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RESEARCH ARTICLE

# Genome wide linkage mapping for black point resistance in a recombinant inbred line population of Zhongmai 578 and Jimai 22

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**Highlights**

- Genotype and environment both contributes to extensive variation of the incidence of black point.
- QTL mapping identified six stable QTLs and five of them were developed into KASP markers.
- Pyramiding favorable alleles of identified QTLs can reduce the incidence of black point.

**Abstract**

Black point is a black discoloration of the grain embryo that reduces the grain quality and commodity grade. Identifying the underlying genetic loci can facilitate the improvement of black point resistance in wheat. Here, 262 recombinant inbred lines (RILs) from the cross of Zhongmai 578/Jimai 22 were evaluated for their black point reactions in five environments. A high-density genetic linkage map of the RIL population was constructed with the wheat 50K single nucleotide polymorphism (SNP) array. Six stable QTLs for black point resistance were detected, *QBp.caas-2A*, *QBp.caas-2B1*, *QBp.caas-2B2*, *QBp.caas-2D*, *QBp.caas-3A*, and *QBp.caas-5B*, which explained 2.1–28.8% of the phenotypic variances. The resistance alleles of *QBp.caas-2B1* and *QBp.caas-2B2* were contributed by Zhongmai 578 while the others were from Jimai 22. *QBp.caas-2B2*, *QBp.caas-2D* and *QBp.caas-3A* overlapped with previously reported loci, whereas *QBp.caas-2A*, *QBp.caas-2B1* and *QBp.caas-5B* are likely to be new. Five kompetitive allele-specific PCR (KASP) markers, *Kasp_2A_BP*, *Kasp_2B1_BP*, *Kasp_2B2_BP*, *Kasp_3A_BP*, and *Kasp_5B_BP*, were validated in a natural population of 165 cultivars. The findings of this study provide useful QTLs and molecular markers for the improvement of black point resistance in wheat through marker-assisted breeding.

**Keywords:** black point, candidate gene, common wheat, kompetitive allele-specific PCR, quantitative trait locus

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## 1. Introduction

Black point, which appears as discoloration of the embryo in wheat grains, has impacts on grain quality and commodity grading (Dexter and Matsuo 1982; Conner *et al.* 2009; Fernandez and Conner 2011). It has become a serious problem in most wheat growing regions worldwide, including China, the USA, Australia, Canada and France (Williamson 1997; Lehmensiek *et al.* 2004; Bensassi *et al.* 2009; Conner *et al.* 2009; Sissons *et al.* 2010; Liu *et al.* 2016). It can reduce grain processing quality, including test weight, falling number, dough stability, total flour yield, and break flour yield (Dexter and Matsuo 1982; Zhai *et al.* 2016) and has restricted trading in the USA (≤4%), Australia (≤5%), Canada (≤10%), and China (≤10%) (Li *et al.* 2014). It affects the germination percentage and causes impaired seedling development, although no significant effect on yield has been reported (Lehmensiek *et al.* 2004; Fernandez *et al.* 2014; Li *et al.* 2014). Furthermore, *Fusarium* and *Alternaria* pathogens associated with black point leads to mycotoxin contamination, which can cause oesophageal cancer and neural tube defects in humans (Williamson *et al.* 1997; Bensassi *et al.* 2009; Kahl *et al.* 2015; Masiello *et al.* 2020).

The formation of black point is extremely complex. Most previous studies reported that black point is caused by the infection of a series of fungi (Ellis *et al.* 1996; Logrieco *et al.* 2003), including *Alternaria alternata*, *Bipolaris sorokiniana*, and *Fusarium proliferatum* (Williamson *et al.* 1997; Kumar *et al.* 2002; Desjardins *et al.* 2007; Bensassi *et al.* 2009; Kahl *et al.* 2015; Somma *et al.* 2019; Masiello *et al.* 2020; Al-Sadi *et al.* 2021; Li *et al.* 2022b). Several other studies reported that high humidity and extremely high temperatures during grain filling triggered enzymatic browning and caused heavy black point (Conner 1989; Mak *et al.* 2006; Fernandez *et al.* 2011; Li *et al.* 2019). Specifically, such stress conditions or disruptions trigger enzymes like peroxidases (POD), polyphenol oxidase (PPO) and lipoxygenase (LOX), which react with phenolic acids to produce quinines, consequently resulting in black point during grain filling (Régnier and Macheix 1996; Williamson *et al.* 1997; Walker and Ferrar 1998; March *et al.* 2007; Fuerst *et al.* 2014; Wei *et al.* 2015). Although several cultivation and chemical strategies are used to control black point (Fuerst *et al.* 2014), breeding resistant cultivars remains the most effective, economical and environmentally friendly approach. Black point resistance is a typical quantitative trait controlled by multiple minor genes (Conner and Kuzyk 1988; Lehmensiek *et al.* 2004; March *et al.* 2008; Liu *et al* 2016, 2017; Li *et al.* 2022b; Tang *et al.* 2022; Gao *et al.* 2023). Thus far, only a few genetic studies on black point resistance have been conducted. For example, Lehmensiek *et al.* (2004) identified nine QTLs on chromosomes 1D, 2A, 2B, 2D, 3D, 4A, 5A and 7A in Cascades/AUS1408 and Sunco/Tasman recombinant inbred line (RIL) populations, which explained 4.5–15.2% of the phenotypic variances. Liu *et al.* (2016) reported nine QTLs for black point resistance on chromosomes 2AL, 2BL, 3AL, 3BL, 5AS, 6A, 7AL, and 7BS in a Linmai 2/Zhong 892 RIL population. Furthermore, Liu *et al.* (2017) identified 25 resistance loci on chromosomes 2A, 2B, 3A, 3B (2), 3D, 4B (2), 5A (3), 5B (3), 6A, 6B, 6D, 7A (5), 7B, and 7D (2) by genome-wide association analysis (GWAS) in 165 wheat accessions, and each explained 7.9 to 18.0% of the phenotypic variances. In addition, nearly 40 loci for the black point reaction on chromosomes 1B, 1D, 2A, 2B, 2D, 3A, 3B, 3D, 4A, 5A, 5B, 6A, 6B, 6D, 7A, and 7D were identified in recent GWAS studies (Li *et al.* 2020a; Lv *et al.* 2020; Tang *et al.* 2022). Gao e*t al.* (2023) identified five QTLs for black point resistance on chromosomes 5A, 5B, and 5D in a RIL population. However, stable loci and available markers for black point resistance breeding are still limited.

With the development of the new generation of sequencing and chip technology, single nucleotide polymorphisms (SNPs) have become widely used for the genetic analysis of complex traits in common wheat (Semagn *et al.* 2014; Wang *et al.* 2014; Rasheed *et al.* 2017; Yang *et al.* 2020; Li L L *et al.* 2021; Li Q Y *et al.* 2021; Shawai *et al.* 2022; Liu *et al.* 2023). Due to the increasing deployment of water and fertilizer, black point has become one of the most important diseases in the Yellow and Huai River Valley Facultative Wheat Region in China (Liu *et al.* 2016, 2017; Li *et al.* 2020b; Lv *et al.* 2020; Gao *et al.* 2023). Therefore, breeding for black point resistant cultivars is important and urgent. In this study, we identified stable loci and candidate genes by genome-wide linkage mapping in a Zhongmai 578/Jimai 22 recombinant inbred lines (RILs) population and developed high-throughput KASP markers for black point resistance breeding.

## 2. Materials and methods

### 2.1. Plant materials and field trials

The 262 RILs used for QTL mapping were derived from a Zhongmai 578 (ZM578)/Jimai 22 (JM22) cross. Zhongmai 578 shows excellent bread-making quality, high and stable yield and broad adaptability with moderate black point resistance. Jimai 22 is characterized by high yield and

broad adaptability with moderate susceptibility to black point. The 262 RILs and two parents were evaluated for black point resistance at Shangqiu (33°43′N, 114°49′E) in Henan Province, China during the 2020–2021 cropping season, at Xinxiang (34°53′N, 113°23′E) in Henan Province during 2020–2021, 2021–2022 and 2022–2023 cropping seasons, and at Dezhou (37°45′N, 116°37′E) in Shandong Province, China during the 2021–2022 cropping season. From mid-May to June, Xinxiang and Dezhou have relatively high temperatures and humidities, which are conducive to the development of black point. The field trials were designed using a Latinized alpha-lattice design with three replications in all locations and cropping seasons. Each plot consisted of six rows that were 3.0 m in length and 1.2 m in width, with 0.2 m row spacing. The field trials were managed according to local practices (Shawai *et al.* 2022; Liu *et al.* 2023).

An association panel used for the validation of QTLs with KASP markers comprised 165 cultivars that were mainly from the Yellow and Huai River Valley Facultative Wheat Region. All accessions were grown at Anyang in Henan Province during the 2012–2013 and 2013–2014 cropping seasons, and at Suixi in Anhui Province, China during 2012–2013, 2013–2014 and 2014–2015. The field trials were conducted in randomized complete blocks with three replications at all locations. Each plot contained three 2 m rows spaced 20 cm apart. Black point incidence for the 165 accessions was reported in a previous study (Liu *et al.* 2017) and black point scores were collected in the same year after seed harvesting.

### 2.2. Evaluation of black point incidence

After harvesting and threshing, 200 grains of each accession were randomly selected and the percentage of kernels with black point symptoms was determined as the incidence (Liu *et al.* 2016). The best linear unbiased estimation (BLUE) values across five environments were used as the phenotypic values for association mapping to eliminate any environmental effects. BLUE estimation was calculated using the MIXED procedure (PROC MIXED) in SAS v9.4 (SAS Institute Inc, Cary, NC, USA, http://www.sas.com).

### 2.3. Statistical analyses

Analysis of variance (ANOVA) was performed using PROC GLM. Environment, genotype and environment interactions, and replicates were treated as random effects when estimating effect sizes and their significance. Broad-sense heritability ($H_b^2$) for black point resistance was estimated using the formula $H_b^2=\sigma^2_g/(\sigma^2_g+\sigma^2_{ge}/e+\sigma^2_\varepsilon/re)$, where $\sigma^2_g$, $\sigma^2_{ge}$ and $\sigma^2_\varepsilon$ are estimates of the genotype, genotype (line)×environment interaction and residual error variances, respectively (Nyquist and Baker 1991), and e and r are the numbers of environments and replicates, respectively.

### 2.4. Linkage mapping

The wheat 50 K SNP markers were used for genotyping the RILs and parents (Liu *et al.* 2023). The binning of redundant markers (BIN) function of IciMapping v4.2 (http://www.isbreeding.net/; Li *et al.* 2007; Meng *et al.* 2015) was used to optimize the polymorphic markers. Linkage groups were generated using Joinmap v4.0 (http://www.kyazma.com; Stam 1993) and drawn using MapChart v2.32 (http://www.earthatlas.mapchart.com; Voorrips 2002). QTL analysis was conducted by inclusive composite interval mapping (ICIM) using IciMapping v4.2 software, with a 0.1 cM walk speed based on 2000 permutations at $P<0.01$ (Li *et al.* 2007). A LOD threshold of 2.8 was set for declaring significant QTLs based on 2,000 permutations at $P=0.05$. QTLs detected in two or more environments were considered stable.

### 2.5. Development and validation of KASP markers

KASP markers were designed using PolyMarker software (http://polymarker.tgac.ac.uk/) for target polymorphic sites. Primer premixes were prepared according to Yang *et al.* (2020) and PCR was performed following Li L L *et al.* (2021). The genotyping function of the software KlusterCaller™ 2.24.0.11 (LGC, Hoddesdon, UK) was used to read the different fluorescence signal values of each sample and analyze the genotypes. KASP markers that could be successfully used for genotyping were validated with a diverse panel of 165 wheat cultivars.

### 2.6. Candidate gene prediction

We aimed to identify candidate genes within stable QTL intervals by integrating annotation information. The selection of candidate genes was primarily based on the following criteria. First, the genes must be located in or adjacent to the physical intervals of the identified QTLs, and the confidence interval of stable genetic loci based on the annotation information from IWGSC was considered, such as genes involved in POD, PPO, LOX metabolism, and stress tolerance-related genes. Second, we determined whether the SNP variations in the initially screened candidate genes were meaningful mutations and excluded those found to be non-significant. Third, they may be differentially expressed in reproductive organs or in grains between contrasting genotypes

based on the database expVIP8. Finally, to provide concise information, only those candidate genes that we considered to be the most reliable are reported here. The flanking sequences corresponding to the SNP markers significantly associated with black point resistance were used to search for candidate genes or putative protein functions of SNP flanking-regions in BLASTx against NCBI (http://www.ncbi.nlm.nih.gov/). Gene expression patterns were analyzed using expVIP8 (http://www.wheat-expression.com/), and genes that were specifically highly expressed in grains or spikes were selected.

## 3. Results

### 3.1. Phenotypic analysis

The black point scores in Zhongmai 578, Jimai 22 and the RILs were evaluated in five environments (Appendix A). Zhongmai 578 and Jimai 22 both tended to be resistant to black point, and the *T*-test results indicated significant differences between the two cultivars in Xinxiang 2021, Xinxiang 2022 and Xinxiang 2023, while no significant differences were observed between them in the remaining environments (Appendix B). The incidence of black point in 262 RILs was continuously distributed, ranging from 0.3–55.6% in Xinxiang 2021, 0–58.3% in Shangqiu 2021, 0.3–27.8% in Xinxiang 2022, 1.3–47.7% in Dezhou 2022, and 0–26% in Xinxiang 2023 (Appendices B and C). The black point incidences for the RIL population were significantly correlated ($r$=0.58–0.90, $P$<0.01) among the five environments, with a high broad-sense heritability ($H_b^2$=0.89) (Table 1). ANOVA indicated significant differences ($P$<0.001) among genotypes, environments, and genotype×environment interactions (Appendix D).

### 3.2. Linkage map

In total, 9,354 (16.9%) out of 55,224 SNP markers from the Illumina 50K SNP array were used for linkage mapping analysis. A high-density genetic map was constructed with 1,501 bin markers including 39 linkage groups (LGs) (Liu *et al.* 2023). The total length of the linkage map was 2,384.95 cM, with a mean chromosome length of 119.2 cM.

### 3.3. Loci for black point resistance

Six QTLs identified in two or more environments were located on chromosomes 2A (*QBp.caas-2A*), 2B (*QBp.caas-2B1* and *QBp.caas-2B2*), 2D (*QBp.caas-2D*), 3A (*QBp.caas-3A*) and 5B (*QBp.caas-5B*) (Fig. 1; Table 2). Among them, *QBp.caas-2A*, *QBp.caas-2D* and *QBp.caas-5B* were more stable, and they were detected in three environments and the BLUE value. *QBp.caas-3A* was detected in two environments and the BLUE value. *QBp.caas-2B1* and *QBp.caas-2B2* seemed to be less repeatable, being significant only in two environments each. Some loci detected in a single environment were located on chromosomes 3B, 4A, 4D, 5D, and 7A with phenotypic variations lower than 2.0%, and they are not discussed in this article. The resistance alleles of *QBp.caas-2B1* and *QBp.caas-2B2* were from Zhongmai 578, whereas those of *QBp.caas-2A*, *QBp.caas-2D*, *QBp.caas-3A*, and *QBp.caas-5B* were contributed by Jimai 22.

### 3.4. Additive effect of the QTLs for black point resistance

To further understand the combined effects of these alleles on the reaction to black point, we examined the number of favorable alleles in each RIL in the Zhongmai 578/Jimai 22 population, which ranged from zero to six (Fig. 2). A significant correlation ($r^2$=0.92) between the BLUE values of black point incidence and the numbers of favorable alleles was observed, indicating that lines with more favorable alleles tended to be more resistant to black point.

### 3.5. Validation of KASP markers

Five KASP markers, *Kasp_2A_BP*, *Kasp_2B1_BP*, *Kasp_2B2_BP*, *Kasp_3A_BP*, and *Kasp_5B_BP*, were designed based on the SNPs tightly linked with *QBp.caas-2A*, *QBp.caas-2B1*, *QBp.caas-2B2*, *QBp.caas-3A*, and *QBp.caas-5B*, respectively, and they showed clear polymorphism between the two parental genotypes and among the 165 wheat varieties (Table 3; Appendices E and F). Association analyses indicated that each of the five QTLs had significant effects on black point in the

**Table 1** Correlation analysis of black point incidence in the Zhongmai 578/Jimai 22 recombinant inbred line (RIL) population among five environments in China

| Environment | Xinxiang 2021 | Xinxiang 2022 | Shangqiu 2021 | Dezhou 2022 |
|---|---|---|---|---|
| Xinxiang, Henan Province, 2022 | 0.79** | | | |
| Shangqiu, Henan Province, 2021 | 0.74** | 0.73** | | |
| Dezhou, Shandong Province, 2022 | 0.81** | 0.90** | 0.74** | |
| Xinxiang, Henan Province, 2023 | 0.58** | 0.61** | 0.61** | 0.60** |

**, significant at $P$<0.01.

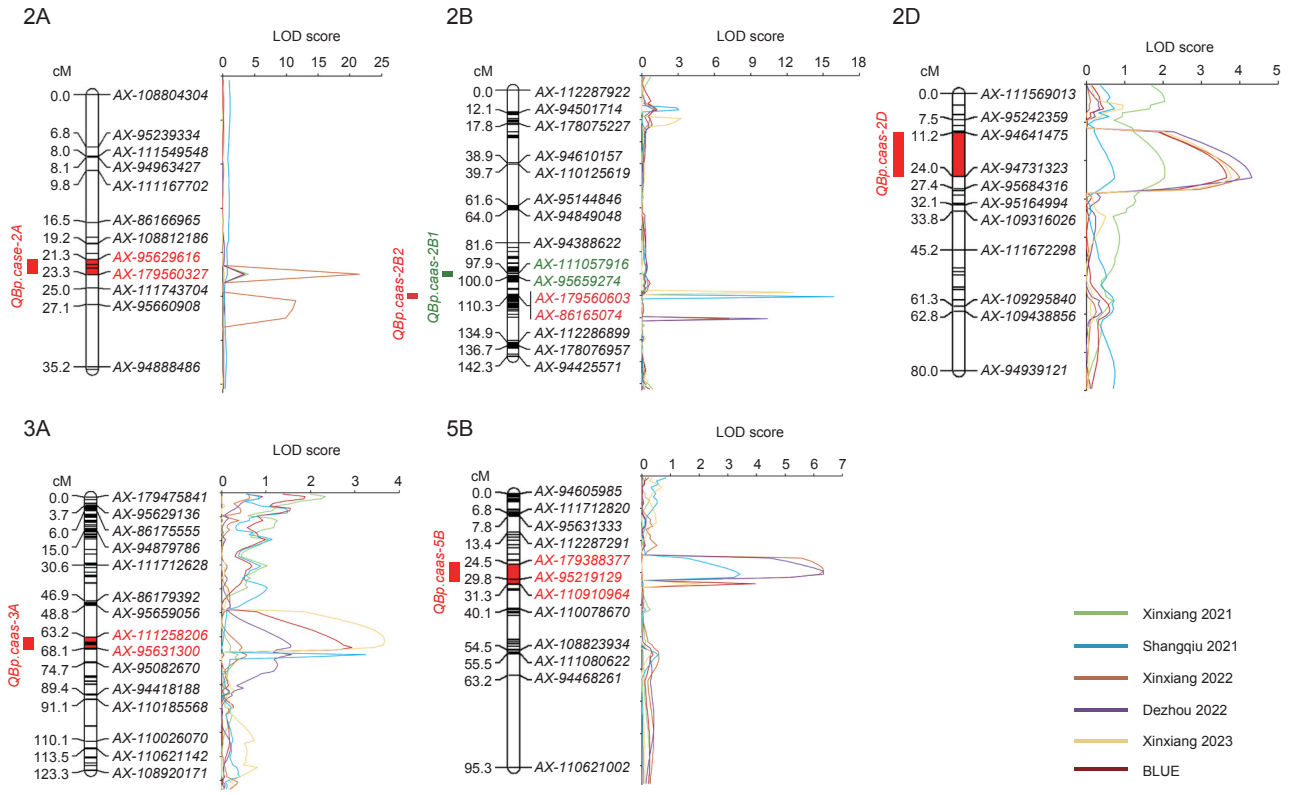


**Fig. 1** Logarithm of odds (LOD) score contours obtained by composite interval mapping of the QTLs for black point resistance in the Zhongmai 578/Jimai 22 recombinant inbred line (RIL) population. Data for Xinxiang (Henan Province, China) 2021, Shangqiu (Henan Province, China) 2021, Xinxiang 2022, Dezhou (Shandong Province, China) 2022, Xinxiang 2023, and the best linear unbiasedestimation (BLUE) values are indicated with deep green, light blue, purple, blue, yellow, and red colors, respectively.

**Table 2** QTL for black point resistance in the Zhongmai 578/Jimai 22 recombinant inbred line (RIL) population across five environments in China[1)]

| QTL[2)] | Marker Interval | Physical position (Mb) | Xinxiang 2021[3)] | | | Xinxiang 2022[3)] | | | Shangqiu 2021[3)] | | | Dezhou 2022[3)] | | | Xinxiang 2023[3)] | | | BLUE | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | LOD | $R^2$ | Add | LOD | $R^2$ | Add | LOD | $R^2$ | Add | LOD | $R^2$ | Add | LOD | $R^2$ | Add | LOD | $R^2$ | Add |
| *QBp.caas-2A* | *AX-95629616–AX-179560327* | 752.1–753.6 | 4.1 | 6.2 | 1.7 | 21.5 | 13.5 | 1.8 | | | | 3.5 | 4.6 | 1.5 | | | | 3.3 | 3.4 | 1.0 |
| *QBp.caas-2B1* | *AX-111057916–AX-95659274* | 159.4–167.5 | | | | | | | 15.9 | 28.8 | –4.8 | | | | 12.5 | 19.7 | –1.8 | | | |
| *QBp.caas-2B2* | *AX-179560603–AX-86165074* | 558.3–563.9 | | | | 7.3 | 4.1 | –1.1 | | | | 10.4 | 15.2 | –2.8 | | | | | | |
| *QBp.caas-2D* | *AX-94641475–AX-94731323* | 74.9–84.8 | | | | 4.0 | 2.1 | 0.7 | | | | 4.3 | 5.7 | 1.7 | 3.8 | 5.4 | 0.9 | 3.7 | 3.8 | 1.1 |
| *QBp.caas-3A* | *AX-111258206–AX-95631300* | 682.3–688.9 | | | | | | | 3.3 | 5.4 | 2.0 | | | | 3.7 | 6.0 | 1.0 | 2.9 | 3.4 | 1.0 |
| *QBp.caas-5B* | *AX-179388377–AX-110910964* | 673.0–679.7 | | | | 6.4 | 3.6 | 0.9 | 3.4 | 5.6 | 2.1 | 6.4 | 8.7 | 2.1 | | | | 4.0 | 4.1 | 1.2 |

[1)] Xinxiang 2021, evaluated at Xinxiang, Henan Province, in the 2020–2021 cropping season; Xinxiang 2022, evaluated at Xinxiang in the 2021–2022 cropping season; Shangqiu 2021, evaluated at Shangqiu, Henan Province, in the 2021–2022 cropping season; Dezhou 2022, evaluated at Dezhou, Shandong Province, in the 2021–2022 cropping season; Xinxiang 2023, evaluated at Xinxiang in the 2022–2023 cropping season; BLUE, the best linear unbiased estimator across five environments.
[2)] QTLs were detected with a LOD threshold of 2.8 for declaring significance based on 2,000 permutations at $P$=0.05.
[3)] LOD, logarithm of odds score; $R^2$, percentage of phenotypic variance explained by the QTL; Add, additive effect of resistance allele.

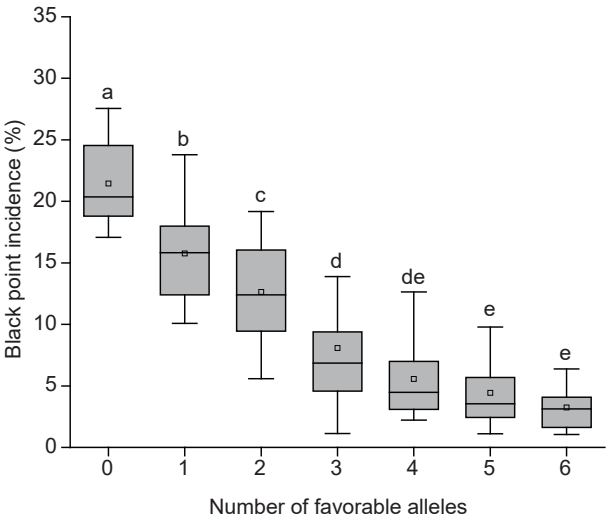


**Fig. 2** Effects of favorable allele combinations on black point incidence among different classes. *X*-axis shows the number of favorable alleles combined in each subset of recombinant inbred lines (RIL). Each of the box plots shows the upper and lower whisker, the 25 and 75% quartiles, the median (as solid line) and the mean (as rectangle). Different letters indicate significant differences at $P<0.05$.

natural population based on genotyping of the KASP markers (Fig. 3). Of them, the resistance alleles of *QBp.caas-2A* (51.5%), *QBp.caas-2B1* (79.4%), *QBp.caas-2B2* (73.3%), *QBp.caas-3A* (55.8%) and *QBp.caas-5B* (57.6%) had higher frequency distributions than their contrasting alleles, suggesting that these resistance alleles were subjected to positive selection.

## 4. Discussion

Wheat black point resistance is a typical complex quantitative trait (Conner and Kuzyk 1988; Lehmensiek *et al.* 2004; March *et al.* 2008; Liu *et al.* 2016, 2017; Li *et al.* 2022a; Tang *et al.* 2022; Gao *et al.* 2023). Environment and genotype have significant impacts on the incidence of black point (Lehmensiek *et al.* 2004; Fernandez *et al.* 2014; Li *et al.* 2014, 2022b; Liu *et al.* 2016; Zhai *et al.* 2016). In this study, the resistance alleles of *QBp.caas-2B1* and *QBp.caas-2B2* were from Zhongmai 578, whereas *QBp.caas-2A*, *QBp.caas-2D* and *QBp.caas-5B* were from Jimai 22, partially accounting for the observed transgressive segregation of the reaction. Most previous studies on black point focused on pathogen identification, biological characteristics, disease cycle and control (Conner 1989; Ellis *et al.* 1996; Desjardins *et al.* 2007; Fernandez *et al.* 2011; Fernandez and Conner 2011; Li *et al.* 2019, 2020a). The identification of QTL underpinning black point resistance and their introgression into varieties are still formidable challenges.

### 4.1. Comparisons with previous reports

In previous studies, resistance loci for black point were mapped in all 21 chromosomes (Li *et al.* 2022a). Three QTLs identified in this study, *QBp.caas-2B2*, *QBp.caas2D* and *QBp.caas-3A*, were co-located with previously

**Table 3** Polymorphic kompetitive allele-specific PCR (KASP) markers used in this study

| SNP marker | KASP marker | Physical position (Mb)[1)] | Primer name[2)] | Sequence (5′ to 3′)[3)] |
|---|---|---|---|---|
| *AX-179560327* | *Kasp_2A_BP* | 753.6 | *Kasp_2A_BPA* | **GAAGGTGACCAAGTTCATGCT**CCTCGACAAGTTTCATGTTCATGT |
| | | | *Kasp_2A_BPB* | **GAAGGTCGGAGTCAACGGATT**CCTCGACAAGTTTCATGTTCATGG |
| | | | *Kasp_2A_BPC* | GCACGCTCTTCAATCTGGGA |
| *AX-111057916* | *Kasp_2B1_BP* | 159.4 | *Kasp_2B1_BPA* | **GAAGGTGACCAAGTTCATGCT**GTCGGTAAGGTCTGCGTGTG |
| | | | *Kasp_2B1_BPB* | **GAAGGTCGGAGTCAACGGATT**GTCGGTAAGGTCTGCGTGTA |
| | | | *Kasp_2B1_BPC* | TGATATGTTTGTCAAATTGGCGA |
| *AX-86165074* | *Kasp_2B2_BP* | 563.9 | *Kasp_2B2_BPA* | **GAAGGTCGGAGTCAACGGATT**TGATACTACACGGTTAATGGCAA |
| | | | *Kasp_2B2_BPB* | **GAAGGTGACCAAGTTCATGCT**TGATACTACACGGTTAATGGCAG |
| | | | *Kasp_2B2_BPC* | GTGTCCTTCAAACGTTCGCA |
| *AX-109313738* | *Kasp_3A_BP* | 685.4 | *Kasp_3A_BPA* | **GAAGGTGACCAAGTTCATGCT**ACTACAGCAACCACAGACCTTT |
| | | | *Kasp_3A_BPB* | **GAAGGTCGGAGTCAACGGATT**ACTACAGCAACCACAGACCTTC |
| | | | *Kasp_3A_BPC* | ATGGCGTCATTGGTGAGACA |
| *AX-179388377* | *Kasp_5B_BP* | 673.0 | *Kasp_5B_BPA* | **GAAGGTGACCAAGTTCATGCT**GACGCAAACGCAAACGGT |
| | | | *Kasp_5B_BPB* | **GAAGGTCGGAGTCAACGGATT**GACGCAAACGCAAACGGC |
| | | | *Kasp_5B_BPC* | TGGCTAGCTGGTCGAATTCA |

[1)] Physical positions (Mb) of the markers were obtained by blasting SNP flanking sequences against the Chinese Spring RefSeq v1.0 sequence (https://urgi.versailles.inra.fr/blast_iwgsc/).
[2)] *A* and *B* indicate allele-specific primers; *C* indicates the common reverse primer.
[3)] The carboxyfluorescein (FAM) and hexachlorofluorescein (HEX) tails used for KASP marker assays are shown in bold text.

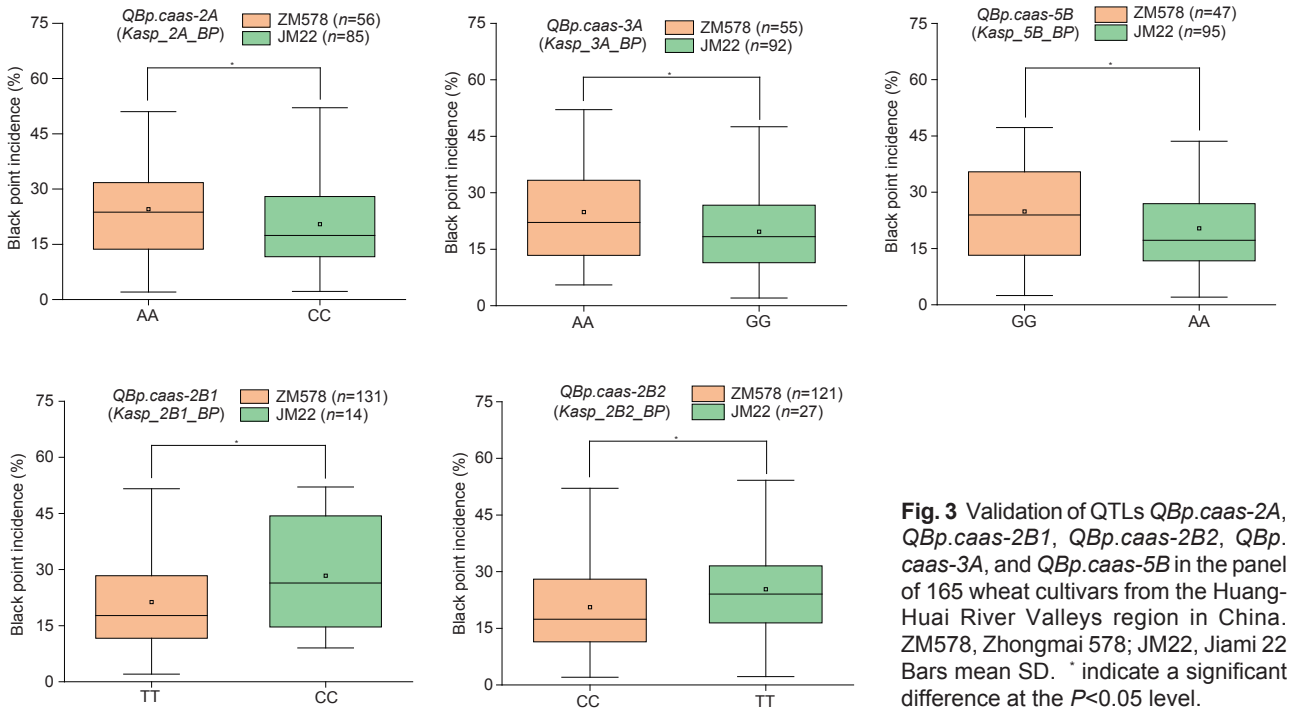


**Fig. 3** Validation of QTLs *QBp.caas-2A*, *QBp.caas-2B1*, *QBp.caas-2B2*, *QBp.caas-3A*, and *QBp.caas-5B* in the panel of 165 wheat cultivars from the Huang-Huai River Valleys region in China. ZM578, Zhongmai 578; JM22, Jiami 22 Bars mean SD. * indicate a significant difference at the $P<0.05$ level.

reported loci, whereas *QBp.caas-2A*, *QBp.caas-2B1* and *QBp.caas-5B* may be new QTLs.

***QBp.caas-2A*** *QBp.caas-2A* was detected at Xinxiang 2021, Xinxiang 2022 and Dezhou 2022, and tightly linked with *AX-95629616* (753.2 Mb) and *AX-179560327* (753.6 Mb). Lehmensiek *et al.* (2004) reported a QTL for black point resistance on chromosome 2A that was tightly linked with *Xgwm294* (709.1 Mb) and *Xgwm312* (716.3 Mb), and explained 13.0% of the phenotypic variances. Liu *et al.* (2016) identified a QTL for black point resistance that was tightly linked with *IWB39123* (706.0 Mb) in a Linmai 2/Zhong 892 RIL population. Furthermore, Zhai *et al.* (2016) detected a QTL closely linked with *RAC875_c58006_436* (715.3 Mb) for PPO activity (*QPPO.caas-2AL*) in a Gaocheng 8901/Zhoumai 16 RIL population. Although *QBp.caas-2A* is near the loci mentioned above, they do not overlap. Thus, *QBp.caas-2A* is probably a new black point resistance locus.

***QBp.caas-2B1*** Wang *et al.* (2021) identified a SNP marker (*AX-173599798*) significantly associated with black point resistance at 2B (185.1–209.1 Mb) from a Shannong 4143/Wanyuanbai 1 population. Lv *et al.* (2020) reported the closely linked marker *Kukri_c20617_17* for black point resistance at 175.6–202.9 Mb on 2B. In this study, *QBp.caas-2B1* was flanked by *AX-111057916* and *AX-95659274* and located at 159.4–167.5 Mb, implying that it could be a new locus.

***QBp.caas-2B2*** Lehmensiek *et al.* (2004) reported a black point resistance QTL closely linked with SSR markers *Xwmc154* (364.5 Mb) and *Xwmc149* (779.1 Mb) in a Sunco/Tasman population. Liu *et al.* (2016) identified a QTL for black point resistance on chromosome 2BL that was closely linked with *IWA243* (563.9 Mb). In this study, *QBp.caas-2B2* was flanked by *AX-179560603* and *AX-86165074* and located at 558.3–563.9 Mb, which overlapped with the loci identified by Liu *et al.* (2016).

***QBp.caas-2D*** *QBp.caas-2D* was tightly linked with *AX-94641475* and *AX-94731323* and located at 74.9–84.8 Mb. Wang *et al.* (2021) detected two QTLs for black point resistance that were closely linked to *AX-109844715* (151.4 Mb) and *AX-95023704* (493.1 Mb) in a Wanyuanbai 1/Shannong 4143 RIL population. In addition, Li *et al.* (2020a) reported *QBB.hau-2D* was closely linked to *AX-110483509* at 79.4–84.1 Mb, which overlapped with *QBp.caas-2D* identified in this study.

***QBp.caas-3A*** Liu *et al.* (2016) identified *QBp.caas-3AL* as tightly linked with *IWA94* (600.6 Mb) and reported the SNP marker *AX-111053669* (9.6 Mb) which was tightly linked to black point resistance (Liu *et al.* 2017). Li *et al.* (2020a) mapped *QBB.hau-3A.1* and *QBB.hau-3A.2* for black point resistance at 682.3 and 743.5 Mb. Lv *et al.* (2020) and Tang *et al.* (2022) reported *QBP.hau-3A* (60.2 Mb) and *Q_bp_5* (150.5–154.4 Mb) that were closely linked to black point resistance. *QBp.caas-3A* identified in this study was located at 682.3–688.9 Mb, which overlapped with *QBB.hau-3A.1* (682.3 Mb).

***QBp.caas-5B*** *QBp.caas-5B* was located in the interval of *AX-179388377* and *AX-110910964* on chromosome 5B (673.0–679.7 Mb), and explained 3.6–8.7% of the phenotypic variances. Li *et al.* (2020a) identified a QTL

for black point resistance that was closely linked to *AX-109321322* and located at 696.1–712.4 Mb. Lv *et al.* (2020) reported a SNP marker (*AX-86162996*) significantly associated with black point resistance at 5B (702.2 Mb), which accounted for 4.8–9.6% of the phenotypic variances. Gao *et al.* (2023) mapped *QBB.hau-5B.1* (604.0–604.2 Mb) and *QBB.hau-5B.2* (620.8–673.4 Mb), which explained 6.2 and 16.9% of the phenotypic variances, respectively, and they are different from the *QBp.caas-5B* identified in this study. Therefore, *QBp.caas-5B* is likely to be a new QTL for black point resistance.

### 4.2. Prediction of candidate genes for black point resistance

Five candidate genes for black point resistance are involved in the oxidase reaction, signal transduction and stress resistance, and show differential expression among the two parental genotypes (Appendices G and H). *TraesCS2A02G537100* for *QBp.caas-2A* encodes superoxide dismutase (SOD), which may influence the oxidation of phenolics by regulating the rate of $H_2O_2$ generation and further causing black point (Mak *et al.* 2006; March *et al.* 2007; Fernandez *et al.* 2014; Fuerst *et al.* 2014; Wei *et al.* 2015). *TraesCS2B02G183700* for *QBp.caas-2B1* is associated with lipoxygenase (LOX), which can catalyze the oxidation of phenolic compounds to brown or black pigments (melanins and quinines) (Walker and Ferrar 1998). *TraesCS2B02G398000* for *QBp.caas-2B2* corresponds to phenylalanine ammonia-lyase (PAL) which may affect black point development by adjusting the contents of phenolic compounds (Régnier and Macheix 1996). *TraesCS3A02G452400* for *QBp.caas-3A* is related to laccase, which may lead to the repeated binding of double bonds of low molecular phenolics to form chromophores (Ge *et al.* 2011). The anabolic process of lignin is one way of responding to environmental pressure. This process facilitates the pigmentation of plant cells (Janusz *et al.* 2020). *TraesCS5B02G507900* for *QBp.caas-5B* corresponds to pectinesterase inhibitor (PMEI). PME is an enzyme acting on pectin, a major component of the plant cell wall. The interplay between PME and PMEI can be a determinant of cell adhesion, cell wall porosity and elasticity, as well as a source of signaling molecules released under cell wall stress (Jolie *et al.* 2010; Wormit and Usadel 2018).

### 4.3. Tracing of resistance alleles

Tracing the resistance alleles back to their origin in the pedigree of Zhongmai 578 and Jimai 22 is important for MAS breeding. In this study, five KASP markers were successfully developed based on the tightly linked SNPs for the corresponding QTL. From the KASP marker genotyping data, the resistance allele contributed by Jimai 22 for *QBp.caas-2A* was inherited from accessions 935106, 865139 and Taishan 5 (Appendices I and J). The resistance allele at *QBp.caas-2B1* could be traced back to Zhongmai 255, Sunstate and finally to Cook (Appendices J and K). The resistance allele at *QBp.caas-2B2* could be traced back to VPM1 or Cook through Sunstate, which originated from Australia (Appendices J and L). *QBp.caas-3A* could be traced back to 865139, and finally to Taishan 5 or $F_{16-71}$ (Appendices J and M). The resistance allele at *QBp.caas-5B* could be traced back to 935106, 865139 and finally to Taishan 5 (Appendices J and N).

### 4.4. Application in wheat breeding for black point resistance

Selecting highly black point resistant lines in the field is difficult because this trait can be assessed only on mature seeds after harvest, and it is largely affected by environments. KASP is a uniplex SNP genotyping platform that offers cost-effectiveness, flexibility and high accuracy in the MAS and fine mapping of genes. In this study, five KASP markers *Kasp_2A_BP*, *Kasp_2B1_BP*, *Kasp_2B2_BP*, *Kasp_3A_BP* and *Kasp_5B_BP,* were successfully developed based on the tightly linked SNPs for the corresponding QTL. Linear regression analysis indicated that the accessions with more favorable alleles tended to be resistant to black point in the RIL and natural populations, indicating that pyramiding the QTL in wheat breeding could effectively improve black point resistance. Thus, black point resistance QTL with stable effects and their corresponding KASP markers could be used for MAS. Several accessions with a high number of resistance alleles and excellent agronomic traits, such as Norin 67, Yumai 21, Yannong 19, Zhongmai 578, Lumai 5 and Zhongmai 871, could be good parental lines for wheat breeding against black point.

## 5. Conclusion

In the present study, six stable QTLs were identified on chromosomes 2A, 2B (2), 2D, 3A, and 5B, and they explained 2.1–28.8% of the phenotypic variances. Five high-throughput KASP markers for black point resistance were developed and validated in a natural population. The resistance QTLs and their corresponding KASP markers, as well as the highly resistant cultivars and lines identified, can be used for the improvement of black point resistance through wheat breeding.

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## Declaration of competing interest

The authors declare that they have no conflict of interest.

**Appendices** associated with this paper are available at https://doi.org/10.1016/j.jia.2023.12.039

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