# Fine Mapping of the QTL cqSPDA2 for Chlorophyll Content in Brassica Napus L.

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# Abstract

**Background:** Chlorophyll is the most important factor enabling plants to absorb, transfer and transform light energy and plays an important role in yield formation. *Brassica napus* is one of the most important oil crops. Qinghai Province is located in the Qinghai-Tibet Plateau in China. Solar radiation and its intensity are higher, and the light duration is longer. Under this light condition, conducting high light efficiency breeding of *Brassica napus* from the perspective of improving photosynthetic efficiency has considerable social and economic value. At present, the fine mapping of chlorophyll quantitative trait loci (QTLs) in many crops has been studied, especially in rice. In *Brassica napus*, there have been studies on the initial location of chlorophyll in seed embryos and pericarps, but there are few reports on the fine mapping of chlorophyll QTLs. We built near-isogenic lines, fine mapped the chlorophyll locus, and evaluated the effect of the dominant locus on agronomic traits.

**Results:** The *cqSPDA2* locus was mapped in an interval of 21.87-22.91 Mb on the A02 chromosome of *Brassica napus* using doubled haploid (DH) lines. To fine map *cqSPDA2*, we built near-isogenic lines and designed Indel primers covering the mapping interval. The 469 individuals in the $BC_3F_2$ population were analyzed using these Indel primers. Among the Indel primers, 15 narrowed the mapping interval to 5.2 cM between Indel3 and Indel15. Next, 16 Indel primers and 19 SSR primers were designed within the new narrow mapping interval, and 7 primers were polymorphic and tightly linked to the *cqSPDA2* locus in the $BC_4F_2$ population. The mapping interval was narrowed to 152 kb on A02 between SSR2 and

Indel15. We found three annotated genes in the mapping interval, including *BnaA02g30260D*, *BnaA02g30290D* and *BnaA02g30310D*, which may be responsible for chlorophyll synthesis by gene expression analysis.

**Conclusions:** The locus *cqSPDA2*, the dominant QTL for chlorophyll content in *Brassica napus*, was fine mapped to the 21.89-22.04 Mb interval on A02. Three annotated genes (*BnaA02g30260D*, *BnaA02g30290D* and *BnaA02g30310D*) which may be responsible for chlorophyll synthesis were found.


# Background

The material basis of crop yield formation is derived from photosynthesis. Studying crop high yield from photosynthesis is becoming a new breeding hotspot [1]. Chlorophyll is the most important factor enabling plants to absorb, transfer and transform light energy and plays an important role in the growth and development of plants [2]. Maintaining a high level of chlorophyll content in leaves is an important factor in increasing photosynthetic activity [3]. In a certain range, there is a positive correlation between chlorophyll content and photosynthetic rate, which directly determines the yield [4, 5]. Therefore, chlorophyll content plays an important role in yield formation [6, 7]. The seedling development of *Brassica napus* leads to a higher yield stability and has a high importance for plant breeders [8]. Chlorophyll content is a quantitative characteristic that is primarily controlled by nuclear genes and has high heritability. In recent years, researchers have analyzed QTLs of chlorophyll content in seedling leaves

of different crops with different populations from different perspectives and made considerable progress, establishing a foundation for future research attempting to elucidate the molecular genetic mechanism of chlorophyll [9–15].

The completion of the whole genome sequencing of *Brassica napus* indicates that research on the *Brassica napus* genome has entered a new era. In recent years, with the rapid development of molecular marker technology, it has become possible to construct a high-density molecular marker genetic map of *Brassica napus*. Therefore, efficient light breeding of *Brassica napus* with chlorophyll QTLs is an important breakthrough in improving the yield potential and direction of high-yield breeding of *Brassica napus* in the future. Qinghai Province is located in the Qinghai-Tibet Plateau in northwestern China. The solar radiation and its intensity during the growth period of crops are higher than those in the interior of China, and the light duration is longer. Under this light condition, performing high light efficiency breeding of *Brassica napus* from the perspective of improving photosynthetic efficiency has strong social and economic value. At present, studies on the initial location of chlorophyll content in seed embryos [16] and pericarps [17] of winter *Brassica napus* have been conducted, QTL loci have been identified under drought and salt stress, and even candidate genes related to salt tolerance have been predicted [18–20]. However, studies on the fine mapping of chlorophyll content QTLs in *Brassica napus* have rarely been reported.

In a previous study, we discovered a dominant QTL named *cqSPDA2* located in 21.87–22.91 Mb on A02 by DH lines from a cross between Zhongshuang11 (ZS11) and QU (under review). In this study, the near isogenic line (NIL) of *cqSPDA2* was constructed by flanking markers closely linked to *cqSPDA2* and backcross selection. The near isogenic lines were analyzed using a molecular marker to further narrow the *cqSPDA2* to the range of 150 kb.

This study established a foundation for future research investigating the cloning of chlorophyll genes controlling photosynthetic function and provided a theoretical basis for improving germplasm resources and selecting new high-yield varieties by molecular markers.

## Results

# Phenotypic and genetic analysis

The first fully developed leaves counting from the top of the 2061 individuals in the $BC_4F_2$ populations at the six-leaf stage were measured by SPAD (SPAD 502, Japan). The Chi-square test showed that the segregation pattern of the chlorophyll content trait was in keeping with the expected Mendelian segregation ratio of 3:1 ($\chi^2 = 2.53$) (SPAD = 43, high chlorophyll content vs. low chlorophyll content) (Fig. 1). Among the random selection of 198 individuals in the $BC_6F_1$ population, the markers SSR2 and Indel100 were used to validate the effect of *cqSPDA2*. The result of a Chi-square test was in keeping with a 1:1 ($\chi^2 = 1.46$; $\chi^2 = 1.82$) (AA:Aa) Mendelian ratio (Additional file 1: Table S1).

# Fine mapping of cqSPDA2

To fine map the *cqSPDA2* locus and identify the candidate genes, 87 primer pairs of Indel markers were designed to uniformly cover the preliminary mapping interval 21.87–22.91 As a result, 28 polymorphic markers were detected with the two parental lines and some DH lines. Twenty-three of these markers were found to be cosegregated in the DH lines. The 469 individuals in the $BC_3F_2$ population were analyzed using these Indel primers. The linkage map constructed using the Indel data and corresponding chlorophyll content phenotypes showed that 15 Indel primers were tightly linked with the *cqSPDA2* locus (Additional file 2: Table S2). The *cqSPDA2* locus was delimited to an interval of 5.2 cM between Indel3 and Indel15 (Additional file 3: Fig. S1). The fragments of the primers indel3, indel6, indel15 and indel 17 near the QTL locus were recovered. TA clone analysis was performed with the PMD18T vector, and the physical location of the region was found to be within the 188-kb range of 21.88–22.07 Mb (Additional file 4: Figure S2).

Next, 16 Indel and 19 SSR primers were designed within the new narrow mapping interval, and 7 primers were polymorphic and tightly linked to the *cqSPDA2* locus (Additional file 5: Table S3). These new primers helped to narrow the interval for 250 individuals in the $BC_4F_2$ population. As a result, the *cqSPDA2* locus was mapped to a 152-kb interval between SSR2 and Indel15 (Fig. 2). BSNP88 and BSNP90 are SNP markers developed in the interval (Additional file 6: Table S4). SSR2 is a codominant marker and closely linked with *cqSPDA2*. Twenty plants with low, medium and high chlorophyll phenotypes in $BC_4F_2$ were selected, and the corresponding genotypes were identified by SSR2. The results showed that the three groups of different phenotypes can be divided into three genotypes: AA, Aa and aa. It is suggested that SSR2 was closely linked with *cqSPDA2* and could be effectively used in MAS breeding (Fig. 3).

# Quantitative RT-PCR of genes in the mapping interval

According to the *Brassica napus* genome annotation database (http://www.genoscope.cns.fr/brassicanapus/), twenty-seven genes were identified in the targeted mapping interval 21.89–22.04 Mb on A02 (Additional file 7: Table S5). The melting curve and amplification curves of twenty-seven genes were analyzed, and the results showed that 24 primers can be used to analyze gene expression (Additional file 8: Table S6). The twenty-four genes and the housekeeping gene *Actin7* were quantified by qRT-PCR (Additional file 9: Table S7). The results showed that the expression levels of three genes (*BnaA02g30260D*, *BnaA02g30290D* and *BnaA02g30310D*) were all higher in ZS11 and $BC_4F_{2:3}$(AA) than in QU and $BC_4F_{2:3}$(aa) at the three stages. There were significant differences between $BC_4F_{2:3}$(AA) and $BC_4F_{2:3}$(aa) at the 6-leaf stage in *BnaA02g30290D and BnaA02g30310D* ($p < 0.05$), and there was a highly significant difference in *BnaA02g30260D* ($p < 0.01$) (Fig. 4). There was no consistent expression of other genes in QU, $BC_4F_{2:3}$(AA), ZS11 and $BC_4F_{2:3}$(aa) at the three stages. Therefore, *BnaA02g30260D, BnaA02g30290D* and *BnaA02g30310D* were likely candidate genes for *cqSPDA2*.

# Agronomic traits analysis

To investigate the effect of *cqSPDA2* on agronomic traits, 50 plants with the AA genotype (high chlorophyll content) and 50 plants with the aa genotype (low chlorophyll content) were selected from the $BC_4F_2$ population by molecular marker and SPAD. We investigated plant height, silique length, number of seeds per silique, number of siliques per plant, 1000-seed weight, and individual plant yield. The results showed that plant height, number of seeds per silique, number of siliques per plant and individual plant yield were highly significantly different between the AA genotype plants and the aa genotype plants ($P < 0.01$). There was a difference in 1000 grain weight but no difference in silique length ($P < 0.05$) (Additional file 10: Table S8).

# Discussion

Leaf is the main photosynthetic organ, and chlorophyll content is an important agronomic trait for plant yield. Ninety to ninety-five percent of plant dry matter is produced by photosynthesis, and crop yield is primarily derived from the photosynthetic products of leaves [21]. Chlorophyll is an important pigment involved in photosynthesis in chloroplasts, which can absorb and transform light energy. Chlorophyll is also an important index to evaluate the photosynthetic capacity of leaves [22]. Increasing crop yield by increasing chlorophyll content is one of the important breeding objectives of high light efficiency breeding [23]. Chlorophyll content is a quantitative characteristic that is primarily controlled by nuclear genes and has high heritability. At present, research on chlorophyll QTLs has been performed on various crops, such as rice [8, 10, 12, 13, 15], wheat [9, 11], beans [24, 25], and cabbage [14], especially rice [13]. There have been studies on the location of chlorophyll in the embryo [16] and pericarp [17] of winter *Brassica napus*. Under drought and salt stress conditions, QTLs were also detected, and even a candidate gene related to salt tolerance was predicted [18–20]. However, there is no report on the fine location of the chlorophyll QTL locus in *Brassica napus*. This report is the first to describe fine mapping of *cqSPDA2* on *Brassica napus*. In this study, the NILs of *cqSPDA2* regions were constructed using recurrent parent ZS11 with flanked markers. Indel and SSR markers were used to scan the populations of $BC_3F_2$ and $BC_4F_2$, and the mapping interval was reduced to 152 kb. This strategy for mapping the target genes is reasonable, inexpensive, and highly efficient. According to the $BC_4F_2$ phenotype and $BC_6F_1$ genotype analysis, the strategy is consistent with the Chi-square test. The results indicated that *cqSPDA2* is the main QTL locus. A positive correlation between *cqSPDA2* and agronomic characteristics, such as yield, was determined through the analysis of NIL.

*BnaA02g30260D*, *BnaA02g30290D* and *BnaA02g30310D* were identified as good candidate genes of *qSPADA2* among the twenty-nine genes annotated from *Brassica napus* genomes in the mapping interval according to the qRT-PCR analysis. *BnaA02g30260D*, which is a disease-resistance protein family, has transmembrane receptor activity, nucleoside-triphosphatase activity, nucleotide binding, and ATP binding function and is involved in signal transduction, defense response, apoptosis, and innate immune response according to the annotations. Further study is needed to determine whether *BnaA02g30260D* affects chlorophyll synthesis. *BnaA02g30290D* is FK506- and rapamycin-binding protein 15 kD-2 (*FKBP15-2*) and has peptidyl-prolyl cis-trans isomerase activity related to protein folding. Luan et al. [26]

found that *AtFKBP15-1* and *AtFKBP15-2* had the highest homology to *FKBP13* and encoded functional homologs of *FKBP13*. *AtFKBP13* was reported to be associated with Rieske protein, both before and after the import of the proteins into the chloroplast stroma. AtFKBP13 can play a role in the downregulation of Rieske protein accumulation. Rieske is a subunit of the cytochrome $b_6f$ complex, which is one of the four complexes of the photosynthetic electron transport chain [27]. It was also reported that *ScFKBP12* was transferred into *Arabidopsis*, chloroplast formation was inhibited and the expression of genes related to chloroplast formation was inhibited [28]. In this study, the expression levels of *BnaA02g30290D* (*AtFKBP15-2*) in NILs (aa) and ZS11 were all higher than those in NILs (AA) and QU at the three stages, which inhibited the formation of chlorophyll and was consistent with the above mentioned results. *BnaA02g30310D* is homologous to *GCH-1* in *Arabidopsis thaliana*. GCH-1 is the first enzyme for tetrahydrobiopterin (BH4) biosynthesis [29]. BH4 is an essential coenzyme for all three kinds of nitric oxide synthase (NOS) [30]. *AtNOA1* (*AtNOS1*) is located in *Arabidopsis* chloroplasts, and *OsNOA1* (*OsNOS1*) is also located in rice chloroplasts [31–33]. Yang et al. [33] found that the chlorophyll content decreased with increasing *OsNOA1* at a low temperature (22 °C). He [34] suggested that *OsNOA1* directly regulates the chloroplast self-coding protein by affecting the function of the chloroplast ribosome and then transmits the signal to the nucleus through the chloroplast retrograde signal pathway mediated by Mg protoporphyrin IX, further affecting the expression of chloroplast protein encoded by the nuclear gene. Qinghai Province is located in the Qinghai-Tibet Plateau. The average temperature during crop growth is lower, and the expression level of *BnaA02g30310D* (*GCH-1*) in NILs (aa) and ZS11 was more strongly expressed at three stages in the study, especially at the six-leaf stage, which is consistent with the results obtained by He [33, 34].

In addition, more research, such as a transgenic complementary test, CRISPR/Cas9, VIGS and RNAi, is warranted to examine whether *BnaA02g30260D*, *BnaA02g30290D* and *BnaA02g30310D* are the target genes of *cqSPDA2*. Analysis of the regulatory network for chlorophyll synthesis will facilitate *Brassica napus* molecular breeding for high yield.

## Conclusions

In the study, we built near-isogenic lines and narrowed the interval of *cqSPDA2* to 152 kb on A02 between SSR2 and Indel15. According to the *Brassica napus* genome annotation database, there were twenty-seven genes in the targeted mapping interval. *BnaA02g30260D*, *BnaA02g30290D* and *BnaA02g30310D* were identified as good candidate genes of *cqSPAD A2* according to the qRT-PCR analysis, which may be responsible for chlorophyll synthesis were found. The dominant locus *cqSPDA2* has positive effects on agronomic traits.

## Methods

# Plant materials

The leaves of ZS11 have low chlorophyll content, and the leaves of QU have high chlorophyll content (Additional file 11: Fig. S3). To investigate the genetic control regulation mechanism for the leaf chlorophyll content trait, we crossed ZS11 with QU to produce $F_1$ populations. In a previous study, the main effect QTL *cqSPDA2* was detected. To obtain a relatively simple genetic background and to fine map *cqSPDA2*, we constructed the near-isogenic line (NIL). The $F_1$ line with the QU genotype in the *cqSPDA2* region was selected and backcrossed with ZS11 for three generations. $BC_3F_1$ individuals were selfed to generate $BC_3F_2$ mapping populations backcrossed with ZS11. The markers Indel1 and Indel87 were used for marker-assisted selection (MAS) of each generation among the segregating progenies.

The $BC_4F_1$ individuals with a QU genetic background in the *cqSPDA2* region selected with the markers indel3 and indel15 were selfed to generate $BC_4F_2$ populations for fine mapping of the *cqSPDA2* locus. The detailed process of population development is illustrated in Additional file 12: Fig. S4. $BC_4F_{2:3}$ (AA genotype with *cqSPDA2* and aa genotype without *cqSPDA2*) were detected for qRT-PCR analysis. The $BC_3F_2$ and $BC_4F_2$ populations were grown at the same density in fields in Yunnan and Xining, respectively. $BC_4F_{2:3}$ and $BC_6F_1$ populations were grown in a greenhouse at the Academy of Agricultural and Forestry Sciences, Qinghai University. Spacing was maintained at 30 cm between rows and 15 cm between plants. Standard crop management practices were followed.

# Phenotypic trait and data analysis

The testing targets were every plant of populations that eliminated diseases and insect pests. According to previous research, we measured the first fully developed leaves counted from the top at the six-leaf stage by SPAD. Each measurement was repeated three times. Statistical analysis was calculated by Excel. Chi-square tests were performed on the segregation data to determine the genetic regulation of the chlorophyll content.

# DNA extraction and development of molecular markers

Total DNA was extracted from fresh leaves using the CTAB method [35]. PCR was performed in a 20-μL reaction solution containing 2 μL DNA, 2 μL 2 mM dNTPs, 2 μL 10 × PCR buffer, 1 μL Taq, 1 μL of 2 μM forward and reverse primers and 12 μL of $ddH_2O$. The PCR program was carried out according to Yang's method with minor modifications [36]. The PCR products were separated on 6% nondenatured polyacrylamide gels and detected by silver staining [37]. Indel (insertion/deletion) markers were developed from the target region to determine recombination sites and the genotype of recombinant progenies based on a previous study. The sequences of SSR markers were designed using SSR Hunter 1.3 and Primer Premier 5.0.

# Mapping of the cqSPDA2 locus

$BC_3F_2$ and $BC_4F_2$ family populations were used to fine map the *cqSPDA2* locus using indel and SSR markers. First, we designed 87 indel markers in the primer mapping interval 21.87–22.91 Mb on the A02 chromosome to map the *cqSPDA2* locus. A linkage map for the *cqSPDA2* locus was constructed using

JoinMap 4.0. The mapping interval for the *cqSPDA2* locus was gradually reduced using the mapping results of the $BC_3F_2$ populations. Finally, indel and SSR markers within the new narrow mapping interval were designed to fine map the *cqSPDA2* locus based on $BC_4F_2$ populations with WinQTLCart2.5. The physical location was obtained by sequence database comparison with *Brassica napus* and analysis. The physical linkage map was made by mapdraw 2.1V.

# TA clone

The specific markers closely linked to *cqSPDA2* were sequenced by NIL population scanning. Specific fragments were collected according to Yi et al. [38]. The product was connected to the pDM18-T vector (Takara), and the transformed clone was detected with primers M13. Six positive clones were randomly selected and sequenced by Sangon Biotech (Shanghai) Co., Ltd. [39].

# Genes in the mapping interval

All genes within the targeted mapping interval on A02 were identified using annotations from *Brassica napus* genomes (http://www.genoscope.cns.fr/brassicanapus/) and annotated according to the annotations of the BRAD (http://brassicadb.org/brad/blastPage.php). The homologous sequences were aligned using BLASTN (http://blast.ncbi.nlm.nih.gov/).

# RNA extraction and qRT-PCR analysis

Total RNA was isolated from leaves (4 leaf stage, 6 leaf stage and squaring stage) of $BC_4F_{2:3}$ and parents using TRNzol-A + Total RNA Reagent (Takara, Dalian, China) according to the manufacturer's protocol. RNA integrity was monitored using 1% agarose gel electrophoresis. cDNA was obtained via reverse transcription of total RNA using the PrimeScript RT reagent Kit (Takara, Dalian, China) and following the manufacturer's instructions.

We performed a quantitative reverse transcription-PCR (qRT-PCR) analysis to determine the genes in the mapping interval. Real-time PCR was conducted using LightCycler 480 II 96-Well PCR Plates (Roche, Rotkreuz, Switzerland). The utilized reaction system contained 10 μL of 2 × SG Fast qPCR Master Mix (B639271, BBI), 2 μL cDNA, and 10 μM gene-specific primers in a final volume of 20 μL. The thermal cycling conditions used were 95 °C for 30 min, followed by 45 cycles at 95 °C for 5 s and 60 °C for 30 s followed by a final extension stage. The housekeeping gene *Actin7* was used as a reference gene for calculating the relative expression levels of each gene.

# Agronomic traits

To evaluate the agronomic efficiency of *cqSPDA2*, 100 individuals (50 AA and 50 aa genotype plants) were sampled using the markers indel3 and indel15 referencing chlorophyll content from the $BC_4F_2$ population, respectively. The agronomic traits investigated were as follows: plant height, total siliques per plant, silique length, seeds per silique, 1000-seed weight, and yield per plant. The mean values, standard deviations and significant differences analysis of all the agronomic traits were compared between AA and aa genotype plants by Minitab16 and Excel2010.

# Abbreviations

QTL
Quantitative trait locus; NIL:Near Isogenic line; DH:Doubled haploid; qRT-PCR:Quantitative real-time PCR; SNP:Single nucleotide polymorphism; InDel:Insertion-deletion; SSR:Simple sequence repeat; SPAD:Soil and plant analyzer development; *FKBP*:FK506- and rapamycin-binding protein; *GCH-1*:GTP cyclohydrolase I; BH4:Tetrahydroboipterin; NOS/NOA:nitric oxide synthase.

# Declarations

### *Ethics approval and consent to participate*

This study does not contain any research requiring ethical consent or approval.

### *Consent for publication*

Not applicable.

### *Availability of data and materials*

All data used during the study are included in this published article and its additional files.

### *Competing interests*

The authors declare no competing financial interests.


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### *Authors' contributions*

YJX, LHD and DDZ conceived and designed the research; YJX and LHD performed the experiments; YJX, LHD and DDZ discussed the results and strategies; YJX analyzed the data and wrote the manuscript. ZZ, XL and LKX corrected the manuscript. All authors read and approved the manuscript.


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# Figures

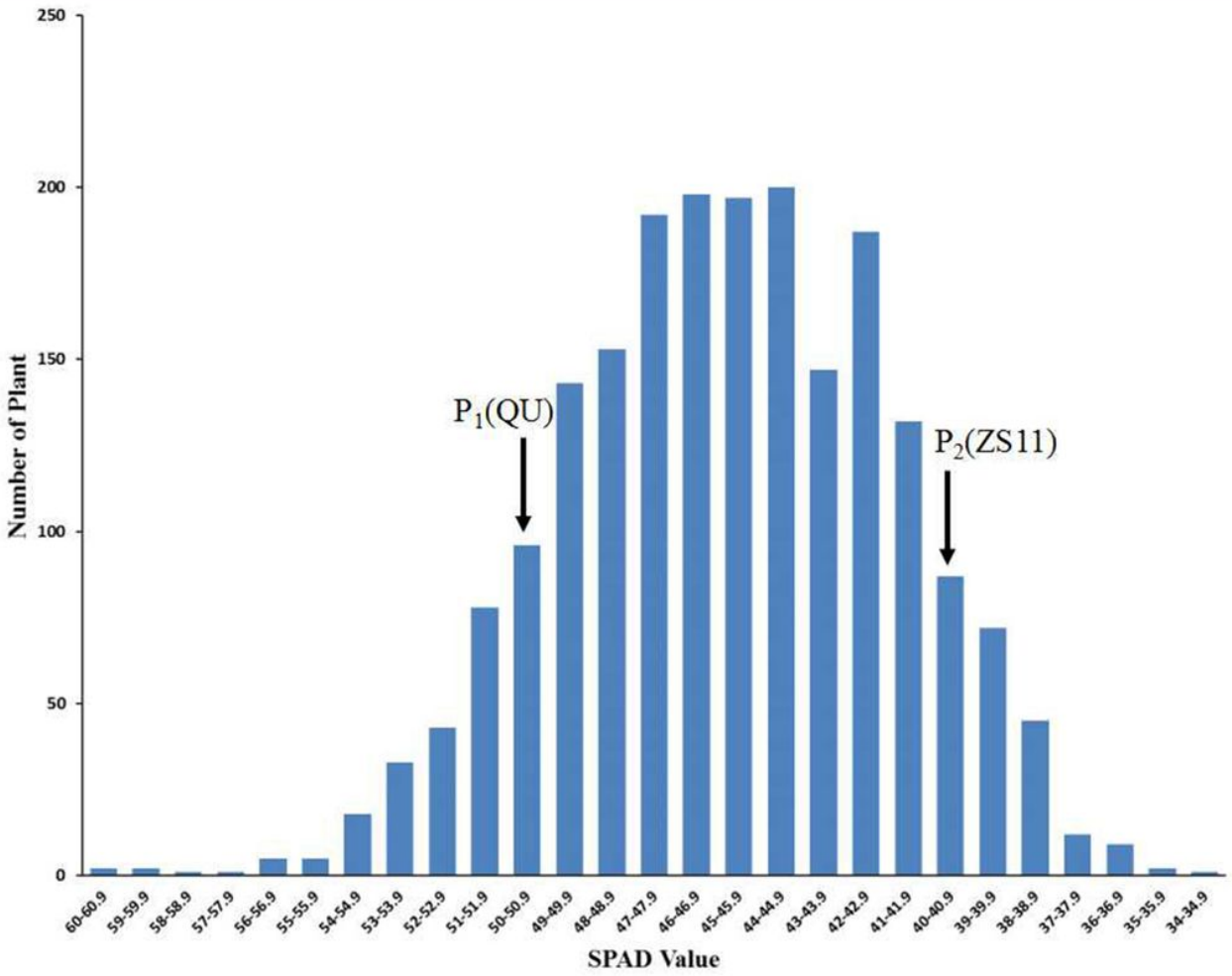


**Fig. 1** Phenotypic frequency distribution of chlorophyll SPAD in the $BC_4F_2$ population

## Figure 1

Phenotypic frequency distribution of chlorophyll SPAD in the BC4F2 population.

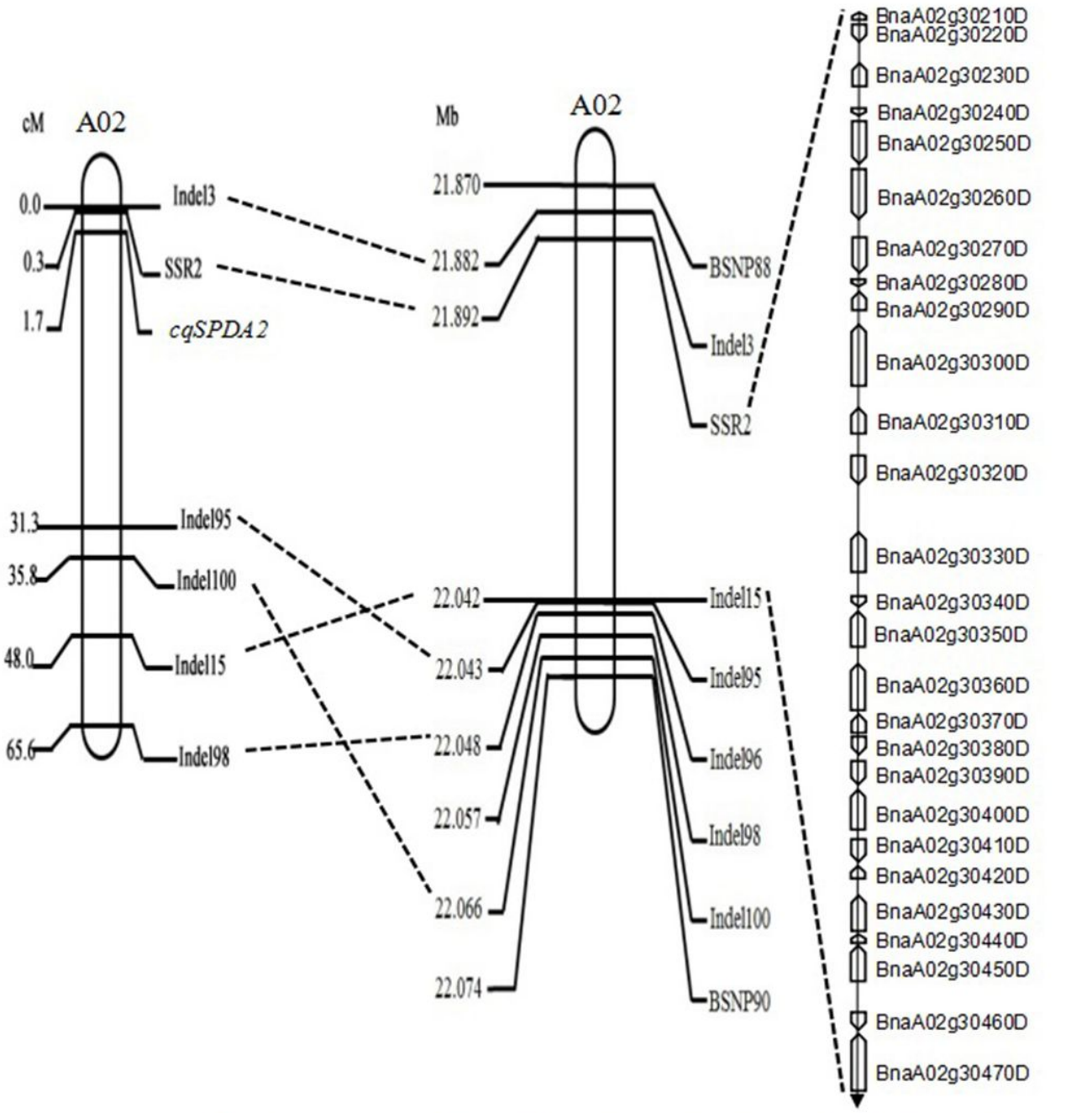


**Fig. 2** Genetic and physical maps of the *cqSPD A2* gene locus and candidate gene analysis

## Figure 2

Genetic and physical maps of the cqSPDA2 gene locus and candidate gene analysis.

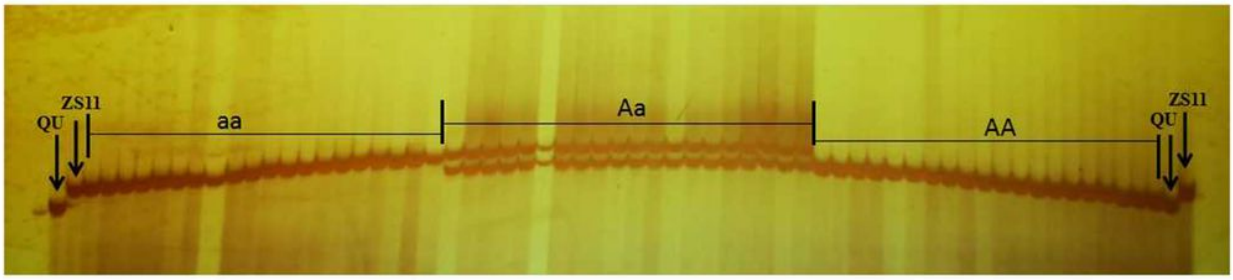


**Fig. 3** Codominant marker and closely linked with cqSPDA2. aa: the mean of SPAD=40.0±0.27; Aa: the mean of SPAD=44.8±1.44; AA: the mean of SPAD=53.3±0.80.

## Figure 3

Codominant marker and closely linked with cqSPDA2. aa: the mean of SPAD=40.0±0.27; Aa: the mean of SPAD=44.8±1.44; AA: the mean of SPAD=53.3±0.80.

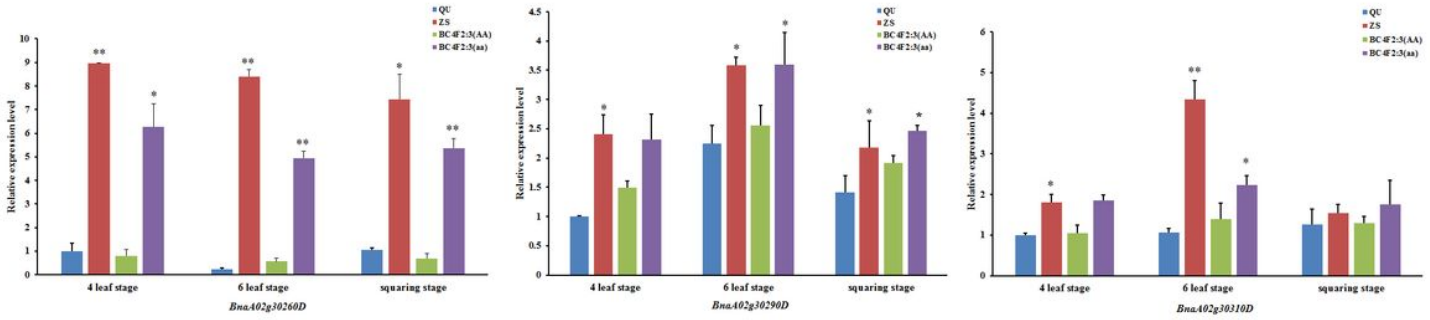


**Fig. 4** Differential expression of 3 genes in the mapping interval in parents and NILs. Values shown are means ± SD (n=3).
* denotes significance at the probability level of 0.05. ** denotes significance at the probability level of 0.01.

## Figure 4

Differential expression of 3 genes in the mapping interval in parents and NILs. Values shown are means ± SD (n=3). * denotes significance at the probability level of 0.05. ** denotes significance at the probability level of 0.01.

# Supplementary Files

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