Molecular Mapping and Characterization of *QBp.caas-3BL* for Black Point Resistance in Wheat (*Triticum Aestivum* L.)

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Abstract

Wheat black point, which occurs in most wheat growing regions of the world, is detrimental to grain appearance, processing and nutrient quality. Mining and characterization of genetic loci for black point resistance is helpful for breeding resistant wheat cultivars. We previously identified a major QTL *QBp.caas-3BL* in a recombinant inbred line (RIL) population of Linmai 2/Zhong 892 across five environments. Here we confirmed the QTL in two additional environments. The genetic region of *QBp.caas-3BL* was enriched with newly developed markers. Using four sets of near isogenic lines *QBp.caas-3BL* was narrowed down to a physical interval of approximately 1.7 Mb, including five annotated genes according to IWGSC reference genome. *TraesCS3B02G404300*, *TraesCS3B02G404600* and *TraesCS3B02G404700* were predicted as candidate genes based on the analyses of sequence polymorphisms and differential expression. We also converted a SNP of *TraesCS3B02G404700* into a breeding-applicable KASP marker and verified its efficacy for marker-assisted breeding in a panel of germplasm. The findings not only lay a foundation for map-based cloning of *QBp.caas-3BL* but also provide a useful marker for selection of resistant cultivars genotypes in wheat breeding.

Key Message

We fine-mapped *QBp.caas-3BL* for black point resistance in an interval of 1.7 Mb containing five high-confidence annotated genes and developed a KASP marker suitable for selection of *QBp.caas-3BL*.

Introduction

Wheat is an important staple crop that provides about 20% of calories for humankind. Black point (also known as kernel smudge) is a serious disease characterized by dark discoloration at the embryo end of wheat grains and occurs in most wheat-growing regions of the world. It causes inferior grain appearance, processing and nutrient quality as well as lower seed vigor (Liu et al. 2016). Mining and characterization of genetic loci for black point resistance is helpful for breeding cultivars with resistance to the disease.

To date, relatively few genetic studies have been performed on black point response in wheat. A total of nine QTL for black point resistance were mapped on chromosomes 1D, 2A, 2B, 2D, 3D, 4A, 5A and 7A (2 QTL) in bi-parent mapping populations derived from crosses Cascades/AUS1408 and Sunco/Tasman, each explaining 4.0-18.4% of the phenotypic variance (Lehmensiek et al. 2004). Twenty-five loci associated with black point response on chromosomes 2A, 2B, 3A, 3B (2), 3D, 4B (2), 5A (3), 5B (3), 6A, 6B, 6D, 7A (5), 7B and 7D (2) explained phenotypic variances ranging from 7.9 to 18.0% (Liu et al. 2017). Recently, Li et al. (2020) detected 23 loci associated with black point response on chromosomes 1B, 1D, 2B (2), 2D, 3A (2), 3B (2), 3D, 4A, 5A (2), 5B (2), 6B (3), 6D, 7A (2) and 7D (2), respectively. Lv et al. (2020) identified 386 marker-trait associations (MTAs) for black point response by genome-wide association analysis and three of the MTAs were further verified in a bi-parental population. These studies laid the foundation for map-based cloning of genes for resistance to black point, but fine mapping of the genetic loci has not been conducted thus far.

In our previous study, nine QTL for black point resistance were mapped on chromosomes 2AL, 2BL, 3AL, 3BL, 5AS, 6A, 7AL (2) and 7BS in a recombinant inbred line (RIL) population derived from the Linmai 2/Zhong 892 cross (Liu et al. 2016). *QBp.caas-3BL* was detected across five environments and explained 6.0-12.2% of the phenotypic variances, suggesting that it was a stable major QTL for black point resistance. Here *QBp.caas-3BL* was confirmed in another two environments. We created a series of near isogenic lines to fine-map *QBp.caas-3BL* and predicted its candidate genes. A cost-effective KASP marker was also developed to improve black point resistance in wheat breeding.

Materials And Methods

Plant materials

To confirm *QBp.caas-3BL*, we planted the Linmai 2/Zhong 892 RIL population in Anyang (Henan province) and Suixi (Anhui province) during the 2018-2019 cropping season. The residual heterozygous recombinant plants for *QBp.caas-3BL* were screened from the RIL population. Four sets of near isogenic lines (NILs) for *QBp.caas-3BL* were identified from the residual heterozygous recombinant plants and used for fine mapping (Table S1). The NILs were grown in single row plots of 2 m with approximately 40 plants per row and 20 cm between rows at Anyang during the 2018-2019 cropping season and Xinxiang (Henan province) during the 2019-2020 cropping season. A panel of 166 wheat cultivars from the Huang-Huai Wheat Region was used to validate the genetic effect of *QBp.caas-3BL* on black point response (Liu et al. 2017).

Phenotypic evaluation for black point

Black point in mature grains was assessed manually at least 15 days after harvest. Each RIL or NIL was scored for black point response (obvious discoloration around the embryos) by counting the number of black point-affected grains among three samples of 200. The percentage of black point-affected kernels was taken as the black point score for each plot. To ensure the accuracy of phenotyping, black point of all materials was scored by the same person under similar illumination conditions.

PCR-based genotyping by sequencing

To enrich the target region of *QBp.caas-3BL*, annotated genes were identified in the physical interval according to the International Wheat Genome Sequencing Consortium (IWGSC) RefSeq v1.1 (http://plants.ensembl.org/index.html). The sequences from some annotated genes were retrieved and aligned to design gene-specific primers by DNAMAN software. The polymorphic sites between parents Linmai 2 and Zhong 892 were used to develop markers for fine mapping of *QBp.caas-3BL*. PCR was performed in reaction mixtures of 15 μl containing 7.5 μl of 2 × Taq PCR Mix (Beijing HT-biotech Co., Ltd., Beijing), 5 pmol of each primer and 50-100 ng of genomic DNA using an ABI Applied Biosystems Veriti 96 Well Thermal Cycler (Gene Co., Ltd., Shanghai). PCR amplification was performed at 95°C for 3 min, followed by 35 cycles (94°C for 30 s, 59-61°C for 30 s and 72°C for 1 min) and a final extension at 72°C for 7 min. Primer pairs and their annealing temperature for PCR were listed in Table S2. PCR products

were recovered from 1.5% agarose gels and purified by TIANgel MIDI Purification Kit (Tiangen, Biotechnology Co., Ltd., Beijing). The sequencing of target PCR products was performed by BGI Genomics Co., Ltd in Beijing.

Development of a KASP marker

PolyMarker software (http://polymarker.tgac.ac.uk/) was used to design KASP markers for target polymorphic sites (Table S3). The PCR cycling conditions were 94°C for 15 min, followed by 10 touchdown cycles (94°C for 20 s; 62°C to 55°C for 60 s), 26 cycles of 94°C for 20 s and 55°C for 60 s, 6 cycles of 94°C for 20 s and 57°C for 60 s, and one final acquisition cycle at 16°C for 60 s (Collins et al. 2018). The KASP platform of LGC Co., Ltd was used to carry out genotyping and statistical analyses.

Quantitative PCR

Grains of Linmai2 and Zhong892 were sampled 25 days after flowering and used to isolate total RNA by a Total RNA Extraction Kit (Beijing TransGen Biotechnology, Cat#ER301-01). cDNA was generated with a PrimeScript RT Reagent Kit plus gDNA Eraser (TaKaRa, Dalian). Quantitative PCR (qPCR) was performed on cDNA samples produced from three biological replicates in a BioRad CFX system using iTaq Universal SYBR Green Supermix (BioRad, Beijing). The primer pairs for gene-specific qPCR were designed according to the gene annotation from RefSeq v1.1 (Table S4). The profile of qPCR was as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 59°C for 20 s. A final dissociation stage was run to generate a melting curve for judgement of amplification specificity. Relative gene expression was normalized to *elongation factor 1 alpha* (*EF1α*) using the $2^{-\Delta\Delta Ct}$ equation.

Linkage mapping and QTL analysis

A genetic linkage map for *QBp.caas-3BL* was constructed using JoinMap 4.0 (http://www.kyazma.com). QTL mapping was conducted by inclusive composite interval mapping (ICIM) in IciMapping 4.0 software (https://www.isbreeding.net) with a LOD threshold of 3.0. The means of phenotypic data for RILs in each environment were used for QTL analysis.

Statistical analysis

Association analysis for declaration of significance at $P<0.05$ in KASP marker validation was conducted by student's *t*-tests in Excel. The Excel software was also used to calculate the means, standard deviation and coefficients of variation of phenotypic data.

Results

Confirmation of QBp.caas-3BL

In the previous study, *QBp.caas-3BL* was identified as a major QTL for black point in the RIL population of Linmai 2/Zhong 892 (Liu et al. 2016). To confirm the presence of *QBp.caas-3BL*, we re-investigated black

point response of the RIL population in two additional environments (2019 Anyang and 2019 Suixi). Based on the phenotypes in these two environments, the QTL *QBp.caas-3BL* was confirmed with a consistent genetic location across environments (Fig. 1A), explaining 6.6–12.4% of the phenotypic variance. These results confirmed that *QBp.caas-3BL* is a stable QTL for black point.

Generation of secondary mapping populations for QBp.caas-3BL

To narrow down the target interval of *QBp.caas-3BL*, we created secondary mapping populations. Based on the genotyping data by the wheat 90K SNP chip, four RILs with residual heterozygosity in the *QBp.caas-3BL* target region were identified using markers. We genotyped approximately 2000 individual plants from the heterozygous recombinant lines using the flanking markers *RAC875_rep_c105184_88* (*M1*) and *Tdurum_contig67350_494* (*M12*) (Fig. 1A; Table S2). Each of the so-called residual heterozygous recombinant lines had homozygous plants with contrasting genotypes in the target region of *QBp.caas-3BL*. Since the plants from each residual heterozygous recombinant line harbored contrasting genotypes in the target region but were homozygous for most other regions of the genome based on SNP arrays, they were treated as NILs of *QBp.caas-3BL*. Four sets of *QBp.caas-3BL* NILs were selected and used to create secondary populations for fine mapping (Table S1).

Fine mapping of QBp.caas-3BL

To enrich the interval of *QBp.caas-3BL*, we identified polymorphic sites of six genes within the target region based on the IWGSC RefSeq v1.1 (http://plants.ensembl.org/index.html) and developed gene-specific markers (Table S2). Six chip-based SNPs that were identified previously as flanking markers of *QBp.caas-3BL* were also converted into sequencing-based markers. Using these markers, we mapped *QBp.caas-3BL* to a smaller genetic region (Fig. 1A). *QBp.caas-3BL* was further delimited in a physical interval between *M8* (*TraesCS3B02G404200*) and *M10* (*TraesCS3B02G404800*) based on genotypic and phenotypic analyses of the secondary mapping populations (Fig. 1B; Table S1). The physical interval spanned 1.7 Mb, including five high-confidence annotated genes, *TraesCS3B02G404300*, *TraesCS3B02G404400*, *TraesCS3B02G404500*, *TraesCS3B02G404600* and *TraesCS3B01G404700* according to RefSeq v1.1 (Fig. 1C).

Prediction Of Candidate Genes

Among the five candidate genes, *TraesCS3B02G404300*, *TraesCS3B02G404400, TraesCS3B02G404500* and *TraesCS3B02G404600* are bZIP family transcription factors that are orthologous to *ABSCISIC ACID-INSENSITIVE 5* (*ABI5*) (Fig. 1C and S1), whereas *TraesCS3B02G404700* is a homolog of *protein phosphatase* (Fig. S2). To determine the candidate gene(s) in *QBp.caas-3BL* we compared the open reading frames (ORFs) of the five genes from parents Linmai 2 and Zhong 892 by sequencing. The ORFs of *TraesCS3B02G404600* and *TraesCS3B02G404700* contained sites polymorphic between Linmai 2 and Zhong 892. The SNP in *TraesCS3B02G404600* was a synonymous mutation, whereas that of *TraesCS3B02G404700* was a nonsynonymous mutation causing a serine-to-alanine substitution (Fig. 2A

and 2B). We also investigated the expression patterns of the five genes in Wheat Expression Browser (http://wheat-expression.com/) and found that all were expressed in grains, suggesting that they could be involved in black point response (Fig. S3-7). All four *ABI5* homologs, *TraesCS3B02G404300*, *TraesCS3B02G404400, TraesCS3B02G404500* and *TraesCS3B02G404600*, have spike- and seed-specific expression patterns, whereas *TraesCS3B02G404700* is highly expressed in all tissues (Fig. S3-7). We further compared the transcriptional levels of the five genes in developing grains using qPCR assays and observed that *TraesCS3B02G404300, TraesCS3B02G404600* and *TraesCS3B02G404700* were differentially expressed between Linmai 2 and Zhong 892 (Fig. 2C). All three differentially expressed genes were down-regulated in Linmai 2 compared to Zhong 892 (Fig. 2C). Thus, each of *TraesCS3B02G404300*, *TraesCS3B02G404600* and *TraesCS3B02G404700* could be the causal gene of *QBp.caas-3BL* based on sequence polymorphism and differential expression.

Development and validation of a KASP marker for QBp.caas-3BL

TraesCS3B02G404700 was identified as a candidate gene for *QBp.caas-3BL*, with a SNP in its ORF between the parents. To develop a breeding-applicable marker for *QBp.caas-3BL*, we converted the SNP into a cost-effective, high-throughput KASP marker (Fig. 2D; Table S3) and validated it in a panel of 166 wheat cultivars from the Huang-Huai Wheat Region, the largest wheat area in China. Association analysis indicated that sequence variation in *TraesCS3B02G404700* was significantly associated with black point response (Table 1; Table S5).

Table 1
Genetic effects of *QBp.caas-3BL* on black point response in a panel of 166 wheat cultivars tested by the KASP marker developed from *TraesCS3B02G404700*

Environment	Genotype	Number of accessions	Mean ± SD (%)	*P*-value
2013AY	0	93	30.5 ± 1.52	0.0002**
	2	73	19.5 ± 0.53	
2014AY	0	93	20.6 ± 2.15	0.046*
	2	73	16.4 ± 0.74	
2013SX	0	93	29.3 ± 0.75	0.0003**
	2	73	19.8 ± 0.56	
0, 2 indicate the genotypes of Linhan 2 and Zhong 892, respectively.				
* and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.				
2013AY, 2014AY and 2013SX represent different cropping seasons in Anyang (AY) and Suixi (SX), respectively.				

Discussion

QBp.caas-3BL has potential to improve black point resistance in wheat breeding

In the present study we confirmed the effect of *QBp.caas-3BL* in additional two environments, in agreement with Liu et al. (2016). *QBp.caas-3BL* was further mapped into a physical interval of approximately 1.7 Mb from which candidate genes were also identified. These results provide important reference information for map-based cloning and marker-assisted selection of *QBp.caas-3BL.* Based on a polymorphic site in *TraesCS3B02G404700*, a candidate gene for *QBp.caas-3BL*, we developed a cost-effective and high-throughput KASP marker. Association analysis showed that *QBp.caas-3BL* had a significant effect on black point resistance using the KASP marker in a diverse panel of 166 wheat cultivars, suggesting that the marker is a breeding-applicable tool for marker-assisted selection of black point resistance. The additional field data obtained in the study confirmed that *QBp.caas-3BL* is a stable major QTL for black point response and could be useful to improve black point resistance in wheat breeding.

Functional prediction of candidate genes for QBp.caas-3BL

The cause of black point is complicated; the symptom have been attributed to fungal infection, enzymatic browning and environmental stress, such as high humidity and temperature (March et al. 2007; Fernandez and Conner 2011; Busman et al. 2012; Fernandez et al. 2014). In this study we mapped *QBp.caas-3BL* to a physical interval of 1.7 Mb, and determined *TraesCS3B02G404300*, *TraesCS3B02G404600* and *TraesCS3B02G404700* as candidate genes. Phylogenetic analysis showed that *TraesCS3B02G404300* and *TraesCS3B02G404600* are probably resulted from tandem duplication events (Fig. S1A). Additionally, it appears that the tandem duplication events have happened in *Triticum dicoccoides* (also known as wild emmer), the putative progenitor of wheat subgenomes A and B, according to BLASTN against WEWSeq_v.1.0 (Genomic sequence) (http://plants.ensembl.org/Triticum_dicoccoides/Tools/Blast/Results?r=3B:639752637-639756075;tl=7tHVuMlVeE1hFS0L-20388519). *TraesCS3B02G404300* and *TraesCS3B02G404600* are orthologous to *ABI5* in Arabidopsis and *OsABI5-1* in rice (Fig. S1A). *ABI5* has pleiotropic effects on plant growth and development and is also involved in response to biotic and abiotic stresses. In Arabidopsis, ABI5 (AT2G36270) is a key regulator in ABA signaling during seed maturation and germination and modulates late embryogenesis abundant genes during both developmental stages (Finkelstein et al. 2000; Ibarra et al. 2016). Bi et al. (2017) showed that AtABI5 played a role in regulating the homeostasis of reactive oxygen species (ROS) by activating *CATALASE1* transcription during seed germination. *OsABI5* (Os01g0859300) was reported to regulate fertility and stress tolerance in rice (Zou et al. 2008). *TaABF1-3A* (*TraesCS3A02G371800*), an ortholog of *TraesCS3B02G404300*, was shown to participate in grain development by physically interacting with *PKABA1*, a signaling component in the ABA-suppression of GA-induced gene expression in cereal grains (Fig. S1; Johnson et al. 2008). Harris et al. (2013) further validated that *TaABF1* functioned as an integrator of ABA and GA signaling in aleurone cells of grains. Recently, overexpression of *TaABI5* (99.4% similarity with *TraesCS3D02G364900* in IWGSC RefSeq v1.1) in Arabidopsis transformants exhibited high sensitivity to ABA and repressed germination (Utsugi et al.

2020). Hence, *TraesCS3B02G404300* and *TraesCS3B02G404600*, homologs of *ABI5*, are probably involved in seed development by modulating ABA and GA signaling.

TraesCS3B02G404700, the third candidate gene of *QBp.caas-3BL*, is orthologous to *OsPP16* in rice, whereas an ortholog is not detected in Arabidopsis based on phylogenetic analyses (Fig. S2). OsPP16 belongs to a dual specificity phosphatase (DUSP) clade (Singh et al. 2010). As a member of the tyrosine phosphatase superfamily, dual specificity phosphatase (DUSP) can dephosphorylate tyrosine, serine and threonine residues, and is involved in many basic physiological activities such as plant growth, development and abiotic/biotic stress response (Jiang et al. 2018). OsPFA-DSP1, an atypical dual-specific phosphatase (PFA-DSP1) subfamily member in rice, may act as a negative regulator in drought stress responses (Liu et al. 2012). DUSP IBR5 in Arabidopsis is involved in regulation of R protein CHS3-mediated temperature dependent responses (Liu et al. 2015). Thus, it appears that *TraesCS3B02G404700* is involved in response to abiotic/biotic stress, a postulated cause of black point.

In summary, the functional prediction of candidate genes in *QBp.caas-3BL* lays a strong foundation for identification of its causal gene and ascertainment of the underlying mechanism. Transgenic experiments are ongoing to validate the functions of the candidate genes.

Declarations

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Conflict of interest:

We declare no conflicts of interest in regard to this manuscript.

Ethical Standards:

These experiments complied with the ethical standards in China.

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Figures

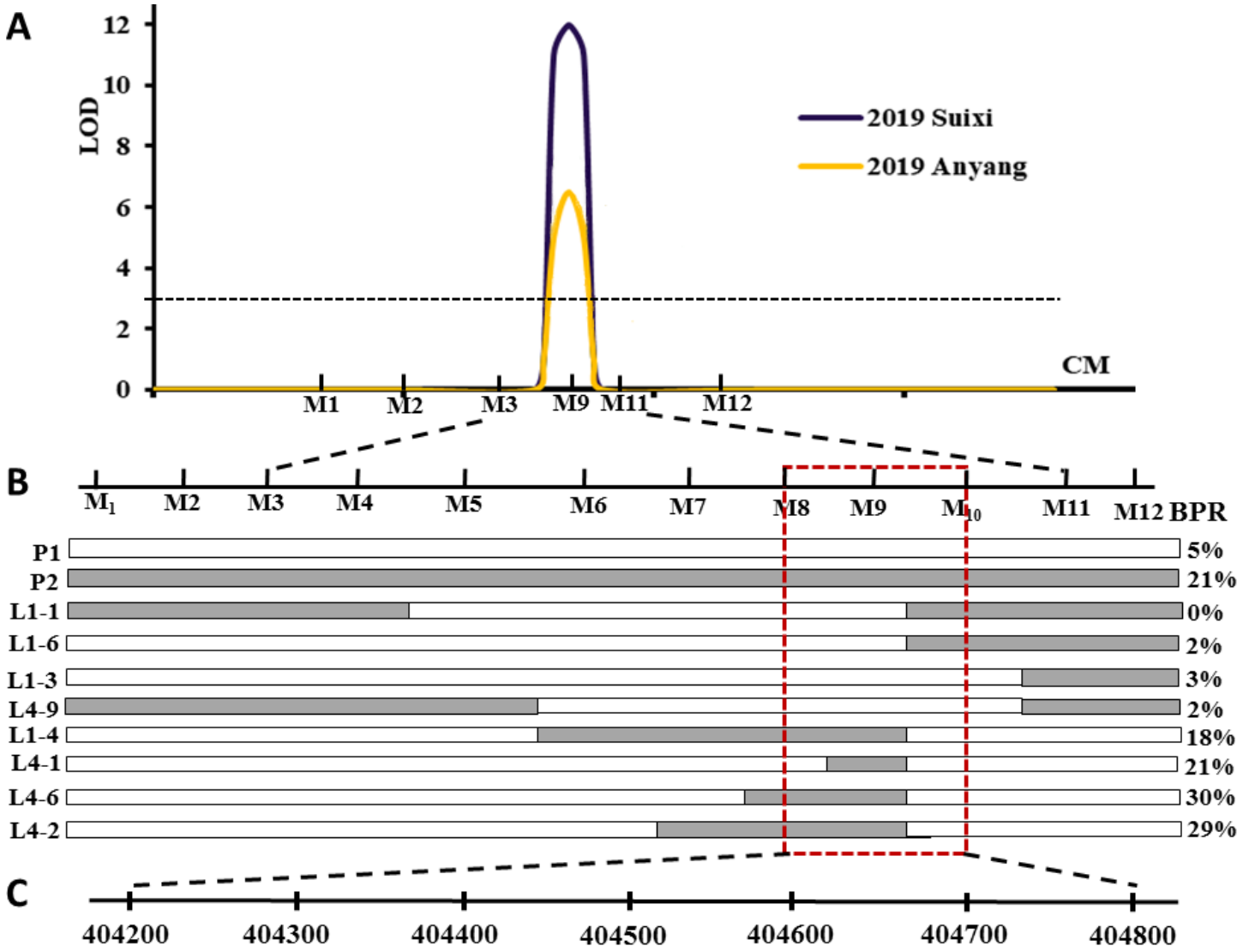

Figure 1

Genetic confirmation (A), fine mapping (B) and physical delimitation (C) of QBp.caas-3BL. M1-M12 are markers that correspond to Table S2; P1 and P2 represent Zhong 892 and Linmai 2, respectively; BPR, black point rate.

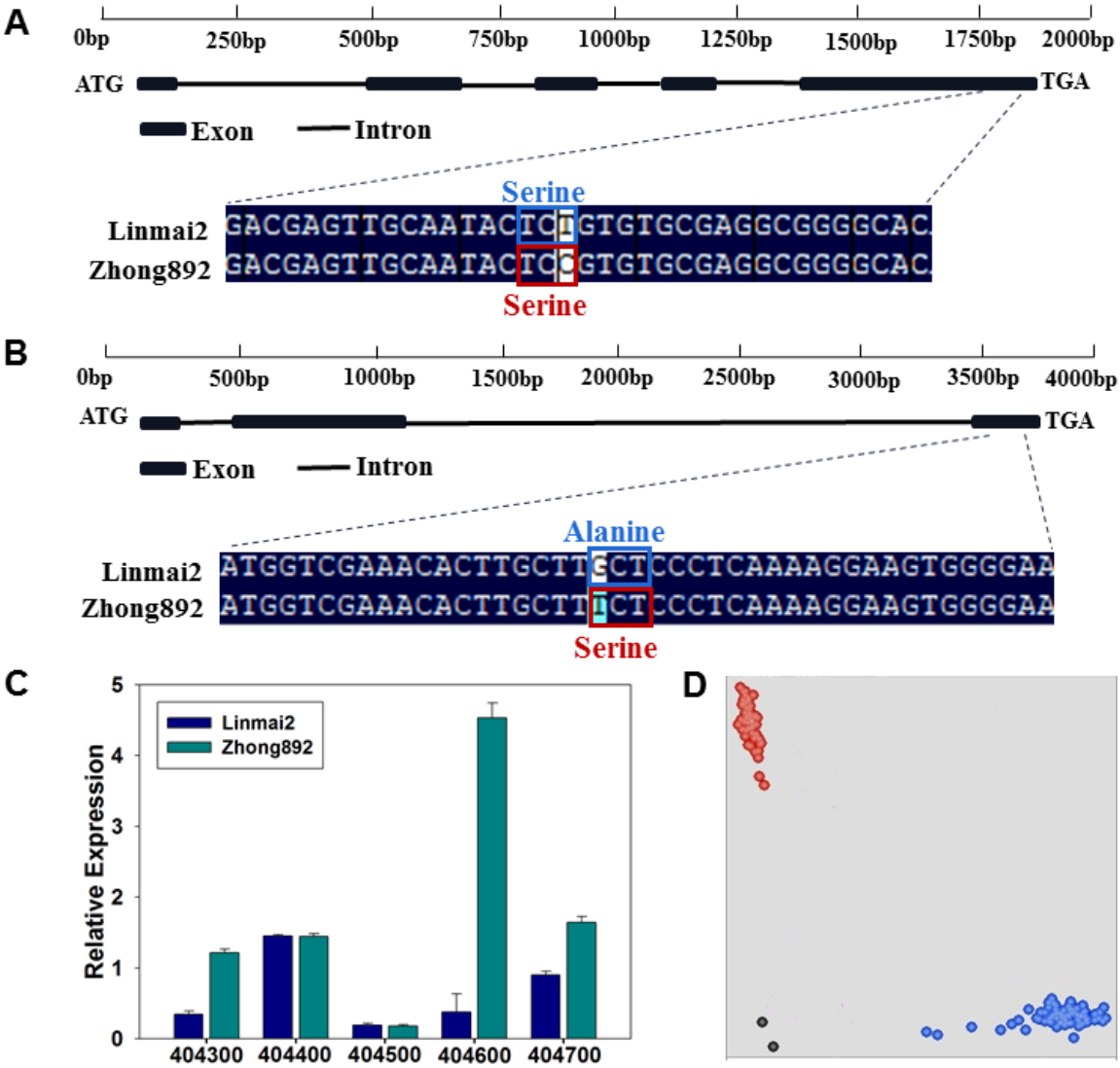

Figure 2

Prediction of candidate genes and development of a breeding marker for QBp.caas-3BL. Polymorphic sites between the parents in open reading frames of genes TraesCS3B02G404600 (A) and TraesCS3B02G404700 (B). (C) Differential expression of the annotated genes in the target region of QBp.caas-3BL in grains 25 days after flowering. (D) Genotyping of 166 wheat cultivars from the Huang-Huai Wheat Region using the KASP marker developed from TraesCS3B02G404700.

Supplementary Files

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