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Genome-wide association studies reveal QTL hotspots for grain brightness and black point traits in barley

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ABSTRACT

Grain kernel discoloration (KD) in cereal crops leads to down-grading grain quality and substantial economic losses worldwide. Breeding KD tolerant varieties requires a clear understanding of the genetic basis underlying this trait. Here, we generated a high-density single nucleotide polymorphisms (SNPs) map for a diverse barley germplasm and collected trait data from two independent field trials for five KD related traits: grain brightness (TL), redness (Ta), yellowness (Tb), black point impact (Tbpi), and total black point in percentage (Tbpt). Although grain brightness and black point is genetically correlated, the grain brightness traits (TL, Ta, and Tb) have significantly higher heritability than that of the black point traits (Tbpt and Tbpi), suggesting black point traits may be more susceptible to environmental influence. Using genome-wide association studies (GWAS), we identified a total of 37 quantitative trait loci (QTL), including two major QTL hotspots on chromosomes 4H and 7H, respectively. The two QTL hotspots are associated with all five KD traits. Further genetic linkage and gene transcription analyses identified candidate genes for the grain KD, including several genes in the flavonoid pathway and plant peroxidase. Our study provides valuable insights into the genetic basis for the grain KD in barley and would greatly facilitate future breeding programs for improving grain KD resistance.

1. Introduction

Grain kernel discoloration (KD), sometimes known as weather staining, is a common quality defect in cereal crops such as barley [1,2], wheat [3,4], and rice [5]. In barley and wheat, this defect mainly manifests in three forms: the discoloration of whole grain from bright color to deep brown or grey color, the darker discoloration at the embryo end of the grain (referred as black point), and the most severe form: greyish hue or visible spot that is often caused by fungal infection [2,4]. The

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most common KD form is the whole grain discoloration, followed by black point, whilst the last form is less prevalent. Grains affected by KD tend to have reduced desirable flavours, varied protein content and lower seed germination rate and seedling vigour [1]. Moreover, cereal grains with KD above a certain threshold can result in quality down-grading in the market due to reduced customer acceptability, and thus causes substantial economic losses [3,4,6].

Both genetic and environmental factors are believed to affect the occurrence of grain KD. Pre-harvest environmental conditions such as rain, humidity, and temperature have been observed to lead to discoloration symptoms such as black point [7,8]. Earlier studies suggested that tolerance to grain KD is genetically inherited, and it has been reported that germplasms of barley and wheat exhibit quantitative genetic variation in KD tolerance [9–11]. Quantitative trait loci (QTL) contributing to grain KD tolerance have been reported in a number of studies using bi-parental mapping. QTL mapping of KD in barley using self-developed double-haploid (DH) and other crossing populations identified QTL on different chromosomes [2,12,13]. Based on the genetic mapping in three different populations, a recent study identified a putative QTL with major effect and a minor QTL for KD on chromosomes 6H and 2H, respectively, in barley [1]. However, the KD trait in the above-mentioned study referred to the discoloration at the whole grain level only. Meanwhile, the black point, which is characterized by the brown-black discoloration at the embryo end of barley or wheat caryopsis, is regarded as a significant defect in grain quality. Several QTL located on multiple chromosomes have been identified for black point in barley [7,11,14] and wheat [10,15]. The genetic basis of grain KD, including whole grain discoloration and black point, has been tentatively discussed. Several studies have shown that grain KD is significantly associated with the total polyphenol and anthocyanin content of the grain [9,16,17]. Candidate genes responsible for flavonoids production in barley grain have been characterized, which include both the structural genes [18,19] and transcription factors [20–22] in the flavonoid biosynthesis pathway. Several studies have provided evidence that the occurrence of black point is likely due to the enzymatic oxidation of polyphenol by plant peroxidases (PPO) instead of fungi infection [8,23].

Genome-wide association analysis (GWAS) is a widely used analytical tool to identify genetic loci associated with important agronomic traits, thus GWAS allows the identification of novel beneficial alleles from germplasm collection [24]. Genome-wide association studies have been used in identifying genetic variants associated with crop agronomic traits, and have delivered significant insight into the genetic control of those traits [25–27]. In barley, GWAS has been used to study flowering time [28,29], grain yield [30,31], disease resistance [32,33], and tolerance to various abiotic stress factors including drought [34,35], frost [36], salinity [37], and acid soil [38]. In addition, several GWAS studies have also been successfully performed on traits associated with grain quality, such as protein content and malting-related factors in barley [39,40].

Grain KD has been the focus in a number of barley genetic studies [1,2,13]. QTL analyses in those previous studies were often based on bi-parental mapping, limited to only a small fraction of allelic diversity being examined. In this study, we aim to identify QTL regions and novel alleles controlling the grain KD trait in barley using GWAS analysis. We assessed a large collection of barley germplasm for five grain KD related traits: grain brightness, redness, yellowness, and two parameters associated with black point in multiple environments. We performed GWAS analyses on these traits using a high-density single nucleotide polymorphisms (SNPs) map, which led to the identification of significant QTL associated with barley grain KD. We verified the associations of SNP and phenotype using a test population with traits value collected in a separate environment using genomic prediction approach. Finally, we discussed the candidate genes underlying the identified QTL that are related to grain KD in barley.

2. Materials and methods

2.1. Barley germplasm, trial environments and weather conditions

A total of 632 barley accessions with diverse geographic origins were used to map the QTL associated with the grain brightness and black point traits. These barley lines were field-grown at two different geographic locations: Katanning (KAT) and Geraldton (NHT) in Western Australia during the 2015 growing season. The climatic conditions of the growing year and planting information were given in Table S1.

Field trials were established in a randomised complete block design with plots of 1.1 m by 5 m laid out in a row column format with partial replication (about 30% of entries were replicated). Seven control varieties were used for spatial adjustment of the experimental data. The plots were cut back to ~3 m length with pathways spray of 1 m on both ends of the plots. All the grains in the plots were harvested from the middle 1.1 m × 3 m plot area and cleaned with Pfeuffer SLN3 seed cleaner (Pfeuffer, Germany) fitted with 2.2 mm sieve size. Trait data were analysed using mixed linear model analysis to determine Best Linear Unbiased Predictions (BLUPs) for each trait for further analysis.

2.2. Phenotyping barley grains for grain brightness and black point

About 50 g of grain sample from each line were used for the measurement of the grain brightness, which was carried out using a Minolta CR-310 chromometer with a CR-A33e projection tube and using a D65 light source setting. Grain brightness associated traits were recorded in Minolta CIE L – grain brightness (T_L), CIE a – redness (T_a), and CIE b – yellowness (T_b). The black point trait was measured using SeedCount image system (Graintec) to record both percent severe and percent mild (T_{bpi}, black point impact, a value calculated based on the occurrence of black point at different severity; T_{bpt}, black point total in percentage). The trait data were subjected to statistical analysis, including analysis of variance (ANOVA) and pairwise correlation of phenotypic traits with PAST 3.0 [41]. Significance was taken at $P < 0.05$. The effect of diseases (e.g. fungal infection) on KD is neglectable due to the strong light and dry growing conditions in our field trials.

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2.3. SNP genotyping by whole genome sequencing

Genomic DNA for each accession was extracted from the leaf at the three-leaf stage. Whole-genome shotgun (WGS) sequencing libraries were prepared following the instruction of the Illumina sequencing library kit (Paired-End Sample Preparation Guide, Illumina, 1,005,063): genomic DNA was fragmented into less than 800 bp using the nebulization technique. The resulted 5′ and 3′ overhangs were repaired using T4 DNA polymerase and 3′ to 5′ exonuclease, followed by 3′-end "A" addition, adapter ligation, PCR-enrichment, and library validation. Libraries were sequenced on the HiSeq 2500 platform (Illumina, Foster City, CA, USA). Sequencing library preparation and sequencing were performed at Beijing Genomic Institute (Shenzhen, China). An average of 3.5 GB sequence data was obtained for each of the 652 barley samples.

SNP calling from the WGS sequencing data was conducted following the best practice protocol recommended by the Broad Institute (https://software.broadinstitute.org), which includes four steps: raw reads filtration, reads mapping, SNP calling, and SNP filtration. Nucleotide bases with low quality in the raw reads were trimmed using 'Trimmomatic' (Version 0.39) [42]. Clean reads were mapped against the barley genome reference (Version 1.0, https://webblast.ipk-gatersleben.de/barley_ibsc) using BWA-MEM (Version 0.7.15) [43]. Uniquely mapped reads were used for SNP calling using GATK (Version 3.8) [44]. SNP variants with mapping quality less than 40, calling quality less than 60 and supporting read number less than 3 were removed. Additional 4260 SNP genotype through target enrichment sequencing of 174 phenology genes for the same population [28] was also incorporated to the SNP marker map. SNPs across all 632 samples with <10% missing values and a minor allele frequency > 1% were retained, making a final number of SNPs of 30,543.

2.4. Genetic heritability analysis and genome-wide association studies

We employed a genome-based restricted maximum likelihood method (GREML-LDMS) to compute the narrow-sense SNP-based heritability (h^2_{SNP}) for each trait. GREML-LDMS corrects biases stemming from linkage disequilibrium [45]. We first computed linkage disequilibrium (LD) scores between SNPs with the block size of 100 kb using GCTA [46], then used GREML (a function within GCTA) to calculate the proportion of variance in a phenotype explained by the SNPs following an LD score regression [45]. We further estimated the genetic correlation between each pair of traits followed the Bivariate GREML procedure using GCTA [46,47].

To identify SNPs that are associated with a particular seed brightness or black point trait, we used a Factored Spectrally Transformed Linear Mixed Model (FaST-LMM) of GWAS analysis, as FaST-LMM can effectively eliminates the influence of genetic similarity between samples [48]. We first reformatted the genetic data into the binary Plink input file format (*.bed, *.bim, and *.fam) using Plink 2.0 [49], then calculated the first five principal eigenvectors from principal components analysis (PCA) using GCTA [46] and used them as covariates in the model as fixed effects in the association analysis in order to account for population structure. GWAS analysis was conducted using program FaST-LMM [48] following the developers' instruction (https://github.com/fastlmm/FaST-LMM/). We used Bonferroni Correction with a significance threshold at P <0.05 to determine significant SNPs. Based on the previous linkage disequilibrium decay calculation (~3.13 cM) in barley [38], the associated SNPs within a close physical distance (<20 Mb) on each chromosome were defined as a QTL region. A genomic region was defined as a KD QTL hotspot if the QTL was identified for all five individual KD trait within 100 Mb.

To verify the effect of SNPs on each trait as revealed in the above-performed GWAS analysis, we test a genomic prediction model following a machine learning procedure [48]. The prediction model considered the effect of each SNP in the dataset, while gene interactions were not modelled. We used the entire SNP dataset and trait value of all accessions in the NHT trial (537 accessions) as the training data. The test data consisted of 30 samples with SNP profile and trait values recorded in a different environment (Gibson, WA; Table S1). The genomic prediction was implemented using program FaST-LMM [48].

2.5. Candidate gene identification

The public barley reference genome annotation dataset (Version r1, https://webblast.ipk-gatersleben.de/barley_ibsc/downloads/) was used for the genome-wide survey of putative candidate genes related with the grain brightness and black point traits. Representative amino acid sequences for the Kyoto Encyclopedia of Genes and Genomes (KEGG) enzyme lists of the phenylpropanoid pathway (KEGG: map00940), flavonoid biosynthesis pathway (KEGG: map00941), and anthocyanin biosynthesis pathway (KEGG: map00942) were used as Blastp query against the barley genome. Candidate genes with the same functional annotation for the top Blastp hit were selected. The putative MYB, MYC, WD40, F3′H, and F3′5′H candidate genes were obtained from previous studies [18,19,50]. The obtained candidate genes and the identified QTL were plotted into a single genetic map using the MapChart tool [51].

2.6. Gene transcription data mining

The transcriptional data of the candidate genes were extracted from the BARLEX expression database (https://apex.ipk-gatersleben.de/apex/f?p=284:10::::::) [52]. The average expression value in FPKM for three biological replicates was calculated. The gene expression level in different tissues was normalized based on the individual gene. The transcriptional heat-map was generated using PAST 3.0 [41].

2.7. Genetic variation identification and marker design

Genetic variations within the candidate genes were obtained from the barley genomic variation database (http://146.118.64.11/BarleyVar/), which was identified based on the whole-genome resequencing data of 21 barley accessions (thirteen commercial varieties, four wild barley from Israel, two wild

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barley from Tibet of China, and two barley landraces). SNP and InDel variants are predicted based on their positions in the annotated gene models and their effects on the coding products through SNPeffect (http://snpeffect.vib.be/). For primer design, template sequences around the InDels in the candidate genes were retrieved from the barley reference genome. Primer design was performed using the primer3 tool (Version 4.0) with the default parameters [53].

3. Results

3.1. Phenotypic variation of kernel discoloration traits

A collection of 632 barley accessions grown independently at two different geographic locations Katanning (KAT) and Geraldton (NHT) was used for the GWAS analysis of the KD traits. After filtration for missing data, 546 and 537 barley lines with complete phenotype data for the five KD traits (T_L, T_a, T_b, T_{bpi}, and T_{bpt}) were obtained for the KAT and NHT trials, respectively (Table S2). The five KD traits displayed considerable phenotypic variation across the germplasm lines at both trials, implying a diverse genetic pool for genetic mapping (Fig. 1). The tolerant and the sensitive barley accessions for the five target KD traits can be found in Table S3. Phenotype comparison showed that T_a, T_{bpi}, and T_{bpt} are significantly different ($P < 0.05$) between KAT and NHT trials, whereas not for T_L and T_b (Fig. 1), suggesting T_a, T_{bpi}, and T_{bpt} may be more affected by environmental factors than T_L and T_b.

To test whether each of the five KD traits may correlate with each other, we conducted a pairwise regression analysis. Results showed that T_L is significantly correlated with T_a, T_{bpi}, and T_{bpt}. T_{bpi}, and T_{bpt} are also correlated (Fig. 2). These observations suggest that both grain redness and black point negatively affect grain brightness. In contrast, T_b seems to be an independent parameter and is not correlated with any of other parameters.

Results were shown for the KAT trial only. Trend lines were fitted if the correlation is significant at $P < 0.05$.

3.2. Genetic heritability

To measure how much the genetic variation contributes to the phenotypic differences of the grain discoloration, and to assess the relative effects of environmental conditions on the target traits, the narrow-sense SNP-based heritability (h^2_{SNP}) of each of the five KD traits was calculated (Table 1). Grain redness T_a displayed the highest heritability (73.74%), followed by T_L (66.93%) and T_b (57.67%), suggesting a clear genetic effect on these traits. In contrast, the two black point parameters T_{bpi} and T_{bpt} showed relatively lower heritability at 18.59% and 20.91%, respectively. The relatively weak heritability of black point indicates that this trait may be more vulnerable to the effect of environmental conditions.

3.3. Genome-wide association analysis for KD traits

A set of 30,543 SNP markers was used for association analysis on the grain KD traits, which corresponds to an average marker spacing of 170 kb. The number of markers on each chromosome ranges from a minimum of 3127 SNPs for chromosome 4H to a maximum of 6054 SNPs for chromosome 7H. After applying statistical threshold ($P < 1.64 \times 10^{-6}$, significant at $P < 0.05$ with Bonferroni Correction for multiple tests) for marker-trait association, a total of 103 SNPs (T_L: 27, T_a: 9, T_b: 40, T_{bpi}: 23, and T_{bpt}: 4) and 164 SNPs (T_L: 39, T_a: 40, T_b: 18,

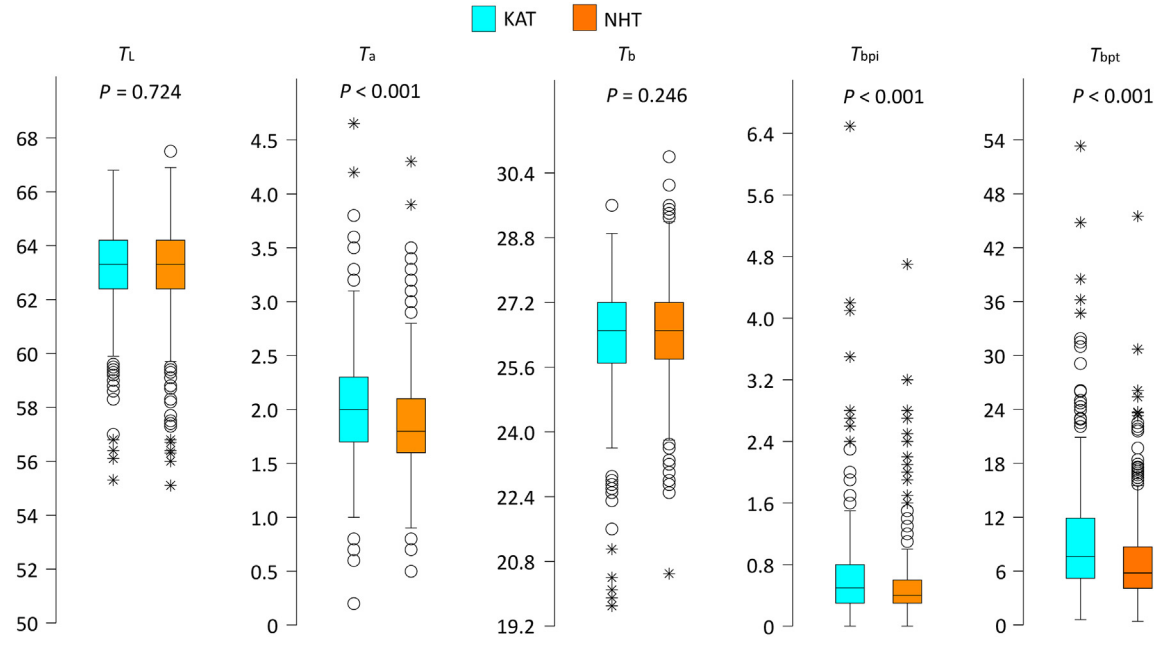

Fig. 1 – Phenotype distribution of the five KD traits in two trials (KAT and NHT). T_L, grain brightness; T_a, redness; T_b, yellowness; T_{bpi}, black point impact value; T_{bpt}, black point total (percentage). T_L, T_a, and T_b are values read from a Minolta CR-310 chromometer.

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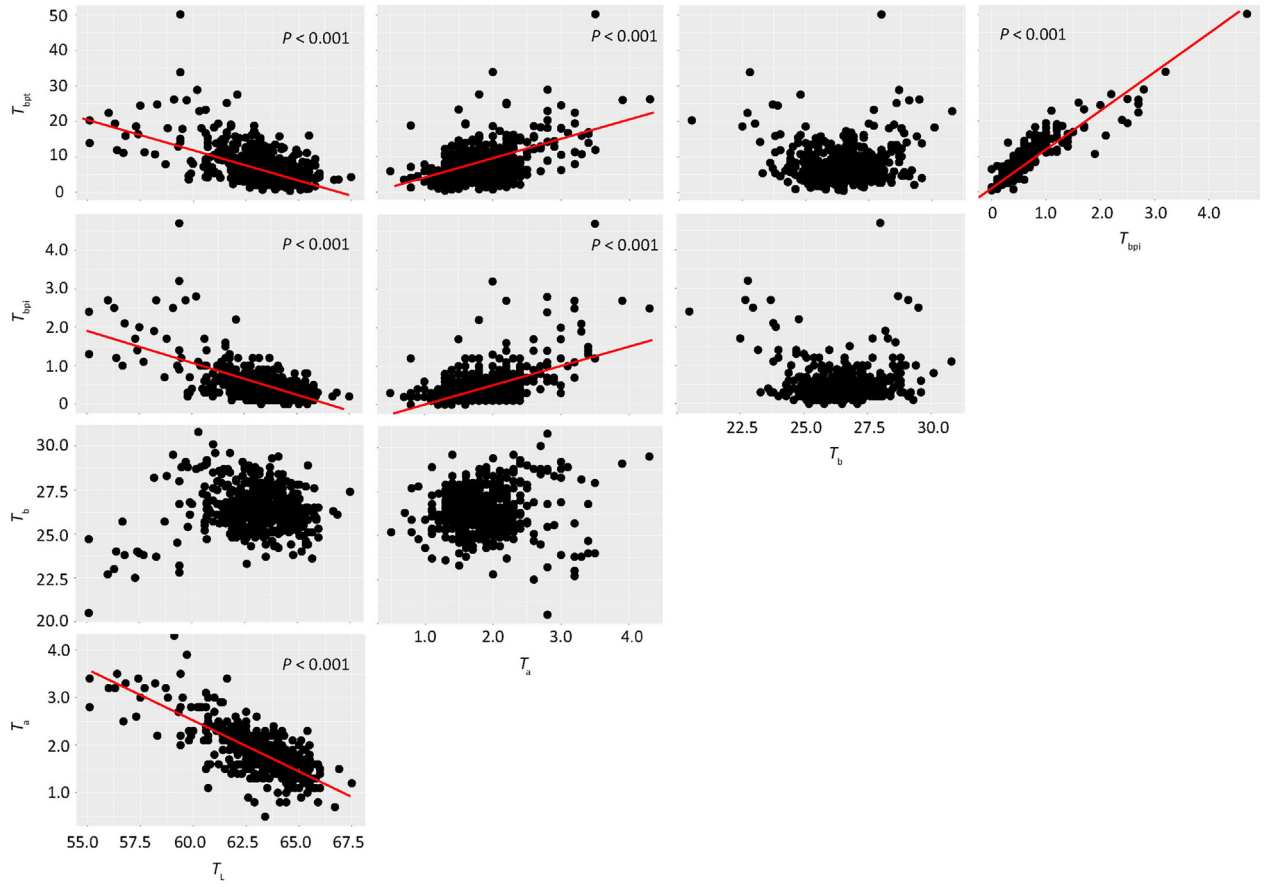

Fig. 2 – Pairwise correlation of the five barley grain KD traits.

T_{bpi}: 53, and T_{bpt}: 14) were identified as being significant for the KAT and NHT trials, respectively (Fig. 3, Table S4). The phenotype of barley lines with specific SNP combinations that were identified as significantly associated with the black point traits were presented in Fig. 4. Generally, the different combinations of these SNP markers could consistently differentiate the most sensitive haplotypes to the black point traits. This led to the identification of 37 QTL altogether at the two trials (Fig. 5, Table S4). At the NHT trial, a major QTL hotspot (QTL32, QTL33, QTL34, QTL35, and QTL36) spanning approximately 100 Mb on chromosome 7H was identified for all the five KD traits (Fig. 3). This QTL hotspot was also detected in the KAT trial, though only associated with T_L and T_b. Another significant QTL hotspot (QTL12, QTL13, QTL14, QTL15, QTL16, and QTL17) on chromosome 4H was associated with four KD traits at the KAT trial, with the exception of T_b (Fig. 3). This QTL hotspot was also associated with T_a and T_{bpi} at the NHT trial.

In addition to the major QTL, several minor QTL were also detected more than once for different KD parameters (Fig. 5). These include QTL31 on chromosome 7H, which was detected for T_L at both KAT and NHT trials. QTL31 was also associated with T_b at the KAT trial, and with T_a at the NHT trial. QTL29 and QTL30 on chromosome 7H were detected specifically for the black point traits (T_{bpi} and T_{bpt}) at both trials. QTL5 for T_{bpt} was detected on chromosome 5H at the NHT trial.

Table 1 – SNP heritability and pairwise genetic correlation between the five KD traits.

	h^2_{SNP}	T_L	T_a	T_b	T_{bpi}
T_L	0.669 ± 0.109				
T_a	0.737 ± 0.108	−0.813 ± 0.034			
T_b	0.577 ± 0.107	0.115 ± 0.067	0.035 ± 0.061		
T_{bpi}	0.186 ± 0.058	−0.716 ± 0.043	0.807 ± 0.050	−0.303 ± 0.097	
T_{bpt}	0.209 ± 0.059	−0.788 ± 0.048	0.690 ± 0.061	−0.050 ± 0.098	0.975 ± 0.007

h^2_{SNP}, narrow-sense SNP heritability; T_L, grain brightness; T_a, grain redness; T_b, grain yellowness; T_{bpi}, black point impact value; T_{bpt}, black point total.

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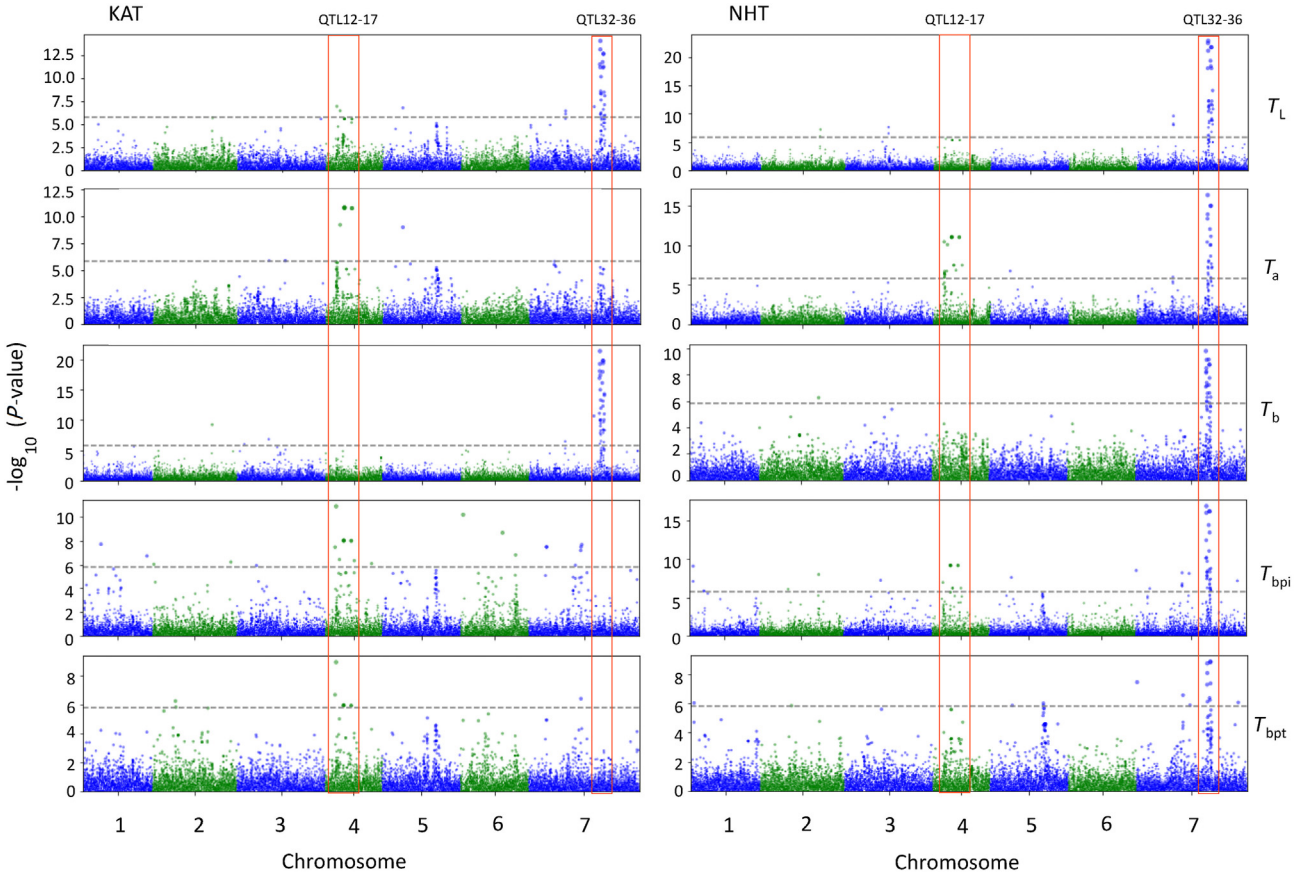

Fig. 3 – Manhattan plots showing SNP effects for give barley grain KD traits. Dotted lines define the threshold of significance at $P = 1.64 \times 10^{-6}$ (equivalent to $P = 0.05$ with Bonferroni correction for multiple tests). Orange frames define two major QTL hotspots in chromosomes 4H and 7H.

3.4. *Association verification through genomic prediction*

In addition to the KAT and NHT trials, a third independent trial (df1) was also carried out on the same germplasm collection. Valid phenotypic and genomic data from this trial was obtained for 30 germplasm accessions. This trial was used as a test population in association verification through genomic prediction. Predicted traits values and observed values are shown in Fig. 6. Genomic prediction achieved 67%–87% accuracy (an accurate prediction was defined by the observed value falling within the predicted phenotype variation range, i.e., mean ± standard deviation. Genomic prediction achieved higher accuracy for the two black point traits (80% and 87% for T_{bpi} and T_{bpt}, respectively) than it did for the three grain brightness traits (67%–73%), which suggests the potential implication of genomic selection in the breeding program involving black point resistance. Note that the standard deviation of a predicted value may be large as only 517 samples were included in the training dataset, and the accuracy derived from 30 accessions need to be interpreted with caution in estimating phenotype values from SNP profiles.

3.5. *Candidate genes associated with QTL*

To identify the possible candidate genes for the KD QTL, we performed a genome-wide survey for candidate genes in three metabolic pathways: phenylpropanoid pathway (KEGG: map00940), flavonoid biosynthesis pathway (KEGG: map00941), and anthocyanin biosynthesis pathway (KEGG: map00942). We also conducted a homology search for a number of transcription factor encoding genes in barley that have previously been characterized to affect anthocyanin production in grains. In addition, candidate genes encoding plant peroxidase (PPO) were extracted from the barley genome based on their functional annotations. In total, 491 genes related to phenolics and flavonoids biosynthesis were found. Among them, 469 genes were mapped to chromosomes 1H – 7H. We plotted these candidate genes and the identified SNP markers in a single map (Fig. 5). Those genes that are located close to the identified QTL were assumed as the candidate genes for the KD traits.

As shown in Fig. 5, candidate genes encoding a flavone synthase (FNS), a flavonoid 3′-hydroxylase (F3′H), seven tandem dihydroflavonol 4-reductase (DFR), and several PPO genes were located at the major QTL hotspot (QTL32–36) on chromosome 7H. In particular, the FNS and F3′H genes overlap with QTL32 and QTL36, respectively, while the seven DFR genes cluster is positioned between QTL34 and QTL35 with close distances (3.9 Mb and 2.4 Mb, respectively). In addition, two, six, and one PPO genes are located close (<5 Mb) to QTL32, QTL33 and QTL35, respectively. QTL32–QTL36 spans the

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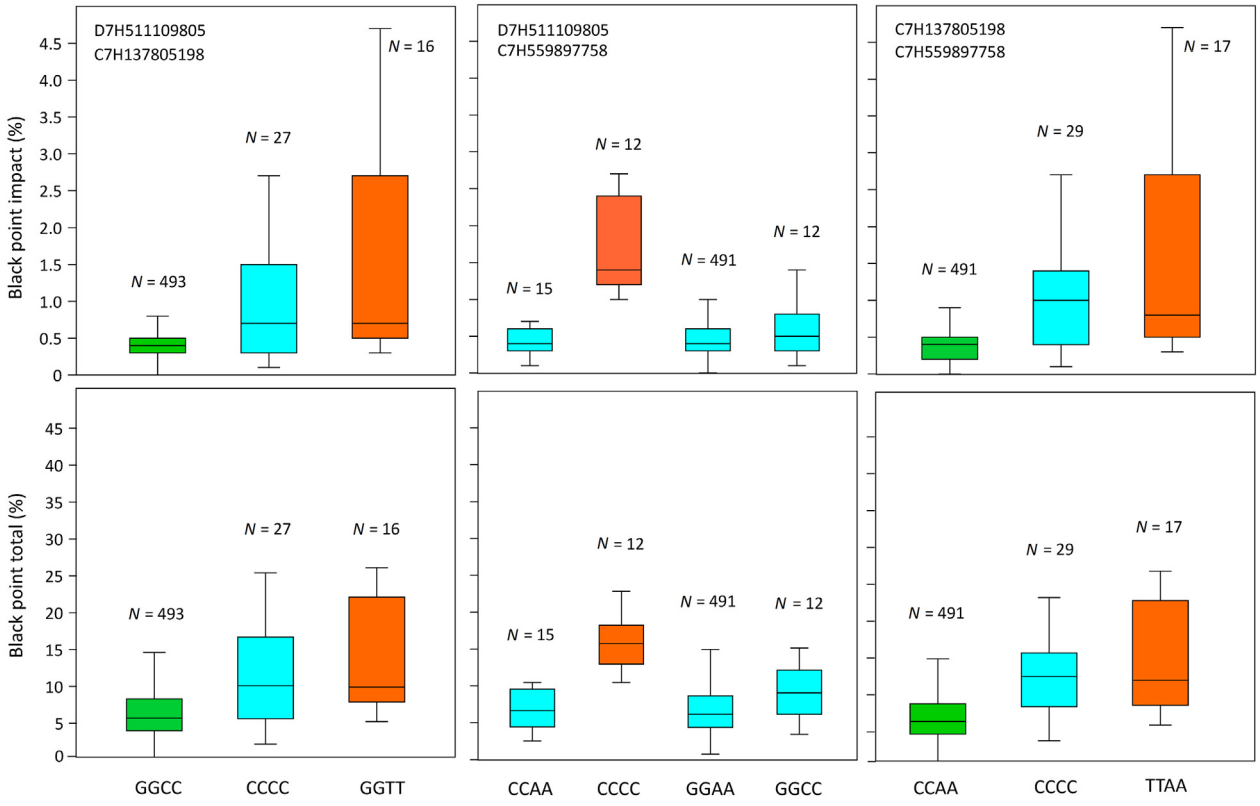

Fig. 4 – Phenotype comparison of different barley haplotypes. Three SNP markers within the major QTL hotspot on chromosome 7H were included: D7H511109805 (G/C, QTL32), C7H137805198 (C/T, QTL29), and C7H559897758 (C/A, QTL34).

genetic region of 497.9–590.5 Mb and was associated with both grain brightness and black point traits. Other minor QTL on chromosome 7H include QTL29 and QTL30 and were associated with black point. Three PPO and one MYB gene were found within 10 Mb distance to these two QTL. For the major QTL hotspots (QTL12, QTL13, QTL14, QTL15, QTL16, and QTL17) on chromosome 4H, two DFR, two PPO, one aldehyde dehydrogenase (ALDH3), and one beta glucosidase (BGSD11) genes were identified close to QTL12, QTL 14, QTL17, and QTL15, respectively. One DFR gene and one 4-coumarate: CoA ligase (4CCLG) gene were located close to QTL13.

The likely candidate genes for the other QTL have also been identified (Table S5). They include flavonoid 3-O-glucosyltransferase (FGT) for QTL1 (black point), anthocyanin 5-aromatic acyltransferase (AN5AT) for QTL2 (black point), DFR for QTL3 (black point), PPO for QTL11 (grain brightness), PPO for QTL18 (black point and grain brightness), MYB for QTL19 (black point), PPO for QTL25 (black point), PPO for QTL27 (black point), cinnamoyl-CoA reductase (CCoAR) for QTL28 (grain brightness), AN5AT and PPO for QTL37 (black point) (Fig. 5).

3.6. Analyses of transcription data of the candidate genes

To further investigate the potential association of those candidate genes with the grain KD traits, we extracted the transcription data of the identified candidate genes from the public database (https://apex.ipk-gatersleben.de/apex/f?p=284:10::::::). A total of fifteen samples from different barley tissues and developmental stages were obtained, five of that are relevant to the grain KD traits (highlighted in the red box in Fig. 7). Majority of the identified flavonoid pathway genes are transcribed in the lemma (LEM), palea (PAL), developing caryopses (CAR5 and CAR15), and embryo tissues. In particular, HORVU4Hr1G007060, HORVU4Hr1G010160, HORVU4Hr1G010250 (associated with QTL13), HORVU4Hr1G082610 (QTL19), HORVU7Hr1G030380, HORVU7Hr1G030480, HORVU7Hr1G030500 (QTL28), and HORVU7Hr1G095900 (QTL36) are highly expressed in the lemma and palea tissues, which may have a direct effect on the grain brightness. HORVU4Hr1G010250 (QTL13), HORVU6Hr1G001270 (QTL23), and HORVU7Hr1G093310, HORVU7Hr1G093360, HORVU7Hr1G093370 (QTL35) are highly transcribed in the embryo. Moreover, another candidate gene HORVU7Hr1G093480 (QTL35) encoding one of the seven tandem DFRs was expressed specifically in the developing caryopses, indicating its likely role in grain KD.

In contrast to the flavonoid pathway genes, most of the identified PPO encoding genes were actively expressed in the embryo. This observation is consistent with their potential association with the black point trait, which occurs in the embryo tip only. These PPO genes were associated with QTL7, QTL18, QTL25, QTL27, QTL29, QTL30, and QTL32–35, which covers the two major QTL hotspots on chromosomes 7H and 4H, respectively. Two PPO encoding genes HORVU4Hr1G022280 (QTL14) and HORVU7Hr1G013470 (QTL27) were transcribed almost specifically

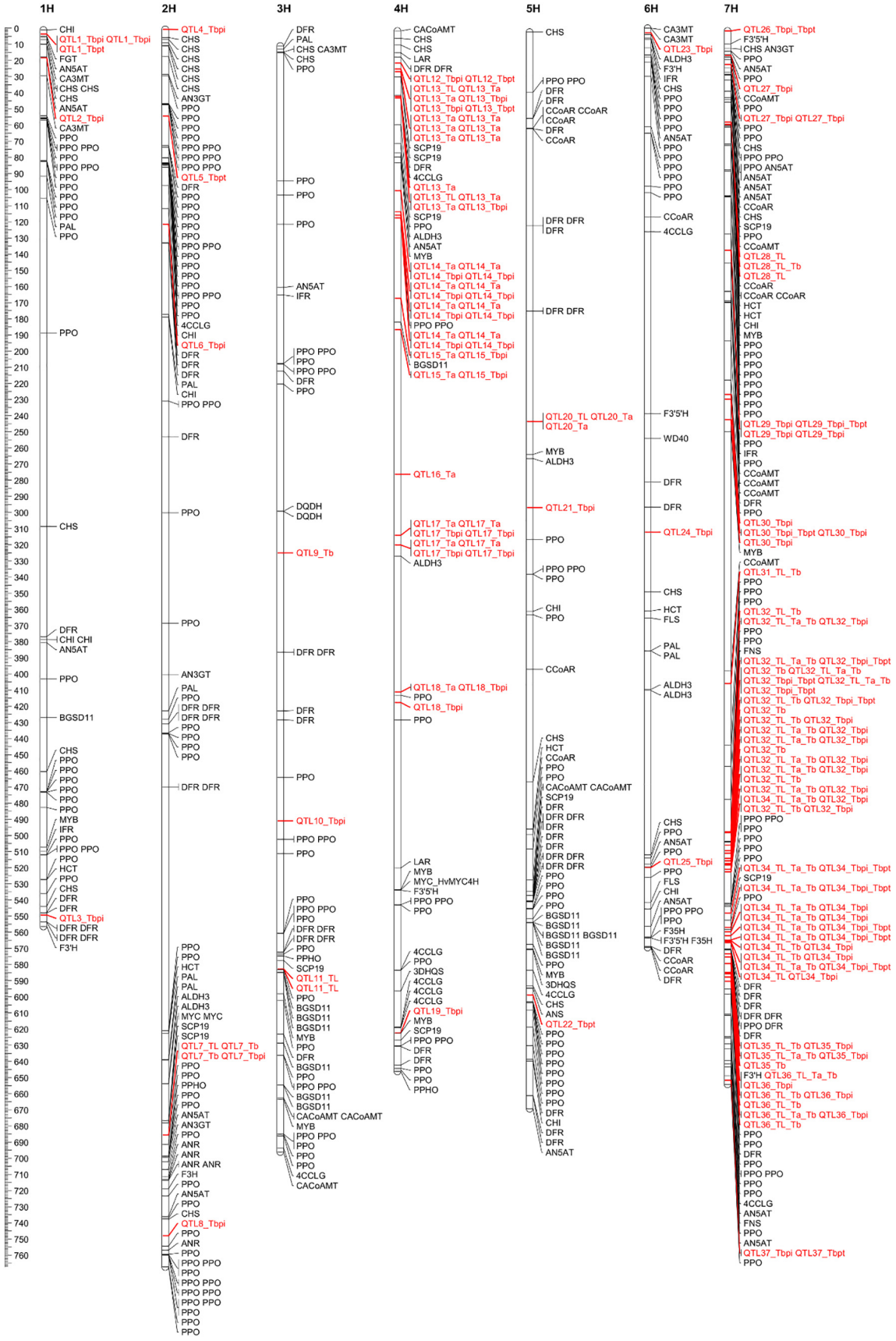

Fig. 5 – Mapping of QTL loci and the predicated candidate genes in the barley genome. Identified QTL from both NHT and KAT were highlighted in red. Predicted candidate genes in phenylpropanoid pathway, flavonoid biosynthesis pathway, and anthocyanin biosynthesis pathway in black. See Table S5 for a full list of the candidate genes, gene functional annotation, and the identified SNPs.

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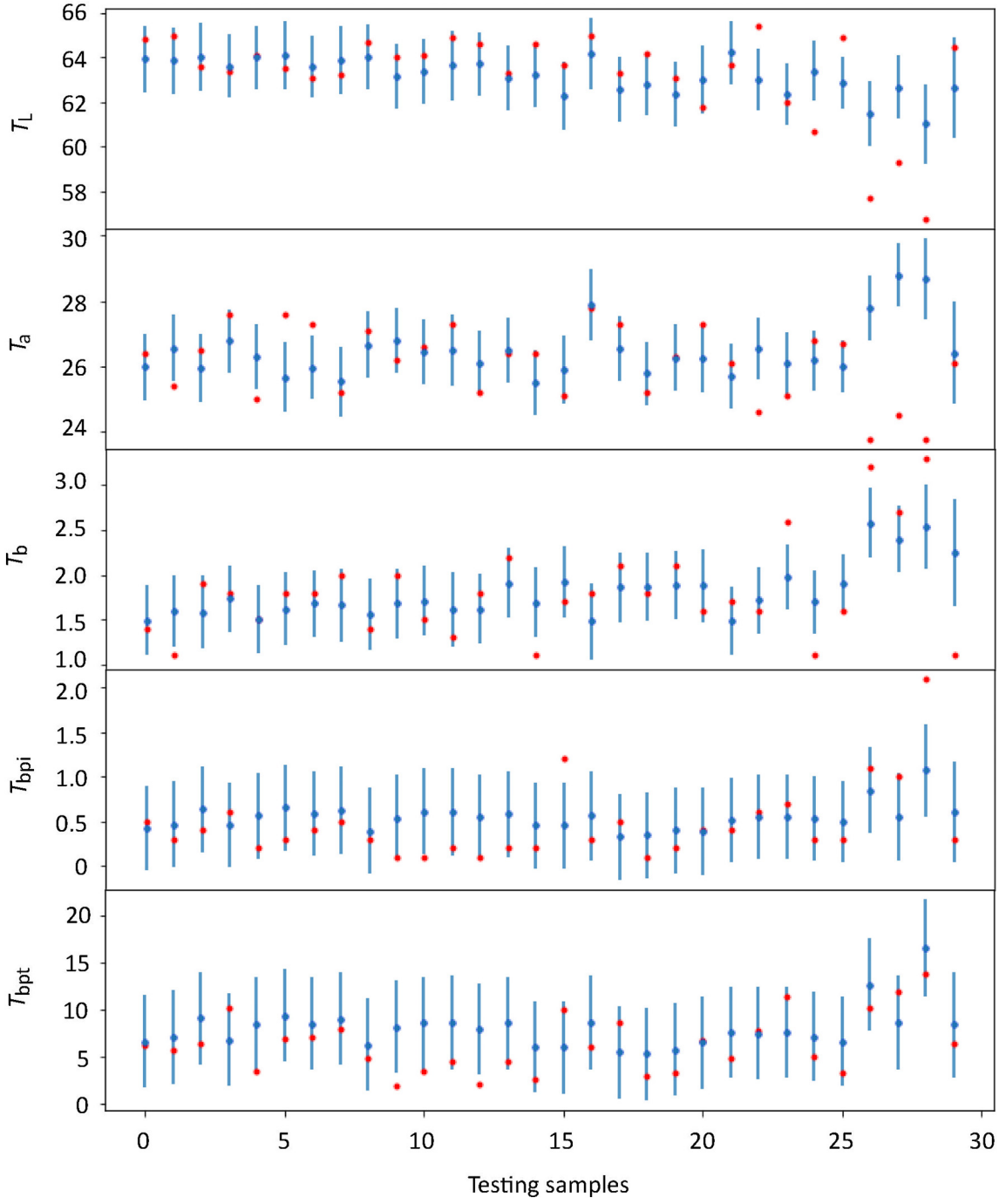

Fig. 6 – SNP - phenotype association verification through genomic prediction for 30 test samples from an independent trial. Red dots represent the observed phenotype values; blue dots and bars represent predicted phenotype value and standard deviation.

in the developing caryopses tissues. One additional PPO gene HORVU7Hr1G013470 associated with QTL14 was highly expressed in the lemma and palea tissues. These findings support the likely role of these PPO genes in the black point trait. In addition to the grain KD related tissues, a notable observation of the identified flavonoid pathway genes and PPO encoding genes was that, many of them were highly expressed in the root tissues (ROO1 and ROO2), which may indicate their possible role in plant stress resistance.

3.7. Genetic variation and marker development

To facilitate future functional validation of the identified candidate genes for the grain KD traits, and also to allow the exploitation of these genes in barley breeding program, the genetic variations within the marked candidate genes (32 in total) in Fig. 7 were identified from our in-house database, which contains the whole-genome sequencing data for 21 barley accessions (13 commercial varieties, 4 wild barley from Israel, 2

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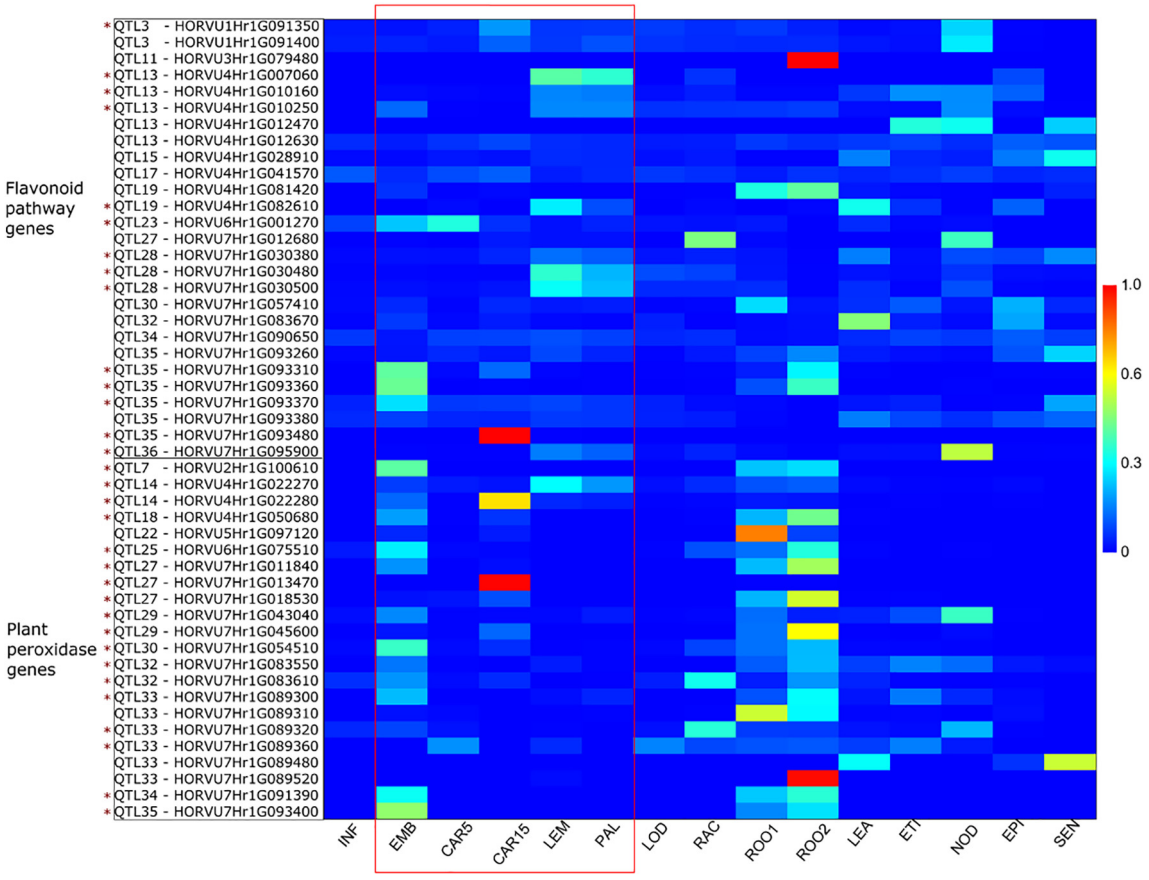

Fig. 7 – Transcriptional heat-map of the grain brightness candidate genes in barley. The upper and lower panels refer flavonoid pathway genes and plant peroxidase genes, respectively. Those genes actively expressed in grain-related tissues were marked with the "*" symbol. The associated QTL number for each gene was labelled before the gene ID. Gene expression value in FPKM was normalized based on individual gene. INF, developing inflorescence 1–1.5 cm; EMB, embryo 4 DPA; CAR5, developing grain 5DPA; CAR15, developing grain 15 DPA; LEM, lemma 42 DPA; PAL, palea 42 DPA; LOD, lodicule 42 DPA; RAC, rachis 35 DPA; ROO1, root 10 cm shoot stage; ROO2, root 28 DPA; LEA, shoot 10 cm stage; ETI, etiolated seedling 10 DPA; NOD, developing tiller 42 DAP; EPI, epidermal strip 28 DPA; SEN, senescing leaf 56 DPA. Samples highlighted in the red box are relevant to grain KD traits.

wild barley from Tibet of China, and 2 barley landraces). At least four InDel variations (average 28) were identified for each candidate gene (Table S6). Twenty-three of these InDel mutations were predicted to cause disruptive frameshift or start-codon loss. An extra 243 InDels were identified in the 3′–/5′- untranslated regions, intron, and splicing sites, which are likely to affect the gene transcription level. The rest InDels are located in either gene upstream or gene downstream region. We went further to design a total of 883 gene-specific markers targeting the identified InDel mutations (Table S6). These InDel markers, despite remaining to be verified, represent a starting point for future grain KD trait analysis and may be utilized in the barley breeding program thereafter.

4. Discussion

Improvement of grain kernel discoloration tolerance has been an important target for crop breeding in barley and wheat. The exploitation of the diverse barley germplasm resource is critical for future breeding as this target may be achieved by introducing KD tolerant alleles into the elite varieties during the breeding process. Despite several previous studies performed on QTL mapping of KD tolerance, particularly for the black point trait, only a limited number of QTL have been identified to date in barley [7,11,14]. The identification of QTL for KD tolerance is challenging for several reasons: the multigenic property of KD, limitation of genetic diversity, and the significant effects imposed by various environmental factors. Most of those previous studies are bi-parental mapping, which is based on segregating populations derived from the crossing of two varieties. Although bi-parental mapping has been useful in genetic studies, its limitation is also apparent: the allelic variations are restricted to the two parents. Recent genetic research and breeding in barley have been greatly accelerated by the revelation of a complete barley reference genome [54]. Whole-genome re-sequencing allows the development of high-density SNP markers to scan an

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extensive collection of barley germplasm to identify beneficial alleles for barley breeding. Our GWAS analyses take the advantage of germplasm that is rich in genetic diversity, thus allow the identification of QTL and the putative candidate genes underlying the KD traits.

Two categories of grain KD traits, whole grain brightness (T_L, T_a, and T_b) and black point (T_{bpi} and T_{bpt}), are examined in the present GWAS study. The whole grain brightness traits were measured using a chromometer with fixed parameters. The same method has been used previously to study grain KD in barley [2,12]. Previously, multiple QTL located on different chromosomes associated with T_L, T_a, and T_b were identified in seven barley DH populations, and QTL associated with more than one grain brightness parameters were found in clusters on the individual chromosome [2]. A similar pattern was observed for the SNP markers associated with the grain brightness in our study. Together, the results suggest that grain brightness, redness, and yellowness may share a common genetic basis. Out of the 37 QTL identified in our study, 18 QTL are associated with one or more grain brightness parameters, which include the two QTL hotspots on chromosomes 7H and 4H, respectively. Some of the previously identified markers, such as Bmac0960 (4H), GMS89 (4H), and Bmag183 (7H) [2], are located close to the two QTL hotspots found in the present study. A dominant QTL on chromosome 4H responsible for the blue aleurone trait in barley was found in several previous studies [2,12,19]. This QTL was not detected in the present study, probably because only two barley accessions in our germplasm collection have the blue aleurone trait. Compared to the bi-parental mapping, our GWAS analyses based on a diverse germplasm collection have detected a much larger number of QTL associated with grain brightness, many of that have not been reported before. We identified the tolerant and sensitive haplotypes for the representative SNP markers associated with the major QTL. These SNP markers provide a valuable tool for the improvement of grain KD resistance in the future barley breeding program.

It is generally agreed that grain brightness is affected by the accumulation of phenolics and flavonoid compounds [55–57]. Candidate genes related with flavonoids production have been previously characterized to affect grain colorations in barley [19,21], wheat [22,58], maize [20,59], and rice [60], including both transcriptional factors and structural genes. In the present study, genes involved in the phenylpropanoid pathway, flavonoid biosynthesis pathway, anthocyanin biosynthesis pathway, and plant peroxidase family were screened for their potential relationship to grain KD. Genes located at the genetic regions close to or at the same region as the identified QTL were assumed to be associated with grain KD. Several structural genes in the flavonoid pathway overlap with or are positioned close to the identified QTL. For example, an F3′H gene on chromosome 7H overlaps with QTL36. A cluster of seven tandem DFR genes plus a PPO gene on chromosome 7H is positioned between QTL34 and QTL35 within less than 5 Mb. An FNS gene overlaps with QTL32 on chromosome 7H. We further narrowed down the candidate genes by restricting to those actively expressed in grain KD relevant tissues, which has enabled the identification of the most likely genes with high confidence. Moreover, the development of additional gene-specific markers would allow functional validation of these candidate genes in future studies, and their application in barley breeding programs thereafter.

Compared to the whole grain brightness traits, the black point is a grain defect that has been under intensive studies in both barley and wheat. However, QTL mapping for black point has been challenging due to the unstable nature of this trait. Black point expression has long been shown to be susceptible to environmental conditions [7,8]. This is consistent with our genetic inheritance analysis, which showed that black point has lower heritability than the whole grain brightness traits. In addition to environmental conditions, phenology developments such as barley heading date and grain maturity are also potential factors affecting grain brightness and black point. The latter usually occurs under certain environmental conditions at the specific grain development stage. In barley, elevated humidity level during grain maturation can significantly increase the occurrence of black point [7]. Thus, attempts to identify QTL associated with black point often fail due to variations in growing conditions. In this study, we found that some grain KD QTL are located close to the key phenology genes identified a previous study [28]. Despite the fact that we tended to harvest barley at late stage to allow full maturity, it is possible that some of the QTL are associated with barley phenology also affects grain development and maturity.

Substantial efforts by plant biologists have been made to create a reliable black point screening system, such as growing plants under controlled high-humidity environments [7]. These pioneering studies have led to the identification of a limited number of QTL for black point. Specifically, five QTL located on barley chromosomes 2HS, 2HC, 3HL, 4HL, and 5HL were identified using an F_2 population derived from tolerant and susceptible parents [11]. One of these QTL (marker interval: bPb4830 – Ebmac708) on chromosome 3HL accounts for 28% of the phenotypic variation. Nine QTL associated with the black point were identified in wheat using a RIL population, with individual QTL explaining 3.7% to 12.7% phenotypic variation [10]. Again, most of those studies are based on bi-parental mapping, with a clear limitation of genetic diversity. Several studies indicated that black point is related to the oxidation of phenolic compounds catalyzed by polyphenol peroxidase or plant peroxidase in the grain [16,17,23]. In the present study, we found that the major QTL hotspot on chromosome 7H was also associated with the black point. However, this association was only detected at one of the two field trials, which may be due to the susceptibility of black point to environmental effects. In contrast, the QTL hotspot on chromosome 4H was detected for black point consistently in the two experiments. One of the notable observations is that many QTL associated with the black point, including the two major QTL hotspots, overlap with the QTL detected for grain brightness. These results suggest that these two categories of grain KD traits may be genetically linked and may share some common genetic bases. This suggestion is supported by previous reports that black point may be associated with phenolic oxidation [16,17,23]. The expression of black point may be related to the phenylpropanoid biosynthesis pathway that controls the production of phenolic compounds. A close link between black

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point and whole-grain brightness can be drawn due to a close relationship between the phenylpropanoid pathway and the flavonoid biosynthesis pathway, the latter of which is believed to have a direct effect on grain brightness. In addition to the overlapping QTL, QTL associated specifically with black point have also been identified. Some of these QTL are closely associated with the PPO or flavonoid pathway genes, whilst no candidate gene was found for other QTL. The present results support the occurrence of black point as a complex trait controlled by multiple metabolic pathways. Future genetic and biochemistry studies are needed to better understand the molecular basis underlying the grain black point.

5. Conclusions

Grain KD represents a significant challenge for barley production due to its negative effect on grain quality. We performed a GWAS analysis of KD in a large barley germplasm collection using high-density SNP markers. A total of 37 QTL were identified for five grain KD traits (T_a, T_b, T_L, T_{bpt}, and T_{bpi}). Compared to the whole grain brightness traits, the black point trait is more susceptible to environmental affection. Two major QTL hotspots associated with all the target KD traits were identified on chromosomes 7H and 4H, respectively, suggesting that whole grain brightness traits and black point may share a related genetic basis. Genetic linkage and gene transcriptional analyses identified strong candidate genes for the grain KD traits. Our study would facilitate future exploitation of barley germplasm as a source of beneficial alleles for grain KD resistance improvement in barley breeding.

Supplementary data for this article can be found online at https://doi.org/10.1016/j.cj.2020.04.013.

Declaration of competing interest

The authors declare no conflicts of interest.

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Author contributions

Chengdao Li devised the idea and designed the project. Camila Hill and Xiaoqi Zhang collected the genotype data. Sharon Westcott, Lee Anne McFawn, and Tefera Angessa collected the phenotype data. Yong Jia, Cong Tan, Tianhua He, and Gaofeng Zhou performed data analysis. Yong Jia wrote the manuscript with Tianhua He's assistance. All authors have contributed to editing the manuscript.

Data availability

Phenotypic data are available as online supplementary material; SNP profiles are available from the corresponding author.

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