.83–11.41	3362–7668	7.66	45.63	-
	FAN	*QFa.GpFp-5H*	JHI-Hv50k-2016-362943	5H	591,069	10.00	200.76–212.60	589,596–598,994	42.69	−28.00	*HvMKK3, HvGAox1*
	Quality score	*QQs.GpFp-5H*	JHI-Hv50k-2016-363828	5H	592,162	16.00	198.12–212.60	587,972–598,994	36.87	−6.78	*HvMKK3, HvGAox1*
	Overall rank	*QOr.GpFp-5H*	JHI-Hv50k-2016-364126	5H	592,490	16.00	200.76–212.60	588,682–598,994	48.27	31.21	*HvMKK3, HvGAox1*
Sensory	Beer color	*QCo.GpFp-5H*	JHI-Hv50k-2016-361935	5H	588,466	14.00	195.05–212.16	586,795–598,994	21.36	−0.33	*HvMKK3, HvGAox1*
	Cereal flavor	*QCe.GpFp-7H*	JHI-Hv50k-2016-511500	7H	619,240	3.52	157.32	619,240	6.88	0.09	-
	Malty flavor	*QMa.GpFp-3H*	JHI-Hv50k-2016-207283	3H	581,633	3.71	138.02–138.90	581,663–582,615	7.04	0.04	-
	Honey flavor	*QHo.GpFp-3H*	JHI-Hv50k-2016-225245	3H	625,551	3.85	192.19–193.94	623,023–625,680	7.11	0.04	-
		QHo.GpFp-5H	JHI-Hv50k-2016-284934	5H	19,967	5.20	32.57–73.05	10,652–491,116	7.73	0.04	*HvAlaAT1, HvDep1*
	Grassy flavor	*QGr.GpFp-2H*	BOPA1_816-265	2H	34,276	3.82	47.05	34,276–34,355	7.81	0.05	-
	Toasted flavor	*QTo.GpFp-3H*	JHI-Hv50k-2016-207283	3H	581,633	4.96	132.30–138.90	577,460–582,615	10.35	0.09	-
		QTo.GpFp-5H	BOPA1_6873-531	5H	592,173	3.65	202.07–205.14	590,798–592,247	7.35	−0.08	-

(*continued on next page*)

Table 3 (*continued*)

Category	Trait	QTL	Peak SNP	Chr.	Position (kb) *	-Log$_{10}$(*P*)	QTL region (cM)	QTL region (kb)*	% Phenotypic variation	Effect	Known gene(s) in QTL
Metabolites	2-methoxy-4-vinylphenol	*QMVP.GpFp-3H*	SCRI_RS_146347	3H	528,679	5.54	86.54–99.28	526,926–548,613	11.68	270460.71	-
		QMVP.GpFp-5H	JHI-Hv50k-2016-367564	5H	597,237	7.87	204.70–212.60	592,087–598,994	17.50	−334270.87	*HvMKK3, HvGAox1*
	Acetic acid, 2-phenylethyl ester	*QPEA.GpFp-5H*	JHI-Hv50k-2016-367061	5H	596,380	7.08	199.44–212.60	588,466–598,994	14.04	−94588275.18	*HvMKK3, HvGAox1*
		QPEA.GpFp-7H	JHI-Hv50k-2016-470701	7H	82,589	6.00	87.87–97.52	66,454–503,740	10.70	−84295784.80	-
	Linalool	*QLOO.GpFp-2H*	JHI-Hv50k-2016-113871	2H	611,171	7.12	106.13–119.29	606,789–624,863	10.95	−395681.89	-
	Ethyl hexonate-like	*QEHEXL.GpFp-6H*	JHI-Hv50k-2016-380526	6H	27,476	3.89	44.89–47.97	25,117–28,720	7.09	7591.54	-
	Oxalic acid dibutyl ester	*QDBOA.GpFp-3H.1*	JHI-Hv50k-2016-195050	3H	527,696	3.38	87.42	527,696	7.37	1600930.34	-
		QDBOA.GpFp-3H.2	JHI-Hv50k-2016-215710	3H	602,711	3.32	168.87–169.31	602,711–603,732	6.71	1523937.55	–

Research Unit) using the same methods, traces to samples from a different crop year (2013) than the samples that were malted and used for nano-brewing (2014). Furthermore, the samples of the parents (Golden Promise and Full Pint) trace to a different location (Lebanon, Oregon). This "imbalance" was an inevitable consequence of the timing and scale of the experiment.

4.1. Malt modification and beer flavor

Due to the large number of grain samples, under-modification of malts used in QTL studies is an inevitable consequence of the need to use automated, high throughput malting systems. It is impossible and unrealistic to optimize malting regimes for each individual grain sample. It is particularly difficult in a case such as the current research, where neither parent is amenable to current malting protocols, which are designed for contemporary varieties. Golden Promise is an heirloom variety that continues to persist in the market due to perceived contributions to flavor; Full Pint is a specialty variety that also has perceived contributions to flavor (Mallet, 2014). This leads to the question – are differences in contributions to beer flavor of barley genotypes artifacts of poor modification? Herb et al. (2017a) adjusted for modification differences and found that flavor differences were still present. Bettenhausen et al. (2020) and Windes et al. (2020) also found differences in flavor in a sample of contemporary varieties, where similar degrees of modification were achieved by tailoring malting protocols to the needs of each variety. Likewise, Craine et al. (2021) used bespoke malting protocols to achieve similar levels of modification in a small set of barley varieties/selections of potential interest to the craft industry. Cumulatively, these results point to subtle, but definitive contributions of barley genotypes to beers made from pale malts, despite the degree of modification. A key follow-up question for future research remains: what are the contributions of barley genotypes to beer flavor when higher color malts are made from these varieties?

4.2. Trained panel sensory analysis of nano beers identifies differences in flavor

The nano-beers for this research were produced using a different protocol than that used by Herb et al. (2017b). Furthermore, not all the same sensory descriptors were used in this study as in prior research. In the current study, Golden Promise was rated higher for cereal, malty, and grassy. Full Pint was rated higher for beer color, honey and toasted. In Herb et al. (2017b), Golden Promise was described as significantly higher for floral and fruit, whereas Full Pint was significantly higher for malt, sweet, toasted, and toffee. These commonalities (e.g. toasted) and differences (e.g. malt) between slightly different beer styles brewed from the same two varieties of barley using a different protocol, underscore the challenges of sensory analysis and the importance of beer style and descriptor lexicon in assessing varietal and environmental contributions to beer flavor. In both Herb et al. (2017b) and in the current study, beers brewed from the progeny showed much more variation than those brewed from the parents (Table 1). As with malting quality, the positive and negative transgressive segregants for flavor descriptors in the progeny suggests that the parents have different alleles at multiple loci determining these attributes.

4.3. Metabolite abundance: barley variety signatures in beer flavor?

The relative abundances of the five metabolites varied between Full Pint and Golden Promise. 2-methoxy-4-vinylphenol (a phenol) and phenylethyl acetate (a benzenoid compound) were more abundant in Full Pint. In Golden Promise, ethyl hexanoate (a lipid ester), linalool (a terpene) and dibutyl oxalate (a carboxylic acid) were more abundant. Bettenhausen et al. (2018) and Windes et al. (2020) also reported that Full Pint beers had higher abundances of benzenoid compounds, phenolics, and lipids and a lower abundance of ethyl hexanoate and many

terpenes. Therefore, these relative abundances of metabolic compounds may be useful chemical signatures for specific varieties. Connecting metabolic signatures with sensory attributes, however, can be more challenging. For example, benzenoid compounds, phenolics, and lipids can lead to a fruity/floral/spicy profile and yet Golden Promise beer, rather than Full Pint beer, was described as being higher for fruity and floral attributes by Carpena et al. (2021). Since the fruity and floral descriptors were not significant in the current research, it is not possible to associate them with metabolite abundance. Furthermore, the abundance of certain metabolites, and corresponding flavors, may be due to the interactions of the malt with other components of the finished beer. The higher abundance of 2-methoxy-4-vinylphenol in Full Pint beer could be due to the by enzymatic decarboxylation of the compound ferulic acid by certain strains of *S. cerevisiae* (Coghe et al., 2004). As with malting quality and sensory attributes, the positive and negative transgressive segregants for metabolite abundance in the progeny suggests that the parents have different alleles at multiple loci determining these attributes.

4.4. Phenotypic correlations set the stage for QTL analysis

Many of the correlations between malting quality traits conform to expectations based on prior literature: for example, barley grain protein was positively correlated with enzymatic traits and negatively correlated with malt extract (Xue et al., 2008). The phenotypic correlations between malting quality traits and sensory traits and between malting quality traits and metabolic compounds need to be considered in view of the malting quality data tracing to malts different than those used for brewing. Nonetheless, the negative correlation between wort β-glucan and PEA (-0.55, $p = 0.00$) could merit further investigation. A genetic basis for this correlation is provided by coincident QTLs for these traits at the QTL hotspot located on the 5H-end region, as described below. The positive correlation between malty and toasted (0.46, $p = 0.00$) is also supported by the overlapping QTLs on 3H. The positive correlation of dibutyl oxalate with linalool (0.50, $p = 0.00$) merits further exploration, given the compound is found in both barley and hops. It has a high affinity for calcium and in the context of beer, precipitated oxalate in the beer leads to particulate and haze formation, gushing, and "beer stone," which is particularly a problem in brewing equipment, the latter being responsible for the blocking of beer piping (Oliver, 2012).

4.5. Putative candidate genes for QTL clusters include genes associated with dwarf growth habit and degree of dormancy

Of particular interest, in terms of QTLs and putative candidate genes, are the clusters (hot spots) of coincident QTLs for multiple traits on chromosomes 3H and 5H. There are putative candidate genes for each of these clusters, based on prior literature, and it is also possible that there are multiple physically linked genes that have roles in determining these QTLs. Additional research will be required to identify the actual gene (or genes) determining each QTL.

The 3H QTL cluster is the most diffuse of the three clusters, and further research would be necessary to assign putative candidate genes to the various malting quality, beer sensory, and beer metabolite QTLs. A possible candidate on this chromosome is the *Sdw1/Denso* locus, where the determinant gene is *HvGA20ox2* (Xu et al., 2017; Jia et al., 2009) and Full Pint has the recessive (dwarfing allele). The *Denso* locus is within the barley protein QTL, where the higher value allele was contributed by Golden Promise. The wild type allele, in this case, was associated with higher grain protein, a trait in malting barley that has an upper limit, depending on beer style. For adjunct malts, 12% is the maximum; lower levels are required for all malt brewing. In addition to pleiotropic effects on grain protein, *Denso* alleles are known to affect a range of other agronomic traits (reviewed by Kuczyńska et al., 2013). Additional research is required to determine if, in the Oregon Promise population, *Denso* is also the determinant of the QTLs for kernel plumpness, kernel weight, and malty flavor, and toasted flavor. If it is, it would be a positive pleiotropic effect of the wild type allele, as Golden Promise has higher value alleles at these QTLs. Golden Promise also contributes the higher value alleles for malty, honey and toasted flavors, as well as for two volatile metabolites (MVP and DBOA) at QTLs distal to *HvGA20ox2*. In addition to *HvGA20ox2*, there are other genes in this 3H QTL region that could have impacts on malting and flavor traits. These include *HORVU.MOREX.r2.3HG0259410,* which encode ethylene-responsive transcription factors, and *HORVU.MOREX.r2.3HG0256690* and *HORVU.MOREX.r2.3HG0256700*, encoding two amino acid permeases. Ethylene stimulates the biosynthesis of gibberellin, a hormone that releases seeds from dormancy (Corbineau et al., 2014). ERFs have also been found to impact kernel size, which would logically also have an impact on kernel weight as well as the protein content of the kernels, as larger kernels tend to have lower protein content (Magliano et al., 2014). Amino acid permeases are involved in nitrogen remobilization (Kohl et al., 2012); nitrogen availability and supply impacts grain protein content, which may affect many malt quality traits (Guo et al., 2019). Assuming that higher levels of all the 3H QTL phenotypes (except perhaps grain protein) are positives, from a breeding standpoint it would seem desirable to maintain the positive relationships by selecting for a large block of this chromosome region with Golden Promise alleles. Interestingly, the variety "Oregon Promise", which was top rated for flavor by a consumer panel (Bettenhausen et al., 2020) has a 100% Full Pint haplotype at all alleles for the chromosome 3H QTLs (Supplementary Table 3). Assuming linkage, rather than pleiotropy, additional research will be required to determine if the agronomic advantages of the *Denso* allele from Full Pint can be combined, via recombination, with the potentially favorable alleles for other traits from Golden Promise.

Potential candidate genes for the mid-5H QTL cluster are *HvDep1* and *HvAlaAT1*. The former is a dwarfing gene – the *Ari-e* locus. Golden Promise has the loss of function dwarfing allele (*ari-e.GP*). Full Pint has the wild type (functional) allele. The Golden Promise allele, the result of an induced mutation, was a breakthrough in reducing plant height and lodging. *HvAlaAT1* is the determinant of *SD1*, a major dormancy gene (Sato et al., 2016). Allele resequencing shows that Full Pint and Golden Promise are identical at the causal SNP in *HvAlaAT1* (Sweeney et al., 2021). While it is possible that regulation of the structural gene could account for differences in dormancy, with pleiotropic effects on malting and sensory traits, this leaves *HvDep1* as the most obvious likely candidate. The *ari-e. GP* allele has negative pleiotropic effects on thousand grain weight and grain length (Wendt et al., 2016). This supports our detection of a QTL for kernel plumpness, with Full Pint contributing the positive (favorable) allele, and barley protein, with Golden Promise contributing the higher value (generally unfavorable) allele. In this same QTL cluster, Full Pint has the higher value and positive allele for grain color; brighter grain has a higher Agtron score. There are no reports of pleiotropic effects of *ari-e. GP* on grain color. Further research is warranted, following a possible lead from rice, where protein and seed color are positively correlated (Tan et al., 2001). Golden Promise contributes the positive allele for honey flavor. Further research is necessary to determine the basis of this QTL, which may relate to grain protein level and sensory panel perceptions. Storage proteins are important in all cereals for the embryo once germination occurs, and these proteins typically have high amounts of the amino acid, proline (Fox, 2010). Although not directly associated with honey flavor, proline has a sweet flavor (Sorensen and Sammis, 2004), which may be chemically altered during the malting process to be similar enough (along with other metabolic factors) to be perceived as a honey flavor by a sensory panel, but further exploration is needed. From a breeding standpoint, moderate grain protein and plump kernels are desirable. Therefore, in this population, the *denso* dwarfing allele on 3H (tracing to Full Pint) would be more favorable than the *arie*-dwarfing allele on 5H. However, selection for the wild type allele at 5H would compromise selection for the coincident honey flavor QTL, where Golden Promise contributes the

favorable allele. Interestingly, the Oregon Promise variety has Full Pint alleles at all markers in this this QTL region (Supplementary Table 3). Perhaps, if honey flavor is desirable in beers, the 3H QTL allele (tracing to Golden Promise) is sufficient.

A putative candidate gene for the end-5H region QTLs is *HvMKK3*, the most cited determinant gene for *SD2* (Nakamura et al., 2016). Full Pint has the most non-dormant *HvMKK3* allele (MKK3_N*) (Sweeney et al., 2021). However, *HvGA20ox1* may also have role in these QTL, as proposed by Nagel et al. (2019). Full Pint and Golden Promise have contrasting alleles at both *HvMKK3* and *HvGA20ox1,* but according to Sweeney et al. (2021) *HvGA20ox* is not a determinant of dormancy and malting quality in North American spring barley germplasm. Precedent for malting quality QTL coincident with the *SD2* locus are provided by Castro et al. (2010), who used a biparental population with Full Pint as a parent and reported that the most QTL, and QTL with the largest effects, were found on the long of chromosome 5H at a location coincident with as *SD2*. In terms of validation, Oregon Promise has Full Pint alleles at all markers in end-5H QTL region (Supplementary Table 3). Castro et al. (2010) also reported QTLs for dormancy and water sensitivity in the *SD2* region – with Full Pint contributing the non-dormant and non-water sensitive alleles. In the current research, absolute dormancy was not encountered, because grain was malted one year after harvest. Furthermore, pre-harvest sprouting and water sensitivity were not observed. As argued by Vetch et al. (2019) and Sweeney et al. (2021), the effects of *SD2* on malting quality traits can be in terms of degree of dormancy. If a lower degree of dormancy is equated with higher germination rate and metabolic activity, then we would expect Full Pint to achieve a greater degree of modification. Indeed, Full Pint has higher value alleles for malt extract, wort protein, S/T, AA, FAN, quality score, and the lower value allele for BG.

5. Conclusions

This work represents an important first step towards integrating malting quality, beer sensory, and metabolomics via an understanding of the determinant genes/QTLs. The data presented herein support that morphological traits (e.g. semidwarf growth habit) and seed physiology traits (e.g. dormancy) may have profound downstream effect of malting quality, beer flavor, and metabolite abundance. QTL data indicate potential causal relationships between beer flavor outcomes and the genes determining malting quality and volatile metabolites. Our results lay the groundwork for future genetics and breeding research, including (i) editing of candidate genes to determine flavor outcomes and (ii) marker assisted selection for key QTL haplotypes in other genetic backgrounds. Further research is also warranted in malting and brewing sciences involving the same genotypes, or subsets thereof. These could include (i) different malt styles (ii) different beer styles, and/or different growing environments.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

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